Iron is a nutrient that is required by most bacteria. However, the bioavailability of iron in an aerobic environment at pH 7 is low since it is primarily available in its oxidized form (Fe$^{3+}$), which is highly insoluble ($\sim 10^{-9}$ M) (5). Under iron depletion conditions many bacteria can synthesize iron-chelating molecules called siderophores. Siderophores are also of particular importance to pathogens during infection due to iron sequestration by host iron-binding proteins (30).

The gram-negative bacterium Pseudomonas aeruginosa produces two siderophores, pyochelin (7) and pyoverdine (25, 41). This opportunistic pathogen infects both burn victims and individuals afflicted with cystic fibrosis, in which it is the leading cause of morbidity and mortality (10). Both pyoverdine and pyochelin are synthesized during pathogenesis (11, 40), although pyoverdine was found to have more prominent effects on virulence (24, 39). Recently, extracellular ferric pyoverdine has also been shown to upregulate the transcription of other Pseudomonas virulence genes, such as the exotoxin A and PrPL endoprotease genes (20).

Structurally, pyoverdines produced by strains of Pseudomonas consist of a peptide chain containing 6 to 12 amino acids, a dihydroxyquinoline chromophore, and an amide-linked acyl side chain (Fig. 1). Both the peptide chain and the chromophore have been proposed to be assembled from amino acid precursors by nonribosomal peptide synthetases (1, 22, 23, 26). The chromophore, for example, is a condensation product of D-tyrosine and L-2,4-diaminobutyrate. Thus, it has been demonstrated that position 4 of the chromophore is labeled with $^{15}$N if the pyoverdine-producing bacteria are fed 1-2,4-[4-$^{15}$N]diaminobutyrate in the media (3). However, the precursor of 1-2,4-diaminobutyrate and the enzyme(s) required for its biosynthesis in fluorescent pseudomonads have not been reported.

In a limited number of cases 1-2,4-diaminobutyrate has also been found to serve both a structural role and a metabolic role in bacteria. For example, 1-2,4-diaminobutyrate is a precursor of the compatible solute ectoine (28, 29) and a metabolite for the biosynthesis of 1,3-diaminopropane in Acinetobacter baumannii (14). In these cases, 1-2,4-diaminobutyrate is formed from aspartate $\beta$-semialdehyde by the enzyme 1-2,4-diaminobutyrate$\alpha$-ketoglutarate 4-aminotransferase. The gene encoding a homologue of this enzyme is found in P. aeruginosa PAO1 at a locus of the genome that contains other pyoverdine synthesis genes (Fig. 2). The homologous gene, termed pvdH, has been inactivated by insertional mutation and has been shown to abolish the formation of fluorescent pyoverdine in P. aeruginosa PAO1 (21, 27). However, the reported knockout mutants have not been complemented with the intact gene in trans; thus, its role in pyoverdine synthesis is not unequivocal. Moreover, the biochemical activity of PvdH has not been demonstrated.

Here we characterized PvdH and showed that it is an aminotransferase that catalyzes the formation of 1-2,4-diaminobutyrate from aspartate $\beta$-semialdehyde and is required for pyoverdine synthesis. We also developed a convenient assay that allows us to determine the true $K_m$ and $k_{cat}$ values for this type of aminotransferase.

**MATERIALS AND METHODS**

**Chemicals.** $\alpha$-Ketobutyrate was purchased from ICN Biomedicals, Inc. *meso*-Diaminopimelate, $\alpha$-homoserine, 1-2,4-diaminobutyrate, NAD$^+$, NADP$^+$, NADPH, NADH, pyridoxal phosphate, $\alpha$-ketoglutarate, pyruvate, and oxaloacetate were purchased from Sigma-Aldrich.

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plasmid was transformed into P. aeruginosa PAO1. P. aeruginosa transformants were plated onto Luria-Bertani (LB) agar containing tetracycline.

