CHAPTER 8

Isolation, Identification and Cloning of Folate Biosynthetic Genes of *Lactococcus lactis* CM28

8.1. Introduction

The nutritional value of fermented foods can be further enhanced by the overproduction of certain components including folate in the fermenting lactic acid bacteria (LAB).The knowledge about the folate biosynthetic genes of the producing bacteria is important for the modulation of its content in fermented foods. Folate biosynthesis is a complex pathway linking several other metabolic pathways. Glycolysis, pentose phosphate pathway and shikimate pathway take part in the synthesis of PABA, a precursor for folate biosynthesis while purine biosynthesis is needed for the production of GTP from which the pteridine portion of folate is derived. A number of additional enzymatic reactions take part in the final assembly of the folate molecule (Sybesma et al., 2006; Wegkamp et al., 2007).

Several *Lactococcus lactis* strains were demonstrated to produce intra- and extracellular folate. The amount and type of folate produced, its accumulation as well as secretion strongly depends on the strain and culture conditions. *L. lactis* is a homofermentative bacterium used extensively in food fermentations and this long history of safe use has rendered it a GRAS status. The relatively simple metabolism and a small genome have made *L. lactis* an excellent choice for metabolic engineering (De Vos & Hugenholtz, 2004; Gaspar et al., 2011).

Gene expression systems have been developed for the controlled over expression of homologous or heterologous genes in *L. lactis*. Nisin controlled gene expression system (NICE) is the best characterized controllable expression system in *L. lactis* which make use of nisin as the inducer (**Fig.1.5**). Nisin is a 34 amino acid lantibiotic commonly used for food preservation (Maischberger et al., 2010). Despite the large homology shown by *L. lactis* strains at genome level there are considerable differences which make characterization of folate genes in each strain important for metabolic engineering approaches (Sybesma et al., 2003a).

The aim of the present study was to isolate and identify folate biosynthetic genes such as (*folKE, folC* and *folA*) of the isolate *L. lactis* CM28 and cloning them into the translational fusion vector (pNZ8148) for further expression studies.

8.2. Materials and Methods

8.2.1. Microorganisms, Plasmids and Culture Conditions

L. lactis CM28 and *L. lactis* NIZO9000 were grown in M17 medium supplemented with 0.5% (w/v) glucose at 37 ºC at static conditions. *E. coli* DH5α was propagated in LB medium at 37 ºC. pTZ57R/T and pNZ9530 vectors were used for cloning the folate genes. and Ampicillin (100 µg/mL) and chloramphenicol (10 µg/mL) were added to the media accordingly while propagating plasmids.

8.2.2. Isolation of Genomic DNA of L. lactis CM28

Total DNA was extracted from culture (1.5 ml) harvested in the mid-log phase (OD600 of 0.5-1) as per the method described in section 2.2.3.a.

8.2.3. PCR Amplification of Folate Biosynthetic Genes

The folate biosynthetic genes *folKE, folC* and *folA* were amplified from the genomic DNA of *L. lactis* CM28 using the specific primers (Sybesma et al., 2003a) as listed in **Table 8.1**. The forward primer *folKE*-F was extended at the 5' end by including an *Nco*I restriction site resulting in a slight modification of the mature gene (represented in italics). The restriction site *Kpn*I was introduced at the 5' end of *folKE*-R. The primers *folC*-F and *folC*-R were extended at 5' end by introducing *Kpn*I and *Xba*I respectively. An *Nco*I site was included at the 5' end of *folA*-F resulting in a slight modification of the mature gene (shown in italics) and the reverse primer contained a *Hind* III site at the 5' end.

Table 8.1. Oligonucleotides used for amplifying folate biosynthetic genes

Primer	**Sequence**
FolKE-F	CATGCCATG*GGG*CAAACAACTTATTTAAGCATGG
FolKE-R	GGGGTACCGATTCTTGATTAAGTTCTAAG
FolC-F	GAAGAGGTACCAGAAGAGTTTAAAAAGTATTATCG
FolC-R	TCTCTAGACTACTTTTCTTTTTTCAAAAATTCACG

FolA-F	GGAATTCCATG*GTT*ATTGGAATATGGGCAGAAG
FolA-R	GCCTCAAGCTTCATGGTTGTTTCACTTTTTC

Restriction sites are underlined

PCR reactions were carried out to a final volume of 50 µL containing a reaction mix of 5 µL 10X PCR buffer for *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 300 µM dNTP mix, 0.4 µM of each primer, 2 U of *Taq* DNA Polymerase and 1 µL of template DNA. Amplification was performed in a thermocycler (Mastercycler EP Gradient, Eppendorf, Germany). A gradient PCR was carried out for the amplification of *folC* gene with annealing temperatures varying from 40 -56°C. The optimized PCR conditions for the amplification of the selected genes are shown in **Table 8.2.**

Table 8.2. Optimum PCR conditions for the selected folate genes of *L. lactis* CM28

PCR step	**Temperature**	**Time**	**Number of cycles**
Initial denaturation	95°C	3 minutes	1
Denaturation	95°C	30 seconds	
Annealing	50°C for *folKE* and *folA*; 40°C for *folC*	30 seconds	30
Extension	72°C	1 minute	
Final extension	72°C	8 minutes	1

Amplicons were analyzed by electrophoresis in 1 % (w/v) agarose gels in 1X TAE buffer. PCR products were purified using GeneJET gel extraction kit (2.2.3.f).

8.2.4. Cloning of Folate Genes into pTZ57R/T Vector

The purified amplicons were ligated into pTZ57R/T vector (**Fig. 8.1**) as per the protocol mentioned in the kit (InsTAclone™ PCR Cloning Kit, Fermentas, USA). The ligation mix consisted of vector and insert in 1:3 ratio, 1 µL ligase and 2 µL buffer (2x). Nuclease free water was added to make up the total volume of 20 µL. This was then incubated at 16 ºC for 9 h and finally ligase was inactivated at 65 °C for 10 min. The T vector constructs were then transformed into cloning host *E. coli* DH5α competent cells as

per the protocol described in 2.2.3.b. After transformation the cells were plated onto LB agar with ampicillin and incubated overnight at 37 °C.

