Pyoverdine synthesis by the Mn(II)-oxidizing bacterium

*Pseudomonas putida* GB-1

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When iron-starved, the Mn(II)-oxidizing bacteria *Pseudomonas putida* strains GB-1 and MnB1 produce pyoverdines (PVD<sub>GB-1</sub> and PVD<sub>MnB1</sub>), siderophores that both influence iron uptake and inhibit manganese(II) oxidation by these strains. To explore the properties and genetics of a PVD that can affect manganese oxidation, LC-MS/MS, and various siderotyping techniques were used to identify the peptides of PVD<sub>GB-1</sub> and PVD<sub>MnB1</sub> as being (for both PVDs): chromophore-Asp-Lys-OHAsp-Ser-Gly-aThr-Lys-cOHOrn, resembling a structure previously reported for *P. putida* CFML 90-51, which does not oxidize Mn. All three strains also produced an azotobactin and a sulfonated PVD, each with the peptide sequence above, but with unknown regulatory or metabolic effects. Bioinformatic analysis of the sequenced genome of *P. putida* GB-1 suggested that a particular non-ribosomal peptide synthetase (NRPS), coded by the operon PputGB1_4083-4086, could produce the peptide backbone of PVD<sub>GB-1</sub>. To verify this prediction, plasmid integration disruption of PputGB1_4083 was performed and the resulting mutant failed to produce detectable PVD.

In *silico* analysis of the modules in PputGB1_4083-4086 predicted a peptide sequence of Asp-Lys-Asp-Ala-Thr-Lys-Orn, which closely matches the peptide determined by MS/MS. To extend these studies to other organisms, various Mn(III)-oxidizing and non-oxidizing isolates of *P. putida*, *P. fluorescens*, *P. marincola*, *P. marincola-syringae* group, *P. mendocina-resinovorans* group, and *P. stutzeri* group were screened for PVD synthesis. The PVD producers (12 out of 16 tested strains) were siderotyped and placed into four sets of differing PVD structures, some corresponding to previously characterized PVDs and some to novel PVDs. These results combined with previous studies suggested that the presence of OHAsp or the flexibility of the pyoverdine polypeptide may enable efficient binding of Mn(III).

**Keywords:** siderophore, pyoverdine, azotobactin, manganese oxidation, iron

INTRODUCTION

The global manganese oxidation-reduction cycle, which depends on microbial activities that increase the manganese oxidation rate by up to 5 orders of magnitude (Hastings and Emerson, 1986; Tebo et al., 2004), strongly influences the cycling of organic compounds, pollutants, and many elements including carbon, arsenic, uranium, and chromium (Tebo et al., 2004). Among the most prevalent Mn(II)-oxidizing bacteria are various *Pseudomonas* species, which oxidize soluble Mn<sup>2+</sup> to insoluble Mn(IV)oxides that accumulate in late logarithmic and early stationary growth phases (Toner et al., 2005). These Mn oxides coat the cells with dark brown precipitates of nanoparticulate MnO<sub>2</sub>, birnessite-type minerals that exhibit large surface areas and efficient adsorption of toxic metals and organics (Villalobos et al., 2003, 2006), contributing to the environmental importance of this process. Oxidation of Mn<sup>2+</sup> by all tested pseudomonads is enzymatic and utilizes oxygen as an electron acceptor (Okazaki et al., 1997; Brouwers et al., 1999; Francis and Tebo, 2001).

The model Mn(II)-oxidizing *Pseudomonas putida* strains GB-1 and MnB1 are typical representatives of the widely-distributed and diverse group of several “fluorescent *Pseudomonas*” species, which synthesize fluorescent iron-chelating compounds (siderophores) called pyoverdines (PVDs) to scavenge iron in iron-starved conditions (Budzikiewicz, 1993; Albrecht-Gary et al., 1994; Schalk et al., 2002). However, PVDs also form strong complexes with Mn(III) and can inhibit the enzymatic formation of MnO<sub>2</sub> by *P. putida* GB-1 and MnB1 (Parker et al., 2004, 2007), raising several interesting questions about the interplay of iron and manganese metabolisms in these organisms and in the environment.

