บทความวิจัย

การโคลนและการวิเคราะห์ลำดับ DNA ของยีน สำหรับ arginine deiminase จากแบคทีเรียแลคติก ที่ใช้ในอุตสาหกรรมอาหาร

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บทคัดย่อ

คณะผู้วิจัยทำการสังเคราะห์ชิ้นส่วน DNA จากแบคทีเรียแลคติกไอโซเลท SC8 ซึ่งเป็นสายพันธุ์ที่ใช้ในอุตสาหกรรมอาหาร จากการวิเคราะห์ลำดับ DNA ขนาด 4,149 bp ดังกล่าวพบว่า ประกอบด้วย 3 open reading frames ได้แก่ *arcA arcB* และ *arcD* (บางส่วน) ตามลำดับ เมื่อทำการวิเคราะห์ลำดับกรดอะมิโนที่แปลรหัสจาก *arcA* พบว่ามีความคล้ายคลึงกับลำดับกรดอะมิโนของเอนไซม์ arginine deiminase จากแบคทีเรียชนิดต่างๆ ที่มีรายงานใน Swiss-Prot Protein Sequence Database ทำให้เชื่อได้ว่า *arcA* น่าจะเป็นยีนสำหรับ arginine deiminase และจากการวิเคราะห์ลำดับกรดอะมิโนที่แปลรหัสจาก *arcB* พบว่ามีความเหมือนกับลำดับกรดอะมิโนของเอนไซม์ ornithine carbamoyltransferase จาก *Lactococcus lactis* subsp. *cremoris* MG1363 ถึง 100% โดยเอนไซม์ดังกล่าวมีการพิสูจน์หน้าที่มาแล้ว นอกจากนี้ในส่วน upstream ของ *arcA* ยังพบบริเวณที่น่าจะทำหน้าที่เป็นโปรโมเตอร์และบริเวณที่คล้ายกับโอเปอเรเตอร์อีก 2 แห่ง คือ Arg box และ Catabolite repression element (Cre) sequence

จากการวิเคราะห์ลำดับนิวคลีโอไทด์ของ 16S rDNA และการศึกษา restriction fragment length polymorphism ของชิ้นส่วนของยีน *gadB* จากเชื้อไอโซเลท SC8 ทำให้จำแนกได้ว่า เชื้อดังกล่าวน่าจะเป็น *Lactococcus lactis* subsp. *cremoris*

คำสำคัญ: แบคทีเรียแลคติก, *Lactococcus lactis,* arginine deiminase, ornithine carbamoyltransferase, glutamate decarboxylase, 16S rDNA

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Cloning and DNA Sequence Analysis of the Putative Arginine Deiminase Gene from a Commercial Strain of Lactic Acid Bacteria

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ABSTRACT

An attempt was made to identify the gene encoding arginine deiminase of a commercial strain of lactic acid bacteria, previously isolated and designated SC8. A DNA sequence of 4,149 bp containing at least 3 putative open reading frames: *arcA, arcB* and *arcD* (partial) was determined. Predicted amino acid sequence of *arcA* showed high similarity with arginine deiminase enzyme in several bacteria from the Swiss-Prot Protein Sequence Database, suggesting that *arcA* is a putative arginine deiminase gene. Translated sequence of *arcB* showed 100% identity to ornithine carbamoyltransferase from *Lactococcus lactis* subsp. *cremoris* MG1363, the function of which has already been established. In addition, putative -35 and -10 promoter regions, a putative Arg box and a putative Catabolite repression element (Cre) sequence were identified upstream from *arcA.*

Based on 16S rDNA sequence analysis and the restriction fragment length polymorphism of PCR-amplified *gadB* gene fragment, the isolate SC8 was identified as a member of *Lactococcus lactis* subsp. *cremoris.*

Keywords: lactic acid bacteria, *Lactococcus lactis,* arginine deiminase, ornithine carbamoyltransferase, glutamate decarboxylase, 16S rDNA

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Introduction

The arginine deiminase (ADI) pathway, formerly referred to as the arginine dihydrolase pathway, involves (i) irreversible conversion of arginine into citrulline and ammonia, (ii) phosphorolysis of citrulline generating ornithine and carbamoylphosphate in a reversible reaction, and (iii) reversible conversion of carbamoylphosphate and ADP into CO_2, ammonia and ATP [1]. These reactions are catalysed by the 3 enzymes: arginine deiminase, ornithine transcarbamylase (also known as ornithine carbamoyltransferase) and carbamate kinase, respectively [1].

The ADI pathway is of interest for a number of aspects. It was suggested that the ADI pathway in a variety of bacteria contributed to bacterial survival of potentially lethal acidification through the production of ammonia to raise the environmental pH values [2]. In addition, ammonia, generated from this pathway, along with acetoin can be used as substrates for the production of tetramethylpyrazine (TMP) [3], an important flavouring compound [4] with medicinal properties [5, 6], by several bacteria. In *Lactococcus lactis,* the ability of the bacteria to hydrolyse arginine (via the ADI pathway) is one of the characteristics used to distinguish between *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris,* in which the former possesses such ability, and the latter does not [7].

The genes involved in the ADI pathway from several bacteria have been characterised. In lactic acid bacteria (LAB), in particular, the genes of *Lactobacillus sakei* [8, 9], *L. hilgardii* [10], *Lactococcus lactis* [11], *Streptococcus gordonii* [12], *S. rattus* [13], *Enteroccoccus faecalis* [14] and *Oenococcus oeni* [15] were reported. The gene for arginine deiminase enzyme *(arcA)* is of particular interest. Since the enzyme plays a role in ammonia generation in an irreversible reaction, over-expression of *arcA* in a high acetoin-producing strain should enhance the production of TMP [11].

Previously, several strains of LAB isolated from fermented food products, which were capable of acetoin production and arginine utilization, were selected. A putative arginine deiminase gene fragment, 670 bp in length, was amplified from one of the isolates, designated SC8 [16]. In this article, cloning of the complete arginine deiminase gene and its downstream region, and analyses of the gene sequences thereof were described. Identification of the isolate SC8 at the species and subspecies levels was also reported.

