Novel pyoverdine biosynthesis gene(s) of *Pseudomonas aeruginosa* PAO

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Conjugational mobilization of a *Pseudomonas aeruginosa* PAO1 cosmid bank (in pMMB33) into a pyoverdine-deficient (*pvd*) mutant harbouring a mutation in the 47 min region of the chromosome yielded one clone which restored yellow-green pigmentation and fluorescence when grown on iron-deficient medium. The relevant pMMB33-derivative cosmid, pPYP17, contained a 15.1 kb insert which was subcloned into pKT240 as a 10.8 kb *SacI*–*Clai* fragment conferring the same phenotype. This derivative, pPYP180, like pPYP17, also conferred an apparent wild-type phenotype on *pvd* mutants previously shown to map genetically in the 23 min region of the *P. aeruginosa* PAO chromosome. Physical mapping indicated that the cloned DNA fragment is located at the 66–70 min region of the PAO chromosome, demonstrating that the restored apparent wild-type phenotype observed for the transconjugants was not the result of a true gene complementation. A gene interruption was obtained by replacing a 0.6 kb *Bgl*II–*Bgl*II region of pPYP180 necessary for the expression of the pigmentation/fluorescence phenotype, by a *Hg*^r interposon (Ω Hg). After conjugational transfer and introduction of the mutagenized fragment into the PAO1 chromosome by gene replacement, pyoverdine-deficient mutants were recovered, indicating that the fragment indeed contained at least one gene involved in pyoverdine synthesis. The yellow-green fluorescent compound produced by such cells harbouring plasmids pPYP17 or pPYP180 differed from pyoverdine in several aspects and was consequently named pseudoverdine. Although pseudoverdine was able to complex iron, it was unable to restore growth to *pvd* mutants in the presence of the iron chelator ethylenediamine di(o-hydroxyphenylacetic acid), or to mediate iron uptake into PAO1. Pseudoverdine lacked a peptide chain but possessed spectral properties similar to pyoverdine, suggesting that it was structurally related to the chromophore of the pyoverdine molecule. The recent structural determination of pseudoverdine as a coumarin derivative confirmed this view and sheds some light on the biosynthetic pathway of the pyoverdine chromophore.

Keywords: *Pseudomonas aeruginosa*, iron metabolism, siderophore, pyoverdine gene(s)

INTRODUCTION

Pseudomonas aeruginosa PAO produces two siderophores in iron-limited media. One is pyochelin, a derivative of salicylic acid (Cox *et al.*, 1981), the other is the biologically more important pyoverdine (Ankenbauer *et al.*, 1985)

Abbreviations: Ap, ampicillin; Cb, carbenicillin; EDDHA, ethylenediamine di(o-hydroxyphenylacetic acid); Km, kanamycin; TOPA, 2,4,5-trihydroxyphenylalanine.

which is composed of a fluorescent chromophore and a peptide arm (Briskot *et al.*, 1989). Other *P. aeruginosa* strains, as well as strains belonging to other fluorescent pseudomonad species (*P. fluorescens*, *P. putida*, *P. syringae*), were found to produce pyoverdines differing in the composition of the peptide arm (Meyer *et al.*, 1987; Cornelis *et al.*, 1989; Budzikiewicz, 1993). As a result, the uptake of ferripyoverdine is usually strain-specific (Hohnadel & Meyer, 1988; Cornelis *et al.*, 1989). Conversely, the chromophore, which is a quinoline derivative, appears to be highly conserved among all the pyoverdines

Table 1. Bacterial strains and plasmids

Strain/plasmid	Characteristics	Reference
<i>E. coli</i>		
S17-1	<i>pro</i> ($\tau_k^- m_k^+$) RP4-2 (Δ TcMu, Km Tn7) integrated in the chromosome	Simon <i>et al.</i> (1983)
<i>Pseudomonas aeruginosa</i>		
PAO1	Prototroph	Holloway (1969)
PAO1293	Prototroph	Ratnaningsih <i>et al.</i> (1990)
PAO6049	<i>met-9011</i> , <i>amiE200</i> , <i>strA</i>	Rella <i>et al.</i> (1985)
PAO6601	Mutants affected in pyoverdine biosynthesis (<i>pvd</i>) obtained by UV mutagenesis of strain PAO6049, except for PAO6602 and PAO6603 which are Tn1733 insertion mutants of PAO6049. <i>pvd</i> mutations were localized to the 47 min region by linkage analysis in R68-45-mediated conjugational crosses, except for PAO6601 and PAO6622 which harbour a 23 min localized mutation*	Hohnadel <i>et al.</i> (1986) Hohnadel (1988)
PAO6602		
PAO6603		
PAO6606		
PAO6609		
PAO6616		
PAO6620		
PAO6622		
PAO6624		
Plasmid		
pMMB33	IncP-4 <i>mob</i> ⁺ Km ^r λ cos	Frey <i>et al.</i> (1983)
pKT240	IncP-4 <i>mob</i> ⁺ Km ^r Cb ^r	Bagdasarian <i>et al.</i> (1983)
pHP45 Ω Hg	pUC18 derivative carrying a Hg ^r gene	Fellay <i>et al.</i> (1987)
pSUP202	pBR325 derivative <i>mob</i> ⁺ Ap ^r CAM ^r Tc ^r	Simon <i>et al.</i> (1986)
pSUP202CD	pSUP202 derivative carrying the 5.1 kb <i>Eco</i> R1 fragment from pPYP17, with the <i>Bg</i> II– <i>Bg</i> II region replaced by the Hg ^r gene of pHP45 Ω Hg	This study
pPYP17	pMMB33 derivative carrying a <i>Sau</i> 3A DNA fragment of 15.1 kb from PAO1	This study
pPYP18	pKT240 derivatives containing different fragments of the pPYP17 insert	This study
pPYP171		
pPYP174		
pPYP177		
pPYP179		
pPYP180		
pPYP181		

* The 23 min and 47 min locations refer to the recalibrated genetic map of O'Hoy & Krishnapillai (1987), recently revised (by Holloway *et al.*, 1994). They correspond to the previously published values, 35 min and 65–70 min respectively (Hohnadel *et al.*, 1986), based on an earlier version of the map (Holloway & Matsumoto, 1984).

characterized to date (Leong, 1986; Michels *et al.*, 1991; Budzikiewicz, 1993).

