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Identification, isolation, and analysis of a gene cluster involved in iron acquisition by *Pseudomonas mendocina* ymp

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Abstract Microbial acquisition of iron from natural sources in aerobic environments is a little-studied process that may lead to mineral instability and trace metal mobilization. *Pseudomonas mendocina* ymp was isolated from the Yucca Mountain Site for long-term nuclear waste storage. Its ability to solubilize a variety of Fe-containing minerals under aerobic conditions has been previously investigated but its molecular and genetic potential remained uncharacterized. Here, we have shown that the organism produces a hydroxamate and not a catecholate-based siderophore that is synthesized via non-ribosomal peptide synthetases. Gene clustering patterns observed in other Pseudomonads suggested that hybridizing multiple probes to the same library could allow for the identification of one or more clusters of syntenic siderophore-associated genes. Using this approach, two independent clusters were identified. An unfinished draft genome sequence of *P. mendocina* ymp indicated that these mapped to two independent contigs. The sequenced clusters were investigated informatically and shown to contain respectively a potentially complete set of genes responsible for siderophore biosynthesis, uptake, and regulation, and an incomplete set of genes with low individual homology to siderophore-associated genes. A mutation in the cluster's *pvdA* homolog (*pmhA*) resulted in a siderophore-null phenotype, which could be reversed by complementation. The organism likely produces one siderophore with possibly different isoforms and a peptide backbone structure containing seven residues (predicted sequence: Acyl-Asp-Dab-Ser-fOHOrn-Ser-fOHorn). A similar approach could be applied for discovery of Fe− and siderophore-associated genes in unsequenced or poorly annotated organisms.

Keywords Siderophore · Iron · Bacteria · Mineral

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Introduction

The need for iron far outstrips its availability in aerobic, pH neutral environments. Microbes living in terrestrial or acquatic environments must acquire iron directly from sources that are dilute, diverse, or for the most part insoluble. Ferric minerals serve as primary sources of nutritional iron for a variety of microbes, including water-borne pathogens and environmental species. Their iron-scavenging activities contribute to global Fe cycling as well as mineral instability over geological time (Cornell and Schwertmann 1996; Hersman et al. 2001; Kraemer 2004).

Because of the potential for microbial impacts on both mineral stability and toxic metal mobilization (Ams et al. 2002; Rosenberg and Maurice 2003),

microbial surveys were carried out as part of the Yucca Mountain Site Characterization Project, an effort aimed at assessing the suitability of this location for the long-term storage (≥10,000 years) of high-level nuclear waste (Kieft et al. 1997). *Pseudomonas mendocina* ymp, an obligate aerobe, was isolated from and found to be ubiquitous in Yucca Mountain volcanic tuff. The organism's ability to aerobically solubilize a variety of Fe-bearing minerals and clays with differing surface properties and solubilities has been the topic of several laboratory-based geochemical studies (Hersman et al. 2000, 2001; Kieft et al. 1997; Maurice et al. 2000, 2001a, b). In spite of the inherent interest in and attention given to this strain, nothing was known of *P. mendocina*'s genetic potential for metal metabolism prior to the beginning of this work. Little is known of the molecular details of nutritional iron acquisition from mineral sources in general (Kraemer 2004).

P. mendocina is at the same time a member of an environmentally ubiquitous, economically important, and metabolically diverse genus. The type species of the genus, *P. aeruginosa*, is very well characterized genetically; the genome for the pathogenic strain PAO1, the first available for the genus (2001), has been the subject of considerable analysis. Moreover, its iron uptake pathways are among the best understood of the prokaryotes (Poole and McKay 2003). *P. putida* KT2440, sequenced in 2002, is likewise one of the better studied strains due to its ability to completely oxidize a large variety of carbon sources. While the exact phylogenetic relationships among *Pseudomonas* sp. remain controversial (Tayeb et al. 2005), these two well-known strains each have important physiological and ecological similarities with *P. mendocina*, *P. putida* and *P. mendocina*, for example, are both saprophytic soil-dwelling organisms that are only very rarely found as pathogens. Both are obligate aerobes, lacking any dissimilatory pathways by which they might reductively acquire iron. *P. aeruginosa*, by contrast, is a facultative anaerobe and opportunistic human pathogen (Nakazawa et al. 1996). Like *P. mendocina*, it is capable of producing extracellular polysaccharide material or alginate, which may be significant for biofilm formation and metal metabolism in pathogenic or environmental contexts (Nakazawa et al. 1996). Unlike either *P. aeruginosa* or *P. putida, P. mendocina* strains lack the characteristic chromophore of pyoverdine siderophores and are therefore non-fluorescent (Nakazawa et al. 1996). While it has been previously shown that *P. mendocina* ymp produces large amounts of at least one non-fluorescent siderophore under iron stress (Ams et al. 2002; Hersman et al. 2000), the siderophore or siderophores have evaded definitive isolation by other workers.

In this study, we sought to learn more about the organism's Fe uptake capabilities, including its siderophore/siderophores, and to isolate and describe the genes involved with siderophore biosynthesis and use. Identifying and localizing siderophore-associated genes in an unsequenced strain is often complicated as simple PCR or hybridization-based strategies are ineffective. Especially problematic are non-ribosomal peptide synthetases (NRPSs): large, single-polymer, multi-domain and multi-functional enzymes often exceeding 250 kDa (Crosa and Walsh 2002; Schwarzer et al. 2003; Weber and Marahiel 2001). Though NRPSs and related polyketide synthetases are of great interest due to their roles in the biosynthesis of siderophores and other natural products, PCR or hybridization-based approaches to their isolation can be non-trivial (Mootz and Marahiel 1997; Schwarzer et al. 2003; Weber and Marahiel 2001). Large numbers of paralogous genes share sequence similarities with the targets but belong to unrelated pathways, leading to unmanageable numbers of false positive hits. Similar problems arise when attempting to isolate associated monooxygenase or acyltransferase enzymes, or regulatory and receptor genes (Thupvong et al. 1999).

Only by using a strategy involving hybridization of multiple genetic probes to the same genomic library could a single cluster of siderophore associated genes be identified. While most of this work was being carried out, no genetic sequence information for the stain was available; hence, the approach described herein may be applied for siderophore or other metabolite-associated gene discovery in other environmentally or medically relevant but unsequenced strains.

