Revealing a New Family of D-2-Hydroxyglutarate Dehydrogenases in Escherichia coli and Pantoea ananatis Encoded by ydiJ

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Abstract: In E. coli and P. ananatis, L-serine biosynthesis is initiated by the action of D-3-phosphoglycerate dehydrogenase (SerA), which converts D-3-phosphoglycerate into 3-phosphohydroxypyruvate. SerA can concomitantly catalyze the production of D-2-hydroxyglutarate (D-2-HGA) from 2-ketoglutarate by oxidizing NADH to NAD+. Several bacterial D-2-hydroxyglutarate dehydrogenases (D2HGDHs) have recently been identified, which convert D-2-HGA back to 2-ketoglutarate. However, knowledge about the enzymes that can metabolize D-2-HGA is lacking in bacteria belonging to the Enterobacteriaceae family. We found that ydiJ encodes novel D2HGDHs in P. ananatis and E. coli, which were assigned as D2HGDHPa and D2HGDHEc, respectively. Inactivation of ydiJ in P. ananatis and E. coli led to the significant accumulation of D-2-HGA. Recombinant D2HGDHEc and D2HGDHPa were purified to homogeneity and characterized. D2HGDHEc and D2HGDHPa are homotetrameric with a subunit molecular mass of 110 kDa. The pH optimum was 7.5 for D2HGDHPa and 8.0 for D2HGDHEc. The Km for D-2-HGA was 208 µM for D2HGDHPa and 83 µM for D2HGDHEc. The enzymes have strict substrate specificity towards D-2-HGA and displayed maximal activity at 45 °C. Their activity was completely inhibited by 0.5 mM Mn2+, Ni2+ or Co2+. The discovery of a novel family of D2HGDHs may provide fundamental information for the metabolic engineering of microbial chassis with desired properties.

Keywords: L-serine biosynthesis; D-3-phosphoglycerate dehydrogenase; D-2-hydroxyglutaric acid; D-2-hydroxyglutarate dehydrogenase

1. Introduction

L-Serine biosynthesis is a substantial metabolic pathway in almost all living organisms, including Escherichia coli and Pantoea ananatis. L-Serine is a pivotal amino acid since it is used in protein synthesis and serves as a direct precursor for the biosynthesis of L-cysteine, L-methionine, L-tryptophan, and glycine. As the primary precursor of glycine, L-serine contributes a one-carbon unit (C1) that acts as a donor in methylation reactions via derivatives of tetrahydrofolate and S-adenosyl methionine. Thus, directly or indirectly, L-serine is a source of one-carbon units for the biosynthesis of various compounds, such as phosphatidylserine, sphingolipids, purines, and porphyrins [1–6]. It was also elucidated recently that L-serine synthesis through D-3-phosphoglycerate dehydrogenase (PHGDH) coordinates nucleotide levels in mammals by maintaining central carbon metabolism [7]. The phosphorylated glycolytic intermediate D-3-phosphoglycerate (3PG) is a bifurcation point of carbon flow for later steps in glycolysis toward pyruvate and L-serine biosynthesis. The pathway to L-serine biosynthesis is one of the main pathways in bacteria growing on sugars. For example, in E. coli, approximately 15% of the assimilated glucose carbon passes through L-serine before incorporation into biosynthetic products [8]. Three enzymes are responsible for bacterial de novo L-serine biosynthesis (Figure 1). D-3-Phosphoglycerate dehydrogenase (SerA, PHGDH) converts 3PG into phosphohydroxypyruvate (PHP), accompanied...
by the reduction of NAD⁺ to NADH. L-Phosphoserine aminotransferase (SerC, PSAT) converts PHP to L-phosphoserine (PS) with the concomitant deamination of glutamate to 2-ketoglutarate (2-KG). Finally, L-phosphoserine phosphatase (SerB, PSP) dephosphorylates PS, yielding L-serine [1–9].

Figure 1. The pathway of L-serine biosynthesis in E. coli. 2-Ketoglutarate reductase activity of SerA is represented; Pi—inorganic phosphate.

PHGDH of E. coli (SerA), like many enzymes that catalyze the first key step in a biosynthetic pathway, is allosterically inhibited by the end product (L-serine) and by glycine, whose biosynthesis is linked to that of L-serine [9–11]. Dehydrogenation of 3PG, catalyzed by SerA, is thermodynamically unfavorable (ΔG° = +33.0 kJ·mol⁻¹); the equilibrium of the reaction lies in the direction of D-3-phosphoglycerate [12]. As conventionally assumed, 3PG dehydrogenation is driven by coupling with reactions catalyzed by L-phosphoserine aminotransferase (SerC) (ΔG° = −11.5 kJ·mol⁻¹) [12] and L-phosphoserine phosphatase (SerB) (ΔG° = −10.2 kJ·mol⁻¹) [13] in L-serine biosynthesis; nevertheless, the calculated sum of ΔG° indicates that these reactions are not thermodynamically favorable [14]. According to the most plausible view, the reaction proceeds in the direction of L-serine synthesis because L-serine, the final product in that direction, is continually utilized in subsequent metabolic steps. This is proposed as a mechanism that conserves 3PG for later steps in glycolysis by using it only when L-serine is required [2,9].

In 1996, Zhao and Winkler [15] discovered that E. coli SerA could utilize 2-ketoglutarate (2-KG) as a substrate in the reverse direction in place of PHP, converting it to D-2-hydroxyglutarate (D-2-HGA) with the concomitant oxidation of NADH to NAD⁺ (Figure 1).

For a long time, the 2-ketoglutarate reductase activity of SerA was considered “promiscuous” and did not draw much attention after being identified. Just recently, the ability to use 2-KG as a substrate has also been reported for human PHGDH (hsPHGDH) [16,17], Saccharomyces cerevisiae (scPHGDH) [18], Arabidopsis thaliana (atPHGDH) [19], Pseudomonas stutzeri (psPHGDH) [14,20], Pseudomonas aeruginosa (paPHGDH) [14], and Acinetobacter denitrificans (adPHGDH) [21]. Those findings led to the hypothetical proposal that D-2-hydroxyglutarate (D-2-HGA) production from 2-KG by PHGDH of E. coli (SerA) is necessary to convert the bound NADH to NAD⁺ in order to shift the reaction to proceed toward L-serine biosynthesis [22]. It is interesting that the ability to reduce 2-KG to D-2-HGA appears to be a common feature of Type II PHGDHs, while Type I or Type III PHGDHs examined so far do not share this feature [2,9,23,24].

