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Identification of Two Dihydrodipicolinate Synthase Isoforms from *Pseudomonas aeruginosa* that Differ in Allosteric Regulation

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Running title

Functional DHDPS enzymes in *Pseudomonas aeruginosa*

Abbreviations

ASA, (S)-aspartate semialdehyde; CD, circular dichroism; DAP, diaminopimelate; DAPDC, diaminopimelate decarboxylase; DHDPS, dihydrodipicolinate synthase; DHDPR, dihydrodipicolinate reductase; HTPA, 4-hydroxy-2,3,4,5-tetrahydro-(2S)-dipicolinic acid; L-lysine, lysine; NADPH, nicotinamide adenine dinucleotide phosphate; NSDAP, N-succinyl-L-L-2,6-diaminopimelate; NSKAP, N-succinyl-L-2-amino-6-ketopimelate; PEP, phosphoenolpyruvate; Pyr4H2C, 1-pyrroline-4-hydroxy-2-carboxylate; SDAP-DS succinyl-diaminopimelate desuccinylase; THDP, 2,3,4,5-tetrahydropicolinate; THPC-NST, 2,3,4,5-tetrahydropyridine-2-carboxylate N-succinyltransferase; NSDAP-AT, N-succinyldiaminopimelate aminotransferase.

Databases

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Allosteric regulation, antibiotic resistance, diaminopimelate, 4-hydroxy-tetrahydrodipicolinate synthase, lysine biosynthesis

Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article.

ABSTRACT

Pseudomonas aeruginosa is one of the leading causes of nosocomial infections, accounting for 10% of all hospital-acquired infections. Current antibiotics against *P. aeruginosa* are becoming increasingly ineffective due to the exponential rise in drug resistance. Thus, there is an urgent need to validate and characterise novel drug targets to guide the development of new classes of antibiotics against this pathogen. One such target is the diaminopimelate (DAP) pathway, which is responsible for the biosynthesis of bacterial cell wall and protein building blocks, namely meso-DAP and lysine. The rate limiting step of this pathway is catalysed by the enzyme dihydrodipicolinate synthase (DHDPS), typically encoded for in bacteria by a single *dapA* gene. Here, we show that *P. aeruginosa* encodes two functional DHDPS enzymes, PaDHDPS1 and PaDHDPS2. Although these isoforms have similar catalytic activities ($k_{\text{cat}} = 29 \text{ s}^{-1}$ and 44 s^{-1} for PaDHDPS1 and PaDHDPS2, respectively), they are differentially allosterically regulated by lysine, with only PaDHDPS2 showing inhibition by the end-product of the DAP pathway ($\text{IC}_{50} = 130 \text{ }\mu\text{M}$). The differences in allostery are attributed to a single amino acid difference in the allosteric binding pocket at position 56. This is the first example of a bacterium that contains multiple bona fide DHDPS enzymes, which differ in allosteric regulation. We speculate that the presence of the two isoforms allows an increase in the metabolic flux through the DAP pathway when required in this clinically important pathogen.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium that causes a wide range of acute and chronic infections. It commonly affects immunocompromised patients, including those with cystic fibrosis, cancer, AIDS and burn wounds [1–4]. *P. aeruginosa* infections have been frequently treated over the past 70 years using antibiotic classes, such as aminoglycosides, carbapenems and cephalosporins that target the synthesis of bacterial cell wall and proteins. However, these anti-pseudomonal agents are becoming increasingly ineffective, due to a combination of intrinsic antibiotic resistance (efflux pumps and membrane permeability) and acquisition of new traits, with up to one quarter of infections classified as extremely drug resistant [5–7]. Nevertheless, the effectiveness of these antibiotics, that have dominated the market since their discovery, provides proof of concept that targeting cell wall and amino acid biosynthesis is an excellent strategy for antibiotic development against *P. aeruginosa*.

A promising, but as yet unexploited, antibiotic target is the diaminopimelate (DAP) pathway, which yields essential metabolic building products, namely meso-DAP and lysine (Fig. 1). In Gram-negative bacteria, including *P. aeruginosa*, meso-DAP is found in the penta-peptide moieties used to crosslink peptidoglycan chains in the cell wall, whilst lysine is critical for protein synthesis [8–12]. Importantly, meso-DAP and lysine are only synthesised in bacteria and plants via the DAP pathway, and thus, chemical inhibition of this pathway is unlikely to result in toxicity to the human host [8,12,13]. The DAP pathway commences with the condensation of pyruvate and (S)-aspartate semialdehyde (ASA) to form 4-hydroxy-2,3,4,5-tetrahydro-(2S)-dipicolinic acid (HTPA) (Fig. 1). This first committed and rate-limiting step is catalysed by 4-hydroxy-tetrahydrodipicolinate synthase (**EC 4.3.3.7**), commonly referred to as dihydrodipicolinate synthase (DHDPS) [9,14]. HTPA is then reduced to 2,3,4,5-tetrahydrodipicolinate (THDP) in an NADPH-dependant reaction by 4-hydroxy-tetrahydrodipicolinate reductase (**EC 1.17.1.8**), commonly known as dihydrodipicolinate reductase (DHDPR) (Fig. 1) [12,13,15,16]. From this point on, the pathway diverges depending on the organism until meso-DAP is formed [12]. Gram-negative bacteria, including *P. aeruginosa*, are proposed to utilise the succinyl pathway to produce LL-DAP (Fig. 1) [12]. LL-DAP is converted to meso-DAP, which is then used in the cross-linking of the peptidoglycan layer [11,17,18]. Finally, meso-DAP is decarboxylated to L-lysine (referred to as lysine herein) by diaminopimelate decarboxylase (DAPDC, **EC 4.1.1.20**) (Fig. 1) [12,13,19]. Interestingly, the final product, lysine, can allosterically inhibit DHDPS from

some bacterial species via a negative feedback loop [20–22]. The current dogma suggests that the DHDPS residue in position 56 (*E. coli* DHDPS numbering) is a key molecular determinant for allosteric regulation [20], as the presence of a histidine or glutamate allows allosteric inhibition, whereas a lysine or arginine at this position results in no inhibition [20].

