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METHANOGENS WITH PSEUDOMUREIN USE DIAMINOPIMELATE AMINOTRANSFERASE IN LYSINE BIOSYNTHESIS

David E. Graham[1,2,*] and **Holly K. Huse**[3]

1*Institute for Cellular and Molecular Biology, University of Texas at Austin, Austin, TX 78712*

2*Department of Chemistry and Biochemistry, University of Texas at Austin, Austin, TX 78712*

3*Microbiology Graduate Program, University of Texas at Austin, Austin, TX 78712*

Abstract

Methanothermobacter thermautotrophicus uses lysine for both protein synthesis and cross-linking pseudomurein in its cell wall. A diaminopimelate aminotransferase enzyme from this methanogen (MTH0052) converts tetrahydrodipicolinate to L,L-diaminopimelate, a lysine precursor. This gene complemented an *Escherichia coli* diaminopimelate auxotrophy, and the purified protein catalyzed the transamination of diaminopimelate to tetrahydrodipicolinate. Phylogenetic analysis indicated this gene was recruited from anaerobic Gram-positive bacteria. These results expand the family of diaminopimelate aminotransferases to a diverse set of plant, bacterial and archaeal homologs. In contrast marine methanogens from the *Methanococcales*, which lack pseudomurein, appear to use a different diaminopimelate pathway for lysine biosynthesis.

1. Introduction

Bacteria and archaea use at least five different biosynthetic pathways to produce lysine [1]. Three of these pathways produce a diaminopimelate (DAP) intermediate (Figure 1), while two others produce an α-aminoadipate intermediate. Genome sequence analysis predicts that most *Crenarchaea* and the euryarchaeal *Thermococcales* produce lysine by a variation of the α-aminoadipate pathway that was first identified in fungi [2]. Alternatively, NMR studies using ^{13}C-labeled glycerol suggested that the euryarchaeon *Haloarcula hispanica* uses a DAP dehydrogenase [3]. Similar studies in the methanogens *Methanospirillum hungatei* and *Methanococcus voltae* using ^{13}C-labeled acetate indicated that these organisms also use a DAP pathway, but could not discriminate among the three DAP pathways [4,5]. Finally, enzyme assays of *Methanothermobacter thermautotrophicus* ΔH extracts detected dihydrodipicolinate synthase and DAP decarboxylase activities that are characteristic of all the DAP pathways [6].

Methanogen genome sequences lack some genes required for lysine biosynthesis. A metabolic reconstruction of *Methanocaldococcus jannaschii* predicted this organism uses an acylated DAP pathway, based on the identification of homologs of the *Escherichia coli asd, dapA, dapB, dapF* and *lysA* genes [7]. However, no *dapD* or *dapC* homologs were found in *M. jannaschii*, and the *dapE* homolog shares only 22% amino acid identity with the *E. coli* protein.

*Corresponding Author. 1 University Station A5300, Austin TX 78712-0165. Tel. 512-471-4491. Fax: 512-471-8696, *E-mail address*: degraham@mail.utexas.edu.

Therefore the pathway from tetrahydrodipicolinate to L,L-diaminopimelate is unresolved; the other methanogen genomes are also missing these genes.

Plants and the bacterium *Chlamydia trachomatis* use an L,L-diaminopimelate aminotransferase enzyme (DapL) to bypass the need for an acyl protecting group in lysine biosynthesis (Figure 1) [8,9]. Sequence similarity searches suggested that *M. thermautotrophicus* and two other members of the *Methanobacteriales* order have homologs of the plant and chlamydial protein that share 39-43% amino acid sequence identity [10]. Unlike other archaea, these methanogens produce pseudomurein –a carbohydrate-based cell wall that is cross-linked through a pentapeptide containing L-alanine, L-lysine and L-glutamate [11]. While most bacteria use DAP in their peptidoglycan, lysine serves the same purpose in some Gram-positive bacterial cell walls and in pseudomurein [12,13]. It is possible that the *Methanobacteriales* ancestor acquired a *dapL* homolog by horizontal gene transfer, along with glycosyltransferase and peptide ligase genes required for pseudomurein formation.

A crystal structure model of the *Arabidopsis thaliana* DapL protein showed that it belongs to the aspartate aminotransferase family of pyridoxal 5′-phosphate dependent proteins [14]. Because members of this enzyme family catalyze a diverse set of transamination reactions [15], careful sequence analysis and genetic or biochemical characterization is often required to identify gene function [16]. The discovery of DapL homologs in cyanobacteria, diverse Gram-positive and Gram-negative bacteria, and the *Methanobacteriales* suggested these organisms also use this pathway for lysine biosynthesis. However, the bacterial and archaeal homologs form a separate, diverged cluster in the aminotransferase phylogeny. No representative gene has been characterized from this group, therefore it is possible that these homologs have a different function.

To test whether the *M. thermautotrophicus* homolog functions as a DAP aminotransferase, we cloned the MTH0052 homolog of *dapL* in *E. coli*. Expressed in an *E. coli* DAP auxotroph, this gene restored the cells' ability to grow in the absence of DAP. Cell-free extracts and affinity purified MTH0052 protein catalyzed the transamination of DAP with 2-oxoglutarate to produce tetrahydrodipicolinate and glutamate. Combined with phylogenetic analysis, these results suggest that lysine biosynthesis in the *Methanobacteriales* proceeds through the DAP aminotransferase pathway using a gene that was acquired from a Gram-positive bacterium by horizontal gene transfer. The other orders of methanogens appear to produce lysine by a different mechanism.