In their recent innovative work, Zhang et al. [14] showed that in Pseudomonas stutzeri A1501, both 3PG dehydrogenation and 2-KG reduction are catalyzed by SerA, and SerA...
couples with the energetically favorable reaction of D-2-hydroxyglutaric acid (D-2-HGA) production from 2KG to overcome the thermodynamic barrier of 3PG dehydrogenation. They also identified a bacterial D-2-HGA dehydrogenase (D2HGDH), a flavin adenine dinucleotide (FAD)-dependent enzyme that subsequently converts D-2-HGA back to 2-KG. Electron transfer flavoprotein (ETF) and ETF-ubiquinone oxidoreductase (ETFQO) are also essential in D-2-HGA metabolism due to their capacity to transfer electrons from D2HGDH. Thus, it was uncovered that D-2-HGA-mediated coupling between SerA and D2HGDH drives bacterial L-serine biosynthesis [14].

As the key enzyme involved in D-2-HGA metabolism, D2HGDHs have been identified and characterized in bacteria (Pseudomonas stutzeri [14,20], Pseudomonas aeruginosa [14,25], Achromobacter denitrificans [21], and Ralstonia solanacearum [26]), yeast (Saccharomyces cerevisiae [18]), plants (Arabidopsis thaliana [27]), and humans [28–30].

Even though SerA of E. coli belongs to Type II PHGDHs and was reported to have the ability to produce D-2-HGA, there are neither D2HGDH nor ETF homologs in E. coli, suggesting the existence of other unknown enzymes in bacteria of the Enterobacteriaceae family involved in the possible D-2-HGA catabolism [14].

Due to that flaw, G. Grant proposed a self-sustaining cycle in E. coli L-serine biosynthesis that results in the conservation of NAD$^+$ and does not require D2HGDH [24]; nevertheless, this proposal does not explain the further fate of D-2-HGA that has to be produced via E. coli SerA during L-serine biosynthesis.

Thus, the goal of our work was to reveal possible mechanisms and enzymes, if any, that can metabolize D-2-HGA in E. coli and P. ananatis bacteria belonging to the Enterobacteriaceae family.

To accomplish this goal, we performed screening of the genomic P. ananatis library for genes that conferred fast growth on D-2-HGA. We detected D2HGDH activity in a crude extract of the clone containing the pSTV29 plasmid with ydiJ. Based on this observation, we proposed that the product of ydiJ can function as D2HGDH. Inactivation of this gene in both E. coli and P. ananatis led to a significant accumulation of D-2-HGA in culture media. These results indicate that YdiJ appears to be the only D2HGDH in E. coli and P. ananatis, providing the way to recuperate D-2-HGA back to 2-KG. The YdiJ genes of E. coli and P. ananatis were sequenced, cloned, and expressed in Escherichia coli as recombinant His-tagged proteins. We provide experimental evidence that ydiJ indeed encodes a novel family of bona fide D2HGDHs. The detailed enzymatic characterization of D2HGDHEc and D2HGDHPa adds a new and interesting member to the D2HGDH family.

2. Materials and Methods

2.1. Chemicals

D-2-Hydroxyglutarate (disodium salt), L-2-hydroxyglutarate (disodium salt), L-malate, D-malate, L-lactate, D-lactate, L-glycerate, D-glycerate, L-tartrate, D-tartrate, pyruvate, glutarate, Phenazine ethosulfate (PES), 2,6-Dichlorophenolindophenol (DCIP), isopropyl-β-D-1-thiogalactopyranoside (IPTG), TRIS, Bis-TRIS, and M9 minimal media were purchased from Sigma-Aldrich (San Luis, MO, USA). Yeast extract and tryptone were obtained from BD Biosciences (San Jose, CA, USA). Other chemicals were of analytical reagent grade.

2.2. Analytical Method

The analysis of organic acids, including D-2-HGA, was carried out by HPLC (Shimadzu system IE-HPLC-ECD) using a CDD-10A conductivity detector. Generally, a Phenomenex Rezex ROA-Organic Acid H+, 8% column was used, and the mobile phase (5 mM p-toluenesulfonic acid monohydrate, 100 mM EDTA-2Na, and 20 mM BIS-TRIS, pH 6.0) was pumped at a flow rate of 0.8 mL/min and a temperature of 40 °C.

2.3. Bacterial Strains, Plasmids, and Growth Conditions

The strains and plasmids used in this study are listed in Table 1. All strains were grown at 34 °C in Luria–Bertani (LB) medium or M9 minimal medium [31] supplemented...
with 25 µg/mL—kanamycin, 25 µg/mL—chloramphenicol, and 20 µg/mL—tetracycline if required. M9 minimal medium with the addition of 5 g/L D-2-Hydroxyglutarate (disodium salt) (D-2-HGA) was used for the selection of clones containing gene bank plasmids with targeted genes growing on D-2-HGA. The test tube fermentation medium for the determination of D-2-HGA accumulation was composed of M9 supplemented with Glucose (40 g/L), CaCO₃ (20 g/L), Tryptone (0.6 g/L), Yeast extract (0.3 g/L), and chloramphenicol 25 µg/mL, and the medium for the determination of the growth rate was composed of M9 supplemented with D-2-HGA 5 g/L.

