Cloning and Sequencing of the *meso*-Diaminopimelate-D-dehydrogenase (*ddh*) Gene of *Corynebacterium glutamicum*

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The *ddh* gene of *Corynebacterium glutamicum* encoding *meso*-diaminopimelate (*meso*-DAP)-D-dehydrogenase (DDH) involved in the lysine biosynthesis was cloned in a DAP auxotroph (*dapD4*) of *Escherichia coli* by complementation of the DAP auxotroph. Deletion analysis revealed that a ~1.7-kb *Xho*I-*Kpn*I fragment contained the *ddh* structure gene. The specific activity of DDH was increased fourteen-fold when *C. glutamicum* was transformed with a recombinant plasmid harbouring the cloned *ddh* gene. Furthermore, the *ddh* gene has been sequenced [S. Ishino *et al.*, *Nucleic Acids Res.*, 15, 3917 (1987)] and some properties of the *ddh* gene are discussed.

Bacteria synthesize lysine by a pathway whose final step is the decarboxylation of *meso*-diaminopimelate (*meso*-DAP). The so called DAP pathway has been well characterized in *Escherichia coli*, as reviewed by Patte.¹⁾ The DAP pathway requires four steps from L- α -amino- ϵ -ketopimelate (L-AKP) to *meso*-DAP. In *Bacillus sphaericus*, an alternative pathway was proposed in which *meso*-DAP is directly formed from L-AKP by reductive amination with NH₃ and NADPH, catalyzed by *meso*-DAP-D-dehydrogenase (DDH) (EC 1.4.1.16)²⁾ and this pathway was proved to be unique and essential.³⁾ We have shown that DDH in addition to the DAP pathway is involved in the lysine biosynthesis in *Corynebacterium glutamicum*^{4,5)} which is an important species in the industrial production of lysine.⁶⁾ It is interesting to elucidate which pathway has the advantage in obtaining a higher yield of lysine.

In this paper, we describe the cloning and sequencing of the *ddh* gene of *C. glutamicum*, as announced in our previous report,²⁹⁾ and its gene dosage effect in *C. glutamicum*. Moreover, we have already presented the sequence of the *ddh* gene and discuss here some

properties of the *ddh* gene. Recently, P. Yeh *et al.*³⁰⁾ reported that the *ddh* gene of *C. glutamicum* (they called this *lys Y* gene) was cloned using the cosmid gene library by complementation of a *dapD8* mutant of *E. coli*.

MATERIALS AND METHODS

Chemicals and enzyme. Restriction enzymes were purchased from Takara Shuzo, Boehringer Mannheim Yamanouchi or Toyobo. T4 ligase, *E. coli* DNA polymerase I and an M13 sequencing kit were from Takara Shuzo. [α -³⁵S]dATP (500 Ci/mmol) was from Amersham. DAP (mixture of L,L-,D,D- and *meso*-isomers), ampicillin (Ap) and spectinomycin (Spc) were from Sigma.

Strains, plasmids and media. *C. glutamicum* KY10755 (Leu⁻, homoserine⁺, S-(2-aminoethyl)-cysteine^R, α -amino- β -hydroxyvalerate^R; a lysine-producing mutant derived from a wild-type strain, ATCC 10132) and RRL-5 (lysozyme^S, r⁻)⁷⁾ were used as a source for DNA and a recipient for transformation, respectively. Culture was done as described in our previous paper.⁴⁾ *E. coli* TM 132 (F⁻, *dapD4*, *hsdR2*, Met⁺, Rif^R, Lac⁺) was constructed in our laboratory by F-mediated conjugation using AT982 (Hfr, *thiI*, *dapD4*)⁸⁾ and a spontaneous rifampicin (Rif) resistant mutant of WA802 (F⁻, *metB1*, Lac⁻, *galK2*, *galT22*, *supE*, *hsdR2*)⁹⁾ and was used as a host for cloning. JM103¹⁰⁾ was used as a host for M13 phage vectors. *E. coli* was grown in L-broth.¹¹⁾ DAP (50 μ g/ml), Ap (50 μ g/ml)

and Spc (100 µg/ml) were added when appropriate. Agar was added at 20 g/l for plates. Plasmids pBR322 and pUC 18 and phage vectors M13 mp18 and mp19 were purchased from Takara Shuzo. A *C. glutamicum* plasmid, pCG11, carrying the Spc resistance gene was described previously.¹²⁾