The PVDs of various fluorescent pseudomonads share the same chromofluorophore [(1S)-5-amino-2,3-dihydro-8,9-dihydroxy-1H-pyrimido-[1,2-a]quinoline-1-carboxylic acid], but can differ in an attached peptide chain that is recognized by a strain-specific PVD uptake receptor on the cell surface (Fuchs et al., 2001; Clement et al., 2004; Schons et al., 2005,
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Shen et al., 2005). Most isolates synthesize and recognize a suite of PVDs, usually with the same peptide but with various modifications including the addition of acyl chains to the chromophore, sulfonation of the chromophore, or the formation of azotobactin, in which an extra 5-membered ring is added to the PVD chromophore (Fuchs et al., 2001). Both the peptide that comprises the backbone of a PVD and the chromophore are synthesized by non-ribosomal peptide synthetases (NRPSs) (Ravel and Cornelis, 2003). NRPSs are large enzymes containing multiple modules; the number and order of these modules generally correlate to the number and order of (modified or unmodified) amino acids in the peptides (Ravel and Cornelis, 2003). Among the domains found within each of the modules, the adenylation domains specifically recognize and activate corresponding amino acids. This property enables in silico predictions concerning the amino acid sequence corresponding to a particular NRPS (Rausch et al., 2005). The sequence of the peptide backbone of a PVD can also be indirectly achieved by siderotyping. Siderotyping compares unknown PVDs with standard PVDs in terms of isoelectric focusing. Siderotyping compares differing structures (which defines the specificity of a strain’s PVD uptake receptor) and, if necessary, mass spectrometric (MS) techniques adapted to PVDs (Fuchs and Budzikiewicz, 2001; Fuchs et al., 2001; Meyer et al., 2008). Among other things, siderotyping rapidly screens whether a strain produces a previously-described or a novel PVD and can aid in determining the sequence of the peptide backbone, confirmed by MS/MS.

The present study aims: (1) to identify NRPSs responsible for synthesis of the peptide backbone of the PVD produced by P. putida GB-1, using genomic and genetic analyses; (2) to describe the structure of this PVD based on in silico predictions coupled with siderotyping and MS/MS determinations; and (3) assess whether there is any correlation between PVD structure or siderotype and the ability to oxidize Mn(II). Additionally, siderotyping of the PVDs synthesized by several other Mn(II)-oxidizing Pseudomonas species from diverse environments was also included to define a set of PVDs with varying peptide composition and differing uptake receptor specificity for use in future investigations of PVD effects on Mn(II) oxidation in pseudomonads.

MATERIALS AND METHODS

IN SILICO ANALYSIS

For phylogenetic analysis, a maximum likelihood tree was constructed with NRPS sequences of known capabilities. Sequences used include the PvdI, J and D proteins of P. aeruginosa PA01 (PA2399-2402), Psyr_1957-1960 from P. syringae pv. B301D (Scholz-Schroeder et al., 2003). To generate a plasmid integration disruption mutation of PputGB1_4083 (PputGB1_4083::pKG220), a homolog of pvdI, an ∼1 kb region within the gene was amplified using primer fliF_2-R (ACGATGTCAGCGCCGACCC). This primer was fortuitously discovered to anneal near the 3’ end of PputGB1_4083 on opposite strands of the DNA, producing a 1 kb product. The PCR product was then cloned into the specialized PCR cloning plasmid pJET1.2/blunt (Fermentas) then subcloned into pKG161, a derivative of pEX18Gm (Table 1) from which the sacB gene had been deleted by digestion with MscI/SnaBI and self-ligation of the plasmid backbone. This plasmid (pKG220) was moved by conjugation into P. putida GB-1 (Geszvain and Tebo, 2010) and transconjugants were screened for Gm resistance. GmR colonies were screened for homologous recombination between the plasmid and the chromosome, resulting in integration of the plasmid into the chromosome, by isolating genomic DNA from candidate colonies and screening by PCR using the M13-F primer, which anneals within the plasmid, and the 4083_1-F primer (GGGCCGACCATCGGTTGAAAG), which anneals within PputGB1_4083 immediately upstream of the region present on pKG220. The ability to amplify an ∼1 kb product with these primers indicated that the plasmid had integrated into the chromosome to generate PputGB1_4083::pKG220.
Table 1 | Bacterial strains and plasmids used in genetic studies in this work.

| Characteristics | References |
|-----------------|------------|
| **P. putida GB-1 STRAINS** | |
| GB-1 | Wild type | Corstjens et al., 1992 |
| KG163 | PputGB1_4083::pKG220, GmR | This work |
| KG165 | glmS::pKG222, GmR | This work |
| **PLASMIDS** | |
| pEX18Gm | Gene replacement vector, GmR, orT, sacB | Hoang et al., 1998 |
| pJET1/2/blunt | Commercial cloning vector | Fermentas |
| pKG161 | pEX18Gm with MscI/SnaBI fragment removed | This work |
| pKG220 | pKG161 with ~1 kb internal fragment from PputGB1_4083 cloned into the BamHI site | This work |
| pKG222 | pKG161 with ~300 bp from the attTn7 region cloned into the BamHI site | This work |

To address the concern that the presence of the plasmid backbone itself could affect the phenotype of the bacteria, we also generated a plasmid integration strain in which the plasmid was inserted downstream from PputGB1_5427 (glmS) in the attTn7 region of the chromosome. This region commonly tolerates insertions without affecting cell growth/behavior (Choi et al., 2005). The attTn7 region was amplified using primers glmS-F (GTT GGGTGTTGTTCGCCAGCAG) and glmS-R (TTCAAGGCACGGG AGGG), and then cloned into pKG161 to generate plasmid pKG222 as described above. Plasmid integrants were generated as above and screened via PCR with the M13-F primer and the attTn7 region primer glmS_3-F (GGGCCGAACACG AACCTGC).