Table 1. Bacterial strains and plasmids used in this study.

| Strain or Plasmid | Description | Source or Reference |
|-------------------|-------------|---------------------|
| **Escherichia coli** | | |
| MG1655 | Wild type | VKPM a |
| DH5α | F⁻ q80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17 (rK-, mK+) phoA supE44 λ thi-1 grrA96 relA1 | Novagen |
| BL21(DE3) | F' ompT hsdS (rB-, mB-) gal dcm (DE3) | Novagen |
| MG1655 Pnlp8-ydiJEc | MG1655 with enhanced expression of ydiJ | This study |
| MG1655 ΔydiJ::Km | MG1655 with inactivated ydiJ | This study |
| **Pantoea ananatis** | | |
| SC17 (AJ13355) | Wild type | [32] |
| SC17(0) | Mutant resistant to lambda Red recombinase selected from SC17 | [33] |
| SC17(0) ΔydiJ::Km | SC17(0) with inactivated ydiJ | This study |
| SC17(0) Pnlp8-ydiJPa | SC17(0) with enhanced expression of ydiJ | This study |
| **Plasmids** | | |
| pMIV-5JS | Vector, SC101 ori, Cm r empty vector | [34] |
| pMIV-Pnlp8-348(Ec) | serA348 (encodes feedback-resistant enzyme [35]) from *E. coli* cloned in pMIV-5JS; the gene is flanked by Pnlp8 (constitutive derivative of nlpD promoter) and TrrnB (rrnB terminator) | This study |
| pSTV29 | Cloning vector; Cm r | TaKaRa Bio |
| pET28b(+) | Expression vector, Km r | Novagen |
| pET-ydiJ_Pa1 | pET28b(+) with cloned ydiJ from *P. ananatis* with 6His on N-end | This study |
| pET-ydiJ_Pa2 | pET28b(+) with cloned ydiJ from *P. ananatis* with 6His on C-end | This study |
| pET-ydiJ_Ec1 | pET28b(+) with cloned ydiJ from *E. coli* with 6His on N-end | This study |
| pET-ydiJ_Ec2 | pET28b(+) with cloned ydiJ from *E. coli* with 6His on C-end | This study |
| pMW-λattL-KmR-λattR | Source of antibiotic resistance marker Km | [33] |
| pMW-λattR-KmR-λattL-Pnlp8 | Source of antibiotic resistance marker Km (for enhancing gene expression) | This study |
| pRSFRedTER | For red-dependent integration of PCR fragments in *Pantoea ananatis* | [33] |
| pKD46 | For red-dependent integration of PCR fragments in *E. coli* | [36] |

a—VKPM, The Russian National Collection of Industrial Microorganisms (WDCM No. 588); Cm r—resistance to kanamycin; Km r—resistance to chloramphenicol.

2.4. DNA Manipulation and Plasmid Construction

Plasmid DNA was isolated using the Cleanup Standard kit (Evrogen, Moscow, Russia). Chromosomal DNAs from *E. coli* and *P. ananatis* were isolated using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Restriction endonucleases, Klenow Fragment, T4 DNA ligase, and Taq DNA polymerases were purchased from Thermo Fisher...
The ydiJ genes from *E. coli* and *P. ananatis* were PCR-amplified by KAPA HiFi DNA Polymerase (Roche, Basel, Switzerland) from genomic DNA of *E. coli* MG1655 and *P. ananatis* SC17 (AJ13355) using the primers listed in Table S1. Inactivation or enhancement of ydiJ expression in *P. ananatis* or *E. coli* was performed using the λ-Red recombination system [33]. The plasmid pRSFRedTER [GenBank: FJ347161], which carries an IPTG-inducible λ-Red gene, was used to allow Red recombination. The plasmid pMW-λattL-Km-R-λattR (Km) was used as a template to provide PCR-generated gene disruption cassettes, and pMW-λattR-Km-R-λattL-P_nlp8 (Km') was used to enhance gene expression [33]. The primers used to generate these cassettes are listed in Table S1.

For overexpression of genes in chromosomes, randomized in -10 and -35 regions, a derivative of the constitutive promoter of the *E. coli* nlpD gene was used in the present work [37]. The antibiotic resistance marker Km was introduced upstream of the promoter. The direction of Km transcription was opposite to the direction of transcription from the P_nlp8 promoter. P_nlp8 is a stronger promoter than P_nlpD [37]. A derivative of the SerA (3-Phosphoglycerate dehydrogenase) enzyme from *E. coli* (SerA348) with N348A replacement was used. The SerA348 enzyme is feedback-resistant to L-serine [38]. To construct the serA348 expression plasmid, a low-copy-number plasmid, pMIV-5JS, was used as a backbone. The PCR fragment containing the P_nlp8serA348 expression cassette was digested using *Pae*I and *Xba*I (these sites were designed based on the 5′ and 3′ ends of the primers) and cloned into the corresponding sites in pMIV-5JS.

To obtain YdiJ with an N-terminal His-tag, the pairs of primers ydiJ_EcF1-ydiJ_EcR1 and ydiJ_PaF1-ydiJ_PaR1 were used for PCR amplification of ydiJ from chromosomes of *E. coli* and *P. ananatis*, resulting in PCR fragments ydiJ_Ec1 and ydiJ_Pa1, respectively. For the C-terminal His-tag in YdiJ, the pairs of primers ydiJ_EcF2-ydiJ_EcR2 and ydiJ_PaF2-ydiJ_PaR2 were used and resulted in ydiJ_Ec2 and ydiJ_Pa2 fragments. After digestion with *Bsp*I19I, the ydiJ_Ec1 fragment was blunted by the Klenow Fragment, digested with *Xho*I, and cloned into *Nde*I site of pET28b (+); the resulting plasmid was pET-ydiJ-Ec1. Plasmids pET-ydiJ-Ec2 and pET-ydiJ-Pa1 were obtained by cloning fragments with ydiJ genes from *E. coli* and *P. ananatis* into *Nco*I, *Bam*HI and *Nde*I, *Xho*I sites of pET28b (+), respectively. The plasmid pET-ydiJ-Pa2 was obtained by cloning the ydiJ_Pa2 fragment digested with *Pst*I and *Xho*I into *Nco*I, *Xho*I sites of pET28b (+). The integrity of the nucleotide sequence of all newly constructed plasmids was confirmed by DNA sequencing. The obtained plasmids were transformed into BL21 (DE3) cells for protein expression.
2.7. Enzyme Overexpression and Purification

