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Different Modes of Diaminopimelate Synthesis and Their Role in Cell Wall Integrity: a Study with *Corynebacterium glutamicum*

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In eubacteria, there are three slightly different pathways for the synthesis of *m*-diaminopimelate (m-DAP), which is one of the key linking units of peptidoglycan. Surprisingly, for unknown reasons, some bacteria use two of these pathways together. An example is *Corynebacterium glutamicum*, which uses both the succinylase and dehydrogenase pathways for m-DAP synthesis. In this study, we clone *dapD* and prove by enzyme experiments that this gene encodes the succinylase (M_r = 24082), initiating the succinylase pathway of m-DAP synthesis. By using gene-directed mutation, *dapD*, as well as *dapE* encoding the desuccinylase, was inactivated, thereby forcing *C. glutamicum* to use only the dehydrogenase pathway of m-DAP synthesis. The mutants are unable to grow on organic nitrogen sources. When supplied with low ammonium concentrations but excess carbon, their morphology is radically altered and they are less resistant to mechanical stress than the wild type. Since the succinylase has a high affinity toward its substrate and uses glutamate as the nitrogen donor, while the dehydrogenase has a low affinity and incorporates ammonium directly, the m-DAP synthesis is another example of twin activities present in bacteria for access to important metabolites such as the well-known twin activities for the synthesis of glutamate or for the uptake of potassium.

The amino acid *meso*-diaminopimelate (m-DAP) is one of the key intermediates of peptidoglycan synthesis. It serves to link the glycan backbones in the cell walls of many bacteria, giving them their shape and rigid structure. In addition, the synthesis of m-DAP is required for protein synthesis, since after decarboxylation, it yields L-lysine. Due to this vital role of m-DAP for bacteria and the facts that mammals neither synthesize nor require m-DAP and that many proven antibiotics act in preventing bacterial cell wall synthesis, the m-DAP biosynthesis pathway is an attractive target for the rational design of new drugs. Accordingly, m-DAP analogs were assayed for inhibition of enzyme activity (9), and the crystal structures of several enzymes of m-DAP synthesis were resolved (2, 3, 19). Interestingly, there are three slightly different pathways of m-DAP synthesis. They all share the first steps for aspartate and pyruvate conversion to L-2,3,4,5-tetrahydrodipicolinate, but they vary in the subsequent conversion to yield m-DAP (Fig. 1): pathway 1, a four-step reaction with succinylated intermediates; pathway 2, another four-step reaction involving acetylated intermediates; and pathway 3, a one-step reaction catalyzed by an ammonium-incorporating dehydrogenase. An example of the respective variant is *Escherichia coli* (pathway 1), *Bacillus subtilis* (pathway 2), and *Bacillus sphaericus* (pathway 3). Each variant pathway (hereafter termed the succinylase, acetylase, and dehydrogenase variant, respectively) has its specific cofactor demand. However, it is still not known whether there is any special reason for this wide range of m-DAP synthesis, whether the respective pathway, for instance, corresponds to a particular need of the bacterium in question.

There is an unusual situation in the gram-positive bacterium *Corynebacterium glutamicum*. The succinylase and dehydrogenase variants operate in this organism side by side (Fig. 1). Due to this situation, *C. glutamicum* is an ideal candidate to be assayed on the relevance of the variant pathways established in bacteria. The m-DAP pathway in *C. glutamicum* has been intensively investigated, since mutants of this bacterium are used to produce the m-DAP-derived L-lysine on a scale of more than 3×10^5 tons/year (t/y) (10). We have studied almost all the enzymes of the m-DAP pathway of L-lysine synthesis (5, 23) and used them for flux increase (5), and we have shown by nuclear magnetic resonance studies that in vivo both the succinylase and dehydrogenase variants contribute to m-DAP formation (12, 27). The relative use of both pathways is dependent on the ammonium concentration. To study a particular function of the two variant pathways in *C. glutamicum*, we had already inactivated the dehydrogenase-encoding *ddh* gene and found reduced L-lysine yields with overproducers, whereas the growth rates were not affected (24). However, it proved difficult to clone the genes for the enzymes of the four-step reaction. Although we were able to clone the desuccinylase-encoding gene *dapE* (28) and we also obtained a DNA fragment which led to succinylase activity, its structure did not correspond to that in the chromosome. As we demonstrated, this can be attributed to the adjacent *aroP* gene which encodes an aromatic amino acid transporter (29). In this study, we describe the cloning of *dapD* (succinylase), as well as the structure of a related gene, and apply gene inactivation to enable physiological experiments. This allows conclusions to be drawn on the use and function of the dehydrogenase and succinylase variants of m-DAP synthesis.