At present, little is known about the biosynthesis of pyoverdine or its regulation. Clusters of genes involved in biosynthesis have been cloned from different fluorescent *Pseudomonas* soil isolates (Loper *et al.*, 1984; Moores *et al.*, 1984; Marugg *et al.*, 1985; O'Sullivan *et al.*, 1990). Cloned DNA fragments of 27.2 kb (O'Sullivan *et al.*, 1990) or 33.5 kb (Marugg *et al.*, 1988) were shown to contain several transcriptional units corresponding to genes involved in pyoverdine biosynthesis, in the dissociation of iron from ferripyoverdine, and in iron regulation. In some instances, the ferripyoverdine receptor gene has also been identified and localized in the same region as the biosynthetic genes (Magazin *et al.*, 1986; Marugg *et al.*, 1989; O'Sullivan *et al.*, 1990). In *P. aeruginosa* PAO, two genetically and phenotypically distinct groups of

pyoverdine-deficient mutants have been recognized. The corresponding loci, probably representing gene clusters, were localized respectively at 35 min (Hohnadel *et al.*, 1986) and 65–70 min (Ankenbauer *et al.*, 1986; Hohnadel *et al.*, 1986) on a previous chromosome map (Holloway & Matsumoto, 1984) of *P. aeruginosa* PAO, but placed in a recalibrated map (O'Hoy & Krishnapillai, 1987; Holloway *et al.*, 1994) to 23 min and 47 min, respectively. More recently, a 103 kb region located at 47 min of the PAO chromosome has been defined as the *pvd* region (Tsuda *et al.*, 1995), containing the few siderophore biosynthesis genes already cloned and found to encode an L-ornithine *N*⁵-oxygenase (*pvdA*, Visca *et al.*, 1994), an expected peptide synthetase (*pvdD*, Merriman *et al.*, 1995) and also the ferripyoverdine receptor gene *fvpA* (Poole *et al.*, 1993b). In this paper we describe the cloning of *P. aeruginosa* PAO gene(s) located in the 66–70 min region of the chromosome (current map, Holloway *et al.*, 1994) and

involved in the synthesis of the chromophore moiety of pyoverdine.

METHODS

Bacterial strains, plasmids and growth conditions. *Escherichia coli* S17-1 (Table 1) was grown in Luria-Bertani (LB) medium supplemented with 10 µg streptomycin ml⁻¹. *Pseudomonas aeruginosa* strains (Table 1) were grown either in LB medium, in King's B (KB) medium or in succinate minimal medium (Meyer & Abdallah, 1978), supplemented with 1 mM methionine for PAO6049 and its derivatives. For growth of plasmid-containing strains (Table 1), antibiotics were used at the following concentrations: ampicillin (Ap) and kanamycin (Km) at 100 µg ml⁻¹ for *E. coli*, carbenicillin (Cb) at 400 µg ml⁻¹ and Km at 1000 µg ml⁻¹ for *P. aeruginosa*. *P. aeruginosa* cells harbouring the ΩHg interposon were selected on HgCl₂-supplemented (15 µg ml⁻¹) succinate medium. Iron supplementation at the appropriate concentration was achieved by adding 2 M FeCl₃ to the sterile succinate medium. Due to an intrinsic instability of the plasmid constructions when introduced into *P. aeruginosa*, cultures for production of pseudoverdine or ferripseudoverdine were inoculated directly from a bacterial suspension in sterile saline of daughter colonies grown on KB agar plates supplemented with Km at 1000 µg ml⁻¹. The daughter colonies were obtained by streaking one single colony displaying the pseudoverdine-positive phenotype on the same medium.

DNA manipulation. *P. aeruginosa* PAO1 chromosomal DNA, purified according to Chesney *et al.* (1979), was partially digested

with *Sau3A* and fragments of approximately 20–30 kb were cloned in the *Bam*HI site of pMMB33 using the procedure described by Frey *et al.* (1983). *In vitro* packaging and infection of *E. coli* S17-1 were performed according to the recommendations of the manufacturer (Gigapack gold, Stratagene). Plasmid DNA was purified from *E. coli* cells according to Clewell & Helinski (1969), or from *P. aeruginosa* strains by the method of Hansen & Olsen (1978). Standard procedures (Sambrook *et al.*, 1989) were used for small-scale plasmid DNA purification, agarose gel electrophoresis, bacterial transformation, maxicell protein expression and subcloning in plasmid pKT240. DNA hybridization (Southern, 1975) was performed using a digoxigenin labelling kit (Boehringer, Mannheim). Detection was done according to Kreike *et al.* (1990).

Recombinant plasmid mobilization. Recombinant plasmids were conjugationally mobilized from *E. coli* S17-1 (using the integrated promiscuous conjugative plasmid RP4-2) to *P. aeruginosa* in mating experiments performed in liquid or solid medium. One hundred microlitres of an overnight (37 °C) culture of *E. coli* cells harbouring pMMB33-derivatives were mixed with the same volume of an overnight culture of *P. aeruginosa* cells, grown at 43 °C without agitation to lower *P. aeruginosa* restriction efficiency (Holloway, 1969). The cell mixture was incubated for 4 h at 37 °C and drops were plated on KB medium supplemented with Ap (100 µg ml⁻¹) and Km (1000 µg ml⁻¹). For the mobilization of pKT240-derivative plasmids, the *E. coli* cells were spotted directly on the selective medium (KB medium supplemented with 100 µg Km ml⁻¹ and

Fig. 1. Restriction map of the original cosmid clone pPYP17 and its derivatives. Cosmid pPYP18 is a deletion derivative of pPYP17 restricted with *Bgl*II and religated. The other constructs are subclones in pKT240. Recovery of the wild-type phenotype (yellow-green pigmentation and fluorescence) by the different transconjugants is indicated on the right.

