The 1.8 Å Crystal Structure of PA2412, an MbtH-like Protein from the Pyoverdine Cluster of Pseudomonas aeruginosa*

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Many bacteria use nonribosomal peptide synthetase (NRPS) proteins to produce peptide antibiotics and siderophores. The catalytic domains of the NRPS proteins are usually linked in large multidomain proteins. Often, additional proteins are coexpressed with NRPS proteins that modify the NRPS peptide products, ensure the availability of substrate building blocks, or play a role in the import or export of the NRPS product. Many NRPS clusters include a small protein of ~80 amino acids with homology to the MbtH protein of mycobactin synthesis in Mycobacteria tuberculosis; no function has been assigned to these proteins. Pseudomonas aeruginosa utilizes an NRPS cluster to synthesize the siderophore pyoverdine. The pyoverdine peptide contains a dihydroxyquinoline-based chromophore, as well as two formyl-N-hydroxyornithine residues, which are involved in iron binding. The pyoverdine cluster contains four modular NRPS enzymes and 10–15 additional proteins that are essential for pyoverdine production. Coexpressed with the pyoverdine synthetic enzymes is a 72-amino acid MbtH-like family member designated PA2412. We have determined the three-dimensional structure of the PA2412 protein and describe here the structure and the location of conserved regions. Additionally, we have further analyzed a deletion mutant of the PA2412 protein for growth and pyoverdine production. Our results demonstrate that PA2412 is necessary for the production or secretion of pyoverdine at normal levels. The PA2412 deletion strain is able to use exogenously produced pyoverdine, showing that there is no defect in the uptake or utilization of the iron-pyoverdine complex.

Iron is an essential cofactor for many proteins, playing both catalytic and structural roles (1–3). Because of the low solubility of free Fe³⁺, many bacteria produce siderophores that bind to iron. The iron-siderophore complex is then actively transported into the cell. Although some small molecules like citrate or salicylate are able to function as lower affinity siderophores, many specialized peptides with extremely high affinity for iron are produced nonribosomally by bacteria. These compounds are produced by nonribosomal peptide synthetases (NRPSs) that exist as modular proteins with multiple catalytic domains joined in a single protein. Often expressed with NRPS genes are other genes that encode proteins involved in additional essential steps including the synthesis of building blocks, siderophore export, import of the Fe³⁺ siderophore complex, or the removal Fe³⁺ from the imported siderophore (4, 5).

Pseudomonas aeruginosa is a bacterial pathogen that commonly causes infections in patients with cystic fibrosis (6). The siderophore pyoverdine (Fig. 1) is synthesized by P. aeruginosa (3) and has been correlated with virulence (7). Pyoverdine contains a cyclic peptide chain as well as a chemically modified dihydroxyquinoline-based chromophore that is responsible for iron binding. The peptide backbone of pyoverdine is synthesized by four large, multi-module NRPS proteins. The pyoverdine molecule contains proteinogenic amino acids as well as the unusual amino acids 2,4-diaminobutyrate and formyl-N-hydroxyornithine. P. aeruginosa produces multiple isoforms of pyoverdine that have been identified by mass spectrometry (8, 9). The peptide backbone, as dictated by the NRPS enzymes, remains constant, but variations in the chromophore and the N-terminal side chain have been observed. Alternate side chains include succinate, succinamide, malate, and γ-glutamate; the latter is believed to be the original amino acid incorporated into the peptide. Although the C-7 position of the chromophore is most commonly a hydride, sulfonyl and chloride groups have also been observed experimentally (8). Finally, pyoverdines isolated from P. aeruginosa have been observed to contain a single or double bond between the C-5 and C-6 position of the chromophore (9).

