A study on native brackish rice-associated *Flavobacterium* spp.: first insights into their ecology, plant functions, eco-physiology, taxonomy, and genomes

by

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- **Rahul R. Menon**

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List of Abbreviations

%	Percentage	Mb	Megabase
µl	microlitre	mg	milligram
µm	micrometer	ml	milli litre
g	Gram	mM	milli Molar
h	Hour	nm	nanometer
Kb	Kilobase	^{o}C	Celsius
M	Molar	rpm	rotation per minute
min	Minutes	w/v	weight/volume
hpi	hours post inoculation		
ATP	Adenosine triphosphate	PCR	Polymerase Chain reaction
BLAST	Basic Local Alignment Search Tool	pH	Potential of Hydrogen
cDNA	complimentary Deoxyribonucleic acid	R2A	Reasoner's 2 Agar
CDS	Coding DNA Sequence	RNA	Ribonucleic acid
CFUs	Colony Forming Units	RNA-seq	RNA Sequencing
DNA	Deoxyribonucleic acid	RND	Resistance Nodulation-Division
dpi	day post inoculation	rRNA	ribosomal RNA
dsDNA	Double-stranded Deoxyribonucleic acid	RT-qPCR	Real-Time quantitative PCR
ERIC	Enterobacterial repetitive intergenic consensus	SEM	Scanning Electron Microscopy
etc.	et cetera	Sl. No.	Serial number
Fig.	Figure	SRA	Sequence Read Archive
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	UV	Ultraviolet
HPLC	High Performance Liquid Chromatography	VTL	Vytila
LPSN	List of Prokaryotic names with Standing in Nomenclature	WT	Wildtype
MLSA	Multilocus Sequence Analysis	FPRM	*F. pokkalii* L1I52^{T} random mutant
NCBI	National Centre for Biotechnology Information		
nm	Nanometer		
NPK	Nitrogen Phosphorus Potassium		
No./no.	Number		
OD	Optical density		
PBS	Phosphate-buffered saline		

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Introduction and Review of Literature

1.1 Introduction

"Microbes will have the last word." – A famous quote by the renowned French biologist Louis Pasteur means: "Microbes (including bacteria) are resilient and omnipresent that have a profound influence on human health, ecosystems, and the planet". Given bacteria's enduring and ubiquitous nature, Pasteur recognized that they play a significant role in the world, often beyond human control. Over the decades, his quote has turned out to be where the world is witnessing the power of microbes as both a boon and a bane to all other life forms, including human beings dwelling on Earth.

My research broadly falls within the beneficial aspects of bacterial interaction relative to host plant health. To be specific, the impact of plant-bacterial interaction within the context of an environment (brackish ecosystem), emphasizing a less-explored bacterial genus, "*Flavobacterium*," was studied in terms of its eco-physiology, host plant growth influence, and related aspects. Therefore, in this introductory chapter, we discuss briefly the current understanding of the position of microbes in plant health, further describing their contribution to abiotic stress tolerance with a significant focus on saline stress, as the research study discussed in this thesis is much inclined towards that subject. Further, we narrow down to a particular bacterial phylum called Bacteroidota that is extensively studied for its role in the gut microbiome, contrasting to the least studied counterparts in plant ecosystems. Despite their abundance in plant habitats, meagre knowledge exists of their function. Within phylum Bacteroidota, the genus *Flavobacterium* is a known group with some plant-beneficial traits, categorizing them under the Plant growth-promoting rhizobacteria (PGPR) group. However, compared to the abundance and unique genetic mechanisms coded in *Flavobacterium* strains for carbon turnover, very little is known about their contribution to host and ecosystem functioning. Therefore, in this research study, we initially aim to understand the ecology of *Flavobacterium* in the brackish rice varieties through an ecological search for the native, novel, and potential *Flavobacterium* through culture-independent and dependent approaches. Further, we attempted to analyze their association with the brackish rice host through a multi-dimensional approach comprising binary-association experiments, genomic and transcriptomic studies, and mutational studies to unravel novel and unknown molecular factors responsible for Plant-*Flavobacterium*-Brackish interaction.

1.2 Plant health and abiotic stress

Plants are complex beings that have evolved to adapt to various environmental challenges over millions of years. The stress composition in any plant habitat is a mixture of biotic and abiotic factors and is often very complex and unknown. However, it is evident that they significantly impact the growth and development of plants (Greco et al., 2012). Salinity is among the most brutal environmental stresses and a complex phenotypic and physiological phenomenon that imposes ion imbalance or disequilibrium, hyper-ionic, and hyperosmotic stress on plants. These disturbances affect the overall metabolic activities in plants. Thus, saline stress is a major cause of crop loss, and the area of agricultural lands affected by salinity increases by a few percent yearly (Pitman and Läuchli, 2002). Hence, it makes it hard to estimate the overall impact because other stresses like drought, heat, or waterlogging often cause saline stress or result from salt stress.

Plant saline stress occurs mainly through osmotic and ionic toxicity (Horie et al., 2012). Osmotic stresses occur when salt concentration around the root's increases, which causes a decrease in water uptake and negatively affects cell elongation and lateral bud development. Ion toxicity starts relatively slowly with increased Na^+ concentrations in leaves, which disrupt metabolic reactions, leading to various adverse effects such as decreased photosynthetic rate (Horie et al., 2012). Osmotic and ionic stresses also cause secondary reactions, including activating calcium signaling, producing reactive oxygen species (ROS), and building abscisic acid (Horie et al., 2012). Other stress, such as drought, flooding, or contamination with heavy metals such as mercury, arsenic, lead, and chromium, demonstrate significant toxicity and may also cause the formation of reactive oxygen species (Forni et al., 2017). As a result, the generated ROS produces secondary DNA damage, including base loss, DNA-protein crosslinks, and double-stranded DNA breakage, ultimately leading to arrested plant development and plant death. However, there are various physiological adaptation mechanisms by which the plants circumvent these abiotic stress factors.

1.2.1 Physiological adaptation of plants to saline stress

Plants have mechanisms to adapt and evolve to different stresses. Hence, plants deal with stress uniquely to create homeostasis with the environment around them. In certain instances, the plant genotypes alter to withstand or overcome abiotic stress. When plants face abiotic stress, they can produce various physical responses to conserve energy and

survive. It includes gene expression changes to reduce their metabolism or tissue composition changes. This phenotypic plasticity, which refers to the ability of an organism to change when faced with environmental pressures, is fueled by the activation of specific genes encoding signal transduction, transcriptional control, ion transporters, and metabolic pathways (Sommer, 2020). Numerous distinct reactions, some of which may occasionally be similar, are brought on by plant exposure to stress. This is because the stress response changes in response to varied stimuli, producing multiple parallels in gene expression, thereby highlighting the overlap between environmental stress factors. Coordination of several biochemical pathways involving ion homeostasis, osmotic adjustment, osmolytes synthesis, scavenging of toxic enzymes/compounds, and hormonal balance governed by a complex interplay between mediators, hormones, transcription factors, and the varied regulatory genes is required to attain tolerance towards saline stress in plants (Hasegawa 2000, Mishra et al., 2006). This adaptation occurs at the molecular scale and entails the activation of gene regulatory pathways, producing defense molecules (Blumwald et al., 2004).

As a result, the genes triggered by salinity are divided into three classes, including those that regulate osmotic adjustment, growth under salinity stress, and salt transport and uptake(Munns, 2005). These genes, belonging to ABA-dependent or ABA-independent and Ca^{2+} signal pathways, are involved in reactive oxygen species (ROS) scavenging, secondary metabolism, etc., that provide tolerance to plants against saline stress (Hussain et al., 2021).

In addition to the physiological responses and adaptation generated from the plant side in response to saline stress, the core microbiota native to the plant also significantly contribute to the stress resilience mechanism. Hence, the upcoming sections discuss the critical role of microbes in plant health.

1.3 General insight into plant health and microbes

In a decade of plant microbiome research, the studies have elevated the understanding of the highly complex microbial assemblages that are associated with different parts of plants (Hardoim et al., 2015; Reinhold-Hurek et al., 2015; Vorholt, 2012). It includes the phyllosphere (above-ground part of the plant), rhizosphere, rhizoplane, and endosphere (below-ground parts of the plant). The microbial component of all these compartments constitutes the plant microbiota, including commensals, mutualistic, symbiotic organisms,

and disease-causing phytopathogens. The beneficial and symbiotic microbes impart several essential functions supporting plant growth and health, ranging from the nutrient supply, stress resilience against biotic and abiotic factors, and defence against phytopathogens (Hardoim et al., 2015; Lemanceau et al.,2017; Unter Brader et al., 2017; Vorholt, 2012) (Fig. 1.1). Since our research is focused on microbes associated with the rhizosphere and root regions of plants, our discussion in the upcoming sections will mainly pertain to the plant root microbiome.

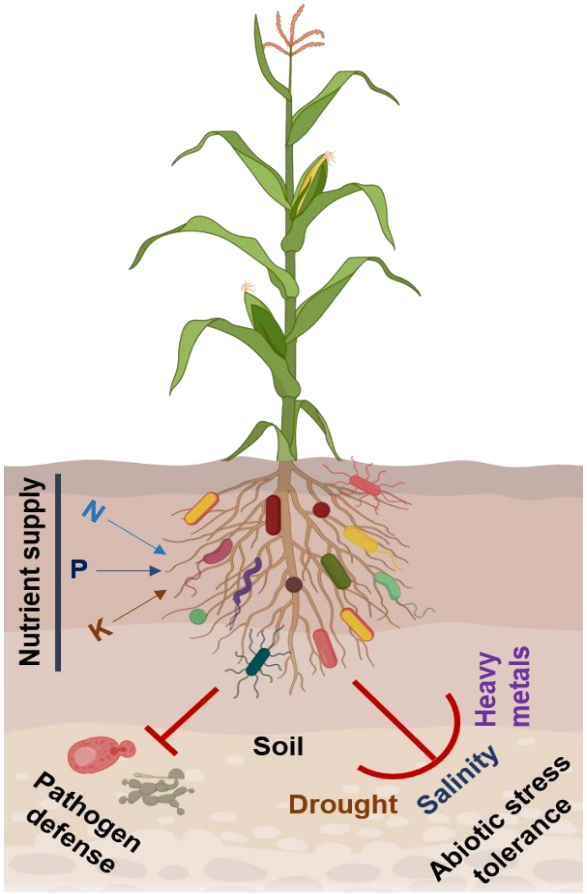


Fig. 1.1. Illustrative figure displaying the different phyto-beneficial functions attributed by the beneficial microbiota ranging from nutrient supply to pathogen defense and tolerance to several abiotic factors such as salinity, drought, heavy metal contamination, and many more.

Deciphering the microbial ecology of plant roots is crucial for understanding plant health, and it is estimated that the current technology-enabled knowledge has only touched the tip of the iceberg regarding the composition and function of the root microbiome (Hacquard et al., 2015). However, the metadata generated from the microbiome studies has identified

members of Pseudomonadota, Bacteriodota, and Actinomycetota to be the dominant players in the rhizosphere and root ecosystems compared to the bulk soil (Coleman-Derr et al., 2016; Fierer et al., 2009; Kolton et al., 2011; Mendes et al., 2011; Peiffer et al., 2013). Although these phyla members are ubiquitous in a wide range of plant species, the community composition and abundance vary within these lineages between the plant species (G. L. Mark et al., 2005; Ofek et al., 2014; Ofek-Lalzar et al., 2014). Moreover, several studies have demonstrated evidence for plant selection of root-associated microbiome(Bai et al., 2015; Bais et al., 2006; Chaparro et al., 2014; Rudrappa et al., 2008). Further, the recruited microbiome will have shifts in the community composition across the different developmental stages of the plant (Chaparro et al., 2014; Reinhold-Hurek et al., 2015).

1.3.1 Role of microbes in plant stress adaptation

Plants are well-known holobionts. "Plant microbiota" refers to the diverse and taxonomically structured microbial communities associated with plants in both natural settings and agricultural fields. It has been nearly ten decades since we first identified the importance of *Rhizobium* in legume plants and almost two decades since we began to understand its host association genetically (Frank, 1889; Roy et al., 2020). Since then, many novel and diverse plant-microbe associations have astounded us with their unique capabilities. These plant-associated microbes found in the vicinity of the plants, such as in the soil, air, water, etc., form close associations with the plant and influence its health by promoting growth through a series of mechanisms that include increasing mineral solubility (Jin et al., 2006; Rodríguez and Fraga, 1999; Roy et al., 2020; Vansuyt et al., 2007), modifying signalings such as phytohormone signaling of auxin (Bashan and Levanony, 1990; Garcia de Salamone et al., 1996), gibberellin(Joo Gil-Jae et al., 2005), and ethylene (Glick BR et al., 1998; Patten and Glick, 2002), directly providing nutrients (Bashan' and Levanony, 1990; Garcia de Salamone et al., 1996), and providing defense against pathogens and mineral solubility (Bakker et al., 2007, 2013; Mazurier et al., 2009). Abiotic stress tolerance, such as saline tolerance, is offered by different plant-associated PGPR (plant growth-promoting rhizobacteria) through diverse amelioration strategies, which include phytohormone production, ACC (1-Aminocyclopropane-1-Carboxylate) deaminase activity, extracellular polysaccharides (EPS) production, osmolytes and antioxidants synthesis, and through uptake of essential nutrients (Fig. 1.2). All these PGP traits contribute significantly towards the adaptation of

plant relative to its challenging stressed ecosystems.

However, it takes years of eco-adaptive evolution by the plants to gain resistance to abiotic stresses such as salinity through the gradual activation of its coded genetic traits and those acquired during evolution. In addition, the eco-adapted and co-evolved microbes complement the saline tolerance activity of plants, thereby initiating a beneficial association with the host plant to assist in its eco-adaptation and health. All these complex processes equip the sensitive plant variety to gain resistance or tolerance against the salinity stress and evolve as a saline-resistant variety through years of adaptive evolution (Fig. 1.2).

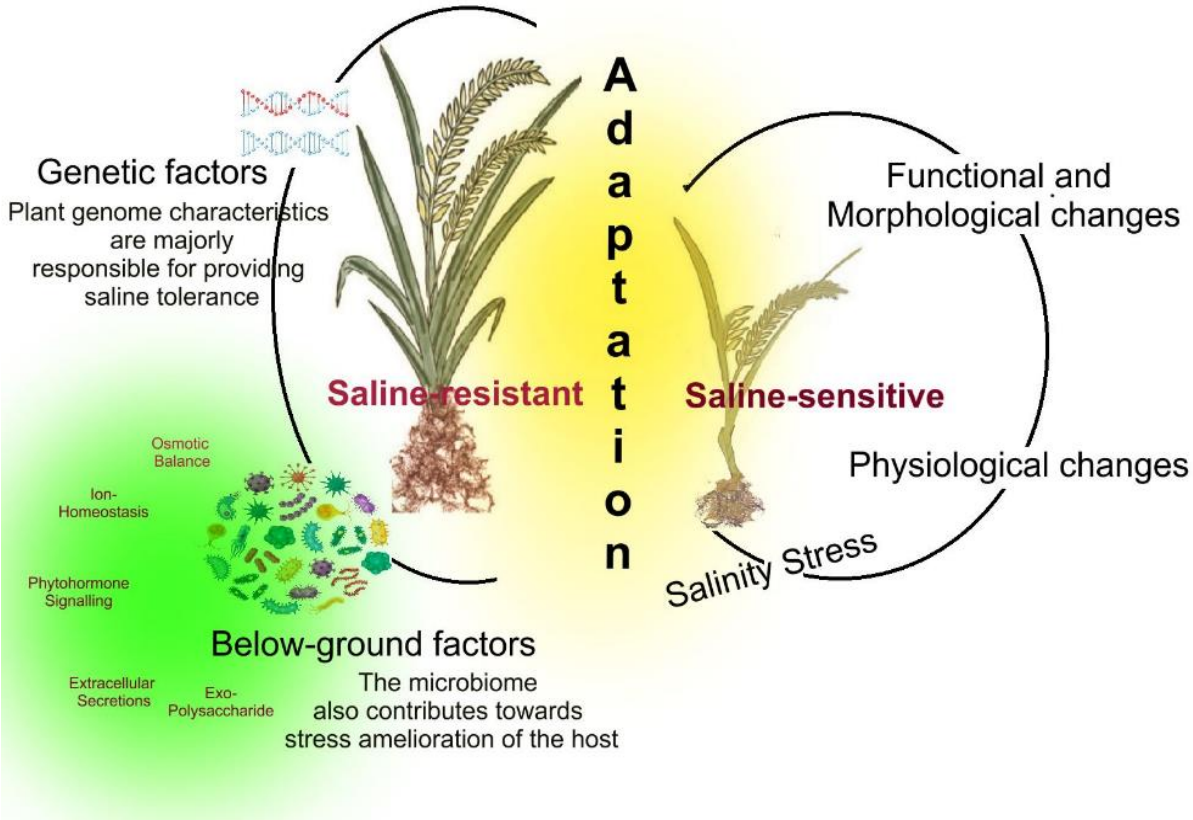


Fig. 1.2. Schematic figure representing the effect of salt stress on a saline-sensitive plant variety and how years of adaptive evolution can equip the saline-sensitive variety to adapt to salt stress and become saline-resistant that is attributed through the combinatorial effect of genotypic factors (expression of the already coded genes in the plant genome or the genes acquired during the adaptive evolution) and the below-ground factors i.e., the native microbiota that contributes largely to the host stress resilience through varied mechanisms such as osmotic balance, ion homeostasis, phytohormone signaling, extracellular secretions, exopolysaccharide synthesis and many more.

1.3.1.1 Phytohormone production and ACC deaminase activity

Plants under saline stress are known to produce a gaseous hormone, ethylene, which, when accumulated, leads to plant damage (Glick, 2004). 1-aminocyclopropane carboxylic acid

(ACC) is the direct precursor of ethylene and is synthesized and modified by the enzymes ACC synthase and ACC oxidase, respectively. The transcription of ACC synthase and ACC oxidase increases under situations of salt stress or other environmental challenges, increasing the quantity of ethylene produced by the plant. Plant-associated microorganisms with ACC deaminase activity constrain the subsequent increase in ethylene levels. The bacterial enzyme ACC deaminase thereby aids plants in reducing various stress-related symptoms, fostering growth (often by lengthening roots and aerial structures), and facilitating adaptability and survival. The PGPR that create ACC deaminase serve as a natural sink for ACC in this aspect (Glick and Penrose, 1998; Glick, 2014). This strategy of saline stress amelioration in plants has been reported in a wide range of bacteria, such as *Pseudomonas* (Zahir et al, 2009; Hernandez et al, 2015; Fathalla and Mageed, 2020), *Bacillus* (Kapadia et al, 2022; Azeem et al, 2022; Fathalla and Mageed, 2020; Yue et al, 2019) *Burkholderia* (Han et al, 2021; Sarkar et al, 2018; Akhtar et al, 2015; Onofre-Lemus, 2009), *Streptomyces* (Yoolong et al, 2019; Jaemsaeng et al, 2018), *Enterobacter* (Liu et al, 2017; Singh et al, 2022), *Leclercia* (Kang et al, 2019) and many more.

The other essential plant hormones that microbes control are indole-3-butyric acid and indole-3-acetic acid, collectively known as auxins. Bacterial genera such as *Actinobacteria* (Myo et al, 2019), *Frankia* (Nouioui et al, 2019), *Kitasatospora* (Shatoury et al, 2013), and *Streptomyces* (Harikrishnan et al, 2014) are known to produce indole-3-acetic acid. *Leclercia adecarboxylata*, capable of producing both ACC deaminase and Indole-3 acetic acid, was reported to help *Solanum lycopersicum* in saline tolerance (Kang et al, 2019). The plant-associated microbes have also been reported to produce cytokinins, which are known to maintain plant totipotency in the root and shoot apical meristems, and gibberellins, which are known to be associated with plant growth. Increased plant growth is positively connected with higher cytokinin and gibberellin levels and, thus, has been indirectly shown to assist in stress tolerance. Abscisic acid, often known as the stress hormone, is increased in the root zone when there is a water shortage due to salinity stress, which aids the plant in reducing the effects of stress. This occurs mainly by the abscisic acid helping in the buildup of calcium cations such as calcium and potassium and compatible solutes like sugars and proline in the root vacuoles, which counteract the adverse effects of excessive salinity. Thus, plant-associated microbes promote stress tolerance by abscisic acid biosynthesis or translocation (Belimov et al, 2014; Numan et al, 2018).

1.3.1.2 Production of extracellular polysaccharides

Exopolysaccharides produced by plant-associated bacteria reduce the effects of salinity stress by chelating free Na^+ ions and making them inaccessible to plants, promoting soil aggregation and stability, boosting biofilm development, and aiding in water retention (Bhagat et al, 2021). There are numerous findings on how exopolysaccharide (EPS)-producing plant growth-promoting bacteria protect plants from higher salt concentrations. *Planococcus* and *Halomonas* are EPS-producing saline-tolerant plant growth-promoting rhizosphere bacteria that were found to stabilize soil structure and increase soil aggregation under high saline conditions, which promoted *Cicer arietinum* growth (Qurashi et al, 2012). The EPS also aided in the colonization of these plant growth-promoting bacteria. EPS-producing strains of *Bacillus* and *Marinobacter* were found to increase the resilience of *Triticum aestivum* to salt and drought conditions by lowering Na^+ absorption and raising the dry weight of roots (Atouei et al, 2019). The lipoxygenase gene expression increased in the presence of EPS-producing *Pseudomonas*, thereby increasing the synthesis of jasmonic acid in *Arabidopsis thaliana.* Jasmonic acid has been known to function as a positive regulator of genes responsible for stress response (Chu et al, 2019; Wang et al, 2020). Compared to the wild-type strain, EPS-deficient *Mesorhizobium* mutants exhibited poor tolerance to saline stress, low antioxidant capacity, and reduced cell motility. This indicates the role of *Mesorhizobium* EPS in adaptation to extreme salinity by maintaining cellular concentrations of Na^+ and antioxidant enzyme activity at optimal levels (Liu et al, 2017). Combined application of melatonin and EPS-producing *Azotobacter* ameliorated saline stress by reducing Cl^- levels in faba beans (Ghani and Attia, 2020). Similarly, an EPS component, galacturonic acid, in *Rhodopseudomonas* EPS was found to chelate Na^+ cations from an aquatic environment and thus contribute towards saline stress tolerance (Nunkaew et al, 2015).

1.3.1.3 Synthesis of osmolytes, antioxidants and nutrient uptake

Highly soluble solutes such as sugars, glucosyl glycerol, betaines, and amino acids are among the organic osmolytes that bacteria synthesize (Ciulla et al, 1997). A small amount of these osmolytes, together with the organic solutes produced by the bacteria, are occasionally taken up by them from their surroundings and are present in their cytoplasm. The development of osmolytes in the cytoplasm contributes to the preservation of the cell's

osmotic balance. Sugars, sugar alcohols, γ-Aminobutyric acid (GABA), glycine, betaine, proline, and glutamate are significant plant osmolytes. These act as osmoprotectants and are major drivers of saline stress tolerance in plants (Chen and Jiang, 2010; Suprasanna et al, 2016). Antioxidants, both enzymatic such as catalase, peroxidase, superoxide dismutase (SOD), ascorbate peroxidase (APX), monodehydro-ascorbate reductase, dehydrogenase ascorbate, and glutathione reductase and non-enzymatic such as ascorbate, glutathione, carotenoids, tocopherols, flavonoids, play a significant part in the protection against saline stress by controlling the levels of reactive oxygen species (ROS) (Sengupta et al, 2016). *Glycyrrhiza glabra* inoculated with *Azotobacter* sp. Under saline, stress was recorded to show increased enzymatic activities of polyphenol oxidase (PPO), peroxidase (POD), and phenylalanine ammonia-lyase (PAL) (Mousavi et al, 2022). Similarly, the lipid peroxidation, relative membrane permeability, and lipoxygenase enzyme activity of plant growth-promoting bacteria inoculated wheat was found to be lower than the uninoculated plants (Singh and Tiwari, 2021). In another experimental setup, ten strains of gram-positive bacilli were found to increase the osmolyte concentrations in saline-stressed *Olea europaea* (Galicia-Campos et al, 2020). *Glutamibacter* and *Pseudomonas* were found to increase the antioxidant concentration and aid in nutrient uptake in saline-stressed *Suaeda fruticosa* (Hidri et al, 2022).

One of the main reasons for the decline in plant growth and productivity under saline stress is a lack of vital nutrients. Due to high osmotic pressure and ion toxicity, salinity decreases the uptake and accumulation of vital plant nutrients which are required to maintain development under stressful circumstances (Meena et al, 2014; Abbas et al, 2015). According to a study, *Achromobacter piechaudii* inoculated tomatoes increase potassium and phosphorus uptake from the soil (Mayak et al., 2004). In another study, the NPK content of wheat leaves under saline stress dramatically increased after being inoculated with *Bacillus aquimaris*, (Upadhyay and Singh, 2015). Plant growth-promoting bacteria are also known to increase the mineral availability of Cu, Fe, Mn, and Zn in plants (Etesami et al, 2014). Siderophore-producing bacteria are known to increase the iron availability to plants and aid growth under saline stress (Kavamura et al, 2013; Ramadoss et al, 2013).

Apart from the possession of individual traits, certain bacteria are reported to aid in the amelioration of saline stress by a combination of the above-mentioned traits. For example,

Burkholderia and *Enterobacter* were shown to have a positive effect on saline stress amelioration by a combination of ACC deaminase activity and EPS production (Akhtar et al, 2015). *Bacillus* and *Arthrobacter* ameliorate saline stress in maize by combining phytohormone production and phosphate solubilization traits (Vanissa et al, 2020). Also, *Bacillus* strains were found to ameliorate saline stress in sunflowers through a combination of ACC deaminase activity and antioxidant production (Yasmeen et al, 2020). Further, *Rhizobium* and *Enterococcus* strains were identified to alleviate saline stress in spring mungbean, and *Pseudomonas* alleviated saline stress in wheat by a combination of ACC deaminase, EPS, and phytohormone production (Kumawat et al, 2021; Singh et al, 2022).

Therefore, a combination of both genotypic factors and the effect of associated microbes significantly impacts a plant's ability to thrive under a particular abiotic stress, such as salt stress (Fig. 1.2). Only a limited number of bacterial genera, such as *Bacillus*, *Pseudomonas*, *Enterobacter, Rhizobium, Azotobacter,* etc., under the phyla Bacillota and Pseudomonadota were considered the main rhizobacterial contributors to plant function until recently. Although various plant metagenome-based investigations made possible by the development of modern sequencing technologies have led to a change in the above paradigm, some bacterial groups remain poorly understood. One understudied but predominant phylum in plant microbiome databases is the phylum Bacteroidetes (recently renamed Bacteroidota). This phylum is extensively studied for its function in the gut microbiome and is a significant contributor to carbon turnover in the human gut. However, their dominance in the plant microbiomes does not correlate with the existing knowledge on their functional role in plants and associated environments, which needs to be studied.

1.4 Phylum Bacteroidota

Bacteroidota are Gram-negative non-motile or gliding motile rods in various environments (B. M. Mark and Zhu, 2013; Paster et al., 1994; Woese, 1987). They have nearly eight orders and around 7,000 species described (Parte et al., 2020). However, when compared to the phyla Pseudomonadota, Actinomycetota, and Bacillota, this phylum has remained understated in various sequence databases despite being abundant in habitats such as the human gut, oceans, soil, etc (Hugenholtz, 2002). Gliding motility has been considered one of the candidate phenotypic traits for its identification (B. M. Mark and Zhu, 2013). Other candidate phenotypic traits of this phylum include the use of flexirubin family pigments as UV

protectants (Arul Aruldass et al., 2018), and their capability to take up complex biopolymers via TonB-dependent outer membrane receptors/transporters (Terrapon et al., 2015; Thomas et al., 2011). Also, the *gld* genes for gliding motility, *flx* for flexirubin biosynthesis, and the *susC/susD* gene pair for intake of polysaccharides are some of the genomic markers that represent these phenotypes (Terrapon et al., 2015; Thomas et al., 2011a). Further, studies were performed to trace the traits that played a significant role in Bacteroidota evolution and adaptive radiation by compiling the genomes of 89 Bacteroidota representatives (Munoz et al., 2020). These traits were those of the type IX secretion system, loss of ACIII-caa3COX super-complex in the anaerobes, and sodium-pumping Nqr-complex I in the marine members (Munoz et al., 2020).

1.4.1 Ecology of phylum Bacteroidota

Although the phylum Bacteroidota are present in diverse environments, they occur predominantly in glycan-rich habitats such as the gastrointestinal tract of humans and animals, aquatic environments, terrestrial environments, and sometimes extreme environments (Anders et al., (2014); Fernández-Gómez et al., (2013); Krüger et al., (2019); Larsbrink and McKee, (2020); Newton et al., (2011); Thomas et al., (2011b)). Recent sequencing technologies have abundantly identified members of the phylum Bacteroidota in aquatic ecosystems such as freshwater and marine environments. The studies using the Fluorescent *in situ* hybridization (FISH) technique revealed Bacteroidota members in the river and lake samples, with the abundance of the phylum increasing with the depth (Glo¨ckner et al., 1999; Šimek et al., 2001). Further research revealed that the planktonic Bacteroidota community of lakes and rivers differed from the surrounding terrestrial and sediment communities (Zwart et al., 2002). Similarly, sequence data obtained from ocean samples have suggested that Bacteroidota are the third-largest phylum found in ocean environments after Pseudomonadota and Cyanobacteria (Glo¨ckner et al., 1999; Kirchman, 2006). Certain research studies have shown that they are essential aspects of the bacterioplankton in coastal areas (Kirchman et al., 2002; Alonso-Sáez and Gasol, 2007). Also, they are identified as critical elements of offshore sediments (Llobet-Brossa et al., 1998; Julies et al., 2010), hydrothermal vents (Pommier *et al.*, 2007), biofilms (Edwards et al., 2010), coral inhabitants (Frias-Lopez et al., 2002; Rohwer et al., 2002), and macroalgal or angiosperm inhabitants (Beleneva and Zhukova, 2006; Staufenberger et al., 2008; Salaun et al., 2010; Crump and

Koch, 2008). *Bacteroidota* members are highly functional in algal blooms, supported by their active genes and proteins identified in metaproteome studies (Kruger et. al., 2019). CARD-FISH observations revealed the presence of *Bacteroidetes* across the North Atlantic Ocean and showed that *Polaribacter* was the most abundant genus (Gomez-Pereira et. al., 2010). Apart from these habitats, phylum Bacteroidota members constitute a predominant component of the soil microbial community. Using various culture-dependent and -independent techniques, they exist in soil samples taken from cultivated fields, greenhouse soils, and uninhabited places.

Further, phylum Bacteroidota is a vital player of the animal microbiota, as they occur predominantly in the gastrointestinal tracts, oral cavities, and dental plaques as commensal microorganisms (Moore et al., 1994; Biagi et. al., 2010; Thomas et al., 2011; Verster et. al., 2017; Radka et. al., 2020, Nasidze et. al., 2009 a, b). An increasing number of studies in the oral cavity and the gastrointestinal tract have been performed due to the ease of accessibility of these samples. They account for roughly half of the 16S rRNA sequences detected in healthy human mucosal tissues (Eckburg et al., 2005). Andersson et al., (2008) studied the throat microbiome using barcoding pyrosequencing and reported that Bacteroidota accounts for 20% of the reads, with the genus *Prevotella* being the second most represented after *Streptococcus*. Similar studies on the throat microbiome using barcoding pyrosequencing reported that Bacteroidota constitutes around 12.7% of the total microbiota (Gong et. al., 2014). The proportions were found to be the same in the distal esophagus (Pei et al., 2004). The throat biodiversity in laryngeal carcinoma patients was studied by Gong et. al., (2013), where the Bacteroidota constituted 15% of the total microbiome.

Phylum Bacteroidota and phylum Bacillota account for more than ninety-eight percent of the 16S rRNA sequences detected in the gut of mammals (Ley et al., 2006). The most colonized portion of the gastrointestinal tract is the large intestine, where high bacterial densities of up to 10^{12} cells/ml can be obtained (Whitman et al., 1998). Members of the phylum Bacteroidota are well-recognized colonizers of the colon. Among this phylum, members of the genus *Bacteroides* are the most abundantly represented in the fecal microbiota (Van den Abbeele et. al., 2013; Eckburg et. al., 2005; Sghir et al., 2000). The colonization by other members of this phyla has occurred in different parts of the gut that vary in pH, nutrients, and oxygen availability. The stomach is a harsh ecological niche,

distinct from the gastrointestinal tract compartments, due to its low pH. Even though *Helicobacter pylori* is a significant component of the stomach microbiota, other Bacteroidota, such as *Prevotella*, *Capnocytophaga*, *Bergeyella*, *Porphyromonas*, and *Tannerella*, were also found (Bik et al., 2006).

Other mammals such as mice (Ridaura et. al., 2013; Brown et. al., 2019), dogs (Manfredi et. al., 2015; Middelbos et al., 2010), pigs (Isaacson and Kim, 2012) and ruminants (Xue et. al., 2020; Leng et al., 2010) have also reported to harbor Bacteroidota as one of their principal microbial constituents. Non-mammals with similar compositions of Bacteroidota population are also reported in birds, echinoderms (Pagán-Jiménez et. al., 2019; Balakirev et al., 2008) and, termites (Schmitt-Wagner et al., 2003).

Even though most Bacteroidota are chemo-organoheterotrophs, this phylum has a varied range of metabolic processes and includes both aerobic (Bernardet, 2011; Nakagawa, 2011; Hahnke R. L. et al., 2016) and anaerobic members (Thomas et al., 2011). Many strains, particularly those from fish, are reported as pathogens causing diseases in marine eukaryotes and are among the greatest threats to ocean life. The pathogenic members of these classes lead either an opportunistic lifestyle that gets involved in polymicrobial diseases by causing secondary infections (Hudson and Egan, 2022). The most widely known pathogens within the phylum Bacteroidota belong to the family *Flavobacteriaceae*, the largest family within the phylum Bacteroidota (Nowlan et al, 2020). Members of the family *Flavobacteriaceae* are predominant in marine ecosystems but are also widespread in terrestrial and freshwater ecosystems (Kirchman, 2002).

1.4.1.1 Phylum Bacteroidota in plants

Besides the abundance of phylum Bacteroidota in the plants, as evident from the microbiome studies, meagre knowledge exists on their role in plant growth and related ecosystem functioning compared to their counterparts from the mammalian gut. However, a recent article discussing the importance of phylum Bacteroidota in host-microbe interactions and ecosystem functioning has highlighted the grey area of Bacteroidota-plant interactions (Pan et al., 2023). Although, few studies have reported the ability of Bacteroidota members to colonize in plants ranging from the model plant, *Arabidopsis thaliana*, to various agriculturally essential crops such as barley, tomato, lettuce, etc. (Cardinale et al., 2015; Escudero-Martinez et al., 2022; Johansen and Binnerup, 2002; Kwak et al., 2018) Also, the

abundance of Bacteroidota members are considered as indicators of good soil quality (Kruczyńska et al., 2023). Moreover, their presence is reported in the metagenomic samples extracted from different plant compartments, such as rhizosphere, endosphere, and phyllosphere, as well as from fruit surfaces (Carrión et al., 2019; Mamphogoro et al., 2020; Mukhtar et al., 2021; Zhang et al., 2019). Interestingly, the relative abundance of Bacteroidota members is reportedly higher in the wild plants compared to their domesticated counterparts, and this phenomenon has been observed across different plant species, including the model plant *A. thaliana,* and other plants such as barley, common bean, lettuce, and sugar beet (Pérez-Jaramillo et al., 2018). The variation in the root architecture and exudate composition between the wild and domesticated plant varieties at different growth stages plays a significant role in shaping the root microbiome, and this is believed to influence the differential abundance of Bacteroidota members between the plant cultivars (Raaijmakers and Kiers, 2020).

Moreover, Bacteroidota members are known for the genetically equipped array of Carbohydrate Active Enzymes (CAZymes) encoding genes organized into Polysaccharide Utilization Loci (PULs) that are capable of degrading complex polysaccharides which are the major components of plant cell walls (Lapébie et al., 2019; McKee et al., 2021). This ability of Bacteroidota strains has made them critical players for carbon turnover in the gut, which is facilitated by the breakdown of the glycans that have been extensively investigated (Lapébie et al., 2019). However, their role in carbon turnover in soil habitats or plant ecosystems has not been deeply studied, which opens a strong possibility for exploration that can resolve many ecological questions. Interestingly, it is observed that the soil-dwelling Bacteroidota members are more motile than their counterparts in the gut to increase their efficiency in hunting for nutrients in complex soil ecosystems (Larsbrink and McKee, 2020). Relatively, these soil-dwelling Bacteroidota candidates are assumed to be probable colonizers of plants in terrestrial habitats, and this increased motility phenotype might offer an advantage in establishing their colonization events with the host.

Despite the above information indicating their close association with plants, their functions remain largely unknown. Among the phylum Bacteroidota, members of the genus *Flavobacterium* represent a significant proportion of the microbiomes of different plant tissues. Moreover, several plant microbiome studies have reported their increased relative

abundance in rhizosphere and rhizoplane regions compared to the soil, possibly indicating their tight association with the host plants, contributing to their involvement in plant functioning (Kolton et al., 2016). However, despite being so dominant in the plant environments, very little is known about the ecology of *Flavobacteirum* members compared to the extensively studied genera such as *Azotobacter*, *Bacillus*, and *Pseudomonas*. Therefore, in the following sections, we discuss the current status of the highly abundant yet poorly explored bacterial genus associated with plants, i.e., the *Flavobacterium*.

1.5 The Genus *Flavobacterium*

Flavobacterium is recognized as one of the most important genera in the family *Flavobacteriaceae* under phylum Bacteroidota due to its large size and diverse habitat presence. They are rod-shaped bacteria, often 2–5 µm long. Certain *Flavobacterium* strains are short rods (1 µm) or filamentous (10 - 40 µm), and some are even known to exhibit pleomorphism (J. Bernardet and Bowman, 2015). Colonies of *Flavobacterium* are characterized by a yellow-orange color attributed to the presence of carotenoid or flexirubin pigments or both (Bernardet,' et al., 1996; J.-F. Bernardet and Bowman, 2006; Reichenbach et al., 1980.). Most species are obligately aerobic, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. A few species may also grow weakly under microaerobic to anaerobic conditions (J. Bernardet and Bowman, 2015). To date, 400 species of the genus *Flavobacterium* have been recognized, with more than 200 validly published species isolated from diverse ecological niches across the Earth as per the data available in LPSN on 27 November 2023 (Parte et al., 2020).

1.5.1 Ecology of genus *Flavobacterium*

Flavobacterium members are reportedly isolated from various habitats, including animals (Bernardet, et al., 1996), soil, marine (Debnath et al., 2019; Fu et al., 2011; Kaur et al., 2012; Weon et al., 2007), and freshwater ecosystems (Cousin et al., 2007; Lee and Jeon, 2018). They have also been reported from extreme habitats like a glacier and contaminated environments (Lata et al., 2012; Yi et al., 2005; Zhu et al., 2013). Further, they are also reported to be isolated from algae and cyanobacterial samples (Cai et al., 2018; Miyashita et al., 2010). Fig. 1.3 illustrates the broad classification of habitats or environments where the presence of members of the genus *Flavobacterium* is detected through both culture-dependent and culture-independent approaches. They are predominantly studied from the aquatic

environments as they are the major causative agents of fish pathogens. Even though their presence is reported to be dominant in plant microbiome studies, information relative to their interaction and host function remains largely under-explored (Fig. 1.3).

1.5.1.1 *Flavobacterium* as a constituent of the human gut and animal microbiome

Members of the genus *Flavobacterium* occur predominantly in the gastrointestinal tracts, oral cavities, and dental plaques of animals and humans. They are present in the gut of mammals and account for a large proportion of the 16S rRNA sequences found in healthy human gut. In a 16S rRNA metagenome study performed from the fecal samples of healthy

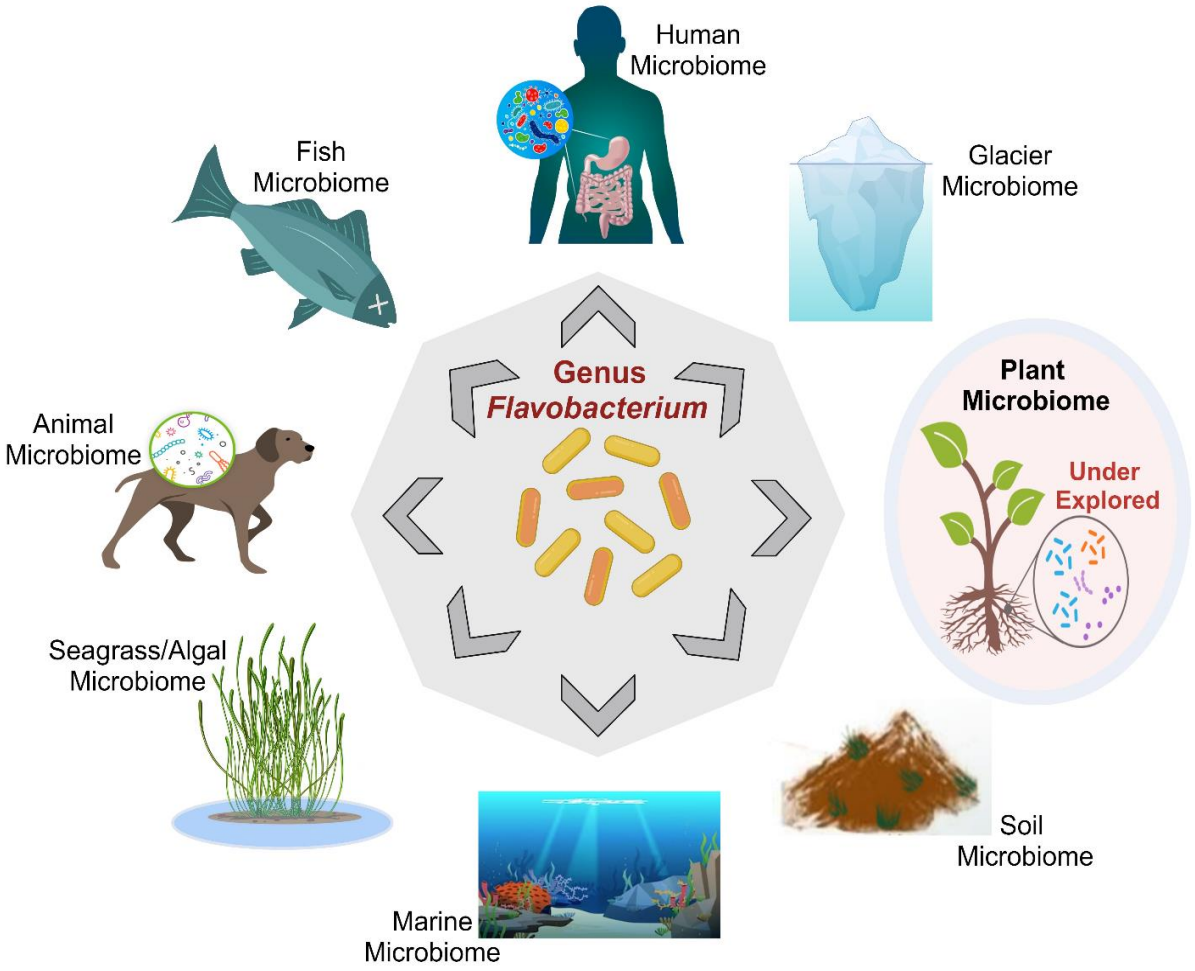


Fig. 1.3. Schematic figure representing the distribution of genus *Flavobacterium* across different habitats on Earth. Contrasting to the dominance of *Flavobacterium* members in the plant microbiome, their role in plant functioning remains largely under-exploited, and hence the plant microbiome component in the figure is highlighted which is also the main subject of the research study in this thesis work.

humans, submitted in the SRA database of NCBI (Bioproject Accession: PRJNA794317 SRA: SRR17439316), genus *Flavobacterium* was found to constitute more than 19% of the

operational taxonomic units (OTUs) belonging to the family *Flavobacteriaceae*. The species recognized in the study are *F. gossypii, F. columnare,* and *F. gillisiae.* In a similar study performed with the adult female gut sample (Bioproject Accession number: PRJNA783959; SRA: SRR17063322), OTUs corresponding to genus *Flavobacterium* constituted around 37% of the total *Flavobacteriaceae* OTUs, with identified species being *F. oryzae, F. petrolei, F. viscosus, F. croceum, F. urocaniciphilum, F. palustre, F. verecundum, F. lutivivi, F. cheniae, F. sasangense, F. araucananum, F. branchiophillum,* and *F. columnare.* Another recent submission (Bioproject Accession: PRJNA911939), which analyzed the microbial alterations on normal subjects inflammatory bowel disease, colorectal adenoma, and colorectal cancer patients, *Flavobacterium* population constituted around 20% of the order Flavobacteriales in healthy people. Further, a comparative study showed that the abundance of *Flavobacterium* members is a sign of healthy individuals (Palmas et al., 2021). In this study, the *Flavobacterium* members were reported as a potential marker with a considerable reduction in the obese microbiomes, confirming that *Flavobacterium* abundance are a sign of healthy individuals (Palmas et al., 2021).

Apart from the human gut, members of the genus *Flavobacterium* is reported to be a component in the gut microbiome of *Caenorhabditis elegans*. The *Flavobacterium* abundance was majorly modulated in the intestinal flora of *C. elegans* upon applying *Spirulina platensis* polysaccharide (Y. Chen et al., 2020). The increased abundance of *Flavobacterium* along with other bacterial groups is assumed to increase the antioxidant activity regulating the ROS-related metabolism in the *C. elegans* (Y. Chen et al., 2020). Another interesting study conducted on the gut microbiome of sea lamprey at different life stages reported a higher abundance of the genus *Flavobacterium* in the larval stages, indicating that the probable functional role of *Flavobacterium* strains is attributed to the growth stage of the host (Mathai et al., 2021).

1.5.1.2 *Flavobacterium* in marine environments/seagrass microbiome

Members of the genus *Flavobacterium* are commonly found in marine environments, including seawater, marine sediments, and marine organisms. Many *Flavobacterium* species are known to play essential roles in the functioning of marine ecosystems by contributing to the cycling of organic matter and nutrients. Primarily, they are involved in degrading complex organic compounds, such as polysaccharides and proteins, abundant in marine

environments. For example, *Flavobacterium algicola* isolated from marine algae is known to degrade algal polysaccharides, essential components of marine organic matter (Miyashita et al., 2010). Later in 2022, it was reported that *F. algicola* harbors a novel metabolic pathway that facilitates the degradation of the algal polysaccharide, carrageenan (Jiang et al., 2022).

Further, the genome analysis of the alga-associated marine *Flavobacterium, Formosa agariphila* KMM 3901^T reveals a potential to degrade a wide range of distinct algal polysaccharides such as carrageenans, laminaris, and xylans (Mann et al., 2013). Other algal-associated *Flavobacterium* strains are *F. litorale* sp. nov. WSW3-B6^T and *F. jocheonensis* UR11^T isolated from the red alga, and green alga *Ulva pertusa*, respectively (Choi et al., 2019; Muhammad Neak, 2022). *Flavobacterium* members have also been isolated from the sediments, such as *Flavobacterium beibuence* sp. nov. F44-8^T from the crude-oil-degrading consortium enriched from the marine sediment in China (Fu et al., 2011). These brief examples demonstrate the diverse roles and potential applications of Flavobacterium species in marine environments.

Additionally, *Flavobacterium* members are also detected in the microbiomes of seagrass. Even though, the published research reports are few, the analysis of the metagenomic datasets submitted to NCBI has detected reasonable amounts of *Flavobacterium* OTUs in the rhizosphere microbiome of seagrass. For example, in a Bioproject submission to NCBI (PRJNA881797) for studying the composition and diversity of Seagrass rhizosphere microbiome, we could identify a significant proportion of *Flavobacterium* OTUs in different metagenomic datasets. The relative abundance (%) of genus *Flavobacterium* OTUs within the family *Flavobacteiaceae* ranged from the least proportion, such as 0.05%, to 22% across the different metagenomic datasets.

1.5.1.3 *Flavobacterium* in fish microbiome

Genus *Flavobacterium* members are well-known for their association with fish and are a prominent causative agent for fish pathogenesis. For example, *Flavobacterium columnare* is a fish pathogen that causes columnaris disease, a bacterial infection that can devastate fish populations (Loch and Faisal, 2015). In addition, other pathogenic strains of the genus *Flavobacterium,* such as *F. psychrophilum,* cause bacterial cold-water disease and rainbow trout fry syndrome, and reported the deletion of a predicted peptidase and cytolysin to reduce

the virulence in trout (Thunes et al, 2022).

Further, many unknown pathogenic species of *Flavobacterium* were revealed during a study conducted to find the causative organism of a disease outbreak in fish(Loch et al., 2013). Therefore, fish diseases caused by pathogenic or opportunistic *Flavobacterium* strains are considered significant threats to aquaculture industries and oceans (Hudson and Egan, 2022). However, most of the *Flavobacterium* infections in fish were reported to be secondary, and the primary infection was known to be caused by viral pathogens (Adamek et al., 2018). Further, the *Flavobacterium* members have been isolated from other related sources. This includes isolating novel species strains, such as *F. channae* and *F. cyclinae*, from the intestines of *Channa sinensis* and *Cyclina argus*, respectively (Kang et. al., 2022).

1.5.1.4 Genus *Flavobacterium* in soil and plants

A culture-based study of the barley rhizosphere microbiome revealed the first evidence of the abundance of the genus *Flavobacterium* in plant ecosystems (Johansen and Binnerup, 2002). Later, the dominance of the genus *Flavobacterium* in various plant sources was evident through several culture-independent studies, and their presence was detected in different compartments of plants such as rhizosphere, rhizoplane, endosphere, and phyllosphere (Bodenhausen et al., 2013; Bulgarelli et al., 2012; Lundberg et al., 2012). For example, they represented a significant proportion in the rhizosphere microbiomes of the model plant *Arabidopsis thaliana* and other plants such as lettuce, maize, peanuts, tomatoes etc. (Bulgarelli et al., 2012; Graber et al., 2010; Haldar et al., 2011; Lundberg et al., 2012). Further, the findings from another research study suggested that *Flavobacterium* abundance is strongly influenced by plant-associated factors and not by soil composition or compost amendment (Tian and Gao, 2014). These findings hinted at the possible tight association of *Flavobacterium* members with its host plants. In support of this, the results from microbiome studies indicated a higher abundance of *Flavobacterium* OTUs in the rhizosphere and rhizoplane compartments compared to the bulk soil (Bodenhausen et al., 2013; Bulgarelli et al., 2012; Lundberg et al., 2012). Further, their abundance was higher in the early and intermediate growth stages of plants compared to late growth stages, where the *Flavobacterium* abundance is decreased (Qin et al., 2016).

Genus *Flavobacterium* strains has been isolated from the soil and different compartments (rhizosphere, phyllosphere, and endosphere) of various plant species. This section provides

insights into the important cultured plant-associated *Flavobacterium* strains and related host functions. Regarding their presence in the terrestrial soil, *Flavobacterium* members have been isolated from the field soil of different countries, mostly reported from South Korea to date. For example, *F. daejeonense, F. suncheonense, F. terrae, and F. cucumis* were isolated from greenhouse soils, and *F. ginsenosidimutans*, *F. panacisoli* from the ginseng field soil in South Korea, respectively, whereas *F. anhuiense* was isolated from the field soil in China, and *F. oryzae* from flooded rice fields of Taiwan (W. M. Chen et al., 2014; Jung et al., 2016; B. Y. Kim et al., 2006; Liu et al., 2008; Weon et al., 2007; Yang et al., 2011). Further, their presence is also found in contaminated soils, such as *Flavobacterium lindanitolerans* isolated from soil contaminated with hexachlorocyclohexane (Jit et al., 2008). Apart from this, *F. johnsoniae*, which is the most studied model organism under the genus Flavobacterium, is predominantly found in soil habitats. *F. johnsoniae* is extensively studied for its unique motility behavior, called gliding motility, which is facilitated by the combined functioning of the gliding motility complex and type IX secretion system (T9SS) (McBride and Nakane, 2015). This motility system is known to have potential applications in biotechnology for creating micro-robots and micro-scale devices (McBride, 2014).

(a) Plant-associated *Flavobacterium* strains and their potential as biocontrol agents

Even though limited knowledge exists on the beneficial influence of *Flavobacterium* strains on host plants, few studies have claimed the potential of *Flavobacterium* strains as effective biocontrol agents. In this regard, a *Flavobacterium* sp. strain F52 isolated from the greenhouse pepper roots was used as the model strain to demonstrate the potential role of gliding-motility and T9SS complex in root colonization and plant defence in tomato plants (Kolton et al., 2014). In this study, the researchers have constructed a gliding and secretion-deficient (*gldJ*) mutant and complemented it to check the comparative colonization in tomato roots, adhesion to seed surfaces, and subsequent response in plant protection against the phytopathogen *Clavibacter michiganensis* (Kolton et al., 2014). Interestingly, they could observe significant differences between the colonization rates of the wild-type and mutant strains of *Flavobacterium* sp. strain F52, indicating that the mutation in the *gldJ* gene impairs the colonization ability of *Flavobacterium* sp. F52 strain (Kolton et al., 2014). The reduced biocontrol activity of the mutated strain compared to the wild-type strain validated the significant role of gliding motility and T9SS complex in host-colonization and its followed

plant protection abilities (Kolton et al., 2014). Further, the findings from an exciting research study published in 2018 strongly supported the potent ability of *Flavobacterium* strains to offer protection against phytopathogen attack in plants(Kwak et al., 2018). In this study, the researchers identified distinct microbiome structures between the pathogen (*Ralstonia solanacearum*) resistant and susceptible tomato varieties while checking the role of native microbiota in disease resistance. The results from the rhizosphere microbiota transplantation experiments and subsequent comparative analysis of rhizosphere metagenomes identified the presence of a *Flavobacterium* strain that was more abundant in the pathogen-resistant rhizosphere microbiome (Kwak et al., 2018). The genome of this *Flavobacterium* strain was assembled from the metagenomes, and they could also culture this potential strain, TRM1. Interestingly, the cultured *Flavobacterium* sp. strain TRM1 could suppress the disease development by *R. solanacearum* in the susceptible plant, as demonstrated in the pot experiments (Kwak et al., 2018). This extensive research study strengthened the biocontrol potential of *Flavobacterium* strains, opening the possibility to explore more on the plant-beneficial aspects of this less-explored bacterial genus. Apart from this, few other studies have indicated the plant-growth-promoting abilities of *Flavobacterium* strains indirectly as biocontrol agents (Alexander and Stewart, 2001; An et al., 2009; Hebbar I et al., 1991; Sang and Kim, 2012). In addition, few studies have reported the *in-vitro* plant-growth beneficial traits of *Flavobacterium* strain that were directly correlated with their beneficial effect on the growth of plants such as canola, corn, and tomato as observed from the plant growth-related parameters (Flynn et al., 2014; Madhaiyan et al., 2010).

(b) Genus *Flavobacterium* and saline stress

Salinity is a major abiotic stress factor that impairs plant growth and productivity. To date, there is not much literature discussing the ability of *Flavobacterium* strains to alleviate the effect of salinity stress on their host plants through effective colonization and subsequent influence on their growth when tested under saline/brackish conditions. However, few earlier studies have briefly reported the effect of certain *Flavobacterium* strains in offering enhanced salt tolerance to plants. For example, *F. crocinum* HYN0056^{T} reportedly offered enhanced salt tolerance to *Arabidopsis* plants as indicated by the upregulation of specific salt-inducible genes in *Arabidopsis* supported with increased survival rate when tested in 250 mM NaCl salt stress (J. eun Kim et al., 2020). However, it is essential to note that the

tested condition for checking the salt stress tolerance is artificial saline, i.e., NaCl, not nature-mimicking/brackish conditions. Therefore, further evidence is required to validate how the strain would complement the plant growth when tested under brackish-mimicking conditions that offer more relevance to the saline enhancement ability of the bacterial strain from an ecological perspective. Extensive trials with customized experimental setups are vital to understanding how to mimic the environmental conditions and how they influence the interaction between the host plant and the associating bacterial group. Research on these aspects is very elusive. Hence, there is a critical need to investigate the plant-microbe interaction relative to the surrounding environment. This approach can impact the resolution of current global challenges, especially the impacts of climate change on crop health, to some extent.

1.6 Brief insights into the grey area of plant-microbe interaction

The research on plant-microbe interaction dates back decades, and enormous progress has been made in understanding how plants respond to microbial colonization in different ecological to investigate since it involves multi-dimensional interaction primarily influenced by several biotic and abiotic factors of nature and the after-effects of climate change being a predominant influencing factor. In contrast, the impact of environmental conditions on plant-microbe conditions (Cheng et al., 2019). Still, the discoveries made in this field astound researchers and interactions is poorly studied, as most of the research is performed under simple niches. Plant- microbe interaction is one of the most complex and trickiest research disciplines to study in laboratory and it is believed that we have witnessed only the tip of an iceberg, with much yet to be discovered. However, sequencing technologies have accelerated our attempts to unravel the secrets of plant- microbial interaction or the microbial ecology of an ecosystem or plant to a more significant microbial interaction or the microbial ecology of an ecosystem or plant to a more significant extent. The bipartite interaction system is the conventional method or commonly followed system for studying Plant-Microbe interaction. It involves the plant as the host and its associated microbe or native microbiota (Fig. 1.4 (a)). Nevertheless, several factors influencing this complex interaction positively or negatively are not investigated, and the surrounding environment or the ecosystem is assumed to be a critical factor influencing the Plant-microbe interaction. Also, it is important to note that it is nearly impossible to mimic the entire ecosystem or

related conditions and comprehensively study every influencing factor. However, in our research study, we mainly attempted to understand the role of the environment as a link of connection in mediating the plant-microbe interaction using different potential and novel strains identified during our extensive cultivation experiments. Briefly, we study the influence of salinity or natural brackish conditions in initiating and establishing an association between a native bacterium and its plant host. Thus, it involves three factors, i.e., the host plant, native microbiota, and the environment (as the influencing factor linking the interaction between the host plant and the native microbiota of the ecosystem) (Fig. 1.4 (b)).

1.7 Aim and Objectives of the Research Study

A brief outline of the ecological distribution of *Flavobacterium* members in diverse

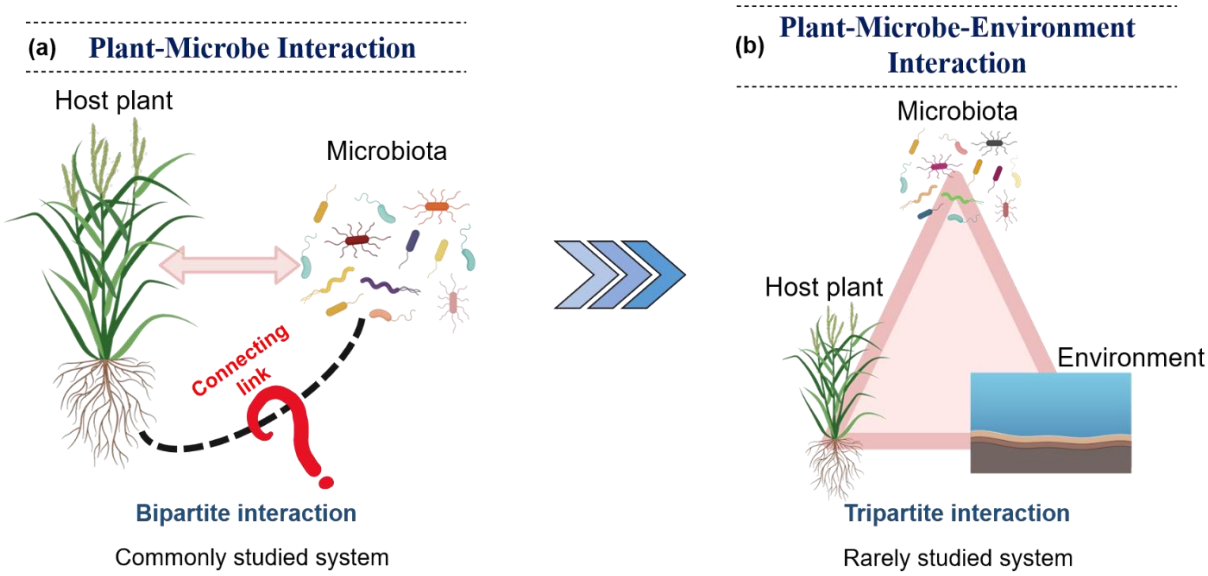


Fig. 1.4. Illustration representing the different systems used to study the interaction between the host plants and its native microbiota: **(a)** Plant-Microbe Interaction, which is a bipartite interaction i.e., involves the study of interaction between two entities/systems involving plant (as the host), and the native microbiota (commonly studied system), and **(b)** Plant-Microbe-Environment Interaction, which is a tripartite interaction i.e., involves the study of interaction between three entities/systems involving plant (as the host), native microbiota, and the environment (as the influencing factor linking the interaction between the host plant and the native microbiota of the ecosystem) (rarely studied system.

ecosystems clearly defines their dominance in plant ecosystems, indicating their firm association with the plants. Despite the abundance in the metagenomic datasets generated from different plant microbiomes, studies on plant-associated *Flavobacterium* strains native to an ecosystem investigating their eco-adaptability and host-functioning are finite. Moreover, the lack of a potential native cultured representative is a significant drawback

limiting their potential to explore plant functions and eco-physiology. As discussed earlier, *Flavobacterium* members are known to have a tight association with the host plants, as indicated through their metagenomic dominance in rhizosphere and rhizoplane compartments compared to bulk soil. However, the influence of *Flavobacterium* dominance on host growth or functioning remains poorly studied. This opens many possibilities to explore and understand the beneficial role of bacteria towards host plant health and fitness relative to the ecological conditions. Hence, my research study attempts to understand the significance of salinity/brackish conditions in bridging the link between the under-explored genus *Flavobacterium* and its host plant, Pokkali rice (Fig. 1.5). Beyond this, we also trace their interaction at the eco-physiological and molecular levels (Fig. 1.5). We assume that similar studies would provide initial insights into understanding the critical contribution of an ecosystem/environment in governing Plant-microbe interactions, at least at the binary level, as a start.

Therefore, to address the major research objectives of my thesis, i.e., understanding Pokkali-*Flavobacterium*-Brackish interaction, we listed out the following objectives or research questions that need to be addressed:

1) Understand the distribution and abundance of the genus *Flavobacterium* across the different compartments (rhizosphere and root) of native brackish rice varieties.
2) Isolation of native *Flavobacterium* strains and characterization of their host-growth benefits and eco-adaptive traits.
3) Taxonomic characterization of novel *Flavobacterium* strains through polyphasic taxonomic approach.
4) Detailed understanding of the unique capabilities of the novel *Flavobacterium strains* relative to host association, plant functions and eco-adaptation through in-depth genomic characterization and its functional validation through omics-based approaches and molecular techniques.
5) Attempts to develop mutants of novel *Flavobacterium* strain developed through a random mutagenesis approach to unravel the key genetic factors involved in Pokkali-*Flavobacterium*-Brackish interaction

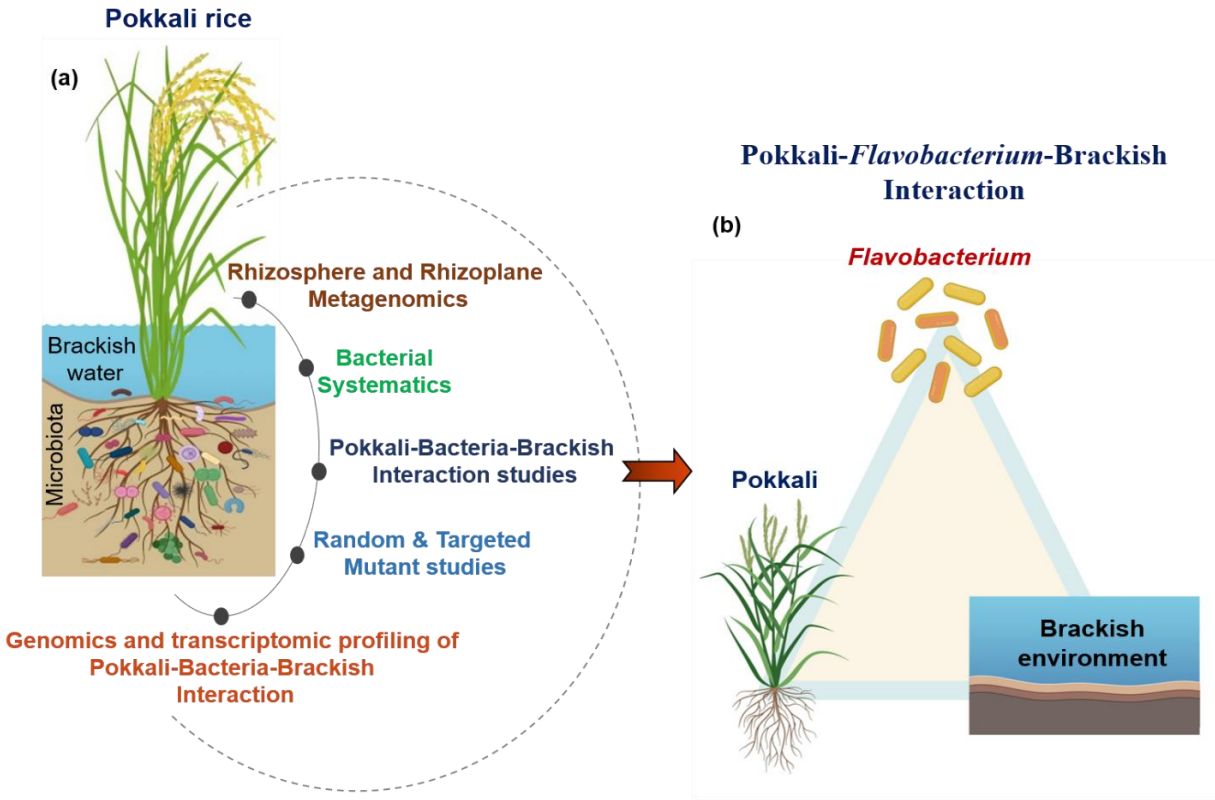


Fig. 1.5. Illustration depicting: **(a)** The Pokkali rice plant in a brackish ecosystem colonized with its native microbiota, which is the main research interest of our group. The major questions pertaining to this complex Plant-Microbe-Environment (i.e., Pokkali-Bacteria-Brackish) interaction are addressed through the listed brief disciplines starting from the broad metagenomics approach narrowing down to identifying a novel and potential bacterial strain of interest and studying its interaction with the host with the environment as the influencing factor through genomics, transcriptomics, and genetics approaches, and **(b)** the major research question of this thesis work which revolves on the tripartite interaction involving **(1)** Pokkali rice as the host plant, **(2)** *Flavobacterium* genus, the targeted bacterial group for studying its host interaction under the influence of the **(3)** native environment, i.e., the brackish ecosystem that is resolved through the different research disciplines practiced in our research lab.

Targeting the above-listed questions or challenges, extensive studies were carried out, and the following findings were organized into the thesis comprising five chapters, which includes three working chapters as represented in Fig. 1.6. Chapter – 1 is the Introduction and Review of Literature that is detailed above in the current chapter addressing the current status of the research pertaining to the subject of the research topic of the thesis work. Chapter – 2 details the metagenomic findings from the brackish rice varieties, focusing on the distribution and abundance of phylum Bacteroidota and genus *Flavobacterium*. Further, a bacteriome analysis derived from the customized, Pokkali soil microcosm-based setup validated the possible enrichment of the genus *Flavobacterium* in brackish rice. Finally, the

culture-dependent screening of brackish rice native *Flavobacteirum* strains is performed with a taxonomically valid publishing of a novel *Flavobacterium* strain (*Flavobacterium pokkalii* sp. nov. L1I52^T). Chapter – 3 discusses the *in-planta* host-growth influence and *in-vitro* host growth influencing traits supported with detailed genomic evidence complementing the plant-associated lifestyle and brackish adaptation of the novel strain, *Flavobacterium pokkalii* sp. nov. L1I52^T. Furthermore, their phenotypic and genotypic traits are validated at the transcript level through molecular approaches such as RNA-Seq-based global transcriptomic analysis and RT-qPCR-based quantitative analysis. Chapter – 4 details the attempts to genetically manipulate *Flavobacterium pokkalii* sp. nov. L1I52^T through a random mutagenesis approach using *pHimarEm-based* transposons.

Interestingly, we discuss the identification of specific novel gene insertions in *Flavobacterium pokkalii* sp. nov. L1I52^T responsible for defective phenotypic traits through loss of function (LoF), such as motility and xylan utilization that are essentially considered significant for host plant association. Finally, chapter 5 summarizes the significant findings

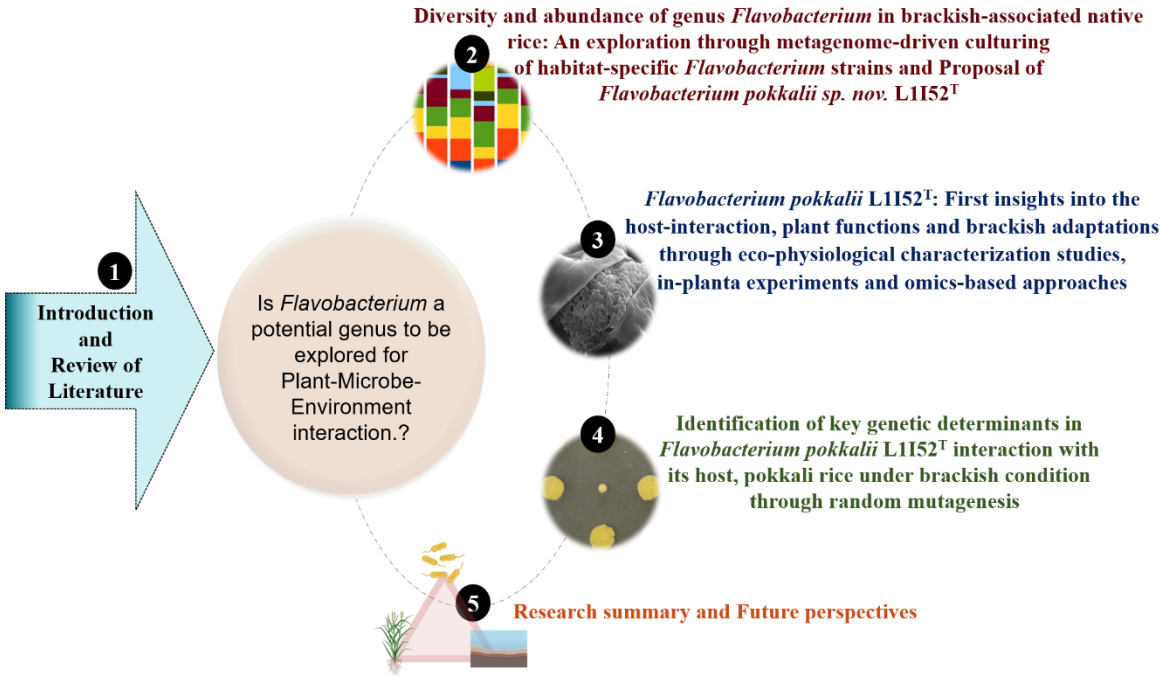


Fig. 1.6. Schematic figure summarizing the organization of the thesis represented by 5 chapters and the respective chapter titles: **Chapter – 1**: Introduction and Review of Literature briefing the existing literature status and current challenges, **Chapters – 2, 3, and 4**: represent the 3 working chapters detailing the findings from the thesis research study by addressing the research questions in the best possible manner, and **Chapter – 5** summarizes the highlights and flashes on the possible future perspectives of this research.

from all the working chapters and highlights the novelty of this research work that has shed light on the critical insights into *Flavobacterium*-Plant-Brackish interaction. Furthermore, we also flash upon the possible prospects of this research to extend deeply in unravelling the molecular mysteries responsible for the interesting environment-influenced association between the novel strain, *Flavobacterium pokkalii* sp. nov. L1I52^{T}, and its host, Pokkali rice.

1.8 References

1. Adamek, M., Teitge, F., Jung-Schroers, V., Heling, M., Gela, D., Piackova, V., Kocour, M., and Steinhagen, D. (2018). *Flavobacteria* as secondary pathogens in carp suffering from koi sleepy disease. *Journal of Fish Diseases*, 41(11), 1631–1642. https://doi.org/10.1111/jfd.12872

2. Alexander, B. J. R., and Stewart, A. (2001). Glasshouse screening for biological control agents of *Phytophthora cactorum* on apple (*Malus domestica*). *New Zealand Journal of Crop and Horticultural Science*, 29(3), 159–169. https://doi.org/10.1080/01140671.2001.9514174.

3. An, Q. D., Zhang, G. L., Wu, H. T., Zhang, Z. C., Zheng, G. S., Luan, L., Murata, Y., and Li, X. (2009). Alginate-deriving oligosaccharide production by alginase from newly isolated *Flavobacterium* sp. LXA and its potential application in protection against pathogens. *Journal of Applied Microbiology*, 106(1), 161–170. https://doi.org/10.1111/j.1365-2672.2008.03988.x.

4. Anders, H., Dunfield, P. F., Lagutin, K., Houghton, K. M., Power, J. F., MacKenzie, A. D., Vyssotski, M., Ryan, J. L. J., Hanssen, E. G., Moreau, J. W., and Stott, M. B. (2014). *Thermoflavifilum aggregans* gen. nov., sp. nov., a thermophilic and slightly halophilic filamentous bacterium from the phylum Bacteroidetes. *International Journal of Systematic and Evolutionary Microbiology*, 64(PART 4), 1264–1270. https://doi.org/10.1099/ijs.0.057463-0.

5. Arul Aruldass, C., Dufossé, L., Azlina Ahmad, W., and Dufosse, L. (2018). Current perspective of yellowish-orange pigments from microorganisms-a review. *Journal of Cleaner Production*, 180, 168–182. https://doi.org/10.1016/j.jclepro.2018.01.093ï.

6. Bai, Y., Müller, D. B., Srinivas, G., Garrido-Oter, R., Potthoff, E., Rott, M., Dombrowski, N., Münch, P. C., Spaepen, S., Remus-Emsermann, M., Hüttel, B., McHardy, A. C., Vorholt, J. A., and Schulze-Lefert, P. (2015). Functional overlap of the *Arabidopsis* leaf and root microbiota. *Nature*, 528(7582), 364–369. https://doi.org/10.1038/nature16192.

7. Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S., and Vivanco, J. M. (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. *Annual Review of Plant Biology*, 57, 233–266). https://doi.org/10.1146/annurev.arplant.57.032905.105159.

8. Bakker, P. A. H. M., Doornbos, R. F., Zamioudis, C., Berendsen, R. L., and Pieterse, C. M. J. (2013). Induced systemic resistance and the rhizosphere microbiome. *Plant Pathology Journal*, 29(2), 136–143. https://doi.org/10.5423/PPJ.SI.07.2012.0111

9. Bakker, P. A. H. M., Pieterse, C. M. J., and Van Loon, L. C. (2007). Induced systemic resistance by fluorescent *Pseudomonas* spp. *Phytopathology*, 97(2), 239–243. https://doi.org/10.1094/PHYTO-97-2-0239

10. Bashan', Y., and Levanony, H. (1990). Current status of *Azospirillum* inoculation technology: *Azospirillum* as a challenge for agriculture1. www.nrcresearchpress.com

11. Bernardet, J., and Bowman, J. P. (2015). *Flavobacterium*. Bergey's Manual of Systematics of Archaea and Bacteria (pp. 1–75). Wiley. https://doi.org/10.1002/9781118960608.gbm00312.

12. Bernardet, J.-F., and Bowman, J. P. (2006). The Genus *Flavobacterium*. *The Prokaryotes* (pp. 481–531). Springer New York. https://doi.org/10.1007/0-387-30747-8_17.

13. Bernardet,', J.-F., Segers, P., Vanca, M., Eyt, ", Berthe, F., Kersters,' And,' K, and Vandamme2, P. (1996). International Union of Microbiological Societies Systematic Bacteriology (40, 68), *The Prokaryotes*. International Journal of Systematic Bacteriology.

14. Blumwald, E., Grover, A., and Good, A. G. (2004). Breeding for abiotic stress resistance: Challenges and Opportunities Climate-Resilient Millet and Peanut View project BNF Crops View project. www.cropscience.org.au.

15. Bodenhausen, N., Horton, M. W., and Bergelson, J. (2013). Bacterial Communities Associated with the Leaves and the Roots of *Arabidopsis thaliana*. *PLoS ONE*, 8(2). https://doi.org/10.1371/journal.pone.0056329.

16. Bulgarelli, D., Rott, M., Schlaeppi, K., Ver Loren van Themaat, E., Ahmadinejad, N., Assenza, F., Rauf, P., Huettel, B., Reinhardt, R., Schmelzer, E., Peplies, J., Gloeckner, F. O., Amann, R., Eickhorst, T., and Schulze-Lefert, P. (2012). Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. *Nature* (Vol. 488, Issue 7409, pp. 91–95). https://doi.org/10.1038/nature11336.

17. Cai, H., Zeng, Y., Wang, Y., Cui, H., and Jiang, H. (2018*). Flavobacterium cyanobacteriorum* sp. nov., isolated from cyanobacterial aggregates in a eutrophic lake. *International Journal of Systematic and Evolutionary Microbiology*, 68(4), 1279–1284. https://doi.org/10.1099/ijsem.0.002664.

18. Cardinale, M., Grube, M., Erlacher, A., Quehenberger, J., and Berg, G. (2015). Bacterial networks and co-occurrence relationships in the lettuce root microbiota. *Environmental Microbiology*, 17(1), 239–252. https://doi.org/10.1111/1462-2920.12686.

19. Carrión, V. J., Perez-Jaramillo, J., Cordovez, V., Tracanna, V., de Hollander, M., Ruiz-Buck, D., Mendes, L. W., van Ijcken, W. F., Gomez-Exposito, R., Elsayed, S. S., Mohanraju, P., Arifah, A., van der Oost, J., Paulson, J. N., Mendes, R., van Wezel, G. P., Medema, M. H., and Raaijmakers, J. M. (2019). Pathogen-induced activation of disease-suppressive functions in the endophytic root microbiome. *Science*, 366(6465), 606–612. https://www.science.org.

20. Chaparro, J. M., Badri, D. V., and Vivanco, J. M. (2014). Rhizosphere microbiome assemblage is affected by plant development. *ISME Journal*, 8(4), 790–803. https://doi.org/10.1038/ismej.2013.196.

21. Chen, W. M., Chen, J. C., and Sheu, S. Y. (2014). *Flavobacterium oryzae* sp. nov., Isolated from a flooded rice field, And emended descriptions of *Flavobacterium flevense*, *Flavobacterium yonginense* and *Flavobacterium myungsuense*. *International Journal of Systematic and Evolutionary Microbiology*, 64, 3701–3708. https://doi.org/10.1099/ijs.0.065524-0.

22. Chen, Y., Wan, X., Wu, D., Ouyang, Y., Gao, L., Chen, Z., El-Seedi, H. R., Wang, M. fu, Chen, X., and Zhao, C. (2020). Characterization of the structure and analysis of the anti-oxidant effect of microalga *Spirulina platensis* polysaccharide on *Caenorhabditis elegans* mediated by modulating microRNAs and gut microbiota. *International Journal of Biological Macromolecules*, 163, 2295–2305. https://doi.org/10.1016/j.ijbiomac.2020.09.041.

23. Cheng, Y. T., Zhang, L., and He, S. Y. (2019). Plant-Microbe Interactions Facing Environmental Challenge. *Cell Host and Microbe* (Vol. 26, Issue 2, pp. 183–192). Cell Press. https://doi.org/10.1016/j.chom.2019.07.009.

24. Choi, H. R., Park, S. H., and Heo, M. S. (2019). *Flavobacterium jocheonensis* sp. nov., isolated from Marine Green Alga Ulva pertusa. *Journal of Microbiology and Biotechnology*, 29(8), 1266–1272. https://doi.org/10.4014/JMB.1812.12011.

25. Coleman-Derr, D., Desgarennes, D., Fonseca-Garcia, C., Gross, S., Clingenpeel, S., Woyke, T., North, G., Visel, A., Partida-Martinez, L. P., and Tringe, S. G. (2016). Plant compartment and biogeography affect microbiome composition in cultivated and native Agave species. *New Phytologist*, 209(2), 798–811. https://doi.org/10.1111/nph.13697.

26. Cousin, S., Päuker, O., Stackebrandt, E. (2007). *Flavobacterium aquidurense* sp. nov. and *Flavobacterium hercynium* sp. nov., from a hard-water creek. *International journal of systematic and evolutionary microbiology*, 57(2), 243-249.

27. Debnath, S. C., Miyah, A. M. A., Chen, C., Sheng, H., Xu, X. W., Wu, Y. H., Zheng, D. Q., Xu, J. Z., Di, Y. N., Wang, P. M., and Shen, L. (2019). *Flavobacterium zhairuonensis* sp. nov., a gliding bacterium isolated from marine sediment of the East China Sea. *Journal of Microbiology*, 57(12), 1065–1072. https://doi.org/10.1007/s12275-019-9194-4.

28. Escudero-Martinez, C., Coulter, M., Alegria Terrazas, R., Foito, A., Kapadia, R., Pietrangelo, L., Maver, M., Sharma, R., Aprile, A., Morris, J., Hedley, P. E., Maurer, A., Pillen, K., Naclerio, G., Mimmo, T., Barton, G. J., Waugh, R., Abbott, J., and Bulgarelli, D. (2022). Identifying plant genes shaping microbiota composition in the barley rhizosphere. *Nature Communications*, 13(1). https://doi.org/10.1038/s41467-022-31022-y.

29. Fernández-Gómez, B., Richter, M., Schüler, M., Pinhassi, J., Acinas, S. G., González, J. M., and Pedrós-Alió, C. (2013). Ecology of marine Bacteroidetes: A comparative genomics approach. *ISME Journal*, 7(5), 1026–1037. https://doi.org/10.1038/ismej.2012.169.

30. Fierer, N., Strickland, M. S., Liptzin, D., Bradford, M. A., and Cleveland, C. C. (2009). Global patterns in belowground communities. *Ecology Letters*, 12 (11), 1238–1249. https://doi.org/10.1111/j.1461-0248.2009.01360.x.

31. Flynn, B., Graham, A., Scott, N., Layzell, D. B., and Dong, Z. (2014). Nitrogen fixation, hydrogen production and N2O emissions. *Canadian Journal of Plant Science*, 94(6), 1037–1041. https://doi.org/10.4141/CJPS2013-210.

32. Forni, C., Duca, D., and Glick, B. R. (2017). Mechanisms of plant response to salt and drought stress and their alteration by rhizobacteria. *Plant and Soil*, 410(1–2), 335–356. https://doi.org/10.1007/s11104-016-3007-x.

33. Frank, B. (1889). Ueber die Pilzsymbiose der Leguminosen. Berichte Der Deutschen Botanischen Gesellschaft, 7(8), 332–346. https://doi.org/10.1111/j.1438-8677.1889.tb05711.x.

34. Fu, Y., Tang, X., Lai, Q., Zhang, C., Zhong, H., Li, W., Liu, Y., Chen, L., Sun, F., and Shao, Z. (2011a). *Flavobacterium beibuense* sp. nov., isolated from marine sediment. *International Journal of Systematic and Evolutionary Microbiology*, 61(1), 205–209. https://doi.org/10.1099/ijs.0.018846-0.

35. Garcia de Salamone, I., Urquiaga, bereiner S., and Boddey, R. (1996). Biological nitrogen fixation in *Azospirillum* strain-maize genotype associations as evaluated by the aSN isotope dilution technique. *Biol Fertil Soils* (Vol. 23).

36. Glick, B. R. (2004). Bacterial ACC Deaminase and the Alleviation of Plant Stress. *Advances in applied microbiology* 56 (2004): 291-312

37. Glick BR, Penrose DM, and Li J. (1998). A Model For the Lowering of Plant Ethylene Concentrations by Plant Growth-promoting Bacteria. *J. theor. Biol* (Vol. 190).

38. Glo¨ckner, F. O., Glo¨ckner, G., Fuchs, B. M., and Amann, R. (1999). Bacterioplankton Compositions of Lakes and Oceans: a First Comparison Based on Fluorescence Situ Hybridization. *Applied And Environmental Microbiology* (Vol. 65, Issue 8).

39. Graber, E. R., Harel, Y. M., Kolton, M., Cytryn, E., Silber, A., David, D. R., Tsechansky, L., Borenshtein, M., and Elad, Y. (2010). Biochar impact on development and productivity of pepper and tomato grown in fertigated soilless media. *Plant and Soil*, 337(1), 481–496. https://doi.org/10.1007/s11104-010-0544-6

40. Greco, M., Chiappetta, A., Bruno, L., and Bitonti, M. B. (2012). *Posidonia oceanica* cadmium induces changes in DNA methylation and chromatin patterning. *Journal of Experimental Botany*, 63(2), 695–709. https://doi.org/10.1093/jxb/err313

41. Hacquard, S., Garrido-Oter, R., González, A., Spaepen, S., Ackermann, G., Lebeis, S., McHardy, A. C., Dangl, J. L., Knight, R., Ley, R., and Schulze-Lefert, P. (2015). Microbiota and host nutrition across plant and animal kingdoms. *Cell Host and Microbe* (Vol. 17, Issue 5, pp. 603–616). Cell Press. https://doi.org/10.1016/j.chom.2015.04.009

42. Haldar, S., Roy Choudhury, S., and Sengupta, S. (2011). Genetic and functional diversities of bacterial communities in the rhizosphere of *Arachis hypogaea*. Antonie van Leeuwenhoek, *International Journal of General and Molecular Microbiology*, 100(1), 161–170. https://doi.org/10.1007/s10482-011-9570-5

43. Hardoim, P. R., van Overbeek, L. S., Berg, G., Pirttilä, A. M., Compant, S., Campisano, A., Döring, M., and Sessitsch, A. (2015). The Hidden World within Plants: Ecological and Evolutionary Considerations for Defining Functioning of Microbial Endophytes. *Microbiology and Molecular Biology Reviews*, 79(3), 293–320. https://doi.org/10.1128/mmbr.00050-14

44. Hasegawa, M., and Bressan, R. (2000). The dawn of plant salt tolerance genetics. *Trends in Plant Science 5*(8), 317-319.

45. Hebbar I, P., Berge ~, O., Heulin, T., and Singh, S. P. (1991). Bacterial antagonists of Sunflower (Helianthus annuus L.) fungal pathogens. *Plant and Soil* (Vol. 133).

46. Horie, T., Karahara, I., and Katsuhara, M. (2012). Salinity tolerance mechanisms in glycophytes: An overview with the central focus on rice plants. *Rice*, 5(1). https://doi.org/10.1186/1939-8433-5-11.

47. Hudson, J., and Egan, S. (2022). Opportunistic diseases in marine eukaryotes: Could Bacteroidota be the next threat to ocean life?. *Environmental Microbiology* (Vol. 24, Issue 10, pp. 4505–4518). John Wiley and Sons Inc. https://doi.org/10.1111/1462-2920.16094.

48. Hugenholtz, P. (2002). Exploring prokaryotic diversity in the genomic era. http://genomebiology.com/2002/3/2/reviews/0003.1http://genomebiology.com/2002/3/2/reviews/0003.

49. Hussain, S., Hussain, S., Ali, B., Ren, X., Chen, X., Li, Q., Saqib, M., and Ahmad, N. (2021). Recent progress in understanding salinity tolerance in plants: Story of Na+/K+ balance and beyond. *Plant Physiology and Biochemistry*, 160, 239–256. https://doi.org/10.1016/j.plaphy.2021.01.029.

50. Jiang, C., Zhang, T., Li, Q., Jiang, H., and Mao, X. (2022). A Novel Carrageenan Metabolic Pathway in *Flavobacterium algicola*. *Applied and Environmental Microbiology*, 88(18). https://doi.org/10.1128/aem.01100-22.

51. Jin, C. W., He, Y. F., Tang, C. X., Wu, P., and Zheng, S. J. (2006). Mechanisms of microbially enhanced Fe acquisition in red clover (*Trifolium pratense* L.). *Plant, Cell and Environment*, 29(5), 888–897. https://doi.org/10.1111/j.1365-3040.2005.01468.x.

52. Jit, S., Dadhwal, M., Prakash, O., and Lal, R. (2008). *Flavobacterium lindanitolerans* sp. nov., isolated from hexachlorocyclohexane-contaminated soil. *International Journal of Systematic and Evolutionary Microbiology*, 58(7), 1665–1669. https://doi.org/10.1099/ijs.0.65578-0.

53. Johansen, J. E., and Binnerup, S. J. (2002). Contribution of Cytophaga-like bacteria to the potential of turnover of carbon, nitrogen, and phosphorus by bacteria in the rhizosphere of barley (*Hordeum vulgare* L.). *Microbial Ecology*, 43(3), 298–306. https://doi.org/10.1007/s00248-002-2006-z.

54. Joo Gil-Jae, Kim Young-Mog, Kim Jung-Tae, Rhee -Koo, and Kim Jin-Ho. (2005). Gibberellins-Producing Rhizobacteria Increase Endogenous Gibberellins Content and Promote Growth of Red Peppers. *The Journal of Microbiology*, 43(6), 510–515.

55. Jung, S. Y., Kim, Y. J., Hoang, V. A., Jin, Y., Nguyen, N. L., Oh, K. H., and Yang, D. C. (2016). *Flavobacterium panacisoli* sp. nov., isolated from soil of a ginseng field. *Archives of Microbiology*, 198(7), 645–651. https://doi.org/10.1007/s00203-016-1216-6.

56. Kaur, I., Kaur, C., Khan, F., and Mayilraj, S. (2012). *Flavobacterium rakeshii* sp. nov., isolated from marine sediment, and emended description of *Flavobacterium beibuense* Fu et al. 2011. *International Journal of Systematic and Evolutionary Microbiology*, 62(12), 2897–2902. https://doi.org/10.1099/ijs.0.035691-0.

57. Kim, B. Y., Weon, H. Y., Cousin, S., Yoo, S. H., Kwon, S. W., Go, S. J., and Stackebrandt, E. (2006). *Flavobacterium daejeonense* sp. nov. and *Flavobacterium suncheonense* sp. nov., isolated from greenhouse soils in Korea. *International Journal of Systematic and Evolutionary Microbiology*, 56(7), 1645–1649. https://doi.org/10.1099/ijs.0.64243-0.

58. Kim, J. eun, Woo, O. G., Bae, Y., Keum, H. L., Chung, S., Sul, W. J., and Lee, J. H. (2020). Enhanced Drought and Salt Stress Tolerance in Arabidopsis by *Flavobacterium crocinum* HYN0056^{T}. *Journal of Plant Biology*, 63(1), 63–71. https://doi.org/10.1007/s12374-020-09236-8.

59. Kirchman, D. L. (2006). The ecology of Cytophagaâ Flavobacteria in aquatic environments. *FEMS Microbiology Ecology*, 39(2), 91–100. https://doi.org/10.1111/j.1574-6941.2002.tb00910.x.

60. Kolton, M., Erlacher, A., Berg, G., and Cytryn, E. (2016). The *Flavobacterium* Genus in the Plant Holobiont: Ecological, Physiological, and *Applicative Insights. Microbial* Models: From Environmental to Industrial Sustainability (pp. 189–207). Springer Singapore. https://doi.org/10.1007/978-981-10-2555-6_9.

61. Kolton, M., Frenkel, O., Elad, Y., and Cytryn, E. (2014). Potential role of flavobacterial gliding-motility and type IX secretion system complex in root colonization and plant defense. *Molecular Plant-Microbe Interactions*, 27(9), 1005–1013. https://doi.org/10.1094/MPMI-03-14-0067-R.

62. Kolton, M., Harel, Y. M., Pasternak, Z., Graber, E. R., Elad, Y., and Cytryn, E. (2011). Impact of biochar application to soil on the root-associated bacterial community structure of fully developed greenhouse pepper plants. *Applied and Environmental Microbiology*, 77(14), 4924–4930. https://doi.org/10.1128/AEM.00148-11.

63. Kruczyńska, A., Kuźniar, A., Podlewski, J., Słomczewski, A., Grządziel, J., Marzec-Grządziel, A., Gałązka, A., and Wolińska, A. (2023). Bacteroidota structure in the face of varying agricultural practices as an important indicator of soil quality – a culture independent approach. *Agriculture, Ecosystems and Environment*, 342. https://doi.org/10.1016/j.agee.2022.108252.

64. Krüger, K., Chafee, M., Ben Francis, T., Glavina del Rio, T., Becher, D., Schweder, T., Amann, R. I., and Teeling, H. (2019). marine Bacteroidetes the bulk of glycan degradation during algae blooms is mediated by few clades using a restricted set of genes. *ISME Journal*, 13(11), 2800–2816. https://doi.org/10.1038/s41396-019-0476-y.

65. Kwak, M. J., Kong, H. G., Choi, K., Kwon, S. K., Song, J. Y., Lee, J., Lee, P. A., Choi, S. Y., Seo, M., Lee, H. J., Jung, E. J., Park, H., Roy, N., Kim, H., Lee, M. M., Rubin, E. M., Lee, S. W., and Kim, J. F. (2018). Rhizosphere microbiome structure alters to enable wilt resistance in tomato. *Nature Biotechnology*, 36(11), 1100–1116. https://doi.org/10.1038/nbt.4232.

