PvdO is required for the oxidation of dihydropyoverdine as last step of fluorophore formation in Pseudomonas fluorescens *

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SUMMARY

Pyoverdines are important siderophores that guarantee iron supply to important pathogenic and non-pathogenic pseudomonads in host habitats. A key characteristic of all pyoverdines is the fluorescent dihydroxyquinoline group that contributes two ligands to the iron complexes. Pyoverdines are derived from the non-ribosomally synthesized peptide ferribactin and their fluorophore is generated by periplasmic oxidation and cyclization reactions of D-tyrosine and L-diaminobutyric acid. The formation of the fluorophore is known to be driven by the periplasmic tyrosinase PvdP. Here we report that the putative periplasmic oxidoreductase PvdO of Pseudomonas fluorescens A506 is required for the final oxidation of dihydropyoverdine to pyoverdine, which completes the fluorophore. The *pvdO* deletion mutant accumulates dihydropyoverdine, and this phenotype is fully complemented by recombinant PvdO. The autooxidation of dihydropyoverdine at alkaline pH and the presence of high copper concentrations can mask this phenotype. Mutagenesis of conserved residues with potential catalytic function identified Glu-260 as essential residue whose mutation abolished function without affecting stability or transport. Glu-260 of PvdO is at the exact position of the active site cysteine in the structurally related formylglycine-generating enzyme. Evolution thus used the same protein fold for two distinct functionalities. As purified PvdO was inactive, additional factors are required for catalysis.

The acquisition of iron is often a limiting factor for bacteria that live in host habitats, including pathogenic as well as mutualistic species (1). Fluorescent pseudomonads warrant their iron supply by pyoverdines, fluorescent siderophores that are derived from non-ribosomally synthesized peptides that are called ferribactins (2) (Figure 1). Ferribactins are transported into the periplasm in acylated form and deacylated thereafter by PvdQ (3–5). In the periplasm, a 5,6-dihydroxyquinoline fluorophore is generated from ferribactin and the conserved glutamic acid residue at the first position of the peptide is modified (6–8). Iron can then be chelated by the two hydroxyl groups of the fluorophore and four further ligands from side chains of the peptide moiety (9, 10). Only one enzyme, PvdP, is currently believed to catalyze the complete oxidation of the fluorophore (6). However, the often found dihydropyoverdine had been postulated in the past to be an obligate intermediate in fluorophore biogenesis (11, 12), and a mutant strain was known that produced dihydropyoverdine (13), suggesting two successive oxidation steps for fluorophore biogenesis. The *pvdP* gene is usually encoded adjacent to the *pvdMNO* operon, which encodes three further periplasmic proteins that might act as enzymes (Figure 2). We recently have shown that PvdN is
responsibility for one of the glutamic acid modifications, the conversion of this residue to succinamide (7). The functions of PvdM and PvdO have not been resolved yet, but based on interposon mutagenesis, both proteins are postulated to be essential for pyoverdine formation (14, 15). The structure of PvdO has been solved recently (16). It has been shown to be structurally related to the formylglycine-generating enzyme (FGE), but since the catalytic residues of FGE are not conserved in PvdO (16), no function could be assigned to this important protein so far. Being a periplasmic protein, PvdO can be expected to play a role in periplasmic pyoverdine maturation processes, which are fluorophore formation and pyoverdine modification (Figure 1).

Here we describe that PvdO from Pseudomonas fluorescens A506 is required for the last oxidation step during fluorophore formation. We found that the dihydro-form of the fluorophore was the final product of a mutant strain with a scarless in-frame pvdO deletion, and this phenotype was fully complemented in trans by pvdO. The requirement of PvdO can be masked by a PvdO-independent oxidation under non-physiological conditions. Purified PvdO from the periplasmic fraction did not contain potentially catalytic metal ions. However, the protein as purified was inactive but active siderophore– in vitro requirement of PvdO can be masked by a PvdO-deletion strain produces a non-fluorescent A pvdO deletion strain produces a non-fluorescent strain, which is due to the known quantitative conversion of [1] to [2] and [3] by PvdN and PtaA (7, 8). The identity of the dihydro-pyoverdine compounds was verified by high-resolution mass spectrometry (calculated exact masses are denoted in brackets; see Ref. 8 for the other pyoverdine masses):

- [5] 1192.560 a.m.u. [1192.560 a.m.i.];
- [6] 1191.528 a.m.u. [1191.528 a.m.u.];
- [7] 1162.550 a.m.u. [1162.549 a.m.u.].

The data not only demonstrated that PvdO is responsible for the final oxidation of dihydro-pyoverdine, but also that the side chain modifying enzymes accept the dihydro-substrate.

PvdO oxidizes dihydro-pyoverdine and thereby completes the formation of the pyoverdine fluorophore– We extracted pyoverdines from culture supernatant of wild type P. fluorescens A506 and the corresponding pvdO deletion strain and examined them by mass spectrometry (Figure 4A). Elution profiles of the respective masses are shown on the left side, and the corresponding mass spectra are depicted on the right. The assigned structures are illustrated in Figure 4B. While the wild type strain produced the well-known pyoverdines PVD-α-ketoglutaric acid [2], PVD-succinamide [3], and PVD-succinic acid [4], the data clearly showed that the pyoverdines of the pvdO deletion mutant were the known dihydro-intermediates of the pyoverdines with the different modifications known for the first residue, namely glutamic acid [5], α-ketoglutaric acid [6], succinamide [7], and succinic acid [8]. The succinic acid form [8] was very low abundant, and some ferribactin [9] was detected in both preparations, especially in the pvdO deletion strain. The glutamic acid variant [1] was not detected in the wild type strain, which is due to the known quantitative conversion of [1] to [2] and [3] by PvdN and PtaA (7, 8). The identity of the dihydro-pyoverdine compounds was verified by high-resolution mass spectrometry (calculated exact masses are denoted in brackets; see Ref. 8 for the other pyoverdine masses):