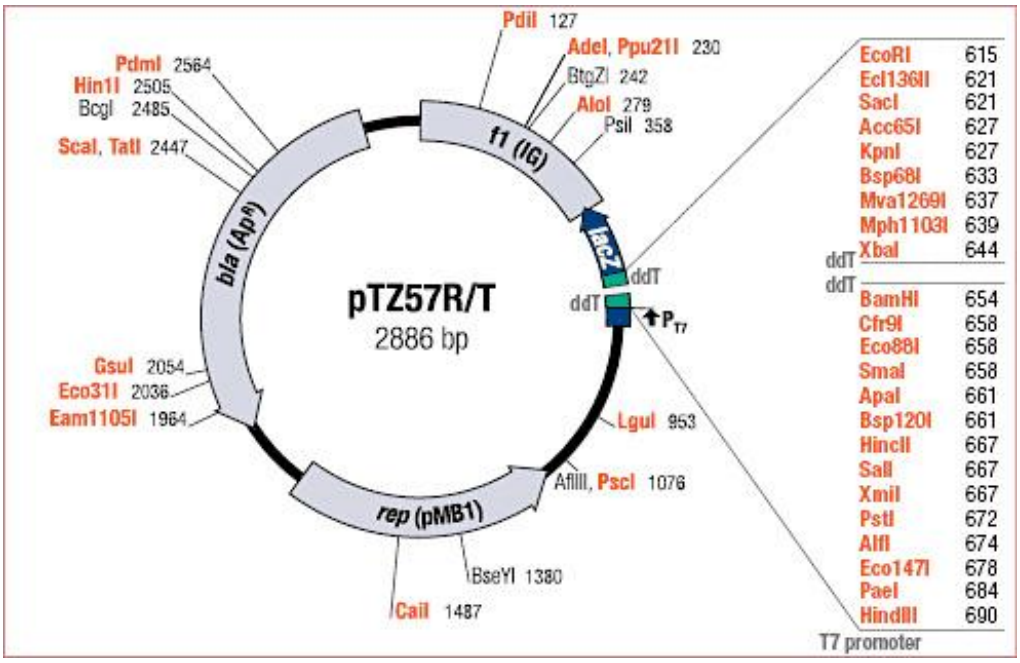


Fig. 8.1. Vector map of pTZ57R/T

8.2.5. Isolation of Recombinant Plasmids from E. coli DH5α Cells

Single colonies were inoculated into LB-ampicillin broth and incubated overnight at 37 °C. Plasmid DNA was isolated using kit obtained from Fermentas (USA) as explained previously (2.2.3.c). Plasmid was loaded on to agarose gel and stained with ethidium bromide for visualization. The clones were confirmed by PCR amplification. The plasmids were then sequenced with an automated sequencer (ABI 3100, Applied Biosystems) using the primers M13 F (5´-GTAAAACGACGGCCAG-3´) and M13 R (5´-CAGGAAACAGCTATGAC-3´). The sequences obtained were analyzed using BLAST algorithm to confirm the identity. Amplification, cloning and sequencing were performed twice in independent experiments.

8.2.6. Cloning of Folate Genes into the Expression Vector pNZ8148

pNZ8148 is a translational fusion vector used in NICE system (Zhou et al., 2006). It is a broad host range vector with *nisA* promoter followed by *Nco*1 restriction site to allow translational fusions at the ATG (**Fig. 8.2**). After sequencing, the T vector clones were digested with appropriate restriction enzymes and cloned into the expression vector

pNZ8148 (digested with suitable restriction enzymes). The ligation mix contained vector and insert in 1:3 ratio, 1 µL ligase and 2 µL buffer (2x). Nuclease free water was added to make up the total volume of 10 µL. The mix was incubated at 16 ºC for 9 h and finally ligase was inactivated at 65 °C for 10 min. The ligation mix was transformed to *L. lactis* NIZO9000 cells by electroporation. The details of electrocompetent cell preparation and electroporation are provided in section 2.2.3.d. *L. lactis* NIZO9000 is the standard host strain for NICE and contains the regulatory genes *nisR* and *nisK* integrated into the *pepN* locus of chromosomal DNA. The recombinant plasmid was isolated as per the protocol described in section 2.2.3.e. and the clone confirmation was done by PCR using the plasmid DNA as template.

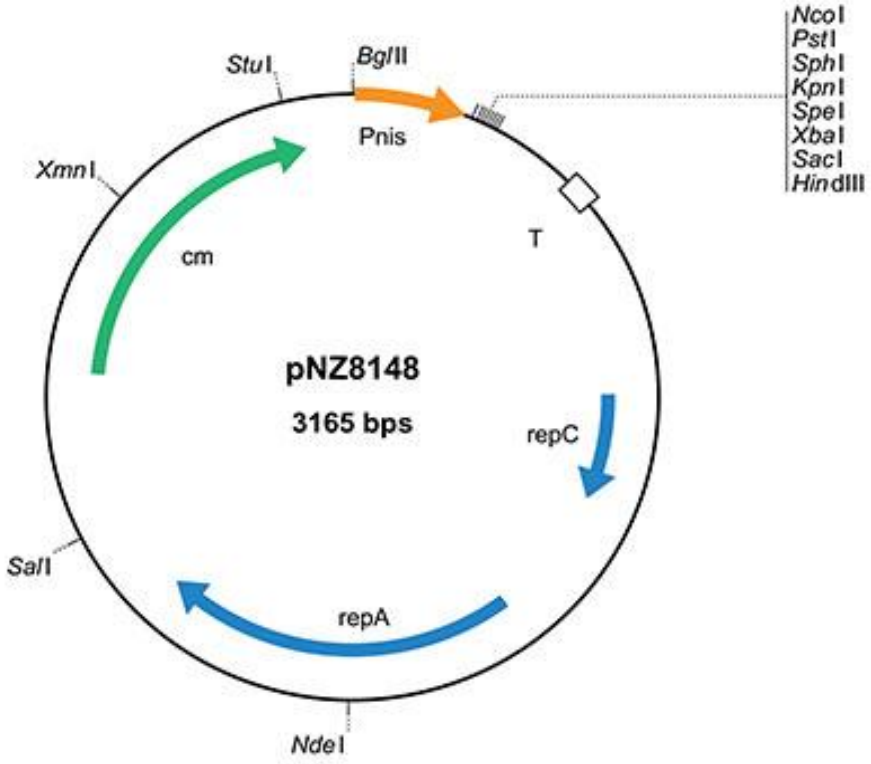


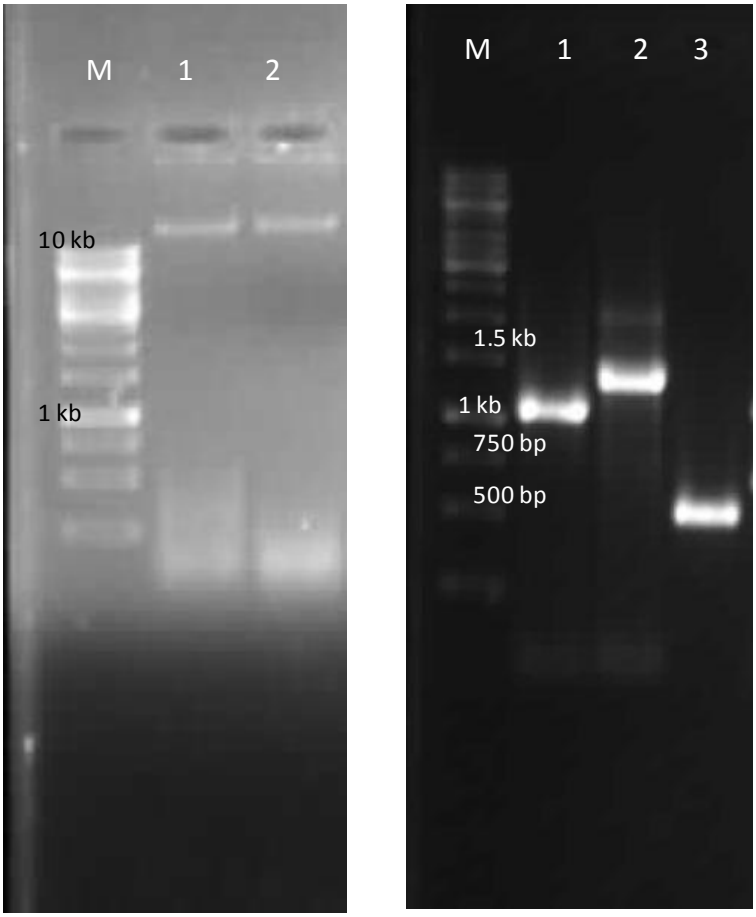
Fig. 8.2. Vector map of pNZ8148

8.3. Results and Discussion

8.3.1. PCR Amplification of Folate Biosynthetic Genes from L. lactis CM28

Genomic DNA was isolated from *L. lactis* CM28 (**Fig. 8.3 (A)** and *folKE, folC* and *folA* genes were amplified from the genomic DNA using the specific primers. The size of

the amplicons was in the expected range of about 1 kb for *folKE*, 1.3 kb for *folC* and 500 bp for *folA* (**Fig. 8.3 (B)**).



