

Siderophore-mediated iron acquisition in the entomopathogenic bacterium *Pseudomonas entomophila* L48 and its close relative *Pseudomonas putida* KT2440

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Abstract *Pseudomonas entomophila* L48 is a recently identified entomopathogenic bacterium which, upon ingestion, kills *Drosophila melanogaster*, and is closely related to *P. putida*. The complete genome of this species has been sequenced and therefore a genomic, genetic and structural analysis of the siderophore-mediated iron acquisition was undertaken. *P. entomophila* produces two siderophores, a structurally new and unique pyoverdine and the secondary siderophore pseudomonine, already described in *P. fluorescens* species. Structural analysis of the pyoverdine produced by the closely related *P. putida* KT2440 showed that this strain produces an already

characterised pyoverdine, but different from *P. entomophila*, and no evidence was found for the production of a second siderophore. Growth stimulation assays with heterologous pyoverdines demonstrated that *P. entomophila* is able to utilize a large variety of structurally distinct pyoverdines produced by other *Pseudomonas* species. In contrast, *P. putida* KT2440 is able to utilize only its own pyoverdine and the pyoverdine produced by *P. syringae* LMG 1247. Our data suggest that although closely related, *P. entomophila* is a more efficient competitor for iron than *P. putida*.

Keywords *Pseudomonas* · Pyoverdine · Pseudomonine · Siderotyping · Mass spectrometry

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Introduction

When fluorescent pseudomonads are grown under iron limiting conditions, they produce a yellow–green, fluorescent siderophore, called pyoverdine (Meyer and Abdallah 1978). The structures of pyoverdines produced by different strains of fluorescent pseudomonads have been characterised. They are made of three distinct structural parts: a small peptide chain of L- and D-amino acids (6–12 amino acids depending on the producing strain), linked to a yellow–green chromophore group and a small dicarboxylic acid or its monoamide connected amidically to the NH₂-group of the chromophore (Teintze et al. 1981; Budzikiewicz

1993, 1997). Pyoverdines contain both catechol and hydroxamate groups that are participating in Fe(III) binding, the chromophore contains one catechol group, and the peptide chain two hydroxamate groups.

Besides pyoverdine, several other secondary siderophores, which have a relatively lower affinity for iron, have been identified in fluorescent pseudomonads. These include thioquinolobactin, pyochelin, pseudomonine, corrugatin and the recently identified ornicorrugatin (Cox and Graham 1979; Risse et al. 1998; Mercado-Blanco et al. 2001; Cornelis and Matthijs 2002; Matthijs et al. 2007, 2008).

The ability to produce the high affinity siderophore pyoverdine in addition to other siderophores of lower affinity makes fluorescent pseudomonads well equipped to compete with other micro-organisms for iron (Ravel and Cornelis 2003).

Another trait that makes fluorescent pseudomonads good competitors for iron is the ability to utilise iron complexes of a variety of different siderophores produced by other micro-organisms, including fungi and bacteria (Poole et al. 1990; Jurkevitch et al. 1992; Meyer 1992; Raaijmakers et al. 1995). Whereas fluorescent pseudomonads are able to utilise siderophores of members of other genera, the pyoverdines produced by fluorescent pseudomonads cannot be used by bacteria outside this group. In this study a genomic, genetic and structural analysis of pyoverdine-mediated iron acquisition in the entomopathogenic bacterium *Pseudomonas entomophila* L48 and *P. putida* KT2440 was undertaken. Genomic and mass analysis showed the presence of the secondary siderophore pseudomonine in *P. entomophila*. In addition the ability to utilize heterologous pyoverdines was investigated revealing a high capacity of *P. entomophila* L48 to utilize pyoverdines produced by other fluorescent pseudomonads. In contrast, the closely related *P. putida* KT2440 was only able to use one heterologous pyoverdine in addition to its own pyoverdine and did not produce any secondary siderophore.

