

Impaired maturation of the siderophore pyoverdine chromophore in *Pseudomonas fluorescens* ATCC 17400 deficient for the cytochrome *c* biogenesis protein CcmC

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Abstract Pyoverdines are the main siderophores of fluorescent pseudomonads. They comprise a quinoline chromophore, a peptide chain, and a dicarboxylic acid or a dicarboxylic acid amide side chain. Each *Pseudomonas* species produces a pyoverdine with a different peptide chain. A cytochrome *c* biogenesis $\Delta ccmC$ mutant of *Pseudomonas fluorescens* ATCC 17400 produces multiple pyoverdine forms, showing differences at the level of the chromophore or the side chain. When grown in the presence of L-cysteine, $\Delta ccmC$ produces only ferribactin, a non-fluorescent precursor of pyoverdine, while addition of oxidized glutathione improves pyoverdine production. We suggest that the conversion of ferribactin to pyoverdine does not take place in the $\Delta ccmC$ mutant because of lack of oxidizing power in the periplasm. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Pyoverdine; Ferribactin; Siderophore; Chromophore; CcmC; *Pseudomonas*

1. Introduction

Free-living aerobes, like the fluorescent pseudomonads, need to produce and excrete high-affinity Fe³⁺-chelating siderophores in order to satisfy their need for iron [1–3]. Under conditions of iron limitation, fluorescent pseudomonads (among others, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Pseudomonas fluorescens*) produce different fluorescent peptidic siderophores, named pyoverdines or pseudobactins [4,5]. *P. fluorescens* ATCC 17400 produces both pyoverdine and another, recently described, non-fluorescent siderophore, quinobactin [6]. Pyoverdines are composed of a conserved dihydroxyquinoline chromophore, a variable peptide chain, comprising six to twelve amino acids, depending on the producing strain, and a so-called ‘side chain’, generally a dicarboxylic acid or a dicarboxylic acid amide [4,5]. The peptide chain comprises L- and D-amino acids, some of them unusual, such as N⁵-hydroxycycloornithine or N⁵-formyl-N⁵-hydroxyornithine [4]. Pyoverdines are fluorescent under ultraviolet (UV) light, a characteristic conferred by the quinolinic chromophore [4,5]. Probable precursors of pyoverdines have been identified, some of them being non-fluorescent [4]. One of

them, dihydropyoverdine, found together with pyoverdine, does not fluoresce because of a difference in the saturation of two carbons in the chromophore [7]. Dihydropyoverdine can be non-enzymatically converted to pyoverdine by oxidation at high pH [7,8]. Dihydropyoverdine has also a lower affinity for iron compared to the normal fluorescent pyoverdine [8]. Other precursors are also found in culture supernatants of fluorescent pseudomonads, such as sulfo-dihydropyoverdine [3]. Finally, non-fluorescent siderophores, named ferribactins, with an incomplete chromophore have been described, and suggested to be precursors of pyoverdines [9].

Pyoverdine isoforms, differing by their side chain attached to the chromophore (succinate or its amide form, malate or its amide form, α -ketoglutarate or glutamate, depending on the growth conditions) can easily be distinguished by isoelectric focusing [10,11]. A *P. fluorescens* mutant has been described that produces less fluorescent pyoverdine, and excretes non-fluorescent as well as fluorescent iron chelators under condition of iron limitation [12,13]. This mutant is deficient in the biogenesis of *c*-type cytochromes because of the disruption of the *ccmC* gene that encodes an inner membrane protein that is a key component in the periplasmic delivery of heme to apocytochromes [14,15].

However, the absence of *c*-type cytochrome synthesis is not the cause for pyoverdine deficiency, since one mutant in the periplasmic Trp₁₂₆ residue of CcmC is cytochrome *c*-deficient, but produces normal amounts of mature pyoverdine [13]. On the other hand, mutation of the residue Trp₁₁₅ particularly affects pyoverdine production but not *c*-type cytochrome synthesis [13], suggesting a dual function of CcmC.

