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The pathway of lysine biosynthesis in the methanococci has not been identified previously. A variant of the diaminopimelic acid (DAP) pathway uses diaminopimelate aminotransferase (DapL) to catalyze the direct conversion of tetrahydrodipicolinate (THDPA) to LL-DAP. Recently, the enzyme DapL (MTH52) was identified in Methanothermobacter thermautotrophicus and shown to belong to the DapL1 group. Although the Methanococcus maripaludis genome lacks a gene that can be unambiguously assigned a DapL function based on sequence similarity, the open reading frame MMP1527 product shares 30% amino acid sequence identity with MTH52. A Δmmp1527 deletion mutant was constructed and found to be a lysine auxotroph, suggesting that this DapL homolog in methanococci is required for lysine biosynthesis. In cell extracts of the M. maripaludis wild-type strain, the specific activity of DapL using LL-DAP and α-ketoglutarate as substrates was 24.3 ± 2.0 nmol min⁻¹ mg of protein⁻¹. The gene encoding the DapL homolog in Methanocaldococcus jannaschii (MJ1391) was cloned and expressed in Escherichia coli, and the protein was purified. The maximum activity of MJ1391 was observed at 70°C and pH 8.0 to 9.0. The apparent Kₘ's of MJ1391 for LL-DAP and α-ketoglutarate were 82.8 ± 10 μM and 0.42 ± 0.02 mM, respectively. MJ1391 was not able to use succinyl-DAP or acetyl-DAP as a substrate. Phylogenetic analyses suggested that two lateral gene transfers occurred in the DapL genes, one from the archaea to the bacteria in the DapL2 group and one from the bacteria to the archaea in the DapL1 group. These results demonstrated that the DapL pathway is present in marine methanogens belonging to the Methanococcales.
Methanogens are strictly anaerobic archaea that obtain all or most of their energy for growth from the production of large quantities of methane. All methanogens belong to the Euryarchaeota and are currently classified in six orders: Methanobacteriales, Methanococcales, Methanomicrobiales, Methanocellales, Methanopyrales, and Methanosarcinales (23, 41, 42).

Biochemical studies of Methanocaldococcus jannaschii and Methanococcus voltae revealed that these organisms derive their L-lysine from a DAP pathway, but the studies did not discriminate among the four DAP pathway variations (2, 9, 10, 32). Genome sequence analysis also suggested a DAP pathway in Methanosarcina mazei belonging to Methanosarcinales (8). Recent studies identified a dapL homolog belonging to the DapL1 group in M. thermautotrophicus. The gene product complemented an Escherichia coli dapD dapE double mutant and catalyzed the transamination of DAP to THDPA, suggesting that Methanobacteriales use the DapL pathway for L-lysine biosynthesis (15, 18). Homologs of asd, dapA, dapB, dapF, and lysA have been identified in the genomes of M. maripaludis and M. jannaschii belonging to the Methanococcales, but homologs responsible for the conversion of THDPA to LL-DAP have not been annotated (4, 17). Here we identified methanococcal DapL homologs and demonstrated that the DapL pathway is present in Methanococcales.

**MATERIALS AND METHODS**

**Chemicals.** A mixture of LL, DD, and meso isomers of 2,6-diaminopimelic acid (ot-DAP) was purchased from Sigma-Aldrich. ll-DAP was a gift from André O. Hudson and Thomas Leustek.