The *E. coli* BL21 (DE3) strain (picked from a single colony) harboring pET-\(ydiJ\)-Ec1, pET-\(ydiJ\)-Pa1 (N-terminal 6His-tags), pET-\(ydiJ\)-Ec2, and pET-\(ydiJ\)-Pa2 (C-terminal 6His-tags) was propagated overnight in Luria–Bertani (LB) medium with 50 µg/mL kanamycin at 30 °C with shaking on a rotatory plate at 240 rpm. All of the overnight growth cultures were used to inoculate 1 L (4 × 250 mL) of fresh LB to a final OD600nm of 0.1 with the same antibiotic to grow until the cell density reached an OD600nm of 0.5–0.6. IPTG was added to the culture at a final concentration of 1 mM, and the growth continued at 30 °C for about 105 min. Cells were harvested by centrifugation at 10,000 \(\times\) g for 10 min at 4 °C, washed with buffer A (20 mM Tris-HCl, 20 mM Imidazole-HCl, and 0.5 M NaCl, pH 8.0), and resuspended in 30 mL of the same buffer. The cells were disrupted by French press (Thermo Fisher) at 4 °C until clear lysate was obtained. The cell debris was then removed by centrifugation at 12,000 \(\times\) g for 20 min at 4 °C. The recombinant YdiJ with a 6x His-tag on its N- or C-terminus was purified using a 5 mL HisTrap HP column (GE Healthcare Life Sciences) equilibrated with buffer A. Unbound protein was washed away with buffer A. The protein fractions were eluted with an imidazole gradient of 0 to 100% buffer B (20 mM Tris-HCl, 500 mM Imidazole-HCl, and 0.5 M NaCl, pH 8.0) and 500 mM elution buffer. The active fraction with the recombinant D2HGDH was pooled and concentrated, and the buffer was changed to 25 mM Tris-HCl, pH 7.5, using a Vivaspin 20 centrifugal concentrator. The purity of the recombinant enzymes was confirmed by 12% SDS-PAGE and non-denaturing 4–20% gradient PAGE.

2.8. Measurement of Enzyme Activity

D-2-Hydroxyglutarate dehydrogenase (D2HGDH) activity was routinely measured by following the reduction of DCIP spectrophotometrically at 600 nm [14]. Reaction mixtures were incubated at 25 °C and contained 50 mM Tris-HCl buffer (pH 7.5), 200 µM phenazine ethosulfate (PES), 100 µM DCIP, 1 mM D-2-HGA (disodium salt), and cell extract (or pure enzyme) in a total volume of 1.0 mL. Phenazine ethosulfate (PES) was used instead of phenazine methosulfate (PMS) due to its higher stability, especially at increased pH and ionic strength [39]. After the determination of the pH optimum, D2HGDH\(\text{Pa}\) was measured in 50 mM Tris-HCl buffer (pH 7.5), while D2HGDH\(\text{Ec}\) was measured in 50 mM Tris-HCl buffer (pH 8.0). The reduction of DCIP was monitored at 600 nm with a thermostated Shimadzu-1800 UV-Vis spectrophotometer (Shimadzu Corp, Kyoto, Japan), converting the absorbance to concentration using a molar extinction coefficient of 22 mM\(^{-1}\)cm\(^{-1}\). One unit (U) of activity was defined as 1 µmol of DCIP reduced per minute. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard. All measured values indicate the means of at least three independent experiments.

2.9. Characterization of the Recombinant D2HGDH (YdiJ)

The native molecular mass of the recombinant D2HGDHE\(\text{c}\) and D2HGDH\(\text{Pa}\) were estimated by non-denaturing 4–20% gradient gel electrophoresis. The protein standards used for the calibration of the gel were albumin (66 kDa), lactate dehydrogenase (140 kDa), catalase (250 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa) (GE Healthcare Life Sciences).

The effects of pH and temperature on recombinant D2HGDHE\(\text{c}\) and D2HGDH\(\text{Pa}\) activity were determined in the standard reaction mixture. To obtain the pH profile, the enzyme was assayed in 100 mM buffer (Bis-Tris–HCl, pH 5.5–7.0) and (Tris–HCl, pH 7.5–9.0).

The temperature optimum was determined at various temperatures up to 60 °C. The temperature influence on protein stability was investigated by means of pure enzyme (0.03 mg/mL incubation in 50 mM Tris-HCl, pH 7.5, at different temperatures for 10 min, after which aliquots were immediately cooled on ice, and the residual activity was assayed. The kinetic parameters for the recombinant D2HGDHE\(\text{c}\) and D2HGDH\(\text{Pa}\) were
determined by measuring their activity at various D-2-HGA concentrations at saturating concentrations of another substrate. The apparent kinetic parameters were calculated by a double-reciprocal Lineweaver–Burk plot.

The effects of different metal ions (0.5 mM MnCl$_2$, 0.5 mM MgCl$_2$, 0.5 mM CoCl$_2$, 0.5 mM NiCl$_2$, and 0.5 mM ZnSO$_4$) or other substrates on the activity of D2HGDH$_{Ec}$ or D2HGDHP$_{Pa}$ were determined using the standard assay protocol at the pH optimum.

2.10. Polyacrylamide Electrophoresis

Analysis by 12% SDS-PAGE was carried out as described elsewhere (https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6201.pdf, accessed on 1 August 2022). Non-denaturing gradient PAGE (4–20%, Bio-Rad Mini-Protean® GTG™ gel, #456-1093) was carried out in standard Tris-Glycine buffer without SDS and reducing agents. Gels were stained with Coomassie brilliant blue R-250 staining solution (Bio-Rad, Hercules, CA, USA).

2.11. Structure-Based Protein Sequence Alignment

Structure-based amino acid sequence alignment was conducted with the CLUSTALX program (ftp://ftp.ebi.ac.uk/pub/software/clustalw2, accessed on 1 June 2022) and ESPript 3.0 web tool (http://escript.ibcp.fr/EScript/EScript/, accessed on 1 June 2022) [40,41].

2.12. Phylogenetic Analysis

The phylogenetic tree of YdiJs from _E. coli_ and _P. ananatis_ was constructed by means of the NCBI BLASTP service to check its distribution among the _Proteobacteria_ phylum.

