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Expression of the *Corynebacterium glutamicum panD* Gene Encoding L-Aspartate-α-Decarboxylase Leads to Pantothenate Overproduction in *Escherichia coli*

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The *Corynebacterium glutamicum panD* gene was identified by functional complementation of an *Escherichia coli panD* mutant strain. Sequence analysis revealed that the coding region of *panD* comprises 411 bp and specifies a protein of 136 amino acid residues with a deduced molecular mass of 14.1 kDa. A defined *C. glutamicum panD* mutant completely lacked L-aspartate-α-decarboxylase activity and exhibited β-alanine auxotrophy. The *C. glutamicum panD* ($panD_{C.g.}$) as well as the *E. coli panD* ($panD_{E.c.}$) genes were cloned into a bifunctional expression plasmid to allow gene analysis in *C. glutamicum* as well as in *E. coli*. The enhanced expression of $panD_{C.g.}$ in *C. glutamicum* resulted in the formation of two distinct proteins in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, leading to the assumption that the $panD_{C.g.}$ gene product is proteolytically processed into two subunits. By increased expression of $panD_{C.g.}$ in *C. glutamicum*, the activity of L-aspartate-α-decarboxylase was 288-fold increased, whereas the $panD_{E.c.}$ gene resulted only in a 4-fold enhancement. The similar experiment performed in *E. coli* revealed that $panD_{C.g.}$ achieved a 41-fold increase and that $panD_{E.c.}$ achieved a 3-fold increase of enzyme activity. The effect of the $panD_{C.g.}$ and $panD_{E.c.}$ gene expression in *E. coli* was studied with a view to pantothenate accumulation. Only by expression of the $panD_{C.g.}$ gene was sufficient β-alanine produced to abolish its limiting effect on pantothenate production. In cultures expressing the $panD_{E.c.}$ gene, the maximal pantothenate production was still dependent on external β-alanine supplementation. The enhanced expression of $panD_{C.g.}$ in *E. coli* yielded the highest amount of pantothenate in the culture medium, with a specific productivity of 140 ng of pantothenate mg (dry weight)$^{-1}$ h^{-1}.

Pantothenate, a member of the vitamin B complex, is synthesized by microorganisms and plants but not by mammals which require it as a nutritional factor. The biosynthesis of pantothenic acid in *Escherichia coli* consists of two convergent pathways requiring four biosynthetic genes (13). The initial step of one branch is the formation of ketopantoate from α-ketoisovalerate, an intermediate of the valine biosynthetic pathway, catalyzed by the *panB*-encoded enzyme ketopantoate hydroxymethyltransferase. Subsequent reduction to pantoate requires the action of the *panE* gene product, ketopantoate reductase. In the second branch of the pathway β-alanine is formed by the α-decarboxylation of aspartate, catalyzed by the *panD*-encoded enzyme L-aspartate-α-decarboxylase. Finally, the *panC* gene product pantothenate synthase performs the combining reaction of the two branches, the ATP-dependent condensation of pantoate and β-alanine to form pantothenate. In subsequent reactions, pantothenate is converted to the essential phosphopantotheine moiety, the acyl group carrier found in acyl carrier proteins and coenzyme A (36). These essential compounds participate in various reactions of intermediary metabolism (1).

The *panD* gene product, L-aspartate-α-decarboxylase, of *E. coli* is an unusual enzyme in that it requires pyruvate as a covalently bound, catalytically active prosthetic group (39). It belongs to a small group of mechanistically related pyruvoyl-dependent enzymes, together with other decarboxylases and reductases from various prokaryotic and eukaryotic sources (for a review, see reference 37). Most if not all of these enzymes are initially translated as inactive precursor proteins (π-proteins) which are proteolytically cleaved at a specific X-Ser bond. Particularly, the *E. coli* L-aspartate-α-decarboxylase is processed at a Gly-Ser bond (21). As a consequence, two dissimilar subunits are produced, a β-subunit with X-OH at its C terminus and an α-subunit with a pyruvoyl group formed from the serine residue at its N terminus. In most of these enzymes, the subunits are closely associated, but usually they are not covalently linked. The determination of the crystal structure of the *E. coli* L-aspartate-α-decarboxylase revealed that the active enzyme is a tetramer which is composed of three α- and β-subunits and an incompletely processed π-protein (2). These findings are consistent with the determination of only three pyruvoyl residues per tetramer in the active recombinant *E. coli* enzyme (21).

Since pantothenate is a nutritional requirement in mammals, the interest in a biotechnological approach for the production of pantothenate is enormous. In this study, we report on the identification of the *Corynebacterium glutamicum panD* gene encoding L-aspartate-α-decarboxylase. We describe the characterization and the directed mutagenesis of the *C. glutamicum panD* gene. Furthermore, we report on its expression in *E. coli* in view of pantothenate production.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study and their sources are listed in Table 1. *E. coli* and *C. glutamicum* strains were routinely cultivated in Luria-Bertani (LB) medium (22) at 37 and 30°C, respectively. During complementation studies, the *panD* mutant strain *E. coli* DV9 was incubated at 39°C because this slightly elevated temperature caused a more-pronounced *panD* auxotrophy. For complementation tests and biochemical studies, *E. coli* cells were grown in Medium E consisting of Medium E salts (38) supplemented with thiamine (0.2 mg/ml) and glucose (4 g/liter). For growth of the methionine auxotrophic strain *E. coli* DV9, 50 μg of methionine per ml was added. Medium CGXII (16) was used for *C. glutamicum* studies as a test

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference[a]
E. coli		
MG1655	Wild type	CGSC
DH5αMCR	*mcrA* Δ(*mrr-hsdRMS-mcrBC*) Δ(*lacZYA-argF*) *lacZ*Δ*M15 hsdR recA1*	10
SCS110	*dam dcm*	14
S17-1	Mobilizing donor strain carrying an integrated RP4 derivative, *recA*	28
DV9	*panD metB1*	CGSC
C. glutamicum		
ATCC 13032	Wild type	ATCC
RES167	Restriction deficient mutant of ATCC 13032, Δ(*cglIM-cglIR-cglIIR*)	University of Bielefeld
ND2	*panD* insertion mutant of ATCC 13032, Cm^r	This work
L. plantarum	Pantothenate auxotroph test organism for quantitation of pantothenate	ATCC
Plasmids		
pUC18	*E. coli* cloning and sequencing vector, Ap^r	40
pK18*mob*	Mobilizable *E. coli* cloning and sequencing vector, Km^r	25
pK18*mobsacB*	pK18*mob* containing *sacB* derivative, Km^r	25
pEC31	pSVB31 containing the chloramphenicol resistance gene *cmx* of pTP10	A. Tauch, University of Bielefeld
pNIC1.3	pUC18 carrying a 7-kb *Sau*3A chromosomal fragment of *C. glutamicum* RES167, Ap^r	This work
pND10	pUC18 with a 3-kb *Eco*RI insert of pNIC1.3 containing the *panD* region, Ap^r	This work
pND10-1	pUC18 with a 1.9-kb *Eco*RI-*Bam*HI insert of pND10, Ap^r	This work
pND10-2	pUC18 with a 1.4-kb *Pst*I-*Bam*HI insert of pND10, Ap^r	This work
pND10-3	pUC18 with a 1.15-kb *Xba*I insert of pND10, Ap^r	This work
pND10-4	pUC18 with a 1.16-kb *Bam*HI-*Eco*RI insert of pND10, Ap^r	This work
pZ8-1	Shuttle expression vector, containing *tac* promoter, *mcs*, *E. coli ori* from pACYC177, *C. glutamicum ori* from pHM1519, Km^r	7
pND-D1	pZ8-1 with *Eco*RI-*Bgl*II PCR amplificate of *E. coli* K-12 *panD* gene, Km^r	This work
pND-D2	pZ8-1 with *Eco*RI-*Pst*I PCR amplificate of *C. glutamicum* RES167 *panD* gene, Km^r	This work
pND12	pK18*mobsacB* with a 2.3-kb *Eco*RI-*Hin*dIII insert of pND10 containing the *panD* region, Km^r	This work
pND12-Cm	pND12 with a 2.3-kb *Pvu*II fragment from pEC31, containing the *cmx* gene, inserted in the *panD* gene, Km^r, Cm^r	This work

