Molecular Cloning and Expression of *dapA*, the Gene for Dihydrodipicolinate Synthetase of *Corynebacterium glutamicum*

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Dihydrodipicolinate Synthetase를 코딩하는 *Corynebacterium glutamicum*의 *dapA* 유전자의 클로닝 및 발현

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ABSTRACT: The *dapA*-complementing gene (L-2, 3-dihydrodipicolinate synthetase: DHDP synthetase, *dapA*) has been cloned by using a cosmid genomic bank of *Corynebacterium glutamicum* JS231 that is a lysine overproducer, AEC (s-(2-aminoethyl)-L-cysteine) resistant mutant. By enzymatic deletion analysis, the DNA region complementing the *Escherichia coli dapA* host could be confined to 4.5 kb *Sal*I-generated DNA fragment. This DNA fragment was inserted into the *C. glutamicum/E. coli* shuttle vector pECCG117 to construct pDHDP5812. The specific activity of DHDP synthetase detected in *C. glutamicum* JS231/pDHDP5812 was increased about 10 fold above that of *C. glutamicum* JS231. The addition of leucine during growth did not repress the expressin of *dapA*, and the enzyme activity was not inhibited by lysine.

KEY WORDS □ *dapA* gene, *Corynebacterium glutamicum*, Cloning, Expression

Coryneform bacteria such as *Corynebacterium glutamicum*, *Brevibacterium lactofermentum*, and *B. flavum* are widely used in industrial production of amino acids (Kinoshita, 1959; Tosaka *et al.*, 1983). Despite of their usefulness in industrial fermentation, the genetic information on the coryneform bacteria has been limited. Therefore, to obtain the improved amino acid producers, classical mutagenesis was applied to the selection of amino acid analog(s)-resistant mutants and auxotrophic mutants. Recently, cloning vectors and transformation systems such as protoplast transformation and electroporation were developed so that recombinant DNA techniques can be used for the breeding of amino acid producing strains using coryneform bacteria (Katsumata *et al.*, 1984; Yoshihama *et al.*, 1985; Santamaria *et al.*, 1984; Santamaria *et al.*, 1985; Wolf *et al.*, 1989). It has been reported that those genes involved in the diaminopimelic acid-lysine biosynthesis of *E. coli* and *C. gultamicum* are scattered along the chromosome in separate transcription units (Bukhari and Taylor, 1972; Theze *et al.*, 1974; Yeh *et al.*, 1988) and feedback inhibition seems to play a prominent role in the regulation of this pathway (Shiio and Miyajima, 1969; Patte *et al.*, 1974). In lysine biosynthetic pathway, the two enzymes,

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aspartokinase (EC 2.7.2.4.) and DHDP synthetase (EC 4.2.1.52.) have been shown to be involved in the rate limiting steps in lysine biosynthesis. The single aspartokinase of *C. glutamicum* that catalyses the phosphorylation reaction from aspartate to aspartyl phosphate is feedback inhibited by lysine and threonine (Tosaka *et al.*, 1978). The DHDP synthetase which is encoded by *dapA* gene is the first enzyme of diaminopimelate and lysine biosynthesis and catalyses the synthesis of dihydrodipicolinate from aspartate semialdehyde that is also the precursor of homoserine (Shedlarski and Gilvarg, 1970). In *E. coli*, it was reported that the activity of DHDP synthetase is sensitive to the inhibition by lysine (Yugari and Gilvarg, 1962). We report here the cloning and expression of *dapA* gene of *C. glutamicum* and the effect of the gene on the production of L-lysine.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids are listed in Table 1. Cells were grown in LB or M9 medium (Maniatis *et al.*, 1982). MC medium, a minimal medium for *C. glutamicum*, contained, per liter: 10 g glucose, 2 g $(NH_4)_2SO_4$, 2 g urea, 0.2 g KH_2PO_4, 0.2 g K_2HPO_4, 0.1 g $CaCl_2$, 100 μg biotin, 100 μg thiamine-HCl, and trace elements. The required supplements were added to growth medium for auxotrophic strains to a final concentration of 100 μg/m*l* for amino acids and 50 μg/m*l* for diaminopimelic acid(DAP). Antibiotics were used at concentration of 50 μg/m*l* for ampicillin(Ap) and kanamycin(Km).