3. Results and Discussion

3.1. Screening the Genomic _P. ananatis_ Library for Genes That Conferred Fast Growth on D-2-HGA

To elucidate the ability of _P. ananatis_ to utilize D-2-HGA as a sole carbon source, we plated a wild-type strain of _P. ananatis_ SC17 (AJ13355) on M9 minimal agar supplemented with 5 g/L D-2-HGA. Tiny colonies appeared after 7–8 days of incubation at 34 °C, so we can conclude that D-2-HGA can support the growth of _P. ananatis_, although it barely serves as a good substrate. To identify _P. ananatis_ genes encoding possible enzymes that take part in D-2-HGA utilization, we screened a multicopy genomic library prepared from wild-type _P. ananatis_ genomic DNA in a pSTV29 vector. _P. ananatis_ strain SC17 (AJ13355) was electroporated with the genome library, and transformed cells were cultured on M9 minimal agar supplemented with 5 g/L D-2-HGA and 25 µg/mL chloramphenicol. Fast-growing colonies were selected after 2–4 days, and the genes responsible for the fast-growing phenotype were identified by sequencing the plasmid DNAs. Of thirty analyzed clones, twenty-nine sequenced fragments had obscure ORF or ORF with predicted membrane proteins that are under our investigation at the moment as possible importers of D-2-HGA. One of the sequenced ORF fragments contained the _ydiJ_ gene (NCBI Reference Sequence: WP_019105315.1) with high similarity to _E. coli_ ydiJ (NCBI Reference Sequence: NP_416202.1), encoding a polypeptide of 1018 amino acids with identities of 79% (Figure 2). The predicted pI values are 7.80 and 7.11 for YdiJ from _P. ananatis_ and _E. coli_, respectively. While D2HGDH activity was not detected in wild-type _P. ananatis_ even when it grew on D-2-HGA, we were able to detect D2HGDH activity at 0.87 × 10$^{-3}$ U/mg protein in a crude extract of a clone containing the pSTV29 plasmid with _ydiJ_. Based on this observation, we proposed that the product of _ydiJ_ could function as D2HGDH, and we assigned YdiJs of _E. coli_ and _P. ananatis_ as D2HGDH$_{Ec}$ and D2HGDHP$_{Pa}$, respectively.

In _E. coli_ and _P. ananatis_, the product of _ydiJ_ was described as a putative cytosolic FAD-linked oxidoreductase of unknown function. It is interesting that YdiJ was recently predicted to be a metalloprotein in _E. coli_ and isolated; the function of the protein was not reported. As isolated, purified YdiJ contains FAD as well as a 4Fe-4S cluster [42]. A comparison of the domain structure of YdiJ and known D2HGDH from _P. stutzeri_ A1501 reveals that YdiJ is about two times longer and contains an additional GlpC superfamily do-
main, which is the membrane-associated subunit of the heterotrimeric glycerol-3-phosphate dehydrogenase complex (Figure 3). Multiple sequence alignment of the first 542 residues (GlcD superfamily, FAD oxidoreductase domain) of YdiJs (D2HGDHs) of \textit{P. ananatis} (Pa) and \textit{E. coli} (Ec) with the whole sequence of D2HGDH of \textit{P. stutzeri} (Ps) reveals their very weak homology, with identities of about 23–25\% (Figure 4).

| YdiJ_{Pa} | 1 | 20 | 30 | 50 | 60 |
|------------|---|----|----|----|----|
| YdiJ_{Ec} | HEP| ISO| SA| YP| TET|

**Figure 2.** Sequence alignment of the YdiJs (D2HGDHs) of \textit{P. ananatis} (Pa) and \textit{E. coli} (Ec). The conserved amino acids are highlighted in shaded red boxes; conserved replacements are highlighted as red letters in blue boxes. The alignment was drawn with ESPRRIPT 3.0.

**Figure 3.** Domain structure of YdiJ of \textit{P. ananatis} (Pa) and \textit{E. coli} (Ec) and D2HGDH of \textit{P. stutzeri} A1501 (Ps).
In *E. coli* and *P. ananatis*, the product of *ydiJ* was described as a putative cytosolic FAD-linked oxidoreductase of unknown function. It is interesting that YdiJ was recently predicted to be a metalloprotein in *E. coli* and isolated; the function of the protein was not reported. As isolated, purified YdiJ contains FAD as well as a 4Fe-4S cluster [42]. A comparison of the domain structure of YdiJ and known D2HGDH from *P. stutzeri* A1501 reveals that YdiJ is about two times longer and contains an additional GlpC superfamily domain, which is the membrane-associated subunit of the heterotrimeric glycerol-3-phosphate dehydrogenase complex (Figure 3). Multiple sequence alignment of the first 542 residues (GlcD superfamily, FAD oxidoreductase domain) of YdiJs (D2HGDHs) of *P. ananatis* (*Pa*) and *E. coli* (*Ec*) with the whole sequence of D2HGDH of *P. stutzeri* (*Ps*) reveals their very weak homology, with identities of about 23–25% (Figure 4).

### 3.2. Determination of Physiological Function of YdiJ In Vivo

To determine the in vivo function of YdiJ in *E. coli* and *P. ananatis*, we inactivated the corresponding genes, resulting in *E. coli* strain MG1655 Δ*ydiJ::Km* and *P. ananatis* strain SC17(0) Δ*ydiJ::Km*. They were evaluated in conditions of test tube fermentation, as described in Materials and Methods. Compared to the parental strains, test tube fermentation of these strains revealed that inactivation of *ydiJ* led to the accumulation of D-2-HGA to 4.2 g L\(^{-1}\) for MG1655Δ*ydiJ::Km* and 2.7 g L\(^{-1}\) for SC17(0)Δ*ydiJ::Km* (Figure 5A). Thus, we demonstrated that disruption of *ydiJ* in both *E. coli* and *P. ananatis* impairs D-2-HGA utilization, which is expected to be produced via 2KG reductase activity of SerA (PHGDH).