A mixture of the different N-acetyl-diaminopimelic acid isomers was prepared by acetylation of ot-DAP with acetic anhydride. To do this, 150 μl of acetic anhydride was added to 3 ml of ot-DAP (190 mg [1 mmol] dissolved in 1 M NaOH) with stirring. After incubation for 1 h at room temperature, thin-layer chromatography (TLC) analysis followed by detection of the amines with ninhydrin revealed the presence of only two positive bands. Isolation of each band from the TLC plate and conversion to the dimethyl trifluoroacetyl derivatives showed that the bottom band was DAP with M+ = 410 m/z with fragment ions at M+–32 = 378 m/z and M+–COOH, at 351 m/z and the upper band was the N-acetyl-DAP derivative with M+ = 356 m/z with fragment ions at M+–31 = 325 m/z and M+–COOH, at 297 m/z. The sample was diluted with water, applied to a Dowex 50 H+ column (1.5 by 16 cm), and eluted with a 400-ml 0 to 2 M HCl gradient. This resulted in complete separation of DAP and N-acetyl-DAP. The N-acetyl-DAP was concentrated by lyophilization. The yield of a soft, white, noncrystalline solid was 15%. Despite the presence of multiple stereoisomers in each sample, only a single TLC spot was observed. The 1H nuclear magnetic resonance (NMR) spectrum of the final product was also consistent with the spectrum of the authentic compound.

A mixture of the different N-succinyl-DAP isomers was prepared by succinylation of ot-DAP as previously described for preparation of N-succinyl-LL-DAP (22). Analysis of the reaction mixture by TLC revealed the presence of three compounds: the unreacted DAP, N-succinyl-LL-DAP, and N,N-disuccinyl-DAP. The first two compounds formed ninhydrin-positive spots after TLC. Despite the presence of multiple stereoisomers, only a single spot was observed by TLC. The TLC plates used for analysis of amino acids were Silica Gel 60 F254 glass plates (5 by 20 cm; EMD Chemicals, Inc., Darmstadt, Germany), and the solvent consisted of acetonitrile, water, and formic acid (88%) (40:10:5, vol/vol/vol). With this solvent system, compounds had the following m/z ratios: the unreacted DAP, m/z 410 with fragment ions at M+–31 = 325 m/z and M+–COOH, at 297 m/z. As the sample was diluted with water, applied to a Dowex 50 H+ column (1.5 by 16 cm), and eluted with a 400-ml 0 to 2 M HCl gradient. This resulted in complete separation of DAP and N-acetyl-DAP. The N-acetyl-DAP was concentrated by lyophilization. The yield of a soft, white, noncrystalline solid was 15%. Despite the presence of multiple stereoisomers in each sample, only a single TLC spot was observed. The 1H nuclear magnetic resonance (NMR) spectrum of the final product was also consistent with the spectrum of the authentic compound.

**FIG. 1. Variations in the DAP pathway for lysine biosynthesis. 1, succinylase pathway; 2, acetylase pathway; 3, aminotransferase pathway; 4, dehydrogenase pathway. Abbreviations and designations: THDPA, t-2,3,4,5-tetrahydrodipicolinate; ll-DAP, ll-2,6-diaminopimelate; meso-DAP, meso-2,6-diaminopimelate; LysC, aspartase kinase; Asd, aspartate semialdehyde dehydrogenase; DapA, dihydrodipicolinate synthase; DapB, dihydrodipicolinate reductase; DapD, THDPA succinylase; DapC, succinyl-DAP aminotransferase; DapE, succinyl-DAP desuccinylase; DapF, DAP epimerase; LysA, DAP decarboxylase; DapL, ll-DAP aminotransferase; Ddh, DAP dehydrogenase.**

**M. maripaludis** was constructed by transforming strain S601 by the pIJA03::mmp1527, which was constructed from the integration vector pIJA03. Plasmid pIJA03 contains multiple-cloning sites MCS1 and MCS2 of pIJA03, respectively. Primer sequences are available upon request. The orientation of each insert was confirmed by restriction mapping.

**Conclusions.** The mmp1527::pac mutant was transformed into M. maripaludis strain S2 by the poly-
ethylen glycol method (35). After transformation, cultures were plated on McC containing puromycin. Puromycin-resistant isolates were restreaked on the same medium, and isolated colonies were then transferred to broth cultures in 5 ml McC containing puromycin. After growth, 3-ml portions of cultures were used for determination of the genotype and phenotype. The remaining culture was used for preparation of frozen stocks (34). The genotype of the mmp1527-pac mutant (S600) was confirmed by Southern hybridization (data not shown).

