Analysis of different DNA fragments of *Corynebacterium glutamicum* complementing *dapE* of *Escherichia coli*

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In *Corynebacterium glutamicum* L-lysine is synthesized simultaneously via the succinylase and dehydrogenase variant of the diaminopimelate pathway. Starting from a strain with a disrupted dehydrogenase gene, three different-sized DNA fragments were isolated which complemented defective *Escherichia coli* mutants in the succinylase pathway. Enzyme studies revealed that in one case the dehydrogenase gene had apparently been reconstituted in the heterologous host. The two other fragments resulted in desuccinylase activity; one of them additionally in succinylase activity. However, the physical analysis showed that structural changes had taken place in all fragments. Using a probe derived from one of the fragments we isolated a 3.4 kb *Bam*HI DNA fragment without selective pressure (by colony hybridization). This was structurally intact and proved functionally to result in tenfold desuccinylase overexpression. The nucleotide sequence of a 1966 bp fragment revealed the presence of one truncated open reading frame of unknown function and that of *dapE* encoding N-succinyl diaminopimelate desuccinylase (EC 3.5.1.18). The deduced amino acid sequence of the *dapE* gene product shares 23% identical residues with that from *E. coli*. The *C. glutamicum* gene now available is the first gene from the succinylase branch of lysine synthesis of this biotechnologically important organism.

Keywords: lysine synthesis, heterologous complementation, diaminopimelate desuccinylase, *Corynebacterium glutamicum*

INTRODUCTION

Bacteria synthesize L-lysine via the diaminopimelate (DAP) pathway. Three variants of this pathway are now known which are established in different organisms (Gilvarg, 1959; Weinberger & Gilvarg, 1970; White, 1983). A peculiarity is the lysine synthesis of the Gram-positive *Corynebacterium glutamicum*, which is used for industrial production of L-lysine (Eggeling, 1994). Interestingly, in this organism two of the three possible variants are present simultaneously, namely the succinylase variant and the dehydrogenase variant (Fig. 1). Both variants are actually used simultaneously *in vivo* as shown by the ^{13}C tracer technique (Sonntag *et al.*, 1993). The flux distribution via both variants is ammonium-

dependent and the overall contribution of the succinylase branch for lysine synthesis is about 70%.

We and others (Ishino *et al.*, 1988; Yeh *et al.*, 1988; Cremer *et al.*, 1990, 1991) have cloned all genes of the DAP pathway via the dehydrogenase variant. We found that the dehydrogenase is dispensable for growth and that its oversynthesis has no positive effect on lysine overproduction, whereas other enzymes increase lysine formation (Cremer *et al.*, 1991). To perform similar experiments with enzymes of the succinylase variant the respective genes are required. However, in two attempts to clone genes of either the succinylase or the dehydrogenase variant, only the *ddh* gene (dehydrogenase) was isolated (Ishino *et al.*, 1988; Yeh *et al.*, 1988). In these experiments *dapD* mutants of *Escherichia coli* (succinylase deficient) were used, showing that the *dapD* mutation can be favourably bypassed in *E. coli* by *ddh* of *C. glutamicum*. However, in order to isolate a gene of the succinylase variant, *E. coli* mutants must be used, because no *C.*

Abbreviation: DAP, diaminopimelate.

The EMBL accession number for the sequence reported in this paper is X81379.

Fig. 1. The split pathway of lysine synthesis in *Corynebacterium glutamicum*. Synthesis of meso-diaminopimelate via the succinylase variant (left) and the dehydrogenase variant (right).

glutamicum DAP-auxotrophs can be isolated (Yeh *et al.*, 1988), probably due to the inability of *C. glutamicum* to take up DAP. In addition, it is not possible to design and use degenerate oligonucleotides for cloning, since currently the *dapD* sequences known from three bacteria do not reveal well-conserved domains (Richaud *et al.*, 1984; Denich *et al.*, 1991; Baril *et al.*, 1992) and the sequences for *dapE* and *dapF* are known only for *E. coli* (Bouvier *et al.*, 1992; Richaud *et al.*, 1987).