Materials and Methods

Bacterial Strains, Media and Plasmid

LAB isolate SC8 was grown statically in MRS medium [17] at 30°C. *Escherichia coli* TOP10 (Invitrogen, California, USA), used as a recipient for recombinant plasmid construction, was grown in Luria Bertani (LB) broth [18] supplemented with 100 µg/ml ampicillin, at 37°C with continuous agitation.

Plasmid pCR2.1-TOPO (Invitrogen, California, USA) was used for cloning of PCR products.

Chromosome Extraction

The method used for extraction of LAB chromosome was a modification of that described by Lewington *et al.* [19]. A 10-ml overnight culture was harvested by centrifugation. The pellet was resuspended in 240 µl of 0.25 M sucrose and 50 mM Tris-HCl (pH 8.0). The suspension was mixed with 50 µl of 10 mg/ml lysozyme and incubated at 37°C for 10 min. The sample was then added with 160 µl of 20% sodium dodecyl sulfate (prewarmed to 37°C), and 80 µl of ice cold 5 M NaCl, consecutively, and incubated on ice for 1 h. Following centrifugation, the supernatant was collected, and subjected to 2 extractions with phenol/ chloroform (1: 1) and 1 extraction with chloroform. The DNA was precipitated by adding 0.1 volume of 3 M sodium acetate and 2 volume of absolute ethanol. The DNA was recovered by centrifugation, and redissolved in sterile DNase-free water.

Polymerase Chain Reaction (PCR)

PCR was conducted with a Mastercycler (Eppendorf, Hamburg, Germany). A 50 µl PCR mixture contained 250 ng DNA template, 1× PCR buffer supplemented with 2 mM of each dNTP, 50 pmol of each primer, and 0.4 U of *Taq* DNA polymerase, in sterile DNase-free water. The mixture was subjected to 30 cycles of denaturation at 92°C for 2 min, primer annealing at 48°C for 2 min, and extension at 72°C for 2-3 min, with an additional extension at 72°C for 10 min following the last cycle.

Oligonucleotide primers used in this research were obtained from BioService Unit, Bangkok, Thailand. The nucleotide sequences of the primers are listed in Table 1.

Construction of Recombinant DNA and Transformation

Cloning of PCR products into pCR2.1-TOPO and transformation of One Shot TOP10 Chemically Competent *E. coli* (*E. coli* TOP10) with the recombinant plasmid were carried out using TOPO TA cloning kit (Invitrogen, California, USA), according to the instructions of the manufacturer.

Table 1 List of primers and their nucleotide sequences.

Primer	Nucleotide sequence	Target	Reference
rdi-fwd1 (forward)	5′-CTTCTTGGGGTTGCTGC-3′	ADI gene cluster	This study
rdi-rev1 (reverse)	5′-AATACTGAGTTTACTTCTGC-3′	ADI gene cluster	This study
rdi-fwd2 (forward)	5′-GGAAAAAATAATGACATCACC-3′	ADI gene cluster	This study
rdi-rev2 (reverse)	5′-TGTTTTCTTCTCTTGAATCCC-3′	ADI gene cluster	This study
fD1 (forward)	5′-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3′	16S rDNA	[23]
rD1 (reverse)	5′-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC-3′	16S rDNA	[23]
gadB21 (forward)	5′-CGTTATGGATTTGATGGATATAAAGC-3′	*gadB*	[25]
GAD7 (reverse)	5′-ACTCTTCTTAAGAACAAGTTTAACAGC-3′	*gadB*	[25]

Plasmid Isolation

Preparation of plasmid DNA was carried out using Wizard Plus SV Minipreps DNA Purification System (Promega, Wisconsin, USA), according to the instructions of the manufacturer.

DNA Sequencing and DNA Sequence Analysis

DNA sequencing of recombinant DNA was performed by BioService Unit, Bangkok, Thailand. Nucleotide sequence data were analysed using programmes from the European Bioinformatics Institute, Cambridge, UK (http://www.ebi.ac.uk/Tools/).

Results

Cloning of the Putative Arginine Deiminase Gene and Its Downstream Region

In a previous study, a PCR fragment of 670 bp was amplified from LAB isolate SC8, and its sequence showed 99.786% identity to arginine diminase gene of *Lactococcus lactis* subsp. *cremoris* MG1363 [16]. In order to amplify the complete arginine deiminase gene and its downstream region from SC8, 2 sets of primers were designed, based on DNA sequence of the genes encoding components of the ADI pathway of *L. lactis* subsp. *cremoris* MG1363 (EMBL

accession number AJ250129), as follows: rdi-fwd1 (forward), nucleotide position 759-775; rdi-rev1 (reverse), 2,533-2,514; rdi-fwd2 (forward), 2,480-2,500; and rdi-rev2 (reverse), 4,906-4,886. Two sets of PCR were carried out: one with primers rdi-fwd1 and rdi-rev1, and the other with primers rdi-fwd2 and rdi-rev2, using chromosomal DNA of SC8 as a template. DNA fragments of 1.7 and 2.4 kb in size, as expected, were generated by the 2 reactions, respectively. The DNA fragments were then cloned into pCR2.1-TOPO, and sequenced. The 2 DNA sequences were assembled, and a total of 4,149 bp of DNA sequence was accomplished.

DNA Sequence Analysis

Analysis of the 4,149 bp DNA sequence was carried out using programmes from the European Bioinformatics Institute. Three open reading frames: *arcA, arcB,* and *arcD* (partial), respectively, were identified. The DNA sequences of the open reading frames were translated, using Transeq programme. The 4,149 bp DNA sequence and translated sequences of the open reading frames are shown in Figure 1.

For each predicted amino acid sequence, search for homologous sequences from the Swiss-Prot Protein Sequence Database was performed using Fasta3 programme. Protein sequences from the database that are highly similar to the translated sequences are listed in Table 2. Search of recognisable protein motifs on each translated sequence was carried out using PPSearch programme. As a result, an aspartate and ornithine carbamoyltransferase signature (FAKTSTRT) was identified on translated *arcB* sequence (Figure 1).