66. Lapébie, P., Lombard, V., Drula, E., Terrapon, N., and Henrissat, B. (2019). Bacteroidetes use thousands of enzyme combinations to break down glycans. *Nature Communications*, 10(1). https://doi.org/10.1038/s41467-019-10068-5.

67. Larsbrink, J., and McKee, L. S. (2020). Bacteroidetes bacteria in the soil: Glycan acquisition, enzyme secretion, and gliding motility. Advances in Applied Microbiology (Vol. 110, pp. 63–98). Academic Press Inc. https://doi.org/10.1016/bs.aambs.2019.11.001.

68. Lata, P., Lal, D., and Lal, R. (2012). *Flavobacterium ummariense* sp. nov., isolated from hexachlorocyclohexane-contaminated soil, and emended description of *Flavobacterium ceti* Vela et al. 2007. *International Journal of Systematic and Evolutionary Microbiology*, 62(11), 2674–2679. https://doi.org/10.1099/ijs.0.030916-0.

69. Lee, Y., and Jeon, C. O. (2018). *Flavobacterium alvei* sp. nov., isolated from a freshwater river. *International Journal of Systematic and Evolutionary Microbiology*, 68(6), 1919–1924. https://doi.org/10.1099/ijsem.0.002768

70. Lemanceau, P., Blouin, M., Muller, D., and Moënne-Loccoz, Y. (2017). Let the Core Microbiota Be Functional. *Trends in Plant Science* (Vol. 22, Issue 7, pp. 583–595). Elsevier Ltd. https://doi.org/10.1016/j.tplants.2017.04.008

71. Liu, H., Liu, R., Yang, S. Y., Gao, W. K., Zhang, C. X., Zhang, K. Y., and Lai, R. (2008). *Flavobacterium anhuiense* sp. nov., isolated from field soil. *International Journal of Systematic and Evolutionary Microbiology*, 58(4), 756–760. https://doi.org/10.1099/ijs.0.65536-0

72. Loch, T. P., and Faisal, M. (2015). Emerging flavobacterial infections in fish: A review. *Journal of Advanced Research* (Vol. 6, Issue 3, pp. 283–300). Elsevier B.V. https://doi.org/10.1016/j.jare.2014.10.009

73. Loch, T. P., Fujimoto, M., Woodiga, S. A., Walker, E. D., Marsh, T. L., and Faisal, M. (2013). Diversity of fish-associated flavobacteria of Michigan. *Journal of Aquatic Animal Health*, 25(3), 149–164. https://doi.org/10.1080/08997659.2012.758189

74. Lundberg, D. S., Lebeis, S. L., Paredes, S. H., Yourstone, S., Gehring, J., Malfatti, S., Tremblay, J., Engelbrektson, A., Kunin, V., Rio, T. G. Del, Edgar, R. C., Eickhorst, T., Ley, R. E., Hugenholtz, P., Tringe, S. G., and Dangl, J. L. (2012). Defining the core *Arabidopsis thaliana* root microbiome. *Nature*, 488(7409), 86–90. https://doi.org/10.1038/nature11237

75. Madhaiyan, M., Poonguzhali, S., Lee, J. S., Lee, K. C., and Sundaram, S. (2010). *Flavobacterium glycines* sp. nov., a facultative methylotroph isolated from the rhizosphere of soybean. *International Journal of Systematic and Evolutionary Microbiology* (Vol. 60, Issue 9, pp. 2187–2192). https://doi.org/10.1099/ijs.0.014019-0

76. Mamphogoro, T. P., Maboko, M. M., Babalola, O. O., and Aiyegoro, O. A. (2020). Bacterial communities associated with the surface of fresh sweet pepper (Capsicum annuum) and their potential as biocontrol. *Scientific Reports*, 10(1). https://doi.org/10.1038/s41598-020-65587-9

77. Mann, A. J., Hahnke, R. L., Huang, S., Werner, J., Xing, P., Barbeyron, T., Huettel, B., Stüber, K., Reinhardt, R., Harder, J., Glöckner, F. O., Amann, R. I., and Teeling, H. (2013). The genome of the alga-associated marine flavobacterium Formosa agariphila KMM 3901T reveals a broad potential for degradation of algal polysaccharides. *Applied and Environmental Microbiology*, 79(21), 6813–6822. https://doi.org/10.1128/AEM.01937-13

78. Mark, B. M., and Zhu, Y. (2013). Gliding motility and por secretion system genes are widespread among members of the phylum Bacteroidetes. *Journal of Bacteriology*, 195(2), 270–278. https://doi.org/10.1128/JB.01962-12.

79. Mark, G.L., Dow, J.M., Kiely, P.D., Higgins, H., Haynes, J., Baysse, C., Abbas, A., Foley, T., Franks, A., Morrissey, J. and O'Gara, F. (2005). Transcriptome profiling of bacterial responses to root exudates identifies genes involved in microbe-plant interactions. *Proceedings of the National Academy of Sciences*, *102*(48), pp.17454-17459.

80. Mathai, P. P., Byappanahalli, M. N., Johnson, N. S., and Sadowsky, M. J. (2021). Gut microbiota associated with different Sea lamprey (*Petromyzon marinus*) Life Stages. *Frontiers in Microbiology*, 12. https://doi.org/10.3389/fmicb.2021.706683.

81. Mazurier, S., Corberand, T., Lemanceau, P., and Raaijmakers, J. M. (2009). Phenazine antibiotics produced by fluorescent pseudomonads contribute to natural soil suppressiveness to Fusarium wilt. *ISME Journal*, 3(8), 977–991. https://doi.org/10.1038/ismej.2009.33.

82. McBride, M. J., and Nakane, D. (2015). *Flavobacterium* gliding motility and the type IX secretion system. *Current Opinion in Microbiology* (Vol. 28, pp. 72–77). Elsevier Ltd. https://doi.org/10.1016/j.mib.2015.07.016.

83. McKee, L. S., La Rosa, S. L., Westereng, B., Eijsink, V. G., Pope, P. B., and Larsbrink, J. (2021). Polysaccharide degradation by the Bacteroidetes: mechanisms and nomenclature. *Environmental Microbiology Reports* (Vol. 13, Issue 5, pp. 559–581). John Wiley and Sons Inc. https://doi.org/10.1111/1758-2229.12980.

84. Mendes, R., Kruijt, M., De Bruijn, I., Dekkers, E., Van Der Voort, M., Schneider, J. H. M., Piceno, Y. M., DeSantis, T. Z., Andersen, G. L., Bakker, P. A. H. M., and Raaijmakers, J. M. (2011). Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science*, 332(6033), 1097–1100. https://doi.org/10.1126/science.1203980.

85. Mishra, N. S., Tuteja, R., and Tuteja, N. (2006). Signaling through MAP kinase networks in plants. *Archives of Biochemistry and Biophysics* (Vol. 452, Issue 1, pp. 55–68). https://doi.org/10.1016/j.abb.2006.05.001.

86. Miyashita, M., Fujimura, S., Nakagawa, Y., Noshizawa, M., Tomizuka, N., Nakagawa, T., and Nakagawa, J. (2010). *Flavobacterium algicola* sp. nov., isolated from marine algae. *International Journal of Systematic and Evolutionary Microbiology*, 60(2), 344–348. https://doi.org/10.1099/ijs.0.009365-0.

87. Muhammad Neak, Han, H. L., L. Y.-J., Ko. J., Nguyen. T. T. H., Kim, S.-G. (2022). *Flavobacterium litorale* sp. nov., isolated from red alga. *International Journal of Systematic and Evolutionary Microbiology*, 72(8).

88. Mukhtar, S., Mehnaz, S., and Malik, K. A. (2021). Comparative Study of the Rhizosphere and Root Endosphere Microbiomes of Cholistan Desert Plants. Frontiers in Microbiology, 12. https://doi.org/10.3389/fmicb.2021.618742.

89. Munns, R. (2005). Genes and salt tolerance: Bringing them together. New Phytologist (Vol. 167, Issue 3, pp. 645–663). https://doi.org/10.1111/j.1469-8137.2005.01487.x.

90. Munoz, R., Teeling, H., Amann, R., and Rosselló-Móra, R. (2020). Ancestry and adaptive radiation of Bacteroidetes as assessed by comparative genomics. *Systematic and Applied Microbiology*, 43(2). https://doi.org/10.1016/j.syapm.2020.126065.

91. Newton, R. J., Jones, S. E., Eiler, A., McMahon, K. D., and Bertilsson, S. (2011). A Guide to the Natural History of Freshwater Lake Bacteria. *Microbiology and Molecular Biology Reviews*, 75(1), 14–49. https://doi.org/10.1128/mmbr.00028-10.

92. Ofek, M., Voronov-Goldman, M., Hadar, Y., and Minz, D. (2014). Host signature effect on plant root-associated microbiomes revealed through analyses of resident vs. active communities. *Environmental Microbiology*, 16(7), 2157–2167. https://doi.org/10.1111/1462-2920.12228.

93. Ofek-Lalzar, M., Sela, N., Goldman-Voronov, M., Green, S. J., Hadar, Y., and Minz, D. (2014). Niche and host-associated functional signatures of the root surface microbiome. *Nature Communications*, 5. https://doi.org/10.1038/ncomms5950

94. Palmas, V., Pisanu, S., Madau, V., Casula, E., Deledda, A., Cusano, R., Uva, P., Vascellari, S., Loviselli, A., Manzin, A., and Velluzzi, F. (2021). Gut microbiota markers associated with obesity and overweight in Italian adults. *Scientific Reports*, 11(1). https://doi.org/10.1038/s41598-021-84928-w

95. Pan, X., Raaijmakers, J. M., and Carrión, V. J. (2023). Importance of Bacteroidetes in host–microbe interactions and ecosystem functioning. *Trends in Microbiology*. Elsevier Ltd. https://doi.org/10.1016/j.tim.2023.03.018

96. Parte, A. C., Carbasse, J. S., Meier-Kolthoff, J. P., Reimer, L. C., and Göker, M. (2020). List of prokaryotic names with standing in nomenclature (LPSN) moves to the DSMZ. *International Journal of Systematic and Evolutionary Microbiology*, 70(11), 5607–5612. https://doi.org/10.1099/ijsem.0.004332

97. Paster, B. J., Dewhirst, F. E., Olsen, I., and Fraser', G. J. (1994). Phylogeny of Bacteroides, Prevotella, and Porphyromonas spp. and Related Bacteria. *Journal of Bacteriology*, *176*(3), pp.725-732.

98. Patten, C. L., and Glick, B. R. (2002). Role of Pseudomonas putida indoleacetic acid in development of the host plant root system. *Applied and Environmental Microbiology*, 68(8), 3795–3801. https://doi.org/10.1128/AEM.68.8.3795-3801.2002

99. Peiffer, J. A., Spor, A., Koren, O., Jin, Z., Tringe, S. G., Dangl, J. L., Buckler, E. S., and Ley, R. E. (2013). Diversity and heritability of the maize rhizosphere microbiome under field conditions. *Proceedings of the National Academy of Sciences*, 110(16), 6548–6553. https://doi.org/10.1073/pnas.1302837110

100. Pérez-Jaramillo, J. E., Carrión, V. J., de Hollander, M., and Raaijmakers, J. M. (2018). The wild side of plant microbiomes. *Microbiome*, 6(1). https://doi.org/10.1186/s40168-018-0519-z

101. Pitman, M. G., and Läuchli, A. (2002). Chapter 1 global impact of salinity and agricultural ecosystems. *Salinity: environment-plants-molecules*, *3*, p.20.

102. Qin, Y., Fu, Y., Dong, C., Jia, N., and Liu, H. (2016). Shifts of microbial communities of wheat (*Triticum aestivum* L.) cultivation in a closed artificial ecosystem. *Applied Microbiology and Biotechnology*, 100(9), 4085–4095. https://doi.org/10.1007/s00253-016-7317-y

103. Raaijmakers, J. M., and Kiers, E. T. (2020). Rewilding plant microbiomes. *Science*, 378(6620), 599–600.

104. Reichenbach, H., Kohl, W., B6ttger-Vetter, A., and Achenbach, H. (1980). Hicrebiolegy Flexirubin-Type Pigments in *Flavobacterium*. *Archives of Microbiology*, *126*, 291-293.

105. Reinhold-Hurek, B., Bünger, W., Burbano, C. S., Sabale, M., and Hurek, T. (2015). Roots Shaping Their Microbiome: Global Hotspots for Microbial Activity. *Annual Review of Phytopathology*, 53, 403–424. https://doi.org/10.1146/annurev-phyto-082712-102342.

106. Rodríguez, H., and Fraga, R. (1999). Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnology Advances*, 17(4-5), 319 - 339.

107. Roy, S., Liu, W., Nandety, R. S., Crook, A., Mysore, K. S., Pislariu, C. I., Frugoli, J., Dickstein, R., and Udvardi, M. K. (2020). Celebrating 20 Years of Genetic Discoveries in Legume Nodulation and Symbiotic Nitrogen Fixation. *The Plant Cell*, 32(1), 15–41. https://doi.org/10.1105/tpc.19.00279.

108. Rudrappa, T., Czymmek, K. J., Paré, P. W., and Bais, H. P. (2008). Root-secreted malic acid recruits beneficial soil bacteria. *Plant Physiology*, 148(3), 1547–1556. https://doi.org/10.1104/pp.108.127613.

109. Sang, M. K., and Kim, K. D. (2012). The volatile-producing *Flavobacterium johnsoniae* strain GSE09 shows biocontrol activity against Phytophthora capsici in pepper. *Journal of Applied Microbiology*, 113(2), 383–398. https://doi.org/10.1111/j.1365-2672.2012.05330.x.

110. Šimek, K., Armengol, J., Comerma, M., Garcia, J. C., Kojecka, P., Nedoma, J., and Hejzlar, J. (2001). Changes in the epilimnetic bacterial community composition, production, and protist-induced mortality along the longitudinal axis of a highly eutrophic reservoir. *Microbial Ecology*, 42(3), 359–371. https://doi.org/10.1007/s00248-001-0014-z.

111. Sommer, R. J. (2020). Phenotypic plasticity: From theory and genetics to current and future challenges. *Genetics*, 215 (1), 1–13). Genetics Society of America. https://doi.org/10.1534/genetics.120.303163.

112. Parte, A., Krieg, N.R., Ludwig, W., Whitman, W.B., Hedlund, B.P., Paster, B.J., Staley, J.T., Ward, N. and Brown, D. eds., (2011). *Bergey's Manual of Systematic Bacteriology: Volume 4: The Bacteroidetes, Spirochaetes, Tenericutes (Mollicutes), Acidobacteria, Fibrobacteres, Fusobacteria, Dictyoglomi, Gemmatimonadetes, Lentisphaerae, Verrucomicrobia, Chlamydiae, and Planctomycetes* (Vol. 4). Springer Science and Business Media.

113. Terrapon, N., Lombard, V., Gilbert, H.J. and Henrissat, B. (2015). Automatic prediction of polysaccharide utilization loci in Bacteroidetes species. *Bioinformatics*, *31*(5), 647-655.

114. Thomas, F., Hehemann, J.H., Rebuffet, E., Czjzek, M. and Michel, G. (2011) Environmental and gut Bacteroidetes: the food connection. *Frontiers in microbiology*, *2*, 93.

115. Tian, Y., and Gao, L. (2014). Bacterial Diversity in the Rhizosphere of Cucumbers Grown in Soils Covering a Wide Range of Cucumber Cropping Histories and Environmental Conditions. *Microbial Ecology*, 68(4), 794–806. https://doi.org/10.1007/s00248-014-0461-y.

116. Unter Brader, G. ¨, Compant, S., Vescio, K., Mitter, B., Trognitz, F., Ma, L.-J., and Sessitsch, A. (2017). Ecology and Genomic Insights into Plant-Pathogenic and Plant-Nonpathogenic Endophytes. https://doi.org/10.1146/annurev-phyto-080516.

117. Vansuyt, G., Robin, A., Briat, J.-F., Curie, C., and Lemanceau, P. (2007). Iron Acquisition from Fe-Pyoverdine by *Arabidopsis thaliana*. / 441 MPMI, 20(4), 441–447. https://doi.org/10.1094/MPMI.

118. Vorholt, J. A. (2012). Microbial life in the phyllosphere. *Nature Reviews Microbiology* Vol. 10 (12), 828–840). https://doi.org/10.1038/nrmicro2910.

119. Weon, H. Y., Song, M. H., Son, J. A., Kim, B. Y., Kwon, S. W., Go, S. J., and Stackebrandt, E. (2007a). *Flavobacterium terrae* sp. nov. and *Flavobacterium cucumis* sp. nov., isolated from greenhouse soil. *International Journal of Systematic and Evolutionary Microbiology*, 57(7), 1594–1598. https://doi.org/10.1099/ijs.0.64935-0.

120. Woese, C. R. (1987). Bacterial Evolution. Microbiological Reviews (Vol. 51, Issue 2).

121. Yang, J. E., Kim, S. Y., Im, W. T., and Yi, T. H. (2011). *Flavobacterium ginsenosidimutans* sp. nov., a bacterium with ginsenoside converting activity isolated from soil of a ginseng field. *International Journal of Systematic and Evolutionary Microbiology*, 61(6), 1408–1412. https://doi.org/10.1099/ijs.0.025700-0.

122. Yi, H., Oh, H. M., Lee, J. H., Kim, S. J., and Chun, J. (2005). *Flavobacterium antarcticum* sp. nov., a novel psychrotolerant bacterium isolated from the Antarctic. *International Journal of Systematic and Evolutionary Microbiology*, 55(2), 637–641. https://doi.org/10.1099/ijs.0.63423-0.

123. Zhang, Q., Acuña, J. J., Inostroza, N. G., Mora, M. L., Radic, S., Sadowsky, M. J., and Jorquera, M. A. (2019). Endophytic Bacterial Communities Associated with Roots and Leaves of Plants

Growing in Chilean Extreme Environments. *Scientific Reports*, 9(1). https://doi.org/10.1038/s41598-019-41160-x.

124. Zhu, L., Liu, Q., Liu, H., Zhang, J., Dong, X., Zhou, Y., and Xin, Y. (2013). *Flavobacterium noncentrifugens* sp. nov., a psychrotolerant bacterium isolated from glacier meltwater. *International Journal of Systematic and Evolutionary Microbiology*, 63(PART6), 2032–2037. https://doi.org/10.1099/ijs.0.045534-0

125. Zwart, G., Crump, B. C., Kamst-van Agterveld, M. P., Hagen, F., and Han, S. K. (2002). Typical freshwater bacteria: An analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. *Aquatic Microbial Ecology*, 28(2), 141–155. https://doi.org/10.3354/ame028141.

Chapter – 2

Diversity and abundance of genus *Flavobacterium* in brackish-associated native rice: An exploration through metagenome-driven culturing of habitat-specific *Flavobacterium* strains and Proposal of *Flavobacterium pokkalii* sp. nov. L1I52^{T}

2.1 Introduction

Microbial communities underpin the existence of life on Earth by governing the global biogeochemical cycles across all ecosystems, impacting the functioning of all life forms ranging from plants to animals and humans. All eukaryotic systems exist as holobionts, wherein the microbial symbionts complement their functioning based on the surrounding environment and allied factors (Bordenstein and Theis, 2015; Simon et al., 2019). The microbial ecology of natural ecosystems is so complex to study since it involves the interaction of thousands of microbes and associated species, both within and between species, as well as their interactions with the host and surrounding environment. Understanding these complex microbial interactions from multiple dimensions is highly challenging. Moreover, a large proportion of this microbial population remains unexplored due to the culturing challenges that constitute the "microbial dark matter," which is paramount in understanding the functioning of holobionts and ecosystem dynamics. It is estimated that only a tiny fraction of the microbial world has been successfully cultured and characterized, leaving the majority of microorganisms undiscovered to date (Adeleke et al., 2023; Unter Brader et al., 2017). However, the advent of next-generation sequencing technologies combined with advanced bioinformatic pipelines has enabled researchers to trace the unexplored microbiomes of diverse ecological niches at a higher resolution within a limited time in a cost-effective manner to some extent (Goodwin et al., 2016). Hence, these advanced sequencing technology-enabled metagenomics have become a breakthrough in tracing the unknown genetic composition and functional potential of complex ecosystems such as soil, oceans, human gut, etc. (Hugenholtz and Tyson, 2008; Yen and Jonson, 2021). Nevertheless, cultured representatives are essential in studying the functional attributes of novel isolates towards host-functioning and eco-adaptation. Hence, understanding the microbiota, i.e., the totality of microorganisms in an ecosystem, is vital. In the context of plants, their microbiota is composed of diverse microbial groups residing in different microenvironments, such as the phyllosphere, roots, rhizosphere, etc. (Vandenkoornhuyse et al., 2015). The heterogeneity observed within the plant microbiome of an organ, for example, in the root, is assumed to be an outcome of its adaptation processes and adjustments to external environmental conditions, thereby allowing for its rapid response to the changing environmental cues. Hence, the habitat of the host plant plays a critical role in shaping its native microbiota (Haichar et al., 2008). Thus, the surrounding ecosystem (both biotic and abiotic factors) largely governs the distribution and abundance of plant

microbiomes, thus influencing plant growth and adaptation.

As an outcome of global climate change over the last two decades, salinity has emerged as a prime factor adversely affecting plant growth and productivity (Ullah et al., 2021). Relating to this, a group of researchers during the IC-MPMI (International Congress on Molecular Plant-Microbe Interactions) meeting in Glasgow, 2019 voted "How does abiotic stress, such as climate change, influence plant-microbe interactions?" as Question No. 2 among the Top 10 unanswered questions in Molecular-Plant Microbe Interactions. In this context, the role of the native plant microbiome and its associated interaction with the host plant relative to the ecosystem is a highly relevant topic to be investigated. Therefore, the most essential prerequisite is identifying an ideal ecosystem relevant to the investigation. In this scenario, coastal agroecosystems are ideal habitats as they are the prime effectors of increasing soil salinization caused by the increased seawater intrusion occurring as part of climate change. Therefore, we chose three agroecosystems of South India: Pokkali, Kaipad, and Kagga, that practice switch farming through rice-shrimp/fish cultivation in alternate seasons of the year for this research. Since farming practices in these agroecosystems are organic and hence devoid of fertilizers, the native microbiota evolved over years of eco-adaptation, and host association is assumed to be undisturbed over the years and is worth investigating.

This research study focuses only on the bacteriome and no other microbial members such as archaea, fungi, viruses, and protozoa. Hence, this chapter provides a preliminary insight into the distribution and abundance of various bacterial phyla residing in the rhizosphere and root compartments of brackish rice varieties (Pokkali, Kaipad, and Kagga) through the 16S rRNA gene amplicon sequencing approach. However, the focus of the analysis is narrowed down to phylum Bacteroidota, emphasizing their metagenomic distribution in plants from earlier studies and its comparison with the metagenomic datasets generated in this study and related comparative inferences. Further, the family-level distribution emphasizing the distribution patterns of the genus *Flavobacterium* is analyzed and discussed in detail. Further, soil-microcosm-based bacteriome analysis is performed to study the influence of brackish conditions on shaping the native microbiome in brackish-associated rice, Pokkali. Finally, a metagenome-driven targeted culturing of habitat-specific native *Flavobacterium* strains through unique isolation strategies was attempted.

2.2 Materials and Methods

2.2.1 Site description, sampling procedure and media conditions

The coastal agroecosystems of South India cultivating saline-tolerant native rice varieties were chosen for sampling in this study. The selected three sites, namely Pokkali rice fields (Kumbalangi: 9.8684353 N 76.2828707 E, and Varappuzha: 10.0784624 N 76.2558175 E; Ernakulam district, Kerala), Kaipad rice fields (12.0267034 N 75.2913545 E; Kannur district, Kerala), and Kagga rice fields (14.425406 N 74.393183 E; Uttara Kannada district, Karnataka), are stretched across the south-western coastal belt of India (Table 2.1). The samples were collected by transferring the rice plants (6-8 rice plants as a bunch with the root-adhered soil) into clean polythene bags from the field. The sampling was performed

Table 2.1. Details of the brackish rice samples collected for the metagenomic study

Rice	Variety	Sample	Year	Geographic coordinates
Pokkali	VTL6	Rhizo-S1	2015	10.0784624 N 76.2558175 E
	Pokkali	Rhizo-S2	2016	9.8684353 N 76.2828707 E
	Pokkali	Root-S1	2017	9.8684353 N 76.2828707 E
	Pokkali	Root-S2	2015	9.8684353 N 76.2828707 E
Kaipad	Ezhome7	Rhizo-S1	2015	12.0267034 N 75.2913545 E
	Kuthiru	Rhizo-S2	2016	12.0267034 N 75.2913545 E
	Orkayama	Root-S1	2015	12.0267034 N 75.2913545 E
	Ezhome7	Root-S2	2015	12.0267034 N 75.2913545 E
Kagga	Kagga	Rhizo-S1	2018	14.425406 N 74.393183 E
	Kagga	Rhizo-S2	2018	14.425406 N 74.393183 E
	Kagga	Root-S1	2018	14.425406 N 74.393183 E
	Kagga	Root-S2	2018	14.425406 N 74.393183 E

during the early reproductive growth stage (booting phase) of the rice growth. The collected rice plants and bulk soil samples were stored at 4ºC until used for experiments. However, for the metagenomic studies, the rhizosphere soil and root portions were segregated separately into sterile tubes and stored at -80ºC until taken for total DNA extraction.The term brackish condition used in the text represents the natural seawater (NSW)-amended conditions (% of natural seawater varies as indicated in respective sections), and the non-

brackish condition means non-seawater or 0% seawater condition. The natural seawater used for all the media preparations and experiments performed in this study was collected from the Arabian Sea (~2 km from the Vizhinjam beach, Thiruvananthapuram, Kerala, India). The collected seawater was filter-sterilized in 8 µm cellulose nitrate filters (Sartorius Stedim Biotech, Germany) to remove particle traces and stored at 4ºC until used.

2.2.2 Soil-microcosm-based condition-driven bacteriome analysis

For this experiment, a customized setup was made using square phytajars from HiMedia, India (Cat No. PW1143), wherein a bored phytajar (12 holes with 6 mm in diameter) was mounted over an unbored phytajar filled with respective sterile nutrient solution. Half-strength hoagland's nutrient solution (½ HNS) from HiMedia, India (Cat. No: TS1094-5L) was used as the base nutrient solution. Three conditions were made using ½ HNS as the base, i.e., 1) ½ HNS (non-saline condition), 2) ½ HNS + 100 mM NaCl (saline condition), and ½ HNS + 30% natural seawater (NSW) (brackish condition). 5-day old sterile Pokkali seedlings were aseptically transferred to the customized hydroponics setups with respective nutrient solutions wherein the root portions of the seedlings remained immersed in the nutrient solution prepared in respective conditions. After 24 h of incubation of seedlings in the respective conditioned nutrient solutions, a soil suspension (1 g /10 ml) prepared from the Pokkali rice rhizosphere (Site: Kumbalangi, Ernakulam, Kerala; Sample ID: S2F2-3) was inoculated to the phytajar setup. The entire setup was incubated in a plant growth chamber (Binder, Germany) in the following growth conditions: 12-h light/12-h dark cycles with a temperature of 30°C and a relative humidity range of 50 %.

2.2.3 Metagenomic DNA isolation and library preparation

The rhizosphere soil and root compartments stored at -80ºC were shifted to room temperature and allowed to thaw for some time. For rhizosphere soil DNA extraction, ~ 250 mg of soil was weighed and subjected to homogenization. The homogenized samples were subjected to extraction using a DNeasy PoweSoil Pro kit (Cat. No: 47014, Qiagen, Germany) as per the manufacturer's instruction. For extracting root DNA, the root samples were washed thoroughly in sterile double distilled water to remove all soil particles from the root surfaces. The thoroughly washed root tissues were homogenized and subjected to extraction following the protocol of the DNeasy Plant mini kit (Cat. No: 69104, Qiagen, Germany). In the case of the soil microcosm-based condition-driven bacteriome analysis, the total DNA was extracted from the roots of seedlings grown in different conditions (as mentioned in the

previous section) on 7th and 14th dpi, respectively, following the protocol of the DNeasy Plant mini kit (Cat. No: 69104, Qiagen, Germany).

Table 2.2. Accession details of the datasets derived from metagenomes of brackish rice varieties and soil-microcosm-based pokkali root bacteriome analyzed in this study

S. No.	Sample name and details	BioProject ID
I	**Brackish rice**	
1	Pokkali (Rhizosphere and root)	PRJNA970257
2	Kaipad (Rhizosphere and root)	PRJNA970551
3	Kagga (Rhizosphere and root)	PRJNA970256
II	**Soil-microcosm-based bacteriome analysis**	
1	7th dpi (Non-saline, Saline, Brackish)	PRJNA1012597
2	14th dpi (Non-saline, Saline, Brackish)	PRJNA1012831

The quality and quantity of all the extracted DNA samples were checked using NanoDrop (Thermo Scientific) and Qubit dsDNA BR assay, respectively. Additionally, the quality of extracted total DNA was visualized by running an agarose gel (0.8%) electrophoresis that was imaged under the ChemiDoc imaging system (Biorad, USA).

25 ng of the total DNA isolated from rhizosphere and root compartments of Pokkali, Kaipad, and Kagga rice varieties were amplified using the 16S rRNA gene primers 341F/785R targeting the V3 – V4 hypervariable region. For the bacteriome analysis, 799F/1391R primer pair targeting the V5 – V6 hypervariable region was used (Beckers et al., 2016; Klindworth et al., 2013). The PCR conditions were as followed: an initial denaturation of 95°C for 5 min followed by 25 cycles of 95°C for 30s, 55°C for 45s, and 72°C for 30s, and a final extension at 72°C for 7 min. The amplicons were purified using AMPure beads to remove unused primers and an additional 8 cycles of PCR were performed using Illumina barcoded adapters to prepare the sequencing libraries. The libraries were sequenced on an Illumina MiSeq platform at the Clevergene sequencing facility, Bangalore. The raw sequence reads generated from this study is submitted to NCBI and the respective BioProject accessions are listed in Table. 2.2.

2.2.3.1 Sequencing data analysis and OTU (Operational taxonomic unit) clustering

The raw reads generated from the Illumina MiSeq runs were trimmed for 20 bp from the 5' end to remove the degenerate primer using TrimmingReads.pl available in NGSQCtoolkit

v.2.3.3. The high-quality reads (Phred score>20 and >70% based) were filtered using IlluQC.pl in NGSQCtoolkit (Patel and Jain, 2012). Further, the high-quality paired-end reads were joined using fastq-join through join_paired_ends.py script from QIIME (Quantitative Insights Into Microbial Ecology) with a minimum overlap of 125 nucleotide and 20% maximum difference between reads (Kuczynski et al., 2012). The OTU picking was conducted using denovo OTU picking strategy with pick.otus.py script in QIIME. Further, the clustering was done at a cutoff of 99% sequence identity. The representative sequences were identified using the pick_rep_set.py script, and the taxonomy assignment of the representative sequences was analyzed using a script called assign_taxonomy.py. The taxonomy assignment was conducted against SILVA 132 database with a minimum sequence similarity of 90% (Quast et al., 2013) . The final OTU table was constructed using make_otu_table.py in QIIME and was used for further metagenome-related analysis at different hierarchies of bacterial taxonomy.

For comparative studies, rice root metagenome data from Edwards et al. 2015 was retrieved from the National Centre for Biotechnology Information, Short Read Archive (NCBI-SRA) under accession no. SRP044745 (Edwards et al., 2015). The Root samples of three different sites (Arbuckle, Davis and Sacramento), and two different rice varieties (IR50 and Nipponbare) were considered for the analysis. The raw data from terrestrial rice root (SRP044745) samples was processed using the same procedure for the brackish-associated root bacteriome analysis. Except that the paired-end reads were joined using fastq-join with a minimum overlap of 200 nucleotides and a 10% maximum difference between reads. The merged contigs were then used for OTU clustering along with brackish-associated rice root samples for further comparative studies.

For the bacterial community analysis, the representative sequences were aligned using align_seq.py and filtered using filter alignment.py scripts available in QIIME. The aligned sequences were used for phylogenetic tree construction using make_phylogeny.py. First, a rarefaction plot was constructed using the OTU table and phylogenetic tree data using alpha_rarefaction.py to understand the microbial communities captured with the given sample size. Next, we analyzed the alpha diversities of the samples at the genus level. Alpha diversities of the observed OTUs were calculated using alpha_diversity.py in QIIME. The boxplot was constructed using ggplot2 in the R package (http://www.R-project.org/). Beta diversity was calculated with weighted_unifrac distance matrices using beta_diversity.py

and visualized with principal coordinate analysis (PCoA) using make_2d_plots.py scripts in QIIME.

The counts for higher taxonomic levels were constructed from the raw OTU table by summing all read counts within a taxon based on the assigned taxonomy using summarize_taxa.py in QIIME. The sequences from chloroplast and mitochondrial sources were removed to understand the bacterial composition without any organelle contamination from the plant. The mitochondria family and chloroplast class were selectively removed from the OTU table using filter_taxa_from_otu_table.py in QIIME. The final OTU table without chloroplast and mitochondria sequences were used for further taxonomic composition analysis at different taxonomic levels. Two sample t-tests were used to analyze the deferentially abundant taxa between the rhizosphere and root samples.

2.2.4 Targeted culturing of native *Flavobacterium* strains from Pokkali rice roots

Pokkali rice plants collected from Kumbalangi rice fields, Ernakulam, Kerala, India, were chosen for the isolation experiments. The root portion was excised from the rice plants and aseptically transferred to sterile petri dishes, where the roots were washed thrice with sterile distilled water to remove the rhizosphere soil portion from the roots. The roots devoid of rhizosphere soil were aseptically transferred to a sterile conical flask and washed with sterile natural seawater (full strength/100%) through manual shaking. Further, 2 gm of washed root pieces were homogenized in a mortar and pestle using sterile 30% NSW under aseptic conditions. The crushed root slurry was transferred to a sterile 50 ml tube (10 ml in 50 ml tube), vortexed for 10 min, and allowed to settle for 15-20 min. The upper aqueous phase was taken as the mother dilution and further diluted up to 10^{-3} dilutions, and several sub-dilutions were also made and spread on customized media: R2A3 (R2A agar supplemented with 30% NSW) and R2A3K (R2A3 agar amended with 50 µg/ml kanamycin antibiotic).

2.2.4.1 L1I52^{T} isolation and culture conditions

The bacteriological medium used for isolating the nine novel strains and their respective isolation source are mentioned in Table 2.3. The strains were routinely sub-cultured in R2A agar medium (Himedia, India) supplemented with 0.5% NaCl (R2AN) or R2A3 at 30°C for 4 days. For long-term preservation, bacterial cells were stored frozen at -80°C in 20% glycerol stocks as cell suspensions. 16S rRNA phylogenetic relatives; *Flavobacterium daejeonense* KACC 11422^{T}, *F. sufflavum* KCTC 52809^{T}, *F. glycines* ICMP 17618^{T}, and *F. nitratireducens* N1^{T} were used as reference type strains for comparative taxonomic studies.

Table 2.3. Isolation details of the novel bacterial strains used in this study

Strain ID	Medium composition	Source	Place and Year of isolation
L1I52^T	50% artificial seawater (HiMedia) + 0.1% malate + 3mM ACC + 1.8% purified agar	Pokkali rhizosphere	Kumbalangi (2014)
NRK F1, F15, F16, F41, F42, F47, F49, F50	R2A broth (HiMedia) + 30% natural seawater + 1.8% purified agar + 50 µg kanamycin antibiotic (R2A3K)	Pokkali root	Kumbalangi (2017)

2.2.5 Polyphasic taxonomic characterization

2.2.5.1 16S rRNA phylogenetic characterization and Multilocus sequence analysis

QIAamp DNA mini kit (Qiagen) was used to isolate the genomic DNA from the overnight-grown bacterial cells. 16S rRNA gene amplification, sequencing, and phylogenetic analysis were performed as described before (Krishnan et al., 2016). Multilocus sequence analysis (MLSA) was performed by using five housekeeping genes, namely, *gyrB, glyA, atpA, dnaK,* and *murG* as described earlier (Nicolas et al., 2008). The primer sequences of the respective housekeeping gene and their PCR amplification conditions are provided in Table 2.4. The amplified PCR products were purified using Qiagen gel extraction kit according to the manufacturer's instructions. The purified PCR products were sequenced directly using the same primer pairs used for gene amplification. Applied Biosystems 3500 DNA sequencer was used for sequencing, following the manufacturer's protocol. For the MLSA-based phylogeny, a concatenated tree comprising the above five housekeeping gene sequences was constructed following the methods described earlier (Rameshkumar et al., 2010). MEGA6.0 package was used for the phylogenetic analysis (Tamura et al., 2013). All the gene sequences generated from this study are submitted to NCBI. The GenBank accessions for the 16S rRNA gene are as follows: L1I52 (KX533956), NRK F15 (MK397018), and NRK F16 (MK397019). The GenBank accessions for the MLSA genes are listed in Table 2.5. The type strain L1I52^T is deposited in the culture collections with the following strain accessions: MTCC 12454, and KCTC 42429.

2.2.5.2 Phenotypic and biochemical characterization

Strains L1I52^T, NRK F15 and NRKF16 were routinely cultivated on R2AN or R2A3 agar at 30°C for all physiological and biochemical tests performed. The cell shape was determined as described previously (Rameshkumar et al., 2016). Motility was checked by pricking the L1I52^T cells onto the centre of R2AN or R2A3 (0.18%) soft agar plate and observed for the migration of cells for a period of 7 days. The presence of flexirubin-type pigments was

Table 2.4. The primer sequences of the five housekeeping genes and their respective PCR cycle conditions. An initial denaturation at 95°C for 5 min and a final elongation of 72°C for 8 min was followed in all PCR reactions

Gene	Primer	Primer sequence (5' – 3')	Amplification conditions
gyrB	*gyrB*F	GTTGTAATGACTAAAATTGGTG	30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, elongation at 72°C for 2 min
	*gyrB*R	CAATATCGGCATCACACAT	
glyA	*glyA*F	AAAGATAGACAAATTCACGG	30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, elongation at 72°C for 2 min
	*glyA*R	GGTGATTTATCATCAAAAGG	
atpA	*atpA*F	CTTGAAGAAGATAATGTGGG	30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min for L1I52^T, at 46°C for 90 sec for ICMP 17618^T, at 50°C for 1 min for KACC 11422^T and N1^T, elongation at 72°C for 2 min
	*atpA*R	TGTTCCAGCTACTTTTTTCAT	
dnaK	*dnaK*F	AAGGTGGAGAAATTAAAGTAGG	30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 90 sec, elongation at 72°C for 2 min
	*dnaK*R	CCACCCATAGTTTCGATACC	
murG	*murG*F	TGGCGGTACAGGAGGACATAT	35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 90 sec, elongation at 72°C for 2 min
	*murG*R	GCATTCTTGGTTTGATGGTCTTC	

Table 2.5. GenBank accessions of the MLSA gene sequences generated from this study

S. No.	Strain	*gyrB*	*glyA*	*atpA*	*dnaK*	*murG*
1	*F. pokkalii* L1I52^T	KX257376	KX257380	KX257384	KX257388	KX257392
2	*F. pokkalii* NRK F15	MK442501	MK442503	MK442505	MK442507	MK442509
3	*F. pokkalii* NRK F16	MK442502	MK442504	MK442506	MK442508	MK442510
4	*F. daejeonense* KACC 11422^T	KX1257377	KX257381	KX257385	KX257389	KX257393
5	*F. glycines* ICMP 17618^T	KX257378	KX257382	KX257386	KX257390	KX257394
6	*F. nitratireducens* N1^T	KX257379	KX257383	KX257387	KX257391	KX257395

checked by flooding the plates with 20% KOH solution. The Gram reaction, catalase and oxidase tests were tested using protocols described previously (Rameshkumar et al., 2014). Growth in different bacteriological media was tested on Luria-Bertani agar, nutrient agar, trypticase soya agar, and ZoBell marine agar. Growth at different temperatures and pH was tested as described previously except R2AN agar medium was used for the analysis (Krishnan et al., 2016). The tolerance to various NaCl concentrations (0.5, 1, 1.5, 2, 2.5, 3% w/v) was tested in R2A broth. The growth in anaerobic conditions was tested as described previously (Rameshkumar et al., 2016) . Citrate utilization, indole production, nitrate reduction, methyl red and Voges Proskauer (MR-VP) tests were carried out as mentioned earlier (Smibert, 1994). Hydrolysis of complex polymers such as starch, pectin, xylan, carboxymethyl cellulose, and chitin was checked on nutrient agar supplemented with 0.5% of each polymer and incubated for 5 days at 30°C. Hydrolysis of esculin, urea, tributyrin, DNA, arginine dihydrolase, lysine decarboxylase and utilization of different sugars (L-arabinose, cellobiose, D-glucose, lactose, D-maltose, D-mannitol, D-raffinose, L-rhamnose, sucrose, D-trehalose, D-xylose), organic acids (citric acid, malic acid, succinic acid) and solvents (ethanol, methanol) as sole carbon source and different amino acids (L-alanine, L-methionine, L-phenylalanine, L-proline, putrescine, L-threonine, L-tryptophan, L-serine, L-valine) as sole nitrogen source were performed by following methods of Krishnan et al (Krishnan et al., 2016). Antibiotic sensitivity tests for the following antibiotics (HiMedia; µg/disc): amoxyclav (30), ampicillin (10), carbenicillin (100), cefozolin (30), chloramphenicol (30), cinoxacin (100), ciprofloxacin (5), clindamycin (2), co-trimaxozole (25), doxycycline hydrochloride (30), erythromycin (15), gentamycin (10), kanamycin (30), linezolid (30), methicillin (5), nalidixic acid (30), netilin (30), ofloxacin (5), oxacillin (1) and penicillin G (10 units), polymixin B (300 units), rifampicin (5), streptomycin (10), tetracycline (30), vancomycin (30) were carried out as described previously (Rameshkumar et al., 2016) , except R2AN was used as the basal medium. Acid production from various carbohydrates was checked using the HiMedia HiCarbohydrate kit, according to the manufacturer's instructions, except that the kits were inoculated with culture suspensions made in 0.85% NaCl (w/v), and incubated at 30°C. The results were noted after 48 h of incubation.

2.2.5.3 Chemotaxonomic characterization

For cellular fatty acid analysis, the cells were grown in R2A agar for 72 hours at 30°C except *F. sufflavum* KCTC 52809^{T} was grown at 28°C because of its inability to grow at 30°C. Cells

were harvested at similar physiological age, and cellular fatty acid methyl esters (FAMEs) were prepared and identified by methods as previously described earlier (Krishnan et al., 2016; Sasser, 1990). For polar lipid analysis, L1I52^T cells were harvested at the logarithmic phase and the pellet was used for polar lipids extraction with methanol/chloroform/0.3 % sodium chloride (2:1:0.8, by vol.) as described earlier by Bligh and Dyer considering the modifications by Card (Bligh and Dyer, 1959; Card, 1973). Lipids were separated using silica gel TLC (Kieselgel 60 F254; Merck) by two-dimensional chromatography using chloroform-methanol-water (65:25:4 by vol.) in the first dimension and chloroform-acetic acid-methanol-water (40:7.5:6:2, by vol.) in the second dimension (Minnikin A' et al., 1984). The dried plates were subjected for spraying with 5 % ethanolic phosphomolybdic acid for total lipids and further characterized by spraying with ninhydrin (specific for amino groups), molybdenum blue (specific for phosphates), Dragendorff (quaternary nitrogen) or α-naphthol (specific for sugars). The respiratory quinones were extracted with chloroform/methanol (2:1, v/v) and analyzed using HPLC as described by (Komagata and Suzuki, 1987).

2.2.5.4 Genomic DNA fingerprinting

A PCR-based genomic DNA fingerprinting using ERIC (Enterobacterial Repetitive Intergenic Consensus) primer pair ERIC1R and ERIC2 was carried out to check the intra-species diversity among the nine novel isolates (Krishnan et al., 2016). Based on the difference in genomic fingerprint patterns, three strains, L1I52^T, NRK F15, and NRK F16, were selected for further comparative characterization studies

2.2.5.4 Draft genome sequencing and genomic characterization

High-quality genomic DNA of the L1I52^T strain was isolated using QIAamp DNA mini kit (Qiagen) according to the manufacturer's instruction and sequenced on Illumina HiSeq 4000 platform using NEXTFlex DNA sequencing library kit as recommended by the manufacturer. Raw reads were trimmed using the software Solexa QA followed by removing reads with quality score lower than 20 and length shorter than 50bps (Cox et al., 2010). The trimmed reads were assembled with IDBA assemble v1.1.1 using the "pre_correction" option (Peng et al., 2012). The ANI (Average Nucleotide Identity) between close genomes was calculated using JSpecies WS online program (Richter et al., 2015), AAI (Average Amino Acid Identity) using the webserver available through http://enve-omics.gatech.edu/, dDDH – In-silico DNA-DNA hybridization score through http://ggdc.dsmz.de/ggdc.php

and Tetra z-score – statistical analysis of tetra-nucleotide usage patterns, using the web-browser available through, http:// jspecies.ribohost.com/jspeciesws/ (Richter et al., 2016; Rodriguez-R and Konstantinidis, 2016). The GenBank accessions of the genomes of L1I52^{T} and its phylogenetic neighbours taken for genome-based phylogenetic analysis is listed in table 3.2 (Chapter - 3).

2.3 Results and Discussion

2.3.1 Initial insights into the metagenomic distribution in brackish rice

The three agroecosystems chosen in this research study – Pokkali, Kaipad, and Kagga- are native to saline-tolerant wild rice varieties cultivated in the coastal stretches of South India (Fig. 2.1). Despite the differences in the geographical location between the three fields, the farming practices followed in these fields are very similar. Notably, all three fields are near the estuaries where river freshwater mixes with the seawater to form brackish water. Hence, during high tides in the oceans, the brackish water from estuaries gushes into the field, creating a brackish water-logged condition in these areas, posing severe saline stress that fluctuates according to the tidal variations. The organic switch-farming practice followed in these fields for centuries makes these sites ideal for microbiome/microbiota research. The lack of fertilizer/chemical usage in these fields is believed to house an undisturbed pool of potential microbes critical for host rice growth benefit and ecosystem functioning. In all three sites, the rice is cultivated in the low-saline phase (May/June – September/October),

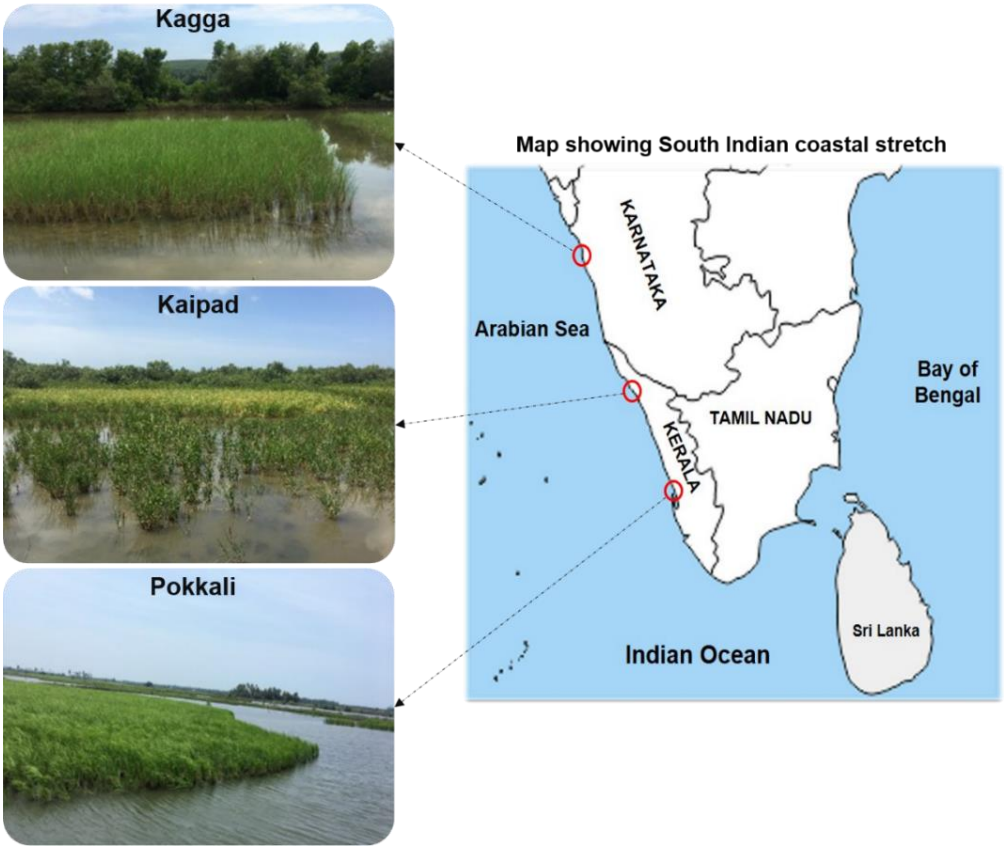


Fig. 2.1. Image depicting the map of South India, showing the geographical locations (marked as red circles) of the sampling sites in this study that is spread across the coastal stretches of Kerala, and Karnataka. The images of the respective fields during the cultivation of brackish rice varieties are also shown in the figure.

followed by shrimp/fish cultivation during the high-saline phase (November/December – April/May) of the year. After harvest, the rice straw and residues serve as the feed for prawn cultivation. On the other hand, the excreta that remains after the prawn harvest serves as the natural fertilizer for rice that augments plant growth and health. Therefore, these brackish rice fields offer a dynamic ecosystem that has the potential to be developed as research model to study the microbiome and underlying plant-microbe interaction under saline-

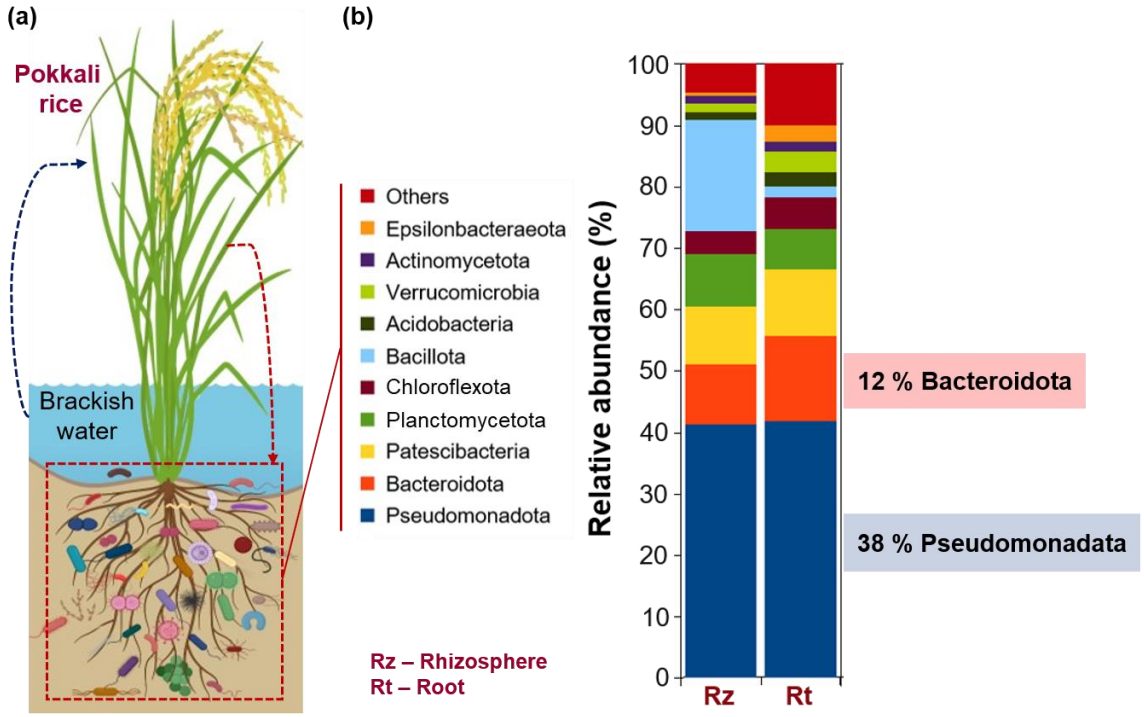


Fig. 2.2. Initial insight from the metagenomic distribution in Pokkali rice: **(a)** Illustrative figure representing the prime concept of the research investigation, which highlights the Pokkali rice growing in the brackish ecosystem. The black dotted arrow pointing upward in the figure indicates the influence of saline condition caused due to the brackish water on the plant, which sensitizes its native rhizobacterial partners to colonize for a beneficial note indicated by the red dotted arrow pointing downward. **(b)** Histogram displaying the relative abundance of phyla in the rhizosphere and root compartments of Pokkali rice obtained through 16S rRNA gene amplicon sequencing (V3 – V4 region). The most abundant phyla, Pseudomonadota and Bacteroidota are highlighted with their relative abundance values.

stressed habitats. Moreover, it is assumed that the native microbiota of these ecosystems will be evolutionary adapted to alleviate saline stress. Hence, it provides the right platform to explore the varied diversity of beneficial rhizobacterial groups that colonize and complement the host plant growth under varying brackish conditions.

Therefore, tracing the bacterial metagenomic diversity from the rhizosphere and roots of the three brackish rice varieties was one of the prime mandates of our research group. The prime research concept here was to understand how the environment i.e., brackish condition,

influences the rice plant to recruit its beneficial rhizobacterial partners, which complement their co-existence in this complex saline ecosystem (Fig. 2.2 (a)). Hence, as a start to this exploration, we performed a pilot experiment wherein the rhizosphere and root compartments of Pokkali rice variety were subjected to 16S rRNA gene amplicon sequencing (V3 – V4 region) using the Illumina MiSeq platform to gain an initial insight into the bacterial distribution. At the phylum level analysis, the phylum Pseudomonadota

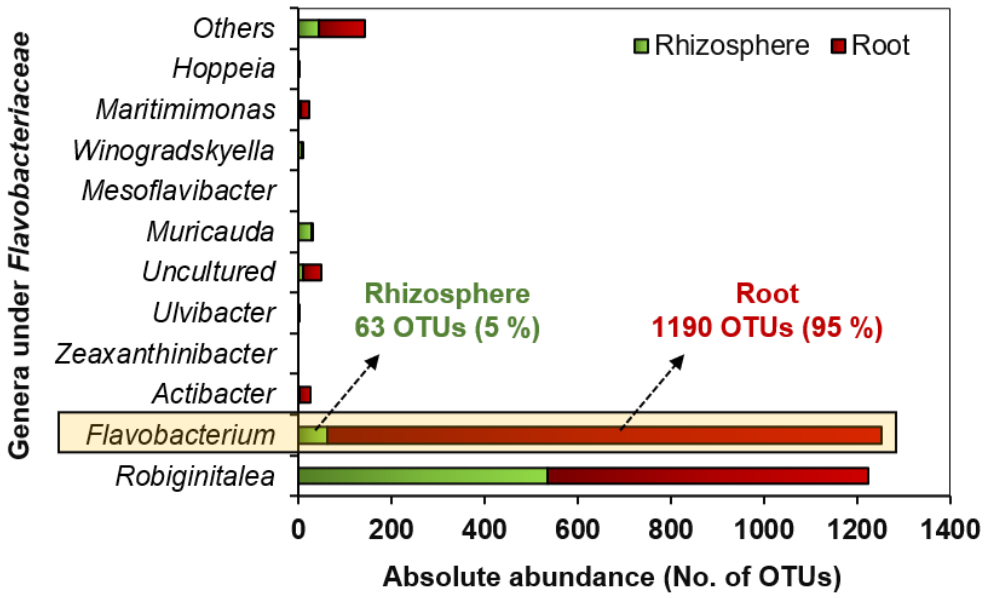


Fig. 2.3. Bar graph representing the absolute abundance of different genera under the family *Flavobacteriaceae* of phylum Bacteroidota. Genera *Flavobacterium* and *Robigintalea* constitute the dominant group. Genus *Flavobacterium* represents higher OTUs in the root compared to the rhizosphere

dominated with 38% of the total bacterial population, followed by 12% of the phylum Bacteroidota (Fig. 2.2 (b)). The subsequent dominant groups were bacterial phyla, such as Patescibacteria, Planctomycetota, and Chloroflexota (Fig. 2.2 (b)). Phylum Bacillota quantified around 15% in the rhizosphere, whereas it was less abundant in the root compartments (Fig. 2.2 (b)). However, among all the observations, the high abundance of phylum Bacteroidota, both in the rhizosphere and root compartments, caught our interest (Fig. 2 (b)). Hence, we further explored the OTU distribution at different bacterial hierarchies (family and genus). An intriguing observation about the bacterial distribution within the family *Flavobacteriaceae* caught our interest during the analysis. This was the high enrichment of OTUs corresponding to the genus *Flavobacterium* in the root compartments of Pokkali rice (Fig. 2.3). The genera *Flavobacterium* and *Robiginitalea* constituted the significant proportion of the family *Flavobacteriaceae* and both these genera constituted almost similar levels of absolute abundance (ranging between 1200 to 1300

OTUs) (Fig. 2.3). However, the intriguing finding in the analysis was the significantly high enrichment of *Flavobacterium* OTUs in root compartments (95%) compared to rhizosphere compartment (5%) which is almost 19 times higher (Fig. 2.3). In contrast, the abundance of genus *Robiginitalea* between the rhizosphere and root compartments were significant, but not enriched highly as observed in genus *Flavobacterium* (Fig. 2.3). Altogether, the preliminary metagenomic distribution-based observation summarizes the fact that genus *Flavobacterium* members establish and maintains a tight association with its host plant, Pokkali rice. Thus, the Flavobacterium members are believed to play a critical role in its host growth relative to the ecological context.

2.3.2 Metagenomic distribution in Pokkali, Kaipad, and Kagga

Based on these initial leads, we performed the 16S rRNA gene amplicon sequencing (V3 – V4 region) for all three brackish rice varieties (Pokkali, Kaipad, and Kagga) of South India using the Illumina MiSeq platform. For this, high-quality total DNA was isolated from the rhizosphere and root compartments of Pokkali, Kaipad, and Kagga rice varieties (Fig. 2.4). In this study, we used 341F/785R 16S rRNA gene primer pair (best recommended for soil and plant-associated bacteriome studies) to amplify the V3 – V4 hypervariable region (Thijs et al., 2017). Since the metagenomic analysis of brackish rice varieties constitutes the major research component of my colleague's PhD research, the raw reads and related parameters of the metagenome datasets are included in the AcSIR research thesis (Kumar, S, AcSIR PhD Thesis, 2024; to be submitted). Moreover, in this research study, the major focus has been on the metagenomic distribution of phylum Bacteriodota and genus *Flavobacterium*.

2.3.2.1 Phylum level rhizobacterial distribution in brackish rice

The diversity and abundance of the rhizobacterial population distributed in the rhizosphere and root compartments of brackish rice varieties were investigated in this study. Initially, we analyzed the taxonomic composition of the rhizobacterial microbiome at the phylum level, where we found Pseudomonadota to be the most dominant phylum, constituting around 32 – 42% of the total population residing in the rhizosphere and root compartments of all three brackish rice varieties. Following this, the dominant group is the phylum Bacteroidota abundant phyla varied between the regions (rhizosphere and root) and rice varieties with Patescibacteria, Planctomycetota, and Chloroflexota as the dominant bacterial groups. Following this, the population distribution was seen among phyla such as Bacillota, Acidobacteria Verrucomicrobia, Epsilonbacteraeota, and Actinomycetota. ranging from 10

– 16% of the total phyla proportion (Fig. 2.4). The proportion of other All these phyla were distributed across the compartments of all rice varieties in more or less similar abundance patterns except for Bacillota and Verrucomicrobia, which showed significant variations between the rhizosphere and root compartments of Pokkali rice (Fig. 2.4). However, our interest caught majorly on the dominance of phylum Bacteroidota in the brackish rice metagenomes.

(a) Phylum Bacteroidota distribution in brackish rice metagenomes

The dominance of phylum Pseudomonadota in the metagenomes of brackish rice varieties was not unusual, as many plant microbiomes studied from diverse habitats have reported their high abundance (Mendes et al., 2013). However, the position of phylum Bacteroidota as the second dominant phylum in the metagenomes of all brackish rice varieties investigated in this research study, irrespective of their distinct geographical locations, was intriguing. Therefore, we traced back to earlier plant microbiome studies to understand the distribution and abundance of the phylum Bacteroidota in plants. Despite the plant varieties

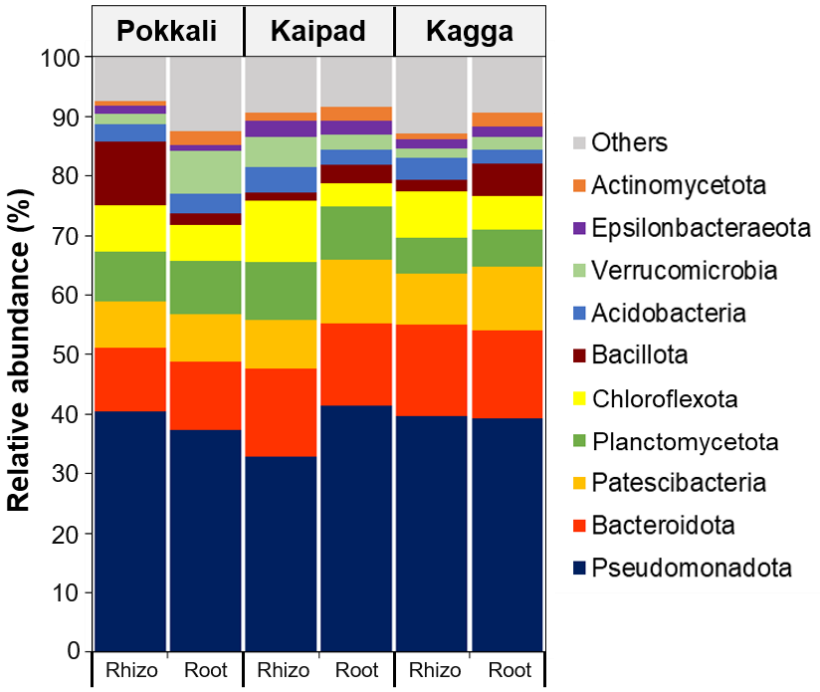


Fig. 2.4. Histograms representing the relative abundance (%) of different phyla residing in the rhizosphere (Rhizo) and root (Root) compartments of brackish rice varieties: Pokkali, Kaipad, and Kagga.

and geographical locations, most of the metagenomic datasets published to date reported the dominance of phyla such as Pseudomonadota, Bacillota, Bacteroidota, and Actinomycetota in the plant microbiomes (Mendes et al., 2013). However, among the dominant groups, the

information on plant-associated Bacteroidota members is very meagre compared to their counterparts in animal and human guts, investigated in detail (Pan et al., 2023).

(b) Varied abundance of phylum Bacteroidota in brackish and terrestrial rice metagenomes

A plant's microbiome largely depends on the geographical location, soil, and host genotype; if it is a crop, the cultivation practice followed for years also matters (Edwards et al., 2015). Interestingly, all these aspects governing the microbiome distribution are new in the case of the sampling sites chosen in this research study, i.e., the brackish agroecosystem cultivating saline-tolerant native rice varieties. These unexplored habitats in a terrestrial-marine juncture are expected to harbor a vast wealth of potential and novel microbiota with unique host-growth influential and eco-adaptive traits. In support of this, we have cultured and characterized several novel and potential brackish-adapted rhizobacterial strains capable of influencing host growth under brackish conditions (Krishnan et al., 2016, 2017, 2018; Menon et al., 2019; Rameshkumar et al., 2016). Further, the metagenome-based studies expanded our knowledge about the uncultured population of the brackish rice varieties. Hence, to know how variedly the microbiomes of brackish rice varieties are distributed (especially on the distribution of phylum Bacteroidota), we compared the brackish rice metagenomes generated in this study with the terrestrial rice metagenomic datasets available in NCBI, generated from the studies by Edwards et al, 2015 (Edwards et al., 2015). Despite being one of the best-cited research papers for rice microbiome, Edwards et al, 2015 analyzed the microbiome distribution along three root niches; rhizosphere, rhizoplane, and endosphere, which provides comprehensive data for comparative metagenomic analysis, and hence the rationale for choosing this dataset for comparative research (Edwards et al., 2015). Therefore, we compared the phylum-level taxonomic distribution in the root compartments of terrestrial and brackish rice metagenomes (Fig. 2.5). Phylum Pseudomonadota was observed to be the most dominant phylum in both datasets, but their abundance varied between the terrestrial and brackish datasets. The relative abundance of phylum Pseudomonadota varied between 40 – 60% in terrestrial rice metagenomes, whereas in brackish rice metagenomes, it ranged between 32 – 42% (Fig. 2.5). Conversely, the abundance of phylum Bacteroidota constituted only 2 – 4% of the total bacterial population in terrestrial rice metagenomes against 10 – 16% in brackish rice metagenomes, which is ~5 times higher (Fig. 2.5). However, the second dominant phylum in terrestrial rice metagenomes was Chloroflexota which was the fifth dominant in the brackish rice metagenome (Fig. 2.5). Even though a high variation was observed in the Bacteroidota

abundance between the terrestrial and brackish rice metagenomes, Edwards et al have reported the enrichment of Bacteroidota members in the rhizocompartments (endosphere, rhizoplane, and rhizosphere) of the terrestrial rice compared to the bulk soil (Edwards et al., 2015). However, in this study, the bacteriome distribution of rhizoplane (mentioned as root) and rhizosphere regions of brackish rice varieties are analyzed. Despite the variations observed in the abundance of Bacteroidota members in terrestrial and brackish rice, their specific enrichment in the rhizocompartments indicates the tight association between this bacterial group and its plant host. Moreover, it reflects the host-association lifestyle of Bacteroidota members, which are capable of breaking down complex polysaccharides such as cellulose, pectin, xylan, etc., available in the roots thought to be mediated by the action of Carbohydrate-active enzymes (CAZymes) encoded in their genome (Grondin et al., 2017).

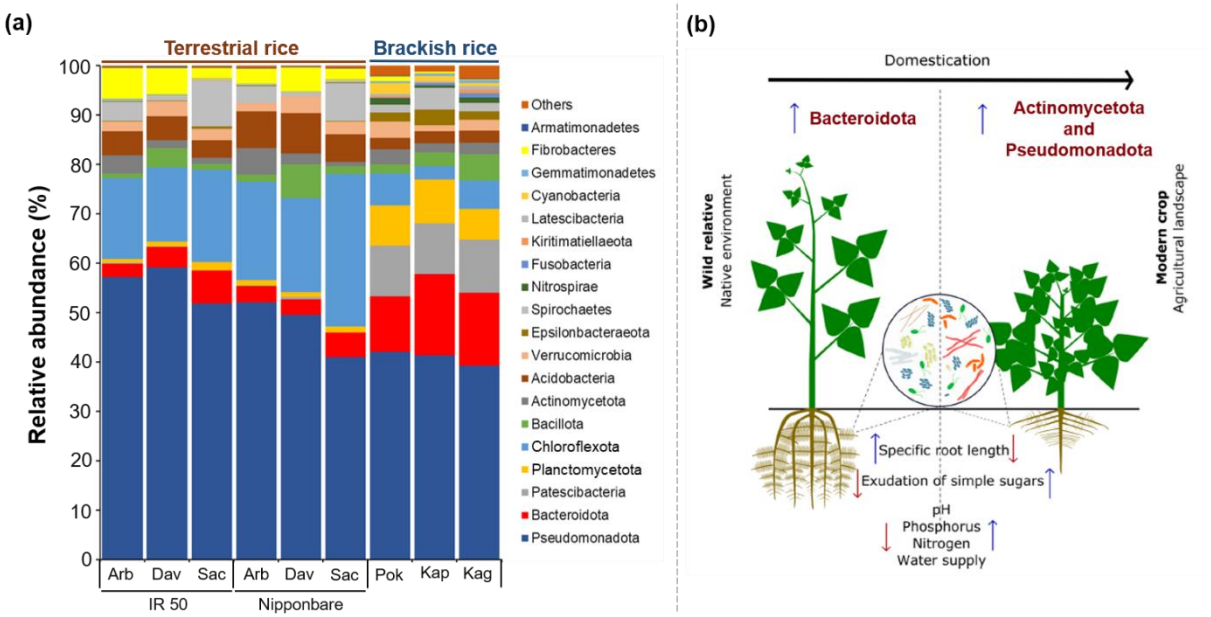


Fig. 2.5. Phylum Bacteroidota (represented in orange colour) are more abundant in wild rive varieties compared to domesticated cultivars: **(a)** Histogram comparing the relative abundance (%) of different phyla distributed along the root compartments of terrestrial rice varieties (IR50 and Nipponbare), and brackish rice varieties (Pokkali, Kaipad, and Kagga), **(b)** Image from Pérez Jaramillo *et al* depicting the higher abundance of Bacteroidota members in wild plant relative compared to its domesticated counterparts.

In 2017, an interesting research work by Pérez Jaramillo et al., revealed a higher abundance of phylum Bacteroidota in the rhizosphere microbiome of the wild common bean (*Phaseolus vulgaris*) compared to its modern cultivar that is dominated by members of phylum Pseudomonadota and Actinomycetota (Pérez-Jaramillo et al., 2018). Complementing this,

earlier metagenomic studies have claimed the compositional changes in the root microbiomes of domesticated plant species of *Arabidopsis*, barley, lettuce, and sugar beet compared to their wild counterparts (Bulgarelli et al., 2015; Cardinale et al., 2015; Schlaeppi et al., 2014; Zachow et al., 2014). Based on these leads and to validate further if plant domestication causes shifts in root microbiomes, Pérez Jaramillo *et al*. 2018 performed a meta-analysis of the root microbiomes of various wild crop plants and their domesticated relatives (Pérez-Jaramillo et al., 2018). During this study, Pérez Jaramillo *et al.* claimed the higher abundance of phylum Bacteroidota members in the roots of wild varieties of barley,

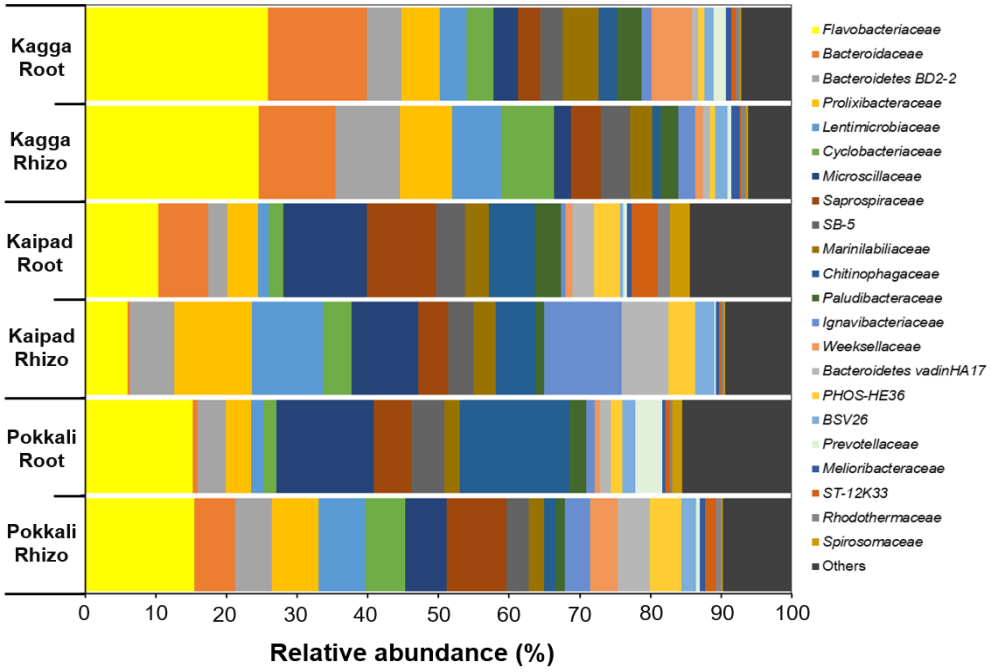


Fig. 2.6. Histogram graph displaying the relative abundance of OTUs distributed across different families within the phylum Bacteroidota.

lettuce, common bean, and *Cardamine hirsuta* contrasting to their domesticated counterparts and *Arabidopsis*, respectively (Pérez-Jaramillo et al., 2018). However, information on the abundance of phylum Bacteroidota in rice varieties was not analyzed or discussed in the meta-analysis study carried out by Pérez Jaramillo *et al*. In this context, the metagenomic analysis of the brackish rice varieties discussed in this study remains highly relevant. Furthermore, it is very important to note that the rice varieties used in our metagenomic study are wild rice cultivars grown in coastal stretches of South India through organic switch farming practice. Hence, it very well correlates with the findings from Pérez Jaramillo *et al,* where they claim a higher abundance of Bacteroidota members in the wild relatives of plants. Thus, the comparative metagenomic analysis shows that Bacteroidota members are

more abundant in brackish rice roots than terrestrial rice roots. One of the primary reasons for this might be the wild cultivar-driven enrichment of Bacteroidota, as identified earlier. However, other influencing factors are yet to be discovered. This preliminary evidence also correlates with the hypothesis proposed by Pérez Jaramillo *et al* i.e., the altered plant physiology and root exudation caused due to crop domestication is assumed to be the driving factor in the enrichment of phylum Bacteroidota members in the wild rice microbiomes. However, more experimental evidence is essential to validate this hypothesis. Despite the dominance of phylum Bacteroidota in the plant microbiomes, very little information exists on their role in plant growth and health. However, very few studies have reported that certain members of phylum Bacteroidota, especially strains belonging to the genus *Flavobacterium* and *Chryseobacterium* can promote plant growth and disease protection (Carrión et al., 2019.; Kwak et al., 2018; Nishioka et al., 2019). Nevertheless, minimal experimental evidence exists to support these claims.

Next, we estimated the relative abundance of different families within the phylum Bacteroidota. The family *Flavobacteriaceae* (represented in yellow colour) was observed to be the dominant family in Pokkali and Kagga samples constituting about 15 – 25% of the total population whereas, in Kaipad samples, the abundance of family *Flavobacteriaceae* was relatively less (Fig. 2.6). Since our major interest was to analyze the distribution of the genus *Flavobacterium,* we estimated the taxonomic composition of different genera within the family *Flavobacteriaceae.*

2.3.2.2 Genus *Flavobacterium* enriches in brackish rice roots

The bacterial distribution at the genus level within the family *Flavobacteriaceae* detected the presence of 10 genera namely, *Actibacter, Flavobacterium*, *Hoppeia, Maritimimonas, Mesoflavibacter, Muricauda, Robiginitalea, Ulvibacter, Wingogradskyella,* and, *Zeaxanthinibacter* distributed along the rhizosphere and root compartments of three rice varieties (Fig. 2.7). Among these, two genera namely, *Robiginitalea,* and *Flavobacterium* dominated the population by constituting a major proportion (ranging from 81 – 98% of the total population) within the family *Flavobacteriaceae.* Fig. 2.7 displays the heat map representing the absolute abundance of OTUs corresponding to different genera residing in the rhizosphere and roots of brackish rice. As evident from the heat map, the absolute abundance of *Flavobacterium* OTUs was higher in the root compartments compared to the rhizosphere across all rice varieties. Interestingly, this observation of enriched *Flavobacterium* OTUs in rice roots was unique compared to the most abundant genus,

Robiginitalea, which was higher in the rhizosphere compartments of Pokkali and Kagga rice varieities (Fig. 2.7).

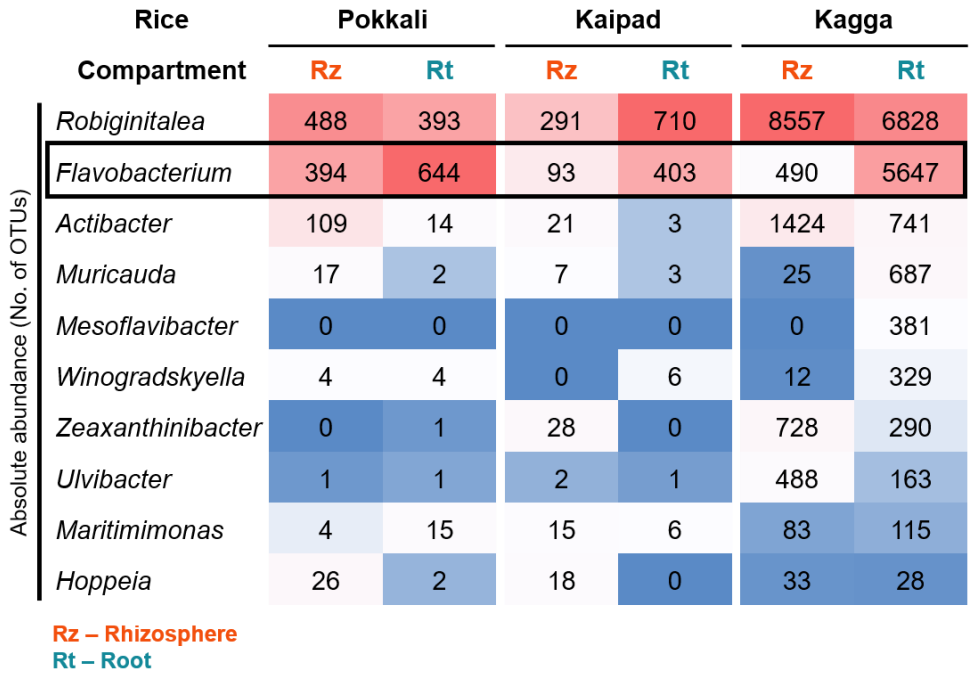


Fig. 2.7. Heat map displaying the absolute abundance of OTUs distributed along different genera within the family *Flavobacteriaceae*. The OTUs corresponding to genus *Flavobacterium* is highlighted in a black box.

Finally, to have a basic understanding of the OTU designation at the species level within the genus *Flavobacterium*, we mapped the 16S rRNA gene sequence of the cultured *Flavobacterium* strains available in the public database to the metagenomic datasets derived from the rhizosphere and root compartments of brackish rice varieties. OTUs with >99% identity and a length ranging from 400 – 426 bp were taken for species-level mapping, and the OTUs not fitting into these criteria were removed. Also, it is important to note that the OTUs corresponding to non-cultured or novel *Flavobacterium* strains will not be detected as the mapping is performed with the only cultured *Flavobacterium* strains available in LPSN (List of Prokaryotic Names with Standing in Nomenclature) during this analysis (Parte et al., 2020). The brackish rice metagenome OTUs were mainly mapped to the following species of *Flavobacterium* genus: *F. aquaticum* JC164, *F. aquaticum* ARSA-111, *F. Cucumis* R-2, *F. cheonhonense* ARSA-15, *F. rakeshii* FCS-5, *F. sediminis* MEBiC07310, *F. shanxiense* YF-2, *F. lacunae* AHQ-46, *F. inkyongensei* IMCC27201, *F. akiainvivens* IK, *F. luticoti* xz20, *F. brevivitae* TTM-43, *F. oceanosedimentum* ATCC 31317, *F. ceti* 454-2, *F. nitratireducens* N1, *F. keumense* K3R-10, *F. haoranii* LQY-7, *F. humicola* UCM-46, *F. eburneum* SA31, *F. anhuiense* D3, *F. lindanitolerans* IP-10, *F. terrae* R2A1-13, *F.*

yanchengense hg, *F. urocaniciphilum* YIT, *F. dongtanense* LW30, *F. seoulense* EM1321, *F. glycines* Gm-149, *F. piscinae* ICH-30, *F. limi* THG-AG6.4, *F. stagni* WWJ-16.

To summarize this section, we observed a significantly higher abundance of genus *Flavobacteirum* OTUs in the roots compared to the rhizosphere regions of brackish rice varieties. This unique observation firmly hints at the tight association between the genus *Flavobacterium* and its host, Pokkali rice, which needs further investigation. To gain hypothetical reasoning for the high abundance of *Flavobacterium* OTUs in the rice roots, we analyzed the abundance of genus *Flavobacterium* members in the metagenomic datasets of terrestrial rice varieties available in NCBI (BioProject ID: PRJDB14860, PRJNA255789, PRJNA864623, PRJNA850879) and compared against the *Flavobacterium* abundance in the metagenomic datasets of brackish rice varieties (PRJNA970257, Pokkali; PRJNA970551, Kaipad; PRJNA970256, Kagga) generated from this study. Interestingly, we observed a reduced abundance of *Flavobacterium* OTUs in the roots of terrestrial rice varieties compared to the brackish rice metagenome, indicating that the brackish condition might

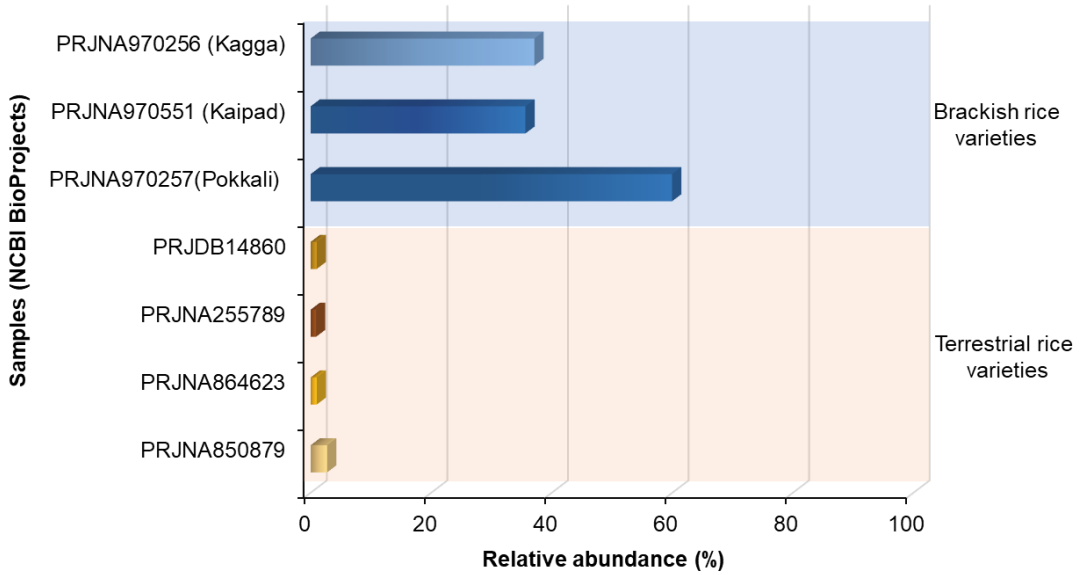


Fig. 2.8. Genus *Flavobacterium* is highly abundant in brackish rice varieties compared to terrestrial rice varieties. Bar graph comparing the relative abundance (%) of genus *Flavobacterium* in terrestrial rice varieties (data taken from NCBI BioProjects) and brackish rice varieties (data from this study).

critically contribute to the enrichment of *Flavobacterium* members in the rice roots. Thus, the rhizobacterial distribution analyzed through the comparative metagenomic approach possibly hints at the crucial role of the ecosystem in enriching *Flavobacterium* members in the brackish-environment-associated rice roots. Therefore, to study, if the surrounding environment. i.e., the brackish condition (natural seawater-amended condition) has any

influence on the enrichment of the *Flavobacterium* in rice roots; a customized experiment was designed wherein the bacterial community of soil was analyzed after growing seedlings in different conditions provided in the gnotobiotic setup. Since we observed a higher abundance of *Flavobacterium* OTUs in the Pokkali rhizosphere, we chose the Pokkali rhizosphere as the soil inoculum for the soil-microcosm experiment (Fig. 2.9). Here, the major challenge to be addressed was to know if *Flavobacterium* dominance influenced by wild/domesticated cultivar-based variation similar to phylum Bacteroidota, or any other factors like environment or salinity is responsible for it. Hence, a soil-microcosm-based bacteriome analysis was designed to address this challenge as a proof of concept (PoC) experiment.

2.3.3 Pokkali soil-microcosm-based bacteriome analysis in rice roots

It is highly challenging to comprehensively understand the microbial ecology of a natural ecosystem, which is dynamic and fluctuating due to the influence of diverse biotic and abiotic factors. However, PoC experiments might help in extrapolation-based hypothetical

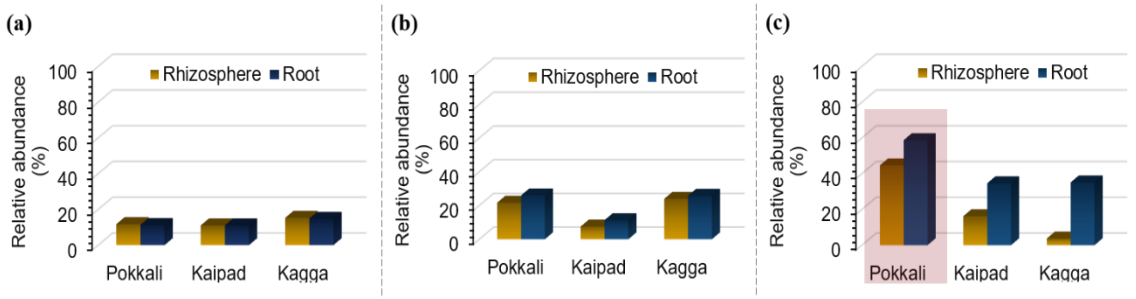


Fig. 2.9. Bar graphs representing the relative abundance (%) of **(a)** phylum Bacteroidota, **(b)** family *Flavobacteriaceae*, and **(c)** genus *Flavobacterium* across the rhizosphere and root compartments of brackish rice varieties: Pokkali, Kaipad, and Kagga.

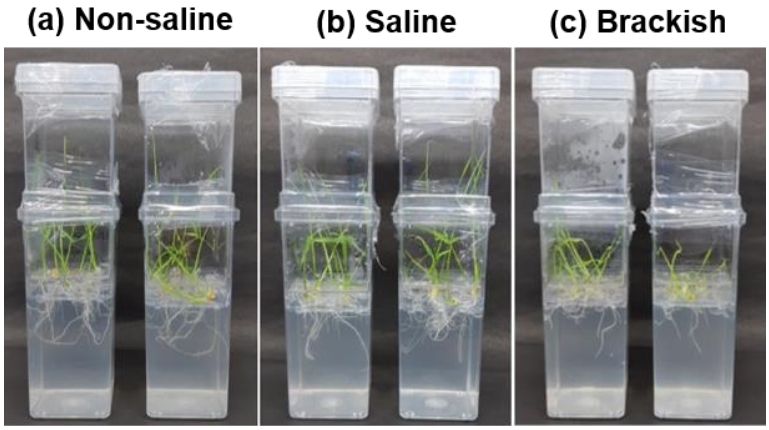


Fig. 2.10. Image representing the customized phytajar setup used for soil-microcosm and hydroponic-based gnotobiotic setup used to perform the bacteriome analysis under different conditions: **(a)** non-saline (0mM NaCl), **(b)** saline (100 mM NaCl), and **(c)** brackish (30% NSW).

reasoning for answering certain complex aspects of microbial ecology. Hence, the Pokkali soil-microcosm-based bacteriome analysis attempted in this study is a PoC experiment. The primary aim of this PoC experiment was to analyze the variation in the rice root colonized bacteriome when the rice seedlings were grown under three different conditions: non-saline (0 mM NaCl), saline (100 mM NaCl), and brackish (30% NSW) when inoculated with a common soil suspension (Fig. 2.10; detailed in method section). The prime interest of our research group was to know if salinity, i.e., the brackish condition, is the influencing factor in the bacterial recruitment process of host plants. Hence, in this study, we attempted to understand if the brackish-mimicking condition (30% NSW condition) drives the enrichment or depletion of any bacterial group compared to the non-brackish conditions (0 mM and 100 mM NaCl). Focusing towards the subject of my research, I was keen to know if the brackish-mimicking condition (30% NSW amended condition) can alter the abundance of genus *Flavobacterium* members in Pokkali seedling roots. Since we wanted to monitor the variation in the abundance of bacterial communities over time across the different conditions, we isolated the total DNA from the seedling's roots at two-time points: the 7th and 14th-day post-inoculation Further, we proceeded with 16S rRNA gene amplicon sequencing targeting the V5 – V6 region of the 16S rRNA gene (see method section for details) . As mentioned earlier, the detailed metagenomic statistics of the experiment will not be detailed in the thesis as the major objective was to analyze the distribution within phylum Bacteriodota (with genus *Flavobacterium* as the major target). Briefly, the alpha diversity analysis between the samples with respective time points indicates that both richness and evenness were higher for brackish conditions, followed by saline and non-saline conditions (Sunitha Kumari, AcSIR PhD Thesis, July 2024; data unpublished to date). The total bacterial diversity was also observed to be increased on the 14th dpi compared to the 7th dpi within each condition (Sunitha Kumari, AcSIR PhD Thesis, July 2024; data unpublished to date). Altogether, it was observed that the bacterial groups prefer to colonize along the roots more in the brackish condition than non-brackish conditions. Further, from the beta-diversity analysis, it was observed that the sample points of brackish condition clustered well separately, indicating their varied species combination relative to the non-brackish conditions (Sunitha Kumari, AcSIR PhD Thesis, July 2024; data unpublished to date).

2.3.3.1 Taxonomic composition of the soil-microcosm-based condition-driven Pokkali seedling bacteriome

The taxonomic composition analysis at the phylum level revealed the presence of 6 major bacterial phyla – Actinomycetota, Bacillota, Bacteroidota, Cyanobacteria, Planctomycetota,

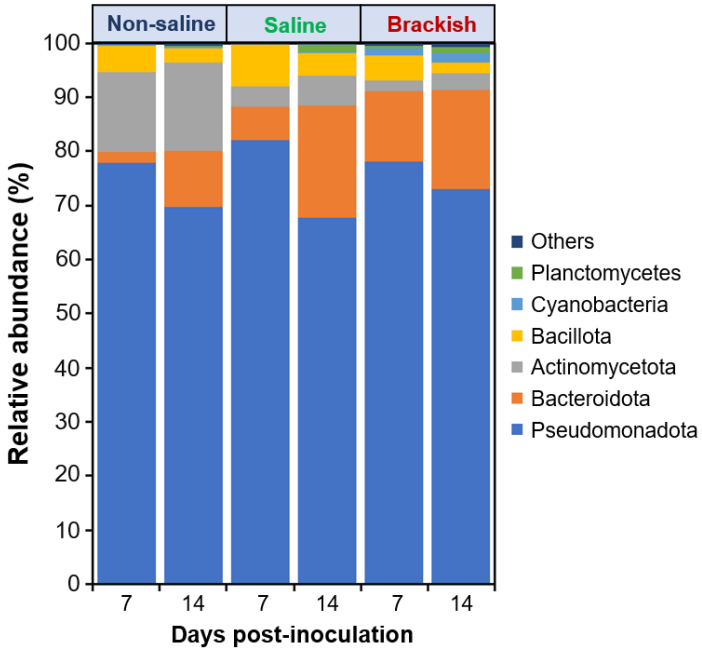


Fig. 2.11. Histogram representing the relative abundance of different phyla distributed along the roots of Pokkali rice seedlings grown under different conditions: non-saline, saline (100 mM NaCl), and brackish (30% natural seawater) conditions.

and Pseudomonadota distributed along the roots of Pokkali rice seedlings grown under different conditions. Even though there was no significant difference in the bacterial composition between the 7th dpi and 14th dpi of each condition, the abundance of certain phyla varied (Fig. 2.11). Most importantly, the OTUs corresponding to phylum Bacteroidota increased from 7th to 14th dpi against a dip observed in the relative abundance of phylum Pseudomonadota (Fig. 2.11). The increase in the phylum Bacteroidota abundance possibly hints at the ability of these bacterial groups to colonize and gradually establish along the host roots over time. Next, we checked the family-level distribution within the phylum Bacteroidota, followed by estimating the absolute abundance of genus *Flavobacterium* OTUs under different conditions at varied time points. Contrasting to the phylum level distribution, a huge variation was observed in the taxonomic composition at the family level between conditions (Fig. 2.12 (a)). In non-saline condition, the population was dominated by the family *Chitinophagaceae* (on both 7th and 14th dpi) (Fig. 2.12 (a)). However, on the

14th dpi, a slight decrease in the overall abundance of *Chitinophagaceae* accommodated two other families: *Prolixibacteriaceae* and *Paludibacteriaceae* into the pool (Fig. 2.12 (a)). In the saline condition (100 mM NaCl), the population was dominated by *Prolixibacteriaceae* and *Paludibacteriaceae* followed by *Weeksellaceae* (Fig. 2.12 (a)). Interestingly, the population in the brackish condition was majorly dominated by the *Flavobacteriaceae*,

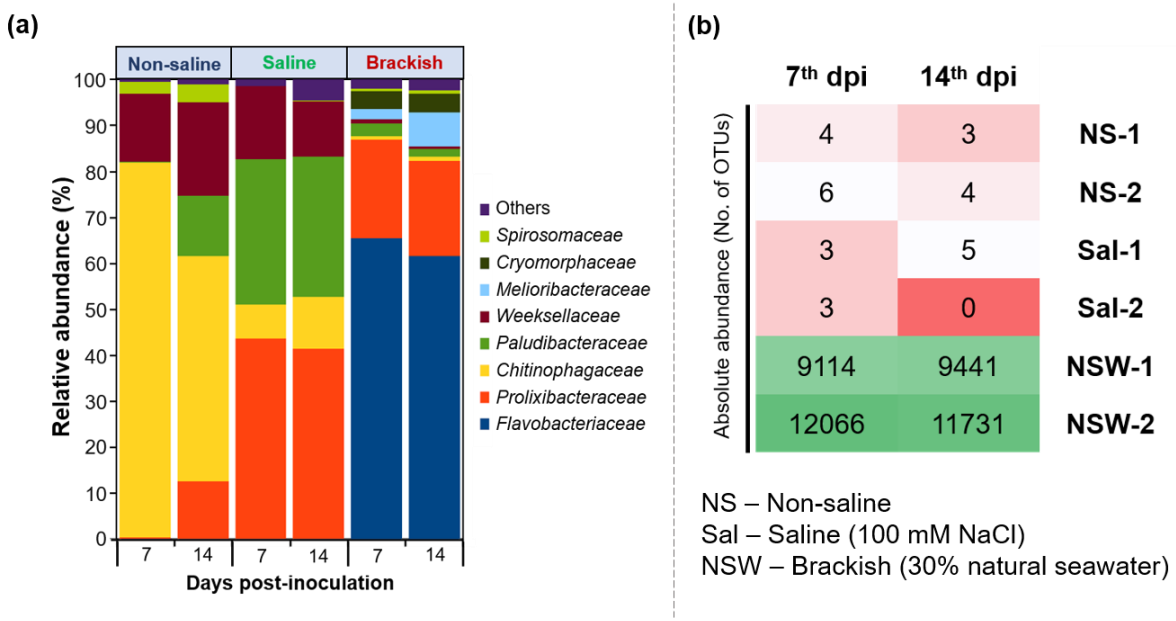


Fig. 2.12. Brackish condition enriches family *Flavobacteriaceae* and genus *Flavobacterium* members in the roots of Pokkali rice seedling: **(a)** Histogram comparing the distribution and relative abundance (%) of different families within the phylum Bacteroidota colonized along the roots of Pokkali seedlings grown in different conditions, **(b)** Heat map displaying the absolute abundance of genus *Flavobacterium* OTUs quantified at varied time points across different conditions.

which constituted around 60 – 65 % (Fig. 2.12 (a)). The dominance of *Flavobacteriaceae* OTUs exclusively in the rice seedlings grown in brackish conditions indicates that the environment provides a selective enrichment for these bacterial groups in the host colonization. Further, as expected, we could quantify a relatively high abundance of OTUs corresponding to the genus *Flavobacterium* in the brackish condition, as indicated in Fig. 2.12 (b). The absolute abundance values listed in Fig. 2.12 (b) clearly represent the extremely high dominance of genus *Flavobacterium* members, which proves a heavy enrichment of this bacterial group on rice roots. This unique observation is assumed to be driven by the 30% NSW offering a brackish condition to the host, i.e., the Pokkali seedlings, which, in response to the brackish condition, is observed to recruitf *Flavobacterium* as its major rhizobacterial partner from family *Flavobacteriaceae.* Next, to understand if the dominance of *Flavobacteriaceae* members alters the distribution of other prominent bacterial families, we estimated the relative abundance of overall family-level distribution

across different conditions at varied time points.