The recent availability of a *ddb* mutant of *C. glutamicum*, constructed by vector integration into the *ddb* gene (Schrumpf *et al.*, 1991), prompted us to start a new attempt at isolating genes of the *C. glutamicum* succinylase variant. Using genomic DNA from this mutant the appropriate *E. coli* mutants should be complemented by either *dapE* or *dapD* of *C. glutamicum*. We describe our findings using this approach.

METHODS

Bacterial strains and growth conditions. All strains and plasmids used are listed in Table 1. *Escherichia coli* was grown on LB medium for cloning purposes (Lennox, 1955), or on BHI medium (Difco) for complementation and enzyme measurements. *Corynebacterium glutamicum* was grown on CgIII (Menkel *et al.*, 1989). When appropriate, kanamycin ($50 \mu\text{g ml}^{-1}$), ampicillin ($40 \mu\text{g ml}^{-1}$) or DAP ($50 \mu\text{g ml}^{-1}$) was added. All strains were grown aerobically at 30°C except for *E. coli* DH5 and *E. coli* NM522, which were incubated at 37°C .

Genetic engineering. DNA was isolated from *C. glutamicum* by a modified alkaline extraction procedure with lysozyme (Schrumpf *et al.*, 1991). All enzymes used were obtained from Boehringer and used as instructed by the manufacturer. DNA fragments were isolated and purified by using GeneClean (Dianova). For DNA hybridization according to Southern (1975), Nytran 13 nylon membranes (Schleicher and Schüll) were used. *E. coli* DH5 and NM522 were transformed by the CaCl_2 method, whereas electroporation was used to transform *C. glutamicum* (Liebl *et al.*, 1989).

Complementation of *E. coli* DAP auxotrophs. In order to establish a cosmid genomic gene bank, total DNA was isolated from *C. glutamicum* AS70 partially digested with *Sau3A* and ligated into *Bam*HI-restricted pHC79. Using the DNA Packaging Kit (Boehringer) the gene bank was established in *E. coli* DH5. Transformation of the DAP auxotrophs *E. coli* RDD32 and RDE51 was done by the following procedure. LB medium plus DAP (50 ml) was inoculated and cells grown overnight at 30°C ; 10 ml of this culture was used to inoculate 400 ml LB medium plus DAP and incubation was continued. The culture was harvested at $\text{OD}_{600} 0.5$ and washed three times with ice-cold glycerol (10%, w/v). The cell pellet was diluted with ice-cold glycerol (10%, w/v) to obtain a final volume of 1 ml, and 100 μl aliquots were frozen at -70°C . For electroporation, 1 μl DNA of the gene bank ($0.5 \mu\text{g } \mu\text{l}^{-1}$) was mixed with 40 μl cells in an ice-cold sterile electroporation cuvette (2 mm electrode gap, Bio-Rad) and pulsed (Bio-Rad, Gene Pulser plus Pulse Controller with the settings 2500 V, 25 μF and 200 Ω). Immediately after the pulse 1 ml prewarmed (30°C) BHI medium was added and the suspension was transferred into a tube containing another 1 ml of prewarmed BHI. After shaking at 30°C for 45 min, 10 μl (*E. coli* RDD32) or 100 μl (*E. coli* RDE51) aliquots were plated on selective agar plates containing ampicillin. Growth was checked after 1, 2 and 4 d.

Colony hybridization. A partial genomic gene bank, consisting of *C. glutamicum* ATCC 13032 *Bam*HI DNA fragments of 2.5–4.3 kb inserted in pUC19, was established in *E. coli* NM522. A total of 740 colonies carrying recombinant plasmids (as confirmed by α -complementation) were transferred on to a nylon membrane (PALL). Denaturation, washing and hybridization with the digoxigenin-labelled probe was performed as described in the standard procedure of the non-radioactive DNA Labelling and Detection Kit from Boehringer.

DNA sequencing and analysis. Deletion clones were prepared by exonuclease III treatment (Promega) of both strands of the 3.4 kb *Bam*HI *dapE* fragment in pUC19. The nucleotide sequence was determined by the method of Sanger *et al.* (1977) with Sequenase version 2.0 from US Biochemicals. Sequence data were compiled and analysed using the HUSAR program package, release 2.0 (EMBL).

