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Cloning and Sequencing of the *ddh* Gene involved in the Novel Pathway of Lysine Biosynthesis from *Brevibacterium lactofermentum*

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The *ddh* gene encoding *meso*-diaminopimelate (*meso*-DAP)-dehydrogenase (DDH) in *Brevibacterium lactofermentum* was isolated by complementation of the *Escherichia coli dapD* mutation. It was supposed from subcloning experiments and complementation tests that the evidence for DDH activity appeared in about 2.5 kb *Xho*I fragmented genome. The 2.5 kb *Xho*I fragment containing the *ddh* gene was sequenced, and an open reading frame of 960 bp encoding a polypeptide comprising 320 amino acids was found. Computer analysis indicated that the deduced amino acid of the *B. lactofermentum ddh* gene showed a high homology with that of the *Corynebacterium glutamicum ddh* gene.

It has been well known that L-lysine biosynthesis proceeds in procaryotes through three different pathways (16) (Fig. 1).

One of them, the diaminopimelate (DAP) pathway involving succinylated intermediates requires four steps from tetrahydrodipicolinate (THDP) to *meso*-diaminopimelate (*meso*-DAP) and has been extensively studied in *E. coli* (1). In *Bacillus sphaericus*, THDP is converted by a single enzymatic step to *meso*-DAP, catalyzed by *meso*-DAP-dehydrogenase (DDH) (19). We refer to this step as the DDH pathway. It has been supposed that the DDH pathway has more merits in non-requiring acetyl-CoA or succinyl-CoA and abbreviating the enzymatic reaction of four steps as found in the DAP pathway. The another pathway involving acetylated intermediates is utilized in certain *Bacillus* species (16).

Corynebacterium glutamicum, a gram-positive bacterium, has both the DAP pathway and DDH pathway (4, 12). This organism is widely used for the industrial production of amino acids because it produces higher levels of the lysine than did the strains utilizing only one pathway (20). There is considerable confusion about the lysine biosynthetic pathway of *Brevibacterium lactofermentum*, a lysine producer. Tosaka and Takinami (18) have reported that lysine biosynthesis in *B. lactofermentum* proceeds through the DAP pathway as found in *E. coli*. In contrast, Misono *et al.* (11) have reported that DDH activity occurs in *Brevibacterium* sp. ICR7000.

We presumed that the DDH pathway in addition to the DAP pathway is utilized by *B. lactofermentum*, and then we measured the DDH activity, and proved the existence of the DDH pathway mentioned in the previous paper (13).

Therefore, characterizing the expression of genes involved in the DDH pathway is an important first step towards understanding the lysine pathway of *B. lactofermentum* and metabolic control for high productivity of lysine.

In this paper, we report the cloning and sequence analysis of a chromosomal DNA fragment bearing the *ddh* gene of *B. lactofermentum*.

MATERIALS AND METHODS

Chemicals and Enzymes

Restriction enzymes were purchased from Promega, Kosco and Boehringer Mannhein. T4 DNA ligase and calf-intestinal alkaline phosphatase were from Promega. Nick translation kit, [α-^{32}P]CTP and [α-^{35}S]ATP were from Amersham. Deletion kit for kilo- sequence and sequencing kit were from Takara and United States Biochemical, respectively. Thiamine, DAP (mixture of LL, DD-and *meso*-isomers), ampicillin (Ap)

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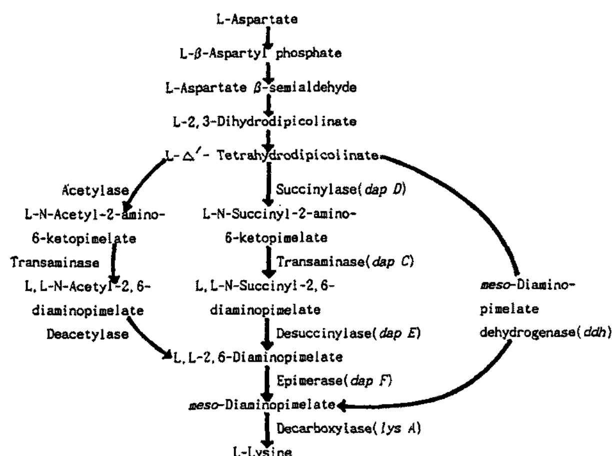

Fig. 1. Biosynthetic pathway of L-lysine in procaryotes.

and tetracyline (Tc) were from Sigma.