**TEST OF NRPS MUTANT**

The wild-type equivalent, glmS::pKG222, and NRPS mutant, pvdI::pKG220, were grown in LB containing 50 μg ml⁻¹ gentamicin and streaked out on LB plates supplemented with 36 μM FeSO₄ with either 100 μM or 1 mM 2‘-2‘ dipyridyl (Lehoux et al., 2000), CAS-CAA (Matthijs et al., 2004), or succinate medium (Meyer et al., 1997) to determine production of PVDs or siderophores.

**COMPARISON OF Mn-OXIDIZING STRAINS**

Table 2 lists the strains examined. For tests of siderophore production, each organism was serially transferred three times in each of two media: low-iron casamino acids medium (Meyer et al., 1997) and succinate minimal medium without added iron (Meyer et al., 1997). Chrom azurol S (CAS) tests of general siderophore presence in centrifuged and filtered culture supernatants were performed by the standard shuttle method (Schwyn and Neillands, 1987). For PVD detection, culture supernatants (ca. pH 7.7) were adjusted to pH 5, 6, or 8 with HCl or NaOH, transferred to quartz cuvettes (1 cm path length) and examined in a SpectraMax M2 scanning spectrophotometer-fluorimeter (Molecular Devices). PVD was identified by its known fluorescence (excitation at 405 nm; emission read at 470 and 535 nm, pH 8) and by its characteristic UV-vis absorbance properties as a function of pH (Albrecht-Gary et al., 1994; Parker et al., 2004, 2007), with screening for absorbance maxima at ca. 364 (pH 5), 380 (pH 6), and 400–405 nm (pH 7.5–8). Isoelectric focusing and biological uptake of ⁵⁹Fe-PVD standards were as previously described (Fuchs et al., 2001; Meyer et al., 2007), with PVD standards from: Pseudomonas putida CFML 90-44, Pseudomonas sp. G76, Pseudomonas sp. G4, P. putida CFML 90-51, P. putida GS43, P. costantinii CFBP 5705T, P. fluorescens W, P. monteilii CFML 90-54, P. putida GS37, P. aeruginosa Pa6, Pseudomonas sp. 2908, P. putida WCS358, P. fluorescens PL7, Pseudomonas sp. B10, P. fluorescens 51W, P. fluorescens Pflii, Pseudomonas sp. CFML 96-188, P. fluorescens Pf12, Pseudomonas sp. D47, P. thivervalensis ML45, P. fluorescens Pf0-1, P. putida AP3, Pseudomonas sp. G85, Pseudomonas sp. F317, and Pseudomonas sp. F360 (abbreviations: CFML, Collection de la Faculté de Médecine de Lille, France; CFBP, Collection Française de Bactéries Phytopathogènes, Angers, France).

**RESULTS**

**IN SILICO IDENTIFICATION OF THE PUTATIVE PVD SYNTHESIS OPERON IN P. putida GB-1**

The genome of P. putida GB-1 encodes an NRPS operon comprised of the genes PputGB1_4086 through PputGB1_4083 (Figure 1A) (Markowitz et al., 2008). These four genes are annotated as encoding NRPSs and have homology to the P. aeruginosa PA01 PVD synthesis genes pvdIJ/F and D. Furthermore, downstream of this operon is a putative TonB-dependent siderophore receptor gene (PputGB1_4082). A gene encoding a homolog of PvdO (PputGB1_4081) which appears to have some role in PVD formation (Yeterian et al., 2010) was also found to be present (Figure 1A). Upstream of the first gene in the putative operon—PputGB1_4086—is a sequence with a perfect match to the PvdS sigma recognition site (TAAAT-N₁₆-CGT) (Ochsner et al., 2002) (Figure 1B). The alternative sigma factor PvdS is an iron-responsive extracytoplasmic function (ECF) sigma (Leoni et al., 2000), suggesting that expression of the genes PputGB1_4086–4083 is regulated by iron concentration as would be expected for a PVD synthesis operon. A PvdS recognition site is located upstream of the pvdI (PA2402) and pvdD (PA2399) genes of P. aeruginosa PA01 as well (Ochsner et al., 2002). PputGB1_3810, a homolog of pvdS (PA2424), was found along with PputGB1_3809, a homolog of pvdL/psvA (PA2424), a putative NRPS for chromophore synthesis (Mossialos et al., 2002) (Figure 1A). Also found in this operon along with NRPSs for PVD peptide backbone synthesis is a homolog of SyrP (PputGB1_4087) which is an Asp hydroxylase required for synthesis of syringomycin (Singh et al., 2008).