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The pyoverdines of the wild type, the ΔpvdO-, the ΔpvdN/ΔpvdO- and the pvdO-
complemented ΔpvdO strains were analyzed by isoelectric focusing, followed by fluorescence detection (Figure 4C, left) or the CAS overlay assay (Figure 4C, right). The fluorescence detection confirmed the pyoverdines detected by mass spectrometry, with the succinic acid and α-ketoglutaric acid forms migrating at the lower part of the gel, and the succinamide and glutamic acid forms migrating at the upper part. Note that the epifluorescence was recorded without filter, as the dihydropyoverdines (middle two lanes) exhibit only a rather weak blue fluorescence that can be thereby detected.

The CAS overlay assay demonstrated iron-binding of all identified siderophores, including the dihydropyoverdines, which agrees with former reports about a complementation of pyoverdine deficiency by dihydropyoverdine as well as with affinity measurements (13).

Non-enzymatic oxidation of dihydropyoverdine masks PvdO activity— The finding that PvdO was required for the final oxidation of dihydropyoverdine in the described cultivations raised the question, why PvdO was not required for the reported PvdP-dependent fluorophore formation from ferrobactin in previous in vitro experiments (6). In that study, it had been clearly shown that the mature fluorophore was generated in assays that contained PvdP as the only enzyme (6). It thus was very likely that the previously applied in vitro conditions had somehow triggered a PvdO-independent completion of fluorophore oxidation. Obvious unusual conditions that had been used in these assays were (i) the alkaline pH 9.0, which is non-physiological as the periplasmic compartment is slightly acidic, and (ii) the inclusion of 250 µM CuSO4, which is a copper stress condition. These conditions had been found to be the optimum conditions for the PvdP-catalyzed reaction (6). As fluorescence is quenched by copper ions, we used the oxidation-dependent increase of chromophore absorbance at 405 nm to examine the potential influence of pH and copper on dihydropyoverdine oxidation (Figure 5A). Non-catalytic oxidation was very rapid at pH 9.0 and this was significantly slowed down by the addition of 250 µM CuSO4. At pH 8, there was essentially the same effect of copper, but autoxidation proceeded at a much slower rate. At pH 7, there was hardly any autoxidation observed. In conclusion, alkaline autoxidation occurs rapidly without involvement of copper as catalyst. The alkaline autoxidation had already been recognized in early studies on the structure of dihydropyoverdine (17). In that study, it was also shown that chelation of iron inhibits the autoxidation (17), and our data indicate that chelation of copper has the same effect.

The assay system for the PvdP-dependent formation of completely oxidized pyoverdine from ferribactin did not monitor the potential formation of a dihydropyoverdine intermediate (6). The rapid autoxidation of this intermediate at the applied non-physiological pH 9 masked the PvdO requirement in these assays.

However, the effect of copper is more complex. When we analyzed the impact of copper on the formation of fluorescent pyoverdines in growing cultures, we noted differential effects (Figure 5B, upper panel). As expected, in the absence of copper, the wild type strain produced fluorescent pyoverdine and the medium of the pvdO deletion strain contained only very little fluorescence due to the formation of dihydropyoverdine. However, abundant copper in the bacterial cultures promoted the oxidation of dihydropyoverdine in the ΔpvdO background, and fluorescence reached the level of the wild type strain at ~300 µM copper. At higher copper concentrations, fluorescence was gradually quenched by copper. The positive influence of copper on dihydropyoverdine oxidation thus had a maximum effect in the range of 200-500 µM copper, which coincides with the reported optimum of PvdP activity at 250 µM copper (6). Therefore, although copper-binding to dihydropyoverdine partially inhibits alkaline autoxidation (Figure 5A), the same high copper concentrations can artificially trigger a dihydropyoverdine-oxidizing activity in the presence of whole cells (see discussion). In control experiments, strains lacking PvdM or PvdP did not produce any fluorescent pyoverdine in the presence of added copper even after prolonged incubations, excluding alternative explanations for fluorescence increase (Figure 5B, lower panel).

PvdO and PvdP generally co-occur in fluorescent pseudomonads— As shown above, PvdO is required for the oxidation of dihydropyoverdine under physiological conditions in P. fluorescens A506. It can be therefore expected that PvdO must always co-occur with PvdP, since PvdP is essential for the
preceding ring formation. We thus examined the presence of pvdO and pvdP in all genomes of Pseudomonas strains available at pseudomonas.org (18) in August 2017. In full agreement with the prediction, these analyses demonstrated a complete co-occurrence of the two genes (Figure 6). Individual mismatches (in 13 out of 3347 analyzed genomes) could all be traced back to unfinished genome assemblies or gaps.

**Mutational analysis of PvdO—** As PvdO is somehow required for the oxidation of dihydropyoverdine in living cells under physiological conditions, it was interesting to investigate its structural and biochemical properties.

