Appl Microbiol Biotechnol (1999) 51: 325–333

ORIGINAL PAPER

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Flux through the tetrahydrodipicolinate succinylase pathway is dispensable for L-lysine production in *Corynebacterium glutamicum*

Received: 20 May 1998 / Received revision: 12 August 1998 / Accepted: 3 September 1998

Abstract The *N*-succinyl-LL-diaminopimelate desuccinylase gene (*dapE*) in the four-step succinylase branch of the L-lysine biosynthetic pathway of *Corynebacterium glutamicum* was disrupted via marker-exchange mutagenesis to create a mutant strain that uses only the one-step *meso*-diaminopimelate dehydrogenase branch to overproduce lysine. This mutant strain grew and utilized glucose from minimal medium at the same rate as the parental strain. In addition, the *dapE*$^-$ strain produced lysine at the same rate as its parent strain. Transformation of the parental and *dapE*$^-$ strains with the amplified *meso*-diaminopimelate dehydrogenase gene (*ddh*) on a plasmid did not affect lysine production in either strain, despite an eightfold amplification of the activity of the enzyme. These results indicate that the four-step succinylase pathway is dispensable for lysine overproduction in shake-flask culture. In addition, the one-step *meso*-diaminopimelate dehydrogenase pathway does not limit lysine flux in *Corynebacterium* under these conditions.

Introduction

The gram-positive microorganism *Corynebacterium glutamicum* is widely used for the overproduction of amino acids such as L-lysine. The development of a genetic toolbox to study this bacterium has been progressing rapidly (Jetten and Sinskey 1995), but the interaction of many intermediate biochemical reactions in the lysine biosynthetic network is still not well understood. Product yield maximization requires optimal coordination of the *C. glutamicum* metabolic network. To this end, experiments were conducted to study the effect of biochemical variations in the reactions of the tetrahydrodipicolinate branchpoint on lysine flux.

At the tetrahydrodipicolinate branchpoint of the L-lysine pathway of *C. glutamicum*, carbon can be shunted either through the one-step *meso*-diaminopimelate (Dap) dehydrogenase pathway (Misono et al. 1979), or through the four-step succinylase pathway (Yeh et al. 1988; Schrumpf et al. 1991) to form the cell-wall component and immediate precursor to lysine, *meso*-Dap (Fig. 1). To assess the role of the dual pathway at this branchpoint, marker-exchange mutagenesis was employed to create a mutant strain that lacks the *dapE* gene and consequently is unable to synthesize *N*-succinyl-LL-Dap desuccinylase, the third enzyme in the four-step succinylase pathway. This mutant strain utilizes only the one-step *meso*-Dap dehydrogenase pathway for lysine synthesis. The *dapE*$^-$ mutant was further characterized by determining pathway fluxes when the strain was transformed with a multicopy plasmid, which amplified the *ddh* gene product, *meso*-Dap dehydrogenase.

Although disruption of the *meso*-Dap dehydrogenase (*ddh*) gene in the one-step pathway has been characterized (Schrumpf et al. 1991), the lysine productivity of a strain harboring a disrupted gene in the four-step succinylase pathway has not yet been studied. The tetrahydrodipicolinate succinylase (*dapD*) and *dapE* genes in the four-step pathway have been cloned in *Corynebacterium*, and disruption of these genes has been shown to alter cell morphology (Wehrmann et al. 1998, 1994). In amplification experiments this group was able to achieve tenfold overexpression of the desuccinylase enzyme, but no data were provided on how its overexpression affected lysine flux (Wehrmann et al. 1994). The

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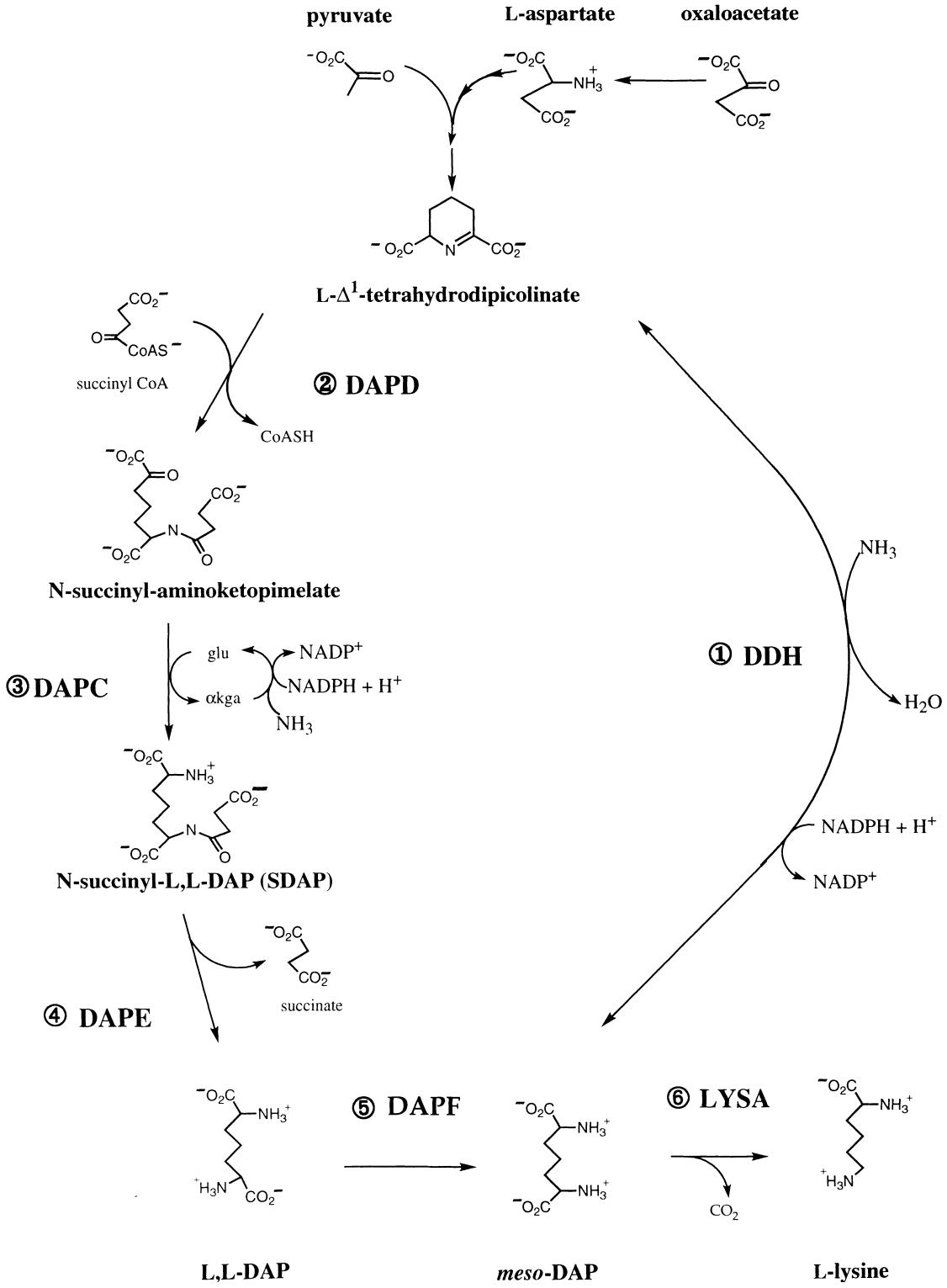