Interestingly, the presence of the family *Flavobacteriaceae* in the brackish condition had a profound effect on the overall family-level distribution compared to the non-brackish conditions. This includes an increased abundance of *Pseudomonadaceae* encoding OTUs in brackish conditions compared to other conditions (Fig. 2.13). Apart from the family *Pseudomonadaceae*, OTUs corresponding to the family *Rhodobactericeae* were also

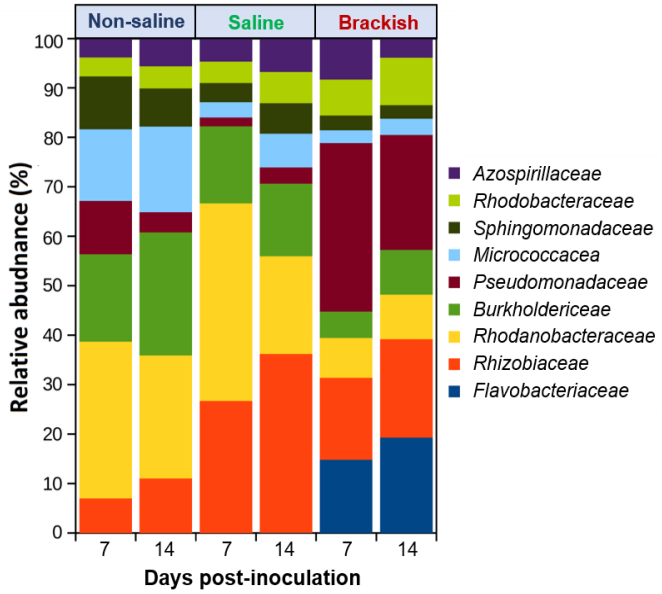


Fig. 2.13. Histogram representing the relative abundance of different bacterial families distributed along the roots of Pokkali rice seedlings grown under different conditions: non-saline, saline (100 mM NaCl), and brackish (30% natural seawater) conditions.

slightly enriched in the brackish conditions (Fig. 2.13). It is very important to note that certain members of the family *Pseudomonadaceae,* especially those belonging to the genus *Pseudomonas,* are known to harbour multi-faceted plant beneficial traits (Pandey and Gupta, 2020; Singh et al., 2022; Zboralski and Filion, 2023). Moreover, we assume that the enrichment of *Pseudomonadaceae* OTUs is largely influenced by the presence of *Flavobactericeae* OTUs in the brackish condition. The hypothesis is that the members of the *Flavobacteriaceae* family coded with an array of CAZymes are capable of breaking down complex sugars/polysaccharides. These CAZyme coding *Flavobacteriaceae* members facilitate the breakdown of complex sugars into simple sugars, the primary carbon source for *Pseudomonadaceae* members. Hence, it is believed that the enrichment of *Flavobactericeae* members dominated by the genus *Flavobacterium* can drive a carbon turnover in the root environments, attracting other beneficial bacterial groups towards the roots. Subsequently, the attracted phytobeneficial bacterial groups can enhance plant growth. Thus, from this bacteriome analysis, we assume that apart from the direct benefits,

Flavobacterium members can induce indirect benefits by attracting other rhizobacteria to impact the host plant growth synergistically.

In summary, the significant observations from Pokkali soil microcosm-based microbiome analysis confirm the influence of brackish condition (30% NSW) in the enrichment of genus *Flavobacterium* along the roots of Pokkali seedlings. Hence, the PoC experiment has proven that the environment is critical to the bacterial dynamics in the plant rhizosphere/root biomes.

2.3.4. Selection and condition-based targeted culturing of brackish rice native *Flavobacterium* strains

The analysis of metagenomic datasets generated from the brackish rice varieties and soil-microcosm-based experiments portrayed the distribution and abundance of the less-explored genus *Flavobacterium* in the brackish agroecosystems. However, to further study their host-association capabilities and related eco-physiological traits, cultured representatives of *Flavobacterium* strains native to the brackish agroecosystems are essential. Interestingly, two significant findings revealed through the metagenomic analysis detailed in the above sections guided us in designing the isolation methods with increased culturing probability of *Flavobacterium* strains. Firstly, the genus *Flavobacterium* OTUs was highly abundant in the root compartments of brackish rice plants compared to the rhizosphere, indicating an unknown tight association between the *Flavobacterium* members and native brackish rice varieties. Secondly, the brackish condition (30% NSW) enriched the *Flavobacterium* population in Pokkali rice seedling roots compared to non-brackish conditions. Based on these leads, we attempted to isolate novel and potent rhizobacterial strains of the genus *Flavobacterium* through unique isolation strategies and customized media combinations (detailed in the method section). Nevertheless, isolating bacterial strains specific to a genus from a sample inhabited by thousands of rhizobacterial groups poses several challenges. But, as mentioned earlier, the findings from the metagenomic data led us to choose rhizosphere-removed root compartments for selectively isolating *Flavobacterium* strains tightly associated with the Pokkali rice. Further, we amended 30% NSW in the isolation medium to offer a brackish-mimicking condition for the increased probability of culturing native brackish-adaptive *Flavobacterium* strains with phytobeneficial properties. Furthermore, based on the report published by Flint K. P., kanamycin antibiotic (50 µg/ml) is an effective agent for the selective enumeration of *Flavobacterium* isolates from water (Flint K. P, 1985). Hence, we used 50 µg/ml kanamycin antibiotic to reduce the noise of

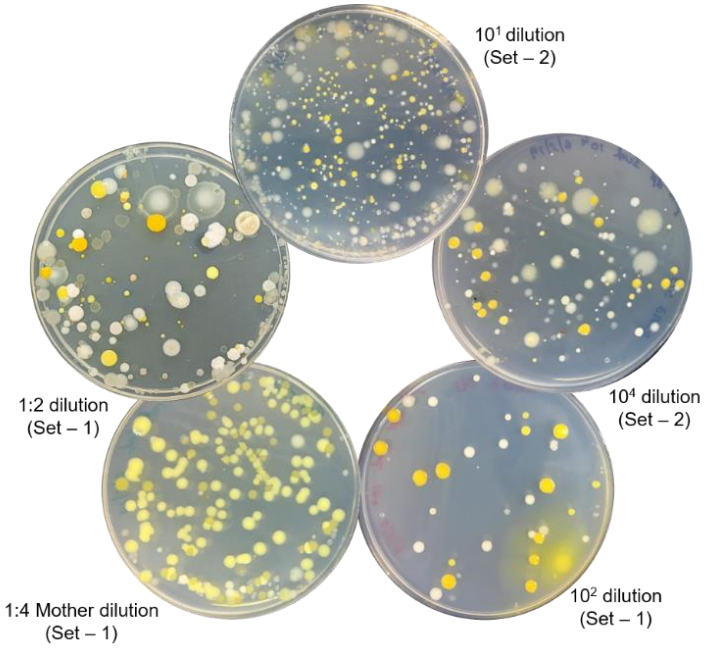


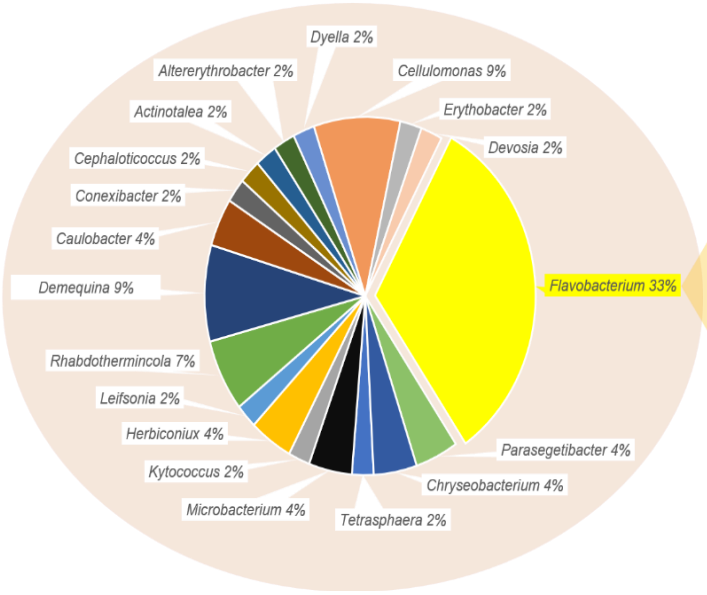
Fig: 2.14. Dilution plate images representing the colony diversity appeared in customized isolation medium amended with 30% NSW and kanamycin antibiotic which recovered maximum yellow-pigmented colonies possibly indicating the strains belonging to genus *Flavobacterium*. The dilution of the resepective isolation plates is indicated in the image near to each plate image.

other bacterial groups during the *Flavobacterium* targeted isolation. However, we also used non-antibiotic plates to evaluate the effect of amending antibiotics in selective isolation of *Flavobacterium* strains. The characteristic yellow-orange pigmentation of *Flavobacterium* strains guided us in the easy picking of the desired colonies from the diverse pool of isolates with varied colony morphotypes (Fig. 2.14). Even though our focus was to isolate the members of the genus *Flavobacterium*, we also picked the non-yellow/orange pigmented colonies from the dilution spread plates to know if the selection-based culturing can provide an advantage in the culturing of genus *Flavobacterium* members. Hence, we picked and sorted yellow-pigmented colonies and other varying colony morphotypes and subjected them to further screening. The isolates were identified through partial 16S rRNA gene-based sequencing and phylogenetic analysis. This indicated that selection-based screening aids in recovering more *Flavobacterium* strains from complex samples, such as plant rhizosphere/roots. The relative 16S rRNA gene sequence similarity % of the identified isolates within the total population are displayed in Fig. 2.15.

The relative proportion of genus *Flavobacterium* corresponding isolates from the 30% NSW/Kanamycin-amended medium is 33%, which is very high when compared to the 2% recovery of *Flavobacterium* isolates from the non-NSW/Kan antibiotic-amended medium. Interestingly, the seawater (30% NSW) and kanamycin antibiotic-amended isolation plates

recovered more yellow-pigmented colonies than the non-selection plates (isolation plates without kanamycin antibiotic).

Next, we performed a nearly full-length sequencing of the 16S rRNA gene using a combination of universal primers targeting different regions to obtain a gene sequence length of ~1.5 kb. Thus, based on the full-length 16S rRNA gene-based phylogeny, we confirmed the isolation of four novel species under the genus *Flavobacterium* (Fig. 2.15). In addition



S. No.	Strain ID	Year	Similarity
1	L1I52	2014	< 98 %
2	NRK F1	2017	
3	NRK F15		
4	NRK F16		
5	NRK F41		
6	NRK F42		
7	NRK F47		
8	NRK F49		
9	NRK F50		
10	NRK1	2017	< 98 %
11	NRK F7	2017	< 98.5 %
12	NRK F30		
13	NRK F39		
14	NRK F44		
15	NRK F10	2017	< 97 %
16	NRK F24		

Fig: 2.15. Pie chart representing the relative abundance (%) distribution of the different genus identified from the isolated pool of strains through selection based-culturing highlighting the novel strains and their partial 16S rRNA gene % similarity.

to the identification of novel species strains, the isolated pool contained two strains (NRK P23, and NRK P43) that could be probable novel genera under phylum *Bacteroidota* (data not shown). Interestingly, 8 strains among the isolated pool were equivalent to a *Flavobacterium* strain designated L1I52^T isolated in 2014 from the rhizosphere of Pokkali rice (Fig. 2.15). Moreover, during our preliminary *in-planta* screening experiments to check the host growth-influencing strains among the isolated pool, the L1I52^T strain was among the strains that positively affected host plant growth, especially on root-related parameters. This gained our interest, and we assumed these strains to be native to the Pokkali rice ecosystem as they were re-isolated after three years from the same site. In addition, it was also worth noting that the current isolation was done from the root compartments, avoiding the rhizosphere region, and we could recover more (eight numbers) equivalent strains of L1I52^T. Further, to confirm whether the 8 newly isolated L1I52^T equivalent strains are clones, we performed a genomic DNA fingerprinting.

2.3.5 Taxonomy characterization of L1I52^T and its equivalent strains

The preliminary analysis done through 16S rRNA gene-based sequencing and phylogeny revealed the identity of the newly isolated eight strains – NRK F1, NRK F15, NRK F16,

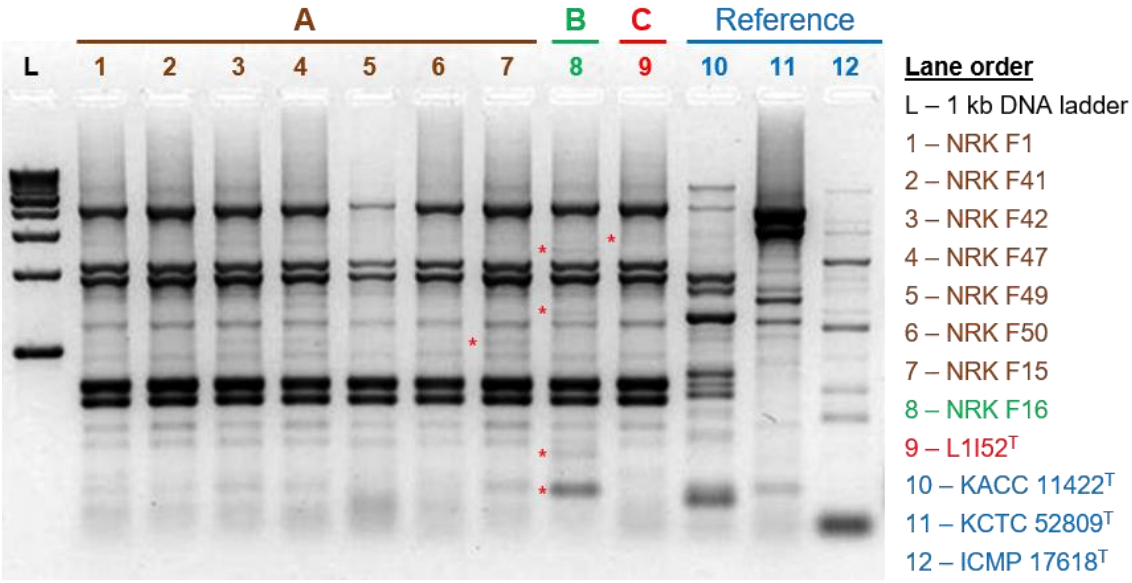


Fig. 2.16. Genomic fingerprint of the nine *Flavobacterium* strains using ERIC primers. Red asterisk indicates the regions with the difference in the banding patterns among the strains

NRK F41, NRK F42, NRK F47, NRK F49, and NRK F50 (isolated in 2017) to be equivalent to L1I52^T (isolated in 2014). However, genomic fingerprinting performed using ERIC (enterobacterial repetitive intergenic consensus) primers confirmed that the strains are not clonal in origin. Three different DNA fingerprint patterns were obtained: pattern A shared by 7 strains (NRK F1, NRK F41, NRK F42, NRK F47, NRK F47, NRK F49, NRK F50 NRK F15), pattern B for NRK F16 and fingerprint pattern C for L1I52^T strains (Fig. 2.16). Therefore, the three strains, L1I52^T, NRK F15, and NRK F16, with distinct genomic fingerprint patterns, were selected for further characterization (Fig. 2.17).

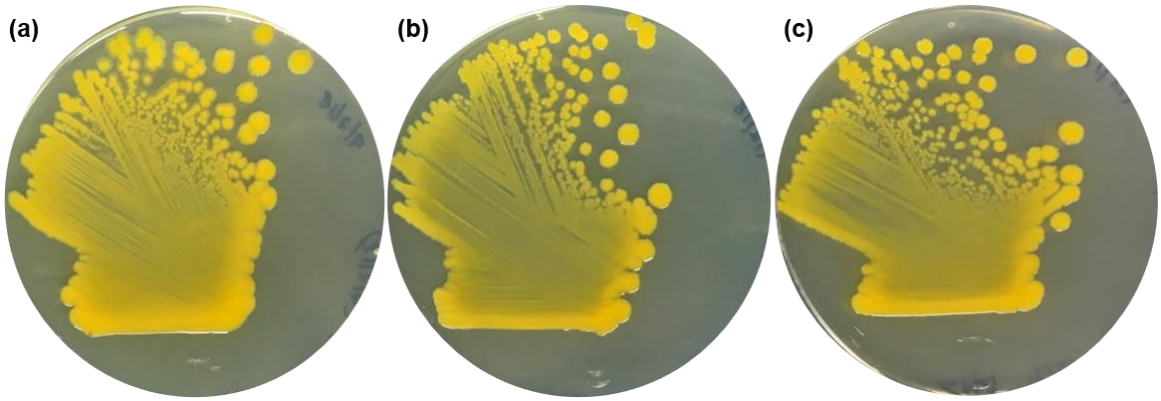


Fig. 2.17. Images showing the matured colonies of fully grown novel *Flavobacterium* strains: **(a)** NRK F15, **(b)** NRK F16, and **(c)** L1522^T in CYG3 agar

2.3.5.1 16S rRNA gene-based phylogenetic analysis

The 16S rRNA gene analysis placed the three strains L1I52^T, NRKF 15 and NRK F16 within the genus *Flavobacterium* sharing the highest 16S rRNA gene sequence similarity to *Flavobacterium sufflavum* BBQ-12^T (97.97%), *F. daejeonense* GH1-10^T (97.64%) and *F. glycines* Gm-149^T (97.00%). The 16S rRNA gene sequence similarities between the novel strains and with other *Flavobacterium* type strains were less than 97%. A 16S rRNA gene

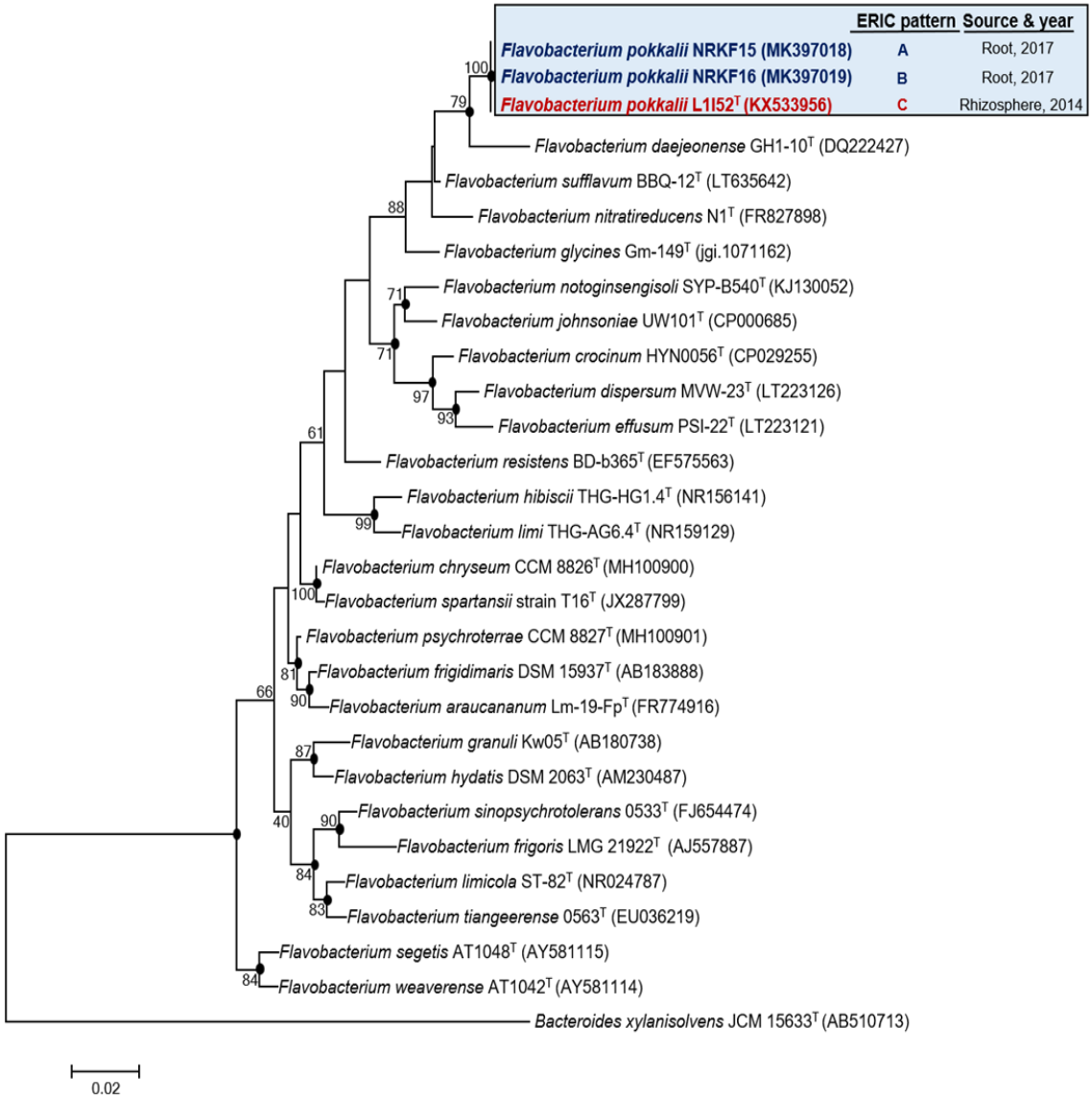


Fig. 2.18. Maximum likelihood tree constructed using 16S rRNA nucleotide sequences showing the phylogenetic position of three novel *Flavobacterium* strains (L1I52^T, NRK F15, and NRK F16) within the genus *Flavobacterium*. The percentages shown at each branch point represent bootstrap values derived from 1000 replications and values more than 60% are indicated. Bar, 0.02 represents positions per nucleotide position. The outgroup used for this analysis was *Bacteroides xylanisolvens* JCM 15633^T. Dark closed circles at each node indicate similar grouping observed in other tree-constructing methods (neighbour joining and minimum evolution).

phylogenetic tree was constructed using three different algorithms (neighboring joining, maximum parsimony and maximum likelihood). Irrespective of the algorithms, the three novel strains clustered stably with *F. daejeonense* GH1-10^T, supported with higher bootstrap values (Fig. 2.18). Despite having higher 16S rRNA gene sequence similarity with *F. sufflavum* BBQ-12^T, the stable clustering of novel *Flavobacterium* strains with *F.* daejeonenseGH1-10^T strain indicate their tight phylogenetic relationship. This also could be correlated to their ecological habitat as *F. daejeonense* GH1-10^T is from greenhouse soil whereas *F. sufflavum* BBQ-12^T is from a freshwater source (Chen et al., 2019). Further, the phylogenetic relationship of the three novel strains L1I52^T, NRKF 15, and NRK F16 was studied in detail by performing MLSA.

2.3.5.2 Multilocus Sequence analysis (MLSA)

For MLSA, five housekeeping genes: *gyrB* (DNA gyrase subunit B), *glyA* (serine hydroxymethyl transferase), *atpA* (ATP synthase subunit alpha), *dnaK* (chaperone protein), and *murG* (N-acetylglucosaminyl transferase) which were shown previously to give higher taxonomic resolution in the genus *Flavobacterium* were selected and used (Mun et al., 2013; Nicolas et al., 2008).

Table 2.6. Multilocus sequence analysis of strain L1I52^T and the type strains of related *Flavobacterium* species based on the individual and concatenated sequences (4598 bp) of five housekeeping genes: *gyrB* (1139 bp), *glyA* (922 bp), *atpA* 9822 bp), *dnaK* (946 bp), and *murG* (776 bp)

Phylogenetic neighbours of L1I52^T	**Pairwise nucleotide sequence similarity (%) with L1I52^T**					
	gyrB	***glyA***	***atpA***	***dnaK***	***murG***	**Concatenated**
F. daejeonense KACC 11422^T	92.36	92.52	94.87	95.54	93.14	93.74
F. sufflavum BBQ-12^T	85.05	92.91	91.74	91.97	87.19	92.91
F. glycines ICMP 17618^T	91.04	93.60	92.67	93.10	86.93	91.75
F. nitriatireducens N1^T	87.97	90.13	94.87	93.52	83.18	90.08

The sequence similarities obtained through the MLSA of the housekeeping genes were identical between the three strains (L1I52^T, NRK F15, and NRK F16), supporting the fact that the three strains belong to the same species. However, they shared low levels of gene sequence similarities ranging from 85 to 92.36% for *gyrB*, 90 to 92.52% for *glyA*, 91 to

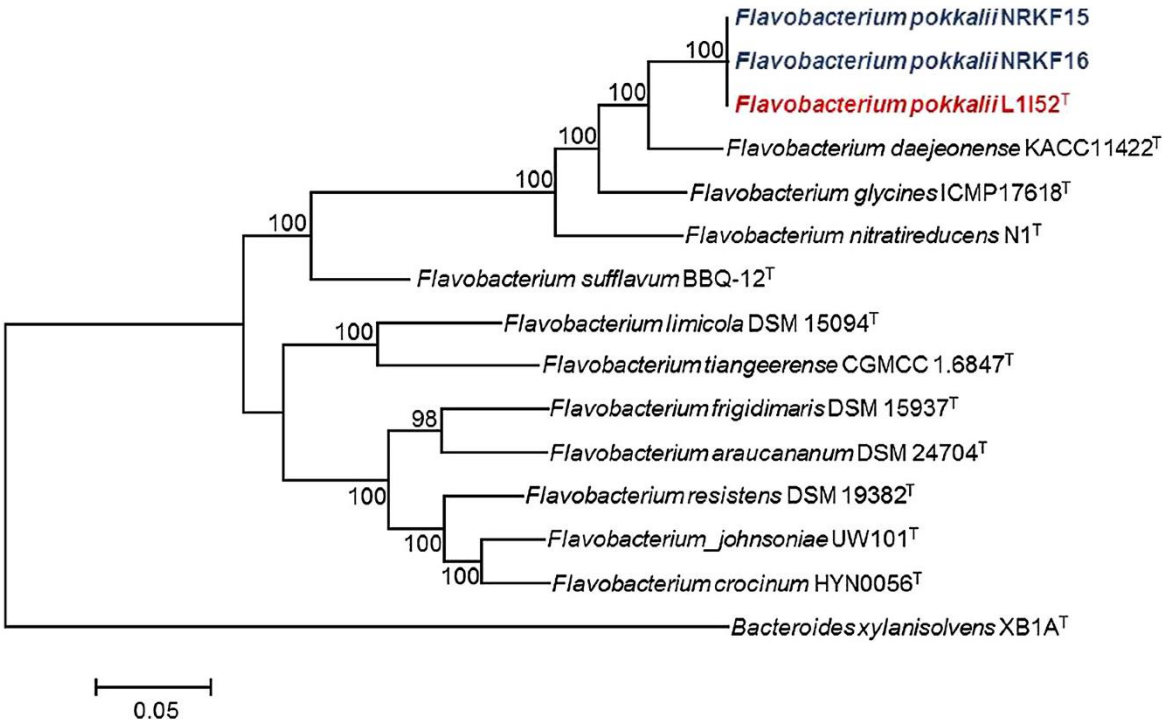


Fig. 2.19. Phylogenetic tree constructed using the concatenated gene sequences (4598 bp) of five MLSA genes (*gyrB, glyA, atpA, dnaK, murG*) displaying the position of novel *Flavobacterium* strains within the *Flavobacteirum* genus. The percentages shown at each branching point represent bootstrap values derived from 1000 replications and values morethan 60% are indicated. Bar, 0.05 represents positions per nucleotide position. The outgroup used for this analysis was *Bacteroides xylanisolvens* XB1A^T. Dark circles at each nodes shows similar grouping seen in other tree constructing methods (neighbour joining and minimum evolution).

94.89% for *atpA*, 91 to 95.24% for *dnaK* and 83 to 93.14% for *murG* with its most closely related *Flavobacterium* type strains (Table 2.6), indicating that strains L1I52^T, NRKF 15 and NRK F16 may belong to a novel *Flavobacterium* species. A maximum likelihood tree was constructed using the concatenated sequences of the five housekeeping genes, and this result showed similar phylogenetic clustering as observed in 16S rRNA gene phylogenetic analysis where the strains L1I52^T, NRKF 15 and NRK F16 formed distinct branch but paired stably with *F. daejeonense* GH1-10^T (Fig. 2.19).

2.3.5.3 Genome-based phylogeny

L1I52^T was identified as the representative strain for accurate taxonomic placement of the three strains, and its draft genome was sequenced. In-silico genome-based comparison showed that the L1I52^T genome shared an average nucleotide identity (ANI) value of 80.2 – 92.7%, average amino acid identity (AAI) values of 83.1 – 94.0%, and digital DNA-DNA hybridization (dDDH) values of 24.0 – 52.6% with its most closely related *Flavobacterium* type strains (Table 2.7; Fig. 2.20). These species percentage values are well below the

threshold values of ANI (<95%), AAI (<95%) and dDDH (<70%) for bacterial species discrimination, supporting the fact that L1I52^T represents a novel species within the genus *Flavobacterium* (Auch et al., 2010; Goris et al., 2007; Konstantinidis and Tiedje, 2007).

Table 2.7. Genome sequence similarity scores between *Flavobacterium* strain L1I52^T and genome sequences of its phylogenetic neighbours with sequenced genomes. The percentage similarities and scores were below the standard species cut-off threshold values.
ANIb – Average nucleotide identity; AAI – Amino acid identity; dDDH – In-silico DNA-DNA hybridization score

Strains	ANIb (%)	AAI (%)	dDDH (%)	Tetra z-score
F. daejeonense DSM17708^T	92.7	94.0	52.6	0.998
F. glycines Gm-149^T	81.8	85.2	25.9	0.986
F. sufflavum BBQ-12^T	80.2	83.1	24.0	0.966

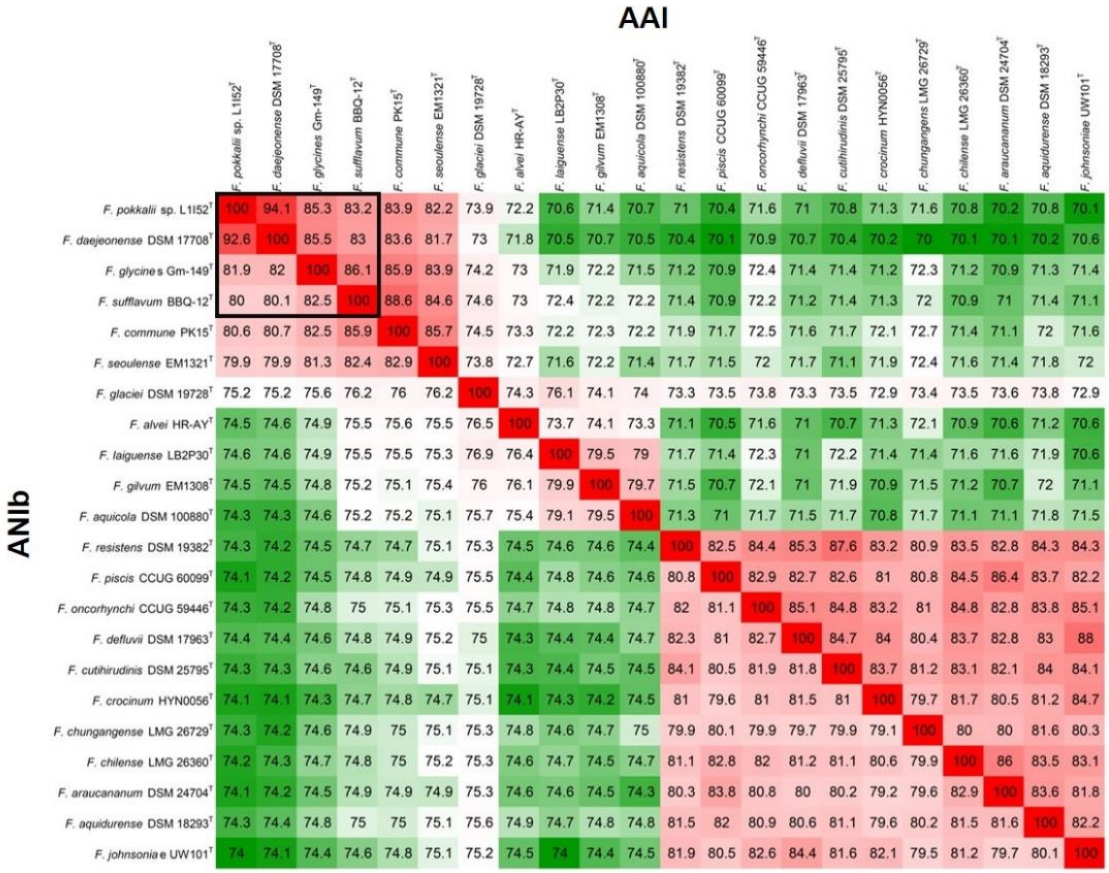


Fig. 2.20. Heatmap displaying the ANI and AAI matrix of L1I52^T and its closely related strains within genus *Flavobacterium.* Heat map shows the pairwise comparisons of the ANI and AAI between each pair of genomes and the numbers in each cell represent ANI and AAI values of respective genomes pairs. The black box represents the comparative ANI and AAI values of L1I52^T and its nearest phylogenetic neighbours.

2.3.5.4 Phenotypic characterization

Three strains, L1I52^T, NRKF 15, and NRKF16, shared very similar phenotypic properties among each other and revealed main characters as described for the genus *Flavobacterium*; cells are Gram-staining negative rods with strict aerobic lifestyle, colonies are deep-yellow pigmented, negative for flexirubin-type pigments, gliding motility was observed, and catalase and oxidase tests were positive (Bernardet et al., 2002). The phenotypic traits that distinguish the three novel strains from their closely related phylogenetic relatives are given in Table 2.8 (Fig. 2.21). Further details on the physiological and biochemical characterization are provided in the species description of the strain L1I52^T.

Table 2.8. Phenotypic characters differentiating L1I52^T from its closest phylogenetic neighbours. Strains: 1 – *Flavobacterium pokkali* L1I52^T, 2 – *F. daejeonense* KACC 11422^T, 3 – *F. sufflavum* BBQ-12^T, 4 – *F. glycines* ICMP 17618^T, 5 – *F. nitratireducens* N1^T. +, positive; -, negative; R, resistant; S, sensitive. All data were obtained in this study.

Phenotypic Characters	**1**	**2**	**3**	**4**	**5**
Isolation source	Pokkali rhizosphere	Greenhouse soil	Freshwater	Soybean rhizosphere	Marine water
DNA G + C content (mol%)	34.9	35.0	34.2	35.6	36.3
Growth in ZoBell marine agar	+	-	-	-	-
Growth in 2% NaCl	+	-	-	-	-
Growth at 37°C	+	+	-	+	+
Growth in R2A broth at 30°C	+	+	-	+	+
Esculin hydrolysis	-	+	+	-	-
Utilization of raffinose	-	+	-	+	+
Acid production from:					
Melibiose	-	+	-	-	-
Raffinose	-	+	-	-	-
Mannose	+	-	-	-	+
Antibiotic sensitivity test					
Penicillin G (P)	S	R	R	R	S
Vancomycin (Va)	R	S	S	R	R

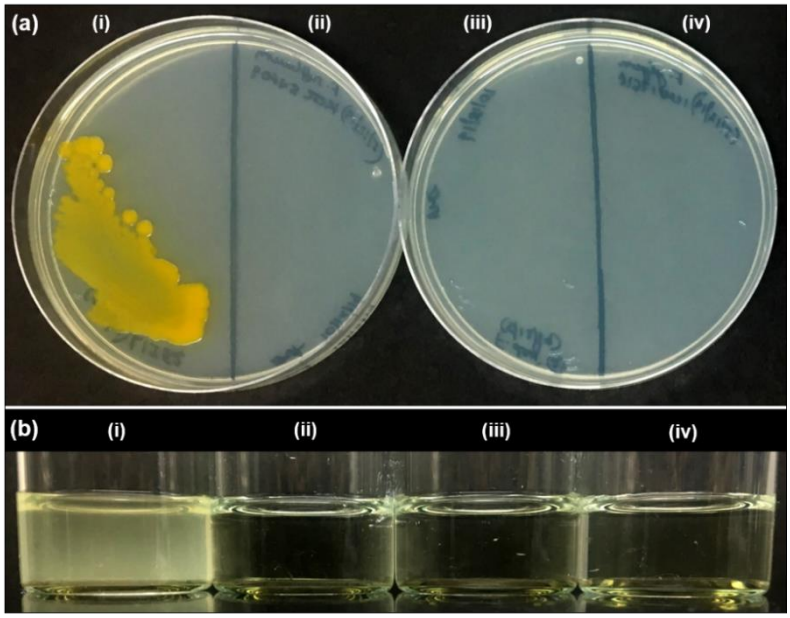


Fig. 2.21. Unique phenotypic characters that differentiate *F. pokkalii* L1I52^T from its phylogenetic neighbours: **(a)** Growth in Zobell marine agar, and **(b)** Growth in R2A broth amended with 2% NaCl. **(i)** *F. pokkalii* L1I52^T, **(ii)** *F. sufflavum* KCTC 52809^T, **(iii)** *F. glycines* ICMP 17618^T, and **(iv)** *F. daejeonense* KACC 11422^T

2.3.5.5 Chemotaxonomy characterization

The major fatty acids of L1I52^T were iso-$C_{15:0}$ (17.10%), summed feature 3($C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH) (24.11%), $C_{16:0}$ (8.95%), anteiso-$C_{15:0}$ (6.90%), and $C_{16:0}$ 3-OH (6.70%), which is also the most abundant fatty acid reported in most members of the genus *Flavobacterium* (Bernardet et al., 2002). A detailed comparison of cellular fatty acid methyl esters of L1I52^T and its closest phylogenetic neighbours are shown in Table 2.9. The polar lipid profile of L1I52^T revealed the presence

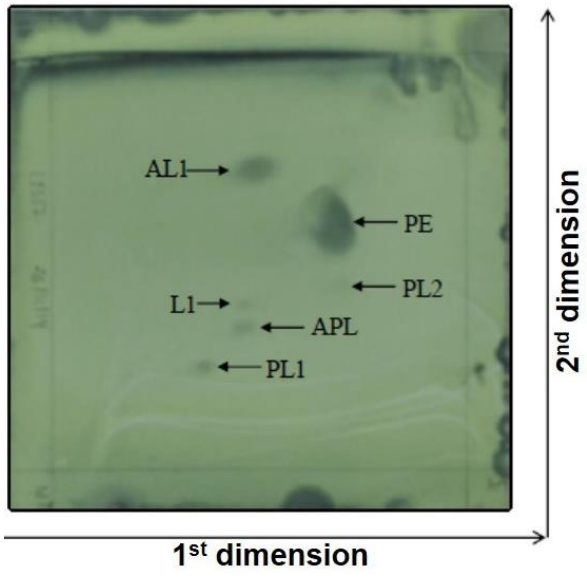


Fig. 2.22. Total polar lipid profile of strain L1I52^T obtained after two-dimensional thin layer chromatography method. The total lipid spots were detected by spraying with 5% ethanoic phosphomolybdic and further characterized by spraying with ninhydrin (specific for amino group), molybdenum blue (specific for phosphates), dragendorff's (quaternary nitrogen) or α-naphthol (specific for sugars). PE, phosphatidylethanolamine; PL, phospholipid; AL, aminolipid; APL, unidentified aminophospholipid.

of phosphatidylethanolamine (PE), a major polar lipid observed in other described members of the genus *Flavobacterium* (Bernardet et al., 2002; Dong et al., 2013). In addition, an unknown aminolipid (AL1) and few unidentified lipids such as phospholipids (PL1 and PL2), lipid (L1), and aminophospholipid (APL) were also observed. (Fig. 2.22)**.** The major respiratory quinone was identified as MK 6.

Table 2.9. Whole cell fatty acid composition of strain L1I52^T and its nearest phylogenetic neighbours. Taxa: 1, L1I52^T; 2, *F. daejeonense* KACC 11422^T; 3, *F. sufflavum* BBQ-12^T 4, *F. glycines* ICMP 17618^T. Only fatty acid percentages amounting to 0.8 % or higher are shown. All data were obtained in this study. Summed features are groups of two or three fatty acids that could not be separated by GC with the MIDI system. Summed feature 1 comprised iso- C15:1/C13:0 3−OH; summed feature 2 comprised iso-C16:1/C14:0 3−OH; summed feature 3 comprised C16:1ω7c/C16:1ω6c and summed feature 9 comprised C16:0 10-methyl.

Fatty acids (%)	1	2	3	4
12:0	0.81	1.11	-	0.86
13:0 iso	-	1.01	TR	1.73
13:0	2.63	1.99	-	2.01
14:0 iso	4.29	4.25	-	3.09
14:0	1.92	1.52	2.72	2.52
15:0 iso	17.10	17.27	20.11	16.30
15:0 anteiso	6.90	6.58	8.77	9.47
15:1 w6c	3.50	7.39	1.15	9.37
16:0 iso	-	1.00	0.84	1.06
16:0	8.95	7.92	9.32	6.20
15:0 iso 3OH	5.88	7.46	5.62	7.94
15:0 2OH	-	-	TR	0.89
17:1 w8c	2.31	1.63	TR	0.95
17:1 w6c	3.67	3.32	1.45	7.66
16:0 3OH	6.70	6.10	6.26	8.21
Summed Feature 1	4.39	7.33	-	-
Summed Feature 2	1.51	1.22	1.18	1.64
Summed Feature 3	24.11	19.96	24.70	18.75
Summed Feature 9	1.47	1.02	1.23	-

2.3.5.6 Species description of L1I52^T

Flavobacterium pokkalii (pok.ka'li.i. N.L. gen. n. pokkalii of Pokkali (a variety of rice) Cells are Gram-negative rods with a strict aerobic lifestyle and are motile by gliding (Fig. 2.23). Colonies are yellow-pigmented/deep-yellow-pigmented, convex, up to 1.5 mm in diameter, and circular with wavy margins after 4 days of incubation in R2AN (R2A + 0.5 %

NaCl), R2A3 (R2A + 30% NSW), CYG3 media at 30 °C. Growth occurs in Luria bertani agar, nutrient agar, TSA agar medium, and Zobell marine agar medium. No growth was observed in the Zobell marine broth medium. Growth occurs at 18 – 37 °C and pH 6.0 – 8.0; optimal growth at 28 – 30 °C and pH 7.0. No growth was observed at 4 °C and 42 °C. Tolerates NaCl up to 3 % (optimal at 0.5 % NaCl). Catalase and oxidase tests are positive. Starch was hydrolyzed, whereas DNA, esculin, gelatin, carboxymethyl cellulose, xylan, and pectin was not hydrolyzed. Able to grow in minimal mineral medium containing plant-based polymers: xylan and pectin as sole carbon source. Negative reactions were observed for arginine dihydrolase, lysine decarboxylase, MR-VP and Indole test. Produces acid from xylose, maltose, fructose, dextrose, galactose, sucrose, L-arabinose, mannose, inulin and D-arabinose whereas lactose, raffinose, trehalose, melibiose, sodium gluconate, glycerol, salicin pulcitol, inositol, sorbitol, mannitol, adonitol, arabitol, erythritol, α-methyl D-glucoside, rhamnose, cellobiose, melezitose, α-methyl D-mannoside, xylitol and sorbose showed negative acid production (Himedia HiCarbohydrate kit). ONPG, citrate and malonate are not utilized as well. Assimilates L-arabinose, glucose, D-fructose, D-maltose, L-rhamnose, sucrose and D-xylose as sole carbon source, weak for D-galactose and D-mannose, but not for D-cellobiose, lactose, D-mannitol, D-raffinose, D-melibiose, D-trehalose and D-sorbitol. Amino acids such as L-methionine, L-phenylalanine, putrescine, L-threonine, L-tryptophan and L-valine were assimilated as sole nitrogen source, but L-alanine, L-proline and L-serine were not assimilated. The type strain is resistant to the following antibiotics; Kanamycin (30), vancomycin (30), oxacillin (1), methicillin (5) and sensitive to: ampicillin (10), gentamycin (10), tetracycline (30), chloramphenicol (30), streptomycin (10), penicillin G (10 units), polymyxin B (300), rifampicin (5), ofloxacin (5), clindamycin (2), carbenicillin (100), ciprofloxacin (5), erythromycin (15), amoxyclav, co-trimaxozole, cefozolin, doxycycline hydrochloride (30) , netillin (30), linezolid (30), nalidixic acid (30), cinoxacin (100).

The major fatty acids were iso-C15:0 (17.10 %), summed feature 3(C16:1 ω7c and/or iso-C15:0 2−OH) (24.11 %), C16:0 (8.95 %), anteiso-C15:0 (6.90 %), and C16:0 3−OH (6.70 %) and the complete cellular fatty acid profile is given in Table 2.8. Other chemotaxonomy properties such as polar lipid profile and respiratory quinone are mentioned in the main text. The type strain can promote Pokkali rice growth and encodes several gene features in their genome that are potentially involved in plant-microbe interactions.

The type strain L1I52^T (=MTCC 12454^T =KCTC 42429^T) was isolated from the rhizosphere of brackish water cultivated saline-tolerant Pokkali rice variety (VTL-8) grown in Kumbalangi Pokkali rice fields, located at Ernakulam district, Kerala, India. The DNA G+C content of L1I52^T is 34.9 % (Menon et al., 2020).

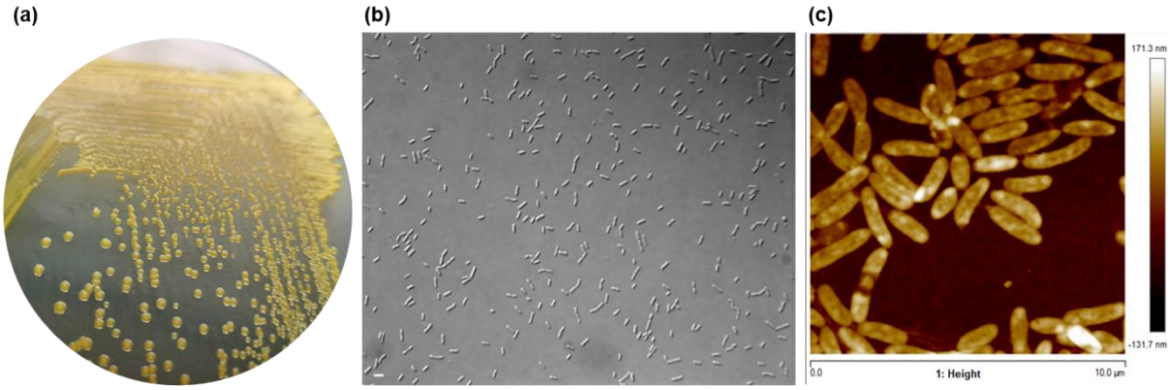


Fig. 2.23. Colony and cell morphotype of L1I52^T strain: **(a)** Image showing the growth of L1I52^T cells in CYG3 agar medium, **(b)** Differential interference contrast image of L1I52^T cells showing rod-shaped morphology captured using Nikon Eclipse 90i microscope under 100X magnification. **(c)** Atomic force microscopic image showing the rod-shaped morphotype of L1I52^T cells without any flagella.

2.4 Highlights and Conclusion

Microbial ecology is among the challenging research disciplines since it involves multi-level interactions between living systems belonging to both prokaryotes and eukaryotes. Moreover, understanding the ecology of a plant is highly complex as the plant hosts their native microbes from the soil, which is considered one of the most dynamic ecosystems on Earth. In addition, if we study the impact of an environment on plant-microbe interaction, it becomes even more complex. In this context, we have chosen the native rice varieties grown in the coastal agroecosystems of South India as they are strongly impacted by the prevailing brackish environments in the fields. Intriguingly, the microbial partners of these saline-tolerant rice varieties remain unexplored to date. In my research, the ecology of a particular bacterial group, *Flavobacterium,* native to these habitats, was studied. Although predominant in plant environments, their impact on plant hosts is elusive compared to their counterparts from other habitats. Therefore, metagenomic profiling was carried out to gain a fundamental insight into the Flavobacterium diversity and abundance in brackish-associated rice. The dominance of this bacterial group in the rice roots and revealing the influence of the environment, i.e., the brackish condition on their tight association, led to the starting point of this research. Further, the isolation of *F. pokkalii* L1I52^{T} through targeted culturing approaches advanced the investigation more deeply relative to its host association, eco-physiological characters, and genetic traits (discussed in the upcoming chapter). Finally, the strain was taxonomically characterized and placed as a novel species within the genus *Flavobacterium* through a polyphasic taxonomic approach and named *Flavobacterium pokkalii* sp. nov. L1I52^{T}. Thus, the findings from this study indicate that *Flavobacterium* strains could be a dominant member in plants native to brackish environments. Moreover, their high dominance in rice roots is expected to significantly influence their host growth and ecosystem functioning, which need extensive investigation. However, I believe certain critical aspects relative to *Flavobacterium*-plant interactions are investigated in the upcoming chapters of my research.

2.5 References

1. Flint, K.P., 1985. A note on a selective agar medium for the enumeration of Flavobacterium species in water. *Journal of applied bacteriology*, *59*(6), pp.561-566.
2. Adeleke, B. S., Muller, D., and Babalola, O. O. (2023). A metagenomic lens into endosphere microbial communities, promises, and discoveries. *Letters in Applied Microbiology* (Vol. 76, Issue 2). Oxford University Press. https://doi.org/10.1093/lambio/ovac030
3. Auch, A. F., von Jan, M., Klenk, H. P., and Göker, M. (2010). Digital DNA-DNA hybridization for microbial species delineation by means of genome-to-genome sequence comparison. *Standards in Genomic Sciences*, *2*(1), 117–134. https://doi.org/10.4056/sigs.531120
4. Beckers, B., Op De Beeck, M., Thijs, S., Truyens, S., Weyens, N., Boerjan, W., and Vangronsveld, J. (2016). Performance of 16s rDNA primer pairs in the study of rhizosphere and endosphere bacterial microbiomes in metabarcoding studies. *Frontiers in Microbiology*, *7* (650). https://doi.org/10.3389/fmicb.2016.00650
5. Bernardet, J. F., Nakagawa, Y., Holmes, B., Bowman, J. P., Bruun, B., Burchard, R. P., Hugo, C. J., Jooste, P. J., Malik, K. A., McMeekin, T. A., Reichardt, W., Reichenbach, H., Segers, P., Wakabayashi, H., Yabuuchi, E., Ursing, J., Segers, P., Suzuki, M., Hinz, K. H., and Nielsen, P. (2002). Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family. *International Journal of Systematic and Evolutionary Microbiology*, *52*(3), 1049–1070. https://doi.org/10.1099/ijs.0.02136-0
6. Bligh, E.G. and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology*, *37*(8), 911-917.
7. Bordenstein, S. R., and Theis, K. R. (2015). Host biology in light of the microbiome: Ten principles of holobionts and hologenomes. *PLoS Biology*, *13*(8). https://doi.org/10.1371/journal.pbio.1002226
8. Bulgarelli, D., Garrido-Oter, R., Münch, P. C., Weiman, A., Dröge, J., Pan, Y., McHardy, A. C., and Schulze-Lefert, P. (2015). Structure and function of the bacterial root microbiota in wild and domesticated barley. *Cell Host and Microbe*, *17*(3), 392–403. https://doi.org/10.1016/j.chom.2015.01.011
9. Card, G.L., 1973. Metabolism of phosphatidylglycerol, phosphatidylethanolamine, and cardiolipin of Bacillus stearothermophilus. *Journal of Bacteriology*, *114*(3), pp.1125-1137.
10. Cardinale, M., Grube, M., Erlacher, A., Quehenberger, J., and Berg, G. (2015). Bacterial networks and co-occurrence relationships in the lettuce root microbiota. *Environmental Microbiology*, *17*(1), 239–252. https://doi.org/10.1111/1462-2920.12686
11. Carrión, V.J., Perez-Jaramillo, J., Cordovez, V., Tracanna, V., De Hollander, M., Ruiz-Buck, D., Mendes, L.W., van Ijcken, W.F., Gomez-Exposito, R., Elsayed, S.S. and Mohanraju, P., 2019. Pathogen-induced activation of disease-suppressive functions in the endophytic root microbiome. *Science*, *366*(6465), pp.606-612.
12. Chen, W. M., Xie, Y. R., Kwon, S. W., and Sheu, S. Y. (2019). *Flavobacterium sufflavum* sp. Nov., isolated from a freshwater lake. *International Journal of Systematic and Evolutionary Microbiology*, *69*(6), 1705–1713. https://doi.org/10.1099/ijsem.0.003382
13. Cox, M. P., Peterson, D. A., and Biggs, P. J. (2010). *SolexaQA: At-a-glance quality assessment of Illumina second-generation sequencing data*. www.libgd.org/.
14. Dong, K., Chen, F., Du, Y., and Wang, G. (2013). *Flavobacterium enshiense* sp. nov., isolated from soil, and emended descriptions of the genus *Flavobacterium* and *Flavobacterium cauense*, *Flavobacterium saliperosum* and *Flavobacterium suncheonense*. *International Journal of Systematic and Evolutionary Microbiology*, *63*(PART3), 886–892. https://doi.org/10.1099/ijs.0.039974-0

15. Edwards, J., Johnson, C., Santos-Medellín, C., Lurie, E., Podishetty, N. K., Bhatnagar, S., Eisen, J. A., Sundaresan, V., and Jeffery, L. D. (2015). Structure, variation, and assembly of the root-associated microbiomes of rice. *Proceedings of the National Academy of Sciences*, *112*(8), E911–E920. https://doi.org/10.1073/pnas.1414592112.

16. Goodwin, S., McPherson, J. D., and McCombie, W. R. (2016). Coming of age: Ten years of next-generation sequencing technologies. *Nature Reviews Genetics* (Vol. 17, Issue 6, pp. 333–351). Nature Publishing Group. https://doi.org/10.1038/nrg.2016.49.

17. Goris, J., Konstantinidis, K. T., Klappenbach, J. A., Coenye, T., Vandamme, P., and Tiedje, J. M. (2007). DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. *International Journal of Systematic and Evolutionary Microbiology*, *57*(1), 81–91. https://doi.org/10.1099/ijs.0.64483-0.

18. Grondin, J. M., Tamura, K., Déjean, G., Abbott, D. W., and Brumer, H. (2017). *Polysaccharide Utilization Loci: Fueling Microbial Communities*. www.cazy.org.

19. Haichar, F. E. Z., Marol, C., Berge, O., Rangel-Castro, J. I., Prosser, J. I., Balesdent, J., Heulin, T., and Achouak, W. (2008). Plant host habitat and root exudates shape soil bacterial community structure. *ISME Journal*, *2*(12), 1221–1230. https://doi.org/10.1038/ismej.2008.80.

20. Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., and Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*, *41*(1). https://doi.org/10.1093/nar/gks808.

21. Komagata, K., and Suzuki, K.-I. (1987). *Lipid and Cell-Wall Analysis in Bacterial Systematics*.

22. Konstantinidis, K. T., and Tiedje, J. M. (2007). Prokaryotic taxonomy and phylogeny in the genomic era: advancements and challenges ahead. *Current Opinion in Microbiology* (Vol. 10, Issue 5, pp. 504–509). https://doi.org/10.1016/j.mib.2007.08.006.

23. Krishnan, R., Lang, E., Midha, S., Patil, P. B., and Rameshkumar, N. (2018). Isolation and characterization of a novel 1-aminocyclopropane-1-carboxylate (ACC) deaminase producing plant growth promoting marine Gammaproteobacteria from crops grown in brackish environments. Proposal for *Pokkaliibacter plantistimulans* gen. nov., sp. nov., *Balneatrichaceae* fam. nov. in the order Oceanospirillales and an emended description of the genus Balneatrix. *Systematic and Applied Microbiology*, *41*(6), 570–580. https://doi.org/10.1016/j.syapm.2018.08.003.

24. Krishnan, R., Menon, R. R., Likhitha, Busse, H. J., Tanaka, N., Krishnamurthi, S., and Rameshkumar, N. (2017). *Novosphingobium pokkalii* sp. nov., a novel rhizosphere-associated bacterium with plant beneficial properties isolated from saline-tolerant pokkali rice. *Research in Microbiology*, *168*(2), 113–121. https://doi.org/10.1016/j.resmic.2016.09.001.

25. Krishnan, R., Menon, R. R., Tanaka, N., Busse, H. J., Krishnamurthi, S., and Rameshkumar, N. (2016). *Arthrobacter pokkalii* sp. nov., a novel plant associated actinobacterium with plant beneficial properties, isolated from saline tolerant pokkali rice, Kerala, India. *PLoS ONE*, *11*(3). https://doi.org/10.1371/journal.pone.0150322.

26. Kuczynski, J., Stombaugh, J., Walters, W. A., González, A., Caporaso, J. G., and Knight, R. (2012). Using QIIME to analyze 16s rRNA gene sequences from microbial communities. *Current Protocols in Microbiology*, *SUPPL.27*. https://doi.org/10.1002/9780471729259.mc01e05s27.

27. Kwak, M. J., Kong, H. G., Choi, K., Kwon, S. K., Song, J. Y., Lee, J., Lee, P. A., Choi, S. Y., Seo, M., Lee, H. J., Jung, E. J., Park, H., Roy, N., Kim, H., Lee, M. M., Rubin, E. M., Lee, S. W., and Kim, J. F. (2018). Rhizosphere microbiome structure alters to enable wilt resistance in tomato. *Nature Biotechnology*, 36(11), 1100–1116. https://doi.org/10.1038/nbt.4232.

28. Mendes, R., Garbeva, P., and Raaijmakers, J. M. (2013). The rhizosphere microbiome: Significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. *FEMS Microbiology Reviews*, 37 (5), 634–663. https://doi.org/10.1111/1574-6976.12028.

29. Menon, R. R., Kumari, S., Kumar, P., Verma, A., Krishnamurthi, S., and Rameshkumar, N. (2019). *Sphingomonas pokkalii* sp. nov., a novel plant associated rhizobacterium isolated from a saline tolerant pokkali rice and its draft genome analysis. *Systematic and Applied Microbiology*, *42*(3), 334–342. https://doi.org/10.1016/j.syapm.2019.02.003.

30. Menon, R. R., Kumari, S., Viver, T., and Rameshkumar, N. (2020). *Flavobacterium pokkalii* sp. nov., a novel plant growth promoting native rhizobacteria isolated from pokkali rice grown in coastal saline affected agricultural regions of southern India, Kerala. *Microbiological Research*, *240*. https://doi.org/10.1016/j.micres.2020.126533.

31. Minnikin A', D. E., O'donnell A' B, A. G., Goodfellow, M., Alderson, G., Athalye, M., Schaal, A., and Parlett, J. H. (1984). An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids. *Journal of Microbiological Methods* (Vol. 2).

32. Mun, S., Lee, J., Lee, S., Han, K., and Ahn, T.-Y. (2013). Phylogeny of Flavobacteria Group Isolated from Freshwater Using Multilocus Sequencing Analysis. *Genomics and Informatics*, *11*(4), 272. https://doi.org/10.5808/gi.2013.11.4.272.

33. Nicolas, P., Mondot, S., Achaz, G., Bouchenot, C., Bernardet, J. F., and Duchaud, E. (2008). Population structure of the fish-pathogenic bacterium *Flavobacterium psychrophilum*. *Applied and Environmental Microbiology*, *74*(12), 3702–3709. https://doi.org/10.1128/AEM.00244-08.

34. Nishioka, T., Marian, M., Kobayashi, I., Kobayashi, Y., Yamamoto, K., Tamaki, H., Suga, H., and Shimizu, M. (2019). Microbial basis of Fusarium wilt suppression by Allium cultivation. *Scientific Reports*, *9*(1). https://doi.org/10.1038/s41598-018-37559-7.

35. Pan, X., Raaijmakers, J. M., and Carrión, V. J. (2023). Importance of Bacteroidetes in host–microbe interactions and ecosystem functioning. *Trends in Microbiology*. Elsevier Ltd. https://doi.org/10.1016/j.tim.2023.03.018.

36. Pandey, S., and Gupta, S. (2020). Evaluation of Pseudomonas sp. for its multifarious plant growth promoting potential and its ability to alleviate biotic and abiotic stress in tomato (*Solanum lycopersicum*) plants. *Scientific Reports*, *10*(1). https://doi.org/10.1038/s41598-020-77850-0.

37. Parte, A. C., Carbasse, J. S., Meier-Kolthoff, J. P., Reimer, L. C., and Göker, M. (2020). List of prokaryotic names with standing in nomenclature (LPSN) moves to the DSMZ. *International Journal of Systematic and Evolutionary Microbiology*, *70*(11), 5607–5612. https://doi.org/10.1099/ijsem.0.004332.

38. Patel, R. K., and Jain, M. (2012). NGS QC toolkit: A toolkit for quality control of next generation sequencing data. *PLoS ONE*, *7*(2). https://doi.org/10.1371/journal.pone.0030619.

39. Peng, Y., Leung, H. C. M., Yiu, S. M., and Chin, F. Y. L. (2012). IDBA-UD: A de novo assembler for single-cell and metagenomic sequencing data with highly uneven depth. *Bioinformatics*, *28*(11), 1420–1428. https://doi.org/10.1093/bioinformatics/bts174.

40. Pérez-Jaramillo, J. E., Carrión, V. J., de Hollander, M., and Raaijmakers, J. M. (2018). The wild side of plant microbiomes. *Microbiome*, 6(1). https://doi.org/10.1186/s40168-018-0519-z.

41. Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., and Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, *41*(D1). https://doi.org/10.1093/nar/gks1219.

42. Rameshkumar, N., Krishnan, R., Lang, E., Matsumura, Y., Sawabe, T., and Sawabe, T. (2014). Zunongwangia mangrovi sp. nov., isolated from mangrove (Avicennia marina) rhizosphere, and emended description of the genus Zunongwangia. *International Journal of Systematic and Evolutionary Microbiology*, *64*(PART 2), 545–550. https://doi.org/10.1099/ijs.0.053512-0.

43. Rameshkumar, N., Lang, E., and Tanaka, N. (2016). Description of Vogesella oryzae sp. nov., isolated from the rhizosphere of saline tolerant pokkali rice. *Systematic and Applied Microbiology*, *39*(1), 20–24. https://doi.org/10.1016/j.syapm.2015.10.003.

44. Rameshkumar, N., Sproer, C., Lang, E., and Nair, S. (2010). *Vibrio mangrovi* sp. nov., a diazotrophic bacterium isolated from mangrove-associated wild rice (Poteresia coarctata Tateoka). *FEMS Microbiology Letters*, *307*(1), 35–40. https://doi.org/10.1111/j.1574-6968.2010.01958.x.

45. Richter, M., Rosselló-Móra, R., Oliver Glöckner, F., and Peplies, J. (2016). JSpeciesWS: A web server for prokaryotic species circumscription based on pairwise genome comparison. *Bioinformatics*, *32*(6), 929–931. https://doi.org/10.1093/bioinformatics/btv681.

46. Rodriguez-R, L. M., and Konstantinidis, K. T. (2016). *The enveomics collection: a toolbox for specialized analyses of microbial genomes and metagenomes*. https://doi.org/10.7287/peerj.preprints.1900v1

47. Sasser, M. (1990). *Bacterial Identification by Gas Chromatographic Analysis of Fatty Acid Methyl Esters (GC-FAME)*.

48. Schlaeppi, K., Dombrowski, N., Oter, R. G., Ver Loren Van Themaat, E., and Schulze-Lefert, P. (2014). Quantitative divergence of the bacterial root microbiota in Arabidopsis thaliana relatives. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(2), 585–592. https://doi.org/10.1073/pnas.1321597111

49. Simon, J. C., Marchesi, J. R., Mougel, C., and Selosse, M. A. (2019). Host-microbiota interactions: From holobiont theory to analysis. *Microbiome*, *7*(1). https://doi.org/10.1186/s40168-019-0619-4

50. Singh, P., Singh, R. K., Zhou, Y., Wang, J., Jiang, Y., Shen, N., Wang, Y., Yang, L., and Jiang, M. (2022). Unlocking the strength of plant growth promoting *Pseudomonas* in improving crop productivity in normal and challenging environments: a review. *Journal of Plant Interactions* (Vol. 17, Issue 1, pp. 220–238). Taylor and Francis Ltd. https://doi.org/10.1080/17429145.2022.2029963.

51. Smibert R. M, and Krieg, N. R. (1994). Phenotypic characterization. in methods for general and molecular bacteriology. *American Society for Microbiology*, *611-651*.

52. Kumari, S (2024; to be submitted). A study on the microbiome of brackish-associated native rice varieties of Southern India: new insights into their genomes, plant-associated lifestyle and brackish adaptation (PhD thesis, AcSIR, Ghaziabad, India).

53. Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, *30*(12), 2725–2729. https://doi.org/10.1093/molbev/mst197.

54. Thijs, S., De Beeck, M. O., Beckers, B., Truyens, S., Stevens, V., Van Hamme, J. D., Weyens, N., and Vangronsveld, J. (2017). Comparative evaluation of four bacteria-specific primer pairs for 16S rRNA gene surveys. *Frontiers in Microbiology*, *8*(MAR). https://doi.org/10.3389/fmicb.2017.00494.

55. Ullah, A., Bano, A., and Khan, N. (2021). Climate Change and Salinity Effects on Crops and Chemical Communication Between Plants and Plant Growth-Promoting Microorganisms Under Stress. *Frontiers in Sustainable Food Systems*, *5*. https://doi.org/10.3389/fsufs.2021.618092.

56. Brader, G., Compant, S., Vescio, K., Mitter, B., Trognitz, F., Ma, L.J. and Sessitsch, A. (2017) Ecology and genomic insights into plant-pathogenic and plant-nonpathogenic endophytes. *Annual Review of Phytopathology*, *55*(1), 61-83.

57. Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., and Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. *New Phytologist* (Vol. 206, Issue 4, pp. 1196–1206). https://doi.org/10.1111/nph.13312

58. Zachow, C., Müller, H., Tilcher, R. and Berg, G. (2014). Differences between the rhizosphere microbiome of Beta vulgaris ssp. maritima—ancestor of all beet crops—and modern sugar beets. *Frontiers in Microbiology, 5*, p.415.https://doi.org/10.3389/fmicb.2014.00415.

59. Zboralski, A., and Filion, M. (2023). *Pseudomonas* spp. can help plants face climate change. *Frontiers in Microbiology*, *14*. https://doi.org/10.3389/fm.

Chapter – 3

Flavobacterium pokkalii L1I52^{T}: First insights into the host-interaction, plant functions and brackish adaptations through eco-physiological characterization studies, in-planta experiments and omics-based approaches

3.1 Introduction

Plants can no longer be considered standalone entities as their microbiota constitute an integral part of the plant system that contributes significantly to their health and stress resilience (Vandenkoornhuyse et al., 2015). Thus, the native microbiota critically impacts plant growth and survival, the two critical components of plant fitness (Vandenkoornhuyse et al., 2015). The rhizobacterial strains belonging to different bacterial phyla generally categorized under plant growth-promoting rhizobacteria (PGPR) might not have a beneficial impact on plant growth beyond its native host or tested plant systems because of their limited eco-adaptive traits that impede their performance when applied to other natural plant ecosystems (Comeau et al., 2021). Hence, it is always essential to isolate and characterize rhizobacterial strains native to the plant host and ecosystem of interest to study their impact on host growth, ecosystem functioning, and related aspects. In this regard, we have isolated a potential novel species belonging to the genus *Flavobacterium,* native to the brackish-adapted wild rice, Pokkali, which is the ecosystem of interest in our research study. The novel species, *F. pokkalii* L1I52^T strain (hereafter mentioned as L1I52^T in the main text except figure and legends) taxonomically described in the previous chapter, was represented in high numbers during isolation from the Pokkali rice roots compared to other pokkali strains, hinting at the possible tight association of L1I52^T strain with its host, Pokkali rice. Further, the influence attributed by the L1I52^T strain on rice growth during the initial *in-planta*-based screening studies promised potential phyto-beneficial aspects to be explored in this novel strain. Hence, in this chapter, we have initially focussed on understanding whether the L1I52^T strain can influence its host growth significantly under varied conditions. To study this, we have depended on *in-planta* studies rather than screening for *in-vitro* PGPR traits that are not considered reliable predictors of plant growth promotion (Smyth et al., 2011). Further, we analyzed if L1I52^T can modulate the host gene responses capable of impacting plant growth and health through molecular and omics-based approaches. Furthermore, the eco-physiological traits of L1I52^T were investigated, which is essential to study its ecological position and associated host-interaction conditions. Finally, we attempted to decode the potential and unique genetic features coded in the L1I52^T genome for its host interaction, beneficial plant functions and eco-adaptation through an in-depth genomic characterization and comparative genomic analysis.

3.2 Material and Methods

3.2.1 L1I52^T – Pokkali *in-planta* studies

3.2.1.1 Pokkali seed sterilization

The Pokkali rice seeds (VTL varieties) were used to check the phyto-stimulatory effects of the novel *Flavobacterium* strain, L1I52^T. The Pokkali rice seeds were surface sterilized following the customized procedures standardized for Pokkali seeds in our lab. Briefly, the seeds were dehulled and washed ~5-7 times with twice-autoclaved distilled water to remove the dust from the seed surfaces. Following this, the seeds were treated with sterilization solution (5% sodium dichloroisocyanurate dihydrate (Sigma) with 100 µl Tween 80 for 100 ml volume) in shaking condition (180 rpm) for 20 min. After this, the seeds were again washed ~8-10 times to remove the sterilization agents from the seed surfaces. Further, the sterilized and washed seeds are incubated in sterile water overnight (dark at room temperature) for sprouting. Finally, the sprouted seeds were placed on clerigel plates and incubated in the dark (room temperature) for ~48 hours for germination. The surface sterilized seeds were used for the following *in-vitro* plate germination and *in-planta* pot studies.

3.2.1.2 *In-vitro* germination experiments

For in vitro plate germination studies, the surface sterilized seeds were immersed in saline solution (0.85%) containing L1I52^T cells (OD_{600} = 0.5, ~10^9 CFU/ml) for three hours. After the required incubation time, L1I52^T treated seeds were briefly dried on a sterile filter paper and aseptically placed on 0.8% water agar plates (8 to 10 seeds per plate). These seeds were then allowed to germinate and grow for seven days at room temperature, and their germination parameters were observed. The sterilized seeds treated with a saline solution without L1I52^T cells served as the control treatments.

3.2.1.3 *In-planta* pot experiments

For the *in-planta* studies, 7-day-old sterile Pokkali rice seedlings with uniform shoot and root lengths were chosen. Further, the root portion of the selected seedlings was treated with saline suspensions of L1I52^T cells, and control treatments were sterile seedlings treated in saline suspensions without L1I52^T and incubated for three hours, as described earlier. After incubation, the seedlings with different treatments were carefully placed in plastic pots containing twice-autoclaved paddy soil (moistened with twice-autoclaved sterile distilled water). The pots were moistened intermittently with twice-autoclaved sterile distilled water and allowed to grow under natural conditions for up to 3 weeks. The plants were sacrificed

after three weeks from transplanting (i.e., a total of 4 weeks from initial sprouting and germination), and various plant growth-related parameters such as shoot length, root length, number of nodal roots, and fresh root weight were quantified. The *in-planta* studies were further repeated but with some modifications from the earlier experiment.

The term brackish condition used in the text represents the natural seawater (NSW)-amended conditions (% of natural seawater varies as indicated in respective sections), and the non-brackish condition means non-seawater or 0% seawater condition.

There were three major differences in the second batch of *in-planta* experiments, which are as follows: (i) the experiment was performed in both brackish (seawater-amended) and non-brackish (0% seawater) conditions contrasting to non-brackish condition alone in the earlier experiment, (ii) soil rite mix:sand (2:1) mixture was used as the substrate instead of natural paddy soil used in the previous experiment, and (iii) sterile nutrient solution, half-strength Hoagland's nutrient solution (½ HNS) was used for both intermittent moistening of plants and for preparing treatment suspensions instead of sterile distilled water and sterile saline suspension, respectively. Here also, uniformly grown seeds were incubated with respective treatments (control and L1I52^T) and transferred to pots filled with sterile soil rite mix:sand (2:1) mixture moistened with ½ HNS and placed in trays flooded with sterile distilled water for non-brackish condition, and sterile 20% NSW for brackish condition and incubated under greenhouse house conditions. After 12 days of incubation, a second round of inoculation (9-Log L1I52^T cells) was given to the growing rice plants along with the nutrient supplementation. Further, the plants were incubated for a total period of 1 month and then sacrificed to quantify different plant growth-related parameters such as shoot length, root length, root fresh weight, leaf chlorophyll content, and total nitrogen, phosphate, and potassium content from the root tissues. The physical parameters such as shoot length, root length, number of nodal roots, and fresh weight of shoot and roots were quantified manually. The chlorophyll content from the leaf portion of rice seedlings were estimated following the methods reported by Arnon with the modification mentioned by et al (Arnon, 1949; J. Zhang et al., 2009). The total nitrogen, phosphate, and potassium (NPK) contents from the dried roots of control and treated seedlings grown in both brackish and non-brackish conditions were quantified as follows: total nitrogen (inorganic and organic) by UV spectrophotometer (Shimadzu, Japan) and ammonia analyzer (Behrotest, Germany), total phosphate by UV spectrophotometer (Shimadzu, Japan), and total potassium by flame photometer (Systronics, India). The sample preparation were carried out according to the standard operating

procedures described by the manufacturer, and the values were represented as mg/g tissue.

All the quantified parameters in both the *in-planta* studies were represented as jittered box plots with appropriate statistical analysis comparing the control and L1I52^T treatment results. The statistical significance was determined through the student's t-test, and the significance was represented as follows: *, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.005.

3.2.2 L1I52^T- Pokkali rice gene modulation studies

3.2.2.1 RT-qPCR-based analysis of host-gene modulation

The host gene modulation by L1I52^T was studied in a hydroponic-based customized phytajar (HiMedia, India) setup. ½ HNS amended with 50% NSW was used as the nutrient solution, and the experiment comprised two treatments: Control (un-inoculated) and test (L1I52^T-inoculated with ~6-Log cells). Both control and test treatments comprised 2 biological replicates, and the test treatments had ~9 Log L1I52^T cells. The entire setup was incubated in customized plant growth racks maintained with light/dark (12h/12h) cycles with a

Table. 3.1. List of genes selected for studying the host gene modulation by L1I52^T cells under tested brackish conditions. The details of the selected genes and their known function in plants are also mentioned in the table.

S. No.	Gene ID	Gene name	Protein name	Known function in Plants
1	LOC4330182	*A1-Ibeta2*	phospholipase A1-Ibeta2, chloroplastic	Lipid metabolism
2	LOC4325760	*CML10_1*	probable calcium-binding protein CML10	Ion homeostasis and stress tolerance
3	LOC4338633	*CML15*	probable calcium-binding protein CML15	Ion homeostasis and stress tolerance
4	LOC4339974	*DREBP-1C*	dehydration-responsive element-binding protein 1C	Abiotic stress tolerance
5	LOC4330306	*DREBP-1G*	dehydration-responsive element-binding protein 1G	Abiotic stress tolerance
6	LOC4341871	*KRP-1*	calcium-binding protein KRP1	Seed development
7	LOC4324791	*NDR-1*	NDR1/HIN1-like protein 10	Stress response
8	LOC4329591	*Ubdcp-27*	U-box domain-containing protein 27	Protein interaction
9	LOC4334593	*ZAT-12*	zinc finger protein ZAT12	Abiotic stress tolerance

temperature range of 26-28ºC and humidity 55–60 %. The total RNA was extracted from the roots of control and test treatments at two time points: 6th hour post-inoculation (hpi) and 3rd dpi (days post-inoculation) using the RNeasy Plant Mini-kit (Qiagen, Germany), as per the manufacturer's instructions. Subsequently, the isolated RNA samples were treated with DNase-I, RNase-free (Thermo Scientific, USA), and subjected to cDNA synthesis, following the procedures outlined in the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, USA). The RNA-converted cDNA samples were subjected to RT-qPCR in QuantStudio^{TM} 5 Real-Time PCR system (Thermo Scientific, USA) using the custom-designed primers specific for genes listed in Table 3.1. The relative expression of the selected genes between the control and test treatments was normalized using GAPDH as the internal gene control. The relative expression was quantified and represented as fold-change ($2^{-\Delta\Delta ct}$). The fold change value >1 was considered up-regulation, whereas <1 was considered down-regulation

3.2.2.2 RNA-Seq-based analysis of host-gene modulation

An RNA-Sequencing (RNA-Seq) approach was chosen to investigate the host gene modulation mediated by L1I52^{T} at the global transcriptome scale. The same setup was used in RT-qPCR-based expression analysis, except the RNA was extracted only at a single timepoint (3rd dpi). The total RNA isolated from samples was treated with DNase-I, RNase-free (Thermo Scientific, USA). Further, stranded TruSeq libraries were generated from the DNase-treated total RNA samples that were subjected to RNA sequencing on Illumina HiSeq platform at Clevergene sequencing facility, Bangalore. High-quality RNA-Seq reads (101 bp end reads; Phred score>30 and >80% based) were filtered using IlluQC.pl in NGSQCtoolkit v2.3.3 (Patel, R. K et al., 2017). The high-quality RNA-Seq reads were then mapped to the *Oryza sativa* Japonica group (Japanese rice) genome assembly using TopHat2 v2.1.1 (D. Kim et al., 2013). Further, the Cufflinks version 2.2.1 was used to assemble the transcript from the mapped sequence data and estimate the abundance of differentially expressed genes (DEGs) (Trapnell et al., 2010). Cuffmerge was used to merge data from different samples, and cuffdiff was used for the differential expression analysis between the samples. Genes with a fold change of >2 and statistical significance of $p<0.05$ were designated significant DEGs. The raw sequence files generated from this study are deposited as BioSamples in NCBI under the BioProject PRJNA1051288.

3.2.3 *In-vitro* plant-growth promoting trait characterization

The *in-vitro* plant beneficial traits of L1I52^{T} were studied as follows. The ability to utilize

ACC as the sole nitrogen source was checked following the protocol of Krishnan et al (Krishnan et al., 2016). The ability of L1I52^T cells to grow in nitrogen-free minimal media was tested as described by Krishnan et al, except the carbon source used was glucose (Krishnan et al., 2016). Siderophore production was checked in R2AN agar medium amended with Chrome Azurol S (CAS) indicator dye, and visualization of a yellow halo zone around the growth was considered positive for siderophore production. The phosphate solubilization ability was checked by growing L1I52^T in pikowskaya agar, and the formation of a halo zone around the colony growth was recorded as positive. Indole-acetic acid (IAA) production was checked according to the methods followed by Menon et al (Menon et al., 2019). The ability to utilize various plant-derived compounds as the sole carbon or nitrogen source was carried out as described by Krishnan et al and the results were recorded after 5 days of incubation (Krishnan et al., 2016). In addition to the sugars, amino acids, and organic acids, the ability of L1I52^T cells to utilize various plant-derived polysaccharides such as carboxy-methyl cellulose, pectin, starch, and xylan was also checked. All the experiments were carried out at 30°C.

3.2.4 Eco-physiological characterization of L1I52^T

3.2.4.1 Growth in ZoBell marine medium

The ability of L1I52^T cells to grow in Zobell Marine Broth 2216 (ZMB; Cat. No: M385, HiMedia, India) amended with 1.8% agar (ZMA) was checked by streaking an active single colony of L1I52^T on ZMA plates and checked for its growth as individual colonies after a week of incubation at 30°C. To confirm its stable growth in ZMA, consecutive subculturing was performed on fresh ZMA plates. Similarly, the ability to grow in ZMB was also tested by inoculating L1I52^T cells into freshly prepared broth and checked for its growth visualized through turbidity after a week of incubation at 30°C. The ability of non-growing L1I52^T cells to grow in ZMB was tested by adding 0.18% sterile molten agar (approx. 40°C) to the already pre-incubated 2 days-old ZMB containing non-growing L1I52^T cells. This experiment setup was incubated for 7 days at 30°C, and the formation of subsurface or surface pellicle near the medium surface was considered positive. This experiment was performed three independent times to validate the repeatability of the results.

3.2.4.2 Growth in NSW amended medium

The effect of NSW on the growth of L1I52^T cells was tested in R2A broth amended with different seawater concentrations, such as 15%. 30%, 50%, 80%, and 100%. For this, active cell suspension of L1I52^T cells was prepared in R2A broth devoid of NaCl or NSW and

inoculated to R2A broth with respective seawater concentrations mentioned above. The growth was determined by measuring the optical density at 600 nm (OD_{600}) in regular intervals. A growth curve was plotted to compare and identify the optimal seawater concentration suitable for L1I52^T growth. Further, the growth of L1I52^T cells was also tested and compared between three conditions: R2A (non-saline), R2AN (R2A + 0.5% NaCl; saline), and R2A3 (R2A + 30% NSW; brackish).

3.2.4.3 Microaerophilic growth and cell motility

To check the micro-aerobic growth ability, L1I52^T cells were inoculated by stabbing into ZoBell marine and R2AN semi-solid agar (0.18%) media that were incubated under static conditions (30ºC) for seven days at 30°C. The formation of a subsurface pellicle near the medium surface was considered positive. To check for the cell movement, *F. pokkalii* L1I52^T cells were pricked on R2AN or R2A3 semi-solid agar (0.18%) plates and incubated at static conditions for seven days at 30°C. The movement of the L1I52^T cells was monitored and imaged.

3.2.4.4 L1I52^T cell survival assay in NSW-amended conditions

(a) Survival in distilled water base

The survival ability of L1I52^T cells was initially checked in two concentrations: half-strength (50% NSW) and full-strength natural seawater (100% NSW) prepared in the distilled water base. 7-log cells of L1I52^T were inoculated to 10 ml of sterile 50% and 100% NSW prepared in glass tubes and incubated in static conditions at 30°C. The survival rate was estimated by quantifying the recovery of *F. pokkalii* L1I52^T cells from both 50% and 100% NSW through dilution spot assay. The recoveries were recorded from 1st dpi to 7th dpi at every 24-hour interval by determining colony-forming units (CFUs) in the R2A3 agar medium. A line plot was generated to analyze the survival rate of *F. pokkalii* L1I52^T cells.

(b) Survival in freshwater base

Further, the survival experiment was repeated using freshwater (FW) as the base instead of distilled water used in the previous survival experiment. The survival rate was tested in different gradients of FW:NSW combinations such as 0% NSW (100% FW), 15% NSW (85% FW), 30% NSW (70% FW), 50% NSW (50% FW), and 100% NSW (0% FW). Like the previous experiment, 7-log cells of L1I52^T were inoculated to 10 ml of different NSW-FW suspensions prepared in glass tubes and incubated in static conditions at room temperature. In this experiment, we monitored the survival rate of *F. pokkalii* L1I52^T cells for a period of 1 month by checking the recoveries at different time intervals: 0th dpi, 2nd dpi,

4th dpi, 7th dpi, 10th dpi, 14th dpi, 16th dpi, 25th dpi, and 28th dpi, and recovery was also checked after 4 months (120th dpi) of undisturbed incubation. Similar to the previous experiment, the survival rate was determined through dilution spot assay and represented as a line plot comparison. The freshwater used in this experiment was collected from Vellayani freshwater lake (coordinates: 8°24′N 76°59′E) in Thiruvananthapuram, Kerala, India.

3.2.4.5 Biofilm formation and quantification

The biofilm formation ability of L1I52^T strain was checked in 3 different conditions: R2A (non-saline), R2AN (saline) and R2A3 (brackish). Active suspension of L1I52^T cells were prepared in the three conditions mentioned above, inoculated to sterile conditions prepared in sterile 48-well microtiter plates, and incubated under static conditions at 30°C for 7 days. After incubation, the media with the grown cells were removed and rinsed with distilled water to remove non-adhered or loosely adhered cells from the wells followed by flooding the wells with filter-sterilized 0.2 % crystal violet stain and incubated at room temperature for 30 min. Excess crystal violet stain was removed by rinsing thrice with distilled water, and the plates were dried at 37°C. The dried wells were flooded with 95% ethanol and incubated at room temperature for 20 – 30 min for complete solubilization of the biofilms, and subsequent spectrophotometric quantification was done at 600nm. Un-inoculated media for the three tested conditions served as the control of the experiment.

3.2.4.6 Seed attachment assay

The surface sterilized Pokkali rice seeds were incubated with L1I52^T (~10^9 CFU/ml) saline suspensions for 3 hours. After the incubation, L1I52^T treated seeds were transferred to a fresh 2 ml sterile vial, and the seeds were vigorously vortexed using 1 ml of sterile saline solution (0.85% NaCl). This procedure was repeated for 4 times and it was done to remove the loosely adhered L1I52^T cells from the seed surfaces. The finally washed seeds were briefly dried on a sterile filter paper before being placed on the R2AN agar plates. These plates were then incubated at 30°C for a week, and the growth of L1I52^T cells as yellow-pigmented colonies around the seed surfaces was observed and recorded positive for its seed attachment ability.

3.2.4.7 Root attachment assay

The root attachment ability of L1I52^T cells was checked in two different conditions: sterile distilled water as non-saline condition and sterile 100% seawater as saline/brackish condition. The root portion of 7-day-old sterile Pokkali rice (VTL-6) seedlings were dipped in respective suspensions containing L1I52^T cells (~10^9 CFU/ml) and incubated for 3 hours

at room temperature. After incubation, root portions were aseptically excised from the shoot portion of seedlings and subjected to vigorous vortex washing with the respective suspension used for attachment. Three rounds of independent washes were performed to remove loosely attached cells and the finally washed root portions were subjected to the following tests:

(a) Recovery of L1I52^T

The final washed root portions were crushed, serially diluted and spot inoculated onto R2AN agar plates. The L1I52^T colonies were counted and determined as CFUs per gram root weight. Simultaneously, we also checked the recovery of L1I52^T cells from respective suspensions in which the roots were incubated to assess the cell viability in both conditions.

(b) SEM imaging

The final washed root portions were excised into small sections of suitable sizes for convenient placement on the SEM stubs. The fixation and imaging of the excised was performed following the procedures of Yuan et al. with some modifications (Yuan et al., 2013). The roots were cut into small portions (~0.5 cm) and placed on a clean cover slip. These roots were then fixed with 2.5% glutaraldehyde solution prepared in 1X PBS buffer, pH 7.4, and incubated overnight at room temperature. Following the overnight incubation, the fixed root sections were dehydrated with increasing concentrations of ethanol prepared in sterile distilled water (in a series of 50%, 60%, 70%, 80%, 90%) and finally in 100% ethanol. Finally, fixed and dehydrated samples were left for air drying. The dried samples were affixed to imaging stubs for gold coating before being examined using SEM (Joel JSM 5600 LV system).

3.2.4.8 L1I52^T-host colonization experiment under customized gnotobiotic setups

The L1I52^T colonization experiment was carried out to study the influence of brackish and non-brackish conditions in aiding the L1I52^T cells in attaching and proliferating along Pokkali seedling roots. The experiment was carried out gnotobiotically in customized phytajar (HiMedia, India) setups with three comparative conditions: 1) ½ HNS (half-strength Hoagland's plant nutrient solution), 2) ½ HNS + 30% NSW (half-strength Hoagland's plant nutrient solution with 30% NSW) and, 3) ½ HNS + 50% NSW (half-strength Hoagland's plant nutrient solution with 50% NSW). 5-day old sterile Pokkali rice seedlings (VTL-6) were placed in phytajars containing the respective nutrient solutions followed by inoculating 6-Log *F. pokkalii* L1I52^T cells. The entire setup was incubated in the plant growth chamber (Binder, Germany) maintained with controlled growth conditions:

14-h light/10-h dark cycles with a temperature of 30°C and relative humidity of 50%. The colonization was monitored up to 7th dpi, and recoveries were performed at intermittent intervals of 1st dpi, 3rd dpi, and 7th dpi. The recovery of L1I52^{T} cells was checked from the crushed roots at the mentioned time points to determine the comparative colonization ability of L1I52^{T} cells between brackish (½ HNS +30% NSW and ½ HNS + 50% NSW) and non-brackish conditions (½ HNS). The recovery colonies from the dilution spot assay were quantified and converted to Log_{10} values of CFU gm root wt^{-1} represented in a line graph plotted through the tested time intervals.

(a) L1I52^{T} abundance in roots through *tuf*-qPCR

The total DNA was extracted from the washed roots excised from seedlings at different time points (0 (3-hour post-inoculation), 4th dpi, and 7th dpi) using the Plant DNA isolation kit (Macherey-Nagel) according to manufacturer's instructions. To quantify the abundance of L1I52^{T} along the roots of Pokkali seedlings, we amplified the total DNA with custom-designed *Flavobacterium* genus-specific *tuf* gene primers (Flavtuf198F – 5' GGATATGGTTGACGATGC 3', Flavtuf274R – 5' CAGGACAGTTATCTCCATC 3') targeting a 76 bp region of the peptide elongation factor Tu (Tuf). To quantify the absolute copies of *tuf* gene, a standard curve was prepared by amplifying *tuf* gene from increasing concentrations of L1I52^{T} genomic DNA (0.05 ng, 0.5 ng, 5 ng, 50 ng, 500 ng). The starting quantities (SQ) values thus obtained was applied to the absolute gene quantification formula, and the gene copies were calculated and represented as Log10 values of tuf gene copies.

(b) SEM imaging

For SEM imaging, the root sections of seedlings grown in the brackish conditions (½ HNS + 30% NSW) was collected on 3rd dpi. The sample preparation and imaging procedures followed were the same as detailed earlier (SEM imaging section; root attachment assay)

3.2.5 Growth in polysaccharide-monosaccharide mixture and HPLC analysis

The growth of L1I52^{T} in different polysaccharides were checked in minimal media with the following composition: K_2HPO_4 – 0.08%, KH_2PO_4 – 0.02%, $MgSO_4.7H_2O$ – 0.02%, $CaCl_2.2H_2O$ – 0.013%, NaCl – 0.5% or NSW – 30%, NH_4Cl – 0.01% as N_2 source, Polysaccharides (xylan (SRL, India), pectin (SRL, India), starch (HiMedia, India), carboxy-methyl cellulose (HiMedia, India)) – 0.1 – 0.5% and monosaccharides (glucose, galactose, rhamnose, xylose) – 0.1 – 0.5% as carbon sources whenever required. Experiments performed under static conditions were carried out in 48 well microtiter plates and shaking condition (180 rpm) experiments in 100 ml flasks with 20 ml working volume at 30°C. The

growth was monitored by checking the absorbance at 600 nm in regular intervals. To validate the L1I52^T preference on polysaccharides over its monosaccharides, 0.5% of both xylan and xylose was given together as a carbon source mixture, and the subsequent utilization was quantified till 36 hours post inoculation with regular time intervals of 12 hours. To quantify the xylose and xylan contents, the filtered samples (0.2 µm filter, Sartorius Stedim Biotech, Germany) were injected using an autosampler program and eluted with deionized water as the mobile phase through Shimadzu (Japan) HPLC system equipped with RPM monosaccharide column with a refractive index (RI) detector. The samples were eluted with deionized water at a flow rate of 0.6 ml/min, and the oven temperature was maintained at 80°C. To determine the retention time (Rt) of xylan and xylose, the standards of two concentrations (2.5% and 5%) were injected. The chromatograms of xylan and xylose of respective samples were compared by considering their peak intensities and peak area to validate how L1I52^T cells preferred the polysaccharide, xylan over its monosaccharide, xylose.