Enzyme assays. Cells were washed with 0.9% NaCl, resuspended in 20 mM Tris/HCl pH 8, and disrupted by sonication. The homogenate was centrifuged for 30 min at 12000 g, and the resulting extract was used to determine DAP

Table 1. Bacterial strains and plasmids

Strain	Relevant genotype/phenotype	Source/reference
<i>E. coli</i>		
DH5	<i>thi-1 endAR1 gyrA96 F⁻ relA1 supE44 recA1 hsdR17 (r⁻m⁺)</i>	Hanahan (1985)
NM522	<i>supE thi Δ(lac-proAB) Δhsd-5 (r⁻m⁻) {F' proAB lacI^qZΔM15}</i>	Gough & Murray (1983)
RDD32	<i>araD139 thi Δ(lac)U168, strA dapD::MuCts</i>	Richaud <i>et al.</i> (1981)
RDE51	<i>araD139 thi Δ(lac)U168 strA dapE::MuCts</i>	Richaud <i>et al.</i> (1981)
<i>C. glutamicum</i>		
ATCC 13032	Wild-type, Nx ^r	Abe <i>et al.</i> (1967)
AS70	ATCC 13032 <i>ddb::pEMddhint</i>	Schrumpf <i>et al.</i> (1991)
Plasmids		
pHC79	<i>cos</i> , Ap ^r Tc ^r , <i>oriV_{E.c.}</i>	Hohn & Collins (1980)
pUC18/pUC19	Ap ^r , <i>oriV_{E.c.}</i>	Yanisch-Perron <i>et al.</i> (1985)
pTG1228	Ap ^r , <i>ddb⁺</i>	Transgene
pJC1	Shuttle vector, Km ^r , <i>oriV_{E.c.}</i>	Cremer <i>et al.</i> (1990)
pD02	Ap ^r , <i>dapD⁺ dapE⁺</i> , pHC79 with <i>C. glutamicum</i> 33.5 kb fragment	This work
pDE07	Ap ^r , <i>dapD⁺ dapE⁺</i> , pHC79 with <i>C. glutamicum</i> 34 kb fragment	This work
pJC dapDE BamHI 2.85	Km ^r , <i>dapD⁺ dapE⁺</i> , pJC1 with <i>C. glutamicum</i> 2.85 kb BamHI fragment from pDE07	This work
pE015	Ap ^r , <i>dapE⁺</i> , pHC79 with <i>C. glutamicum</i> 31 kb fragment	This work
pJC dapE 1.9	Km ^r , <i>dapE⁺</i> , pJC1 with <i>C. glutamicum</i> <i>dapE</i> 1.9 kb fragment from pE015	This work
pJC dapE BamHI 3.4	Km ^r , <i>dapE⁺</i> , pJC1 with <i>C. glutamicum</i> <i>dapE</i> 3.4 kb BamHI fragment	This work

dehydrogenase activity (EC 1.4.1.16) (Cremer *et al.*, 1988). Gel filtration of the crude extract, substrate synthesis for the succinylase assay, and the activity measurements of succinylase (DapD) and *N*-succinyl diaminopimelate desuccinylase (DapE; EC 3.5.1.18) were performed as described previously (Schrumpf *et al.*, 1991). Protein concentrations were determined by the biuret reaction (Gornall *et al.*, 1949).

RESULTS

Complementation of *E. coli* DAP auxotrophs with DNA from *C. glutamicum* *ddb*

A cosmid genomic gene bank of *C. glutamicum* AS70 (with *ddb* interrupted by vector sequences) was constructed in pHC79 using *E. coli* DH5 as host. From this bank we isolated 15 cosmids which were able to complement *E. coli*