Fig. 1 Genes and corresponding enzymatic reactions at the tetrahydrodipicolinate branchpoint in the L-lysine pathway of *Corynebacterium glutamicum*. Derivatives of the precursors pyruvate and oxaloacetate condense to form the branchpoint intermediate tetrahydrodipicolinate. The one-step pathway transforms tetrahydrodipicolinate to *meso*-diaminopimelate (*meso-DAP*) via the activity of (*1*) *meso*-α-ε-diaminopimelate D-dehydrogenase (*DDH*; EC 1.4.1.16) encoded by the *ddh* gene. The four-step succinylase pathway contains the following enzymes: (*2*) tetrahydrodipicolinate *N*-succinyltransferase (*DAPD*; EC 2.3.1.89) encoded by the *dapD* gene, (*3*) *N*-succinyl-LL-diaminopimelate aminotransferase (*DAPC*; EC 2.6.1.17) encoded by the *dapC* gene, (*4*) *N*-succinyl-LL-diaminopimelate desuccinylase (*DAPE*; EC 3.5.1.18) encoded by the *dapE* gene, and (*5*) LL-diaminopimelate epimerase (*DAPF*; EC 5.1.1.7) encoded by the *dapF* gene. The common product of the dual pathway, *meso*-diaminopimelate, is transformed to L-lysine by the activity of (*6*) *meso*-α-ε-diaminopimelate decarboxylase (*LYSA*; EC 4.1.1.20) encoded by the *lysA* gene

dapE gene was chosen for disruption in this report because of evidence suggesting that it may have a rate-controlling role in the four-step pathway (see below).

N-Succinyl-Dap desuccinylase has been purified to homogeneity in *Escherichia coli* (Lin et al. 1988). The *dapE* gene has been compared to the *argE* gene, which encodes acetylornithine deacetylase in the ornithine biosynthesis pathway of *E. coli*, and it is hypothesized that these deacylases may have a common evolutionary origin because of similarities in their amino acid sequences (26% homology) and because both gene products require a cobalt cofactor (Boyen et al. 1992). Wu et al. (1992) found that the *msgB* gene, which encodes a protein that suppresses the temperature-sensitive *grpE* heat-shock gene, has complete amino acid sequence homology to the *E. coli dapE* gene. There has been no analogous research published on either the *argE* or *msgB* genes in *Corynebacterium*.

The *meso*-Dap dehydrogenase (*ddh*) gene in the one-step pathway has been cloned and sequenced in *C. glutamicum* (Ishino et al. 1987; Yeh et al. 1988). Previous experiments (Yeh et al. 1988; Cremer et al. 1991) have shown that overexpression of the *ddh* gene on a plasmid results in 18- to 20-fold amplification of *meso*-Dap dehydrogenase activity, but the increase in activity of this enzyme has no effect on lysine production in wild-type *Corynebacterium* containing both the succinylase and *meso*-Dap dehydrogenase pathways. The effect of *meso*-Dap dehydrogenase amplification on lysine production in a *Corynebacterium* strain containing only the *meso*-Dap dehydrogenase pathway has not been described. The present study extends the characterization of lysine-production pathways by investigating the effect of varying *meso*-Dap dehydrogenase activity in *dapE*-disruption strains that cannot utilize the succinylase pathway to produce lysine.

Previous research indicates that when the *ddh* gene is disrupted in *C. glutamicum*, cell growth is not affected, but the lysine titer decreases two- to fourfold (Schrumpf et al. 1991). Schrumpf et al. also found that ddh^- mutants accumulated up to 25 mM cytosolic *N*-succinyl-LL-Dap, while the wild type only accumulated trace amounts. This observation led to the hypothesis that *N*-succinyl-Dap desuccinylase activity in the four-step pathway may limit lysine production. A subsequent publication (Sonntag et al. 1993) with the same ddh^- strain revealed that lysine production varied significantly with culture conditions.