Materials and methods

Growth of *P. mendocina* ymp under Fe-deficient and Fe-replete conditions

P. mendocina ymp was obtained from Dr. Larry Hersman (Los Alamos National Lab, Biology

Division) and maintained as a frozen stock in 30% glycerol (−80°C). Cultures were grown in the presence and absence of iron, using conditions previously shown to induce vigorous siderophore production in the latter case (Hersman et al. 2000). Fe deficient medium was prepared by adding to 1.0 l of distilled, deionized water the following analytical-grade ingredients (Fluka Chemie, Buchs, Switzerland): 0.5 g of K_2HPO_4, 0.5 g KCl, 1.0 g of NH_4Cl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $CaCl_2$, 5.0 g of succinic acid disodium salt anhydrous ($C_4H_4Na_2O_4$), and 0.125 ml of trace elements (0.005 g of $MnSO_4$ H_2O, 0.0065 g of $CoSO_4 \cdot 7H_2O$, 0.0023 g of $CuSO_4$, 0.0033 g of $ZnSO_4$, and 0.0024 g of MoO_3 per 100 ml of distilled, deionized water). The pH of the medium was approximately 7.2. For Fe-replete cultures, 90 μM $[Fe(III)EDTA]^-$ was added as an iron source.

For isolation of total cellular proteins: one-liter cultures were grown in a shaker incubator at 100 rpm at 32°C for 48 h. Cells were then pelleted and stored at −80°C. For monitoring growth curves and producing siderophore-containing supernatants: 50 ml cultures were grown in a shaker incubator at 100 rpm at 30°C for 54 h. Cell-free supernatants were collected and immediately analyzed for siderophore content.

Assays for total siderophore, catecholate, and hydroxamate

P. mendocina ymp cultures (50 ml) were grown under iron-replete and iron-depleted conditions (as described above) until stationary phase. Cell-free culture supernatants were subjected to the chrome azurol S (CAS) assay performed according to Schwyn and Nielands (1987). Briefly, 500 μl of cell-free supernatant (or a dilution thereof) was added to 500 μl of CAS assay solution and incubated for 1 h at room temperature (assay solution: 6 ml of 10 mM hexadecyltrimethylammonium bromide (HDTMA), 1.5 ml of 1 mM $FeCl_3$, 7.5 ml of 2 mM CAS, 4.307 g of piperazine, 6.25 ml of 12 M HCl, diluted to 100 ml in deionized water; final pH of 5.6). Absorbance spectra for CAS solution-supernatant mixtures were then measured at 630 nm. The siderophore concentration was approximately quantified via comparison to desferrioxamine standards (Sigma).

The Arnow and ferric perchlorate tests were used to assay the type of siderophore (catecholate or hydroxamate) produced by *P. mendocina* ymp. The Arnow method used to detect catecholate-type siderophores gives catechol a yellow color in the presence of a nitrite-molybdate reagent (10 g of sodium nitrite and 10 g of sodium molybdate in 100 ml of deionized water), changing to an orange-red color under strongly basic conditions (Arnow 1937). Briefly: to 1.0 ml of culture supernatant, 1.0 ml each of of 0.5 N HCl, nitrite-molybdate reagent, and 1.0 N NaOH were added and the reaction absorbance measured at 510 nm. Using catechol as a standard, a lower-limit of 18 μM catechol was detectable by this method. Hydroxamate siderophores were detected by the ferric perchlorate test, which gives a yellow-orange color as a positive indicator (Atkin et al. 1970). The assay was carried out by adding 0.5 ml of culture supernatant to 2.5 ml of 5 mM $FeCl_3$ in 0.1 M $HClO_4$ (pH 2). Absorbances of reaction mixtures were measured at 425 nm. A lower concentration of 20 μM desferrioxamine was detectable by the ferric perchlorate test.

Electrophoresis of total cellular proteins

Approximately 1 ml protein cell pellets were thawed and resuspended in 6X protein gel loading buffer (0.1 M Tris–Cl/SDS pH 6.8, 20% glycerol, 4% sodium dodecyl sulfate (SDS), 2% 2-mercaptoethanol, 0.001% bromophenol blue, 49% distilled water). Samples were boiled for 5 min and 60, 40, and 20 μg were loaded onto 4% and 7.5% SDS polyacrylamide gels, respectively, each with 3.65% acrylamide stacking gels. Proteins were electrophoretically separated (15 mA) for 1 h (7.5% SDS polyacrylamide gel) and for 2.5 h (4% SDS polyacrylamide gel) and stained with coomassie blue (50% methanol, 0.05% Coomassie brilliant blue R-250, 10% acetic acid, and 40% distilled water) for 3.5 h following fixing (50% methanol, 10% acetic acid, and 40% distilled water) for 30 min. Gels were destained overnight (distilled water) and imaged using a Kodak Imaging System instrument (Rochester, NY).

General DNA manipulations and sequence analysis

Genomic DNA from *P. mendocina* ymp was prepared according to Promega's Wizard Genomic DNA

Purification Kit. Cosmid and plasmid DNA were manipulated and purified by standard techniques (Sambrook et al. 1989). *Escherichia coli* DH5α (Invitrogen) and JM109 (Promega, Madison, WI) were routinely used as host strains for cloning DNA fragments. The pUC19 and pGEM-T vectors (Promega) were used for cloning DNA fragments. Restriction enzymes and DNA/gel purification kits were purchased from Promega and used according to the manufacturer's instructions. Nucleotide sequences were determined by automated sequencing at SeqWright DNA Technology Services (Houston, TX). Cosmid clones were restriction mapped by digestion with HindIII, BamHI, EcoRI, or PstI and electrophoresed on 0.7–1.0% agarose gels at 100 V for 2.5 h (or until separation of DNA fragments was optimal).

Dot blot hybridization

Samples of denatured genomic DNA from *P. mendocina* ymp and *E. coli* DH5α (used as a control) were dotted onto nylon membranes (Hybond-N^+, Amersham Biosciences, Pittsburgh, PA). DNA was fixed to the membrane by baking at 120°C for 30 min and prehybridized at 37°C in EasyHyb hybridization solution (Roche Applied Science) for 30 min. Hybridization with digoxigenin-labeled probes (10 ng/ml, see below) was carried out overnight at 37°C. High stringency washes were done three times for 5 min in wash solution (2X SSC (0.3 M sodium chloride, 0.03 M sodium citrate), 0.1% SDS) at 25°C, followed by two washes for 15 min in 0.5X SSC, 0.1 %SDS at 65°C. Detection was carried out according to the manufacture's recommendation (Roche Applied Science).