For preparation of recombinant MJ1391 in E. coli. The M. jannaschii MJ1391 gene was cloned from genomic DNA. PCR was performed as described previously (16) using an annealing temperature of 45°C. The primers introduced an Ndel site and a BamHI site at the 5' and 3' ends, respectively. The amplified PCR product was purified with a QIAquick spin column (Invitrogen), digested with restriction enzymes Ndel and BamHI, and then ligated into compatible sites in plasmid pT7-7 (Novagen) by using bacteriophage T4 DNA ligase (Invitrogen) to obtain plasmid pMJ1391. DNA sequences were verified by dye terminator sequencing at the University of Iowa Genetics Resource Center.

Cloning, expression, and purification of recombinant MJ1391 in E. coli. The MJ1391 was grown in 100 ml of McC to an absorbance of 600 nm of ~0.4. The cells were collected by centrifugation at 10,000 × g for 30 min at 4°C and resuspended in 2 ml of buffer containing 100 mM Tris (pH 8.5). The cells were lysed by freezing them at −20°C. After thawing, the cell lysate was incubated with 20 U of RNase (Promega) at 37°C for 15 min to digest the DNA. Unbroken cells were removed by centrifugation at 8,000 × g for 30 min at 4°C. The protein concentrations were determined by the bicinchoninic acid (BCA) protein assay (Pierce) (31).

Preparation of M. maripaludis cell extract. M. maripaludis was grown in 100 ml of McC to an absorbance of 600 nm of ~0.4. The cells were collected by centrifugation at 10,000 × g for 30 min at 4°C and resuspended in 2 ml of buffer containing 100 mM Tris (pH 8.5). The cells were lysed by freezing them at −20°C. After thawing, the cell lysate was incubated with 20 U of RNase (Promega) at 37°C for 15 min to digest the DNA. Unbroken cells were removed by centrifugation at 8,000 × g for 30 min at 4°C. The protein concentrations were determined by the bicinchoninic acid (BCA) protein assay (Pierce) (31).

RESULTS AND DISCUSSION

The Δmmp1527 mutant of M. maripaludis is a lysine auxotroph. Sequence similarity searches identified DapL homologs in Methanococcales that exhibit 27 to 33% amino acid sequence identity with the DapL protein in M. thermautotrophicus (MTH52). Since DapL is an amiotransferase that is closely related to many enzymes with other catalytic activities (18), this level of relatedness was not sufficient to predict the function of the Methanococcales homologs. Therefore, the gene encoding the DapL homolog in M. maripaludis (MMP1527) was deleted by gene replacement with the pac cassette, which encodes puromycin resistance in methanococci. Deletion of MMP1527 was confirmed by Southern hybridization (data not shown). Since the downstream genes were transcribed on the strand opposite the MMP1527 strand, this mutation was not expected to be polar and affect transcription of downstream genes.

The Δmmp1527 mutant, strain S600, was unable to grow in the absence of exogenous l-lysine (Fig. 2). Unlike some amino acids, lysine can be readily taken up from the medium by methanococci (44). When 1 mM l-lysine was added to McNA, S600 grew at a rate comparable to that of wild-type strain S2. Addition of the other 19 standard amino acids to McNA did not result in growth. To confirm that these differences were due to deletion of MMP1527, the S601 complementation strain was constructed by transforming plasmid pMEV2-MMP1527 into S600. In this strain, MMP1527 was expressed from a strong methanococcal promoter. S601 grew in the absence of exogenous l-lysine (Fig. 2). These results indi-
cated that MMP1527 was required for L-lysine biosynthesis and that MMP1527 was not essential for the biosynthesis of other amino acids.