Construction of a pvdH::Gmr insertion mutant. The gene replacement strategy of Schweizer and Hoang (35) was used for creation of knockout mutations in pvdH. Briefly, a 855-bp gentamicin resistance cassette was excised by SmaI digestion from pUCGm and ligated to the SacI restriction site of pvdH. The gentamicin cassette was inserted at position 1019 of the 1440-bp pvdH gene. The pvdH::Gmr construct in pT7-7 was then subcloned into pEX18Tc by using XbaI and HindIII, transformed into E. coli JM109, and subsequently transformed into E. coli SM10. The pEX18Tc (pvdH::Gmr) plasmid was introduced into P. aeruginosa PAO1 by electroporation. Recombinant cells by biparental mating and gentamicin-resistant P. aeruginosa strains were selected. Correct gene replacement was ascertained by PCR of the pvdH gene and Southern blotting of digested genomic DNA by using a DIG High Prime DNA labeling and detection starter kit I (Roche) according to the manufacturer’s instructions.

Characterization of pvdH::Gmr and ΔpvdH::Gmr mutants. Pyoverdine production by P. aeruginosa pvdH::Gmr and ΔpvdH::Gmr mutants was assessed by growing the mutants in iron-limiting Casamino Acids (CAA) broth (17). The same mutants were also grown in CAA broth supplemented with 1.5 mM 1,2-diaminobutyrate. The presence of pyoverdine in culture supernatant was detected spectrophotometrically at 405 nm (37) and visually by green fluorescence under UV light. Pyoverdine production was also assessed in the pvdH::Gmr mutant that contained the wild-type copy of pvdH inserted into the expression vector pVL731. The same mutant transformed only with the vector pVL731 served as a negative control. The abilities of the pvdH::Gmr mutant and the complemented mutant to grow on LB agar containing the iron chelator 2,2’-dipyridyl (0.6 mM) were also determined.

Purification of recombinant homoserine dehydrogenase. E. coli BL21(DE3) containing the hom6 gene, encoding homoserine dehydrogenase from Saccharomyces cerevisiae, was obtained from G. Wright, McMaster University. Homoserine dehydrogenase was expressed by using a previously reported protocol (16). The cell pellet from a 1-liter culture was resuspended in 10 ml of 20 mM HEPES buffer (pH 7.5) and passed through a French press cell three times. Buffers containing 20 mM sodium HEPES (pH 7.5) were used throughout the purification procedure. Chromatography was performed by using an AKTA Explorer with resin columns and columns from Amersham Pharmacia Biotech. Crude cell extracts were applied to a Source 15Q anion-exchange column and eluted with a linear gradient of a buffer containing 0 to 0.25 M NaCl in 10 column volumes. Homoserine dehydrogenase did not bind to the column and was eluted during the initial column wash. Fractions containing homoserine dehydrogenase activity were pooled and concentrated by using an Amicon cell equipped with a YM10 filter to 3 ml and then loaded onto a Superdex 200 column equilibrated with 20 mM HEPES buffer (pH 7.5) containing 0.15 M NaCl. Active fractions were pooled and concentrated as described above to 3 ml, divided into aliquots, and stored at −80°C in 20 mM HEPES buffer (pH 7.5) with 5% glycerol.

Purification of PlDl. pvdH was overexpressed in P. aeruginosa PAO1 by using the expression vector pVL731. Protein expression was induced at the log phase with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Mixtures (total volume, 1 ml) for standard coupled assays to determine fractions containing PvdH during purification contained 0.15 mM pyridoxal phosphate, 10 mM 3-ketoglutarate, 0.2 mM NAD(P)H, 10 mM 1,2-diaminobutyrate, 0.03 mg of homoserine dehydrogenase, partially purified PvdH, and 1 mM phosphate buffer (pH 7.5). The decrease in absorbance at 340 nm due to NADH oxidation in the assay was monitored spectrophotometrically.