Putative -35 and -10 promoter regions preceding *arcA* and putative ribosome binding sites [20], preceding the start codons, were identified manually (Figure 1). In addition, in the vicinity of the promoter, an operator-like region highly resembling the Catabolite repression element (Cre) sequence of Gram-positive bacteria [21] and a putative Arg box resembling the consensus Arg box sequence of *E. coli* [22] were found (Figure 1).

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                                                    Arg box
  1 CTTCTTGGGGTTGCTGCTCCTGAAAATATGTAAAAATATACTTAATTATTTAATCTCTTA
 61 TCAAAAAGAATTTAATTCTTGCTGATGAGAGATTTTTTTGTTTATTTTTTAAAACATTTT
121 GTAAAATAAGTATTTTAATAAAAAACTAAGAATCCCATGATAAGCAAAGGAGTGCATAAA
181 CGTGAAATTGTCAGAATATAAAAAAACATGTATAAATGAATAAACCTTTGTAAAGTGTAG

             -35                        -10        Cre sequence
241 CATTAGTGCTCGACAAAAAATATGCATAGATGTATAATTTCCTTGTAAACGATTCTAATA
301 ATACTGAATCGAAATCAGAAAGGATACTCCAAAATGAACAATGGAATTAATGTTAACTCA
                                  M  N  N  G  I  N  V  N  S

361 GAAATTGGGAAATTAAAATCAGTCCTTCTCCACCGCCCAGGTGCAGAGGTAGAGAATATT
    E  I  G  K  L  K  S  V  L  L  H  R  P  G  A  E  V  E  N  I

421 ACCCCAGACACAATGAAACAGCTTTTATTTGATGATATTCCATATCTCAAAATTGCTCAA
    T  P  D  T  M  K  Q  L  L  F  D  D  I  P  Y  L  K  I  A  Q
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 481 AAAGAGCATGATTTCTTTGCTCAAACATTGCGTGACAATGGTGCTGAAACTGTTTATATC
     K  E  H  D  F  F  A  Q  T  L  R  D  N  G  A  E  T  V  Y  I

 541 GAAAATCTTGCAACAGAAGTTTTTGAAAAATCATCTGAAACAAAAGAAGAGTTTTTAAGC
     E  N  L  A  T  E  V  F  E  K  S  S  E  T  K  E  E  F  L  S

 601 CATTTGTTGCATGAAGCAGGTTACCGTCCAGGACGTACTTATGATGGATTGACTGAATAT
     H  L  L  H  E  A  G  Y  R  P  G  R  T  Y  D  G  L  T  E  Y

 661 TTAACTTCAATGCCAACAAAAGATATGGTTGAAAAAGTCTATGCCGGTGTTCGTAAAAAT
     L  T  S  M  P  T  K  D  M  V  E  K  V  Y  A  G  V  R  K  N

 721 GAATTGGATATCAAACGCACAGCACTTAGTGACATGGCAGGTTCTGATGCAGAAAATTAT
     E  L  D  I  K  R  T  A  L  S  D  M  A  G  S  D  A  E  N  Y

 781 TTCTACCTCAACCCATTACCAAATGCTTACTTCACACGTGACCCACAAGCTTCAATGGGT
     F  Y  L  N  P  L  P  N  A  Y  F  T  R  D  P  Q  A  S  M  G

 841 GTCGGAATGACTATTAATAAAATGACTTTCCCAGCACGTCAACCTGAAAGCTTGATTCCC
     V  G  M  T  I  N  K  M  T  F  P  A  R  Q  P  E  S  L  I  P

 901 GAATATGTGATGGCTAACCATCCACGTTTCAAAGACACTCCAATCTGGCGTGATCGTAAT
     E  Y  V  M  A  N  H  P  R  F  K  D  T  P  I  W  R  D  R  N

 961 CATACTACTCGTATTGAAGGTGGTGATGAATTAATTCTTAATAAGACAACTGTAGCAATC
     H  T  T  R  I  E  G  G  D  E  L  I  L  N  K  T  T  V  A  I

1021 GGGGTTTCAGAACGTACTTCATCTAAAACAATTCAAAATCTTGCTAAAGAATTATTTGCA
     G  V  S  E  R  T  S  S  K  T  I  Q  N  L  A  K  E  L  F  A

1081 AATCCACTTTCAACATTTGATACAGTGCTTGCGGTTGAAATCCCTCATAACCATGCAATG
     N  P  L  S  T  F  D  T  V  L  A  V  E  I  P  H  N  H  A  M

1141 ATGCACTTGGATACTGTATTTACAATGATTAACCATGATCAATTTACAGTTTTCCCAGGA
     M  H  L  D  T  V  F  T  M  I  N  H  D  Q  F  T  V  F  P  G

1201 ATTATGGATGGTGCAGGTAACATCAACGTCTTCATTCTTCGTCCTGGTCAAGATGGTGAA
     I  M  D  G  A  G  N  I  N  V  F  I  L  R  P  G  Q  D  G  E

1261 GTTGAAATTGAACATTTGACAGATCTTAAAGCAGCCCTTAAGAAAGTCTTGAACCTTTCA
     V  E  I  E  H  L  T  D  L  K  A  A  L  K  K  V  L  N  L  S

1321 GAACTTGACTTGATTGAATGTGGTTCAGGTGACCCAATTGCCGCTCCTCGTGAACAATGG
     E  L  D  L  I  E  C  G  S  G  D  P  I  A  A  P  R  E  Q  W

1381 AATGATGGTTCAAATACCCTTGCTATTGCTCCAGGAGAAATCGTTACTTATGACCGTAAC
     N  D  G  S  N  T  L  A  I  A  P  G  E  I  V  T  Y  D  R  N

1441 TATGTAACTGTTGAACTTTTGAAAGAACATGGTATTAAAGTTCATGAAATTCTTTCAAGT
     Y  V  T  V  E  L  L  K  E  H  G  I  K  V  H  E  I  L  S  S

1501 GAACTTGGTCGTGGTCGTGGTGGAGCGCGTTGTATGTCACAACCACTTTGGCGTGAAGAT
     E  L  G  R  G  R  G  G  A  R  C  M  S  Q  P  L  W  R  E  D

