Involvement of a transformylase enzyme in siderophore synthesis in Pseudomonas aeruginosa

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Fluorescent pseudomonads produce yellow-green siderophores when grown under conditions of iron starvation. Here, the characterization of the pvdF gene, which is required for synthesis of the siderophore pyoverdine by Pseudomonas aeruginosa strain PAO1, is described. A P. aeruginosa pvdF mutant was constructed and found to be defective for production of pyoverdine, demonstrating the involvement of PvdF in pyoverdine synthesis. Transcription analysis showed that expression of pvdF was regulated by the amount of iron in the growth medium, consistent with its role in siderophore production. DNA sequencing showed that pvdF gives rise to a protein of 31 kDa that has similarity with glycinamide ribonucleotide transformylase (GART) enzymes involved in purine synthesis from a wide range of eukaryotic and prokaryotic species. Chemical analyses of extracts from wild-type and pvdF mutant bacteria indicated that the PvdF enzyme catalyses the formylation of N5-hydroxyornithine to give rise to N5-formyl-N5-hydroxyornithine, a component of pyoverdine. These studies enhance understanding of the enzymology of pyoverdine synthesis, and to the best of the authors’ knowledge provide the first example of involvement of a GART-type enzyme in synthesis of a secondary metabolite.

Keywords: pyoverdine, glycinamide ribonucleotide transformylase, secondary metabolite synthesis, evolution of biosynthetic pathways

INTRODUCTION

Siderophores – chelating compounds that scavenge iron (Fe3+) ions from the environment – are secreted by very many micro-organisms (Drechsel & Winkelmann, 1997). Fluorescent pseudomonads are characterized by the production of yellow-green siderophores termed pyoverdines or pseudobactins. These molecules are thought to be associated with biocontrol of fungal pathogens in the biosphere (Leong, 1986), and pyoverdine is required for virulence of the opportunistic mammalian pathogen, Pseudomonas aeruginosa (Meyer et al., 1996). They consist of a quinoline-type chromophore attached to a dicarboxylic acid and a short (6–12 residue) strain-specific peptide (Abdallah, 1991; Budzikiewicz, 1993). For P. aeruginosa strain PAO1, the peptide contains two residues each of d-serine, l-threonine and Nδ-formyl-Nδ-hydroxyornithine and one residue each of l-arginine and l-lysine (Fig. 1). The presence of unusual amino acids suggests that the peptide moieties of pyoverdines are synthesized by a non-ribosomal mechanism, and the finding that genes required for pyoverdine synthesis by P. aeruginosa probably encode peptide synthetase (Merriman et al., 1995; Lehoux et al., 2000) is consistent with this. Synthesis of one of the amino acids, Nδ-formyl-Nδ-hydroxyornithine, requires the product of the pvdA gene, which catalyses the hydroxylation of ornithine and is essential for pyoverdine production (Visca et al.,

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Abbreviations: EDDA, ethylenediamine(o-hydroxy)phenylacetic acid; GART, glycinamide ribonucleotide transformylase.
The GenBank accession number for the sequence reported in this paper is U07359.
Synthesis of the chromophoric component of pyoverdine requires at least four genes, pvcA–D, which are thought to be arranged in an operon (Stintzi et al., 1999).

Two other genes that are required for pyoverdine synthesis have been characterized. The pvdS gene encodes a protein, PvdS, that is an alternative sigma factor and is required for expression of all other pvd genes that have been examined (Cunliffe et al., 1995; Leoni et al., 1996, 2000; Miyazaki et al., 1995; Wilson & Lamont, 2000), as well as being involved in expression of the gene encoding exotoxin A (Ochsner et al., 1996). PvdS is also required for expression of the ptxR gene, which encodes a transcriptional activator required, in turn, for expression of the pvc genes (Stintzi et al., 1999; Vasil et al., 1998). Expression of pvdS is repressed by the Fur repressor protein in iron-rich cells, explaining the absence of pyoverdine synthesis under these conditions (Leoni et al., 1996; Ochsner et al., 1996). The pvdE gene encodes an ABC-type transporter protein and may be required for secretion of pyoverdine or a precursor (McMorran et al., 1996).