Construction of the genomic library of *P. mendocina* ymp

Genomic DNA from *P. mendocina* ymp was prepared according to Promega's Wizard Genomic DNA Purification Kit. DNA (150 μg) was sheared by passing it through a 25-gauge syringe three times. Sheared genomic DNA was separated on a 1% low melting point (LMP) agarose gel at 80 V for 3 h. DNA fragments ranging from 30 to 50 kb were purified and end repaired according to the Epicentre (Madison, WI) pWEB-TNC Cosmid Cloning kit procedure. The end-repaired DNA fragments were blunt end ligated to a SmaI-digested pWEB vector overnight at room temperature. The ligated DNA was packaged into phage using MaxPlax Packaging Extracts (Epicentre) following the recommendations of the manufacturer and transformed into XL-1 Blue (Stratagene) cells on LB plates containing 100 μg/ml of ampicillin. Approximately 1000 colonies were picked, replated and then stored at 4°C. Original cosmid library clones were resuspended in 2 ml of sterile liquid LB media for each plate and then replated on LB containing 100 μg/ml of ampicillin to produce an amplified library. The amplified library was resuspended in liquid LB containing 20% glycerol and stored at −80°C.

Preparation of DIG-labeled probes

PCR-amplified or restriction-digested DNA fragments were used as template DNA for generating digoxigenin (DIG)-labeled probes. DNA fragments were labeled according to the manufacture's recommendation (Roche Applied Science, Indianapolis, IN). To construct probes from PCR products, primers (Table 1) were developed from a conserved region within each gene of interest and amplified by PCR using genomic DNA from the sequenced, saprophytic soil organism *P. putida* KT2440 (ATCC #77095) genomic DNA as a template (strain acquired from ATCC). The target size of each PCR product was between 350 and 1000 bp. Primers to detect NRPS-encoding genes were designed to amplify highly conserved adenylation and thiolation domains (Crosa and Walsh 2002; Mootz and Marahiel 1997). Primers for amplification of ornithine monooxygenase genes (*pvdA* homologs) targeted highly conserved and functionally essential NAD(H)-binding and FATGY domains (Yamada et al. 2003). Specific NRPS and *pvdA* probes were generated using genomic DNA from the sequenced, saprophytic soil organism *P. putida* KT2440 (ATCC #77095). The *fur* gene was directly PCR amplified from *P. mendocina* ymp genomic DNA. Because *fur* has a high degree of conservation and single genomic copy, it was possible to develop degenerate primers from ClustalW alignments of several sequenced Pseudomonad *fur* homologs. The *fpvA* and *fptA* genes were previously cloned from *P. aeruginosa* and received as a kind gift

Table 1 Primers used in this study[a]

Primer	Sequence (5′–3′)
Fur-F1	ATGGTTGAAAATAGCGAA
Fur-R1	CTTCTTSTTGCGBACGTA
PvdS-F1	GCGGAAGACGTGGTGCAGGATGC
PvdS-R1	TCGGCGACACGCCCAGTTCCTT
PvdA-F1	GGCGTTCATCGACCTCAACGACA
PvdA-R1	TAACCGGTGGCSAGSAYMACBGCG
NRPS-F1	ACYGARACCGCSATCAACGTYACC
NRPS-R1	SGARTGMCCRCCRAGSKCRAAGAA
Iuc-F1	CCGGTGCATCCGTGGCAG
Iuc-R1	CGCACGCCGTCATGGAAGTC
PmhS-F	ATGGAATGTTCTCGACAGTCA
PmhN-R	CACACATACAGCGTCGCTTC
PvdA-F	CCTTCTACCCCAGTCGTGAA
PvdA-R	GAACATCGAAGCGACCAACT

[a]B = C or G or T; S = C or G; Y = C or T; R = A or G; K = G or T

from Prof. D. Castignetti (Loyola University of Chicago) (Ankenbauer and Quan 1994; Poole et al. 1993). The *fpvA* gene was digested with BstXI and Bsu36I to produce a 1.7 kb DNA fragment and *fptA* was digested with SacII to produce a DNA fragment size of 1.8 kb. Each digested DNA fragment was gel purified and DIG-labeled as described above.

Screening of the *P. mendocina* ymp genomic library

One-hundred fifty colonies were picked randomly, representing 96.5% (Wahl et al. 1987) of the ~5.0 Mbp genome of *P. mendocina* ymp. Each colony was grown overnight at 37°C in 10 ml of LB containing 100 μg/ml of ampicillin. Cosmid DNA was purified from each culture by the alkaline lysis procedure (Sambrook et al. 1989). Two strategies were used to detect and isolate genes from the cosmid library: Altschul et al. (1997) membrane hybridization, or (Ams et al. 2002) direct PCR. For hybridization, approximately 2 μl of extracted DNA (500 ng/μl) was dotted on a nylon membrane and fixed according to the previously described dot blot method. Hybridization and detection were likewise carried out as described for dot blots against total genomic DNA. The second method used a PCR strategy to screen the genomic library, employing the same primer sets used to generate the hybridization probes. The 150 purified cosmid DNAs were collected into six groups of twenty-five. Each group was screened with the appropriate pair of primers by PCR (94°C for 5 min, 94°C for 30 s, 45°C for 30 s, 72°C for 45 s for 30 cycles, and 72°C for 10 min). The resultant PCR products were separated on 2.0% agarose gels. Members of the group that yielded an amplified PCR product were subjected to a second round of PCR to isolate the individual *fur*-containing cosmid DNA. A similar direct amplification procedure was attempted for the other genes in Table 1, but none yielded a unique or easily analyzed product.