Preparation of DNA fragments and plasmids. Chromosomal DNA was purified from the protoplasts of *C. glutamicum* KY10755 by the method of Saito and Miura.¹³⁾ Plasmids were isolated by the alkaline extraction procedure.¹⁴⁾ Restriction fragments for use in M13 cloning were isolated by agarose gel electrophoresis and electroelution onto dialysis membranes.¹⁵⁾

Transformation. *E. coli* TM132 and JM103 were transformed by the CaCl₂ method of Cohen *et al.*¹⁶⁾ and *C. glutamicum* RRL-5, by the protoplast transformation method of Katsumata *et al.*¹⁷⁾

Cloning procedure for the *ddh* gene. Chromosomal DNA of *C. glutamicum* KY10755, a lysine-producing mutant, was partially digested with the restriction endonuclease *Eco*RI and ligated into the *Eco*RI site of PBR322. The ligation mixture was used to transform *E. coli* TM132. The transformed cells were incubated in L-broth containing DAP at 30°C for 16 hr, washed, plated on L-broth agar containing Ap and incubated at 30°C for 1–2 days. The resultant colonies (Ap^R, DAP⁺) were then screened by the activity staining method (see below) to select positive clones with DDH activity. From one positive clone, a plasmid was obtained and designated pCD1 (Figs. 1 and 2).

Construction of the shuttle plasmid harboring the *ddh* gene. A *C. glutamicum*–*E. coli* shuttle plasmid carrying the *ddh* gene was constructed as follows. The plasmid pCD1 was partially digested with *Pst*I and ligated into the unique *Pst*I site of pCG11. The ligation mixture was used to transform *E. coli* TM132. One of the resultant plasmids from Spc^R, DAP⁺ and Ap^S transformants was designated pCD2. The restriction analysis of pCD2 revealed that it consists of pCG11 and all of pCD1 whose junctions are within the β-lactamase gene originating from pBR322 (Fig. 1).

DNA sequencing. DNA was sequenced by the M13 cloning protocols of Messing *et al.*¹⁰⁾ and the dideoxy method of Sanger *et al.*¹⁸⁾ G-C rich regions were resequenced with dITP in place of dGTP as described by Mills and Kramer.¹⁹⁾

Activity staining for DDH. Activity staining for DDH using the crude extracts of *E. coli* and *C. glutamicum* was done after polyacrylamide gel electrophoresis as described previously.⁴⁾

Enzyme purification and assay. DDH from *C. glutamicum* KY10755 was purified to homogeneity by the procedure of Misono *et al.*²⁴⁾ DDH and glutamate dehydrogenase (GDH) activities were measured by the procedure of Misono *et al.*²⁾ and Shimizu *et al.*,²⁰⁾ respectively, using the crude extracts. Protein was measured by the method of Lowry *et al.*³¹⁾

Amino acid sequence analysis. NH₂-terminal sequence of the purified DDH was identified by stepwise Edman degradation with an automated gas-phase sequencer (Applied Biosystems model 470A).²¹⁾ Resultant phenylthiohydantoin were identified by HPLC on a C₈ reversed-phase column (Senshu Kagaku, SEQ-4, C₈, 0.46 × 30 cm, particle size 7 µm) at 40°C using a gradient to acetonitrile in 40 mM sodium acetate buffer (pH 4.9) for elution.

RESULTS

*Cloning and localization of the *ddh* gene*

Some genes of *C. glutamicum* have been expressed in *E. coli*.^{22,23)} DDH catalyzes the reaction of *meso*-DAP formation from L-AKP. Then, we considered that if the *ddh* gene was expressed in *E. coli*, the DAP deficiency would be satisfied if it was caused by a mutation in the *dapC*, *dapD*, *dapE* or DAP epimerase gene. This is because a series of the four genes are essential in supplying *meso*-DAP from L-AKP in *E. coli*. Thus, the *ddh* gene of *C. glutamicum* was cloned in a *dapD4* mutant of *E. coli* which was thought to be a suitable recipient.

The activity staining was done for twenty Ap^R and DAP⁺ transformants, resulting in the detection of DDH activity in all twenty. From one positive clone, a plasmid was isolated and designated pCD1 (Fig. 1). The plasmid pCD1 conferred both Ap^R and DAP⁺ phenotypes and directed DDH synthesis upon retransformation (Figs. 1 and 2). This plasmid was approximately 12 kb in length, consisting of the vector and an 8-kb *Eco*RI insert (tandem *Eco*RI fragments of 5.3 kb and 2.7 kb).