RDD32 (*dapD*) or *E. coli* RDE51 (*dapE*). By retransformation, three types of complementing cosmids could be identified which conferred a stable DAP prototrophy. Type I, represented by cosmids pD02 and pD03, showed complementation of both *E. coli* mutants, while pDE07 (the only type II cosmid) also showed cross-complementation but shared no common restriction pattern with type I cosmids. Cosmid pE015, as well as cosmids pE06, pE09, pE011 and pE012 (type III cosmids), complemented *E. coli* RDE51 (*dapE*) only. It is noteworthy that all type I and type II cosmids led to better growth of the *E. coli* mutants (colonies appeared after 1–2 d incubation) than type III cosmids (colonies appeared only after 4 d incubation). Therefore, we initially chose the type I cosmid pD02, complementing both *E. coli* mutations, for further analysis. However, after a series

Table 2. Enzyme activities in *C. glutamicum* due to a subclone obtained from cosmid pDE07 (type II)

Strains were grown overnight in CgIII medium. The activity is expressed in $\mu\text{mol min}^{-1}$ (mg protein) $^{-1}$. Results are from three separate experiments. ND, Not determined.

Strain	Succinylase (DapD)	Desuccinylase (DapE)	Dehydrogenase (Ddh)
ATCC 13032(pJC1)	0.005	0.021	0.114
ATCC 13032(pJC dapDE BamHI 2.85)	0.027	0.148	0.148
ATCC 13032(pJC dapDE BamHI 2.85 _{inv})	0.028	0.156	0.176
AS70 (<i>ddh</i>)	0.005	ND	< 0.0005

Fig. 2. Southern blot analysis of genomic DNA from *C. glutamicum* strains ATCC 13032 (lane 1) and AS70 (lane 2), cosmid DNA of pDE07 (lane 3) and pE015 (lane 4), and plasmid DNA of pUC19 dapDE 2.85 (lane 5). DNA was *Bam*HI-restricted and probed with a digoxigenin-labelled internal 0.9 kb *Sal*I fragment isolated from the 2.85 kb dapDE *Bam*HI fragment.

of biochemical and genetic investigations we had to assume (not shown) that in the type I cosmids the *ddh* gene was functionally reconstituted.

Analysis of type II cosmid pDE07

We investigated cosmid pDE07 in detail since it enabled growth of the *E. coli* strains with the *dapD* or *dapE* mutation, suggesting that the two genes might be linked to each other. For subcloning, cosmid pDE07 was

digested with *Bam*HI, fragments were ligated with pUC18, and after transformation 38 ampicillin-resistant DAP-prototrophic clones of RDD32 were obtained. Several plasmids were isolated and proved also to transform RDE51 to DAP prototrophy. The plasmids contained a 2.85 kb *Bam*HI fragment which was ligated into the *C. glutamicum*/*E. coli* shuttle vector pJC1 yielding pJC dapDE *Bam*HI 2.85 and pJC1 dapDE *Bam*HI 2.85_{inv} (with the insert in the inverse orientation). After introduction of these plasmids into *C. glutamicum* ATCC 13032, enzyme activities in the recombinant strains were determined (Table 2). In this case dehydrogenase (Ddh) activity was comparable to the wild-type level, and the expected DapD oversynthesis and that of DapE was also obtained. To probe the integrity of the cloned fragment a Southern blot analysis with chromosomal DNA of the wild-type was performed. This analysis, however, showed hybridization to a 3.4 kb *Bam*HI fragment (Fig. 2) instead of the expected cloned 2.85 kb *Bam*HI fragment. Therefore, the isolated *Bam*HI fragment was structurally altered, and we had to concentrate on a type III cosmid.

Analysis of type III cosmid pE015

Subcloning of pE015 led to a 1.9 kb *Sau*3A fragment inserted in vector pJC1. The restriction pattern of this fragment resembled in part that of the 2.85 kb *Bam*HI fragment originating from type II cosmid. Its structural integrity was confirmed by extended Southern blot studies (data not shown). With the aim to clone *dapD* also, and based on the assumption that *dapD* and *dapE* might be located close to each other, we isolated the *dapE* locus again, but avoided a functional selection.