HEPES (20 mM, pH 7.5) was used throughout the purification procedure. Crude cell extracts from a 2-liter culture were applied to a Source 15Q anion-exchange column equilibrated with buffer containing 0.1 M NaCl. The enzyme was eluted with a 10-column-volume 0.15 to 0.35 M NaCl gradient. PvdH eluted at approximately 0.22 M NaCl. Fractions containing the expected activity were pooled and concentrated; the protein concentration was kept less than 1 mg/ml to prevent undesired precipitation of proteins. The concentrated fraction was loaded onto a Superdex 200 column equilibrated with buffer containing 0.15 M NaCl. Activity-containing fractions were pooled and concentrated as described above, divided into aliquots, and stored at −80°C in 20 mM HEPES (pH 7.5) containing 10% glycerol and 0.5 mM pyridoxal phosphate. The enzyme exhibited the same specific activity for at least 1 month when it was stored under these conditions.

Determination of protein concentration, purity, and molecular mass. Protein concentrations were determined by the Bradford assay (4) by using bovine serum albumin as the standard. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed, and gels were stained blue by using established procedures (19). The Benchmark protein ladder (Invitrogen) containing proteins having molecular masses ranging from 10 to 220 kDa

FIG. 2. Location of P. aeruginosa PAO1 genome containing the pvdH gene (PA2413). The promoters of pvdH and PA2412, indicated by vertical lines, contain iron starvation box consensus sequences, suggesting that they are both regulated by the sigma factor, PvdS (21).
RESULTS

Construction and analysis of pvdH insertion mutant. The pvdH gene from P. aeruginosa PAO1 was inactivated by insertion of a gentamicin cassette. When grown in iron-limiting CAA broth, the mutant did not produce pyoverdine, as determined by the absence of green fluorescence of the medium under UV light and the characteristic absorbance peak of pyoverdine at 405 nm (37). In addition, the mutant did not grow on LB agar containing the iron chelator 2,2'-dipyridyl. However, the mutant was able to produce pyoverdine and grow in media containing dipyridyl if it was complemented with an intact pvdH gene introduced in trans by using the broad-host-range plasmid pVLT31. Pyoverdine production was also restored in the mutant if it was grown in the presence of 1.5 mM pure 1,2,4-diaminobutyrate in the medium (Fig. 3). Together, these results are consistent with the prediction that PvdH is involved in the production of 1,2,4-diaminobutyrate for pyoverdine synthesis.

Analysis of asd insertion mutant. Aspartate β-semialdehyde dehydrogenase is an enzyme that catalyzes the formation of aspartate β-semialdehyde from aspartyl phosphate (Fig. 4) (13). Aspartate β-semialdehyde is a metabolic precursor for the amino acids lysine, threonine, isoleucine, methionine, and meso-diaminopimelate. The latter compound is a component of the peptidoglycan of P. aeruginosa. Accordingly, the asd knockout mutant was viable only if meso-diaminopimelate is added to CAA media. We reasoned that if 1,2,4-diaminobutyrate is formed from aspartate β-semialdehyde, the asd knockout mutant should also be pyoverdine negative. Indeed, when the asd mutant was grown in CAA broth containing only meso-diaminopimelate, no pyoverdine was produced. Pyoverdine production could be restored if the same mutant was grown in CAA broth containing 1,2,4-diaminobutyrate (Fig. 3). Surprisingly, in the presence of 1,2,4-diaminobutyrate, meso-diaminopimelate did not have to be added to the media for growth of the mutant. This could be explained by a reverse reaction catalyzed by PvdH, converting a fraction of the 1,2,4-diaminobutyrate in the media to aspartate β-semialdehyde, which could then be used for meso-diaminopimelate biosynthesis.