3.2.6 Root macerate (RM) preparation from Pokkali seedlings roots and L1I52^T growth in RM amended conditions

10-day old sterile Pokkali seedlings were used to prepare root macerate (RM). The root portions of the seedlings were excised and transferred aseptically to a sterile mortar and pestle, where it was ground with sterile distilled water. The crushed root macerate suspension was centrifuged to remove the root debris materials and then syringe filtered through 0.2 µm filters (Sartorius Stedim Biotech, Germany) and stored at -80°C until used. The total proteins and sugars of the prepared root macerate were estimated using Bradford's method and the phenol-sulphuric acid method, respectively. The influence of root macerate in L1I52^T growth was checked in three different conditions as follows: 1) R2A3-d (½ strength 1/10 R2A3), 2) R2A3-d + 50% RM, and 3) 50% RM alone without any amended media components incubated under static condition at 30°C. The root macerate-influenced growth was validated by determining the CFUs recovered from each condition at regular intervals.

3.2.7 L1I52^T draft genome sequencing and analysis

High-quality genomic DNA of the novel strain, L1I52^T and other *Flavobacterium* strains isolated from Pokkali roots (NRK F7, NRK F10, and NRK 1) used in this study was isolated using QIAamp DNA mini kit (Qiagen) according to the manufacturer instructions. The libraries were prepared using NEXTFlex DNA sequencing library kit follwoing the

procedures recommended by the manufacturer and sequenced on Illumina HiSeq 4000 platform. Raw reads were trimmed using the software Solexa QA [reference], followed by removing reads with quality scores lower than 20 and lengths shorter than 50bps. The trimmed reads were assembled with IDBA assemble v1.1.1 [reference] using the "pre_correction" option. The genome's completeness, contamination, and heterogeneity were checked using the checkM tool (Parks et al., 2015).

Gene prediction and annotation was performed using the ClassicRAST annotation scheme available in the RAST Server (Rapid Annotations using Subsystems Technology (Aziz et al., 2008). The resultant subsystem category distribution with the generated subsystem feature counts was analyzed and compared manually. Further, the sequence-based option available in RAST server was employed to have a detailed comparative genomic analysis wherein the required bacterial genomes were compared. For comparative study, either the annotated genomes available in the RAST database were used, or the FNA file (FASTA) containing the genome sequences of the required bacterial strains were retrieved from NCBI and annotated in RAST for subsequent analysis.

For pangenome analysis, we used anvi'o pangenomic workflow, which uses BLAST search to assess the similarity between each pair of amino acid sequences among all genomes and then resolves the resultant graph into gene clusters using the Markov Cluster algorithm (Eren et al., 2015). The pangenome diagram was finally visualized using anvi'o graphical interface depicting the basic genomic comparison between L1I52^T and its nearest relatives: *F. daejeonense* DSM 17708^T, *F. suffalvum* BBQ-12^T, and *F. glycines* Gm-149^T. Further, a BLAST similarity search was conducted in RAST to identify the unique genes of L1I52^T in comparison with its nearest reference strains (Aziz et al., 2008). A sequence identity below 50 % cut-toff was considered as a unique gene in L1I52^T (Chaudhari et al., 2016). The unique genes are grouped by at least 3 numbers of functional genes and gene proximity of maximum 2 nucleotide gap within the cluster. The gene organizations of unique gene clusters were constructed using genoPlotR in the R package (http://www.R-project.org/).

The Carbohydrate-active enzymes (CAZyme) coding genes present in all the genomes analyzed in this study were predicted through dbCAN3 database (automated carbohydrate-active enzyme and substrate annotation) using HMMER tool (E-value < 1e-15, coverage > 0.35) and further verified by the prediction from the DIAMOND tool (E-value < 1e-102) (Zheng et al., 2023). The CAZyme prediction was further verified using NCBI and KEGG annotation. Sulfatase classes were identified using the BLAST search in the NCBI-

conserved domain database. The gene organization of the CAZyme-organized PULs was constructed using genoPlotR in the R package (http://www.R-project.org/). The genome sequences of L1I52^{T} and other *Flavobacterium* strains isolated from Pokkali rice are deposited in NCBI with the GenBank accessions mentioned in Table 3.2. The GenBank accessions of the *Flavobacterium* strains used for the comparative genomic analysis are also listed in Table 3.2.

Table. 3.2. GenBank accession details of the *Flavobacterium* strains used for comparative genomic analysis in this study. The asterisk symbol (*), marked near the GenBank accession numbers represent the genomes submitted for this study.

S. No.	Bacterial strains	GenBank accession no.
1	*Flavobacterium pokkalii* L1I52^{T}	NZ_NASZ00000000*
2	*Flavobacterium* sp. NRK F7	JAMXLB000000000*
3	*Flavobacterium* sp. NRK F10	JAMXLC000000000*
4	*Flavobacterium* sp. NRK1^{T}	JAMXLA000000000*
5	*F. daejeonense* DSM17708^{T}	AUDK00000000
6	*F. sufflavum* BBQ-12^{T}	SACJ01000000
7	*F. glycines* Gm-149^{T}	FNEO01000000
8	*Flavobacterium johnsoniae* UW101	NC_009441
9	*Flavobacterium* sp. F52	NZAKZQ00000000
10	*Flavobacterium* sp. URHB0058	AUEU00000000
11	*Flavobacterium* sp. WG21	NZMYW00000000
12	*Flavobacterium* sp. CF136	NZAKJZ00000000
13	*Flavobacterium rivuli* DSM 21788	NZKB899988
14	*Flavobacterium* sp. B17	BACY00000000
15	*Flavobacterium soli* DSM 19725	AUGO00000000
16	*Flavobacterium* sp. ACAM 123	NZAJXL01000285
17	*Flavobacterium frigoris* PS1	NZAHKF00000000
18	*Flavobacterium branchiophilum* FL-15	NC016001
19	*Flavobacterium columnare* ATCC 49512	NC016510
20	*Flavobacterium antarcticum* DSM 19726	NR042998
21	*Flavobacterium indicum* GPTSA100-9	NC017025
22	*Flavobacterium psychrophilum* JIP02/86	NC009613

3.3 Results and Discussion

3.3.1 L1I52^T promotes Pokkali rice growth

3.3.1.1 L1I52^T influences Pokkali seed germination and enhances plant growth

Most studies on the beneficial aspects of plant-microbe interactions are confined to understanding the *in-vitro* plant-beneficial traits of the bacteria. However, studying the *in-vitro* traits alone will not solve the challenges faced in the real scenario of plant-microbe interactions, where many factors, especially the surrounding environment, are highly influential. Moreover, certain bacterial strains that show potential *in-vitro* plant growth-promoting properties also fail to influence plant growth positively when tested in pots or fields. This indicates that *in-vitro* analysis is not the real predictor for tracing the plant growth-promoting abilities of bacteria. Hence, we started our investigation to trace the potential plant growth influencing strains through *in-planta* experiments. During our initial *in-planta* screening for identifying potential novel PGPR native to brackish-adapted Pokkali rice, we identified L1I52^T as a potential strain among the many tested isolates and were shortlisted for further studies. Interestingly, the novel *Flavobacterium* strain showed an enhanced plant growth promotion in Pokkali rice. To further substantiate this finding, we

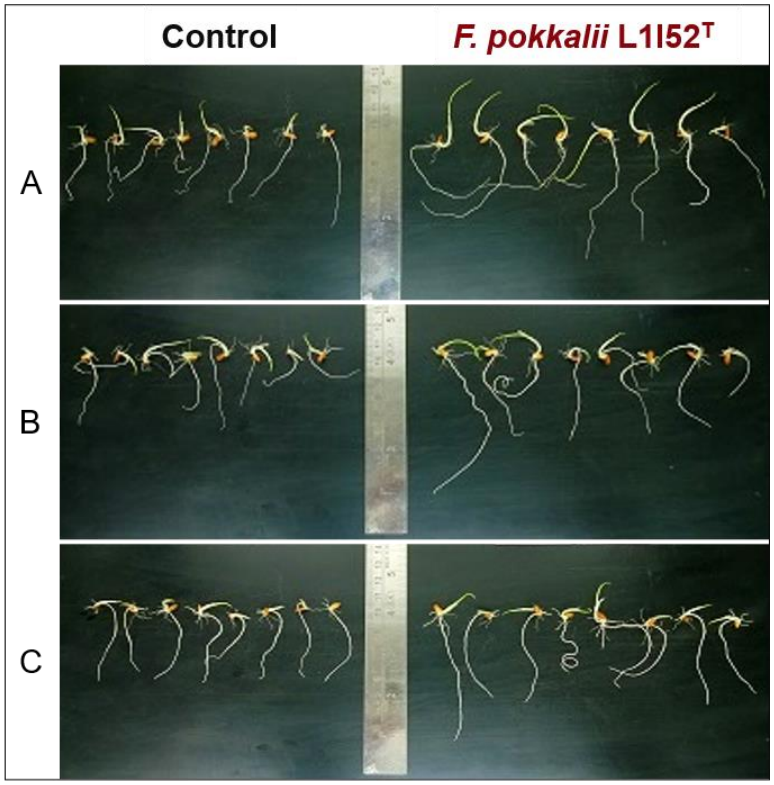


Fig. 3.1. *F. pokkalii* L1I52^T influences the germination of Pokkali rice seeds. Images taken on 7th dpi. A, B, and C represent biological replicates. Enhanced root length is observed for seedlings germinated from Pokkali seeds treated with *F. pokkalii* L1I52^T cells compared to the un-treated (control) seeds.

investigated in detail on the potential ability of L1I52^T strain to promote Pokkali rice growth. At first, the surface sterilized Pokkali seeds were, treated with L1I52^T ($0D_{600}$ = 0.8; ~10^9 CFU/ml) and subsequently transferred to the water agar plates. After seven days of incubation, we observed a significant increase in root and shoot length of Pokkali rice seeds treated with L1I52^T compared to non-treated control seeds (Fig. 3.1). Similar results were observed in repeated experiments suggesting L1I52^T can enhance the growth of germinating seeds and early seedling growth. Next, we performed *in-planta* pot experiments for an extended time period to understand if a similar influence is observed at the later growth stages of Pokkali rice. For this, we incubated the roots of uniformly grown seven days old Pokkali rice seedlings with L1I52^T ($0D_{600}$ = 0.8; ~10^9 CFU/ml) for ~3 hours and then transferred them to pots filled with twice-autoclaved paddy field soil (see method section

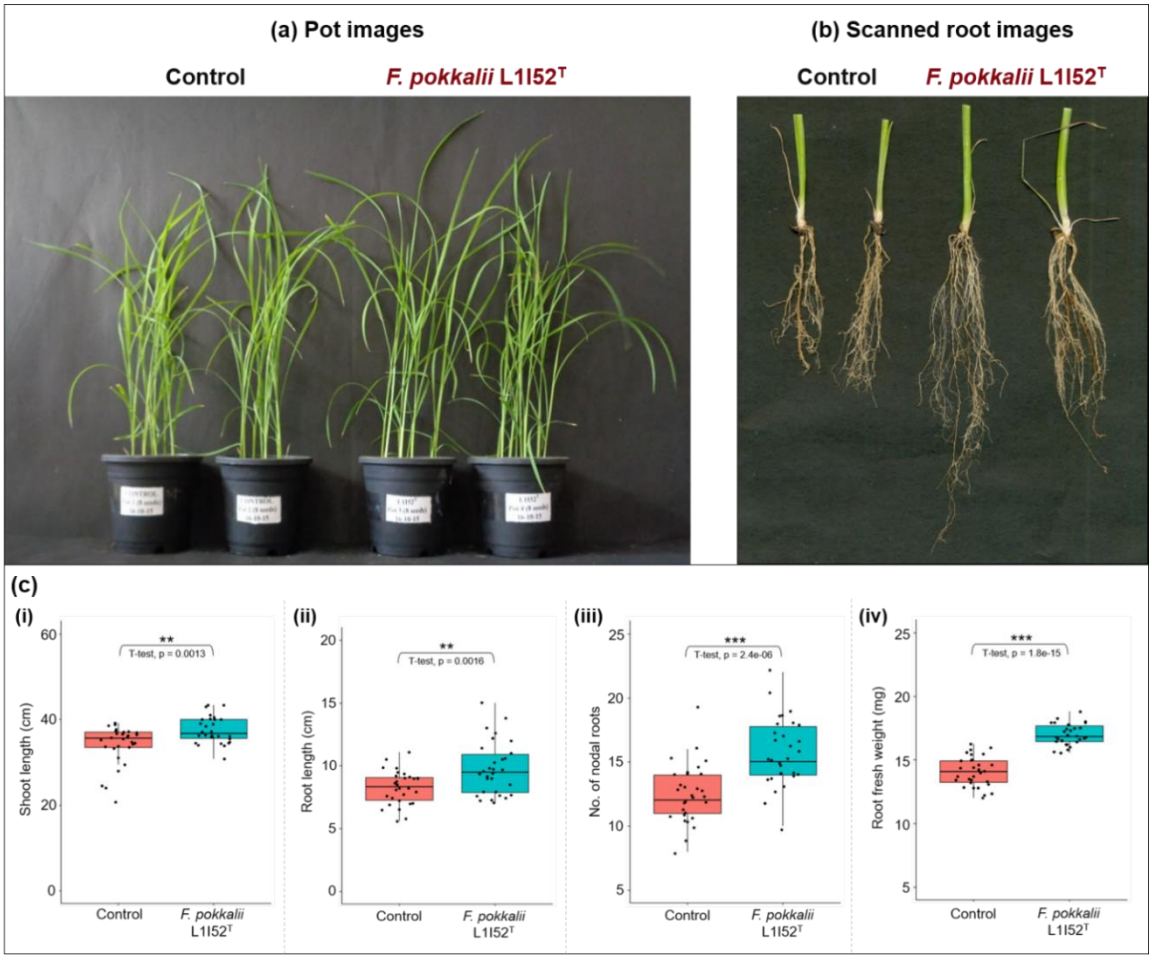


Fig. 3.2. *F. pokkalii* L1I52^T influences Pokkali rice growth under natural conditions: **(a)** Pot images, **(b)** scanned root images showing comparative growth differences between Pokkali rice plants grown from control and *F. pokkalii* L1I52^T-treated seedlings, and **(c)** Boxplots representing the comparison between the plant growth-related parameters: **(i)** shoot length, **(ii)** root length, **(iii)** no. of nodal roots, and **(iv)** root fresh weight.
Statistical significance represented by *, p-value<0.05; **, p-value<0.01, ns, non-significant.

for details). After four weeks of inoculation, L1I52^T-treatment showed a significant enhancement in the plant growth, as clearly visible in the pot images displaying an increased growth in the shoot portion of the rice plants supported with a significant increase in the shoot length between both treatments (Fig. 3.2 (a, c)). Moreover, the root growth of the rice plants was significantly enhanced upon L1I52^T-treatment compared to the control treatment, as seen in the scanned root images and especially on the root-related parameters such as root length, number of nodal roots, and root fresh weight that was highly significant (Fig. 3.2 (b, c)). Altogether, these results demonstrated the beneficial effects of a novel *Flavobacterium* strain, L1I52^{T,} in promoting the growth of Pokkali rice grown in autoclaved paddy soil and incubated in natural conditions. Although several studies reported the occurrence of *Flavobacterium* strains in plant habitats, their beneficial role in the influence of host growth remained largely unknown. However, in a few studies *Flavobacterium* strains from various terrestrial plant rhizospheres, the knowledge has documented their plant growth-promoting abilities indirectly as a plant-protecting biocontrol agent and directly as a plant growth promoter (Alexander and Stewart, 2001; An et al., 2009; Flynn et al., 2014; Hebbar I et al., 1991; Madhaiyan et al., 2010; Sang and Kim, 2012). Hence, the preliminary findings from this *in-planta* experiments extend the plant host range and plant growth-promoting ability of this genus to a native rice variety traditionally grown in brackish environments for the first time. However, to validate the host growth influencing the capacity of L1I52^T, *in-planta* experiments were performed in different conditions discussed in the upcoming sections.

3.3.1.2 L1I52^T enhances Pokkali rice growth under brackish (20% NSW) and non-brackish (0% NSW) conditions

The observations and inference from the initial experiment point towards the potential of the novel strain L1I52^T in influencing its host-plant growth. Therefore, our next target was to investigate if similar effects were observed in the host plant growth upon treatment with *F. pokkalii* L1I52^T cells under further controlled conditions. For this, the experiment was performed in sterile soilrite: sand mixture instead of paddy soil to avoid the influence of any nutrients or organic content from the soil, if any. Further, this experiment was performed under two conditions: (i) brackish (pots incubated in trays flooded with sterile 20% NSW), and (ii) non-brackish condition (pots incubated in trays flooded with sterile 0% NSW (distilled water)) to mimic both brackish and non-brackish conditions in the best possible way. Interestingly, more significant plant growth parameters and associated phenotypes were observed in the plants grown under brackish conditions compared to non-brackish conditions (Fig. 3.3; Fig. 3.4). For example, the increase in shoot length of L1I52^T treatments

was more evident from the shoot growth phenotype observed in the pot images (Fig. 3.3 (a); 3.4 (a)), uprooted images (Fig. 3.3 (b); Fig.3.4 (b)) supported with a significant increase in the shoot length of seedlings treated with L1I52^T grown under brackish condition (20% NSW) compared to non-brackish conditions (0% NSW) (Fig. 3.3 (d-i); Fig. 3.4 (d-i)). More importantly, L1I52^T-treatments significantly enhanced the root growth-related parameters ii, iii)). The positive influence of L1I52^T strain on the root length of Pokkali rice under both conditions is visible from the scanned root images as well (Fig. 3.3 (c); Fig. 3.4 (c)). Further,

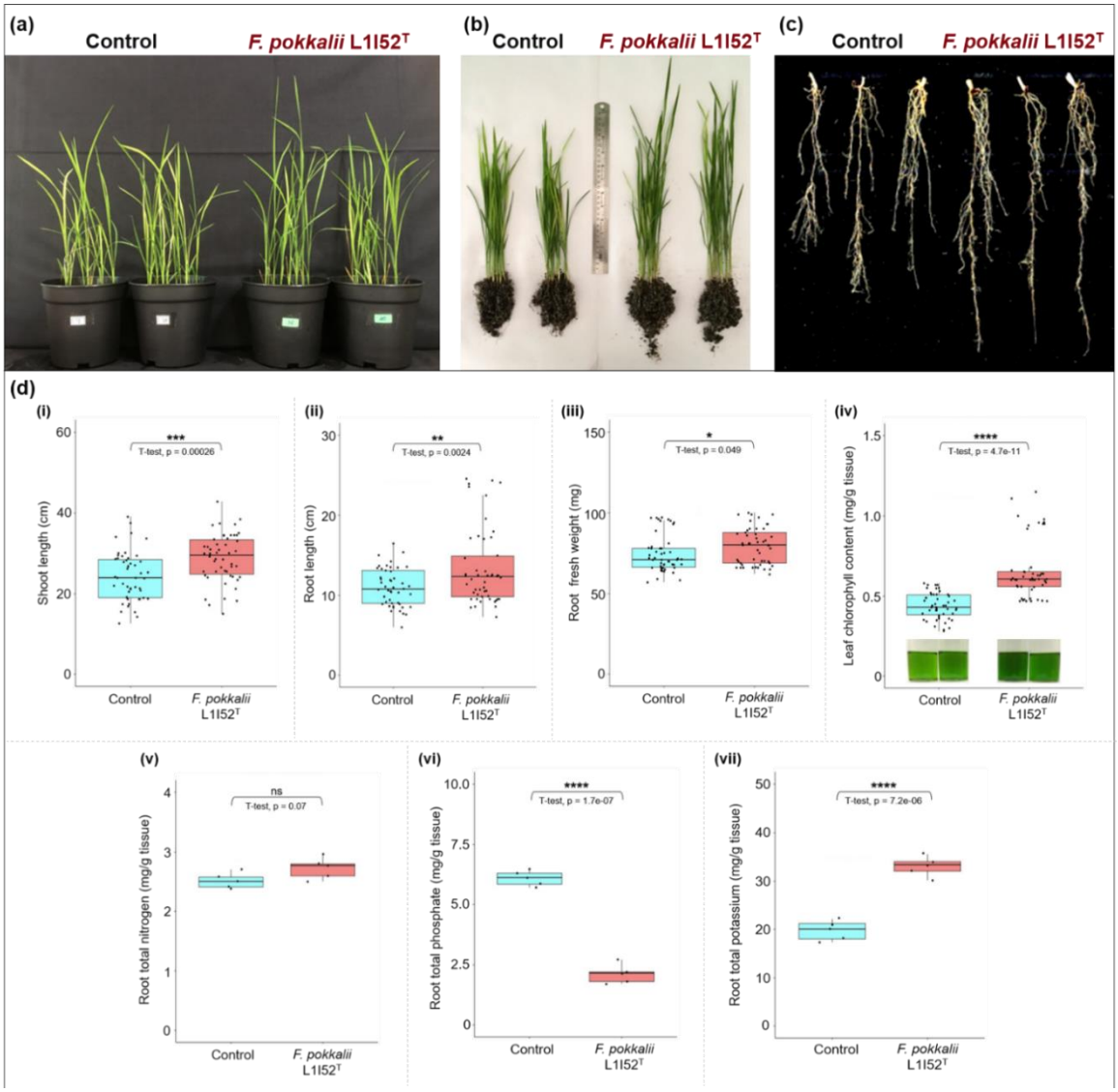


Fig. 3.3. *F. pokkalii* L1I52^T enhances Pokkali rice growth under brackish (20% NSW) conditions: **(a)** Pot images, **(b)** Uprooted plant images, and **(c)** scanned root images showing comparative growth differences between Pokkali rice plants grown from control, and *F. pokkalii* L1I52^T-treated seedlings, and **(d)** Boxplots representing the comparison between the the plant growth-related parameters: **(i)** shoot length, **(ii)** root length, **(iii)** root fresh weight, **(iv)** leaf chlorophyll content, **(v)** total root nitrogen, **(vi)** root total phosphate, and **(vii)** root total potassium. Each jitter point in the box plot represents one plant and all parameters are quantified from 5 replicates each comprising 10 plants. Each jitter in nitrogen, phosphate and potassium contents represent the average of 10 plants.
Statistical significance represented by *, p-value<0.05; **, p-value<0.01, ns, non-significant.

the positive influence of L1I52^T treatment on Pokkali rice growth was evident in the leaf chlorophyll estimation supported by a highly significant increase in the quantified chlorophyll content as well as high variation in the intensity of the extracted pigments between L1I52^T and control treatments as seen in Fig. 3.3 (d-iv); Fig. 3.4 (d-iv). Apart from the growth-related parameters, we also quantified the NPK (nitrogen, phosphate, and potassium) content from the root tissues of L1I52^T and control treatments grown in both conditions. The nitrogen content remains unaffected irrespective of the treatments under

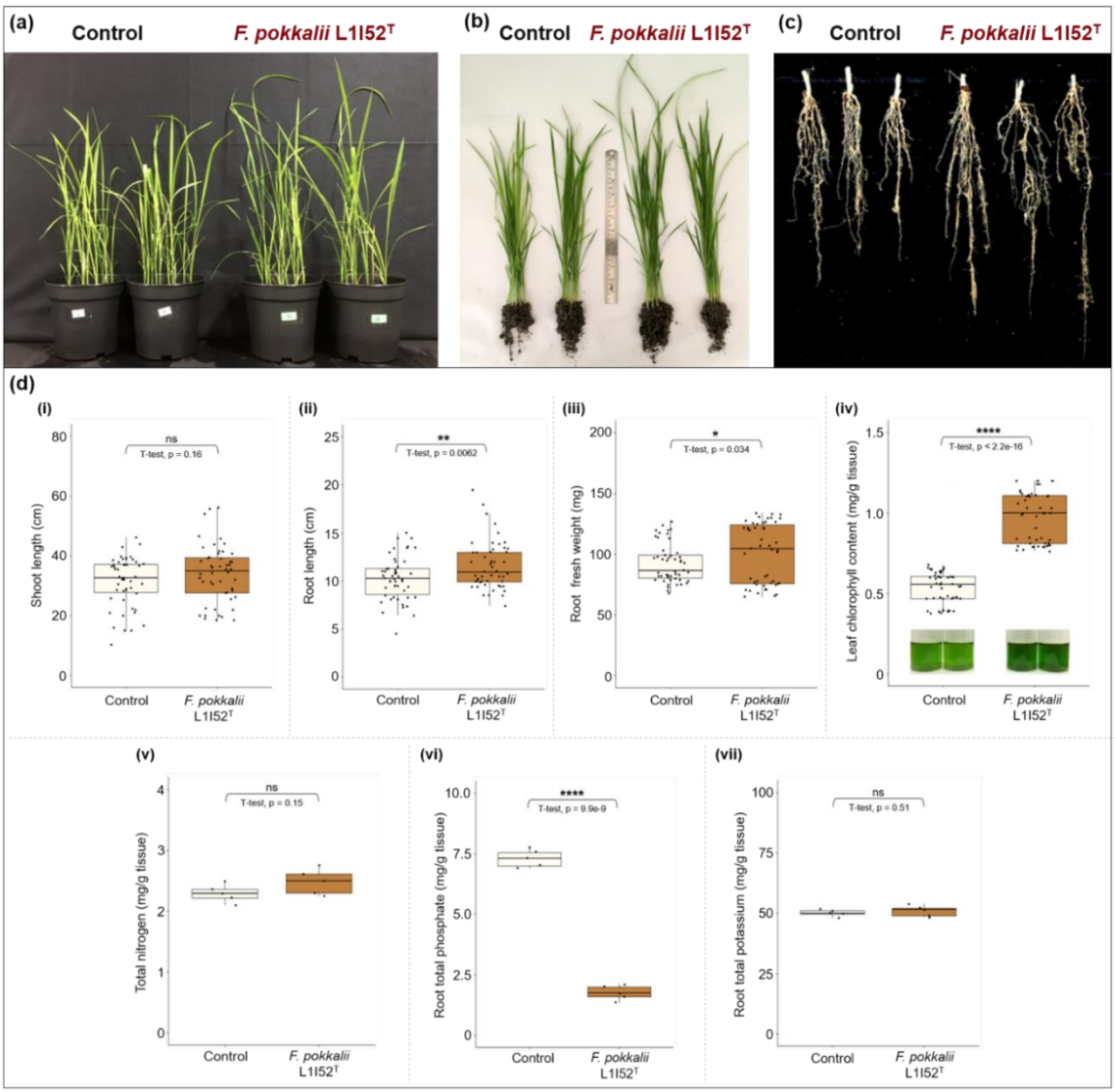


Fig. 3.4. *F. pokkalii* L1I52^T enhances Pokkali rice growth under non-brackish (0% NSW) conditions: **(a)** Pot images, **(b)** Uprooted plant images, and **(c)** scanned root images showing comparative growth differences between Pokkali rice plants grown from control, and *F. pokkalii* L1I52^T-treated seedlings, and **(d)** Boxplots representing the comparison between the the plant growth-related parameters: **(i)** shoot length, **(ii)** root length, **(iii)** root fresh weight, **(iv)** leaf chlorophyll content, **(v)** total root nitrogen, **(vi)** root total phosphate, and **(vii)** root total potassium. Each jitter point in the box plot represents one plant and all parameters are quantified from 5 replicates each comprising 10 plants. Each jitter in nitrogen, phosphate and potassium contents represent the average of 10 plants.
Statistical significance represented by *, p-value<0.05; **, p-value<0.01, ns, non-significant.

both conditions (Fig. 3.3 (d-v); Fig. 3.4 (d-v)). In contrast, the phosphate content was significantly reduced in plants grown from L1I52^T treatment compared to control treatments in both conditions (Fig. 3.3 (d-vi); 3.4 (d-vi)). The reduced phosphate content in the roots of L1I52^T treatment hints at the possible competition for phosphate between the L1I52^T cells and its host, Pokkali rice. However, further experiments are required to validate this reasoning. Interestingly, we could identify a critical observation wherein the total potassium content in the roots of plants grown from L1I52^T treatment was higher (mean of 3mg/g tissue) compared to control treatment (mean of 19.7 mg/g tissue) under brackish condition (Fig. 3.3 (d-vii); Fig. 34 (d-vii)). The increased potassium content indicates the uptake of K^+ ions, one of the primary mechanisms to circumvent the increased salt stress caused by high salinity. Supportively, the potassium content from roots of seedlings grown from L1I52^T-treatment and control treatments under non-brackish conditions showed nearly similar values, indicating that the uptake of K^+ ions was not complemented upon L1I52^T treatment since there was no saline stress. These preliminary findings strongly suggest that L1I52^T enhances Pokkali rice growth, as indicated by increased growth parameters under brackish and non-brackish conditions. However, the growth enhancement was higher under the brackish condition, indicating that the native environment might favour L1I52^T cells more to interact and contribute to its hostplant growth. Additionally, the significant increase in the total potassium content of roots upon L1I52^T treatment under brackish condition compared to non-brackish conditions possibly indicates an unknow role of L1I52^T cells in alleviating the saline stress of the host. Furthermore, to confirm that the colonized L1I52^T cells are the main contributor to enhanced plant growth and associated parameters, a crushed recovery was performed from the roots of Pokkali rice seedlings after uprooting. The high abundance of yellow-pigmented colonies in the dilution plates hinted at the presence of the treated *Flavobacterium* strain, L1I52^T. Further, to confirm, we performed an ERIC primer pair-based genomic DNA fingerprinting from the total DNA isolated from L1I52^T-treated seedlings grown in both brackish and non-brackish conditions roots after uprooting. Fig. 3.5 represents the gel image showing the ERIC fingerprint pattern comparison between the genomic DNA of the L1I52^T strain and the total DNA of L1I52^T-treated rice plants grown in brackish and

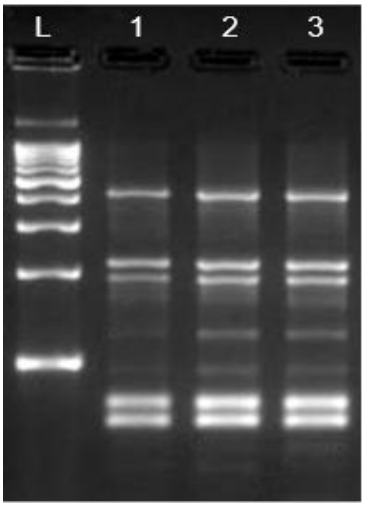


Fig. 3.5. Gel image showing the comparison of ERIC fingerprint pattern between 1, genomic DNA of L1I52^T, and the total DNA from L1I52^T-treated plants grown under 2, brackish condition, and 3, non-brackish condition. L, 1 kb DNA ladder.

non-brackish conditions. The fingerprint patterns shared high similarity, confirming the presence of L1I52^T in the roots of Pokkali rice plants grown for 1 month in both conditions. To the best of our knowledge, this is the first study investigating the influence of an environment in mediating beneficial host plant growth by a *Flavobacterium* strain. However, further in-depth studies are mandatory to validate these preliminary findings at the genetic and physiology levels.

3.3.2 L1I52^T modulates host gene expression under the brackish condition

3.3.2.1 RT-qPCR-based analysis of host-gene response in Pokkali rice roots upon L1I52^T colonization

The results and inference from the *in-planta* studies firmly validated the positive influence of L1I52^T on Pokkali rice growth. Even though L1I52^T mediated host growth influence in the tested brackish and non-brackish conditions, an increased significant influence was observed and quantified when tested under brackish conditions. Moreover, the host ecosystem of the rice plant, Pokkali, and the bacterium tested, L1I52^T, is brackish. Considering all these facts, we designed an experiment to investigate if L1I52^T cells can modulate the host gene responses upon colonization when the seedlings are grown under brackish conditions. In this experiment, we employed the same hydroponic-based phytajar setup used for the binary association experiment, and ½-HNS (without N) amended with 50% NSW was used as the testing condition. The eukaryotic genes picked in this gene expression analysis were based on the leads obtained from one of our earlier transcriptomic studies wherein a subset of eukaryotic genes showed significant gene modulations upon colonization by one of our potential novel bacterial strain (*Pokkaliibacter plantistimulans* L1E11^T) under the same brackish conditions tested (data not shown). The eukaryotic genes selected for analysis and their known functions from the literature are listed in the table. 3.1. The *GAPDH* gene was used as the internal control for quantifying the relative expression of the test genes between the two tested conditions. We opted for two-time points, 6-hour post-inoculation and 72-hour post-inoculation (3rd dpi), to analyze the gene expression modulation in Pokkali rice host. Fig. 3.6 summarizes the gene expression in L1I52^T colonized Pokkali roots against the un-inoculated (control) Pokkali roots represented as fold change ($2^{-\Delta\Delta ct}$) quantified on 6th hpi and 72nd hpi. The initial time point showed downregulation of all the tested nine genes in L1I52^T colonized Pokkali roots, whereas, in 72 hpi, the gene expression modulates variedly for different genes of seedling roots colonized by L1I52^T cells (Fig. 3.6). Among the tested nine genes listed in Table 3.1, seven genes were upregulated (Fig. 3.5). The two genes, Dehydration-Responsive Element

Binding Protein (*DREB*) *1C* and *DREB*P-*1G* showed the highest modulation with an up-regulation of almost 5.4-fold and 5.7-fold, respectively (Fig. 3.6). The other four genes, *A1-beta2*, *CML10_1*, *CML15*, and *NDR-1*, showed a moderate up- regulation ranging from 1.6 to 2 folds (Fig. 3.6). However, among the remaining 3 genes, *ZAT-12* showed a slight up-regulation of ~1.2 fold with a good variation among the replicates, and *KRP-1* was observed to be down-regulated whereas, no signal was detected for *Ubcdp-27* gene at 72 hpi time point (Fig. 3.6).

(a) L1I52^T modulates the *DREB* gene expression in Pokkali rice plants under the brackish condition

The highly up-regulated genes *DREB1C* and *DREB*P1G are transcription factors belonging to the *DREB* family, well-known for stress tolerance in plants (Lata and Prasad, 2011). Moreover, *DREB* genes function as master regulators that modulate the expression of an array of downstream stress-inducible genes that code for multiple stresses, including salinity, drought, temperature, etc (Sarkar et al., 2019). Although the specific role of *DREB1C* and *DREB1G* in saline stress tolerance was not studied well until recently, a research study by Wang H et al have validated their role in rice salt stress tolerance through loss-of-function (LOF) mutant studies (H. Wang et al., 2022). However, only a few studies have reported the influence of bacterial colonization in modulating the *DREB* gene expression in plants, thereby conferring salt tolerance to plants. For example, *Glutamibacter* sp. YD01 inoculated

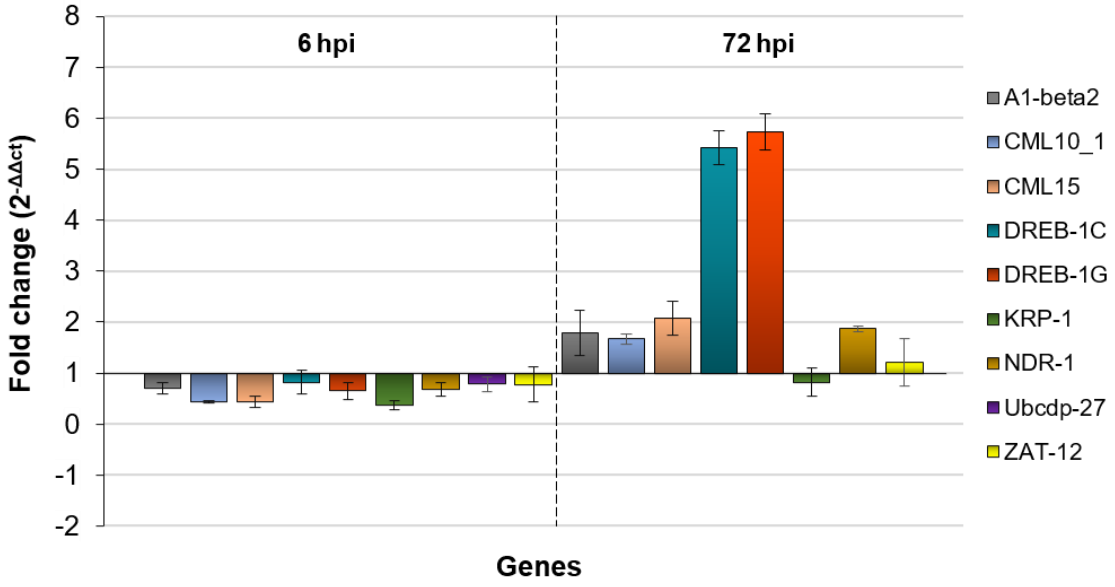


Fig. 3.6. *F. pokkalii* L1I52^T modulates host gene responses under brackish (50% NSW) condition: Bar graph representing the quantitative expression (expressed as fold-change ($2^{-\Delta\Delta ct}$)) of eukaryotic (Pokkali rice) genes modulated in the presence of *F. pokkalii* L1I52^T normalized against the expression in un-inoculated (control) plant expression using GAPDH as internal control gene.

with rice under salt stress induced the expression of *OsDREB2A* gene, offering salt tolerance to plants compared to the un-inoculated control rice plants (Ji et al., 2020). Further, a *DREB* family gene, *DREB2b* was upregulated in *Arabidopsis* upon colonization by *Enterobacter* sp. EJ01 compared to un-inoculated *Arabidopsis* seedlings on exposure to salt stress (K. Kim et al., 2014). Furthermore, a study has reported that *Bacillus megaterium* BP17 colonizes *Arabidopsis thaliana* Col 0 and upregulated a *DREB* subfamily transcription factor encoding gene that was reported to enhance salt stress (Vibhuti et al., 2017). All these studies indicate that *DREB* gene families are probable candidates that are differentially expressed upon certain beneficial bacterial associations complementing salt stress tolerance in plants. Supportive to this limited knowledge of *DREB* gene expression upon bacterial association, our preliminary findings on *DREB1C* and *DREB1G* gene expression modulation provide added evidence. However, we have demonstrated this L1I52^T modulated gene expression in Pokkali rice under natural mimicking-brackish conditions instead of artificial saline or NaCl that is studied to date. Hence, our study finds a significant relevance where the influence of the ecosystem is mimicked in the best possible way to understand the plant-microbe interactions at the molecular level.

Apart from *DREB* family genes, other genes also exhibited moderate gene expression modulation in Pokkali seedling roots colonized by L1I52^T cells. However, not many studies have demonstrated their role in bacteria-influenced gene expression modulation despite the information on their involvement in stress response and ion homeostasis (Table. 3.1). Also, the exact function of the A1beta-2 gene remains unclear. However, this altered gene expression from seedlings colonized by L1I52^T indicates that the novel *Flavobacterium* strain can influence the host gene responses under the tested brackish condition (50% NSW). Based on these preliminary leads, we performed an RNA-seq-based global transcriptome analysis to identify differentially expressed genes (DEG) in the host plant, Pokkali rice, with and without L1I52^T cells under the same brackish condition (50% NSW).

3.3.2.2 Insights into Pokkali rice root transcriptome responses upon colonization by L1I52^T under brackish condition

A high-throughput RNA sequencing was used to identify differentially expressed genes (DEGs) in Pokkali rice roots upon colonisation by L1I52^T. In this experiment, the transcript responses from the roots of Pokkali rice seedlings grown in brackish condition (half-strength Hoagland's nutrient solution amended with 50% NSW) in the presence (test treatment) and absence (control treatment) of L1I52^T cells were analysed (3rd dpi) (see method section for

details). Each treatment had two biological replicates. To identify the DEGs from the dataset, a fold change of >2 (Log2FC>1) with an FDR (False Discovery Rate) adjusted *p*-value of <0.05 was set. These criteria identified around 2710 genes differentially expressed between the control and L1I52^{T}-treated Pokkali seedling roots. Further screening from this pool of transcripts revealed 45 DEGs with high significance (expresseion level indicated by Log10 (FPKM+1)) (Fig. 3.7). Interestingly, most of these DEGs belonged to the light-harvesting complex (LHC) and Calvin cycle, constituting the integral photosynthesis components that regulate plant growth and development. Thirteen genes belonging to the light-harvesting complex (LHC) were highly expressed in L1I52^{T}-colonized Pokkali rice roots compared to the control (Fig. 3.7). It included the following: LOC4324599 (chlorophyll a-b binding protein (CAB) 2, chloroplastic), LOC4343709 (photosystem I CAB 2, chloroplastic), LOC4333359 (CAB protein, chloroplastic), LOC4346803 (CAB 1, chloroplastic), LOC4340892 (CAB 1B-21, chloroplastic), LOC4343583 (CAB CP29.1, chloroplastic), LOC4328623 (CAB 8, chloroplastic), LOC4343604 (CAB of LHCII type III, chloroplastic), LOC4350176 (CAB CP26, chloroplastic), LOC4345663 (CAB P4, chloroplastic), LOC4324705 (CAB protein of LHCII type 1), LOC4344899 (photosystem II 10 kDa polypeptide, chloroplastic isoform X1), and LOC4336028 (CAB protein CP-24, chloroplastic) (Fig. 3.7). The Light-harvesting chlorophyll a-b binding proteins (Lhc) are a class of antennae proteins of the LHC that serve in both photosystems (PS), but mainly in the photosystem II (PSII) (Umate, 2010). They are primarily associated with synthesis of plant pigments such as chlorophyll and xanthophyll, and further complement in photoprotection under stress conditions (Umate, 2010). Additionally, 6 major enzyme-encoding genes involved in the Calvin cycle were up-regulated in the L1I52^{T}-colonized roots compared to the control. It includes LOC4352014 (ribulose bisphosphate carboxylase (RuBisco) small chain A, chloroplastic) and LOC4352021 (RuBisco small chain, chloroplastic) that contribute to the RuBisco catalytic activity (Fig. 3.7). Further, LOC4351224 (ribulose bisphosphate carboxylase/oxygenase (Rubisco) activase (RCA), chloroplastic), known for its role in photosynthesis regulation, was also up-regulated in rice roots upon L1I52^{T} colonization (Fig. 3.7). RCA is a multiple responder to abiotic stress in rice(Chen et al., 2015). Next, the LOC4336044 gene encoding glyceraldehyde-3-phosphate dehydrogenase A (GAPDH), chloroplastic that catalyses the single reductive step in the Calvin cycle of photosynthetic carbon assimilation, was highly expressed in the presence of L1I52^{T} (Fig. 3.7) (Sparla et al., 2005). The LOC4349897 coding for fructose-bisphosphate aldolase (FBA), chloroplastic, a key plant enzyme involved in carbohydrate metabolism

including glycolysis, gluconeogenesis, and the Calvin cycle, thus contributing towards the plant growth and development, was highly expressed in L1I52^T-colonized roots (Lv et al., 2017). Within the Calvin cycle, FBA catalyses the formation of fructose 1,6-bisphosphate and sedoheptulose 1,7-bisphosphate (SBPase). Further, LOC4335227 coding SBPase, chloroplastic, was also highly expressed upon L1I52^T colonisation (Fig. 3.7). SPBase activity regulates photosynthetic carbon fixation, influencing plant growth and yield ((Ding et al., 2016; Driever et al., 2017)). The overexpression of the SPBase encoding gene in transgenic plants has been identified to have profound effects on plant growth and conferore tolerance to abiotic stress (Ding et al., 2016; Driever et al., 2017). All the above-mentioned 6 protein-encoding genes involved in the Calvin cycle have been reported to be affected by salt stress, as inferred from the proteome response studies performed in several plants (H. Zhang et al., 2012). Hence, it is assumed that the increased expression of these genes upon L1I52^T-colonization would offer enhanced saline tolerance to the host rice plants. Furthermore, two genes, LOC4324479 and LOC4342370, coding for oxygen-evolving enhancer protein 1 (OEE1), chloroplastic, and OEE2 chloroplastic, respectively, are expressed more in L1I52^T-colonized rice roots (Fig. 3.7). These genes are reportedly known to play essential roles through overexpression in plant responses to salt stress, and in our analysis, the expression of these protein-encoding genes was further enhanced upon L1I52^T colonization (Saleethong et al., 2016; Zhou et al., 2023).Glycine-rich proteins (GRP) that contain more than 60% glycine constitute a major part of plant cell walls that actively participate in plant defence mechanisms, especially different abiotic stress. Relative to saline stress, studies have shown that overexpression of GRPs has offered higher salt tolerance in transgenic lines compared to wild-type plants (Ortega-Amaro et al., 2015; Sanan-Mishra et al., 2002; C. Wang et al., 2012). In this regard, two genes coding for glycine-rich cell wall structural proteins: LOC9269383 (glycine-rich cell wall structural protein (GRP)) and LOC4348767 (GRP- 2) were up-regulated in L1I52^T-colonized roots cells compared to the non-colonised control rice roots. (Fig. 3.7).

Another important enzyme related to cell wall development up-regulated upon L1I52^T colonisation was LOC4331102, which codes for cinnamoyl-CoA reductase 1-like protein (Fig. 3.7). This protein is a critical member of lignin biosynthesis and contributes to defence signalling (Kawasaki et al., 2005). The altered expression of other genes between non-colonised (control) and L1I52^T-colonized Pokkali roots is represented in the heatmap (Fig. 3.7). The up-regulation of LHC- and Calvin cycle-associated genes upon L1I52^T

colonisation possibly hints at the ability of this novel *Flavobacterium* strain to modulate the gene responses of the host plant, especially those related to its photosynthesis, and associated plant growth.

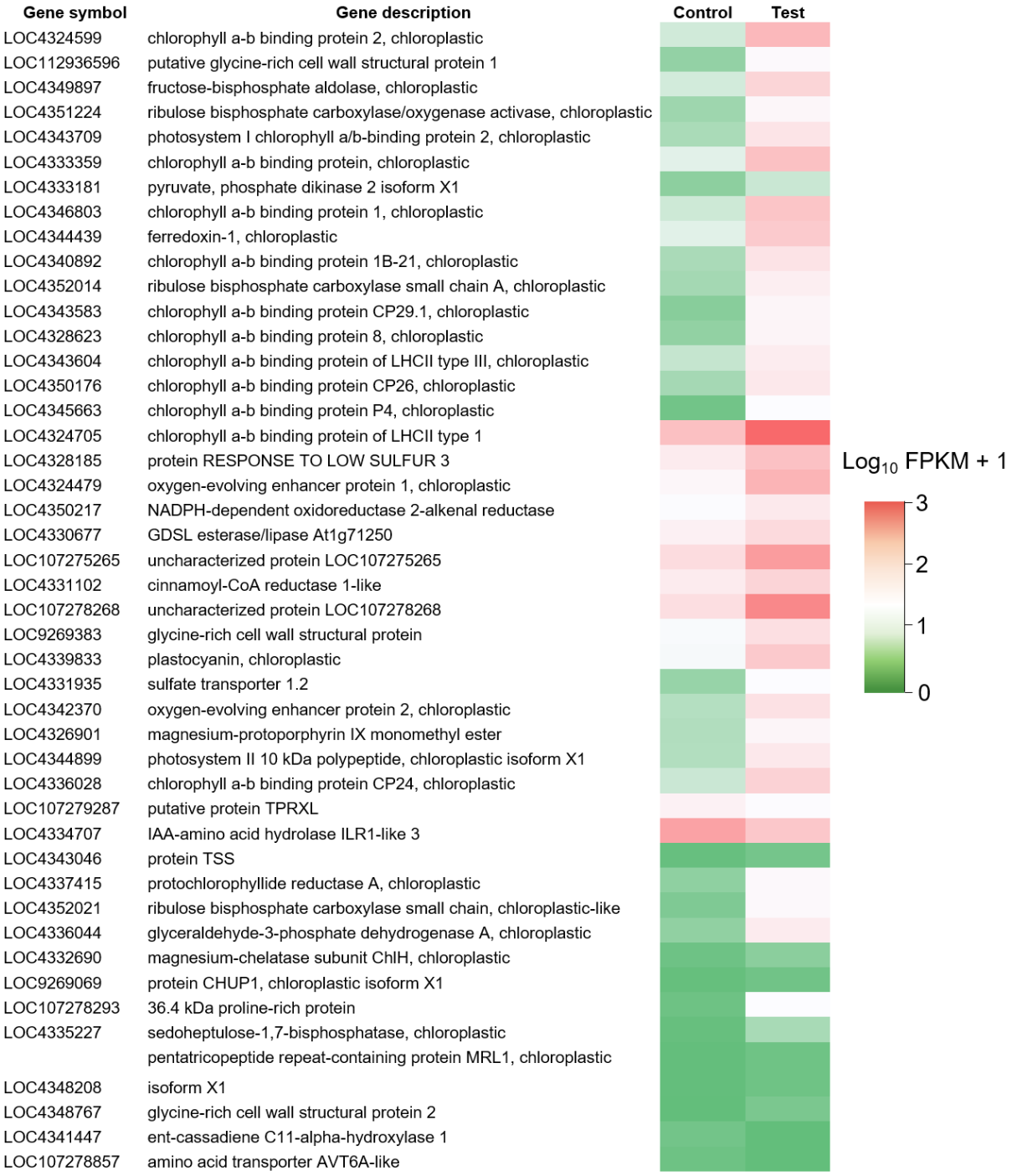


Fig. 3.7. Heatmap representing the differentially expressed genes (DEGs) modulated between the non-colonized (control) and L1I52^{T}-colonized (test) Pokkali rice roots of seedlings grown in brackish condition (50% NSW). The presented 45 genes (Log10 (FPKM+1)) in the heatmap are a part of the 2710 up-regulated and down-regulated significant DEGs (Log2FC; FDR<0.05)

Finally, the host-gene expression data quantified through the RT-qPCR (selected set of 8 rice genes as mentioned earlier) was correlative with the expression profile derived from the RNA-Seq-derived transcriptome data (Table. 3.3). Thus, these gene expression profile comparisons could be considered as a validation for the host-gene modulation by L1I52^T cells. Summarising the findings, we hypothesize that the enhanced plant growth-related parameters and chlorophyll content quantified from the *in-planta* pot studies performed under brackish conditions could be a probable outcome contributed by the functions of these up-regulated genes upon L1I52^T colonization (Table 3.3; Fig. 3.7).

Table. 3.3. Table comparing the expression profile of the modulated genes in Pokkali rice roots upon colonization by L1I52^T as validated through RT-qPCR and RNA-Seq-based approaches. Among the test 8 genes, 6 genes displayed similar expression profile in both RT-qPCR and RNA-Seq-based analysis.

S. No.	RT-qPCR analysis			RNA-Seq analysis			
	Genes	$2^{-\Delta\Delta ct}$	Gene-expression	Control	L1I52^T	Log2FC	Gene-expression
1	***A1-beta2***	1.7865	Up-regulation	146.273	145.104	-0.01158	Down-regulation
2	***CML10_1***	1.67	Up-regulation	96.7804	124.199	0.359871	Up-regulation
3	***CML15***	2.0765	Up-regulation	147.244	178.022	0.273842	Up-regulation
4	***DREBP-1C***	5.4265	Up-regulation	102.571	147.026	0.519446	Up-regulation
5	***DREBP-1G***	5.7285	Up-regulation	7.9264	8.92148	0.170619	Up-regulation
6	***KRP-1***	0.8215	Down-regulation	233.476	245.553	0.072763	Up-regulation
7	***NDR-1***	1.866	Up-regulation	100.106	152.909	0.61114	Up-regulation
8	***ZAT-12***	1.2205	Up-regulation	151.281	184.431	0.285848	Up-regulation

3.3.3 Eco-physiological traits of L1I52^T

3.3.3.1 L1I52^T grows optimally in brackish conditions

Saline-tolerant Pokkali rice cultivated in brackish water submerged fields encounter fluctuations in the ion content, caused due to seawater intrusions, thereby posing varied salinity, majorly represented by NaCl. Thus, adaptation and tolerance to changing salinity stress is highly essential for its microbial inhabitants to survive and adapt gradually. Earlier studies have indicated that most terrestrial *Flavobacterium* strains are susceptible to NaCl compared to their marine counterparts, which can tolerate NaCl above 2%. Hence, we initially checked the NaCl tolerance L1I52^T cells along with their phylogenetic neighbours.

The results showed that L1I52^T cells could tolerate up to 3% NaCl while the nearest phylogenetic neighbours from terrestrial habitat (*F. daejeonense* KACC 11422^T, and *F. glycines* ICMP 17618^T) and freshwater habitat (*F. sufflavum* KCTC 52809^T) failed to grow in 3% NaCl amended broth (Fig. 3.8) (Menon et al., 2020). Likewise, the growth of L1I52^T was also checked in varying natural seawater (NSW) concentrations: 15%, 30%, 50%, 80%, and 100% NSW prepared in R2A broth. Interestingly, 30% NSW was the optimum among the tested seawater concentrations, followed by 50%, 15%, and 80% NSW concentrations (Fig. 3.9 (a)). However, weak growth was comparatively observed in 100% NSW-amended

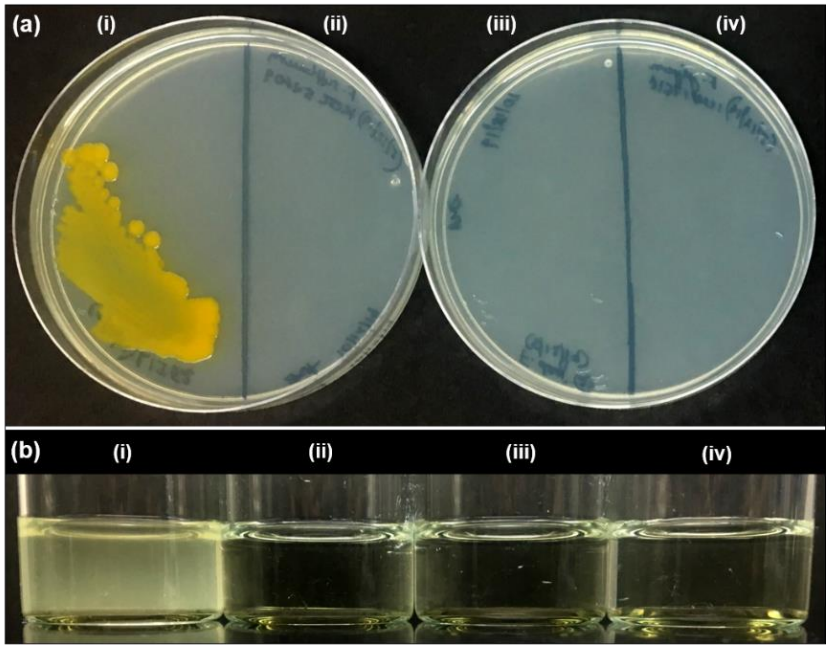


Fig. 3.8. *F. pokkalii* L1I52^T cells grow in brackish conditions. Unique phenotypic characters that differentiate *F. pokkalii* L1I52^T from its phylogenetic neighbours: **(a)** Growth in Zobell marine agar, and **(b)** Growth in R2A broth amended with 2% NaCl.
(i) *F. pokkalii* L1I52^T, **(ii)** *F. sufflavum* KCTC 52809^T, **(iii)** *F. glycines* ICMP 17618^T, and **(iv)** *F. daejeonense* KACC 11422^T

condition after seven days of incubation. This probably indicates that L1I52^T is a terrestrial bacterium that evolved with probable brackish adaptive traits required for its survival and fitness to sustain itself in brackish environments. Next, we compared the growth of L1I52^T cells between R2A3 (R2A + 30% NSW) and artificial saline (R2AN; R2A + 0.5% NaCl), and non-saline (R2A) conditions. Interestingly, we observed that L1I52^T cells grew optimally in the R2A3 condition compared to R2AN and R2A conditions, which indicates that the brackish condition offered an optimal growth condition for the L1I52^T cells (Fig. 3.9 (b)). Also, it is essential to note that the difference in the absorbance (OD_{600}) corresponding to the R2A3 condition in the two graphs is due to the change in inoculum size

and the working volume used in the experiments (Fig. 3.9 (a, b)). Thus, the growth studies indicate that L1I52^T cells can grow in variable seawater concentrations with altered growth rates, a trait typically known for bacteria originating from brackish environments. Altogether, the findings indicate the potential ability of this novel *Flavobacterium* strain to balance its growth based on the fluctuating brackish conditions. Supportively, other novel strains isolated from the Pokkali rhizosphere (*Novosphingobium pokkalii* L3E4^T, and

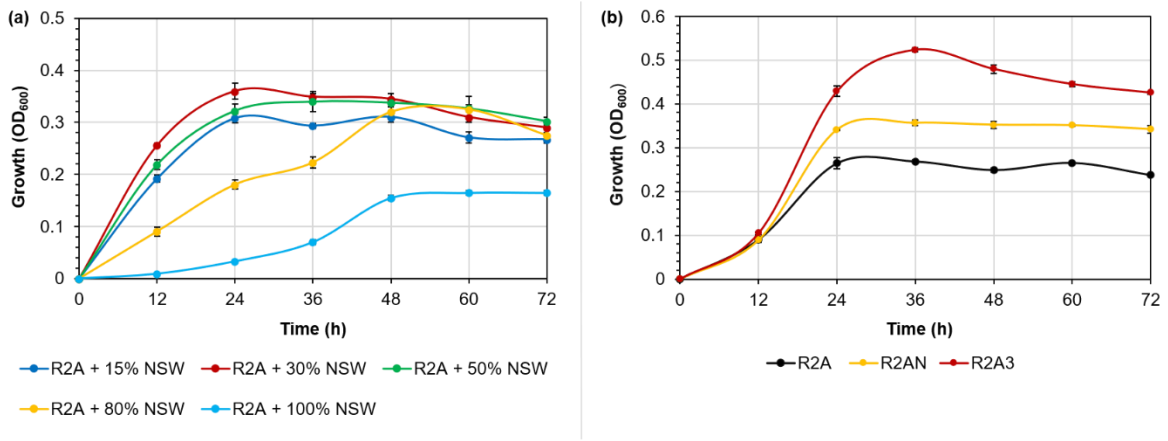


Fig. 3.9. *F. pokkalii* L1I52^T grows optimally in brackish (30% seawater/NSW-amended) condition: **(a)** Growth curve showing the growth of *F. pokkalii* L1I52^T cells in R2A broth base amended with varied seawater concentrations (15%, 30%, 50%, 80%, and 100%), **(b)** Growth curve of *F. pokkalii* L1I52^T cells in R2A, R2AN (R2A + 0.5% NaCl), and R2A3 (R2A + 30% NSW)

Sphingomonas pokkalii L3B27^T) by our group also showed growth in seawater amended medium. Hence, the growth ability in seawater-amended conditions could be regarded as an eco-adaptive trait unique to rhizobacterial strains native to Pokkali rice.

3.3.3.2 Growth physiology of L1I52^T in synthetic marine media

Further, we studied the growth ability of L1I52^T cells in a synthetic marine medium to expand the understanding of their saline-adaptation physiology. For this, we used the Zobell Marine Broth 2216 medium, which mimics the seawater composition and is hence widely used for the cultivation of marine bacteria. Interestingly, we observed an altered phenotype exhibited by the L1I52^T cells when grown in different conditions, i.e., in broth and solid/semi-solid states of Zobell marine media. Intriguingly, L1I52^T cells grew very well on ZoBell marine broth amended with agar (ZMA) but failed to grow in ZoBell marine broth (ZMB) after repeated inoculations (Fig. 3.10 (a, b-i)). Surprisingly, the growth of L1I52^T cells was restored upon the addition of 0.18% soft agar to the ZMB, forming a subsurface pellicle after seven days of incubation, as visibly seen in Fig. 3.10 (b-ii, iii). This experiment was repeated three independent times, and similar results were obtained, indicating that

L1I52^T cells can survive as non-growing cells in ZMB. However, these non-growing cells could multiply and grow in ZMB upon supplementation of agar, which acts as a substrate for the cells to attach and multiply. This finding attracted us to relate this unique growth lifestyle as a possible niche adaptation of L1I52^T cells that might contribute to its ability to survive as planktonic (non-dividing) cells in the brackish water in the absence of the host plant (during the prawn cultivation season of Pokkali farming). Subsequently, when the Pokkali cultivation starts, the survived planktonic L1I52^T cells attach to the Pokkali rice

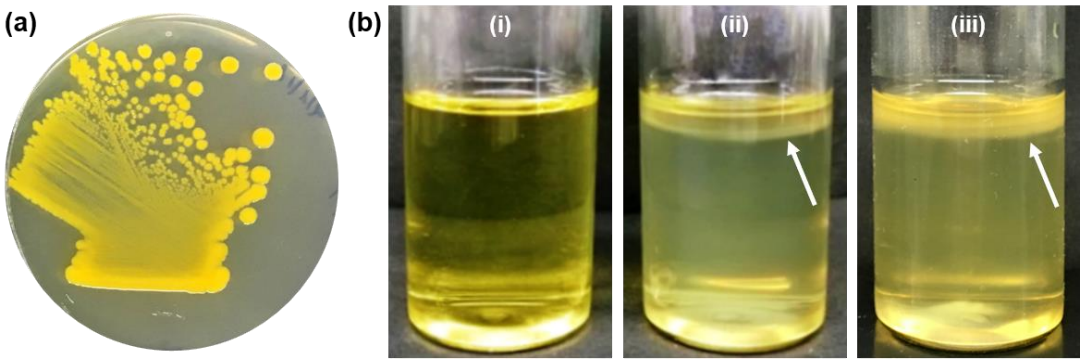


Fig. 3.10. Physiological growth lifestyle of *F. pokkalii* L1I52^T in ZoBell marine media: **(a)** Positive growth in ZoBell marine agar (ZMA) after 4 days at 30°C, **(b)** Growth phenotype in Zobell marine broth (ZMB): **(i)** Negative growth in ZMB after 7 days of incubation at 30°C, (ii, iii) Positive growth of *F. pokkalii* L1I52^T cells in ZMB after addition of 0.18% agar after 7 days at 30°C. Arrow's in b-ii, iii images denote the positive growth of *F. pokkalii* L1I52^T cells as indicated by sub-surface pellicle.

roots and colonize to establish themselves. This might be one of the strategies that enables this potential *Flavobacterium* strain to survive under non-host conditions. Further, their survival ability in brackish (seawater-amended) conditions is discussed in the upcoming section, supporting their potential to remain as planktonic cells for longer, enabling their long-term co-adaptation in this unique brackish ecosystem. Moreover, the culturing of L1I52^T equivalent strains from the Pokkali roots after 4 years supports evidence that strengthens the claim that the L1I52^T strain is a native of Pokkali rice and ecosystem. These findings highlight an important adaption trait of L1I52^T cells that enables survival as non-growing cells under varying seawater concentrations. This eco-physiological trait is assumed to help them survive in brackish conditions during stages when it is not attached to the host rice roots. A similar adaptation mechanism has been reported in certain marine bacterial groups associated with algae, diatoms, and open surface seawater of different oligotrophic marine environments. However, the information on plant-associated *Flavobacterium* strains isolated from brackish environments are not reported to date. To our knowledge, this may be the first data about the unique physiological lifestyle of a

Flavobacterium strain.

3.3.3.3 Brackish condition enhances biofilm formation, seed attachment, and root attachment abilities of L1I52^T

Biofilm formation is regarded as a characteristic attribute in many rhizobacteria that significantly contributes to firm plant-bacterial interaction (Knights et al., 2021). Also, the role of biofilm-forming rhizobacteria has been studied for its advantage in aiding tolerance to different abiotic stresses (Bhagat et al., 2021; Karimi et al., 2022). More importantly, the biofilm formation ability of rhizobacteria assists in the initial attachment to host plant root surfaces, which is critical for establishing plant-bacterial interaction (Amaya-Gómez et al., 2020). In this context, we tested the biofilm formation ability of L1I52^T cells under three different *in-vitro* conditions: R2A, R2AN (R2A + 0.5% NaCl, and R2A3 (R2A + 30% NSW). Interestingly, we found that the biofilm formation ability of L1I52^T cells was enhanced in the brackish condition i.e., R2A3 condition (Fig. 3.11 (a)). This observation supports the eco-adaptive behaviour of L1I52^T cells. However, these are *in-vitro* condition-tested observations, and in nature, it will not be the artificial abiotic surfaces and media-equivalent conditions available for the bacteria to form biofilm or initiate initial attachment to plant surfaces. The adhesion of bacterial cells to seeds is a critical first step during bacterial colonization of any rhizobacteria to the plant media or nutrients (see methods section for details). The major aim of this experiment was to analyze if L1I52^T cells were capable of binding to sterile Pokkali rice seed surfaces under brackish conditions. Even after several rounds of vigorous vortexing and washing, growth of L1I52^T colonies were observed

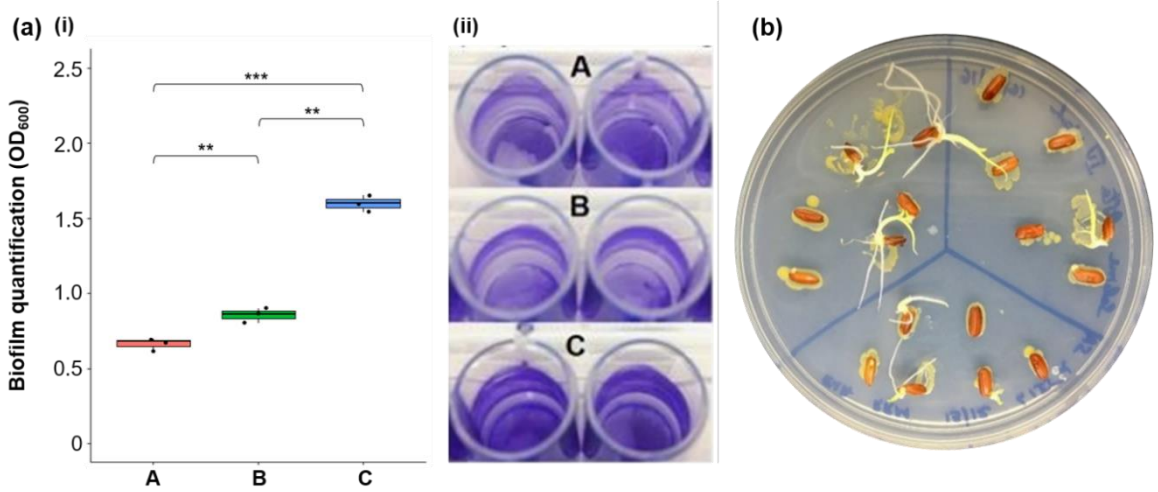


Fig. 3.11. (a) Brackish condition enhances *in-vitro* biofilm formation by *F. pokkalii* L1I52^T: **(i)** Box plot representing the biofilm quantification, and (ii) Image showing crytal-violet stained rings by *F. pokkalii* L1I52^T cells when grown under different conditions: A –R2A, B – R2AN, C – R2A3, **(b)** *F. pokkalii* L1I52^T cells firmly attach to seed surfaces in the 100% NSW condition. Statistical significance represented by *, p-value<0.05; **, p-value<0.01; ***, p-value<0.01.

around most of the seeds placed in R2A3 agar medium (Fig. 3.11 (b)), suggesting that L1I52^T Rather, it might be during the seedling growth stage of rice plants. Moreover, the bacterial attachment to roots is considered the first physical step in many plant-microbe interactions (Wheatley and Poole, 2018). Hence, we wanted to know whether L1I52T cells can attach to Pokkali seedling root surfaces under brackish conditions. To check this, we performed a root- attachment assay wherein we compared the attachment ability of L1I52^T cells to the roots of Pokkali seedlings incubated in brackish and non-brackish conditions. The dilution spot assay could only recover L1I52^T cells from the crushed roots of seedlings

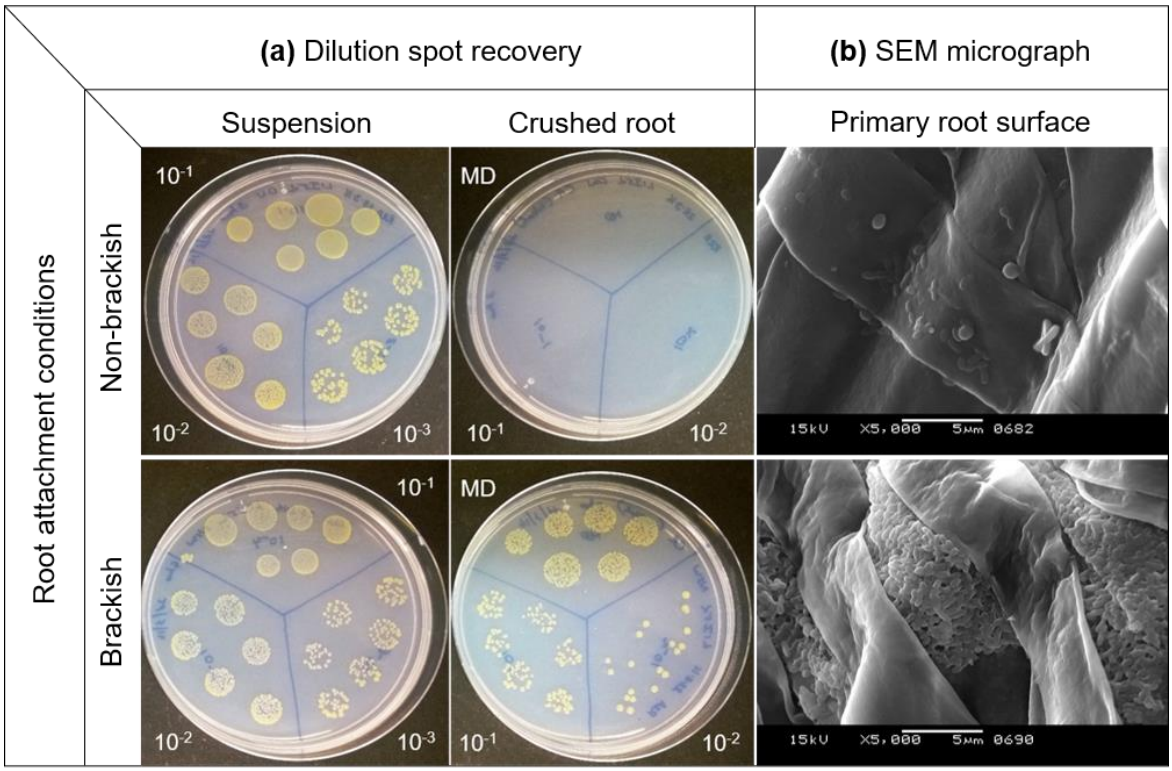


Fig. 3.12. Brackish condition enhances root attachment ability of *F. pokkalii* L1I52^T cells: **(a)** Dilution spot assay recoveries of *F. pokkalii* L1I52^T cells indicating the strong attachment of *F. pokkalii* L1I52^T cells on Pokkali rice roots in brackish (100% NSW) condition whereas no colonies recovered from non-brackish (0% NSW) condition, **(b)** SEM micrographs showing the root sections of *F. pokkalii* L1I52^T attached Pokkali rice roots incubated in 0% (non-brackish) and 100% NSW (brackish) conditions.

incubated in brackish condition (100% seawater). In contrast, no cells were recovered from the crushed roots of seedlings incubated in non-brackish condition (0% NSW) (Fig. 3.12(a)). Intriguingly, as evident from Fig. 3.12 (a), L1I52^T cells recovered from the suspensions of non-brackish conditions contrasting to the crushed roots, where no cells were recovered, means that even though L1I52^T cells survived in non-brackish conditions, they require a brackish condition for the attachment to root surfaces (Fig. 3.12 (a)). To validate this observation further, we performed the SEM imaging of the L1I52^T cell attached seedling

roots incubated in both conditions as checked for recovery. Correlatively, in the SEM micrographs as well, we could observe only a countable number of cells in one location on the primary root surface of seedlings incubated in non-brackish conditions, contrasting to heavily attached L1I52^T cells along the primary root surfaces in brackish condition (Fig. 3.11 (b)). This observation was unique, which signifies the importance of brackish condition for this novel *Flavobacterium* strain to attach with its host at the initial growth stage, which helps in its colonization. Similar ability of root attachment in 100% seawater condition was observed in *N. pokkalii* L3E4^T, a novel plant-beneficial rhizobacterial isolated from Pokkali rhizosphere earlier (Krishnan et al., 2017). Hence, we assume that brackish conditions play a crucial role in the root attachment process of rhizobacteria strains native to Pokkali ecosystem, which is a key step to the successful host-root colonization.

3.3.3.4 Brackish condition enhances the host root colonization by L1I52^T

Colonization of plant roots is a critical step in plant-microbe interaction, and several factors influence this multi-stage process (Badri et al., 2009). Among these factors, the environment or the surrounding ecosystem is thought to be highly significant in guiding bacterial host colonization (Badri et al., 2009). Hence, the colonization efficiency of L1I52^T cells was studied under an *in-planta* hydroponic setup using Hoagland's nutrient solution as the base maintained at different conditions: non-brackish (0% NSW) and brackish conditions (30% and 50% NSW) (detailed in method section). The colonization efficiency was analysed by quantifying the L1I52^T cells recovered from the roots of seedlings grown in different conditions at varied time intervals. Interestingly, increased recovery of L1I52^T cells was observed in brackish conditions compared to non-brackish/non-saline conditions. The experiment started with 6.3-Log cells of L1I52^T, and the recoveries on 1st dpi (24 h post-inoculation), in non-brackish condition (0 % NSW), and 30% brackish condition was recorded at 6.8-Log cells whereas, in 50% brackish condition, it was slightly higher than recorded at 7-Log cells (Fig. 3.13 (a)). Further, the recovery on 3rd dpi (72 h post-inoculation), the recovery rates from brackish conditions: 30% NSW and 50% NSW was quantified to be 7.4-Log, and 7.7-Log L1I52^T cells respectively, whereas, in non- brackish condition, the recovery dropped to 6.4-Log cells (Fig. 3.13 (a)). Finally, the recovery checked after 1 week (7th dpi/168 h post-inoculation) recorded a further increased colonization of L1I52^T cells in brackish conditions: 30% NSW and 50% NSW at 7.7-Log, and 8.2-Log cells, respectively (Fig. 3.13 (a)). However, in the case of non-brackish condition, the recovered cells were maintained at 6.4-Log till 7th dpi, equivalent to the 3rd

dpi recovery (Fig. 3.13 (a)). Further, to support the proliferation of L1I52^T cells along the roots of Pokkali seedlings over time, we performed a qPCR-based quantification L1I52^T-*tuf* gene from the total DNA isolated from the roots of seedlings grown in 30% NSW brackish condition. The *tuf* gene copies were quantified at three different time points: 0th (3 h post-inoculation), 4th, and 7th dpi. As expected, the *tuf* gene copies corresponding to the L1I52^T cell load along the Pokkali seedling root increased from 0 to 4th to 7th dpi, as represented in 3.13 (b). The increased *tuf* gene copies firmly validated the proliferation ability of L1I52^T cells in the roots of Pokkali seedlings, supporting the colonization assay. Finally, to further

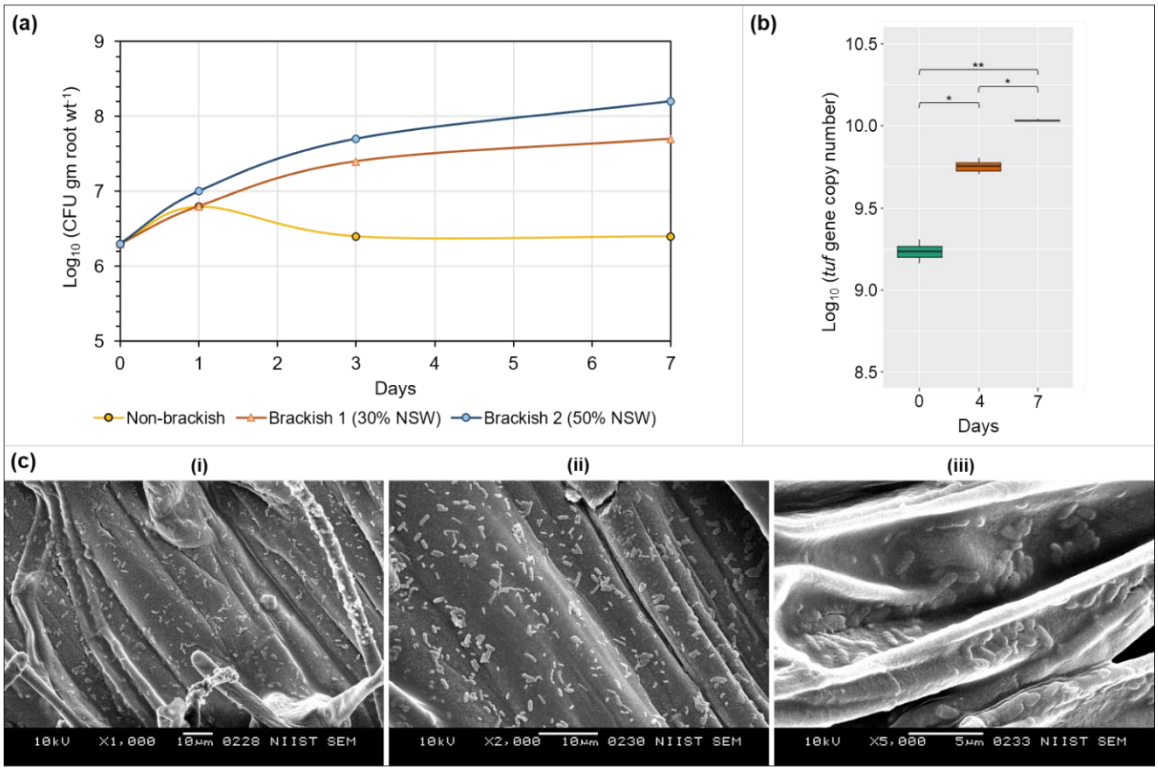


Fig. 3.13. Brackish condition favours the colonization of *F. pokkalii* L1I52^T cells along the host roots: **(a)** Recovery of *F. pokkalii* L1I52^T cells from the colonization assay performed in non-brackish, and brackish conditions (30%, and 50% NSW), **(b)** Box plot representing the *tuf* gene copy numbers quantified from total DNA through qPCR on 0 (3 h post-inoculation, 4th, and 7th dpi indicating the proliferation of *F. pokkalii* L1I52^T cells along the Pokkali seedling roots, and **(c) - i, ii, iii** SEM micrographs displaying the colonization of rod-shaped *F. pokkalii* L1I52^T cells along the roots of Pokkali seedlings.

support the colonization of L1I52^T cells along the roots of Pokkali seedlings, a SEM-based imaging was performed on the root sections of Pokkali rice seedlings colonized by L1I52^T cells grown in 30% NSW brackish condition (3rd dpi). The SEM micrographs from different root sections taken at different magnifications clearly showed a very good population of rod-shaped L1I52^T cells supporting the enhanced colonization of the novel *Flavobacterium* strain on its host plant root (3.13 (c)). Altogether, the findings from colonization assay

validated through crushed root recovery, *tuf*-gene based qPCR detection and SEM imaging confirmed that brackish conditions significantly enhance the root colonization ability of L1I52^T cells.

The critical findings from *in-vitro* and *in-planta*-based eco-physiological characterization studies imply the link mediated by the brackish condition between L1I52^T cells and its host, Pokkali rice. Even though previous studies from our group have reported preliminary insights on the impact of seawater on root attachment by other rhizobacterial strains, a detailed characterization, as performed in L1I52^T, is lacking. Therefore, further in-depth studies with potentially diverse rhizobacterial strains are necessary to unravel the fundamental link between brackish environments and plant-microbe interaction.

3.3.4 Plant growth promoting traits of L1I52^T

Next, we checked for in vitro plant beneficial properties such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, phosphate solubilization, growth in nitrogen-free minimal medium, hydrogen cyanide, indole acetic acid (IAA) and siderophore production in L1I52^T to relate possible plant beneficial traits mediating the growth promotion. In this analysis, L1I52^T did not show any promising beneficial plant traits that can be correlated to its improved plant growth promotion in Pokkali rice. However, L1I52^T exhibited weak phosphate solubilization and siderophore production (refer to Fig. 3.18 in genome section). Similar results were observed in other studies, wherein the reported plant-associated *Flavobacterium* strains displayed weak or negative reaction to most of the in vitro PGPR traits tested (Soltani et al., 2010). These results further emphasize that plant-associated *Flavobacterium* species are not well known for their multiple phytobeneficial traits, unlike the other rhizobacteria belonging to genera such as Pseudomonadota, and Bacillota, that possess multiple PGP traits (Lugtenberg and Kamilova, 2009). Because of this, the plant-beneficial aspects of *Flavobacterium* strains are not fully explored despite being dominant members in various plant rhizospheres. Moreover, their dom

3.3.5. Genomic traits of L1I52^T

3.3.5.1 General genome features

The high-quality draft genome of L1I52^T comprised a single circular chromosome of size 3,894,211 bp (69 contigs with length>500 bp) with an overall G+C content of 34.9% (Fig. 3.14; Table. 3.4). The genome was predicted to encode 3,236 protein-coding genes, 69 tRNA genes, and one rRNA operon with no evidence of plasmids. Through CheckM analysis, the genome completeness was estimated to be 99.65%, with contamination of 0.07%, indicating

the high quality of the draft genome. The general genome features of L1I52^T and its phylogenetic neighbours are listed in Table. 3.3. Further, the subsystem features analyzed through RAST-based analysis are compared in detail between L1I52^T, its phylogenetic neighbours, and other native *Flavobacterium* strains isolated from Pokkali roots.

Table 3.4. General features of *F. pokkalii* L1I52^T genome

Genome features	Value
Genome size (bp)	3,894,211
No. of contigs	69
DNA coding (bp)	3,458,312
DNA G+C (%)	34.94
Total genes	3,287
Protein coding genes	3,236
RNA genes	51
Hypothetical proteins	965
Genes with function prediction	2,271
Genes with Pfam domains	881

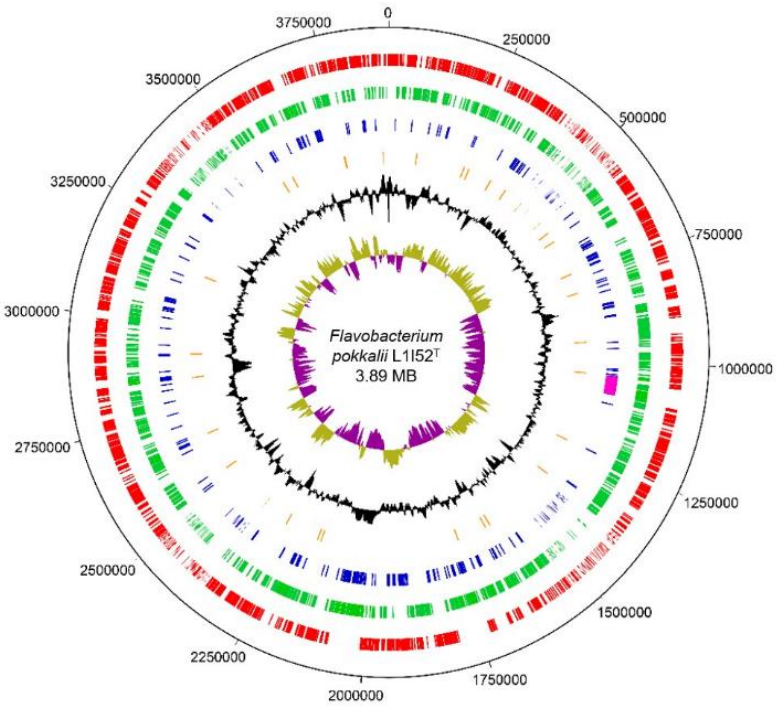


Fig. 3.14. Circular genome map of *F. pokkalii* L1I52^T strain: Ring 1 – GC content, Ring 2 – GC skew, Ring 3 – tRNA genes, Ring 4 – Polysaccharide utilization loci's (PULs) (Magenta block: Xylan PUL), Ring 5 – Predicted CDS in the counter-clockwise and Ring 6 – predicted CDS in clockwise direction.

To begin, we used SEED subsystem-based functional categorization system to obtain initial insight into the broad classification of L1I52^T genome features relative to its metabolism, host functions and niche adaptation as a plant-associated rhizobacteria. This analysis revealed that the major portion of the annotated protein-coding genes were assigned to the metabolism of carbohydrates (386), and amino acids and their derivatives (250), indicating L1I52^T specialized in the uptake of plant-derived substrates. In addition, the genome was represented by the highest number of genes involved in the metabolism of DNA (77), RNA (118), and proteins (154), reflecting its active cellular machinery. Interestingly, the genome was found to contain very few genes related to prophages and transposable elements, a feature commonly found in most genomes of plant endophytic bacteria (Mitter et al., 2013). Although, phages and mobile elements are not quite often reported in *Flavobacterium* genomes and if it is present the number of mobile elements is not directly linked to its genome size (Tekedar et al., 2017). However, the presence of few mobile elements in the L1I52^T genome may indicate low genetic recombination events and lateral gene transfer, reflecting its high genome stability and possible adaptation to a restricted host environment (Krause et al., 2006). The distribution of genes in L1I52^T and its phylogenetic neighbours classified under different SEED functional category subsystems are listed in Table 3.5.

3.3.5.2 Energy metabolism

The L1I52^T genome harbours a complete set of genes coding for all central carbohydrate metabolic pathways; glycolysis (Embden-Meyerhof-Parnas) pathway, citrate (Krebs) cycle, and pentose phosphate (PP) pathway. However, the genome annotated to have an incomplete Entner-Doudoroff (ED) pathway but encodes genes for 2-dehydro-3-deoxygluconokinase and 2-dehydro-3-deoxyphosphogluconate aldolase which converts 2-dehydro-3-deoxygluconate and 2-dehydro-3-deoxyphosphogluconate to pyruvate, respectively. To convert pyruvate into acetyl-CoA, the genome encodes pyruvate dehydrogenase and possesses genes required for synthesizing fatty acids using acetyl-CoA. The Krebs cycle is found to be intact but without a glyoxylate pathway. The presence of an intact PP pathway in the genome of LI152^T reveals its potential uptake of plant-derived pentose sugars. In complement, the genome codes for genes that transport and metabolise pentose sugars such as L-arabinose, D-ribose, and D-xylose. Notably, based on the cell fluxes metabolic modelling, the plant sugars such as xylose, L-rhamnose, D-ribose, maltose, and maltohexaose were the most uptaken plant carbohydrates from the environment. These genome findings suggest that plant-derived carbohydrates are the most preferred substrates

for L1I52^T growth in the plant rhizosphere. These genome-based inferences supported the results of the phenotypic assays where L1I52^T showed growth on minimal media amended with plant-derived polysaccharides such as pectin and xylan. Additionally, plant-derived monosaccharides such as L-arabinose, D-fructose, galactose, D-glucose, D-mannose, L-rhamnose and L-xylose, the disaccharides maltose and D-sucrose were also utilized. However, the inference from growth experiments implies that L1I52^T cells grow very well in the presence of polysaccharides compared to monosaccharides (discussed in PUL section).

3.3.5.3 Respiration

Several genes encoding for enzymes that generate energy via oxidative phosphorylation pathway were identified in the genome of L1I52^T, which is indicative of aerobic respiration and it is in line with most reported *Flavobacterium* strains (Bernardet et al., 2002). The L1I52^T genome encoded genes for complex I (NADH dehydrogenase; NuoA-N genes), complex II (Succinate dehydrogenase; SdhCAB genes), complex IV (Cytochrome c oxidase; CyoE, CoxCAB and COX15 genes) and complex V (F-type ATPase), but did not contain genes for complex III. Further, the genome harboured cbb3-type cytochrome C oxidase, an enzyme primarily induced under oxygen-limiting conditions and critical for bacterial respiration in microaerobic environments (Pitcher et al., 2002). Moreover, in the case of Pokkali fields, the rhizosphere regions and root surfaces of Pokkali rice roots will possess limited oxygen due to the continuous logging of brackish water. Further, to support this view, we proved experimentally that L1I52^T cells prefer to position themselves in a microaerobic condition indicated by the formation of a subsurface pellicle when tested in various semisolid nutrient media such as R2A, and ZMA (Fig. 3.15). Interestingly, microaerophile growth is observed as a characteristic phenotype in many plant-associated bacteria, especially endophytic diazotrophs, where they use root microaerophilic environments to fix atmospheric nitrogen (Hurek et al., 1995). However, no experimental or genomic evidence was observed supporting the anaerobic growth for L1I52^T.

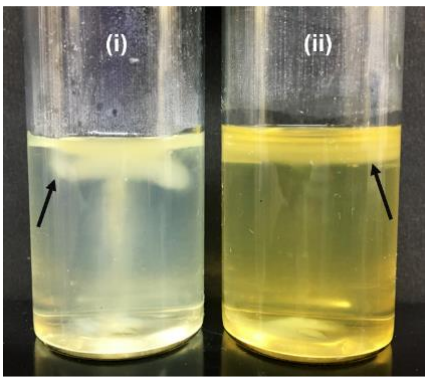


Fig. 3.15. *F. pokkalii* L1I52^T form sub-surface pellicle in semi-solid nutrient media: **(i)** R2A, **(ii)** ZMA, indicating the micro-aerobic growth lifestyle.

3.3.5.4 Host-Interactions traits of L1I52^T

The association of a beneficial bacteria with its host plant is a multi-stage process that bypasses several barriers. It starts from 1) detecting the signal/chemical cue released from the host plant, followed by 2) the journey of bacteria towards the host plant tissue (root/leaf), where 3) the bacteria manage to defend and evade the host plant defence mechanisms, and finally proceed to 4) the successful colonization and establishment in the target region of the host plant. To facilitate the above-briefed process, the bacteria must be equipped with various genetic traits coding for chemotaxis, motility, host-defence evasion, surface adhesion, colonization and establishment that function sequentially.

(a) Motility, adhesion, and secretion systems

Bacterial motility is one of the most common traits used by several rhizobacteria to colonize their host plant roots successfully (Santoyo et al., 2021; Turnbull et al., 2006). Among various motility mechanisms identified in bacteria, flagellar-driven motility is the most common, and through our genome analysis, it was found that L1I52^T cells lacked flagellar apparatus genes. Hence, we checked for the presence of genes relating to gliding motility (Gld), a type of motility seen in most of the phylum Bacteroidota members, including *Flavobacterium* strains wherein the cells tend to move rapidly over solid surfaces without the aid of flagella (McBride and Nakane, 2015; Nan et al., 2014). In the L1I52^T genome, 14 gliding associated genes (*gld*); *gldA, gldB, gldC, gldD, gldE, gldF, gldG, gldH, gldI, gldJ, gldK, gldL, gldM,* and *gldN* were predicted, and they share a high degree of gene organisation and homology (amino acid similarities ranging from 63 to 84 %) with *Flavobacterium johnsoniae* (Fig. 3.16). Furthermore, we identified two additional genes that encode for cell surface adhesins: SprB and RemA, genes which were previously shown to be involved in surface recognition, adhesion, and gliding motility (Johnston et al., 2018). In accordance with these gene findings, experimentally, we confirmed the gliding motility of L1I52^T cells on soft R2A3 agar (Fig. 3.17).

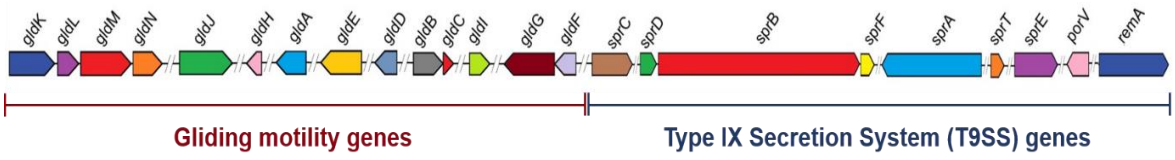


Fig. 3.16. Gene organization of Gliding motility gene complex and Type IX Secretion System (T9SS) encoded in *F. pokkalii* L1I52^T genome.

Protein secretion systems in bacteria play a vital role in modulating their behaviours while interacting with their hosts or surrounding environments (Tseng et al., 2009). The L1I52^T genome contain genes coding for membrane proteins SecA, SecY, SecE and SecG of the general secretion (Sec) and the TatA and TatC genes of the twin-arginine translocation (Tat) pathways where these systems actively participate in the transport of folded and unfolded proteins across the cytoplasmic membrane. Further, our analysis predicated genes that encode for proteins belonging to Por secretion systems (PorSS), also referred to as type IX secretion system (T9SS); GldK, GldL, GldM, GldN, SprA, SprE, and SprT, which are commonly found in most well studied environmental and fish pathogenic *Flavobacterium* strains where these secretion systems are shown to play an essential role in gliding motility, polysaccharides degradation and pathogenesis (Veith et al., 2017). Notably, the most common secretion systems reported in many plant-associated bacteria; type III, IV, and VI, were absent in the L1I52^T genome (Lucke et al., 2020). These results signify that the mode of transport of proteins in L1I52^T is limited only to the general secretion and type IX systems, as observed in other *Flavobacterium* strains (Fig. 3.16).

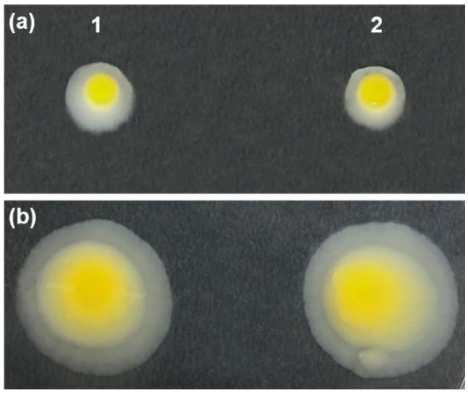


Fig. 3.17. Image representing the motility of *F. pokkalii* L1I52^T cells in R2A3 soft agar (0.18% agar) captured on **(a)** 2nd dpi, **(b)** 5th dpi. 1, and 2 indicate the replicates.