We here present evidence that the different iron-chelating molecules excreted by a $\Delta ccmC$ mutant of *P. fluorescens* are all pyoverdine forms differing either by their chromophore side chain or by the saturation of the carbon atoms of the chromophore.

Furthermore, growth and pyoverdine maturation in the $\Delta ccmC$ mutant is negatively affected by a reducing agent (L-cysteine), while addition of an oxidizing agent (oxidized glutathione) improves the yield of native pyoverdine.

2. Materials and methods

2.1. Organisms and culture conditions

P. fluorescens ATCC 17400 wild-type and the $\Delta ccmC$ mutant [12,13] were grown during 48 h on the low-iron casamino acid medium

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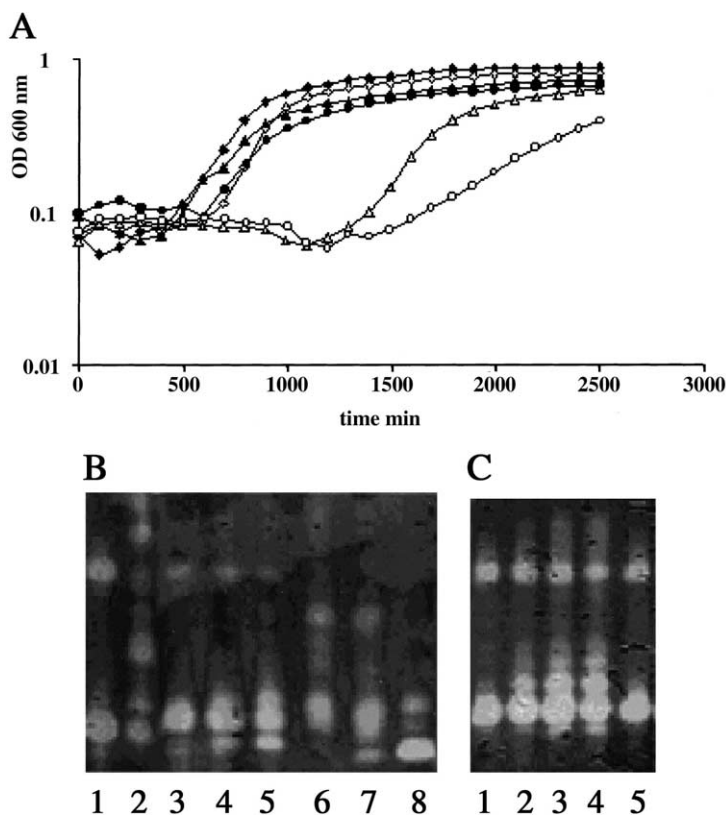


Fig. 1. A: Growth of wild-type (black symbols) and $\Delta ccmC$ mutant (white symbols) in CAA medium (\diamond, \blacklozenge), CAA plus 5 mM L-cysteine ($\triangle, \blacktriangle$), and CAA plus 10 mM L-cysteine (\circ, \bullet). B: IEF-CAS of concentrated pyoverdines from 48 h CAA cultures. Lane 1, pyoverdines from wild-type grown in CAA; lane 2, pyoverdines from $ccmC$ mutant grown in CAA; lanes 3–5, pyoverdines from wild-type grown in CAA in the presence of 2, 4, and 6 mM L-cysteine, respectively; lanes 6–8, pyoverdines from $ccmC$ mutant grown in CAA in the presence of 2, 4, and 6 mM L-cysteine, respectively. C: IEF-CAS of concentrated pyoverdines from 48 h CAA cultures from $Cytc^-$, PVD^+ Trp_{126} $CcmC$ mutant grown in CAA (lane 1), and grown in CAA in the presence of 2, 4, and 6 mM L-cysteine (lanes 2–4, respectively); lane 5, pyoverdines from wild-type grown in CAA.