Expression, purification, and determination of the molecular mass of PvdH. PvdH was initially expressed in E. coli BL21(DE3) under control of the T7 promoter from plasmid pT7-7. However, the expression levels were low, and the PvdH protein was insoluble (data not shown). The low levels of expression may have been due to the presence of arginine (CGG and AGG), glycine (GGA), and proline (CCC) codons that are rare in E. coli (18). Introduction of the expression plasmid into E. coli BL21(DE3) RP, which contains a greater number of copies of tRNA for the codons AGG and CCC, did not significantly improve PvdH expression, and the protein remained insoluble. PvdH was then transferred to P. aeruginosa PAO1 and expressed in a soluble form under control of the tac promoter from plasmid pVLT31. PvdH was purified from crude extracts by using anion-exchange and gel filtration chromatography (Table 1). The enzyme was purified up to 221-fold with a yield of 7%. The final enzyme preparation was judged to be more than 90% pure by SDS-PAGE analysis (Fig. 5).

During purification, fractions containing PvdH were faintly yellow, with an absorbance maximum at 410 nm. This is consistent with the presence of the cofactor pyridoxal phosphate.

PvdH has a molecular mass of 50 kDa as determined by SDS-PAGE. This is consistent with the calculated molecular mass based on the amino acid sequence, 50.2 kDa. The relative
molecular mass of the native enzyme, as determined by gel filtration, was approximately 183 ± 6.6 kDa, suggesting that the enzyme forms a homotetramer.

Coupled assay to determine PvdH activity. PvdH was predicted to catalyze the reversible transamination of L-2,4-diaminobutyrate (Fig. 4). L-Aspartate-semialdehyde is not available commercially. Therefore, the activity of PvdH was measured in the reverse direction by using L-2,4-diaminobutyrate as the amino donor. A novel coupled assay involving the use of homoserine dehydrogenase from S. cerevisiae was developed. Homoserine dehydrogenase catalyzes the reversible conversion of L-aspartate-semialdehyde to L-homoserine, with concomitant oxidation of NADH, which can be monitored spectrophotometrically at 340 nm. Homoserine dehydrogenase from S. cerevisiae is a highly specific enzyme, accepting only the amino acids L-aspartate-semialdehyde and L-homoserine as substrates (15), and thus it is suited for use in this coupled assay.

The reliability of this assay was assessed by using several criteria. The concentrations of homoserine dehydrogenase and NADH in each coupled assay were confirmed to be in excess, since addition of higher concentrations of these constituents did not result in higher PvdH activity. In addition, a linear relationship between the concentration of PvdH and the resultant PvdH activity was verified (data not shown). Finally, if the enzyme reaction was allowed to proceed to completion (end point assay), the amount of NADH consumed, calculated from the decrease in absorbance at 340 nm, corresponded to the total amount of L-2,4-diaminobutyrate in the assay mixture (99.7%), thus demonstrating that there was stoichiometric detection of products. This is in line with previous studies which showed that the equilibrium of the reaction catalyzed by homoserine dehydrogenase strongly favors L-aspartate-semialdehyde reduction at pH 7.5 (16).

Kinetic analysis of PvdH. PvdH activity was measured by using L-2,4-diaminobutyrate as the amino donor and α-ketoglutarate, pyruvate, oxaloacetate, or α-ketobutyrate as the amino acceptor. A Lineweaver-Burk plot of the data obtained with α-ketoglutarate has a series of

TABLE 1. Purification of PvdH from P. aeruginosa PA01

| Step       | Protein (mg) | Activity (U) | Sp act (U/mg) | Recovery (%) | Purification (fold) |
|------------|--------------|--------------|---------------|--------------|---------------------|
| Cell extract | 796          | 1.42         | 0.01          | 100          | 1                   |
| Source 15Q | 44           | 0.2          | 1.72          | 14           | 172.2               |
| Superdex 200 | 26           | 0.09         | 2.21          | 1            | 220.7               |

*One unit was the amount of enzyme required to oxidize 1 μmol of NAD(P)H/min at pH 7.5
against the reciprocal of the mM (\(1/F\)) diaminobutyrate concentration. The Pyruvate 6.195 \(\text{-Ketoglutarate 0.18}\) specificity constant (\(K_m\)). The enzyme from Halomonas elongata (33% identity). Neither of the enzymes aminotransferases from A. baumannii (51% identity) and Halomonas elongata (33% identity). Neither of the enzymes from these bacteria has been associated with siderophore synthesis. The enzyme from A. baumannii is involved in the synthesis of 1,3-diaminopropane (14), while the enzyme from H. elongata catalyzes the first step in the ectoine biosynthesis pathway (28).