Bacterial Strains and Plasmids

A wild-type strain, *B. lactofermentum* ATCC13869 (KCTC1844), was the DNA donor for the genomic library. *E. coli* JM83 (*ara, rspL,* △(*lac-proAB*)ϕ80, *lacZ* △M15) and *E. coli* JM109 (*recAl, supE44, endA1, hsdR17, gyrA96, relA1, thi*△(*lac-proAB*)F′[*traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*△M15]) were used as the host for the *B. lactofermentum* ATCC13869 genomic library and the host for propagating plasmids, respectively. *E. coli* AT986 (*dapD8, relA1, spoT1, thi-1,* λ$^-$), obtained from Bachmann, Yale University, U. S. A. (2), was the recipient for transformation with library DNA. Plasmids pBR322 and pBluescriptII SK+ were used as the cloning vector.

Media and Growth Condition

LB medium (14) was used to grow *E. coli* and *B. lactofermentum* ATCC13869. M9 medium (14) was used as minimal medium for the growth of *E. coli.* DAP (50 μg/ml), thiamine (0.1 mM), Ap (50 μg/ml) and Tc (15 μg/ml) were added to culture media when appropriate. *E. coli* and *B. lactofermentum* ATCC 13869 were grown under aerobic conditions at 37°C and 30°C, respectively.

Isolation of DNA and Transformation

Chromosomal DNA from *B. lactofermentum* ATCC 13869 was obtained by the method of Hintermann *et al.* (6), except that lysozyme was added to a final concentration of 10 mg/ml. A large-scale preparation of plasmid DNA and rapid analysis of recombinant plasmids were carried out by the alkaline lysis procedure (3). *E. coli* strains were transformed by either the $CaCl_2$ (14) or the hexaminecobalt chloride method (5).

Construction of a *B. lactofermentum* Genomic Library

B. lactofermentum ATCC13869 DNA was partially digested with *Sau*3AI, and large restriction fragments (> 4 kb) were isolated by sucrose density gradient centrifugation. The *Sau*3AI fragments were then ligated with pBR322 DNA which was digested with *Bam*HI and dephosphorylated with calf intestinal alkaline phosphatase (14). The ligation mixture was used to transform *E. coli* JM83. The library obtained consisted of approximately 5,800 independent colonies of which 99% contained a *B. lactofermentum* insert. Analysis of several representative clones showed that the average size of the plasmid inserts was 10 kb.

Nick translation and Southern hybridization

An insert fragment of recombinant plasmid was labeled by nick translation to a specific activity of 2× 10^8 cpm/μg with [α-^{32}P]CTP. The labeled DNA was used for hybridization at 42°C with blotted DNA fragment on a nylon membrane according to the procedure of Southern (17).

Enzyme Assay

DDH activity was determined at 25°C by measuring the rate of an increase in the absorbance at 340 nm, essentially as described by Yeh *et al.* (20). The reaction mixture contained 10 mM DAP, 0.1 mM $NADP^+$, 0.5 M glycine-KCl-KOH buffer (pH 10.5) and the crude extract in a final volume of 1.0 ml. One unit of enzyme was defined as the amount of enzyme that catalyzes the formation of l μM of NADPH per minute. Specific activity was expressed as units per milligram of total protein. The amounts of protein in the crude extracts were measured by the method of Lowry *et al.* (9) with bovine serum albumin as a standard.

Construction of Deleted Subclones

Deleted subclones were generated with a deletion kit for kilo-sequence according to the manufacturer's instructions. The insert fragment containing the *ddh* gene was digested with *Eco*RV and *Pst*I and deleted by exonucleaseIII at about 150~200 bp/30 seconds at 37°C.

DNA Sequencing

The DNA sequence was determined by the dideoxynucleotide chain-terminating method of Sanger *et al.* (15). The sequencing reaction for each deleted subclone was performed according to the protocols given by U. S. Biochemical. The deleted subclones were independently subjected to autosequencing by an Applied Biosystem Autosequencer (Model 373A) with Automated Laser Fluorescent (A. L. F.).

Sequence Analysis

Computer analysis of nucleotide sequence and deduced amino acid sequence was carried out using the IBI/MacVector™ 4.0 sequence analysis software.

RESULTS

Cloning of the *B. lactofermentum ddh* Gene

Competent cells of *E. coli* AT986 were transformed with the *B. lactofermentum* genomic library constructed with pBR322 and plated on supplemented M 9 plates without DAP. After two days of incubation at 37°C, the eight Ap^R and DAP^+ transformants were selected. The specific activity of DDH was measured for eight Ap^R and DAP^+ transformants, resulting in the detection of DDH activity in all eight. From the eight Ap^R and DAP^+ transformants, plasmids were isolated and digested with *Bam*HI. Restriction enzyme analysis of plasmids DNA revealed identical restriction patterns indicating the presence of an identical plasmid in these transformants.