400 µg Cb ml⁻¹) first inoculated with a lawn of *P. aeruginosa* cells subcultured at 43 °C. After incubating for 2 d at 37 °C, transconjugants were screened for their colour and fluorescence under UV light at 365 nm.

Mutagenesis by marker exchange. The 5.0 kb *EcoRI*–*EcoRI* fragment from pPYP17 (Fig. 1) was cloned into the gene replacement vector pSUP202 (Simon *et al.*, 1986), and the recombinant vector subsequently digested with *Bgl*II to remove the 0.6 kb *Bgl*II–*Bgl*II fragment essential for pseudoverdine production. A 5 kb *Bam*HI fragment carrying the Hg^r gene of pHP45ΩHg (Fellay *et al.*, 1987) was inserted into the *Bgl*II-restricted plasmid, yielding pSUP202CD. Following transfer into *E. coli* S17-1, the vector was mobilized into PAO1 by conjugation and Hg^r clones were randomly picked up and analysed for their sensitivity towards Cb. PAO1 clones, resulting from an homologous recombination between the *EcoRI* fragment of pSUP202CD and the corresponding chromosomal region, will be Hg^r but will lack the Cb^r determined by the pSUP202 suicide vector. The *pvd* phenotype expected from the disruption of the *Bgl*II–*Bgl*II fragment by insertion of the Hg^r gene was assessed by spectrometric and fluorimetric analysis of the recombinant strain growth supernatants. Southern analysis of the *Bgl*II-, *EcoRI*- or *Xho*I-digested chromosomal DNA of the putative mutants was carried out using a 4.8 kb digoxigenin-labelled Hg^r-containing *Hind*III fragment of pHP45ΩHg as probe.

PFGE. The physical mapping of the pyoverdine genes within pPYP17 was done by digestion of cosmid DNA with *Sac*I and *Cla*I (Fig. 1) and the 10.8 kb fragment was isolated from low-melting-point agarose, labelled with ³²P and used as a probe in Southern hybridization against PAO1293 genomic DNA digested with *Spe*I. Fragments were separated by PFGE as described by Ratnaningsih *et al.* (1990).

Other techniques. The purification procedure for pyoverdines and electrophoretic analysis were as described previously (Meyer & Abdallah, 1978). The protocol used for iron uptake experiments has been described elsewhere (Meyer *et al.*, 1990). UV-visible spectra were obtained from a Uvikon 930 (Kontron) spectrophotometer.

RESULTS

Construction of a *P. aeruginosa* PAO genomic bank and complementation of *pvd* mutants

A PAO genomic bank was constructed by cloning 20–30 kb fragments from a partial *Sau*3A digest of chromosomal DNA into the broad-host-range cosmid pMMB33 (Frey *et al.*, 1983). After *in vitro* packaging and infection of *E. coli* S17-1, about 2000 Km^r colonies were picked and individually transferred into microtitre plates containing appropriate growth medium. Twelve clones were chosen at random for plasmid DNA isolation. Eleven of the clones were found to contain inserts of more than 20 kb (data not shown).

For complementation experiments, the pyoverdine-deficient mutant PAO6606, whose mutation maps at 47 min, was chosen because of its complete lack of fluorescence when grown in succinate medium (Hohnadel *et al.*, 1986). The 2000 clones were individually mated with this mutant. Transconjugants were obtained at a high frequency (80%). When grown on KB medium, one of them produced an intense pigmentation and

Fig. 2. Southern hybridization of *P. aeruginosa* PAO1 DNA with the digoxigenin-labelled 0.6 kb *Bgl*II fragment from a pUC19 construct containing the *Bgl*II–*Bgl*II fragment from pPYP17. PAO1 DNA was digested with *Bam*HI (lane 1), *Bgl*II (2), *Eco*RI (3), *Xho*I (4), *Hind*III (5), *Bam*HI and *Hind*III (6), *Bam*HI and *Xho*I (7), *Bam*HI and *Bgl*II (8), *Hind*III and *Xho*I (9), and *Hind*III and *Eco*RI (10). The *Bam*HI–*Bgl*II digestion (8) was done as a control for complete digestion (absence of partial fragments) when using a single restriction buffer (One-Phor-All). *Hind*III-digested λ DNA was used as a size marker.

fluorescence, similar to the wild-type phenotype due to pyoverdine excretion. This clone contained a cosmid, named pPYP17, which harboured an insert of 15.1 kb. The genomic bank was also used in complementation assays with the pyoverdine-deficient strains PAO6601 and PAO6622, two mutants having mutations in the 23 min chromosomal region (Hohnadel *et al.*, 1986). Transconjugants displaying the wild-type phenotype were not recovered from these two strains.

Restriction analysis and subcloning of the pPYP17 insert

Fig. 1 shows a restriction map of the 15.1 kb insert of pPYP17. Since the size of the insert was found to be too small for a cosmid (total size of the cosmid: 28 kb), we considered that the actual cosmid pPYP17 resulted from deletions in the original cosmid clone. Fig. 2 shows a Southern blot with PAO1 DNA digested with different restriction enzymes in single or in double digests. The gel-purified 0.6 kb *Bgl*II–*Bgl*II fragment from the 15.1 kb insert of pPYP17 (Fig. 1), subcloned in pUC19 and labelled with digoxigenin, was used as probe. Hybridization with chromosomal DNA digested with *Bam*HI, *Bgl*II, *Xho*I, *Bam*HI + *Hind*III and *Bam*HI + *Xho*I (Fig. 2, lanes 1, 2, 4, 6 and 7, respectively) produced positive signals at positions corresponding to those predicted from the pPYP17 restriction map (11, 0.6, 5.7, 5.8 and 4 kb, respectively). However, a single band of more than 15 kb clearly hybridized with the probe in the

EcoRI digestion (Fig. 2, lane 3), while according to the restriction map, a fragment of 5 kb was expected. Another discrepancy was found in the case of *HindIII* (Fig. 2, lane 5); the hybridization occurred with a band of 7 kb, suggesting the existence of a second *HindIII* site in the chromosomal DNA, absent from pPYP17. The position of this *HindIII* site, close to the left *XhoI* site of pPYP17, was confirmed by double digestion with *XhoI* and *HindIII* (Fig. 2, lane 9); the mobility of this band on the gel was similar to that observed for the DNA digested with *XhoI* (Fig. 2, lane 4). The existence and position of this unexpected *HindIII* site was confirmed by the *EcoRI*–*HindIII* double digestion (Fig. 2, lane 10) which gave a band at 5.3 kb. Hybridization with another probe corresponding to the left 1.5 kb *EcoRI*–*BamHI* fragment of pPYP17 gave two signals with the genomic DNA cut with the same enzymes, one at 5 kb and the other at 0.5 kb, confirming the existence of a deletion in cosmid-cloned DNA which occurred to the left of the *BamHI* fragment (results not shown).