Studies have shown that some aquatic *Flavobacterium* strains are known to act as severe fish pathogens (Wahli and Madsen, 2018). Considering the isolation source of L1I52^{T,} i.e., Pokkali rice fields where shrimps/fish are cultivated in the alternate seasons, the presence of any effector proteins of virulence factors in the L1I52^T genome was investigated. However, the genome showed no virulence genes relating to fish pathogenicity. It did not encode any genes for the type III secretion system, an essential virulent system primarily coded in gram-negative bacterial pathogens. Therefore, it was confirmed that L1I52^T is not an opportunistic fish pathogen. Further, the L1I52^T genome lacked dedicated genes coding for protein secretion systems, which export effector proteins into host cells and cause virulence or damage to the plant cells, thus supporting the claim that L1I52^T is a beneficial rhizobacterium.

The findings from root attachment, and colonization experiments showed that L1I52^T can

efficiently attach and colonise the rice roots. Surprisingly, no genes encoding for pili, fimbriae, or cellulose fibrils were identified in the genome of L1I52^{T,} which are previously identified in other well-studied plant growth-promoting rhizobacterial genomes (Krause et al., 2006). Max Kolten et al showed that mutation of a gilding motility gene, especially (*gldJ*) in *Flavobacterium* strain F52, resulted in the loss of root colonization and disease suppression (Kolton et al., 2014). Thus, we hypothesize that gliding motility and its co-regulated type IX secretion system might be involved in the plant root attachment and colonization of L1I52^{T}. However, further molecular experiments are necessary to validate this hypothesis. L1I52^{T} genome harbours gene clusters coding for lipopolysaccharide and exopolysaccharide synthesis that is assumed to play a critical role in the biofilm formation ability, which aids in the host colonization and establishment of L1I52^{T} cells.

(b) Signal transduction mechanisms

L1I52^{T} genome harboured many genes coding for signal transduction systems, reflecting their active interaction with the host. Two-component systems comprising sensor histidine kinases and response regulator proteins are among the most important players that mediate signal transduction in bacterial systems. In this regard, L1I52^{T} harboured 52 genes corresponding to the two-component sensor and associated regulator. Among these, 3 component sensor systems are located within polysaccharide utilization loci (PULs) coding for starch, xylogalacturonan, and mannan, whereas one is located within the phosphate transport system (pts) operon. The L1I52^{T} genome harbour LuxR coding genes, and all of them are included in the two-component system. Among these, one LuxR coding gene is in proximity with the gliding motility complex genes, *gldB,* and *gldC,* another LuxR is near to nitrate reductase genes, ammonium transporter, and nitrogen regulatory protein P-II, and one is near the two-component system located with the pts operon. In addition, the L1I52^{T} genome encodes 20 genes coding for extracytoplasmic function (ECF) sigma factors that enable the bacteria to alter their gene expression in response to host environmental stimuli. Among these, 8 ECF sigma factors are next to anti-sigma factors, which indicates the tight regulation of gene expression based on environmental stimuli. Interestingly, 3 ECF sigma factors are located within the PUL coding for plant polymers such as pectin, arabinoxylan, and rhamnogalacturonan. Furthermore, L1I52^{T} genome encodes 9 copies of genes coding for Crp/Fnr family transcriptional regulators known to respond to environmental stimuli activating and repressing several functional genes in bacteria (Fu et al., 2023). Certain PGPR strains have reported the expression of transcriptional regulators belonging to LuxR and

Crp/Fnr families in response to salt stress (Oshone et al., 2017). Hence, the presence of these genes in L1I52^T strains is assumed to mediate similar transcript response-driven gene functions.

Chemotaxis is one of the critical phenomena wherein the bacterial cells sense chemical gradients in the surrounding environment and move towards their favourable conditions. This is regarded as one of the major mechanisms by which rhizobacterial colonizers propel toward their host plants for their association, which is facilitated by their motility genes. L1I52^T harbours major chemotaxis-related genes such as *cheB, cheR, cheY,* and *cheA,* also found in the model strain, *F. johnsoniae.* Altogether, these genomic findings indicate that a strong signal transduction mechanism is coded in the L1I52^T genome to facilitate its host and ecological interaction efficiently.

(c) Eukaryotic-like proteins (ELPs)

The enrichment of *Flavobacterium* members in the brackish rice roots followed by the influence of brackish water in OTU enrichment, as evidenced by the metagenome and soil-microcosm-based bacteriome analysis, encouraged to investigate deeply on the host-interaction associated molecular factors coded in L1I52^T genome. Bacterial-host plant colonization involves distinct stages, which include: (i) bacterial movement towards the host plant by root exudate sensing, (ii) adherence or interaction with the host, (iii) cell wall disruption and modification for host cell entry, (iv) host immune defence evasion, and (v) population establishment in the host. In this regard, we checked whether L1I52^T possessed any eukaryotic-like proteins (ELPs), which might complement any of the above-briefed stages that lead to L1I52^T-Pokkali root colonization. Hence, based on the NCBI protein database and UniProt BLAST analysis, around 42 genes possessing 26 different types of ELP domains were identified to be encoded in the L1I52^T genome (Table 3.6). Thus, based on previous literature reports, we elaborate on certain ELP domains that we assume to be very important in mediating the L1I52^T – Pokkali rice interaction process.

(i) Host interaction or binding proteins

1. Ankyrin repeat domains (ANKs) - Ankyrin repeats are a class of ELPs known for participating in the interactions of bacterial symbionts with their eukaryotic hosts by mimicking or manipulating host functions (Carolin Frank, 2019). Few studies have reported the widespread presence of these protein domains in *Methylobacterium* genus and its homologs in certain plant-associated *Pseudomonas* species (Koskimäki et al., 2015a). For example, *Methylobacterium extorquens* DSM 1360 is known to promote growth in scots

pine seedlings strongly, and it is thought that the ELPs, which include ANK domain-containing proteins, play a predominant role in mediating these symbiotic interactions (Pohjanen et al., 2014). Interestingly, in L1I52^T, 2 ankyrin repeat-containing protein domains which are assumed to be involved in L1I52^T-Pokkali interaction processes were identified (Table 3.6). Moreover, through comparative genome analysis, we could identify the presence of ankyrin repeat domain-containing proteins in the nearest phylogenetic neighbours and other *Flavobacterium* strains compared (other Pokkali-associated strains and other terrestrial strains as described earlier) as well, which indicates their widespread presence within the *Flavobacterium* genus.

2. Tetratricopeptide repeat, Leucine-rich repeat, and fibronectin type III domain-containing proteins - Tetratricopeptide repeats (TPR) are structural motifs known to occur widely in prokaryotes and eukaryotes. Despite their widespread occurrence in different bacterial species, their role in symbiotic interaction with plants remains unexplored. However, the enrichment of TPR repeats has been identified during the metagenome analysis from a model sponge *Cymbastela concentrica* (Thomas et al., 2010). In a subsequent study, an uncultured δ-proteobacterium from *C. concentrica* reported to encode TPR proteins (Liu et al., 2011). Further, other ELPs, such as leucine-rich repeat and fibronectin type III domain-containing proteins, have also been identified during the single-cell genome analysis of candidate phylum Poribacteria from sponge *Aplysina aerophoba* (Siegl et al., 2011). Fibronectin type III domain proteins are known for their potential role in host cell binding and subsequent colonization (Hoffmann et al., 2011). In this regard, the L1I52^T genome encodes 12 copies of TPR, and 2 copies of fibronectin type III domain-containing protein (Table. 3.6). However, their functional role relative to host plant-bacterial interaction remains elusive. In the case of Leucine-rich repeat (LRR) proteins, they are effector proteins known for host defence suppression and are widespread ELPs in bacteria. After host defence evasion, any plant-associating bacteria must suppress the host defence mechanisms to establish its population in the host. LRRs domains have been reported previously in pine bud endophyte *Pseudomonas synxantha* DSM13080, pine symbiont *M. extorquens* DSM 13060, and its homologs have been identified in other plant-associated bacterial strains as well (Koskimäki et al., 2015). Similarly, L1I52^T harboured an LRR encoding protein with a signal peptide, which is likely to be involved in mediating host defence suppression, and this was found to be 100% similar to its nearest phylogenetic neighbour, *F. daejeonense* DSM 17707^T (Table. 3.6).

(ii) Cell wall disruption/modification and host cell entry

1. Glycoside hydrolases (GH) - Glycoside hydrolases (GHs) are plant cell wall degrading enzymes that act on various plant-derived polymers such as cellulose, xylan, pectin etc. Some bacterial endophytes produce several GH variants, namely endoglucanases, xylanases, pectinases, etc., that facilitate their initial entry into the host through plant cell wall disruption. For example, endoglucanase activity-deficient mutants of *Azoarcus* sp. BH72 showed decreased ability to colonize rice roots and shoots, contrasting to its wild-type strain (Reinhold-Hurek et al., 2006). In the case of shoot symbiont, *M. exotorquencs* DSM 13060, the activity of GH enzymes is reportedly involved in the active degradation of the host cell wall, which mediates their entry (Koskimäki et al., 2015). Similarly, in L1I52^T, we could identify 16 GHs, which included GH28 (14), GH35 (1) and GH100 (1) (Table 3.6). Among these, 13 GH28s were coded for polygalacturonase activity, and 1 GH28 was coded for hydrolase activity. Some GHs possessed a signal peptide that aids in their extracellular secretion followed by their initial interaction with the host, i.e., Pokkali roots through host cell wall disruption, thereby complementing L1I52^T in its initial host cell entry process.

2. Pectinesterases - Pectinesterases are enzymes that catalyse the de-esterification of polygalacturonans, thereby playing a significant role in cell wall modification and thus affecting the host colonization processes. Interestingly, the L1I52^T genome harboured 5 copies of pectinesterase coding genes (Table 3.6). It is important to note that similar numbers of pectinesterase coding genes were present in its phylogenetic neighbours. However, these genes were absent in other *Flavobacterium* strains isolated from Pokkali, which differentiates them from the L1I52^T strain and might contribute to L1I52^T-Pokkali interaction.

(iii) Host defence evasion and suppression

1. Catalase peroxidase - L1I52^T, thus interacting with the host Pokkali, will be encountered with several defence mechanisms generated as a result of host cell wall disruption and modification. Thus, the reactive oxygen species (ROS) generated because of oxidative stress can damage the bacterial cells. To counter this, we could identify a copy of *katG* (catalase-peroxidase domain-containing gene) in L1I52^T, which coded for a bifunctional enzyme with catalase and broad-spectrum peroxidase activity; which is assumed to play a significant role in neutralizing the oxidative stress caused by the host generated ROS during cell evasion (Table 3.6). Thus, it protects the bacterial cells and associated enzymes from oxidative damage by catalysing the reduction of hydrogen peroxide generated by the host.

3.3.5.5 Plant beneficial functions

It is known that rhizobacteria involved in symbiotic interactions with plants often benefits the hosts by performing essential plant growth functions that complement the plant growth (Bhat et al., 2022; Mendes et al., 2013). Experimental evidence from our *in-planta* assays suggested that L1I52^T cells promote rice plant growth, despite the absence of critical genes coding various plant beneficial properties such as fixation of atmospheric nitrogen, production of phytohormones (Indole acetic acid or cytokinin), hydrogen cyanide (HCN), acetoin, 2,3-butanediol, and reduction of plant stress ethylene by 1-aminocyclopropane-1-carboxylate (ACC) deaminase. This observation at the genomic level was well supported by our *in-vitro* phenotypic assays, where L1I52^T showed negative to most of the plant growth beneficial traits tested. However, in this context, it is important to note that the genome of the well-known grass endophyte, *Azoarcus olearius* BH72 strain, did not possess genes encoded for PGPR functions such as indole acetic acid (IAA) synthesis nor phosphate solubilisation, but still they promoted the plant growth (Hurek et al., 1994). Likewise, the genome of *Methylobacterium extorquens* DSM 13060 lacks genes known to encode for plant growth promoting traits but still promotes plant growth without producing any plant stimulating factors (Koskimäki et al., 2015). Therefore, it might be possible that L1I52^T uses an unknown mechanism by which it promotes plant growth when they mutually interact with the plant roots, which must be investigated further in detail.

(a) Nitrogen and phosphate metabolism

For nitrogen metabolism, the genome encodes a nitrate/nitrite transporter and an assimilatory nitrate reductase (NarB) and nitrite reductase (NirBD) encoding genes, later both involved in the reduction of nitrate to ammonia and not to nitric oxide. In addition, the L1I52^T genome encodes for a functional GS/GOGAT pathway (glutamine synthetase (GlnA) and glutamate synthase (GltBD) known to assimilate ammonia.

Phosphate is an essential macronutrient required by plants, and bacteria play a major role in the solubilization and mobilization of phosphate (Tian et al., 2021). During environmental Pi scarcity, the bacteria employ numerous strategies to mobilize phosphate. The L1I52^T genome is equipped with a high-affinity Pi transport system encoded by *pstSCAB* genes regulated by the two-component system encoded by *phoUR* induced under low phosphate concentrations. L1I52^T also encodes a low-affinity Pi-transporter, a homolog of PitA that is known to be expressed constitutively. Further, the phosphate mineralization and mobilization are likely to be mediated by the activity of certain alkaline phosphatases during

Pi scarcity. However, a unique phosphate-insensitive bacterial phosphatase, *pafA,* that functions independently of the phosphate availability was identified in L1I52^T genome. The discovery of PafA as a highly active enzyme is studied extensively for its role in facilitating the rapid mineralization of bioavailable Pi (Lidbury et al., 2022). Moreover, this is regarded as a characteristic trait assumed to be coded in most plant-associated Flavobacterium spp., which demonstrated high activity towards both artificial and natural organophosphorus substrates (Lidbury et al., 2022). Further, the L1I52^T genome encodes *phnABP* genes of the phosphonate-related gene system (*phn*) that facilitates the phosphonate metabolism to phosphate and encodes a cope of phytase. Furthermore, we could identify potential genes coding for enzymes such as polyphosphate kinase (EC. 2.7.4.1) and exopolyphosphatase (3.6.1.11) that store energy and phosphate in polyphosphate. All these suggest that an efficient mechanism for phosphate metabolism is encoded in L1I52^T. Supportively, through *in-vitro* plate assay we could identify a weak solubilization of phosphate indicated by a halo zone around the spotted L1I52^T colony after incubation for 3 weeks (Fig. 3.18 (a)).

(b) Iron acquisition

Iron is scarce in the rhizosphere because of its low solubility and the fact that bacteria residing in the rhizosphere compete for iron. The L1I52^T genome harboured 17 protein-encoding genes assigned to the SEED subsystem iron acquisition and metabolism category.

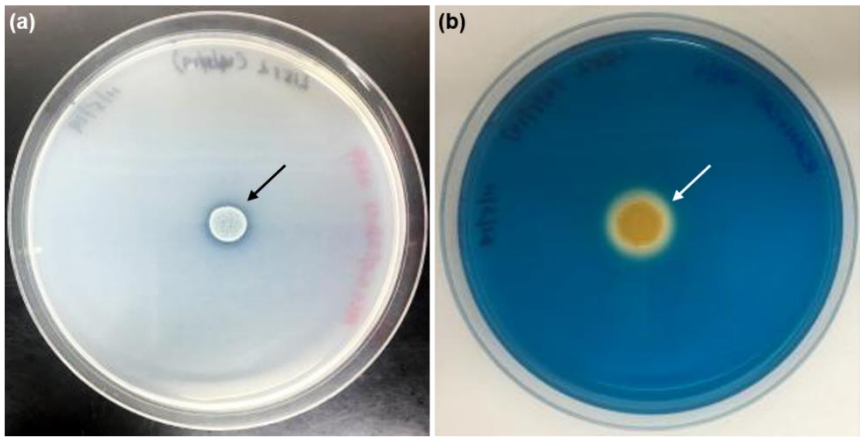


Fig. 3.18. *In-vitro* PGP traits of *F. paokkalii* L1I52^T strain: **(a)** Phosphate solubilization in pikovskaya's agar plate, and **(b)** Siderophore production in R2AN+CAS agar plate after 3 weeks of incubation at 30°C.
The black arrow and white arrow in fig. (a) and (b) indicate the halo zones representing solubilization of phosphate and siderophore production around the growth of *F. pokkalii* L1I52^T colony, respectively

The genes include aerobactin siderophore receptors lutA and other TonB-dependent receptors, which are assumed to be involved in environmental iron uptake. However, the genome lacks genes involved in siderophore synthesis, as observed in other pathogenic

Flavobacterium (Guan et al, 2013) and plant-beneficial rhizobacterial genomes (Eastman et al, 2014). However, a very weak siderophore activity was observed after 3 weeks of incubation in the *in-vitro* plate assay (Fig. 3.18 (b)).

(c) Vitamin biosynthesis

The synthesis of vitamins by rhizobacteria is a phyto-beneficial trait that contributes to host growth and development. L1I52^T encodes the entire set of genes for the biosynthesis of biotin (H), thiamine (B1), folic acid (B9) and pantothenate (B5) (Table 3.7).

3.3.5.6 Comparative genomic analysis reveals unique traits in L1I52^T

(a) Genome size-based ecological niche differentiation in *Flavobacterium* strains

The genomic traits reflect the functional potential and lifestyle of an organism. The advent of sequencing technologies and rapid advancements in bioinformatic pipelines have made genomics research very cheap, reliable, and highly informative. This has led to the generation of huge genome information in the past decade that has decoded the functional potential of many critical bacterial strains. In this regard, a comparative genomic study was published by Kolton, M. et al in 2013, claiming that the terrestrial and aquatic *Flavobacterium* strains can be segregated into two distinct clades reflecting their varied niche adaptation based on their coded genetic traits in glycan metabolism-related genes that is reflected in their genome size (Kolton et al., 2013). The comparative genome analysis performed in this study revealed that terrestrial *Flavobacterium* strains have larger genomes than their aquatic environment counterparts (Kolton et al., 2013). The larger genome size in terrestrial *Flavobacterium* strains is occupied by a higher abundance and diversity of genes coding for carbohydrate metabolism (Kolton et al., 2013). The findings from the study indicate that the physiology of the *Flavobacterium* genus is strongly influenced by its ecology (Kolton et al., 2013). Fig. 3.19, which compares the average genome sizes of different *Flavobacterium* strains (selected from Kolton et al., 2013) belonging to terrestrial and aquatic clade, clearly indicate the gradual increase in the genome size from aquatic clade

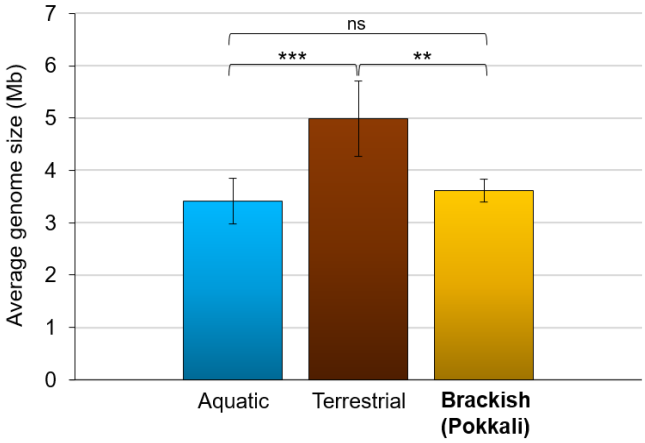


Fig. 3.19. Bar graph comparing the average genome size (Mb) of *Flavobacterium* genus representative strains belonging to different clades based on their isolation habitats such as Aquatic, Terrestrial, and Brackish (Pokkali).

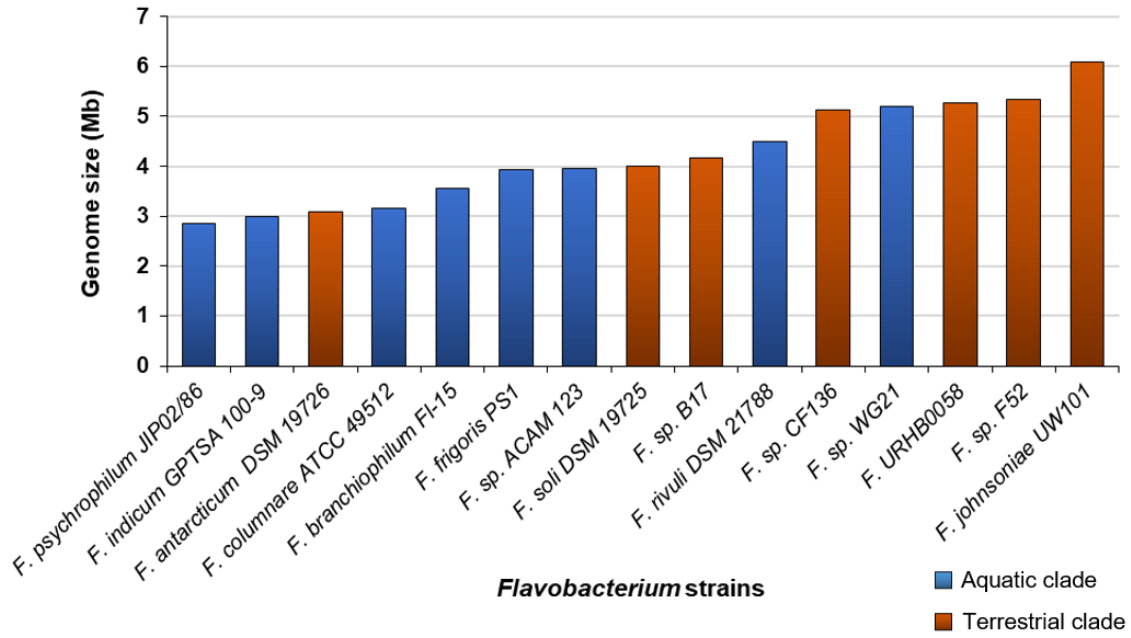


Fig. 3.20. Bar graph comparing the genome size (Mb) of *Flavobacterium* genus representative strains belonging to aquatic clade and terrestrial clades, which indicate an increased genome size for terrestrial clade strains compared to aquatic clade strains with exceptions of a few strains showing varied genome size despite their habitat

(genome size ranging from 2.86 to 3.96 Mb) to terrestrial clade (genome size ranging from 4.0 to 6.1 Mb) (Fig. 3.19). However, certain strains such as *F. antarticum, F. rivuli,* and *F.*

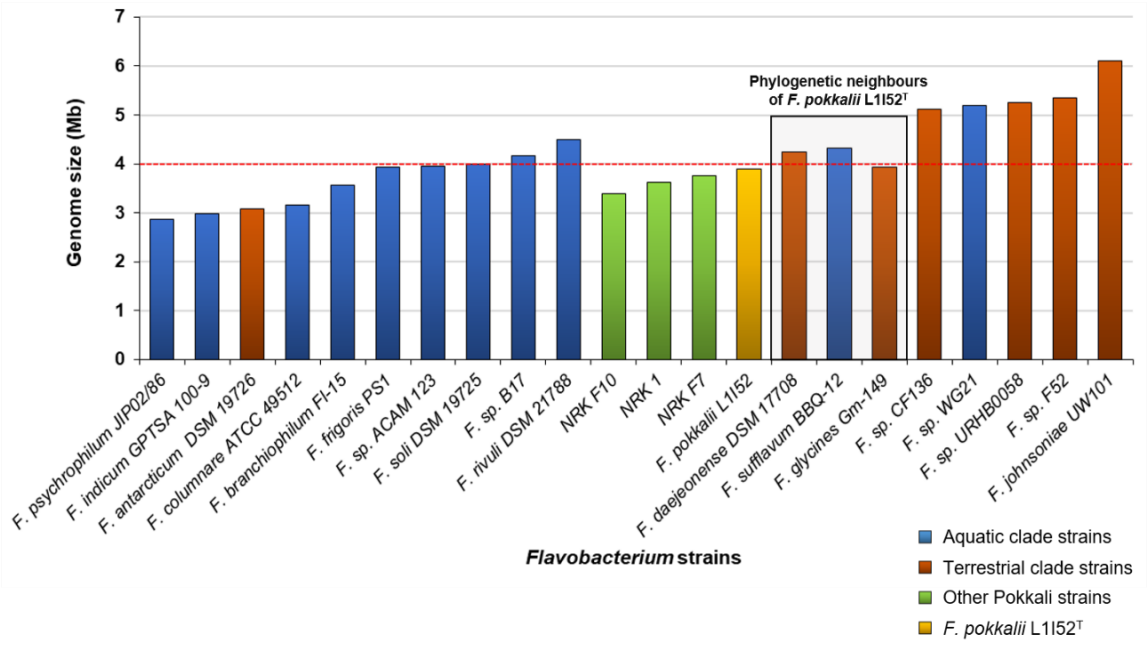


Fig. 3.21. Bar graph comparing the genome size (Mb) of *Flavobacterium* genus representative strains belonging to aquatic clade and terrestrial clades with the strains isolated from Pokkali rice, including *F. pokkalii* L1I52^{T} to analyze how the brackish-plant associated strains are placed in the genus *Flavobacterium* based on their genome sizes.

sp. WG21 are some exceptions that do not follow the criteria of habitat-based genome size variation (Fig. 3.20). The *Flavobacterium* strains from the brackish (Pokkali) habitat showed a reduced average genome size of 3.61 Mb (ranging from 3.4 to 3.89) compared to the terrestrial clade average of 4.99 Mb (ranging from 4 to 6.1 Mb) (Fig. 3.19). The intriguing observation was the significant difference in *Flavobacterium* strains from Kolton et. al, 2013 with *Flavobacterium* strains isolated from Pokkali and its nearest phylogenetic neighbour to have a clear resolution in the genome comparison. The comparative bar graph clearly showed the placement of *Flavobacterium* strains from Pokkali with genome size well aligning with or slightly below certain aquatic clade strains (Fig. 3.21). However, compared to terrestrial *Flavobacterium* strains, the size of *Flavobacterium* strains from Pokkali was much lesser, with a reduction of ~1 Mb (Fig. 3.21). Among the phylogenetic neighbours, two strains belong to the terrestrial clade (*F. daejeonense* DSM17708^T from greenhouse soil, and *F. glycines* Gm-149^T from soybean rhizosphere), and 1 strain belong to aquatic clade (*F. sufflavum* BBQ-12^T from freshwater lake). L1I52^T genome size (3.89 Mb) is small compared to *F. daejeonense* DSM17708^T (4.24 Mb) and *F. sufflavum* BBQ-12^T (4.33 Mb), whereas the size of *F. glycines* Gm-149^T is almost similar with a slight difference (3.94 Mb) (Fig. 3.21). Altogether, L1I52^T and other *Flavobacterium* strains from Pokkali have a reduced genome size compared to other terrestrial *Flavobacterium* strains, with *F. glycines* Gm-149^T only as the exception (Fig. 3.21). Since the Pokkali ecosystem encounters frequent brackish water intrusion caused by tide variations, Pokkali ecosystem can be regarded as a juncture between terrestrial and marine, i.e., a terrestrial habitat frequently influenced by marine-driven brackish conditions. So, this brackish-adapted terrestrial environment is assumed to be distinct from the normal terrestrial environments and, hence, its microbial inhabitants. Therefore, these unique ecological conditions could be critical for housing plant-associated *Flavobacterium* strains showing similar genome sizes to aquatic clade *Flavobacterium* strains despite being isolated from terrestrial habitats. However, to confirm if the *Flavobacterium* strains from Pokkali rice possess genetic traits of terrestrial clade *Flavobacterium* strains, further detailed analysis of their carbohydrate utilization potential must be decoded, especially for the novel strain, L1I52^T, which is identified as a host-growth influencing strain with characteristic plant-colonization traits among the isolated strains. However, based on the results from our *in-planta* studies and genome size-based predictions, we assume that the other *Flavobacterium* strains from Pokkali (NRK F7, NRK F10, and NRK 1) are not plant-associated strains despite being isolated from the Pokkali rice roots.

(b) SEED subsystem-based whole genome functional gene categories

The literature studies to date have provided enough evidence that the *Flavobacterium* genomes harbour variedly sized genomes based on their niche adaptation. Interestingly, the L1I52^T genome harbours a smaller genome than its terrestrial counterparts. It shares a similar size range with its aquatic counterparts, mostly within 4 Mb (Fig. 3.21). However, the findings from our *in-planta* experiments and eco-physiology studies strongly suggest that the L1I52^T strain is a potential terrestrial clade strain with host-growth influencing traits. Hence, to understand how variedly the genetic functions are coded under broad functional categories that correlate with the data from functional studies, we compared the subsystem feature-based functional categories between Pokkali strains (NRK F7, NRK F10, NRK 1) and the taxonomically characterized novel strain, L1I52^T, along with its phylogenetic neighbours, and certain representative strains from terrestrial and aquatic clade (Fig. 3.22). Among the many subsystem features, we selected 9 major features that majorly influence the organism's lifestyle, metabolism, and adaptation. This includes (i) carbohydrates, (ii) protein metabolism, (iii) virulence, disease, and defense, (iv) respiration, (v) membrane transport, (vi) stress response, (vii) nitrogen metabolism, (viii) sulfur metabolism, and (ix) phosphorus metabolism. Further, we constructed a heatmap based on the total number of genes encoded under each of these subsystem features coded in various *Flavobacterium* strains for an easy comparative analysis based on their abundance (Fig. 3.22). The genome size of each strain is included in the heat map diagram to correlate the genome size with its encoded subsystem features, especially the carbohydrate and protein metabolism potential known to vary in the *Flavobacterium* strains based on their habitats (Kolton et al., 2013). The major aim of this comparative analysis was to see how the isolated *Flavobacterium* strains NRK F7, NRK F10, and NRK 1, especially the novel strain L1I52^T, placed themselves within the genus in terms of their functional potential. Compared to its nearest phylogenetic neighbours, L1I52^T coded a slightly higher number of carbohydrate-related genes than its terrestrial clade relatives: *F. daejeonense* DSM 17708^T and *F. glycines* Gm-149^T, whereas its aquatic clade relative, *F. sufflavum* BBQ-12^T harbored higher carbohydrate-related genes which is an exception (Fig. 3.22). Importantly, the abundance of carbohydrate-related genes is high, likely in proportion with the terrestrial clade Flavobacterium strain despite the smaller genome size of L1I52^T than the terrestrial clade strains. Interestingly, the protein metabolism-related genes are comparatively reduced in L1I52^T and its nearest phylogenetic neighbour, *F. daejeonense* DSM 17708^T, compared to

other terrestrial clade strains and aquatic clade strains (Fig. 3.22). This is assumed to be one of the reasons for the reduced genome size of the L1I52^T genome despite coding for a higher number of carbohydrate-related genes. Further, the number of genes related to membrane transport and virulence, disease, and defence categories align with the terrestrial clade strain *Flavobacterium* sp. CF136 and phylogenetic neighbour strains, whereas slightly higher in the terrestrial clade *Flavobacterium* sp. F52, and *F. johnsoniae* UW101^T strains (Fig. 3.22). However, a reduced number of genes were encoded in the membrane transport category of aquatic clade strains, displayed by a clear colour differentiation in the heat map (Fig. 3.22). Furthermore, genes related to plant functions such as nitrogen, sulphur, and phosphorous metabolism were enriched in L1I52^T, its phylogenetic neighbours, and other terrestrial clade strains, whereas it was found to be depleted in aquatic clade strains, including other Pokkali strains (NRK F7, NRK F10, NRK 1) (Fig. 3.22). Moreover, it is important to note that the subsystem distribution in L1I52^T followed a very similar pattern with its nearest phylogenetic neighbour, *F. daejeonense* DSM 17708^T (Fig. 3.22). This indicates a tight relation between L1I52^T and *F. daejeonense* despite their isolation source and habitat differences. However, the genome size of L1I52^T remains reduced with a reduction of almost 0.37 Mb compared to *F. daejeonense* DSM 17708^T which probably indicates the possible adaptation of L1I52^T relative to its brackish ecosystem that lies in the juncture of terrestrial and marine ecosystem contrasting to the standard terrestrial habitat which is the native of *F. daejeonense* DSM 17708^T. All these indicate that a brackish-adapted terrestrial strain with a plant-associated lifestyle, for example, L1I52^T, might have a varied genome size between the terrestrial clade strains and aquatic clade strains with associated functional traits.

Based on these function-based genomic predictions, it is well confirmed that *Flavobacterium* strains NRK F7, NRK F10, and NRK 1 are different from strain L1I52^T despite their similar isolation host, pokkali rice. Strain L1I52^T harboured almost two times higher carbohydrate-related genes compared to other *Flavobacterium* strains (NRK F7, NRK F10, and NRK 1) isolated from Pokkali, which hints at the possible host growth influence uniquely attributed by L1I52^T (Fig. 3.22). Therefore, to understand how L1I52^T and other Pokkali strains are ecologically placed within the genus *Flavobacterium*, we constructed a phylogenetic tree using L1I52^T, NRK F7, NRK F10, NRK 1 strains along with its phylogenetic neighbours and other related strains of the genus *Flavobacterium* (Fig. 3.23). As validated earlier in the taxonomic characterization section, L1I52^T clustered closely with *F. daejeonense* DSM 17708^T from greenhouse soil as the nearest relative,

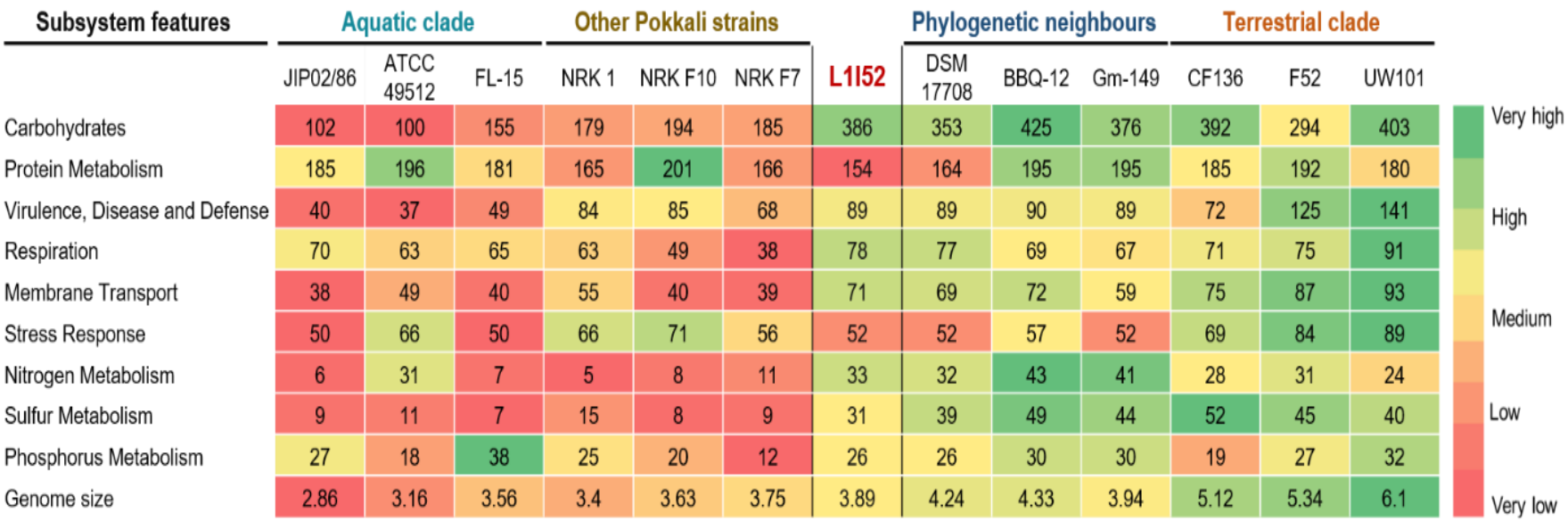


Fig. 3.22. Heat map comparing the gene distribution within the subsystem features of strain *F. pokkalii* L1I52^{T}, its phylogenetic neighbours, other *Flavobacterium* strains isolated from pokkali (from this study) and representative *Flavobacterium* strains belonging to aquatic and terrestrial clade

followed by *F. sufflavum* BBQ-12^T and *F. glycines* Gm-149^T from freshwater lake and soybean rhizosphere, respectively (Fig. 3.23). However, other Pokkali strains clustered phylogenetically within the aquatic clade of the genus *Flavobacterium* (Fig. 3.23). For example, *Flavobacterium* sp. NRK F7 clustered with *F. jejuense* EC11^T isolated from the

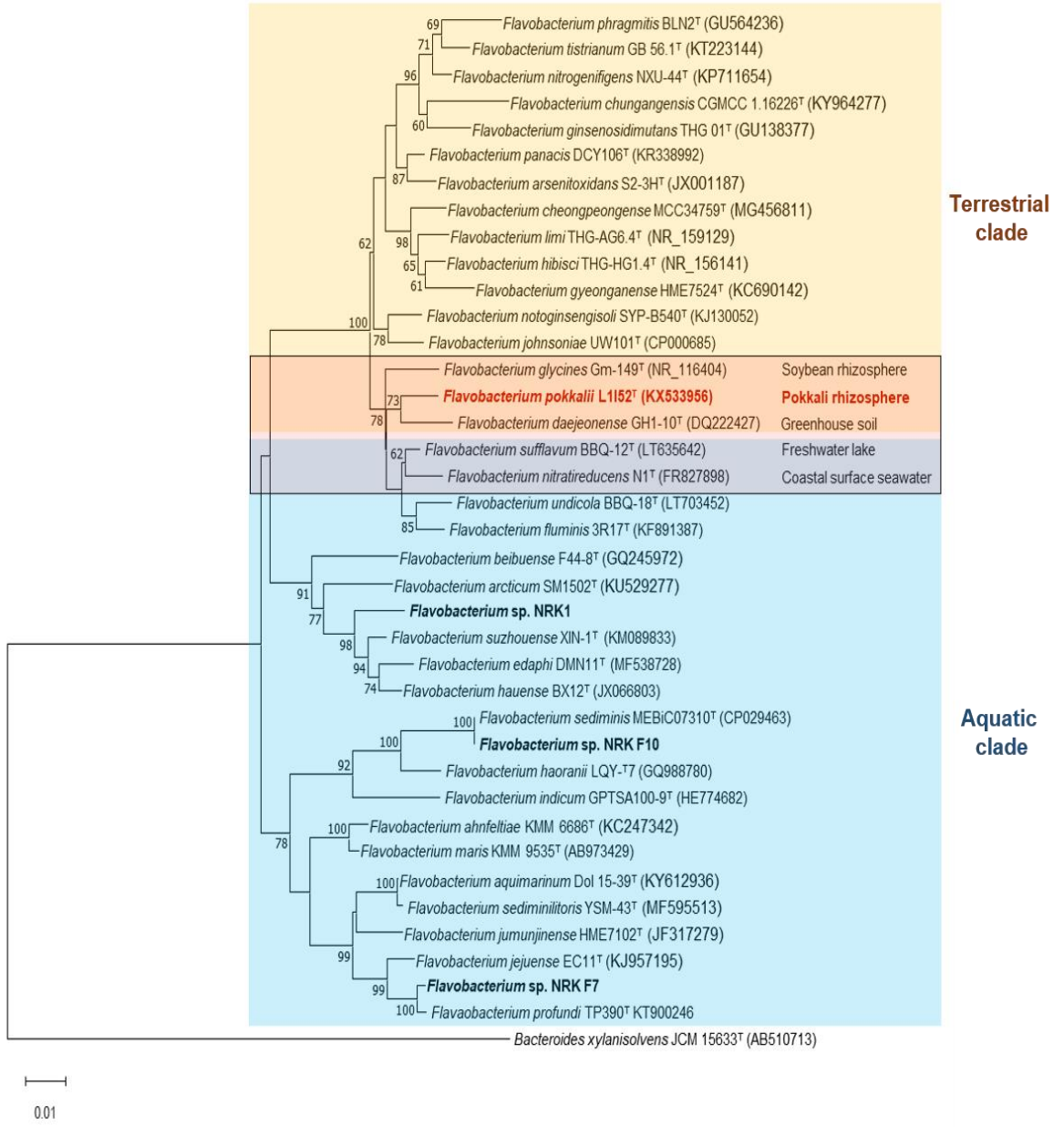


Fig. 3.23. Neighbour-joining tree constructed using the 16S rRNA gene sequences showing the phylogenetic positions of different *Flavobacterium* strains isolated from Pokkali rhizosphere/roots: *F. pokkalii* L1I52^T (labelled in red font), *Flavobacterium* sp. strains - NRK F7, NRK F10, NRK 1 (labelled in black bold font) within the different habitat-based clades (terrestrial clade and aquatic clades strains highlighted in yellow and blue boxes, respectively) within the genus *Flavobacterium*. The box representing strains from both terrestrial and aquatic clades are nearest relatives of *F. pokkalii* L1I52^T strain belonging to varied habitats as mentioned in the figure. The percentages shown at each branch point represent bootstrap values derived from 1000 replications, and values more than 60 % are indicated. Bar, 0.01 represents positions per nucleotide position. *Bacteroides xylanisolvens* JCM 15633^T was used as the outgroup.

marine brown alga *Ecklonia cava*, NRK F10 was 100% identical to *F. sediminis* MEBiC07310^T isolated from tidal flat sediment (Fig. 3.23). However, NRK F10 (isolated in 2017) was a probable novel species until the *F. sedminis* MEBiC07310^T strain was validly published in 2018.

Flavobacterium sp. NRK 1 was isolated from a farmland river sludge sample (Fig. 3.23). Altogether, the phylogeny places the *Flavobacterium* strains, NRK F7, NRK F10, and NRK 1 in the aquatic clade, contrasting with L1I52^T that is clustered within the terrestrial clade of *Flavobacterium* with close relatives from the aquatic clade as well (Fig. 3.23). Hence, we can claim that L1I52^T is a terrestrial clade strain with aquatic adaptations native to brackish ecological niche. As discussed earlier, the terrestrial clade *Flavobacterium* strains are specialized in carbohydrate metabolism mediated by an array of CAZymes. Hence, the CAZyme distribution and associated potential of L1I52^T strain in polysaccharide utilization was investigated.

3.3.5.7 Carbohydrate Active Enzymes (CAZymes)

A broad distribution of the genetic traits coded in different *Flavobacterium* strains from the RAST subsystem-based functional prediction. However, a detailed targeted analysis is essential to have an in-depth understanding of the functional categories. Here, we were more eager to know about the distribution of the genes coding for carbohydrate utilization, which is regarded as a major determinant of terrestrial adaptation and plant-associated lifestyle. Therefore, we used dbCan3 server to annotate and identify the genes that code for different CAZymes. Here, we mainly investigated the details of how these CAZymes are categorized in L1I52^T genome under different types such as Glycosyl hyrolases (GH), Glycosyl transferases (GT). Polysaccharide lyases (PL), Carbohydrate esterases (CE), and Carbohydrate-binding modules (CBM) and respective families of each type. The abundance and diversity of CAZyme families indicate an organism's carbohydrate utilization/degradation potential. Therefore, we performed a comparative analysis, where we chose all the isolated Pokkali strains from this study along with the phylogenetic neighbours of L1I52^T isolated from plant-associated habitats and a few other representative *Flavobacterium* strains isolated from plant roots and soil (Fig. 3.24). It is important to note that the comparative analysis performed here is based on the proportion of total CAZyme coded relative to the coding sequence (CDS) of the genome represented in percentage (%). Compared to its phylogenetic neighbour, *F. daejeonense* DSM 17708^T, L1I52^T harboured ~3% higher proportion of CAZymes whereas *F. glycines* Gm-149 harboured ~0.4%

CAZyme higher (Fig. 3.24). The near similar proportion of CAZyme abundance between L1I52^T, and *F. glycines* Gm-149 strains is assumed to be due to their close association with the host plants as they were isolated from the rhizosphere regions of Pokkali rice and soybean, respectively. However, a higher proportion was not found in other Flavobacterium strains isolated from plant roots (Fig. 3.24). The model strain *F. johnsoniae* UW101^T harboured a lower proportion of CAZymes (6.65%), reflecting their probable non-host-associated lifestyle in soil habitats. The most interesting observation from this comparative analysis was the significantly high variation observed between the CAZyme proportions of L1I52^T and other *Flavobacterium* strains isolated from Pokkali roots (NRK F7, NRK F10, NRK 1). The CAZyme proportion in these strains was nearly half (4.36% - 5.19%) compared to 10.33% of CAZyme harboured in L1I52^T (Fig. 3.24). This major difference validates the non-plant-associated lifestyle of NRK F7, NRK F10, and NRK 1, which also failed to show host growth influence during our initial *in-planta*-based screening studies. Therefore, these three strains are not considered to be a native of the Pokkali rice plant and their presence on Pokkali roots or rhizoplane region as obtained during the isolation may be due to some unknown factors.

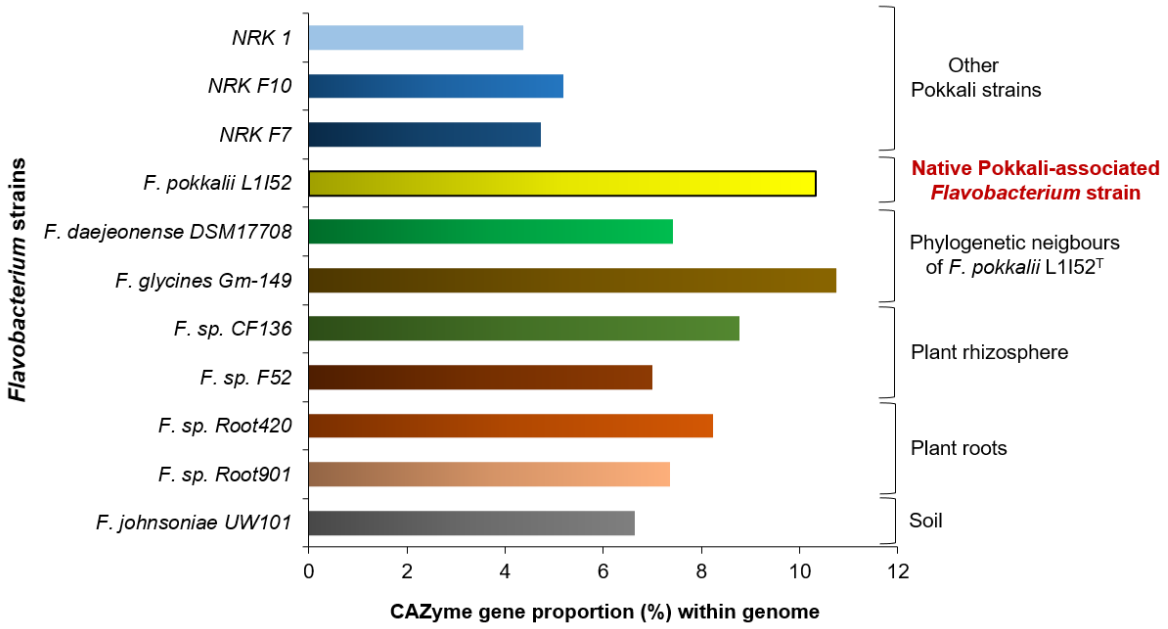


Fig. 3.24. Bar graph comparing the average genome size (Mb) of *Flavobacterium* genus representative strains belonging to aquatic clade and terrestrial clades with the strains isolated from Pokkali rice, including *F. pokkalii* L1I52^T to analyze how the brackish-plant associated strains are placed based on their genome sizes

3.3.5.8 Polysaccharide utilization loci (PULs)

The high number of CAZyme coding genes encoded in the genome of L1I52^T encouraged us to explore their carbohydrate metabolism potential more. The organization of CAZyme encoding genes into regulated, colocalized gene clusters for the efficient saccharification of complex carbohydrates is a known characteristic trait of phylum Bacteroidota members, and they have been extensively studied for their role in the gut carbon turnover through glycan breakdown (Lapébie et al., 2019). The characteristic feature of a gene cluster to be recognized as a PUL is the presence of at least one pair of a gene coding for SusC-/SusD-

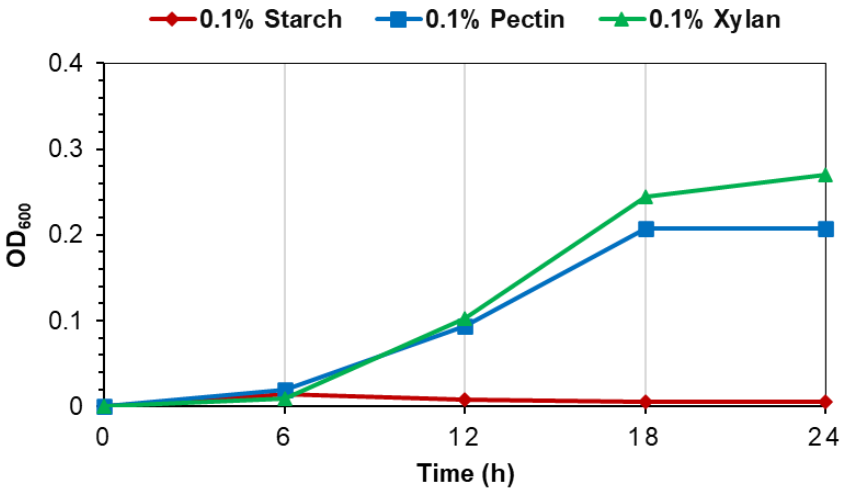


Fig. 3.25. Comparative growth of *F. pokkalii* L1I52^T cells in minimal media amended with different plant-based polysaccharides (0.1% of starch, pectin, and xylan) as carbon sources.

like binding proteins and TonB-dependent receptor/transporter (TBDT) linked to genes coding for predicted polysaccharide degrading enzymes (Lapébie et al., 2019). As per the defined criterion, the genome of L1I52^T was predicted to have 16 PULs with more than 60% of the predicted CAZymes, i.e., 113 GHs, and 15 PLs encoded within the PULs. In addition, 21 out of 39 TonB-systems (TBTS) are located within predicted PULs, indicating their essential role in the transport of saccharides. Next, we determined the functional-based analysis of PULs to understand the substrates or polysaccharides that each PUL can utilize or degrade based on the conjoint action of different CAZymes organized within the PUL gene cluster. Interestingly, we found that the L1I52^T genome is well-equipped with PULs capable of utilizing several important plant-related polysaccharides. Most importantly, the L1I52^T genome encoded PULs assumed for the utilization of major plant polymers such as starch, pectin, and xylan. To understand the polysaccharide utilization ability of L1I52^T cells, we checked their growth in minimal media amended with respective polymers. L1I52T cells efficiently utilized pectin and xylan among the tested polymers, whereas starch was not

utilized (Fig. 3.25). However, all the genes required for a functional starch PUL (PUL-1) were coded in the L1I52^T genome (Fig. 3.26). We assume the negative growth of L1I52^T cells in starch-amended minimal media would be a condition-specific trait that requires further media optimization.

(a) Starch PUL (PUL-1)

L1I52^T genome harboured a PUL coding for starch utilization, which included three copiesof GH13 CAZyme coding for α-amylases that are known to break the α-1,4 bonds in starch (reference). In addition, single copies of other CAZymes, such as GH97 coding for α-glucosidase and Carbohydrate Esterase 1 (CE1), complement the function. The cluster

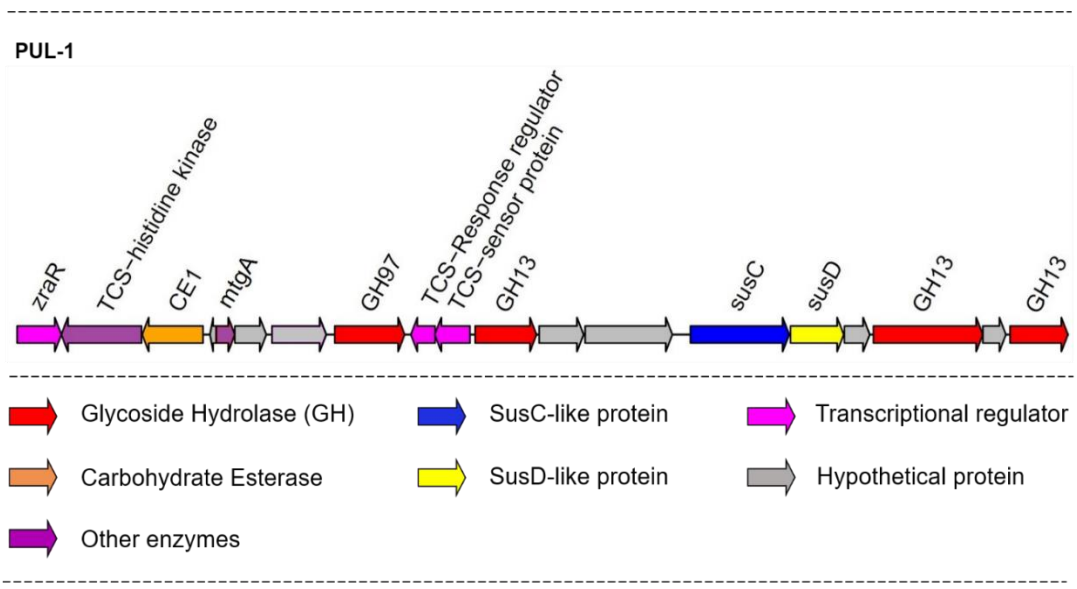


Fig. 3.26. Gene organization of PUL-1 (Starch) in *F. pokkalii* L1I52^T genome. Gene's belonging to different CAZyme categories and respective families are mentioned in the PUL along with other subset of genes as indicated in the legend with different colours.

encodes a pair of *susC/D*-like genes and is controlled by sigma-54 dependent transcriptional regulator, *zraR* (3.26). Further, the cluster encodes a pair of two-component system (TCS) genes coding a response regulator, LytT, and a sensor histidine kinase, LytS (Fig. 3.26). Despite the presence of all genes necessary for a functional PUL for starch utilization, the growth of L1I52^T cells was not detected in the starch-amended minimal media under the tested conditions (Fig. 3.25). We assume that a better-optimized media composition might initiate the starch utilization potential of L1I52^{T,} which needs to be investigated further.

(b) Pectin PULs (PUL-2 (A, B, C))

The L1I52^T genome harboured three PULs specialized for the utilization of plant polymers and pectin (Fig. 3.27). Polygalacturonase (PG) which is the major hydrolytic enzyme that

acts on polygalacturonic acid by hydrolyzing α-1,4 glycosidic bonds of the pectic acid (Tucker, 2004). Moreover, L1I52^{T} harbours endo-PG (EC 3.2.1.15), known for the efficient hydrolysis of pectic acid facilitated through the random degradation of pectin chains (Tucker, 2004). Further, it includes other glycosyl hydrolases such as GH88 coding for d-4,5-unsaturated β-glucuronyl hydrolase (EC 3.2.1.-), GH43 coding for arabinofuranosidase (EC 3.2.1.55), and GH2 coding for β-galactosidase (E.3.2.1.23) that target the pectin side

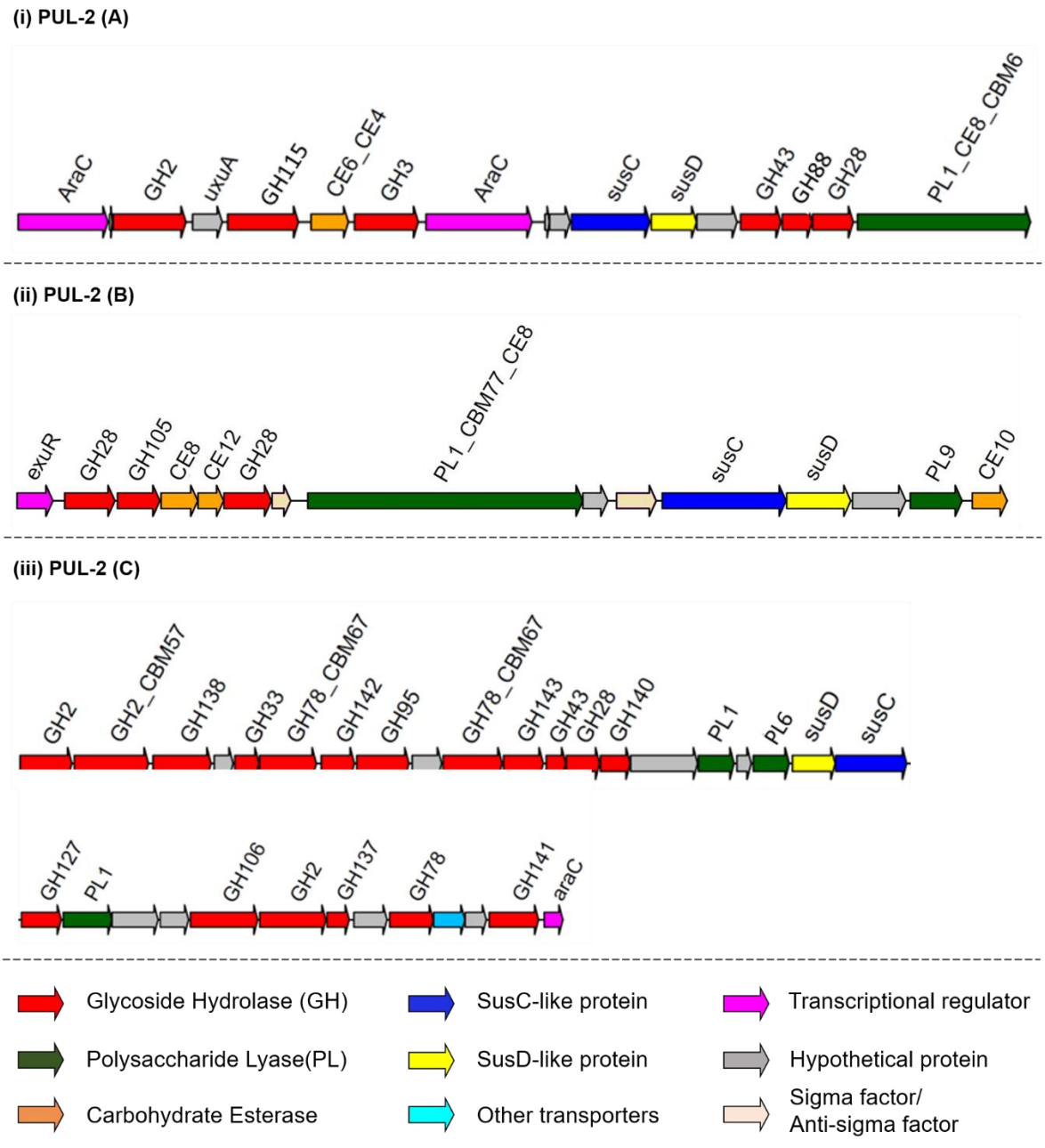


Fig. 3.27. Gene organization of (i) PUL-2 (A), PUL-2 (B), and PUL_2 (C) (Pectin) in *F. pokkalii* L1I52^{T} genome. Gene's belonging to different CAZyme categories and respective families are mentioned in the PUL along with other subset of genes as indicated in the legend with different colours.

chains (Fig. xx). Further, to enhance the pectin degradation, a copy of the polysaccharide lyase gene, PL1 coding for pectinesterase family protein containing CE8 and CBM6 domains, are also present in the cluster (Fig. 3.27 (a)). A pair of DNA-binding response regulators of the AraC family function as the transcriptional regulator of PUL-2 (A) (Fig. 3.27 (a)). The second pectin PUL (PUL-2 (B)) encodes two copies of GH28 for efficient breakdown of pectic polysaccharides and 1 copy of GH88 for the side chain hydrolysis (Fig. 3.27 (b)). Further, the PUL-2 (B) gene cluster harboured two PLs: PL1 containing CE8 and CBM77 domains, PL9 and three carbohydrate esterases: CE8 with pectinesterase activity, CE12 with rhamnogalacturonan acetyltransferase activity, and CE10 with alpha/beta hydrolase activities that perform to enhance the pectinolytic activities (Fig. 3.27 (b)). The PUL-2 (B) gene cluster expression is regulated by the LacI family transcriptional regulator, ExuR (Fig. 3.27 (b)). Finally, the third pectin PUL (PUL-2 (C)) is a huge gene cluster harbouring almost 33 genes comprising a long array of GH coding CAZyme genes (Fig. 3.27 (c)). Majorly, it contains single copies of GH28 coding for PG, and GH106 accompanied by three copies of GH78 (two copies contain a CBM6 domain) coding α-L-rhamnosidase for the efficient breakdown of pectin. For the side chain breakage, PUL-2 (C) is equipped with two copies of GH2 (one copy with malectin-domain containing CBM57) coding for β-galactosidase (GalB), GH95 coding for α-L-fucosidase and GH43, and GH127 coding for α-L-arabinofuranosidase (Fig. 3.27 (c)). Further, the cluster includes three copies of polysaccharide lyases (two copies of PL1, and a single copy of PL6) (Fig. 3.27 (c)). The cluster is regulated by an AraC family transcriptional regulator (Fig. 3.27 (c)).

Altogether, it is assumed that the presence of these three PULs encoding for pectin utilization complements the growth of L1I52^{T} cells in pectin-amended media. However, the comparative analysis found almost all genes corresponding to the three pectin PULs in its phylogenetic neighbours and other terrestrial strains such as *Flavobacterium* sp. F52, and *Flavobacterium* sp. CF136 (plant-associated) and *F. johnsoniae* UW101^{T} (soil). However, the genes corresponding to two PULs: PUL-2 (A) and PUL-2 (C), were absent in the other three Pokkali-isolated *Flavobacterium* strains: NRK1, NRK F7, and NRK F10. Hence, it is assumed that the presence of more than one pectin PUL might be a characteristic feature of plant-associated or standard terrestrial *Flavobacterium* strains, unlike the other *Flavobacterium* strains isolated from Pokkali rice (NRK1, NRK F7, and NRK F10).

(c) Xylan PUL (PUL-3)

Xylan is a highly complex heteropolysaccharide predominantly found in plants. In L1I52^T genome, we could identify a major PUL dedicated for xylan utilization that comprised almost 31 genes (Fig. 3.28). Xylan PUL (PUL-3) cluster harbours two copies of GH10 CAZyme coding for endo-1,4-beta-xylanase that are known for the efficient breakdown of xylan backbone through the endo hydrolysis of 1,4-beta-glycosidic bonds connecting xylose subunits to its respective xylooligosaccharides and xylose (reference) (Fig. 3.28). Further, PUL-3 encodes various GH encoding genes belonging to different families: six copies of GH43 (2 copies contain CBM6 domain) coding for beta-xylosidase, GH3 coding for 1,4-β-

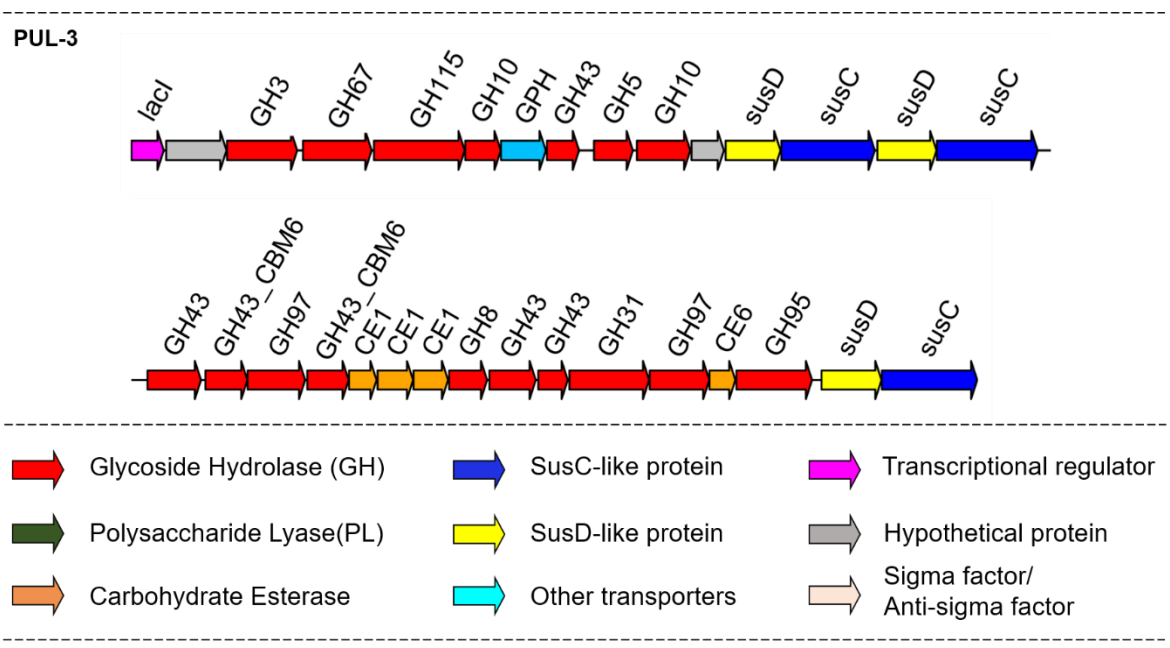


Fig. 3.28. Gene organization of PUL-3 (Xylan) in *F. pokkalii* L1I52^T genome. Gene's belonging to different CAZyme categories and respective families are mentioned in the PUL along with other subset of genes as indicated in the legend with different colours.

xylosidase, GH5 coding for cellulase family GH with O-glucosyl hydrolase activity, GH31 coding for alpha-xylosidase, GH67, and GH115 coding for alpha-glucuronidase, GH95 coding for alpha-L-fucosidase and two copies of GH97 coding for α-glucosidase (Fig. 3.28). Further, the PUL-3 cluster encodes three copies of CE1 and one copy of CE6, which is known to perform key roles in xylan degradation by removing acetyl and feruloyl moieties (Fig. 3.28). LacI family transcriptional regulator controls the expression of the xylan cluster, PUL-3 (Fig. 3.28). Interestingly, PUL-3 harbours three pairs of *susC/D*-like genes, two of which are tandemly arranged within the cluster (Fig. 3.28). Interestingly, the PUL-3 cluster shares good gene synteny with the xylan degradation clusters: PUL 43, and the larger xylan cluster (PUL-XylL) of the human gut members, *Bacteroidetes xylanisolvens* XB1A^T, and

Bacteroidetes ovatus ATCC 8483 strains which are known for potential (Despres et al., 2016; Rogowski et al., 2015). Fig. 3.29 shows the gene synteny between the xylan cluster (PUL-3) of L1I52^T strain and PUL-XylL of *B. xylanisolvens* XB1A^T. Moreover, the core xylan utilization cluster mentioned in PUL43 and PUL-XylL xylan clusters of *Bacteroidetes* strains which includes the tandem repeats of *susC/D*-like gene pair, a hypothetical protein, and GH10 along with its adjacent glycosyl hydrolase coding genes such as GH43, GH43-CBM6, and GH97 are present in PUL-3 of L1I52^T arranged in the same pattern (Despres et al., 2016) (Fig. 3.29). Hence, we assume that PUL-3 of L1I52^T also follow a similar

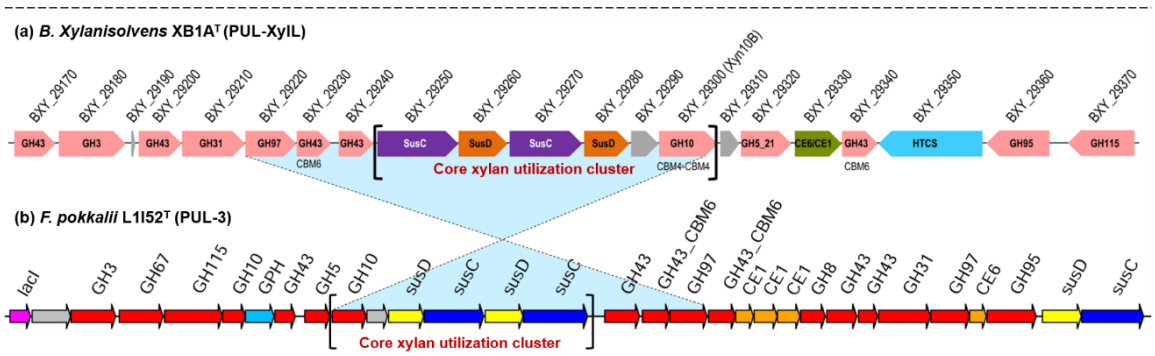


Fig. 3.29. Image representing the gene synteny of xylan PULs encoded in the genomes of *B> xylanisolvens*XB1AT (PUL-XylL), and *F. pokkalii* L1I52^T (PUL-3) which highlights the major genes shared between the PUL highlighted in blue shading with the presence of core xylan utilization cluster marked within the brackets.

mechanism to carry mediate the utilization of xylan. Further, to validate the xylan utilization ability of L1I52^T, we checked if the concentration of xylan influences the growth of L1I52^T cells compared to its monosaccharide, xylose. For this, we compared the growth rates of L1I52^T cells in minimal media amended with 0.1% (lower concentration) and 0.5% (higher concentration) of xylan (polysaccharide) and xylose (monosaccharide) as sole carbon sources. Interestingly, the growth of L1I52^T was enhanced ~2 times in 0.5% compared to 0.1% of xylan, whereas the varied concentration of its monosaccharide, xylose, did not enhance the growth (Fig. 3.30). This observation indicates their high efficiency in utilizing polysaccharides compared to monosaccharides. Further, we were interested in

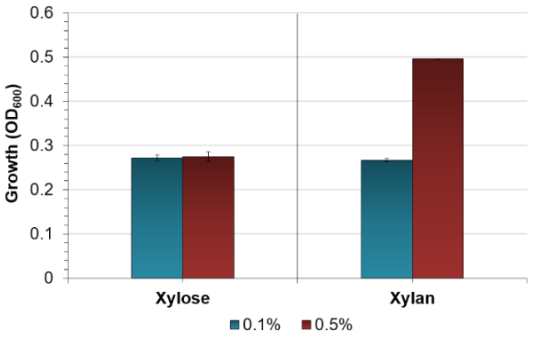


Fig. 3.30. Xylan concentration enriches the growth of *F. pokkalii* L1I52^T cells: Bar graph representing the growth comparison of *F. pokkalii* L1I52^T cells between xylan (polysaccharide) and its monosaccharide (xylose) amended as sole carbon sources at different concentrations (0.1% and 0.5%) in the minimal media base.

whether the brackish condition significantly influences the L1I52^{T} growth. For this, we checked the cell growth in minimal media amended with xylan and xylose as a sole carbon source under two conditions: 1) artificial saline (0.5% NaCl), and 2) brackish condition (30% NSW). Interestingly, xylan was utilized in NaCl and NSW amended conditions whereas xylose was utilized only in NaCl-amended conditions (Fig. 3.31 (a, b)). This preliminary data pertaining to the enhanced utilization of xylan in the brackish condition (30% NSW) supports the eco-adaptive trait of the novel *Flavobacterium* strain.

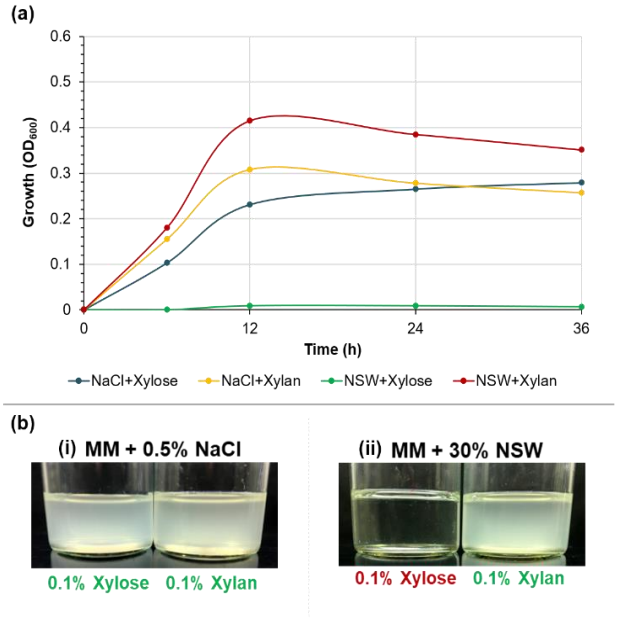


Fig. 3.31. Comparative growth represented by **(a)** line plot, **(b)** images of *F. pokkalii* L1I52^{T} cells in minimal media amended with 0.5% xylan, and 0.5% xylose as sole carbon sources in the presence of **(i)** artificial saline (0.5% NaCl), and **(ii)** brackish conditions (30% NSW)

To finally confirm the selective utilization of xylan by L1I52^{T}cells, an experiment was performed wherein a mixture of both xylan and xylose was supplemented as the carbon source, and the xylan and xylose contents in the media was quantified through High-performance liquid chromatography (HPLC). For this, we collected the samples at 0th h to know the initial amount of xylose and xylan present in the media and at 12th, 24th h to know the utilisation rate. The sampling points are marked in the black dotted circle in Fig. 3.32 (a). The collected samples were subjected to an HPLC analysis, and the peaks were detected based on the retention time (tR) obtained for xylan and xylose standards that appeared at 8.9 and 16.4, respectively. We did a preliminary quantification based on the peak area from the chromatograms to determine the reduction of xylan and xylose from the supplemented mixture over the time range 0 - 12 - 24 h. Interestingly, the peak area of xylan reduced 50% from 0 – 24 h time interval whereas the peak area of xylose showed and low reduced of only

17% (Fig. 3.32 (b, c)). Hence, the growth of L1I52^T cells observed in the media supplemented with xylan/xylose mixture is assumed to be contributed majorly by the xylan.

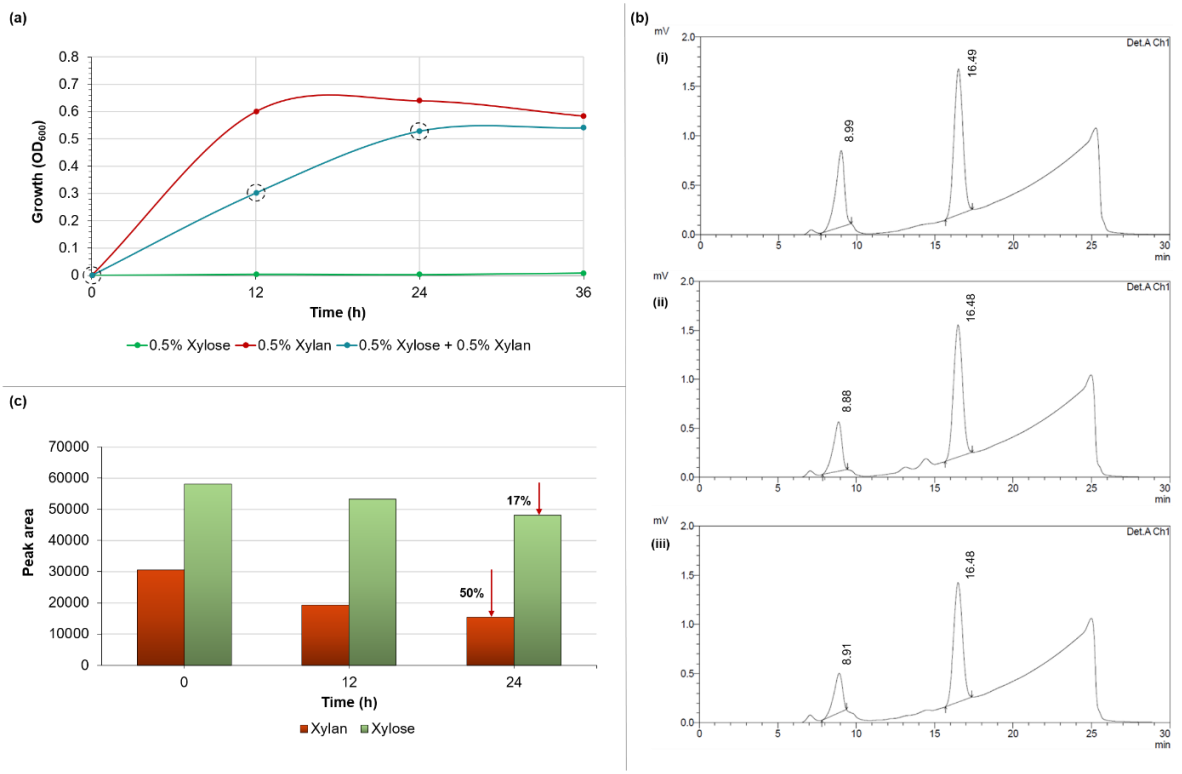


Fig. 3.32. *F. pokkalii* L1I52^T prefers polysaccharides over monosaccharide: **(a)** Bar graph depicting the growth of *F. pokkalii* L1I52^T cells in minimal media amended with xylan, xylose, and xylan-xylose mixture as carbon sources. The dotted circles on 0.5% Xylose-Xylan data line indicate the time-points sampled for HPLC detection of xylose and xylan, **(b)** HPLC chromatograms indicating the peaks of xylan (R_t – 8.9) and xylose (R_t – 16.4) detected from the xylan-xylose sample mixture, **(c)** Bar graph comparing the reduction in peak areas of xylan and xylose at different time points (0 h, 12 h, and 24 h) from the xylan-xylose sample mixture.

Further, this observation validates the preference for polysaccharide (xylan) by L1I52^T cells over its monosaccharide (xylose), especially in the brackish conditions, which is assumed to be an eco-adaptive driven trait of the novel *Flavobacterium* strain. However, a slight reduction in the peak area of xylose was also observed during the HPLC analysis despite the absence of growth in the media (Fig. 3.32 (b, c)).

(d) Other major PULs in the L1I52^T genome

Apart from the above-detailed PULs, the L1I52^T genome encodes another array of CAZyme-organized PULs that can utilize different plant-derived substrates. There are 8 PULs that are specific for a variety of plant-derived substrates such as Arabinoxylan, Dextran, Galactan, Levan, α-mannan, Rhmanogalacturonan and Xylogalacturonan (Fig. 3.33; Fig 3.34). Among this, two PULs (PUL–9 (A) and PUL–9 (B)) were coded for rhamnogalacturonan

polysaccharide harbouring critical CAZyme genes such as GH28, GH43, GH75, GH105, and GH106 (Fig. 3.34 (ii, iii)). Further, PUL 9 (B) encoded three copies of Polysaccharide Lyases (PL) belonging to different families such as PL4, PL6, and PL22 coding for Rhamnogalacturonan endolyase (EC 4.2.2.23), Alginate lyase (EC 4.2.2.3), and Oligogalacturonate lyase (EC 4.2.2.6), respectively (Fig. 3.34 (iii)). Additionally, the cluster contained three copies of CE encoding CAZyme genes belonging to two families, CE8 and CE12 (Fig. 3.34 (iii)). The cluster harboured two copies of *sus-C/-D*-like genes and a GntR

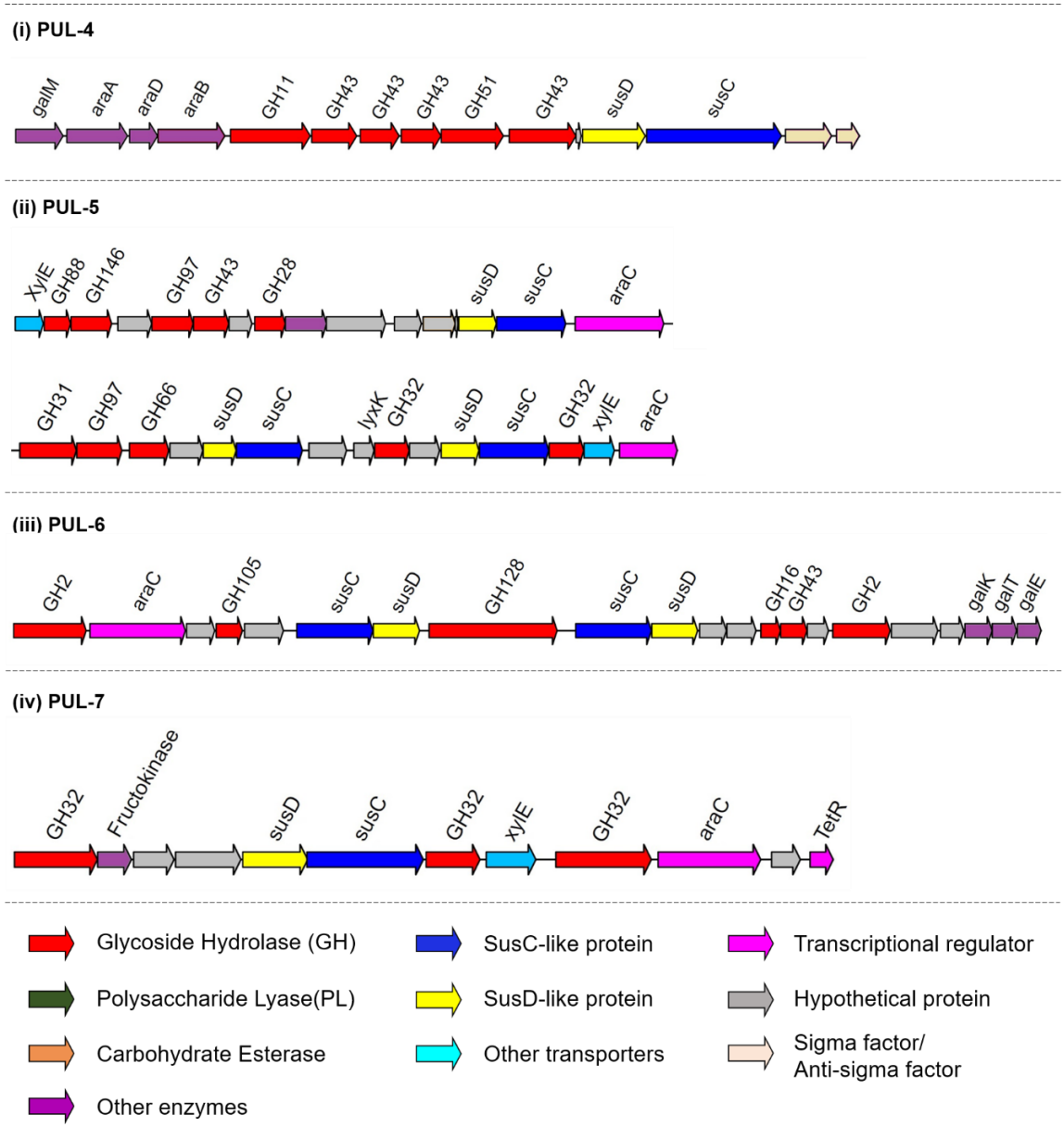


Fig. 3.33. Other major PULs coded in L1I52^{T} genome: **(i)** PUL-4 (Arbinoxylan), **(ii)** PUL-5 (Dextran), **(iii)** PUL-6 (Galactan), and **(iv)** PUL-7 (Levan). Gene's belonging to different CAZyme categories and respective families are mentioned in the PUL along with other subset of genes as indicated in the legend with different colours.

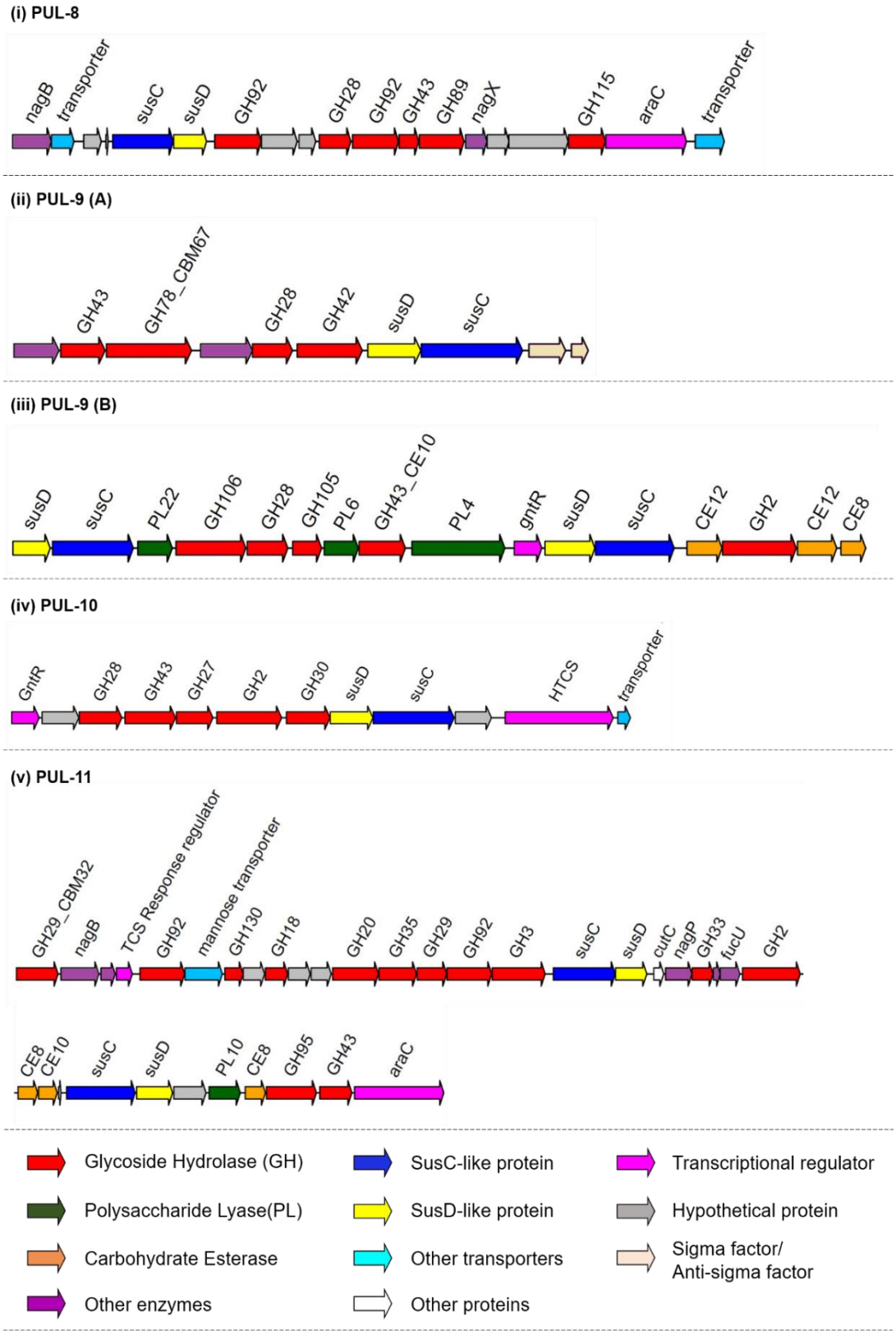


Fig. 3.34. Other major PULs coded in L1I52^{T} genome: **(i)** PUL-8 (Alpha-mannan), **(ii)** PUL-9 (A) (Rhamnogalacturonan), **(iii)** PUL-9 (B) (Rhamnogalacturonan), **(iv)** PUL-10 (Xylogalacturonan), and **(v)** Chitin. Gene's belonging to different CAZyme categories and respective families are mentioned in the PUL along with other subset of genes as indicated in the legend with different colours.

family transcriptional regulator (Fig. 3.34 (iii)). Altogether, an efficient system for the utilization of rhamnogalacturonan polysaccharide is coded in L1I52^T, which requires functional validation. Other major PULs were those coding for plant polysaccharides such as Arabinoxylan, dextran, and galactan, among which dextran and galactan-specific PULs harboured multiple repeats of *sus-C/-D* like genes indicating their high biding and uptake ability for coded polysaccharides (Fig. 3.33).

Interestingly, the L1I52^T genome encoded specialized PUL for the breakdown of chitin polymer (Fig. 3.34 (v)). The cluster harboured a copy of GH18 and GH20 coding for Chitinase (EC 3.2.1.14), and β-N-acetylhexosaminidase (EC 3.2.1.52), respectively (Fig. 3.34 (v)). Further, the cluster encoded a copy of *nagB* gene coding for glucosamine-6-phosphate deaminase (EC 3.5.99.6) (Fig. 3.34 (v)). The cluster is further supported with an array of CAZymes belonging to different families such as GH2, GH3, GH29 (2 copies), GH33 GH35, GH92 (2 copies), GH130, and 1 single copy of PL10 (Fig. 3.34 (v)). The cluster also contained a copy of the sodium-dependent mannose transporter and a single copy of *nagP* gene coding for the transport of N-acetyl glucosamine (Fig. 3.34 (v)). A copy of a two-component sensor protein and a response regulator is also coded in the cluster (Fig. 3.34 (v)). The PUL harbours 2 copies of *SusC-/D*-like genes, and the expression is regulated by the DNA-binding response regulator gene, *araC* (Fig. 3.34 (v)). All these indicate a functional chitin PUL capable of chitin depolymerization. However, more validation studies are required for its functional confirmation. The presence of chitin-specific PUL in the plant-associated L1I52^T strain was highly intriguing. However, we assume this characteristic PUL in L1I52^T might be an ecologically evolved adaptive trait acquired over years of its inhabitance in this complex ecosystem where shrimps are also cultivated in the alternate season of the year. Shrimps and other fishes are known for their high chitin content. L1I52^T is known for its survival ability as planktonic cells without the plant host, Pokkali for many months, and during this stage, the bacteria might have evolved to depolymerize chitin that is assumed to be a major polymer in the Pokkali rice fields during shrimp cultivation season.

In nature, polysaccharides will not be available in pure forms of pectin or xylan for bacteria to utilize; rather, they will be a mixture of complex polysaccharides. Therefore, to validate the polysaccharide utilization potential, the ability of L1I52^T cells to utilize the root macerate (RM) prepared from 10-day-old Pokkali seedlings was checked. For this, we inoculated L1I52^T cells into customized media combinations, which included 3 conditions: (i) diluted R2A3 (½ strength 1/10 R2A3) – R2A3-d, (ii) R2A3-d + 50% RM, and (iii) 50% RM without

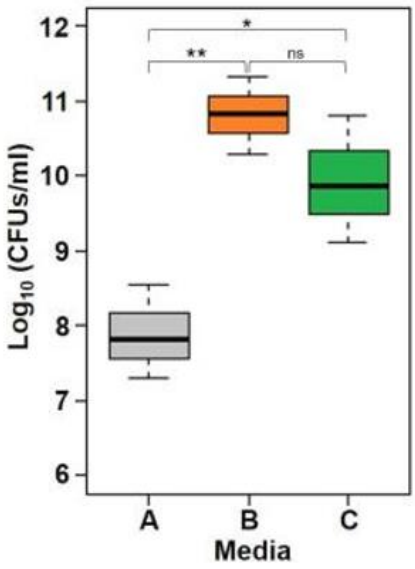


Fig. 3.35. *F. pokkalii* L1I52^T efficiently utilizes Pokkali seedling root macerate: Box plot representing the recovery of L1I52^T cells (represented as Log_{10} (CFUs/ml) from customized media's: A, R2A-d; B, R2A3-d + 50% RM; C, 50% RM. Statistical significance represented by *, p-value<0.05; **, p-value<0.01, ns, non-significant.

any media base or nutrient supplements. L1I52^T cells were recovered from different conditions through dilution spot assay the quantified. Interestingly, the RM prepared from sterile Pokkali seedlings influenced the growth of L1I52^T cells, which showed a 3-fold increase in the recovery of L1I52^T cells compared to the media base, i.e., R2A3-d without RM (Fig. 3.35). Intriguingly, 50% RM alone could influence the growth of L1I52^T cells with a 2-fold increase compared to the media without RM (Fig. 3.35). These results support the efficient polysaccharide utilization potential of the novel *Flavobacterium* strain, which prefers plant-derived materials over synthetic media. These observations substantiate their position in the ecosystem relative to the host association and eco-adaptation. Overall, these physiological results display an interesting ecological adaptive lifestyle of this novel *Flavobacterium* strain, L1I52^T, which must be studied further in detail, particularly with its plant-associated lifestyle under brackish conditions.

