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Dual diaminopimelate biosynthesis pathways in *Bacteroides fragilis* and *Clostridium thermocellum*

André O. Hudson [a], Ayelet Klartag [b,1], Charles Gilvarg [b], Renwick C.J. Dobson [c], Felipe Garbelini Marques [d,2], Thomas Leustek [d,*]

[a] School of Biological and Sciences, Rochester Institute of Technology, Rochester, NY 14623, USA
[b] Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA
[c] Department of Biochemistry and Molecular Biology, University of Melbourne, Melbourne, Australia
[d] Biotech Center and Department of Plant Biology and Pathology, Rutgers University, New Brunswick, NJ 08901, USA

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ABSTRACT

Bacteroides fragilis and *Clostridium thermocellum* were recently found to synthesize diaminopimelate (DAP) by way of *LL*-DAP aminotransferase. Both species also contain an ortholog of *meso*-diaminopimelate dehydrogenase (Ddh), suggesting that they may have redundant pathways for DAP biosynthesis. The *B. fragilis* Ddh ortholog shows low homology with other examples of Ddh and this species belongs to a phylum, the *Bacteriodetes*, not previously known to contain this enzyme. By contrast, the *C. thermocellum* ortholog is well conserved with known examples of Ddh. Using *in vitro* and *in vivo* assays both the *B. fragilis* and *C. thermocellum* enzymes were found to be authentic examples of Ddh, displaying kinetic properties typical of this enzyme. The result indicates that *B. fragilis* contains a sequence diverged form of Ddh. Phylogenomic analysis of the microbial genome database revealed that 77% of species with a Ddh ortholog also contain a second pathway for DAP biosynthesis suggesting that Ddh evolved as an ancillary mechanism for DAP biosynthesis.

1. Introduction

The diaminopimelic acid (DAP) pathway serves for the biosynthesis of lysine in bacteria, some archaea, plants and algae [1]. The pathway is also necessary for peptidoglycan biosynthesis [2]. The DAP pathway exists as four variants all of which follow the same general schema [3] (Supplementary Fig. 1). First, tetrahydrodipicolinate (THDPA) is produced from aspartate. Then THDPA is converted to *meso*-diaminopimelate (*m*-DAP). Finally, *m*-DAP is converted to lysine. The variant DAP pathways differ in the mechanism by which *m*-DAP is produced from THDPA. The most complex of the variants utilizes succinylated or acetylated intermediates and require 4 enzymes, THDPA acyltransferase (DapD) (EC:2.3.1.117), N-acyldiaminopimelate aminotransferase (DapC) (EC:2.6.1.11 and 2.6.1.17), acyl-diaminopimelate desuccinylase (DapE) (EC:3.5.1.18), and diaminopimelate epimerase (DapF) (EC:5.1.1.7) to produce *m*-DAP from THDPA. The other two variant pathways bypass the acylated intermediates with either a *L,L*-diaminopimelate aminotransferase (DapL) (EC 2.6.1.83) in conjunction with DAP epimerase (DapF) (EC 5.1.1.7) or a *m*-DAP dehydrogenase (Ddh) (EC 1.4.1.16).

DapL was recently described in diverse bacterial species, the *Methanobacteriaceae* and plants [3–9]. This enzyme catalyzes 2-oxoglutarate dependent transamination of *L,L*-DAP, and in most cases shows strong specificity for the *L,L*-DAP isomer. Genomic data shows that where it exists, DapL almost always occurs as the sole pathway for *m*-DAP synthesis. There are no examples in the microbial genome database of DapL being coincident in the same species with one of the acyl-DAP pathways.

Ddh catalyzes the reversible, $NADP^+$-dependent, oxidative deamination of *m*-DAP. The enzyme was first discovered in *Bacillus sphaericus*, *Corynebacterium glutamicum*, and *Brevibacterium* sp. [10–14]. It is a dimer of approximately 70 kDa and shows stereo specificity for the *m*-DAP isomer. Ddh has weak sequence homology with other amino acid dehydrogenases. Based on structural similarity *C. glutamicum* Ddh resembles dihydrodipicolinate reductase (DapB) (EC:1.3.1.26), the enzyme catalyzing the preceding step in the DAP pathway, prompting Scapin et al. [15] to propose that DapB and Ddh evolved from a common ancestral enzyme.