Using plasmid pKT240 as a vector, several subclones with inserts of different sizes were obtained (Fig. 1) and introduced in PAO6606 following mobilization from *E. coli* S17-1. Of these, only pPYP180, harbouring the 10.8 kb *SacI*–*Clal* fragment derived from the 15.1 kb pPYP17 insert, conferred the same pigmented/fluorescent phenotype to the mutant as did pPYP17. Clones harbouring the 6 kb *XhoI* fragment (in pPYP171 and pPYP177, see Fig. 1) produced a faint yellowish fluorescence, whereas the deletion of the 0.6 kb *BglII*–*BglII* fragment from pPYP17 (Fig. 1, pPYP18) resulted in the total loss of the fluorescent phenotype.

When pPYP17 and pPYP180 were mobilized into the other pyoverdine-deficient mutants PAO6602, PAO6603, PAO6609, PAO6616, PAO6620 and PAO6624 (all carrying mutations mapping in the same 47 min region of the chromosome as PAO6606), the transconjugants displayed the wild-type (i.e. pigmentation/fluorescence) phenotype. The other constructions (pPYP18, pPYP171, pPYP174, pPYP177, pPYP179 and pPYP181) did not restore fluorescence. Surprisingly, the transfer of pPYP17 or pPYP180 into a mutant with a mutation mapping in the 23 min region of the chromosome (PAO6622) also produced transconjugants with wild-type pigmentation and fluorescence.

Chromosomal location of the 10.8 kb *SacI*–*Clal* fragment of pPYP17

Physical mapping by PFGE indicated that the 10.8 kb *SacI*–*Clal* fragment from pPYP17 hybridized with the 320 kb *SpeI* fragment F of the PAO chromosome (Holloway *et al.*, 1994) which is located in the 66–70 min region of the current physical and genetic map of PAO (Holloway *et al.*, 1994). This differs from previously identified locations of *pvd* biosynthesis loci at 23 and 47 min (Ankenbauer *et al.*, 1986; Hohnadel *et al.*, 1986; Visca *et al.*, 1994; Merriman *et al.*, 1995; Tsuda *et al.*, 1995).

Fig. 3. Southern hybridization of the restricted chromosomal DNA of a Hg^r Cb^s *pvd* PAO-transconjugant (lanes 2–4) and of the restricted plasmid DNA of pSUP202CD (5–7). The DNA was digested with *BglII* (2, 5), *EcoRI* (3, 6) and *XhoI* (4, 7) and hybridized at high stringency with the digoxigenin-labelled 4.75 kb *HindIII* fragment of pHP45 Ω Hg carrying the Hg^r gene. Digoxigenin-labelled *HindIII*-digested λ DNA was used as a size marker (1).

Mutagenesis by marker exchange

To confirm that *P. aeruginosa* chromosomal DNA present in pPYP17 carries gene(s) involved in pyoverdine biosynthesis, a 0.6 kb *BglII* fragment of pPYP17 was replaced with the Hg^r interposon of pHP45 Ω Hg and introduced into the chromosome of *P. aeruginosa* PAO1 via gene replacement. Of 200 Hg^r *P. aeruginosa* PAO clones screened, four lacked the plasmid-associated Cb resistance, indicating replacement of the *BglII* fragment with the Ω Hg element. Screening for production of pyoverdine revealed two phenotypes amongst the mutant clones: complete lack of pyoverdine-associated pigmentation/fluorescence or slight fluorescence and pigmentation. Repeated subculturing of the mutants revealed some instability with respect to pigmentation with some increase in pigmentation/fluorescence noted. In no case, however, did the insertion mutants demonstrate wild-type pigmentation/fluorescence. To confirm the expected site-specific recombination event, Southern analysis was carried out using the Hg^r -containing fragment of pHP45 Ω Hg as probe. Fig. 3 illustrates the results obtained with the *BglII*, *EcoRI* or *XhoI* digests of the chromosomal DNA of such an insertion mutant (lanes 2–4) or of the plasmid pSUP202CD (lanes 5–7). Both *EcoRI* digests revealed a 3 kb fragment, demonstrating that the Ω Hg interposon was effectively present in the chromosomal DNA. Furthermore, the *XhoI* digestion of that DNA produced a single *XhoI* fragment of 10.1 kb, corresponding to the PAO1 5.7 kb *XhoI* fragment (Fig. 2) augmented

Fig. 4. (a) Absorption spectrum of pyoverdine (thin line) and pseudoverdine (thick line) as measured in growth supernatants (pH adjusted to 7.5) of PAO1 and PAO6624(pPYP17), respectively. (b) Absorption spectrum of ferrispseudoverdine as a function of pH. The iron-supplemented (200 μ M) succinate medium growth supernatant of PAO6624(pPYP17) was diluted fivefold with 0.1 M phosphate buffer with pH adjusted (final value) to 6.87, 6.64, 6.37, 6.15 and 6.01 for, respectively, curves 1–5.

in size by the integrated interposon fragment (5 kb) replacing the *Bgl*II fragment (0.6 kb).