(e) Sulfatase genes and associated PULs in L1I52^T

The L1I52^T genome harboured 14 genes coding for sulfatases. Interestingly, its nearest phylogenetic neighbours, *Flavobacterium* strains isolated from Pokkali roots (NRK F7, NRK F10, NRK1), and other terrestrial *Flavobacterium* strains (*F. johnsoniae* UW101^T, F52, CF136) possessed significantly less (< 4 no's) sulfatase coding genes. However, sulfatase coding genes have been identified in higher numbers in certain *Flavobacterium* strains isolated from marine or algal-associated habitats that are predicted to degrade algal

polysaccharides such as alginate, laminarin, agar, fucoidan, etc (Mann et al., 2013; Sun et al., 2016). More importantly, all the 14 sulfatase genes coded in the L1I52^{T} genome are organized within 4 different PULs arranged with an array of CAZymes including GHs, PLs, and Sus-C/-D like binding proteins and transcriptional regulator (Fig. 3.36). This further

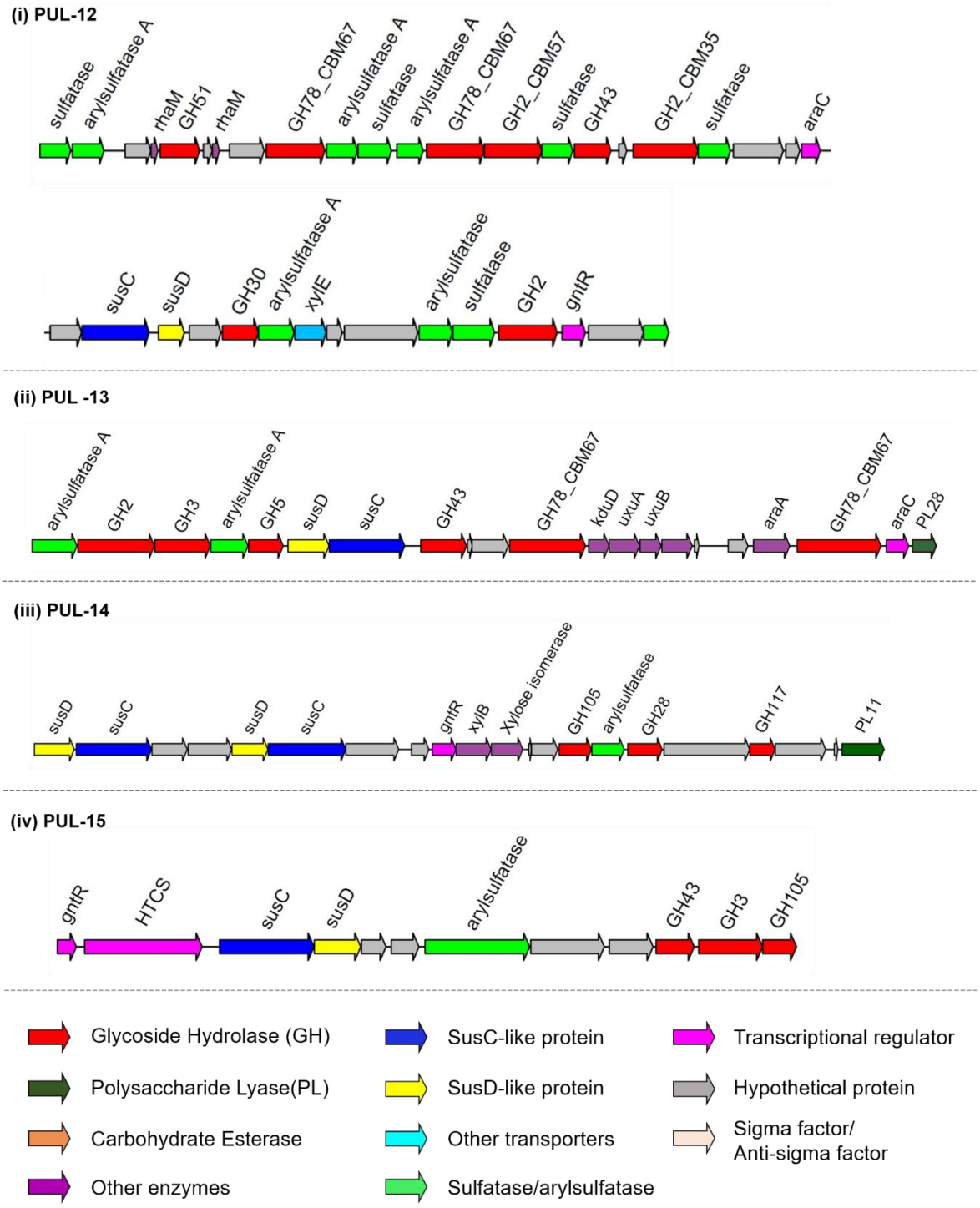


Fig. 3.36. Sulfated PULs coded in L1I52^{T} genome. Gene's belonging to different CAZyme categories and respective families are mentioned in the PUL along with other subset of genes as indicated in the legend with different colours.

indicates their probable role in the potential degradation of non-plant- based polysaccharides. Among these, PUL-12 is identified as xylose and rhamnose-specific sulfated PUL which consist of β-xylosidase (GH43), xylanase (GH30), endo- β-xylanase (GH51), α-L-rhamnosidase (GH78), β-galactosidase (GH2) and L-rhamnose mutarotase. A xylan transport gene *xylE* was also identified within the PUL, involved in the uptake of plant materials. PUL-12 contains 10 sulfatases, which include nine choline sulfatase genes and one arylsulfatase gene. Additionally, the cluster comprises a sulfatase modifying factor 1 reported to be involved in the regulation of sulfatase production. The presence of CAZyme genes and all other important genes required for the sulfatase production indicates that this PUL might be functional. Further, PUL-13 is identified as rhamnose-specific sulfated PUL which contain α-L-rhamnosidase (GH78), α-L-rhamnose isomerase for rhamnose degradation controlled by an araC transcriptional regulator (Fig. 3.36). The two sulfatases identified in this PUL correspond to choline-sulfatases. However, sulfatase modifying factor could not be identified in this PUL. PUL-14 is assumed to be a rhamnogalacturonan-specific PUL with only 1 copy of choline-sulfatase genes adjacent to GH28 CAZyme gene coding for polygalacturonase along with GH117 and PL11 CAZyme coding for rhamnogalacturonan lyase. This cluster harbours 2 copies of *susC-/D*-like genes controlled by a GntR family transcriptional regulator coding gene (Fig. 3.36). Finally, PUL-15 contained a sulfatase gene harboured with CAZyme genes, such as GH43, GH3, and GH88, with a single copy of s*usC-/D*-like genes controlled by GntR family transcriptional regulator coding gene (Fig. 3.36).

These results indeed point out that L1I52^T is not specialized in targeting sulfated algal-derived polysaccharides although it is well known that the flooded rice fields are known to harbour cyanobacteria (Ariosa et al, 2004; Irisarri et al, 2001). Thus, a higher proportion and greater diversity of CAZymes in the L1I52^T genome reflects its catabolic potential for degrading primarily on plant-related polysaccharides. Further, these conclusions were well supported by the results of the peptidase/GHs ratio, where the L1I52^T genome had a ratio of 0.96 peptidase/GHs, a low ratio typically found in all terrestrial *Flavobacterium* genomes known to have a higher preference for utilizing plant-derived polysaccharides (Kolton et al, 2013). These results point out that L1I52^T is not specialized in targeting sulfated algal polysaccharides despite four sulfated PULs. However, a detailed investigation is necessary to claim these aspects.

3.3.5.9 Pangenome analysis identifies unique genetic traits in L1I52^T

To have a brief insight into the uniquely coded genetic traits in L1I52^T, we performed a pan-genome analysis using anvi'o pangenome pipeline wherein we compared L1I52^T with its

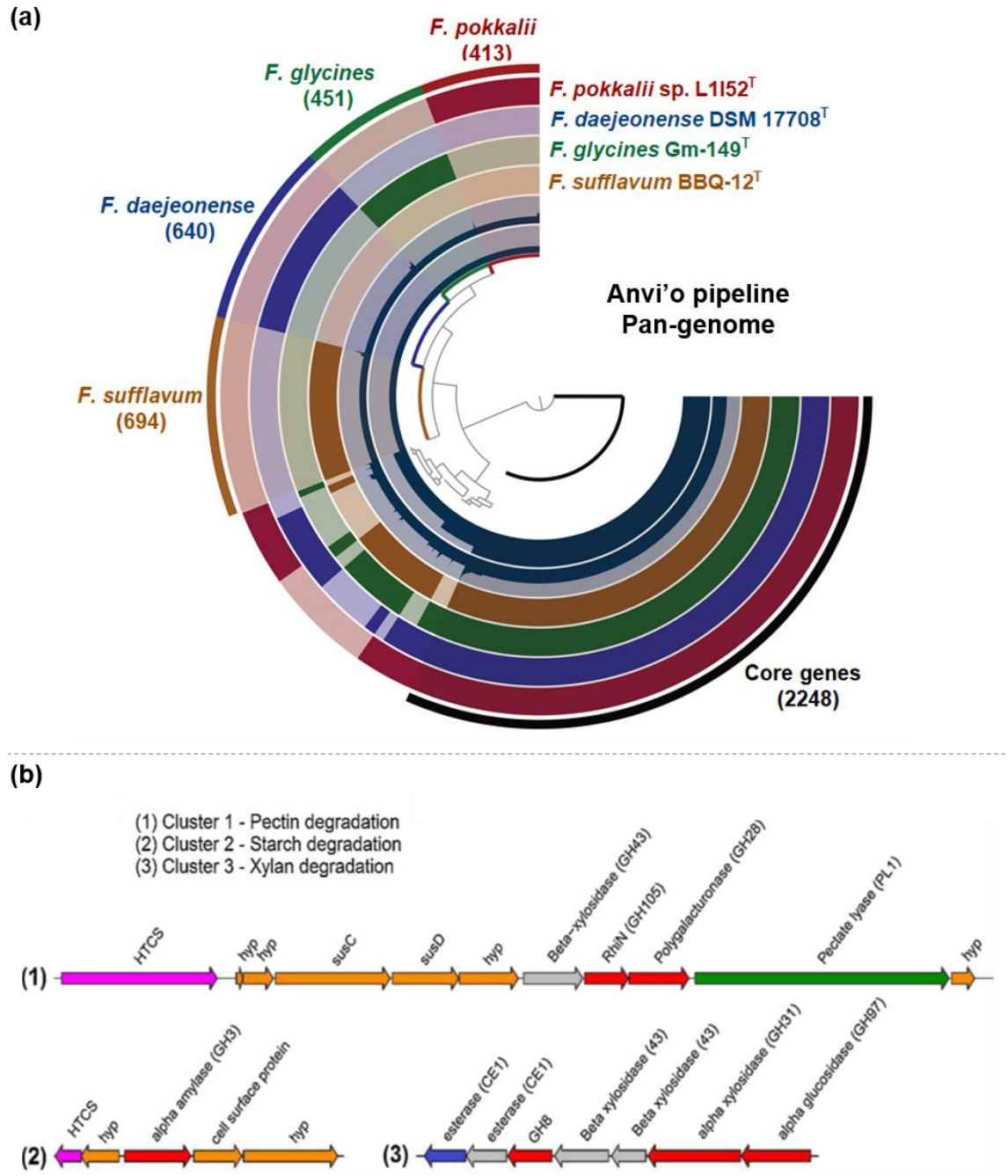


Fig. 3.37. (a) Pangenome map generated using four genomes of genus *Flavobacterium* type strains - *F. pokkalii* sp. L1I52^T, *F. daejeonense* DSM 17708^T, *F. glycines* Gm-149^T, and *F. sufflavum* BBQ-12^T. The dendrogram in the inner region represents the relationship between the 5339 gene clusters consisting of 13,781 genes found in all four genomes analyzed. The genome map represents the core genes common in all strains and unique genes present in each strain. Dark-coloured regions in circles represent unique genes in each genome, and light-colored regions represent the genes commonly shared among the genomes. **(b)** Unique gene clusters in *F. pokkalii* L1I52^T compared to its phylogenetic neighbours.

nearest phylogenetic neighbours (*F. daejeonense* DSM 17708^T, *F. glycines* Gm-149^T, and *F. sufflavum* BBQ-12^T. From the analysis, around 2248 genes were shared among all four strains, constituting the core genes assumed to share similar functions. Further, around 413 genes were identified to be unique in L1I52^T compared to the other three genomes (Fig. 3.37 (a)). From detailed analysis, it was found that most of the genes were designated as hypothetical proteins, indicating that certain genes/gene clusters were designated to code for polysaccharide degradation specific for plant polymers such as pectin, starch, and xylan (Fig. 3.37 (b)). However, the presence of the genes/gene clusters coding for starch, pectin, and xylan has already been discussed in the previous sections. The presence of sulfatase gene coding PULs also accounts for a unique feature of L1I52^T genome. However, their functional role relative to their eco-physiology needs to be investigated. Interestingly, certain unique genes corresponded to code for sodium homeostasis, which possibly indicates the unique brackish-adaptive traits of L1I52^T compared to its phylogenetic neighbours. Based on the critical leads obtained from the pan-genome analysis, we understood that polysaccharide utilization and brackish adaptation are critical traits that place L1I52^T unique from its phylogenetic neighbours and other *Flavobacterium* strains (Fig. 3.37).

3.3.6.10 Brackish-adaptive traits in L1I52^T genome and associated survival physiology

From the eco-physiological characterization studies, it is conclusive that the tested brackish conditions influence the growth, survival, and host-association abilities of L1I52^T cells (discussed in earlier sections). Further, the influence of seawater in the Pokkali rice root attachment and colonization compared to artificial saline conditions led to the realization that L1I52^T cells rely on the ionic salts of seawater compared to sodium ions alone as in artificial or synthetic saline conditions.

Altogether, the observations and findings relating to the brackish adaptive phenotypes position L1I52^T as a unique eco-physiological spot within the genus *Flavobacterium*. In support of this view, the L1I52^T genome was deeply characterized for its sodium extrusion/intake combined with inorganic compatible solute uptake/extrusion mechanisms, and organic compatible solute transport and biosynthesis.

(a) Na^+ extrusion and uptake

The Na^+ extrusion is regarded as a key homeostatic process for maintaining the ionic balance in bacterial cells during high saline conditions in the external environment. Pokkali ecosystems are known for fluctuating brackish conditions that witness high saline phases. To circumvent this, L1I52^T genome is equipped with an array of genes to facilitate the Na^+

mechanism (Fig. 3.38). Most importantly, L1I52^T genome harbours Na^+-translocating NADH-quinone reductases (Na^+-NQR) coding gene complex (*nqrABCDEF*) comprising 6 subunits (A, B, C, D, E, F), which is a characteristic feature identified in several marine bacteria (Kogure, 1998; Minato et al., 2014) (Fig. 3.38; Table. 3.8). This is assumed to function as the primary Na^+ extrusion pump in L1I52^T during high saline conditions. The energy generated from this redox reaction pumps the sodium ions from the inner to the outer membrane of the cell thereby maintaining the cell homeostasis. Interestingly, this gene complex was absent in other known plant-associated/soil *Flavobacterium* strains including the closest phylogenetic neighbours except *F. daejeonense* DSM17708^T, which is the closest relative of L1I52^{T}able. x. Further to support this, there are 2 copies of genes coding for NhaA-type antiporters that act as secondary Na^+ pumps that extrude Na^+ ions in exchange for H^+ ions (Fig. 3.38; Table. 3.8). Further, to complement the cell homeostasis under low

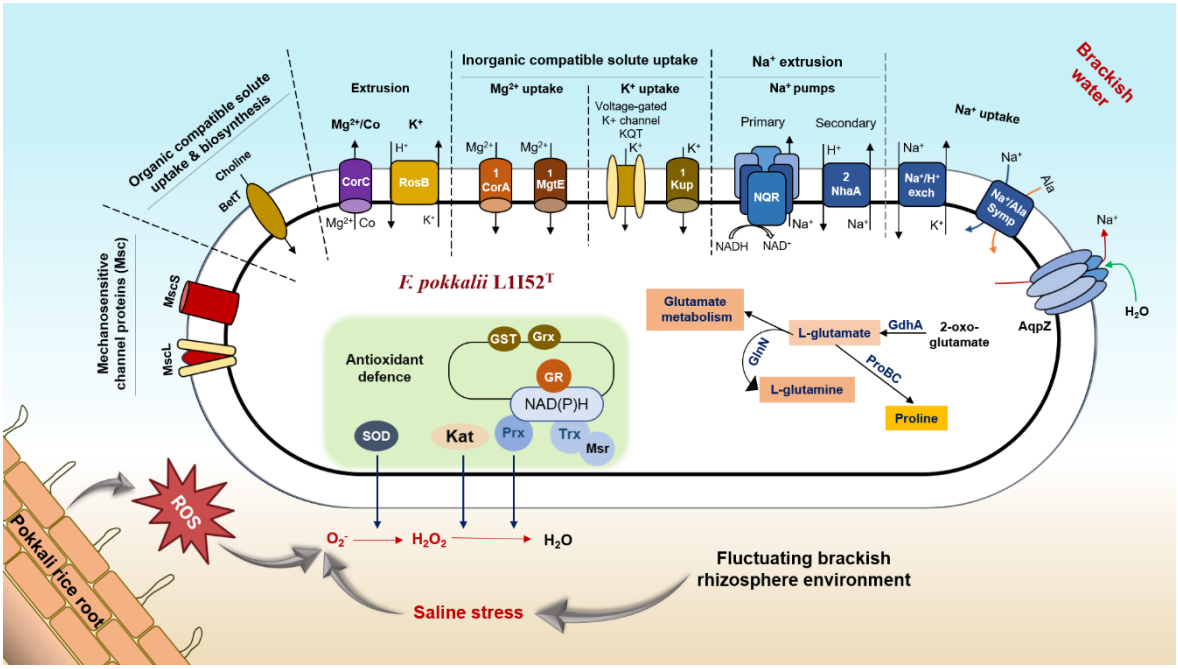


Fig. 3.38. Schematic diagram depicting the brackish adaptive traits and ROS detoxification mechanism coded in *F. pokkalii* L1I52^T genome to circumvent the fluctuating brackish conditions prevailing in the Pokkali rice fields and the resultant ROS aroused from the environmental and host plant defence caused due to the saline stress

saline conditions, L1I52^T is also coded with Na^+ ion uptake genes (Fig. 3.38, Table. 3.8). This includes a few genes coding for symporter proteins that co-transport Na^+ ions along with other sugars or amino acids such as hexose, myo-inositol and alanine in L1I52^T (Fig. 3.38; Table. 3.8). In addition, the genome also contains a copy of genes coding for sodium iodide symporter and Na^+/H^+ exchanger proteins (Fig. 3.38; Table. 3.8).

(b) Inorganic ion uptake and extrusion

Another mechanism bacteria use to maintain the ionic balance under high saline/brackish conditions is the uptake of inorganic ions such as K^+, Mg^{2+}, etc. To complement K^+ uptake, the L1I52^T genome is equipped with a potassium voltage-gated channel subfamily protein, KQT. Further, single copies of the Kup system potassium uptake protein and K^+ channel protein accompany the K^+ ion uptake process (Fig. 3.38; Table. 3.8). Next, to complement the cell homeostasis through Mg^{2+} uptake, the genome is coded with the following genes: *mgtE* gene coding for magnesium transport protein, and *corA* gene coding for magnesium/cobalt transport protein CorA (Fig. 3.38; Table. 3.8). Further, L1I52^T genome also encodes for genes that function to extrude inorganic ions when needed to maintain the osmotic balance. This includes a copy of *rosB* gene coding for potassium/proton antiporter protein, RosB, and two copies of *corC* gene that codes for the uptake of $Mg^{2+/}$Co ions (Fig. 3.38; Table. 3.8).

(c) Organic compatible solute transport and biosynthesis

Further, to complement the stress adaptation, the L1I52^T genome harbours genes that code to synthesize osmoprotectants such as glutamate, glutamine, and proline. The genome harboured *gdhA* gene encoding NADP-specific glutamate dehydrogenase that converts 2-oxo-glutamate to L-glutamate and glutamine synthetase III that converts L-glutamate to L-glutamine (Fig. 3.38; Table. 3.8). The glutamate also serves as the precursor for proline synthesis, and L1I52^T encodes *proC* gene coding for pyrroline-5-carboxylate reductase and *proB* coding for glutamate-5-kinase that are the key enzymes known to catalyze the conversion of glutamate to proline (Fig. 3.38; Table. 3.8).

(d) Mechanosensitive ion channels

Due to climatic shifts, the fluctuating brackish conditions in the Pokkali fields may suddenly shift from saline or hyper-saline conditions to non-saline or hypo-saline conditions, giving rise to an osmotic down shock to the inhabitant microbiota. These drastic environmental shifts are balanced by robust mechanisms that include the accumulation of compatible solutes and mechanisms to release osmolytes during hypo-osmotic conditions. In this regard, the mechanosensitive ion channel proteins play a vital role as primary osmolyte release valves, causing turgor pressure reduction (Booth, 2014; Bootha and Blount, 2012). Hence, the L1I52T genome harbours 6 genes coding for mechanosensitive ion channel family proteins to counter this hypo-osmotic stress. Among these, 1 gene (*mscL*) code for large-conductance mechanosensitive ion channel protein (Fig. 3.38; Table. 3.8). Certain organisms that dwell

in marine habitats are known to have lost *mscL* gene (Penn and Jensen, 2012). Since L1I52^T cells are exposed to shifts between saline and non-saline conditions, the presence of mscL gene is assumed to be critical for survival in the brackish fluctuating Pokkali ecosystem. Supportive to this extensive gene repertoire for brackish adaptation, L1I52^T survives in brackish conditions as planktonic cells.

(e) Other transporters coded in the L1I52^T genome

Apart from the dedicated transport systems available for the uptake and extrusion of different ions, L1I52^T harbours genes for several transporter proteins. In detail, the following transporters were identified for organic compounds: N-acetylglucosamine, Di-/tripeptide, drug/metabolite, preQ0, RND multidrug efflux, biopolymer, chromate, hexuronate, methionine, methylglutaconyl-CoA, sialic acid, ribose, ribosyl nicotamide, vitamin B12 and L-isoaspartate, O-Methyltransferase. Further, ion transporters for ammonium, iron, manganese, molybdenum, phosphate, potassium, sulfate, zinc, nitrate/nitrite, and Lead/ cadmium/ zinc/ mercury were also identified. Furthermore, thirty-nine ATP-Binding cassette (ABC) transporters were identified for phosphonate, maltose, molybdenum, methionine, iron, ribose, nitrate, lipopolysaccharide, polysaccharide, multi-sugar binding protein, and vitamin B12.

(f) Brackish survival physiology of L1I52^T

In nature, bacterial cells are known to exist in two modes of growth, i.e., in the unicellular life phase, where the cells are free-swimming/free-living, which is called the planktonic state. In contrast, another form is the multicellular life phase, wherein the cells form

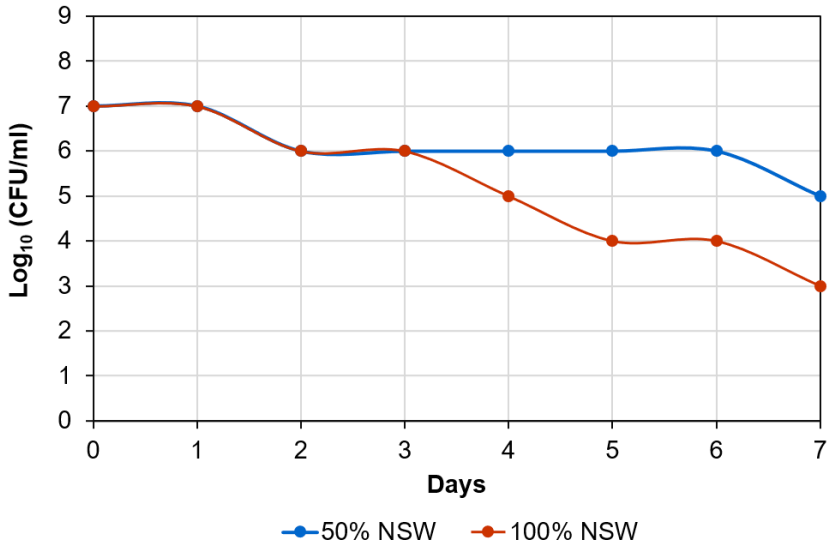


Fig. 3.39. Line plot representing the survival rate of *F. pokkalii* L1I52^T cells in half-strength (50%), and full-strength (100%) NSW for a period of 1 week (7 days)

aggregates that live in a biofilm or in a surface-associated matrix; for example, the bacterial cells attach to plant root surfaces or in the compartments as aggregates or groups of cells which establish their population in the host thus resulting in their host-associated form. The Pokkali rice cultivation exists only for one-third of the duration (i.e., 4 months) of a year, and for the remaining bacterial groups that are closely associated with Pokkali rice, an efficient mechanism is highly mandatory for their survival in free-living form until they are associated with the Pokkali rice host in the next season. Hence, to test this hypothesis, we first examined the survivability of L1I52^T cells in varying natural seawater concentrations

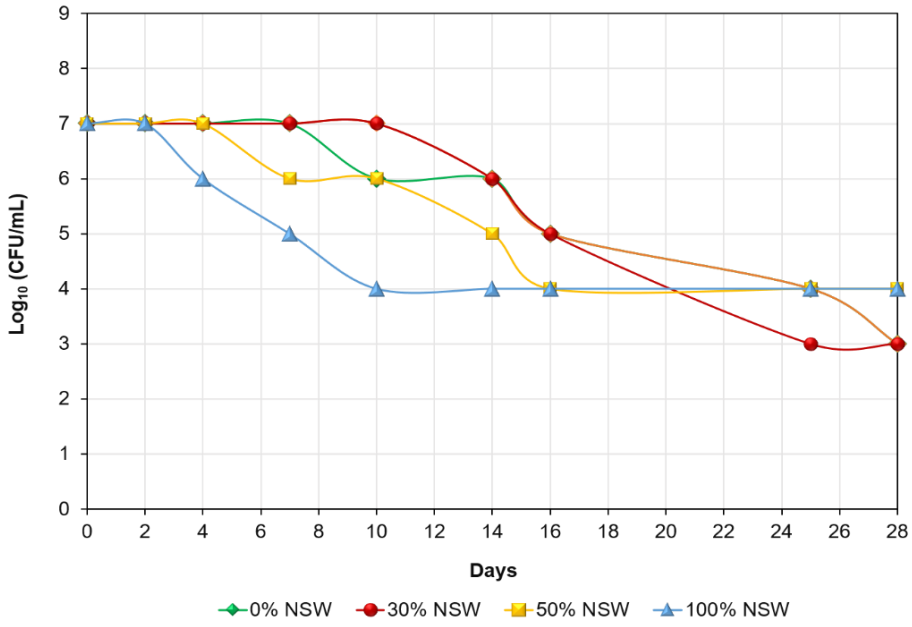


Fig. 3.40. Line plot representing the survival rate of *F. pokkalii* L1I52^T cells in varying seawater concentration (0% 30%, 50% and 100% NSW) prepared in fresh water base for a period of almost 1 month (28 days).

(devoid of any nutrients) as a free-living planktonic form. For this purpose, the L1I52^T cells (~ 7 Log CFUs/ml) were inoculated into half and full-strength natural seawater solutions (NSW) and incubated under static conditions. Further, the viability of L1I52^T cells was determined by quantifying the CFUs represented as $\log_{10}$ of CFUs/ml calculated from the dilution spot assay performed at every 24-hour time interval (see methods section). The recovery data showed no significant reduction in the viability of L1I52^T cells for a period of 6 days in half-strength NSW. On the other hand, a dramatic decrease in cells was observed in full-strength NSW from 7-Log to 3-Log CFUs/ml from 3rd dpi to 7th dpi (Fig. 3.39), The declining survival rate in full-strength NSW condition was in correlation with our growth experiment results wherein we observed weak growth in full-strength NSW amended media that indicating a possible negative growth effects or growth inhibition on L1I52^T cells. The

survival experiments we performed in half-strength and full-strength NSW was prepared in the distilled water base. However, in nature, for example, in the case of Pokkali ecosystem, the brackish water logging in the fields will be a mixture of seawater and freshwater, having an entirely different ionic composition. Hence, we checked the survival rate of L1I52^T cells in suspensions prepared with varying seawater concentrations (0%, 15%, 30%, 50%, and 100%) in the freshwater (FW) base to mimic the natural brackish conditions in the best possible way (see details in method section). Intriguingly, the survival pattern of L1I52^T cells displayed a different scenario when performed using a FW base. The experiment was initiated with 7-log cells, and the same survival rate was observed for a longer duration of 10 days in 30% NSW condition (i.e., 30% NSW and 70% FW) that gradually dropped to 6-Log cells on 14th dpi (Fig. 3.40). Hence, 30% NSW condition was identified to be the best condition for survival during the initial phase of the experiment (i.e., up to 14 days/2 weeks). The survival in 0% NSW (i.e., 100 % fresh water) condition showed an initial drop from 7-Log cells to 6-Log cells on 10th dpi followed by a second drop on 16th dpi to 5-Log cells (Fig. 3.40). Further, the survival in 50% NSW (i.e., 50% NSW and 50% FW) showed a first decline from 7-Log cells to 6-Log cells on 7th dpi followed by a second decline to 5-Log declined at steady pattern from 7-Log cells to 4-Log cells on 4th dpi which followed almost similar patterns as observed in our previous experiment (Fig. 3.40). cells on 14th dpi (Fig. 3.40). However, the survival rate of 4-Log cells was maintained till 28th dpi without any dip (Fig. 3.40). In the case 30% NSW condition after 14th dpi, a sudden fall from 6-Log cells to 5-Log cells was observed within 2 days (16th dpi) followed by a gradual decrease to 3-Log cells when observed on 28th dpi (Fig. 3.40). Similarly, in 0% NSW condition, 5-Log cells recorded in 16th dpi made a gradual reduction to 3-Log cells by 28th dpi (Fig. 3.40). Similar to the 30% NSW, we observed a sudden fall in 50% NSW condition from 5-Log on 14th dpi to 4-Log cells on 16th dpi followed by which it was maintained at the same rate till 28th dpi as equivalent to 100% NSW condition (Fig. 3.40). Despite the initial fall in the survival of L1I52^T cells in 50%, and 100% NSW conditions, the survival recovery recorded during the longer periods of incubation indicate a firm survival mechanism adapted by the novel *Flavobacterium* strain. Since the survival of L1I52^T cells in 15% NSW followed a recovery pattern as observed in 30% NSW, we haven't included it in the figure as the data overlaps. Out of curiosity, the experiment setup was left undisturbed for a long period of 4 months, and we further checked the recovery to determine the survival rate from all the tested conditions. Interestingly, L1I52^T survived in all the tested conditions. The survival rate of L1I52^T was as follows: 2-Log cells in 0% NSW (i.e., 100% FW) and 100% NSW (i.e., 0%

FW), whereas the survival rate in the brackish conditions (15%, 30%, and 50% NSW) were recorded at 3-Log cells (Fig. 3.40)

3.3.6.11 ROS-detoxification mechanism in L1I52^T

In response to various biotic and abiotic stress, including salinity, pathogen attack, or early rhizobacteria-root colonization events, plants actively produce reactive oxygen species (ROS) as one of their primary defence mechanisms (Choudhury et al., 2017; You and Chan, 2015). to establish efficient colonization with the Pokkali rice roots, L1I52^T cells must overcome the rhizosphere oxidative stress induced by the host plant or the surrounding environment. Hence, to outcompete the oxidative stress from the host, L1I52^T cells are equipped with an array of anti-oxidant enzyme encoding genes (Fig. 3.38; Table. 3.9). It includes *katG* gene coding for catalase/peroxidase HPI, a bifunctional enzyme with catalase and peroxidase activity (Fig. 3.38; Table. 3.9). In addition, L1I52^T genome harbour genes coding for 3 copies of superoxide dismutase (SOD; EC 1.15.1.1): 2 copies of Mn-SOD and 1 copy of Cu/Zn-SOD, 2 copies (1 copy of Bcp-type and 1 copy of Tpx-type) of thiol peroxidase (EC 1.11.1.15), 3 copies of Alkyl hydroperoxide reductase subunit C-like protein (peroxiredoxin), 1 copy of 4-carboxymuconolactone decarboxylase domain/alkyl hydroperoxidase AhpD family core domain protein , 1 copy of glutathione peroxidase (GPx; EC 1.11.1.9), 2 copies of glutathione S-transferase-related transmembrane protein (EC 2.5.1.18), 5 copies of thioredoxin (Trx) and 2 copies of thioredoxin reductase (TrxR; EC 1.8.1.9) that reduces the thioredoxin (Fig. 3.38; Table. 3.9). Further, it encodes 2 copies of peptide methionine sulfoxide reductase (MsrA and MsrB) (Fig. 3.38; Table. 3.9). Finally, the hydrogen peroxide-inducible genes activator, *oxyR* that activates the expression of several hydrogen peroxide inducible genes such as *katG, ahpC.,* etc., is also present in L1I52^T genome (Table. 3.9). All these genes contribute to the antioxidant defence mechanism of L1I52^T that can detoxify the reactive oxygen species (ROS) produced by the host Pokkali rice plants during their colonization events.

3.4 Highlights and Conclusion

Studying eco-physiology and host association of a wild bacterial strain is exceptionally challenging. However, studyin these aspects is necessary to understand how this novel strain would impact its host plant or ecosystem functioning. Hence, this chapter discussed the potential host growth influencing phenotypes and associated host gene-modulation abilities of L1I52^T under brackish conditions. The host-gene modulation abilities of L1I52^T studied through RT-qPCR and RNA-Seq approaches identified critical host genes important for coping with abiotic stress and plant growth. Further, the eco-physiological trait characterization confirmed the crucial role of brackish conditions in growth and survival and important host association traits such as biofilm formation, root attachment, and subsequent colonization of the novel strain, L1I52^T. These findings correlated well with the metagenomic evidence that showed high dominance of *Flavobacterium* OTUs in rice roots cultivated in brackish conditions (discussed in Chapter 2). Further, a high-quality draft genome analysis and comparative genomics identified efficient genetic systems in L1I52^T for its carbohydrate utilization and ecological adaptation. This encouraged us to explore its unique polysaccharide utilization ability, wherein we could discover novel aspects relating to polysaccharide utilization, especially on xylan. However, the genome is encoded with a vast array of plant substrate-based PULs, which demands extensive investigation at the genomic and molecular levels. Being a terrestrial clade strain, the unique brackish adaptive traits coded in L1I52^T to circumvent the fluctuating saline conditions are worth noting. Thus, L1I52^T can be firmly considered a terrestrial *Flavobacterium* strain with unique aquatic or marine adaptive traits, for which the host, Pokkali rice, and brackish ecosystem would have been the sources of its adaptive evolution. To the best of my knowledge, this is the first report on a *Flavobacterium* strain to be studied for its host plant functions and associated interaction through eco-physiological characterization studies combined with omics-based approaches. The findings revealed the crucial role of the brackish conditions in mediating *Flavobacterium*-plant interactions. Hence, I believe that the L1I52^T-Pokkali-brackish interaction can be considered as a model system for the future to investigate the influence of brackish environments on *Flavobacterium* strains to mediate its beneficial functions on the host functioning and vice versa through advanced proteomic and metabolomic approaches.

Table 3.5. List of different SEED subsystem-based functional categories and the number of genes associated with each category in L1I52^T genome.

S. No.	SEED subsystem based-functional categories	Number of genes
1	Cofactors, Vitamins, Prosthetic Groups, Pigments	207
2	Cell Wall and Capsule	80
3	Virulence, Disease and Defense	89
4	Potassium metabolism	7
5	Photosynthesis	0
6	Miscellaneous	22
7	Phages, Prophages, Transposable elements, Plasmids	16
8	Membrane Transport	71
9	Iron acquisition and metabolism	9
10	RNA Metabolism	118
11	Nucleosides and Nucleotides	76
12	Protein Metabolism	154
13	Cell Division and Cell Cycle	27
14	Motility and Chemotaxis	3
15	Regulation and Cell signalling	17
16	Secondary Metabolism	5
17	DNA Metabolism	77
18	Fatty Acids, Lipids, and Isoprenoids	59
19	Nitrogen Metabolism	33
20	Dormancy and Sporulation	4
21	Respiration	78
22	Stress Response	52
23	Metabolism of Aromatic Compounds	15
24	Amino Acids and Derivatives	250
25	Sulfur Metabolism	31
26	Phosphorus Metabolism	26
27	Carbohydrates	386

Table 3.6. List of different categories of Eukaryotic-like protein (ELPs) coded in L1I52^T genome.

S. No.	RAST ID	RAST annotation	NCBI Ptn ID
(a)	**Host interaction or binding proteins**		
(i)	**Ankyrin repeat domains (ANKs)**		
1	L1I52_800	Ankyrin 1	WP_188220666.1
2	L1I52_1609	ankyrin repeat protein	WP_188219783.1
(ii)	**Tetratricopeptide repeat (TPR)**		

1	L1I52_106	TPR domain protein	WP_188219292.1
2	L1I52_120	TPR domain protein	WP_188219299.1
3	L1I52_178	TPR domain protein	WP_055091768.1
4	L1I52_181	TPR domain protein	WP_188219346.1
5	L1I52_1000	TPR domain protein	WP_188220835.1
6	L1I52_1094	TPR domain protein	WP_188220892.1
7	L1I52_1316	TPR domain protein	WP_055092985.1
8	L1I52_1354	TPR domain protein	WP_055092700.1
9	L1I52_2083	TPR domain protein	WP_188221440.1
10	L1I52_2367	TPR domain containing sensor histidine kinase	WP_188219958.1
11	L1I52_2642	TPR domain protein	WP_188220035.1
12	L1I52_2694	TPR domain protein	WP_188220075.1
(iii)	**Leucine-rich repeat**		
1	L1I52_567	leucine-rich repeat domain-containing protein	WP_055093818.1
(iv)	**Fibronectin**		
1	L1I52_339	fibronectin type III domain-containing protein	WP_188219463.1
2	L1I52_963	fibronectin type III domain-containing protein	WP_188220805.1
(b)	**Host defence evasion**		
1	L1I52_2091	Catalase (EC 1.11.1.6) / Peroxidase (EC 1.11.1.7)	WP_238929420.1
(c)	**Cell wall disruption/modification and host cell entry**		
(i)	**Glycoside hydrolases (GH)**		
1	L1I52_1262	glycosyl hydrolase family 28 protein	WP_238929395.1
2	L1I52_1441	glycoside hydrolase family 28 protein	WP_188221145.1
3	L1I52_1531	glycoside hydrolase family 28 protein	WP_188219729.1
4	L1I52_1629	glycoside hydrolase 100 family protein	WP_188219797.1
5	L1I52_2952	glycoside hydrolase family 28 protein	WP_188220290.1
6	L1I52_2986	glycosyl hydrolase family 28 protein	WP_238929371.1
7	L1I52_3037	glycosyl hydrolase family 28 protein	WP_188220363.1
8	L1I52_3116	beta-galactosidase; Glyco_hydro_35	WP_188220425.1
9	L1I52_3202	glycoside hydrolase family 28 protein	WP_188220492.1
10	L1I52_427	glycoside hydrolase family 28 protein	WP_188219541.1
11	L1I52_577	glycosyl hydrolase family 28 protein	WP_188219639.1
12	L1I52_595	glycoside hydrolase family 28 protein	WP_188219653.1
13	L1I52_599	glycoside hydrolase family 28 protein	WP_188219657.1
14	L1I52_746	glycoside hydrolase family 28 protein	WP_188220632.1
15	L1I52_147	glycoside hydrolase family 28 protein	WP_188219320.1
16	L1I52_3050	glycosyl hydrolase family 28 protein	WP_188220373.1
(ii)	**Pectinesterases**		
1	L1I52_3128	pectinesterase family protein	WP_188220435.1
2	L1I52_3135	pectinesterase family protein	WP_188220440.1

3	L1I52_438	pectinesterase family protein	WP_188219550.1
4	L1I52_597	pectinesterase family protein	WP_188219655.1
5	L1I52_1442	pectinesterase family protein	WP_188221146.1

Table 3.7. List of vitamin biosynthesis gene clusters identified in the genome of L1I52^T.

S. No.	RAST ID	RAST annotation	NCBI Ptn ID
(a)	**Biotin (H)**		
1	L1I52_peg.835	8-amino-7-oxononanoate synthase (EC 2.3.1.47)	WP_188220694.1
2	L1I52_peg.837	Dethiobiotin synthetase (EC 6.3.3.3)	WP_188220695.1
3	L1I52_peg.838	Adenosylmethionine-8-amino-7-oxononanoate aminotransferase (EC 2.6.1.62)	WP_188220696.1
4	L1I52_peg.839	3-oxoacyl-[acyl-carrier-protein] synthase, KASII (EC 2.3.1.179)	WP_188220697.1
5	L1I52_peg.840	Putative regulatory protein	WP_188220698.1
(b)	**Thiamine (B1)**		
1	L1I52_peg.278	Thiamine-monophosphate kinase (EC 2.7.4.16)	WP_055091326.1
2	L1I52_peg.1634	Sulfur carrier protein ThiS	WP_055094267.1
3	L1I52_peg.1635	Hydroxymethylpyrimidine phosphate synthase ThiC (EC 4.1.99.17)	WP_188219802.1
4	L1I52_peg.1636	Thiamin-phosphate pyrophosphorylase (EC 2.5.1.3)	WP_188219803.1
5	L1I52_peg.1637	Hydroxymethylpyrimidine phosphate kinase ThiD (EC 2.7.4.7)	WP_188219804.1
6	L1I52_peg.1638	Thiamin-phosphate pyrophosphorylase (EC 2.5.1.3)	WP_188219805.1
7	L1I52_peg.1639	Thiazole biosynthesis protein ThiG	WP_188219806.1
8	L1I52_peg.1640	2-iminoacetate synthase (ThiH) (EC 4.1.99.19)	WP_18821980.1
9	L1I52_peg.1641	Sulfur carrier protein adenylyltransferase ThiF	WP_188219808.1
(c)	**Folic acid (B9)**		
1	L1I52_peg.450	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase (EC 2.7.6.3) / Deoxyadenosine kinase (EC 2.7.1.76) / Deoxyguanosine kinase (EC 2.7.1.113)	WP_188219561.1
2	L1I52_peg.1918	Dihydropteroate synthase (EC 2.5.1.15)	WP_188221327.1
3	L1I52_peg.1460	Dihydrofolate reductase (EC 1.5.1.3)	WP_188219683.1
4	L1I52_peg.975	Dihydrofolate synthase (EC 6.3.2.12) @ Folylpolyglutamate synthase (EC 6.3.2.17)	WP_188220816.1
5	L1I52_peg.74	Dihydroneopterin aldolase (EC 4.1.2.25)	WP_188219265.1
6	L1I52_peg.934	GTP cyclohydrolase I (EC 3.5.4.16) type 1	WP_188220773.1
7	L1I52_peg.2157	GTP cyclohydrolase I (EC 3.5.4.16) type 1	WP_188221499.1
8	L1I52_peg.2300	Methylenetetrahydrofolate dehydrogenase (NADP+) (EC 1.5.1.5) / Methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9)	WP_055093388.1
(d)	**Pantothenate (B5)**		

1	L1I52_peg.1944	Dephospho-CoA kinase (EC 2.7.1.24)	WP_188221346.1
2	L1I52_peg.659	Ketol-acid reductoisomerase (EC 1.1.1.86)	WP_188220564.1
3	L1I52_peg.828	Pantoate--beta-alanine ligase (EC 6.3.2.1)	WP_188220690.1
4	L1I52_peg.754	Phosphopantetheine adenylyltransferase (EC 2.7.7.3)	WP_05509005.1
5	L1I52_peg.1939	Phosphopantothenoylcysteine decarboxylase (EC 4.1.1.36) / Phosphopantothenoylcysteine synthetase (EC 6.3.2.5)	WP_188221341.1
6	L1I52_peg.3053	3-methyl-2-oxobutanoate hydroxymethyltransferase (EC 2.1.2.11)	WP_055091060.1
7	L1I52_peg.1164	Branched-chain amino acid aminotransferase (EC 2.6.1.42)	WP_188220929.1

Table 3.8. List of brackish-adaptation genes coded in L1I52^T genome.

S. No.	RAST gene ID	RAST annotation	NCBI protein ID
(a)	**Na extrusion**		
(i)	**Na^+-NQR**		
1	L1I52_2941	Na^+-translocating NADH-quinone reductase subunit A	WP_188220281.1
2	L1I52_2942	Na^+-translocating NADH-quinone reductase subunit B	WP_188220282.1
3	L1I52_2943	Na^+-translocating NADH-quinone reductase subunit C	WP_188220283.1
4	L1I52_2944	Na^+-translocating NADH-quinone reductase subunit D	WP_188220284.1
5	L1I52_2945	Na^+-translocating NADH-quinone reductase subunit E	WP_188220285.1
6	L1I52_2946	Na^+-translocating NADH-quinone reductase subunit F	WP_188220286.1
(ii)	**Na^+ Antiporters**		
1	L1I52_481	Na^+/H^+ antiporter NhaA type	WP_188219586.1
2	L1I52_2675	$Na+^+H^+$ antiporter NhaA type	WP_055090856.1
3	L1I52_1377	sodium/hydrogen exchanger	WP_188221094.1
4	L1I52_362	Na^+/H^+-exchanging protein (Na^+/H^+ antiporter)	WP_157502711.1
5	L1I52_2663	Aquaporin Z	WP_188220053.1
(b)	**K^+ uptake**		
1	L1I52_1345	Potassium voltage-gated channel subfamily KQT; possible potassium channel, VIC family	WP_055092682.1
2	L1I52_1709	Kup system potassium uptake protein	WP_188221193.1
3	L1I52_1961	potassium channel protein	WP_188221360.1
(c)	**Mg^{2+} uptake**		
1	L1I52_805	Mg/Co/Ni transporter MgtE / CBS domain	WP_055094962.1
2	L1I52_1894	Magnesium and cobalt transport protein CorA	WP_188221308.1
(d)	**Na^+ uptake**		
1	L1I52_1300	putative sodium/hexose cotransport protein	WP_188221036.1
2	L1I52_3219	SSS sodium solute transporter superfamily	WP_188220508.1
3	L1I52_1234	sodium-solute symporter, putative	WP_188220987.1
4	L1I52_1718	sodium/alanine symporter family protein	WP_188221177.1

5	L1I52_2902	Sodium iodide symporter	WP_188220248.1
6	L1I52_1377	sodium/hydrogen exchanger	WP_188221094.1
7	L1I52_569	Sodium/myo-inositol cotransporter	WP_055093822.1
8	L1I52_1047	Sodium/iodide co-transporter	WP_188220851.1
9	L1I52_1189	Sodium/bile acid symporter family	WP_188220945.1
10	L1I52_3034	Sodium-dependent transporter	WP_188220360.1
(e)	**Mg^{2+} Extrusion**		
1	L1I52_996	Magnesium and cobalt efflux protein CorC	WP_188220832.1
2	L1I52_1488	Magnesium and cobalt efflux protein CorC	WP_055094509.1
(f)	**Osmoprotectants (Glutamate, glutamine, proline)**		
1	L1I52_232	NADP-specific glutamate dehydrogenase (EC 1.4.1.4)	WP_188219382.1
2	L1I52_1224	Glutamine synthetase type III, GlnN (EC 6.3.1.2)	WP_188220979.1
3	L1I52_804	Pyrroline-5-carboxylate reductase (EC 1.5.1.2)	WP_026714317.1
(g)	**Mechanosensitive ion channels**		
1	L1I52_360	Small-conductance mechanosensitive channel	WP_188219480.1
2	L1I52_1079	Large-conductance mechanosensitive channel; mscL	WP_188220880.1
3	L1I52_1397	Small-conductance mechanosensitive channel	WP_188221112.1
4	L1I52_1892	Small-conductance mechanosensitive channel	WP_188221306.1
5	L1I52_1923	Mechanosensitive ion channel family protein	WP_188221331.1
6	L1I52_2329	Potassium efflux system KefA protein / Small-conductance mechanosensitive channel	WP_055093452.1

Table 3.9. ROS detoxifying genes coded in $L1I52^T$ genome.

S. No.	RAST ID	RAST annotation	NCBI Ptn ID
1	L1I52_2091	Catalase (1.11.1.16)/Peroxidase (EC.1.11.1.17)	WP_238929420.1
2	L1I52_271	Manganese superoxide dismutase (EC 1.15.1.1)	WP_055091267.1
3	L1I52_1311	Manganese superoxide dismutase (EC 1.15.1.1)	WP_188221052.1
4	L1I52_1154	Superoxide dismutase [Cu-Zn] (EC 1.15.1.1)	WP_055090347.1
5	L1I52_1169	Thiol peroxidase, Bcp-type (EC 1.11.1.15)	WP_055090252.1
6	L1I52_1251	Thiol peroxidase, Tpx-type (EC 1.11.1.15)	WP_055091085.1
7	L1I52_1248	Alkyl hydroperoxide reductase subunit C-like protein (peroxiredoxin)	WP_055091083.1
8	L1I52_2151	Alkyl hydroperoxide reductase subunit C-like protein (peroxiredoxin)	WP_188221493.1
9	L1I52_2635	Alkyl hydroperoxide reductase subunit C-like protein (peroxiredoxin)	WP_188220031.1
10	L1I52_744	4-carboxymuconolactone decarboxylase domain/alkylhydroperoxidase AhpD family core domain protein	WP_188220631.1
11	L1I52_893	Glutathione peroxidase family protein	WP_188220745.1

12	L1I52_1390	Probable glutathione S-transferase-related transmembrane protein (EC 2.5.1.18)	WP_188221106.1
13	L1I52_3023	Probable glutathione S-transferase-related transmembrane protein (EC 2.5.1.18)	WP_188220350.1
14	L1I52_2497	Alkyl hydroperoxide reductase and/or thiol-specific antioxidant family (AhpC/TSA) protein	WP_188221604.1
15	L1I52_1915	Thioredoxin family protein	WP_188221324.1
16	L1I52_1616	Thioredoxin	WP_026712617.1
17	L1I52_396	Thioredoxin	WP_024980008.1
18	L1I52_733	Thioredoxin	WP_026714507.1
19	L1I52_1249	Thioredoxin	WP_055091084.1
20	L1I52_1482	Thioredoxin Disulfide Isomerase	WP_055094520.1
21	L1I52_2052	Thioredoxin reductase (EC 1.8.1.9)	WP_055094593.1
22	L1I52_2838	Thioredoxin reductase (EC 1.8.1.9)	WP_188220202.1
23	L1I52_2210	Peptide methionine sulfoxide reductase MsrA (EC 1.8.4.11)	WP_188219845.1
24	L1I52_2932	Peptide methionine sulfoxide reductase MsrB (EC 1.8.4.12)	WP_055092557.1
25	L1I52_2147	Peptide methionine sulfoxide reductase MsrA (EC 1.8.4.11)	WP_188221490.1
26	L1I52_2148	Peptide methionine sulfoxide reductase MsrB (EC 1.8.4.12)	WP_188221491.1
27	L1I52_1160	Hydrogen peroxide-inducible genes activator (OxyR)	WP_055090265.1

3.5 References

1. Alexander, B. J. R., and Stewart, A. (2001). Glasshouse screening for biological control agents of *Phytophthora cactorum* on apple (*Malus domestica*). *New Zealand Journal of Crop and Horticultural Science*, 29(3), 159–169. https://doi.org/10.1080/01140671.2001.9514174

2. Amaya-Gómez, C. V, Porcel, M., Mesa-Garriga, L., Gómez-Álvarez, M. I., Zhou, N.-Y., and Tong, S. J. (2020). A Framework for the Selection of Plant Growth-Promoting Rhizobacteria Based on Bacterial Competence Mechanisms. https://doi.org/10

3. An, Q. D., Zhang, G. L., Wu, H. T., Zhang, Z. C., Zheng, G. S., Luan, L., Murata, Y., and Li, X. (2009). Alginate-deriving oligosaccharide production by alginase from newly isolated Flavobacterium sp. LXA and its potential application in protection against pathogens. *Journal of Applied Microbiology*, 106(1), 161–170. https://doi.org/10.1111/j.1365-2672.2008.03988.x

4. Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in beta vulgaris. Plant physiology volume (Vol. 24, Issue 1). https://academic.oup.com/plphys/article/24/1/1/6076157

5. Aziz, R. K., Bartels, D., Best, A., DeJongh, M., Disz, T., Edwards, R. A., Formsma, K., Gerdes, S., Glass, E. M., Kubal, M., Meyer, F., Olsen, G. J., Olson, R., Osterman, A. L., Overbeek, R. A., McNeil, L. K., Paarmann, D., Paczian, T., Parrello, B., … Zagnitko, O. (2008). The RAST Server: Rapid annotations using subsystems technology. *BMC Genomics*, 9. https://doi.org/10.1186/1471-2164-9-75

6. Badri, D. V., Weir, T. L., van der Lelie, D., and Vivanco, J. M. (2009). Rhizosphere chemical dialogues: plant-microbe interactions. *Current Opinion in Biotechnology* (Vol. 20, Issue 6, pp. 642–650). https://doi.org/10.1016/j.copbio.2009.09.014

7. Bernardet, J. F., Nakagawa, Y., Holmes, B., Bowman, J. P., Bruun, B., Burchard, R. P., Hugo, C. J., Jooste, P. J., Malik, K. A., McMeekin, T. A., Reichardt, W., Reichenbach, H., Segers, P., Wakabayashi, H., Yabuuchi, E., Ursing, J., Segers, P., Suzuki, M., Hinz, K. H., and Nielsen, P. (2002). Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae and* emended description of the family. *International Journal of Systematic and Evolutionary Microbiology*, 52(3), 1049–1070. https://doi.org/10.1099/ijs.0.02136-0

8. Bhagat, N., Raghav, M., Dubey, S., and Bedi, N. (2021). Bacterial exopolysaccharides: Insight into their role in plant abiotic stress tolerance. *Journal of Microbiology and Biotechnology* (Vol. 31, Issue 8, pp. 1045–1059). Korean Society for Microbiology and Biotechnology. https://doi.org/10.4014/jmb.2105.05009

9. Bhat, B. A., Tariq, L., Nissar, S., Islam, S. T., Islam, S. U., Mangral, Z., Ilyas, N., Sayyed, R. Z., Muthusamy, G., Kim, W., and Dar, T. U. H. (2022). The role of plant-associated rhizobacteria in plant growth, biocontrol and abiotic stress management. *Journal of Applied Microbiology* (Vol. 133, Issue 5, pp. 2717–2741). John Wiley and Sons Inc. https://doi.org/10.1111/jam.15796

10. Booth, I. R. (2014). Bacterial mechanosensitive channels: Progress towards an understanding of their roles in cell physiology. *Current Opinion in Microbiology* (Vol. 18, Issue 1, pp. 16–22). Elsevier Ltd. https://doi.org/10.1016/j.mib.2014.01.005

11. Bootha, I. R., and Blount, P. (2012). The MscS and MscL families of mechanosensitive channels act as microbial emergency release valves. *Journal of Bacteriology* (Vol. 194, Issue 18, pp. 4802–4809). https://doi.org/10.1128/JB.00576-12

12. Carolin Frank, A. (2019). Molecular host mimicry and manipulation in bacterial symbionts. *FEMS Microbiology Letters* (Vol. 366, Issue 4). Oxford University Press. https://doi.org/10.1093/femsle/fnz038

13. Chaudhari, N. M., Gupta, V. K., and Dutta, C. (2016). BPGA-an ultra-fast pan-genome analysis pipeline. *Scientific Reports*, 6. https://doi.org/10.1038/srep24373

14. Chen, Y., Wang, X. M., Zhou, L., He, Y., Wang, D., Qi, Y. H., and Jiang, D. A. (2015). Rubisco activase is also a multiple responder to abiotic stresses in rice. *PLoS ONE*, 10(10). https://doi.org/10.1371/journal.pone.0140934

15. Choudhury, F. K., Rivero, R. M., Blumwald, E., and Mittler, R. (2017). Reactive oxygen species, abiotic stress and stress combination. *Plant Journal*, 90(5), 856–867. https://doi.org/10.1111/tpj.13299

16. Comeau, D., Balthazar, C., Novinscak, A., Bouhamdani, N., Joly, D. L., and Filion, M. (2021). Interactions Between Bacillus Spp., Pseudomonas Spp. and *Cannabis sativa* Promote Plant Growth. *Frontiers in Microbiology*, 12. https://doi.org/10.3389/fmicb.2021.715758

17. Despres, J., Forano, E., Lepercq, P., Comtet-Marre, S., Jubelin, G., Chambon, C., Yeoman, C. J., Berg Miller, M. E., Fields, C. J., Martens, E., Terrapon, N., Henrissat, B., White, B. A., and Mosoni, P. (2016). Xylan degradation by the human gut *Bacteroides xylanisolvens* XB1A^{T} involves two distinct gene clusters that are linked at the transcriptional level. *BMC Genomics*, 17(1). https://doi.org/10.1186/s12864-016-2680-8

18. Ding, F., Wang, M., Zhang, S., and Ai, X. (2016). Changes in SBPase activity influence photosynthetic capacity, growth, and tolerance to chilling stress in transgenic tomato plants. *Scientific Reports*, 6. https://doi.org/10.1038/srep32741

19. Driever, S. M., Simkin, A. J., Alotaibi, S., Fisk, S. J., Madgwick, P. J., Sparks, C. A., Jones, H. D., Lawson, T., Parry, M. A. J., and Raines, C. A. (2017). Increased sbpase activity improves photosynthesis and grain yield in wheat grown in greenhouse conditions. Philosophical Transactions of the Royal Society B: *Biological Sciences*, 372(1730). https://doi.org/10.1098/rstb.2016.0384

20. Eren, A. M., Esen, O. C., Quince, C., Vineis, J. H., Morrison, H. G., Sogin, M. L., and Delmont, T. O. (2015). Anvi'o: An advanced analysis and visualization platformfor 'omics data. *PeerJ*, 2015(10). https://doi.org/10.7717/peerj.1319

21. Flynn, B., Graham, A., Scott, N., Layzell, D. B., and Dong, Z. (2014). Nitrogen fixation, hydrogen production and N2O emissions. *Canadian Journal of Plant Science*, 94(6), 1037–1041. https://doi.org/10.4141/CJPS2013-210

22. Fu, T., Fan, Z., Li, Y., Li, Z., Zhao, H., Feng, Y., Xue, G., Cui, J., Yan, C., Gan, L., Feng, J., Yuan, J., and You, F. (2023). Roles of the Crp/Fnr Family Regulator ArcR in the Hemolysis and Biofilm of Staphylococcus aureus. *Microorganisms*, 11(7). https://doi.org/10.3390/microorganisms11071656

23. Hebbar I, P., Berge ~, O., Heulin, T., and Singh, S. P. (1991). Bacterial antagonists of Sunflower (Helianthus annuus L.) fungal pathogens. *Plant and Soil* (Vol. 133).

24. Hoffmann, C., Ohlsen, K., and Hauck, C. R. (2011). Integrin-mediated uptake of fibronectin-binding bacteria. *European Journal of Cell Biology* (Vol. 90, Issue 11, pp. 891–896). https://doi.org/10.1016/j.ejcb.2011.03.001

25. Hurek, T., Van Montagu, M., Kellenberger, E., and Reinhold-Hurek, B. (1995). Induction of complex intracytoplasmic membranes related to nitrogen fixation in Azoarcus sp. BH72. *Molecular Microbiology* (Vol. 18, Issue 2).

26. Ji, J., Yuan, D., Jin, C., Wang, G., Li, X., and Guan, C. (2020). Enhancement of growth and salt tolerance of rice seedlings (Oryza sativa L.) by regulating ethylene production with a novel halotolerant PGPR strain Glutamicibacter sp. YD01 containing ACC deaminase activity. *Acta Physiologiae Plantarum*, 42(4). https://doi.org/10.1007/s11738-020-3034-3

27. Johnston, J. J., Shrivastava, A., and McBride, M. J. (2018). Untangling *Flavobacterium* johnsoniae gliding motility and protein secretion. *Journal of Bacteriology*, 200(2). https://doi.org/10.1128/JB.00362-17

28. Karimi, E., Aliasgharzad, N., Esfandiari, E., Hassanpouraghdam, M. B., Neu, T. R., Buscot, F., Reitz, T., Breitkreuz, C., and Tarkka, M. T. (2022). Biofilm forming rhizobacteria affect the physiological and biochemical responses of wheat to drought. *AMB Express*, 12(1). https://doi.org/10.1186/s13568-022-01432-8

29. Kawasaki, T., Koita, H., Nakatsubo, T., Hasegawa, K., Wakabayashi, K., Takahashi, H., Umemura, K., Umezawa, T., and Shimamoto, K. (2005). Cinnamoyl-CoA reductase, a key enzyme in lignin biosynthesis, is an effector of small GTPase Rac in defense signaling in rice. https://www.pnas.org

30. Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., and Salzberg, S. L. (2013). TopHat2: Accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biology*, 14(4). https://doi.org/10.1186/gb-2013-14-4-r36

31. Kim, K., Jang, Y. J., Lee, S. M., Oh, B. T., Chae, J. C., and Lee, K. J. (2014). Alleviation of salt stress by Enterobacter sp. EJ01 in tomato and Arabidopsis is accompanied by up-regulation of conserved salinity responsive factors in plants. *Molecules and Cells*, 37(2), 109–117. https://doi.org/10.14348/molcells.2014.2239

32. Knights, H. E., Jorrin, B., Haskett, T. L., and Poole, P. S. (2021). Deciphering bacterial mechanisms of root colonization. *Environmental Microbiology Reports* (Vol. 13, Issue 4, pp. 428–444). John Wiley and Sons Inc. https://doi.org/10.1111/1758-2229.12934

33. Kogure, K. (1998). Bioenergetics of marine bacteria Kazuhiro Kogure. *Curr Opin Biotechnol*, 9, 278–282. http://biomednet.com/elecref/09581669009002?8

34. Kolton, M., Frenkel, O., Elad, Y., and Cytryn, E. (2014). Potential role of flavobacterial gliding-motility and type IX secretion system complex in root colonization and plant defense. *Molecular Plant-Microbe Interactions*, 27(9), 1005–1013. https://doi.org/10.1094/MPMI-03-14-0067-R

35. Kolton, M., Sela, N., Elad, Y., and Cytryn, E. (2013). Comparative Genomic Analysis Indicates that Niche Adaptation of Terrestrial Flavobacteria Is Strongly Linked to Plant Glycan Metabolism. *PLoS ONE*, 8(9). https://doi.org/10.1371/journal.pone.0076704

36. Koskimäki, J. J., Pirttilä, A. M., Ihantola, E. L., Halonen, O., and Carolin Frank, A. (2015). The intracellular scots pine shoot symbiont Methylobacterium extorquens DSM13060 aggregates around the host nucleus and encodes eukaryote-like proteins. *MBio*, 6(2). https://doi.org/10.1128/mBio.00039-15

37. Krause, A., Ramakumar, A., Bartels, D., Battistoni, F., Bekel, T., Boch, J., Böhm, M., Friedrich, F., Hurek, T., Krause, L., Linke, B., McHardy, A. C., Sarkar, A., Schneiker, S., Syed, A. A., Thauer, R., Vorhölter, F. J., Weidner, S., Pühler, A., … Goesmann, A. (2006). Complete genome of the mutualistic, N2-fixing grass endophyte Azoarcus sp. strain BH72. *Nature Biotechnology*, 24(11), 1385–1391. https://doi.org/10.1038/nbt1243

38. Krishnan, R., Menon, R. R., Likhitha, Busse, H. J., Tanaka, N., Krishnamurthi, S., and Rameshkumar, N. (2017). *Novosphingobium pokkalii* sp. nov., a novel rhizosphere-associated bacterium with plant beneficial properties isolated from saline-tolerant pokkali rice. *Research in Microbiology*, 168(2), 113–121. https://doi.org/10.1016/j.resmic.2016.09.001

39. Krishnan, R., Menon, R. R., Tanaka, N., Busse, H. J., Krishnamurthi, S., and Rameshkumar, N. (2016). *Arthrobacter pokkalii* sp. nov., a novel plant associated actinobacterium with plant beneficial properties, isolated from saline tolerant pokkali rice, Kerala, India. *PLoS ONE*, 11(3). https://doi.org/10.1371/journal.pone.0150322

40. Lapébie, P., Lombard, V., Drula, E., Terrapon, N., and Henrissat, B. (2019). Bacteroidetes use thousands of enzyme combinations to break down glycans. *Nature Communications,* 10(1). https://doi.org/10.1038/s41467-019-10068-5

41. Lata, C., and Prasad, M. (2011). Role of DREBs in regulation of abiotic stress responses in plants. *Journal of Experimental Botany* (Vol. 62, Issue 14, pp. 4731–4748). https://doi.org/10.1093/jxb/err210

42. Lidbury, I. D. E. A., Scanlan, D. J., Murphy, A. R. J., Christie-Oleza, J. A., Aguilo-Ferretjans, M. M., Hitchcock, A., and Daniell, T. J. (2022). A widely distributed phosphate-insensitive phosphatase presents a route for rapid organophosphorus remineralization in the biosphere. *Proceedings of the National Academy of Sciences*, 119(5), 1–10. https://doi.org/10.1073/pnas.2118122119/-/DCSupplemental

43. Liu, M. Y., Kjelleberg, S., and Thomas, T. (2011). Functional genomic analysis of an uncultured δ-proteobacterium in the sponge *Cymbastela concentrica*. ISME Journal, 5(3), 427–435. https://doi.org/10.1038/ismej.2010.139

44. Lucke, M., Correa, M. G., and Levy, A. (2020). The Role of Secretion Systems, Effectors, and Secondary Metabolites of Beneficial Rhizobacteria in Interactions With Plants and Microbes. *Frontiers in Plant Science* (Vol. 11). Frontiers Media S.A. https://doi.org/10.3389/fpls.2020.589416

45. Lugtenberg, B., and Kamilova, F. (2009). Plant-growth-promoting rhizobacteria. *Annual Review of Microbiology* (Vol. 63, pp. 541–556). https://doi.org/10.1146/annurev.micro.62.081307.162918

46. Lv, G. Y., Guo, X. G., Xie, L. P., Xie, C. G., Zhang, X. H., Yang, Y., Xiao, L., Tang, Y. Y., Pan, X. L., Guo, A. G., and Xu, H. (2017). Molecular characterization, gene evolution, and expression analysis of the fructose-1, 6-bisphosphate Aldolase (FBA) gene family in wheat (*Triticum aestivum* L.). Frontiers in Plant Science, 8. https://doi.org/10.3389/fpls.2017.01030

47. Madhaiyan, M., Poonguzhali, S., Lee, J. S., Lee, K. C., and Sundaram, S. (2010). *Flavobacterium glycines* sp. nov., a facultative methylotroph isolated from the rhizosphere of soybean. *International Journal of Systematic and Evolutionary Microbiology* (Vol. 60, Issue 9, pp. 2187–2192). https://doi.org/10.1099/ijs.0.014019-0

48. Mann, A. J., Hahnke, R. L., Huang, S., Werner, J., Xing, P., Barbeyron, T., Huettel, B., Stüber, K., Reinhardt, R., Harder, J., Glöckner, F. O., Amann, R. I., and Teeling, H. (2013). The genome of the alga-associated marine *Flavobacterium Formosa agariphila* KMM 3901^{T} reveals a broad potential for degradation of algal polysaccharides. Applied and *Environmental Microbiology*, 79(21), 6813–6822. https://doi.org/10.1128/AEM.01937-13

49. McBride, M. J., and Nakane, D. (2015). *Flavobacterium* gliding motility and the type IX secretion system. *Current Opinion in Microbiology* (Vol. 28, pp. 72–77). Elsevier Ltd. https://doi.org/10.1016/j.mib.2015.07.016

50. Mendes, R., Garbeva, P., and Raaijmakers, J. M. (2013). The rhizosphere microbiome: Significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. *FEMS Microbiology Reviews* (Vol. 37, Issue 5, pp. 634–663). https://doi.org/10.1111/1574-6976.12028

51. Menon, R. R., Kumari, S., Kumar, P., Verma, A., Krishnamurthi, S., and Rameshkumar, N. (2019). *Sphingomonas pokkalii* sp. nov., a novel plant associated rhizobacterium isolated from a saline tolerant pokkali rice and its draft genome analysis. *Systematic and Applied Microbiology*, 42(3), 334–342. https://doi.org/10.1016/j.syapm.2019.02.003

52. Menon, R. R., Kumari, S., Viver, T., and Rameshkumar, N. (2020). *Flavobacterium pokkalii* sp. nov., a novel plant growth promoting native rhizobacteria isolated from pokkali rice grown

in coastal saline affected agricultural regions of southern India, Kerala. *Microbiological Research*, 240. https://doi.org/10.1016/j.micres.2020.126533

53. Minato, Y., Fassio, S. R., Kirkwood, J. S., Halang, P., Quinn, M. J., Faulkner, W. J., Aagesen, A. M., Steuber, J., Stevens, J. F., and Häse, C. C. (2014). Roles of the sodium-translocating NADH:Quinone oxidoreductase (Na +-NQR) on Vibrio cholerae metabolism, motility and osmotic stress resistance. *PLoS ONE*, 9(5). https://doi.org/10.1371/journal.pone.0097083

54. Mitter, B., Petric, A., Shin, M. W., Chain, P. S. G., Hauberg-Lotte, L., Reinhold-Hurek, B., Nowak, J., and Sessitsch, A. (2013). Comparative genome analysis of Burkholderia phytofirmans PsJN reveals a wide spectrum of endophytic lifestyles based on interaction strategies with host plants. *Frontiers in Plant Science*. https://doi.org/10.3389/fpls.2013.00120

55. Nan, B., McBride, M. J., Chen, J., Zusman, D. R., and Oster, G. (2014). Bacteria that Glide with Helical Tracks. *Current Biology* (Vol. 24, Issue 4). https://doi.org/10.1016/j.cub.2013.12.034

56. Ortega-Amaro, M. A., Rodriguez-Hernandez, A. A., Rodriguez-Kessler, M., Hernandez-Lucero, E., Rosales-Mendoza, S., Ibanez-Salazar, A., Delgado-Sanchez, P., and Jimenez-Bremont, J. F. (2015). Overexpression of AtGRDP2, a novel glycine-rich domain protein, accelerates plant growth and improves stress tolerance. *Frontiers in Plant Science*, 1–16. https://doi.org/10.3389/fpls.2014.00782

57. Oshone, R., Ngom, M., Chu, F., Mansour, S., Sy, M. O., Champion, A., and Tisa, L. S. (2017). Genomic, transcriptomic, and proteomic approaches towards understanding the molecular mechanisms of salt tolerance in Frankia strains isolated from Casuarina trees. *BMC Genomics*, 18(1). https://doi.org/10.1186/s12864-017-4056-0

58. Parks, D. H., Imelfort, M., Skennerton, C. T., Hugenholtz, P., and Tyson, G. W. (2015). CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Research*, 25(7), 1043–1055. https://doi.org/10.1101/gr.186072.114

59. Penn, K., and Jensen, P. R. (2012). Comparative genomics reveals evidence of marine adaptation in Salinispora species. *BMC Genomics*, 13(1). https://doi.org/10.1186/1471-2164-13-86

60. Pitcher, R.S., Brittain, T. and Watmugh, N.J. (2002). Cytochrome cbb 3 oxidase and bacterial microaerobic metabolism. *Biochemical Society Transactions*, *30*(4), pp.653-658.

61. Pohjanen, J., Koskimäki, J. J., Sutela, S., Ardanov, P., Suorsa, M., Niemi, K., Sarjala, T., Häggman, H., and Pirttilä, A. M. (2014). Interaction with ectomycorrhizal fungi and endophytic methylobacterium affects nutrient uptake and growth of pine seedlings in vitro. *Tree Physiology*, 34(9), 993–1005. https://doi.org/10.1093/treephys/tpu062

62. Reinhold-Hurek, B., Maes, T., Gemmer, S., Van Montagu, M., and Hurek, T. (2006). An Endoglucanase Is Involved in Infection of Rice Roots by the Not-Cellulose-Metabolizing Endophyte *Azoarcus* Sp. Strain BH72. / 181 *MPMI*, 19(2), 181–188. https://doi.org/10.1094/MPMI

63. Rogowski, A., Briggs, J. A., Mortimer, J. C., Tryfona, T., Terrapon, N., Lowe, E. C., Baslé, A., Morland, C., Day, A. M., Zheng, H., Rogers, T. E., Thompson, P., Hawkins, A. R., Yadav, M. P., Henrissat, B., Martens, E. C., Dupree, P., Gilbert, H. J., and Bolam, D. N. (2015). Glycan complexity dictates microbial resource allocation in the large intestine. *Nature Communications*, 6. https://doi.org/10.1038/ncomms8481

64. Saleethong, P., Roytrakul, S., Kong-Ngern, K., and Theerakulpisut, P. (2016). Differential Proteins Expressed in Rice Leaves and Grains in Response to Salinity and Exogenous Spermidine Treatments. Rice Science, 23(1), 9–21. https://doi.org/10.1016/j.rsci.2016.01.002

65. Sanan-Mishra, N., Tuteja, N. and Sopory, S.K. (2002). Salinity-and ABA-induced up-regulation and light-mediated modulation of mRNA encoding glycine-rich RNA-binding

protein from Sorghum bicolor. *Biochemical and Biophysical Research Communications*, *296*(5), 1063-1068.

66. Sang, M. K., and Kim, K. D. (2012). The volatile-producing *Flavobacterium johnsoniae* strain GSE09 shows biocontrol activity against Phytophthora capsici in pepper. *Journal of Applied Microbiology*, 113(2), 383–398. https://doi.org/10.1111/j.1365-2672.2012.05330.x

67. Santoyo, G., Urtis-Flores, C.A., Loeza-Lara, P.D., Orozco-Mosqueda, M.D.C. and Glick, B.R. (2021). Rhizosphere colonization determinants by plant growth-promoting rhizobacteria (PGPR). *Biology*, *10*(6), p.475.

68. Sarkar, T., Thankappan, R., Mishra, G. P., and Nawade, B. D. (2019). Advances in the development and use of DREB for improved abiotic stress tolerance in transgenic crop plants. *Physiology and Molecular Biology of Plants* (Vol. 25, Issue 6, pp. 1323–1334). Springer. https://doi.org/10.1007/s12298-019-00711-2.

69. Siegl, A., Kamke, J., Hochmuth, T., Piel, J., Richter, M., Liang, C., Dandekar, T., and Hentschel, U. (2011). Single-cell genomics reveals the lifestyle of Poribacteria, a candidate phylum symbiotically associated with marine sponges. *ISME Journal*, 5(1), 61–70. https://doi.org/10.1038/ismej.2010.95.

70. Smyth, E. M., McCarthy, J., Nevin, R., Khan, M. R., Dow, J. M., O'Gara, F., and Doohan, F. M. (2011). In vitro analyses are not reliable predictors of the plant growth promotion capability of bacteria; a Pseudomonas fluorescens strain that promotes the growth and yield of wheat. *Journal of Applied Microbiology*, 111(3), 683–692. https://doi.org/10.1111/j.1365-2672.2011.05079.x.

71. Soltani, A.-A., Khavazi, K., Asadi-Rahmani, H., Omidvari, M., Abaszadeh Dahaji, P., and Mirhoseyni, H. (2010). Plant Growth Promoting Characteristics in Some Flavobacterium spp. Isolated from Soils of Iran. *Journal of Agricultural Science*, 2(4). https://doi.org/10.5539/jas.v2n4p106.

72. Sparla, F., Zaffagnini, M., Wedel, N., Scheibe, R., Pupillo, P., and Trost, P. (2005). Regulation of photosynthetic GAPDH dissected by mutants. *Plant Physiology*, 138(4), 2210–2219. https://doi.org/10.1104/pp.105.062117.

73. Sun, C., Fu, G. Y., Zhang, C. Y., Hu, J., Xu, L., Wang, R. J., Su, Y., Han, S. B., Yu, X. Y., Cheng, H., Zhang, X. Q., Huo, Y. Y., Xu, X. W., and Wu, M. (2016). Isolation and complete genome sequence of *Algibacter alginolytica* sp. nov., a novel seaweed-degrading Bacteroidetes bacterium with diverse putative polysaccharide utilization loci. *Applied and Environmental Microbiology*, 82(10), 2975–2987. https://doi.org/10.1128/AEM.00204-16

74. Tekedar, H. C., Karsi, A., Reddy, J. S., Nho, S. W., Kalindamar, S., and Lawrence, M. L. (2017). Comparative genomics and transcriptional analysis of Flavobacterium columnare strain ATCC 49512. *Frontiers in Microbiology*, https://doi.org/10.3389/fmicb.2017.00588

75. Thomas, T., Rusch, D., DeMaere, M. Z., Yung, P. Y., Lewis, M., Halpern, A., Heidelberg, K. B., Egan, S., Steinberg, P. D., and Kjelleberg, S. (2010). Functional genomic signatures of sponge bacteria reveal unique and shared features of symbiosis. *ISME Journal*, 4(12), 1557–1567. https://doi.org/10.1038/ismej.2010.74

76. Tian, J., Ge, F., Zhang, D., Deng, S., and Liu, X. (2021). Roles of phosphate solubilizing microorganisms from managing soil phosphorus deficiency to mediating biogeochemical p cycle. *Biology* (Vol. 10, Issue 2, pp. 1–19). MDPI AG. https://doi.org/10.3390/biology10020158

77. Trapnell, C., Williams, B. A., Pertea, G., Mortazavi, A., Kwan, G., Van Baren, M. J., Salzberg, S. L., Wold, B. J., and Pachter, L. (2010). Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nature Biotechnology*, 28(5), 511–515. https://doi.org/10.1038/nbt.1621

78. Tseng, T. T., Tyler, B. M., and Setubal, J. C. (2009). Protein secretion systems in bacterial-host associations, and their description in the Gene Ontology. *BMC Microbiology*, (9). https://doi.org/10.1186/1471-2180-9-S1-S2

79. Tucker, G. (2004). Improving fruit and vegetable texture by genetic transformation. *Texture in foods*, *2*, pp.321-341.

80. Turnbull, G. A., Morgan, J. A. W., Whipps, J. M., and Saunders, J. R. (2006). The role of bacterial motility in the survival and spread of Pseudomonas fluorescens in soil and in the attachment and colonisation of wheat roots. *FEMS Microbiology Ecology*, 36(1), 21–31. https://doi.org/10.1111/j.1574-6941.2001.tb00822.x

81. Umate, P. (2010). Genome-wide analysis of the family of light-harvesting chlorophyll a/b-binding proteins in Arabidopsis and rice. *Plant Signaling and Behavior*, 5(12), 1537–1542. https://doi.org/10.4161/psb.5.12.13410

82. Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., and Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. *New Phytologist* (Vol. 206, Issue 4, pp. 1196–1206). https://doi.org/10.1111/nph.13312

83. Veith, P. D., Glew, M. D., Gorasia, D. G., and Reynolds, E. C. (2017). Type IX secretion: the generation of bacterial cell surface coatings involved in virulence, gliding motility and the degradation of complex biopolymers. *Molecular Microbiology* (Vol. 106, Issue 1, pp. 35–53). Blackwell Publishing Ltd. https://doi.org/10.1111/mmi.13752

84. Vibhuti, M., Kumar, A., Sheoran, N., Nadakkakath, A. V., and Eapen, S. J. (2017). Molecular Basis of Endophytic Bacillus megaterium-induced Growth Promotion in Arabidopsis thaliana: Revelation by Microarray-based Gene Expression Analysis. *Journal of Plant Growth Regulation*, 36(1), 118–130. https://doi.org/10.1007/s00344-016-9624-z

85. Wahli, T., and Madsen, L. (2018). Flavobacteria, a Never Ending Threat for Fish: a Review. *Current Clinical Microbiology Reports* (Vol. 5, Issue 1, pp. 26–37). Springer. https://doi.org/10.1007/s40588-018-0086-x

86. Wang, C., Zhang, D. W., Wang, Y. C., Zheng, L., and Yang, C. P. (2012). A glycine-rich RNA-binding protein can mediate physiological responses in transgenic plants under salt stress. *Molecular Biology Reports*, 39(2), 1047–1053. https://doi.org/10.1007/s11033-011-0830-2

87. Wang, H., Lu, S., Guan, X., Jiang, Y., Wang, B., Hua, J., and Zou, B. (2022). Dehydration-Responsive Element Binding Protein 1C, 1E, and 1G Promote Stress Tolerance to Chilling, Heat, Drought, and Salt in Rice. *Frontiers in Plant Science*, 13. https://doi.org/10.3389/fpls.2022.851731

88. Wheatley, R. M., and Poole, P. S. (2018). Mechanisms of bacterial attachment to roots. *FEMS Microbiology Reviews* (Vol. 42, Issue 4, pp. 448–461). Oxford University Press. https://doi.org/10.1093/femsre/fuy014

89. You, J., and Chan, Z. (2015). Ros regulation during abiotic stress responses in crop plants. *Frontiers in Plant Science* (Vol. 6, Issue DEC). Frontiers Research Foundation. https://doi.org/10.3389/fpls.2015.01092

90. Yuan, J., Ruan, Y., Wang, B., Zhang, J., Waseem, R., Huang, Q., and Shen, Q. (2013). Plant growth-promoting rhizobacteria strain bacillus amyloliquefaciens njn-6-enriched bio-organic fertilizer suppressed fusarium wilt and promoted the growth of banana plants. *Journal of Agricultural and Food Chemistry*, 61(16), 3774–3780. https://doi.org/10.1021/jf400038z

91. Zhang, H., Han, B., Wang, T., Chen, S., Li, H., Zhang, Y., and Dai, S. (2012). Mechanisms of plant salt response: Insights from proteomics. *Journal of Proteome Research* (Vol. 11, Issue 1, pp. 49–67). https://doi.org/10.1021/pr200861w

92. Zhang, J., Han, C., and Liu, Z. (2009). Absorption spectrum estimating rice chlorophyll concentration: Preliminary investigations. *Journal of Plant Breeding and Crop Science* (Vol. 1, Issue 5). http://www.academicjournals.org/jpbcs

93. Zheng, J., Ge, Q., Yan, Y., Zhang, X., Huang, L., and Yin, Y. (2023). DbCAN3: Automated carbohydrate-Active enzyme and substrate annotation. *Nucleic Acids Research*, 51(W1), W115–W121. https://doi.org/10.1093/nar/gkad328

94. Zhou, H., Liu, M., Meng, F., Zheng, D., and Feng, N. (2023). Transcriptomics and physiology reveal the mechanism of potassium indole-3-butyrate (IBAK) mediating rice resistance to salt stress. *BMC Plant Biology*, 23(1). https://doi.org/10.1186/s12870-023-04531-1

Chapter – 4

Identification of key genetic determinants in *Flavobacterium pokkalii* L1I52^{T} interaction with its host, pokkali rice under brackish condition through random mutagenesis

4.1 Introduction

Understanding the genetics of a bacterial strain is highly mandatory to unravel critical aspects ranging from its growth, metabolism, physiology, and host interaction to its potential role in host health and influence on ecosystem functioning. The essential tools to study bacterial genetics include gene transfer ability between the bacterial strains, mutant generation, development of recombinant strains, and the ability to do complementation studies for functional validation (Maloy, 2013). These tools have been finely honed for a selected group of model bacterial strains such as *Escherichia coli, Bacillus subtilis, Salmonella enterica, etc* (Maloy, 2013). Even though these genetic tools and related concepts developed for model bacterial strains are readily applicable to other bacterial strains belonging to different phyla or genera with customized experimental conditions, certain species of bacteria require highly customized tools and procedures. The genus *Flavobacterium* within the phylum Bacteriodota is one such bacterial group that poses severe challenges during its genetic manipulation. It is assumed that factors such as cell wall structure, complex physiology, and lack of well-established genetic tools or methods specific to the strain restrict the genetic manipulation of *Flavobacterium* strains. Since certain aquatic *Flavobacterium* strains, such as *F. columnare, F. psychrophilum*, etc, are major causative agents of fish pathogenesis, genetic tools were developed to trace the molecular factors responsible for their virulence mechanism (Alvarez et al., 2004; Staroscik et al., 2008). However, this did not solve the genetic manipulation challenges faced in other *Flavobacterium* strains. In 1995, McBride and his group developed genetic tools using Tn*4351* transposon to study the gliding motility mechanism in *F. johnsonaie* (earlier known as *Cytophada johnsonae*). Until then, the knowledge of gliding motility was limited almost entirely to one species, *Myxococcus xanthus,* primarily because of the wide variety of techniques available for the genetic manipulation of *M. xanthus*. Since then, McBride and his group conducted extensive studies to unravel the gliding motility mechanism using both targeted and random mutagenesis approaches. However, the degree of randomness in the mutant insertions was much less, with Tn*4351* transposons inducing multiple insertions in the same gene, which makes the random mutant screening more laborious. These drawbacks demanded a much more efficient genetic tool that could mainly increase the randomness, thereby increasing the possibility to identify novel gene insertions. During this, McBride's group developed mariner transposons that increased the randomness of the mutation-induced with which he, along with his collaborators, decoded the function of a large set of

genes that led to the discovery of the Type IX secretion system (T9SS) that conjointly works with the gliding motility complex. However, most of the genetic tools developed in F. johnsoniae have mostly been used to decode the molecular factors that deepen the understanding of gliding motility linked with T9SS. Despite this, the information relative to their host interaction, especially related to terrestrial clade *Flavobacterium* strains, remained largely elusive, irrespective of their high abundance in the rhizosphere and root compartments of different plant varieties (Bodenhausen et al., 2013; Schlaeppi et al., 2014). In 2014, Kolton, M et al generated *gldJ* mutant and complement strains of terrestrial *Flavobacterium* sp. F52 isolated from roots of green pepper to demonstrate the role of gliding motility and T9SS complex in tomato root colonization and plant defence (Kolton et al., 2014). Nevertheless, Kolton, M et al studied the role of the already discovered *gldJ* gene, which is required for gliding motility and chitinase secretion in *F. johnsoniae* (Braun and McBride, 2005; Kolton et al., 2014). However, the findings from this study further supported the fact that the gliding motility complex and T9SS are vital gene components that can influence multiple functions involving host colonization and subsequent health (Kolton et al., 2014). Despite all these discoveries, the molecular information pertaining to *Flavobacterium* – plant interaction was again restricted to gliding motility complex and T9SS. Therefore, the major aim of this chapter was to attempt genetic manipulation of the novel *Flavobacterium* strain, *F. pokkalii* L1I52^{T} (will be mentioned as L1I52^{T} or WT strain in the main text except legends) to identify novel genetic determinants with loss-of-function (LoF) phenotypes and to observe the associated colonization ability with its host, Pokkali rice. For this, the mariner-based transposons developed by McBride's group were used with certain modifications in the growth conditions and conjugation procedures (Braun et al., 2005) (see method section for details). The findings from this study unravelled novel genes that contribute to the interaction of *Flavobacterium* members with their host plants. However, in-depth molecular studies are required to validate these preliminary findings. Hence, this study may open new avenues in understanding *Flavobacterium*–plant interaction, thus deepening the knowledge of the molecular factors governing their cross-talk.

4.2 Materials and Methods

4.2.1 Strain details and media conditions

The strains used in this study are L1I52^T (WT) strain, *E. coli* S17-1 λ *pir* strain harboring pHimarEm1 plasmid (kanamycin resistance in *E. coli* strains and erythromycin resistance in *Flavobacterium* strains) and *E. coli* EC100D *pir*$^+$ strain with kanamycin resistance (both the *E. coli* strains were gifted by Prof. Mark J McBride, Distinguished Professor, Department of Biological Sciences, University of Wisconsin, Milwaukee). *E. coli* strains were grown in Luria-Bertani (LB) medium at 37ºC, and the L1I52^T strain was grown in a modified Casitone-yeast extract (CYE) medium (Mcbride and Kempf, 1996). The composition of the modified CYE medium: casitone-yeast extract-glucose amended with 30% natural seawater (NSW) abbreviated as CYG3 is as follows (g/L): 10 g of casitone, 5 g of yeast extract, 1 g of glucose, and 30% NSW. Liquid cultures were incubated with shaking at 180 rpm. Solid CYG3 medium contained 18 g/L of bacteriological agar (HiMedia, India). Higher agar concentrations of 20 g/L were used for conjugation experiments since these conditions offered higher conjugation efficiency (Mcbride and Kempf, 1996). *F. pokkalii* L1I52^T was routinely sub-cultured and maintained for conjugation experiments in CYG3 agar medium incubated at 30ºC. The plasmids or transposons were selected by supplementing antibiotics with the following concentrations (µg / ml): erythromycin, 100; gentamycin, 10; and kanamycin, 30.

4.2.2 Random mutagenesis

4.2.2.1 Biparental Conjugation

For conjugation experiments, *E. coli* S17-1 λ *pir* strain harbouring pHimarEm1 plasmid and L1I52^T were used as the donor and recipient strains, respectively. Both the strains were grown to the mid-exponential phase in respective media and growth conditions, as mentioned in the previous section. The grown cells of both the donor and recipient strains were harvested by centrifugation and mixed in 1:5 and 1:10 (donor: recipient) ratios, and approximately 9-Log cells were spotted on N_{66}® Posidyne® Positive Zeta membrane (0.2 µm; 47 mm) (Cat. No: NRZ047100; PALL Life Sciences, USA) placed in CYG3 agar medium (2% agar). After 18 hours of incubation at 30ºC, the co-cultured cells were scraped off the plates, diluted in CYG3 broth, and the dilutions (mother dilution; MD, 1:1 and 1:2) were spread plated on CYG3 agar medium amended with selection (erythromycin) and counter-selection (gentamycin) antibiotics. Plates were incubated for 4 to 5 days at 30ºC.

The yellow-pigmented colonies grown on the selection plates were the mutant strains of *F. pokkalii* L1I52^T, termed FPRM (*F. pokkalii* L1I52^T random mutants). Thus, the mutants were designated as FPRM, followed by a number. The FPRM strains thus obtained were picked and smeared on CYG3 agar medium containing erythromycin for mutant confirmation. The FPRM strain was maintained on CYG3 agar medium amended with erythromycin selection, and long-term storage was done in 20% glycerol suspensions stored at -80ºC.

4.2.2.2 Rescue cloning

(a) Genomic DNA isolation, restriction digestion and ligation

The genomic DNA of the selected FPRM strains (based on defective motility and xylan utilization followed by delayed Pokkali root colonization) were isolated using QIAamp® DNA Mini Kit (Cat No. 51304; Qiagen, Germany) according to the manufacturer's instructions. Further, 2 µg of the genomic DNA was used as the template for a restriction digestion reaction that was set up with 2 µl NsiI restriction enzyme (Cat. No: R0127S; New England Biolabs, USA) as detailed earlier (Braun et al., 2005). The restriction enzyme-digested product was further subjected to a ligation reaction using T4 DNA ligase (5U/µl) as detailed in the manufacturer's instructions (Cat. No: EL0011; Thermo Fisher Scientific, USA).

(b) Competent cell preparation and transformation

The chemically competent cells of the *E. coli* EC100D *pir*$^+$ strain was prepared for transforming the ligated products, thereby to rescue clone the gene insertions of the selected FPRM strains. An active E. coli EC100D pir$^+$ strain colony was inoculated to 5 ml of LB broth. 1 ml of overnight culture was inoculated to 50 ml LB broth grown at 37ºC/180 rpm until the OD_{600} reached 0.3. The grown cells were incubated in ice for 10 to 15 minutes, transferred to a 50 ml tube, and harvested at 1,600 × g for 10 minutes at 4ºC. 10 ml of sterile 0.1 M $CaCl_2$ was added to the pellet, slowly resuspended using cut-end tips, incubated in ice for 10 minutes, and centrifuged at 1,100 × g for 10 minutes at 4ºC. The resulting pellet was resuspended in 750 µl 0.1 M $CaCl_2$ and 250 µl 60% glycerol using cut-end tips. 100 µl of the competent cell suspensions were aliquoted to sterile 1.5 ml tubes and stored at -80ºC until used. The transformation of the ligated product to the prepared competent cells of *E. coli* was performed following the high efficiency transformation protocol (C29871) V.1 of New England Biolabs (NEB) (*New England Biolabs*, 2014). Briefly, 4 µl of the ligated product

was added to the competent cell mixture, flicked 4 to 5 times, and placed on ice for 30 minutes. The heat shock treatment was given at 42ºC for 30 seconds, followed by a snap-chill on ice for 5 minutes. 900 µl of sterile LB broth was added to the mixture, and the tube was shaken at 37ºC/200 rpm for 60 minutes. The transformed and recovered cells were plated on LB agar medium supplemented with kanamycin antibiotic (30 µg/ml) and incubated at 37ºC. The overnight-grown transformed *E. coli* colonies were picked and smeared on fresh LB agar selection plates for the following experiments.

4.2.2.3 Gene-insert identification

(a) Plasmid isolation and outward sequencing

The rescued *E. coli* clones harboring plasmids were subjected to plasmid isolation. Active *E. coli* clones harboring the plasmid with the transposon, and the flanking gene insert regions were inoculated to fresh LB broths. Following the manufacturer's instructions, the plasmid DNA was isolated using Qiagen® Plasmid Mini Kit (Cat. No. 12125; Qiagen, Germany). The plasmid quality was checked by performing an agarose (0.8%) gel electrophoresis, and the concentration was quantified using the NanoDrop® ND-1000 UV-Vis spectrophotometer (Thermo Scientific, USA) per the instrument guidelines. The plasmid DNA was used as the template to setup a PCR reaction using the outward sequencing primer pair: IR1-609, 5' TGGGAATCATTTGAAGGTTGG 3' and IR2-615, 5' CGGGTATCGCTCTTGAAGGG 3' (Braun et al., 2005) with the following PCR conditions: initial denaturation at 95 ºC for 3 minutes followed by 30 cycles of 3 steps: denaturation at 95ºC for 1 minute, annealing at 62ºC for 1 minutes and extension at 72ºC for 3 minutes and a final extension at 72ºC for 8 minutes. The amplified products were visualized through agarose (1%) gel electrophoresis and documented through a ChemiDoc MP imaging system (Biorad, USA) according to the instrument guidelines. The quality-checked amplified products were used as templates for sequencing PCR using IR1-609 and IR2-615 primer pairs performed using the BigDye^{TM} Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA) according to manufacturer instructions. Further, the samples were purified, precipitated and resuspended in Hi-DiTM Formamide (Thermo Fisher Scientific, USA) for injecting into the automated capillary electrophoresis system, Applied Biosystem 3500 Genetic Analyzer (Thermo Fisher Scientific, USA).

(b) Mapping of sequences to *F. pokkalii* L1I52^{T} genome for insert region identification

The sequences obtained were checked for quality and subjected to the reference genome (*F.*

pokkalii L1I52^T genome; NCBI accession no.: NASZ00000000) mapping to identify the mutated gene regions responsible for defective function in the FPRM strains compared to the *F. pokkalii* L1I52^T WT strain. For mapping, we used the BLAST algorithm in NCBI.

4.2.3 Phenotype screening for loss-of-function (LoF) FPRM strains

The FPRM strains carrying random gene insertions were screened for two different LoF phenotypes i.e., defective in motility and xylan-utilization, compared to the *F. pokkalii* L1I52^T WT strain. For screening motility phenotype, the WT and FPRM strains were pricked on CYG3 soft-agar medium (0.3% bacteriological agar) and incubated at 30ºC under static conditions. After 5 days of incubation, the FPRM strains that showed defective motility compared to the WT strain were considered motility-deficient FPRM strains.