2. Materials and Methods

2.1. Plasmids and Strains

Escherichia coli MG1655 (CGSC 7740) was obtained from the *E. coli* Genetic Stock Center (Yale). *E. coli* DH5α (Invitrogen) was used as a general cloning host, and *E. coli* LMG194 (Invitrogen) was used for protein expression. *E. coli* ATM769 (MG1655 Δ*dapD*::Tet Δ*dapE*::Kan), *E. coli* ATM782 (MG1655 Δ*dapE*::Kan), ATM780 (*Chlamydia trachomatis* gene CT390 in pUC19), and pNEA15 (*E. coli dapE* in pBAD18) were gifts from Anthony Maurelli (Uniformed Services University of the Health Sciences) [9]. Plasmid pBAD/HisA was from Invitrogen. *Methanothermobacter thermautotrophicus* ΔH cells were a gift from Eric Johnson and Biswarup Mukhopadhyay (Virginia Polytechnic Institute and State University). Chromosomal DNA was purified from *Methanococcus maripaludis* S2 cells by standard methods and from *M. thermautotrophicus* as described by Mukhopadhyay et al. [17].

The gene encoding protein MTH0052 (RefSeq accession NP_275195.1) was amplified from *M. thermautotrophicus* DNA by PCR using oligonucleotide primers 5MTH0052X and 3MTH0052H (Operon). The PCR product was cloned into XhoI and HindIII sites of vector

pBAD/HisA to produce vector pDG428. The gene encoding protein MMP1527 (RefSeq accession NP_988647.1) was amplified from *M. maripaludis* DNA using primers 5MMP1527XN and 3MMP1527H. The PCR product was cloned into XhoI and HindIII sites of pBAD/HisA to produce pDG422. The gene encoding protein MMP1398 (RefSeq accession NP_988518.1) was amplified from *M. maripaludis* DNA using primers 5MMP1398XN and 3MMP1398H. The PCR product was cloned into XhoI and HindIII sites of pBAD/HisA to produce pDG419. Oligonucleotide sequences are shown in Supplementary Table 2. Recombinant DNA was sequenced at the Institute for Cellular and Molecular Biology Core Labs DNA Sequencing facility (UT-Austin).

2.2. Complementation

Plasmids were transformed into *E. coli* by electroporation. Cells were grown aerobically in LB Miller broth containing ampicillin (100 μg ml^{-1}) and DL-α,ε-diaminopimelate (100 μg ml^{-1}) at 37°C. For complementation, this broth was supplemented with L-arabinose (0.2% w/v). Overnight cultures were streaked onto selective agar medium and incubated at 37°C for 22 h.

2.3 Protein expression and purification

E. coli LMG194 (pDG428) cells were grown with continuous shaking at 37°C (250 rpm) in LB broth with ampicillin. When cultures reached an optical density at 600 nm of 0.6 to 0.7, protein expression was induced by the addition of L-arabinose (0.2% w/v). After four hours of incubation with the inducer, cells were harvested by centrifugation and stored at -20°C. Cell lysis, Ni^{2+}-affinity chromatography and sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) were performed using standard methods [16].

2.4 Aminotransferase activity analysis

The formation of tetrahydrodipicolinate was measured using *o*-aminobenzaldehyde to form a yellow-colored derivative [18]. Neither the structure nor molar absorbance coefficient of this derivative is known, therefore aminotransferase activity is expressed in absorbance units (AU). Standard reactions included 1 mM DL-α,ε-diaminopimelate (Sigma), 4 mM potassium 2-oxoglutarate, 10 mM *o*-aminobenzaldehyde (Sigma) dissolved in acetonitrile, 100 mM Hepes/KOH (pH 7.5) and enzyme. The absorbance of the product derivative at 440 nm was measured in a quartz cuvette using a DU-800 spectrophotometer (Beckman) equipped with a Peltier heated stage at 37°C. Reactions with alternative amine acceptors included oxaloacetate or 2-oxoadipate [19] instead of 2-oxoglutarate.

For product analysis, reactions containing 1 mM DAP and 4 mM 2-oxoglutarate were incubated with His_6-MTH0052 protein for 3 h at 50°C. The reaction product (20 μl) was mixed with 40 μl of 5 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl for 10 min at room temperature. The hydrazone derivatives were separated by reversed phase HPLC using an octadecylsilane column (Axxi-Chrom, 250 by 4.6 mm) with a guard column (Phenomenex, 4 by 3 mm). Chromatography was performed using an isocratic elution with buffer A for 3 min, followed by a gradient to 100% buffer B over 12 min. Buffer A contained 25 mM ammonium phosphate (pH 2.5) and 8% (v/v) acetonitrile. Buffer B contained 25 mM ammonium phosphate (pH 2.5) and 80% (v/v) acetonitrile. DNPH derivatives were detected by their absorbance at 360 nm.

For mass spectrometry, enzymatic reaction mixtures were evaporated to dryness by heating under a flow of nitrogen, and derivatized with trifluoroacetic anhydride (TFAA) for 30 min at 55°C. Excess TFAA was evaporated and the residue was dissolved in water. The acyl derivatives were analyzed by liquid chromatography-mass spectrometry (LC-MS) using a Thermo LTQ-XL instrument. The sample was applied to a reversed phase column (Thermo Hypersil Gold 3 μm; 50 × 2.1 mm) and eluted with a gradient from 5% to 80% acetonitrile with 0.1% formic acid in water at a flow rate of 0.5 ml min^{-1}. Ultraviolet-visible light absorption

was measured using a photodiode array detector, and electrospray ionization mass spectrometry was performed in the negative ion mode. Tandem mass spectra (MS/MS) were acquired using collision-induced dissociation of the $[M-H]^-$ ions.