[a] Abbreviations: CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.; ATCC, American Type Culture Collection.

medium. The antibiotics used for plasmid selection were ampicillin (100 μg/ml) and kanamycin (50 μg/mg for *E. coli*, 25 μg/ml for *C. glutamicum*). *C. glutamicum* ND2, which carries a *panD* gene interrupted by a chloramphenicol resistance cassette, was always grown in a medium supplemented with 10 μg of chloramphenicol per ml to maintain the *panD* mutation and, when appropriate, 100 μg of β-alanine or pantothenate per ml was added.

DNA isolation, transfer, and manipulation. *E. coli* DH5αMCR was used for routine recombinant DNA experiments. Plasmid DNA of *E. coli* was prepared by the alkaline lysis method (22) modified for *C. glutamicum* by using 20 mg of lysozyme per ml of lysis buffer HB1 at 37°C for 2 h. Chromosomal DNAs of *E. coli* MG1655 and *C. glutamicum* RES167 were isolated as described elsewhere (34). DNA restriction, modification, analysis by agarose gel electrophoresis, and ligation were carried out according to standard procedures (22). Restriction endonucleases, T4 DNA ligase, and Klenow polymerase were obtained from Pharmacia (Freiburg, Germany) or from Boehringer (Mannheim, Germany) and used as recommended by the manufacturer. *E. coli* and *C. glutamicum* were transformed by electroporation (12, 33) by using the Bio-Rad Gene Pulser system (Bio-Rad, Munich, Germany). DNA restriction fragments were purified from agarose gels by means of the Nucleotrap Extraction Kit for Nucleic Acids (Macherey-Nagel, Düren, Germany).

Isolation of the *C. glutamicum panD* gene by complementation of an *E. coli panD* mutant. A *C. glutamicum* ATCC 13032 gene bank was constructed by cloning 7- to 10-kb genomic DNA fragments obtained by partial *Sau*3A digestion into the *Bam*HI site of pUC18. The ligation mix was electroporated into DH5αMCR, the plasmids were reisolated, and this gene bank was transformed into *E. coli* DV9. The resulting electroporation mixture was washed twice with Medium E and subsequently used to inoculate a liquid Medium E containing methionine (50 μg/ml) and ampicillin (100 μg/ml). Only complemented transformants can grow in this medium when incubated at 39°C, and single transformants were isolated after 48 h by plating the resulting *E. coli* DV9 culture on selective LB agar plates. Plasmids of selected transformants were isolated, and complementation was confirmed by retransformation of these plasmids into *E. coli* DV9. Further *Eco*RI subcloning of one insert of a plasmid into plasmid pK18*mob* (25) and subsequent complementation analysis led to the isolation of a complementing plasmid, designated pND10, with a chromosomal insert of 3 kb.

DNA sequencing and sequence analysis. For DNA sequencing, suitable restriction fragments of the 3-kb *Eco*RI insert of plasmid pND10 were subcloned into the *E. coli* sequencing vector pUC18. Plasmid DNA was isolated by using the Qiagen (Hilden, Germany) Plasmid Mini-Kit according to the manufacturer's protocol. The nucleotide sequence was determined on the A.L.F. Express DNA sequencer (Pharmacia, Freiburg, Germany) by the dideoxy chain-termination method (23), with the modifications of Zimmermann et al. (41), by using the Cy5-AutoRead Sequencing kit from Pharmacia. The DNA sequence data were processed by using the Staden computer program, version 97-0 (31). Similarity comparisons of nucleotide and deduced amino acid sequences with entries in databases were performed by using the BLAST search programs (9). Global multiple alignments between protein sequences were computed by using the CLUSTAL W program (35).

Construction of *panD* expression plasmids. The *C. glutamicum* and the *E. coli panD* genes, including their native ribosomal binding sites, were amplified by PCR by using synthetic oligonucleotides deduced from the nucleotide sequences of the genes (18). PCR experiments were carried out with a PCT-100 thermocycler (MJ Research, Inc., Watertown, Mass.) with *Taq* DNA polymerase (Gibco-BRL, Karlsruhe, Germany). The initial denaturation was conducted at 94°C for 2 min followed by 90 s of denaturation, 90 s of annealing at the primer-dependent temperature T_m (2AT+4GC) of −5°C (32), and 90 s of extension at 72°C. This cycle was repeated 35 times and completed by an extension step for 10 min at 72°C.

The primers pand-Ec1 (5′-GAATTCGACAGGGTAGAAAGGTAGA-3′) and pand-Ec2 (5′-AGATCTGGGATAACAATCAAGCAACC-3′) introduce flanking *Eco*RI and *Bgl*II sites to the *E. coli panD*. By using these sites, the amplified DNA fragment derived from *E. coli* MG1655 chromosomal DNA was cloned into *Eco*RI- and *Bam*HI-digested pZ8-1, resulting in plasmid pND-D1. Accordingly, the primers pand-Cg1 (5′-CATCTCACGCTATGAATTCT-3′) and pand-Cg2 (5′-ACGAGGCCTGCAGCAATA-3′) introduce *Eco*RI and *Pst*I sites to the *C. glutamicum* RES 167 *panD* gene. Plasmid pND-D2 originated from an *Eco*RI-*Pst*I ligation of this amplified product into pZ8-1. The inserts of the plasmids pND-D1 and pND-D2 were sequenced in order to verify the correctness of these constructs.