To better understand the molecular events involved in pyoverdine biosynthesis, we have now characterized a further gene, named pvdF, that is required for this process.

**METHODS**

**Growth conditions.** Strains of *Escherichia coli* were grown in Luria (L) broth (Sambrook et al., 1989) and strains of *P. aeruginosa* were grown in brain–heart infusion broth (Gibco-BRL), King’s B broth (King et al., 1954) or minimal medium (Sambrook et al., 1989), solidified by addition of agar and supplemented as necessary with ampicillin, chloramphenicol, kanamycin and tetracycline as described previously (Cunliffe et al., 1995). Media were also supplemented with FeCl₃ (60 μg ml⁻¹), ethylenediamine-o-hydroxy)phenylacetic acid (EDDA) (200 μg ml⁻¹) and adenine (0.1 mM) as required.

**Measurement of pyoverdine.** To measure pyoverdine production, bacteria were grown in King’s B broth (King et al., 1954) to stationary phase and the absorbances of culture supernatants were measured between 350 and 500 nm. Pyoverdine has a characteristic absorbance spectrum in this range, with a peak at 403 nm (Hohnadel et al., 1986). **Nucleic acid methodology.** Enzymes were purchased from Boehringer Mannheim, except for the Klenow fragment of DNA polymerase I (Amersham) and T4 polynucleotide kinase (New England Biolabs) and were used under the conditions recommended by the manufacturers. Preparation of plasmid DNA, treatment of DNA with enzymes, subcloning of DNA and transformation of plasmid constructs into *E. coli* were carried out using standard methods (Sambrook et al., 1989). DNA molecules were end-polished by using T4 DNA polymerase and the Klenow fragment (Sambrook et al., 1989). Oligonucleotides were obtained from Otagoligos (Centre for Gene Research, Otago University, Dunedin, New Zealand) and Macromolecular Resources (Colorado State University, USA).

For DNA sequencing, plasmid DNA was prepared using the Wizard Plus Minipreps DNA Purification System (Promega). The sequences of both DNA strands were determined by using chemically synthesized oligonucleotides, and PsfI and KpnI restriction fragments of pSOT3 (Rombel & Lamont, 1992), in conjunction with an automated sequencer. The sequence was determined across all restriction sites used in subcloning. The sequences were analysed by standard methods with version 8.1-UNIX of the Computer Genetics Group package (Derevets et al., 1984) in conjunction with other programs as described previously (Merriman et al., 1995); sequence alignments were performed using pileup (Derevets et al., 1984) and residues were highlighted using BoxShade (http://www.ch.embnet.org/software/BOX_form.html). Southern analysis was done by standard methods (Sambrook et al., 1989) with chromosomal DNA prepared by the method of Chen & Kuo (1993), and Northern (RNA) analysis was done as described previously (Rombel et al., 1995).

**Creating a mutation in pvdF.** A pvdF mutant strain of *P. aeruginosa* was constructed using a strategy similar to one we have used previously for mutating genes in *P. aeruginosa* (Cunliffe et al., 1995; Markie et al., 1986). Plasmid pSOT3 DNA was treated with *Xho*I, which has a unique site within pvdF, and ligated to a kanamycin resistance cassette (kan) that had been purified from agarose following SalI digestion of pUC18-19Km (Markie et al., 1986). The ligated DNA was transformed into *E. coli* MC1061 (Casabdan & Cohen, 1980) with selection for Ap′ and Km′ bacteria. Plasmid DNA was prepared from one transformant, and restriction analysis confirmed that the kan gene had been inserted correctly into pvdF; the resulting plasmid was named pSOT3Km. Purified pSOT3Km DNA was treated with SalI to release a fragment containing the mutated pvdF gene. This fragment was end-polished and subcloned into pSUP202 (Simon et al., 1986) which had been treated with EcoRI and end-polished. The DNA was transformed into *E. coli* MC1061 to give Ap′ Km′ Te′ transformants; restriction analysis confirmed that the plasmid DNA of one such transformant (pSUPKm) consisted of pSUP202 carrying the mutated pvdF gene. This plasmid was transferred by triparental conjuction into *P. aeruginosa* PAO1 (Holloway, 1955) in conjunction with helper plasmid pRK2013 (Figurski & Helinski, 1979) as described previously (Merriman & Lamont, 1993), with selection for Te′ Km′ bacteria which arose following recombination of the plasmid into the chromosome. A second recombination event was required for excision of the plasmid and loss of the wild-type pvdF gene, and bacteria in which this had occurred were identified as being Te′ Km′.