1561 TTGTAATCTGAAGACTTTTACATAATGTTCTTGAATTTAACTACTAAATTTAAAAACAAG
     L  *

1621 TGAATTTATAAATTTAGTTTTATAAGTTCGTAAAATTAAAGATGAAAGTAGCTCATTAAT
1681 GTAATTTAAAAAATTCATTAAATATTTAAAAATAGAA AAAGGA AAAAAATAATGACATCA
                                                    M  T  S

1741 CCACTTATTACAAAAGCAGAAGTAAACTCAGTATTCCAAGGTCGTAGCTTGCTTGCTGAA
     P  L  I  T  K  A  E  V  N  S  V  F  Q  G  R  S  L  L  A  E

1801 AAAGATTTTACACCAGCTGAAATTAACTACCTTGTTGATTTTGGTCTTCATTTGAAAGCA
     K  D  F  T  P  A  E  I  N  Y  L  V  D  F  G  L  H  L  K  A
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1861 TTGAAACAACAAAATATTCCTCATCACTATCTTGAAGGTAAAAATATTGCCTTGTTATTT
     L  K  Q  Q  N  I  P  H  H  Y  L  E  G  K  N  I  A  L  L  F

1921 GCAAAAACTTCAACTCGTACACGTGCCGCATTTACAACTGCTGCCATTGACCTTGGTGCT
     A  K  T  S  T  R  T  R  A  A  F  T  T  A  A  I  D  L  G  A

1981 CATCCTGAATATCTTGGTGCAAATGATATCCAACTCGGAATCAAAGAATCAACAGAAGAT
     H  P  E  Y  L  G  A  N  D  I  Q  L  G  I  K  E  S  T  E  D

2041 ACAGCACGTGTTCTTGGTTCAATGTTTGATGCTATTGAACGTCGTGGATTTTCTCAAAAA
     T  A  R  V  L  G  S  M  F  D  A  I  E  R  R  G  F  S  Q  K

2101 GAAGTTGAAGATTTGGCAAAATACTCTGGTGTTCCAGTTTGGAATGGTTTGACAGATGAT
     E  V  E  D  L  A  K  Y  S  G  V  P  V  W  N  G  L  T  D  D

2161 TGGCATCCAACACAAATGATTGCTGACTTTATGACGGTAAAAGAAAACTTTGGTTACCTT
     W  H  P  T  Q  M  I  A  D  F  M  T  V  K  E  N  F  G  Y  L

2221 AAAGGGTTGACATTAGTTTACGTTGGTGATGGTCGTAACAACATGGCAAATTCACTCATC
     K  G  L  T  L  V  Y  V  G  D  G  R  N  N  M  A  N  S  L  I

2281 GTAACTGGTTCTATGCTTGGTGTAAATGTTCATATCGTTGCTCCAGATTCACTTCATCCT
     V  T  G  S  M  L  G  V  N  V  H  I  V  A  P  D  S  L  H  P

2341 TCTAAAGAAGTTATGGATATTGCCAATAAATTTGCTGAAAAATCAGGTGCTAAACCTCTT
     S  K  E  V  M  D  I  A  N  K  F  A  E  K  S  G  A  K  P  L

2401 GCAACTTCTAATATTGAAGAAGGTGTTAAAGGTGCTAACATTATTTATTCAGACGTTTGG
     A  T  S  N  I  E  E  G  V  K  G  A  N  I  I  Y  S  D  V  W

2461 GTATCTATGGGAGAATCTAACTGGGAAGAACGTGTTAAACTTTTGACACCATACCGCATC
     V  S  M  G  E  S  N  W  E  E  R  V  K  L  L  T  P  Y  R  I

2521 ACAATGGATATGTTGAAAATGACAGGAAATGCTGACAACGGTAAACTTATCTTTATGCAC
     T  M  D  M  L  K  M  T  G  N  A  D  N  G  K  L  I  F  M  H

2581 TGCTTACCAGCCTTCCATGACACTGAAACTGAATATGGTAAAGAAATCAAAGAAAAATAT
     C  L  P  A  F  H  D  T  E  T  E  Y  G  K  E  I  K  E  K  Y

2641 GGTTTGACAGAAATGGAAGTTACTGACGAAGTTTTCCGTTCTAAATATGCTCGTCAATTT
     G  L  T  E  M  E  V  T  D  E  V  F  R  S  K  Y  A  R  Q  F

2701 GAAGAAGCAGAAAATCGTATGCACTCAATCAAAGCAATTATGGCTGCAACTTTGGGTAAT
     E  E  A  E  N  R  M  H  S  I  K  A  I  M  A  A  T  L  G  N

2761 TTATTTATCCCAGCAGTTCCTGAAGATTTTAAATAATTAAAATAGATAATTTGTCAGTTT
     L  F  I  P  A  V  P  E  D  F  K  *

2821 ACTGACAGAGCTGTCAGTAAACTGATAGATTTTGATTTAAGTCGTTGAAAGCAGTCTAAG
2881 ATAGTTTTTAGACTGTTTTGACGGTTAAAATCATTGAAAGGATTGATATGGACGCAGAAA
                                                  M  D  A  E  N

2941 ATAAAAAAGGAATTGGCCTTGCTGCTTTAGTTGCGATTATTGTTTCTGGAGCGATTGGTG
       K  K  G  I  G  L  A  A  L  V  A  I  I  V  S  G  A  I  G  G

3001 GTGGGGTATTCAACCTCTCTAACGATTTAGCAACAAATGCATCCACCTGGTGGTGTTGTC
       G  V  F  N  L  S  N  D  L  A  T  N  A  S  P  G  G  V  V

3061 ATTTCATGGATCGTTATTGGTTTTGGTATATTAATGTTGGTTTTATCACTGAATCATTTG
     I  S  W  I  V  I  G  F  G  I  L  M  L  V  L  S  L  N  H  L

3121 GTGGTAAACAAACCTGAACTTTCAGGTGTGTCAGATTATGCACGTGCTGGTTTTGGTAAT
     V  V  N  K  P  E  L  S  G  V  S  D  Y  A  R  A  G  F  G  N