2.5 Phylogenetic analysis

An alignment of 34 homologs of the diaminopimelate aminotransferase proteins was prepared using the T-Coffee program (ver. 4.96) [20]. From the full alignment 409 positions were chosen for phylogenetic analysis. The phylogeny was inferred using the proml program (with 100 bootstrap replicates) from the Phylip package (ver. 3.67) [21]. This program used the Jones-Taylor-Thornton model of amino acid changes and assumed a γ-distribution of rates (α=2.4) approximated by three states. Complete organism names and sequence accession numbers are listed in the Supplementary Material.

3. Results

3.1 MTH0052 gene complements dapDE mutations

The *E. coli* strain ATM769 (*dapD dapE*) is a diaminopimelate auxotroph and requires supplemental DAP for growth on LB medium [9]. When expressed from the arabinose-inducible P_{BAD} promoter in plasmid pDG428, the MTH0052 gene complements this mutation (Figure 2). The ATM769 (pDG428) strain grows as well as wild-type *E. coli* MG1655 and the ATM769 (pATM780) strain that expresses the chlamydial homolog CT390. As expected, the ATM769 (pDG428) strain failed to grow without arabinose. With the arabinose inducer, the ATM769 (pDG428) strain had a specific growth rate of $0.95 \pm 0.007\ h^{-1}$, compared to a rate of $0.99 \pm 0.03\ h^{-1}$ for the same strain supplemented with DAP. Therefore expression of the MTH0052 protein substitutes for the succinyl-DAP enzymes of *E. coli*.

3.2 MTH0052 protein catalyzes the diaminopimelate aminotransferase reaction

In reactions containing DAP, 2-oxoglutarate and *o*-aminobenzaldehyde, cell-free extracts of the *E. coli* ATM769 strain expressing the MTH0052 protein catalyzed the transamination reaction with an activity of 25 ± 9 milliAU min^{-1} mg^{-1} total protein (Figure 3). For comparison, cell-free extracts of the same strain with the empty pBAD/HisA vector had no significant activity (2 ± 5 milliAU min^{-1} mg^{-1} total protein) after an initial burst of color formation.

To confirm that DAP-AT activity was catalyzed by the heterologously expressed MTH0052 protein, the His_6-MTH0052 protein was partially purified by nickel affinity chromatography. The protein had an apparent mass of 47 kDa determined by SDS-PAGE, close to its expected mass of 50.6 kDa. It had an absorption maximum at 412 nm, characteristic of PLP bound to lysine as a Schiff base. This protein catalyzed the transamination of DAP with a specific activity of 0.35 ± 0.12 AU min^{-1} mg^{-1}. It has maximal aminotransferase activity using 2-oxoglutarate as an amino group acceptor. No activity was observed when oxaloacetate replaced 2-oxoglutarate, although 2-oxoadipate could substitute with 21% relative activity.

The products of the MTH0052 DAP aminotransferase reaction were analyzed by liquid chromatography. Carbonyls derivatized with DNPH formed the hydrazones of the 2-oxoglutarate substrate and the 2-oxo-6-aminopimelate product, which were resolved by HPLC (Figure 4). Reaction mixtures treated with TFAA formed *N*-acyl derivatives that were analyzed by LC-MS and tandem MS/MS. Using this method, the derivatives had the following retention times, UV-visible absorption maxima and mass spectral data. Peaks corresponding to the molecular ions ($[M-H]^-$) are shown first, followed by characteristic complexes (usually $[2M-H]^-$), and ion fragments listed in decreasing order of intensity. The acyl derivative of glutamate eluted at 0.55 min (233 nm) producing peaks at 242, 485, 113 and 216 *m/z*; MS/MS of the ion at 242 *m/z* produced peaks at 199 and 225 *m/z*. The acyl derivative of tetrahydrodipicolinate

eluted at 0.88 min (243 nm) producing peaks at 266, 533, 113, and 222 *m/z*; MS/MS of the ion at 266 *m/z* produced peaks at 223, 113 and 153 *m/z*. The diacyl derivative of DAP eluted at 1.4 min (229 nm) producing peaks at 381, 763, 113 and 240 *m/z*); MS/MS of the ion at 381 *m/z* produced peaks at 207, 268, 240, 338, 324, 137 and 113 *m/z*.

3.3 Diaminopimelate and lysine biosynthesis in the Methanococcales

Both *M. jannaschii* and *Methanococcus maripaludis* are marine methanogens that belong to the order *Methanococcales*. Their genomes encode homologs of the *asd*, *dapA*, *dapB*, *dapF* and *lysA* genes that compose a gapped diaminopimelate biosynthetic pathway, as in *M. thermautotrophicus* (see Supplementary Table 1). The closest *M. maripaludis* homolog of MTH0052 is MMP1527 (29% amino acid identity). But expression of the MMP1527 protein in *E. coli* ATM769 (pDG422) failed to rescue growth in the absence of diaminopimelate. Therefore the MMP1527 protein probably belongs to a different group of aminotransferases.