To determine the influence of the inactivation of *ydiJ* or its enhanced expression on D-2-HGA accumulation in strains with high-level expression of *serA*, we constructed the plasmid pMIV-P\(_{nlp8}\)serA348 with high-level expression of a feedback-resistant variant of *E. coli* SerA (SerA348) [38] by introducing a P\(_{nlp8}\)serA348 cassette in pMIV-5JS to enhance D-2-HG synthesis in cells. The plasmid pMIV-P\(_{nlp8}\)serA348 was transformed into *P. ananatis* SC17(0), SC17(0)Δ*ydiJ::Km*, SC17(0)P\(_{nlp8}\)ydiJ\(_{Pa}\), *E. coli* MG1655, MG1655Δ*ydiJ::Km*, and MG1655P\(_{nlp8}\)ydiJ\(_{Ec}\), and the resulting strains were evaluated in test tube fermentation. D-2-HGA accumulation was determined after fermentation for all tested strains and is shown in Figure 5B. Inactivation of *ydiJ* led to an enormous accumulation of D-2-HGA to 25 g L\(^{-1}\) for MG1655Δ*ydiJ::Km* and 12 g L\(^{-1}\) for SC17(0)Δ*ydiJ::Km*. 

**Figure 4.** Multiple sequence alignment of the first 542 residues (GlcD superfamily, FAD oxidoreductase domain) of YdiJ (D2HGDHs) of *P. ananatis* (*Pa*) and *E. coli* (*Ec*) with the whole sequence of D2HGDH of *P. stutzeri* (*Ps*). The alignment was drawn with ESPRRIPT 3.0.
The oligomeric status of D2HGDH (Figure 6A). The oligomeric status of D2HGDH (Table 1) or pET-BL21 (DE3)-harboring plasmid pET-P. ananatis SC17(0)/pMIV-Pnlp8-serA348 and E. coli MG1655/pMIV-Pnlp8-serA348 with various allelic stages of the ydiJ gene: enhanced expression of the ydiJ gene (Pnlp8-ydiJ), expression of native ydiJ (ydiJ-wt) and inactivation of ydiJ (ΔydiJ).

3.3. Recombinant Enzyme Purification and Characterization

Although four plasmids were constructed for the overexpression of ydiJ from E. coli and P. ananatis, many attempts to isolate the proteins failed because they tended to form inactive inclusion bodies without the detection of corresponding D2HGDH activity in the soluble fraction and the corresponding protein band on SDS-PAGE. We performed a number of approaches to improve the expression of recombinant YdiJ in soluble form, which include changing the E. coli host, cultivation conditions, such as expression temperature, medium composition, the timing of induction, and inducer concentration. Finally, we found that decreasing the induction time to 90–100 min and quickly starting the purification procedure led to the most reliable results, although even under these conditions, most of the recombinant YdiJ from both E. coli and P. ananatis precipitated into inclusion bodies. The E. coli BL21 (DE3)-harboring plasmid pET-ydiJ_Ec (N-terminus 6xHis-tagged D2HGDHEc, Table 1) or pET-ydiJ_Pa2 (C-terminus 6xHis-tagged D2HGDHPa, Table 1) did not produce a detectable protein band in the soluble fraction corresponding to the size of the fusion protein under various experimental conditions.

Recombinant C-terminus 6xHis-tagged D2HGDHEc (E. coli, ydiJ_Ec2, Table 1) and D2HGDHPa N-terminus 6xHis-tagged D2HGDHPa (P. ananatis, ydiJ_Pa1, Table 1) were purified to homogeneity. The molecular mass of both enzymes was determined to be approximately 110.0 kDa by SDS-PAGE, which compares well to the predicted value (113 kDa) (Figure 6A). The oligomeric status of D2HGDHEc and D2HGDHPa was confirmed by non-denaturing 4–20% gradient PAGE, which showed one protein band for both D2HGDHEc and D2HGDHPa with a molecular mass of approximately 440 kDa, suggesting that the native enzyme forms a homotetramer in solution (Figure 6B). Unfortunately, gel filtration chromatography on a HiLoadTM 10/300 Superdex 200 column (GE Healthcare) resulted in a diffuse non-symmetrical peak, possibly due to protein aggregation in standard elution conditions.

The final specific activity of the purified D2HGDHEc was 1.12 U mg⁻¹ and 1.01 U mg⁻¹ for D2HGDHPa. The enzymes showed Michaelis–Menten kinetics. The apparent Kₘ value for D-2-HGA was 83 µM for D2HGDHEc and 208 µM for D2HGDHPa (Figure S1). All steady-state kinetic parameters of D2HGDHEc and D2HGDHPa are shown in Table 2.
Figure 6. SDS-PAGE (A) and non-denaturing 4–20% gradient PAGE (B). (A) M—molecular weight marker (Spectra™ Multicolor Broad Range Protein Ladder, Thermo Scientific™, #26634); 1—crude extract of recombinant D2HGDH\textsubscript{Pa}; 2—purified D2HGDH\textsubscript{Pa}; 3—crude extract of recombinant D2HGDH\textsubscript{Ec}; 4—purified D2HGDH\textsubscript{Ec}; (B) M—molecular weight marker (GE Healthcare HMW Calibration Kit proteins, # 17-0445-01); 1—crude extract of recombinant D2HGDH\textsubscript{Pa}; 2—purified D2HGDH\textsubscript{Pa}.

### Table 2. Steady-state kinetic parameters of known D2HGDHs toward D-2-HGA.

| Enzyme Family          | Mw (Da)       | \(K_m\) (D-2-HGA) (\(\mu\)M) | \(V_{max}\) (D-2-HGA) (\(\mu\)M/min·mg\(^{-1}\)) | \(k_{cat}\) (s\(^{-1}\)) | \(k_{cat}/K_m\) (s\(^{-1}/\mu\)M\(^{-1}\)) | Reference |
|------------------------|---------------|--------------------------------|---------------------------------|-----------------|-----------------|-----------|
| VAO/PCMH Flavoprotein  |               |                                |                                 |                 |                 |           |
| D2HGDH                 | 51,086.14     | 170 ± 20                       | 4.56 ± 0.60                     | 7.9 ± 1.05      | 45.4            | [14,20]   |
| D2HGDH                 | 51,286.52     | ~580                           | NR                              | ~0.8            | 1.37            | [27]      |
| A. thaliana Dld2       | 59,282.45     | 28 ± 8                         | NR                              | 0.18 ± 0.03     | 7               | [18]      |
| S. cervisiae Dld3      | 55,241.00     | 130 ± 9                        | NR                              | 6.6 ± 0.5       | 50 ± 2          | [18]      |
| D2HGDH                 | 51,286.52     | 60                             | NR                              | 11.2 ± 0.4      | 186             | [25]      |
| P. aeruginosa D2HGDH   | 50,405.42     | 31.6                           | 40.6                            | 6.9             | 215             | [21]      |
| A. denitrificans D2HGDH| 50,444.64     | 433                            | NR                              | 4.86            | 11              | [26]      |
| R. solanacearum D2HGDH | 56,416.06     | 38 ± 0.3                       | NR                              | 0.51 ± 0.01     | 13              | [29]      |
| Homo sapiens D2HGDH    |               | 120 ± 10                       | 2.29 ± 0.03                     | 1.98 ± 0.03     | 17              | [28]      |