**Chemical determinations.** Wild-type *P. aeruginosa* PAO1 and the pvdF mutant strain were grown in succinate medium.
(Meyer & Abdallah, 1978) to early stationary phase (OD₆₀₀ 1.0–1.2). Cell lysates were prepared by sonication and samples were incubated at 20 °C for 12–16 h after the addition of an equal volume (2 ml) of sodium phosphate buffer (100 mM, pH 7.0) containing 2 mM sodium pyruvate and 4 mM l-ornithine as described previously (Visca et al., 1994). The amounts of hydroxylamine nitrogen were then determined using the Csaky test as modified by Gillam et al. (1971) with hydroxylamine hydrochloride as a standard. The combined amounts of hydroxamate and hydroxylamine nitrogen were determined using a modification of the acid hydrolysis method of Gibson & McGrath (1970), which converts hydroxamate groups into hydroxylamines. Samples were heated at 130 °C for 30 min in the presence of 12 M HCl and neutralized by the addition of an excess of CaCO₃. The amounts of hydroxylamine nitrogen were then assayed, with each assay being carried out in triplicate. Assays were also carried out with a P. aeruginosa PAO1 pvdS mutant that does not express genes involved in pyoverdine synthesis (Cunliffe et al., 1995; Leoni et al., 1996; Miyazaki et al., 1995) in order to determine hydroxylamine nitrogen generated through other pathways. The levels of reactive material obtained with this strain (equivalent to approx 62 nmol hydroxylamine without hydrolysis, and 77 nmol hydroxylamine with hydrolysis) were subtracted from those obtained with the wild-type and pvdS mutants in order to determine the amounts of pyoverdine-related reactive material for these strains.

RESULTS

Sequence analysis

We have previously isolated a clone, λOT1 (Fig. 2), that contains DNA required for synthesis of pyoverdine (Rombel & Lamont, 1992). Subsequent studies showed that this clone hybridized with five distinct iron-regulated transcripts from P. aeruginosa (Rombel et al., 1995), with three of these corresponding to the pvdD, fpvA and pvdE genes (McMorran et al., 1996; Merriman et al., 1995; Poole et al., 1993). The first aim of this study was to characterize the gene corresponding to another of the iron-regulated transcripts. This transcript (1200 bases) corresponded to a 1.4 kb SalI fragment (Fig. 2).

The DNA sequence of the 1.4 kb SalI fragment was determined and is the same as that recently determined as part of the Pseudomonas genome sequencing project (Stover et al., 2000). Analysis of the sequence revealed one complete extended ORF, which was named pvdF. This ORF showed similar codon usage to other genes from P. aeruginosa (West & Iglewski, 1988) and so was highly likely to be expressed. The corresponding protein, PvdF, has 275 amino acids and a predicted molecular mass of 30985 Da. The pvdF DNA sequence has a G+C content of 61%, which is at the lower end of the range of genes from P. aeruginosa (West & Iglewski, 1988). The proposed translational start site of pvdF is preceded by a consensus ribosome-binding site (GGGAA) 7 bp upstream of the stop codon (Yager & von Hippel, 1987) and consists of a stem–loop structure with a predicted stability of −23.8 kJ mol⁻¹, followed by a thymine-rich sequence.