E. coli strain ATM782 lacks the *dapE* gene that encodes an *N*-succinyl-DAP desuccinylase. The *M. maripaludis* protein MMP1398 is 21% identical to *E. coli* DapE, and both are annotated as succinyl-DAP desuccinylase enzymes. Therefore it was surprising that expression of MMP1398 from plasmid pDG419 failed to complement the *dapE* mutation. Only expression of the native *dapE* in strain ATM782 (pNEA15) restored growth in the absence of DAP. Although the DAP aminotransferase circumvents the entire succinyl-DAP pathway in *E. coli*, expression of neither MTH0052 nor the chlamydial homolog complemented the *dapE* mutation.

While these results do not identify the functions of the MMP1527 or MMP1398 proteins, they suggest that the *Methanococcales* use a different pathway than *M. thermautotrophicus* to transaminate tetrahydrodipicolinate to L,L-diaminopimelate. No homolog of DAP dehydrogenase was identified in the *M. maripaludis* genome. Furthermore, no DAP dehydrogenase activity was detected in spectrophotometric assays containing 0.2 mg ml^{-1} *M. maripaludis* cell-free extract, 3 mM DAP, 1 mM NAD^+ or $NADP^+$, and 50 mM glycine/NaOH (pH 10).

3.4 Methanobacteriales acquired DAP aminotransferase by horizontal gene transfer

Phylogenetic analysis of the DAP aminotransferase family identified orthologs in all three genomes of the sequenced *Methanobacteriales*: *M. thermautotrophicus*, *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* (Figure 5). These genes share 63-73% nucleotide sequence identity. The three proteins are more closely related to the bacterial and plant DAP aminotransferases than they are to aminotransferase homologs from other archaea. They also share up to 64% nucleotide sequence identity with homologs from the anaerobic δ-proteobacteria and Gram-positive bacteria. The sporadic phylogenetic distribution and the unusually high similarity between the archaeal and bacterial homologs indicate that these genes were horizontally transferred into numerous lineages. A phylogeny constructed using nucleotide sequences from a subset of genes represented in Figure 5 confirmed the specific relationship between the methanobacterial *dapL* and clostridial homologs (Supplementary Figure 1). The genomes that have DAP aminotransferase homologs lack *dapD* or *dapE* homologs that are required for the succinyl-DAP pathway.

4. Discussion

Convergent pathways for the production of lysine have evolved several times, although in most organisms there is little redundancy in lysine biosynthesis. Archaea also have two different classes of lysyl-tRNA synthetases [22]. Horizontal gene transfer has subsequently shuffled these modular pathways and displaced ancestral modes of lysine formation. Crenarchaea and

the deeply-branching euryarchaeal *Thermococcales* use an α-aminoadipate pathway to produce lysine. Most methanogens have a DAP pathway, but they differ in their enzymology for transaminating tetrahydrodipicolinate to form DAP. We have shown here that *Methanobacteriales* have a DAP aminotransferase to catalyze this reaction; the analogous reactions in other methanogens have not yet been identified. Therefore the DAP aminotransferase was probably acquired by the *Methanobacteriales* ancestor through horizontal gene transfer from an anaerobic bacterium.

The previous identification and characterization of DAP aminotransferases in plants and *Chlamydia* led us to question how widespread is this pathway. Homologs of *dapL* were noted in all cyanobacterial genomes [8], yet phylogenetic analysis rejects the model that plants acquired *dapL* through chloroplast endosymbiosis (Figure 5). The *M. thermautotrophicus* protein is the first characterized member of this archaeal and bacterial clade that also includes homologs from anaerobic bacteria. The DapL phylogeny does not identify a single gene donor to the *Methanobacteriales* lineage, and there is only weak bootstrap support for grouping the three *Methanobacteriales* homologs. However, gene order is conserved: the orthologs in both *M. thermautotrophicus* and *M. stadtmanae* are adjacent to glutamyl-tRNA synthetase genes. Therefore most of the divergence in gene sequences is probably due to the high G+C base composition in MTH0052 (53%) versus *M. stadtmanae* (31%) and *M. smithii* (33%) orthologs. Supporting this artifact, a phylogeny derived from an alignment of DNA sequences groups the MTH0052 gene with homologs from the high-%G+C Gram-negative bacteria to the exclusion of the other methanogen homologs (Supplementary Figure 1). The most closely related bacterial homologs come from anaerobic Gram-positive or δ-proteobacterial lineages. These organisms share the same ecological niche, and could also be the donors of peptide ligase homologs (MurC, MurE and MurF) in the *Methanobacteriales* that are probably responsible for pseudomurein biosynthesis.