Characterization of the fluorescent pigment produced by transconjugants harbouring pPYP17 or pPYP180

During the 24 h of growth at 37 °C in succinate medium supplemented with 1 mM methionine and 500 μ g Km ml^{-1} , the transconjugants harbouring pPYP17 or pPYP180 accumulated a strongly fluorescent yellow-green extracellular pigment. This pigment appeared similar to PAO1 pyoverdine except that it was slightly greener. This small, subjective difference was confirmed

by comparing the UV-visible absorption spectra of the supernatants, at pH 7.5, which showed an absorption maximum at 418 nm for the PAO6624(pPYP17) supernatant, compared with the typical absorption maximum at 405 nm obtained at the same pH with the PAO1 growth medium containing authentic pyoverdine (Fig. 4). At other pH values, the absorption maximum of the compound produced by PAO6624(pPYP17) showed even more pronounced shifts compared to those observed for pyoverdine: 422 nm at pH 8.8 (407 nm for pyoverdine), 365 nm at pH 5.0 (385 nm for pyoverdine).

Upon iron addition (FeCl_3 2 M, 1 ml per 500 ml medium), a pyoverdine-containing supernatant rapidly turns from yellow-green to brown, together with the disappearance of fluorescence, as a result of the formation of the iron(III) complex of pyoverdine. Ferripyoverdine is characterized by an absorption maximum at 403 nm which is pH-independent (Meyer & Abdallah, 1978). Addition of FeCl_3 to the culture supernatant of the transconjugant led to a similar colour change, indicating that the fluorescent compound was also able to complex iron. However, the UV-visible absorption spectrum of this iron complex had an absorption maximum which was highly sensitive to pH since it shifted from 417 nm at pH 7.0 to 368 nm at pH 6.0 (Fig. 4). The probability that this compound, which we termed pseudoverdine, was not pyoverdine, was confirmed by electrophoresing the fluorescent compounds excreted by the pyoverdine-producer PAO6049 and the PAO6624(pPYP17) and PAO6624(pPYP180) transconjugants. Pseudoverdine did not migrate during electrophoresis at pH 5.0 (the fluorescent spot remained at the origin after a 30 min electrophoresis), whereas the pyoverdines found in the PAO6049 (or PAO1) supernatant migrated towards the cathode to produce two well separated fluorescent spots (data not shown) corresponding to the different forms of pyoverdine found in a wild-type PAO growth supernatant (Budzikiewicz, 1993). Similarly, simultaneous production of pyoverdine and pseudoverdine occurred in a pyoverdine-producing strain (PAO1 or PAO6049) containing pPYP17 (or pPYP180), since two fluorescent spots migrated to the expected positions for pyoverdine and one supplementary fluorescent spot stayed at the origin as for pseudoverdine.

Purification and biological and chemical properties of pseudoverdine

Initial attempts to purify pseudoverdine were performed using a procedure shown to be successful for pyoverdine purification (Meyer & Abdallah, 1978). The brown iron complex (ferrispseudoverdine) was extractable from NaCl-saturated culture supernatant using chloroform/phenol. Like ferripyoverdine, it re-dissolved in water following the addition of diethylether to the organic phase. However, the chromatographic behaviour of ferrispseudoverdine on CM-Sephadex differed from that of ferripyoverdine since it bound irreversibly to CM-Sephadex, as well as to DEAE-Sephadex and to a C18-reversed phase column. Thus, the free ligand (pseudoverdine) was purified from the culture supernatant by repeated gel

Fig. 5. Pseudoverdine and pyoverdine chromophore structures and hypothetical biosynthesis pathway correlating the two compounds.

filtration on Sephadex G-25. An alternative method of pseudoverdine purification described elsewhere (Longerich *et al.*, 1993), involved the isolation of the dark-brown precipitate of ferripseudoverdine which formed following a 10-fold concentration of the culture supernatant from PAO6624(pPYP17) grown in 200 μ M iron-supplemented succinate medium (in an iron-rich medium the culture turned brown instead of yellow-green as for an iron-poor medium, indicating that the pseudoverdine production, contrary to pyoverdine production, was not iron-regulated; see Discussion). Chlorhydric or iodhydric hydrolysis of Sephadex G-25-purified pseudoverdine did not reveal the presence of any amino acid residue in the molecule. The spectral and electrophoretic properties of the purified pseudoverdine were unchanged from those described above for the transconjugant culture supernatants. The complete chemical structure of pseudoverdine was determined and found to be 3-formylamino-6,7-dihydroxycoumarin (Longerich *et al.*, 1993). A hypothetical biogenetic correlation between pseudoverdine and the pyoverdine chromophore has been subsequently proposed (Fig. 5; Longerich *et al.*, 1993).

Although pseudoverdine, like pyoverdine, was apparently able to complex iron(III), it was unable to promote the

growth of *P. aeruginosa* under iron starvation conditions, or to promote the uptake of iron in iron-deficient cells. Supplementation of the growth medium with the strong iron chelator ethylenediamine di(*o*-hydroxyphenylacetic acid) (EDDHA, 1 mg ml⁻¹) inhibited the growth of the pyoverdine-less mutants (Hohnadel *et al.*, 1986) as well as the growth of such mutants harbouring pPYP17 or pPYP180 (data not shown). Growth of mutants or transconjugants could, however, be restored by the addition of purified pyoverdine (10 μ M, final concentration), but not pseudoverdine, to the EDDHA/succinate medium (Hohnadel *et al.*, 1986). Finally, when pseudoverdine was added as the chelator instead of pyoverdine in iron uptake assays, it efficiently solubilized the labelled iron, but, unlike pyoverdine, failed to promote iron uptake (data not shown).