3181 ATGGTTGGTTTTATCTCAGGATGGGGTTATTGGTTATCTGCTTGGGCAGGTAATATTGCC
     M  V  G  F  I  S  G  W  G  Y  W  L  S  A  W  A  G  N  I  A
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3241 TTTGCTGTTTTGATGATGACTGCCGTTGACTATTTCTTCCCCGGCGTTTTTCAAGCTAAG
     F  A  V  L  M  M  T  A  V  D  Y  F  F  P  G  V  F  Q  A  K

3301 AATGGCTCATTGACCATTCTGTCAGTAATTGTTGTTTCAATTGTTTCGTGGGGATTGACT
     N  G  S  L  T  I  L  S  V  I  V  V  S  I  V  S  W  G  L  T

3361 TTACTTGTTATGCGAGGTGTTGAAGGGGCTGCTGCCATTAATGCAATTGTCCTTGTTGCA
     L  L  V  M  R  G  V  E  G  A  A  A  I  N  A  I  V  L  V  A

 421 AAATTGATTCCCTTGTTTGTCTTTGTTATTGCTGGAATAGTTACTTTCAAAGCTGGAGTC
     K  L  I  P  L  F  V  F  V  I  A  G  I  V  T  F  K  A  G  V

3481 TTTAGTGCTCACTTTTGGCAAAATTTCGTTGCGAATACAAATGCTGATGGCGTAATTAAG
     F  S  A  H  F  W  Q  N  F  V  A  N  T  N  A  D  G  V  I  K

3541 AGTTTAACTTGGTCTAATATGACTGGCGGAGATTTATTCAGTCAAGTCAAAGGTTCACTC
     S  L  T  W  S  N  M  T  G  G  D  L  F  S  Q  V  K  G  S  L

3601 ATGGTTATGATTTGGGTCTTCGTCGGAATCGAAGGGGCTGCTATGATGGGTGACCGTGCC
     M  V  M  I  W  V  F  V  G  I  E  G  A  A  M  M  G  D  R  A

3661 AAACGTAAATCAGATGCTGGTAAAGCTTCAATTCTCGGTTTGATTGCCTTGTTAGTGATT
     K  R  K  S  D  A  G  K  A  S  I  L  G  L  I  A  L  L  V  I

3721 TATATCTTGCTCTCACTATTGCCATTTGGTTTTATGAGCCAACAAGAACTAGCTAATACT
     Y  I  L  L  S  L  L  P  F  G  F  M  S  Q  Q  E  L  A  N  T

3781 GGTCAACCAGGTTTGGTTCATATTTTGAACGCTATGGTTGGCGGTTGGGGTGGTTCACTA
     G  Q  P  G  L  V  H  I  L  N  A  M  V  G  G  W  G  G  S  L

3841 ATGGCCATTGGTCTTGTGATTTCACTTCTTGGAGCTTGGTTGTCATGGACAATGCTTCCC
     M  A  I  G  L  V  I  S  L  L  G  A  W  L  S  W  T  M  L  P

3901 GTTGAAGCAACACAACAATTATCAGAACAAAAATTACTTCCTAGTTGGTTTGGTAAACTT
     V  E  A  T  Q  Q  L  S  E  Q  K  L  L  P  S  W  F  G  K  L

3961 AATGACAAAGGTGCACCTAAAAATTCACTTTTGCTGACACAATTGATTGTTCAAATTTTC
     N  D  K  G  A  P  K  N  S  L  L  L  T  Q  L  I  V  Q  I  F

4021 TTGATTGTCACTTATTTTGTAGCTGATGCTTACAATGTCTTTGTTTACCTTTGTACCGCT
     L  I  V  T  Y  F  V  A  D  A  Y  N  V  F  V  Y  L  C  T  A