Gene knockout studies have shown either directly or indirectly that the *dapA* gene encoding DHDPS is essential in a number of bacterial species, including *Salmonella typhimurium* [23], *Bacillus subtilis* [24], *Escherichia coli* [25] and *Streptococcus pneumoniae* [26]. However, a recent study demonstrated that a *dapA* knockout in *P. aeruginosa* results in no change in bacterial counts or virulence [27]. Interestingly, unlike other bacterial species that encode for a single *dapA* gene, there are four annotated *dapA* genes in *P. aeruginosa* genomes. Thus far, only the *dapA2* gene product (**GenBank: PA1010**) from *P. aeruginosa* has been functionally and structurally characterised as a DHDPS enzyme, displaying similar catalytic properties to other bacterial orthologues [28]. Interestingly, despite *dapA3* (**GenBank: PA1254**) being annotated as a *dapA* gene, it has been shown to have 1-pyrroline-4-hydroxy-2-carboxylate (Pyr4H2C) deaminase (**EC 3.5.4.22**) activity [29]. It remains to be explored whether this gene product has Pyr4H2C deaminase/DHDPS dual activity. The proposed DHDPS activity for the products of *dapA1* (**GenBank: PALES_34361**) and *dapA4* (**GenBank: PA0223**) genes is also yet to be elucidated.

In this study, we set out to validate the existence of multiple isoforms of DHDPS from *P. aeruginosa* and compare the aforementioned four putative *dapA* genes and their products by employing a combination of bioinformatics, RT-PCR, circular dichroism spectroscopy, enzyme kinetics, complementation assays, allosteric inhibition assays, mutagenesis studies, X-ray crystallography and homology models. Firstly, we show that the presence and expression of the *dapA* genes vary between *P. aeruginosa* strains. Subsequently, we expressed and purified the 4 *dapA*-encoding recombinant proteins and showed that only the *dapA1* and *dapA2* gene products (referred to as PaDHDPS1 and PaDHDPS2, respectively) have bona fide DHDPS activity. Moreover, these isoforms display similar catalytic efficiencies but are differentially allosterically regulated by lysine, with only PaDHDPS2 subject to allosteric inhibition. We then employed site-directed mutagenesis, X-ray crystallography and homology models to further investigate the molecular determinants defining lysine-mediated allosteric inhibition. Thus, this work provides important insights

into the future design of *P. aeruginosa* DHDPS inhibitors and, importantly, the metabolic regulation of the DAP pathway in this pathogen.

RESULTS

Annotated dapA genes in *P. aeruginosa* genomes

To identify the genes encoding for DHDPS in *P. aeruginosa*, we searched for annotated dapA genes in the Pseudomonas Genome Database [30] and NCBI. The search yielded 4 putative dapA genes, of which dapA2 (**PA1010**), dapA3 (**PA1254**) and dapA4 (**PAO223**) were universally present. The dapA2 gene was found to be consistently located in the same operon in all annotated Pseudomonas species. In contrast, dapA1 (**PALES_34361**) was found in 144 of 335 (43%) complete Pseudomonas species genomes available through the Pseudomonas Genome Database (version 18.1; 2019-03-12; <http://www.pseudomonas.com/> [30]), and is widely distributed across species with 57 *P. aeruginosa* isolates and 83 isolates from other Pseudomonas species. A comparison between PAO1 and LESB58 showed the dapA1 gene is inserted as a 1180 base pair fragment, which contains a putative promoter and the gene itself. This fragment and the site of insertion is highly conserved with isolates of the Liverpool Epidemic Strain (LES), such as the 7 genome sequenced isolates found in cystic fibrosis (CF) patients in both the UK and Canada [31], and CF isolates including NCTC 13618 and NCTC 13620 [32]. This does not appear to be part of any of the known or predicted genomic islands or other mobile genetic elements within this epidemic clone [33]. The fragment also appears in isolates from other high-risk international clones, such as the colistin-resistant NDM-1 expressing ST654 isolate described previously [34]. It remains unclear whether this is conserved in all isolates of this sequence type. Next, we set out to assess whether the annotated dapA genes were indeed expressed in *P. aeruginosa* using RT-PCR. The two strains employed in this study were (i) PAO1, a standard lab strain that contains dapA2, dapA3 and dapA4, and (ii) 372261, a clinical strain that has the 4 dapA genes annotated. The RT-PCR analyses showed that all dapA genes present are transcribed in PAO1 during exponential phase, while only dapA1, dapA2 and dapA4 are expressed in 372261 (Fig. 2).

To assess whether the dapA gene products are likely to encode for functional DHDPS enzymes, sequence alignments were performed at the protein sequence level and compared to the sequence of *E. coli* (Ec) DHDPS (Fig. 3). The amino acid sequences of the putative DHDPS enzymes show a high degree of variation, with identity ranging from 19-32% (Fig. 3). Interestingly, only the dapA1 and dapA2 gene products were shown to have the 7 residues

that are critical for DHDPS catalytic function [35], with dapA3 and dapA4 gene-encoded proteins containing only 2 and 4 of these residues, respectively (Fig. 3). This suggests that only dapA1 and dapA2 encode for functional DHDPS enzymes. We therefore set out to express, purify and characterise these dapA gene products to validate their putative DHDPS function.