To identify proteins similar to PvdF, the predicted amino acid sequence was compared to protein sequence databases using PSI-BLAST (Altschul et al., 1997). All of the most similar proteins were glycaminamide ribonucleotide transformylase (GART; EC 2.1.2.2) enzymes from a very wide variety of species, including bacteria, fungi, plants and animals and an alignment of PvdF and some GART enzymes is shown in Fig. 3. Pairwise alignments using GAP (Devereux et al., 1984) showed that PvdF has 23–27% identity and 48–58% similarity with various GART enzymes including those shown in Fig. 3. GART is involved in the de novo synthesis of purines, catalysing the formylation of glycaminamide ribonucleotide to formylglycinamide ribonucleotide in conjunction with the cofactor N₁⁰-formyltetrahydrofolate (Buchanan & Hartman, 1959). Several of the residues identified as being important for the function of the E. coli version of GART (Almassy et al., 1992; Chen et al., 1992; Warren et al., 1996; Shim & Benkovic, 1999; Greasley et al., 1999) are present in the PvdF sequence (Fig. 3). This suggests that PvdF possesses a similar catalytic activity.

Involvement of pvdF in pyoverdine synthesis

It was expected that the product of the pvdF gene was involved in pyoverdine metabolism since the transcript corresponding to this gene is strongly iron-regulated (Rombel et al., 1995) and the sequence is adjacent to

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Fig. 2. The DNA used in this study. Clone λOT1 was originally isolated by Rombel & Lamont (1992). The positions of the pvdD, fpvA, pvdE and pvdF genes (McMorran et al., 1996; Merriman et al., 1995; Poole et al., 1993; this study) and an iron-regulated transcript of 1200 bases (Rombel et al., 1995) are shown. The lower part of the figure represents a 1.4 kb SalI fragment for which the sequence was determined as part of this study. B. BamHI; E. EcoRI; K. KpnI; P. PstI; S. SalI.

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other pyoverdine genes on the *P. aeruginosa* chromosome. However, the similarity of the *pvdF* gene product to GART enzymes raised the possibility that the gene was involved in purine synthesis. To investigate the function of *pvdF*, a mutant strain lacking this gene was constructed through an approach that we have used previously for generating mutations in chromosomal genes of *P. aeruginosa* (Cunliffe et al., 1995).

The *pvdF* coding sequence was disrupted *in vitro* by the insertion of a kanamycin resistance (*kan*) cassette derived from pUC18-19Km (Markie et al., 1986) into the XhoI site of the *pvdF* gene. The wild-type gene was then replaced with the mutant version as described in the Methods. Southern blotting, and also PCR with primers specific to *pvdF* and the *kan* cassette, followed by sequencing of the PCR product, confirmed that the expected recombination events had taken place (data not shown); the mutant strain was named PAO1 *pvdF*.

The *pvdF* mutant was analysed for production of pyoverdine and the ability to make purines. King's B broth...
medium (King et al., 1954) stimulates production of pyoverdine, observed as a yellow-green fluorescent compound. No pyoverdine synthesis was detectable when PAO1pvdF was grown on solid or in liquid King’s B, as observed previously with mutations in other pyoverdine genes (Cunliffe et al., 1995; McMorrân et al., 1996; Merriman et al., 1995), whereas the wild-type strain made large amounts of pyoverdine. In addition, the pvdF mutant strain was unable to grow on King’s B agar containing EDDA, an iron-chelating agent that prevents the growth of pyoverdine-deficient strains, whereas the isogenic wild-type strain grew on this medium. These data show that PAO1pvdF was unable to make pyoverdine. However, this strain was able to grow on EDDA-containing medium when pyoverdine was supplied from wild-type cells, indicating that its ability to take up and utilize ferri-pyoverdine was not affected. Reintroduction of the pvdF gene that had been cloned into plasmid pUCP22 (West et al., 1994) restored the ability of PAO1pvdF to make pyoverdine, confirming the involvement of pvdF in pyoverdine synthesis.

To test the possible involvement of the pvdF gene product in purine synthesis, the mutant strain and the isogenic wild-type strain were inoculated onto minimal medium lacking purines. Both strains showed good growth, indicating that the PvdF protein is not essential for purine synthesis by P. aeruginosa. Analysis of the P. aeruginosa genome sequence (Stover et al., 2000) showed that a gene located at nt 1032713–1032048 in the genome encodes an enzyme that is 53% identical (72% similar) to GART from E. coli and is likely to be the GART enzyme of P. aeruginosa; this sequence was used in the alignment (Fig. 3).