Ddh has a restricted phylogenetic distribution. Until now it was thought to be relegated to the *Firmicutes* [16]. In some cases Ddh

Abbreviations: THDPA, tetrahydrodicolinate; DAP, diaminopimelate *m*- and *L,L*-isomers; Ddh, *m*-DAP dehydrogenase; DapL, *L,L*-diaminopimelate aminotransferase; DapD, THDPA acyltransferase

* Corresponding author at: Rutgers University, Biotech Center, 59 Dudley Road, New Brunswick, NJ 08901-8520, USA. Tel.: +1 732 932 8165x327; fax: +1 732 932 0312.
E-mail address: leustek@aesop.rutgers.edu (T. Leustek).
[1] Present address: Department of Human Molecular Genetics and Biochemistry, Sackler School of Medicine, Tel-Aviv University.
[2] Present address: Departamento de Genética, Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo.

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occurs as the only route for *m*-DAP biosynthesis. However, an example of pathway redundancy exists in *C. glutamicum*, which contains in addition to Ddh, the four enzymes of the acyl pathway [17]. Although the two pathways are redundant, Ddh is largely responsible for the high rate of lysine synthesis in this species [18].

In the course of their analysis of the phylogenomic distribution of DapL Hudson et al. [6] noted that *Clostridium thermocellum*, a Firmicute, contained a well conserved example of Ddh, suggesting that this species also has redundant pathways for DAP synthesis. Curiously, it was also noted that *Bacteroides fragilis* contained an ORF that might be a Ddh, but was significantly diverged from known examples of this enzyme, and moreover, this species does not belong to a phylum known to contain Ddh. Therefore, the aim of the present study was to determine whether the orthologs identified from *B. fragilis* and *C. thermocellum* are indeed functional examples of Ddh, and if so, to re-examine the phylogenomic distribution of this enzyme and to determine the frequency with which Ddh co-exists in the same species with a second pathway for DAP biosynthesis.

2. Experimental procedures

2.1. Bioinformatic methods

Orthologous protein sequences were identified by searching the microbial genome database at NCBI (http://www.ncbi.nlm.nih.gov/) with blastp using the default settings. Protein alignment was performed using ClustalW [19]. Phylogenetic trees were constructed with MEGA 4.1 using the default settings [20]. Transcriptome data from the Gene Expression Omnibus database at NCBI https://www.ncbi.nlm.nih.gov/geo/ was used to examine *dap* gene expression.

2.2. Orf cloning

The Ddh ortholog from *B. fragilis* NCTC9343 (Bf3481) and *C. thermocellum* ATCC27405 (Cthe_0922) was amplified in a 50 μL reaction. The reaction contained 12 pmol each of forward and reverse primers, 1 mM $MgSO_4$, 0.5 mM of each of the four deoxynucleotide triphosphates, 0.5 ng of genomic DNA and 1 unit of Platinum *Pfx* DNA polymerase (Invitrogen, #11708-013). PCR was run at 1 cycle at 94 °C, 2 min, followed by 36 cycles of 94 °C for 15 s, 60 °C for 30 s and 72 °C for 2 min. The primers used to amplify Bf3481 were GGGGGGATCC**ATG**AAAAAAGTAAGAGCAGCCATCGT and GGGGGTCGAC**TTA**TACCAGGTGGCCGATCCATTCTTCACG; Cthe_0922 CCCCGGATCC**ATG**GGAGGAGTTACTTTGGAAAAGATAAGGATAGGTATTGTGGG and CCCCGTCGAC**TAC**AATAAACTTCTCCTGAGTTCTTCGGCAGATTTGGGGGAAAG. The translation initiation and termination codons that define the ORFs are underlined. Genomic DNA from *B. fragilis* NCTC9343 was kindly provided by Dr. Sheila Patrick (Queen's University of Belfast) and *C. thermocellum* genomic DNA was purchased from ATCC (product #27405D). Amplified DNA fragments were digested with *BamH*I and *Sal*I and ligated into pET30a (Novagen, #69909-3) to produce the plasmids pET30a-BfDdh and pET30a-CtDdh. The recombinant proteins expressed from these plasmids contain at their amino terminus a hexahistidine and S-TAG epitope. The cloned PCR products were sequenced using the ABI prism 3100 genetic analyzer and were found to be identical to the accession sequences.

2.3. Functional complementation

Plasmids used for functional complementation of *E. coli dap* mutants were produced by subcloning the *Xba*I and *Sal*I fragment from pET30a-BfDdh or pET30a-CtDdh into pBAD33 [21] to produce pBAD33-BfDdh and pBAD33-CtDdh. The fusion proteins produced from the pBAD33 construct are identical to those produced from the pET30a constructs.