One of the clones with DDH activity designated

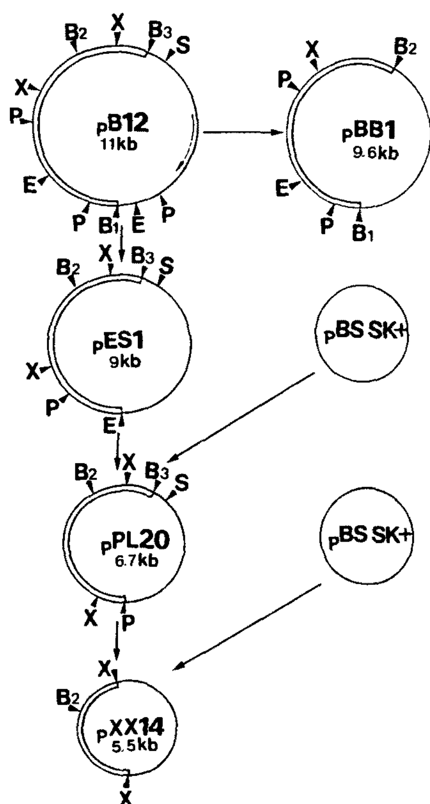

Fig. 2. Schematic diagram showing the construction of plasmids.

The pES1 and pBB1 were derived from pB12 after deletion of the *Eco*RI fragment and deletion of the *Bam*HI₂/*Bam*HI₃ fragment, respectively. The pPL20 was constructed by *Pst*I/*Sal*I fragment insertion to the pBluescriptII SK+ and pXX14 by *Xho*I fragment insertion into the pBluescriptII SK+. B, *Bam*HI; E, *Eco*RI; P, *Pst*I; X, *Xho*I; pBS SK+, pBluescriptII SK+.

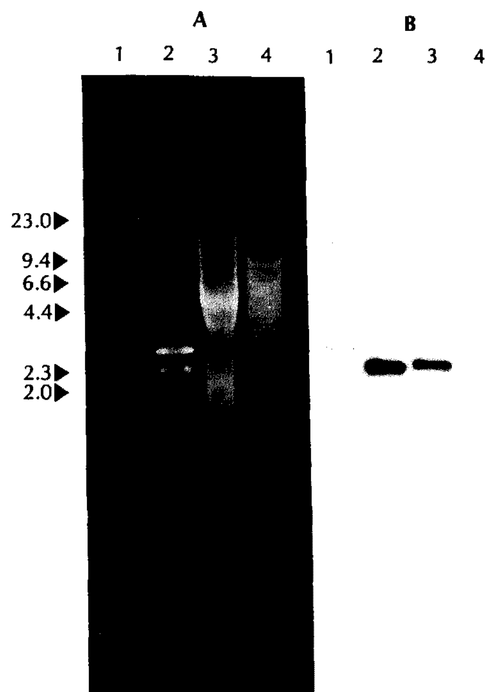

Fig. 3. Identification of the plasmid pXX14 with Southern blot hybridization.

The chromosomal DNA of *Brevibacterium lactofermentum* was digested with *Xho*I. The α-^{32}P labeled 2.5 kb insert fragment from pXX 14 was hybridized to chromosomal DNA.

A: Agarose gel electrophotogram

Lane 1. λ DNA digested with *Hin*dIII

Lane 2. pXX14 digested with *Xho*I

Lane 3. *B. lactofermentum* chromosomal DNA digested with *Xho*I

Lane 4. *E. coli* AT986 chromosomal DNA digested with *Xho*I

B: Autoradiogram

Table 1. Expression of *B. lactofermentum ddh* gene in *E. coli*.

Strain/plasmid	Relevant genotype	Specific activity (U^a/mg of total protein)
Brevibacteium lactofermentum ATCC 13869	wild type	6.7
E. coli JM109 (pBR322)	Ap^+	ND^b
E. coli JM109 (pBluescriptII SK+)	Ap^+	ND
E. coli JM109 (pB12)	Ap^+ ddh^+	5.0
E. coli JM109 (pES1)	Ap^+ ddh^+	6.3
E. coli JM109 (pPL20)	Ap^+ ddh^+	7.3
E. coli JM109 (pXX14)	Ap^+ ddh^+	6.2

[a]1 unit: the amount of enzyme that catalyzes the formation of 1 μM of NADPH per min. [b]ND: not detected.