Expression, purification and characterisation of dapA gene products

The putative dapA genes were overexpressed in *E. coli* and purified to >98% homogeneity as described in the Methods (Fig. 4A). The secondary structure of the recombinant proteins was assessed using circular dichroism (CD) spectroscopy. The CD spectra show a broad minimum spanning approximately 208 to 225 nm, which is consistent with the α/β secondary motif reported for the previously characterised *P. aeruginosa* DHDPS enzyme (i.e., the dapA2 gene product), as well as other bacterial orthologues (Fig. 4B) [28, 36-38]. Having confirmed the recombinant proteins were folded, they were next assessed for DHDPS activity using the qualitative o-aminobenzaldehyde assay, which measures activity based upon the production of a purple chromophore [20,39,40]. Both dapA1 and dapA2 gene products are shown to produce a purple colour relative to the positive control EcDHDPS, while the dapA3 and dapA4 gene products result in no discernible colour change (Fig. 5A). To further validate this, the DHDPS activity of all four gene products was quantitatively assessed using the DHDPS-DHDPR coupled assay [38,41,42]. The dapA1 and dapA2 gene products are shown to have comparable activity to EcDHDPS, whilst the dapA3 and dapA4 gene products have no detectable activity (Fig. 5B).

To confirm that neither the dapA3 or dapA4 gene products could complement DHDPS activity *in vivo*, a complementation assay was performed using the AT997r⁻ *E. coli* strain that lacks a functional dapA gene [43-45]. The pET28a vectors containing the dapA1-4 genes were transformed into the AT997r⁻ strain and expression was induced using IPTG. As expected, all cells grew on the DAP supplemented media (Fig. 6A), while only the cells transformed with the pET28a-dapA1 or pET28a-dapA2 plasmids were able to grow without DAP (Fig. 6B). This demonstrates that only the dapA1 and dapA2 genes encode for functional DHDPS enzymes - PaDHDPS1 and PaDHDPS2.

Catalytic efficiency of PaDHDPS1 and PaDHDPS2

Having determined that the recombinant PaDHDPS1 and PaDHDPS2 enzymes were homogenous, folded and catalytically active, we set out to determine their enzyme kinetic parameters using the DHDPS-DHDPR coupled assay [38,41,46]. Initial rates were monitored whilst varying the concentrations of the DHDPS substrates, pyruvate and ASA (Fig. 7). The resulting Michaelis-Menten curves were analysed globally to a bi-substrate ping-pong model without substrate inhibition to yield the kinetic parameters summarised in Table 1. The K_M values for PaDHDPS1 were 230 μM for pyruvate and 120 μM for ASA, whilst for PaDHDPS2 the K_M values were 150 μM and 53 μM , respectively (Table 1). The turnover numbers for the two isoforms were comparable, with PaDHDPS1 having a k_{cat} of 29 s^{-1} compared to 44 s^{-1} for PaDHDPS2 (Table 1). Interestingly, the enzyme kinetic data reported here for PaDHDPS2 is significantly different to an earlier report that employed pseudokinetic analyses, where only one substrate was titrated at a time (Table 1) [28].

Allosteric inhibition by lysine

In order to determine whether PaDHDPS1 and PaDHDPS2 are allosterically inhibited by lysine, the end product of the DAP pathway (Fig. 1), the DHDPS-DHDPR coupled assay was performed in the presence of increasing lysine concentrations. We show for the first time that PaDHDPS2 is inhibited by lysine with an IC_{50} value of 130 μM (Fig. 8), consistent with other bacterial DHDPS enzymes [20,26,39]. On the other hand, PaDHDPS1 is shown to be insensitive to lysine even up to 2 mM (Fig. 8).

To probe the molecular determinants for this differential allosteric regulation, we compared the amino acid sequences of PaDHDPS1 and PaDHDPS2 with other bacterial DHDPS enzymes. This revealed that a histidine at position 56 (*E. coli* DHDPS numbering), which has been shown to be the key molecular determinant for lysine inhibition [20], is found in PaDHDPS2 but not PaDHDPS1 (Fig. 9A). Interestingly, PaDHDPS1 has a glutamine at the equivalent position, which has not been observed in previously characterised DHDPS enzymes (Fig. 9A). Other known lysine insensitive DHDPS enzymes, including *Legionella pneumophila* and *Coxiella burnetii* [20], contain a lysine and arginine at the equivalent position, respectively (Fig. 9A). To confirm the effect of these residues at position 56 in lysine-mediated allosteric inhibition, three mutants were generated; PaDHDPS2-H56Q, PaDHDPS2-H56K and PaDHDPS2-H56R. As predicted, mutant enzymes lacking a histidine at position 56 are insensitive to lysine even at 2 mM concentration (Fig. 9B). Moreover, to

examine why the presence of these residues at position 56 result in lysine insensitivity in DHDPS enzymes, we determined the crystal structure of PaDHDPS2-H56Q to 1.9 Å resolution (**PDB ID: 6P90**, Fig. 10A). The data collection, processing and scaling are presented in Table 2, and refinement statistics in Table 3. An overlay with the lysine-bound PaDHDPS2 structure (**PDB ID: 3PUO**) demonstrates that a glutamine at this position causes steric hindrance with a lysine ligand in the allosteric site (Fig. 10B). Furthermore, homology models of PaDHDPS2-H56K (Fig. 11A) and PaDHDPS2-H56R (Fig. 11B) generated using SWISS-MODEL predict that a lysine or arginine at this position adopts a similar conformation to glutamine, also resulting in steric clash.

DISCUSSION

Multiple DHDPS isoforms have been commonly observed in plants [47-49], and the retention of the genes have been attributed to a beneficial increase in metabolic flux [50]. However, bacteria typically contain only a single *dapA* gene. Even when multiple *dapA* genes have been annotated in a bacterium, such as in *Agrobacterium tumefaciens*, only one *dapA* gene has been shown to have bona fide DHDPS activity [36]. In this study, we identified and characterised the 4 putative *dapA* genes encoding DHDPS proteins in *P. aeruginosa*.