**GENERATION OF AN NRPS MUTANT**

If the PputGB1_4086–4083 operon encodes the synthetic machinery of the PVD peptide in P. putida GB-1, disruption of this operon should lead to a loss of PVD synthesis, as has been shown to occur following mutation of the PVD peptide NRPS genes pvdIJ and pvdD in P. aeruginosa PA01 (Merriman et al., 1995;
Lehoux et al., 2000). Plasmid integration disruption was therefore performed on PputGB1_4083, which encodes a homolog to pvdI (PA2402 in P. aeruginosa PAO1). As would be expected, the plasmid integration mutant (KG163) did not fluoresce and lacked yellow-green pigments whereas a control strain in which the plasmid was integrated into the attTn7 site (KG165) did, suggesting the NRPS mutant KG163 did not produce PVDs (Figures 2A,B).

KG163 was also grown in increasing amounts of the iron-chelator dipyridyl to verify the decreased ability to synthesize PVD, which is needed to compete for iron under these conditions. As shown in Figure 2C, the growth of the NRPS mutant is inhibited by increasing amounts of dipyridyl while growth of KG165 was not. This result supports the conclusion that the inability to acquire iron and not toxicity of dipyridyl was responsible for the growth defect of the NRPS mutant, KG163. Based on these findings, we conclude that PputGB1_4083 is required for PVD synthesis.

**IN SILICO ANALYSES OF ADENYLATION DOMAINS OF NRPSs**

To determine the peptide sequence of PVDGB−1, *in silico* analyses were performed using the NRPSpredictor website (http://www-ab.informatik.uni-tuebingen.de/software/NRPSpredictor) (Rausch et al., 2005). In *in silico* analyses predicted 8 adenylation modules within the 4 genes of the putative PVD peptide synthesis operon (PputGB1_4086–4083) (Figure 1B). Also, it was possible to generate a preliminary prediction of the sequence of amino acids in the peptide: Asp1−Xxx2−Asp3−Ser4−Ala5−Xxx6−Thr7−Xxx8 (where Xxx indicates amino acids that could not be predicted using NRPSpredictor). To further investigate the nature of the amino acids recognized by each of the adenylation domains in the putative PVD peptide synthesis operon of *P. putida* GB−1, we compared the amino acid sequence of each domain to that of other pseudomonad PVD synthesis proteins that produce PVDs with known structures. In this analysis, most of the adenylation modules from *P. putida* GB−1 fell into distinct clusters with

### Table 2 | Properties of various *Pseudomonas* sp. strains examined.

| Strain | *Pseudomonas* species or group (gp), from 16S rRNA* | Forms MnO2 | CAS reactionb (PVD reaction)b | Siderotypec | 59Fe-PVD uptake (% homologous uptake)d | Isolated frome (references in footnote) |
|--------|--------------------------------------------------|------------|--------------------------------|------------|--------------------------------------|----------------------------------------|
| CFML 90-45 | putida | No | CAS+ (PVD+) | 1 | CFML 90-51 (>90%) | Clinical specimen |
| CFML 90-48 | putida | No | CAS+ (PVD+) | 1 | CFML 90-51 (>90%) | Clinical specimen |
| CFML 90-49 | putida | No | CAS+ (PVD+) | 1 | CFML 90-51 (>90%) | Clinical specimen |
| CFML 90-50 | putida | No | CAS+ (PVD+) | 1 | CFML 90-51 (>90%) | Clinical specimen |
| CFML 90-51 | putida | No | CAS+ (PVD+) | 1 | CFML 90-51 (100%) | Clinical specimen |
| GB-1 | putida | Yes | CAS+ (PVD+) | 1 | CFML 90-51 (95%) | Freshwater sediment |
| MnB1 | putida | Yes, at low O2 | CAS+ (PVD+) | 2 | F317 (91%) | Soil, toluate deg |
| ATCC 55241 | fluorescens biotype II BNL-IVC | No | CAS+ (PVD+) | 3 | No match to known PVD | Radiowaste leachate |
| ISO6 | fluorescens-syringae gp. | Yes, at low O2 | CAS+ (PVD+) | 4 | D47, SB8.3 (~50% each) | Sediment, mine drainage |
| PCP1 | fluorescens-syringae gp. | Yes | CAS+ (PVD+) | 4 | Metallogenium particles | Metallogenium particles |
| MG1 | fluorescens-syringae gp. | Yes | CAS+ (PVD+) | NT1 | Metallogenium particles | Metallogenium particles |
| ISO1 | fluorescens-syringae gp. | Yes | CAS+ (PVD−) | NA3 | NA | Pulpmill effluent |
| GP1 | stutzeri gp. | Yes | CAS− (PVD−) | NA | NA | Marine bay, suboxic |
| SJ85-2B | marincola | Yes | CASNT (PVD−) | NA | NA | Sediment, mine effluent |
| PCP2 | mendocina-resinovorans gp. | Yes | CAS− (PVD−) | NA | NA | Sediment, mine effluent |