Generation and phenotypic screening of a *P. mendocina* ymp Pmen1887 (*pmhA*) mutant

A mutation was created within *P. mendocina* ymp locus Pmen1887 (*pmh*A gene, *vide infra*) via the gene replacement strategy described by Hoang et al. (1998). Specific PCR primers PmhS-F and PmhN-R (Table 1) were used to amplify a ~5.0 kb fragment containing the *pmh*A gene from *P. mendocina* ymp genomic DNA, which was cloned into a suicide plasmid containing a tetracycline resistance marker, pEX18Tc (Hoang et al. 1998). A gentamycin resistance gene (Hoang et al. 1998) was inserted into a single BstEII restriction site within *pmhA* gene, resulting in plasmid pND100. pND100 was electroporated into *P. mendocina* ymp (Choi et al. 2006) and selected on LB agar plates containing 50 μg/ml of gentamycin. Gentamycin resistant *P. mendocina* ymp colonies that were also tetracycline (100 μg/ml) sensitive were potential *pmhA* mutants. PCR was performed with primers PvdA-F and PvdA-R upstream and downstream of the gentamycin cassette to verify the mutation within the chromosome of potential *P. mendocina* ymp mutant colonies. Disrupted *pmhA* PCR products contained an additional 1.0 kb fragment, which indicated the integration of a gentamycin marker into *pmhA*. Approximately 87% of *P. mendocina* ymp isolated colonies that were gentamycin resistant and tetracycline sensitive were found to *pmhA* mutants. An unmarked *pmhA* mutant was isolated by the FLP-mediated marker excision

procedure described by Hoang et al. (1998). *pmhA* mutant cultures (50 ml) were grown in Fe-replete and Fe-depleted conditions according to methods described above. Cultures were sampled at stationary phase (54 h) and assayed for cell density (600 nm) and siderophore production (CAS assay). Finally, the excised fragment containing the *pmhA* gene was inserted into a broad-host range plasmid (Hoang et al. 1998). The plasmid was re-introduced into the *pmhA*(−) strain via electroporation and the culture screened for siderophore production as described.

Results

Analysis of siderophore production under Fe deficient conditions

Optical densities of *P. mendocina* ymp cultures grown in iron replete and iron depleted media were followed by absorbance at 600 nm and siderophore production was monitored by using the CAS assay (Fig. 1). Previously, Ams et al. have shown siderophore biosynthesis by *P. mendocina* ymp is at its highest production between 2 and 3 days under the growth conditions used and begins to decrease dramatically over time (Ams et al. 2002). Therefore, cultures were stopped at the 54 h time point once stationary growth phase occurred and CAS-reactive material was detected in the supernatant. Cell-free supernatants of *P. mendocina* ymp cultures in iron-depleted media revealed over a 10-fold increase in siderophores (~90 μM desferrioxamine equivalents) over $[Fe(III)EDTA]^-$ supplemented media (~7 μM desferrioxamine equivalents). Further investigation aimed at determining if the siderophore produced by *P. mendocina* ymp is a catecholate (Arnow method) or hydroxamate (ferric perchlorate test) type. Supernatants of iron depleted *P. mendocina* ymp 54 h cultures were positive for a hydroxamate siderophore, but not a catecholate type of siderophore. To ensure that low siderophore concentration was not the reason for a negative Arnow test, the lower limit of detection of catechol by the Arnow method and CAS assay was determined, and shown to be 18–20 μM. Hence the siderophore concentration observed in the *P. mendocina* ymp supernatant was approximately 5X higher than the lower detectable limit.

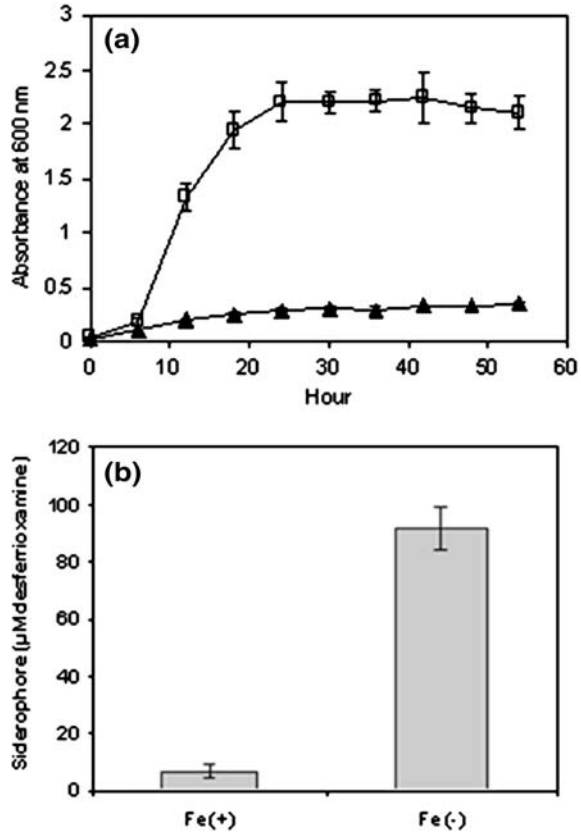

Fig. 1 (**a**) Growth curves for *P. mendocina* ymp grown under Fe-deficient (▲) and Fe-replete (□) conditions (see text). (**b**) Supernatants from both cultures were collected at 54 h and subjected to the CAS assay, with results as shown. Data and error bars represent the means and standard deviations of three replicate measurements

Identification of iron acquisition genes

P. mendocina ymp cultures were grown in the absence or presence of iron to screen for the production of high molecular weight proteins such as NRPS that should be induced under iron-depleted conditions (Georges and Meyer 1995). Total protein was isolated from each culture and separated by one-dimensional denaturing SDS-PAGE (Fig. 2). Two proteins of exceptionally high molecular weight (approximately 280 and 510 kDa based on comparisons to standards) were identified exclusively in the iron-starved culture. Identical growth conditions are known to strongly induce production of a siderophore or siderophores (Hersman et al. 2000). As expression of these proteins was induced by iron starvation, and as few other cellular proteins are expected to be of comparable