Materials and methods

Pyoverdine typing and isolation from different *Pseudomonas* strains

A large collection of about 160 *Pseudomonas* strains isolated at the source (site W2) and the mouth (site

W15) of the Woluwe River (Brussels, Belgium) was subjected to isoelectric focusing (IEF) analysis of culture supernatants according to Koedam et al. (1994) (S. Matthijs, unpublished data). The strains were grouped according to their IEF profile and each profile was assigned a type number. *P. aeruginosa* strains producing type I, II and III pyoverdine were given the same type number, the pyoverdines coming from other *Pseudomonas* were numbered starting from type 1, type 2, Strains producing a unique siderotype were subsequently selected (Table 1; Fig. 1) and the pyoverdine was re-isolated by a medium scale purification to confirm the siderotype and to obtain a salt-free sample for mass analysis. Therefore 100 ml cultures were grown for 42–48 h at 26°C in 1 l Erlenmeyers. Subsequently the cultures were centrifuged for 15 min at 10,000g, the supernatant was filtered with a 0.2 µm Minisart Satorius filter (Stedim Biotech) and applied to a C₋₁₈ column that was activated with methanol and washed with distilled water. Elution was done with acetonitrile/H₂O (70/30%). The siderotypes were confirmed by IEF and the mass of the pyoverdine was determined by LC/MS or MS/MS.

To identify the pyoverdines produced by the Woluwe isolates, pyoverdine was isolated from reference strains (producing an already characterized pyoverdine) and the siderotype and the mass of the pyoverdine was compared with the result of the reference strain pyoverdine. In case the IEF profile and the mass were identical they were considered as producing the same pyoverdine. These reference strains included: *P. aeruginosa* PAO1 (type I, Briskot et al. 1986), *P. aeruginosa* 7NSK2 (type II, de Chial et al. 2003), *P. aeruginosa* 59.20 (type III, de Chial et al. 2003), *P. fluorescens* SBW25 (type 1, Moon et al. 2008), *P. putida* G4R (type 3, Salah-el-Din et al. 1997), *P. fluorescens* CHA0 (type 6, Wong-Lun-Sang et al. 1996), PL7 (type 7, Barelmann et al. 2002), B10 (type 9, Teintze et al. 1981), *P. fluorescens* A6 (CFBP 2392; type 32, Beiderbeck et al. 1999), *P. fluorescens* W (type 37, Demange et al. 1990). To increase the number of unique pyoverdines, following reference strains were included in this study: *P. fluorescens* Pf0-1 (type 14, Meyer et al. 2008), *P. syringae* subsp. *syringae* (type 15, Jülich et al. 2001), and *P. fluorescens* ATCC 17400 (type 16, Demange et al. 1990).