4081 GTTATCATGATTTGTTATGCCTTAGTTGGTTTATATCTCTTTAAATTAGGGATTCAAGAG
     V  I  M  I  C  Y  A  L  V  G  L  Y  L  F  K  L

4141 AAGAAAACA
```

Figure 1 The 4,149 bp DNA sequence and translated sequences of the open reading frames (including stop codons): *arcA,* position 334-1,566; *arcB,* 1,732-2,796; and *arcD* (partial) 2,928-4,128. Nucleotide at position 3,043 (in bold print and underlined), possibly a PCR generated error, was excluded from translation. Binding sites for primers rdi-fwd1 and rdi-rev2 at the beginning and the end of the sequence, respectively, are underlined. Putative -35 and -10 promoter regions, putative Arg box and putative Cre sequence, all preceding *arcA,* are double-underlined. Putative ribosome binding sites, preceding each start codon, are shown in bold print. Aspartate and ornithine carbamoyltransferase signature (FAKTSTRT) on translated *arcB* sequence is in bold print.

Table 2 Amino acid sequences from the Swiss-Prot Protein Sequence Database that are homologous to the translated sequences of the open reading frames in SC8.

Translated sequence	% similarity	% identity	Organism/ gene product	Size (amino acids)	Swiss-Prot accession number
ArcA	99.756	99.512	*Lactococcus lactis* subsp. *cremoris* MG1363/ arginine deiminase	410	Q9K576
	99.268	98.537	*Lactococcus lactis* subsp. *lactis* IL1403/arginine deiminase	410	P58013
	83.373	60.000	*Lactobacillus hilgardii* X1B/ arginine deiminase	418	Q8G999
	83.619	59.902	*Lactobacillus sakei*/arginine deiminase	409	O53088
	82.169	56.386	*Bacillus cereus* ATCC10987/ arginine deiminase	410	Q73E87
ArcB	100.000	100.000	*Lactococcus lactis* subsp. *cremoris* MG1363/ornithine carbamoyltransferase	354	Q9K575
	99.718	99.718	*Lactococcus lactis* subsp. *lactis* ML3/ornithine carbamoyltransferase	354	P0C2U1
	100.000	99.153	*Lactococcus lactis* subsp. *lactis* IL1403/ornithine carbamoyltransferase	354	P0C2U0
	92.733	75.000	*Lactobacillus hilgardii* X1B/ ornithine carbamoyltransferase	344	Q8G998
	92.582	76.261	*Lactobacillus sakei*/ornithine carbamoyltransferase	337	O53089
ArcD	82.952	51.908	*Lactobacillus sakei*/arginine/ ornithine antiporter	478	O53092
	76.203	41.013	*Clostridium perfringens*/ arginine/ornithine antiporter	478	Q46170
	73.367	35.678	*Clostridium perfringens*/ Putative amino acid transporter	481	P0C217

16S rDNA Sequence Analysis

PCR amplification of 16S rDNA fragment of SC8 was carried out using primers fD1 and rD1, as described by Weisburg *et al.* [23]. The PCR fragment (of 1,572 bp) was cloned into pCR2.1-TOPO and sequenced. Analysis of the DNA sequence, using FASTA programme, revealed high similarity with 16S rDNA sequences of several strains of *Lactococcus lactis* from the European Molecular Biology Laboratory (EMBL) database, as shown in table 3. Since it has been generally accepted that 99% similarity can be used as a cutoff value for 16S rDNA-based bacterial identification at the species level [24], on this basis, we identified SC8 as a member of the species *L. lactis.*

Table 3 16S rDNA sequences of several organisms from the EMBL Database that are highly similar to the 16S rDNA sequence of SC8 (1,502 bp in size, fD1-and rD1-binding sites excluded).

Organism	EMBL accession No.	DNA size (bp)	% identity
Lactococcus lactis subsp. *lactis* IL1403	AE006456	1502	99.933
Lactococcus lactis subsp. *lactis* SCC43K	AY626141	1502	99.867
Lactococcus lactis SL3	AY675242	1502	99.867
Lactococcus lactis subsp. *lactis* NCDO 604T	AB100803	1499*	99.933
Lactococcus lactis subsp. *lactis* bv. diacetylactis ATCC 13675	AB100805	1499*	99.933
Lactococcus lactis subsp. *lactis* bv. diacetylactis NIAI N-7	AB100799	1499*	99.933
Lactococcus lactis subsp. *lactis* bv. diacetylactis NIRD DRC-2	AB100801	1499*	99.933
Lactococcus lactis subsp. *lactis* bv. diacetylactis NIRD DRC-1	AB100800	1499*	99.933
Lactococcus lactis SL1	AY675241	1502	99.800
Lactococcus lactis subsp. *lactis* NIAI 527	AB100795	1499*	99.867
Lactococcus lactis subsp. *cremoris* MG1363	AM406671	1502	99.268

Note: * Partial sequence; no gap was added upon the analysis.

Identification of SC8 Based on *gadB* Gene Fragment

Lactococcus lactis SC8 was further identified at the subspecies level, based on the method described by Nomura *et al.* [25]. According to this method, the 2 subspecies of *Lactococcus lactis* can be distinguished by the restriction fragment length polymorphism of PCR-amplified glutamate decarboxylase gene *(gadB)* fragment, in which the PCR fragment generated from *L. lactis* subsp. *lactis* is ~600 bp in size digestible with *Ase*I, whereas that of *L. lactis* subsp. *cremoris* is ~560 bp without *Ase*I restriction site (except *L. lactis* subsp. *cremoris* MG1363).

A PCR fragment was generated from SC8 chromosomal DNA using primers gadB21 and GAD7. The fragment was 564 bp in size, and *Ase*I site was not presence in the fragment (Figure 2). These results concurred with the characteristics of *L. lactis* subsp. *cremoris* (Table 4), therefore suggesting that isolate SC8 is probably a member of *L. lactis* subsp. *cremoris.*

In addition, the DNA sequence was translated, and the amino acid sequence there of was compared against the Swiss-Prot Protein Sequence Database using Fasta3 programme. It was found that the translated sequence showed high similarity with the sequences of several glutamate decarboxylase enzymes from the database (data not shown), the highest being with the enzymes from *L. lactis* subsp. *cremoris* MG1363 (Swiss-Prot accession number O30418) (94.915% similarity, 89.831% identity) and *L. lactis* subsp. *lactis* IL1403 (Swiss-Prot accession number Q9CG20) (94.915% similarity, 86.441% identity), respectively.

```
  1 CGTTATGGATTTGATGGATATAAAGCTATTCATGAGAGAACGCATAAAGTAGCCATGTAT
                               I  H  E  R  T  H  K  V  A  M  Y

 61 TTAGCAGAAGAAATTGAAAAAACAGGAATGTTTGAGATTATGAACGATTGGTCACAATTG
    L  A  E  E  I  E  K  T  G  M  F  E  I  M  N  D  W  S  Q  L

121 CCAATTGTCTGCTACAAATTAAAAGAAAATTCAAATCTTGGTTGGAATCTTTATGATTTG
    P  I  V  C  Y  K  L  K  E  N  S  N  L  G  W  N  L  Y  D  L

181 GCAGATCGTTTATTTAATGAAGGGATGGCAAGTGCCTGCTTATCCACTTCCTAAAAATTT
    A  D  R  L  F  N  E  G  M  A  S  A  C  L  S  T  S  *