DISCUSSION

P. aeruginosa PAO strains harbouring a cosmid derivative containing a 15.1 kb fragment of PAO1 chromosomal DNA (pPYP17) or a pKT240-plasmid derivative containing a 10.8 kb fragment originating from the 15.1 kb insert in pPYP17 (pPYP180) were found to produce a pyoverdine-like compound, pseudoverdine, which was excreted in amounts equivalent to pyoverdine produced by the wild-type strain grown in succinate medium. As judged by the colour and fluorescence of the culture supernatants, these plasmids restored an apparently wild-type phenotype to PAO pyoverdine-less mutants. Originating from the 66–70 min region of the bacterial chromosome, as demonstrated by PFGE physical mapping, the 10.8 kb fragment (or its 15.1 kb precursor fragment) led to the production of pseudoverdine by *pvd* mutants with mutations mapped to the 47 min and the 23 min regions of the bacterial chromosome. Thus, the apparently restored wild-type phenotype did not result from gene complementation but rather, as discussed below, from a deregulated expression of the genes in the 10.8 kb *SacI*–*Clal* DNA fragment. This was confirmed by the excretion of pseudoverdine, together with pyoverdine, by the wild-type PAO1 strain when the cosmid pPYP17 (or pPYP180) had been introduced into it. Due to their strong similarities in UV-visible absorption spectral properties, a simple quantification of the respective production of these two compounds by PAO1(pPYP17) was not possible. However, it should be emphasized that the absorbance in the 400–420 nm region observed for the culture supernatant of PAO1(pPYP17) was not increased compared to that of the wild-type strain, suggesting that pseudoverdine production reduced pyoverdine biosynthesis. This, together with the similarities in fluorescence and colour of the two compounds, as well as their iron-binding abilities, suggested that pseudoverdine might be related to the chromophore of pyoverdine.

Pseudoverdine was found to differ from the biologically active pyoverdine in several aspects: it did not restore the growth of pyoverdine-deficient mutants in EDDHA-supplemented media and it did not mediate iron uptake by

iron-starved cells. These results, however, do not disagree with a chromophore-related structure for pseudoverdine since it is well established that ferripyoverdine recognition by its specific outer-membrane receptor is mediated by the peptide moiety of the molecule (Hohnadel & Meyer, 1988) which is absent from pseudoverdine. Interestingly, both pseudoverdine and pyoverdine were excreted into the growth medium, suggesting that excretion of pyoverdine (Poole *et al.*, 1993a) is independent of the peptide moiety. Pseudoverdine, a coumarin derivative (3-formylamino-6,7-dihydroxycoumarin, Longerich *et al.*, 1993), possesses an *ortho*-dihydroxyl group in common with the chromophore of pyoverdine, 5-amino-2,3-dihydro-8,9-dihydroxy-1*H*-pyrimido-[1,2*a*] quinoline-1-carboxylic acid (Fig. 5, Michels *et al.*, 1991). From a chemical point of view the coumarin compound may be formed by an internal cyclization of 2,4,5-trihydroxyphenylalanine (TOPA). A hypothetical scheme for the biosynthesis of this molecule and its relationship to the pyoverdine chromophore has been proposed (Fig. 5, Longerich *et al.*, 1993): it suggests that during the biogenesis of the chromophore, TOPA condenses with diaminobutyric acid leading to a precursor of the quinoline chromophore. Thus, pseudoverdine should not be considered as a direct biosynthetic precursor of the pyoverdine chromophore but, rather, as a precursor side-product resulting from an internal condensation of TOPA when the reaction with diaminobutyrate does not occur. Thus, the genes present on the 10.8 kb DNA fragment may be related to enzymes involved in the biosynthesis of TOPA from tyrosine. Preliminary biochemical experiments have already confirmed this view in that supplementation of cultures of PAO6624(pPYP17) or PAO1 with ¹⁴C-tyrosine produced radioactive pseudoverdine and pyoverdine, respectively, labelled at the chromophore (A. Stintzi & J.-M. Meyer, unpublished results). Thus, tyrosine which has already been described as a precursor for the pseudobactin (pyoverdine) chromophore of *P. fluorescens* B10 (Nowak-Thomson & Gould, 1994), appears to be a common precursor of the chromophore of both pyoverdine and pseudoverdine. It should also be pointed out that the chromophore of azotobactin, a pyoverdine-like pigment produced by *Azotobacter vinelandii*, is partially derived from 3,4-dihydroxyphenylalanine (Fukasawa & Goto, 1973).

The results of the marker exchange experiments demonstrate conclusively that gene(s) present on pPYP180 are involved in pyoverdine biosynthesis. It should be emphasized, however, that the resulting *pvd* mutants were phenotypically unstable, yielding the reappearance of some fluorescence in their culture supernatants. This might explain the lack of previously reported *pvd* mutants with lesions mapping to this region of the chromosome since selection of *pvd* mutants is usually based on the absence of pigmentation and fluorescence.

Thus, it can be concluded that pyoverdine biosynthesis genes are dispersed around the *P. aeruginosa* PAO chromosome, present at least at three different loci including the 23 min (Hohnadel *et al.*, 1986) and 47 min regions (Ankenbauer *et al.*, 1986; Hohnadel *et al.*, 1986; Tsuda *et al.*, 1995) as demonstrated by genetic mapping and the

66–70 min region, as demonstrated in the present study by physical mapping.

Pseudoverdine produced by pPYP17 or pPYP180 transconjugants was expressed constitutively, and was not dependent on the iron content of growth medium. In this respect it differed markedly from pyoverdine biosynthesis which like all siderophores, is strongly iron-regulated (Meyer *et al.*, 1987). In *E. coli* the regulation of the different iron uptake systems is negatively controlled and depends on a repressor molecule, the Fur (ferric uptake regulation) protein which acts with iron(II) as a corepressor (Bagg & Neilands, 1987). Such negative control has also been recognized for a soil fluorescent *Pseudomonas* isolate (O'Sullivan *et al.*, 1990) and a *P. aeruginosa* *fur* gene has recently been cloned (Prince *et al.*, 1993). The constitutive expression of pseudoverdine could be explained by titration of a Fur-like repressor protein by the multiple copies (20–30, Jeenes *et al.*, 1986) of the cloning vector pKT240 in *P. aeruginosa*. This explanation is, however, unlikely since we have shown that the synthesis of the ferripyoverdine receptor in the pseudoverdine-producing transconjugants remained under iron control (Hohnadel, 1988). Transcription of the pseudoverdine gene(s) from a plasmid promoter appears very unlikely: first, the cloning sites *Cla*I and *Sac*I in pKT240 are in the coding regions of *kan* and *str* genes (Bagdasarian *et al.*, 1983; Pouwels *et al.*, 1985) and, secondly, the DNA sequences in the 5' region of *Sac*I and in the 3' region of *Cla*I could be deleted, as is the case with pPYP180, without affecting the expression of pseudoverdine. We consider that the missing DNA in the pPYP17 cosmid, as detected by the discrepancy in the restriction map left of the *Bam*HI site in a Southern blot with PAO1 genomic DNA, has either placed promoter sequences directly upstream of the gene(s) contained on the fragment or resulted in the loss of regulatory regions upstream of the promoter. Work is in progress to more precisely characterize this DNA fragment and to determine the biosynthetic pathway of pseudoverdine.