(A) **(B)**

Fig. 8.3. (A) Genomic DNA of *L. lactis* CM28 (M – 1 kb DNA ladder, Lane 1 and 2 – genomic DNA) **(B)** PCR amplification of folate biosynthetic genes (M- 1 kb DNA ladder, Lane 1 – *folKE*, Lane 2 – *folC*, Lane 3 – *folA)*

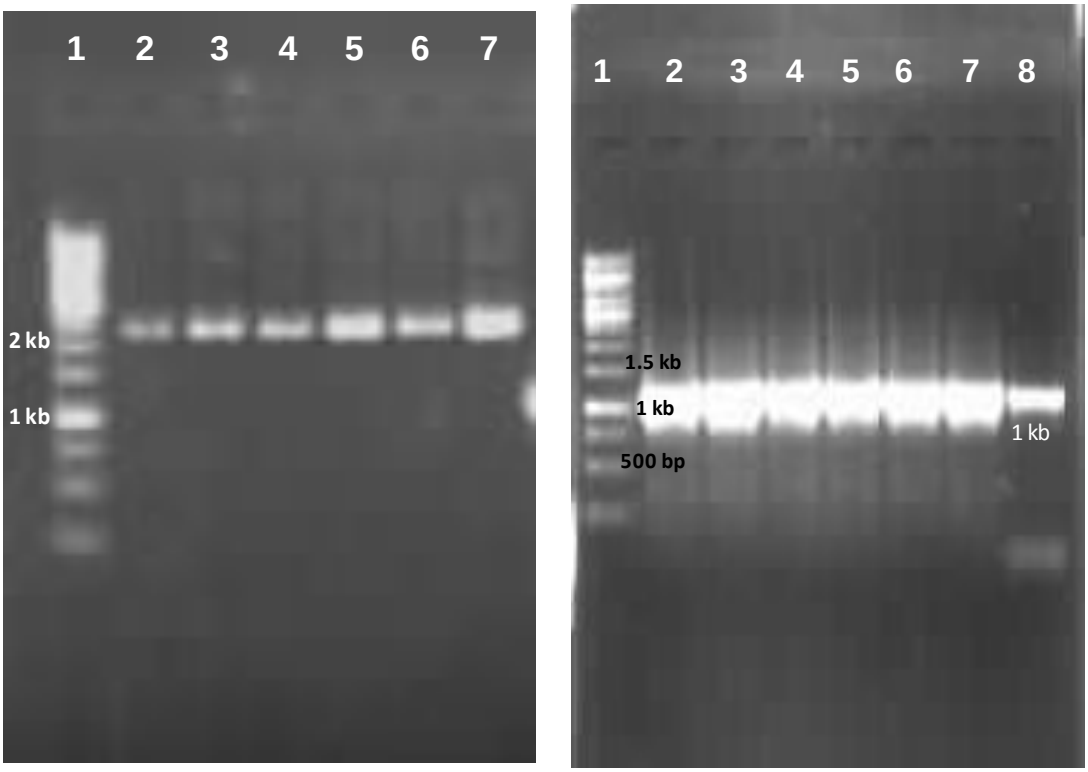
L. lactis strains were reported to contain the complete set of genes for folate biosynthesis which are organized in a folate gene cluster consisting of *folA*, *folB*, *folKE*, *folP*, *ylgG*, and *folC* genes (Sybesma et al., 2003a; Wegkamp et al., 2004). The biosynthetic pathway includes seven consecutive steps catalyzed by the enzymes coded by these genes. An overview of folate biosynthesis and folate gene cluster of *L. lactis* is provided in section 1.3.9.

8.3.2. Cloning of Folate genes into T vector and Sequencing

The amplicons purified by gel elution were cloned into pTZ57R/T vector and the clones were confirmed by PCR. The isolated plasmids were sequenced and the sequences were confirmed by BLAST analysis.

a. TA Cloning and Sequencing of folKE Gene

Recombinant plasmids isolated from the transformed *E. coli* colonies are represented **Fig. 8.4 (A)** and **Fig. 8.4 (B)** shows the clone confirmation by PCR. The positive clones were sequenced and the BLAST results indicated 99% identity towards *folKE* gene of *L. lactis* strain (**Fig. 8.5**).



(A) **(B)**

Fig. 8.4 (A) T vector clone of *folKE* (Lane 1 – 1 kb DNA ladder, Lane 2 to 7 – recombinant plasmids) **(B)** Clone confirmation by PCR (Lane 1- 1kb DNA ladder, Lane 2 to 7 – clone confirmation by PCR, Lane 8 – control PCR using genomic DNA of CM28)