We homology-modeled the structure of PvdO from *P. fluorescens* A506 (PvdOA506) to the known structures of PvdO from *P. aeruginosa* PA01 (PvdOPAO1; 73% identity; PDB 5HHA) and the structurally related formylglycine generating enzyme (FGE; 25% identity; PDB 2Q17) from *Streptomyces coelicolor*. Neither PvdOPAO1 nor FGE contain a redox active cofactor associated with the purified proteins used in crystallographic analyses. However, FGE is suggested to employ a copper cofactor to oxidize cysteine to formylglycine (19), and according to a structural analysis study on PvdOPAO1, FGE is the only so-far known protein that is structurally related to PvdO, although active site cysteines of FGE are not conserved in PvdO (16). The PvdOPAO1- and FGE-based structural models of PvdOA506 are in most parts highly similar with respect to the backbone structure and differ mainly in the conformation of surface loop structures (Video S1). The FGE-derived model was suggestive for a potential His/His/Glu non-heme iron-binding site, that resembled known sites (20, 21). These residues (His-188, His-218, Glu-260) are highly conserved (Figure 7D), as are several other potentially important candidate residues (Glu-179, Asn-210, Tyr-215, Asp-216, Met-217, Asp-257) that were included in a mutational analysis. Also, several non-conserved residues with potential metal-chelating propensity were included as negative controls (Gln-187, Met-214). As in the case of PvdO from *P. aeruginosa* (16), all six cysteines of *P. fluorescens* A506 form disulfides and thus are unlikely to be involved in metal binding. Exchanged residues are indicated in the sequence of Figure 7D as well as in the structural model in Figure 7E.

Using our functional complementation system described above, we examined for all these individual PvdO variants their ability to complement the ΔpvdO phenotype in terms of pyoverdine production (Figure 7A, upper part); their stability (Figure 7A, lower part), and their transport into the periplasm (Figure 7B). Fortunately, while a number of exchanges caused an instability of the protein and consequently a loss of functionality, several of the exchanges, namely PvdO(D257A) and PvdO(E260A), abolished complementation of the mutant phenotype without compromising protein stability. The transport of these variants into the periplasm was unaffected, indicating that the non-functionality was not caused by a mislocalization. As expected from the stability analysis shown in Figure 7A, the other PvdO variants were not detected in any subcellular fraction (Figure 7B), indicating degradation to non-detectable levels. From all these biochemical data, it can be concluded that the residues Asp-257 and Glu-260 are essential for dihydropyoverdine oxidation in living *P. fluorescens* A506 cells, without being required for protein integrity or subcellular targeting.

As complementary analysis, we also evaluated pyoverdine formation during growth on iron-depleted or iron-limited solid media (Figure 8). As expected, *pvdO*-deleted strains or strains containing inactivated or destabilized PvdO variants grew with reduced rate under iron-depleted conditions (i.e. in the presence of the chelator EDDHA, Figure 8AC), and could not form fluorescent pyoverdine halos (Figure 8CD). Some fluoreuscence surrounding the strain with the D257A variant indicates a residual activity that had not been detected with planctonic growth. In all other cases, the results of this assay were only confirmative. Together, the *in vivo* data described above indicated that the two acidic residues Asp-257 and Glu-260 of PvdO were important for the oxidation of dihydropyoverdine in living cells.

Based on this knowledge, we then carried out analyses *in vitro*. We purified wild type PvdO and the E260A variant from the periplasmic fraction of *E. coli* harboring a *pvdO* expression system (Figure 7C). The protein migrated near 35 kDa, which is slightly above the calculated mass of 30 kDa for the Strep-tagged protein. Using ICP-MS, we did not
detect any potentially redox-active iron-, copper-, cobalt- or manganese ions, nor zinc ions as constituent of the purified proteins. Therefore, as discussed in more detail below, either a cofactor was not stably assembled or catalysis does not depend on metal ions. The activity of purified PvdO and its variant PvdO(E260A) was not detectable in vitro (Figure S1), which can have various reasons (see discussion).

DISCUSSION

PvdO is required for dihydropyoverdine oxidation—

The functional role of PvdO has been matter of speculations since its importance had been recognized by Ochsner et al. in 2002 (15). It was known that no fluorescent pyoverdine could be formed in a pvdO mutant strain that had been generated by interposon mutagenesis (15, 14). However, the deletion strains were not complemented and the fact that pvdO is organized in the pvdMNO operon made the interpretations difficult due to possible polar effects. PvdN - which is encoded by the same operon and had also been believed to be essential for pyoverdine formation – is in fact not essential and catalyzes the conversion of the fluorophore-attached glutamic acid to succinamide (7). To clarify this issue for PvdM and PvdO, we carried out in-frame scar-less deletions of these two genes, analyzed their phenotypes and successfully complemented them. While PvdM turned out to be indeed essential for pyoverdine formation, the ΔpvdO phenotype was more complex: There was still some growth possible on iron-depleted medium, albeit the typical yellow-greenish pyoverdine fluorescence was absent from this mutant (Figure 3). This prompted us to investigate the spectrum of potential pyoverdine precursors produced by that strain, and we discovered that PvdO is required for the oxidation of dihydropyoverdine in vivo under physiological conditions (Figure 4). The pvdO gene fully complemented the ΔpvdO phenotype and produced wild type levels of the oxidized pyoverdine fluorophore. Thus, no secondary mutations had caused the phenotype of the gene deletion. The role in fluorophore biogenesis was unexpected, since the early postulated dihydropyoverdine intermediate (11, 12) had not been considered as relevant anymore when PvdP had been shown to catalyze the complete conversion of ferribactin to fluorescent pyoverdine (6). In that in vitro study, however, the full oxidation had been demonstrated in assays that contained 250 µM CuSO4 at pH 9.0. As we observed that dihydropyoverdine is rapidly oxidized at pH 9.0 even in the presence of 250 µM copper (Figure 5A), and as PvdO is responsible for the oxidation of dihydropyoverdine under physiological conditions, it is likely that dihydropyoverdine rather than pyoverdine is the product of PvdP. However, it is noteworthy that a growing ΔpvdO strain could generate fluorescent pyoverdine in the presence of 200-500 µM CuSO4. This copper effect most likely relates to living cell constituents, and it is intriguing that the optimal concentration in the published PvdP assays is the same as in our in vivo assays (Figure 5B). It thus might be speculated that an occupancy of low-affinity copper sites on cellular proteins such as PvdP can contribute – beside alkaline autoxidation – to an artificial full oxidation to pyoverdine under these conditions. Since PvdO strictly co-occurs with PvdP in fluorescent pseudomonads (Figure 6), it is clear that the oxidation of dihydropyoverdine must generally be catalyzed in a PvdO-dependent manner under physiological conditions not only in P. fluorescens but also in the other species.