The 8-kb fragment from pCD1 was subcloned in pUC18 to localize the gene coding for DDH and the plasmids pEE1, pEP1, pKSP1, pXH1 and pXK1 were obtained (Fig. 3). These deletion studies and the results of the activity staining (Fig. 2) indicate that the cod-

FIG. 1. Isolation of the *ddh* Gene of *C. glutamicum* and Construction of a *C. glutamicum*-*E. coli* Shuttle Plasmid.

Thin line, pBR322 DNA; thick line, pCG11 DNA; boxed line, chromosomal DNA carrying the *ddh* gene from *C. glutamicum* KY10755.

ing region for DDH is between the *Kpn*I and *Xho*I sites (~1.7 kb).

Sequencing of the *ddh* gene

Based on the report that the molecular weight of the purified DDH from *C. glutamicum* ATCC 10132 (a wild-type strain from which the DNA passenger was derived) is

70,000 and the DDH consists of two identical subunits,²⁴⁾ the coding region was estimated to be about 1 kb. Interruption at the *Hind*III or the *Sph*I site in the *Kpn*I-*Xho*I fragment led to a loss of DDH activity (Figs. 1, 2), suggesting that both sites are in the *ddh* gene. Since the *Sph*I site is ~0.2 kb from the *Xho*I site, the coding region which was predicted

to be ~1kb may be adjacent to the *Xho*I site. Hence, in sequencing, some region beyond the *Xho*I site should be added to the

FIG. 2. Activity Staining for DDH after Polyacrylamide Gel Electrophoresis.

Crude extracts (~20 µg of protein) of *C. glutamicum* KY10755 (the passenger of DNA) and *E. coli* TM132 carrying subclones of the plasmid pCD1 (see Fig. 1) were electrophoresed on native polyacrylamide gel and activity stained for DDH. The arrow indicates the position of DDH.

*Kpn*I–*Xho*I fragment to investigate the structures of 3' and 5' noncoding regions.

The *Kpn*I–*Xho*I fragment from pXK1 and a small region from pEP1 expanding beyond the *Xho*I site were sequenced by the strategy shown in Fig. 3, and the nucleotide and the deduced amino acid sequences have been already reported.²⁹⁾ There is a possible long open reading frame of 1,125 nucleotides extending from positions 618 to 1742, which is consistent with the results of the deletion studies shown in Fig. 3. In this open reading frame are five ATG codons at positions 618, 690, 699, 723 and 783 upstream from the *Hind*III site (position 840), at which interruption resulted in no expression of the *ddh* gene (Figs. 2, 3).

To decide which ATG is the real initiation codon, DDH of *C. glutamicum* KY10755 was purified (data not shown) and the NH₂-terminal amino acids were sequenced. The first nine residues were identified as Thr, Asn, Ile, Arg, Val, Ala, Ile, Val and Gly in this order. This corresponds to the deduced amino acid sequence²⁹⁾ only when the translation is initiated at position 783. Thus, we have concluded that the initiation codon for the open reading frame

FIG. 3. Physical Map, Deletion Analysis and Sequencing Strategy of *C. glutamicum* DNA Containing the *ddh* Gene.

The physical map of the plasmid pCD1 is shown on the top line. The subclones of pCD1 are designated pEE1, pEP1, pXH1, pKSP1 and pXK1. The respective inserts are indicated by the bracketed lines. The insert of pXK1 is enlarged to show the details of restriction sites. The arrows indicate the direction and extent of sequencing from restriction sites of DNA fragments prepared from pXK1 and pEP1 ((a), (b)). The shaded region represents the DDH coding region. Growth of TM132 carrying each plasmid was observed on a L-both agar plate. +, growth; -, no growth. E, *Eco*RI; Bg, *Bgl*II; P, *Pst*I; S, *Sac*I; X, *Xho*I; H, *Hind*III; Sp, *Sph*I; K, *Kpn*I; Pv, *Pvu*II; N, *Nsi*I; R, *Rsa*I; Hc, *Hinc*II; B, *Bam*HI.

is the ATG at 783.

The coding region consists of 960 nucleotides, corresponding to a protein of 320 amino acid residues. The NH₂-terminal residue of the purified DDH was found to be threonine, suggesting that the first methionine residue is removed after the initiation of translation. Therefore, the subunit of DDH is calculated to be 35,099.

Upstream from the coding region is a potential Shine-Dalgarno (SD) sequence of GGAGG, which is preceded by promoter-like sequences. The coding region is followed by a typical ρ -independent terminator sequence.²⁵⁾

Expression of the cloned ddh gene in C. glutamicum

C. glutamicum RRL-5 was transformed with pCD2, the *C. glutamicum*-*E. coli* shuttle plasmid carrying the *ddh* gene. The specific activity

of DDH was increased about fourteen-fold in the transformant probably, due to the gene dosage effect. In contrast, there are no significant differences in the specific activity of GDH between the host and the transformants (Table I).