Transcriptional analysis of pvdF

Transcription of pvdF was analysed using Northern blotting (Fig. 4). A single hybridizing band was obtained with RNA extracted from iron-starved cells and no bands were detectable in RNA from cells grown in high-iron medium. This is consistent with previous results (Rombel et al., 1995) and confirms that the level of iron regulates pvdF. The size of the hybridizing band, 0.95 kb, was less than that estimated previously (1.2 kb), although it corresponds well with the size of pvdF. The value determined here is likely to be more accurate as it was obtained by comparison with RNA molecular mass standards; previous estimates were made using the rRNA bands (Rombel et al., 1995).

Enzymic activity of PvdF

Analysis of the sequence of PvdF showed that it has similarity to enzymes that catalyse the formylation of glycinamide ribonucleotide. Examination of the structure of pyoverdine (Fig. 1) showed that the only likely reaction involving formylation at a nitrogen atom was in the conversion of N⁵-hydroxyornithine, formed by the product of the pvdA gene (Visca et al., 1994), to N⁵-formyl-N⁵-hydroxyornithine. We therefore hypothesized that pvdF catalyses this reaction in wild-type bacteria, whereas a pvdF mutant strain would synthesize N⁵-hydroxyornithine but would be unable to synthesize N⁵-formyl-N⁵-hydroxyornithine. To test this hypothesis, we prepared cell extracts from wild-type and pvdF bacteria, incubated them with ornithine to allow synthesis of N⁵-hydroxyornithine and N⁵-formyl-N⁵-hydroxyornithine, and then measured the amounts of hydroxylamine nitrogen present after, or without, acid hydrolysis as described in Methods. Hydrolysis converts hydroxamates into hydroxylamines so that the values obtained without hydrolysis correspond to the amount of hydroxylamines and the values obtained after hydrolysis correspond to the total amount of hydroxylamine and hydroxamate nitrogen. If the hypothesis were correct the amount of hydroxylamine nitrogen (N⁵-hydroxyornithine) would be the same for the pvdF mutant whether or not hydrolysis was carried out, as no N⁵-formyl-N⁵-hydroxyornithine would be formed; for the wild-type, values would be higher after hydrolysis as N⁵-formyl-N⁵-hydroxyornithine would be formed in the cell extract.

For wild-type bacteria, cell extracts contained 3.3 ± 2.0 nmol ml⁻¹ (mean ± sp) hydroxylamine nitrogen in the absence of acid hydrolysis. This increased to 10.1 ± 3.8 nmol ml⁻¹ following hydrolysis, showing that a significant amount of hydroxamate nitrogen was present. The hydroxylamine nitrogen detected in the absence of acid hydrolysis may reflect incomplete synthesis of N⁵-formyl-N⁵-hydroxyornithine from N⁵-hydroxyornithine. By contrast, the amount of hydroxylamine nitrogen in cell extracts from the pvdF mutant bacteria was 10.0 ± 6.8 nmol ml⁻¹ in the absence of acid hydrolysis.
hydrolysis and this did not change significantly following hydrolysis (116 ± 7.0 nmol ml⁻¹). This indicates that these bacteria were unable to form N⁴-formyl-N⁵-hydroxyornithine and consequently accumulated N⁵-hydroxyornithine. The amounts of hydroxylamine formed by the pvdF cell extracts were comparable to the amounts obtained with wild-type extracts after acid hydrolysis, showing that the mutant bacteria were not defective for hydroxylamine synthesis. These data support the hypothesis that PvdF catalyses the formation of N⁴-formyl-N⁵-hydroxyornithine from N⁵-hydroxyornithine.