Although required, the exact mode by which PvdO contributes to the oxidation of dihydropyoverdine remains unclear—

We initially expected a transition metal cofactor as constituent of PvdO and thus homology-modeled the structure with the related S. coelicolor FGE structure and later also with the recently published P. aeruginosa PvdO structure as templates to identify potential metal binding sites. Several residues were possibly involved in an iron-binding site but there was no good evidence for any copper site. The mutational analysis included therefore residues with potential metal-binding properties in the environment of the suspected iron site and we examined the influence of the mutations on fluorescent pyoverdine formation as well as on stability (Figure 7, 8). The exchange of the presumed histidine ligands strongly affected PvdO stability, which argued against a primarily catalytic function. Only the mutation of two acidic residues, Asp-257 and Glu-260, resulted in complete (E260A) or strongly reduced (D257A) functionality without any significant effect on protein stability. We had analyzed not only wild type PvdO but also the E260A variant, as we suspected Glu-260 to be an essential ligand for the supposed metal ion. To
our surprise, neither the wild type nor the mutated protein contained any detectable metal ions, although PvdO had been purified under mild conditions by affinity chromatography. The absence of metals pointed to a mechanism similar to the metal-independent alkaline autoxidation (17) in which the deprotonation of the catechol is the initializing step for the oxidation. It is proposed that unpaired electrons that are generated during the oxidation are stabilized between the neighboring catechol hydroxylates, which promotes the oxidation (17). To this mechanism fits the essential role of Glu-260, and also the significant contribution of a second carboxylic acid, Asp-257. If PvdO directly catalyzes the oxidation of dihydropyoverdine, it likely enables the deprotonations at pH 6.5 in the periplasm, stabilizes the oxyanions and catalyzes the electron transfer to an unknown electron acceptor (Figure 9A). Once the ring system is oxidized, tautomizations would readily generate the 5,6-dihydroxyquinoline fluorophore. However, since we could not reconstitute the activity in vitro, PvdO most likely needs to interact with another component in the periplasm to perform its function. Obviously, this could be a specific electron acceptor that channels the electrons into the respiratory electron transport chain. The inactivity of purified PvdO can be taken as argument against a more direct electron transfer to oxygen as implied by an autoxidation-like mechanism. It certainly also cannot be excluded that an unknown cofactor is required that is lost during purification or that PvdO is a protein that enables another enzyme to oxidize dihydropyoverdine, although the active site conservation argues for a direct enzymatic involvement (see below).

**EXPERIMENTAL PROCEDURES**

**Strains and growth conditions**—The strain *P. fluorescens* A506 was utilized for all physiological assays. *Escherichia coli* DH5α λ pir+ was employed for cloning and *E. coli* Rosetta 2 (DE3) pLysSRA2E2 for protein production. The *E. coli* strains were cultivated at 37 °C and *P. fluorescens* strains at 30 °C unless noted otherwise. LB-Medium (1% (w/v) tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract) was used for standard cultivations. Appropriate antibiotics were supplemented at following final concentrations: 100 µg/ml ampicillin, 25 µg/ml chloramphenicol, 50 µg/ml kanamycin, 20 µg/ml tetracycline. The pyoverdine-plate assay and the relative pyoverdine quantification assay were performed as previously described (7, 8).

**Genetic methods and plasmids**—Construction of all plasmids and scar- and marker-less deletions were performed as previously described (7, 8). All primers used for this study are listed in Table 1.

**Biochemical methods**—SDS-PAGE, successive Western blotting, and in-gel colloidal Coomassie staining for protein analysis, were performed according to standard protocols (23–26). Development of Western blots was performed

Glu-254 of PvdO_PAO1, which corresponds to Glu-260 of PvdO_A506, had been already recognized to be at the position of the active site cysteine in human FGE (16). Although no function had been assigned to this residue in that study, the corresponding surface cleft was proposed to represent a binding site for an unknown substrate. Therefore, nature apparently used the same protein fold and the same active site region to generate two distinct functionalities for distinct subcellular compartments. While the cytoplasmic FGEs can use cysteines for active site metal coordination (22), periplasmic PvdO does not have this cysteine and does therefore not bind copper at that position. Instead, PvdO function requires a glutamic acid that has been placed at the exact position of the copper ligands in FGEs. This finding strongly argues for a direct enzymatic function of PvdO, which remains to be demonstrated in future. This will help to ultimately clarify the mechanism by which dihydropyoverdine is oxidized.
Dihydropyoverdine oxidation by PvdO

according to the manufacturer’s instructions using a StrepMAB-Classic monoclonal antibody (IBA, Göttingen, Germany) for detection of Strep-tagged proteins. As secondary antibody, anti-mouse-HRP conjugate (Carl Roth, Karlsruhe, Germany) was employed. Images were recorded using the MF-ChemiBIS 4.2 imaging system (DNR Bio-Imaging Systems, Jerusalem, Israel).