DISCUSSION

We report here the cloning and sequencing of the *ddh* gene of *C. glutamicum* in *E. coli*. The plasmids harboring the cloned gene directed the synthesis of DDH in *E. coli* (Fig. 2). It has already been reported that the cloned genes from *C. glutamicum*, a homoserine dehydrogenase-homoserine kinase (HD-HK) operon²²⁾ and the prephenate dehydrogenase gene (*pheA*),²³⁾ are functional in *E. coli* and inversely the gene from *E. coli* can be expressed in *C. glutamicum*.⁷⁾ Hence, it is likely that the regulatory regions responsible for transcription and translation resemble each other.

The DNA sequence upstream from the coding region contains a potential SD sequence (GGAGG) and promoter-like sequences (putative -10 sequence; TAAGCT and -35; TCATCA) for *E. coli*, as has been reported in the HD-HK operon of *C. glutamicum*.²²⁾

The G+C content of chromosomal DNA from *C. glutamicum* is 53%²⁶⁾ and that of the

TABLE I. GENE DOSAGE EFFECT OF THE *ddh* GENE IN *C. glutamicum*

Strain (plasmid)	Specific Activity (U/mg-protein)	
	DDH	GDH
RRL-5	0.16 (1) ^a	0.08 (1) ^a
RRL-5 (pCG11)	0.17 (1.1)	0.078 (0.98)
RRL-5 (pCD2)	2.22 (13.9)	0.076 (0.95)

^a Relative activity.

TABLE II. CODON USAGE OF THE *ddh* GENE

Phe	TTT	1	Ser	TCT	1	Tyr	TAT	0	Cys	TGT	0
	TTC	14		TCC	7		TAC	8		TGC	3
Leu	TTA	1		TCA	2	Term	TAA	1	Term	TGA	0
	TTG	3		TCG	1		TAG	0	Trp	TGG	2
	CTT	3	Pro	CCT	3	His	CAT	0	Arg	CGT	0
	CTC	5		CCC	1		CAC	13		CGC	16
	CTA	0		CCA	10	Gln	CAA	4		CGA	2
	CTG	8		CCG	0		CAG	11		CGG	1
Ile	ATT	2	Thr	ACT	0	Asn	AAT	0	Ser	AGT	0
	ATC	13		ACC	20		AAC	11		AGC	2
	ATA	0		ACA	1	Lys	AAA	0	Arg	AGA	0
Met	ATG	8		ACG	1		AAG	11		AGG	0
Val	GTT	5	Ala	GCT	7	Asp	GAT	7	Gly	GGT	4
	GTC	12		GCC	15		GAC	22		GGC	15
	GTA	3		GCA	9	Glu	GAA	11		GGA	6
	GTG	7		GCG	3		GAG	5		GGG	0

G+C content: 60% (chromosome: 53%).

sequenced 1,829 base fragment was 56%. Within the fragment, the coding region (60%) was much more G+C-rich than the 5' non-coding region (52%). As shown in Table II there is a strong codon bias, generally preferring C in the third position in comparison with 16, 19, and 10% for A, G, and T, respectively. Such a strong bias is observed in the *phe A* gene,²³⁾ but not in the HD-HK operon of *C. glutamicum*.²²⁾

The NH₂-terminal residue of DDH was found to be threonine but not methionine. This suggests that the methionine residue is removed after the initiation of translation. Although nothing is known about the mechanism, a methionine aminopeptidase may be present in *C. glutamicum* as has been reported in *E. coli*.²⁷⁾

In this cloning experiment, no *dapD* gene of *C. glutamicum* could be cloned. The failure in cloning of the *dapD* gene is probably due to the interruption of the essential region for the gene expression by *EcoRI* and we are now attempting to clone the *dapD* gene of *C. glutamicum*.

Lysine has been widely supplied on an industrial scale by the fermentation process using mutants of glutamic acid bacteria²⁸⁾ represented by *C. glutamicum*. In the lysine biosynthesis in *C. glutamicum* DDH supplies *meso*-DAP, the precursor of lysine. A fourteen-fold increase in the specific activity of DDH was observed after the introduction of the cloned gene into a nonproducer (Table I). We have an interest in its effect on lysine productivity and the introduction of the gene into some lysine-producing mutants is in progress. The results will give significant information on strain improvement.

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