(CAA) [16] for maximal production of pyoverdines. For purification of pyoverdines, the two strains were grown in the synthetic minimal succinate or glutamate medium [17]. These media were amended with reducing or oxidizing agents as mentioned in Section 3.

Cultures were grown in a Bioscreen apparatus (Life Technologies) using the following parameters: shaking for 10 s every 3 min; reading every 20 min; temperature, 28°C; volume of culture, 300 μ l. As inoculum, an overnight culture of PAO1 in CAA was diluted in order to achieve a final optical density (OD) at 600 nm of 0.01. Each culture was replicated three times and each experiment was performed in triplicate.

2.2. Isoelectric focusing of pyoverdines

Pyoverdines were purified from 500 ml of glutamate minimal medium supernatant of a 48 h culture at 28°C by C18 chromatography. Briefly, 500 ml of sample were poured onto a 1 \times 4 cm C18 column, washed twice with 10 volumes of distilled water, and eluted with 1 ml of 50% methanol and evaporated in a Speed-Vac apparatus.

Pyoverdines (20 μ l of a 10 mM solution) were loaded on an IEF gel (pH 3.5–10 ampholine PAG-plate, Pharmacia). Electrophoresis was done as previously described [10,11]. After electrophoresis, the fluorescence due to pyoverdines was visualized by exposure to UV light on a transilluminator, and the gel was put in contact with a chrome azurol S agarose (CAS agarose) gel [18] in order to detect non-fluorescent siderophores [10].

2.3. Separation of pyoverdines by high-performance liquid chromatography (HPLC) and analysis by mass spectrometry and NMR

The pyoverdine isoforms were separated as ferri-complexes on Nucleosil-100 C18, 250 \times 4 mm (Knauer), detection 254 nm, gradient in 50 mM CH_3COOH with CH_3OH (3–30%), during 30 min, at a rate of

0.7 ml/min. Afterwards ferripyoverdines were de-complexed by passage through a Sep-Pak RP18 cartridge. They were first adsorbed on the column and rinsed with 6.5% (w/v) oxalate solution (pH 4.3), washed with water and de-sorbed with CH_3OH/H_2O 1:1 v/v, and brought to dryness. The pyoverdines were dissolved in $CH_3OH/H_2O/CH_3COOH$ (50:50:0.1 v/v) and introduced into the electrospray ionization (ESI) source of a Finnigan MAT (Bremen, Germany) 900ST mass spectrometer with an electrostatic/magnetic analyzer quadrupole ion trap geometry. Fragmentation of the protonated molecular ions was achieved by collision activation in the quadrupole unit and in the ion trap. 1H -NMR data were obtained with a Bruker (Karlsruhe, Germany) DRX 300 instrument, solvent D_2O .

2.4. Periplasmic extracts

Cell pellets from exponential phase growth cultures in CAA were harvested by centrifugation for 5 min at 4000 $\times g$ and suspended in 1/10 volume of 30 mM Tris-HCl pH 7.5, 20% sucrose and 1 mM EDTA. Cells were finally harvested by 20 min centrifugation at 4000 $\times g$ and carefully suspended in 1/100 volume of ice-cold distilled water, followed by the addition of $MgCl_2$ to a final concentration of 1 mM. After 10 min incubation on ice, the supernatants corresponding to the periplasmic fraction are collected by centrifugation for 5 min at 10 000 $\times g$.

Protein concentration was determined by the DC protein assay from Bio-Rad.

2.5. DTNB assays for free sulfhydryl groups

Determination of total sulfhydryl groups in the periplasmic fractions was carried out using the Ellman's assay [19]. Reduced glutathione (GSH, Sigma) was used as standard using a range of 100–1000 μ M in 20 mM triethanolamine-HCl (TEA, Sigma). 20- μ l samples were added to 75 μ l of 30 mM Tris-HCl pH 8.2, 25 μ l of 150 μ M

5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB, Sigma) and 400 μ l of methanol. The samples were centrifuged at $3000\times g$ for 5 min at room temperature and extinction measured at 412 nm.