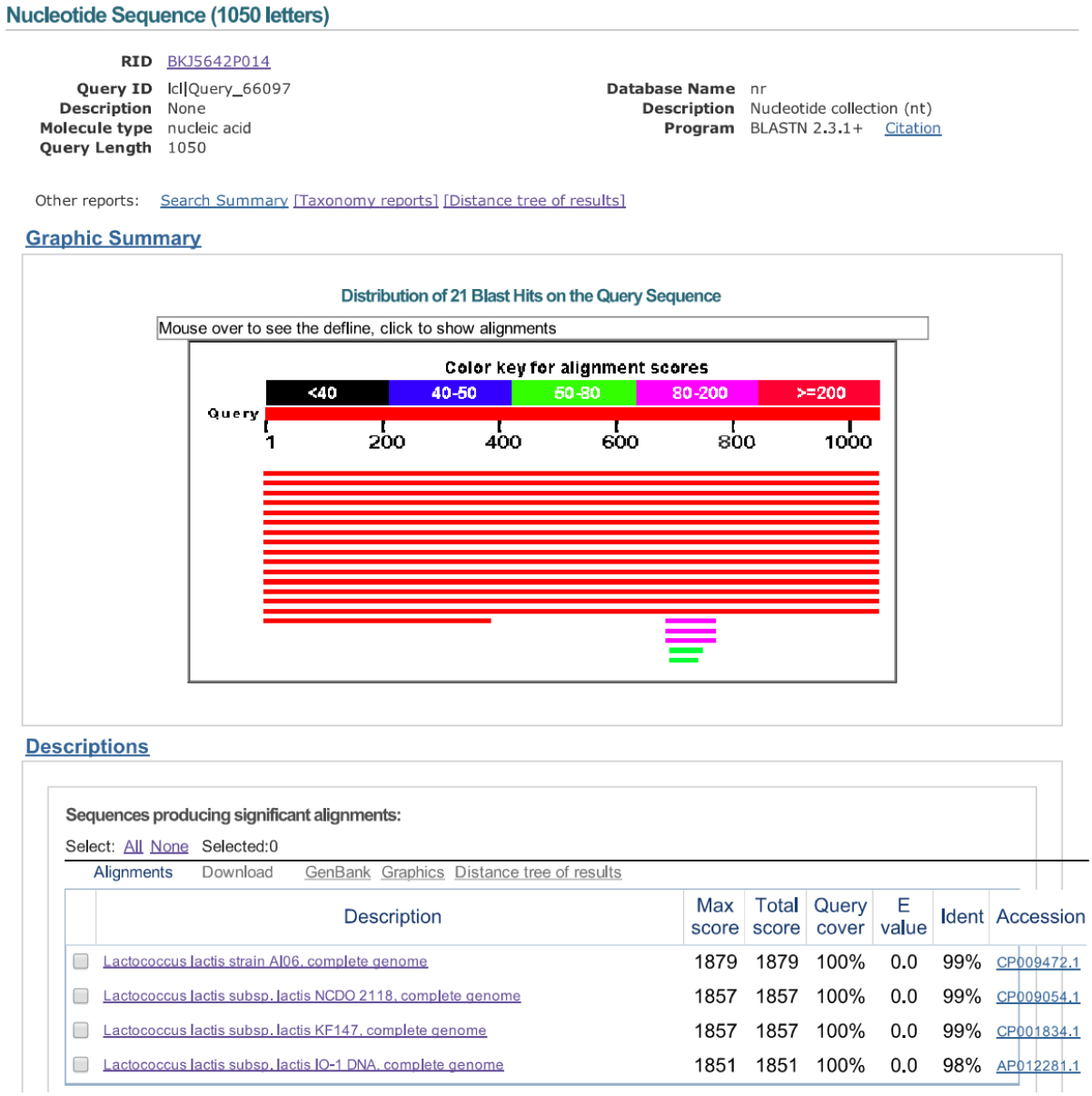


Fig. 8.5. BLAST analysis of *folKE* gene sequence

folKE is reported to encode a bifunctional protein where *folK* (EC 2.7.6.3) encodes the protein 2-amino-4-hydroxy-6- hydroxymethyl dihydropteridine pyrophosphokinase and *folE* (EC 3.5.4.16) encodes GTP cyclohydrolase I which is the first enzyme in the folate biosynthesis (Rossi et al., 2011). GTP cyclohydrolase I catalyses the reaction from GTP to dihydroneopterine triphosphate in which the five-membered imidazole ring of GTP is opened, C8 is expelled as formate, and a six-membered dihydropyrazine ring is formed using C1 and C2 of the ribose moiety of GTP. 2-amino-4-hydroxy-6-hydroxymethyl dihydropteridine pyrophosphokinase catalyses the reaction in which 6-hydroxymethyl-7,8-dihydropterin is converted to 6- hydroxymethyl-7,8-dihydropterin

pyrophosphate (de Crécy-Lagard et al., 2007). The analysis of nucleotide sequence of *folKE* gene confirmed its bifunctional nature as there was no stop codon at the end of *folK* or a start codon at the beginning of *folE*. The 1050 bp sequence obtained was then translated to obtain the amino acid sequence. The sequence of the polypeptide with 349 amino acids beginning at the first ATG codon in the open reading frame is shown (**Table 8.3**). The predicted molecular mass of the protein was 40.3 kDa which corresponded to the combined molecular weight of the proteins encoded by *folK* and *folE*.

Table 8.3. Nucleotide and amino acid sequence of *folKE* gene of *L. lactis* CM28

GenBank Accession No. KU214676

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ATGCAAACAACTTATTTAAGCATGGGAAGTAATATTGGTGACCGTCAGTATTATTTACATGAAGCCATTCGT
TTATTGGGAAAACACCCTAAAATTATGATTGAAAAAGTATCAAATTTTTATGAAAGTACTCCAGTCGGCGGC
GTCAAACAAGATGATTTTACTAATTTGGTATTAAAGGTGGCAACGCTACTTGAACCTTTGGAATTATTATCT
TTTATTCATGAAGTTGAGCTATCTTTGAACCGTGAGCGAAAAATTCATTGGGGGCCAAGAACAATTGATATT
GATATTATTTTCTATAACGACTTAGAAATGCAAGAAGAAAACTTGGTTATTCCACATAAAGAAGCTTTTAAT
CGTCTTTTTGTCTTGAAACCTATTTTTGAATTGATTGATAAAGACTTTAAATATTATGCGTCAATAGAAAAA
GCAATAGCCGAACTTTCAGTAAGTGAACAAGAGCTCCATGTGATAAAAGAAGAAAAAATACCGAGAAATCGT
ATTGAAGATGCCGTTAAAGAGATTCTCTTTGCAGTAGGTGAAAATCCAAATCGAGAAGGATTACTTGAAACT
CCAGCGAGAGTAGCTAAAATGTATGAAGAAATTCTTTCATCACAACGCTTAAGCAAGTTTAATGAGTATAAA
CTTTTTGAAATTGATTCTTCTAAAACGGATTCAATTGTGTTGATTAAAGATATTCCTTTTTATTCAATGTGT
GAGCATCATATGTTACCATTTTTCGGGAAAGCACATGTTGCCTATATCCCAGCTGATGGAAAAATTATTGGC
TTGTCAAAAATTCCCCGTTTAGTTGATTATGTTTCGCGCAAACTCTCGGTTCAAGAAAATATCACTCATGAT
ATTGGAGATATTTTGACTGATATTTTGAATCCTAAAGGAGTGGCAGTTCTTGTTGAAGGACGTCATATGTGC
GTTGAAATGCGTGGAGTAAAAAAAGTAAATTCTATTACTAAAACTTCTTATTTTTTAGGTGAATTTATAGAA
AATAACGAAAAAAGAATGGAATTTTTAGAAAGTCTTTTATGA
MQTTYLSMGSNIGDRQYYLHEAIRLLGKHPKIMIEKVSNFYESTPVGGVKQDDFTNLVLKVATLLEPLELL
SFIHEVELSLNRERKIHWGPRTIDIDIIFYNDLEMQEENLVIPHKEAFNRLFVLKPIFELIDKDFKYYASI
EKAIAELSVSEQELHVIKEEKIPRNRIEDAVKEILFAVGENPNREGLLETPARVAKMYEEILSSQRLSKFN
EYKLFEIDSSKTDSIVLIKDIPFYSMCEHHMLPFFGKAHVAYIPADGKIIGLSKIPRLVDYVSRKLSVQEN
ITHDIGDILTDILNPKGVAVLVEGRHMCVEMRGVKKVNSITKTSYFLGEFIENNEKRMEFLESLL*
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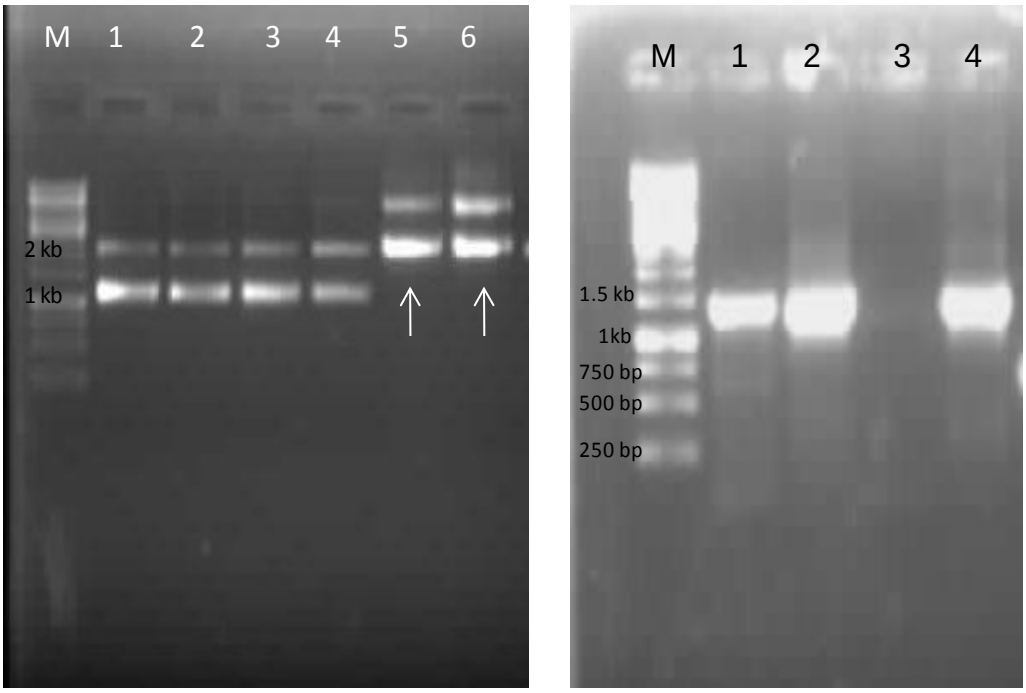
b. TA Cloning and Sequencing of folC Gene