Alignments of multiple *P. aeruginosa* strains demonstrate that *dapA2*, *dapA3* and *dapA4* are universally present, whereas *dapA1* is annotated in only 47% of strains. Moreover, the fragment containing the *dapA2* gene appears in isolates from high-risk international clones. However, the significance of this additional copy of the gene in these epidemic strains is unknown. Employing DHDPS catalytic activity and complementation assays, it was revealed that only *dapA1* and *dapA2* encode functional DHDPS enzymes. These enzymes have similar catalytic efficiencies, which are consistent with other DHDPS orthologues [16,36,51,52]. Thus, *P. aeruginosa* is the first reported bacterium to have two functional DHDPS enzymes.

Interestingly, PaDHDPS1 was shown in this study to be insensitive to allosteric regulation, whilst PaDHDPS2 displays mid-micromolar inhibition by lysine. Mutagenesis experiments confirmed that the differential regulation can be attributed to a single residue at position 56 (*E. coli* DHDPS numbering), which has been shown to be critical for allosteric inhibition [20]. Specifically, mutation of histidine 56 to a glutamine in PaDHDPS2, the equivalent residue in PaDHDPS1, results in the abolishment of lysine inhibition. The presence of a glutamine at this position has never been reported in other bacterial or plant DHDPS

enzymes, and the crystal structure of PaDHDPS2-H56Q demonstrates that steric hindrance prevents lysine binding into the allosteric cleft. Furthermore, mutation of histidine 56 in PaDHDPS2 to a lysine or arginine, which are residues found in other lysine insensitive DHDPS orthologues, also abolish lysine inhibition. These findings further support the current dogma regarding the importance of position 56 as an allosteric determinant in bacterial DHDPS enzymes [20], but also expands it by adding glutamine to the list of residues that prevent allosteric inhibition. The allosteric site of DHDPS enzymes has recently been targeted for the development of new antibacterial agents with the discovery of a bis-lysine compound that inhibits *Campylobacter jejuni* DHDPS in vitro [53]. The presence of DHDPS enzymes that are not inhibited by lysine, such as PaDHDPS1, indicates that this approach cannot be employed to develop broad spectrum bacterial DHDPS inhibitors.

Importantly, we speculate that the presence of two differentially regulated DHDPS enzymes in *P. aeruginosa* allows a means to control the flux of pyruvate and ASA into the DAP pathway as observed in plants [48,49,54]. Pyruvate and ASA are used in multiple other biosynthetic pathways, including alanine and lactate synthesis [55-57], as well as threonine, isoleucine and methionine synthesis [58-60]. Limiting ASA and pyruvate entry into the DAP pathway ensures substrate availability for other pathways. This principle is supported by previous studies showing that single *dapA* gene knockouts of the diploid plant, *Arabidopsis thaliana*, result in increased levels of both aspartate and pyruvate derived amino acids [48,49,54]. Allosteric regulation is also hypothesised to affect the metabolic flux of other biosynthetic pathways in *P. aeruginosa* that have multiple genes encoding for each enzyme, including the shikimate pathway responsible for the synthesis of aromatic amino acids [61]. The fitness advantages associated with having two differentially regulated DHDPS enzymes in this important pathogen remains to be elucidated.

METHODS

Sequence Analysis

Sequence alignments were performed using the ClustalW algorithm [62] incorporated in BIOEDIT (v7.2.0). Protein sequences were obtained from UniProt for *E. coli* DHDPS (UNIPROT ID: P0A6L2), *L. pneumophila* DHDPS (UNIPROT ID: Q5ZT5A) and *C. burnetii* DHDPS (UNIPROT ID: Q83CA6). All alignments used *E. coli* residue numbering. Putative annotated *dapA* genes were identified in the genome for PAO1 (*dapA*1, 3 and 4)

with an additional *dapA* gene (*dapA2*) identified in the annotation of strain LESB58. The distribution of the genes was assessed using BLASTN searches of the complete *Pseudomonas* species genomes available on the *Pseudomonas* Genome Database (<https://www.pseudomonas.com/search/sequences>; DB version 18.1; searched 7 June 2019). The genome context was explored by aligning the sequences of LESB58 flanking the *dapA2* gene with PAO1 as a reference and with other genomes, using MegAlign (DNASTAR Lasergene v14).

Protein Expression and Purification

Recombinant proteins were expressed in *E. coli* BL21 (DE3) cells as hexa-histidine-tagged constructs and purified using immobilised metal affinity chromatography (IMAC) as previously described [16,63,64]. In brief, the synthetic genes coding for the *P. aeruginosa* proteins and the PaDHDPS2 mutants H56Q and H56K were purchased from Bioneer (Bioneer Pacific, Kew East, Victoria, Australia) ligated in the pET28a expression vector. The PaDHDPS2-H56R mutant was generated using QuikChange mutagenesis (Stratagene) as per manufacturer's protocol. Primers are listed in Table 4. Recombinant proteins were produced in *E. coli* BL21 (DE3) cells upon treatment with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) in Luria-Bertani (LB) broth at 16 °C for 18 hrs for the *P. aeruginosa* proteins or 37 °C for 4 hrs for the *E. coli* proteins. Cells were harvested by centrifugation at $5,000 \times g$ at 4 °C and sonicated in 20 mM Tris, 150 mM NaCl, 20 mM imidazole, pH 8.0 using a Vibra Cell VC40 (Sonics & Materials, Newtown, Connecticut, USA). Recombinant His-tagged proteins were purified using a 5 mL IMAC column (GE Healthcare, Silverwater, NSW, Australia) and stored in 20 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 8.0 or 20 mM Tris, 150 mM NaCl, pH 8.0 for EcDHDPS and EcDHDPR.