2.6. Quantification of pyoverdines by fluorescence and CAS liquid assay

Pyoverdines present in the culture supernatants from 24 h cultures grown in CAA were quantified by measuring the fluorescence emitted at 460 nm after excitation at 405 nm in a Shimadzu spectrofluorimeter. The supernatants were diluted in 10 mM Tris-HCl pH 7.5. The values were normalized for the biomass as expressed by the $OD_{600\text{ nm}}$ of the cultures.

The siderophore production in culture supernatants was measured using the CAS assay developed by Schwyn and Neilands [18]. The percentage of siderophore units, proportional to the discoloration of the CAS solution were calculated using the formula $[(A_r - A_s) / A_r] \times 100$, where A_r is the $OD_{630\text{ nm}}$ of the reference sample (CAA medium plus CAS assay solution) and A_s the $OD_{630\text{ nm}}$ of the sample (culture supernatants plus CAS assay solution). The measurements were made after 10 min of incubation.

2.7. Construction of the pBBRLacZ2

The 4.6-kb *KpnI*-*Sall* fragment of the pBgal-Basic cloning vector (Clontech), that carries the promoterless *lacZ* gene, was inserted into the corresponding sites of the pBBR1mcs [20]. The resulting plasmid, pBBRLacZ2, allows constitutive expression of *lacZ* in *P. fluorescens*.

3. Results

3.1. IEF patterns of pyoverdines from wild-type and $\Delta ccmC$ and effect of cysteine

Addition of 5 or 10 mM L-cysteine to the CAA medium did not affect the growth of the wild-type, but clearly inhibited the growth of the $\Delta ccmC$ mutant (Fig. 1A).

Fig. 1B shows the pattern of pyoverdines present in the supernatant of wild-type and $\Delta ccmC$ mutant in function of the concentration of L-cysteine in the medium. As previously described, two major forms of wild-type pyoverdines can be distinguished on IEF-CAS (lane 1), while up to seven forms can be detected in the case of the $\Delta ccmC$ mutant (lane 2). Addition of L-cysteine to the cultures causes little changes to

the wild-type pyoverdine pattern, namely the presence of two supplementary bands at the highest cysteine concentration (Fig. 1B, lanes 3–5), but has a dramatic effect on the pattern of the $\Delta ccmC$ mutant, causing a reduction in the number of isoforms (Fig. 1B, lanes 6–8). The pattern of the pyoverdines produced by the Trp₁₂₆ CcmC mutant (Cyt^{c-}, PVD⁺) [13] was similar, but slightly more affected than the wild-type by incubation with L-cysteine (Fig. 1C, lanes 1–4).

3.2. Separation and identification of pyoverdine forms from wild-type and $\Delta ccmC$ mutant

HPLC on C18 columns separates the pyoverdines present in the supernatant of the $\Delta ccmC$ mutant into seven major peaks at 3.04, 7.13, 8.23, 9.23, 10.17, 11.20, and 17.36 min (results not shown). The major peaks were peaks 3, 4, 5 and 6. Peak 7 could not be well separated from peak 6. By ESI/mass spectrometry (ESI/MS), peak 3 was found to have a mass of 692 Da, which corresponds to the pyoverdine with glutamic acid as side chain (Fig. 2, structure 4). Peak 4 has a mass of 685 Da and corresponds to pyoverdine with malamide as side chain (Fig. 2, structure 3). Peak 5 has a mass of 677 Da and corresponds to pyoverdine with succinamide as side chain (Fig. 2, structure 1). The major component of peaks 6 and 7 has a mass of 640.4 Da and it could correspond to pyoverdine with the chromophore of azotobactin (Fig. 2, structure 5). Present as minor components, corresponding to HPLC peaks 6 and 7, were two ions with a mass of 717.9 Da and 733 Da. These two components could correspond to dihydropyoverdine sulfonic acid with succinamide (Fig. 2, structure 6), and glutamic acid (Fig. 2, structure 7) as chromophore side chains, respectively.