Directed *panD* mutagenesis in the chromosome of *C. glutamicum* ATCC 13032. A defined chromosomal mutation of the *panD* gene of *C. glutamicum* RES167 was constructed by insertional inactivation of the gene by using the *sacB* system, which enables positive selection of an allelic exchange by homologous recombination (25). For this purpose, a 2.3-kb *Eco*RI-*Hin*dIII fragment of plasmid pND10, carrying the *panD* gene of *C. glutamicum* ATCC 13032, was ligated into the *Eco*RI-*Hin*dIII-digested vector pK18*mobsacB*. The resulting plasmid pND12 has a single *Xba*I positioned in the *panD* gene and was thus a suitable insertion site. To prevent methylation and thus inhibition of the *Xba*I cleavage site, plasmid pND12 was transformed into *E. coli* SCS110 (14), a *dam*- and *dcm*-deficient strain. The plasmid was reisolated and cleaved with *Xba*I, and overhanging DNA ends were filled in with Klenow enzyme. For the chloramphenicol (*cmx*) gene cassette, the restriction fragments of *Pvu*II-digested vector pEC31 were separated in a 0.8% agarose gel, and the 2.3-kb fragment carrying the chloramphenicol gene was isolated as described above. Integration of the *cmx* gene into *panD* was achieved by ligation of the *Xba*I- and Klenow-treated plasmid pND12 and the *cmx* gene fragment. The ligation mixture was electroporated into *E. coli* DH5αMCR, and recombinant transformants were selected on LB agar containing 10 μg of chloramphenicol per ml. *E. coli* S17-1 was subsequently transformed with the resulting plasmid, termed pND12-Cm. After mobilization of pND12-Cm into *C. glutamicum* RES167 (24), the plasmid can establish itself only by homologous integration into the chromosome. The resulting *C. glutamicum* strain carried the *cmx*-interrupted *panD* copy and the wild-type gene separated by vector sequences. Excision of the vector was selected for by growing the cells on LB agar containing 10% sucrose. Cells able to grow on this medium have lost the plasmid due to a second crossover event that either restored the wild-type gene or led to a mutant strain carrying a *panD* gene interrupted by the inserted *cmx* gene. Southern hybridization with *Hin*dIII-digested chromosomal DNAs of sucrose-resistant clones and labeled pEC31 DNA were performed to distinguish between both genomic situations and to verify the construction of the *panD*::*cmx* insertion (22). The resulting *panD* mutant was designated *C. glutamicum* ND2. DNA labeling and hybridization was performed with the Nonradioactive DNA Labeling and Detection Kit from Boehringer (Mannheim, Germany) as recommended by the manufacturer.

Preparation of crude extracts of bacterial cell cultures. *E. coli* and *C. glutamicum* whole-cell extracts were prepared from cells grown to late logarithmic phase in selective Medium E and CGXII, respectively. For the *panD* mutant *C. glutamicum* ND2, in addition to 10 μg of chloramphenicol per ml, the medium was supplemented with 50 μg of pantothenate per ml. At optical densities measured at 580 nm (OD_{580}) of 2.3 and 10 for *E. coli* and *C. glutamicum*, respectively, 60-ml portions of cells were harvested by 15 min of centrifugation at 5,500 × *g*. All of the following procedures were carried out on ice. The pellet was carefully rinsed with distilled H_2O, resuspended in 10 ml of distilled H_2O, and subsequently centrifugated for 10 min at 5,500 × *g* and 4°C. After the supernatant was completely discarded, the pellet was resuspended in 1 ml of extraction buffer (0.1 M potassium phosphate, pH 6.8; 0.5 mM dithiothreitol), and a 1.2-ml cell suspension was added to a RiboLyser BLUE tube (Hybaid, Ltd., Teddington, United Kingdom) containing a silica-ceramic matrix. Cell disruption was performed by using the RiboLyser instrument at a speed rating of 6.5 for 30 s (*E. coli*) or two times for 30 s with a 3-min incubation on ice in between (*C. glutamicum*). Subsequently, 2 μg of DNase was added, and cell debris was removed by centrifugation. Protein concentrations of the clear crude extracts were determined by using the Bio-Rad Protein Assay, and bovine serum albumin was used as the standard.

SDS-PAGE analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 10 to 20% polyacrylamide gradient gel (Amresco, Solon, Ohio) with a Tris-glycine buffer system (17). The gels were stained with Coomassie blue (22), and the molecular masses of the enzyme subunits were derived from the relative mobilities of standard proteins. The low-range Kaleidoscope Prestained standards were purchased from Bio-Rad Laboratories (Munich, Germany).

Aspartate decarboxylase activity assay. L-Aspartate-α-decarboxylase activity was assayed by evaluating the conversion of aspartate to β-alanine as determined by high-pressure liquid chromatography (HPLC) analysis. The standard reaction mixture, modified from the reaction conditions previously described (6), contained 1.2 mM aspartate, extraction buffer, and 0.05 mg of *C. glutamicum* or 0.55 mg of *E. coli* crude extract in a final volume of 100 μl. Immediately before incubation at 37°C for 1 h (the enzyme activity was linear with time for at least 2 h [data not shown]), 40 μl of the reaction mixture was mixed with the same volume of 3% perchloric acid in water containing 393 μM δ-amino-*n*-valeric acid as an internal standard. The precipitated protein of these solutions was removed by centrifugation, and 50 μl of supernatant was added to 100 μl of sodium borate buffer (0.4 M, pH 9.5) to guarantee a pH above pH 8. These t_0 samples were kept at 4°C until used for HPLC analysis. After incubation, 50 μl of the assay solution was mixed with 50 μl of stop solution. The further treatment of these t_1 samples was as described for the t_0 samples. The β-alanine concentration of the samples was determined by using an automated reversed-phase HPLC system with precolumn derivatization by the OPA method (4). Specific activities were expressed in units per milligram, with the units equaling nanomoles of β-alanine formed (t_1 [β-alanine] − t_0 [β-alanine]) per minute.

Pantothenate assay. Pantothenate was determined by a microbiological assay by using the pantothenate auxotrophic strain *Lactobacillus plantarum* ATCC 8014 as the test organism (8). The Bacto-Pantothenate Assay Medium used for the assay was purchased from Difco Laboratories. For pantothenate synthesis studies, Medium E containing kanamycin was inoculated with overnight-grown *E. coli* cultures to an initial OD_{580} of 0.1. After incubation for 5 h, the cell-free medium was filter sterilized, and the pantothenate concentration was determined according to the method set forth in the Difco Manual. A standard curve with 0 to 10 ng of pantothenic acid per ml was set up for each assay. *E. coli* dry cell mass was determined by measuring the OD_{580}, with a value of 1 corresponding to 0.37 mg (dry mass)/ml.

Nucleotide sequence accession number. The nucleotide sequence of the *panD* region of *C. glutamicum* ATCC 13032 has been deposited in the GenBank database under accession number AF116184.

RESULTS

A 3-kb DNA fragment of *C. glutamicum* complemented an *E. coli panD* mutant. In order to isolate the *C. glutamicum panD* gene, we followed a strategy based on the complementation of the *panD* mutant strain *E. coli* DV9. A *C. glutamicum* gene bank using the *C. glutamicum* strain RES 167 was constructed by cloning of 7- to 10-kb partially *Sau*3A-digested DNA fragments ligated into the *Bam*HI site of pUC18. The direct complementation of the *E. coli panD* mutant strain DV9 was not successful, probably due to restriction of *C. glutamicum* DNA in *E. coli* (33). The *C. glutamicum* gene bank was therefore established in the *E. coli* strain DH5αMCR, which is characterized by defects in *Eco*K-specific restriction as well as in methylated-DNA restriction (10). Subsequently, total plasmid DNA from this library was reisolated and used to transform *E. coli* DV9. Although the *panD* mutant strain *E. coli* DV9 is able to grow on an unsupplemented solid Medium E, it exhibits a β-alanine auxotrophy when incubated in liquid Medium E at 39°C. Therefore, selection for complementation was performed in liquid Medium E in the presence of ampicillin. Recombinant plasmid DNA was isolated from three transformants as outlined in Materials and Methods. To confirm complementation, the isolated plasmid DNA, each with an insert length of approximately 7 kb, was retransformed into strain DV9, and the prototrophic growth phenotype in the test medium was analyzed.