In addition to the NRPS proteins, 11 other proteins have been identified that are essential for proper pyoverdine production (10). Genetic deletion of any of these proteins prevents...
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FIGURE 1. Chemical structure of pyoverdine from P. aeruginosa PA01. The structure of pyoverdine is shown. The residues that form the peptide are labeled. DAB, 2,4-diaminobutyrate; fOHOrn, formyl-N-hydroxyornithine. The peptide is cyclized via an amide linkage between the side chain of Lys8 and the carboxyl group of Thr11. Variants of pyoverdine that have been identified include alternate N-terminal carboxylate, R1, referred to as the side chain that can include succinate, succinamide, malate, and glutamate residues; alternate constituents at C-7; and a single or double bond between C-5 and C-6.

Pyoverdine production and growth in the presence of the iron chelator EDDHA. Finally there are four proteins, PvcABCDD, that have been implicated in chromophore maturation (11), although the essential role of these proteins has been questioned (3). Because the enzymes that catalyze siderophore synthesis have been targeted recently for antibiotic development (12), insights into the pyoverdine synthetic pathway may identify new antibiotic targets.

Many NRPS clusters contain a small ∼80-amino acid protein belonging to the MbtH family, named after the MbtH protein from the mycobactin cluster of Mycobacteria tuberculosis (4). These proteins are ∼8–9 kDa in size, generally have calculated pI values ranging from 4.0 to 5.5, and exhibit no homology to proteins of known structure. Escherichia coli contains a member of this family with the cluster of genes regulated for synthesis and import of the siderophore enterobactin (2, 13). This protein, encoded by the ybdZ gene, is located on a polycistronic operon that contains the fes and entF genes. The fes gene encodes an enterobactin esterase that is responsible for release of iron from the imported enterobactin (14), whereas entF encodes the multidomain EntF NRPS protein that catalyzes the final steps in the synthesis of enterobactin (15, 16). E. coli proteins have been identified for all steps of enterobactin synthesis, and the necessary proteins for import, export, and cleavage of the enterobactin molecule have also been identified (1, 5). The functional roles of the YbdZ protein and all of the members of the MbtH family therefore remain unknown.

We have initiated a structural study of the proteins involved in siderophore production in E. coli and P. aeruginosa. We have recently determined the structures of the E. coli EntA (17) and EntB proteins (18). Here we present the 1.8 Å crystal structure of PA2412, the P. aeruginosa MbtH-like protein that is coregulated with other pyoverdine producing proteins (10). Additionally, growth experiments in the presence and absence of an iron chelator demonstrate that PA2412 is necessary for growth under iron-depleted conditions. The addition of pyoverdine extracted from culture supernatants of wild-type cells was able to support the growth of the PA2412 mutant cell line, demonstrating that the defect caused by PA2412 deletion is in production, stabilization, or export of the mature pyoverdine molecule and not in the import and utilization of the iron-siderophore complex.

EXPERIMENTAL PROCEDURES

Protein Production and Purification—The gene encoding PA2412 was obtained commercially from a distributor (Open Biosystems, Huntsville, AL) for the cDNA library of P. aeruginosa genes (19). The gene was amplified with PCR to incorporate restriction sites at the 5′ and 3′ ends and subcloned into a modified pET15b plasmid that contains a 5×His affinity tag and a TEV protease recognition site in place of the thrombin site (20). The final plasmid (pED235) was transformed into BL21(DE3) for protein production. Protein expression was induced by addition of 0.75 mM isopropyl β-D-thiogalactopyranoside to the growth medium. The cells were grown at 16 °C, and induction was continued overnight. The cells were harvested by centrifugation, and all of the remaining purification steps were performed at 4 °C. The cells were lysed by sonication in a lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 10 mM imidazole, 0.2 mM tris-carboxyethylphosphine, and clarified by centrifugation at 45,000 rpm for 45 min. The cell lysate was passed over a His-trap (GE-Healthcare) immobilized metal ion affinity column and washed with lysis buffer containing 50 mM imidazole, and bound proteins were eluted with the same buffer containing 300 mM imidazole. The protein was dialyzed against TEV cleavage buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.2 mM tris-carboxyethylphosphine, and 0.5 mM EDTA). After 24 h, TEV protease was added to the dialysis at a ratio of 1:100. The cleavage continued for 18 h; the clarified protein was passed over the His-Trap column a second time to remove uncleaved protein, the TEV protease, and contaminating proteins. The protein in the column flow-through was dialyzed against final crystallization buffer (20 mM Tris, pH 7.5, 0.2 mM tris-carboxyethylphosphine) and concentrated to ~6 mg/ml using an extinction coefficient at 280 nm of 20940 M−1 cm−1. Selenomethionine (SeMet) labeled protein was produced by the metabolic inhibition method (21) and purified in the same manner as wild-type protein. Both wild type and SeMet were subjected to matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Roswell Park Proteomics Facility, Buffalo, NY). The experimentally determined molecular mass of the native protein was 8646 Da (the calculated molecular mass was 8638 Da), confirming no chemical modifications to the recombinant protein and that the selenium incorporation occurred at all three Met residues with greater than 80% occupancy.
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Crystallographic data for selenomethionine and native data sets