*Based on 16S rRNA sequence (Francis and Dodge, 1998; Francis and Tebo, 2001; Meyer et al., 2007).

bThe chrom azurol S (CAS) method, which depends on the ability of siderophores to displace Fe from its CAS complex, is a general assay for siderophores (Schwyn and Neilands, 1987). The presence of pyoverdine-group siderophores (PVD) was detected from UV-vis absorption and fluorescence spectra (Parker et al., 2007). A strain was scored positive (+1) if a CAS reaction or PVD was detected.

cBased on the isoelectric focusing pattern of each strain's fluorescent PVD and confirmed by each strain's uptake of FePVD standard that was taken up in greatest amount. (% uptake compared to that of the homologous standard strain).

dBased on 16S rRNA sequence (Francis and Dodge, 1998; Francis and Tebo, 2001; Meyer et al., 2007); freshwater pipe encrusted with MnO2, Germany (Schweisfurth, 1973); freshwater sediment, Green Bay of Lake Michigan, USA (Francis and Tebo, 2001); laboratory variant selected by Brandy-Toner in the Garrison Sposito laboratory, University of California Berkeley, USA; marine fjord, oxic-oxic-anoxic interface, Saanich Inlet, Vancouver Island, BC, Canada (Emerson et al., 1982; Francis and Tebo, 2001; Romanenko et al., 2009); Metallogenium particles from Horsetooth Reservoir, Fort Collins, CO, USA (Francis and Tebo, 2001); pulpmill effluent, Grande Prairie, AB, Canada (Francis and Tebo, 2001); radiowaste leachate, low-level radioactive waste leachate, Brookhaven Natl. Lab., USA (Francis and Dodge, 1998); sediment, mine drainage, Pinal Creek, Globe, AZ, USA, downstream from a Cu mine (Fuller and Harvey, 2000; Francis and Tebo, 2001); soil, toluate deg, soil enrichment for degradation of toluate, Osaka, Japan (Nakazawa, 2002; Regenhardt et al., 2002).

eClinical specimen or from associated medical environment, Collection de la Faculté de Médecine de Lille, France (Meyer et al., 2007); freshwater pipe encrusted with MnO2, Germany (Schweisfurth, 1973); freshwater sediment, Green Bay of Lake Michigan, USA (Francis and Tebo, 2001); laboratory variant selected by Brandy-Toner in the Garrison Sposito laboratory, University of California Berkeley, USA; marine fjord, oxic-oxic-anoxic interface, Saanich Inlet, Vancouver Island, BC, Canada (Emerson et al., 1982; Francis and Tebo, 2001; Romanenko et al., 2009); Metallogenium particles from Horsetooth Reservoir, Fort Collins, CO, USA (Francis and Tebo, 2001); pulpmill effluent, Grande Prairie, AB, Canada (Francis and Tebo, 2001); radiowaste leachate, low-level radioactive waste leachate, Brookhaven Natl. Lab., USA (Francis and Dodge, 1998); sediment, mine drainage, Pinal Creek, Globe, AZ, USA, downstream from a Cu mine (Fuller and Harvey, 2000; Francis and Tebo, 2001); soil, toluate deg, soil enrichment for degradation of toluate, Osaka, Japan (Nakazawa, 2002; Regenhardt et al., 2002).

fNA, not applicable because that organism does not make PVD.
Clusters of putative genes likely to be involved in PVD synthesis in P. putida GB-1. Identification is based on in silico comparison to known PVD-related genes, as described in the text. Genes are annotated according to Ravel and Cornelis (2003). Color schemes are based on Ravel and Cornelis (2003). Numbers on top are locus tags for P. putida GB-1 (PputGB1 numbers). Length is not to scale.

The 8 predicted adenylation domains found in the putative NRPSs (PputGB1_4083-4086) in P. putida GB-1, and the resulting peptides of each adenylation domain predicted using NRPSpredictor and cluster analysis in comparison with the reported peptide sequence of PVD_CFML90-51. The NRPS genes are shown in green with the adenylation domains boxed in blue.

FIGURE 1 | (A) Clusters of putative genes likely to be involved in PVD synthesis in P. putida GB-1. Identification is based on in silico comparison to known PVD-related genes, as described in the text. Genes are annotated according to Ravel and Cornelis (2003). Color schemes are based on Ravel and Cornelis (2003). Numbers on top are locus tags for P. putida GB-1 (PputGB1 numbers). Length is not to scale. (B) The 8 predicted adenylation domains found in the putative NRPSs (PputGB1_4083-4086) in P. putida GB-1, and the resulting peptides of each adenylation domain predicted using NRPSpredictor and cluster analysis in comparison with the reported peptide sequence of PVD_CFML90-51. The NRPS genes are shown in green with the adenylation domains boxed in blue.