To define the complementing region by subcloning, the DNA of one of these retransformed plasmids, designated pNIC1.3, was *Eco*RI digested, and the resulting fragments were cloned into the vector pK18*mob*. The subclones were electroporated into DV9, and complemented clones were selected in a liquid test medium containing kanamycin. As a result, plasmid pND10 carrying a 3-kb *Eco*RI insert was identified to complement the *panD* auxotrophy of *E. coli* DV9 (Fig. 1).

The *orf3* region carried by the *C. glutamicum* 3-kb *Eco*RI insert of plasmid pND10 was sufficient to complement an *E. coli panD* mutant. The entire chromosomal DNA fragment carried by plasmid pND10 was sequenced in order to identify the *panD* complementing region. Computer-assisted analysis of the assembled 3,060-bp DNA sequence for regions with high coding probability (31) yielded five putative open reading frames (ORFs), termed *orf1* to *orf5* (Fig. 1), with *orf1* being incomplete on the sequenced DNA fragment. In order to assign the *panD* gene to one of these ORFs, the 3-kb *Eco*RI insert of pND10 was digested with the restriction enzymes *Bam*HI-*Eco*RI, *Bam*HI-*Pst*I, and *Xba*I. The DNA fragments were cloned into the vector pUC18, resulting in plasmids pND10-1, -2, -3, and -4 (Fig. 1). In contrast to pND10-1 and -2, plasmids pND10-3 and -4 did not complement the *E. coli panD* mutant DV9. From these results it can be deduced that a DNA fragment containing the nearly complete *orf3* is necessary to correct the *panD* mutation of *E. coli* DV9.

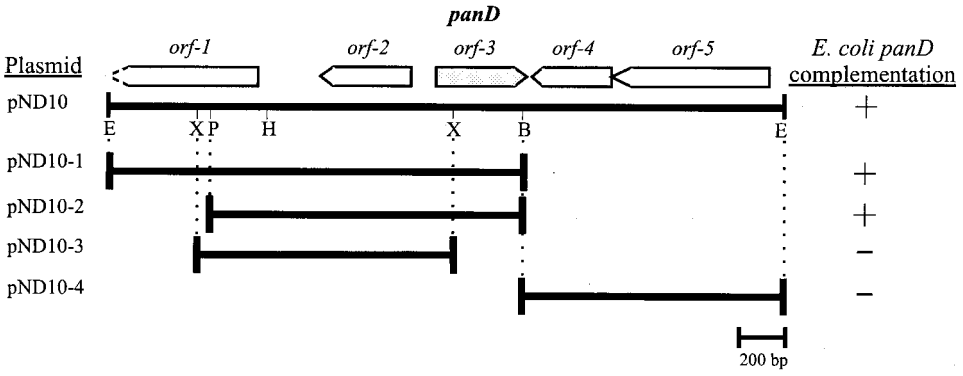

FIG. 1. Complementation tests of the *E. coli panD* mutant DV9 by a 3-kb *C. glutamicum* ATCC 13032 DNA fragment and various deletions thereof. A restriction map of the 3-kb DNA insert of pND10 is shown. The positions of *orf1* (incomplete), *orf2*, *orf3*, *orf4*, and *orf5* are indicated by open arrows. DNA fragments used for *E. coli panD* complementation studies are shown in solid lines. *orf3*, which is sufficient for complementation of the *E. coli panD* mutant DV9, is designated *panD*. Abbreviations: E, *Eco*RI; X, *Xba*I; P, *Pst*I; H, *Hin*dIII; B, *Bam*HI. Symbols: +, complementation; −, no complementation.

The *C. glutamicum orf3*, complementing an *E. coli panD* mutant, encoded a protein with similarities to bacterial L-aspartate-α-decarboxylases. The DNA sequence of the DNA fragment containing *orf3* and the deduced amino acid sequence are shown in Fig. 2. Analysis of the coding probability suggested that the translation of *orf3* starts at an ATG codon at nucleotide positions 1517 to 1519 and ends with a TAG stop codon at nucleotides 1925 to 1927. The entire *orf3* coding region covers 411 nucleotides and encodes a protein of 136 amino acids with a calculated molecular mass of 14,147 Da. Twelve nucleotides upstream of the putative start codon a 5′-AAGGA-3′ sequence was found which matches perfectly the 3′ end of the 16S rRNA from *C. glutamicum* (15) and probably acts as the ribosome binding site. A putative promoter region displaying strong similarities to the −10 and −35 consensus promoter sequences of *E. coli* (11) and *C. glutamicum* (20) is located 35 bp upstream of the putative start codon. Downstream of the stop codon no palindromic sequence suitable for a ρ-independent termination signal could be identified.

Similarity comparisons with the deduced amino acid sequences of the five ORFs present on the 3-kb insert of pND10 with gene databases were performed by using the BLAST search programs (3, 9). Consistent with the complementation analysis described above, the amino acid sequence of the *orf3* gene displayed strong similarities to the amino acid sequences of bacterial L-aspartate-α-decarboxylases. The best overall

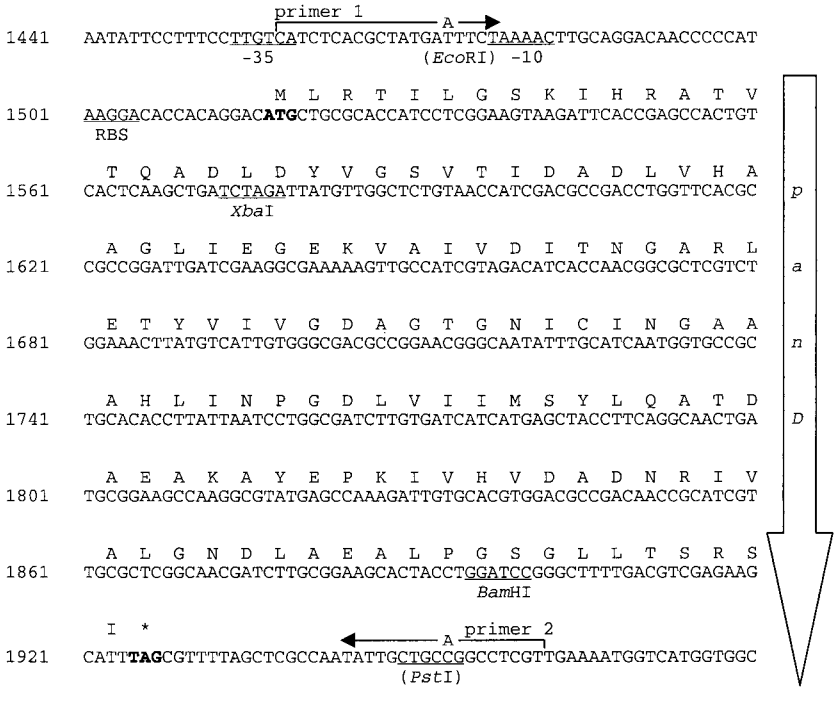

FIG. 2. Nucleotide sequence and deduced amino acid sequence from nucleotides 1441 to 1980 of the 3-kb insert of plasmid pND10 representing the *panD* gene of *C. glutamicum*. The putative promoter, a possible ribosome binding site, and relevant restriction sites are underlined, whereas the putative start and stop codons are indicated by boldface letters. The deduced amino acid sequence is shown above the DNA sequence. The oligonucleotides used as primers for PCR amplification of the *panD* gene are indicated by arrows. Suitable restriction sites, given in parentheses, were introduced into the primers by a T-to-A exchange at position 1473 and a C-to-A exchange at position 1953.