The T vector clones of *folC* gene of *L. lactis* CM28 were identified by an up shift in the gel and were further confirmed by PCR (**Fig. 8.6**). The T vector clones were then sequenced followed by BLAST analysis. The sequence showed maximum identity (97%) towards *folC* gene of *L. lactis* strains. The results are presented in **Fig. 8.7**. The nucleotide (1284 bp) and the deduced amino acid sequence of *folC* gene of *L. lactis* CM28 are shown in **Table 8.4**. The open reading frame encodes 427 amino acids with a predicted molecular weight of 47 kDa.



(A) **(B)**

Fig. 8.6 (A) T vector clone of *folC* (M – 1 kb DNA ladder, Lane 1 to 4 – T vector without insert, Lane 5 and 6 – T vector clones (up shift)) **(B)** Clone confirmation by PCR
(M- 1kb DNA ladder, Lane 1 – control PCR with genomic DNA, Lane 2 and 4 – clone confirmation)

Nucleotide Sequence (1284 letters)

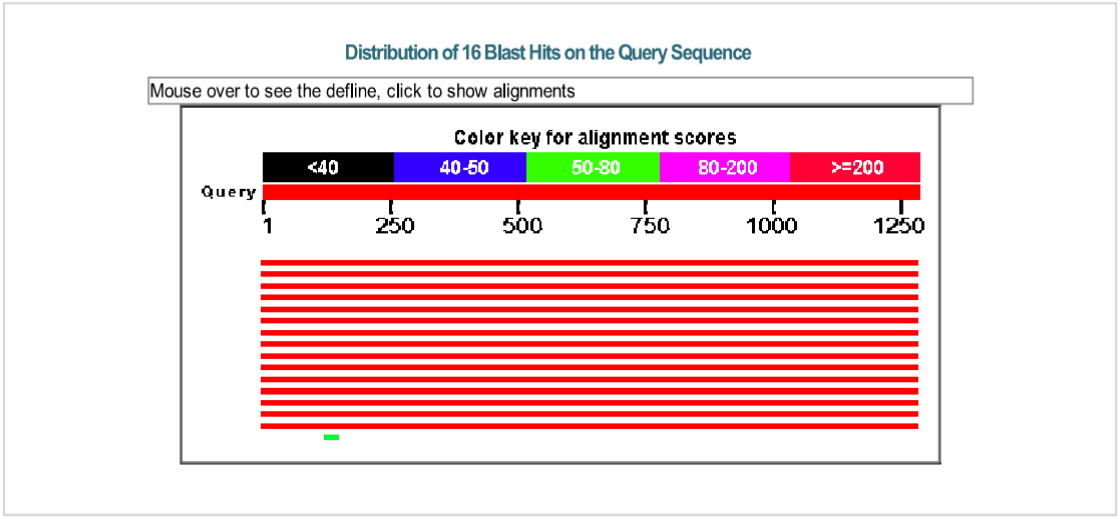
RID CUXNAJFH014

Query ID lcl|Query_7191
Description None
Molecule type nucleic acid
Query Length 1284

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.3.1+ Citation

Other reports: Search Summary [Taxonomy reports] [Distance tree of results]

Graphic Summary



Descriptions

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Lactococcus lactis subsp. lactis IO-1 DNA, complete genome	2126	2126	99%	0.0	97%	AP012281.1
Lactococcus lactis strain AI06, complete genome	2115	2115	99%	0.0	96%	CP009472.1
Lactococcus lactis subsp. lactis CV56, complete genome	2104	2104	99%	0.0	96%	CP002365.1
Lactococcus lactis subsp. lactis Il1403, complete genome	2104	2104	99%	0.0	96%	AE005176.1
Lactococcus lactis subsp. lactis strain S0, complete genome	2093	2093	99%	0.0	96%	CP010050.1