| Peak | Inflection | Remote | Native |
|------|------------|--------|--------|
| Wavelength | 0.97910 Å | 0.97934 Å | 0.9641 Å | 0.97931 Å |
| Resolution | 1.9 Å | 2.0 Å | 2.0 Å | 1.8 Å |
| Rmerge | 51.6 Å | 51.6 Å | 51.7 Å | 51.5 Å |
| Rmerge (highest resolution shell) | 32.6 Å | 32.6 Å | 32.6 Å | 32.6 Å |
| Completeness | 7.8% (14.1%) | 4.3% (11.3%) | 5.6% (14.1%) | 7.2% (30.4%) |
| I/σ | 99.9% (99.9%) | 99.9% (99.9%) | 99.9% (99.9%) | 96.3% (80.2%) |
| No. of observations | 31,553 | 31,319 | 32,254 | 34,285 |
| No. of reflections | 6914 | 6917 | 6916 | 7822 |
| Anomalous phasing power | 6.21 | 1.90 | 3.11 |
| Isomorphic phasing power | 1.95 | 3.44 |

* Rmerge = \( \frac{\sum |I_{h} - I_{h}'|}{\sum I_{h}} \) where \( I_{h} \) and \( I_{h}' \) are the individual and mean intensities of all equivalent reflections, respectively.

* The values for the highest resolution shell are given in parentheses.

* Anomalous and isomorphic phasing powers are defined as the mean value of \( F_{c}\)/E and \( F_{c}'\)/E where \( F_{c}\) is the calculated anomalous scattering structure factor, \( F_{c}\) is the calculated heavy atom structure factor and E is the lack of closure error.

Crystallographic data (Table 1) were collected at Beam-line F2 of the Cornell High Energy Synchrotron Source.

Determination of the three-dimensional structure of PA2412—The structure of PA2412 was determined by multiwavelength anomalous dispersion methods using SeMet-labeled protein. Initial phases were determined by BnP (24), which identified the locations of the two internal methionines; the N-terminal methionine was not identified. The final solvent flattened phases from BnP were submitted to RESOLVE (25, 26) for automated chain tracing, which located 60 residues, Ile\(^{10}\)–Pro\(^{26}\), Tyr\(^{28}\)–His\(^{66}\), and four residues of alanine that could not be placed in the sequence. The model was completed through iterative refinement with REFMAC5 (27) and manual model building with COOT (28). The final model contains residues 8–68 and 76 molecules of solvent. Residues 1–7 and 69–72, as well as the side chain of residue Asp\(^{9}\), were disordered. The final refinement statistics for PA2412 are shown in Table 2. The atomic coordinates and structure factors for PA2412 have been deposited in the Protein Data Bank (accession code 2PST).