The molecular weight of native PvdH from P. aeruginosa PAO1 determined by gel filtration suggested that the protein forms a homotetramer. Similarly, the recombiant enzyme from A. baumannii has also been reported to be a homotetramer (14). However, this contrasts with the L-2,4-diaminobutyrate aminotransferase of H. elongata, which forms a homohexamer (28).

Both continuous (2, 32) and discontinuous (14, 28) assays have been used to test aminotransferase activity. In continuous reactions, glutamate and malate dehydrogenases have been coupled with aspartate aminotransferases from beef liver and pig heart, respectively. Solely discontinuous assays have been used to determine the activity of 1,2,4-diaminobutyrate aminotransferases from A. baumannii and H. elongata (14, 28). The continuous assay described in this report provides a more convenient and rapid method to obtain substrate specificity constants for keto acids in the reverse reaction. This also allowed us to determine the true \(K_m\) and \(k_{cat}\) values for this aminotransferase.

Kinetic parameters for the 1,2,4-diaminobutyrate aminotransferase from H. elongata have not been reported. A previous study of the enzyme from A. baumannii, on the other hand, yielded apparent \(K_m\) values of 4.30 mM for L-2,4-diaminobutyrate and 1.46 mM for \(\alpha\)-ketoglutarate (14). In contrast, the true \(K_m\) values for L-2,4-diaminobutyrate and \(\alpha\)-ketoglutarate amino acceptor was too low (0.14 ± 0.016 U/mg at 30 mM oxaloacetate) for determination of kinetic parameters. No activity was detected when \(\alpha\)-ketobutyrate was used as the amino acceptor.

**DISCUSSION**

The chromophore of pyoverdine is produced from condensation of L-2,4-diaminobutyrate and D-tyrosine (3). We demonstrated in this study that L-2,4-diaminobutyrate is synthesized from aspartyl phosphate in P. aeruginosa. This conversion requires two enzymatic activities, aspartate \(\beta\)-semialdehyde dehydrogenase and an aminotransferase, which are encoded by \(asd\) and \(pvdH\), respectively.

PvdH exhibits high amino acid sequence similarity (~85%) to its homologues in *Pseudomonas fluorescens*, *Pseudomonas syringae*, and *Pseudomonas putida*. The genes encoding PvdH homologues in these *Pseudomonas* species are in a genome locus containing other pyoverdine synthesis genes (31). PvdH exhibits lower sequence identity with L-2,4-diaminobutyrate aminotransferases from *A. baumannii* (51% identity) and *Halomonas elongata* (33% identity). Neither of the enzymes from these bacteria has been associated with siderophore synthesis. The enzyme from *A. baumannii* is involved in the synthesis of 1,3-diaminopropane (14), while the enzyme from *H. elongata* catalyzes the first step in the ectoine biosynthesis pathway (28).

| Amino acceptor | \(K_m\) for amino acceptor (mM) | \(K_m\) for L-2,4-diaminobutyrate (mM) | \(k_{cat}\) (s\(^{-1}\)) | \(k_{cat}/K_m\) for amino acceptor (mM\(^{-1}\) s\(^{-1}\)) | \(k_{cat}/K_m\) for L-2,4-diaminobutyrate (mM\(^{-1}\) s\(^{-1}\)) |
|---------------|---------------------------------|--------------------------------------|-----------------|---------------------------------|---------------------------------|
| \(\alpha\)-Ketoglutarate | 0.18 ± 0.0075 | 0.26 ± 0.0076 | 1.39 ± 0.032 | 7.61 | 5.34 |
| Pyruvate | 6.195 ± 0.196 | 0.291 ± 0.012 | 1.15 ± 0.026 | 0.183 | 3.95 |