Table 1 List of strains used in this study

Strain	Genotype or relevant characteristics	Source or reference
<i>P. entomophila</i> L48	Wild type	Vodovar et al. (2006)
1C11	<i>pvsA</i> ::Tn5, pyoverdine-negative Tn5 mutant of <i>P. entomophila</i> , Tc ^R	This study
1E10	PSEEN3234::Tn5, pyoverdine-negative Tn5 mutant of <i>P. entomophila</i> , Tc ^R	This study
1H12	PSEEN3231::Tn5, pyoverdine-negative Tn5 mutant of <i>P. entomophila</i> , Tc ^R	This study
1G9	PSEEN3234::Tn5, pyoverdine-negative Tn5 mutant of <i>P. entomophila</i> , Tc ^R	This study
3C6	<i>pvdA</i> ::Tn5, pyoverdine-negative Tn5 mutant of <i>P. entomophila</i> , Tc ^R	This study
<i>ΔpmsCEAB</i>	<i>P. entomophila</i> pseudomonine-negative mutant	This study
pUK21	Cloning vector; <i>lacZx</i> ; Km ^R	Vieira and Messing (1991)
pME3087	Suicide vector, ColE1 replicon, RK2-Mob, Tc ^R	Voisard et al. (1994)
<i>P. putida</i> KT2440	Wild type	DSMZ
3E2	PP_4220::Tn5, pyoverdine-negative Tn5 mutant of <i>P. putida</i> , Tc ^R	This study
5A12	PP_4220::Tn5, pyoverdine-negative Tn5 mutant of <i>P. putida</i> , Tc ^R	This study
5C10	PP_4218::Tn5, pyoverdine-negative Tn5 mutant of <i>P. putida</i> , Tc ^R	This study
W2Aug1	<i>Pseudomonas</i> sp., isolate from the Woluwe	This study
W2Aug36	<i>Pseudomonas</i> sp., isolate from the Woluwe	This study
W2Dec18	<i>Pseudomonas</i> sp., isolate from the Woluwe	This study
W2Dec33	<i>Pseudomonas</i> sp., isolate from the Woluwe	This study
W2Dec36A	<i>Pseudomonas</i> sp., isolate from the Woluwe	This study
W2Feb31B	<i>Pseudomonas</i> sp., isolate from the Woluwe	This study
W2Jun14	<i>Pseudomonas</i> sp., isolate from the Woluwe	This study
W15Apr2	<i>Pseudomonas</i> sp., isolate from the Woluwe	This study
W15Aug26	<i>P. aeruginosa</i> isolate from the Woluwe	Pirnay et al. (2005)
W15Aug23	<i>P. aeruginosa</i> isolate from the Woluwe	Pirnay et al. (2005)
W15Aug24	<i>P. aeruginosa</i> isolate from the Woluwe	Pirnay et al. (2005)
W15Feb38	<i>Pseudomonas</i> sp., isolate from the Woluwe	This study
Pf0-1	<i>P. fluorescens</i>	S. B. Levy, Tufts University School of Medicine
Pf5	<i>P. fluorescens</i>	A. Sarniguet, INRA, France
Pf17400	<i>P. fluorescens</i> ATCC 17400, egg yolk isolate	BCCM/LMG
CFBP 2392	<i>P. fluorescens</i> (A6)	CFBP
LMG 2112	<i>P. agarici</i> type strain	BCCM/LMG
LMG 21623	<i>P. brassicacearum</i> type strain	BCCM/LMG
LMG 23068	<i>P. brenneri</i> type strain	BCCM/LMG
LMG 14562	<i>P. fluorescens</i>	BCCM/LMG
LMG 21624	<i>P. kilonensis</i> type strain	BCCM/LMG
LMG 21606	<i>P. libanensis</i> type strain	BCCM/LMG
LMG 1247	<i>P. syringae</i> subsp. <i>syringae</i> type strain	BCCM/LMG
LMG 20222	<i>P. vancouverensis</i> type strain	BCCM/LMG

Table 1 continued

Strain	Genotype or relevant characteristics	Source or reference
<i>E. coli</i> DH5 α	<i>supE44</i> Δ <i>lacU169</i> (Φ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Hanahan (1983)

BCCM/LMG Belgian co-ordinated collections of micro-organisms, DSMZ German collection of microorganisms and cell cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH)

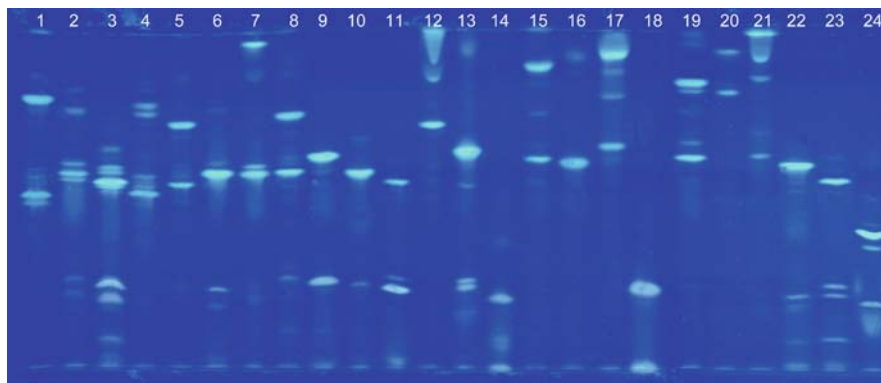


Fig. 1 Isoelectrofocusing patterns of pyoverdines produced by strains W15Aug26 (lane 1), W15Aug23 (lane 2), W15Aug24 (lane 3), W2Dec36A (lane 4), W2Aug1 (lane 5), *P. putida* KT2440 (lane 6), *P. libanensis* LMG 21606^T (lane 7), *P. fluorescens* 5 (lane 8), W15Feb38 (lane 9), W2Aug36 (lane 10), W2Feb31B (lane 11), W15Ap2 (lane 12), *P. fluorescens* Pf0-1 (lane 13), *P. syringae* subsp. *syringae* LMG 1247^T (lane