The accumulation of intracellular *N*-succinyl-Dap is not unique to the *C. glutamicum* ddh^- strain. Gilvarg (1959) found that *N*-succinyl-Dap accumulates in a Dap-requiring mutant of *E. coli*. In addition, Sundharadas and Gilvarg (1967) found that Dap auxotrophs of *Bacillus megaterium* accumulate *N*-acetyl-Dap in their cytosol. Both *E. coli* and *B. megaterium* utilize only the four-step pathway to synthesize lysine. These results support the hypothesis that the *N*-succinyl-LL-Dap desuccinylase reaction may be a rate-controlling step in the four-step pathway to *meso*-Dap and lysine biosynthesis, but they do *not* imply that the four-step pathway limits lysine production when a cell also has access to the one-step *meso*-Dap dehydrogenase pathway.

The objectives of this work were to elucidate the dependence of lysine synthesis on the two pathways by constructing *dapE*-disruption mutant strains and establishing the effect of *dapE* disruption on cellular growth, glucose uptake and lysine production. The phenotype of the *dapE*-disruption mutant was characterized in the presence of varying levels of *meso*-Dap dehydrogenase activity. Our results indicate that the *dapE* gene is dispensable for lysine overproduction in shake-flask cultures, and amplification of the *ddh* gene has no effect on lysine production in *Corynebacterium* that utilizes only the one-step *meso*-Dap dehydrogenase pathway.

Materials and methods

Bacterial strains, plasmids and culture conditions

The strains and plasmids used or constructed in this study are listed in Table 1. This parental strain, *C. glutamicum* ATCC 21253, is a homoserine, or threonine plus methionine, auxotrophic strain that overproduces and excretes lysine after the depletion of threonine from the extracellular medium. *C. glutamicum* was routinely grown at 30 °C in LB (per liter: 10 g tryptone, 5 g NaCl, and 5 g yeast extract) supplemented with 5 g D-glucose/l. Minimal medium modified from Kiss and Stephanopoulos (1991) contained, per liter, 20 g D-glucose, 200 mg $MgSO_4 \cdot 7H_2O$, 1 g NaCl, 75 mg Na_2 EDTA $\cdot 2H_2O$, 25 mg $FeSO_4 \cdot 7H_2O$, 50 mg $CaCl_2 \cdot 2H_2O$, 1.2 g trisodium citrate $\cdot 2H_2O$, 10 ml trace mineral salts, 8 g K_2HPO_4, 1 g KH_2PO_4, 150 mg L-threonine, 80 mg L-methionine, 100 mg L-leucine, 15 g $(NH_4)_2SO_4$, 1 mg biotin, and 1 mg thiamine $\cdot$ HCl. The 100× trace mineral salts solution contained, per liter, 200 mg $MnSO_4 \cdot H_2O$, 200 mg $FeCl_3 \cdot 6H_2O$, 50 mg $ZnSO_4 \cdot 7H_2O$, 20 mg $Na_2B_4O_7 \cdot 10H_2O$, 20 mg $CuCl_2 \cdot 2H_2O$, and 10 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$. Cells used for enzyme assays were grown to mid-exponential phase in 50–100 ml of either complex or minimal medium. Growth and amino acid production studies were performed exclusively in 50 ml minimal medium.

E. coli strains were grown in LB at 37 °C. When necessary, 50 mg/l ampicillin, 25 mg/l kanamycin, or 20 mg/l chloramphenicol antibiotics were added to the medium for selection pressure.

DNA techniques

Standard protocols (Sambrook et al. 1989) were used for the construction, purification, and analysis of plasmid DNA. Restriction enzymes, T4 DNA ligase, and other reagents were purchased from New England Biolabs or Boehringer Mannheim. DNA fragments were isolated from 1% agarose gels using the QIAEX II Gel Extraction Kit for DNA purification (Qiagen). *Corynebacterium* was transformed via electroporation as described in Jetten et al. (1995).

The polymerase chain reaction (PCR) was used to amplify the *N*-succinyl-LL-Dap desuccinylase (*dapE*) gene as a 1.4-kb fragment of DNA. Flanking primers (Gibco BRL) were selected on the basis of the sequence published by Wehrmann et al. (1995). The upstream primer sequence was 5′-**CTGCAG**CGATATTTGCGATTCCAACTGCT-3′, while the downstream primer sequence was 5′-**CTGCAG**CGCATTTCTAGCAAGCGT-3′. An artificial *Pst*I site (in bold type) was inserted at the 5′-terminal ends of both primers to facilitate subsequent cloning steps. The amplified *dapE*

Table 1 Bacterial strains and plasmids. *ATCC* American Type Culture Collection, Rockville, Md, USA; *BGSC* Bacillus Genetic Stock Center, Columbus, Ohio, USA. *Km*^{r}, *Ap*^{r}, *Cm*^{r}, *Spec*^{r}, *Rf* resistance against kanamycin, ampicillin, chloramphenicol, spectinomycin and rifampicin respectively