3.3. Identification of the major pyoverdine form from $\Delta ccmC$ mutant grown in the presence of reducing or oxidizing agents

As already mentioned, and shown in Fig. 1, addition of

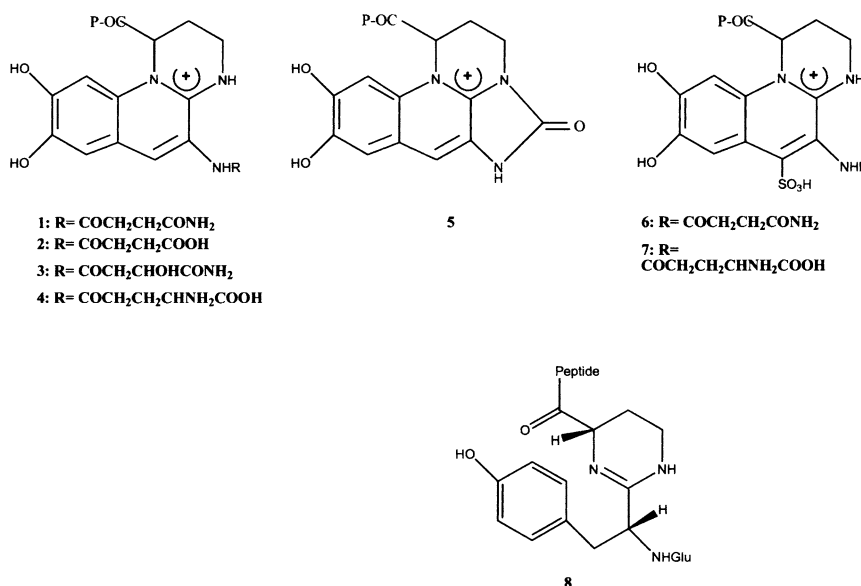


Fig. 2. Schematic representations of the different types of pyoverdine chromophoric parts (chromophore and side chain) from $\Delta ccmC$ mutant separated by HPLC and analyzed by ESI/MS. Structure 1, pyoverdine with succinamide as side chain; structure 2, pyoverdine with succinic acid as side chain; structure 3, pyoverdine with malamide as side chain; structure 4, pyoverdine with glutamic acid as side chain; structure 5, azotobactin; structure 6, dihydropyoverdine sulfonic acid with succinamide as side chain; structure 7, dihydropyoverdine sulfonic acid with glutamic acid as side chain. Structure 8 represents ferribactin.