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REFERENCES

- Ankenbauer, R., Sriyosachati, S. & Cox, C. D. (1985). Effects of siderophores on the growth of *Pseudomonas aeruginosa* in human serum and transferrin. *Infect Immun* **49**, 132–140.
- Ankenbauer, R., Hanne, L. F. & Cox, C. D. (1986). Mapping of

- mutations in *Pseudomonas aeruginosa* defective in pyoverdine production. *J Bacteriol* **167**, 7–11.
- Bagdasarian, M. M., Amann, E., Lurz, R., Rückert, B. & Bagdasarian, M. (1983). Activity of the hybrid *trp-lac* (*tac*) promoter of *Escherichia coli* in *Pseudomonas putida*. Construction of broad-host-range, controlled-expression vectors. *Gene* **26**, 273–282.
- Bagg, A. & Neilands, J. B. (1987). Ferric uptake regulation protein acts as a repressor, employing iron(II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli*. *Biochemistry* **26**, 5471–5477.
- Briskot, G., Taraz, K. & Budzikiewicz, H. (1989). Pyoverdine-type siderophores from *Pseudomonas aeruginosa*. *Liebigs Ann Chem* **375**–384.
- Budzikiewicz, H. (1993). Secondary metabolites from fluorescent pseudomonads. *FEMS Microbiol Rev* **104**, 209–228.
- Chesney, R. H., Scott, J. R. & Vapnek, D. (1979). Integration of the plasmid prophages P1 and P7 into the chromosome of *Escherichia coli*. *J Mol Biol* **130**, 161–173.
- Clewell, D. B. & Helinski, D. R. (1969). Supercoiled circular DNA–protein complex in *Escherichia coli*: purification and induced conversion to an open circular DNA form. *Proc Natl Acad Sci USA* **62**, 1159–1166.
- Cornelis, P., Hohnadel, D. & Meyer, J. M. (1989). Evidence for different pyoverdine-mediated iron uptake systems among *Pseudomonas aeruginosa* strains. *Infect Immun* **57**, 3491–3497.
- Cox, C. D., Rinehart, K. L., Moore, M. L. & Cook, J. C. (1981). Pyochelin: novel structure of an iron-chelating growth promoter for *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **78**, 4256–4260.
- Fellay, R., Frey, J. & Krisch, H. (1987). Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for *in vitro* insertional mutagenesis of Gram-negative bacteria. *Gene* **52**, 147–154.
- Frey, J., Bagdasarian, M., Feiss, D., Franklin, F. C. & Deshusses, J. (1983). Stable cosmid vectors that enable the introduction of cloned fragments into a wide range of Gram-negative bacteria. *Gene* **24**, 299–308.
- Fukasawa, K. & Goto, M. (1973). Biosynthesis of a heterocycle formed by iron-deficient *Azotobacter vinelandii* strain O. *Biochim Biophys Acta* **320**, 545–548.
- Hansen, J. B. & Olsen, R. H. (1978). Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. *J Bacteriol* **135**, 227–238.
- Hohnadel, D. (1988). *Metabolisme du fer chez Pseudomonas aeruginosa: approche physiologique, biochimique et génétique du système pyoverdine*. PhD Thesis, Université Louis Pasteur, Strasbourg.
- Hohnadel, D. & Meyer, J. M. (1988). Specificity of pyoverdine-mediated iron uptake among fluorescent *Pseudomonas* strains. *J Bacteriol* **170**, 4865–4873.
- Hohnadel, D., Haas, D. & Meyer, J. M. (1986). Mapping of mutations affecting pyoverdine production in *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* **36**, 195–199.
- Holloway, B. W. (1969). Genetics of *Pseudomonas*. *Bacteriol Rev* **33**, 419–443.
- Holloway, B. W. & Matsumoto, H. (1984). *Pseudomonas aeruginosa*. In *Genetic Maps* 1984, pp. 194–197. Edited by S. J. O'Brien. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Holloway, B. W., Römling, U. & Tümmler, B. (1994). Genomic mapping of *Pseudomonas aeruginosa* PAO. *Microbiology* **140**, 2907–2929.
- Jeenes, D. J., Soldati, L., Baur, H., Watson, J. M., Mercenier, A., Reimann, C., Leisinger, T. & Haas, D. (1986). Expression of biosynthetic genes from *Pseudomonas aeruginosa* and *Escherichia coli* in the heterologous host. *Mol Gen Genet* **203**, 421–429.
- Kreike, C. M., De Koning, J. R. A. & Krem, F. A. (1990). Non-radioactive detection of single-copy DNA–DNA hybrids. *Plant Mol Biol Rep* **8**, 172–179.
- Leong, J. (1986). Siderophores: their biochemistry and possible role in the biocontrol of plant pathogens. *Annu Rev Phytopathol* **24**, 187–209.
- Longerich, I., Taraz, K., Budzikiewicz, H., Tsai, L. & Meyer, J.-M. (1993). Pseudoverdin, a compound related to the pyoverdine chromophore from a *Pseudomonas aeruginosa* strain incapable to produce pyoverdins. *Z Naturforsch* **48c**, 425–429.
- Loper, J. E., Orser, C. S., Panopoulos, N. J. & Schroth, M. N. (1984). Genetic analysis of fluorescent pigment production in *Pseudomonas syringae* pv. *syringae*. *J Gen Microbiol* **130**, 1507–1515.
- Magazin, M. D., Moores, J. C. & Leong, J. (1986). Cloning of the gene coding for the outer membrane receptor protein for ferric pseudobactin, a siderophore from a plant growth-promoting *Pseudomonas* strain. *J Biol Chem* **261**, 795–799.
- Marugg, J. D., van Spanje, M., Hoekstra, W. P. M., Schippers, B. & Weisbeek, P. J. (1985). Isolation and analysis of genes involved in siderophore biosynthesis in plant-growth-stimulating *Pseudomonas putida* WCS358. *J Bacteriol* **164**, 563–570.
- Marugg, J. D., Nielander, H. B., Horrevoets, A. J. G., van Megen, I., van Genderen, I. & Weisbeek, P. J. (1988). Genetic organization and transcriptional analysis of a major gene cluster involved in siderophore biosynthesis in *Pseudomonas putida* WCS358. *J Bacteriol* **170**, 1812–1819.
- Marugg, J. D., de Weger, L. A., Nielander, H. B., Oorthuizen, M., Recourt, K., Lugtenberg, B., van der Hofstadt, G. A. J. M. & Weisbeek, P. J. (1989). Cloning and characterization of a gene encoding an outer membrane protein required for siderophore-mediated uptake of Fe³⁺ in *Pseudomonas putida* WCS358. *J Bacteriol* **171**, 2819–2826.
- Merriman, T. R., Merriman, M. E. & Lamont, I. L. (1995). Nucleotide sequence of *pvdD*, a pyoverdine biosynthetic gene from *Pseudomonas aeruginosa*: PvdD has similarity to peptide synthetases. *J Bacteriol* **177**, 252–258.
- Meyer, J. M. & Abdallah, M. A. (1978). The fluorescent pigment of *Pseudomonas fluorescens*, biosynthesis, purification and physico-chemical properties. *J Gen Microbiol* **107**, 319–328.
- Meyer, J. M., Hallé, F., Hohnadel, D., Lemanceau, P. & Ratefiarivelo, H. (1987). Siderophores of *Pseudomonas* – biological properties. In *Iron Transport in Microbes, Plants and Animals*. pp. 188–205. Edited by G. Winkelmann, D. van der Helm & J. B. Neilands. Weinheim: VCH Verlagsgesellschaft.
- Meyer, J. M., Hohnadel, D., Khan, A. & Cornelis, P. (1990). Pyoverdine-facilitated iron uptake in *Pseudomonas aeruginosa*: immunological characterization of the ferripyoverdine receptor. *Mol Microbiol* **4**, 1401–1405.
- Michels, J., Benoni, H., Briskot, G., Lex, J., Schmickler, H., Taraz, K., Budzikiewicz, H., Korth, H. & Pulverer, G. (1991). Isolation and spectroscopic characterization of the pyoverdine chromophore and of its 5-hydroxy analogue. *Z Naturforsch* **46c**, 993–1000.
- Moores, J. C., Magazin, M., Ditta, G. S. & Leong, J. (1984). Cloning of genes involved in the biosynthesis of pseudobactin, a high-affinity iron transport agent of a plant growth-promoting *Pseudomonas* strain. *J Bacteriol* **157**, 53–58.
- Nowak-Thomson, B. & Gould, S. J. (1994). Biosynthesis of the pseudobactin chromophore from tyrosine. *Tetrahedron* **50**, 9865–9872.
- O'Hoy, K. & Krishnapillai, V. (1987). Recalibration of the