MATERIALS AND METHODS

Strains, plasmids, and growth. The bacterial strains and the most relevant plasmids are given in Table 1. *C. glutamicum* was grown at 30°C on minimal medium CGXII (16) or brain heart infusion (BHI; Difco), with the indicated supplements. For enzyme determinations, cells were grown on complex medium CGIII (13). *E. coli* strains were grown at 37°C, except for RDD32 *dapD*, which was grown at 30°C. When appropriate, ampicillin and kanamycin were used at concentrations of 40 and 50 μg ml^{-1}, respectively. Growth was monitored by measuring absorbance at 600 nm of diluted cultures (PM6; Zeiss, Oberkochen, Germany). The isolation of clones complementing the *dapD* mutation in *E. coli* RDD32 was performed as described previously (28).

Genetic techniques, sequencing, and hybridization. Standard procedures were used for the isolation and in vitro recombination of DNA. *E. coli* DH5 and S17-1

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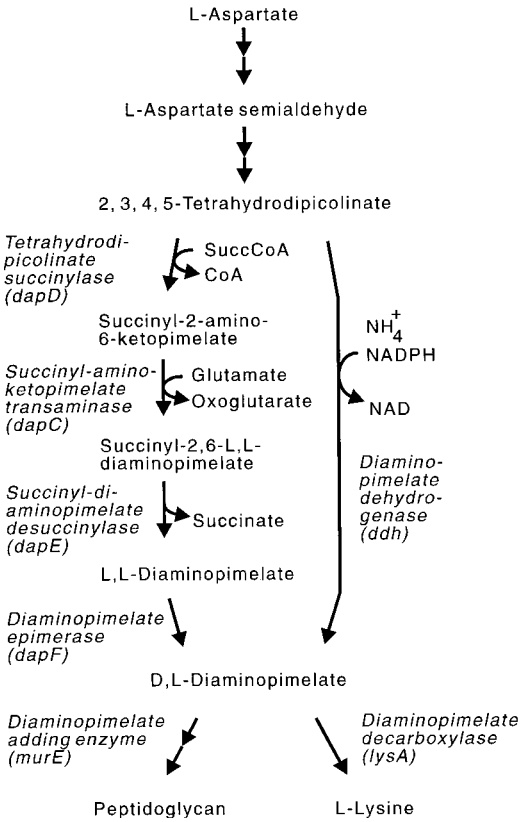

FIG. 1. The split pathway of m-DAP synthesis for the formation of m-DAP and L-lysine as present in *C. glutamicum*. On the left is shown the succinylase variant, on the right the dehydrogenase variant. Some bacteria have only the succinylase or dehydrogenase variant. The third acetylase variant is comparable to the succinylase variant but uses acetyl groups instead of succinyl groups. SuccCoA, succinyl coenzyme A; CoA, coenzyme A.

were transformed by the $CaCl_2$ method, whereas electroporation was used to transform *E. coli* RDD32 and the *C. glutamicum* strains (11). To transfer nonreplicative plasmids into *C. glutamicum*, conjugation was done as previously described (20), and clones with integrated vectors were selected by resistance to kanamycin (15 μg ml^{-1}).

The DNA sequence was determined by the dideoxy-chain termination method, using the AutoRead Sequencing kit and the A.L.F. sequencer from Pharmacia (Freiburg, Germany). The sequences were derived from deletion clones, but two fluorescein-labelled primers (5′-CGCGGTCGGCGTCACCG-3′ and 5′-GCCT CCTCAACAATGTCGT-3′) were made to verify the sequence of a region with an extraordinarily high G+C content. The structural integrity of cloned fragments and of the appropriate chromosomal deletions was verified by DNA hybridization, using digoxigenin-labelled DNA as a hybridizing probe.

Construction of inactivation and deletion mutants. To construct the *orf3* deletion mutant, the 479-bp *Kpn*I fragment was deleted from the 2.5-kb *Eco*RI fragment of the cluster carrying *orf3* as well as the adjacent 5′ regions of *aroP* and *dapE* (Fig. 2). This 2.0-kb Δ*orf3* fragment was ligated with pK18mobsacB, and the resulting plasmid was used for intergeneric conjugation to recombine with the chromosome of *C. glutamicum*. Using the *sacB* gene of the integrated vector, clones were selected for a second recombination (21). An attempt was made to construct a *dapD* deletion mutant. A pK18mobsacB derivative harboring a *dapD* gene with the 457-bp *Eco*47III-*Nsi*I fragment deleted was made for this purpose. However, of 24 recombinants tested, the second recombination always yielded the reconstituted wild-type situation. This is indicative of a selective pressure for the presence of *dapD*. Therefore, this gene was disrupted by use of the internal 330-bp *Pst*I-*Sal*I *dapD* fragment which was ligated to pEM1 (Table 1). To construct the *dapE* inactivation mutant, the internal 298-bp *Hin*dII *dapE* fragment was ligated to pEM1 and used for conjugation. Similarly, *orf2* was inactivated by use of the internal 321-bp *Sgr*AI-*Bam*HI *orf2* fragment ligated to pEM1.