Circular Dichroism (CD) Spectroscopy

CD spectroscopy was performed using 1 mm quartz cuvettes in the Aviv Model 420 CD spectrometer as previously described [20,35,42]. Protein samples were prepared in 20 mM NaH_2PO_4 , 50 mM KF, pH 8.0 at $150\text{--}200 \mu\text{g}\cdot\text{mL}^{-1}$. Wavelength scans were conducted between 190 and 260 nm in 1 nm increments with 4 s averaging time at a temperature of 20 °C. Data were fitted using the CDPro software employing the CONTINLL algorithm and SP22X database [65,66].

RT-PCR

P. aeruginosa strains PAO1 and 372261 were grown in LB broth to an optical density at 600 nm (OD_{600}) of 0.5 before harvesting via centrifugation at $3,000 \times g$ for 10 min followed by lysis using sonication. RNA was isolated using the PureLink RNA Mini Kit (Thermo Fisher Scientific Australia, Scoresby, Victoria, Australia) as per manufacturer's protocol. An iScript reverse transcription supermix was used to create a cDNA library using 500 ng of RNA per reaction. PCR was then performed using a Thermo Fisher Scientific PCR master mix following the manufacturer's protocol. Briefly, each reaction contained ~120 ng cDNA template and 0.4 μ M primer DNA. Primers were designed to amplify specific 100 bp regions in each *dapA* gene. Primers are listed in Table 5. PCR conditions involved 35 cycles of the following: 95 °C for 5 min, 95 °C for 15 sec, 58 °C for 20 sec, 72 °C for 90 sec and a final extension step of 72 °C for 5 min. PCR products were run on a 1% (w/v) agarose gel for 45 min at 90 V using GelGreen nucleic acid stain (Biotium, Hayward, California, USA).

o-Aminobenzaldehyde Assay

The colourimetric o-aminobenzaldehyde (o-ABA) assay was used to qualitatively assess DHDPS activity [20,40]. Assays were performed in 96 well plates with the addition of 100 μ L of mastermix containing 200 mM Tris pH 6.8, 30 mM pyruvate, 10 mM o-ABA and 5 mM ASA and reactions initiated with 5 μ L of 0.5 mg·mL⁻¹ of protein sample. Reactions were incubated for 30 min at 37 °C before they were terminated with 10 μ L of 10% (v/v) trichloroacetic acid. Recombinant *E. coli* DHDPS was used as a positive control and *E. coli* DHDPR as a negative control. DHDPS enzyme activity was confirmed by observation of a purple chromophore, whilst absence of colour indicated no DHDPS activity.

Enzyme Kinetics

DHDPS enzyme activity was quantified using the DHDPS-DHDPR coupled assay [37,38,41] that measures substrate turnover spectrophotometrically at $Abs_{340\text{ nm}}$ ($\epsilon_{340\text{ nm}} = 6220\text{ M}^{-1}\text{ cm}^{-1}$) via the associated oxidation of NADPH as previously reported. Assays were performed in triplicate ($n = 3$) at 37 °C in a temperature-controlled Cary 4000 UV/Vis spectrophotometer (Varian, Mulgrave, Victoria, Australia). All reactions were incubated at 37 °C for 12 min before initiating the reaction by the addition of ASA.

Preliminary DHDPS enzyme activity determination of the *dapA1-4* gene products was performed using excess amounts of pyruvate (4 mM) and ASA (1.25 mM) with a final protein concentration of 6.5 $\mu\text{g}\cdot\text{mL}^{-1}$ and 4.1 $\mu\text{g}\cdot\text{mL}^{-1}$ for EcDHDPS as previously reported [39]. For the determination of the Michaelis-Menten constant (K_M) determination, pyruvate and ASA were varied simultaneously ranging from 0.125 – 4 mM and 0.05 – 1 mM, respectively. PaDHDPS1 and PaDHDPS2 were used at a final concentration of 5 $\mu\text{g}\cdot\text{mL}^{-1}$. Initial velocity data were analysed using ENZFITTER (Biosoft, Cambridge, UK) and fitted to the ping-pong mechanism using Equation 1.

Equation 1:

$$v = (V_{\max} * A * B) / (K_{M,A} * B + K_{M,B} * A + A * B)$$

Where v = initial velocity, $V_{\max}$ = limiting maximal velocity, A = [pyruvate], B = [ASA], $K_{M,A}$ = limiting Michaelis-Menten constant for A, $K_{M,B}$ = limiting Michaelis-Menten constant for B.

Lysine inhibition was measured by titrating concentrations of lysine ranging from 0.078 – 2 mM against PaDHDPS1, PaDHDPS2, PaDHDPS2-H56Q, PaDHDPS2-H56K and PaDHDPS2-H56R. Initial velocity data for PaDHDPS2 were analysed using GraphPad Prism and fitted to the log(inhibitor) vs response (variable slope) using Equation 2, while PaDHDPS1 data was fitted using liner regression. Data were plotted using GraphPad Prism version 7.03.

Equation 2:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogIC}_{50} - X) * \text{HillSlope})})$$

Where X = log of lysine concentration, Y = response, Top and Bottom = Plateaus in same units as Y , Hill slope = slope factor.

Complementation Assay

The pET28a vector containing each of the 4 *dapA* genes and an empty vector were transformed into the *E. coli* *dapA* knockout strain AT997r⁻ [43-45,51] for the complementation assay as previously described [35]. The transformed cells were grown at 37 °C in LB supplemented with 0.5 mM DAP and 50 $\text{mg}\cdot\text{mL}^{-1}$ kanamycin until an OD_{600} of 0.6 was attained. At this point, the cells were harvested by centrifugation ($3,000 \times g$, 10 min, 4

°C) before resuspension in either LB with 0.5 mM DAP or 0.5 mM IPTG to induce protein expression. After incubation at 37 °C for 15 min, 20 µL of the culture was plated onto LB agar containing 50 mg·mL⁻¹ kanamycin with or without 0.5 mM DAP before overnight incubation.