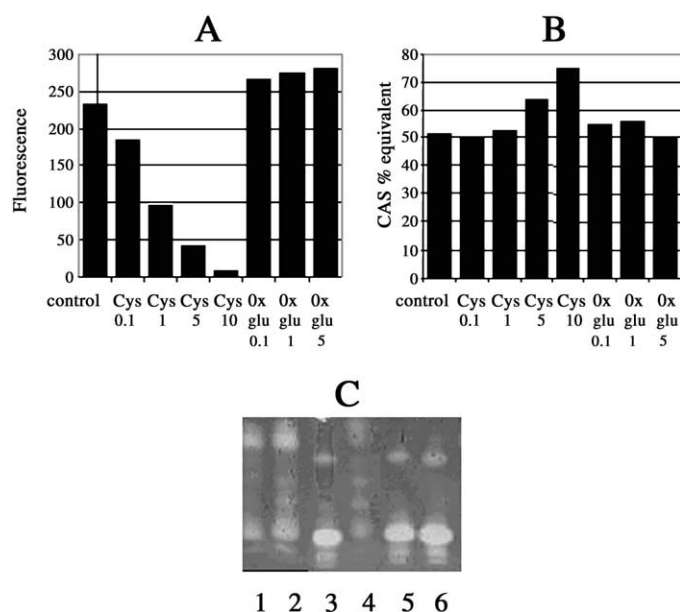


Fig. 3. A: Fluorescence of culture supernatant of the $\Delta ccmC$ mutant grown on minimal glutamate medium without addition (control), with addition of L-cysteine at 0.1, 1, 5 and 10 mM (Cys 0.1, 1 and 5) or oxidized glutathione at 0.1, 1 and 5 mM (ox-glu 0.1, 1 and 5). The cultures were grown for 48 h. B: Total siderophore activity as measured by the CAS discoloration assay. The percentage of siderophore units normalized on the $OD_{600\text{ nm}}$ was determined in each sample using the CAS assay. The order is the same as in A. C: IEF-CAS of concentrated pyoverdines from 48 h CAA cultures. Lane 1, pyoverdines from wild-type grown in CAA; lane 2, pyoverdines from *ccmC* mutant grown in CAA; lanes 3 and 4, pyoverdines from wild-type grown in CAA in the presence of 1 and 5 mM oxidized glutathione, respectively; lanes 5 and 6, pyoverdines from mutant *ccmC* grown in CAA in the presence of 1 and 5 mM oxidized glutathione, respectively.

L-cysteine, a reducing agent, to the culture medium of the $\Delta ccmC$ mutant affects the pattern of pyoverdine isoforms separated by IEF. We therefore decided to compare the effect of the addition of an oxidizing agent (oxidized glutathione) to the culture on the pattern of pyoverdines from the wild-type and the mutant. Oxidized glutathione had no effect on the growth of the wild-type or the $\Delta ccmC$ mutant (results not shown), but resulted in an increase of the fluorescence in the culture supernatant, while, conversely, addition of increasing concentrations of L-cysteine caused a strong reduction of the fluorescence (Fig. 3A). The total amount of siderophore is however not affected by these conditions, since the iron-chelating activity measured by the CAS assay remains more or less constant for all the samples (Fig. 3B). This means that the proportion of siderophores with a fluorescent chromophore (pyoverdines) increases in the presence of an oxidizing agent and decreases in the presence of a reductant. The effect of the addition of oxidized glutathione on the pattern of wild-type and $\Delta ccmC$ pyoverdines is shown in Fig. 3C. As seen in Fig. 3C, lanes 5 and 6, addition of oxidized glutathione changes the pattern of pyoverdines from the $\Delta ccmC$ mutant while it does not affect the wild-type pattern (Fig. 3C, lanes 3 and 4). Addition of increasing concentrations of L-cysteine or oxidized glutathione to purified pyoverdines from the wild-type or the $\Delta ccmC$ mutant followed by 24 h incubation did not affect the intensity of fluorescence or the pattern of pyoverdine isoforms on IEF-CAS (results not shown).

In the sample obtained from the culture medium containing 2 mM oxidized glutathione, according to the mass spectrum obtained by ESI, three isoforms of the pyoverdine *P. fluorescens* ATCC 17400 [21] with a succinamide, a succinic acid and a glutamic acid side chain are present (Fig. 2). Upon collision-induced decomposition of the molecular species, the ions typical for this pyoverdine are obtained. In the sample isolated

from the medium with 5 mM L-cysteine only minute amounts of the pyoverdines could be detected. The main siderophore is the corresponding ferribactin (Fig. 2, structure 8). Collision-induced fragmentation yields the ions characteristic for this chromophore [22], the presence of which is further confirmed by a $^1\text{H-NMR}$ spectrum showing the AA'BB' pattern of the *p*-substituted benzene ring of Tyr (dd's at 6.85 and 7.17 ppm, apparent J_2 8.5 Hz). In addition the signals of the amino acids in the peptide chain can be seen.

3.4. Assessment of the periplasm reducing power in wild-type and $\Delta ccmC$ mutant

The periplasmic fraction of the $\Delta ccmC$ mutant is more reductive (425 pmol of SH groups by μg of protein) compared to the wild-type one (260 pmol/ μg). These fractions were prepared from cells carrying the pBBrLacZ2, that allows a constitutive β -galactosidase expression, characterized by dark blue colonies on X-gal-containing plates. There is no significant contamination of the periplasmic extracts with cytoplasmic proteins since the β -galactosidase activity [23] of these extracts is below 1×10^{-3} units per μg of protein. All the values are the averages of two independent experiments.