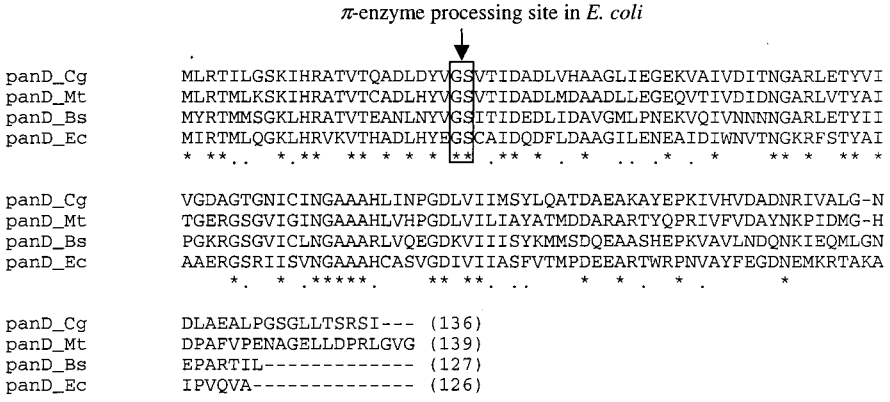

FIG. 3. Multiple alignment of the deduced amino acid sequence of the *C. glutamicum* (Cg) PanD protein with sequences of the homologs from *M. tuberculosis* (Mt; GenBank number Z95557), *B. subtilis* (Bs; GenBank number Z99115), and *E. coli* (Ec; GenBank number L17086). Numbers to the right indicate the lengths of the proteins. The box marks the conserved -G-S- sequence which serves as the proenzyme cleavage site (arrow) in *E. coli* (30). Identical (*) and similar (.) amino acids are marked.

alignment was observed with the PanD protein from *Mycobacterium tuberculosis*, with 62% identical and 77% similar amino acids. Due to the strong amino acid similarities and according to the complementation studies, the *orf3* gene was termed *panD*. As far as the complementation of the *E. coli panD* mutant is concerned, it should be noted that the *Bam*HI site, used to construct the plasmids pND10-1 and -2 in the complementation studies described here, is located within the 3′ end of the *panD* gene. Therefore, the gene products derived from these plasmids are fusions of *panD*- and vector-encoded amino acids. In these proteins, the last eight amino acids of the PanD protein are replaced by 19 vector-encoded amino acids containing three homologous and three similar amino acids (data not shown). Interestingly, the resulting fusion proteins remained functional, since the plasmids pND10-1 and pND10-2 were able to restore the *panD* mutation of *E. coli* DV9.

Comparison of the deduced amino acid sequences of the remaining four putative ORFs present on the 3-kb insert of pND10 revealed no significant similarities to entries in the databases.

The *C. glutamicum panD* gene directed the formation of two proteins. A multiple alignment of PanD amino acid sequences of *C. glutamicum*, *E. coli*, *Bacillus subtilis*, and *M. tuberculosis* (Fig. 3) revealed that the specific Gly-Ser sequence which serves as a posttranslational cleavage site in the *E. coli* PanD protein (21) is conserved in all aligned protein sequences at amino acid positions 24 and 25. These findings indicate that the *C. glutamicum* protein might be equally processed, resulting in the formation of putative α- and β-subunits with calculated molecular masses of 11.5 and 2.7 kDa, respectively. Due to the strong similarity between the *E. coli panD* ($panD_{E.c.}$) and *C. glutamicum panD* ($panD_{C.g.}$) genes, we were interested in analyzing the product of $panD_{C.g.}$ in comparison to that of $panD_{E.c.}$ both in *C. glutamicum* and *E. coli*. For this purpose, both *panD* genes were cloned into a shuttle expression vector which allows study of expression in *E. coli* as well as in *C. glutamicum* strains. The *E. coli-C. glutamicum* shuttle expression vector pZ8-1 proved to be suitable for our studies (Fig. 4). It contains the *tac* promoter in front of the gene to be cloned, the origin of vegetative replication in *E. coli* derived from the vector pACYC177 (5), the *C. glutamicum* replication region of plasmid pHM1519 (19), and a kanamycin resistance gene. Designed PCR primers were used to clone the *C. glutamicum* and *E. coli panD* genes with their endogenous Shine-Dalgarno sequences into the expression vector pZ8-1 (Fig. 4). The resulting plasmids pND-D1, containing $panD_{E.c.}$, and pND-D2, containing $panD_{C.g.}$, were transformed into the *E. coli panD* mutant DV9, and the expression of the genes was proved by complementation. Subsequently, the plasmids pND-D1 and pND-D2, as well as plasmid pZ8-1, were introduced into the *C. glutamicum* RES167 and into *E. coli* MG1655 by electroporation. The resulting strains were cultured in a selective minimal medium, and crude protein extracts were analyzed by SDS-PAGE. In the extracts of *C. glutamicum* RES167 harboring pND-D2, a protein band with the expected size of 14 kDa, resembling the unprocessed PanD protein, was not detected, but two extra protein bands were observed (Fig. 5). The molecular mass of the larger protein was approximately 11 kDa, coinciding with the calculated molecular mass of the putative α-subunit, assuming that the *C. glutamicum* PanD protein is processed at the conserved cleavage site. The mass of the second band was far below 8.3 kDa. Due to the inaccuracy of the size determination of proteins smaller than 6 kDa in this PAGE system, the exact size of this protein could not be specified. Even when the SDS concentration of the sample buffer was increased to 4% and a Tris-tricine gel system was used (26), the molecular mass could not be determined exactly (data not shown), probably because of remaining secondary structures in the protein subunit. This smaller protein is likely to represent the β-subunit with a predicted mass of 2.7 kDa, assuming that Gly-24–Ser-25 serves as the processing site of the π-protein in *C. glutamicum*. These findings imply that the *C. glutamicum panD* gene product is initially translated as a π-protein which is subsequently processed to form two putative subunits of 11 kDa and of less than 8.3 kDa. The fact that no third extra protein band, corresponding to the unprocessed 14-kDa π-protein, could be identified in the crude extracts leads to the assumption that the product of the gene borne by pND-D2 is efficiently processed in *C. glutamicum*. In the extract of *C. glutamicum* containing pND-D1, as well as in the extracts of *E. coli* harboring pND-D1 or pND-D2, no extra protein bands could be detected by SDS-PAGE (data not shown).