X-Ray Crystallography

PaDHDPS2-H56Q was crystallised using the hanging-drop vapour diffusion method in Hampton 24-well crystallisation plates. Diffraction-quality crystals of PaDHDPS2-H56Q grew after 7 days in drops consisting of equal volumes (1 µL) of PaDHDPS2-H56Q (12 mg·mL⁻¹ in 20 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 8.0) and reservoir (100 mM Tris pH 8.5, 200 mM MgCl₂, PEG 8000) solutions equilibrated against 500 µL of reservoir solution. Crystals were soaked in reservoir solution containing 20% (v/v) glycerol prior to data collection. X-ray diffraction experiments were performed at the micro-focus beamline (MX2) of the Australian Synchrotron [67]. The crystals were then flash-cooled in liquid nitrogen and transferred to a stream of nitrogen gas at 100K. X-ray data were collected at a wavelength of 0.9537 Å at 100K using an EIGER-16M detector with 0.1° oscillation and 0.1 s exposure of 0% attenuated beam per frame. The data were indexed and integrated with XDS [68] and scaled using AIMLESS [69]. Data collection and processing statistics are detailed in Table 2. The crystal belongs to space group P2₁. Molecular replacement was performed using the MR phasing protocol of the Auto-Rickshaw software [70-71] with wild-type PaDHDPS2 employed as the search model (**PDB ID: 3QZE**). The resulting model from Auto-Rickshaw was refined using REFMAC5 [72] and manual building was carried out in Coot [73]. The quality of the structure was determined by MolProbability [74]. The refinement statistics are summarised in Table 3. Images were generated using PYMOL version 1.3.

Homology Modelling

Homology models of PaDHDPS2-H56K and PaDHDPS2-H56R were generated using SWISS-MODEL [75-77] employing the highest resolution PaDHDPS2 structure (**PDB ID: 3QZE**) as a template. The lowest energy rotamer for the residue at position 56 are shown aligned to the lysine-bound PaDHDPS2 structure (**PDB ID: 3PUO**) using PYMOL version 1.3.

AUTHOR CONTRIBUTIONS

T.P.S.C., M.A.P., R.E.I., L.J.B. and J.M.S. planned the experiments; R.E.I., S.P. and C.J.H. performed the experiments; T.P.S.C., M.A.P., R.E.I., S.P. and L.J.B. analysed the data; T.P.S.C., M.A.P., J.M.S. and R.E.I. wrote the paper.

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TABLES

Table 1. Kinetic parameters for PaDHDPS1 and PaDHDPS2 obtained in this study compared to parameters obtained for PaDHDPS2 in Kaur et al (2011). Data shown as mean $\pm$ standard deviation (n = 3).

Kinetic Parameter	PaDHDPS1	PaDHDPS2	PaDHDPS2*
K_M Pyruvate (μM)	230 (± 15)	120 (± 8.0)	900 (± 13)
K_M ASA (μM)	150 (± 9.0)	53 (± 3.0)	170 (± 20)
k_{cat} (s^{-1})	44 (± 1.0)	29 (± 0.50)	NR
k_{cat}/K_M Pyruvate ($\text{s}^{-1}\text{M}^{-1}$)	1.9×10^5	2.4×10^5	NR
k_{cat}/K_M ASA ($\text{s}^{-1}\text{M}^{-1}$)	2.9×10^5	5.5×10^5	NR

*Data obtained from [24], NR = not reported.

Table 2. Data collection and processing statistics for PaDHDPS2-H56Q.

Diffraction source	MX2, Australian Synchrotron
Wavelength (Å)	0.953654
Temperature (K)	100
Detector	EIGER-16M
Crystal-to-detector distance (mm)	150
Total rotation range (°)	180
Space group	P2 ₁
a, b, c (Å)	43.2, 123.3, 57.4
α , β , γ (°)	90.0, 107.6, 90.0
Mosaicity (°)	0.23
Resolution range (Å)	20–1.9 (1.94–1.90)
Total no. of reflections	282620 (18555)
No. of unique reflections	44202 (2979)
Completeness (%)	98.8 (98.5)
Multiplicity	6.4 (6.2)
$\langle I/\sigma(I) \rangle$	8.9 (2.6)
CC _{1/2}	0.994 (0.913)
R _{merge}	0.121 (0.448)
Overall B factor from Wilson plot (Å ²)	31.0

Table 3. Refinement statistics for PaDHDPS2-H56Q.

Resolution range (Å)	20–1.9
No. of reflections, working set	43257
No. of reflections, test set	945
Final R_{cryst}	0.186
Final R_{free}	0.219
No. of non-H atoms	
Protein	4408
Glycerol	12
Chloride	2
Water	302
Total	4726
R.m.s. deviations	
Bonds (Å)	0.009
Angles (°)	1.644
Average B factors (Å ²)	
Protein	31.084
Glycerol	41.557
Chloride	40.440
Water	35.396
Ramachandran plot	
Most favoured (%)	98.0
Allowed (%)	2.0
PDB code	6P90

Table 4. Mutagenesis primers employed to generate PaDHDPS2-H56R.

Primer Sequence (5' - 3')	
Forward	GTTAGATGTTGAAGAACGTATTCAGGTTATACGCC
Reverse	GGCGTATAACCTGAATACGTTCTTCAACATCTAAC

Table 5. Primers used for RT-PCR experiments.