Cell disruption and enzyme assays. Cells of *C. glutamicum* were washed with 0.9% NaCl, resuspended in 0.1 M potassium phosphate buffer, pH 7, and the optical density was adjusted to an absorbance at 600 nm of 100. Sonication was done for 10 min with a microtip-equipped Branson sonifier at an output of 2.5 and with the duty cycle set to 20%. The homogenate was centrifuged for 30 min (12,000 × *g*, 4°C), and the protein concentration was determined by the biuret reaction. The tetrahydrodipicolinate succinylase (EC 2.3.1.117), *N*-succinyl-diaminopimelate desuccinylase (EC 3.5.1.18), and diaminopimelate epimerase (EC 5.1.1.7) activities were quantified as described previously (23).

Quantification of amino acids and ammonium. Cells were separated from the medium and inactivated by silica oil centrifugation with the additional extraction and neutralization procedure previously described (23), yielding cellular and extracellular fractions. Amino acids were quantified as their *ortho*-phthaldialdehyde derivatives by automatic precolumn derivatization and separation by reversed-phase chromatography with fluorescence detection. Quantification of ammonium concentrations was done enzymatically by using glutamate dehydrogenase.

Electron microscopy. Cells were grown on Luria-Bertani medium (LB) containing 10% sucrose and 1.6% agar. After growth for 18 h at 30°C, the cells were resuspended in 200 μl of water to give a final absorbance at 600 nm of 4 to 6. One microliter of this cell suspension was placed onto a 5-mm^2 piece of a filter (Nucleopore, Sartorius, Germany), which was dried overnight. The cells were fixed with 2.5% glutaraldehyde, washed twice with water, and subsequently dehydrated in a graded series of solutions with increasing concentrations of acetone (20, 40, 60, 80, and 100%). After the cells were coated with gold, the morphology of the specimen was resolved with a scanning electron microscope (S360; Leika Cambridge, Mass.).

Nucleotide sequence accession number. The nucleotide sequence of *dapD* and its flanking regions has been deposited in the EMBL database under accession number AJ004934.

TABLE 1. Strains and selected plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference
E. coli		
DH5	Cloning strain	
S17-1	Mobilizing donor strain	26
RDD32	*dapD*::MuCts	17
C. glutamicum		
ATCC 13032	Type strain	
ATCC 13032 *dapD*	*dapD*::pEMdapD$_{int}$ Km^r	This work
ATCC 13032 *dapE*	*dapE*::pEMdapE$_{int}$ Km^r	This work
ATCC 13032 *orf2*	*orf2*::pEMorf2$_{int}$ Km^r	This work
ATCC 13032 Δ*orf3*	Deletion of the 479-bp *Kpn*I fragment	This work
Plasmids		
pJC1	Shuttle vector, Km^r	4
pEM1	Integration vector, mobilizable, nonreplicative in *C. glutamicum*, Km^r	23
pK18mobsacB	Replacement vector, mobilizable, nonreplicative in *C. glutamicum*, Km^r	21
pJCdapD1.55	pJC1::*dapD*	This work
pJCorf3/Bam3.4	pJC1::*orf3*/Bam3.4	This work

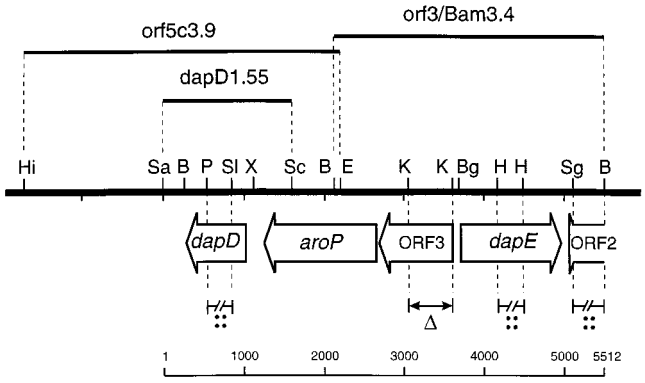

FIG. 2. Overview of the *dapDE* locus of *C. glutamicum*. The thick black line shows the general arrangement with selected restriction sites. Selected fragments used in this study are shown over the line, and the regions used to construct specific mutants, either by gene disruption (::) or gene deletion (△), are shown under the line. The scale at the bottom of the figure shows length (in base pairs). Abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dII; Hi, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Sa, *Sac*I; Sc, *Sca*I; Sg, *Sgr*AI; Sl, *Sal*I; X, *Xho*I.