The set of conserved amino acid positions in an alignment of DapL homologs includes all of the active site residues identified in the *A. thaliana* crystal structure model [14]. This protein family evolved from an aspartate aminotransferase ancestor, and the two enzymes remain highly similar. Several amino acid substitutions may be responsible for the change in substrate specificity: *A. thaliana* DapL residues Glu^{97} and Asn^{309} are highly conserved in DapL sequences, but not in aspartate aminotransferases. Both residues form hydrogen bonds with the γ-carboxylate of glutamate bound in a co-crystal structure model. These residues could also recognize the amino group of L-2-oxo-6-aminopimelate and LL-DAP. By analogy with aspartate aminotransferase, the DapL enzyme probably transfers an amino group from pyridoxamine to L-2-oxo-6-aminopimelate. Chemically synthesized tetrahydrodipicolinate forms an equilibrium mixture of the enamine and acyclic forms shown in Figure 1 [23,24]. Future studies of inhibitors of this protein and co-crystal structure models may test whether DapL can catalyze the ring-opening reaction of tetrahydrodipicolinate, or whether its true substrate is the ketone form. Tetrahydrodipicolinate is unstable at neutral pH [23], so the evolution of the acylated pathway, using the DapD, DapC and DapE proteins to protect the ring-open form, suggests that substrate sequestration and isomerization could be rate-limiting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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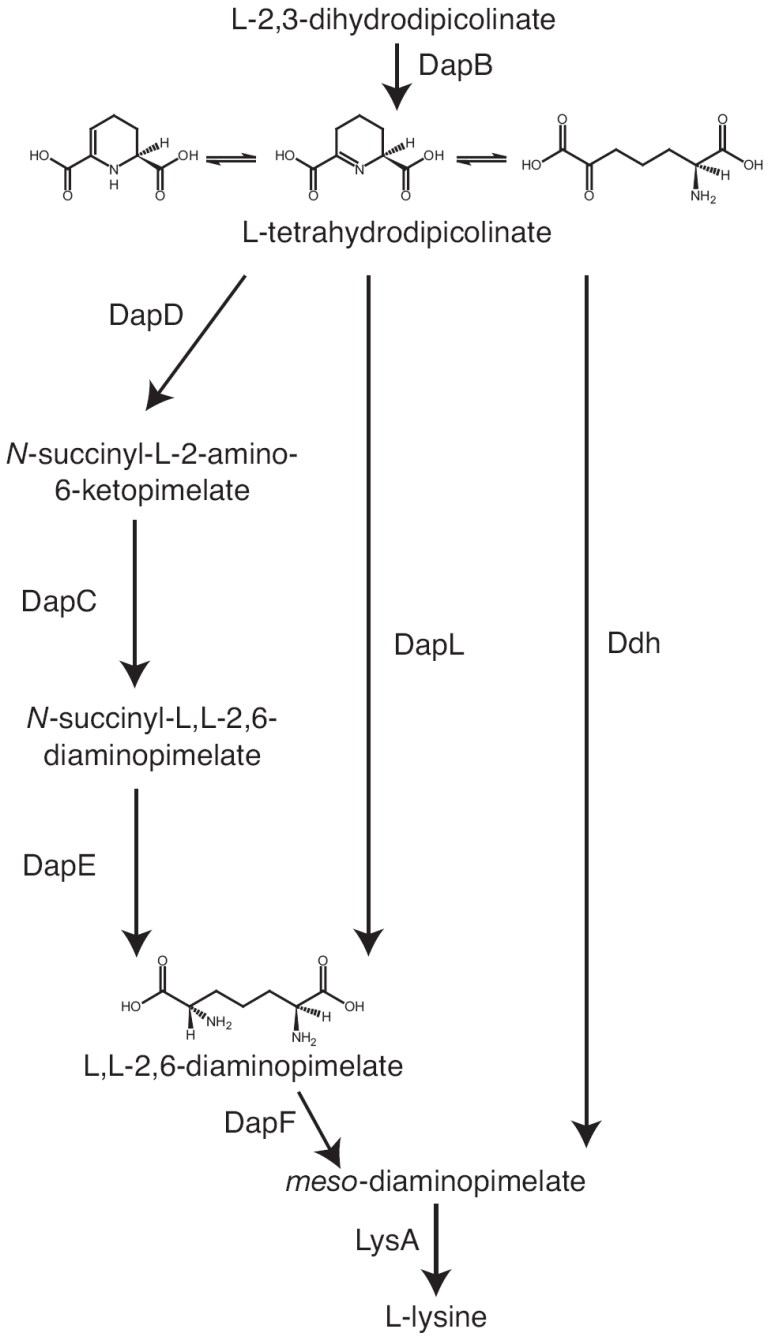

Figure 1.

Alternative biosyntheses of *meso*-diaminopimelate from tetrahydrodipicolinate [8,25]. L-Tetrahydrodipicolinate exists in equilibrium with the enamine product and the acyclic L-2-oxo-6-aminopimelate product [24]. In the succinylate pathway (left), the acyclic form is protected by the tetrahydrodipicolinate *N*-succinyltransferase enzyme (DapD; EC 2.3.1.117). *N*-Succinyl-L,L-diaminopimelate aminotransferase (DapC; EC 2.6.1.17) transfers an amino group from L-glutamate, and then *N*-succinyl-L,L-diaminopimelate desuccinylase (DapE, EC 3.5.1.18) deprotects the product. The L,L-diaminopimelate epimerase enzyme (DapF; EC 5.1.1.7) produces *meso*-diaminopimelate for lysine and peptidoglycan biosynthesis. Some Gram-positive bacteria use an acetyl protecting group instead of the succinyl group.

Alternatively, L,L-diaminopimelate aminotransferase (DapL; EC 2.6.1.83) directly transaminates tetrahydrodipicolinate to produce L,L-diaminopimelate. Finally, some bacteria use a diaminopimelate dehydrogenase enzyme (Ddh; EC 1.4.1.16) to reductively aminate tetrahydrodipicolinate.