4. Discussion

The absence of the CcmC protein results in a pleiotrophic phenotype that includes, among others, a decreased production of the siderophore pyoverdine [12] and a decreased capacity to utilize wild-type ferripyoverdine as a source of iron, despite a normal capacity of uptake of labelled ^{59}Fe -pyoverdine [12]. Another puzzling phenotype of the $\Delta ccmC$ mutant is the accumulation in the supernatant of different pyoverdine isoforms that are not detected in the wild-type. We therefore wanted to know whether these different pyoverdine isoforms

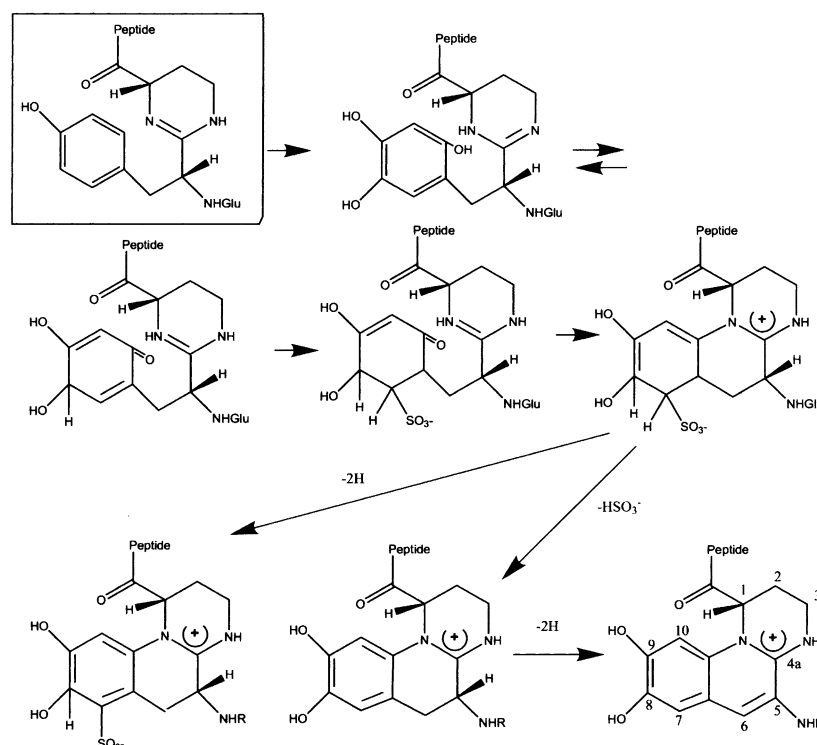


Fig. 4. Proposed scheme of the reactions leading to the formation of the chromophore, starting from ferribactin (top left, framed) and ending up with pyoverdine (bottom right).

could be the result of an incomplete synthesis of pyoverdine. Pyoverdines comprise a quinoline chromophore attached to a side chain and to a peptide chain [5]. The biosynthesis of the peptide chain is the result of the action of peptide synthetases that work via a thiotemplate mechanism [5]. Concerning the biosynthesis of the chromophore, there is evidence for the following scheme: to the N-terminus of the pyoverdine peptide chain the amino acid sequence L-Dab-D-Tyr- γ -L-Glu is added and Dab and Tyr are condensed to a tetrahydropyrimidine ring [9,24]. Metabolites containing this partial structure, referred to as ferribactins [9], have been found to accompany, and are likely to be the precursors of the pyoverdines (Fig. 2, structure 8) [24,25]. Transformation of the ferribactin to the native pyoverdine containing the chromophore involves probably oxidation of Tyr to 2,4,5-trihydroxy-phenylalanine (TOPA) [9,24,25]. A further step is ring closure that leads to dihydropyoverdines [7,9], which are then oxidized to the pyoverdines [1–4]. The Glu attached to the original Tyr by its γ -carboxyl group can be transformed to α -ketoglutaric acid, succinamide and malamide; the two amides may be hydrolyzed to the free acids [26]. This strongly suggests that a peptide synthetase is also likely to be involved in the biosynthesis of the chromophore. Another set of genes, from *P. aeruginosa*, the *pvcABCD* cluster, also seems to be needed for the biosynthesis of the chromophore in this bacterium [27,28]. However, a BLAST search revealed no closely similar proteins in the genomes of *P. fluorescens* Pf0 (<http://www.jgi.doe.gov>), *P. putida* KT 2440 or *Pseudomonas syringae* (<http://www.ncbi.nlm.nih.gov>) (unpublished results).