FIGURE 2 | KG163 (PVD synthesis mutant of P. putida GB-1) and KG165 (WT equivalent) grown in succinate medium and visualized under normal light (A) and UV (B). KG163 and 165 grown on LB supplemented with 36 μM FeSO₄ and different amounts of 2′-2′ dipropyridyl (C). Plate on the left has 100 μM and the right has 1 mM dipyridyl while the left side of each plate is the WT equivalent and on the right side of each plate is the NRPS mutant.

FIGURE 2 | KG163 (PVD synthesis mutant of P. putida GB-1) and KG165 (WT equivalent) grown in succinate medium and visualized under normal light (A) and UV (B). KG163 and 165 grown on LB supplemented with 36 μM FeSO₄ and different amounts of 2′-2′ dipropyridyl (C). Plate on the left has 100 μM and the right has 1 mM dipyridyl while the left side of each plate is the WT equivalent and on the right side of each plate is the NRPS mutant.

known adenylation modules in other organisms (Figure 3). The cluster analysis confirmed the conclusions of NRPS predictor for modules m1 (Asp), m3 (Asp), and m4 (Ser), but suggested a Lys at m7 instead of the Thr chosen by NRPS predictor (Figure 1B). For the three cases in which NRPS predictor failed to select an amino acid, the identifications by cluster analysis were: m2 (Lys), m6 (Thr), and m8 (Orn) (Figure 1B). More precisely, module m6 clusters with other Thr-incorporating modules from five different pseudomonads, and it appears to be distinct from the allo-Thr incorporating modules of SypC, although these belong to the more distantly related organism Pseudomonas syringae pv. syringae B301D. Module m8 falls into a cluster with two P. aeruginosa PAO1 modules that incorporate N⁵-formyl-N⁵-hydroxyornithine (hfOrn) but is located on a long branch and therefore might direct incorporation of a different amino acid. However, the P. putida GB-1 genome encodes a homolog to PvdA (PputGB1_2120), which is an enzyme responsible for the modification of ornithine to OHOrn (Visca et al., 2007) and thus strain GB-1 is predicted to have the biochemical potential to generate OHOrn. Module m5 appears to cluster somewhat near several Ala-incorporating modules from Pseudomonas syringae pv. syringae B301D, but lies on a long branch outside of that cluster, which may reflect a lack of sufficiently homologous sequences to accurately root this branch on the tree.

CHEMICAL COMPOSITION AND SIDEROTYPING OF PVD_GB_1 AND PVD_MnB1

The PVDs of strains P. putida GB-1 and MnB1 were siderotyped in comparison to those of several isolates of known PVD structure (Table 2). Strains GB-1 and MnB1 were found to be in the same siderotype as P. putida CFML 90-51, which has been reported to have the following PVD peptide sequence: chromophore-Asp-Lys-OHAsp-Ser-Gly-aThr-Lys-cOHOrn (D-amino acids underlined) (Sultana et al., 2000). Since this sequence was strikingly similar to that predicted for PVD_GB-1 by cluster analysis, it is included in Figure 1A for comparison. To determine whether this sequence also applied to strains GB-1 and MnB1, mass spectrophotometric (MS/MS) analysis was used to obtain molecular weights and also to identify the secondary MS/MS fragments of
each PVD in partially purified preparations from the *P. putida* strains GB-1 and MnB1, in comparison to parallel preparations from *P. putida* CFML 90-51 (Table 3). The PVDs of all three organisms shared a major MS peak at the monoisotopic m/z of 1250–1251 Da (Table 3). This value, as well as the weights of secondary MS/MS fragmentation products, corresponded to the peptide sequence given above. Our data agreed with the previous report for PVD_{CFML,90−51} (Sultana et al., 2000), except that the variable acyl side chain in our samples from strain GB-1 was malic acid amide (mala) instead of malic acid (mal) and the acyl side chain in our CFML 90-51 preparation was ~50% mala and ~50% mal, in contrast to the previous report in which only mal was found with CFML 90-51 (Sultana et al., 2000). Interestingly, MS/MS samples from all three strains, CFML 90-51, MnB1, and GB-1, also contained a major monoisotopic peak at m/z 1161 Da, which was identified to contain the same PVD peptide as in the 1250 Da peak, but with the PVD chromophore replaced by an azotobactin chromophore, a structure that is frequently co-produced with pyoverdine (Fuchs et al., 2001). Material from each peak at m/z 1250, 1161, and 1333 Da was further fragmented and analyzed by MS/MS. Since these secondary fragments from the three tested siderotype n° 1 strains were indistinguishable (data not shown), we conclude that these strains produced the same general set of PVD-type siderophores including sulfonated and non-sulfonated PVD and azotobactin.