Subcellular fractionation of P. fluorescens A506 was performed as reported previously (27) with minor modifications: cultures (50 ml) were grown to OD_{600} of 1 and a 1 ml fraction-volume was used throughout. The samples were not further concentrated by precipitation for further analysis.

The subcellular fractionation of E. coli was carried out as described earlier, with minor modifications (28). Briefly, ten 100 ml cultures in 0.5 l Erlenmeyer flasks with four baffles each were inoculated with 4 ml of the appropriate over-night culture and incubated at 30 °C and ~180 rpm until the OD_{600} reached 0.6. Then the expression was induced with 1 mM IPTG for 3 h maintaining the incubation conditions. Subsequently, cells were sedimented in 250 ml centrifugation-buckets for 10 min at 3,857 x g and 4 °C. The pellets were resuspended in 40 ml TES buffer (20 % (w/v) sucrose, 10 mM Tris buffer pH 8.0, 1 mM EDTA) and incubated at room temperature for 10 min. The cells were transferred to 45 ml centrifugation buckets and sedimented for 10 min at 2,990 x g and 4 °C. Pellets were then resuspended in 1.5 ml ice cold 5 mM MgSO_4 each and transferred to 2 ml plastic tubes. Resuspended cells were then incubated for 20 min on ice and subsequently centrifuged for 10 min at 4 °C and 16,060 x g. The supernatants (periplasmic fraction) were collected and immediately sterile filtered through a 0.2 μm PES filter. The protein was purified utilizing one 1.5 ml Strep-Tactin® Superflow® column (IBA, Göttingen, Germany) according to the manufacturer’s instructions using 100 mM Tris-HCl buffer pH 8.0 with 150 mM NaCl as running buffer. After elution, the elution-fractions E3 and E4 were combined and diluted with 100 mM Tris-HCl pH 8.0 to lower the NaCl concentration to 50 mM for subsequent ICP-MS analysis. The protein was then concentrated using a Vivaspin® 6 concentrator (cutoff 10 kDa, Sartorius, Göttingen, Germany). The protein-concentration was estimated via absorption at 280 nm using a NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific). Samples were subsequently analyzed by ICP-MS (SpurenAnalytisches Laboratorium Dr. Baumann; Pirkensee, Germany).

A Jasco V-650 spectrophotometer (Jasco, Gross-Umstadt, Germany) was used for in vitro dihydropyoverdine oxidation assays with the following setting: wavelength 405 nm, bandwidth 2 nm, measurement interval 1 min, total measurement time 5 h, temperature 20 °C, stirrer 800 rpm. As buffers for the assay, 50 mM HEPES pH 7, 50 mM HEPES pH 8, and 50 mM CHES pH 9 were used. The assays started with addition of 10 µl freshly thawed dihydropyoverdine stock solution to a final assay volume of 2 ml in the respective buffer. After 1 min of incubation time, CuSO_4 was added from a 100 mM stock solution to a final concentration of 250 µM. The absorptivity caused by CuSO_4 was subtracted from the respective kinetics measurement from the time point of the addition on.

In vitro activity assays were carried out in 50 mM PIPES pH 6.5 in a final total volume of 2 ml. For each measurement, 10 µl of the dihydropyoverdine stock solution were mixed with reaction buffer and the assay was started after 1 min of pre-equilibration time by adding 100 µl the protein (0.4 mg/ml in elution buffer) to a final concentration of 20 µg/ml. As blank, the same volume of elution buffer was used and blank data were subtracted. The settings for the Jasco V-650 spectrophotometer were set as for the above described dihydropyoverdine oxidation assays.

Pyoverdine isolation, the isoelectric focusing assay in conjunction with the chrome azurol S overlay assay, and the UPLC-MS-analysis were carried out as reported previously (7, 8).

Bioinformatic methods–The initial classification of PvdO was performed employing the InterPro webservice (29, 30). To generate the sequence logo, PvdO homologs were identified by searching through all amino acid sequences of all complete- and draft genomic sequences deposited at pseudomonas.com (18) with phmmer v 3.1b (31). The redundancy was subsequently reduced by using the CD-HIT algorithm from the CD-HIT Suite (32), utilizing a similarity threshold of 0.95. Sequences were aligned with MAFFT v. 7.310 (33) using the G-INS-i setting. The resulting sequence alignment was visualized using WebLogo 3 (34), plotting the probability of the occurrence of each amino acid at each position.
The distribution analysis was performed by searching the already downloaded sequences from pseudomonas.com (18) with phmmer v 3.1b (31). Utilizing an in-house developed software, a taxonomy table reduced to species-level was constructed and the identified homologs were classified accordingly. Subsequently, the number of found homologs in one species was divided by the total number of organisms attributed to the same species, giving the percentage of occurrence. To model the structure of PvdO, the SWISS-MODEL webservice (35) was used and in conjunction with UCSF Chimera (36) and APBS (37), the models were visualized. Sequence identities were calculated from alignments generated using CLUSTAL Ω (38).