Transformation

E. coli cells were transformed by using the calcium chloride method (Cohen *et al.*, 1972) or electroporation (Dower *et al.*, 1988). Transformation of *C. glutamicum* by electroporation was conducted using the Gene Pulser apparatus (Bio-Rad) with 0.2 cm cuvette (Bio-Rad). Overnight culture of *C. glutamicum* strains were inoculated to 1 *l* of LB medium with initial OD_{562} nm of 0.07-0.1. The bacterial cells were cultivated to an OD_{562} nm of 0.3 and then Penicillin G was added to a final concentration of 0.3 U/m*l* for the preparation of competent cells. The cells were chilled on ice after the OD_{562} nm of cells reached 0.6 and centrifuged at 4,000×g for 15 min at 4°C. The cells were then suspended in 1 *l* of cold 1 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid(HEPES) buffer (pH 7.0) and centrifuged as above. The cell pellet was resuspended in 500 m*l* of cold deionized water, centrifuged, and resuspended in 20 m*l* of 10% glycerol. After washing twice with 10% glycerol, the cells were resuspended in a final volume of 2-3 m*l* of cold 10% glycerol. The number of cells should be at least $2\text{-}4\times10^{10}$/m*l*. 1-2 μ*l* of DNA (10-100 ng) was added to 40 μ*l* of the cell suspension. Electrical conditions for the transformation were basically same as those for *E. coli*, e.g., 2.5 kv using 25 uF capacitor, and a resistance of 200 ohm in parallel with the sample. After pulse, 1 m*l* of SOC medium (2% Bacto-tryptone, 0.5% Bacto-yeast extract, 25 mM KCl, 10 mM $MgCl_2$, 10 m $MgSO_4$ 20 mM glucose) was added, and incubated for 1 h with

Table 1. *Bacterial strains and plasmids used in this study*

Strain/Plasmid	Characteristics	Source
C. glutamicum		
JS231	Leu^-, Hse , AEC^r, AHV^r	This Lab.
LR2-2	AEC^r, AHV^r	This Lab.
ATCC13032	Wild type	ATCC
E. coli		
AT997	*dapA*	Bukhari & Taylor (1971)
Plasmids		
pBluescriptII KS^+	Ap^r, 2.9 kb	Stratagene
pHC79	Cosmid Vector, Ap^r	Boehringer
pECCG117	Km^r, 5.8 kb Shuttle vector	This work
pDH1	pBluescriptII KS^+ carrying *dapA* gene of *C. glutamicum*	This work
pDHDP5812	pECCG117 carrying *dapA* gene of *C. glutamicum*	This work

Symbols used for relevant genotypes and phenotypes were as follows: Km, Kanamycin; Ap, Ampicillin; AEC, s-(2-aminoethyl)-L-cysteine; AHV, α-amino-β-hydroxyvaleric acid shaking at 200 rpm. Then aliquot was plated on the selective medium.

Preparation of cell-free extracts and enzyme assay

The cells were harvested after cultivation in the LB or minimal media up to mid-exponential phase, washed twice with 100 mM Tris-HCl buffer (pH 7.4), and disintegrated by sonication for 15 min under cooling at 4℃. The cell debris was removed by centrifugation at 14,000×g for 30 min at 4℃ and the supernatant was used as crude extract for enzyme assay. DHDP synthetase activity was measured by the o-aminobenzaldehyde assay method (Yugari and Gilvarg, 1962). Units are given in increment of optical density of 540 nm per minute per milligram of protein. Protein concentration was determined by using the Bradford procedure (1976).

DNA manipulation and isolation

Total genomic DNA and plasmid DNA from *C. glutamicum* were isolated and purified as previously described (Noh *et al.*, 1990). Isolation of plasmid DNA or cosmid DNA from *E. coli* were carried out by alkaline lysis metod (Birnboim and Doly, 1979). To construct the genomic bank of *C. glutamicum*, chromosomal DNA from *C. glutamicum* JS231 was partially digested with *Sau* 3A and fragment ranging from 20-40 kb were isolated from LMP agarose. *Sau* 3A-digested DNA from *C. glutamicum* JS231 and *Bam*HI-digested dephosphorylated cosmid pHC79 were ligated at 12℃ with T4 DNA ligase for 16 h. The ligated DNA was packaged in phage particles by an *in vitro* packaging system (Davis *et al.*, 1980). *In vitro* packaging extract, restriction endonucleases, and alkaline phosphatase were purchased from Boehringer Mannheim or from New England Biolabs and used as recommended by the manufacturers.

Hybridization experiment

Transfer of DNA from 0.8% agarose gels to nitrocellulose filters and hybridization were carried out as described by Maniatis *et al.* (1982).

Plasmid stability test

C. glutamicum cells harboring the plasmids was grown overnight at 32℃ in 20 m*l* of LB medium with the kanamycin (300 m*l* shaker flask); 0.02 m*l* of this culture was transferred to 20 m*l* of fresh LB medium without antibiotics. After incubation for 12-18 h at 32℃, the culture was diluted in the same manner into fresh antibiotic-free medium and grown again for 12-18 h. This cycle, which give almost 6-7 generation, repeated until at least 40 generations of growth under nonselective conditions were reached. Appropriate dilutions of cultures were plated on selective and nonselective agar plates and incubated 3 days at 32℃. The plasmid stability was determined by the percentage of the antibiotic-resistant cells. Resistant and sensitive colonies were occasionally examined for the presence of plasmid DNA by the alkaline lysis method.