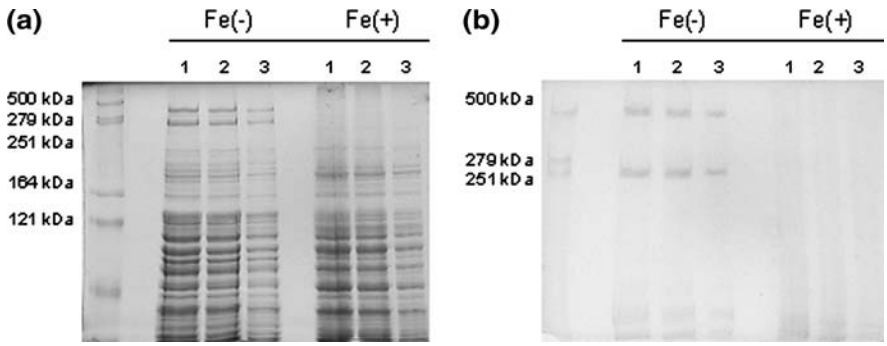

Fig. 2 7.5% (**a**) and 4% (**b**) acrylamide gels of total protein extracted from *P. mendocina* ymp, grown with and without iron. (**a**) Total protein content for cells grown under iron-replete (Fe+) and iron-deficient (Fe−) conditions. (**b**) The same samples were electrophoresed for 2.5 h at 15 mA to provide maximal separation of high molecular weight proteins. Lane 1: 60 μg total protein; 2: 40 μg; 3: 20 μg. Molecular weight markers are as labeled

molecular weight in their monomeric/denatured form (Georges and Meyer 1995), this result immediately suggested a potential NRPS-dependent rather than an NRPS-independent synthetic (NIS) pathway for siderophore biosynthesis by *P. mendocina* ymp. A dot blot hybridization assay was used to confirm the identification of nonribosomal peptide synthetase genes within the *P. mendocina* ymp genomic DNA and identify further genes potentially involved in siderophore biosynthesis. Construction of DIG labeled probes and dot blot optimization were carried out as described in Section "Materials and methods". Seven different gene probes (*fur*, *pvdS* (regulatory proteins), *fpvA*, *fptA* (receptors), NRPS, *pvdA*, and *iucA* (NIS-pathway siderophore biosynthesis)) were generated from sequenced *Pseudomonas* sp. and used to identify homologous genes within the *P. mendocina* ymp genome. Representative results for dot blot optimization and positive/negative controls are shown in Fig. 3. Results of the dot blots suggested that *P. mendocina* ymp contains homologous genes for the regulation and biosynthesis of a siderophore as well as receptors to transport a siderophore-iron complex (Table 2). The biosynthetic pathway appears to be NRPS-dependent rather than NRPS-independent. A pathway of the latter type would be indicated by a positive hybridization result with the *iucA* probe (Challis 2005).

Sequence analysis of an isolated *fur* homolog

Two degenerate primers (Fur-F1 and Fur-R1) were used to amplify a homologous *fur* gene PCR product

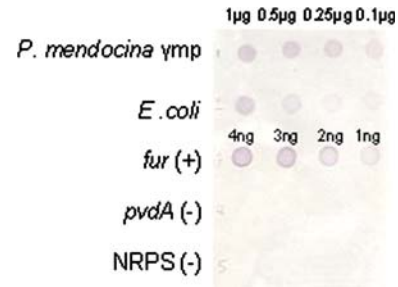

Fig. 3 Representative dot blot hybridization of *P. mendocina* ymp and *E. coli* DH5α genomic DNA with a *fur* gene probe. PCR amplified products of *fur*, *pvdA*, and NRPS DNA fragments were used as controls for optimizing the hybridization conditions. The *E. coli fur* gene has 73% nucleotide identity to the gene later amplified from *P. mendocina* ymp

from *P. mendocina* ymp genomic DNA. The PCR product was sequenced and the deduced amino acid sequence showed 94% and 59% identity to *P. aeruginosa* and *E. coli* Fur proteins, respectively. Alignment of these amino acid sequences with *P. mendocina* ymp Fur showed a conserved regulatory iron-binding site (HHDH motif; residues 86–89), which is consistent with sequenced Fur proteins from other Pseudomonads (Pohl et al. 2003). Sequence analysis also revealed a conserved Cys-92, but not a Cys-95 (ClustalW alignment not shown). Both cysteine residues at these amino acid positions have been shown to be essential in binding a structural Zn^{2+} in *E. coli* Fur (Gonzalez de Peredo et al. 1999; Jacquamet et al. 1998). The Fur protein from *P. aeruginosa*, however, likewise contains only one of the two conserved cysteines (the second one) (Lewin et al. 2002). The significance of the Ser

Table 2 Results of hybridization of DIG-labeled probes to genomic DNA

Gene Probes	Description	*P. mendocina* ymp
fur	Global Fe sensor/ transcription factor	+[a]
fpvA	Ferripyoveridine receptor	+
fptA	Ferripyochelin receptor	+
pvdS	Sigma factor	+
pvdA	*N*-ornithine oxygenase	+
NRPS	Non-ribosomal peptide synthase (NRPS)	+
iucA	NRPS-independent aerobactin synthetase	–

[a]Detected by PCR and sequenced from *P. mendocina* ymp genomic DNA

residue at 95 position in *P. mendocina's* Fur is unknown, and beyond the scope of this study. The cosmid library of *P. mendocina* ymp was screened through PCR to isolate an individual clone containing the *fur* homolog. The isolated cosmid was partially sequenced to determine the upstream regulatory region of the *fur* gene. A 19-bp palindromic sequence having high similarity to a consensus *E. coli fur* box sequence and somewhat lower homology to the consensus sequence for *P. aeruginosa* was found 59 base pairs upstream of the *fur* gene start codon (Fig. 4) (de Lorenzo et al. 1987; Ochsner and Vasil 1996). Though Fur is not auto-regulated in *P. aeruginosa*, it is apparently preceded by a *fur* box in *P. mendocina* and *E. coli* (de Lorenzo et al. 1988; Ochsner et al. 1999).

Localization and analysis of siderophore-related gene clusters

The cosmid library of *P. mendocina* ymp was screened using the five different gene probes (*fpvA, fptA, pvdS, pvdA*, and NRPS) that yielded a positive dot blot result against *P. mendocina* ymp genomic DNA. Resulting numbers of positive hybridization events per 150 cosmids (96.5% genome coverage) are given in Fig. 5. A comparison of screening results for *pvdS*, *pvdA*, and NRPS gene probes taken together identified two individual cosmids that hybridized with all three (Fig. 5a). The *fpvA*/*fptA* probes likewise hybridized to the same two cosmids (Fig. 5b). Restriction mapping showed that the two isolated cosmids had no common restriction digestion fragments (data not shown). Therefore, these cosmids very likely contain two separate genomic DNA fragments. Hence two independent gene clusters potentially involved in iron uptake were identified and localized to two separate cosmids.