To visualize the genomic contexts of pvdO in multiple organisms, the MGcV webservice (39) was used.
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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

MTR performed the experiments, prepared the figures, and analyzed the data together with TB. GD performed the MS analyses. TB conceived and coordinated the study and TB and MTR wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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Dihydropyoverdine oxidation by PvdO

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FOOTNOTES

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The abbreviations used are:  a.m.u., atomic mass units; CAS, chrome azurol S;  CHES, N-cyclohexyl-2-aminoethanesulfonic acid;  DH-PVD, dihydropyoverdine; EDDHA, ethylenediamine di(o-hydroxy)phenylacetic acid; EDTA, ethylenediaminetetraacetic acid; FGE, formylglycine-generating enzyme; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HR-MS, high-resolution mass-spectrometry; ICP-MS, inductively coupled plasma mass spectrometry; IEF, isoelectric focusing; IPTG, isopropyl β-D-1-thiogalactopyranoside; MS, mass-spectrometry; NRPS, non-ribosomal peptide synthetase; OD, optical density; PVD, pyoverdine; UPLC, ultra-performance liquid chromatography;

TABLES

Table 1: Primers used in this study

| Name                  | Sequence                                                                 | Restriction site | Purpose                                      |
|-----------------------|--------------------------------------------------------------------------|------------------|----------------------------------------------|
| pEXH5-RBS-F-MR        | GGCGCGGGATCCCGTTTAACCTTAAAGGAGATATAC                                       | BamHI            | Forward primer for subcloning from pEXH5 into pME6010 |
| pEXH5-strep-term-R-MR | CCCCCTGAAATTCCAAAAAAGC CCGCCCTGCAGGGGCGGGG TTTTTTTTTTTACTTTGACA CTCGGGTTGGCTCC | EcoRI            | Reverse primer for subcloning from pEXH5 into pME6010 |
| pEXPT7-HA-term-R-MR   | CCCCCTGAAATTCCAAAAAAGC CCGCCCTGCAGGGGCGGGG TTTTTTTTTTTAATGCTGACCCGACGTTCGCTTC | EcoRI            | Reverse primer for subcloning from pEXH5 into pME6010 with HA-tag |
| PfA506-dpvdNO-F2-MR   | CTAAACACCTTGAAAGTGAAGTC CAGTACCCGGCCGAACTCTAAATA TCTGAGG                      |                  | Forward primer for *pvdMNO* right flanking region |
| PfA506-matPvdO-strep-R-MR | CCGATTAAGCCTTTTACTTTTCC GAACTGCGGGTGCTCCAGAG TTCCGGTACTACCCCGGAAAACC AATCCCATC | HindIII          | Reverse primer for cloning PvdO-coding region into pEXH5 |
| PfA506-pvdM-DF-MR     | ACCGTCGTGTTGTCGAAAGTAG                                                   |                  | *pvdM* genomic deletion control primer |
| PfA506-pvdM-DR-MR     | GGCCGCAGTGTTGGCTCTTGA GGTTC                                                   |                  | *pvdM* genomic deletion control primer |
| PfA506-pvdM-F1-MR     | CGCCCTAAAGCTTTTATGGGCTGGT GCAAGAAGAAAGGAAATCG                                 | HindIII          | Forward primer for *pvdM* left flanking region |
| PfA506-pvdM-F2-MR     | CCTCACACGCTGAGTTGGGCA ATGACAACAGCCTACACCA CGAAGTAAAGGCGTTCGGCCGGGTTGGCAAAGG |                  | Forward primer for *pvdM* right flanking region |
| PfA506-pvdM-F3-MR     | TCAAGGGTGATAGCGAAAAATGC CTTGCAAAAGGGGCGTGATAT ATC                           | NdeI             | Forward primer for cloning PvdM-coding region into pEXH5 |
| PfA506-pvdM-HA-R-MR   | GGCTAAAGCCTTTTAGGGCTGT GAAGTGCGGCAAGGTTGCC GGGCTTTTGGAC                      | HindIII          | Reverse primer for cloning PvdM-coding region into pEXH5 |
| Primer Name | Sequence | Description |
|-------------|----------|-------------|
| PFA506-PvdM-R1-MR | TGTCATGGCACATCAGTCAGTTGAGG | Reverse primer for pvdM left flanking region |
| PFA506-PvdM-R2-MR | ACTGTTGACTCTTGGACGTTGCAATTGAGGTTTCT | Reverse primer for pvdM right flanking region |
| PFA506-pvdO_D216A-F-MR | CTGTCTTCATGTATGGCAATTGCAAGGC | Primer for D216A exchange by oePCR |
| PFA506-pvdO_D216A-R-MR | CCGTGACTGACATTGACATGCAAGGCACGTTTCT | Primer for D216A exchange by oePCR |
| PFA506-PvdO_D257A-F-MR | GCAATGCTGAGGGGCAGAAGGC | Primer for D257A exchange by oePCR |
| PFA506-PvdO_D257A-R-MR | CGGGCCAGCATTTCTGGCTATTCGAGG | Primer for D257A exchange by oePCR |
| PFA506-PvdO_E179A-F-MR | CCCTTCTCCAGGCGGCAAGGC | Primer for E179A exchange by oePCR |
| PFA506-PvdO_E179A-R-MR | CACATCAGCCCAAGGC | Primer for E179A exchange by oePCR |
| PFA506-pvdO_E260A-F-MR | GGAGAATGCTGAGGGGCAGAAGGC | Primer for E260A exchange by oePCR |
| PFA506-pvdO_E260A-R-MR | CATACATTGACATTGACATGCAAGGC | Primer for E260A exchange by oePCR |
| PFA506-PvdO_H188A-F-MR | CCTACACGGAGGCAAGGC | Primer for H188A exchange by oePCR |
| PFA506-PvdO_H188A-R-MR | AGGTCATGGGTGCAAGGC | Primer for H188A exchange by oePCR |
| PFA506-pvdO_M214A-F-MR | GTACAGTGTTGCGGCCCTGGGCATGCTGTA | Primer for M214A exchange by oePCR |
| PFA506-pvdO_M214A-R-MR | CATACATTGACATTGACATGCAAGGC | Primer for M214A exchange by oePCR |
| PFA506-PvdO_M217A-F-MR | CATACATTGACATTGACATGCAAGGC | Primer for M217A exchange by oePCR |
| PFA506-pvdO_M217A-R-MR | GTACAGTGTTGCGGCCCTGGGCATGCTGTA | Primer for M217A exchange by oePCR |
| PFA506-pvdO_N210A-F-MR | AGGTCATGGGTGCAAGGC | Primer for N210A exchange by oePCR |
| PFA506-pvdO_N210A-R-MR | CATACATTGACATTGACATGCAAGGC | Primer for N210A exchange by oePCR |
| PFA506-pvdO_Q187A-F-MR | GTACAGTGTTGCGGCCCTGGGCATGCTGTA | Primer for Q187A exchange by oePCR |
| PFA506-pvdO_Q187A-R-MR | CATACATTGACATTGACATGCAAGGC | Primer for Q187A exchange by oePCR |
| PFA506-pvdO_Y215A-F-MR | GTACAGTGTTGCGGCCCTGGGCATGCTGTA | Primer for Y215A exchange by oePCR |
| PFA506-pvdO_Y215A-R-MR | CATACATTGACATTGACATGCAAGGC | Primer for Y215A exchange by oePCR |
| PFA506-PvdO-DF-MR | GTACAGTGTTGCGGCCCTGGGCATGCTGTA | pvdO genomic deletion control primer |
| PFA506-PvdO-DR-MR | CATACATTGACATTGACATGCAAGGC | pvdO genomic deletion control primer |
| PFA506-PvdO-F1-MR | TTAGGCTGATCAGCCGAAGGGGCGGCTGATGATCAGG | BamHI | Forward primer for pvdO left flanking region |
| PFA506-PvdO-F2-MR | TCCCTTTGGAAGAGAACGCTCATTGAGGTTTCT | BamHI | Forward primer for pvdO right flanking region |
| PFA506-PvdO-F-MR | TTACCGCTTATGAGCGCGATCATTGAGGTTTCT | Ndel | Forward primer for cloning PvdO-coding region into pEXH5 |
| PFA506-PvdO-R1-MR | CATACATTGACATTGACATGCAAGGC | Ndel | Reverse primer for pvdO left flanking region |
| PFA506-PvdO-R2-MR | GACCGCGATCTCGGTGTCGTTGACATGCAAGGC | HindIII | Reverse primer for pvdO right flanking region |
| PFA506-PvdP-DF-MR | GTACAGTGTTGCGGCCCTGGGCATGCTGTA | pvdP genomic deletion control primer |
| PFA506-PvdP-DR-MR | CCGGCGCGATCTCGGTGTCGTTGACATGCAAGGC | pvdP genomic deletion control primer |
### Dihydropyoverdine Oxidation by PvdO