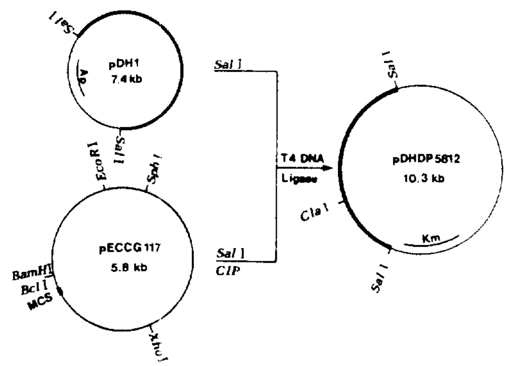

Fig. 1. *Subcloning of dapA gene in E. coli/C. glutamicum shuttle vector pECCG117. The plasmid with dapA gene in an orientation opposite to that in pDHDP5812 was designated pDHDP5813. The heavy line indicates the C. glutamicum JS231 chromosomal DNA fragment. (Km; kanamycin, Ap; ampicillin, CIP; calf intestine phosphatase, MCS; multiple cloning site)*

RESULT

Cloning of the gene for DHDP synthetase

Assuming that a strain harboring a plasmid carrying *dapA* gene would overproduce the corresponding enzyme, and would improve the productivity of lysine in *C. glutamicum* host, we searched for the clone in a genomic library of *C. glutamicum* JS231 constructed in the cosmid vector pHC79. 12 clones harboring chimeric plasmids complementing *dapA* mutant of *E. coli* AT997 were isolated and the plasmids were subjected to restriction anaysis. One of cosmids that carry a common DNA region was subcloned into the plasmid vector pBluescriptII KS⁺ to reduce the size of the frgment carrying the *dapA* gene. The clone overproducting the DHDP synthetase (7 to 10-fold, depending on the growth phase) was selected, and its plasmid, pDH1, was

Table 2. *Specific activity of DHDP synthetase in C. glutamicum JS231 and E. coli*

Strain	Plasmid	Culture media	DHDP synthetase activity in the presence of (unit/mg protein/min)	
			0 mM Lys	10 mM Lys
E. coli				
AT997	None	LB+DAP	10	ND[a]
AT997	pDHDP5812	LB	70	68
C. glutamicum				
JS231	None	Minimal	75	74
JS231	pDHDP5812	Minimal	765	760
JS231	pDHDP5812	Minimal+10 mM Leucine	770	767

[a]Not detected

analyzed. ^{32}P-labeled *Sal*I fragment containing *dapA* gene was used as a probe to hybridize with chromosomal DNA of *C. glutamicum* digested with *Sal*I. The probe hybridized with the *C. glutamicum* JS231 chromosome fragment of the same size as the cloned fragment (4.5 kb) (data not shown), indicating that no cross rearrangement had occurred during the construction of cosmid library and after transformation of *C. glutamicum* and *E. coli*.

Subcloning in *E. coli/C. glutamicum* shuttle vector

Plsmid pECCG117 was constructed by combining *Bam*HI-digested pACYC177 and *Bcl*I-digested cryptic plasmid pCG1 from *C. glutamicum* ATCC13058, deleting β-lactamase region by double digestion with *Bgl*I and *Bgl*II followed by blunt-end ligation, and introducing the multiple cloning site of pBluescriptII KS$^+$ into *Bst*EII site of pACYC177 by blunt-end ligation. The *dapA* gene was then inserted to the *Sal*I site of pECCG117 to form pDHDP5812.

Expression of *dapA* gene in *C. glutamicum* and *E. coli*

As described above, pDHDP5812 complemented the *dapA* mutation of *E. coli* AT997. This indicated that the *dapA* gene of *C. glutamicum* JS231 was contained in pDHDP 5812 and that the gene was expressed in *E. coli* AT997. This result was also proved by assaying the DHDP synthetase activity in *C. glutamicum* and *E. coli* AT997. No detectable activity was observed in *E. coli* AT997. In contrast, *C. glutamicum* JS231 /pDHDP5812 and *E. coli* AT997/pDHDP5812 showed high DHDP synthetase activity. The DHDP synthetase activity was 10 times higher in *C. glutamicum* JS231 than in control strains (Table 2). The expression of this gene was not repressed by leucine, and the enzyme activity was not inhibited by 10 mM of lysine. Also, the plasmid stability of pDHDP5812 and suhuttle vector pECCG117 was examined. After 10 generations, 40-50% cells lost the recombinant plasmid pDHDP5812 although shuttle vector pECCG117 was maintained for up to 40 generations.