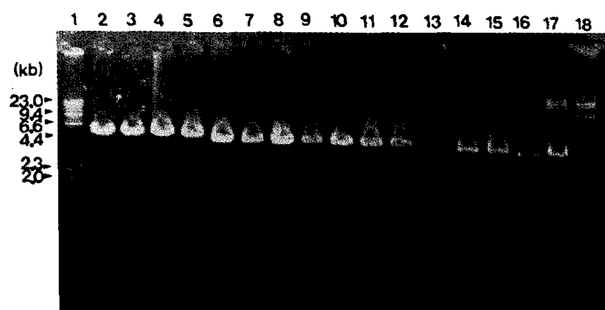

Fig. 4. Plasmids DNA pattern of deleted subclones constructed by exonucleaseIII.

The ~2.5 kb *Xho*I fragment was deleted by exonuclease III at about 150~200 bp/30 seconds at 37°C. Fifteen deletion mutant DNAs were digested with *Xho*I.

Lane 1. λ DNA digested with *Hin*dIII

Lane 2-16. Deletion DNA samples (No. 1~15) digested with *Xho*I

Lane 17. pBluescriptII SK+ digested with *Xho*I

Lane 18. λ DNA digested with *Hin*dIII

pB12 was selected for further studies. A restriction map of pB12 is shown in Fig. 2. This plasmid contained an *Bam*HI insert of approx. 6.6 kb.

Subcloning of the *ddh* Gene

To better localize the *ddh* gene, we subcloned the 6.6 kb fragment from pB12 and obtained four plasmids pBB1, pES1, pPL20 and pXX14 (Fig. 2). The plasmids pES1, pPL20 and pXX14 showed DDH activity. However, there was no activity in the plasmid pBB1 containing the *Bam*HI$_1$-*Bam*HI$_2$ fragment of the pB12. The complementation tests for each subclone indicate that the region showing DDH activity is within the ~2.5 kb XhoI fragment (13).

To confirm whether the insert fragment of pXX14 had originated from the chromosomal DNA of *B. lactofermentum* ATCC13869, a hybridization experiment was performed. *B. lactofermentum* chromosomal DNA was digested with *Xho*I and hy-

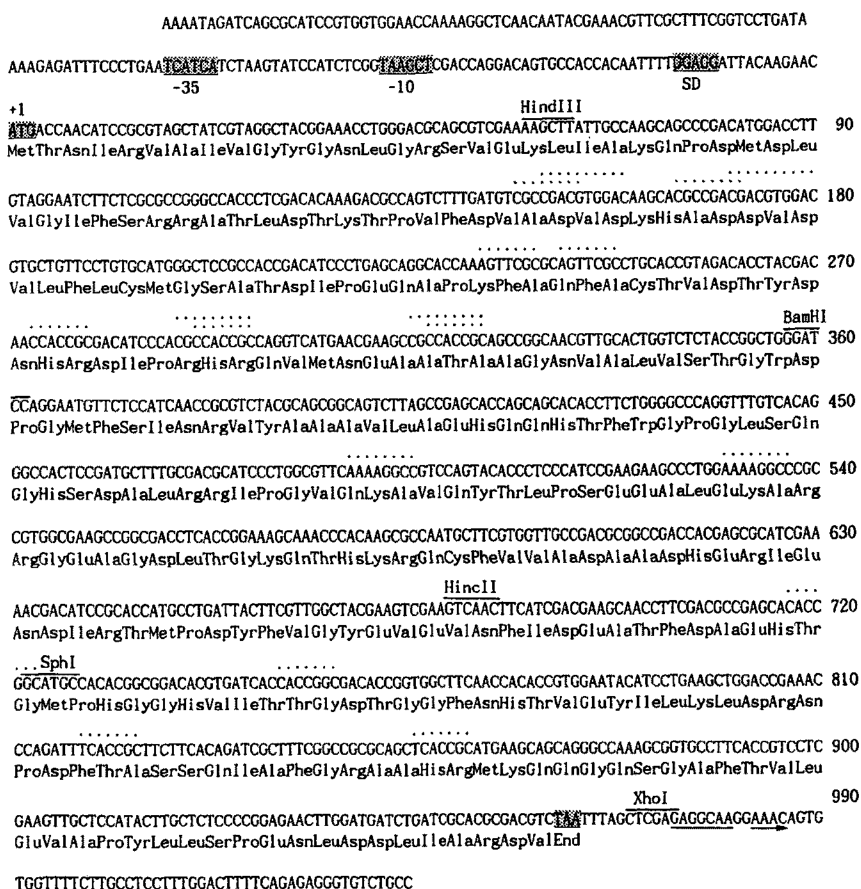

Fig. 5. Nucleotide sequence of *B. lactofermentum ddh* gene and the deduced amino acid sequence.