RESULTS

Cloning and chromosomal localization of *dapD*. In previous work we had isolated cosmid pDE07 carrying *C. glutamicum* DNA, which complemented the *dapD* mutation in *E. coli* RDD32 (28). Although the insert was structurally altered, the complementing function was subcloned and sequenced in an attempt to obtain access to *dapD* which is of particular interest, since its gene product initiates entry into the succinylase variant (Fig. 1). Surprisingly, the complementing function consisted of sequences identical to the first part (109 amino acids) of *orf3* at the *dapE* locus of *C. glutamicum* (Fig. 2) but fused with unknown sequences. To explain this puzzling information, we carried out intensive Southern blot analyses, eventually resulting in the structurally intact fragment *orf5*c3.9. Subcloning and enzyme experiments yielded a 1.55-kb *Sac*I-*Sca*I fragment, which gave rise to an 11-fold increase in succinylase activity on plasmid pJC1 in *C. glutamicum* (Table 2). The nucleotide sequence of this fragment revealed an open reading frame of 690 nucleotides. In accord with the genetic and functional experiments, it was designated *dapD*. This *C. glutamicum* gene has an unusually high G+C content of 61%, while the neighboring sequences have the genome's G+C content of 54%. This analysis shows that *dapD* is close to *orf3* and *dapE*. The chromosomal organization of the entire *dapDE* locus of 5,512 bp thus derived is shown in Fig. 2.

TABLE 2. Tetrahydrodipicolinate succinylase (DapD) and succinyldiaminopimelate desuccinylase (DapE) activities in *C. glutamicum* strains derived from the wild type

C. glutamicum strain	Sp act (μmol min^{-1} mg of $protein^{-1}$)	
	Succinylase	Desuccinylase
ATCC 13032(pJC1)	0.008	0.021
ATCC 13032(pJCdapD1.55)	0.093	ND[a]
ATCC 13032 *dapD*	<0.001	ND
ATCC 13032 *dapE*	ND	<0.001
ATCC 13032(pJCorf3/Bam3.4)	0.009	ND
ATCC 13032 Δ*orf3*	0.012	0.018

[a] ND, not done.

Similarity and function of DapD and other proteins encoded by genes of the *dap* locus. The deduced amino acid sequence of *dapD* has only 21% identical amino acid residues with DapD of *E. coli* (Fig. 3), while other structural homologs of the *E. coli* polypeptide have more than 60% identical amino acids (18). Astonishingly, the two gene products derived from *dapD* and *orf3* of *C. glutamicum* have a high degree (46%) of identity with each other (Fig. 3). Even on the nucleotide level, a high degree of identity is present (not shown), which was apparently one prerequisite for the artificial clone pDE07 being a fusion of both genes. Both the *dapD*- and *orf3*-derived polypeptides display the imperfect tandem-repeated heptapeptide sequence (I/L/V)-G-X_4-I. This encodes a left-handed parallel β helix characteristic of most acyltransferases (2). However, *orf3*-carrying fragments do not result in succinylase activity (Table 2). Nevertheless, a transcript initiating 74 nucleotides in front of *orf3* was formed (Northern and primer extension analyses not shown). An *orf3* deletion mutant was constructed as well as an *orf2* insertion mutant (for details, see Materials and Methods). These mutants exhibited no phenotype. These mutants were not altered in succinylase, *N*-succinylaminoketopimelate transaminase, or diaminopimelate epimerase activity, thus excluding further genes of the succinylase pathway from being located at the *dapDE* locus analyzed.