A *C. glutamicum panD* mutant lacked L-aspartate-α-decarboxylase enzyme activity and exhibited β-alanine auxotrophy. The *E. coli* gene *panD* encodes the enzyme L-aspartate-α-decarboxylase, which is responsible and essential for the synthesis of β-alanine, a direct precursor of pantothenate. A mu-

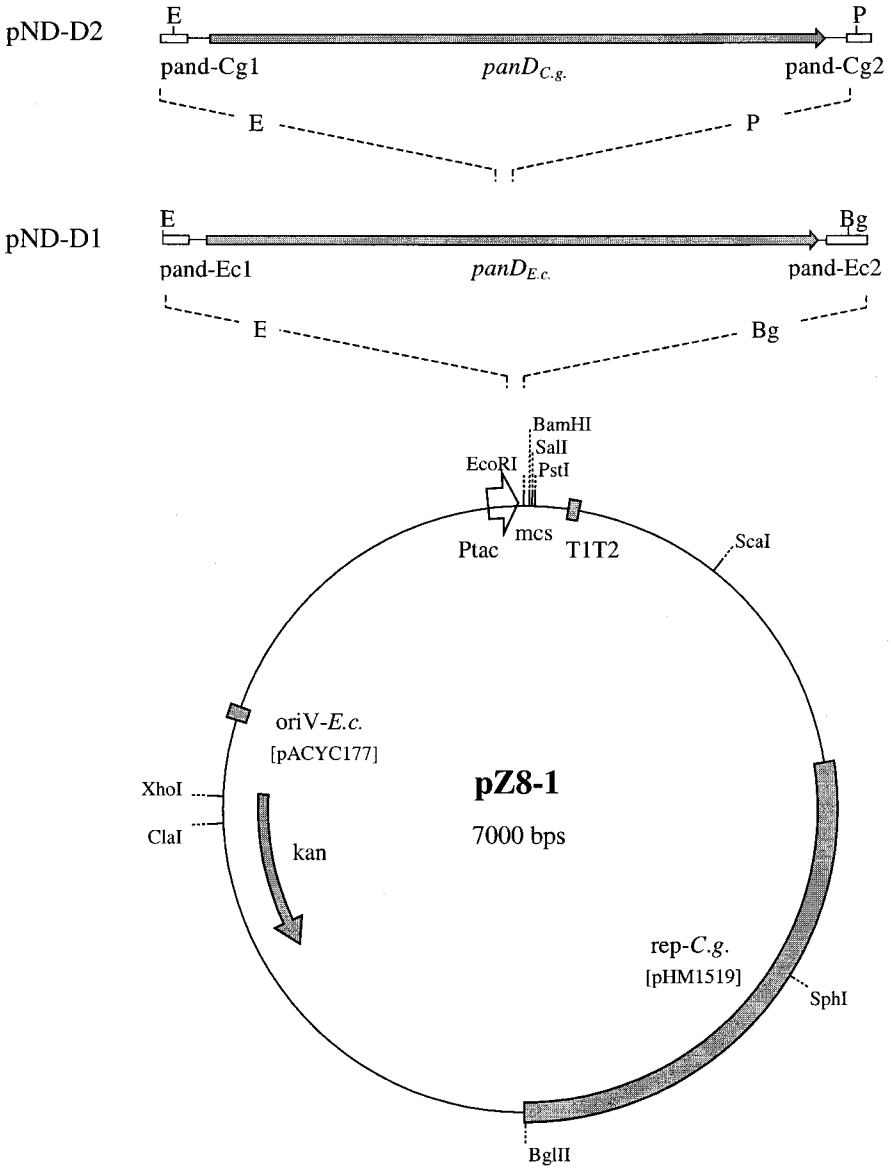

FIG. 4. Construction of the *panD* expression plasmids pND-D2 and pND-D1 by using the *E. coli-C. glutamicum* shuttle expression vector pZ8-1. The restriction sites introduced by PCR-primers pand-Cg1 and -2 and pand-Ec1 and -2 (open boxes) were used to clone the PCR-amplified $panD_{C.g.}$ and $panD_{E.c.}$ genes (filled arrows, not drawn to scale), with *Bgl*II being cloned into the *Bam*HI site of pZ8-1. Only selected single restriction sites of pZ8-1 are shown. The origin of vegetative replication (oriV-*E.c.*) is derived from plasmid pACYC177, and the minimal replicon (rep-*C.g.*) for replication in *C. glutamicum* is from plasmid pHM1519. Abbreviations: Ptac, *tac* promoter; T1T2, *rrnB* ribosomal terminator; kan, kanamycin resistance determinant of Tn*903*; mcs, multiple cloning site of pUC18; E, *Eco*RI; P, *Pst*I, Bg, *Bgl*II.

tation of this gene results in a β-alanine or pantothenate auxotrophic phenotype (6). To enable biochemical evidence for the *panD* gene function, defined *C. glutamicum panD* mutants were generated and analyzed biochemically as well as phenotypically. Mutagenesis was performed by inserting a chloramphenicol (*cmx*) resistance gene cassette derived from plasmid pEC31 into the *Xba*I site of the *panD* gene. Subsequent gene replacement was performed by using the pK18*mobsacB* vector system (25) to select for homologous recombination events. The resulting *panD* mutant strains carried a defective *panD* gene with an inserted *cmx* gene cassette. We isolated chloramphenicol-resistant mutants, and one of these strains, designated *C. glutamicum* ND2, was chosen for further analysis.

Biochemical evidence for the function of *panD* was given by an assay of L-aspartate-α-decarboxylase enzyme. The L-aspartate-α-decarboxylase activity can be measured by its ability to convert aspartate into β-alanine. In this work, we developed an HPLC-based enzyme assay. A standard reaction mixture containing a crude extract of the strain to be tested and aspartate was incubated for 1 h, and the amount of β-alanine formed was quantified by HPLC. *C. glutamicum* RES167 revealed a specific L-aspartate-α-decarboxylase activity of 0.09 U/mg, where as in the *panD* mutant strain *C. glutamicum* ND2 no L-aspartate-α-decarboxylase activity could be detected. Obviously, *panD* is the only gene responsible for L-aspartate-α-decarboxylase activity in *C. glutamicum*.

Furthermore, nutritional requirements of the *C. glutamicum panD* mutant strain ND2 on solid as well as in liquid medium were studied. The experiments revealed that in both cases only

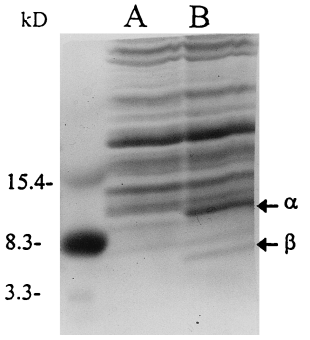

FIG. 5. SDS-PAGE analysis of protein extracts of a *C. glutamicum* strain overexpressing the $panD_{C.g.}$ gene. Crude extracts from *C. glutamicum* RES167 harboring plasmid pZ8-1 as a control (lane A) or pND-D2 carrying $panD_{C.g.}$ (lane B) were mixed with loading buffer containing 2% SDS and boiled for 10 min before being subjected to SDS–10 to 20% polyacrylamide gel. Arrows indicate the putative α- and β-subunits of the *panD* gene product. Molecular mass standards are given on the left side.

minimal medium supplemented with β-alanine or pantothenic acid corrected the phenotype of strain ND2. The obtained results are in accordance with the growth properties of the *E. coli panD* mutant strain DV9. These findings and the fact that transformation of ND2 with plasmid pND-D2, containing the $panD_{C.g.}$ gene, complemented the β-alanine auxotrophy demonstrated that the *panD* gene is responsible for the β-alanine formation in *C. glutamicum*.