The deduced amino acid sequence is given below the nucleotide sequence. The nucleotide sequences corresponding to the putative Shine-Dalgarno (SD) sequence, promoter-like sequence (-10 and -35 sequence), initiation codon and stop codon are underlined with shadow. The lines above the nucleotide sequence indicate the recognition sites for the restriction endonuclease found. The inverted repeat structure for the transcription terminator is indicated by horizontal arrows. The dotted lines indicate the repeated base sequence, which occured at (i) 147-154 and 165-172, (ii) 150-159 and 171-180, (iii) 233-239 and 242-248, (iv) 273-279 and 291-297, (v) 273-279 and 317-323, (vi) 289-297 and 315-323, (vii) 488-495 and 530-537, (viii) 717-723 and 750-756 and (ix) 818-824 and 855-861.

Table 2. Codon usage of the *ddh* gene.

TTT Phe :1	TCT Ser:2	TAT Tyr:0	TGT Cys:0
TTC Phe :14	TCC Ser :6	TAC Tyr :8	TGC Cys :3
TTA Leu :1	TCA Ser :2	TAA Stop:1	TGA Stop:0
TTG Leu :4	TCG Ser :1	TAG Stop:0	TGG Trp :2
CTT Leu :2	CCT Pro :3	CAT His :0	CGT Arg :1
CTC Leu :5	CCC Pro :1	CAC His :3	CGC Arg :15
CTA Leu :0	CCA Pro :9	CAA Gln :4	CGA Arg :2
CTG Leu :8	CCG Pro :1	CAG Gln :11	CGG Arg :1
ATT Ile :1	ACT Thr :0	AAT Asn :0	AGT Ser :0
ATC Ile :14	ACC Thr :20	AAC Asn :11	AGC Ser :2
ATA Ile :0	ACA Thr :1	AAA Lys :0	AGA Arg :0
ATG Met :8	ACG Thr :1	AAG Lys :11	AGG Arg :0
GTT Val :5	GCT Ala :7	GAT Asp :7	GGT Gly :4
GTC Val :12	GCC Ala :16	GAC Asp :21	GGC Gly :15
GTA Val :4	GCA Ala :8	GAA Glu :12	GGA Gly :6
GTG Val :6	GCG Ala :3	GAG Glu :5	GGG Gly :0

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Bl ddh  MTNIRVAIVGYGNLGRSVEKLIAKQPDMDLVGIFSRRATLDTKTPVFDVA   50
Cg ddh  **************************************************
Bl ddh  DVDKHADDVDVLFLCMGSATDIPEQAPKFAQFACTVDTYDNHRDIPRHRQ  100
Cg ddh  **************************************************
Bl ddh  VMNEAATAAGNVALVSTGWDPGMFSINRVYAAAVLAEHQQHTFWGPGLSQ  150
Cg ddh  **************************************************
Bl ddh  GHSDALRRIPGVQKAVQYTLPSEEALEKARRGEAGDLTGKQTHKRQCFVV  200
Cg ddh  ***********************D**************************
Bl ddh  ADAADHERIENDIRTMPDYFVGYEVEVNFIDEATFDAEHTGMPHGGHVIT  250
Cg ddh  ************************************S*************
Bl ddh  TGDTGGFNHTVEYILKLDRNPDFTASSQIAFGRAAHRMKQQGQSGAFTVL  300
Cg ddh  **************************************************
Bl ddh  EVAPYLLSPENLDDLIARDV  320
Cg ddh  ********************
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Fig. 6. Comparison of the deduced amino acid sequence between *B. lactofermentum ddh* (B1 ddh) and *C. glutamicum ddh* genes (Cg ddh).

The identical amino acids are marked by asterisks(*).

bridized with [α-^{32}P]CTP labeled ~2.5 kb *Xho*I fragment of pXX14. As shown in Fig. 3, a single band which was found in hybridization with *Xho*I-digested *B. lactofermentum* chromosomal DNA fragments, corresponded to the size of the insert fragment of pXX14. This result confirmed that the cloned DNA fragment originated from *B. lactofermentum* genomic DNA.