An inactive succinylase pathway results in higher ammonium requirements. To examine the function of the succinylase variant, we used vector integration to disrupt *dapD* in the wild-type strain of *C. glutamicum*. The enzyme assay revealed the absence of succinylase activity in the *C. glutamicum dapD* mutant constructed (Table 2). Similarly, a *C. glutamicum dapE* mutant strain was constructed which was proven by enzyme activity determinations to be without desuccinylase activity. Both mutants and the wild type were grown on salt medium CGXII as well as on complex medium BHI. On both these media, neither the growth rate nor the final cell density was affected (not shown). However, when we added 4% glucose to BHI, we noted that growth of both mutants was arrested at a cell density (A_{600}) of 15, whereas with the wild type, a much higher cell density of 50 was obtained. Since the salt medium CGXII contains 300 mM ammonium but BHI is not supplemented with ammonium, we studied growth of the *dapD* mutant as a function of the ammonium concentration (Fig. 4). In BHI plus 4% glucose, the addition of increasing ammonium concentrations resulted in increasing cell yields of the *dapD* mutant. At 100 mM ammonium sulfate, the cell yield was comparable to that of the wild type, indicating that the dehydrogenase variant can compensate for the loss of the succinylase activity only at high ammonium concentrations. A direct determination of ammonium in the culture of the *dapD* mutant (BHI without ammonium added) confirms that initially a low concentration of about 5 mM ammonium is present, which is reduced to about 0.45 mM when the *dapD* mutant ceases growth (Fig. 4). With the *dapE* mutant, a comparable growth dependence on the ammonium supply was detected (not shown). These data conclusively show that the succinylase variant is required for the use of organic nitrogen compounds of the complex medium. Accordingly, on a salt medium where free ammonium was replaced by 5 mM L-glutamate as a nitrogen source, the *dapD* and *dapE* mutants of *C. glutamicum* were unable to grow within 2 days, whereas growth of the wild type was completed after 15 h (not shown).

Intracellular amino acid concentrations. The restricted growth and its correlation with the ammonium concentration could be due to either a limiting L-lysine supply or a shortage of m-DAP (Fig. 1). Intracellular amino acid concentrations were determined in an attempt to quantify these amino acids.

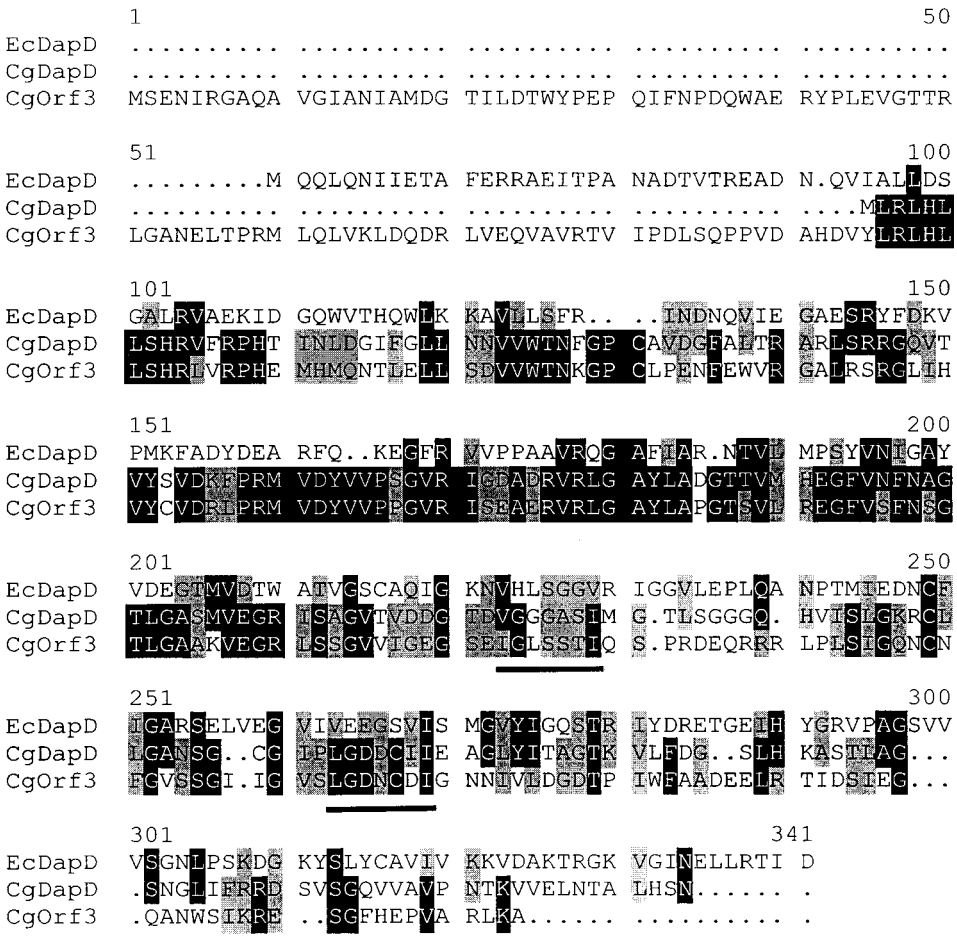

FIG. 3. Direct comparison of three sequences. The deduced amino acid sequences of the *dapD* gene product of *C. glutamicum* (CgDapD) and of *E. coli* (EcDapD) and of the *orf3* gene product of *C. glutamicum* (CgOrf3) are compared. Amino acids that are identical (black boxes) or similar (shaded boxes) are indicated. Although the DapD polypeptides from *C. glutamicum* and *E. coli* both represent functional tetrahydrodipicolinate succinylases, their structural similarity is less than that of the DapD and Orf3 polypeptides from *C. glutamicum*. The bars represent the imperfect tandem-repeated heptapeptide sequence characteristic of acyltransferases (2).