Fig. 8.7. BLAST analysis of *folC* gene sequence

Table 8.4. Nucleotide and amino acid sequence of *folC* gene of *L. lactis* CM28

GenBank Accession No. KX013556

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ATGTCTATAGAAGAAGCATTGGAATGGATACATTCACGTTTAAAATTTAATATTCGCCCAGGCCTAAGTCGT
GTTTCGGCCCTTTTAGAATTGCTTGGTCATCCAGAAGAGTCTTTGTCAATGATTCACGTTGCTGGAACAAAT
GGAAAAGGCTCCACAGTCGCTTTCACACGCTCAATCTTTATGCAGGCAGGTCTGAAGGTTGCTTCCTTCACA
AGTCCTTTCATCACCACTTTTGGTGAGCGGATGTCGATTAATGCACTCCCGATTGCTGAAGATAAATTAATT
TATTATGTAGAAATGATCCAGCCTCTTGTTGCTGAACTTGATAAAGATGCTGAACTGACTGGAATTACCGAA
TTTGAAATTATCACGGTAATGGCTTTTAAATATTTCGCTGATGAGCAGGTTGATTTAGCGGTTATTGAAGTT
GGTTTAGGTGGACTTCTTGATTCAACAAATGTGATTAAACCTGTTGTTTCTGGAATCACAACAATCGGTTTA
GATCATATTGATATTCTTGGTTCGACGATTGAAGAAATTGCAGCTCAAAAGGCTGGAATTATTAAACCAGGA
ATTCCAGTAGTTGTTGGAAATATTGAGTTAAAAGCACTTCGGGTTATATGGGAAGTGGCTCGAAAAAATACA
GCGCGTGTTTATCAATTTCCATATGATTATCGTACGGAAGTGGAAGAACACGAACATTTCAATTTCTTTTCT
GGTCAAGAAGCAATATTGGATATTGAAAAATCTTTAGTTGGCTTACATCAAATAGAAAATGCTGGTATGGCT
ATTGAACTTTCTCTGGTTTATGCAAGTAAGGTTGGGATTGAATTAACTGAGGATGTGATTCGCTCTGGAATT
CGTGAGGCTTTTTGGCCCGTTGGTATGGAAAAATTAGGTGAAAAACCATTCATTTCACTGGATGGTGTTCAT
AATGTTCATGGGAGGATTGGTTGGCTTGAAACCTTTAGTTTGGAGTTTCCAGATAAAAAGATTCCAATCATT
TTTTCAGTTATTATTCCAAAAGATATTAGTCAAATGATAAAAATGCTTCAAACTGTGAAAAATTCCCATTGG
ATTTGGACAACTTTGGATTATCCAAAAGCTTTGAATTTGGGAGATTTTCAAAACTTGGAAGAAGAAGGGGTT
GAATTGGCTCCAAGTTGGGAATTAGCTTTAGTTCGTGCGCAAAAAAATTTAGCTGAAGATGATTTGTTATTA
GTTACAGGCTCTCTCTATTTCTCATCTCAAGTTCGTGAATTTTTGAAAAAAGAAAAGTAG
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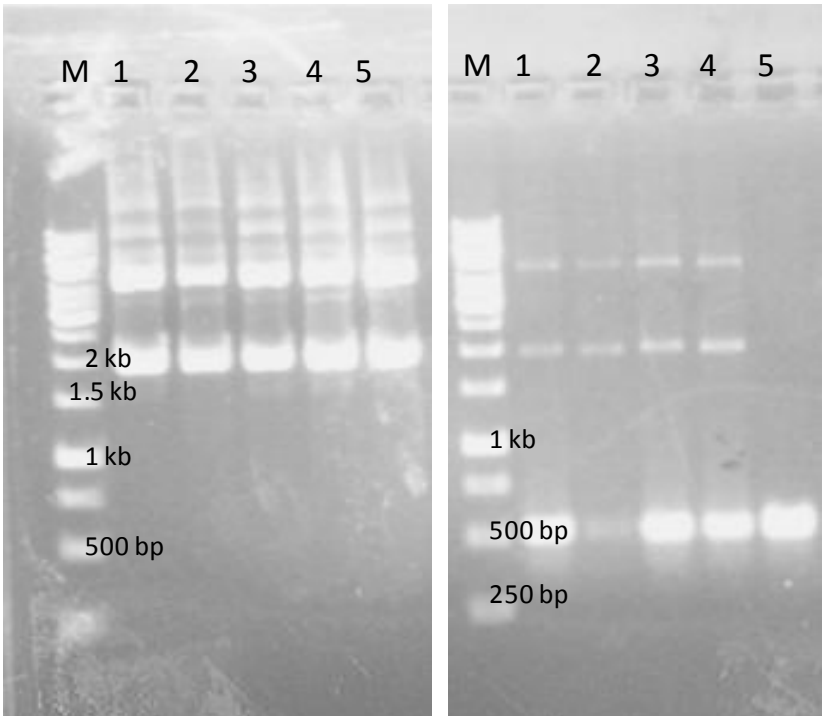
MSIEEALEWIHSRLKFNIRPGLSRVSALLELLGHPEESLSMIHVAGTNGKGSTVAFTRSIFMQAGLKVASFT
SPFITTFGERMSINALPIAEDKLIYYVEMIQPLVAELDKDAELTGITEFEIITVMAFKYFADEQVDLAVIEV
GLGGLLDSTNVIKPVVSGITTIGLDHIDILGSTIEEIAAQKAGIIKPGIPVVVGNIELKALRVIWEVARKNT
ARVYQFPYDYRTEVEEHEHFNFFSGQEAILDIEKSLVGLHQIENAGMAIELSLVYASKVGIELTEDVIRSGI
REAFWPVGMEKLGEKPFISLDGVHNVHGRIGWLETFSLEFPDKKIPIIFSVIIPKDISQMIKMLQTVKNSHW
IWTTLDYPKALNLGDFQNLEEEGVELAPSWELALVRAQKNLAEDDLLLVTGSLYFSSQVREFLKKEK*
```

folC gene is predicted to encode both dihydrofolate synthetase (EC:6.3.2.12) and folyl polyglutamate synthetase (EC:6.3.2.17) (section 1.3.9). Bacteria which biosynthesize folate *de novo* have bifunctional folylpolyglutamate synthetase-dihydrofolate synthetase enzymes, whereas non producers like *Lb. casei* lack dihydrofolate synthetase activity. Both enzymes contribute to the folate biosynthesis and modification. Mammalian folylpolyglutamate synthetases are also monofunctional (Toy & Bognar, 1990; Wang et al., 2010). Folylpolyglutamate synthetase is responsible for the elongation of glutamyl chain which usually varies from 1-10 glutamate residues. The metabolism of folates to polyglutamyl folates have a key role in the regulation of folate homeostasis and for the normal functioning of one carbon metabolism (Shane, 1989). It was reported that most of the polyglutamyl folates are retained intracellularly which can be metabolically utilized after deconjugation by the mammalian deconjugase. However, increased production of polyglutamyl folates might increase the folate concentration delivered by the probiotic bacteria as it is retained intracellularly during growth (Sybesma et al., 2003c).