241 GGAAAATGAAATCATTCAACGTTTAGTAATTCGAGCAGATTTCGGGATGAATATGGCATT
301 TAACTATGTTCAAGATATACAAGAAGCAATTGATGCACTAAACAAGGCTCATATTCTATT
361 TCATCAGGAACCTGAAAATAAAACATATGGCTTTACTCACTAAAAATAAAAGCGATATAT
421 CTTAGATATATCGCTTTTATTTTATTTTAAGCTATTTACTAATTAGCTTATCGCCTATTC
481 TTTTCATAGTATTTATCCAAAATTTCCATTTTTAAAGGAGTAATTTTAGATAGAGGGGCT
541 GTTAAACTTGTTCTTAAGAAGAGT
```

Figure 2 The 564 bp PCR-amplified *gadB* fragment of SC8 and its predicted amino acid sequence. Binding sites for primers gadB21 and GAD7 at the beginning and the end of the sequence, respectively, are underlined. The location where the presence or absence of *Ase*I restriction site (ATTAAT) is determined, is double underlined.

Table 4 Comparison of *gadB* fragments from different subspecies of *L. lactis*

Organism	*gadB* fragment size		Digestion with *Ase*I	Reference
	~ 600 bp	~ 560 bp		
L. lactis subsp. *lactis*				
ATCC 9936	+	-	+	[25]
ATCC 19435	+	-	+	[25]
IL1403	+	-	+	[25]
NIAI 527	+	-	+	[25]
L. lactis subsp. *lactis* bv. diacetylactis				
ATCC 13675	+	-	+	[25]
DRC1	+	-	+	[25]
NIAI 01-7	+	-	+	[25]
L. lactis subsp. *cremoris*				
ATCC 19257	-	+	-	[25]
H-61	-	+	-	[25]
HP	-	+	-	[25]
MG1363	-	+	+	[25]
L. lactis SC8	-	+	-	This study

DNA Sequence Accession Numbers

The DNA sequences reported in this article have been submitted to the EMBL database, and have been assigned the following accession numbers: ADI gene cluster, AM944596; 16S rDNA, AM944595; and *gadB* fragment, AM944594.

Conclusions and Discussion

Previously, a PCR-amplified DNA fragment was generated from a commercial strain of LAB, designated SC8. The PCR fragment was suggested to contain an internal fragment of arginine deiminase gene, as its sequence showed high similarity with the gene from several bacteria from the EMBL database [16]. Since the PCR fragment was 99.786% identical to arginine deiminase gene from *Lactococcus lactis* subsp. *cremoris* MG1363 [11], 2 sets of primers were designed from the gene sequence of *L. lactis* subsp. *cremoris* MG1363 (EMBL accession number AJ250129) for PCR amplification of putative arginine deiminase gene and its downstream region from SC8. Two DNA fragments generated by the PCRs were cloned and

sequenced. The DNA sequences were assembled, and a total of 4,149 bp of DNA sequence was accomplished. Analysis of the 4,149 bp DNA sequence revealed 3 open reading frames, namely *arcA, arcB* and *arcD* (partial), respectively.

Predicted amino acid sequence of *arcA* (designated ArcA) showed high similarity with arginine deiminase enzyme from several bacteria, particularly those of *L. lactis* subsp. *cremoris* MG1363 (99.756% similarity, 99.512% identity) and *L. lactis* subsp. *lactis* IL1403 (99.268% similarity, 98.537% identity). This result strongly suggested that *arcA* is a putative arginine deiminase gene.

Translated sequence of *arcB* (designated ArcB) showed 100% identity to ornithine carbamoyltransferase from *L. lactis* subsp. *cremoris* MG1363, the function of which had already been established [11]. In addition, an aspartate and ornithine carbamoyltransferase signature (FAKTSTRT) was also found on the sequence. Therefore, it can be concluded that *arcB* encodes an ornithine carbamoyltransferase gene.

The presence of a putative promoter upstream from *arcA* and the absence of a putative promoter upstream from *arcB* suggest that *arcA* and *arcB* may be co-transcribed. In addition, it was suggested that in Gram-positive bacteria Cre sequence may be part of a global catabolite repression system, in which a repressor, activated by the presence of glucose or other readily metabolized carbohydrates, would bind to the Cre region and block the transcription [21]. Therefore, the presence of a Cre sequence in the vicinity of the promoter suggests that transcription of *arcAB* may be repressed in the presence of glucose. In Gram-positive bacteria, arginine repressor was reported to have 2 functions: as a repressor for arginine biosynthetic enzymes and an activator for the catabolic enzymes [22]. In *Bacillus licheniformis,* an Arg box sequence was identified upstream from the *arc* promoter, and it was demonstrated that arginine repressor binds to the Arg box in the presence of arginine [26]. Therefore, the presence of an Arg box upstream from the promoter suggests that an arginine regulator protein may play a role in transcription activation of *arcAB* in the presence of arginine. This finding is consistent with the study of Crow and Thomas [1] which reported that arginine deiminase and ornithine transcarbamylase activities of *L. lactis* can be induced by glucose limitation, and by the presence of arginine.

Predicted amino acid sequence of *arcD* (designated ArcD) showed high similarity with arginine/ornithine antiporters from *Lactobacillus sakei* and *Clostridium perfringens,* with 82.952% and 76.203% similarity, respectively. At the DNA level, however, *arcD* sequence shows over 94% identity to arginine/ornithine antiporter genes from several *L. lactis* (data not shown). These results strongly suggested that *arcD* is a putative arginine/ornithine antiporter gene.

SC8 was identified as a member of *L. lactis* based on 16S rDNA sequence analysis. It is interesting to note that 16S rDNA sequence of SC8 was highly similar to those of *L. lactis* subsp. *lactis* compared with those of *L. lactis* subsp. *cremoris,* and SC8, being able to utilize arginine [16], would traditionally have been identified as *L. lactis* subsp. *lactis* [7]. However, bacterial identification at the subspecies level based on 16S rDNA divergence has not been established, and, according to the restriction fragment length polymorphism of PCR-amplified *gadB* fragment, SC8 showed characteristics of *L. lactis* subsp. *cremoris,* rather than those of *L. lactis* subsp. *lactis.* Therefore, at this stage, we were inclined to conclude that SC8 is a member of *L. lactis* subsp. *cremoris.* Similarly, *L. lactis* MG1363, an Arg+ strain, was initially identified as *L. lactis* subsp. *lactis.* However, it was later found that, on the basis of DNA homology, this stain is closely related to *L. lactis* subsp. *cremoris* rather than *L. lactis* subsp. *lactis,* and, therefore, it was reclassified as *L. lactis* subsp. *cremoris* [27].