The $\Delta ccmC$ mutant seems to accumulate pyoverdines with modified chromophores, and differing by their side chains (glutamate, succinate, succinamide, malamide). The presence of azotobactin that results from the cyclization of the side

chain has also been established. In all instances, the core precursor of the quinolinic chromophore was present, indicating that the mutation did not affect the synthesis of the chromophore itself, but affected further modifications of this precursor. The presence of ferribactin and dihydropyoverdine is also indicative of an incomplete maturation of the pyoverdine [3,24].

We propose that some modifications of the chromophore moiety, such as the ring closure, the conversion of dihydropyoverdine sulfonic acid to pyoverdine, and the modification of the side chain, could be the result of a series of oxidation-reduction reactions that take place in the periplasm (Fig. 4). This hypothesis seems to be supported by our results indicating that addition of reducing or oxidizing agents to the culture medium influences the maturation of the pyoverdine chromophore in the $\Delta ccmC$ mutant. These reactions could involve some hemoproteins, since we recently identified heme as a necessary component for pyoverdine biosynthesis [29]. The CcmC protein has been suggested by some to be a heme transporter, while others suggest that it could transport a reducing agent to the periplasm (recently reviewed in [15]). Our data are not in conflict with the first hypothesis, but seem to contradict the second since we found that the periplasm of the $\Delta ccmC$ mutant is more reducing, while the converse would have been expected if CcmC would be involved in the transport of a reducing agent [30]. However, care should be taken when trying to compare results obtained in very different bacteria, namely *P. fluorescens* and *Paracoccus denitrificans* [30]. We have also recently observed that the amount of heme in the cells is strongly decreased in the $\Delta ccmC$ mutant, while addition of the *P. aeruginosa hemH* gene in *trans* results in increased pyoverdine production by $\Delta ccmC$ (unpublished results, manuscript in preparation). The *P. fluorescens* $\Delta ccmC$

mutant was also found to give a reddish fluorescence under UV light, resulting in the appearance of pink colonies, especially on iron supplemented medium, an indication that it accumulates porphyrins. The same phenomenon was observed for *helABCD* mutants (equivalent of *ccmABC*) of *Rhodobacter capsulatus* [31]. Our previous description of some *ccmC* mutants defective for cytochrome *c* biogenesis but not for pyoverdine production or maturation [13] suggests that hemoproteins, other than *c*-type cytochromes, are important for pyoverdine production/maturation. The fact that some reducing or oxidizing agents affect the IEF pyoverdine pattern of the $\Delta ccmC$ mutant is one argument in favor of the hypothesis that CcmC, directly or indirectly, influences the redox balance of the periplasm [15,30]. In this regard, it is interesting to mention that CydDC, an *Escherichia coli* transporter needed for cytochrome *bd* assembly, also seems to control the redox status of the periplasm [32,33]. In this case, however, absence of CydDC seems to increase the oxidative power of the periplasm.

In conclusion, we demonstrated that the absence of CcmC only affects the conversion of ferribactin to pyoverdine, probably by affecting oxidative steps that take place in the periplasm. Our results also confirm that ferribactin is indeed the precursor of pyoverdine.

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