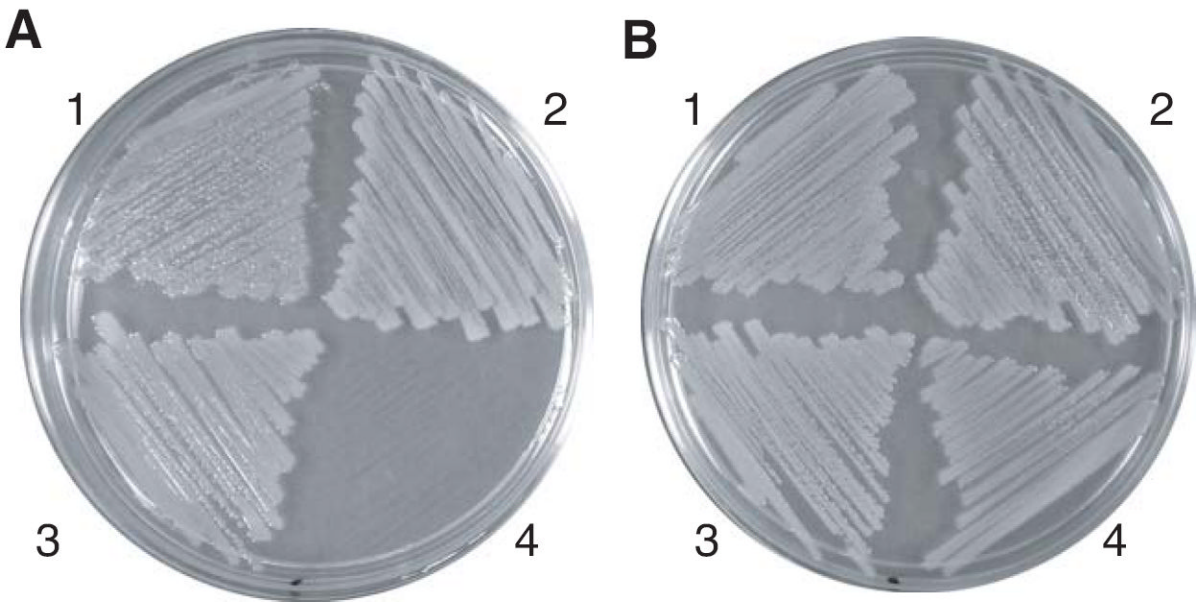

Figure 2.

Expression of MTH0052 complemented diaminopimelate auxotrophy in *E. coli* ATM769. Part A, LB agar supplemented with ampicillin (100 μg ml^{-1}) and arabinose (0.02% w/v) supported the growth of ATM769 (pDG428) in sector 1, ATM769 (pATM780) in sector 2, MG1655 (pBAD/HisA) in sector 3, but not ATM769 (pBAD/HisA) in sector 4. Part B, The same medium as in part A supplemented with DL-α,ε-diaminopimelate (100 μg ml^{-1}) supported the growth of all the strains listed for part A.