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References

1. Crow, V. L. and Thomas, T. D. 1982. Arginine Metabolism in Lactic Streptococci. *Journal of Bacteriology* 150(3): 1024-1032.
2. Marquis, R. E., Bender, G. R., Murray, D. R. and Wong, A. 1987. Arginine Deiminase System and Bacterial Adaptation to Acid Environments. *Applied and Environmental Microbiology* 53(1): 198-200.
3. Demain, A. L., Jackson, M. and Trenner, N. R. 1967. Thiamine-dependent Accumulation of Tetramethylpyrazine Accompanying a Mutation in the Isoleucine-Valine Pathway. *Journal of Bacteriology* 94(2): 323-326.
4. Maga, J. A. and Sizer, C. E. 1973. Pyrazines in Foods. *CRC Critical Reviews in Food Technology* 4: 39-115.
5. Feng, J., Liu, R., Wu, G. and Tang, S. 1996. Pretreatment with Tetramethylpyrazine Increases the Release of PGI_2 and Decreases TXA_2 Release in Isolated Rat Heart. *Planta Medica* 62: 379-381.
6. Pang, P. K. T., Shan, J. J. and Chiu, K. W. 1996. Tetramethylpyrazine, a Calcium Antagonist. *Planta Medica* 62: 431-435.

7. Schleifer, K. H., Kraus, J., Dvorak, C., Kilpper-balz, R., Collins, M. D. and Fischer, W. 1985. Transfer of *Streptococcus lactis* and Related Streptococci to the Genus *Lactococcus* gen. nov. *System. Appl. Microbiol.* 6: 183-195.
8. Zuniga, M., Champomier-Verges, M., Zagorec, M. and Perez-Martinez, G. 1998. Structural and Functional Analysis of the Gene Cluster Encoding the Enzymes of the Arginine Deiminase Pathway of *Lactobacillus sake. Journal of Bacteriology* 180(16): 4154-4159.
9. Zuniga, M., Miralles, M. C. and Perez-Martinez, G. 2002. The Production of *arcR,* the Sixth Gene of the *arc* Operon of *Lactobacillus sakei,* Is Essential for Expression of the Arginine Deiminase Pathway. *Applied and Environmental Microbiology* 68(12): 6051-6058.
10. Arena, M. E., Manca de Nadra, M. C. and Munoz, R. 2002. The Arginine Deiminase Pathway in the Wine Lactic Acid Bacterium *Lactobacillus hilgardii* X_1B: Structural and Functional Study of the *arcABC* Genes. *Gene* 301(1-2):61-66.
11. Aungpraphapornchai, P. 2000. Genetic Manipulation of Genes and Enzymes Involved in Flavour Generation by *Lactococcus lactis.* Ph.D. Thesis. The University of East Anglia, UK.
12. Dong, Y., Chen, Y.-Y. M., Snyder, J. A. and Burne, R. A. 2002. Isolation and Molecular Analysis of the Gene Cluster for the Arginine Deiminase System from *Streptococcus gordonii* DL1. *Applied and Environmental Microbiology* 68(11): 5549-5553.
13. Griswold, A., Chen, Y.-Y. M., Snyder, J. A. and Burne, R. A. 2004. Characterization of the Arginine Deiminase Operon of *Streptococcus rattus* FA-1. *Applied and Environmental Microbiology* 70(3): 1321-1327.
14. Barcelona-Andres, B., Marina, A. and Rubio, V. 2002. Gene Structure, Organization, Expression, and Potential Regulatory Mechanisms of Arginine Catabolism in *Enterococcus faecalis. Journal of Bacteriology* 184(22): 6289-6300.
15. Tonon, T., Bourdineaud, J.-P. and Lonvaud-Funel, A. 2001. The *arcABC* Cluster Encoding the Arginine Deiminase Pathway of *Oenococcus oeni,* and Arginine Induction of a CRP-Like Gene. *Research in Microbiology* 152: 653-661.
16. Aungpraphapornchai, P., Sangobpun, N. and Sopha, P. 2007. Isolation of Lactic Acid Bacteria from Fermented Foods Which Have Potential for Tetramethylpyrazine Production. *Srinakharinwirot Science Journal* 23(1): 94-108.
17. de Man, J. D., Rogosa, M. and Sharpe, M. E. 1960. A Medium for the Cultivation of Lactobacilli. *Journal of Applied Bacteriology* 23: 130-135.
18. Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular Cloning: a Laboratory Manual, Vol. 1, 2^{nd} ed. Cold Spring Habor Laboratory Press, USA.
19. Lewington, J., Greenaway, S. D. and Spillane, B. J. 1987. Rapid Small Scale Preparation of

Bacterial Genomic DNA, Suitable for Cloning and Hybridization Analysis. *Letters in Applied Microbiology* 5: 51-53.

20. van de Guchte, M., Kok, J. and Venema, G. 1992. Gene Expression in *Lactococcus lactis. FEMS Microbiology Reviews* 88: 73-92.
21. Weickert, M. J. and Chambliss, G. H. 1990. Site-directed Mutagenesis of a Catabolite Repression Operator Sequence in *Bacillus subtilis. Proc. Natl. Acad. Sci. USA* 87: 6238-6242.
22. Maas, W. K. 1994. The Arginine Repressor of *Escherichia coli. Microbiological Reviews* 58(4): 631-640.
23. Weisburg, W. G., Barns, S. M., Pelletier, D. A. and Lane, D. J. 1991. 16S Ribosomal DNA Amplification for Phylogenetic Study. *Journal of Bacteriology* 173(2): 697-703.
24. Drancourt, M., Bollet, C., Carlioz, A., Martelin, R., Gayral, J.-P. and Raoult, D. 2000. 16S Ribosomal DNA Sequence Analysis of a Large Collection of Environmental and Clinical Unidentifiable Bacterial Isolates. *Journal of Clinical Microbiology* 38(10): 3623-3630.
25. Nomura, M., Kobayashi, M. and Okamoto, T. 2002. Rapid PCR-Based Method Which Can Determine Both Phenotype and Genotype of *Lactococcus lactis* Subspecies. *Applied and Environmental Microbiology* 68(5): 2209-2213.
26. Maghnouj, A., Cabral, T. F. D., Stalon, V. and Wauven, C. V. 1998. The *arcABDC* Gene Cluster, Encoding the Arginine Deiminase Pathway of *Bacillus licheniformis,* and Its Activation by the Arginine Repressor ArgR. *Journal of Bacteriology* 180(24): 6468-6475.
27. Godon, J.-J., Delorme, C., Ehrlich, S. D. and Renault, P. 1992. Divergence of Genomic Sequences between *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris. Applied and Environmental Microbiology* 58(12): 4045-4047.

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