Strain or plasmid	Relevant characteristics	Source or reference
Corynebacterium glutamicum		
21253	L-Lysine-overproducing strain, *hom*$^-$	ATCC
212DAPEM	*dapE*$^-$ derivative of 21253, Cm^r	This work
ASO19-E12	Restriction-deficient and Rf^r derivative of ATCC 13059	Follettie 1989
Escherichia coli		
DH5α	*lacZ* Δ*M15*, *recA*$^-$, *hsdR*$^-$	Hanahan, 1983
S17-1	*hsdR*, *recA*, *tra* from RP4-2 integrated in chromosome	Schafer et al. 1990
Bacillus sphaericus		
13A5	*ddh*$^+$, *dapE*$^-$	BGSC
Plasmids		
pBluescript KS	Cloning vector, Ap^r	Stratagene
pPCR-Script	Cloning vector, Ap^r	Stratagene
pSUP301	pACY177 derivative, *mob*, Ap^r, Km^r	Schafer et al. 1990
pMT1	*E. coli* (Ap^r)/*C. glutamicum* (Km^r) shuttle vector	Follettie et al. 1993
pMG110	pUC4 derivative, Ap^r, *pneo::cat* Cm^r	Gubler et al. 1994
pAL214	pBluescript KS derivative, Spec^r, Ap^r	This work
pDDHHind	pBluescript KS derivative, *ddh*$^+$, Ap^r	This work
pCSR1	pMT1 derivative containing *ddh* from pDDHHind	This work
pDapEScript	pCR-Script derivative, *dapE*$^-$, Ap^r	This work
pDapESm	pDapEScript derivative, *dapE*$^-$, Ap^r, Spec^r	This work
pDapSupSm	pSUP301 derivative, *dapE*$^-$, Spec^r	This work
pDapCmSup	pSUP301 derivative, *dapE*$^-$, Cm^r	This work

PCR product was inserted into an *Srf*I-digested pCR-Script cloning vector according to the ligation protocol outlined in the Stratagene pCR-Script Amp SK(+) Cloning Kit (Stratagene Cloning Systems, La Jolla, Calif.). The resulting 4.4-kb plasmid was named pDapEScript. A similar PCR strategy was used to amplify the *meso*-Dap dehydrogenase (*ddh*) gene as a 1.8-kb fragment of DNA. The following primers were designed for the *ddh* gene amplification using the sequence published by Ishino et al. (1987): 5′-CCTCTTG**CTGCAG**CGAAGACAC-3′ and 5′-CGGGATTTT**CTGCAG**CCCAGGT-3′. The amplified *ddh* PCR product was inserted via *Pst*I digestion into a pBluescript vector that was modified by removal of the *Hin*dIII site (pDDHHind).

PCR amplification was performed using the TwinBlock System microprocessor-controlled temperature cycler. The 100-µl reaction mixtures contained 10 mM TRIS/HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 100 pmol primers, 20 nmol each deoxynucleoside triphosphate, 2.5 units *Taq* DNA polymerase (Boehringer Mannheim), and 1 µg genomic template DNA. The QIAamp Tissue Kit for chromosomal DNA preparation (Qiagen) was used to isolate *C. glutamicum* genomic DNA from 5-ml overnight cultures. After a hot start at 94 °C for 1 min, amplification was performed during 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C (*dapE*) or 68 °C (*ddh*) for 2 min, and extension at 72 °C for 3 min. The final cycle extension time at 72 °C was 10 min.

The strategy for the construction of the mobilizable plasmid pDapCmSup used for disruption of the *dapE* gene is given in the legend of Fig. 2. Mobilization of the gene-disruption plamid pDapCmSup from *E. coli* to *C. glutamicum* via marker-exchange mutagenesis was performed as described previously (Schafer et al. 1990; Schwarzer and Puhler 1991; Gubler et al. 1994). The donor:recipient ratio was 1:1, and the recipients were heat-treated at 49 °C for 9 min. *C. glutamicum* transconjugants were selected on LB/agar containing, per liter, 25 mg nalidixic acid and 10 mg chloramphenicol. Positive double-crossover candidates (*dapE*$^-$) were distinguished from single-crossovers (*dapE*$^+$) by their inability to grow on 6 mg/l kanamycin. Transconjugants were further verified by Southern blot analysis using the Genius System from Boehringer Mannheim following their Users' Guide for filter hybridization.

The plasmid pCSR1 contains the intact *meso*-diaminopimelate dehydrogenase (*ddh*) gene on a vector that replicates in *Corynebacterium*. This plasmid was constructed by excising the PCR-amplified *ddh* gene from the pBluescript-derived vector pDDHHind and inserting the gene into the *Kpn*I-*Xba*I-digested *E. coli*-*C. glutamicum* shuttle vector, pMT1.

Enzyme assays

Assays were carried out with cell-free extracts, which were prepared as described by Jetten and Sinskey (1993), with the exception that the homogenization buffer consisted of 100 mM HEPES (pH 7.8), 200 mM KCl and 1 mM 2-mercaptoethanol was added to protect sulfhydryl enzymes. The protein concentration of the extracts was determined by the method of Bradford (1976). All enzyme activities were determined at room temperature in a Hewlett-Packard 8452A diode-array spectrophotometer.

The chemicals used for enzymatic assays were purchased from Sigma Chemical Co., with the exception of the substrate *N*-succinyl-LL-Dap. This was synthesized by anhydride acylation and partially purified by ion-exchange chromatography according to the method described by Bouvier et al. (1992).