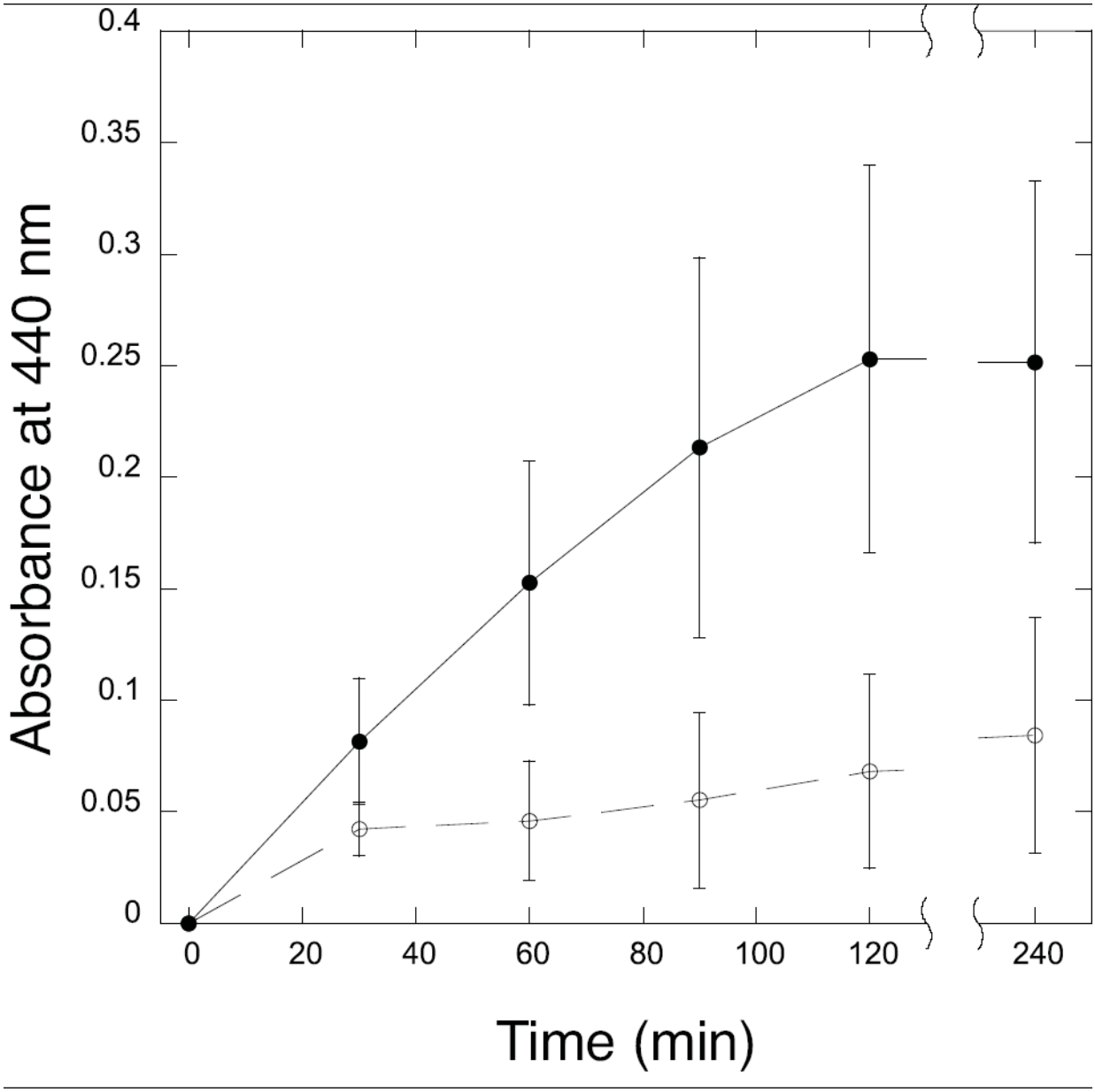

Figure 3.

Reaction profile of diaminopimelate aminotransferase activity in *E. coli* ATM769 (pDG429) extracts expressing MTH0052 (filled circles) and *E. coli* ATM769 (pBAD/HisA) vector control extracts (open circles). Cell-free extract (100 μg) was incubated with 4 mM 2-oxoglutarate, 2 mM DL-α,ε-diaminopimelate (racemic), 50 μM pyridoxal 5′-phosphate, 10 mM *o*-aminobenzaldehyde and 100 mM Tris/HCl (pH 8) in a 300 μl reaction at 37°C. The absorbance of the yellow *o*-aminobenzaldehyde derivative was measured at 440 nm. Error bars show the standard deviation for three replicates of each sample.

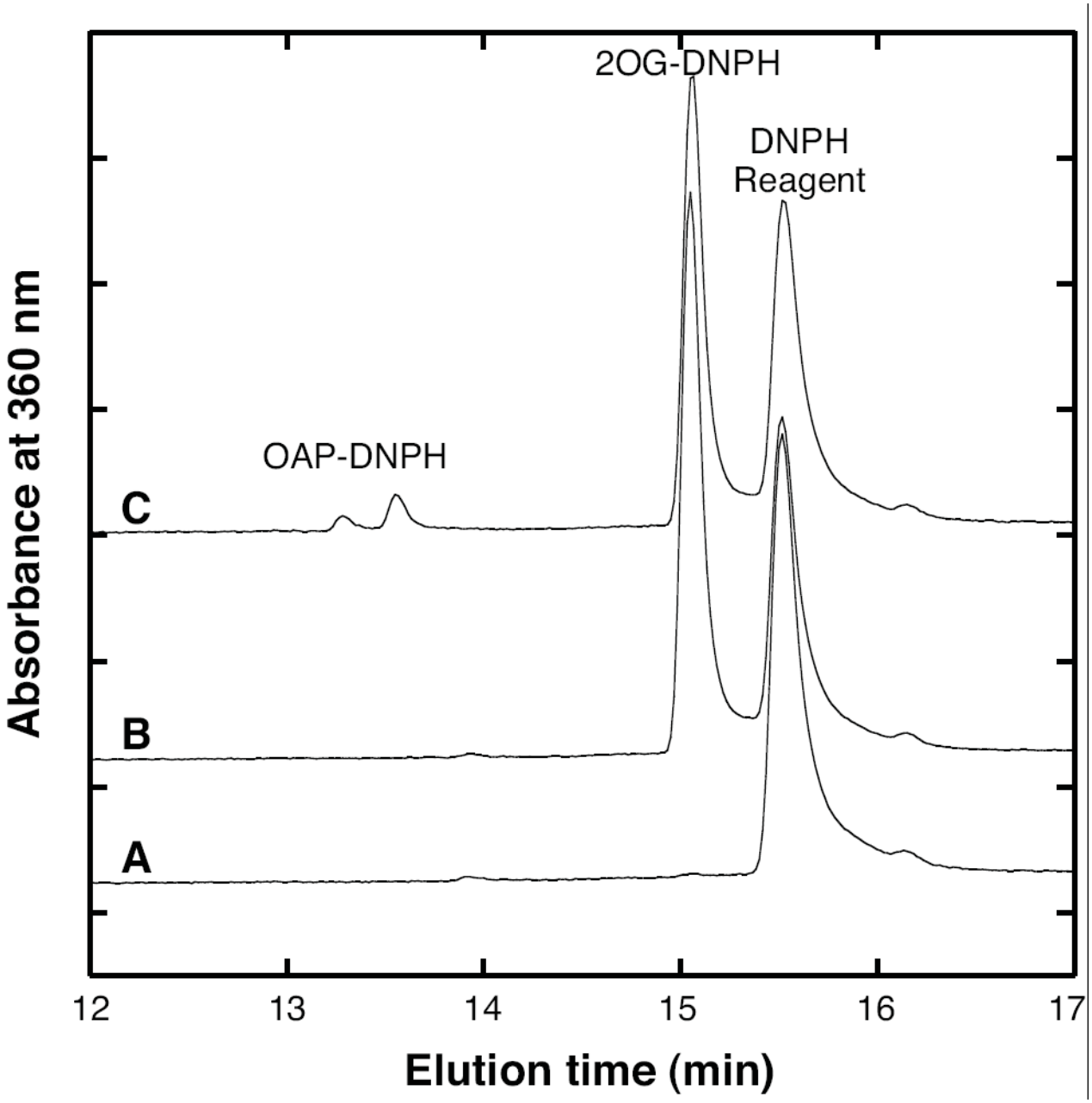

Figure 4.