Primer Target		Primer Sequence (5' - 3')
dapA1	Forward	TACTACATCCGCCCCCTCCC
	Reverse	CACCGGTGCGATAGGGAATG
dapA2	Forward	CGATCCCGCAGATCCTCTAC
	Reverse	ATGATGTTTCGGCACCTTGGA
dapA3	Forward	TGCCGGTGATGGTCTACAAC
	Reverse	AAGCAGACGATGTTCTCGCA
dapA4	Forward	CCCGGGTTGTTGTAGAGCAT
	Reverse	GCCGATCTCCTACTGGAAGC

FIGURE LEGENDS

Figure 1. Diaminopimelate (DAP) pathway in Gram-negative bacteria. Dihydrodipicolinate synthase (DHDPS) catalyses the first step in the pathway, involving the condensation of pyruvate and (S)-aspartate semialdehyde (ASA) to the product 4-hydroxy-2,3,4,5-tetrahydro-(2S)-dipicolinic acid (HTPA). HTPA is subsequently converted to 2,3,4,5-tetrahydrodipicolinate (THDP) by dihydrodipicolinate reductase (DHDPR) in an NADPH-dependent reaction. THDP undergoes several enzymatic steps in the succinyl sub-pathway, before meso-DAP is formed, which can be utilised for cell wall synthesis or be further decarboxylated to lysine for protein synthesis. The pathway can be allosterically regulated by lysine via the inhibition of DHDPS (dashed line).

Figure 2. RT-PCR analyses showing the expression of *dapA2*, *dapA3* and *dapA4* in PAO1 (A), and *dapA1*, *dapA2* and *dapA4* in 372261 (B).

Figure 3. Multiple sequence alignment of the four annotated *dapA* gene products from *P. aeruginosa* (DapA1 UNIPROT ID: A0A3G5V4H5, DapA2 UNIPROT ID: Q9I4W3, DapA3 UNIPROT ID: Q9I490, DapA4 UNIPROT ID: Q9I6R5) compared to the single *dapA* gene product from *E. coli* (UNIPROT ID: P0A6L2). Identical residues are shown by a dot (.), while gaps are shown by a dash (-). Active site residues shown to be important for DHDPS function are denoted with asterisks and conserved residues highlighted by grey boxes. Sequence alignment was performed using the ClustalW algorithm incorporated in BIOEDIT (v7.2.0).

Figure 4. (A) SDS-PAGE summary showing the pure recombinant *P. aeruginosa* *dapA1*, *dapA2*, *dapA3* and *dapA4* gene products (lanes 2, 3, 4 and 5, respectively) with the expected molecular weights between 35-40 kDa as shown by the Broad Range Molecular Weight (10-200 kDa) ladder (lane 1). (B) Circular dichroism spectroscopy analyses of recombinant *dapA1* (●), *dapA2* (■), *dapA3* (▲) and *dapA4* (▼) encoding proteins, with the mean residue ellipticity ($M^{-1} cm^{-1}$) shown as a function of wavelength (nm). The data was fitted using the CONTINILL database using the SP22x reference set (line).

Figure 5. (A) Qualitative o-aminobenzaldehyde assay of the *P. aeruginosa* proteins. Purple colour indicates DHDPS activity. (I) *E. coli* DHDPS positive control; (II) *E. coli* DHDPR

negative control; (III) *P. aeruginosa* dapA1 gene product; (IV) *P. aeruginosa* dapA2 gene product; (V) *P. aeruginosa* dapA3 gene product; (VI) *P. aeruginosa* dapA4 gene product. **(B)** The DHDPS-DHDPR coupled assay was used to quantitatively assess DHDPS catalytic activity for the putative dapA gene products from *P. aeruginosa* compared to the EcDHDPS control (Ec-dapA).

Figure 6. Complementation assay performed with the DHDPS deficient AT997r- *E. coli* cells plated on $50 \mu\text{g}\cdot\text{mL}^{-1}$ kanamycin with 0.5 mM DAP **(A)** or without DAP **(B)**. Each sector contains At997r⁻ cells transformed with either (1) pET28a-dapA1, (2) pET28a-dapA2, (3) pET28a-dapA3, (4) pET28a-dapA4 or (5) no insert. (6) represents an empty sector (no bacteria).

Figure 7. Enzyme kinetic profiles of recombinant PaDHDPS1 and PaDHDPS2. The initial velocity is plotted for PaDHDPS1 **(A)** and PaDHDPS2 **(B)** at varying pyruvate concentrations of 4 mM ($\square$), 2 mM ($\diamond$), 1 mM (∇), 0.5 mM (Δ), 0.25 mM ($\square$), 0.125 mM ($\circ$). The initial velocity is plotted for PaDHDPS1 **(C)** and PaDHDPS2 **(D)** at varying ASA concentrations of 1.0 mM ($\square$), 0.8 mM (∇), 0.4 mM ($\diamond$), 0.2 mM (Δ), 0.1 mM ($\blacksquare$) and 0.05 mM ($\bullet$). The global nonlinear best-fit using the ENZFITTER software v2.0.18 (Biosoft) were fitted to a bi-substrate ping-pong model without substrate inhibition and resulted in R^2 values of 0.98 and 0.97 for PaDHDPS1 and PaDHDPS2, respectively. Data are represented as mean $\pm$ standard deviation ($n = 3$).

Figure 8. Inhibition curves of DHDPS enzymes in the presence of lysine. Activity remains constant for PaDHDPS1 (purple) in the presence of increasing lysine concentrations. This is in contrast to PaDHDPS2 (green), which displays an IC_{50} of 130 μM . Data are represented as mean and $\pm$ standard deviation ($n = 3$).