For screening xylan utilization phenotype, the FPRM strains along with the WT strain were smeared on minimal media (g/L) – K_2HPO_4 - 0.8, KH_2PO_4 - 0.2, $MgSO_4.7H_2O$ - 0.2, $CaCl_2.2H_2O$ - 0.13, NH_4Cl – 1, purified agar, 18 (HiMedia, India) amended with 30% NSW and 0.1 % Xylan (Cat No: 91186; SRL, India) as sole carbon source. After 5 days of incubation at 30ºC, the visible growth equivalent to the WT strain was considered positive for xylan utilization. In contrast, the absence of visual growth was considered defective in xylan utilization. Further, a secondary growth validation was also performed to confirm the defective function of xylan utilization. For this, the FPRM strains that failed to grow in the minimal media agar amended with xylan were further checked in minimal media broth amended with 0.1% xylan, and the growth was compared against the growth of the WT strain. The selected FPRM strains defective in motility and xylan-utilization compared to the WT strain were short-listed for the colonization assay. For routine experiments, the FPRM strains were maintained in CYG3 agar medium amended with erythromycin selection, and for long-term preservation, the strains were suspended in 20% glycerol and stored at -80ºC.

4.2.4 Host root colonization assay of FPRM strain

4.2.4.1 Colonization assay of FPRM strains and WT strain

The colonization ability of the short-listed FPRM strains with LOF phenotypes (motility and xylan utilization) were compared with L1I52^T WT strain. To perform this, a hydroponic-based gnotobiotic setup was used, wherein half-strength Hoagland's nutrient solution amended with 50% NSW was used as the hydroponic solution. Sterile Pokkali rice seedlings were placed in sterile tubes containing 10 ml of the nutrient solution. The colonization assay

was initiated with a cell load of 5-Log cells of the selected FPRM strains and WT strain in respective tubes containing seedlings, and the entire setup was incubated in customized plant growth racks maintained with light/dark (12h/12h) cycles under 28°C. The recovery was recorded from the crushed root of seedlings on 3rd dpi (72-hour post-inoculation) through dilution spot assay. Finally, the recovered colonies were estimated through colony counting and represented as Log_{10} values of CFU gm root weight^{-1}. The recovery values of FPRM strains that were quantified below the cut-off value of 6-Log cells on 72-hpi inoculation were considered colonization-deficient.

4.2.4.2 Colonization validation experiment

In this experiment, only the root tip portion (~ 1 cm) of the seedlings was dipped in the hydroponic nutrient solution containing different bacterial strains in the customized phytajar setup. After 48 hours of incubation, the root portions were excised from the seedlings and washed in sterile physiological saline (0.85% NaCl) to remove loosely adhered cells from the root surface. Further, the washed roots were excised into four sections: section-1, the top-most portion of the root (the most distant point from the root tip portion); section-2, the portion just below the section-1; section-3, the portion below section-2 (the portion above the root tip portion); and section-4, root tip portion dipped in the nutrient solution. The excised root sections were placed on CYG3 agar media with erythromycin selection (for FPRM strains) and without selection for WT strain. Based on the visible growth around the root sections, colonization ability was inferred. This was performed as a secondary validation experiment to confirm the results of the primary colonization experiment performed.

4.3 Results and Discussion

4.3.1 Generation of *F. pokkalii* L1I52^T random mutants

To unravel the key genetic determinants critical for host interaction by L1I52^T, a random mutagenesis approach was of high mandate. However, multiple conjugations performed to induce random mutations through transposon insertion using *E. coli* S17-1 λ pir harbouring Tn7 plasmid failed. In this context, it is important to note that genetic manipulation of certain *Flavobacterium* strains poses challenges that might be attributed to factors such as complex physiology, cell wall structure, lack of well-established genetic tools or methods specific to the strain, etc (Thunes et al., 2022). Despite these challenges, we succeeded in mutating the L1I52^T strain through a *mariner* transposon-based mutagenesis technique. For this, we used the *E. coli* S17-1 λ pir strain harbouring pHimar EM1 specifically developed by the McBride research group to study the genetic determinants of gliding motility in the model strain, *F. johnsoniae* (Braun et al., 2005). Our initial attempts for conjugation followed the procedures detailed in earlier papers from McBride's research group (Hunnicutt and Mcbride, 2000; Mcbride and Kempf, 1996). However, following this, we failed to mutate L1I52^T cells. Hence, we modified the media combination and altered the conditions used for conjugation to check whether these changes could introduce random mutations in L1I52T. Briefly, we used a modified CYE medium (CYG3) to grow *F. pokkalii* L1I52^T cells (composition detailed in

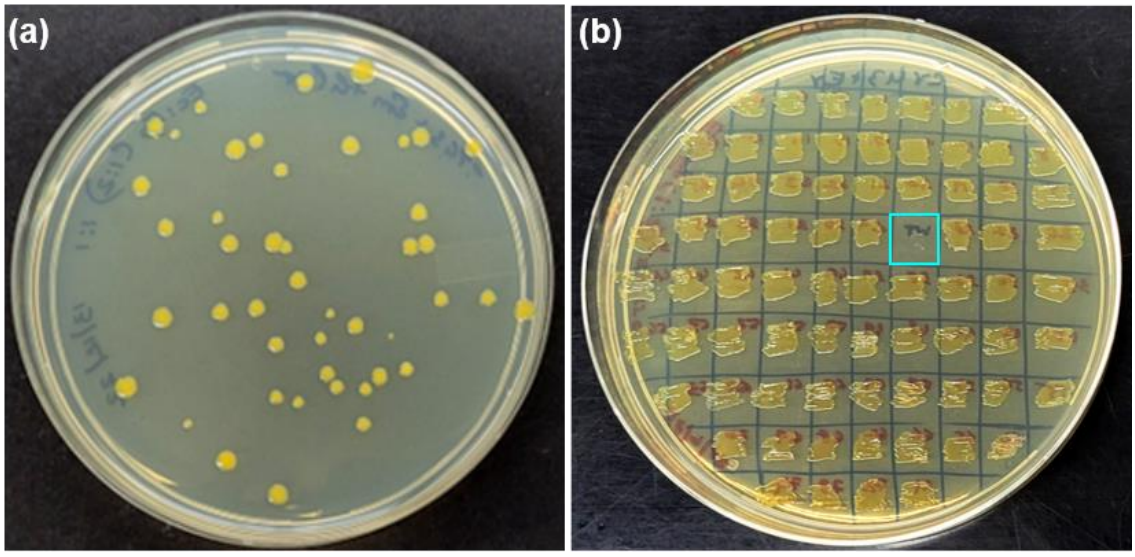


Fig. 4.1. Generation of *F. pokkalii* L1I52^T random mutants: **(a)** Conjugation spread plate showing *F. pokkalii* L1I52^T random mutants with erythromycin antibiotic resistance emerged on 4th dpi after the spread plate from conjugation mixture (*E. coli* S17-1 lambda-pir:*F. pokkalii* L1I52^T) recovery, **(b)** Patches of *F. pokkalii* L1I52^T random mutants on CYG3 agar media amended with erythromycin antibiotic. The blue box in image (b) shows the growth absence indicating the sensitivity of *F. pokkalii* L1I52^T WT strain towards the erythromycin antibiotic.

the method section). Further, a different donor: recipient ratio, i.e., 1:5 and 1:10 (*E. coli*: *F. pokkalii* L1I52^T), was optimized to induce random insertions in *F. pokkalii* L1I52^T strain instead of 1:1 ratio used earlier in *F. johnsoniae* strain (Mcbride and Kempf, 1996). Furthermore, the *HimarEm* mutants were selected on the CYG3 agar medium amended with erythromycin instead of the PY2 agar medium used for *F. johnsoniae* (Braun et al., 2005). In addition to erythromycin selection, we also amended gentamycin as a counter-selection to inhibit the growth of *E. coli* S17-1 cells while recovering the co-cultured conjugation spots. Finally, we picked the yellow-coloured FPRM strains (*F. pokkalii* L1I52^T random mutants) grown on the selection plates after five days of incubation at 30ºC. (Fig. 4.1 (a)). The picked FPRM strains were further grown as patches for further experiments on the CYG3 agar medium with erythromycin selection, where the WT strain failed to grow (Fig. 4.1 (b)). Finally, the WT strain, *F. pokkalii* L1I52^T, and mutant strain, FPRM-166 was, streaked aside on both R2A3 agar medium without any antibiotic selection (the routine agar

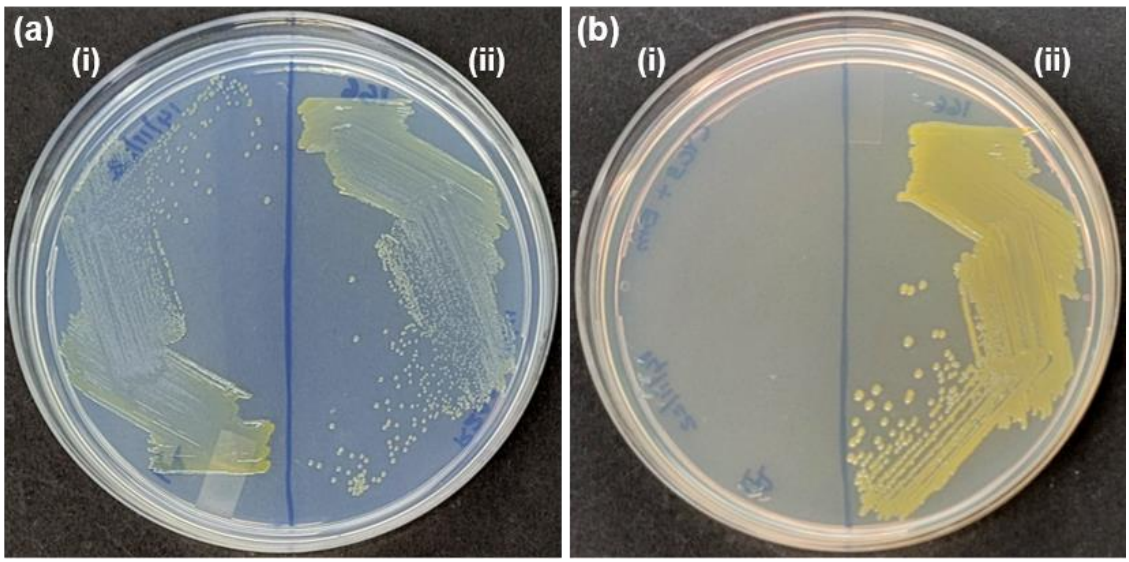


Fig. 4.2. Comparative growth morphotype of **(i)** WT strain, *F. pokkalii* L1I52^T, and **(ii)** the mutant strain, FPRM-166 on **(a)** R2A3 agar medium (imaged on 5th dpi), and **(b)** CYG3 agar medium with erythromycin (imaged on 3rd dpi).

medium used for sub-culturing and maintenance of *F. pokkalii* L1I52^T WT strain) and CYG3 agar medium with erythromycin selection to observe if any morphotype changes or growth differences exist between the WT strain and the mutant strain. Both the WT strain and FPRM-166 strain had equivalent growth morphotype colonies in the R2A3 agar medium (Fig. 4.2 (a)). Similar growth morphotypes were also observed for other selected FPRM strains (data not shown). However, the WT strain failed to grow in the CYG3 agar medium with erythromycin selection, indicating strong antibiotic sensitivity, whereas FPRM-166

harbouring the transposon insertion grew very well (Fig. 4.2 (b)).

4.3.2 Defective phenotype/function screening of FPRM strains

Screening the mutants for a defective phenotype or function caused due to a LoF of a gene is critical to identifying potential mutants generated through random approaches. Hence, the major target of this study was to identify molecular factors critical for host interaction/colonization by L1I52^T. Since host root colonization by rhizobacteria is a multi-stage process that involves several important steps, two major phenotypes that could be directly correlated with the L1I52^T – Pokkali interaction/colonization were selected. This included motility, a very well-known mechanism for host root colonization that is being extensively studied in the case of Rhizobacteria–plant interaction and in the molecular studies related to the genus *Flavobacterim.* The second phenotypic character screened for was selected and designed based on the unique ability of *F. pokkalii* L1I52^T cells to utilize xylan i.e., we screened for mutants that failed to utilize xylan. Approximately 6500 FPRM strains (random mutants of L1I52^T) were generated from 24 conjugations of 8 independent experiments that were screened for defective in motility and xylan-utilization followed by Pokkali root colonization

4.3.2.1 Motility-deficient FPRM strains

We screened the defective motility phenotype in FPRM strains using a standardized soft-agar based motility assay. Briefly, the active cells of L1I52^T WT strain, and FPRM strains were priced in CYG3 soft-agar medium, and the motility was monitored for a period of 5 days under static conditions at 30ºC. The motility of FPRM strains were compared with the WT strain and inferred as motility-deficient or not based on the diameter of visible cell movement from the pricked spot. Through motility screening, we could obtain 7 FPRM strains defective in motility compared to the L1I52^T WT strain (Fig. 4.3). Fig. 4.3 (a-i, ii) represent the 3 FPRM strains (FPRM-166, FPRM-424, and FPRM-628) defective in motility when imaged on 3rd dpi and 5th dpi compared to the WT strain and other motile FPRM strains. Fig. 4.3 (b) represents 4 FPRM strains (FPRM-478, FPRM-586, FPRM-497, and FPRM-628) defective in motility compared to the WT strain and other motile FPRM strains imaged after 5 days of incubation. The motility-defective phenotype of the selected FPRM strains mentioned above was validated and confirmed thrice. Next, we checked the host root colonization ability of these FPRM strains with defective motility.

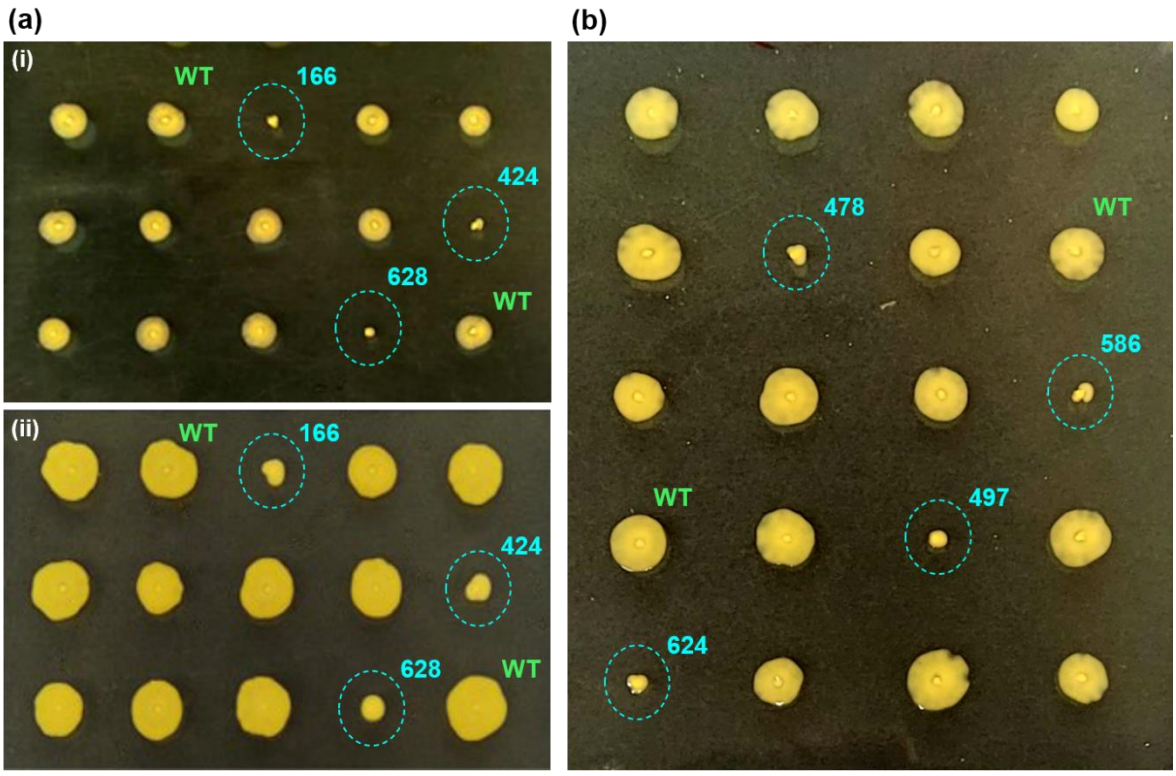


Fig. 4.3. Screening of *F. pokkalii* L1I52^T random mutants (FPRM) for defective motility phenotype: **(a)** Three motility-deficient FPRM strains (FPRM-166, FPRM-424, and FPRM-628) marked in blue dotted circles with respective mutant strain ID displays defective motility when imaged on **(i)** 3rd dpi, and **(ii)** 5th dpi, respectively, **(b)** Four FPRM strains (FPRM-478, FPRM-586, FPRM-497, and FPRM-624) showing defective motility compared to WT strain when imaged on 5th dpi. *F. pokkalii* L1I52^T is labelled WT in green font in the images.

4.3.2.2 Xylan-utilization deficient FPRM strains

As discussed in the earlier chapter, L1I52^T harbours a functional PUL specific for utilizing or breaking down xylan polysaccharide (refer to Chapter 3). In support of this, we also experimentally validated the efficient utilization of xylan by L1I52^T cells compared to its monosaccharide, xylose (refer to Chapter 3). This clearly indicates the affinity of L1I52^T towards complex sugars such as xylan. Supportively, earlier studies have also claimed the specialized ability of *Flavobacterium* members to metabolize complex sugars that are constituents of plant cell walls (Kolton et al., 2013). In this regard, L1I52^T cells efficiently utilized the root macerate of Pokkali seedlings, indicating their ability to utilize plant cell wall constituents (refer to Chapter 3). All these findings suggest that polysaccharide utilization might be one of the many influential factors of host plant colonization in L1I52^T. Hence, we assumed that mutants defective in xylan utilization might have a compromised advantage compared to the WT strain during the host root colonization. However, this hypothetical assumption required an experimental validation that motivated us to screen for defective xylan utilization in FPRM strains. For this, the FRPM strains and the WT strain,

L1I52^T, were smeared on minimal media amended with 0.1% xylan as the sole carbon source. Moreover, we used high-grade purified agar (Cat. No: HiMedia, India) for this screening procedure to avoid the least probability of false-positive results that could arise

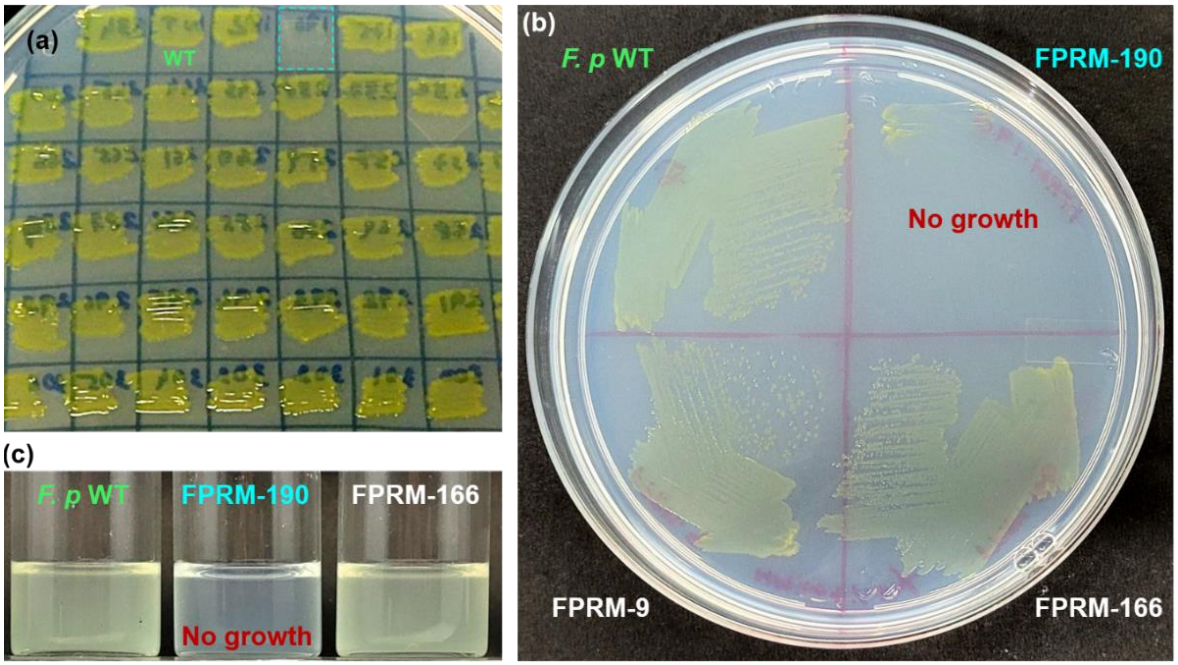


Fig. 4.4. Screening of *F. pokkalii* L1I52^T random mutants (FPRM) for deficiency in xylan-utilization phenotype: **(a)** Growth smears of FPRM strains on xylan-amended (0.1%) minimal media indicating the defective growth of FPRM-190 (marked in blue dotted box) compared to other FPRM strains (not defective for xylan-utilization) and WT strain (labeled in green), **(b)** Plate showing the comparative growth of *F. p* WT strain and other non-defective FPRM strains for xylan utilization with FPRM-190 strain defective for xylan-utilization, **(c)** Image representing the defective growth of FPRM-190 in xylan-amended (0.1%) broth compared to *F. p* WT strain and FPRM-166 (defective mutant for motility).

due to the carbon trace contamination present in normal-grade bacteriological agar. Through this screening method of xylan utilization, we could identify two FPRM strains (FPRM-190, and FPRM-432) defective in xylan utilization (Fig. 4.4 (a, b); Fig. 4.5 (a, b)). Further, to validate this phenotype more firmly, the xylan-utilization by FPRM-190 and FPRM-432 was checked in minimal media broth amended with 0.1% xylan (Fig. 4.4 (c); Fig. 4.5 (c)).

4.3.3 Rescue cloning of FPRM strains with defective phenotypes

To identify the transposon insertion regions in the selected FPRM strains defective in motility and xylan utilization, we performed rescue cloning using the *E. coli* EC100D *pir*$^+$ strain (refer to method section for procedure). The rescued *E. coli* clones carrying the gene insert regions in their plasmids were selected in LB agar medium amended with kanamycin strains were subjected to plasmid DNA isolation. The plasmid from the clones rescued from

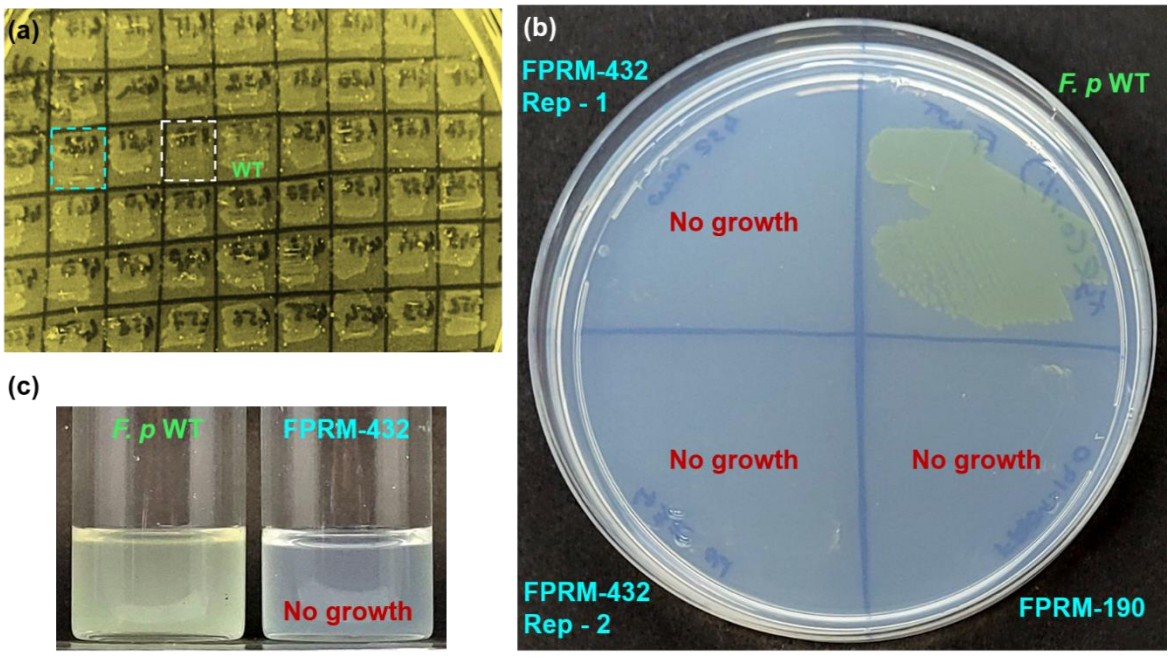


Fig. 4.5. Screening of *F. pokkalii* L1I52^T random mutants (FPRM) for deficiency in xylan-utilization phenotype: **(a)** Growth smears of FPRM strains on xylan-amended (0.1%) minimal media indicating the defective growth of FPRM-432 (marked in blue dotted box) compared to FPRM-190 (defective in xylan utilization; marked in white-dotted box), other FPRM strains (not defective for xylan-utilization) and WT strain (labelled in green), **(b)** Plate showing the comparative growth of *F. p* WT strain and xylan-utilization defective FPRM strains - FPRM-190, and FPRM-432 **(c)** Image representing the defective growth of FPRM-432 in xylan-amended (0.1%) broth compared to *F. p* WT strain.

different FPRM strains had different molecular weights, indicating the variation in the gene insert regions (Fig. 4.7). The plasmid DNA was further amplified using the outward sequencing primers (details provided in the method section); resulting in different amplicon sizes (Fig. 4.8). Next, we proceeded to Sanger-based sequencing of rescued plasmids using the outward sequencing primer pair and mapped the sequence to the high-quality draft genome of L1I52^T (refer to method section for detailed protocol).

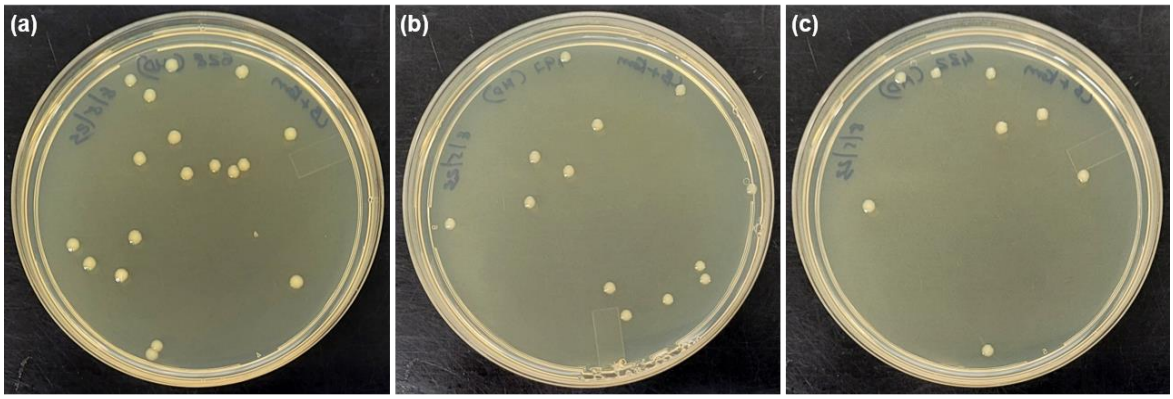


Fig. 4.6. Representation images of the rescued clones containing the FPRM strain insert regions: **(a)** FPRM-628, **(b)** FPRM-166, and **(c)** FPRM-432

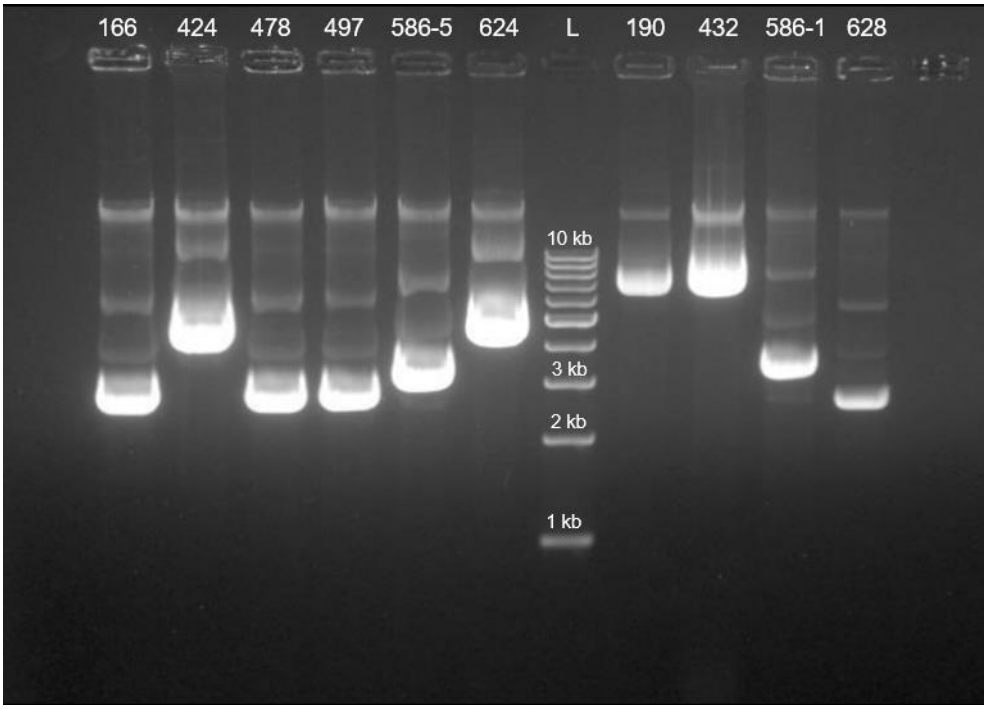


Fig. 4.7. Gel image showing the plasmids isolated from the rescued clones of selected FPRM strains. The varied size of plasmids between the samples indicates the differences in the insert sizes. The labels on top of each well indicate the FPRM strain no.

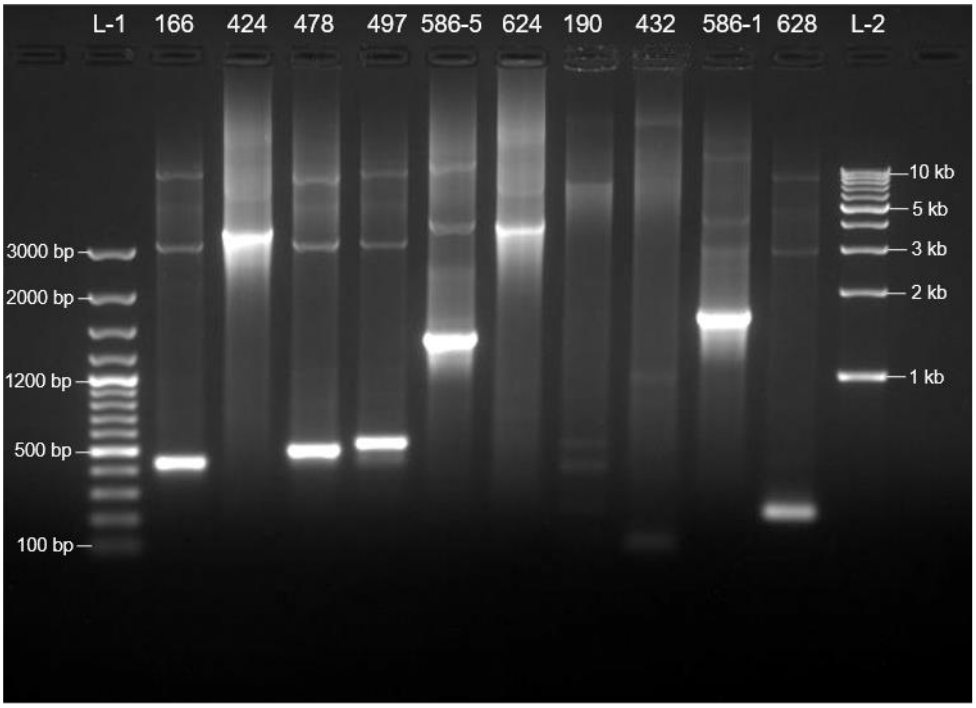


Fig. 4.8. Gel image showing the amplification using outward sequencing primer pair: IR1-609 and IR2-615 using the plasmid as the template. L-1 and L-2 represent the DNA ladders, 100 bp plus and 1 kb DNA respectively. The varied amplicon sizes for different samples indicate the probable insert regions that was subjected for identification through sequencing.

4.3.4 Mariner mutagenesis identifies novel genetic determinants in FPRM strains

The random mutagenesis performed using mariner transposons identified novel gene-insert regions that caused defective motility and xylan utilization phenotypes in *F. pokkalii* L1I52^T strain under the conditions tested. The details of the transposon inserted-gene regions identified by genome mapping are detailed in Table. 4.1. Among the 10 rescued plasmids attempted for transposon insert-gene region identification through outward sequencing, clone 586-1 failed. However, among the 9 rescued plasmids identified for their transposon insert-gene regions, 7 FPRM strains were identified to be novel gene insert regions (Table. 4.1). The remaining two FPRM strains harboured random mutations in the gliding motility complex genes, *gldF,* and *gldK* that are already reported for defective gliding motility when mutated as studied in the model strain *F. johnsoniae* (Hunnicutt et al., 2002). Interestingly, through the random mutagenesis of *F. pokkalii* L1I52^T strain, we could identify 6 novel genes that influenced the motility and xylan utilization of *F. pokkalii* L1I52^T strain (Table 4.1).

Although we could identify FPRM strains with critical defective phenotypes as mentioned above, the major target of this random mutagenesis was to identify potential FPRM strains exhibiting a defective or delayed host colonization function. Hence, to investigate the colonization capacity of the selected 9 FPRM strains, we performed a colonization assay comparing the colonization of FPRM strains with the WT strain. For this, we performed hydroponic-based binary-association experiments in gnotobiotic setups wherein the colonization of FPRM strains was compared against the colonization of the WT strain, *F. pokkalii* L1I52^T. The comparative colonization was quantified in seawater-amended conditions (50% NSW in ½ HNS w/o N) wherein the crushed root recoveries from roots of seedlings inoculated with different FPRM strains and WT strain was quantified. The FPRM strains that displayed recoveries similar or even <1-Log cells to the WT strain were not considered colonization-deficient, whereas those FPRM strains that showed significantly marginal differences (>1-Log cells) in their crushed root recoveries were considered colonization-deficient FPRM strains. Further, to confirm that the reduced or delayed colonization of the portential FPRM strains are not biased with the method or experimental setup, a secondary validation experiment was performed to confirm the colonization ability (refer to method section for details). Hence, in the below sections, the novel gene insertions

Table 4.1. Details of the *F. pokkalii* L1I52^{T} random mutants (FPRM strains)

S. No.	FPRM strain ID	Mutated gene	Protein name	Known or predicted function	NCBI protein ID
I	**Motility-deficient mutants**				
1	FPRM-166 *tpr*::Tn*HEm1*	*tpr*	Tetratricopeptide repeat protein	Numerous vital cell functions	WP_188219292
2	FPRM-424 *gldF*::Tn*HEm1*	*gldF*	Gliding motility-associated ABC transporter permease subunit GldF	Gliding motility	WP_188220449
3	FPRM-478 *ats*::Tn*HEm1*	*ats*	DegT/DnrJ/EryC1/StrS family aminotransferase	Multiple functions	WP_188219565
4	FPRM-497 *surA*::Tn*HEm1*	*surA*	Peptidylprolyl isomerase	Known for functions related to motility, biofilm formation etc	WP_188219703
5	FPRM-586 *gta*::Tn*HEm1*	*gta*	Glycosyltransferase	Biosynthesis of complex carbohydrates	WP_188221661
6	FPRM-624 *ftsK*::Tn*HEm1*	*ftsK*	DNA translocase FtsK	Chromosome segregation and cell division	WP_188221000
7	FPRM-628 *ats*::Tn*HEm1*	*ats*	DegT/DnrJ/EryC1/StrS family aminotransferase	Multiple functions	WP_188219565
II	**Xylan utilization-deficient mutants**				
8	FPRM-190 *gldK*::Tn*HEm1*	*gldK*	Gliding motility lipoprotein GldK	Gliding motility	WP_188219629
9	FPRM-432 *voc*::Tn*HEm1*	*voc*	VOC family protein	Involved in many biological functions	WP_188220563

responsible for the defective phenotype in FPRM strains accompanied by their colonization ability with the host seedlings are discussed.

4.3.4.1 Tetratricopeptide repeat protein (*tpr*::Tn*HEm1*; FPRM-166)

The *F. pokkalii* L1I52^{T} mutant, FPRM-166 (*tpr*::Tn*HEm1)*, defective in motility, harbored a transposon-insert in the gene coding for a tetratricopeptide repeat protein (TPR) (NCBI protein ID: WP_188219292) (Fig. 4.9; Table. 4.1). Generally, TPRs are structural motifs present in a wide range of proteins with multiple functions ranging from prokaryotes to eukaryotes (D'Andrea and Regan, 2003). Only very few studies have reported the involvement of TPRs in bacterial motility. For example, the lyme disease-causing spirochete *Borrelia burgdorferi* encodes a TPR domain protein that exhibited profound effects on flagellar assembly, morphology, and motility upon mutation (Moon et al., 2018). Interestingly, from the colonization assay, we could recover only 5.7 -Log cells of FPRM-166 from the roots of

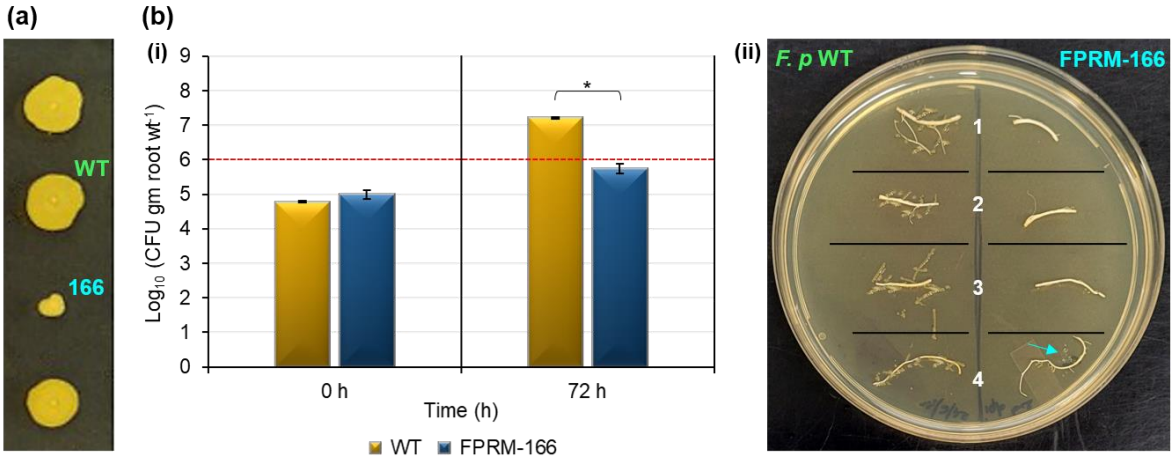


Fig. 4.9. Motility-defective mutant, FPRM-166 (*tpr*::Tn*HEm1*) displays reduced host root colonization: **(a)** Image showing the defective motility of FPRM-166 compared to the WT strain and other FPRM strains, **(b)** Bar graph indicating the reduced colonization of FPRM-166 (below the cut-off value of 6-Log cells) compared to the WT strain and the statistical significance between the recovery is indicated in the figure, **(c)** Plate image showing the colonization validating experiment observation indicating the presence of colony growth around all 4 root section in case of WT strain whereas FPRM-166 presence is represented only by a poor colony growth in the section-4.
Statistical significance is represented as follows: *, $p<0.05$.

Pokkali seedling on 3rd dpi which is quantified to have almost 1.5-Log reduced colonization compared to the WT strain (7.2-Log cells) (Fig. 4.9 (b)). The recovery data in the graph is an average of three biological replicates, indicating the reduced colonization of FPRM-166. Supportively, FPRM-166 strain showed a very weak colonization in the validation experiment (Fig 4.9 (c)). Contrastingly, we observe an efficient colonization of the WT strain even in the section-1 of the root. This indicates that the WT cells have made an active

movement through the root surfaces to colonize the topmost section of the root despite not having any direct contact with the nutrient solution-containing bacteria. Hence, the defective motility phenotype followed by a reduced host root colonization make FPRM-166 an ideal mutant to be studied further. To the best of the knowledge to date, this is the first report for a defective motility function causing altered host root colonization ability in a *Flavobacterium* strain due to a mutation in the gene region coding for TPR. More molecular studies and validation experiments are required to further elaborate on the identified novel function at the gene level through complementation experiments and confirmation at the protein level.

4.3.4.2 DegT/DnrJ/EryC1/StrS family aminotransferase (ats::*RnHEm1*; FPRM-478, FPRM-628)

Two *F. pokkalii* L1I52^{T} mutants, FPRM-478 and FPRM-628, exhibited defective motility compared to the WT strain (Fig. 4.10 (a)). The outward sequencing and genome mapping indicated the transposon-insertion region within the gene coding for DegT/DnrJ/EryC1/StrS

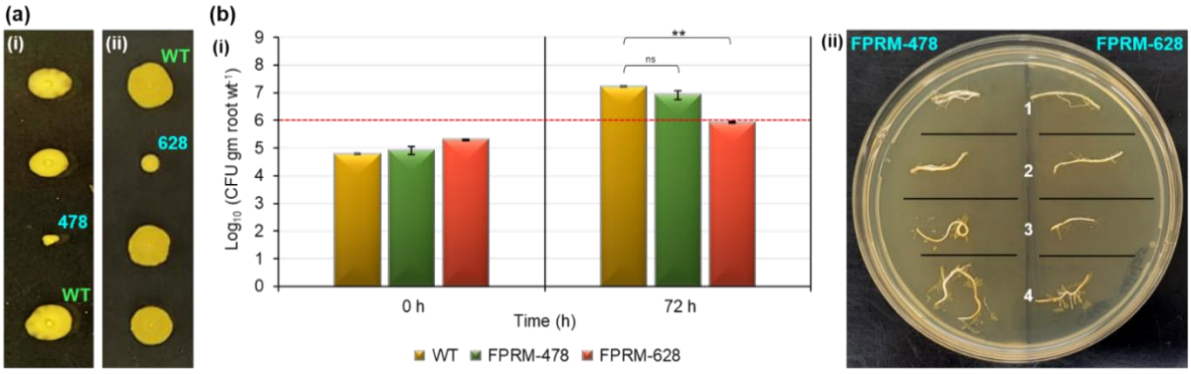


Fig. 4.10. Motility-defective mutants, FPRM-478, and FPRM-638 (ats::*RnHEm1*) displays altered host root colonization: **(a)** Image showing the defective motility of FPRM-478, and FPRM-628 strains compared to the WT strain and other FPRM strains, **(b)** Bar graph comparing the host root colonization of FPRM-478, and FPRM-628 strains (recovery showing above and below the cut-off value of 6-Log cells, respectively) with the WT strain **(c)** Plate image showing the colonization validating experiment observation with the colony growth around roots of section 3 and 4 only indicating a reduced colonization.
Statistical significance is represented as follows: **, $p<0.005$; ns, non-significant.

family aminotransferase enzyme (Table. 4.1). They are members of pyridoxal-phosphate-dependent aminotransferase enzymes with a variety of molecular functions. To the best of our knowledge, the role of these enzyme-encoding genes relative to bacterial motility is not reported to date to the best of our knowledge. However, during the screening for motility-defective mutants and associated mutant identification, these two FPRM strains were represented with the insertion in the same gene exhibiting a defective motility phenotype

compared to the WT strain. This strongly hints that apart from other functions, DegT/DnrJ/EryC1/StrS family aminotransferase encoding gene influences bacterial motility, which is a critical factor in host interaction. Further, we checked if the colonization ability of these mutants is impaired or delayed due to defective motility. Interestingly, we found that FPRM-478 exhibits good colonization, showing a recovery of 6.9-Log cells, which is just showing a 0.3-Log cell reduction compared to the WT strain (7.2-Log cells), whereas FPRM-628 displayed a slightly weakened colonization recording a recovery of 5.9-Log cells which is 1.3 Log cells (just below the ss reduced compared to the WT strain, that is slightly below the cut-off value of 6-Log cells (indicated with a red dotted line in Fig. 4.10 (b). However, a 1-Log cell difference in the colonization recoveries between the counterpart strains, FPRM-478 and FPRM-628, is surprising despite their similar gene insertion region affecting the motility phenotype. Further, colonization recovery results correlated well with the observations from the validation experiment wherein the growth around the root section-2 was higher in FPRM-478 compared to FPRM-628, as clearly seen in the images (Fig. 4.10 (c)). Hence, it confirms that the two FPRM strain carrying transposon insertion in the same gene have similar phenotype defectiveness and exhibit a slight variation in the colonization for which a valid reason is unknown, which requires detailed investigation. However, the role of this gene in *Flavobacterium* motility and associated colonization is firmly validated, which requires molecular validations in future.

4.3.4.3 Peptidylprolyl isomerase (*surA*::Tn*HEm1*; FPRM-497)

Mutations in the FPRM-497 strain exhibited a motility-deficient phenotype, and the transposon-insert region in the genome coded for peptidylprolyl isomerase protein (Table. 4.1). In general, peptidylprolyl isomerases PPIase are multi-functional proteins that are mainly involved in protein folding and secretion in bacteria (Rasch et al., 2018). While studying the gliding motility mechanism of *F. johnsoniae*, it was identified that GldI shared sequence similarity to peptidylprolyl isomerases involved in protein folding, but many gliding Bacteroidota members lacked GldI orthologs (Johnston, 2019). However, these strains are known to have other periplasmic peptidylprolyl isomerases that perform the same function (Johnston, 2019). In the case of L1I52^{T}, we could identify the presence of the GldI encoding gene as well as the other 6 genes coding for peptidylprolyl isomerase (WP_188221618, WP_188220972, WP_188220973, WP_188219858, WP_188219932) including the gene coding for peptidylprolyl isomerase (WP_188219703) that was mutated in FPRM-497 causing defective motility. Several research studies have been published

reporting the role of PPIases involvement in bacterial motility. For example, parvulin PrsA2 was identified for its role in motility of *Listeria monocytogenes,* which was validated by generating a PrsA2-deficient mutant displaying reduced motility (Zemansky et al., 2009). A similar link was observed between the Mip-like protein and *Burkholderia pseudomallei* motility (Norville et al., 2011). In 2016, Skagia A and colleagues identified the link between bacterial motility and a protein called Cyclophilin PpiB belonging to peptidylprolyl isomerases (Skagia et al., 2016). During their investigation, the Δ*ppiB* deletion strain of *E. coli* developed enhanced motility and biofilm formation compared to its wild-type strain (BW25113). The complementation experiments further validated that the deletion of *ppiB* gene caused the increased motility in the deletion mutant (Skagia et al., 2016). Contrastingly, in our studies, the mutation in the gene coding for peptidylprolyl isomerases caused defective motility, as seen in *L. monocytogenes* and *B. pseudomallei* (Norville et al., 2011; Zemansky et al., 2009). Even though there are research studies linking the molecular connection between PPIases and bacterial motility, this is the first study reporting the role of PPIase-encoding genes in bacterial motility and associated host colonization ability. Nevertheless, detailed studies in future could enhance the involvement of these multi-functional proteins in *Flavobacterium* – plant interaction.

4.3.4.4 Glycosyltransferase (*gst*::Tn*HEm1;* FPRM-586-5)

The FPRM-586 strain deficient for motility harboured a transposon-insert mutation in the gene coding for glycosyltransferase (Table. 4.1). Studies relating the bacterial motility function with glycosyltransferase coding genes are very rare. However, findings from Lu et al. have claimed that exopolysaccharide biosynthesis genes are required for motility in *Myxococcus xanthus* (Lu et al., 2005). It is important to note that *M. xanthus* also translocate by means of flagellum-independent gliding motility, which is similar to the motility mechanism in *F. pokkalii* L1I52^{T}. Hence, it could be assumed that glycosyltransferase coding genes contribute to the movement of organisms driven by the gliding motility mechanism as it involves translocation over solid surfaces. In the comparative colonization assay, the recovery of FPRM-586 was 6.2-Log cells, which is just above the cut-off value of 6-Log cells, but 1-Log cells less compared to the WT recovery (7.2-Log cells) from roots (Fig. 4.10). Despite the reduction in recovery, FPRM-586 was not considered as colonization-deficient mutants because it fails to meet our criteria. However, the defective motility phenotype induced upon transposon insertion in the glycosyltransferase coding gene is novel information relative to genus *Flavobacterium*. Hence, this opens a new avenue

to explore the role of glycosyltransferase coding- or related genes in bacterial motility and associated colonization traits.

4.3.4.5 DNA translocase FtsK (*ftsK*::Tn*HEm1*; FPRM-624)

FPRM-624 harbour mutation in the gene coding for DNA translocase FtsK that impaired the motility compared to the WT strain (Table. 4.1). In general, FtsK is a multi-functional protein that contributes significantly in cell division and chromosome segregation (Capiaux et al., 2002). However, their function linked to bacterial cell motility has been poorly studied. Nevertheless, a research note published by Kinscherf et al reported a Tn5 insertion in the *ftsK* gene of *Pseudomonas syringae* pv. *syringae* B728a have several impaired functions compared to its wild type (Kinscherf et al., 2000). This included impaired brown spot lesion formation on *Phaseolus vulgaris*, the ability to grow within bean leaves, and swarming ability on semisolid agar (Kinscherf et al., 2000). The swarming motility function was restored upon complementation, confirming the functional influence of the *ftsK* gene in motility (Kinscherf et al., 2000). Pertaining to other plant-associated bacterial groups, particularly within the genus *Flavobacterium*, a mutation in the *ftsK* gene (FPRM-624) altering the motility phenotype is a first-time report. Following this, we checked if the defective motility phenotype caused by an insertion in the *ftsK* gene altered the the colonization ability of FPRM-624 or not. We recovered 6.4-Log cells from the roots of Pokkali seedlings which was above the cut-off value (Fig. 4.12). Despite a slight reduction in the recovery compared to the WT strain, FPRM-624 was not considered colonization-deficient (Fig. xx). However, the role of the *ftsK* gene in *Flavobacterium* motility seems very interesting and in-depth molecular studies could resolve the mutli-dimensional functional potential for the *ftsK* gene.

4.3.4.6 VOC family protein (*voc*::Tn*HEm1;* FPRM-432)

Among the 6 novel gene insertions identified through the random mutagenesis, the FPRM-432 strain exhibited a defective xylan-utilization phenotype caused by a transposon-insertion in the gene coding for VOC (vicinal oxygen chelate) family protein (Table. 4.1). To the best of our knowledge, no studies to date have reported the role of the VOC family protein-encoding gene to have a functional role in the xylan utilization or breakdown. Further, to know if the transposon insertion has any effect on the host root colonization, we performed a colonization comparison with the WT strain. Interestingly, FPRM-432 recorded a very reduced recovery of 3.9-Log cells from Pokkali seedling roots on 3rd dpi. This is 2.1-

Log cells less than the cut-0ff value of 6-Log cells and around 3.2-Log cells reduced compared to the WT strain recovery (7.2-Log cells) (Fig. 4.11 (b)). Moreover, among all the FPRM strains, the FPRM-432 strain displayed the weakest host root colonization, indicating that the transposon insertion in the VOC family protein-encoding gene that impaired the xylan-utilization ability had profound effects on the host root colonization as well (Fig. 4.11 (b, c)). Further, the observations from the validation experiment also firmly supported the weak colonization of FRPM-432 wherein we could observe only very few growth around the root section-4, and no sign of any growth was observed in the areas around other root

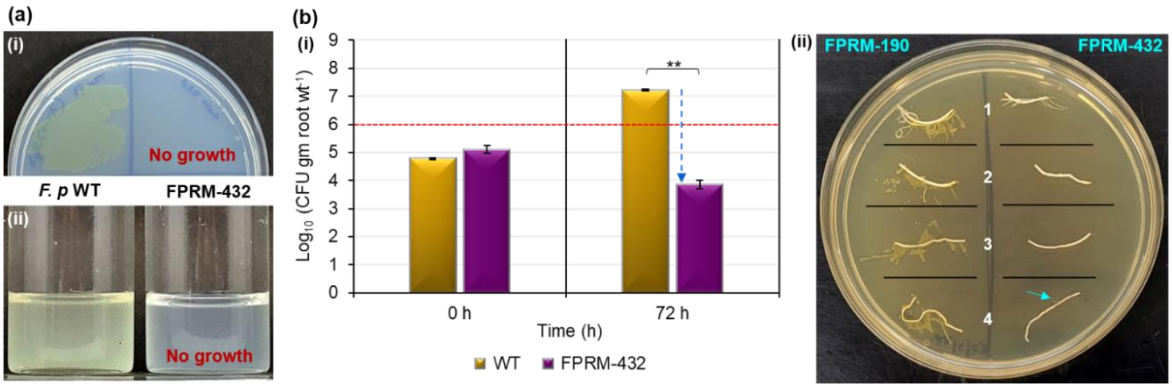


Fig. 4.11. Xylan utilization-deficient mutant, FPRM-432 (*voc*::Tn*HEm1*) exhibits highly reduced host root colonization: **(a)** Images showing the defective xylan-utilization of FPRM-432 in xylan-amended minimal **(i)** agar medium, and **(ii)** broth compared to the WT strain, **(b)** Bar graph indicating highly reduced colonization of FPRM-432 (3-Log cells < than the WT strain) compared to WT strain, **(c)** Plate image of colonization validation experiment observation which show good colony growth around roots for the xylan-utilization mutant, FPRM-190 against the xylan utilization-deficient and colonization deficient mutant, FPRM-432 with no colony growth in sections, 1, 2, and 3 whereas very few colonies were observed in the root section 4 indicated by blue arrow.
Statistical significance is represented as follows: **, $p<0.005$.

sections. These findings clearly validated that the FPRM-432 strain has a highly compromised host root colonization compared to the WT strain and other FPRM strains. Interestingly, the deficient xylan-utilization phenotype has added a novel finding relative to the function of this gene. More studies are required to validate this novel, yet preliminary finding further.

4.3.5 Mariner mutagenesis identified novel gene insertion with LoF and defective colonization

Fig. 4.12 represents the consolidated recovery data obtained from the colonization assay comparing the crushed root recoveries of the FPRM strain and WT strain when recorded on 72 hpi. The graph clearly indicates the load of cells available during the starting point of the

experiment and how these cells were established along the roots of the host seedlings over time. The FPRM strains that recorded a recovery below the cut-off value of 6-Log cells (indicated by a red-dotted line in the graph) were considered colonization deficient. The colonization-deficient strains are FPRM-166, FPRM-628, and FPRM-432, indicated by a red arrow in the figure. FPRM-166 and FPRM-478 are defective in motility, whereas FPRM-432 is defective in xylan utilization. Table. 4.1 summarizes the entire details of the FPRM strains investigated in this study identified through mariner mutagenesis

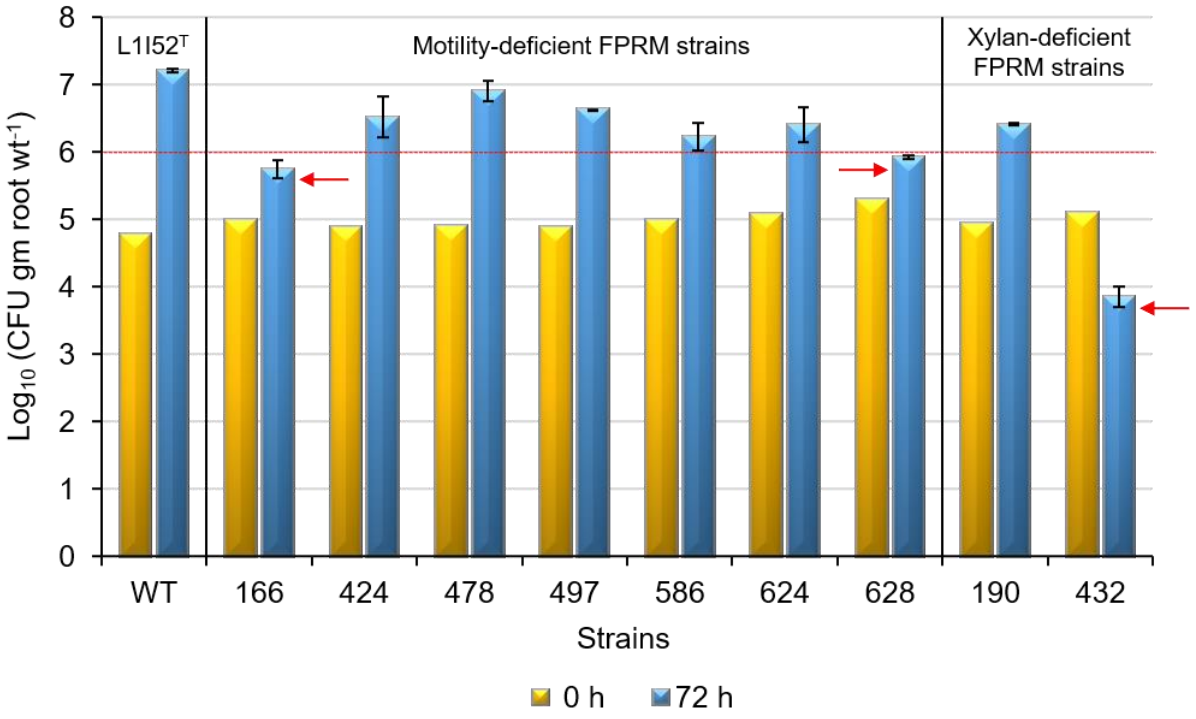


Fig. 4.12. Bar graph representing the comparative colonization recovery of *F. pokkalii* L1I52^T WT and FPRM strains (motility- and xylan utilization-deficient mutants). The red dotted line on the graph represents the cut-off value chosen for categorizing the FPRM strains as colonization-efficient or colonization-deficient. The red arrow marks in the graph indicate the reduced colonization recovery of three FPRM strains (FPRM-166, FPRM-628, and FPRM-432) compared to the WT strain and other FPRM strains. 72 h recovery values for all strains are derived from three biological replicates indicated with respective standard error bars.

4.4 Highlights and Conclusion

The major objective of generating the random mutants of the novel plant-associated *Flavobacterium* strain, L1I52^T, was to identify vital and novel genetic determinants that guide the host association or colonization process. Hence, in this regard, we assumed that motility phenotype could be one essential colonization trait critical for any rhizobacteria to establish its population with the host under favourable conditions. Moreover, the motility phenotype has already been established and used widely as a potential screening method to identify plant-colonization deficient mutants in several rhizobacterial strains. In addition, the second phenotype chosen for mutant screening purpose was the xylan-utilization ability, which was based on the potential ability of the L1I52^T strain to consume xylan as a sole carbon source, assuming that it could contribute to its host colonization. Thus, the mutants (FPRM strains) that were defective in motility and xylan-utilization were subjected to rescue cloning for insert region identification and finally checked for their colonization ability compared to the WT strain using customized setups and experimental procedures.

Through our LOF screening, we could identify a total of 7 FPRM strains defective in motility and 2 FPRM strain defective in xylan-utilization. Further, through colonization assay experiments, we could identify three FPRM strains, two motility-deficient mutants, FPRM-166 (*tpr*::Tn*HEm1*), FPRM-628 (*ast*::Tn*HEm1*), and one xylan-utilization deficient mutant, FPRM-432 (*voc*::Tn*HEm1*) to have colonization deficiencies or delayed colonization compared to the WT strain when quantified on 3rd dpi (72 hpi) (Fig. 4.12). Hence, the comparative colonization recoveries inference indicates that the above-mentioned three FPRM strains profoundly influence host root colonization, which needs to be investigated in detail. Intriguingly, all three FPRM strains deficient in host root colonization harboured novel gene insertions for both the phenotype screened (motility and xylan-utilization) and the associated colonization function with respect to the genus *Flavobacterium.* Further, the colonization validation experiments also displayed observations aligning with the deficient or deficient colonization of the three strains, FPRM-166, FPRM-628 and FPRM-432. Hence, it indicates that the transposon-introduced mutations have firmly disabled the colonization function in the mutants. Among the 3 colonization-deficient FPRM strains, FPRM-432 showed the least colonization (<4-Log cells), indicating that the xylan utilization ability has negatively impacted the root colonization of FPRM-432 and this has been clearly reflected in the observation of the colonization validation experiment as well. This finding encouraged us to explore more on studying how polysaccharide utilization would affect the host

interaction mechanism of a bacteria, especially the members of the genus *Flavobacterium* as they are prominent candidates known for efficient carbohydrate utilization potential.

To conclude, the generation of L1I52^T random mutants (FPRM strains) have provided novel insights into the *Flavobacterium* – rice interaction studies performed under brackish-mimicking conditions wherein we could identify three novel gene insertions that could alter the host colonization ability of this novel plant-associated *Flavobacterium* strain, *F. pokkalii* L1I52^T. Apart from these findings, the mariner-based mutagenesis studies identified novel gene regions that could impair the motility and xylan-utilization abilities of *F. pokkalii* L1I52^T. From the findings, it is understood that apart from the genes belonging to the gliding motility complex and T9SS, certain other genes also contribute to the motility of *Flavobacterium* strains. Nevertheless, gene complementation assays and in-depth molecular studies combined with proteomic analysis are necessary to validate their functional role relative to their eco-physiology and host-associated lifestyle. However, these preliminary findings and success in the genetic manipulation of *F. pokkalii* L1I52^T offer confidence in attempting similar modified approaches to study the genetics of tough-to-manipulate bacterial groups.

Gene insert region sequences of FPRM strain

>FPRM-166 (*tpr*::Tn*HEm1*)

5'GTATTTCCTGTCGGCTTTTCGGTTCATTTGTACCACCGCTTCATAATAATTTCTTGCCAACTCATT
TTCTCCTTTTTCCTGATACAATTGTCCTAAAATAAAACGGTATCGGGCTTTGTCATTATTATGTGTA
CTAAAATTTACGGCTTTTTTCAGTTTGACAATCGCACTGTCTTTTTGTTCCATGTTCAAAAACGAA
GCGGCCAAAAGCGCATTGGCATCAGCCAAAACCTGTTTTTTAGGCTGGTTTTCCTCAAGCATCTT
AGTAATATTTTTAATTACTAACAGGTTGGCTGATAAGTCCCCGGTCTAACAAAGAAAAACACATTT
TTTTGTGAAAATTCGTTTTTATTATTCAACATAGTTCCCTTCAAGAGCGATACCCCTCGAATTGAC
GCGTCGAATTAATTCCGCGAACCCCAGAGTCCCGCTCAGAAGAACTCGTCAAGAAGGCGAGAG
GGAGGCGATGCGCTGCGAATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGCGGTCAGCCCA
TTCGCCGCCAAGCTCTTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGGTCCGCCA
CACCCAGCCGGCCACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGATATTCGGCAAG
CAGGCATCGCCATGGTCACGACGAGATCCTCGCCGTCGGGCATCCGCGCCTTGAGCCTGGCGAA
CAGTTCGGCTGGCGCGAGCCCTGATGCTCTTCGTCCAGATCATCCTGATCGAACAAGGACCGGCT
TCATCCGAGTACGTGCTCGCTCGATGCGATGTTTCCGCTGTGTCGAATGGGCAAGGTAGCCGGAA
TCAAGCGTATGCAGCCCGCCCGCATTGCATCAGCCATGATGATACTTTC 3'

>FPRM-424 (*gldF*::Tn*HEm1*)

5’ATTTTCACAAAAAAATGTGTTTTTCTTTGTTAGACCGGGGACTTATCAGCCAACCTGTTAATAAT
CAGAATTGCAATTGTAACAAATACCGATTTAAGATTTCTTTTTTTAGCAGTTGTCATTACAATTTGA
AGGATTTAAGTTGGTAAACAGTAAACGAAAGAAACGCAATCGTAAGACTGATAAAATAGATCATA
TCTCTGGTGTCAATTACACCGCGGCTCATACTTTTAAAGTGATTTTGCATTCCTAAAGCCGAAATT
GCCGAAGAAAAAGAAGGAATAATTGTGGCAAAACCTTCAAATCCAAAATAAAAAAAGAAGCAT
AAAAATACCGCCAGAATGAAAGCAACAATCTGATTATCGGATAGGGTAGAAGTAAAAATTCCGAT
GGCGGAATAAGCGGCAATTAAAAATAGTAATCCAAAATAAGAACCAACAGTGCTTCCCATATCGA
TATTGCGGGGCGGGCATTCCTAAACTGGAAATCACCTGAACGTAAATAAAAGTTGGCAAAATTGC
CATCCAAATAAGCAACATGGCTCCGATAAATTTTCCATTAGTTATTTGCCAAACAGACAAGGGTTT
GGTGAGTAGTAATTCAAGTGTTCCTTGTCTTCTTTCGTCTGAAAAACTACGCATAGTTACTGCCGG
AATTAAGAAAACAAATATCCAGGGAGCCAGAGTAAAAAACGGAGTCATATCGGCAAATCCGGAA
TTTGGGATGTTGTATTCACCATTAAAAACCCAAAGGAATAATCCGTTGATGATTAAAAAAATGGC
GATACTAAATAACCTATGGGCGAACCAAAAAGGATTTAATTTCACGTAATAATAGGGCTTTCATAT
ATGTTTATTCGTGTATTGTAATTTGGGTTATCGAAATTTATTTTTAA 3’

>FPRM-478 (*ats*::Tn*HEm1*)

5’AAAAATGTGTTTTTCTTTGTTAGACCGGGGACTTATCAGCCAACCTGTTAGACAGTGTTCAGGC
TGCGGTTTTGAATGCTAAATTGCCTTTTTTGAACGATTATAATACAGCTCGTCGATTGGCTGCAAG
CAAGTATAATGCTGCTTTATCAGCTCATGCTAATATTATAACTCCTTCATTTGATGCAAATGAAAAT
AATCATGTTTTTCATCAATATACCTTAAGAATTATTGGTAAAGACAGAAACGGATTGATGCAACAT
TTGTTAGACAAAGGAATTCCTTGTGCTATTTATTATCCAATTCCTTTGCATTCGCAAAAAGCTTATG
CAGATGTTCGTTATAAAGAAGAAGATTTTCCGGTAACCAATCAATTAGTTCAGGAAGTGATTTCG
TTGCCAATGCATATTATTTTTATATAGTACCAAC 3’

>FPRM-497 (*surA*::Tn*HEm1*)

5’TTCCACTACTCTTGGTTCCTGACCTACTCCTCCATTTAATACCGGATTATCTAAGGTAATATTCGT
AGCCTGTTCTACTTTACTTCCTGTTGCTTTAGCGATAGCCTCTAAAGAAGAAGCTGTCATTTTAGC
TTTCAACATTTCAGCTTTTTTCGCTTTTTTCAATTTTGACTCAATAAACGGTCTTACCTGTTCTATC
GGAGCTAAACCTGAATCGTCAATGTTTTTCACCGTAGCAATTACATGACCTAAATTAGCTACTTCA
AAACGTTTTACTGAACCTATTTTAGTATCGTCATCAAAAGCCCATCTTACAATAGCTCTTTGATTT
3’

>FPRM-586-5 (*gst*::Tn*HEm1*)

5’TTTCAATTATTATTGTTACATATAATGCCTGAAAAGCATCTCTGGGAGCATTAATGGAAAGTCTTC
AAACTAGAATCAATGATCAAACAGAAGTCATTGTAATCGATGGGCTTTCGACTGATAGTACCATG
GTTATTCTTCATGAATATTATAAATGTATATCGAAAGTTATTTCAGAAAAAGATTCGGGAATATATG
ATGCAATGAATAAAGGAATTTCAATGGCCTCAGGGAAATATGTTCTTTTTTTAGGTGCAGACGATC
GACTGTTGATAAATCTTGAGGAACTGTCAATGTTTTTGAAAGACGATAAAACTATATATTATGGAG
ATGTTTTATTATCACCCTCAAATAAAATTTACGGAGGGAAGTTTAATACCGCAAAATTGATAAATA
GGAATATTTGTCATCAAAGTATTTTATTTCCGAAGGCAGTATTTGAAAAATATCAATACGGCAAAG
ATTTTAGATTAATGGAGGATTATGCACTTAATTTAAAATTATGGGCTTCAAAAGAATTTAAATTTAC
TTATTTAGAA 3’

>FPRM-624 (*ftsK*::Tn*HEm1*)

5’AAATGTGTTTTTCTTTGTTAGACCGGGGACTTATCAGCCAACCTGTTACCTTTCATGTTAGTTTC
AGAAGCTTCTTTTAAAACTTTCATGATTTCTTCGTAAGAAGTTTCTTTAGCTAATTTTACAGTTAA
ATCTACTGTAGAAACGTCAGCAGTAGGAACACGGAAAGCCATACCTGTTAATTTTCCATTCAAAG
CATATCCAGAATATTAACCGCTCTTGCTTTACTTCCTTCAAAAGGCCCTACAATACCGGCAGCTTC
TAATTGATCAATTAATCGACCGGCGCGGTTGTAACCCAATTTTAGTTTTCGCTGTAGCAAAGAAGC
CGAGCCCTGTTGCGCGTTAACGATAATTTCGGCTGCTTCTCTGAACAAGGTATCTCTTTCAGAAAT
ATCAATATCAAGATTGATGCCATTCTCTTCGCCAACATATTCCGGAAGTAAATAAGCAGTTGCATA
AGCTTTTTGAGAACCAATAAAATCAGTGATTTTTTCTACTTCAGGGGTGTCAATGAAAGCACATT
GTACACGTACCACATCATTTCCGTTAGTGTACAATAAATCTCCTCGTCCAATTAACTGATCAGCTC
CCTGCGTGTCCAAAAATAGTTCTGGAAATCAATTTTAGAAGTTACTCTAAAAGCAATTCGGGCAG
GGAAGTTGGCTTTAATTAAACCTGTAATAACGTTTACCGAAGGTCTTTGTGTAGCATAAATCAAGT
GGATTCCAATGGCTC 3’

>FPRM-628 (*ats*::*RnHEm1*)

5’GCACGAATCACTTCCTGAACTAATTGATTGGTTACCGGAAAATCTTCTTCTTTATAACGAACATC
TGCATAAGCTTTTTGCGAATGCAAAGGAATTGGATAATAAATAGCACAAGGAATTCCTTTGTCTAA
CAAATGTTGCATCAATCCGTTTCTGTCTTTACCAATAATTCTTAAGGTATATTGATGAAAAACATGA
TTATTTTCATTTGCATCAAATGAAGGAGTTATAATATTAGCATGAGCTGATAAAGCAGCATTATACT
TGCTTGCAGCCAATCGACGAGCTGTATTATAATCGTTCAAAAAAGGCAATTTAGCATTCAAAACC
GCAGCCTGAACACTGTCTAACAGGTTGGCTGATAAGTCCCCGG 3’

>FPRM-190 (*gldK*::Tn*HEm1*)

5’TTTCACAAAAAAATGTGTTTTTCTTTGTTAGACCGGGGACTTATCAGCCAACCTGTTACAGTAT
GGCCGGAAATGTTTCGGAGTGGACAGACTCTGCTTAGATCCAAAAGCTTATGAATATGTATCTAC
AATGAACCCAAATGTTTTATATGCATATTATTTTTATATAGTACCAACCTTCAAATGATTCCCAAAA
A 3’

>FPRM-432 (*voc*::Tn*HEm1*)

5’GTTTAAAATTCATCACAATCACTTTATTCAAGAACCAGAGCAATCCCTATTAAAATCCCTTCTGG
ACCTCCAATATAGCAAAGTCTGTAAGCATCCTGATACCGGACAACATCATCAACAAGTTGCGCAC
CGTGTTTATAAAGTCTTTCTAAAGTATCCTCTATGTCACTAACGGCAAACATGACACGTAAATAAC
CTAAGGCATTTACCGGAGCGGTACGATGGTCTTCGATAACTTTTGGAGAAATAAACTGAGACAAT
TCCAAGCGGCTGTTACCATCCGGTGTTACCATCATAGCAATTTCAACAGTTTGATTACCCAGTCCG
GTAACACGACCAGCCCATTCTCCCTTAATTATGGCTCTTCCTTCCAACTCCAAACCGAGTTTGTGA
AAAAAAGGAATTACATTATCCAGCGATTCCACGACGGGCCCGGGGGTTATCCATTCGAAGTAATT
GCTTTT 3’

4.5 References

1. Alvarez, B., Secades, P., McBride, M. J., and Guijarro, J. A. (2004). Development of Genetic Techniques for the Psychrotrophic Fish Pathogen Flavobacterium psychrophilum. *Applied and Environmental Microbiology*, 70(1), 581–587. https://doi.org/10.1128/AEM.70.1.581-587.2004

2. Bodenhausen, N., Horton, M. W., and Bergelson, J. (2013). Bacterial Communities Associated with the Leaves and the Roots of Arabidopsis thaliana. *PLoS ONE*, 8(2). https://doi.org/10.1371/journal.pone.0056329

3. Braun, T. F., Khubbar, M. K., Saffarini, D. A., and McBride, M. J. (2005). *Flavobacterium johnsoniae* gliding motility genes identified by mariner mutagenesis. *Journal of Bacteriology*, 187(20), 6943–6952. https://doi.org/10.1128/JB.187.20.6943-6952.2005

4. Braun, T. F., and McBride, M. J. (2005). Flavobacterium johnsoniae GldJ is a lipoprotein that is required for gliding motility. *Journal of Bacteriology*, 187(8), 2628–2637. https://doi.org/10.1128/JB.187.8.2628-2637.2005

5. Capiaux, H., Lesterlin, C., Pérals, K., Louarn, J. M., and Cornet, F. (2002). A dual role for the FtsK protein in Escherichia coli chromosome segregation. *EMBO reports* (Vol. 3, Issue 6).

6. D'Andrea, L. D., and Regan, L. (2003). TPR proteins: The versatile helix. *Trends in Biochemical Sciences* (Vol. 28, Issue 12, pp. 655–662). Elsevier Ltd. https://doi.org/10.1016/j.tibs.2003.10.007

7. Hunnicutt, D. W., Kempf, M. J., and McBride, M. J. (2002). Mutations in Flavobacterium johnsoniae gldF and gldG disrupt gliding motility and interfere with membrane localization of GldA. *Journal of Bacteriology*, 184(9), 2370–2378. https://doi.org/10.1128/JB.184.9.2370-2378.2002

8. Hunnicutt, D. W., and Mcbride, M. J. (2000). Cloning and Characterization of the Flavobacterium johnsoniae Gliding-Motility Genes gldB and gldC. *Journal Of Bacteriology* (Vol. 182, Issue 4).

9. Johnston, J.J. (2019). Flavobacterium Gliding Motility: From Protein Secretion to Cell Surface Adhesin Movements (Doctoral dissertation, The University of Wisconsin-Milwaukee).

10. Kinscherf, T.G., Hirano, S.S. and Willis, D.K. (2000). Transposon insertion in the ftsK gene impairs in planta growth and lesion-forming abilities in *Pseudomonas syringae* pv. syringae B728a. *Molecular plant-microbe interactions*, *13*(11), pp.1263-1265.

11. Kolton, M., Frenkel, O., Elad, Y., and Cytryn, E. (2014). Potential role of flavobacterial gliding-motility and type IX secretion system complex in root colonization and plant defense. *Molecular Plant-Microbe Interactions*, 27(9), 1005–1013. https://doi.org/10.1094/MPMI-03-14-0067-R

12. Kolton, M., Sela, N., Elad, Y., and Cytryn, E. (2013). Comparative Genomic Analysis Indicates that Niche Adaptation of Terrestrial *Flavobacteria* Is Strongly Linked to Plant Glycan Metabolism. *PLoS ONE*, 8(9). https://doi.org/10.1371/journal.pone.0076704

13. Lu, A., Cho, K., Black, W. P., Duan, X. Y., Lux, R., Yang, Z., Kaplan, H. B., Zusman, D. R., and Shi, W. (2005). Exopolysaccharide biosynthesis genes required for social motility in *Myxococcus xanthus*. *Molecular Microbiology*, 55(1), 206–220. https://doi.org/10.1111/j.1365-2958.2004.04369.x

14. Maloy, S. (2013). Bacterial Genetics. In Brenner's Encyclopedia of Genetics: Second Edition (pp. 265–270). Elsevier Inc. https://doi.org/10.1016/B978-0-12-374984-0.01702-2

15. Mcbride, M. J., and Kempf, M. J. (1996). Development of Techniques for the Genetic Manipulation of the Gliding Bacterium *Cytophaga johnsonae*. *Journal of Bacteriology* (Vol. 178, Issue 3).

16. Moon, K. H., Zhao, X., Xu, H., Liu, J., and Motaleb, M. A. (2018). A tetratricopeptide repeat domain protein has profound effects on assembly of periplasmic flagella, morphology and motility of the lyme disease spirochete *Borrelia burgdorferi*. *Molecular Microbiology*, 110(4), 634–647. https://doi.org/10.1111/mmi.14121

17. New England Biolabs. (2014). https://doi.org/10.17504/protocol

18. Norville, I. H., Harmer, N. J., Harding, S. V., Fischer, G., Keith, K. E., Brown, K. A., Sarkar-Tyson, M., and Titball, R. W. (2011). A *Burkholderia pseudomallei* macrophage infectivity potentiator-like protein has rapamycin-inhibitable peptidylprolyl isomerase activity and pleiotropic effects on virulence. *Infection and Immunity*, 79(11), 4299–4307. https://doi.org/10.1128/IAI.00134-11

19. Rasch, J., Ünal, C.M., Klages, A., Karsli, Ü., Heinsohn, N., Brouwer, R.M.H.J., Richter, M., Dellmann, A. and Steinert, M. (2019). Peptidyl-prolyl-cis/trans-isomerases Mip and PpiB of *Legionella pneumophila* contribute to surface translocation, growth at suboptimal temperature, and infection. *Infection and immunity*, *87*(1), pp.10-1128.

20. Schlaeppi, K., Dombrowski, N., Oter, R. G., Ver Loren Van Themaat, E., and Schulze-Lefert, P. (2014). Quantitative divergence of the bacterial root microbiota in Arabidopsis thaliana relatives. *Proceedings of the National Academy of Sciences*, 111(2), 585–592. https://doi.org/10.1073/pnas.1321597111

21. Skagia, A., Zografou, C., Vezyri, E., Venieraki, A., Katinakis, P., and Dimou, M. (2016). Cyclophilin PpiB is involved in motility and biofilm formation via its functional association with certain proteins. *Genes to Cells*, 21(8), 833–851. https://doi.org/10.1111/gtc.12383

22. Staroscik, A. M., Hunnicutt, D. W., Archibald, K. E., and Nelson, D. R. (2008). Development of methods for the genetic manipulation of Flavobacterium columnare. *BMC Microbiology*, 8. https://doi.org/10.1186/1471-2180-8-115

23. Thunes, N.C., Conrad, R.A., Mohammed, H.H., Zhu, Y., Barbier, P., Evenhuis, J.P., Perez-Pascual, D., Ghigo, J.M., Lipscomb, R.S., Schneider, J.R. and Li, N. (2022). Type IX secretion system effectors and virulence of the model *Flavobacterium columnare* strain MS-FC-4. *Applied and Environmental Microbiology*, *88*(3), pp.e01705-21.

24. Zemansky, J., Kline, B. C., Woodward, J. J., Leber, J. H., Marquis, H., and Portnoy, D. A. (2009). Development of a mariner-based transposon and identification of Listeria monocytogenes determinants, including the peptidyl-prolyl isomerase PrsA2, that contribute to its hemolytic phenotype. *Journal of Bacteriology*, 191(12), 3950–3964. https://doi.org/10.1128/JB.00016-09.

Chapter – 5

Research summary and Future perspectives

5.1 Research summary

"If you can't explain it simply, you don't understand it well enough." – A famous quote by the greatest and most influential scientist of all time, Albert Einstein, highlighted that true understanding of a topic allows a person to explain it in a clear and simple manner. Hence, I believe the findings from this research are scientifically presented with appropriate validations and statistics for ease of understanding. That being so, this chapter summarises the significant conclusions from different chapters discussed earlier, and their relevance pertaining to the current research context.

This research explores the ecology of the genus *Flavobacterium* in brackish-associated native rice varieties through a culture-independent metagenomic approach. The novel plant-associated *Flavobacterium* strain, *F. pokkalii* L1I52^T (hereafter mentioned as L1I52^T in the main text except titles, figures and legends), discovered during this study unravels the host-association traits, host-gene modulation abilities and environmental adaptation features for a plant-associated *Flavobacterium* strain as never studied before. This is facilitated through *in-planta* and *in-vitro* eco-physiological characterization studies validated with in-depth genomic analysis and host-transcriptome profiling. Further, mariner transposon-based random mutagenesis revealed important genetic determinants critical for L1I52^T-Pokkali rice interaction under brackish conditions. The sections briefed below provide insights into the significant discoveries and highlights of this research that has deciphered the host-plant interaction of the genus *Flavobacterium*, especially in brackish environments.

5.1.1 *Flavobacterium:* An abundant rhizobacteria in the roots of brackish-associated native rice varieties

The genus *Flavobacterium* strains are known for their ubiquitous habitat presence, including extreme environments, and their tolerance to cope wide range of environmental stresses makes them abundant members of soil microbiomes and forest litter (Herzog et al., 2019; Kolton et al., 2013; Larsbrink and McKee, 2020; Mukhtar et al., 2021; Ventosa et al., 2015; Zhu et al., 2013). However, their existence in plants or crops native to brackish-associated environments remains elusive. In this context, we traced the distribution of the genus *Flavobacterium* (phylum Bacteroidota) in the brackish-associated native rice varieties (Pokkali, Kaipad, and Kagga) of South India through a 16S rRNA amplicon sequencing-based metagenomic approach. The high abundance of genus *Flavobacterium* OTUs in the root sections compared to the rhizosphere across all the three rice varieties tested, despite their distant geographical locations, was an intriguing observation. Moreover, this

metagenomic dominance on roots hinted at the possible tight association of *Flavobacterium* members with its host plants. Further, to understand if the environment, i.e., brackish condition, is the prime factor driving *Flavobacterium* abundance in brackish-associated native rice varieties, a 16S rRNA amplicon sequencing was performed on the roots of Pokkali seedlings grown in a customized soil-microcosm setup maintained under different conditions: non-brackish (0% NSW, 100mM NaCl) and brackish (30% NSW). Interestingly, the metagenomic dataset analysis yielded a significantly high abundance of *Flavobacterium* OTUs in the roots of seedlings grown in brackish conditions (30% NSW) compared to non-brackish conditions (0% NSW and 100 mM NaCl). These observations implied that brackish environments have a substantial impact in mediating *Flavobacterium*-rice interaction. Thus, the intriguing observations from the 16S rRNA metagenomic dataset analysis were validated with soil microcosm-based bacteriome analysis findings. However, to expand the knowledge further on their host-association benefit and ecological adaptation, a native cultured representative strain was of high mandate. Hence, a targeted-culturing approach was attempted, combining customized media combinations and unique isolation strategies to isolate native *Flavobacterium* strains from the Pokkali rice roots

5.1.2 *Flavobacterium pokkalii* sp. nov. L1I52^T – A native of Pokkali rice

The seawater-dependent and selection-based targeted culturing from pokkali rice roots yielded a pool of novel *Flavobacterium* strains. A strain designated L1I52^T was identified as a potent rice root colonizer with host-growth enhancing abilities and, therefore, subjected to further investigation. The higher representation of L1I52^T-equivalent strains (NRKF1, NRK F15, NRK F16, NRK F41, NRK F42, NRK F47, NRK F49, and NRK F50) indicated their presence in brackish rice roots thus indicating a close association with the host, Pokkali rice.

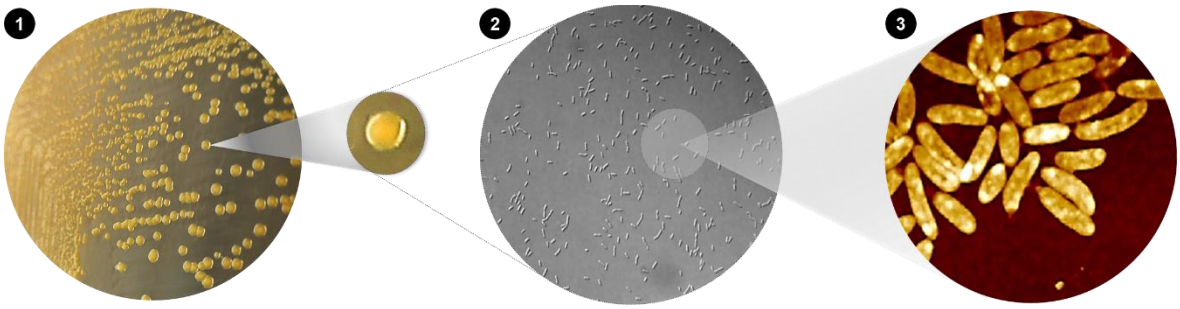


Fig. 5.1. *F. pokkalii* L1I52^T: **(1)** Yellow pigmented colonies of *F. pokkalii* L1I52^T in CYG3 agar plates with a magnified image of a single colony, **(2)** Rod shaped cells of *F. pokkalii* L1I52^T imaged under a differential interference contrast microscopy, and **(3)** Atomic force microscopy (AFM) image displaying the rod-shaped morphology of *F. pokkalii* L1I52^T without flagella.

The observations from phenotypic studies hinted at the unique eco-adaptive traits in L1I52^T, such as increased tolerance to NaCl and ability to grow in seawater-amended medium compared to its nearest phylogenetic relatives. Based on the phylogenetic analysis using 16S rRNA and Multilocus sequence analysis performed using 5 housekeeping genes *(gyrB, glyA, atpA, dnaK* and *murG)* placed, the isolated nine strains as a novel species within the genus *Flavobacterium* with *F. daejeonense* GH1-10^T, *F. sufflavum* BBQ-12^T, and *F. glycines* Gm-149^T as their nearest phylogenetic neighbours. Finally, based on the genotypic, phenotypic, chemotaxonomic, and genome-based taxonomic characterization, the novel *Flavobacterium* species was proposed to name *Flavobacterium pokkalii* sp. nov. L1I52^T (= MTCC 12454^T = KCTC42429^T) (Menon et al., 2020) (Fig. 5.1).

5.1.3 *F. pokkalii* L1I52^T: A potent phyto-beneficial rhizobacterium with host-gene modulation ability, plant-material uptake, and brackish-adaptive traits

The *in-planta* studies and *in-vitro* characterization of plant interaction traits tested under eco-mimicking conditions further confirmed the potential of L1I52^T to enhance the growth of host, Pokkali rice under brackish and non-brackish conditions. The host gene expression analysis tested through RT-qPCR analysis revealed the potential ability of L1I52^T in modulating the expression of important abiotic stress-related genes (A1-beta2, CML10_1, CML15, DREB-1C, DREB-1G, NDR-1) under tested brackish conditions (50% NSW) (Fig. 5.2). Further, the eco-physiological characterization studies revealed the critical influence of brackish conditions on essential host-colonization traits such as biofilm formation, seed attachment, root attachment, and subsequent colonization. In addition, the brackish condition also supported the survival of L1I52^T cells under planktonic conditions (non-colonized state). Altogether, the importance of the environment, i.e., the brackish condition in mediating the interaction between L1I52^T and its host, Pokkali rice, and the subsequent positive influence on host plant growth is firmly validated. Further, the survival ability of L1I52^T as planktonic cells and the isolation of its equivalent strains from the Pokkali rice roots after four years provide sufficient evidence to position L1I52^T as a native of Pokkali rice ecosystem.

Through detailed phylogenetic and comparative genomic analysis, we could confirm that L1I52^T is a terrestrial clade *Flavobacterium* strain harbouring a vast array of CAZyme-organized PULs specific for the uptake and utilization of different plant-derived polysaccharides (discussed in chapter – 3). This unique potential of L1I52^T is assumed to play a critical role in the carbon turnover of the host ecosystem by breaking down complex

polysaccharides to their monosaccharide sugars. This enables the availability of simple sugars for their energy metabolism and for other bacterial groups to utilize and establish in the plant rhizosphere, thereby providing a synergistic beneficial effect to the host plants. This hypothetical explanation correlates with the soil-microcosm-based bacteriome analysis findings discussed in chapter 2. During the bacterial community analysis at the family level, an increased abundance of family *Pseudomonadaceae* OTUs was recorded upon the enrichment of *Flavobacteriaceae* OTUs in roots of Pokkali rice seedlings grown in brackish conditions. In contrast, the abundance of *Psudomonadaceae* OTUs was much lower in non-brackish conditions. Hence, based on this comparative metagenomic analysis, it is observed that the brackish conditions favour the enrichment of *Flavobacteriaeae* OTUs in Pokkali seedlings roots, which in turn enriches the *Pseudomonadaceae* population. It is assumed that *Flavobacteriaceae* members mediate a carbon turnover in the rice roots facilitated by polysaccharide breakdown, thereby attracting the *Pseudomondaceae* members to consume the simple sugars derived from polysaccharide breakdown, thus resulting in the increased abundance of *Pseudomonadaceae* members in brackish conditions. Moreover, the preference for complex polysaccharides by *Flavobacterium* members offers them an advantage in rhizosphere competence, as most of the dominant bacterial groups in plant rhizosphere feed on simple sugars. The high affinity of L1I52^T towards xylan polysaccharide compared to its monosaccharide, xylose, is one of the best experimental proofs for this (discussed in Chapter – 3). This supports the claim for the specialized capacity of *Flavobacterium* strains in utilizing macromolecules against the low molecular mass substances such as plant exudate compounds used by phylum Pseudomonadota members, the major bacterial constituents in plant-associated habitats (Olsson and Persson, 1999). This phenomenon of metabolic niche partitioning is well supported by the fact that a higher abundance of *Flavobacterium* members is recorded in the root environments where exudates do not exist (Georges et al., 2014). This is in accordance with the metagenomic findings of this study that identified a high abundance of *Flavobacterium* OTUs in the roots compared to the rhizosphere (discussed in Chapter – 2). Furthermore, all the equivalent strains of L1I52^T were cultured from the root sections of Pokkali rice (discussed in Chapter – 2), firmly supporting their tight association with the host and thus reflecting their positioning within the terrestrial clade.

Another important observation during the comparative genomic analysis was the reduced genome size (3.89 Mb) of the L1I52^T strain compared to its phylogenetic neighbours and

other terrestrial counterpart strains. Despite harbouring a relatively small genome (< 4 Mb) compared to other terrestrial clade *Flavobacterium* strain (> 4 Mb), the L1I52^T genome exhibited efficient carbohydrate utilization, driven by almost equivalent or even more CAZyme coding PULs than their terrestrial counterparts (discussed in Chapter 3). Hence, we assume the reduced genome size of L1I52^T that marks below 4 Mb (a characteristic genome size cut-off for aquatic clade *Flavobacterium* strains) might be an evolved trait for its adaptation in an ecosystem like Pokkali rice fields, which lies in a juncture between the terrestrial and marine habitats (discussed in Chapter – 3). However, other *Flavobacterium* strains, NRK F7, NRK F10, and NRK 1, isolated from Pokkali rice roots harboured genomes of size < 4 Mb, but they were devoid of carbohydrate utilization genes, which categorized them as probable aquatic clade strains.

The L1I52^T genome is equipped with an efficient genetic system to countervail the fluctuating saline conditions prevailing in brackish environments in Pokkali rice fields to maintain cell homeostasis. This coordinated mechanism is driven by numerous genes that code for various genes coding for various anti-porter, transporter, symporter, and exchanger proteins that function to extrude and uptake different ions such Na^+, K^+, Mg^{2+}, and Ca^{2+}, along with osmo-protectant (glutamate, glutamine, proline) synthesis genes thus maintaining the ionic balance according to the fluctuating external environment. Among these, the presence of Na^+-translocating NADH:quinone oxidoreductase (Na^+-NQR), a characteristic gene reported mostly in marine bacterial strains supported their brackish-adaptation feature. Furthermore, an efficient anti-oxidant defence mechanism is assumed to detoxify the reactive oxygen species (ROS) generated from the host plant, Pokkali rice and surrounding brackisch environments.

5.1.4 *Himar* transposon-based random mutagenesis identifies novel gene insertions in *F. pokkalii* L1I52^T with defective phenotype and associated functions

Genetic manipulation of *Flavobacterium* strains is regarded as challenging due to many limitations, as detailed earlier. However, with repeated attempts performed with modified conditions and methods, the L1I52^T strain was genetically manipulated through a random approach employing *HimarEm1* transposon (discussed in Chapter – 4). Screening of around 6500 L1I52^T mutants (FPRM strains), identified 9 FPRM strains with a LoF phenotype and associated function. Among these, 7 FPRM strains (FPRM-166, FPRM-424, FPRM-478, FPRM-497, FPRM-586, FPRM-624, and FPRM-628) were defective in motility, and 2 FPRM strains (FPRM-190 and FPRM-432) were deficient in xylan utilization. Among these,

7 motility-deficient FPRM strains, FPRM-424 (*gldF*::Tn*HEm1)* harboured transposon insertion in *gldF* gene, already reported for its role in *Flavobacterium* motility as identified in *F. johnsoniae* strain (Hunnicutt et al., 2002). However, other 6 transposon insertions marked novel gene locations, which are reported for the first time for the defective motility phenotype, especially in the genus *Flavobacterium.* This includes 2 insertions in the gene coding for DegT/DnrJ/EryC1/StrS family aminotransferase (FPRM-478 / *ats*::Tn*HEm1*; FPRM-628 / ats::*RnHEm1*) and single insertions in the following 4 genes coding for: (i) tetratricopeptide repeat protein (FPRM-166 / *tpr*::Tn*HEm1*), (ii) peptidylprolyl isomerase (FPRM-497 / *surA*::Tn*HEm1*), glycosyltransferase (FPRM-586 / *gst*::Tn*HEm1*), (iii) DNA trsanslocase FtsK (FPRM-624 / *ftsK*::Tn*HEm1*). Further, among the 2 FPRM strains defective in xylan utilization, FPRM-190 (*gldK*::Tn*HEm1*) had a transposon insertion in the *gldK* gene, which is reportedly known for motility function in *F. johnsoniae*. However, FPRM-432 harboured an insertion in a gene coding for VOC family protein (*voc*::Tn*HEm1*), which is the first report for the role of this protein affecting the xylan-utilization potential of a bacterial strain.