The *E. coli* Δ*dapD*/*dapE* double mutant (AOH1) [6] was transformed with either pBAD33, pBAD33-BfDdh or pBAD33-CtDdh. Colonies were selected on LB agar supplemented with 50 μg mL^{-1} DAP (Sigma-Aldrich # D-1377), 50 μg mL^{-1} kanamycin and 34 μg mL^{-1} chloramphenicol. Individual colonies were then replicated onto LB agar supplemented with 0.2% ($^{w}/_{v}$) arabinose, plus or minus DAP at 50 μg mL^{-1}. The cultures were grown at 30 °C for 24 h.

2.4. Protein expression and purification

The plasmids pET30a-BfDdh and pET30a-CtDdh were transformed into *E. coli* BL21-CodonPlus-RIPL strain (Stratagene, #230280). Protein expression was induced in cultures grown in LB broth containing 50 μg mL^{-1} kanamycin and 34 μg mL^{-1} chloramphenicol at 37 °C to an OD_{600nm} of 0.6 by addition of 0.5 mM IPTG for 4 h at 30 °C. The cells were harvested by centrifugation at 10,000 ×*g* at 4 °C and lysed by sonication in a solution of 50 mM sodium phosphate and 300 mM NaCl (pH 8.0). Sonication was performed with a Heat Systems, Inc. XL-2020 sonicator 5 min process time in pulses of 30 s sonication followed by 30 s cooling. The beaker containing the cell suspension was place in a beaker of slush ice for cooling. The soluble protein was collected and incubated with Talon Metal Affinity Resin (Clontech # 8901-2) for 30 min at 4 °C. The resin was washed 3 times with sonication buffer containing 10 mM imidazole (pH 8.0), and bound protein eluted using sonication buffer containing 300 mM imidazole (pH 8.0). The eluate was concentrated and the buffer exchanged using an Amicon Ultra 10,000 molecular weight cutoff filter device. Storage buffer contained 100 mM Tris–HCl (pH 8.0) containing 1 mM DTT, 1 mM EDTA and 30% ($^{v}/_{v}$) glycerol.

Preparation of *C. glutamicum* Ddh (CgDdh) was from plasmid pET28-CgDdh (provided by Dr. David Roper, University of Warwick) in *E. coli* strain BL21 (DE3) grown in LB broth with 50 μg mL^{-1} kanamycin at 37 °C to absorbance 0.6 at 600 nm. Expression was induced with 0.1 mM IPTG for 2 h at 25 °C. The cells were lysed as described earlier, but in 100 mM Tris–HCl (pH 8.0), 1 mM DTT, and 1 mM EDTA. The soluble protein was concentrated with an Amicon Ultra 10,000 filter and the buffer exchanged with 100 mM Tris–HCl (pH 8.0) containing 1 mM DTT, 1 mM EDTA and 30% ($^{v}/_{v}$) glycerol. Since Ddh comprised approximately 90% of the soluble protein fraction (data not shown) it was not further purified.

Preparation of *Arabidopsis thaliana L,L*-diaminopimelate aminotransferase (AtDapL) was performed as described in Hudson et al. (2006).

2.5. Estimation of molecular weight

The molecular weight of the pure recombinant Bf-Ddh was estimated using both SDS-PAGE and by size exclusion HPLC. For size exclusion HPLC, elution was performed with buffer containing 50 mM sodium phosphate (pH 6.5), 100 mM NaCl, and 0.01% ($^{w}/_{v}$) NaN_3. One hundred micrograms of pure recombinant protein was analyzed on a Phenomex Biosep SEC S2000 column. The elution profile was measured at 280 nm over a 15-minute period.

2.6. CD analysis

Spectra were collected between wavelengths of 200 and 240 nm in a Jasco J-715 CD spectrometer at 25 °C using a 1 mm path length quartz curvette, 1 nm step size, 1 nm bandwidth, and 2 s averaging time. Spectra for CtDdh and BfDdh (in 100 mM Tris–HCl, 1 mM EDTA, 1 mM DDT, pH 8.0) were recorded at a protein concentration of 16 μM and 14 μM, respectively. CD spectra were analyzed by non-linear least-squares regression using the CONTINLL algorithm and various reference databases available with the CDPro software package (available from http://lamar.colostate.edu/~sreeram/CDPro/main.html).