![FIG. 6. Lineweaver-Burk and secondary plots of PvdH activities with L-2,4-diaminobutyrate and \(\alpha\)-ketoglutarate as substrates. (A) Lineweaver-Burk plot showing the relationship of PvdH activity and L-2,4-diaminobutyrate concentration. The \(\alpha\)-ketoglutarate concentration was fixed at 100 \(\mu\)M (●), 150 \(\mu\)M (○), 200 \(\mu\)M (♦), 300 \(\mu\)M (◇), or 1 mM (■). (B) \(y\) intercepts from the Lineweaver-Burk graph plotted against the reciprocal of the \(\alpha\)-ketoglutarate concentration.](http://jb.asm.org/)}
were lower by an order of magnitude at 0.26 mM and 0.18 mM, respectively, for PvdH. The $K_{cat}$ for pyruvate with the A. baumannii enzyme has not been reported; however, the specific activity for pyruvate was reported to be 4% of the activity obtained with $\alpha$-ketoglutarate. This specific activity was determined by using 10 mM keto acid. In contrast, the $K_{cat}$ of PvdH when pyruvate was used as the amino acceptor is similar to the value obtained with $\alpha$-ketoglutarate (Table 2). By using the kinetic parameters determined for PvdH, the specific activity of PvdH with 10 mM pyruvate and 10 mM 1,2,4-diaminobutyrate was calculated to be 52% of the activity obtained with 10 mM $\alpha$-ketoglutarate, which is significantly greater than the corresponding relative activities reported for the A. baumannii enzyme.

PvdH can also utilize oxaloacetate as an amino acceptor, although the specific activity is 20-fold lower than that with $\alpha$-ketoglutarate. No activity was observed with $\alpha$-ketobutyrate. It appears that carbon chain length and the presence of the distal carboxyl group of the keto acids have important bearing on substrate specificity. For example, $\alpha$-ketobutyrate lacks a distal carboxyl group and is one carbon shorter than $\alpha$-ketoglutarate. Although oxaloacetate is also one carbon shorter than $\alpha$-ketoglutarate, it possesses a distal carboxyl group, and the enzyme shows some activity with this amino acceptor. It is, however, possible that a bulky carboxyl group at the $\beta$ carbon position of the keto acid, like that in oxaloacetate, also results in an unfavorable steric interaction; therefore, the specific activity with this substrate is lower than that with $\alpha$-ketoglutarate. Thus, higher activity was observed with pyruvate, which does not contain a distal carboxyl group or a $\beta$-substituent. The absence of a distal carboxyl group combined with a shorter side chain has a prominent effect on the $K_{cat}$ for pyruvate, which is 34-fold higher than that for $\alpha$-ketoglutarate.

We found that 1,2,4-diaminobutyrate, which is required for pyoverdine synthesis, is produced by the aminotransferase PvdH. It has been predicted that during the synthesis of the pyoverdine chromophore, $\alpha$-ketoglutarate, it possesses a distal carboxyl group, and the enzyme shows some activity with this amino acceptor. It is, however, possible that a bulky carboxyl group at the $\beta$ carbon position of the keto acid, like that in oxaloacetate, also results in an unfavorable steric interaction; therefore, the specific activity with this substrate is lower than that with $\alpha$-ketoglutarate. Thus, higher activity was observed with pyruvate, which does not contain a distal carboxyl group or a $\beta$-substituent. The absence of a distal carboxyl group combined with a shorter side chain has a prominent effect on the $K_{cat}$ for pyruvate, which is 34-fold higher than that for $\alpha$-ketoglutarate.

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