Isolation of *dapE* by colony hybridization

In order to isolate the chromosomal 3.4 kb *Bam*HI fragment a partial gene bank consisting of *Bam*HI fragments of *C. glutamicum* wild-type DNA was constructed in pUC19. This gene bank was screened with an 800 bp *Eco*RI/*Xho*I internal fragment of the 1.9 kb *dapE* complementing insertion originating from type III cosmid pE015 (see Methods). Three of the 740 colonies tested reacted positively and plasmid isolation gave the intact 3.4 kb *Bam*HI fragment inserted in pUC19. This

Fig. 3. Map of the chromosomal DNA of *C. glutamicum* in pUC19 *dapE* *Bam*HI 3-4. Top: restriction map. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; S, *Sal*I; X, *Xho*I. Centre: extent of exonuclease-III-generated deletions of pUC19 *dapE* *Bam*HI 3-4. Numbers refer to deletion clones $\Delta 75$, $\Delta 81$ and $\Delta 92$. The DapE phenotype conferred on strain *E. coli* RDE51 is indicated. Bottom: sequencing strategy using exonuclease-III-generated deletions.

plasmid, pUC19 *dapE* *Bam*HI 3-4, was able to complement *E. coli* RDE51 (*dapE*), but, unexpectedly, not *E. coli* RDD32 (*dapD*). However, in this case, DNA hybridization analysis confirmed the structural integrity of this fragment (data not shown). The fragment was ligated with pJC1 to yield pJC *dapE* *Bam*HI 3-4, which was used to transform the wild-type of *C. glutamicum* to kanamycin resistance. In the resulting recombinant strain the desuccinylase activity was determined. The specific activity was $0.221 \mu\text{mol min}^{-1} \text{mg}^{-1}$, whereas the plasmid without insert yielded a specific activity of $0.021 \mu\text{mol min}^{-1} \text{mg}^{-1}$. This DapE oversynthesis is functional proof that *dapE* resides on the cloned fragment. With the same insert in the opposite orientation with respect to the vector a similar high specific activity was obtained, verifying that *dapE* was cloned with its functional promoter. In order to localize the *dapE* gene more precisely within the 3.4 kb *Bam*HI fragment, exonuclease-III-generated deletion clones were made and analysed for their *dapE* complementation ability (Fig. 3). While deletion $\Delta 81$ was still able to complement *dapE*, the 205 bp shorter deletion fragment $\Delta 92$ lacked this ability.

Nucleotide sequence analysis

The complete nucleotide sequence of fragment $\Delta 81$ was determined on both strands using the sequencing strategy shown in Fig. 3. The sequence obtained is shown in Fig. 4. Two open reading frames (ORFs) were found, extending from nucleotide 162 to nucleotide 1268 and from nucleotide 1333 to the end of the fragment (nucleotide 1666). Since the complete ORF spans the DNA region functionally proven to result in desuccinylase activity, it was designated *dapE*. Preceding *dapE*, a typical Shine-Dalgarno sequence, 5'-GTAGG-3' (Moran *et al.*, 1982), is located 9 nucleotides in front of the most probable translational start site, GTG, at position 162 (Fig. 4). ORF2 starts downstream of *dapE*, following a short intergenic region of 61 bp. Due to the codon preference, which resembles moderately expressed genes of *C. glutamicum* (Eikmanns, 1992), and a perfect ribosomal binding site, 5'-AGGAGG-3', ORF2 most

probably represents a coding region. Since we were not able to detect secondary structures reflecting a transcriptional termination site downstream of *dapE*, a transcriptional readthrough from *dapE* to ORF2 is possible. Comparison of the deduced amino acid sequences showed no significant identity with known proteins in the case of ORF2. However, DapE shares 23% identical amino acids with the corresponding polypeptide from *E. coli* (Bouvier *et al.*, 1992). Assuming initiation at position GTG 162, the *C. glutamicum* *dapE* gene product consists of 369 amino acids, with a molecular mass of 39 942 Da, which is comparable with that of 41 129 Da deduced for the *E. coli* polypeptide.