c. TA Cloning and Sequencing of folA Gene

The T vector clones of *folA* gene were confirmed by PCR (**Fig. 8.8**). BLAST analysis showed maximum identity towards *folA* gene of *L. lactis* sub. *lactis* which encodes the protein dihydrofolate reductase (**Fig. 8.9**). The nucleotide (507 bp) and amino acid sequences are represented in **Table 8.5**. The deduced amino acid sequence contained 168 amino acids with a predicted molecular weight of 19.7 kDa and showed significant homology to bacterial dihydrofolate reductase gene.



(A) **(B)**

Fig. 8.8 (A) T vector clone of *folA* **(**M - 1 kb DNA ladder, Lane 1 to 5 - T vector clones) **(B)** Clone confirmation by PCR (M- 1kb DNA ladder, Lane 1 to 5 - clone confirmation by PCR)

Dihydrofolate reductase (EC 1.5.1.3) is one of the key enzymes in folate metabolism and catalyzes the NADPH dependent reduction of dihydrofolate to tetrahydrofolate (section1.3.9). It maintains the intracellular pools of tetrahydrofolates and its derivatives which are essential cofactors in one carbon reactions (Leszczyńska et al., 1995).

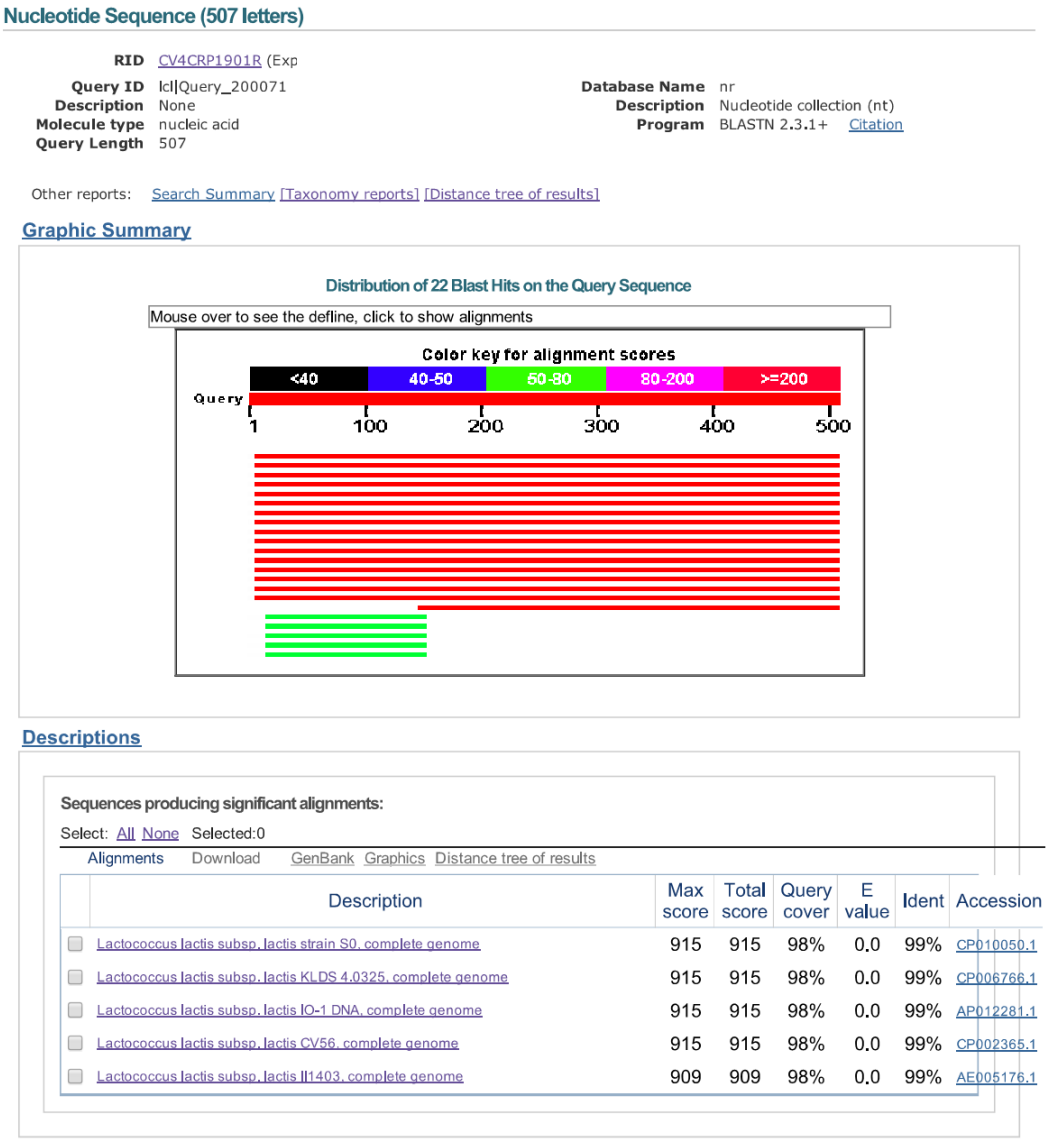


Fig. 8.9. BLAST analysis of *folA* gene sequence

Table 8.5. Nucleotide and the deduced amino acid sequence of *folA* gene of *L. lactis* CM28

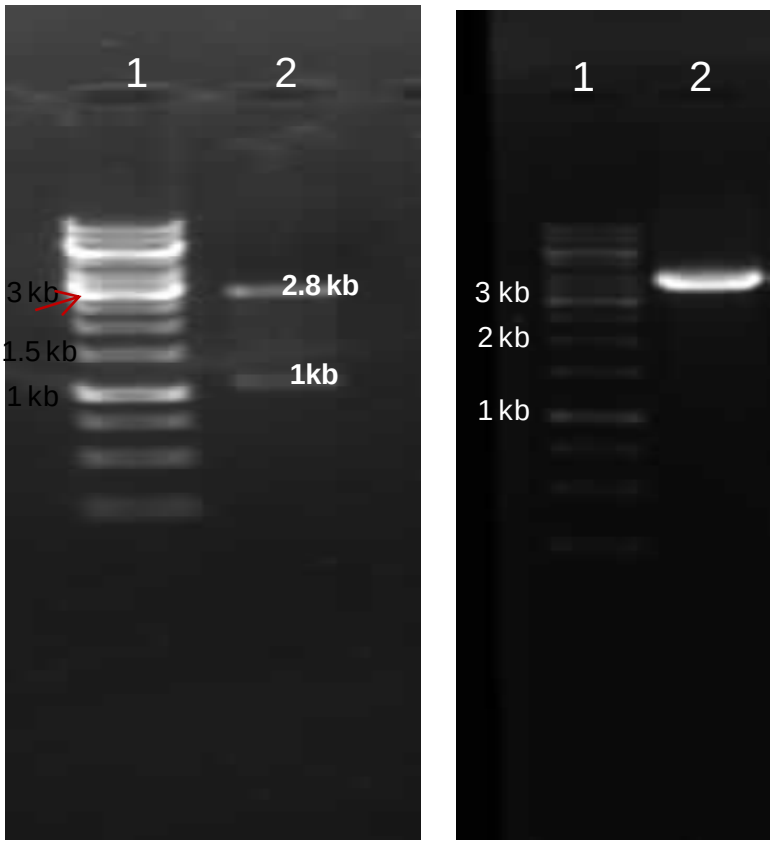
GenBank Accession No. KX013557

ATGATAATTGGAATATGGGCAGAAGATGAAGCAGGTCTTATCGGCGAAGCTGATAAAATGCCTTGGTCCTTA
CCTGCTGAACAACAACATTTTAAAGAAACAACCATGAATCAAGTGATTTTGATGGGACGAAAAACGTTTGAA
GGAATGAATAAACGTGTATTGCCAGGGAGAATAAGTATTATTTTAACTCGCGATGAAACTTATCAATCAGAT
AATGAAAAAGTGCTCATCATGCACAGCCCTAAGGAAGTTCTAGATTGGTACCATAAGCAAAATAAAGACTTA
TTTATCACAGGAGGAGCTGAAATTTTAGCCCTTTTTGAATCTGAACTTGAATTGCTCTATCGAACAGTTGTT
CATGAAAAATTTAAAGGAGATACTTATTTTCCAAGTACATTTGACTTTGGAAGATTTAAGCTAGTCTCTGAA
AAATTTCACGATAAAGATGAGCGGAATTCTTATACTTTTACAATTAAAAAATATGAAAAAGTGAAACAACCA
TGA

MIIGIWAEDEAGLIGEADKMPWSLPAEQQHFKETTMNQVILMGRKTFEGMNKRVLPGRISIILTRDETYQS
DNEKVLIMHSPKEVLDWYHKQNKDLFITGGAEILALFESELELLYRTVVHEKFKGDTYFPSTFDFGRFKLV
SEKFHDKDERNSYTFTIKKYEKVKQP*

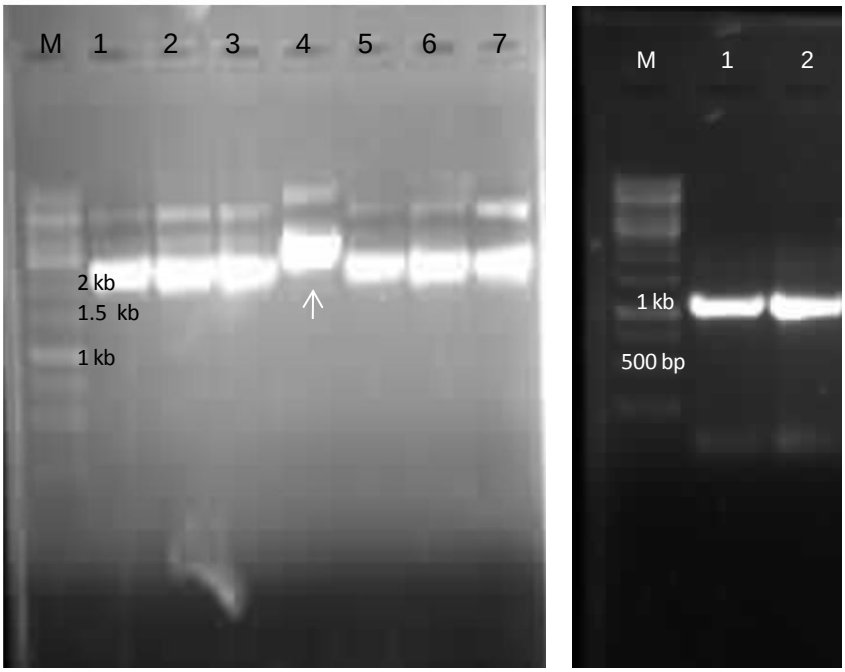
8.3.3. Cloning of Folate Genes into the Expression Vector pNZ8148

The T vector clones of *folKE* and *folC* genes were digested with suitable restriction enzymes and were cloned to linearized pNZ8148 thereby placing the genes under the *nisA* promoter. TA clone of *folKE* was digested with *Nco*1 and *Kpn*1 and that of *folC* was digested with *Kpn*1 and *Xba*1. The digestion products were run on agarose gel and the released insert was gel eluted. The purified inserts were individually cloned on pNZ8148 (digested with appropriate restriction enzymes). The ligation mix was then transformed into the expression host *L. lactis* NIZO9000 by electroporation. The clones were then confirmed by PCR. The results are presented in **Fig. 8.10, 8.11** and **8.12**. The initial attempts to clone *folA* into the expression vector were not successful and hence omitted from the studies.



(A) (B)

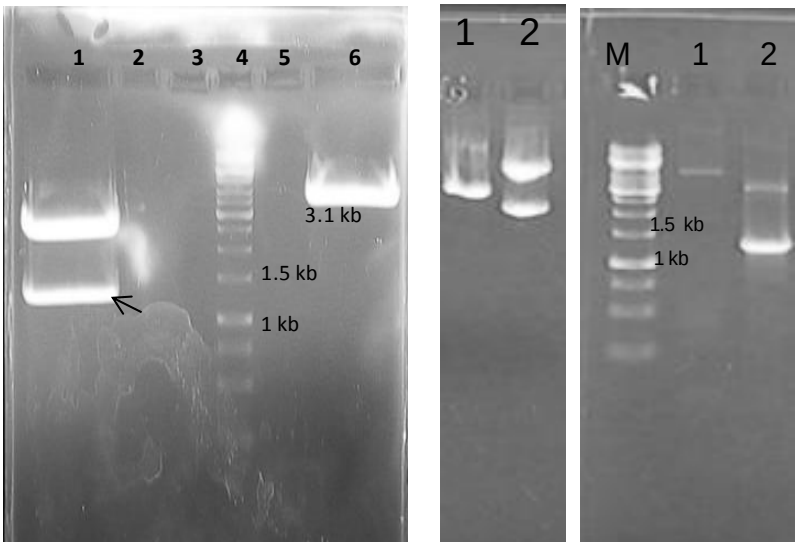
Fig. 8.10. Preparation of insert and vector for cloning of *folKE* gene into pNZ8148