Extraction of Pyoverdine from Wild-type PA01 Culture Supernatants—Pyoverdine was obtained from culture medium using the method of Budzikiewicz and colleagues (29). Briefly, the cells were grown in SM9 medium (29) to saturation. The cells were removed from solution by centrifugation, and 10–30 ml of medium was passed through a 1-ml octyl Amprep minicolumn (GE Healthcare, Piscataway, NJ). The bound material was washed with 2 ml of H\(_{2}\)O and eluted with 50% methanol. The eluate was dried by evaporation and resuspended in H\(_{2}\)O. An estimate of the pyoverdine concentration in the resuspended material was determined by absorbance at 405 nm using an extinction coefficient of 19000 M\(^{-1}\) cm\(^{-1}\) (30). Growth of wild-type cells in the presence of FeCl\(_{3}\) yields a very slight change in absorbance at 405 nm (data not shown), demonstrat-

| TABLE 1 | Crystallographic data for selenomethionine and native data sets |
|---------|-----------------------------|
| Wavelength | 0.97910 Å |
| Resolution | 1.9 Å |
| Rmerge | 51.6 Å |
| Rmerge (highest resolution shell) | 32.6 Å |
| Completeness | 7.8% (14.1%) |
| I/σ | 99.9% (99.9%) |
| No. of observations | 31,553 |
| No. of reflections | 6914 |
| Anomalous phasing power | 6.21 |
| Isomorphic phasing power | 1.95 |

| TABLE 2 | Refinement statistics |
|---------|-----------------------------|
| Resolution range | 50.0–1.8 Å |
| R<sub>crys</sub> | 19.2% |
| R<sub>crys</sub> (highest resolution shell) | 25.3% |
| R<sub>free</sub> (highest resolution shell) | 23.2% |
| Wilson B factor | 19.6 Å<sup>2</sup> |
| Average B factor, protein (all, main chain, side chain) | 19.3 Å<sup>2</sup>, 18.5 Å<sup>2</sup>, 20.1 Å<sup>2</sup> |
| Average B factor, solvent* | 34.6 Å<sup>2</sup> (76) |
| Root mean square deviation bond lengths, angles | 0.011 Å, 1.3° |

* The total number of atoms used in calculation are in parentheses.
tively, were identified as pyoverdines. Analysis confirmed the presence of the pyoverdines in the medium used for the supplementation experiments described above.

Growth Experiments of Wild-type and a Deletion Mutants of PA2411 and PA2412—Growth experiments were performed with knock-out mutants (10) of both PA2411 and PA2412 (generously provided by Dr. Michael Vasil, University of Colorado Health Sciences Center). For growth experiments, the cells were grown in SM9 medium (29) containing EDDHA (Wallace Laboratories, El Segundo, CA) or FeCl₃ (Sigma). In the experiments shown, EDDHA was used at 600 μM to allow some cell growth to occur; at a concentration of 1 mM, all growth of the mutant strain was inhibited. Cell growth was monitored at A₅₉₅.

To measure pyoverdine production, the cells were clarified from solution by centrifugation, and the culture supernatant was analyzed for absorbance at 405 nm.

RESULTS

Structure Determination and Description—The gene encoding PA2412 was obtained commercially and amplified with PCR to incorporate appropriate restriction sites for subcloning. The fragment was subcloned into a modified pET15b plasmid that contains a substituted TEV protease cleavage site in place of the standard thrombin cleavage site. The recombinant PA2412 was purified by two affinity chromatography steps with an intervening TEV cleavage step to remove the histidine tag. The final protein contains the 72 amino acids with two residues, a glycine and a histidine, remaining on the N terminus after protease cleavage. Native and SeMet-labeled protein were crystallized by microbatch methods. The three-dimensional structure was determined by multiwavelength anomalous dispersion diffraction experiments. Two of the three selenium positions were identified, and the experimental phases were used for automated and manual model building. Iterative refinement and model building of the structure continued to completion.