Host colonization is the most critical step of any rhizobacterial strain to establish its interaction with the host. Moreover, the findings from root-colonization experiments have proven that L1I52^{T} cells are efficient root colonizers of its host, Pokkali rice. Therefore, we compared the root colonization ability of the selected 9 FPRM strains with the WT strain to know if any of the mutants defective in motility or xylan utilization has altered the colonization ability of the mutant strains. In this regard, we performed a root colonization experiment in hydroponic-based gnotobiotic setups and the observations from this experiment were further validated with a secondary colonization validation experiment. Through the colonization screening of 9 FPRM strains, we could identify two motility-deficient mutants, FPRM-166 and FPRM-628, and one xylan-utilization deficient mutant, FPRM-432, exhibiting colonization deficiency. Among these, FPRM-432 was the most deficient in host root colonization compared to the other 2 mutants. This proves that motility and xylan utilization are critical phenotypes that contribute primarily to the host colonization of a bacteria. Altogether, these findings have expanded the knowledge of the important genetic factors that could probably contribute to *Flavobacterium*-plant interaction, which was restricted within the gliding motility complex and type IX secretion system to date.

5.1.5 Molecular interaction of *F. pokkalii* L1I52^T-Pokkali rice-brackish environment

A hypothetical illustration that details the molecular interaction of L1I52^T with its host plant, Pokkali rice, and surrounding brackish environments has been conceived based on the eco-physiological, genomic and transcriptomic findings (Fig. 5.2). The numbers mentioned in black-coloured circles in Fig. 5.2 (indicated as numbers within brackets in the write-up) indicate the sequence of molecular events assumed to occur during this L1I52^T-Pokkali rice-brackish interaction which detailed as follows. The fluctuating brackish condition in the Pokkali rice fields induces saline stress in Pokkali rice plants **(1)** that causes an altered root exudation **(2)** (Fig. 5.2). Further, the saline-stress-induced root exudation is sensed by L1I52^T through its signal transduction mechanism **(3)** that activates the movement of L1I52^T towards Pokkali rice roots mediated by the combined functioning of gliding motility complex and T9SS **(4)** (Fig. 5.2). Following this, the molecular patterns from the L1I52^T cells is detected by Pokkali rice root cells which in turn activates a cascade of molecular events inside the root cell **(5)** (Fig. 5.2). This includes the up-regulation of salt stress-responsive genes for enhanced saline tolerance **(6)**, up-regulation of host-genes involved in LHC complex and Calvin cycle in the chloroplast thus contributing towards plant growth **(7)**, and induction of pattern-triggered immunity (PTI) elevating the oxidative stress **(8)** (Fig. 5.2). To outcompete this oxidative stress, the ROS scavenging mechanism in L1I52^T, majorly the peroxidase and SOD is assumed to play a critical role **(9)** and the eukaryotic like-proteins (ELPs) function to evade other host-defences response and is expected to mediate cell-wall disruption/modification for the host-cell entry by L1I52^T **(10)** (Fig 5.2). Further, the CAZyme-coded functional PULs mediate the uptake of complex plant-based polysaccharides **(11)**, its breakdown to oligosaccharides **(12)** and finally to monosaccharides which enters into the energy metabolism pathways of L1I52^T cell **(13)** (Fig. 5.2). Furthermore, L1I52^T circumvent the saline stress caused due to the fluctuating brackish conditions through efficient genetic systems coded for brackish-adaptive **(14)** and antioxidant defence mechanisms **(15)** (Fig. 5.2). Nevertheless, additional confirmational studies at the transcriptomic and proteomic level would provide firm support to the hypothesis of L1I52^T-Pokkali-brackish interaction.

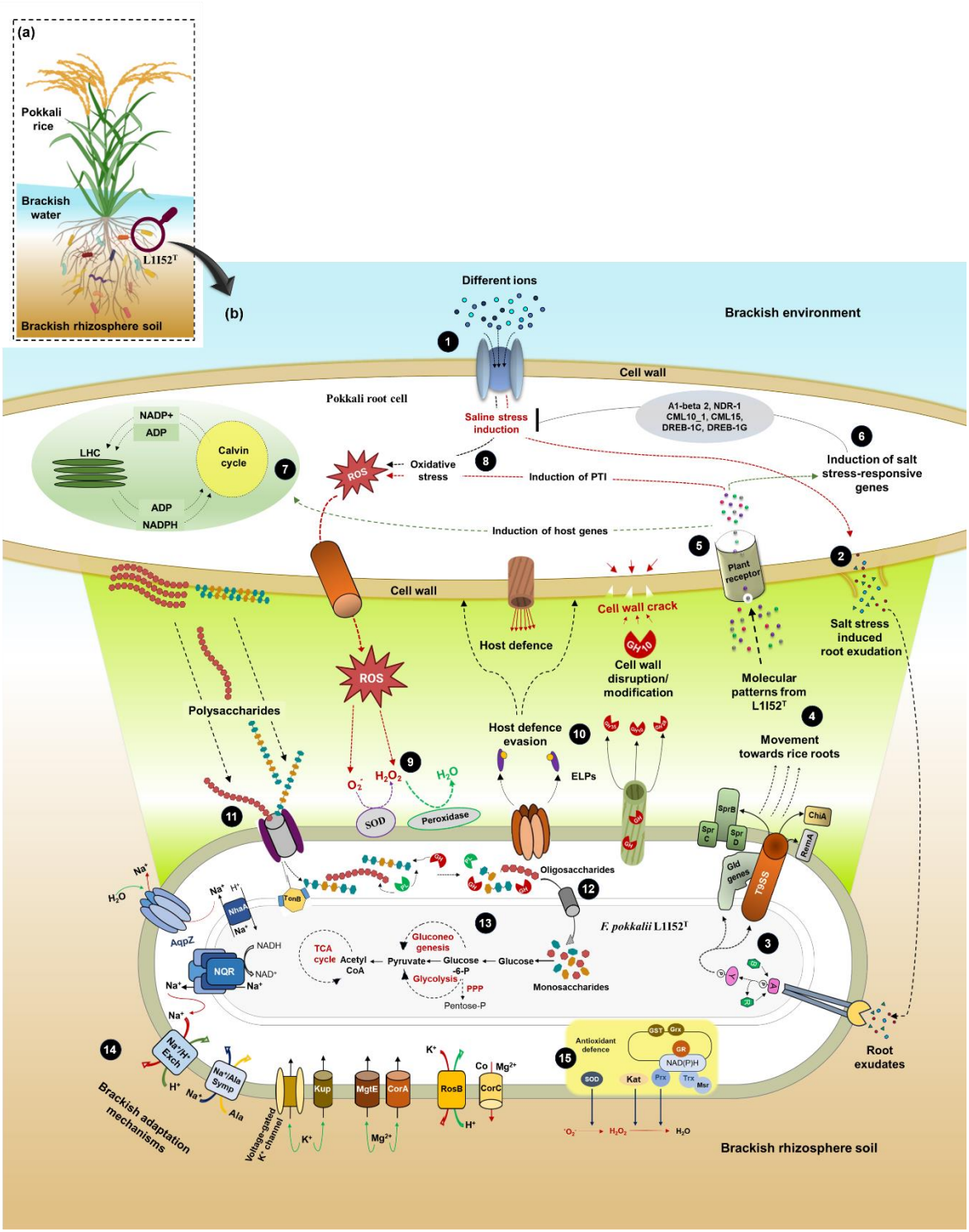


Fig. 5.2. Schematic figure illustrating the molecular interaction between *F. pokkalii* L1I52^T and its host, Pokkali rice under brackish-associated environments: **(a)** Representation of a Pokkali rice plant with its native microbiota and the magnifying lens focused in figure point towards a detailed hypothetical insight into the **(b)** Molecular interaction of *F. pokkalii* L1I52^T with its host, Pokkali rice and surrounding brackish environment conceived based on the eco-physiological, genomic and transcriptomic research findings. The different numbers marked in black-coloured circles from 1 to 15 indicate the possible sequence of molecular events happening during the interaction between *F. pokkalii* L1I52^T with Pokkali rice (highlighted in green colour) and the surrounding brackish environment.

5.2 Future perspectives

The findings from the research have shed potential insights into plant–microbe interaction related to the genus *Flavobacterium*. The *Flavobacterium* abundance in native rice varieties of coastal agro-ecosystems and the influence of brackish conditions on their host root enrichment has been unravelled through 16S rRNA metagenomics. This unique dominance of *Flavobacterium* members might have critical implications to be studied upon understanding the microbial ecology of plants exposed to increasing saline stress, which is the primary cause of global climate change. However, this hypothetical projection requires pervasive research on the saline stress-affected habitats across the globe. Moreover, the findings from this research substantiate the fact that apart from members belonging to genera such as *Azospirillum, Bacillus, Enterobacter, Pseudomonas, Rhizobium,* etc., a large group of rhizobacterial strains with potential phyto-beneficial traits remain unexplored in nature.

The isolation of L1I52^T and its associated studies performed in eco-mimicking conditions firmly validated the importance of an environment in mediating plant-microbe interactions, a rarely visited interface in plant-microbe interaction studies. However, in natural ecosystems, the interactions will happen at the multi-tropic scale and not at the binary scale, as demonstrated in this research. However, studies at the binary scale are necessary to identify potential and novel strains native to unexplored ecosystems, like L1I52^T isolated from Pokkali. Thus, the identified potential strains can be tested for their inter-bacterial compatibility and later subjected to a multi-tropic interaction study with the host. However, this demands a rigorous research attempt to merge high-end culturomics with transcriptomic, proteomic and metabolomic approaches.

The host-gene modulation ability of L1I52^T revealed through RT-qPCR and RNA-Seq approaches hinted at the mutual molecular interaction occurring between L1I52^T strain and its host, Pokkali rice in the presence of brackish environments as detailed in Fig. 5.2. More importantly, the novel genetic determinants identified through L1I52^T random mutagenesis revealed new insights relative to *Flavobacterium*-plant molecular interaction. The findings revealed that the genetic factors critical for *Flavobacterium*-plant interaction are not only confined to the gliding motility complex and T9SS as identified to date, but several other genetic factors contribute to this, as identified in this study. However, function-restoring complementation studies are required to validate these findings. The entire workflow of this research is summarized in Fig. 5.3.

Understanding the microbial ecology of native plants or crops is highly relevant since there is a high demand to increase the scope for sustainable agriculture to countervail the effect of climate change-driven saline stress occurring globally. This has led to an increased soil salinization, affecting the crop yield and has caused a gradual decline in the land area available for farming. Hence, understanding the ecology of the challenged habitat and targeting the cultivation of the metagenome-driven potential rhizobacterial population is assumed to be the preliminary step to resolving this global problem sustainably. As a country with a long coastal stretch, soil salinization severely affects India, making the coastal agro-ecosystems unsuitable for farming. The declining rate of Pokkali rice farming is one of the best examples of this. Hence, it is highly mandated to identify potential rhizobacterial strains native to these ecosystems that can enhance host growth and circumvent saline stress simultaneously. The identified potential strains could be developed as a phyto-beneficial consortium mediating host growth enhancement when applied to the natural ecosystem through enhanced carbon turnover, nutrient supply, or assisting in any plant-beneficial function.

5.3 References

1. Georges, A. A., El-Swais, H., Craig, S. E., Li, W. K. W., and Walsh, D. A. (2014). Metaproteomic analysis of a winter to spring succession in coastal northwest Atlantic Ocean microbial plankton. *ISME Journal*, 8(6), 1301–1313. https://doi.org/10.1038/ismej.2013.234.

2. Herzog, C., Hartmann, M., Frey, B., Stierli, B., Rumpel, C., Buchmann, N., and Brunner, I. (2019). Microbial succession on decomposing root litter in a drought-prone Scots pine forest. *ISME Journal*, 13(9), 2346–2362. https://doi.org/10.1038/s41396-019-0436-6.

3. Hunnicutt, D. W., Kempf, M. J., and McBride, M. J. (2002). Mutations in Flavobacterium johnsoniae gldF and gldG disrupt gliding motility and interfere with membrane localization of GldA. *Journal of Bacteriology*, 184(9), 2370–2378. https://doi.org/10.1128/JB.184.9.2370-2378.2002.

4. Kolton, M., Sela, N., Elad, Y., and Cytryn, E. (2013). Comparative Genomic Analysis Indicates that Niche Adaptation of Terrestrial *Flavobacteria* Is Strongly Linked to Plant Glycan Metabolism. *PLoS ONE*, 8(9). https://doi.org/10.1371/journal.pone.0076704.

5. Larsbrink, J., and McKee, L. S. (2020). Bacteroidetes bacteria in the soil: Glycan acquisition, enzyme secretion, and gliding motility. *Advances in Applied Microbiology* (Vol. 110, pp. 63–98). Academic Press Inc. https://doi.org/10.1016/bs.aambs.2019.11.001.

6. Menon, R. R., Kumari, S., Viver, T., and Rameshkumar, N. (2020). *Flavobacterium pokkalii* sp. nov., a novel plant growth promoting native rhizobacteria isolated from pokkali rice grown in coastal saline affected agricultural regions of southern India, Kerala. *Microbiological Research*, 240. https://doi.org/10.1016/j.micres.2020.126533.

7. Mukhtar, S., Mehnaz, S., and Malik, K. A. (2021). Comparative Study of the Rhizosphere and Root Endosphere Microbiomes of Cholistan Desert Plants. *Frontiers in Microbiology*, 12. https://doi.org/10.3389/fmicb.2021.618742.

8. Olsson, S. and Persson, P. (1999). The composition of bacterial populations in soil fractions differing in their degree of adherence to barley roots. *Applied Soil Ecology*, *12*(3), pp.205-215.

9. Ventosa, A., de la Haba, R. R., Sánchez-Porro, C., and Papke, R. T. (2015). Microbial diversity of hypersaline environments: A metagenomic approach. *Current Opinion in Microbiology* (Vol. 25, pp. 80–87). https://doi.org/10.1016/j.mib.2015.05.002.

10. Zhu, L., Liu, Q., Liu, H., Zhang, J., Dong, X., Zhou, Y., and Xin, Y. (2013). *Flavobacterium noncentrifugens* sp. nov., a psychrotolerant bacterium isolated from glacier meltwater. *International Journal of Systematic and Evolutionary Microbiology*, 63(PART6), 2032–2037. https://doi.org/10.1099/ijs.0.045534-0

Abstract

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Title of the thesis: **A study on native brackish rice-associated *Flavobacterium* spp.: first insights into their ecology, plant functions, eco-physiology, taxonomy, and genomes**

Bacteria engaged in mutualistic or commensal relationships with plants provide plant-beneficial functions such as nutrient supply, stress alleviation, phytopathogen defence and many more. Hence, the importance of sustainable agriculture has generated enormous information on plant-microbe interactions (PMI). However, most of the PMI research has been focusing on the interactions between rhizobacterial strains of Pseudomonadota and Bacillota phyla with terrestrial habitat crops or model plants, which do not always correlate with the PMI occurring in natural ecosystems. In contrast, the PMI in brackish habitats, an interface between terrestrial and marine environments where seawater intrusions are increasing as a cause of global climate change, is poorly studied.

To address the unexplored sides of PMI, the native rice varieties (Pokkali, Kaipad, and Kagga) traditionally cultivated in brackish-associated environments along the coastal belts of South India were chosen for my study. As a start to this, the bacterial communities residing in the rhizosphere and roots of these rice varieties were profiled through a 16S rRNA amplicon sequencing approach. The analysis revealed a high abundance of OTUs belonging to the genus *Flavobacterium* in the roots (average relative abundance of 40%) compared to the rhizosphere (average relative abundance of 20%), indicating a tight association between *Flavobacterium* and its plant host. Further investigation on the 16S rRNA amplicon datasets derived from the soil-microcosm-driven pokkali seedling community revealed a significantly higher abundance of *Flavobacterium* OTUs in tested brackish conditions compared to non-brackish conditions. These metagenome-based findings divulged the critical link between the brackish environment and *Flavobacterium*-rice interaction.

The targeted isolation strategies identified a novel *Flavobacterium* strain designated L1I52^T that enhanced pokkali rice growth. Further, the in-planta studies carried out in tested brackish and non-brackish conditions revealed the ability of L1I52^T to enhance pokkali rice growth. In support of this, RT-qPCR and RNA-Seq-based analysis identified the modulation of important host genes in pokkali rice upon colonization by L1I52^T. This included differentially expressed genes (DEGs) contributing towards the saline stress tolerance and photosynthesis that is assumed to complement the host growth. Furthermore, eco-physiological studies confirmed the influence of tested brackish conditions on growth, survival, and essential host-association traits of L1I52^T, such as biofilm formation, host root attachment, and subsequent colonization. A high-quality genome (3.89 Mb) analysis revealed potential genetic systems coded for host association and eco-adaptation. For host association, L1I52^T majorly comprised the gliding motility complex and T9SS for cell movement and protein secretion, CAZyme organized PULs for plant-derived substrate uptake, and Eukaryotic-like proteins (ELPs) to mediate host-defence evasion and associated interaction. Further, L1I52^T harboured genes coding for Na^+-NQR sodium pump, antiporters, symporters, and exchanger proteins to facilitate the uptake and extrusion of different ions (Na^+, K^+, Mg^{2+}). L1I52^T also encoded genes coding for the synthesis of osmoprotectants. These brackish-adaptive traits are assumed to play an important role in the ion homeostasis essential for L1I52^T survival during fluctuating brackish conditions.

Random mutagenesis attempts with a *mariner*-based transposon, *pHimarEm1* identified key genetic determinants in L1I52^T-pokkali-barckish interaction. The L1I52^T random mutants (FPRM strains) screened for LoF phenotype identified 7 FPRM strains defective in motility and 2 FPRM strains deficient in xylan utilization. Rescue cloning and sequencing identified 6 FPRM strains with novel gene insertions. Among these, 3 FPRM strains harbouring novel transposon insertions in regions coding for tetratricopeptide repeat protein (FPRM-166), amidotransferase family protein (FPRM-628), and vicinity oxygen chelate (VOC) family protein (FPRM-432) exhibited defective root colonization ability compared to the WT strain.

The phylogenetic analysis using 16S rRNA gene and Multilocus sequence analysis performed using 5 housekeeping genes *(gyrB, glyA, atpA, dnaK* and *murG)* placed L1I52^T and its equivalent strains as a novel species under the genus *Flavobacterium*, with *F. daejeonense* GH1-10^T, *F. sufflavum* BBQ-12^T, and *F. glycines* Gm-149^T as the nearest phylogenetic neighbours. Finally, based on the genotypic, phenotypic, chemotaxonomic, and genome-based characterization, the novel *Flavobacterium* species was proposed to be named as *Flavobacterium pokkalii* sp. nov. L1I52^T (= MTCC 12454^T = KCTC42429^T).

In summary, this research provides the first insights into the ecology of the genus *Flavobacterium* in rice crops associated with brackish environments. The inferences from eco-physiology, genome, transcriptome, and genetic manipulation studies emphasized the potential of L1I52^T-pokkali-brackish interaction to be explored as a model system to study the impact of *Flavobacterium* members on host functions and eco-adaptation.

List of Publications

(a) Emanating from the thesis work

1. Menon RR, Kumari S, Viver T, Rameshkumar N. *Flavobacterium pokkalii* sp. nov., a novel plant growth promoting native rhizobacteria isolated from pokkali rice grown in coastal saline affected agricultural regions of southern India, Kerala. Microbiological Research. 2020 Nov 1;240:126533.

2. Menon RR, Kumari S, Rameshkumar N. Metagenomic evidences showing environment-driven enrichment of *Flavobacterium* in rice roots with eco-physiological, genomic and transcriptomic insights of *F. pokkali*– pokkali rice-brackish interaction. (Manuscript to be communicated)

(b) Emanating from other works

1. Kumari S, **Menon RR**, Suresh GG, Krishnan R, Rameshkumar N. Characterization of a novel root-associated diazotrophic rare PGPR taxa, *Aquabacter pokkalii* sp. nov., isolated from pokkali rice: new insights into the plant-associated lifestyle and brackish adaptation. BMC genomics. 2024 Apr 29;25(1):424.

2. Menon RR, Kumari S, Kumar P, Verma A, Krishnamurthi S, Rameshkumar N. *Sphingomonas pokkalii* sp. nov., a novel plant associated rhizobacterium isolated from a saline tolerant pokkali rice and its draft genome analysis. Systematic and applied microbiology. 2019 May 1;42(3):334-42.

List of International workshops attended

1. Menon RR. Elected participant in European Molecular Biology Organization (EMBO) practical course in "Metabolite and Species Dynamics in Microbial Communities". Jointly organized by European Molecular Biology Laboratory, EMBL Advancer Training Centre, Heidelberg, Germany, and Bangalore Life Science Cluster (BLiSC) Campus, Bangalore, India. 16 – 21 October 2022. (One among the selected 10 candidates from India)

List of International conferences attended

1. Menon RR, Kumari S, N. Rameshkumar. First cultured representative of a novel *Verrucomicrobia* from brackish-associated pokkali rice, India: An insight into their Ecology, Phylogeny, Host interactions, Brackish adaptation, and Genome. Microverse I online symposium, Jena, Germany. 1 – 3 September 2020. (Digital poster presentation).

2. Menon RR, Kumari S, Prabhu Ram K, N. Rameshkumar. Molecular characterization and genome analysis of novel *Ciceribacter* strain isolated from brackish rice crop, Pokkali. International Symposium on Microbial Technologies, Sustainable Development of Energy, Environment, Agriculture and Health, Mahendergarh, Haryana, India. 15 – 18 November 2019. (Poster presentation).

3. Gayathri GS, Kumari S, **Menon RR**, N. Rameshkumar. A potent *Burkholderia* with broad spectrum anti-microbial activity from land race rice variety Aiswarya of saline-affected area and its genome analysis. International Symposium on Microbial Technologies, Sustainable Development of Energy, Environment, Agriculture and Health, Mahendergarh, Haryana, India. 15 – 18 November 2019. (Poster presentation).

4. Kumari. S, **Menon RR**, N. Rameshkumar Deciphering the microbiomes of brackish grown wild rice varieties – Pokkali and Kagga from Southern India. International Symposium on Microbial Technologies, Sustainable Development of Energy, Environment, Agriculture and Health, Mahendergarh, Haryana, India. 15 – 18 November 2019. (Poster presentation).

5. Menon RR, Tomeu Viver, N. Rameshkumar. Native novel *Flavobacteirum* from brackish pokkali rice: An insight into their genome, phytobeneficial properties, and ecospecific adaptations. International Conference on Microbiome Research, Pune, Maharashtra, India. 19 – 22 November 2018. (Poster presentation).

1. Menon RR, Kumari S, N. N. Rameshkumar, "First cultured representative of a novel *Verrucomicrobia* from brackish-associated pokkali rice, India: An insight into their Ecology, Phylogeny, Host interactions, Brackish adaptation, and Genome" at Microverse I Symposium on Microbial and Biomolecular Interactions, Jena, Germany during 1 – 3 September 2020 (Digital poster presentation).

Abstract

Pokkali, a GI (Geographical indication) tagged rice crop cultivated organically along the coastal belts of Kerala, India is well known for its saline tolerance and these fields are highly prone to seawater intrusions. This farming practice dating back to centuries is assumed to host several indigenous microbes that are believed to play a potential role in plant's health and productivity under brackish conditions. In connection, a metagenomic analysis was performed on this pokkali rice to decipher its unexplored root and rhizosphere associated microbes. Among the various phyla detected in this analysis, the bacterial phylum *Verrucomicrobia* enriched higher in roots than the rhizosphere indicating their host association. Literature survey on *Verrucomicrobia*-plant interactions under brackish conditions are very much limited. Also, limited cultured representatives of *Verrucomicrobia* strains restrict the possibilities to study their eco-physiological importance and host interactions. Using a novel in-planta enrichment method we successfully isolated a Verrucomicrobial strain (NRK V7) from pokkali roots and 16S rRNA analysis indicated it to be a novel genus in the family *Opitutaceae.* In-vitro binary association studies showed enhanced colonization of NRK V7 cells in pokkali roots under brackish conditions. Strict preference for natural seawater over pure NaCl for growth and host colonization hints its adaptation in brackish conditions. Further, a complete genome analysis of NRK V7 revealed genetic traits important for host interaction such as the nifHDK operon, genes coding for plant polysaccharide utilization and transport, and secretion system genes enabling to interact with its surrounding environment and host plant. It also contains chemotaxis protein-coding genes which are placed within the flagellar motility gene cluster that hints the ability of cells to sense root exudates and thereby colonize the host. In conclusion, NRK V7 is the first cultured representative aerobic *Verrucomicrobia* strain from Indian origin with a diazotrophic lifestyle; also, our study provides more information on how *Verrucomicrobia* interacts with plants under brackish conditions.

Keywords: Brackish, pokkali, *Verrucomicrobia*, phylogeny, in-planta, NRK V7, genome.

2. Menon RR, Kumari S, Prabhu Ram K, N. Rameshkumar, "Molecular phylogeny, host-association and genome characterization of a novel marine adapted plant-beneficial *Ciceribacter* from brackish grown wild pokkali rice" at International Symposium on Microbial Technologies, Sustainable Development of Energy, Environment, Agriculture and Health, Mahendergarh, Haryana, India during 15 – 18 November 2019 (Poster presentation)

Abstract

Several rice varieties tolerant to brackish water stress are cultivated along the coastal agro-ecosystems of India. Pokkali, a GI tagged rice crop of Kerala is known for its brackish and submerged tolerance posed due to frequent sea water intrusions. In account of its unique geographical location, the microbiome of pokkali is assumed to be very unique that may address the challenges of sustainable agriculture in saline-stressed farm lands to some extent. Among the several potential isolates screened, L1K22 and L1K23^T showed very good growth in media containing 100% natural sea water. Moreover, hydroponic based binary association studies performed under gnotobiotic conditions confirmed that these strains require at least 20% NSW to remain viable as planktonic cells followed by its subsequent colonization with the host plant, pokkali compared to non-saline conditions. 16S rRNA gene based phylogenetic analysis confirmed the strains to be a member of *Ciceribacter* genus with only 3 species described till date. Further, Multi-locus sequence analysis comparison between L1K23^T and closest reference strain, *Ciceribacter lividus* MSSRFBL1^T comprising six housekeeping genes (atpD, glnAII, glnA, recA, rpoB, thrC) and genomic DNA fingerprinting confirmed the strain to be a probable novel species under the genus. The 4.80 Mbp draft genome of L1K23^T harboured several plant-beneficial and brackish adaptive genes. More importantly, L1K23^T genome coded for flagellar motility, chemotaxis (CheA, CheD, CheY, CheB, CheW, CheR) and tight adherence (tad) operon genes assumed to play a major role in their host colonization. In addition to type I secretion system, L1K23^T genome coded for type VI secretion system aiding for its competitive colonization in the rhizosphere. Additionally, genes for ectoine degradation and transport and glycine betaine uptake signifies the brackish adaptive traits of L1K23^T strains. Further studies are ongoing to understand the impact of salinity in its host association and influence.

Keywords: Brackish; Pokkali; Natural Sea water, *Ciceribacter*, Genome

3. Suresh GG, Kumari S, **Menon RR**, N. Rameshkumar. "A potent Burkholderia with broad spectrum antimicrobial activity from landrace rice variety Aiswarya of saline affected area and its genome analysis" at International Symposium on Microbial Technologies, Sustainable Development of Energy, Environment, Agriculture and Health, Mahendergarh, Haryana, India during 15 – 18 November 2019 (Poster presentation)

Abstract

Plant-microbe interaction plays a major role for the adaptability and productivity of plants under varying environmental conditions. The study on inland plants that grow under saline affected areas largely remains unexplored. Hence, the plant probiotic studies of these ecosystems will definitely offer an opportunity to obtain novel biologically active metabolites and biostimulant that can be of agricultural benefit. During the screening for microbial cultures producing antimicrobial agents from the rhizosphere of this saline affected inland rice, we have isolated a potential culture named as Bmkn7 that showed broad spectrum antifungal and antibacterial activity against wide range of phytopathogens (*Macrophomina phaseolina, Alternaria alternata, Fusarium oxysporum, Escherichia coli, Staphylococcus aureus* etc.). The 16srRNA gene base phylogeny and draft genome analysis confirmed the strain to be a member of *Burkholderia* genus within cenocepacia complex. The characteristic feature of this Bmkn7 is its antagonistic effect in agar but not in broth. Interestingly, Bmkn7 also elicited antimicrobial activity in presence of 20% natural seawater. Majority of the *Burkholderia* species reported till date has siderophore mediated antimicrobial activity. As expected, siderophore production was confirmed in Bmkn7 by CAS assay. However, the siderophore inhibitory assay done by amending 100µM iron to the CAS assay also showed antimicrobial effect against phytopathogens confirming that the antagonistic action is non-siderophore mediated. This led us to sequence the draft genome of Bmkn7 where we could identify certain unknown clusters that might encode for novel compounds analysed through antiSMASH. Finally, in-planta studies with Bmkn7 confirmed that the strain doesn't have any negative effect on rice seed germination and growth. Altogether, the above findings summarise the possibility of a novel antimicrobial compound from a rice associated *Burkholderia*, for which further analytical based characterisation studies are underway.

Keywords: Inland rice; *Burkholderia*; Antimicrobial; Seawater; Novel compound

4. Kumari S, **Menon RR**, N. Rameshkumar, "Deciphering the microbiomes of brackish grown wild rice varieties – Pokkali and Kagga from southern India" at International Symposium on Microbial Technologies, Sustainable Development of Energy, Environment, Agriculture and Health, Mahendergarh, Haryana, India during 15 – 18 November 2019 (Poster presentation)

Abstract

Our current understanding about the plant associated rhizobacteria and their beneficial functions were largely based on studies from terrestrial crop plants. However, little is known from crop plants growing naturally in coastal saline stress soils as in this case wild local saline tolerant rice varieties of southern India remains largely elusive. In this context, Illumina MiSeq platform based 16S rRNA gene-based amplicon sequencing targeting the V3-V4 region was performed. The processing of the raw data and downstream analysis were carried out using NGSqc toolkit (Quality check) and QIIME pipeline. The 16S rRNA amplicon sequencing generated 68,90,497 number of high-quality reads that were merged into 28,61,801 number of contigs. The alpha rarefaction curve suggests that sufficient sequencing depth was attained to capture the actual microbial diversity within these samples. Further, alpha diversity and shannon index value is higher for the rhizosphere whereas the evenness is higher for the root samples. The principal coordinate analysis (PCoA) plot representing beta diversity of the samples clustered together for replicates suggesting that there is no much variation between the replicate samples. Initial metagenome analysis revealed that Proteobacteria, Bacteroidetes and Planctomycetes are the most dominant phylum in both pokkali and kagga rice varieties. In addition, the taxa's enriched in the root of Kagga include Firmicutes and Actinobacteria whereas Nitrospirae, Chloroflexi and Acidobacteria were found to be depleted in the root compared to rhizosphere. Similarly, in pokkali sample, Verrucomicrobia and Actinobacteria were enriched taxa's whereas Chlorobi and Nitrospirae remain depleted in root. As a starting point, studying the diversity of plant microbiota of this ecosystem represents a key step towards improving our basic understanding about the ecology of plant associated microbes in brackish environments. Furthermore, capitalizing these brackish adapted microbes as bio-inoculants may lead for improvised sustainable agriculture applications in coastal saline affected regions.

Keywords: Pokkali; Kagga; Metagenome; QIIME; microbiome

5. Menon RR, Viver T, N. Rameshkumar, "Native novel *Flavobacterium* from brackish pokkali rice: An insight into their genome, phytobeneficial properties and ecospecific adaptations" at International Conference on Microbiome Research, Pune, Maharashtra, India during 19 – 22 November 2018 (Poster presentation)

Abstract

Little is known about the genus *Flavobacterium* and its plant beneficial interactions despite several studies reveal that they occur in various plant rhizospheres. Here, we report on the occurrence and genetic diversity of several *Flavobacterium* species from pokkali rice grown in brackish environments. In-depth functional characterisation and genome analysis was performed with a *Flavobacterium* strain L1I52^T. On the basis of polyphasic taxonomy (phenotypic, genotypic and chemotaxonomic) and genome data, we propose to classify the strain L1I52^T as *Flavobacterium pokkalii* sp.nov. *In vitro* and *in vivo* plant growth studies proves the ability of L1I52^T to stimulate plant growth in pokkali rice. Notably, L1I52^T can survive, grow and attach to pokkali rice roots in presence of varying seawater conditions, a key adaptation trait required to associate and survive with brackish pokkali rice. The draft genome analysis of L1I52^T (3.84Mbp) revealed features with higher adaptation to plant carbohydrate metabolism. The genome harbors a greater diversity of 293 carbohydrate-active enzymes (CAZymes) which includes 163 glycoside hydrolases and 21 polysaccharide lyases. Moreover, L1I52^T genome have the highest proportion of CAZymes (9%) among so far reported rhizosphere associated *Flavobacterium* genomes. Among sixteen polysaccharide utilization loci (PULs) predicted, three putative PULs were identified to utilize plant polysaccharides such as xylan, pectin, and starch. These genomic inferences supported the phenotypic growth experiment observations. In conclusion, this study provides the first information on an interesting native phytobeneficial *Flavobacterium* for which further experiments are ongoing to understand its plant-associated lifestyle under brackish environmental conditions.

Keywords: Rhizosphere, Brackish, Pokkali, *Flavobacterium*.

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Flavobacterium pokkalii sp. nov., a novel plant growth promoting native rhizobacteria isolated from pokkali rice grown in coastal saline affected agricultural regions of southern India, Kerala

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ABSTRACT

Nine plant-associated bacterial strains designated as L1I52^T, NRK F1, NRK F15, NRK F16, NRK F41, NRK F42, NRK F47, NRK F49, and NRK F50 originating from the roots and rhizosphere region of a coastal saline tolerant pokkali rice were taxonomically characterized in this study. Genomic fingerprinting using Enterobacterial Repetitive Intergenic Consensus (ERIC) primers discriminated the nine strains based on the DNA fingerprint patterns indicating that they were not clonal in origin. Phylogenetic analysis using 16S rRNA and other five housekeeping genes (*gyrB, glyA, atpA, dnaK* and *murG*) revealed that the novel strains constituted a single novel species within the genus *Flavobacterium*. In all tree construction methods, the novel strains formed a distinct phylogenetic branch, with *Flavobacterium daejeonense* GH1-10^T, *F. sufflavum* BBQ-12^T, and *F. glycines* Gm-149^T as their nearest phylogenetic neighbours. However, average nucleotide identity (ANI), average amino acid identity (AAI) and digital DNA-DNA hybridization (dDDH) comparison between the draft genomes of L1I52^T (representative isolate) and it's nearest phylogenetic neighbours were well below the proposed threshold values (< 95 % and < 70 %) used for species discrimination. Thus, based on the phenotypic, genotypic and chemotaxonomic data obtained in this study, we describe a novel *Flavobacterium* species for which we propose the name *Flavobacterium pokkalii* sp.nov., with strain L1I52^T (=MTCC 12454^T=KCTC 42429^T) as the type strain. In addition, L1I52^T is a potential plant growth promoting rhizobacteria as they can promote pokkali rice growth and we identified several plant associated gene features in the genome of L1I52^T that are potentially involved in plant microbe interactions.

1. Introduction

Plant roots and its immediate surrounding soil referred as rhizosphere were colonized by a diverse group of rhizobacteria which are now considered as an important tool to increase crop productivity in an eco-friendly manner. Many of these plant associated rhizobacteria can establish active mutualistic relationships with their host plants and provide specific advantages to the hosts in many ways. This includes better nutrient acquisition, promotes plant growth through production of phytohormones, enhanced tolerance to abiotic stresses and protection against various soil-borne pathogens (Lugtenberg and Kamilova, 2009; Berendsen et al., 2012). Although, the importance of plant associated rhizobacteria was getting more appreciation recently. Our current understanding about these plant associated rhizobacteria and their beneficial functions were largely based on studies from cultivated and model crop plants grown from terrestrial ecosystems. However, little is known from native crop plants cultivated in coastal saline affected agricultural soils, as in this case, pokkali rice, remains largely unknown.

Pokkali is a unique geographical indexed rice variety traditionally grown in water-logged coastal saline affected agro-ecosystems of Kerala, southern India. These rice varieties are well-known for its remarkable tolerance to coastal salinity and were popularly used as a gene donor in many plant breeding programs to develop improved rice cultivars which are tolerant to salinity (Gregorio et al., 2002; Kurotani et al., 2015). Apart from its genetic and physiological abilities to resist salinity, it is speculated that microbes associated in the rhizosphere region of this rice plant might play a major role in the alleviation of salinity stress as well as its health. Thus, in an effort to get more insight

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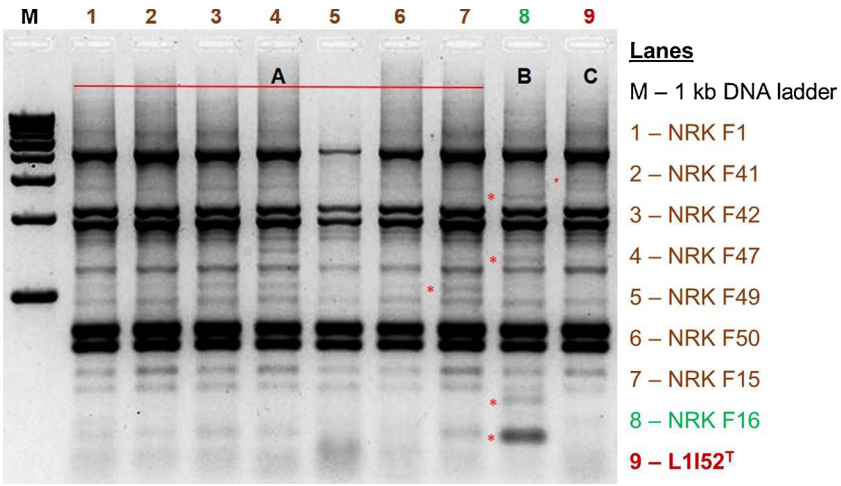

Fig. 1. Genomic fingerprint of the nine *Flavobacterium* strains using ERIC primers. Red asterisks indicate the regions were the differences in the banding patterns among the strains were observed.

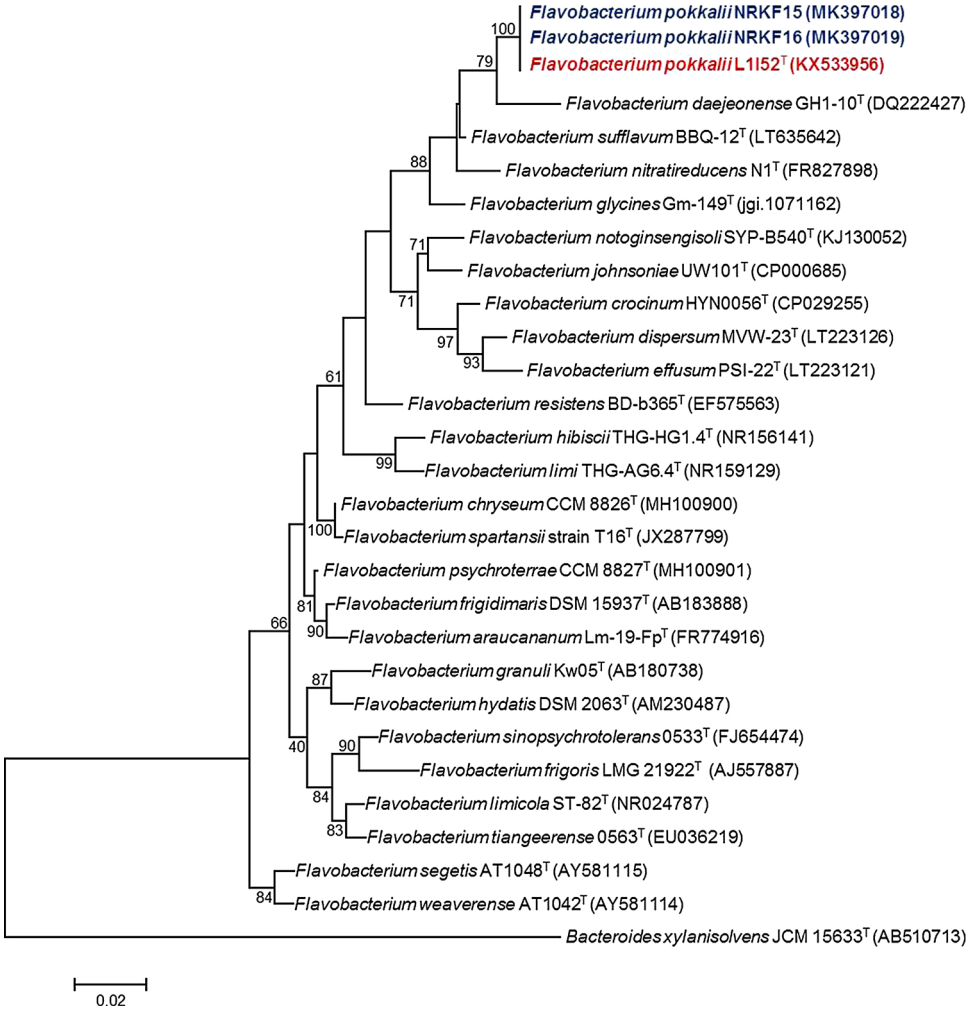

Fig. 2. Maximum likelihood tree constructed using 16S rRNA nucleotide sequences showing the phylogenetic position of the three novel *Flavobacterium* strains (L1I52^T, NRK F15, and NRK F16) within the *Flavobacterium* genus. The percentages shown at each branch point represent bootstrap values derived from 1000 replications and values more than 60 % are indicated. Bar, 0.02 represents positions per nucleotide position. *Bacteroides xylanisolvens* JCM 15633^T was used as an outgroup. Dark closed circles at each node indicate similar grouping observed in other tree constructing methods (neighbour joining and minimum evolution).

into the rhizosphere associated plant beneficial bacteria of this unique saline tolerant rice variety, we isolated and characterised many native isolates which showed potential plant growth beneficial properties, and some of them were taxonomically validated as novel species (Rameshkumar et al., 2016; Krishnan et al., 2016, 2017; 2018; Menon et al., 2019).

Table 1
Multilocus sequence analysis of strain L1I52^T and the type strains of related *Flavobacterium* species based on the individual and concatenated sequences (4598 bp) of five housekeeping genes: gyrB (1139 bp), glyA (922 bp), atpA (822 bp), dnaK (946 bp), murG (776 bp).

Phylogenetic neighbours of L1I52^T	Pairwise nucleotide sequence similarity (%) with L1I52^T					
	gyrB	*glyA*	*atpA*	*dnaK*	*murG*	Concatenated
F. daejeonense KACC 11422^T	92.36	92.52	94.87	95.54	93.14	93.74
F. sufflavum BBQ-12^T	85.05	92.91	91.74	91.97	87.19	92.91
F. glycines ICMP 17618^T	91.04	93.60	92.67	93.10	86.93	91.75
F. nitriatireducens N1^T	87.97	90.13	94.87	93.52	83.18	90.08

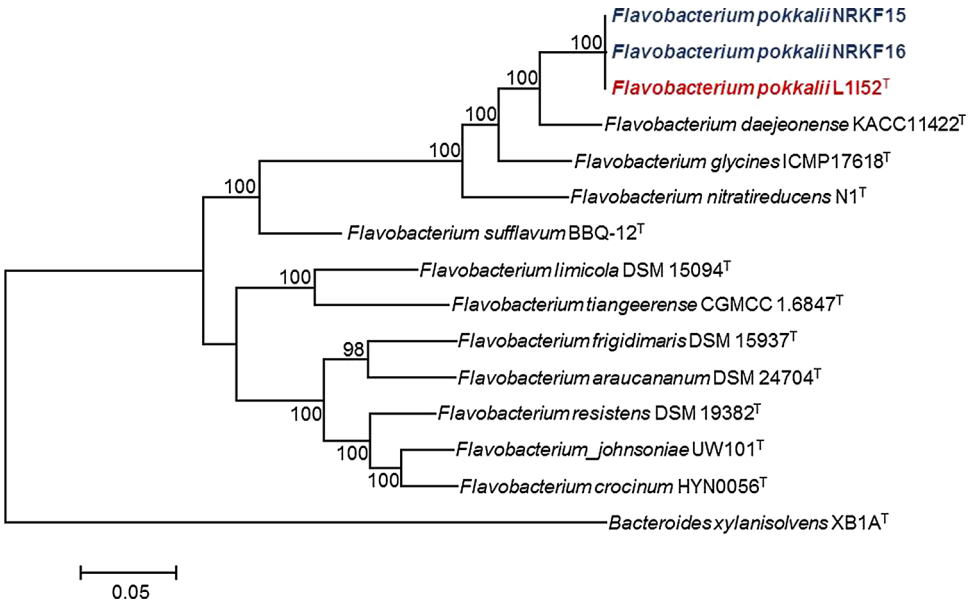

Fig. 3. Maximum likelihood tree constructed using the concatenated sequences (4598 bp) of five housekeeping genes: gyrB (1139 bp), glyA (922 bp), atpA (822 bp), dnaK (946 bp), murG (776 bp) showing the phylogenetic position of the three novel *Flavobacterium* strains (L1I52^T, NRK F15, and NRK F16) within the *Flavobacterium* genus. The percentages shown at each branching point represent bootstrap values derived from 1000 replications and values more than 60 % are indicated. Bar, 0.05 represents positions per nucleotide position. *Bacteroides xylanisolvens* JCM 15633^T was used as an outgroup. Dark closed circles at each node indicate similar grouping observed in other tree constructing methods (neighbour joining and minimum evolution).

Table 2
Predicated average nucleotide identity (ANIb), average amino acid identity (AAI) and digital DNA-DNA hybridisation (dDDH) values between L1I52^T and its closely related type strains of *Flavobacterium* species.

Strains	ANIb (%)	AAI (%)	dDDH (%)
F. daejeonense DSM17708^T	92.7	94.0	52.6
F. glycines Gm-149^T	81.8	85.2	25.9
F. sufflavum BBQ-12^T	80.2	83.1	24.0

ANIb – Average nucleotide identity, calculation based on BLAST+, http://jspecies.ribohost.com/jspeciesws/.
AAI – Amino acid identity, http://enve-omics.ce.gatech.edu/aai/.
Tetra z-score – statistical analysis of tetra-nucleotide usage patterns, http://jspecies.ribohost.com/jspeciesws/.
dDDH – In-silico DNA-DNA hybridization score, http://ggdc.dsmz.de/ggdc.php.

In the present study, we choose to characterise yet another nine rhizobacterial strains designated as L1I52^T isolated from pokkali rhizosphere, 2014 and NRK F1, NRK F15, NRK F16, NRK F41, NRK F42, NRK F47, NRK F49, and NRK F50 isolated from pokkali roots, 2017 of the genus *Flavobacterium*. Because a) these novel rhizobacterial strains could not be assigned to any particular *Flavobacterium* species based upon initial 16S rRNA gene sequence analysis, b) among the nine novel *Flavobacterium* strains, L1I52^T was able to promote pokkali rice growth, and c) only very few reports exits on the occurrence, taxonomy, and plant beneficial properties of *Flavobacterium* from brackish associated crop plants. Based on the genomic characterisation including whole genome sequences, phylogenetic and phenotypic data obtained from this study, we propose to classify strain L1I52^T and its equivalent strains as a novel *Flavobacterium* species, for which the name *Flavobacterium pokkalii* sp. nov., is proposed. In addition, L1I52^T can promote pokkali rice growth and its genome analysis showed that it possesses several gene features which are previously reported to be functional during mutualistic plant interactions.

2. Materials and methods

The bacteriological media used for isolating the nine novel strains and their respective isolation sources were mentioned in Supplementary table S1. The procedure referred in (Krishnan et al., 2018) was followed to isolate the novel strains. The strains were routinely sub-cultured in R2A agar medium (Himedia, India) supplemented with 0.5 % NaCl (R2AN) or 30 % natural sea water (R2A3) at 30 °C for 4 days. For long term preservation, bacterial cells were stored frozen at −80 °C in 20 % glycerol stocks as cell suspensions. 16S rRNA phylogenetic relatives; *Flavobacterium daejeonense* KACC 11422^T, *F. sufflavum* KCTC 52809^T and *F. glycines* ICMP 17618^T were used as reference type strains for comparative taxonomic studies.

QIAamp DNA mini kit (Qiagen) was used to isolate the genomic DNA from an overnight grown bacterial culture. 16S rRNA gene amplification, sequencing and phylogenetic analysis was performed as described before (Krishnan et al., 2016). Multilocus sequence analysis (MLSA) was performed by using five housekeeping genes namely, *gyrB, glyA, atpA, dnaK,* and *murG* as described earlier (Nicolas et al., 2008). The primer sequences of the respective housekeeping gene and their PCR amplification conditions are provided in Supplementary table S2. The amplified PCR products were purified using Qiagen gel extraction kit according to manufacturer's instructions. The purified PCR products were sequenced directly using the same primer pairs used for gene amplification. Applied Biosystems 3500 DNA sequencer was used for sequencing, following the manufacture's protocol. For the MLSA based

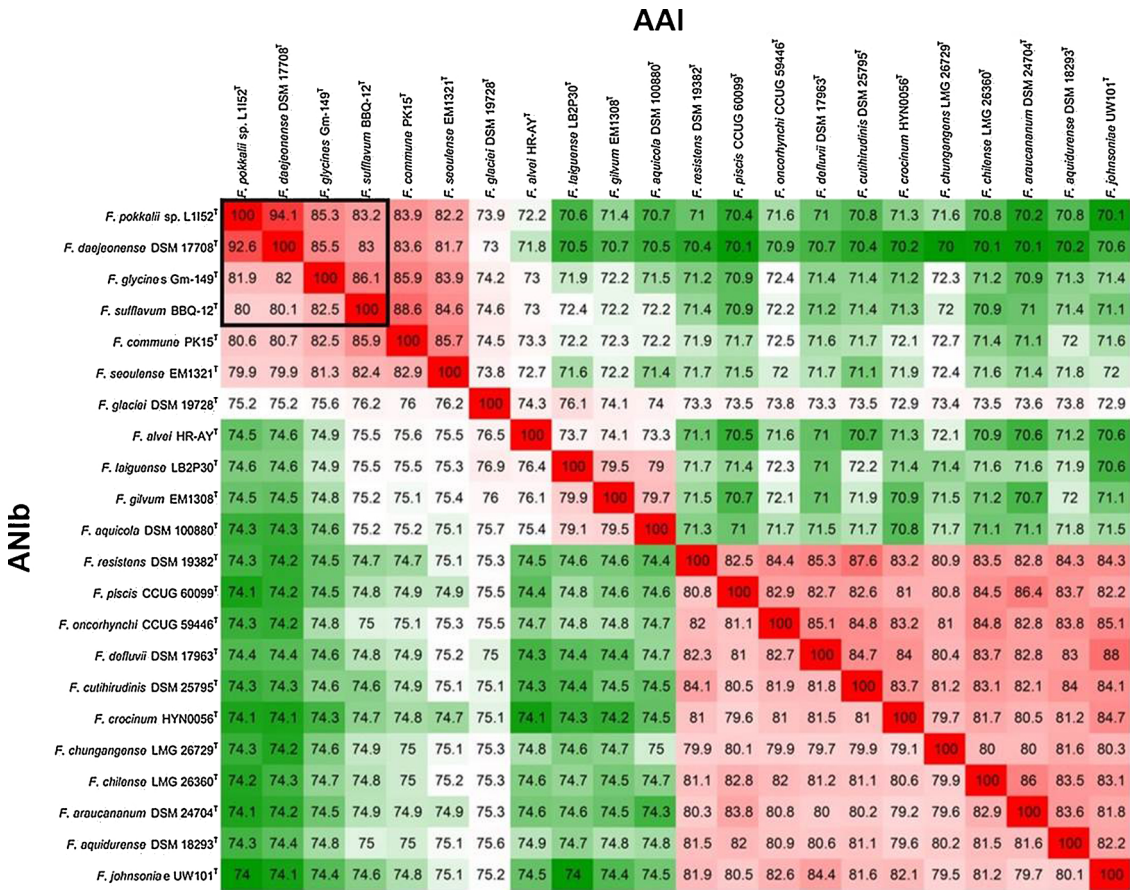

Fig. 4. Heatmap displaying the ANI and AAI matrix of L1I52^T and its closely related strains within *Flavobacterium* genus. Heat map shows the pairwise comparisons of ANI and AAI between each pair of genomes and the numbers in each cell represent ANI and AAI values of respective genome pairs. The black box represents the comparative ANI and AAI values of L1I52^T and it's nearest phylogenetic neighbours.

phylogeny, a concatenated tree comprising the above five housekeeping gene sequences was constructed by following the methods as described (Rameshkumar et al., 2010). MEGA6.0 package (Tamura et al., 2013) was used for the phylogenetic analysis. All the gene sequences were submitted to NCBI with the following accessions, 16S rRNA gene: KX533956, MK397017 to MK397024; MLSA genes: KX257376 to KX257395, MK442501 to MK442510.

A PCR based genomic DNA fingerprinting using ERIC1R and ERIC2 primers (Krishnan et al., 2016) was carried out to check the intra-species diversity among the nine novel strains.

Strains L1I52^T, NRK F15 and NRK F16 were routinely cultivated on R2AN (R2A + 0.5 % NaCl) or R2A3 (R2A + 30 % NSW) agar at 30 °C for all physiological and biochemical tests performed. The cell shape was determined as described previously (Rameshkumar et al., 2016). Motility was checked by pricking the L1I52^T cells onto the centre of R2AN or R2A3 (0.18 %) soft agar plate and checked for the cell migration as indicated by radial concentric circles after a period of 7 days. Presence of flexirubin-type pigments were checked by flooding the plates with 20 % KOH solution. The Gram reaction, catalase and oxidase tests were tested using protocols described previously (Rameshkumar et al., 2014). Growth in different bacteriological media was tested on Luria-Bertani agar, nutrient agar, trypticase soya agar and ZoBell marine agar. Growth at different temperature and pH was tested as described previously (Krishnan et al., 2016), except R2AN agar medium was used for the analysis. The tolerance to various NaCl concentrations (0.5, 1, 1.5, 2, 2.5, 3% w/v) was tested in R2A broth. The growth in anaerobic conditions was tested as described previously (Rameshkumar et al., 2016). Citrate utilization, indole production, nitrate reduction, methyl red and Voges Proskauer (MR-VP) tests were carried out as mentioned earlier Smibert (1994). Hydrolysis of complex polymers such as starch, pectin, xylan, carboxymethyl cellulose and chitin was checked on nutrient agar supplemented with 0.5 % of each polymer and incubated for 5 days at 30 °C. Hydrolysis of esculin, urea, tributyrin, DNA, arginine dihydrolase, lysine decarboxylase and utilization of different sugars; L-arabinose, cellobiose, D-glucose, lactose, D-maltose, D-mannitol, D-raffinose, L-rhamnose, sucrose, D-trehalose, and D-xylose, organic acids; citric acid, malic acid, and succinic acid and solvents; ethanol and methanol as sole carbon source and different amino acids; L-alanine, L-methionine, L-phenylalanine, L-proline, putrescine, L-threonine, L-tryptophan, L-serine and L-valine as sole nitrogen source were performed by following the methods of Krishnan et al (2016). Antibiotic sensitivity tests for the following antibiotics (Hi-Media; µg/disc): amoxyclav (30), ampicillin (10), carbenicillin (100), cefozolin (30), chloramphenicol (30), cinoxacin (100), ciprofloxacin (5),clindamycin (2), co-trimaxozole (25), doxycycline hydrochloride (30), erythromycin (15), gentamycin (10), kanamycin (30), linezolid (30), methicillin (5), nalidixic acid (30), netilin (30), ofloxacin (5), oxacillin (1) and penicillin G (10 units), polymixin B (300 units), rifampicin (5), streptomycin (10), tetracycline (30), vancomycin (30) were carried out as described previously (Rameshkumar et al., 2016), except R2AN was used as the basal medium. Acid production from various carbohydrates was checked using the HiMedia HiCarbohydrate kit, according to the manufacturer's instructions, except that the kits were inoculated with culture suspensions made in 0.85 % NaCl (w/v), and incubated at 30 °C. The results were noted after 48 h of incubation.

For cellular fatty acid analysis, cells were grown in R2A agar for 72 h at 30 °C except *F. sufflavum* KCTC 52809^T was grown at 28 °C because of its inability to grow at 30 °C. Cells were harvested at similar

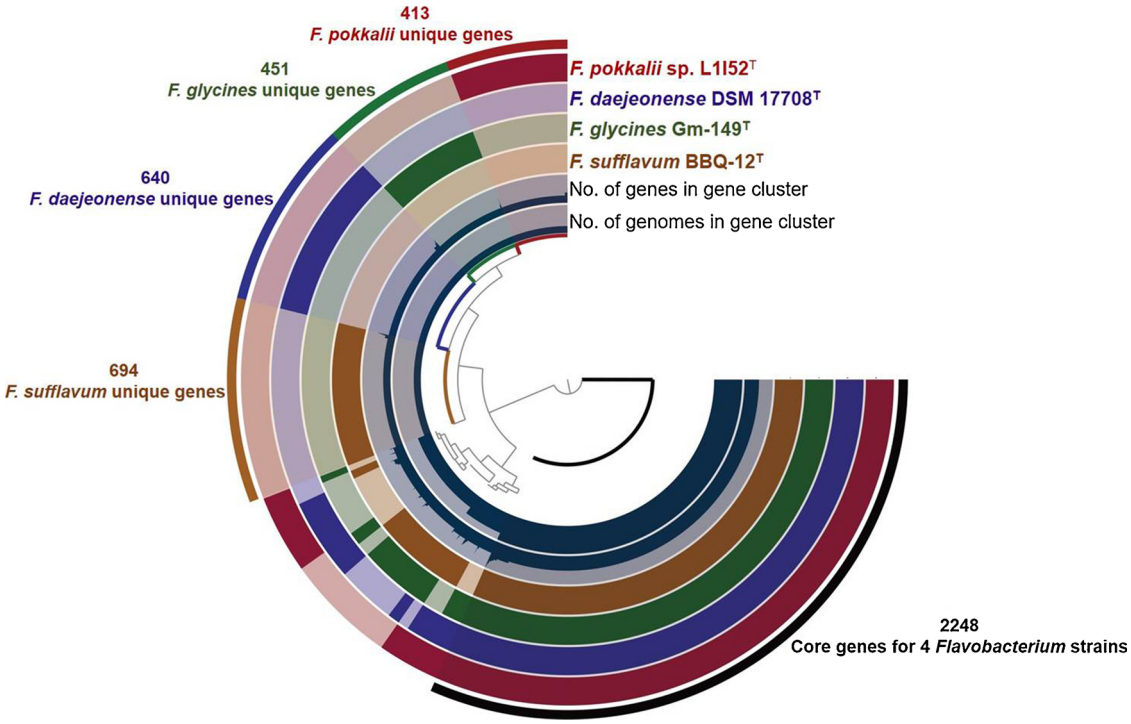

Fig. 5. Pangenome map generated using four genomes of *Flavobacterium* type strains - *F. pokkalii* sp. L1I52^T, *F. daejeonense* DSM 17708^T, *F. glycines* Gm-149^T and *F. sufflavum* BBQ-12^T. Dendrogram in the inner region represents the relationship between the 5339 number of gene clusters consists of 13,781 genes found in all four genomes analyzed. The genome map represents the core genes common in all strains and unique genes present in each strain. Dark colored regions in circles represent unique genes present in each genome and light colored regions represent the genes that are commonly shared among the genomes.

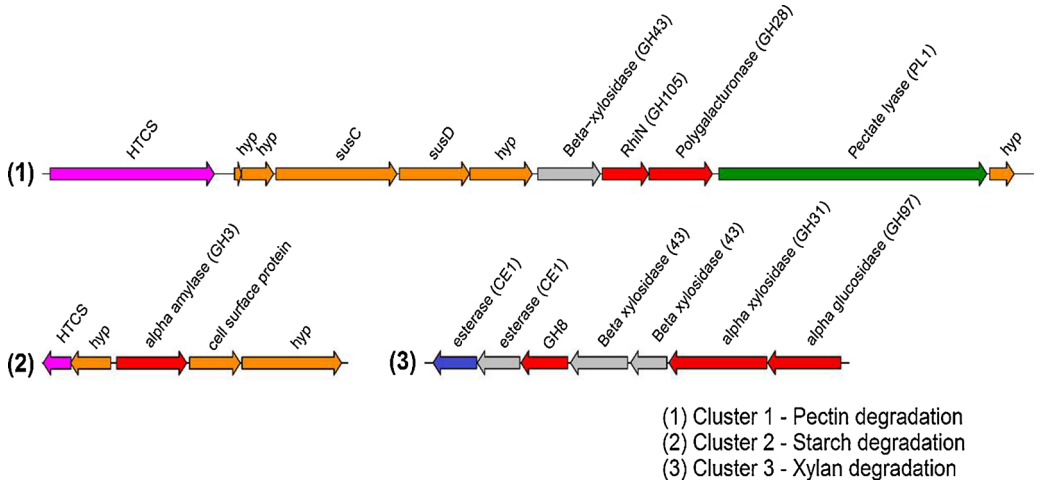

Fig. 6. Unique gene clusters predicated in L1I52^T genome but absent in *F. daejeonense* DSM 17708^T genome.

physiological age and cellular fatty acid methyl esters (FAMEs) were prepared and identified by methods as previously described (Sasser (1990); Krishnan et al., 2016). For polar lipid analysis, L1I52^T cells were harvested at logarithmic phase and the pellet was used for polar lipids extraction with methanol/chloroform/0.3 % sodium chloride (2:1:0.8, by vol.) as described by Bligh and Dyer (1959) considering the modifications of Card (1973). Lipids were separated using silica gel TLC (Kieselgel 60 F254; Merck) by two-dimensional chromatography using chloroform-methanol-water (65:25:4 by vol.) in the first dimension and chloroform-acetic acid-methanol-water (40:7.5:6:2, by vol.) in the second dimension Minnikin et al (1984). The dried plates were subjected for spraying with 5 % ethanolic phosphomolybdic acid for total lipids and further characterized by spraying with ninhydrin (specific for amino groups), molybdenum blue (specific for phosphates), Dragendorff (quaternary nitrogen) or α-naphthol (specific for sugars).The respiratory quinones were extracted with chloroform/methanol (2:1, v/v) and analysed using HPLC as described by Komagata and Suzuki (1988).

High quality genomic DNA of L1I52^T strain was isolated using QIAamp DNA mini kit (Qiagen) according to manufacturer's instruction and sequenced on Illumina HiSeq 4000 platform using NEXTFlex DNA sequencing library kit as recommended by the manufacturer. Raw reads were trimmed using the software Solexa QA (Cox et al., 2010) followed by removing reads with quality score lower than 20 and length shorter than 50bps. The trimmed reads were assembled with IDBA assemble v1.1.1 (Peng et al., 2012) using the "pre_correction" option. Gene prediction and annotation was performed using

Table 3
Functional categorization of genes/gene clusters predicted in L1I52^T genome as identified through RAST analysis that relates to a plant associated lifestyle.

Functional category	Components	Associated gene no.	Function in strain L1I52^T
I. Carbodydrate metabolism			
1. Tricarboxylic acid cycle	Catabolic genes	16	Carbon metabolism
2. Glycolysis and 3. Gluconeogenesis	Catabolic genes	14	
II. Polysaccharide utilization	Catabolic genes	411	Sensing, uptake and utilization of different polysaccharides
1. Polysaccharide utilization loci's			
III. Transport systems			
3.Transporters	MFS genes	28	Transporters involved in plant material uptake
	RND genes	37	
	ABC genes	64	
4. Phosphate mobilization	Transporters	6	Phosphate uptake
5. Iron uptake	Aerobactin siderophore receptor IutA and TonB dependent receptors	17	Ion uptake to compete with pathogens
IV. Motility and aero-tolerance			
6. Gliding motility	Gliding motility and T9SS	24	Motility
7. Aero-tolerance response	Cbb3 cytochrome oxidase	5	Growth in limited oxygen
V. ROS stress response and brackish adaptation			
8. Detoxification and ionic balancing	SOD, peroxidase, catalase, Thioredoxin and Thioredoxin reductase	22	Detoxification of ROS response by the host plant
9. Brackish adaptation	Ion channels, Multisubunit Na+/H + antiporter, Na(+)-translocating NADH-quinone reductase and Aquaporin Z	29	Involved in osmoregulation

PTS, Phosphotransferase System; MFS, The major facilitator superfamily; RND, Resistance-Nodulation-Division; SOD, superoxide dismutase, T9SS; Type IX secretion system.

Table 4
Phenotypic characters differentiating L1I52^T from the phylogenetically closest *Flavobacterium* type strains as evident from 16S rRNA analysis. Strains: 1 – *Flavobacterium pokkalii* L1I52^T, 2 – *F. daejeonense* KACC 11422^T, 3 – *F. sufflavum* BBQ-12^T, 4 – *F. glycines* ICMP 17618^T, 5 – *F. nitratireducens* N1^T. All data's were obtained in this study. +, positive; -, negative; R, resistant; S, sensitive.

Phenotypic Characters	1	2	3	4	5
Isolation source	Pokkali rhizosphere	Greenhouse soil	Freshwater	Soybean rhizosphere	Marine water
DNA G + C content (mol%)	34.9	35.0	34.2	35.6	36.3
Growth in ZoBell marine agar	+	–	–	–	–
Growth in 2% NaCl	+	–	–	–	–
Growth at 37 °C	+	+	–	+	+
Growth in R2A broth at 30 °C	+	+	–	+	+
Esculin hydrolysis	–	+	+	–	–
Utilization of raffinose	–	+	–	+	+
Acid production from:					
Melibiose	–	+	–	–	–
Raffinose	–	+	–	–	–
Mannose	+	–	–	–	+
Antibiotic sensitivity test					
Penicillin G (P)	S	R	R	R	S
Vancomycin (Va)	R	S	S	R	R

RAST Server (Rapid Annotations using Subsystems Technology (Aziz et al., 2008). The ANI (Average Nucleotide Identity) between close genomes was calculated using JSpecies WS online program (Richter et al., 2015) and AAI (Average Amino Acid Identity) using the web-server available through http://enve-omics.gatech.edu/ (Rodriguez-R and Konstantinidis, 2016). For pangenome analysis, we used anvi'o pangenomic workflow which uses BLAST search to assess the similarity between each pair of amino acid sequences among all genomes, and then resolves the resultant graph into gene clusters using the Markov Cluster algorithm (Eren et al., 2015). The pangenome diagram was finally visualized using anvi'o graphical interface. Further, BLAST similarity search was conducted in RAST to identify the unique genes of L1I52^T in comparison with *F. daejeonense* DSM 17708^T (Emms and Kelly, 2015). A sequence identity below 50 % cut-off was considered as unique gene in L1I52^T (Chaudhari et al., 2016). Finally, the unique genes are grouped by at least 3 numbers of functional genes and gene proximity of maximum 2 nucleotide gap within the cluster. The gene organizations of unique gene clusters are constructed using genoPlotR in R package (http://www.R-project.org/). The draft genome of L1I52^T was deposited in NCBI (accession: NASZ00000000; BioProject: PRJNA379921).

3. Results and discussion

3.1. Isolation

During our initial *in planta* screening for identifying possible novel plant growth promoting rhizobacteria from brackish associated pokkali rice, we shortlisted strain L1I52^T among many tested strains because it showed an enhanced pokkali rice growth (Fig. S1). Also, initial 16S rRNA gene sequence analysis showed that strain L1I52^T could not be assigned to any particular *Flavobacterium* species. These observations made us to re-isolate L1I52^T like strains and for this purpose, pokkali rice rhizosphere samples were collected and a routine serial dilution procedure was followed as described in method section. This procedure yielded several novel *Flavobacterium* strains (data not shown) including eight strains designated as NRK F1, F15, F16, F41, F42, F47, F49, and F50 whose 16S rRNA gene sequences (> 1300bp) shared 100 % sequence similarity with L1I52^T, indicating that they all belong to same species. However, genomic fingerprinting by ERIC primers revealed that strains NRK F1, NRK F41, NRK F42, NRK F47, NRK F50 and NRK F15 exhibited similar fingerprinting patterns. However, strains NRK F15, NRK F16 and L1I52^T displayed different DNA fingerprinting patterns between each other indicating that they are not clonal in origin

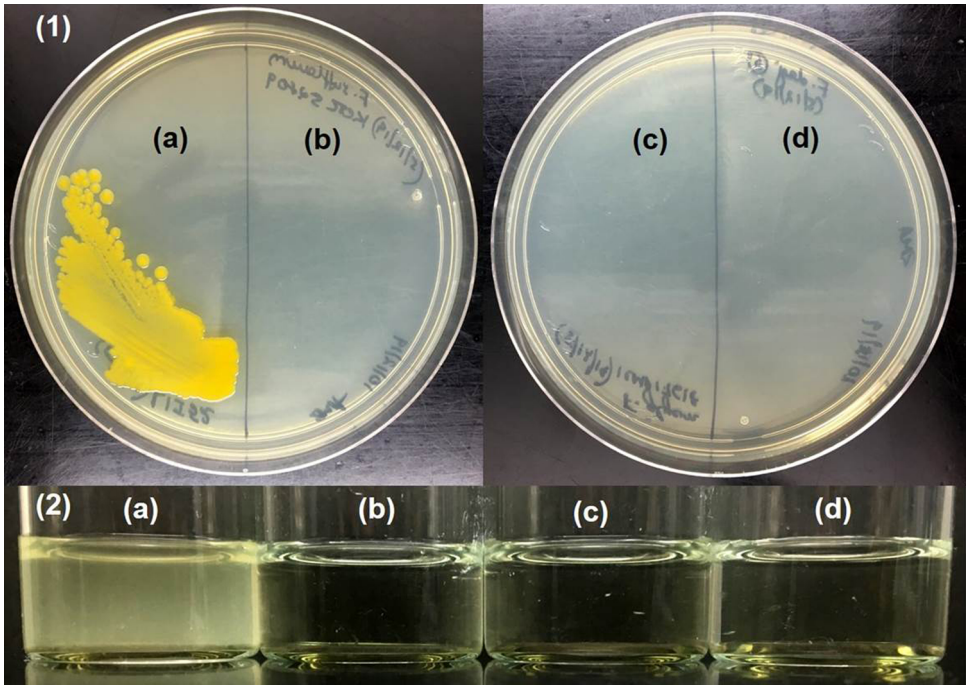

Fig. 7. Useful phenotypic characters differentiating L1I52^T from its nearest phylogenetic neighbours: (1) Growth in ZoBell marine agar at 30 °C after 4 days; (2) growth in 2% NaCl containing R2A broth at 30 °C after 4 days. (a) L1I52^T, (b) *F. sufflavum* KCTC 52809^T, (c) *F. glycines* ICMP 17618^T, (d) *F. daejeonense* KACC 11422^T.

Table 5
Whole cell fatty acid composition of strain L1I52^T and the type strains of related *Flavobacterium* species. Taxa: 1, L1I52^T; 2, *F. daejeonense* KACC 11422^T; 3, *F. sufflavum* BBQ-12^T 4, *F. glycines* ICMP 17618^T. Only fatty acid percentages amounting 0.8 % or higher are shown. All data's were obtained in this study.

Fatty acids (%)	1	2	3	4
12:0	0.81	1.11	–	0.86
13:0 iso	–	1.01	TR	1.73
13:0	2.63	1.99	–	2.01
14:0 iso	4.29	4.25	–	3.09
14:0	1.92	1.52	2.72	2.52
15:0 iso	17.10	17.27	20.11	16.30
15:0 anteiso	6.90	6.58	8.77	9.47
15:1 w6c	3.50	7.39	1.15	9.37
16:0 iso	–	1.00	0.84	1.06
16:0	8.95	7.92	9.32	6.20
15:0 iso 3OH	5.88	7.46	5.62	7.94
15:0 2OH	–	–	TR	0.89
17:1 w8c	2.31	1.63	TR	0.95
17:1 w6c	3.67	3.32	1.45	7.66
16:0 3OH	6.70	6.10	6.26	8.21
Summed Feature 1[a]	4.39	7.33	–	–
Summed Feature 2[b]	1.51	1.22	1.18	1.64
Summed Feature 3[c]	24.11	19.96	24.70	18.75
Summed Feature 9[d]	1.47	1.02	1.23	–

Summed features are group of two or three fatty acids that could not be separated by the GC with the MIDI system. Summed feature 1 comprised iso-$C_{15:1}/C_{13:0}$ 3−OH; summed feature 2 comprised iso-$C_{16:1}/C_{14:0}$ 3−OH; summed feature 3 comprised $C_{16:1}\omega7c/C_{16:1}\omega6c$ and summed feature 9 comprised $C_{16:0}$ 10-methyl.

(Fig. 1). Nevertheless, based on the ERIC-DNA fingerprinting patterns, three strains namely L1I52^T, NRK F15 and NRK F16 were shortlisted for further taxonomic characterization.

3.2. Phylogenetic analysis

The 16S rRNA gene analysis placed the three strains L1I52^T, NRK F15 and NRK F16 within the genus *Flavobacterium* sharing highest 16S rRNA gene sequence similarity to *Flavobacterium sufflavum* BBQ-12^T (97.97 %), *F. daejeonense* GH1-10^T (97.64 %) and *F. glycines* Gm-149^T (97.00 %). The 16S rRNA gene sequence similarities between the novel strains and with other *Flavobacterium* type strains were less than 97 %. A 16S rRNA phylogenetic tree was constructed using three different algorithms (neighbouring joining, maximum parsimony and maximum likelihood). Irrespective of the algorithms adopted the three novel strains clustered stably with *F. daejeonense* GH1-10^T, as supported by higher bootstrap values (Fig. 2). Further, the phylogenetic relationship of the three novel strains L1I52^T, NRK F15 and NRK F16 were studied in more detail by performing mutlilocus sequence analysis (MLSA). For this study, five housekeeping genes; *gyrB* (DNA gyrase subunit B), *glyA* (serine hydroxymethyl transferase), *atpA* (ATP synthase subunit alpha), *DnaK* (chaperone protein) and *murG* (N-acetylglucosaminyl transferase) which were shown previously to give higher taxonomic resolution in the genus *Flavobacterium* (Nicolas et al., 2008; Mun et al., 2013) were selected and used. This MLSA analysis showed that the sequence similarities between the three strains for all the housekeeping genes were observed to be identical, supporting the fact that the three strains belong to the same species. However, they shared low levels of gene sequence similarities ranging from 85 to 92.36 % for *gyrB*, 90–92.52% for *glyA*, 91–94.89% for *atpA*, 91–95.24% for *DnaK* and 83–93.14% for *murG* with its most closely related *Flavobacterium* type strains (Table 1), indicating that strains L1I52^T, NRK F15 and NRK F16 may belong to a novel *Flavobacterium* species. A maximum likelihood tree was constructed using the concatenated sequences of the five housekeeping genes, and this result showed similar phylogenetic clustering as observed in 16S rRNA analysis that strains L1I52^T, NRK F15 and NRK F16 formed distinct branch but paired stably with *F. daejeonense* GH1-10^T (Fig. 3).

3.3. Genome analysis

For accurate taxonomic placement of the three strains, L1I52^T was identified as the group representative and its draft genome was sequenced. In-silico genome comparisons showed that L1I52^T genome shared an average nucleotide identity (ANI) values of 80.2–92.7 %, average amino acid identity (AAI) values of 83.1–94.0 % and digital

DNA-DNA hybridization (dDDH) values of 24.0–52.6 % with its most closely related *Flavobacterium* type strains (Table 2; Fig. 4). These percentage values are well below the recommended threshold values of ANI (< 95 %), AAI (< 95 %) and dDDH (< 70 %) for bacterial species discrimination (Goris et al., 2007; Konstantinidis and Tiedje, 2007; Auch et al., 2010), further supporting the fact that L1I52^T represents a novel species within the genus *Flavobacterium*.

A genome comparison was carried out using anvio's pipeline to identify genome regions which can be used to differentiate L1I52^T from its closest relatives. This analysis showed that L1I52^T genome shared 2248 genes with other three *Flavobacterium* genomes and had 413 unique strain specific genes (Fig. 5). Further, L1I52^T shared more genomic regions with *F. daejeonense* DSM 17708^T than with other two *Flavobacterium* genomes reflecting their close phylogenetic relationship. Based on this observation, we compared these two closely related genomes to determine to what extent the genome composition of L1I52^T can provide information about its habitat adaptions since L1I52^T was isolated from pokkali rhizosphere and *F. daejeonense* DSM 17708^T was from green house soil. This analysis showed that the genome size of L1I52^T (3.89Mb) is smaller than that of *F. daejeonense* DSM 17708^T (4.28Mb). Also, phage, prophage and transposable elements are higher in *F. daejeonense* DSM 17708^T (52) when compared to L1I52^T (16). These informations clearly reflect L1I52^T may have a restricted host adaptative lifestyle. Also, we identified 520 unique genes in L1I52^T genome which are absent in *F. daejeonense* DSM 17708^T. These unique genes were grouped into 17 different clusters according to their gene proximity and functional annotation (most of the hypothetical proteins were excluded) (Table S3). Among the identified gene clusters, three of them encode for degradation and utilization of various plant based polysaccharides such as pectin, starch and xylan (Fig. 6). This finding corroborates the positive utilisation of pectin, starch and xylan by L1I52^T in phenotypic tests. Furthermore, the L1I52^T genome contains genes involved in utilisation of plant derived carbohydrates, plant polysaccharides, gliding motility, chemotaxis, transporters for uptake of plant derived substances, protection against environmental and plant host stress and other functions which probably give L1I52^T an advantage to colonise and mutually interact with its host plant under brackish conditions (Table 3).

3.4. Phenotypic characterisation

Three strains L1I52^T, NRK F15 and NRK F16 shared very similar phenotypic properties among eachother and revealed main characters as described for the genus *Flavobacterium*; cells are Gram-staining negative rods (Fig. S2) with strict aerobic lifestyle, colonies are deep-yellow pigmented, negative for flexirubin-type pigments, gliding motility was observed, catalase and oxidase tests are positive (Bernardet et al., 2002). The useful phenotypic traits that distinguish our three novel strains from its most closely related phylogenetic relatives are given in (Table 4; Fig. 7). Further details on the physiological and biochemical characteristics were provided in L1I52^T species description.

The major fatty acids of L1I52^T were iso-$C_{15:0}$ (17.10 %), summed feature 3($C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2 − OH) (24.11 %), $C_{16:0}$ (8.95 %), anteiso-$C_{15:0}$ (6.90 %), and $C_{16:0}$ 3 − OH (6.70 %), which is also the most abundant fatty acid reported in most members of the genus *Flavobacterium* (Bernardet et al., 2002). Detailed comparison of cellular fatty acid methyl esters of L1I52^T and its closest phylogenetic neighbours are shown in Table 5. The polar lipid profile of L1I52^T revealed the presence of phosphatidylethanolamine (PE), a major polar lipid observed in other described members of the genus *Flavobacterium* (Bernardet et al., 2002; Dong et al., 2013). In addition, an unknown aminolipid (AL1) and few unidentified lipids such as phospholipids (PL1 and PL2), lipid (L1), and amino phospholipid (APL) were also observed (Fig. S3). The major respiratory quinone was identified as MK 6.

In conclusion, based on the results of genotypic, chemotaxonomic and phenotypic analysis, we describe a novel *Flavobacterium* species represented by strain L1I52^T for which we propose the name *Flavobacterium pokkalii* sp. nov. Further experiments are ongoing to determine the novel genetic traits of L1I52^T that involves in plant beneficial effects and mutual interactions under brackish conditions using pokkali rice as a host plant.

3.5. Species description of L1I52^T

Flavobacterium pokkalii (pok.ka'li.i. N.L. gen. n. pokkaliiof pokkali (a variety of rice)

Cells are Gram-negative rods with strict aerobic lifestyle and are motile by gliding. Colonies are deep-yellow pigmented, convex, upto 1.5 mm in diameter and circular with wavy margins after 4 days of incubation in R2AN (0.5 % NaCl) medium at 30 °C. Growth occurs in Luria Bertani agar, nutrient agar, TSA agar and ZoBell marine agar medium. No growth was observed in ZoBell marine broth medium. Growth occurs at temperature between 18 to 37 °C (optimal at 28 − 30 °C), pH 6.0–8.0 (optimal at pH 7.0). No growth was observed at 4 °C and 42 °C. Tolerates NaCl upto 3% (optimal at 0.5 % NaCl). Catalase and oxidase tests are positive. Starch was hydrolysed whereas DNA, esculin, gelatin, carboxymethyl cellulose, xylan and pectin was not hydrolysed. Negative reactions were observed for arginine dihydrolase, lysine decarboxylase, MR-VP and indole test. Produces acid from xylose, maltose, fructose, dextrose, galactose, sucrose, L-arabinose, mannose, inulin and D-arabinose whereas lactose, raffinose, trehalose, melibiose, sodium gluconate, glycerol, salicin pulcitol, inositol, sorbitol, mannitol, adonitol, arabitol, erythritol, α-methyl D-glucoside, rhamnose, cellobiose, melezitose, α-methyl D-mannoside, xylitol and sorbose showed negative acid production (Himedia HiCarbohydrate kit). ONPG, citrate and malonate are not utilized. Positive utilisation for sugars: L-arabinose, D-glucose, D-maltose, L-rhamnose, sucrose and D-xylose as sole carbon source, but did not utilise D-cellobiose, lactose, D-mannitol, D-raffinose, and D-trehalose. Amino acids such as L-methionine, L-phenylalanine, putrescine, L-threonine, L-tryptophan and L-valine were utilised as sole nitrogen source but L-alanine, L-proline and L-serine were not utilised. The type strain is resistant to the following antibiotics; Kanamycin (30), vancomycin (30), oxacillin (1), methicillin (5) and sensitive to: ampicillin (10), gentamycin (10), tetracycline (30), chloramphenicol (30), streptomycin (10), penicillin G (10 units), polymyxin B (300), rifampicin (5), ofloxacin (5), clindamycin (2), carbenicillin (100), ciprofloxacin (5), erythromycin (15), amoxyclav (30), co-trimaxozole (25), cefozolin (30), doxycycline hydrochloride (30), netillin (30), linezolid (30), nalidixic acid (30), cinoxacin (100). The major fatty acids were iso-$C_{15:0}$ (17.10 %), summed feature 3($C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2 − OH) (24.11 %), $C_{16:0}$ (8.95 %), anteiso-$C_{15:0}$ (6.90 %), and $C_{16:0}$ 3 − OH (6.70 %) and the complete cellular fatty acid profile is given in Table 5. Other chemotaxonomy properties such as polar lipid profile and respiratory quinone are mentioned in the main text. The type strain can promote pokkali rice growth and encodes several gene features in their genome that are potentially involved in plant microbe interactions.

The type strain L1I52^T (=MTCC 12454^T =KCTC 42429^T) was isolated from the rhizosphere of brackish water cultivated saline-tolerant pokkali rice variety (VTL-8) grown in Kumbalangi pokkali rice fields, located at Ernakulam district, Kerala, India. The DNA G + C content of L1I52^T is 34.9 %.

Author contributions

Conceived and designed the experiments: R.R.M, N.R. Performed the experiments: R.R.M, S.K, T.T, N.R. Analyzed the data: R.R.M, S.K, T.T, N.R. Contributed reagents/materials/analysis tools: NR, TT. Wrote the paper: NR with inputs from R.R.M, S.K, T.T.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2020.126533.

References

Auch, A.F., Jan, M., Klenk, H.P., Göker, M., 2010. Digital DNA-DNA hybridization for microbial species delineation by means of genome-to-genome sequence comparison. Stand. Genomic Sci. 2 (1), 117. https://doi.org/10.4056/sigs.531120.

Aziz, R.K., Bartels, D., Best, A.A., DeJongh, M., Disz, T., Edwards, R.A., Formsma, K., Gerdes, S., Glass, E.M., Kubal, M., Meyer, F., 2008. The RAST server: rapid annotations using subsystems technology. BMC Genomics 9 (1), 75. https://doi.org/10.1186/1471-2164-9-75.

Berendsen, R.L., Pieterse, C.M., Bakker, P.A., 2012. The rhizosphere microbiome and plant health. Trends Plant Sci. 17 (8), 478–486. https://doi.org/10.1016/j.tplants.2012.04.001.

Bernardet, J.F., Nakagawa, Y., Holmes, B., 2002. Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family. Int. J. Syst. Evol. Microbiol. 52 (3), 1049–1070. https://doi.org/10.1099/ijs.0.042887-0.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37 (8), 911–917. https://doi.org/10.1139/y59-099.

Card, G.L., 1973. Metabolism of phosphatidylglycerol, phosphatidylethanolamine, and cardiolipin of *Bacillus stearothermophilus*. J. Bacteriol. 114 (3), 1125–1137.

Chaudhari, N.M., Gupta, V.K., Dutta, C., 2016. BPGA-an ultra-fast pan-genome analysis pipeline. Sci. Rep. 6 (1), 1–10. https://doi.org/10.1038/srep24373.

Cox, M.P., Peterson, D.A., Biggs, P.J., 2010. SolexaQA: At-a-glance quality assessment of Illumina second-generation sequencing data. BMC Bioinformatics 11 (1), 485. https://doi.org/10.1186/1471-2105-11-485.

Dong, K., Chen, F., Du, Y., Wang, G., 2013. *Flavobacterium enshiense* sp. nov., isolated from soil, and emended descriptions of the genus *Flavobacterium* and *Flavobacterium cauense*, *Flavobacterium saliperosum* and *Flavobacterium suncheonense*. Int. J. Syst. Evol. Microbiol. 63 (3), 886–892. https://doi.org/10.1099/ijs.0.039974-0.

Emms, D.M., Kelly, S., 2015. OrthoFinder: solving fundamental biases in whole genome comparisons dramatically improves orthogroup inference accuracy. Genome Biol. 16 (1), 157. https://doi.org/10.1186/s13059-015-0721-2.

Eren, A.M., Esen, Ö.C., Quince, C., Vineis, J.H., Morrison, H.G., Sogin, M.L., Delmont, T.O., 2015. Anvi'o: an advanced analysis and visualization platform for 'omics data. PeerJ. 3, e1319. https://doi.org/10.7717/peerj.1319.

Goris, J., Konstantinidis, K.T., Klappenbach, J.A., Coenye, T., Vandamme, P., Tiedje, J.M., 2007. DNA–DNA hybridization values and their relationship to whole-genome sequence similarities. Int. J. Syst. Evol. Microbiol. 57, 81–91. https://doi.org/10.1099/ijs.0.64483-0.

Gregorio, G.B., Senadhira, D., Mendoza, R.D., Manigbas, N.L., Roxas, J.P., Guerta, C.Q., 2002. Progress in breeding for salinity tolerance and associated abiotic stresses in rice. Field Crops Res. 76 (2–3), 91–101. https://doi.org/10.1016/S0378-4290(02)00031-X.

Komagata, K., Suzuki, K.I., 1988. 4 Lipid and cell-wall analysis in bacterial systematics. Method. Microbiol 19, 161–207. https://doi.org/10.1016/S0580-9517(08)70410-0. Academic Press.

Konstantinidis, K.T., Tiedje, J.M., 2007. Prokaryotic taxonomy and phylogeny in the genomic era: advancements and challenges ahead. Curr. Opin. Microbiol. 10, 504–509. https://doi.org/10.1016/j.mib.2007.08.006.

Krishnan, R., Menon, R.R., Tanaka, N., Busse, H.J., Krishnamurthi, S., Rameshku-mar, N., 2016. *Arthrobacter pokkalii* sp nov, a novel plant associated *Actinobacterium* with plant beneficial properties, isolated from saline tolerant pokkali rice, Kerala. India. PloS One 11 (3), e0150322. https://doi.org/10.1371/journal.pone.0150322.

Krishnan, R., Menon, R.R., Busse, H.J., Tanaka, N., Krishnamurthi, S., Rameshku-mar, N., 2017. *Novosphingobium pokkalii* sp nov, a novel rhizosphere-associated bacterium with plant beneficial properties isolated from saline-tolerant pokkali rice. Res. Microbiol. 168 (2), 113–121. https://doi.org/10.1016/j.resmic.2016.09.001.

Krishnan, R., Lang, E., Midha, S., Patil, P.B., Rameshkumar, N., 2018. Isolation and characterization of a novel 1-aminocyclopropane carboxylate (ACC) deaminase producing plant growth promoting marine *Gammaproteobacteria* from crops grown in brackish environments. Proposal for *Pokkaliibacter plantistimulans*gen. nov., sp. nov.,*Balneatrichaceae* fam. nov. in the order *Oceanospirillales* and an emended description of the genus *Balneatrix*. Syst. Appl. Mirobiol 41 (6), 570–580. https://doi.org/10.1016/j.syapm.2018.08.003.

Kurotani, K.I., Yamanaka, K., Toda, Y., Ogawa, D., Tanaka, M., Kozawa, H., Nakamura, H., Hakata, M., Ichikawa, H., Hattori, T., Takeda, S., 2015. Stress tolerance profiling of a collection of extant salt-tolerant rice varieties and transgenic plants over-expressing abiotic stress tolerance genes. Plant Cell Physiol. 56 (10), 1867–1876. https://doi.org/10.1093/pcp/pcv106.

Lugtenberg, B., Kamilova, F., 2009. Plant-growth-promoting rhizobacteria. Annu. Rev. Microbiol. 63, 541–556. https://doi.org/10.1146/annurev.micro.62.081307.162918.

Menon, R.R., Kumari, S., Kumar, P., Verma, A., Krishnamurthi, S., Rameshkumar, N., 2019. *Sphingomonas pokkalii* sp. nov., a novel plant associated rhizobacterium isolated from a saline tolerant pokkali rice and its draft genome analysis. Syst. Appl. Mirobiol. 42 (3), 334–342. https://doi.org/10.1016/j.syapm.2019.02.003.

Minnikin, D.E., O'Donnell, A.G., Goodfellow, M., Alderson, G., Athalye, M., Schaal, A., Parlett, J.H., 1984. An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids. J. Microbiol. Methods 2, 233–241. https://doi.org/10.1016/0167-7012(84)90018-6.

Mun, S., Lee, J., Lee, S., Han, K., Ahn, T.Y., 2013. Phylogeny of *Flavobacteria* group isolated from freshwater using multilocus sequencing analysis. Genomics Inform. 11 (4), 272. https://doi.org/10.5808/GI.2013.11.4.272.

Nicolas, P., Mondot, S., Achaz, G., Bouchenot, C., Bernardet, J.F., Duchaud, E., 2008. Population structure of the fish-pathogenic bacterium *Flavobacterium psychrophilum*. Appl. Environ. Microbiol. 74 (12), 3702–3709. https://doi.org/10.1128/AEM.00244-08.

Peng, Y., Leung, H.C., Yiu, S.M., Chin, F.Y., 2012. IDBA-UD: a de novo assembler for single-cell and metagenomic sequencing data with highly uneven depth. Bioinformatics 28 (11), 1420–1428. https://doi.org/10.1093/bioinformatics/bts174.

Rameshkumar, N., Sproer, C., Lang, E., Nair, S., 2010. *Vibrio mangrovi* sp. nov., a diazotrophic bacterium isolated from mangrove-associated wild rice (*Poteresia coarctata Tateoka*). FEMS Microbial. Lett. 307 (1), 35–40. https://doi.org/10.1111/j.1574-6968.2010.01958.

Rameshkumar, N., Krishnan, R., Lang, E., Matsumura, Y., Sawabe, T., Sawabe, T., 2014. *Zunongwangia mangrovi* sp. nov., isolated from mangrove (*Avicennia marina*) rhizosphere, and emended description of the genus *Zunongwangia*. Int. J. Syst. Evol. Microbiol. 64 (2), 545–550. https://doi.org/10.1099/ijs.0.053512-0.

Rameshkumar, N., Lang, E., Tanaka, N., 2016. Description of *Vogesella oryzae* sp.nov., isolated from the rhizosphere of saline tolerant pokkali rice. Syst. Appl. Mirobiol. 39 (1), 20–24. https://doi.org/10.1016/j.syapm.2015.10.003.

Richter, M., Rosselló-Móra, R., Oliver Glöckner, F., Peplies, J., 2015. JSpeciesWS: a web server for prokaryotic species circumscription based on pairwise genome comparison. Bioinformatics 32 (6), 929–931. https://doi.org/10.1093/bioinformatics/btv681.

Rodriguez-R, L.M., Konstantinidis, K.T., 2016. The enveomics collection: a toolbox for specialized analyses of microbial genomes and metagenomes (No. e1900v1). PeerJ Prepr. https://doi.org/10.7287/peerj.preprints.1900v1.

Sasser, M., 1990. Technical Note# 101: Bacterial Identification by Gas Chromatographic Analysis of Fatty Acids Methyl Esters (GC-FAME). MIDI Inc., North Newark.

Smibert, R.M., 1994. Phenotypic Characterization. Methods for General and Molecular Bacteriology.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30 (12), 2725–2729. https://doi.org/10.1093/molbev/mst197.