**COMPARISON OF Mn-OXIDIZING AND Mn-NON-OXIDIZING STRAINS**

*Pseudomonas* isolates from diverse habitats and taxonomic groupings were characterized with respect to MnO2 formation and siderophore production (Table 2). The strains comprised six 16S rRNA groupings: *P. putida*, *P. fluorescens*, *P. maricola*, *P. fluorescens-syringae* group, *P. mendocina-resinovorans* group, and *P. putida* group (Table 2). When iron starved, most strains were positive in the chrome azurol S (CAS) assay (Table 2), a standard method that detects the production of most types of siderophores.
The majority also formed PVD, as indicated by their release of green fluorescent compounds into the medium under iron-limiting, but not iron-replete, conditions (Table 2). The presence of PVD in culture fluids was confirmed by examination of fluorescence and absorbance spectra at pH 5, 6, and 8, both in the absence and presence of iron (Parker et al., 2004, 2007); in all positive cases the spectra were those expected for PVD (data not shown because they were so standard).

When the above strains were siderotyped based on the isoelectric focusing (IEF) patterns of their PVDs (Fuchs et al., 2001; Meyer, 2007), seven P. putida isolates showed identical IEF patterns (Table 2); these strains included two Mn(II) oxidizers (strains GB-1 and Mnb1) and five isolates that did not detectably oxidize Mn(II) (the five CFML strains in Table 2). These seven strains also incorporated $^{59}$Fe-PVD from the reference organism CFML 90-51 as efficiently as their own $^{59}$Fe-PVD (Table 2), but did not internalize 24 other $^{55}$Fe-PVD standards of other siderotypes (see Methods). Based on our data and previous characterizations of the five CFML strains (Meyer et al., 2007), we conclude that these 7 isolates are of the same siderotype, here designated siderotype n$^1$ (Fuchs et al., 2004, 2007); in all positive cases the spectra were those expected for PVDGB$^1$ and PVDGB$^2$ (Fuchs et al., 2004, 2007); in all positive cases the spectra were those expected for PVDGB$^1$ and PVDGB$^2$ (Fuchs et al., 2004, 2007).

In silico examination of the P. putida GB-1 genome also identified candidates (Figure 1) for many of the other genes needed for the synthesis, modification, and expression of PVD$_{GB-1}$, including a NRPS for chromosome synthesis (PputGB1$_{3809}$), a TonB-dependent siderophore receptor gene (PputGB1$_{4082}$), an Asp hydroxylase (PputGB1$_{4087}$), an Orn hydroxylase (PputGB1$_{2120}$), and an iron-responsive ECF alternative sigma factor (PputGB1$_{3810}$) with recognition sites found in the promoters upstream of most of the above genes.

Based on siderotyping followed by MS/MS analysis (Tables 2, 3), we have determined the peptide sequences of PVD$_{GB-1}$ and PVD$_{Mnb1}$ to be: chromohem-Asp-Lys-OHAsp-Ser-Gly-aThr-Lys-cOHOrn, identical to that reported (Sultana et al., 2000) and confirmed here (Table 3) for PVDCFML90–51. The OHAsp and aThr in PVDCFML90–51 are known to be D isomers (Sultana et al., 2000). PVD$_{GB-1}$ and PVD$_{Mnb1}$ probably also contain these two D amino acids because the degree of uptake of $^{59}$Fe-labeled PVDCFML90–51 by strains GB-1, Mnb1, and CFML 90-51 was similar (Table 2), suggesting that the cellular uptake receptors of strains GB-1 and Mnb1 did not detect a steric difference between PVDCFML90–51 and the endogenous PVD of each strain. However, the presence of D amino acids in PVD$_{GB-1}$ and PVD$_{Mnb1}$ has not been directly tested.

The peptide sequence above suggests a metal-binding pocket formed by three moieties: (1) the catecholate of the chromophore, (2) the cyclic hydroxamate from cOHOrn, and (3) the α-OH-carboxylate from OHAsp. It is not yet clear how this structure, including the presence of a (OH)carboxylate donor group, leads to the higher thermodynamic stability constants for Mn(III), as compared to Fe(III), reported for these siderophores at physiological and alkaline pH (Parker et al., 2004; Harrington et al., 2012). However, an influence of OHcarboxylate on the preferential binding of Mn(III) has been recently suggested by an investigation showing that siderophores containing solely hydroxamates (e.g., desferrioxamine B, DFOB) or solely catecholates (e.g., protocelin) bind Fe(III) more strongly than Mn(III), whereas rhizoferrin, which complexes metals via a mixture of carboxylate and (OH)carboxylate groups, binds Mn(III) more strongly than Fe(III) (Harrington et al., 2012), as do several aminocarboxylate ligands (Hamm and Suwyn, 1967; Ahrland et al., 1990; Martell and Smith, 2003). Based on K-edge EXAFS comparisons of various Fe(III)- and Mn(III)-siderophore complexes in solution, Harrington et al. (2012) proposed that the greater flexibility of carboxylates vis-à-vis hydroxamates or catecholates allows the former to accommodate the Jahn-Teller-distorted coordination that is characteristic of Mn(III) but not Fe(III). This result suggests that (OH)carboxylates within siderophores, perhaps including the siderotype n$^1$ PVDs studied here, may affect the preferential binding of Mn(III). However, the situation for pyoverdines probably also involves additional factors, because two
mixed-moiety pyoverdines (PVD<sub>CFML90–51</sub> and PVD<sub>Pa1</sub>) both preferentially bound Mn(III) and both seemed to accommodate Jahn-Teller distortion, even though PVD<sub>CFML90–51</sub> contains a (OH)carboxylate but PVD<sub>Pa1</sub> does not (Harrington et al., 2012). Perhaps these PVDs gain flexibility from their mixed donor groups, their polypeptide structure, or some other factor.