DapL activity in *M. maripaludis* cell extract. The specific activities of DapL in cell extracts of *M. maripaludis* strains S2, S600, and S601 using 0.5 mM LL-DAP as the substrate were 24, 2, and 36 nmol min⁻¹ mg⁻¹ of protein, respectively. These results confirmed the presence of DapL activity in *M. maripaludis* and its attribution to MMP1527.

The *M. maripaludis* wild-type S2 cell extract was examined for substrate specificity. When DAP was replaced in assays with acetyl-DAP (2 mM) and succinyl-DAP (2 mM), the specific activities were 4 nmol min⁻¹ mg⁻¹ of protein. The absence of detectable activity suggested that *M. maripaludis* DapL did not use acetyl-DAP or succinyl-DAP as a substrate.

The MJ1391 gene product catalyzed the diaminopimelate aminotransferase reaction. The DapL homolog from *M. jannaschii* (MJ1391) was expressed in *E. coli* and was partially purified by heat denaturation of *E. coli* proteins, followed by anion-exchange chromatography to obtain a purity of ~70%. The presence of PLP in the recombinant MJ1391 protein was confirmed by the fluorescent excitation and emission spectra of the PLP-cyanide product. Analysis of the spectra indicated that there was 18.7 µmol of PLP/mg protein (data not shown). Using a molecular mass of 47.9 kDa for the protomer, this corresponded to 0.89 PLP molecule per protomer.

The recombinant MJ1391 protein catalyzed the transamination of LL-DAP, as determined by the DapL activity assay (Fig. 3A). Moreover, the MJ1391 protein and the substrates α-KG and LL-DAP were stable at 100°C for at least 30 min (Fig. 3B). The level of activity with acetyl-DAP (2 mM) or succinyl-DAP (2 mM) as the substrate was below the detection limit (0.05 µmol min⁻¹ mg⁻¹ of protein⁻¹) (data not shown).

The DapL activity of the MJ1391-derived enzyme was examined under different reaction conditions. The optimum temperature for the DapL activity of MJ1391 was ~70°C, and no activity was detected at temperatures below 45°C (Fig. 4A). At 85°C, the optimum growth temperature of *M. jannaschii*, the DapL activity was 85% of the maximum activity. The optimum pH was ~8.5. About 40% of the maximum activity and 50% of the maximum activity were observed at pH 7.0 and 10.0, respectively (Fig. 4B). All subsequent assays were performed at 70°C and pH 8.5. Monovalent anions frequently stimulate enzymes from methanococci (45). However, addition of 100 mM KCl, K₂SO₄, KH₂PO₄, NaCl, Na₂SO₄, NaH₂PO₄, MgCl₂, or MgSO₄ had no effect on the enzyme activity (data not shown).

The kinetic constants of MJ1391 were within the ranges for DapL homologs from other sources (18, 24). The $K_m$ values for...
Methanococcales members of the analysis of DapL identified homologs in all sequenced genomes of microbial DapL contains only Glu97. Further study is aspartate aminotransferases, is conserved in all DapL homologs.

Asn309, and Lys129, are important for LL-DAP and glutamate binding (37). Lys129, which is found at similar positions in organisms), respectively. The $V_{\text{max}}$ was $0.39 \pm 0.01 \mu$mol min$^{-1}$ mg of protein$^{-1}$ (0.25 to 10.6 $\mu$mol min$^{-1}$ mg of protein$^{-1}$ for other organisms). The $k_{\text{cat}}$ for MJ1391 was 18.3 min$^{-1}$ after correction for a purity of 70%.

Phylogenetic analysis of DapL homologs. Phylogenetic analysis of DapL identified homologs in all sequenced genomes of members of the Methanococcales, including M. jannaschii, Methanocaldococcus infernus, Methanocaldococcus fervens, Methanocaldococcus vulcanius, M. maripaludis, M. voltae, Methanocaldococcus aeolicus, and Methanocaldococcus vannielli. The sequences exhibited 74 to 89% amino acid sequence identity. According to the phylogenetic tree topology, these methanococcal DapL homologs did not cluster with the DapL1 and DapL2 groups, with which they exhibited 29 to 33% and 30 to 42% amino acid sequence identity, respectively (Fig. 5A).