14), *P. fluorescens* ATCC 17400 (lane 15), W2Dec33 (lane 16), W2Jun14 (lane 17), *P. brassicacearum* LMG 21623^T (lane 18), *P. kilonensis* LMG 21624^T (lane 19), *P. agarici* LMG 2112^T (lane 20), *P. vancouverensis* LMG 20222^T (lane 21), *P. brenneri* LMG 23068^T (lane 22), *P. fluorescens* LMG 14562 (lane 23), *P. entomophila* L48 (lane 24)

Large scale pyoverdine purification

For the pyoverdine growth stimulation experiments a large scale pyoverdine purification was done from previously selected strains (Woluwe isolates and reference strains) producing a specific siderotype (Table 2). Therefore pyoverdine was purified from 42 to 48 h old culture supernatant of the *Pseudomonas* strains grown at 26°C in 5 l Erlenmeyer flasks containing 1 l of iron-poor casamino acids (CAA) medium at 150 rpm. Bacterial cells were removed by centrifugation at 10,000g during 15 min. After filtration the supernatant was passed on a C₋₁₈ column that was activated with methanol and washed with distilled water. Elution was done with acetonitrile/H₂O (70/30%). Most of the acetonitrile was evaporated with a rotavapor and the samples were lyophilized. The pyoverdine was quantified spectrophotometrically by measuring the absorption at 405 nm (Höfte et al. 1993) and adjusted to a

concentration of 8 mM. Aliquots of the pyoverdine were stored at -20°C.

Mass analysis

HRMS-data were obtained with a QT of Micro mass spectrometer (positive ion mode). Under standard measurement conditions the sample was dissolved in CH₃CN/H₂O (1:1) containing 0.1% formic acid. Reserpine was used as lock signal.

Pyoverdine-isoelectrofocusing

Isoelectrofocusing was done according to Koedam et al. (1994). Therefore 1.5 μ l of an 8 mM pyoverdine stock (obtained by large scale pyoverdine purification) was loaded on the IEF gel. The digital photograph presented (Fig. 1) consists out of 2 IEF gels, which were combined with the aid of Adobe Photoshop Elements.

Table 2 List of strains with the type of pyoverdine produced and the monoisotopic mass