The final structural model (Fig. 2) of PA2412 contains residues 9–68 with the N-terminal 8 residues and the C-terminal 4 residues disordered. The protein is shaped like a thin arrowhead with dimensions of ~25 × 43 × 13 Å. The protein contains a central three-stranded antiparallel β-sheet, organized in a β₂-β₁-β₃ topology, followed by two α-helices. One helix, located at residues 44–54, packs against the sheet, whereas the final two-turn helix at residues 61–67 forms the point of the arrowhead.

A structural comparison with proteins in the Protein Data Bank was performed with DALI (31). The search identified two potential homologs, the catalytic domain of the TevI intron endonuclease (32) and the N-terminal domain of the Saccharomyces cerevisiae RNase H1 (33). Both are larger proteins in which the structural match with PA2412 is based on the central three-stranded sheet and the larger helix. Over the core aligned sequences between the two hits, the proteins exhibit very weak structural and sequence conservation. The TevI endonuclease aligns over 47 residues with a root mean square deviation for Ca positions of 2.2 Å and sequence identity of 2%. The RNase H1 protein aligns over 39 residues with an root mean square deviation of 3.2 Å and a sequence identity of 10%. Both proteins have larger domains that are not included in PA2412, and none of the conserved sequence motifs of PA2412 (see below) is present in the structural homologs.

Locations of Conserved Sequences—As noted above, PA2412 is part of a larger family (13) of MbtH-like proteins belonging to COG3251 (cluster of orthologous genes). These proteins have been identified in a number of NRPS clusters that are involved in the synthesis of peptide antibiotics and siderophores. PA2412 exhibits >80% sequence identity with the other members from the Pseudomonas genus and ~25–60% identity with most other members of the MbtH-like family. It has been noted (34) that the family members from enterobacteria such as Salmonella enterica and E. coli are less well conserved than most proteins.
An examination of an alignment of the sequences of all 155 members of the family identifies several well conserved regions of the protein; representative sequences are shown in Fig. 3A. Perhaps most interesting are three tryptophan residues that are present throughout the family. Trp25 and Trp35 are universally conserved in all family members; Trp55 is present in all sequences except for two shorter proteins that appear to terminate before this residue. The residues surrounding these tryptophan residues are also very highly conserved. The sequence surrounding Trp25 is $S_{\alpha}W_{\beta}P$ where $\alpha$ is an aliphatic residue like Ile, Leu, or Val. This region lies on strand $\beta_2$ of the central sheet (Fig. 3). The consensus sequence around Trp35 is $P_{\alpha}X_{\beta}W_{\gamma}$ and falls on the turn that precedes strand $\beta_3$. The side chain indole rings of Trp25 and Trp35 are nearly parallel and form a small pocket about 7 Å across. This pocket is bordered on one side by Pro32 and on the other by Ser23. The base of the pocket is formed in PA2412 by Val15, a residue from strand $\beta_1$ that is always present as a Leu or Val residue. Interestingly, $F_\alpha - F_\gamma^\prime$ difference electron density exists in this pocket for both the native protein crystallized with pentaerythritol propoxylate 426 as the precipitant and SeMet protein, which crystallized in 2-methyl-2,4-pentanediol. Attempts to build a molecule of 2-methyl-2,4-pentanediol or succinate into this density did not fit satisfactorily, and the exact chemical nature of this group remains to be determined. Also positioned on the first strand is Asn17, a residue that is conserved in $\approx 97\%$ of the MbtH-like family members.