The ends of the ~40 kb cosmids were sequenced and analyzed informatically using BLAST (Altschul et al. 1997) and the Joint Genome Institute's draft annotation of the *P. mendocina* ymp genome (http://genome.ornl.gov/microbial/pmen). A diagram depicting the size and orientations of ORFs within each cosmid is presented in Fig. 6. Putative *fur* box sequences (>54%) were identified using Vector NTI software (Invitrogen). The gene with highest sequence similarity to each ORF detected by gene probes (*pvdA, pvdS, NRPS, fpvA/fptA*) is shown in Table 3. The matching of gene probes to the specific ORFs is based on shared sequences between the probe/ORF. Prediction of possible structural elements encoded by the two NRPSs was carried out using the NRPS Domain Search (http://www.nii.res.in/nrps-pks.html) and NRPSpredictor (http://www-ab.informatik.uni-tuebingen.de/toolbox/) programs, available from National Institute

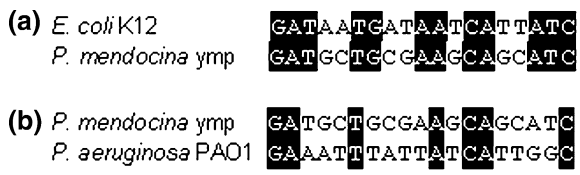

Fig. 4 Boxshade alignment comparing regulatory *fur* box sequences of *E. coli* K12 and *P. mendocina* ymp (**a**) and *P. mendocina* ymp and *P. aeruginosa* PAO1 (**b**)

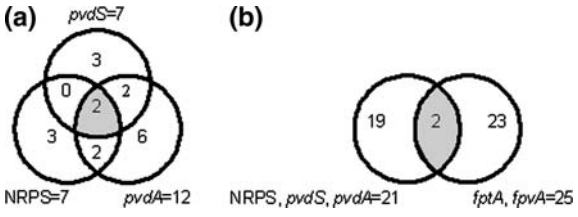

Fig. 5 Venn diagrams showing the number of individual cosmids yielding positive hybridization results with biosynthesis-associated *pvdS*, NRPS, and *pvdA* probes against the genomic library of *P. mendocina* ymp. (**a**) The combined biosynthetic probe results are compared with the number of cosmids detected by receptor gene probes *fptA* and *fpvA* (**b**). The shaded region within the circles represents the number of cosmids that overlapped with all six gene probes

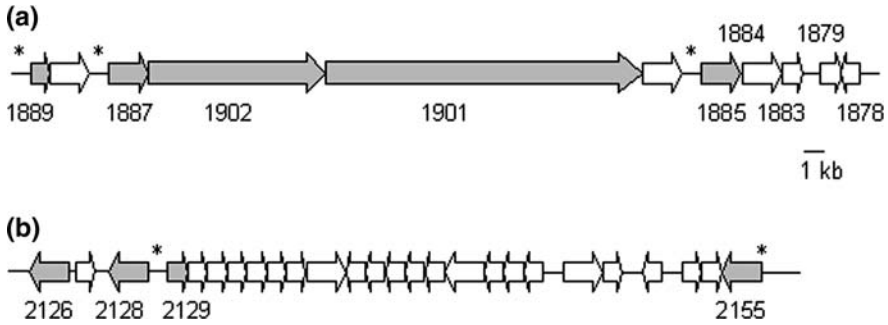

Fig. 6 A schematic representation of the gene clusters (**a**, **b**) isolated from the *P. mendocina* ymp cosmid library. Each arrow signifies an ORF and indicates the direction of transcription. Grey arrows specify ORFs that were detected by gene probes used in the genomic library hydridizations. The locations of putative regulatory Fur-binding sequences (*) are also shown. Fur-binding sequences have >50% similarity to the *E. coli* consensus fur-box (GATAATGATAATCATTATC)

Table 3 Predicted homology of ORFs detected by genomic library hybridization

Cosmid[a]	ORF	Probe[b]	Homology with known proteins
(a)	Pmen1889	*pvdS*	43% identity with RNA polymerase sigma-70 factors
	Pmen1887	*pvdA*	55% identity with lysine/ornithine *N*-monooxygenase
	Pmen1902	*NRPS*	42% identity to nonribosomal peptide synthetases
	Pmen1901	*NRPS*	49% identity to nonribosomal peptide synthetases
	Pmen1885	*fptA/fpvA*	84% identity with ferrichrome iron receptors
(b)	Pmen2126	*NRPS*	90% identity with acetyl-coenzyme A synthetases
	Pmen2128	*pvdS*	91% identity with sigma-54 transcriptional regulators
	Pmen2129	*pvdA*	87% identity with phenylalanine monooxygenases
	Pmen2155	*fptA/fpvA*	65% identity to methyl-accepting chemotaxis proteins

[a] Cosmid (a) is depicted in Fig. 6a, and (b) in Fig. 6b

[b] Sequences with regions of strong homology to the given probes were identified within the specified ORFs

of Immunology, New Delhi, India, and the Center for Bioinformatics Tübingen (ZBIT), University of Tübingen, Germany. Analyses indicate a siderophore consisting of seven polymerized residues encoded by seven separate domains (Fig. 7).

To determine whether the isolated gene cluster was involved in siderophore biosynthesis, a mutation was constructed within the Pmen1887 locus (*pmhA* gene) in the *P. mendocina* genome as described in Section "Materials and methods" (Fig. 6a). Wild-type *P. mendocina* ymp and the mutant were grown in Fe-replete and Fe-depleted liquid cultures and siderophore production was assayed during stationary phase of growth (54 h). Under Fe-replete conditions, both wild-type and the *pmhA* mutant grew equally well (data not shown) and exhibited very low supernatant siderophore concentrations as measured via the CAS assay (Table 4). However, under

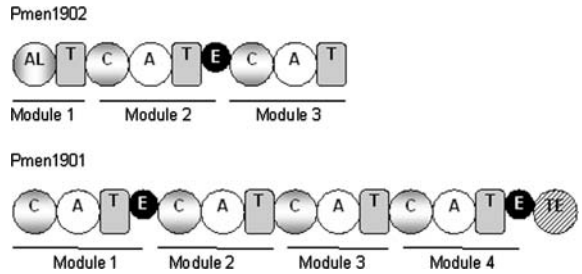

Fig. 7 Domain organization of NRPS genes Pmen1902 and Pmen1901 of *P. mendocina* ymp. AL, acyl CoA ligase; T, 4′phosphopantheteine binding; C, condensation; A, adenylation; E, epimerization; and TE, thioesterase