The enzymatic activity of *N*-succinyl-LL-Dap desuccinylase (EC 3.5.1.18) was measured with a ninhydrin assay as described by Kindler and Gilvarg (1960). The assay reaction mixtures contained 10.4 µmol *N*-succinyl-LL-Dap, 1 µmol $CoCl_2$, 20 µmol NH_4OH, 200 mM TRIS/HCl buffer at pH 8.0, and crude extract in a total volume of 550 µl. The reaction was initiated with crude extract and incubated at 37 °C. The ninhydrin assay was carried out on initial samples at $t = 0$ and samples taken after 30 min as a final assay time. The oxidative deamination enzymatic activity of the reversible *meso*-Dap dehydrogenase (EC 1.4.1.16) was determined by measuring the increase in absorbance of NADPH at 340 nm as *meso*-Dap is converted to tetrahydrodipicolinate. The reaction conditions were those described by Misono et al. (1979).

Determination of amino acids and glucose

Amino acids were analyzed as *ortho*-phthaldialdehyde derivatives by reversed-phase chromatography on an AminoQuant column at 40 °C in a Hewlett-Packard series 1050 high-pressure liquid chromatography (HPLC) system. Glucose was determined by HPLC on

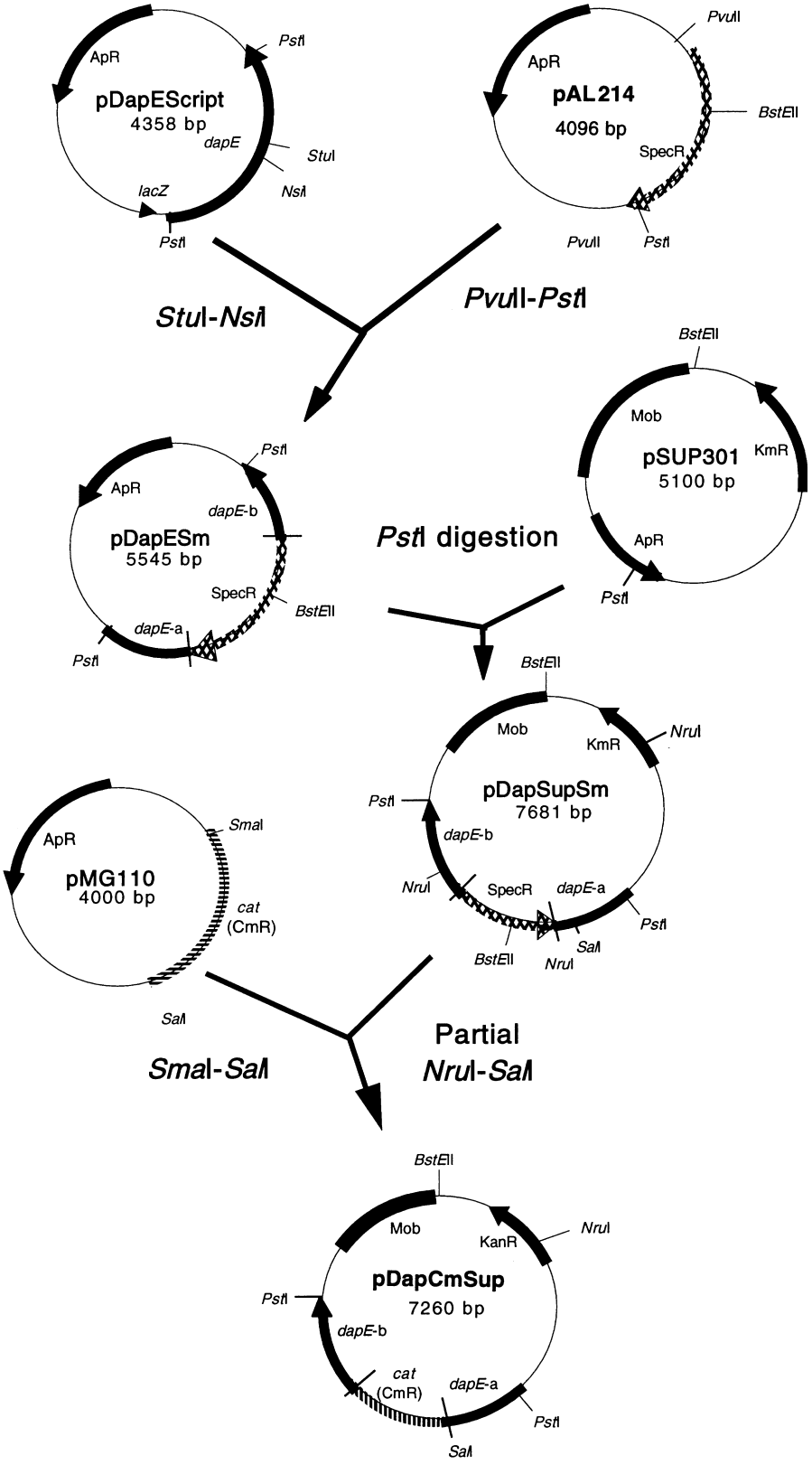

Fig. 2 Cloning strategy for the construction of the mobilizable plasmid used for *dapE* disruption. A 1.3-kb spectinomycin marker was removed from pAL214 via *Pst*I-*Pvu*II digestion, and this marker was used to replace an 85-bp *Nsi*I-*Stu*I internal fragment in the *dapE* gene of pDapEScript, thus rendering the *dapE* gene nonfunctional in plasmid pDapESm. The mobilizable plasmid pDapSupSm was constructed by ligating the 2.6-kb *Pst*I fragment from pDapESm into the *Pst*I site of the vector pSUP301. When initial unsuccessful transconjugation attempts were conclusively attributed to the presence of spectinomycin resistance in *C. glutamicum* wild-type strains, the spectinomycin marker in pDapSupSm was replaced by chloramphenicol. The mobilizable plasmid pDapCmSup was constructed by ligating the 1.1-kb *Sma*I-*Sal*I fragment from pMG110 carrying the chloramphenicol-resistance marker into the partially digested *Nru*I-*Sal*I sites of pDapSupSm. The plasmid pDapCmSup was used for successful transconjugation of *C. glutamicum* 21253 and subsequent disruption of the *dapE* gene to form the strain 212DAPEM

a BioRad Aminex HPX-87H reverse-phase column at 45 °C with a mobile phase consisting of 5 mM H_2SO_4.