Enzyme Activity

The specific activity of the DDH was determined from crude extracts of the *B. lactofermentum* ATCC 13869 and *E. coli ddh* transformants. The data show that *E. coli ddh* transformants have a level of DDH activity equal to that of activity found in the wild type of *B. lactofermentum* ATCC13869. On the other hand, no activity was detected in *E. coli* JM109 harboring pBluescriptII SK+ or pBR322 (Table 1).

According to the methods of Misono and Soda (10) and Ishino *et al.* (7), the activity staining was done for the plasmid pB12, pESI, pPL20 and pXX14. *B. lactofermentum* ATCC13869 and *E. coli* AT986 were used as positive and negative controls, respectively. In the previous results, a specific band of DDH activity was observed in the strains carrying each subclone except *E. coli* AT986 (13).

Nucleotide Sequence and Deduced Amino Acid Sequence Analysis of the *B. lactofermentum ddh* Gene

To determine the DNA sequence of the *B. lactofermentum ddh* gene, we constructed fifteen deleted subclones with the 2.5 kb *Xho*I fragment of pXX14 by exonucleaseIII (Fig. 4). The sequencing reaction was performed on deleted subclone series by the method of Sanger *et al.* (15). Both autoradiographed sequencing and autosequencing by A. L. F. showed good results (data not shown).

Analysed nucleotide sequence for the possible protein-encoding region has revealed an open reading frame (ORF) of 960 bp within the ~2.5 kb *Xho*I fragment. The nucleotide sequence of the *ddh* gene, as well as the deduced amino acid sequence, are shown in Fig. 5. The deduced amino acid sequence indicates a protein comprising 320 amino acids with a molecular weight of 35,195.

The putative promoter regions (-10 and -35 regions), as well as the potential Shine-Dalgarno (SD) sequence, were located upstream from the ATG initiation codon. To investigate the sequence of 3′ noncoding region beyond the *Xho*I site, the *Xho*I-*Bam*HI$_3$ fragment present in pPL20 was sequenced. The DNA sequence downstream contained a palindromic sequence which may function as a transcription terminator. The repeated base sequences were found in the coding region of the *ddh* gene (Fig. 5).

Codon usage and frequency of appearance in the *ddh* gene are shown in Table 2. There appears to be a strong preference for C in the third position by comparison with A, T and G.

The deduced amino acid sequence of the *B. lactofermentum ddh* gene when compared with that of *C. glutamicum ddh* gene showed a high homology (Fig. 6).

DISCUSSION

To clarify that the DDH pathway, in addition to the DAP pathway, is involved in lysine biosynthesis of *B. lactofermentum*, we cloned the *ddh* gene encoding *meso*-DAP-dehydrogenase (DDH) and determined

the nucleotide sequence of the *ddh* gene from *B. lactofermentum.*

From a genomic library of *B. lactofermentum*, we isolated eight Ap^R and DAP^+ transformants by heterologous complementation of an *E. coli dapD* mutation. These eight Ap^R and DAP^+ transformants all showed DDH activity and contained the insert fragment of identical size.

It was strange that no *dapD* gene could be cloned. In the cloning experiment of *C. glutamicum ddh* gene as reported by Ishino *et al.* (8), no activity for the *dapD* gene was detected in twenty clones. This is probably due to the disruption of the *dapD* gene by the digestion of restriction enzyme.

The coding region of the *B. lactofermentum ddh* gene is composed of 960 bp which encodes 320 amino acids. We found the repeated base sequence, which is probably the functional domain within the coding region. This is uncommon in procaryotes when compared to eukaryotes. Comparison of the nucleotide sequence and deduced amino acid sequence between *B. lactofermentum ddh* and *C. glutamicum ddh* genes revealed very high homology.

Schrumpt *et al.* (16) reported that the inactivation of the DDH pathway in *C. glutamicum* resulted in an accumulation of N-succinyl-diaminopimelate and reduction of lysine production. Production of high amounts of lysine in glutamic acid bacteria is probably due to existence of an additional DDH pathway because the DDH pathway is essential for converting intermediates to *meso*-DAP when metabolic flux is increased.

We have found here that the DDH pathway in addition to DAP pathway can operate in *B. lactofermentum* for *meso*-DAP and L-lysine production. We have an interest in the role and function of the *ddh* gene for lysine production in the DDH pathway of *B. lactofermentum* and will perform further studies on it.

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