DISCUSSION

The complexity of pyoverdines indicates that a large number of enzymes are likely to be required for their synthesis and, consistent with this, genetic studies indicate that a large number of genes are involved (Tsuda et al., 1995; Stintzi et al., 1999). The analysis of gene sequences, coupled to genetic and biochemical studies, provides insights into the pathways of pyoverdine synthesis. We have shown here that the pvdF gene is required for pyoverdine synthesis in P. aeruginosa PAO1. The sequence of pvdF indicates that its gene product catalyses formylation of N⁵-hydroxyornithine to give rise to the N⁴-formyl-N⁵-hydroxyornithine residues that are present in pyoverdine from P. aeruginosa strain PAO1. This likelihood is strongly supported by analyses of the amounts of hydroxylamine and hydroxamate residues formed by extracts of wild-type and PvdF bacteria in the presence of ornithine. These analyses indicate that the mutant strain is able to form N⁵-hydroxyornithine but is unable to convert this into N⁴-formyl-N⁵-hydroxyornithine. Overall, the data indicate that the N⁴-formyl-N⁵-hydroxyornithine residues present in pyoverdinePAO1 are formed from ornithine by the sequential action of PvdA (forming N⁵-hydroxyornithine) and PvdF (forming N⁴-formyl-N⁵-hydroxyornithine) and are then incorporated into pyoverdine by a peptide synthetase enzyme.

Micro-organisms produce a wide range of secondary metabolites and many of the genes that are involved are clearly members of multi-gene families that are specific to secondary metabolite synthesis. These include the peptide synthetase and polyketide synthetase families that are involved in very many pathways of secondary metabolism (Zuber & Marahiel, 1997; Khosla et al., 1999). In contrast to these gene families, the pvdF gene has similarity to GART, an enzyme of primary metabolism. However, pvdF is required for synthesis of pyoverdine, a secondary metabolite, and not for purine synthesis. This indicates that an enzyme involved in primary metabolism has been co-opted into the biosynthetic pathway for production of a secondary metabolite. So far as we are aware, this is the first report of the involvement of a GART-like enzyme in synthesis of a secondary metabolite. The PvdF sequence has three residues that are known to be involved in catalysis in E. coli GART, and also a high level of similarity at sites that are likely to interact with the formyl tetrahydrofolate co-substrate (Fig. 3), suggesting that PvdF may utilize the same co-substrate. A complete characterization of the reaction catalysed by PvdF will require purification of the protein. However, some of the differences between PvdF and GART enzymes presumably reflect the involvement of a different substrate. A number of inhibitors of GART enzymes have been identified (Kamen, 1997; Takimoto, 1997) and it will be of interest to determine whether any of these inhibit PvdF, or whether information on GART–inhibitor interactions can enable development of inhibitors of PvdF.

PvdF is only slightly more similar to the likely GART enzyme of P. aeruginosa than to the other GART enzymes shown in Fig. 3 so that either pvdF is the product of duplication of the GART-encoding gene followed by very extensive sequence divergence, or it was acquired by horizontal gene transfer. Siderophores produced by other species of bacteria, including ornibactin from Burkholderia cepacia (Stephan et al., 1993) and exochelin MS from Mycobacterium smegmatis (Sharman et al., 1995), also contain N⁴-formyl-N⁵-hydroxyornithine residues and it remains to be determined whether these bacteria contain enzymes with similarities to PvdF.

P. aeruginosa contains at least one other documented example of a gene of secondary metabolism with a clear homologue in a primary metabolic pathway. Synthesis of the phenazine pigment pyocyanin involves an anthranilate synthase encoded by the phnA and phnB genes, and this anthranilate synthase has a high level of similarity to enzymes involved in tryptophan synthesis in P. aeruginosa and other organisms (Essar et al. 1990); indeed, the phnAB gene pair was able to complement mutations in the corresponding trp genes in E. coli. Intriguingly, PhnA and B were more similar to tryptophan anthranilate synthase from E. coli than the corresponding enzyme from P. aeruginosa, suggesting that horizontal gene transfer may have played a role in the evolution of the pathway for synthesis of this secondary metabolite.

In summary, the data presented here provide further insights into the pathways of siderophore synthesis in P. aeruginosa, and also provide the first example of the involvement of a GART-like enzyme in secondary metabolite synthesis. Analysis of the complete sequences of microbial genomes is likely to provide other examples of enzymes from pathways of primary metabolism that have been incorporated into pathways of secondary metabolite synthesis.

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