Results

Site-specific disruption of the *dapE* gene in *C. glutamicum*

In order to disrupt *dapE* with a selectable marker, the *cat* cartridge from pMG110 (Fig. 2) was used to substitute for an 85-bp DNA fragment of the *dapE* gene in plasmid pDapEScript. The disrupted *dapE* gene was transferred to the mobilizable vector pSUP301, and the final plasmid, pDapCmSup, was mobilized from *E. coli* S17-1 into *C. glutamicum* 21253. After conjugation and DNA exchange between the *dapE* fragments on pDapCmSup and the chromosomal copy of the *dapE* gene, strain 21253 gained chloramphenicol resistance in conjunction with inactivation of the *dapE* gene. Desirable double-cross-over mutants were isolated from single-cross-over mutants by verifying chloramphenicol resistance and kanamycin sensitivity on complex medium plates.

Disruption of the *dapE* gene was further confirmed by Southern blot analysis comparing the genomic DNA of the mutant strain 212DAPEM to the parent strain 21253, as shown in Fig. 3. Two independent probes were used for verification. The *dapE* gene probe indicated that the gene was internally disrupted by a 1.1-kb fragment of DNA, and the *cat* gene probe verified that the 1.1-kb internal fragment was the chloramphenicol resistance marker DNA.

To further verify that the *dapE* gene was disrupted in strain 212DAPEM, in vitro enzymatic assays were performed on the crude extract of 21253, 212DAPEM, and two control strains. *B. sphaericus* 13A5 served as a negative control because this strain does not utilize the four-step succinylase pathway and therefore does not contain a *dapE* gene. Conversely, *E. coli* DH5α served as a positive control because this strain does not utilize the *meso*-Dap dehydrogenase pathway, so it contains all four genes in the four-step succinylase pathway. The *N*-succinyl-Dap desuccinylase assay results shown in Table 2 confirm that strain 212DAPEM does not have the desuccinylase activity. Assay controls indicate that the cofactor requirement is strict. The activity of this enzyme drops by 50% without the divalent cobalt ion.

Amplification of *meso*-Dap dehydrogenase in the *dapE*-disruption strain, 212DAPEM

Overexpression of the *ddh* gene obtained from PCR amplification was achieved by transforming *C. glutamicum* with plasmid pCSR1. Table 2 shows that strains

Fig. 3A, B Southern blot analysis validating mutant construction. Two Southern blots provide evidence for the disruption of the *dapE* gene in the chromosomal DNA of *C. glutamicum* 21253. Genomic DNA from both the parent and mutant strains was digested with *Pst*I, subjected to agarose gel electrophoresis, blotted onto a nitrocellulose filter, and hybridized with a digoxigenin-11-dUTP probe. **A, B** Lanes: *1* the size marker fragments of labeled λ DNA cleaved with *Hin*dIII, *2* the *Pst*I-digested DNA of parent-type 21253 (wild type), 3–10 the *Pst*I-digested DNA of *dapE*$^-$ mutant candidates. Strain 212DAPEM containing the DNA (lane *9*) was selected (on the basis of results of the *N*-succinyl-LL-dipimelate desuccinylase enzymatic assay) for further experimentation. The two panels show the results of two independently labeled DNA probes. **A** The probe specific for the *dapE* gene was isolated from the polymerase chain reaction product. When genomic DNA was probed with the *dapE* gene, the fragment of DNA in strain 21253 containing the gene is highlighted at 6.8 kb. The 1.1-kb displacement of the DNA fragments shown in lanes 3–10 corresponds to integration of a piece of DNA at the *dapE* gene location in the chromosome of the mutant strain candidates. **B** The probe specific for the chloramphenicol gene (*cat*) was isolated from pMG110 to confirm antibiotic marker integration into the *Corynebacterium* chromosome. When genomic DNA was probed with the *cat* gene, the restriction pattern reveals that this 1.1-kb increase in the mutant DNA fragment size (3–10) is indeed due to *cat* integration, which is noticeably absent from the wild-type DNA shown in *2*