(A) Restriction digestion of T vector clone of *folKE* showing insert release (Lane 1 – 1 kb DNA ladder, Lane 2 – insert release) (B) Restriction digestion of vector (Lane 1- 1 kb DNA ladder, Lane 2 – linearized vector)



(A) (B)

Fig. 8.11. Cloning of *folKE* gene into pNZ8148

(A) Isolation of recombinant plasmid (M - 1 kb DNA ladder, Lane 4 - recombinant plasmid (up shift) rest of the lanes contained vector without insert), (B) Clone confirmation by PCR (M – 1 kb DNA ladder, Lane 1 – *folKE* gene amplified from the recombinant plasmid), Lane 2 – control PCR using genomic DNA)



(A) (B) (C)

Fig. 8.12. Cloning of *folC* gene into pNZ8148

(A) Preparation of insert and vector for cloning of *folC* gene into pNZ8148 (Lane 1 – insert release, Lane 4 – 1 kb DNA ladder, Lane 6 – linearized vector) (B) Isolation of recombinant plasmid (Lane 1 – recombinant plasmid indicated by an up shift in the gel, Lane 2 – vector without insert) (C) Clone confirmation by PCR (M – 1 kb DNA ladder, Lane 2 – *folC* gene amplified from the recombinant plasmid)

The NICE system is a tightly controlled gene expression tool based on the auto-regulation mechanism of the nisin biosynthesis (Kuipers et al., 1995; Zhou et al., 2006). The activity of *nisA* promoter is dependent on a two component signal transduction system consisting of a histidine protein kinase, *NisK*, and a response regulator *NisR* that responds to the extracellular inducer nisin (Eichenbaum et al., 1998). When the gene of interest is placed under the control of P*nisA* promoter on the plasmid (eg. pNZ8148) and transformed into a *nisR/K* strain, expression of that particular gene can be induced by sub inhibitory amounts of nisin (Mierau & Kleerebezem, 2005). The binding of nisin to the N-terminal domain of *nisK* results in the autophosphorylation of *nisK*. This results in phosphorylation of *nisR* which then acts as a transcription activator of *nisA* and induces gene expression (Zhou et al., 2006).

8.4. Conclusion

The folate biosynthetic genes *folKE*, *folC* and *folA* were isolated from the indigenous isolate *L. lactis* CM28 and confirmed the identity by sequencing and BLAST analysis. Further, the bifunctional genes *folKE* and *folC* were cloned into the expression vector pNZ8148 and transformed into the host strain *L. lactis* NIZO9000. Subsequent studies on the expression of these genes by nisin induction are in progress. To express the folate genes in other hosts including the indigenous isolates *nisR/K* gene must be integrated into the chromosome and this is relatively complicated. To reduce the complexity of the NICE system implementation, a dual-plasmid NICE system consisting of two compatible replicons, a plasmid carrying the *nisR/K* regulatory genes (regulatory plasmid) and an expression vector carrying the gene of interest is required (Pavan et al., 2000). But the major limitation with this approach was reduced transformation efficiency as well as the slow growth of the derivatives. All these expression studies required more time and could not be completed during the time of this thesis. However, further studies in this area are being carried out by our research group and expecting promising results in the near future.