The third conserved region occurs between the two helices of the protein and appears to orient properly the C-terminal helix. Although less well conserved than the other two regions, the most common region around Trp55 is $W_{\gamma}T_{\delta}D_{\epsilon}$. In this motif, the mostly buried Trp55 is followed by Asp57, which forms an ionic interaction with Arg59. Following Arg59 is a proline residue that initiates the C-terminal helix. In PA2412, the side chain of Asp68 from this helix also forms an interaction with the side chain of Arg59. Although the Asp57, Arg59, and Asp68 are not conserved as highly as several of the other residues, the DXR motif is present in $\approx 75\%$ of the family members; the Asp68 homolog is an Asp or Glu in about 40% of the family members, with the other polar residue being well represented in other protein sequences.
Growth Experiments with PA2412 Deletion Mutant—To further elucidate the role of the PA2412 protein, we obtained a mutant strain of *P. aeruginosa* that was deleted for the PA2412 protein (10). Because the PA2412 gene is cotranscribed with the PA2411 gene, we obtained the PA2411 deletion mutant as well to confirm there were no polar effects on the downstream gene. The cells were grown in SM9 medium in the absence or presence of the chelator EDDHA and monitored for both growth and pyoverdine production (Fig. 4). In the absence of a metal chelator (solid lines), all three cell lines grew to saturation in minimal medium, demonstrating that trace iron is present to support growth. A yellow-green color, attributable to pyoverdine, was present in the wild-type and PA2411 cultures, although not in the PA2412 deletion mutant.

In the presence (Fig. 4, dashed lines) of an iron chelator, EDDHA, growth of the wild-type and PA2411 deletion mutant was slowed, but growth to saturation was still achieved. Growth of the PA2412 deletion mutant, in contrast, was very limited. At 600 μg/ml EDDHA, cell growth occurred, however not to saturation; at 1 mg/ml EDDHA, growth of the PA2412 deletion mutant could be completely suppressed. A slight increase in the absorbance at 405 was observed with the PA2412 deletion mutant (solid circles) and the PA2411 deletion mutant (solid squares) are indistinguishable.

We then asked whether exogenously produced pyoverdine could be used to support growth of the PA2412 deletion mutant in the presence of the EDDHA chelator. Pyoverdine was produced by growing wild-type cells in SM9 medium to saturation. The medium was collected, and the pyoverdine molecule was enriched using a rapid small scale protocol with hydrophobic chromatography (29).
The material from the wild-type culture medium was analyzed by nano-LC/MS/MS to determine the presence of the pyoverdines and which form or forms were present. Both the $m/z$ of ions and the relative polarities of two doubly charged compounds ($m/z$ 695 and 707), which eluted at 35.26 and 20.93 min, respectively, under current chromatographic conditions, suggested that these compounds were possibly pyoverdines (Fig. 5). Interpretation of the acquired MS2 fragmentation spectra of these two candidate ions (Fig. 6) confirmed that these two ions were from two variations of pyoverdines. The structures of these pyoverdines were determined by ion chemistry interpretation. The structure-informative MS2 fragment ions, the confirmed pyoverdines structures, and the deduced fragmentation mechanisms for major fragments are shown in Fig. 7.

The crude isolate of pyoverdine was added to SM9 medium to a final concentration of 40 $\mu$M (0.053 mg/ml), based on absorbance at 405 nm, in the presence of EDDHA. Pyoverdine was able to support growth of the PA2412 deletion mutant in the presence of EDDHA in liquid culture (Fig. 8) or on agar plates (not shown). Of note, the deletion mutant grew more quickly in the presence of supplemented pyoverdine than the wild-type culture grew in the chelator alone. This demonstrates a lag time required to synthesize the pyoverdine siderophore by the wild-type cells.

To confirm that the pyoverdine molecule was the active component of the purified material, we performed a "mock" extraction of pyoverdine from wild-type cells grown in the presence of FeCl$_3$ and tested the ability of this material to confer growth of the PA2412 deletion mutant on agar plates containing SM9 medium and EDDHA (data not shown). Although the pyoverdine-enriched sample obtained from iron-deplete medium supported vigorous growth of the deletion mutant at 24 h on an agar plate containing EDDHA, supplementing the plate with an equivalent volume of the material obtained from cells grown in Fe$^{3+}$-replete medium showed only very limited growth of the PA2412 deletion cells at 48 h. This limited growth likely arises from the low levels of pyoverdine present in the "mock" enrichment sample.
and demonstrates that no other component present in the crude isolate is responsible for the growth.