The methanococcal enzymes were also distinguished from the DapL1 and DapL2 groups by comparison of conserved amino acid residues. Based on the crystal structure of DapL from A. thaliana, three conserved residues in DapL1, Glu97, Asn309, and Lys129, are important for L-L-DAP and glutamate binding (37). Lys129, which is found at similar positions in aspartate aminotransferases, is conserved in all DapL homologs. DapL2 lacks the other two residues, Glu97 and Asn309 (18). Methanococcal DapL contains only Glu97. Further study is needed to understand how the substrates bind to DapL2 and methanococcal DapL.

The phylogeny of the bacterial and archaeal DapL proteins in the ME tree was not correlated with the 16S rRNA-based phylogeny, indicating that lateral gene transfers (LGTs) may have made substantial contributions to the evolution of DapL in some lineages. The evolutionary history of DapL homologs was evaluated by comparison of the ratios of their evolutionary distances (RED) with those of control genes which were believed to have undergone primarily vertical evolution (11). If the evolutionary rate of DapL was constant and LGTs did not occur, then a plot of the DapL evolutionary distances ($E_d$s) against the control $E_d$s should be linear. As shown in Fig. 5B, the intradomain comparisons for each DapL group were close to a trend line indicative of a constant rate of DapL evolution relative to that of the control genes. Moreover, comparisons among the bacterial DapL1, the archaeal DapL2, and the methanococcal DapL lay on the same trend line, indicating that these groups did not obtain the gene from an LGT. However, the interdomain comparisons between bacteria and archaea within each of the DapL1 and DapL2 groups resulted in $E_d$s that were lower than the values expected, and bacterial and archaeal intradomain comparisons for each of the groups resulted in $E_d$s that were higher than the values expected for normal vertical evolution. These results suggested that interdomain LGTs occurred within both groups, that there was one transfer from the archaeal DapL2 group to form the bacterial DapL2 group, and that there was one transfer from the bacterial DapL1 group to form the archaeal DapL1 group (Fig. 5C). Lastly, comparisons between the bacterial DapL2 group and the archaeal DapL1 group are on the central trend line because each group resulted from LGTs but the LGTs were in opposite directions. In toto, these results suggested that in DapL evolution, while there were two LGT events, there were not discontinuities in the rate of evolution or the formation of paralogous gene families.

RED analyses that included the uncharacterized DapL paralogs of E. coli (b2379) and Bacillus subtilis (BSU37690) showed that these paralogs had lower $E_d$s with archaeal DapL2 and methanococcal DapL groups and higher $E_d$s with bacteria DapL than expected, suggesting that these paralogs may have had an archaeal origin (data not shown).

The selective pressure for some organisms to obtain and maintain the DapL pathway instead of the acylation pathways is unclear. THDPA is unstable at neutral pH and at equilibrium forms a mixture of the cyclic compound THDPA and the acyclic compound L-$\epsilon$-keto-$\epsilon$-aminopimelate (5, 6). The equilibrium favors cyclic THDPA, while the acyclic form containing the keto group is the substrate for transamination. Therefore, acylation may facilitate the transamination by exposing the keto group at the expense of one succinyl-CoA or acetyl-CoA (15, 18, 19). However, whether cyclic THDPA is the actual substrate of DapL or DapL catalyzes the ring-opening reaction awaits further investigation (37, 38).

In conclusion, methanococci use the DapL pathway for L-lysine biosynthesis, as a deletion mutation of the dapL homolog in M. maripaludis resulted in lysine auxotrophy and the purified homologous protein in M. jannaschii catalyzed the DAP aminotransferase reaction. Moreover, activities with succinylated and acetylated DAP were not observed for M. marip-
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