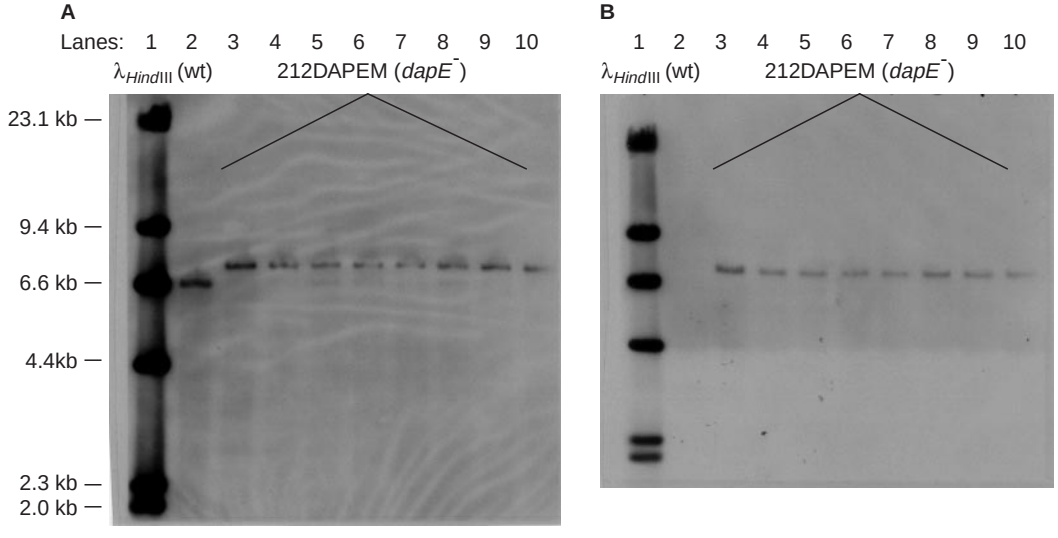

Table 2 Specific enzymatic activities. Specific activities of *meso*-diaminopimelate (*Dap*) and succinyl-Dap desuccinylase in various *C. glutamicum* crude extracts are compared to the activity in *E. coli* and *B. sphaericus* control extracts. These activity results show the average of three independently prepared crude extracts, and the background activity (minus crude extract, substrates, or cofactors) has been subtracted from the final value. Dehydrogenase error ≤ 5%; desuccinylase error ≤ 40%

Strain	Specific enzyme activities (nmol min^{-1} mg $protein^{-1}$)	
	Dehydrogenase	Desuccinylase
C. glutamicum 21253	123	2.5
21253 (pCSR1)	985	1.9
212DAPEM	115	< 0.5
212DAPEM (pCSR1)	970	< 0.5
B. sphaericus 13A5	200	< 0.5
E. coli DH5α	< 55	10.0

transformed with pCSR1 have an eightfold higher *meso*-Dap dehydrogenase activity than the corresponding strains without the plasmid. *B. sphaericus* positive controls exhibited 60%–70% higher *meso*-Dap dehydrogenase activity than *C. glutamicum* strains, consistent with literature data (Misono et al. 1979; Schrumpf et al. 1991).

Impact of *dapE* disruption on cell growth, glucose uptake, and L-lysine production

Wild-type and mutant strains were grown in 50 ml minimal medium shake-flask cultures to determine the effect of the *dapE* gene disruption on growth, glucose uptake, and lysine production rates in *C. glutamicum*. Growth profiles were similar for all four strains, and data obtained during the exponential phase yielded equivalent estimates for the maximum specific growth rate (μ_{max} = 0.20/h), as shown in Fig. 4. The glucose uptake rates shown in Fig. 5 are another indication that

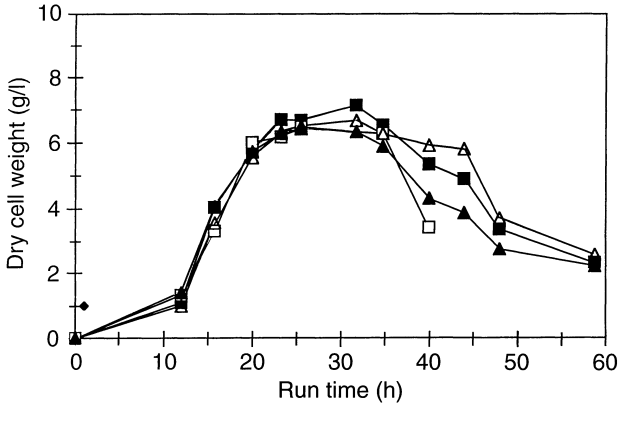

Fig. 4 Comparison of growth in *C. glutamicum* 21253 (■) and 212DAPEM (▲). □, △ Strains containing the plasmid pCSR1 (amplified *ddh*). Increases in cell density were measured by absorbance (*A*) at 660 nm and converted to dry cell weight (DCW) on the basis of the correlation DCW = 0.28 × *A* (Gubler et al. 1994)

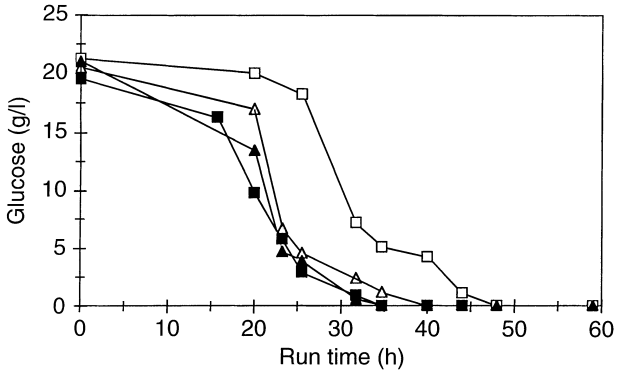

Fig. 5 Comparison of glucose uptake profiles in *C. glutamicum* 21253 (■) and 212DAPEM (▲). □, △ Strains containing the plasmid PCSR1 (amplified *ddh*)

the phenotype of 212DAPEM (*dapE*) is indistinguishable from the parent-type 21253. Although there was an initial lag in the glucose consumption profile of strain 21253 (pCSR1), the maximum specific glucose uptake rate during exponential phase was comparable for all strains (q_{glu} = 0.3–0.4 g glucose g dry cell $weight^{-1}$ h^{-1}), indicating that the absence of the *dapE* gene does not affect glucose-intensive cellular processes required for cell maintenance or survival.