DISCUSSION

Although *C. glutamicum* has been biochemically proven to possess (Schrumpf *et al.*, 1991) and use (Sonntag *et al.*, 1993) the succinylase pathway, in two attempts to clone corresponding genes only *ddh* of the dehydrogenase pathway was isolated (Ishino *et al.*, 1988; Yeh *et al.*, 1988). In this work we were again faced with considerable problems when starting with a dehydrogenase-negative strain. First, restoration of dehydrogenase activity in *E. coli* was obtained, and second, repeatedly structurally altered DNA fragments were found. It is unclear why both type I cosmids carry a functional *ddh* gene. Most probably homologous recombination in the *E. coli* host resulted in deletion of the integration vector pEM1, thereby restoring dehydrogenase activity. This points to strong selection pressure by the DAP-auxotrophic mutants.

With respect to the structural alterations of fragments carrying genes of the succinylase variant, only cloning without functional selection was successful in obtaining a larger fragment with intact *dapE*. This could in principle be due to the possibility that the gene products of *dapD* and *dapE* itself or that of a closely related gene(s) are disadvantageous for the host upon overexpression. It is known that *dapE* of *E. coli* is able to suppress a mutation of the heat-shock gene *grpE* (Wu *et al.*, 1992), which at

Fig. 4. Sequence of the *dapE* gene locus, with deduced polypeptide sequences and selected restriction sites. Putative ribosome-binding sites (RBS) of the corresponding coding regions and starting points of the deletion clones $\Delta 81$ and $\Delta 92$ are indicated.

least shows that enzymes of the succinylase variant may have other as yet unknown functions. But in the case of *dapE* of *C. glutamicum* it seems to be neighbouring

sequences which are responsible for problems in cloning. It is interesting that in spite of various attempts with a probe of the type II cosmid we failed to isolate *dapD* by

hybridization (not shown). Due to this fact, together with the problems in cloning the large fragment with *dapE*, and the type II cosmid complementing both *dapD* and *dapE*, it is tempting to speculate that the difficulties in cloning can be attributed to an identical detrimental gene.

These discoveries might be one reason why Ishino *et al.* (1988) and Yeh *et al.* (1988) were not able to isolate succinylase genes. Another reason is the apparently poorer promoter recognition of certain *C. glutamicum* genes in *E. coli*. *dapE* thus apparently leads only to weak expression in *E. coli* since growth due to the *dapE*-containing fragment only results after 4 d. Similarly, other amino acid biosynthetic genes of *C. glutamicum* are only weakly expressed in *E. coli*, such as *ilvA* and *ilvBN* (Cordes *et al.*, 1992), *lysC* (Kalinowski *et al.*, 1990), or *dapA* and *dapB* (Cremer *et al.*, 1990). The current mapped promoters of *C. glutamicum* lack the features typically attributed to *E. coli* σ^{70} or *B. subtilis* σ^{43} promoters (Schwinde *et al.*, 1993) and no specific consensus sequence can be given to identify a possible promoter directly in front of *dapE*.

Heterologous complementation resulted above all in the DNA probe for cloning the structurally and functionally intact *dapE* gene. Analysis of the *dapE* sequence showed that the *N*-succinyldiaminopimelate desuccinylase (DapE) of *C. glutamicum* shares 23 % identical amino acids with the only known corresponding polypeptide, whose gene was isolated from *E. coli* (Bouvier *et al.*, 1992). This low, but significant, degree of identical residues was also reported for the polypeptides of other amino acid biosynthetic enzymes from *C. glutamicum* (LysC, Kalinowski *et al.*, 1990; IlvA, Möckel *et al.*, 1992; LeuA, Pátek *et al.*, 1994), whereas higher identities were found if the polypeptides involved in central metabolism of both organisms are compared (Schwinde *et al.*, 1993). This shows that in spite of the specific dehydrogenase pathway for Gram-positive bacteria, the DapE structure of *C. glutamicum* has evolved away from that of *E. coli* to the same extent as that of other amino acid biosynthesis genes. The cloning of *dapE* now achieved will permit further molecular studies on flow changes in the unusual lysine synthesis pathway of *C. glutamicum*. Moreover, a fine-structural analysis of a type II cosmid with succinylase and desuccinylase activity will enable further studies on the chromosomal loci of the respective genes.

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