Fe-depleted conditions, the mutant exhibited a marked deficiency in siderophore production relative to the wild-type (Table 4). This suggests that the *pmhA* gene is essential for siderophore biosynthesis. Complementation of the mutant with the *pmhA* gene

Table 4 Siderophore concentrations in the supernatants of *P. mendocina* ymp, *pmhA* mutant, and *pmhA* mutant complement cultures

Strains	Siderophore (μM desferrioxamine)	
	Fe-replete	Fe-deplete
P. mendocina ymp	6.7 ± 2.6	91.3 ± 7.5
Δ*pmh*A	5.6 ± 4.1	6.6 ± 2.9
Δ*pmh*A :: *pmhA*	5.9 ± 2.9	87.6 ± 11.2

Results are shown from three independent experiments

on a plasmid restored siderophore production to normal wild-type levels under Fe-depleted conditions (Table 4).

Discussion

Microbial uptake of iron from natural sources has important geochemical and environmental repercussions, both for global iron cycling and trace metal mobilization (Cornell and Schwertmann 1996; Hersman et al. 2001). *P. mendocina* ymp is an environmentally important strain known to accelerate Fe dissolution and metal mobilization from a variety of distinct minerals and clays (Hersman et al. 2001, 2000; Kieft et al. 1997; Maurice et al. 2000, 2001a, b). Here, we have begun to probe the organism's capabilities for Fe metabolism on the molecular level.

Prior work indicated that the organism could produce large amounts of a siderophore or siderophores under Fe-limited conditions (Hersman et al. 2000). Attempts by others to HPLC-isolate the siderophore yielded five discrete peaks eluting with retention times spanning 55–70 min. Each peak contained an Fe-binding molecule according to the CAS assay. Mass spectrometry of the products indicated identical amino acid compositions and molecular weights (1152 Da) for the products; however, further structural information could not be obtained (Hersman et al. 2000).

We sought to further describe the siderophore by locating and analyzing the genes encoding siderophore biosynthesis, regulation, and uptake in *P. mendocina* ymp. Supernatants from cells grown under Fe-deficient conditions (Fig. 1) were first subjected to colorimetric tests, which indicated that the siderophores contained hydroxamate but not catecholate Fe-binding moieties. One-dimensional denaturing electrophoresis of the proteins prepared from the same cells showed two large molecular weight (280, 510 kDa) proteins produced specifically under Fe-deficient conditions (Fig. 2). This suggested that the cells might be producing siderophores from a NRPS-dependent rather than a NRPS-independent pathway.

A set of genes representing elements of a siderophore-associated iron uptake pathway common to Pseudomonad sp. and therefore potentially found in *P. mendocina* ymp was used to localize a siderphore-associated gene cluster to a particular cosmid or cosmids (Table 2). In this study, only the *fur* gene could be directly PCR-amplified from the *P. mendocina* ymp genomic library, or uniquely identified via library hybridization methods, likely because of its high degree of conservation and single genomic copy.

Comparisons between sequenced Pseudomonad genomes, however, indicate that iron acquisition genes, including genes encoding biosynthetic enzymes, regulatory sigma factors, and receptors, are often organized into one or more (up to three) clusters with sometimes strongly conserved gene synteny (Ravel and Cornelis 2003). The gene probe results were therefore used together to triangulate on a specific cluster of genes that are required for siderophore biosynthesis and regulation. Separately, *fpvA* and *fptA* gene probe results showed a high number and high mutual overlap in positive hits (Fig. 5), as expected from prior work (Chi et al. 2005; Thupvong et al. 1999). The *fpvA* and *fptA* gene probe results were therefore grouped. Overlapping the positive hits of the receptors with those for siderophore biosynthesis and regulatory sigma factors allowed for the identification of two cosmids each containing a positive hit to at least one gene of each type. Restriction mapping of the 40 kb inserts suggested the two are non-identical.

The sequences of the two cosmids were analyzed. The organization of the genes on each is shown in Fig. 6, and the closest homolog of each of the predicted proteins is given in Table 3. The first gene cluster (Fig. 6a) shows similarities between the siderophore biosynthesis gene clusters found in *P. aeruginosa* PAO1, *P. fluorescens* Pf-5, *P. syringae* pv. tomato str. DC3000, and *P. putida* KT2240. Although the organization of the gene cluster from *P. mendocina* ymp is not identical to any of other siderophore biosynthesis gene clusters, they all

contain genes encoding nonribosomal peptide synthetases (here, Pmen1902, Pmen1901), lysine/ornithine monooxygenase (Pmen1887), an RNA-polymerase sigma factor (Pmen1889), and a TonB-dependent receptor (Pmen1885). The cluster from *P. mendocina* ymp additionally contains genes encoding a peptide exporter and 2,4-diaminobutyrate transaminase (Pmen1888 and Pmen1886, respectively). The latter is often found as part of siderophore biosynthesis genes from other Pseudomonads, and is required for synthesis of the pyoverdine chromophore (Ravel and Cornelis 2003; Vandenende et al. 2004; Wandersman and Delepelaire 2004). Downstream of the TonB-dependent receptor (Fig. 6a) are other genes encoding enzymes involved in iron metabolism including a hydroxamate-dependent iron ABC transport system (Pmen1884, Pmen1883 and Pmen1878) and a ferric reductase (Pmen1879).

In order to confirm the involvement of the Fig. 6a genes in siderophore biosynthesis, a mutation was made to knock out the gene corresponding to locus number Pmen1887. Based on homology, this gene is predicted to encode an ornithine monooxygenase. A knock-out of a homologous, siderophore-associated amine monooxygenase in *P. aeruginosa* (*pvdA*) has been shown to have a siderophore-null phenotype (Banin et al. 2005). This is because amine monooxygenation is critical for generating the iron-chelating hydroxamate portions of the pyoverdine and pyochelin siderophores in this species. Knocking out Pmen1887 similarly resulted in a siderophore(−) phenotype in *P. mendocina* ymp, indicating that the corresponding gene is essential for production of a functional siderophore. This strongly suggests that it and the contiguous genes constitute a siderophore-associated gene cluster. Pmen1887 has consequently been named *pmhA*, for P*seudomonas* m*endocina h*ydroxamate.