Figure 6 shows the lysine production profiles for the four strains. Although the time profiles differ somewhat, lysine secretion rates, final titers (1.2 g/l), and yields (0.06 mol lysine/mol glucose) are statistically equivalent, indicating that the lysine-producing capability of *C. glutamicum* is not impaired by the absence of the desuccinylase. This result was not affected by *ddh* overexpression. Our results thus demonstrate that amplifying enzymatic activity does not guarantee higher pathway flux, even in the absence of competing pathways.

Discussion

In order to probe the role of the dual pathway in lysine synthesis in *C. glutamicum*, a *dapE*$^{-}$-disruption mutant

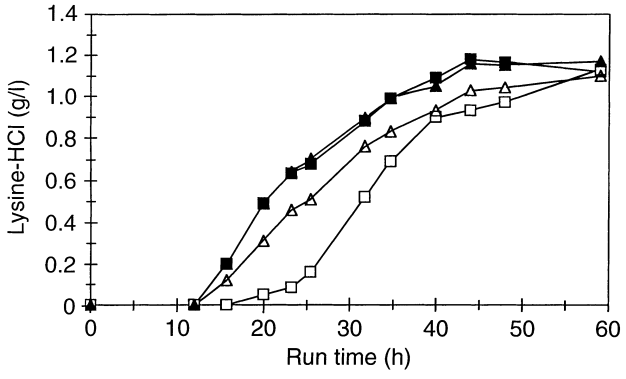

Fig. 6 Comparison of lysine production rates in *C. glutamicum* 21253 (■) and 212DAPEM (▲). □, △ Strains containing the plasmid pCSR1 (amplified *ddh*)

was constructed and compared with the parent-type *C. glutamicum* 21253 phenotype. The disruption was confirmed by antibiotic selection, Southern blots, and enzymatic assays. The absence of *N*-succinyl-Dap desuccinylase activity had no effect on the growth rate, which is consistent with the results of Wehrmann et al. (1998), and no effect on the glucose-uptake rate. In addition, strain 212DAPEM (*dapE*$^-$) produced lysine at a rate and yield similar to those of the parental strain 21253. Although a *dapE*$^-$ strain grown in limiting ammonium may have a different morphology and increased susceptibility to lysis in comparison to the parent type (Wehrmann et al. 1998), these characteristics do not affect the lysine productivity of the mutant when grown in minimal medium with sufficient ammonium to sustain normal growth. The one-step *meso*-Dap dehydrogenase pathway apparently compensates for the lack of the succinylase pathway flux to lysine.

Although these results show that the *dapE* gene is dispensable for lysine production, it is important to note that the absence of the *dapE* gene does not imply that the entire four-step succinylase pathway is dispensable. Currently it is not clear if there are other cellular functions for the intermediates upstream of LL-Dap in the four-step pathway, and it is possible that, even in the *dapE*$^-$ strain, the biosynthesis of these upstream intermediates is sufficient to meet cellular demands. It is not clear if a larger-scale fed-batch or continuous culture of the *dapE*$^-$ strain would elicit different physiological effects due to variations in intracellular fluxes. The results shown in this study simply demonstrate that flux through the four-step pathway is not necessary for *meso*-Dap or lysine production in small-scale batch shake flasks.

An eightfold amplification of *meso*-Dap dehydrogenase activity in strains 21253 and 212DAPEM also had no effect on growth, glucose uptake or lysine production. As shown in Table 2, the specific enzymatic activity of *meso*-Dap dehydrogenase under saturating in vitro conditions in parental strain 21253 is 123 nmol NADPH min^{-1} mg total protein^{-1}. Assuming an equimolar conversion of NADPH to lysine in the reversible dehydrogenase reaction, and assuming that the dry cell weight (DCW) is 50% protein, this enzymatic rate corresponds to a specific lysine productivity of 0.54 g lysine g DCW^{-1} h^{-1} which is 50-fold higher than the activity needed for the production rates (approx. 0.012 g lysine/g DCW^{-1} h^{-1}) observed in this study. In fact, this wild-type *meso*-Dap dehydrogenase activity is fourfold higher than the maximum production rates (approx. 0.15 g lysine g DCW^{-1} h^{-1}) observed in larger-scale fed-batch cultures of *C. glutamicum* 21253 (Kiss and Stephanopoulos 1991). Since the wild-type *meso*-Dap dehydrogenase activity is already sufficient to meet the cellular demand for lysine, it is not surprising that amplification of the *ddh* gene has no effect on lysine production rates. It should be noted that the correlation of enzymatic activity and lysine production is valid even when the succinylase pathway is not used, as shown by the profiles of strain 212DAPEM. The results of this genetic study support the hypothesis that *meso*-Dap dehydrogenase is not a rate-limiting step in lysine biosynthesis in small-scale cultures. The correlation between the in vitro measurement and calculated flux capacity of the *meso*-Dap dehydrogenase enzyme suggests that the rate of formation of the tetrahydrodipicolinate precursor may limit lysine production in this system.

Wehrmann et al. (1994) were successful in achieving a tenfold overexpression of the *N*-succinyl-Dap desuccinylase activity, but they do not report the effects of this amplification on lysine production. Since all enzymes in the four-step succinylase pathway have low activity and are produced in relatively low abundance (Chatterjee and White 1982; Lin et al. 1988; Schrumpf et al. 1991), they would probably require simultaneous amplification to effect higher flux through the pathway.

Acknowledgements We are grateful to Divya Janardhana for technical assistance. This research was supported by the National Science Foundation (grant BES-9634569) and the Department of Energy BES (grant DE-FG02-94ER14487). C. Shaw-Reid was the recipient of a National Science Foundation Graduate Fellowship and a Ford Foundation Dissertation Fellowship.

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