Table 3. *Production of lysine by recombinant C. glutamicum*

Strain	Plasmid	Lysine concentration (g/*l*)
LR2-2	None	12
LR2-2	pDHDP5812	18.5
ATCC13032	None	Trace
ATCC13032	pDHDP5812	Trace

Each strain was cultured at 30℃ in a flask containing 20 m*l* of lysine production medium which contained 75 g of sucrose, 20 g of $(NH_4)_2SO_4$, 5 g of yeast extract, 4 g of urea, 2.5 g of NaCl, 0.6 g of $MgSO_4 \cdot 7H_2O$, 0.6 g of KH_2PO_4, 300 μg of biotin, 500 μg of thiamine-HCl, 40 g of $CaCO_3$ and trace elements per liter. If necessary, amino acids and kanamycin were added as a final concentration of 20 μg/m*l* and 50 μg/m*l*, respectively.

The amount of lysine was determined by HPLC.

Overproduction of L-lysine using *C. glutamicum* strains carrying pDHDP5812

The wild-type strain *C. glutamicum* ATCC13032 and *C. glutamicum* LR2-2, homoserine revertant of *C. glutamicum* JS231 were used instead of *C. glutamicum* JS231 as the host to exclude any unexpected effect of the mutagenesis on the expression of *dapA* gene and to clearly examine the effect of this gene expression on the production of lysine in homoserine prototroph. The plasmid, pDHDP5812, isolated from *E. coli* mutant of *E. coli* DH5 was used to transform *C. glutamicum* strains by electroporation. Results of production of L-lysine using these transformants are summarized in Table 3. It is quite clear that a large increase in lysine production was observed with the strain *C. glutamicum* LR2-2 harboring pDHDP5812, although the strain *C. glutamicum* ATCC13032 carrying pDHDP5812 did not produce much lysine. At the end of the cultivation, cells were diluted and plated on LB agar plates and LB agar plate containing kanamycin; in several experiments, about 80% of the bacteria were found to remain Km-resistance in the selective fermentation medium.

DISCUSSION

The gene encoding the DHDP synthetase, the first enzyme involved in the diaminopimelate and lysine biosynthetic pathway of *C. glutamicum* was cloned, and subcloned in the shuttle vector pECCG117. The expression of this gene was not repressed by leucine, though in *B. lactofermentum* an eight-fold repression by leucine has been reported (Tosaka *et al.*, 1978). The enzyme activity was also not inhibited by 10 mM lysine. The *dapA* gene of *E. coli* has been also located on a 2.8 kb *Pst*I fragment subcloned from a bacteriophage into plasmid pBR322 (Richard *et al.*, 1981), and it has been reported that the corresponding enzyme is inhibited by lysine (Yugari and Gilvarg, 1962). Mutants with feeback resistant DHDP synthetase have not been found, despite of the use of strong selective agents in *E. coli*. It is clear that the lysine production by recombinant *E. coli* carrying *dapA* gene of *E. coli* was much lower than that obtained even by regulatory and auxotrophic mutant of *Corynebacterium* and *Brevibacterium* strains (Reverend *et al.*, 1982). Because the *dapA* gene of *C. glutamicum* is not under the regulation of lysine, its expression in *C. glutamicum* or *E. coli* may be useful to increase the production of lysine. The fermentation result showed that DHDP synthetase catalyses the rate limiting step in lysine biosynthesis after aspartokinase. On the other hand, the increasing the *dapA* gene dosage in wild type *C. glutamicum* ATCC13032 results in a no parallel increase of DHDP synthetase activity and lysine excretion. At the same time, we expect that amplification of other lysine biosynthetic enzymes such as aspartokinase would be effective for the further improvement of lysine biosynthesis, and further studies are being carried out.

적 요

라이신 생산균주이며 AEC(s-(2-aminoethyl)-L-cysteine) 내성 균주인 *Corynebacterium glutamicum* JS231의 cosmid genomic bank를 사용하여 *dapA*(L-2,3-dihydrodipicolinate synthetase : DHDP synthetase, *dapA*) 유전자를 클로닝 하였다. 클로닝된 유전자는 4.5 kb *Sal*I-DNA 절편에 존재하며, 이를 *C. glutamicum* 및 *E. coli*에서 발현되는 셔틀 벡터 pECCG117에 도입하여 재조합 플라스미드 pDHDP5812를 얻었다. pDHDP5812에 의해 형질전환된 *C. glutamicum*/pDHDP5812 균주의 DHDP synthetase 비효소활성은 모 균주인 *C. glutamicum* JS231에 비해 10배 증가하였으며, 이 효소는 루이신을 첨가하여도 그 합성이 억제(repression) 되지 않았다. 또한 효소의 활성은 라이신에 의해 저해(feedback inhibition) 되지 않았다.

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