| Fe\(_3\)S\(_2\) FAD-linked oxidoreductase [42] |
|-----------------------------------------------|
| D2HGDH (Ydij) E. coli                        | 113,247.68 | 83 ± 5 | 1.15 | 13.9 | 170 | This study |
| D2HGDH (Ydij) P. ananatis                   | 113,453.43 | 208 ± 10 | 1.17 | 9.7 | 50 | This study |

NR—not reported.
The $K_m$ value of D2HGDHeC for D-2-HGA (83 $\mu$M) is a little bit higher than that determined for D2HGDHeS from the bacteria P. aeruginosa (60 $\mu$M) [25] and A. denitrificans (32 $\mu$M) [21] but lower than that demonstrated for P. stutzeri (170 $\mu$M) and R. solanacearum (433 $\mu$M) [14,20,26]. The $K_m$ value of D2HGDHPa for D-2-HGA (208 $\mu$M) is basically within the range of characterized D2HGDHeS from P. stutzeri, A. thaliana, S. cerevisiae, R. solanacearum, and Homo sapiens, as demonstrated in Table 2.

The catalytic efficiency of D2HGDHeC (170 s$^{-1}$mM$^{-1}$) is very close to those in P. aeruginosa (186 s$^{-1}$mM$^{-1}$) and A. denitrificans (230 s$^{-1}$mM$^{-1}$) and higher than those of all other known D2HGDHeS. In contrast, the catalytic efficiency of D2HGDHPa (50 s$^{-1}$mM$^{-1}$) is much lower and comparable to the efficiency of characterized D2HGDHeS from P. stutzeri, S. cerevisiae, R. solanacearum, and Homo sapiens.

3.4. Effects of pH and Temperature

The effects of pH and temperature on the activity of D2HGDHeC and D2HGDHPa were determined using the standard assay protocol. The results showed that the optimum pH was 7.5 for D2HGDHPa and 8.0 for D2HGDHeC (Figure 7A), which are in the range of all known D2HGDHeS listed in Table 2. The temperature optimum was essentially the same (45 °C) for both enzymes (Figure 7B). This value is the same as for D2HGDHeS from R. solanacearum and much lower than those for D2HGDHeS from P. stutzeri (70 °C) [14,26]. Heat-inactivation studies revealed that D2HGDHPa and D2HGDHeC have the same thermostability range (20–25 °C) and showed instability during incubation at temperatures higher than 25 °C. D2HGDHPa and D2HGDHeC activity decreased almost linearly in the temperature range from 30 °C to 60 °C (Figure 7C). This range is very close to that of D2HGDHeS in P. stutzeri, which also lost activity at temperatures higher than 37 °C [14].

![Figure 7.](image_url)

**Figure 7.** (A) Effects of pH on the activity of recombinant D2HGDHeC and D2HGDHPa; (B) effects of temperature on the activity of recombinant D2HGDHeC and D2HGDHPa; (C) temperature stability of recombinant D2HGDHeC and D2HGDHPa. D2HGDHPa is depicted by dark circles (●). D2HGDHeC is depicted by open circles (○).

3.5. Effects of Metal Ions on D2HGDH Activity

The effects of different cations (0.5 mM MnCl$$_2$$, 0.5 mM MgCl$$_2$$, 0.5 mM CoCl$$_2$$, 0.5 mM ZnSO$$_4$$, and 0.5 mM NiCl$$_2$$) on enzyme activity were studied, and the results indicate that D2HGDHPa and D2HGDHeC activity was completely inhibited by the addition of 0.5 mM Mn$^{2+}$, Ni$^{2+}$, or Co$^{2+}$ and partially inhibited by 0.5 mM Zn$^{2+}$ (Table 3). It was shown that for D2HGDHeS from P. stutzeri, Zn$^{2+}$ positively influenced activity at a concentration of 10 μM but inhibited D2HGDHeS activity at a concentration of 10 μM [20]. D2HGDHeS from S. cerevisiae (Dld2) is stimulated by 5 μM Zn$^{2+}$, whereas Co$^{2+}$, Mn$^{2+}$, Mg$^{2+}$, and Ca$^{2+}$ did not affect its activity at this concentration. For Dld3, Zn$^{2+}$ and Co$^{3+}$ stimulated D2HGDH activity to a similar extent at the low and high metal concentrations tested, whereas Mn$^{2+}$, Mg$^{2+}$, and Ca$^{2+}$ did not significantly affect its activity [18]. Rat liver D2HGDHeS is stimulated by 100 μM Zn$^{2+}$, Co$^{3+}$, and Mn$^{2+}$, but not by Mg$^{2+}$ or Ca$^{2+}$ [35].
Table 3. Effect of metal ions on the activity of D2HGDH Ec and D2HGDHPa.

| Metal Ions | E. coli | P. ananatis |
|-----------|---------|------------|
| None      | 100.0   | 100.0      |
| Mg^{2+}   | 95.0    | 93.0       |
| Zn^{2+}   | 73.0    | 34.0       |
| Mn^{2+}   | 0.0     | 0.0        |
| Co^{2+}   | 0.0     | 0.0        |
| Ni^{2+}   | 0.0     | 0.0        |