Enhanced expression of the *C. glutamicum panD* gene resulted in a significantly higher L-aspartate-α-decarboxylase activity in *E. coli* as well as in *C. glutamicum*. Since we obtained biochemical evidence that *panD* encodes the enzyme L-aspartate-α-decarboxylase, we addressed the question of whether enhanced expression of *panD* results in an increased synthesis of β-alanine. In order to compare the enhanced expression of $panD_{C.g.}$ to that of the $panD_{E.c.}$, we examined the effect of both genes expressed from plasmid pZ8-1 (Fig. 4) in *C. glutamicum* and *E. coli*. Crude cell extracts of the control strains *C. glutamicum* RES167 and *E. coli* MG1655, each harboring the expression vector pZ8-1, gave similar specific activity values of 0.12 and 0.08 U/mg, respectively (Table 2). The specific activities of *E. coli* crude extracts are in accordance with values obtained by Williamson and Brown (39).

For comparative studies, the specific and relative L-aspartate-α-decarboxylase activities of *E. coli* and *C. glutamicum*, harboring the expression plasmid pND-D1 ($panD_{E.c.}$) or pND-D2 ($panD_{C.g.}$), were determined (Table 2). Surprisingly, in *E. coli* the $panD_{C.g.}$ construct pND-D2 provided a 43-fold-greater level of enzyme activity over that found in the wild type, whereas the $panD_{E.c.}$ construct pND-D1 promoted only a 3-fold rise in activity. In *C. glutamicum*, the difference between the activities resulting from $panD_{E.c.}$ and $panD_{C.g.}$ expression was even more drastic. The activity level provided by pND-D2 was 288-fold greater than that measured in the corresponding wild type, compared to a mere 4-fold rise promoted by pND-D1. The different plasmid copy number and *tac* promoter strength in the diverse organisms might influence *panD* overexpression but could not account for the general expression variance between both *panD* genes in the same organism.

Pantothenate production in *E. coli* was increased by the enhanced expression of the *C. glutamicum panD* gene. We determined, whether the enhanced L-aspartate-α-decarboxylase activity of *E. coli* due to the expression plasmids pND-D1 ($panD_{E.c.}$) and pND-D2 ($panD_{C.g.}$) results in an increase of pantothenate production. *E. coli* cells with plasmid pZ8-1, pND-D1, or pND-D2 were grown to late logarithmic phase in Medium E, and the cell-free supernatant was assayed for pantothenate by using the microbiological *Lactobacillus* assay. The results obtained from these studies were in accordance with the relative decarboxylase activities of the corresponding constructs in *E. coli*. Again, the *C. glutamicum panD* gene was the most efficient gene, since its overproduction provided a 10-fold increase of the pantothenate concentration over that found in the control, whereas the homologous construct pND-D1 promoted only a 3-fold rise in pantothenate synthesis (Fig. 6). The amount of pantothenate produced in strains expressing the *E. coli panD* gene could be increased by external β-alanine supplementation. In contrast to this, a further increase of pantothenate synthesis by the addition of β-alanine to the cultures harboring pND-D2 could not be achieved. Apparently, the expression of the *C. glutamicum panD* gene in *E. coli* resulted in a saturation of the β-alanine level needed for pantothenate biosynthesis (Fig. 6). Our results demonstrate that β-alanine supplementation, external or through $panD_{C.g.}$ expression, can boost the pantothenate production of *E. coli* up to approximately 700 ng of pantothenate per mg (dry weight). This corresponds to a specific productivity of 140 ng of pantothenate per mg (dry weight) per h.

Equivalent *panD* expression studies were also carried out with *C. glutamicum*. But since the intrinsic pantothenate production of *C. glutamicum* strains were 100- to 1,000-fold lower than that of the *E. coli* strains (data not shown), the measurement of pantothenate production by using the *Lactobacillus* assay was practically impossible. Therefore, no further attempts were made to study pantothenate production in *C. glutamicum*.

DISCUSSION

The *panD* gene is not clustered with known pantothenate biosynthesis genes in *C. glutamicum*. We describe in this study the isolation and characterization of the first *C. glutamicum* gene, designed *panD*, involved in pantothenate biosynthesis. The *C. glutamicum panD* gene was identified by functional complementation of an *E. coli panD* mutant. Similarity comparisons of the amino acid sequence deduced from *panD* revealed significant similarities to L-aspartate-α-decarboxylases encoded by *panD* genes from various microorganisms. In *E. coli* as well as in *B. subtilis*, *panD* is closely linked to *panB* and *panC*, which encode further pantothenate biosynthesis enzymes (18). Our sequence data revealed that four further ORFs, located adjacent to *panD*, do not show similarity to known genes involved in the pantothenate pathway. Therefore,

TABLE 2. Specific and relative L-aspartate-α-decarboxylase activities in crude extracts of *E. coli* and *C. glutamicum* carrying additional copies of $panD_{E.c.}$ or $panD_{C.g.}$

Plasmid	Gene	L-Aspartate-α-decarboxylase activity[a] in:			
		E. coli		*C. glutamicum*	
		Sp act (U/mg)	Rel act	Sp act (U/mg)	Rel act
pZ8-1		0.08 ± 0.009	1	0.12 ± 0.041	1
pND-D1	$panD_{E.c.}$	0.22 ± 0.019	3	0.45 ± 0.018	4
pND-D2	$panD_{C.g.}$	3.42 ± 0.293	43	34.55 ± 2.516	288

[a] Specific activities were determined in crude extracts of cells grown in minimal medium as described in Materials and Methods. Units are in nanomoles of β-alanine formed per minute. The results are mean values of five independent assays. Rel act, relative activity.

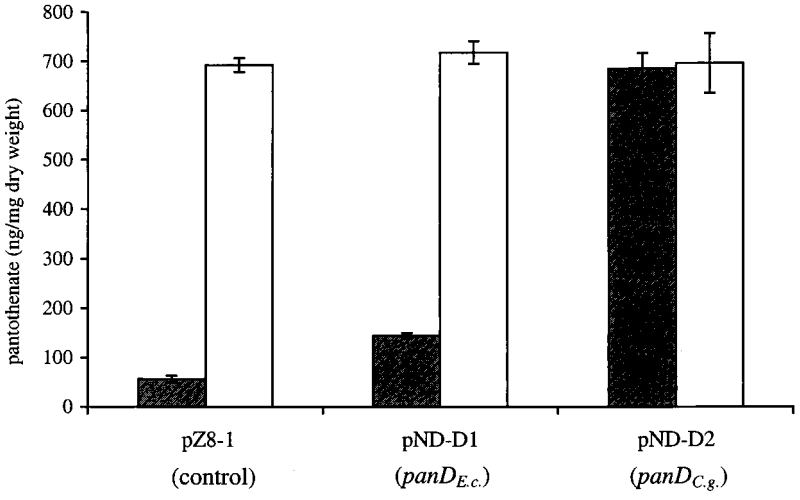

FIG. 6. Pantothenate production in *E. coli* carrying extra copies of *E. coli panD* or *C. glutamicum panD* with or without β-alanine supplementation. The pantothenate accumulation in the supernatant of *E. coli* containing the given plasmids is shown. *E. coli* cells were grown for 5 h in Medium E without supplementation (solid bars) and in medium supplemented with 100 μg of β-alanine per ml (open bars). The results are the means of results of at least four independent pantothenate assays. The pantothenate concentration of each assay was obtained as a mean value of three independent determinations by using the *Lactobacillus* assay.

in *C. glutamicum* the *panD* gene is evidently not linked to other known pantothenate synthesis genes.