For this purpose cells, were grown on BHI plus glucose for 10 h when growth had already been arrested (Fig. 4). In all strains analyzed (*dapE* mutant, *dapD* mutant, and wild type), L-lysine was present at a high concentration of 69 ± 12 mM, whereas m-DAP was below the detection limit of 0.4 mM (intracellular). Therefore, L-lysine is available for the cell. It was apparently taken up via the well-known lysine import carrier of *C. glutamicum*. In the complex medium, L-lysine is present as a proteinogenic amino acid, whereas this is not the case for m-DAP. Therefore, m-DAP was added, but it did not result in increased growth of either of the mutants, which is not surprising due to the apparent inability of *C. glutamicum* to take up m-DAP (31). Interestingly, in the cytosol of the *dapE* mutant, a new amino acid was detected; this amino acid was not found in the wild type or the *dapD* mutant. Comparison with standards identified this derivative as L,L-*N*-succinyl-DAP, which reached a concentration as high as 115 mM after 5 h of growth on BHI plus glucose. This shows the high affinity of the succinylase toward its substrate tetrahydrodipicolinate (25), although no further flux through the succinylase variant is possible in the *dapE* mutant analyzed.

Cell wall integrity of the *dapD* mutant. Since we noted higher protein yields with the *dapD* mutant than with the wild type during preparation of cell extracts for enzyme assays, we quantified the efficiency of cell disruption by sonication. For

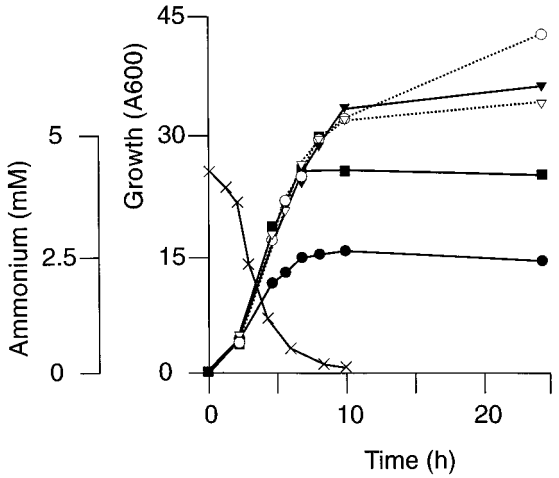

FIG. 4. Growth of two strains of *C. glutamicum*. The wild-type strain (open symbols) and the *dapD* mutant of *C. glutamicum* (solid symbols) are shown. Cells were grown in rich BHI medium plus 4% glucose without $(NH_4)_2SO_4$ (circles), with 20 mM $(NH_4)_2SO_4$ (squares), and with 100 mM $(NH_4)_2SO_4$ (triangles). Growth was measured by monitoring A_{600}. In addition, the decrease in the concentration of free ammonium (×) for the *dapD* mutant without $(NH_4)_2SO_4$ is shown.

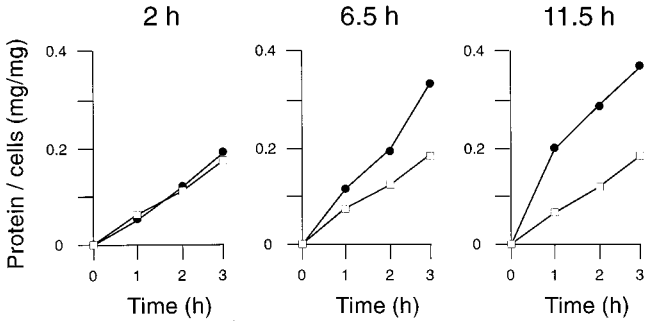

FIG. 5. Kinetics of cell disruption. Cell disruption was measured by the release of protein from cells of the wild type (□) and the *dapD* mutant (●) of *C. glutamicum*. Cells were harvested after 2, 6.5, and 11.5 h of growth in rich BHI medium plus glucose (4%). The cells were disrupted by sonication.

this purpose, cells of the *dapD* mutant grown on BHI were removed at three different times from the culture, and aliquots were disrupted for either 1, 2, or 3 min. As quantified by the protein released from the disrupted cells (Fig. 5), the efficiency of disruption of the mutant is identical with the control when the strains were grown for 2 h. At this time point, the growth rates of the strains are comparable (Fig. 4). However, an approximately twofold increase in the disruption efficiency of the *dapD* mutant is perceptible when the strains were grown for 6.5 h. This sensitivity of the *dapD* mutant to mechanical stress is increased even more when the ammonium concentration in the growth medium at the cultivation time was 0.45 mM. The *dapE* mutant behaved similarly (not shown).