| Primer Set                | Sequence                                | Enzyme | Description                           |
|---------------------------|-----------------------------------------|--------|---------------------------------------|
| PfA506-PvdP-F1-MR         | TGCTCTAAGCTTTTTGGTCGAC                 | HindIII| Forward primer for \( pvdP \) left flanking region |
|                           | ATCTGCGACACGTCGATGATC                  |        |                                       |
|                           | AC                                      |        |                                       |
| PfA506-PvdP-F2-MR         | ACGCTTTAAAAACGTGTTGCAG                |        | Forward primer for \( pvdP \) right flanking region |
|                           | GCGCTCTAGG                              |        |                                       |
| PfA506-PvdP-R1-MR         | GCGCCTGCAACACGTTTTAAA                |        | Reverse primer for \( pvdP \) left flanking region |
|                           | GCGTTGTCATTGGCTACCTTAA                |        |                                       |
|                           | GGAACGGC                               |        |                                       |
| PfA506-PvdP-R2-MR         | TTTAACCCCGGGAGGTACTGG            | XmnI   | Reverse primer for \( pvdP \) right flanking region |
|                           | GTAGCCTGTCCAGGCGTATCG                 |        |                                       |
|                           | ACGAG                                   |        |                                       |
Dihydropyoverdine oxidation by PvdO