MS/MS analysis of the PVDs from P. putida strains GB-1 and MnB1 also indicated that each strain produced a set of three PVD-type siderophores sharing the same peptide tail but with differently modified chromophores: “classical” PVD, sulfonated PVD, and azotoactin (Table 3). Since all three are strongly fluorescent and since fluorescence was undetectable in the mutant KG163 (Figure 2B), it is likely that the peptide tail of all three PVD types in strain GB-1 is synthesized through the same NRPS operon, PputGB1-4083-4086, which makes sense since the peptides of all three PVD types showed identical MSMS fragmentation patterns. However, subsequent modifications could be subject to differing regulatory or catalytic pathways. It is currently unknown whether these three differing PVD types affect Mn metabolism or the complexation of Mn vis à vis Fe similarly or differently.

Azotobactin and PVD are both known to complex various metal cations (Braud et al., 2009; Wichard et al., 2009). However, azotobactin can also bind oxyanions such as molybdate and vanadate (Wichard et al., 2009). In contrast, the predominant PVD of P. aeruginosa PAO1 is not able to form complexes with vanadate, whereas the other main siderophore of strain PAO1, pyochelin, can bind oxyanions (Baysse et al., 2000). This observation is consistent with other reports that PVDs do not play an important role with oxyanions (Wichard et al., 2009). Therefore, one function of azotobactin in P. putida GB-1 and related strains might be to complex oxyanions for uptake or detoxification, as was suggested for Azotobacter vinelandii (Wichard et al., 2009). Alternatively, sulfonated PVDs and azotoactin could be precursors or byproducts of PVD synthesis (Fuchs et al., 2001; Baysse et al., 2002). Further studies need to be performed to elucidate the respective roles of these siderophores of these organisms, especially with regard to manganese oxidation.

Since the ability to oxidize Mn(II) occurs very commonly, but nonetheless sporadically, among a wide variety of Pseudomonas species (Francis and Tebo, 2001), it was no surprise that multiple PVD siderotypes were identified among the phylogenetically diverse Mn(II)-oxidizing pseudomonads tested here (Table 2). Within P. putida, two siderotypes were identified (Table 2): n° 1 including strains CFML 90–51 and GB-1 and n° 2 consisting of strain KT2440. Although not included in this research, P. putida ATCC 12663 is also capable of oxidizing Mn(II), is closely related to P. putida GB-1 based on 16S data (Francis and Tebo, 2001), but produces a PVD that has been previously shown to be different from PVD<sub>CFML90–51</sub> (Meyer et al., 2007). Since PVD<sub>CFML90–51</sub> and PVD<sub>GB-1</sub> are indistinguishable by MS/MS (Table 3) and siderotyping (Table 2), PVD<sub>GB-1</sub> and PVD<sub>ATCC12663</sub> cannot be the same. Therefore, even among the Mn(II)-oxidizing P. putida at least three differing PVDs exist: PVD<sub>GB-1</sub>, PVD<sub>KT2440</sub>, and PVD<sub>ATCC12663</sub>. This situation is in agreement with the conclusion of Meyer et al. (2007) that P. putida as currently defined is heterogeneous with respect to siderotype. It is also notable that the siderotype of a Mn oxidizer can be the same as that of a strain that does not oxidize Mn(II), as for P. putida GB-1 and CFML 90–51 (Table 2).

In summary, this study has combined in silico, genetic and chemical (siderotyping and MS/MS) approaches to explore the synthesis and nature of the suite of related PVDs ("classic" PVD, azotoactin, and sulfonated PVD) that were produced by the model Mn(II)-oxidizing organism Pseudomonas putida GB-1 at our growth conditions. In silico analysis indicated that position PputGB1_4083-4086 of the GB-1 genome contained NRPSs that could synthesize a peptide chain consistent with the PVD<sub>GB-1</sub> peptide determined by MS/MS (chromophore-Asp-Lys-OHAsp-Ser-Gly-aThr-Lys-cOHOrn). Furthermore, mutation at PputGB1_4083 prevented PVD synthesis. A diverse selection of Mn-oxidizing Pseudomonas species were found to comprise at least three distinct PVD siderotypes, indicating differences in PVD structure and PVD uptake specificity that can be exploited in future studies concerning the ways that various PVDs can influence Mn metabolism, especially Mn(II) oxidation, in pseudomonads and other bacteria.

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