DISCUSSION

We present here the three-dimensional crystal structure of a member of the MbtH-like family of proteins. These proteins are present in numerous NRPS clusters encoding the synthetic proteins for both antibiotic and siderophore peptides. The novel structure is characterized by a three-stranded antiparallel β-sheet and two α-helices. Additionally, we report here growth conditions in which both wild-type and a PA2412 deletion strain grow to saturation, yet only the wild-type cells produce significant amounts of pyoverdine. The addition of exogenously produced pyoverdine, however, restores growth of the deletion strain.

The conserved residues identified by comparison of the sequences of numerous family members all lie on one face of the PA2412 molecule (Fig. 3). The other face of the protein, which is formed primarily by residues from strand β3 and the longer α-helix, is not very well conserved. This raises the possibility that PA2412 may function to interact with a conserved component of these NRPS clusters. Because the peptide products of these clusters are diverse, both in chemical structure and in function, it seems unlikely that the PA2412 protein homologs all maintain the ability to interact with the peptide products.

During revision of this manuscript, the structure of the PA2412 protein determined by NMR by the Northeast Structural Genomics Consortium was released from the Protein Data Bank (accession code 2GPF), allowing comparisons of our crystallographic model with the structure determined by NMR. The ensemble of NMR models was superimposed with our structure. The models all align precisely over the core of the protein, from residues 10 to 54. This core encompasses the central three-stranded β-sheet and the first α-helix. The N-terminal 10 residues, of which seven were disordered in the crystallographic model, was modeled in multiple orientations in the NMR model. The short C-terminal helix, which is well ordered in the crystal structure and forms the point of the arrowhead-shaped protein (Fig. 2A), also is modeled in multiple conforma-
ions in the ensemble of NMR models. In most of the models, the C terminus adopts a random coil, often folding back on helix α1. In several models of the NMR ensemble, the C terminus does form a helix, as observed in the crystal structure. To assess whether the C terminus of the crystallographic model was influenced by interactions with neighboring molecules in the crystal lattice, we carefully examined the model for interactions within the molecule as well as with crystallographic neighbors. The PA2412 molecule forms several intramolecular ionic interactions between Arg residues on the C terminus including interactions from Arg59 to Asp68, from Arg64 to Glu20, and from Arg59 to Asp57. These interactions appear to maintain the orientation of the C terminus of PA2412. The C terminus also exhibits limited intermolecular interactions with van der Waals’ contacts occurring between His68 and Met36 of a neighboring molecule, and between Leu63 and Met67 of one molecule and Ile51 of the neighbor.

The function of PA2412 has not been elucidated, and given its novel fold, homology studies lend little insight. However, the results presented here allow us to define more narrowly the steps in the pathway for siderophore production and utilization where the PA2412 protein is essential. We divided the process into five steps: The cell must sense iron-deficient conditions and signal the production of all the proteins necessary for siderophore biosynthesis. These proteins will then produce the siderophore, and the siderophore is secreted from the cell. Finally, the iron-siderophore complex is then actively transported into the cell where the iron ion is now rendered available for use.

Although previous results (10) demonstrate that a PA2412 deletion mutant could neither grow under iron limiting conditions nor produce pyoverdine, our studies have uncoupled the production and use of pyoverdine to provide insight into the role of the PA2412 protein. The experiments presented here extend previous reports that PA2412 is necessary for pyoverdine production. In the studies of Ochsner et al. (10), no pyoverdine was detected in culture medium. In the presence of the iron chelator, however, a cell containing a defect in any of these five steps, including the ability to import and use the Fe$^{3+}$-pyoverdine complex, would be unable to grow, and therefore pyoverdine would not be detected in medium supernatants. We show here that in the absence of a chelator the PA2412 deletion strain grows to saturation, yet pyoverdine is not detected in the medium. Under identical conditions, the wild-type cell line produces significant amounts of pyoverdine. This suggests that the PA2412 deletion mutant contains a defect in synthesis or export of the pyoverdine molecule. More importantly, we have shown directly that the addition of pyoverdine is able to support growth of the PA2412 deletion mutant in the presence of the iron chelator. This demonstrates conclusively that the PA2412 protein is not involved in uptake or utilization of the Fe$^{3+}$-pyoverdine complex.