Reaction products of MTH0052 derivatized with 2,4-dinitrophenylhydrazine (DNPH) and separated by reversed-phase HPLC. Curve **A** shows only a DNPH reagent peak from a control reaction with His_6-MTH0052 enzyme alone. Curve **B** shows the 2-oxoglutarate (2-OG) DNPH hydrazone from a control reaction with 2-OG and DAP substrates. Curve **C** shows that a reaction containing His_6-MTH0052 protein, DAP and 2-OG produced tetrahydrodipicolinate in equilibrium with 2-oxo-6-aminopimelate (OAP), which forms hydrazones with DNPH reagent.

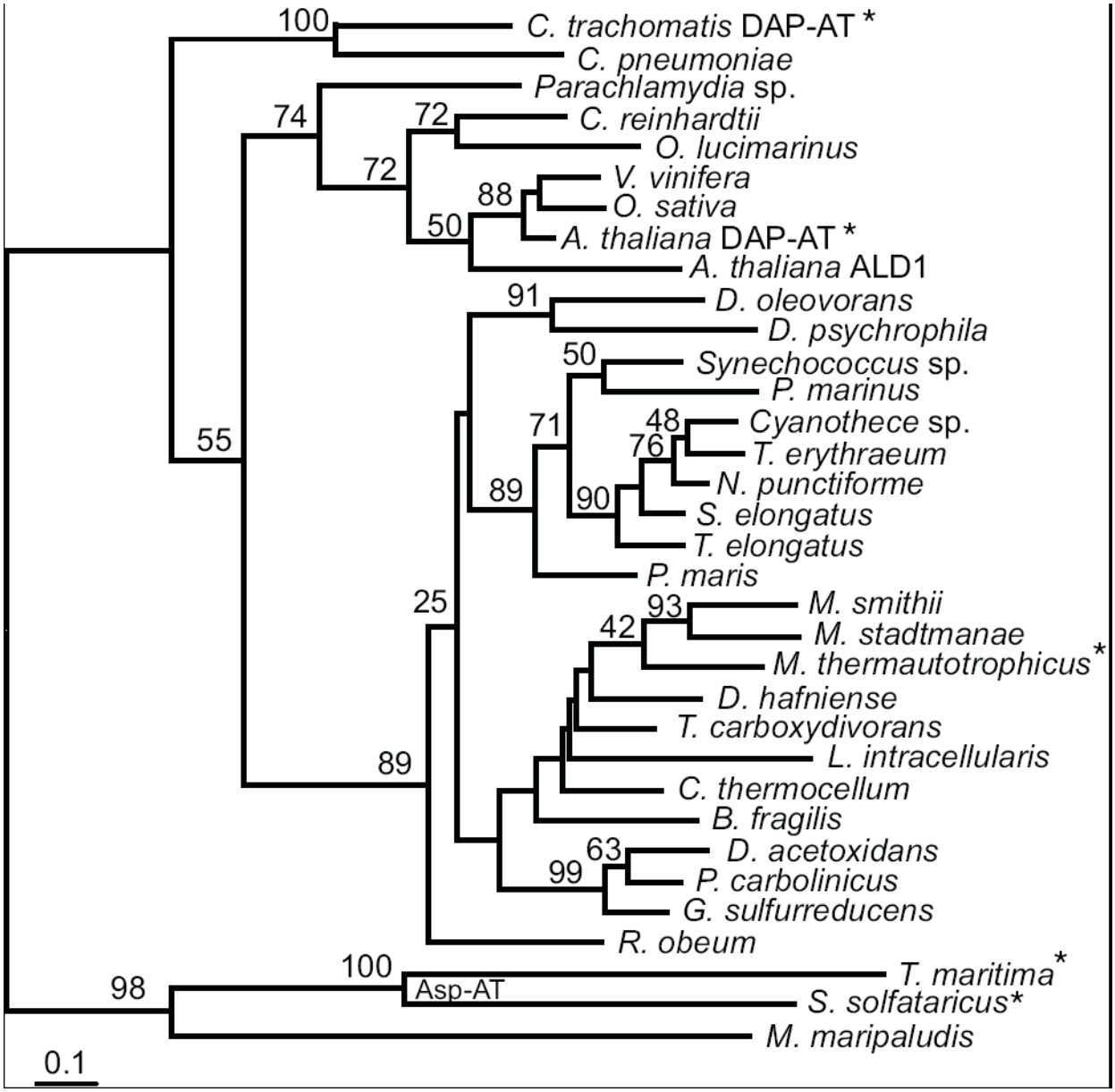

Figure 5.

Phylogram of the DapL homologs inferred by the protein maximum likelihood method. Bootstrap values from are shown near branches supported by a plurality of 100 trees. This tree is rooted using known aspartate aminotransferases enzymes and the closest homolog from *M. maripaludis* (MMP1527). Enzymes whose functions have been experimentally confirmed are indicated by an asterisk. The scale bar indicates one amino acid substitution per 10 positions. Sequence details are described in the Supplementary materials.