Pseudomonas aeruginosa PAO strain chromosome map in time units using high-frequency-of-recombination donors. *Genetics* **115**, 611–618.

O'Sullivan, D. J., Morris, J. & O'Gara, F. (1990). Identification of an additional ferric-siderophore uptake gene clustered with receptor, biosynthesis and *fur*-like regulatory genes in fluorescent *Pseudomonas* sp. strain M114. *Appl Environ Microbiol* **56**, 2056–2064.

Poole, K., Heinrichs, D. E. & Neshat, S. (1993a). Cloning and sequence analysis of an EnvCD homologue in *Pseudomonas aeruginosa*: regulation by iron and possible involvement in the secretion of the siderophore pyoverdine. *Mol Microbiol* **10**, 529–544.

Poole, K., Neshat, S., Krebs, K. & Heinrichs, D. E. (1993b). Cloning and nucleotide sequence analysis of the ferripyoverdine receptor gene *fpvA* of *Pseudomonas aeruginosa*. *J Bacteriol* **175**, 4597–4604.

Pouwels, P. H., Enger-Volk, B. E., Brammar, W. J. (eds) (1985). *Cloning Vectors. A Laboratory Manual*. Amsterdam: Elsevier.

Prince, R. W., Cox, C. D. & Vasil, M. L. (1993). Coordinate regulation of siderophore and exotoxin A production: molecular cloning and sequencing of the *Pseudomonas aeruginosa fur* gene. *J Bacteriol* **175**, 2589–2598.

Ratnaningsih, E., Darmsthiti, S., Krishnapillai, V., Morgan, A., Sinclair, M. & Holloway, B. W. (1990). A combined physical and genetic map of *Pseudomonas aeruginosa* PAO. *J Gen Microbiol* **136**, 2351–2357.

Rella, M., Mercenier, A. & Haas, D. (1985). Transposon insertion mutagenesis of *Pseudomonas aeruginosa* with a *Tn5* derivative: application to physical mapping of the *arc* gene cluster. *Gene* **33**, 293–303.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (eds) (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Simon, R., Priefer, U. & Pühler, A. (1983). A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Bio/Technology* **1**, 784–791.

Simon, R., O'Connell, M., Labes, M. & Pühler, A. (1986). Plasmid vectors for the genetic analysis and manipulation of Rhizobia and other Gram-negative bacteria. *Methods Enzymol* **118**, 640–659.

Southern, E. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98**, 503–517.

Tsuda, M., Miyazaki, H. & Nakazawa, T. (1995). Genetic and physical mapping of genes involved in pyoverdine production in *Pseudomonas aeruginosa* PAO. *J Bacteriol* **177**, 423–431.

Visca, P., Ciervo, A. & Orsi, N. (1994). Cloning and nucleotide sequence of the *pvdA* gene encoding the pyoverdine biosynthetic enzyme L-ornithine *N*⁵-oxygenase in *Pseudomonas aeruginosa*. *J Bacteriol* **176**, 1128–1140.

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