It is unlikely that PA2412 catalyzes a synthetic step in pyoverdine synthesis. Although the synthetic pathway for pyoverdine is not completely elucidated, many of the necessary catalytic steps have been assigned to members of the pyoverdine synthetin clusters (3). The structure of PA2412 illustrates no obvious active site that contains likely catalytic groups. Indeed, several NRPS clusters that contain an MbtH-like protein are very well characterized. All of the steps in the synthesis of the E. coli siderophore enterobactin have been identified (2, 5), as have the steps in mycobactin synthesis in M. tuberculosis (4). Interestingly, the MbtH-like protein in the cluster for the synthesis of balhimycin, designated Orf1, has been shown to be unnecessary for production of balhimycin (34). In this study, a zone of inhibition was observed around a deletion mutant. Unlike our observation with PA2412 and pyoverdine production, the balhimycin antibiotic was detected in culture medium of the orf1 deletion mutant at levels comparable with those produced by wild-type cells. Stegmann et al. noted (34), however, that the Amycolatopsis balhimycina genome contains two additional MbtH-like proteins that may function in place of the Orf1 protein.

Very recently, two reports have provided additional insights into MbtH-like proteins in Streptomyces coelicolor (35, 36), which expresses multiple MbtH-like proteins, in two different NRPS clusters. Lautru et al. (35) demonstrated that production of both coelichelin and the calcium-dependent antibiotic were dependent on the expression of either of two MbtH-like proteins; only by knocking out both mbtH genes could production of either NRPS product be abolished. No effects on RNA levels were identified for remaining genes in the clusters, suggesting that the MbtH-like proteins do not play a role in transcriptional regulation. In the second report, Wolpert et al. (36) used a heterologous system that allows studies of the chlorobiocin cluster in S. coelicolor M512. Deletion of the MbtH homolog CloY did not affect chlorobiocin in the wild-type heterologous host. Deleting the host mbtH homologs, however, resulted in a >100× reduction in production of the chlorobiocin. Because the cloY gene is colocalized with the synthetic proteins that are responsible for the aminocoumarin group of the chlorobiocin, the cloY mutant was tested for antibiotic production in feeding studies with aminocoumarin. The production of antibiotic was restored, demonstrating the involvement of the CloY protein in the formation of the aminocoumarin moiety.

The exact function of the MbtH-like family members remains unknown. These recent reports note the cross-talk that exists between different MbtH-like proteins from different synthetic clusters. It is interesting to note that PA2412 is the only family member encoded by the genome of P. aeruginosa. Wolpert et al. (36) suggest that CloY protein may play a role in catalysis or other regulatory roles, for example, catalyzing a post-translational modification or regulating protein-protein interactions. The absence of aminocoumarin moieties, or indeed any common chemical functionality, in the products of other NRPS clusters that contain MbtH-like proteins, including pyoverdine, seems to argue against a direct catalytic role.

The work reported here extends efforts from our lab and others to characterize structurally and functionally the proteins from NRPS clusters that are involved in peptide synthesis, as well as auxiliary roles in synthesis of nonproteinogenic amino acids or other building blocks. Siderophore synthesis has recently been identified as a target for antibiotic development (12, 37, 38). In these studies, the NRPS adenylation domain has served as the target for inhibitor design. Understanding the complete pathways for siderophore synthesis, maturation,
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transport, and utilization may provide additional targets for the development of novel antibacterials.

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