The *panD* gene is the only gene in *C. glutamicum* encoding L-aspartate-α-decarboxylase. In *E. coli*, β-alanine is synthesized by the decarboxylation of aspartate, catalyzed by the *panD*-encoded enzyme L-aspartate-α-decarboxylase (13). Evidence for an identical function of the *C. glutamicum panD* gene was given by a defined *C. glutamicum panD* mutant which totally lacked L-aspartate-α-decarboxylase enzyme activity. Phenotypically, this mutant was characterized by a strict auxotrophy for β-alanine or pantothenate. These results demonstrate that, as in *E. coli*, the *C. glutamicum panD* gene encodes the L-aspartate-α-decarboxylase enzyme, which is essential for β-alanine synthesis. Furthermore, it indicates that an alternative pathway for β-alanine synthesis is not present in *C. glutamicum*. This is in accordance with results with *E. coli* showing that an earlier proposed alternative β-alanine pathway (29) does not really play a role (6).

The *C. glutamicum* PanD protein is posttranslationally processed, leading to the formation of two subunits. The *E. coli* L-aspartate-α-decarboxylase is a member of a group of mechanistically related enzymes which contain a covalently bound pyruvoyl group involved in catalysis (39). This pyruvoyl group is generated at a specific internal serine residue of the inactive precursor protein (π-protein), with the coincident cleavage at the amino acid residue just preceding the serine residue (37). Since the *E. coli* activation site is conserved in the deduced *C. glutamicum panD* gene product, we assume, that this enzyme represents another member of the group of pyruvoyl-dependent enzymes and that the enzyme is processed in a similar manner. This idea was supported by the detection of two proteins in extracts of *panD* expressing *C. glutamicum* cells. The mass of approximately 11 kDa for the larger protein is consistent with the predicted α-subunit mass of 11.5 kDa and therefore this protein probably represents the α-subunit. The smaller protein band most likely represents the β-subunit with the expected size of 2.7 kDa. A 14-kDa protein band representing the *C. glutamicum* π-enzyme was not detectable in these extracts. Even if there are low amounts of unprocessed protein which are not visible in total protein extracts, the absence of a distinct π-protein band indicates an efficient cleavage process. In contrast to our results, the *E. coli* π-enzyme was shown to be only slowly processed (21). In crude extracts of *panD* expressing *E. coli* cells, a third protein band corresponding to the unprocessed 13.8-kDa π-enzyme, was detectable by SDS analysis, and it was shown that only 10% of the π-protein was processed in these cells (21).

From our results we conclude that the *panD* gene product of *C. glutamicum* is posttranslationally processed, resulting in the formation of an α-subunit and a β-subunit and that the cleavage of the π-enzyme is very efficient. To prove these assumptions, the amino acid sequences of the *C. glutamicum* α- and a β-subunits should be determined in further studies. Additionally, the processing theory might be verified by the determination of a pyruvoyl group at the N terminus of the α-subunit.

The *panD* gene of *C. glutamicum* is superior to the *panD* gene of *E. coli* in directing L-aspartate-α-decarboxylase activity. Evidence for the biochemical function of the *panD* gene product was achieved by specific L-aspartate-α-decarboxylase activity studies. In comparative analysis, the *E. coli panD* and the *C. glutamicum panD* gene expression by means of the constitutive *tac* promoter was studied in *E. coli* as well as in *C. glutamicum*. It was surprising that overexpression of the *panD* genes resulted in strikingly different enzyme activities, depending on the expression in *E. coli* or *C. glutamicum*. In both strains, the *E. coli* PanD promoted only a moderate increase of activity, whereas the *C. glutamicum* PanD elevated the enzyme activity by means of orders.

Several reasons can account for this strong difference in activity. In the first place, a reason for the particularly weak *E. coli* PanD activity becomes obvious when looking at the ribosome binding site upstream of the *panD* start codon. The 5′-AAGGT-3′ sequence (30) differs from the perfect *E. coli* Shine-Dalgarno site 5′-AAGGA-3′ (27) in one nucleotide, whereas the *C. glutamicum panD* gene is preceded by a perfect 5′-AAGGA-3′ site 11 bp upstream of the start codon. Considering that not only a strong promoter but also a strong translational initiation reflected by an optimal ribosome binding site is essential for high-level gene expression, the low activity of the *E. coli* aspartate decarboxylase could be due to a weak translation of the gene. Second, the π-enzyme processing mechanism has to be considered. In vitro studies have demon-

strated that overexpressed and isolated L-aspartate-α-decarboxylase from *E. coli* is only slowly processed by an autocatalytic mechanism (21), whereas in *C. glutamicum* the PanD enzyme was shown to be efficiently processed. Recently, the in vivo cleavage mechanism of the inactive proenzyme has been elucidated by crystal structure determination (2). The structural information suggests that the enzyme is activated solely by protein self-processing. They found no evidence for the existence of a specific factor, a consideration set up by Ramjee and coworkers (21), which catalyzes the correct procession of the π-enzyme to form the active α- and β-subunits. Since nothing is known about the cleavage process in *C. glutamicum*, it is conceivable that the mechanism of *C. glutamicum* PanD processing is more efficient than that of *E. coli* PanD.

Enhanced expression of the *C. glutamicum panD* gene in *E. coli* overcomes the β-alanine limitation in pantothenate biosynthesis. One of the reasons for identifying the *C. glutamicum panD* gene was to investigate whether enhanced expression of this gene corrects the β-alanine limitation in the pantothenate biosynthetic pathway and consequently leads to increased pantothenate synthesis. Although, as determined by the enhanced expression of the *C. glutamicum panD* gene in *C. glutamicum*, the PanD enzyme activity was found to be drastically increased, significant pantothenate synthesis was not detectable in the *Lactobacillus* assay. The low level of pantothenate synthesis in *C. glutamicum* indicates that other enzymes involved in pantothenate synthesis limit the production rate in *C. glutamicum*.

However, in *E. coli* enhanced expression of the *panD* genes of both *C. glutamicum* and *E. coli* promoted a significant increase of pantothenate production. We elucidated whether the expression of *panD* resulted in a surplus of β-alanine in the culture media or whether this metabolite is still the rate-limiting precursor in the biosynthesis of pantothenate. From our results we can conclude that the L-aspartate-α-decarboxylase reaction promoted by the *E. coli panD* gene is still limiting but that the PanD enzyme from *C. glutamicum* produced sufficient β-alanine for pantothenate synthesis. Since expression of *C. glutamicum panD* in *E. coli* abolished the limiting effect of this precursor, in this strain the maximal pantothenate production rate could be achieved without external β-alanine supplementation. Obviously, the *C. glutamicum panD* gene is suitable for the construction of a simple *E. coli* pantothenate producer. Isolation of additional *C. glutamicum* genes involved in this biosynthetic pathway and investigations of whether overproduction of these genes in combination with *C. glutamicum panD* are able to channel the flux toward pantothenate in *E. coli*, as well as in *C. glutamicum* production, will be left for the future.

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