Morphology of the *dapD* and *dapE* mutants. Cells grown on rich medium with excess carbon were prepared for raster electron microscopy. The wild type is rod shaped and about 1.4 to 2 μm long (Fig. 6). In contrast, cells of the *dapD* mutant are elongated (up to 6 μm long) and often club shaped at their ends. The *dapE* mutant is also altered in its morphology. In this case, the mutation results in a rather coccoidal cell form. This altered cell morphology is again indicative of a substantially altered peptidoglycan structure.

DISCUSSION

There are only a few cases where two synthesis pathways for a cellular building block exist in one organism. As can be concluded from individual enzyme measurements (1, 15), m-DAP can, however, be synthesized in some bacteria via two variant pathways simultaneously. A nuclear magnetic resonance analysis for *C. glutamicum* indicated that in vivo, both the succinylase and dehydrogenase variants of m-DAP synthesis contribute to the synthesis of m-DAP (27). In the *dapD* and *dapE* mutants with no succinylase pathway, growth is clearly affected, as well as the cell wall rigidity and the shape. These latter criteria are indicative of altered cross-linking of the peptidoglycan, which in *C. glutamicum* is exclusively via m-DAP (22). These consequences of the succinylase variant inactivation on the structure of *C. glutamicum* are comparable to that resulting from antibiotics applied to *E. coli* and interfering with peptidoglycan synthesis. The difference in the morphology of the two *C. glutamicum* mutants is not surprising, since many cytoplasmatic and membrane-located steps are involved in murein synthesis, including synthesis, hydrolysis, and turnover of murein and its precursors to enable controlled enlargement of the sacculus during growth (8). One reason for the different morphology of the *dap* mutants could be the extremely high