Figure 9. **(A)** Multiple sequence alignment of allosteric site residues in PaDHDPS1 (UNIPROT ID: A0A3G5V4H5), PaDHDPS2 (UNIPROT ID: Q9I4W3), EcDHDPS (UNIPROT ID: P0A6L2), *Legionella pneumophila* (Lp) DHDPS (UNIPROT ID: Q5ZT5A) and *Coxiella burnetii* (Cb) DHDPS (UNIPROT ID: Q83CA6), with enzymes known to be inhibited by lysine underlined. The residue at position 56 (*E. coli* numbering) is highlighted by the grey box. Sequence alignment was performed using the ClustalW

algorithm incorporated in BIOEDIT (v7.2.0). **(B)** Plot of activity remaining for PaDHDPS1 (purple), PaDHDPS2 (green), PaDHDPS2-H56Q (blue), PaDHDPS2-H56K (red) and PaDHDPS2-H56R (yellow) in the presence of 2 mM lysine. Data are represented as mean $\pm$ standard deviation (n = 3).

Figure 10. **(A)** Electron density at position 56 in the PaDHDPS-H56Q crystal structure (**PDB ID: 6P90**). **(B)** Overlay of the residue at position 56 in lysine-bound PaDHDPS2 (**PDB ID: 3PUO**, green) and the crystal structure of PaDHDPS-H56Q (**PDB ID: 6P90**, blue). Images were generated using PYMOL v1.3.

Figure 11. Overlay of the residue at position 56 in lysine-bound PaDHDPS2 (**PDB ID: 3PUO**, green) and the homology models for **(A)** PaDHDPS2-H56K (red) and **(B)** PaDHDPS2-H56R (yellow). Images were generated using PYMOL v1.3.

FIGURES

FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5

FIGURE 6

FIGURE 7

FIGURE 8

FIGURE 9

FIGURE 10

FIGURE 11

0 10 20 30 40 ** 50 60 70 80

EcDHDPS -----MFTGSIVAIVTPMDEKGNVCRASLKKLIDYHVASGTSIAIVSVGTTGESATLNHDEHADVMMTLELADGRIPVIAGTGANATAEA
DapA1 ---MSS.Q.IW.PL...FR-H.EIDF.A.PR.L.HLLNA.VAGV.VC...A.A.E.G.QLQLLDAV..RVAPER-.AM.LAG.NQ.QL
DapA2 ---.IA..M..L...F.AQ.RLDWD..A..V.F.LQE..N...A...DVE..IQ.IRRVVDQVK.....S.R..
DapA3 MSKHVNW.S.VFP.VT.QFNADF.S.NLEETHV.SNL.RD.V.GL.VC.SV..NTS.TIE.KMA.TEVAKDASG..V...C.IAEFTS.G.
DapA4 ---MSASIH.I.GYTI..FAAD.GLDLPA.GRS.ERLIDG.VH..APL.S...G.Y.SDP.WDE..DF..KTVAH.V.T.VSVSDLT..KT

90 100 ** 110 120 130 * 140 150 160* 170

EcDHDPS I.SLTQRFNDSGIVGCLTVTPYNNRPSQEGLYQHFKAIAEHTDLPQILYNVPSRTGCDLLPETVGRLA-KVKNIIGIKEATGNLTRVNQIK
DapA1 LAFQREVLERP.A.L.VPA...I....A.VRF.HGV.DASSV.L.V.DI.Y...VRMEL..LR.IV-DHPR.AA..DCG.S.ENT--LA
DapA2 VA..EAAKSG.ADA..L.....K.T...M...RH...AVAI.....G..S..M.....E..S...P.....D.Q.AKEVI
DapA3 AKVAKAVERV.VD.IMVMPALVYSSKPHETA.E..RSV.SG...VMV..N.PIYRN.VT.DILVS..-DCE..VCF.DSS.DTR.FIDVR
DapA4 VRR.A.FAESL.AEAVMVLPI.S.WKLNEAEVF..YR.VG.AIGV.VM...N.GTS.I.MSV.LIL.IVRE.D.VTMV..S..DIQ.MHKLR

180 190 200 210 220 230 240 250 260

EcDHDPS ELVSDDFVLLSGDDASALDFMQLGGHGVISVTANVAAHDMAQMCKLAAEGHFAEARVINQRLMPLHNKLFVEPNPIPVKWACKELGLVAT
DapA1 L.ADGQVAV.A.E.LQMF-----
DapA2 .R.GK...LVY.....T.VEL.L...K.N.....PRA.SDL.AA.MR.DA.A..A..D.....KA..I.S.....LH.M..IP-
DapA3 NQ.GER.I.FA.L.DVV.ESVAV.AQ.W..GMS..FPKEGETIFR..RA.R...MP.YEW...ILH-.DAR.DLVQCIKL.E..AGRGS
DapA4 L.GEGRVPFYN.CNPL..EAFVA.AK.WC.AAP.LIPTLNG.LYQAVLD.DLEK..ALFY.QL..LDFILRRGL.TTI.AGLG-.SGLEV

270 280 290 300

EcDHDPS DTLRLPMTPTDSGRETVRAALKHAGLL-----
DapA1 -----
DapA2 EGI...L.WLSPRCH.PL.Q.MRQT.V.A-----
DapA3 ALT.P.RLALRGAD..H.ERIMAK.LASRPALPDVGL
DapA4 GAP...VQALDTE.CRYLQGL.EELR-----

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A **B**

A **B**

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A

B

C

D

A

	50		60
			
<u>EcDHDPS</u>	TLNHDE	HADV	V
<u>PaDHDPS2</u>	. . DVE . .	IQ . I	
PaDHDPS1	A . E . G .	QLQLL	
LpDHDPS	. . S . S .	KLL . I	
CbDHDPS	. ITAE .	RHKII	

B

A **B**

A **B**

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