3.6. Substrate Specificity

Substrate specificity screening revealed that D2HGDH Ec and D2HGDHPa have strict substrate specificity and only exhibited distinct activity towards D-2-HGA, but there was no detectable activity on L-2-hydroxyglutarate, L-malate, D-malate, L-lactate, D-lactate, L-tartrate, D-tartrate, L-glycerate, D-glycerate, glutarate, or pyruvate at final concentrations of 5–10 mM. This result is similar to those obtained for bacterial D2HGDHs from *A. denitrificans* [21] and *R. solanacearum* [26] and for plant D2HGDH from *A. thaliana* [27], which also demonstrated a strict preference for D-2-HGA. Conversely, D2HGDHs from bacteria *P. stutzeri* [14,20] and *P. aeruginosa* [25] demonstrated high detectable D-malate oxidizing activity, comparable to that of D-2-HGA. Moreover, it was proposed that D2HGDH evolved as an enzyme for both D-malate and D-2-HGA dehydrogenation in *P. stutzeri* A1501, and the enzyme participates in both the core metabolic pathway for L-serine biosynthesis and utilization of extracellular D-malate [20]. D2HGDH from *S. cerevisiae* (Dld2) displayed promiscuous activity toward D-malate, D-lactate, and even L-2-hydroxyglutarate. In addition to D-2-HGA and D-malate, Dld3 showed substantial activity with D-lactate and lower activity with D-glycerate; however, it displays a higher stereoselectivity for D-2-hydroxyacids over L-2-hydroxyacids than Dld2 [18]. Human D2HGDH exhibits high specific activities towards both D-2-HGA (2.02 ± 0.04 µmol/min/mg) and D-malate (2.52 ± 0.05 µmol/min/mg) and weak activity towards D-lactate (0.16 ± 0.01 µmol/min/mg) and L-2-hydroxyglutarate (0.06 ± 0.01 µmol/min/mg) [28,29]. D2HGDH isolated from rat liver also oxidized D-malate, D-lactate, and *meso*-tartrate [35].

3.7. Phylogenetic Analysis of YdiJ-Like Enzymes

Analysis of YdiJ distribution confirmed that it could be D2HGDH for a large group in the *Proteobacteria* phylum, namely, for the class gamma *Proteobacteria*, with maximal identity to the *Enterobacteraeaceae* family (Figure 8). Of 1000 analyzed species of the *Enterobacteraeaceae* family, all had enzymes with a identity of more than 49% to D2HGDHPa and D2HGDHEc, and all had Type II SerA, which was able to generate D-2-HGA from 2KG.

D2HGDHPa is also an enzyme from the *Proteobacteria* phylum. From 1000 analyzed species of the *Proteobacteria* phylum, the coexistence of D2HGDHPa-like enzymes and Type II SerA was about 30%, and the coexistence of D2HGDHPa-like or D2HGDHEc-like enzymes and Type II SerA in this phylum was more than 65% (Tables S2 and S3). There are probably only two types of D2HGDH that exist in the *Proteobacteria* phylum. Thus, D2HGDHPa-like or D2HGDHEc-like enzymes are predominant in the *Proteobacteria* phylum.
obacteriaceae family, all had enzymes with an identity of more than 49% to D2HGDH Pa and D2HGDH Ec, and all had Type II SerA, which was able to generate D-2-HGA from 2KG.

Figure 8. Phylogenetic analysis of D2HGDH Pa distribution in Proteobacteria phylum. Green color: Enterobacteriaceae; black-yellow color: gamma Proteobacteria; fuchsia color: alpha Proteobacteria.

4. Conclusions

In the present study, we report the identification and biochemical characterization of a novel family of D2HGDHs encoded by ydiJ in P. ananatis and E. coli. D-2-HGA has been detected in various organisms, including Homo sapiens, Arabidopsis thaliana, and Saccharomyces cerevisiae, and extensively studied after being identified as an “oncometabolite” in humans [14,16,45]. Although D-2-HGA has also been found at low detectable levels in bacteria such as Pseudomonas [46] and E. coli [47], it did not draw much attention because it was not considered a core metabolite. Recently, Zhang et al. [14] showed that D-2-HGA is a “normal” metabolite that is simultaneously produced by SerA (Type II PHGDH) and catabolized by D2HGDH without accumulation in bacterial metabolism in P. stutzeri. Moreover, they concluded that coupling between D-3-phosphoglycerate dehydrogenase and D2HGDH drives bacterial L-serine synthesis. In our study, we also demonstrated that D-2-HGA is a normal metabolite in P. ananatis and E. coli produced during L-serine synthesis by SerA and is subsequently converted back to 2KG via D2HGDH encoded by ydiJ. The physiological molecule that functions as the primary electron acceptor during D-2-HGA oxidation by YdiJ in P. ananatis and E. coli is unknown and requires further investigation (Figure 9). The discovery of a novel D2HGDH encoded by ydiJ adds a new and interesting member to the D2HGDH family and may provide fundamental information for metabolic engineering of microbial chassis with desired properties. Previously, we
demonstrated the great impact of the overexpression of ydiJ on the production of L-cysteine or L-methionine by a P. ananatis-based microbial platform [48].

![Schematic diagram of L-serine biosynthesis in P. ananatis and E. coli, associated with recycling of D-2-HGA by YdiJ (D2HGDH). GDH—glutamate dehydrogenase; P_i—inorganic phosphate.](https://www.mdpi.com/article/10.3390/microorganisms10091766/s1)

**Figure 9.** Schematic diagram of L-serine biosynthesis in *P. ananatis* and *E. coli*, associated with recycling of D-2-HGA by YdiJ (D2HGDH). GDH—glutamate dehydrogenase; P_i—inorganic phosphate.

**Supplementary Materials:** The following supporting information can be downloaded at: [https://www.mdpi.com/article/10.3390/microorganisms10091766/s1](https://www.mdpi.com/article/10.3390/microorganisms10091766/s1). Table S1: Primers used in this study; Table S2: Distribution of YdiJ-like enzymes with identity to YdiJ_Ec of more than 50% among the *Proteobacteria* phylum (taxid_1224) constructed by means of the NCBI BLASTP service; Table S3: Taxonomy presentation of distribution of YdiJ-like enzymes with identity to YdiJ_Ec of more than 50% among the *Proteobacteria* phylum (taxid_1224) constructed by means of the NCBI BLASTP service; Figure S1: Lineweaver–Burk plot for purified D2HGDH

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