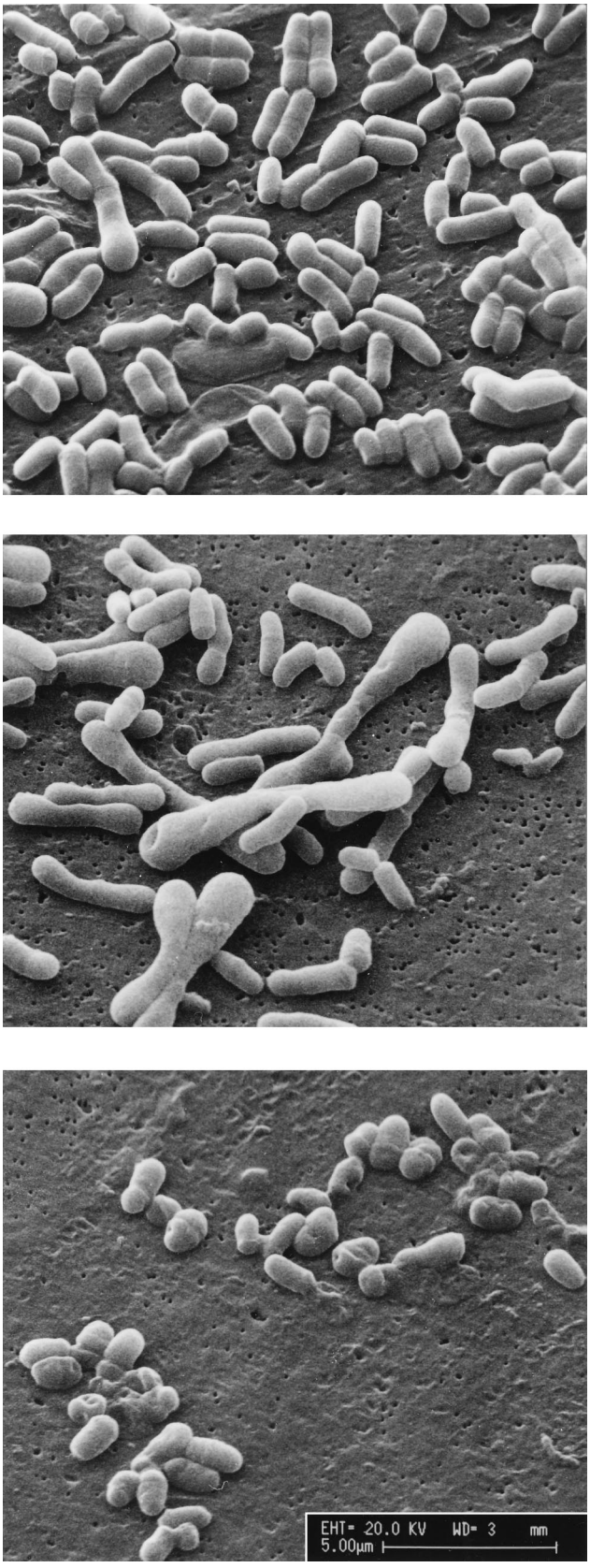

FIG. 6. Electron micrographs of wild-type and mutant strains of *C. glutamicum*. Wild-type (top) and *dapD* (middle) and *dapE* (bottom) mutants grown under identical conditions are shown. Bar, 5 μm.

accumulation of L,L-*N*-succinyl-DAP in the cytosol of the *dapE* mutant, which is, of course, not the case in the *dapD* mutant.

The fact that possession of the dehydrogenase variant alone has severe consequences on the growth of *C. glutamicum* shows that this variant is not suited for all growth conditions of the cell, such as very low ammonium concentrations. This is in agreement with the low affinity (K_m of 34 mM) of the dehydrogenase toward ammonium (14). Thus, this variant is not suitable for providing the basic metabolite flux toward m-DAP formation. In fact, even at the very high ammonium concentration of 600 mM assayed, the flux via the dehydrogenase contributes only 51% to total m-DAP formation (27). In contrast to the situation with the dehydrogenase, possession of the succinylase alone has less-pronounced effects. In *ddh* mutants, we found decreased accumulation of final L-lysine (23) and occasionally noted a prolonged lag phase after inoculation, especially with poor carbon sources like acetate. Although there is still no experimental evidence, it is conceivable that energetic aspects determine the flux partition. Note that in several aspects, the two pathways of nitrogen incorporation into m-DAP resemble other systems where two pathways exist: the systems Trk (low affinity) and Kdp for potassium uptake or glutamate dehydrogenase (low affinity) and glutamine synthetase for ammonium assimilation. These systems share the properties that one enzyme has a low affinity but is constitutively formed, whereas the other has a high affinity and is energetically more costly. The same holds for m-DAP formation, where the dehydrogenase has the low affinity and the enzyme is constitutively formed in the two different organisms measured (6, 15), but the succinylase has the high affinity and is the one which is energetically more costly (25). Although the glutamate dehydrogenase in *E. coli* was originally considered to be a dispensable enzyme, it was demonstrated only recently that with high ammonium availability and limited energy, a mutant without glutamate dehydrogenase has a growth disadvantage (7). In view of this, the presence of the two pathways of m-DAP synthesis together represent another case where the flexibility of the cell increases in response to a limited supply of energy.

How is m-DAP made in bacteria other than *C. glutamicum*? The following situations are shown to exist: only one variant (the succinylase, acetylase, or dehydrogenase variant) is present or two variants (the succinylase and dehydrogenase variants or the acetylase and dehydrogenase variants) are present. The existence of two variants together is not restricted to *C. glutamicum*, but it is also the case in *Bacillus macerans*, for instance (1). In accordance with the present analysis, there is of course a special problem with those bacteria that make m-DAP exclusively via the dehydrogenase variant. This is the case, for example, in *B. sphaericus* or *Bacillus pasteurii*. In fact, it is noted that *B. pasteurii* required an elevated level of ammonium supply for growth (22) and that organic nitrogen sources could not easily replace ammonium (30).

In conclusion, the availability of m-DAP is critical to decide on the structure and finally the proliferation of the cell. Therefore, it is not surprising that there are several ways to provide this important molecule in bacteria. In particular, the possession of two pathways together ensures supply of m-DAP under a variety of conditions. This is the case in *C. glutamicum* and probably also in several other bacteria (1, 15). The dehydrogenase variant operates at a high concentration of free ammonium. Its exclusive presence restricts growth in environments with low concentrations of free ammonium. Instead, environments rich in organic nitrogen require the succinylase pathway to be active for optimal growth. Since the succinylase variant is energetically more expensive than the dehydrogenase variant, the use of the dehydrogenase variant could be more favorable in a situation where energy is limited. Thus, both pathways together enable the optimal adaption of the cell to different environments and the dynamic response to changes in the environmental conditions.

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