The Pmen1902 and Pmen1901 genes identified by the NRPS probe are predicted to encode high molecular weight proteins of 352.7 and 591.6 kDa, respectively. These numbers are approximately 70–80 kDa larger than the apparent masses of two high molecular weight proteins observed by SDS-PAGE in iron-starved cultures of *P. mendocina* ymp (Fig. 2). Nonetheless, the Pmen1902 and Pmen1901 encoded proteins are most likely those identified in Fig. 2, where the discrepancy in molecular weights is potentially due to difficulties in resolving the very high molecular weight bands and estimating their masses from standards. The domain structures of Pmen1902 and Pmen1901 are characteristic of NRPSs (Stachelhaus et al. 1999) (Fig. 7). Informatic analysis using the Non-Ribosomal Peptide Synthetase DataBase (NRPSDB, www.nii.res.in/nrps-pks.html) indicates that Pmen1902 and 1901 can be resolved into 3 and 4 modules, respectively. Further analysis of the domain structure of Pmen1902 (directly upstream of Pmen1901) and Pmen1901 appears to indicate an interaction between the two NRPS. Pmen1901 starts with a condensation domain, suggesting that the natural product made by Pmen1902 is passed directly to Pmen1901 for additional processing. Further, Pmen1901 ends with a thioesterase domain, which catalyzes the release of the product from the NRPS and in some cases a cyclization at its tail end. Hence the two gene products together appear to generate a single natural product.

Each NRPS module incorporates a single substrate into a peptide chain (Ansari et al. 2004), where the substrate's identity is encoded by a series of 10 amino acid residues lining the specificity pocket of the module's adenylation domain (http://www-ab.informatik.uni-tuebingen.de/toolbox/) (Conti et al. 1997; Rausch et al. 2005). Specificity pockets and their corresponding substrates were predicted for each module via sequence comparisons using the NRPS-predictor software. Results are shown in Table 5. Although the sequences of the specificity pockets themselves could be obtained in every case, no reliable substrate predictions could be made for Pmen1902-module1, Pmen1902-module3, Pmen1901-module2, and Pmen1901-module4. Hence a peptide sequence of X-Asp-X-*Ser*-X-*Ser*-X is predicted by this program, where X = an unassigned residue and where the underlined residues occur in modules that contain epimerization domains. These residues, both serines, are therefore D-amino acids. D-serine residues are found in a variety of known Pseudomonad siderophores (Meyer 2000).

In order to gain some insight into possible substrates for the remaining domains, we examined the specificity pockets in several Pseudomonad NRPS sequences that construct siderophores of *known* structure (Table 5) (Mossialos et al. 2002). Comparing these to the unassigned modules above yields the following predicted peptide sequence: Acyl-Asp-Dab-*Ser*-fOHOrn-*Ser*-fOHOrn, where acyl = an acyl

Table 5 Predicted residues incorporated by NRPS adenylation domains of *P. mendocina* ymp

ORF	Module	10 amino acid code[a]	Predicted amino acid[a]	NRPS gene containing a similar amino acid code (residue incorporated/10 amino acid code)[b,c]
Pmen1902	1	DMMSASSLY	No match	*pvdL*(Acyl-cap/DMGSDSCLTK)
Pmen1902	2	DLTKIGHVGK	Asp	
Pmen1902	3	DIWELTADDK	No match	*pvdL*(Dab/DIWELTADK)
Pmen1901	1	DVWHVSLIDK	Ser	
Pmen1901	2	DGEACAGVTK	No match	*pvdJ*(fOHOrn/DGEVCGGVTK)
Pmen1901	3	DVWHVSLIDK	Ser	
Pmen1901	4	DGEACAGVTK	No match	*pvdJ*(fOHOrn/DGEVCGGVTK)

[a] Amino acid codes were identified via NRPS gene alignments made by NRPSpredictor Program. Predictions of the encoded amino acid were generated from the same program (see text)

[b] When an encoded amino acid could not be predicted using this program, a manual method was applied. Amino-acid-encoding regions from several Pseudomonad-derived NRPSs producing structurally characterized siderophores were identified using the same program (from *P. aeruginosa* PAO1, *P. aeruginosa* ATCC27853, *P. aeruginosa* Pa6, *P. fluorescens* 9AW, *P. putida* CFB2461; see Thupvong et al. (1999). These were compared manually with the coding regions identified for the pair of *P. mendocina* NRPSs. Best matches are shown

[c] Dab:2,4-diaminobutyric acid, fOHOrn: N^5-hydroxy-N^5-formyl-L-ornithine

capping group, Dab = 2,4-diaminobutyrate, fOHOrn = N^5-hydroxy-N^5-formyl-L-ornithine. To date, no similar siderophore peptide sequence has been identified in other Pseudomonads. The final ornithine residue in the series would likely be hydroxylated by Pmen1887 and cyclized by the terminal thioesterase domain. Interestingly, Pmen1886, an open reading frame found in the center of the siderophore biosynthesis cluster, appears to encode an L-2,4-diaminobutyrate:α-ketoglutarate 4-aminotransferase. This enzyme converts aspartate β-semialdehyde to L-2,4-diaminobutyrate, which subsequently can condense with L-tyrosine to yield the characteristic chromophore of pyoverdine siderophores (Bockmann et al. 1997). L-2,4-diaminobutyrate is also found in the peptide backbone of some siderophores, often with the 2-amino group removed. *P. mendocina* is nonfluorescent and its siderophore-containing supernatants do not bear the characteristic pyoverdine absorbance at 405 nm (data not shown). Hence Dab would be expected to be part of the backbone of this organism's siderophore.

The sequence above could bind Fe(III) via the two ornithine-derived hydroxamate groups and potentially via the carboxylic acid of aspartate. More commonly, an α-hydroxy-carboxylate rather than carboxylic acid would however serve as the chelating group. Three-dimensional molecular models of Acyl-Asp-Dab-*Ser*-fOHOrn-*Ser*-fOHOrn with an added α-hydroxyl group on the aspartic acid, either computationally generated and energy minimized (MM2) or built manually from model kits, very easily accommodate a central Fe(III) via these three chelating groups. The predicted molecular weight of this sequence (~792 amu if acyl = 3-formylpropanamide) does not match that previously measured by LC–MS for the siderophore (Hersman et al. 1993). Reasons for the discrepancy are unclear but should be resolved by future structural characterization of the siderophore.

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