FIGURES

Figure 1: Schematic overview of pyoverdine biosynthesis stages. PvdO is a putative oxidoreductase and – being a periplasmic protein – implied in fluorophore formation or pyoverdine modification. NRPS, non-ribosomal peptide synthetase.
Figure 2: Comparison of genomic environments of \textit{pvdO} in representative \textit{Pseudomonas} genomes.
Figure 3: Evidence for a non-fluorescent pyoverdine generated in the pvdO deletion strain. Growth of strain A506 (WT) and its derivatives deleted in pvdM (ΔpvdM), pvdO (ΔpvdO), pvdP (ΔpvdP), or pvdN and pvdO (ΔpvdNO) on pyoverdine-inducing solid CAA medium without or with EDDHA for additional iron-depletion. Indicated complementation strains are included in the analysis. Growth on EDDHA-containing plates correlates with the formation of pyoverdines and (in case of the ΔpvdO strain) with dihydropyoverdine, as monitored by fluorescence (lower panel).
Figure 4: PvdO catalyzes the oxidation of dihydropyoverdine. (A) Analysis of pyoverdines produced by wild type *P. fluorescens* A506 (upper part) and the corresponding dihydropyoverdines (DH-PVD) produced by the *pvdO* deletion strain (lower part). Chromatograms on the left depict the elution profiles of the numbered compounds that are assigned in (B) and (D). (C) Detection of fluorescence (left) or iron-binding capacity in a CAS overlay assay (right) after isoelectric focussing of the pyoverdines of indicated strains. WT, wild type A506; Δ*pvdO*, strains with *pvdO* deletion; Δ*pvdO*/Δ*pvdN*, strain with an additional *pvdN* deletion; Δ*pvdO*/*pvdO*, *pvdO*-complemented *pvdO* deletion strain.
Figure 5: Non-physiological oxidation of dihydropyoverdines by alkaline autoxidation in vitro and copper-induced oxidation in growing cultures. (A) pH-dependent autoxidation of dihydropyoverdine as monitored by absorbance-increase at 405 nm. Kinetics of assays containing 250 µM CuSO₄ (dashed lines) are directly compared with kinetics in the absence of copper. Copper has been added after 1 min of incubation. The data of the copper-containing assays were corrected for copper absorption. See method section for details. (B) Pyoverdine fluorescence of culture supernatants of wild type A506, its ΔpvdO derivative, and the ΔpvdO-complemented ΔpvdO strain after 24 h of growth in CAS medium supplemented with indicated CuSO₄ concentrations (upper panel). Control measurements, showing the absence of fluorescence with strains deleted in pvdM (=ΔpvdM) or pvdP (=ΔpvdP) after 48 h incubation (lower panel). Error bars are derived from triplicate assays of independent cultures. Fluorescence was normalized to the wild type levels in the absence of copper. The decrease of fluorescence at higher copper concentrations is due to the quenching of pyoverdine fluorescence by copper ions.
Figure 6: Co-occurrence of PvdO and PvdP homologs within the genus *Pseudomonas*. Numbers behind the bar-graph indicate the total number of genomes analyzed for the respective species. Note that the two genes co-occurred in 3334 out of 3347 genomes, and the 13 exceptions were attributed to unfinished genomes.
Figure 7: Effects of alanine exchanges of residues with potential catalytic function. (A) Effect of indicated single alanine exchanges on the formation of fluorescent pyoverdine, and control of the presence of the respective PvdO variants in the periplasm by SDS-PAGE/Western-blotting. (B) Subcellular fractionation of all analyzed PvdO variants. Cytoplasmic (C), membrane (M) and periplasmic (P) fractions were analyzed for the presence of PvdO by SDS-PAGE/Western blotting. Note that in case of stable PvdO variants, the protein is exclusively detectable in the periplasm, as expected for Sec transport. (C) Coomassie-stained PvdO preparations that had been used for metal detections. (D) Sequence logo of the mutated regions (see methods for details). Exchanged residues in PvdO506 are colored red and distinct regions are highlighted in yellow and blue. The residue numbers on top of the sequence logo indicate the residue position in PvdO506. (E) Homology model of the PvdO506 structure based on the known structure of PvdO from P. aeruginosa (PDB 5HHA). Exchanged residues are highlighted in red. left: overview of the structure; right: close up view. The model was visualized using Chimera (36). Masses of marker proteins (in kDa) are indicated on the left side of blots.
Figure 8: Analysis of physiological functionality of PvdO variants with single amino acid exchanges. (A) and (C) are CAA plates containing iron-depleting EDDHA, (B) and (D) are CAA plates without EDDHA. (A) and (B) show the growth of spotted cultures on the respective media, whereas (B) and (D) detect the fluorescence caused by produced pyoverdines. The amino acid exchanges and control strains are denoted in (B). All plates contain the same organization of spotted strains.
Figure 9: Role of PvdO in fluorophore formation of pyoverdines, and identification of functionally important residues in a potential active site cavity of PvdO. (A) Biosynthetic reactions leading from ferribactin to pyoverdine via the intermediate dihydropyoverdine. PvdP is required for the initial oxidative cyclization reaction that results in the catechol system. PvdO is required for the final oxidation, resulting in the mature fluorophore. (B) The two active site residues Asp-257 and Glu-260 (red) and their position on the surface of PvdO relative to the other residues mutated in this study (blue). (C) Left: The active site cavity shown by the APBS (37)-calculated electrostatic potential-colored surface (scale from -10 = red, over 0 = white, to +10 = blue). Surface exposed side chain regions of Asp-257 and Glu-260 are colored purple. Middle and right: Superposition of the structure from FGE and PvdO_{A506} structural model, highlighting the respective active site residues at overlapping positions. The active site Cys-272 of FGE is at exactly the same position as Glu-260 in PvdO.
PvdO is required for the oxidation of dihydropyoverdine as last step of fluorophore formation in *Pseudomonas fluorescens*
Michael T Ringel, Gerald Draeger and Thomas Brüser

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