Strain pyoverdine isolated from	Dominant monoisotopic mass	Molecular mass (a)	Type	Pyoverdine peptide chain
<i>P. aeruginosa</i> W15Aug26		1,333	Type I	Ser-Arg-Ser-FOHOrn-(Lys-FOHOrn-Thr-Thr) (b)
<i>P. aeruginosa</i> W15Aug23		1,091	Type II	Ser-FOHOrn-Orn-Gly-aThr-Ser-cOHOrn (b)
<i>P. aeruginosa</i> W15Aug24		1,173	Type III	(Ser-Dab)-FOHOrn-Gln-Gln-FOHOrn-Gly (b)
<i>Pseudomonas</i> sp. W2Dec36A	1,188.16	1,189	Type 1	Ser-Lys-Gly-FOHOrn-(Lys-FOHOrn-Ser) (b)
<i>Pseudomonas</i> sp. W2Aug1	1,262.62 + 1,276.64		Type 2	
<i>P. putida</i> KT2440	1,072.45	1,073	Type 3	Asp-Orn-(OHAsp-Dab)-Gly-Ser-cOHOrn (b)
<i>P. libanensis</i> LMG 21606 ^T	1,119.48		Type 5	
<i>P. fluorescens</i> 5	1,286.84	1,287	Type 6	Asp-FOHOrn-Lys-(Thr-Ala-Ala-FOHOrn-Lys) (b)
<i>Pseudomonas</i> sp. W15Feb38	1,045.52	1,046	Type 7	Ser-AOHOrn-Ala-Gly-aThr-Ala-cOHOrn (b)
<i>Pseudomonas</i> sp. W2Aug36	988.44	989	Type 9	eLys-OHAsp-Ala-aThr-Ala-cOHOrn (b)
<i>Pseudomonas</i> sp. W2Feb31B	1,246.58		Type 11	
<i>Pseudomonas</i> sp. W15Apr2	1,462.88		Type 13	
<i>P. fluorescens</i> Pf0-1	1,382.76 + 1,398.85	1,381	Type 14	Ala-AcOHOrn-Orn-Ser-Ser-Ser-Arg-OHAsp-Thr
<i>P. syringae</i> LMG 1247 ^T	1,123.10	1,123	Type 15	eLys-OH Asp-Thr-(Thr-Ser-OH Asp-Ser)
<i>P. fluorescens</i> ATCC 17400	1,300.28	1,299	Type 16	Ala-Lys-Gly-Gly-OHAsp-(Gln-Dab)-Ser-Ala-cOHOrn
<i>Pseudomonas</i> sp. W2Dec33	1,107.66		Type 20	
<i>Pseudomonas</i> sp. W2Jun14	1,159.22		Type 22	
<i>P. brassicacearum</i> LMG 21623 ^T	1,133.45		Type 23	
<i>P. kilonensis</i> LMG 21624 ^T	1,318.67 + 1,333.68	1,318	Type 32	Lys-AOHOrn-Gly-aThr-Thr-Gln-Gly-Ser-cOHOrn (b)
<i>P. agarici</i> LMG 2112 ^T	ND		Type 35	
<i>P. vancoverensis</i> LMG 20222 ^T	1,042.43		Type 36	
<i>P. brenneri</i> LMG 23068 ^T	1,186.50 + 1,202.50	1,187	Type 37	Ser-Dab-Gly-Ser-OHAsp-Ala-Gly-Ala-Gly-cOHOrn (b)
<i>P. fluorescens</i> LMG 14562	1,217.96		Type 39	
<i>P. entomophila</i> L48	1,313.60 + 1,329.60	1,314	Type 41	Ala-Asn-Dab-OHHis-Gly-Gly-Ala-Thr-Ser-cOHOrn

(a) The molecular mass is given based on the masses published in Meyer et al. (2008)

(b) The structure was deduced from the observation that the IEF profile and the mass of the pyoverdine was identical to the relevant reference strain
 ND not determined

Purification and structure determination of pyoverdine of *P. entomophila*

Pyoverdine purified during the medium scale pyoverdine purification was subjected to mass analysis. Mass spectral data were obtained with a MAT 900 ST instrument providing an electrostatic/magnetic analyzer (EB) geometry connected to an octapole collision cell and a quadrupole ion trap (QIT), and equipped with an ESI II ion source (Finnigan MAT, Bremen, Germany); spray voltage 3.4–3.6 kV, capillary temperature 230°C. Source conditions were set to minimize fragmentation, resolution ca. 5,000 (10% valley). The samples were dissolved in water, methanol, and trifluoroacetic acid 50:50:0.1 (v/v). Fragmentation induced by low energy collision activation (CA) was effected in the octapole unit and in the QIT ($\sim 2 \times 10^{-3}$ Pa He as bath gas diffusing in the collision octapole).

Screening for pyoverdine-negative mutants

The plasposon mutagenesis method (Dennis and Zylstra 1998) was used to generate transposon insertions in the chromosome of *P. entomophila* L48 and *P. putida* KT2440. Mid-log phase cultures of *E. coli* SM10 (λ pir), the host of the plasposon pTnmod-OTc, was mixed with strain *P. entomophila* in a 1:1 ratio. *P. entomophila* was kept at 45°C for 20 min just before mixing of both strains in order to inactivate its restriction system. After over night incubation on LB at 26°C, transposon insertions in strain *P. entomophila* were selected on CAA supplemented with 150 $\mu\text{g ml}^{-1}$ tetracycline and 25 $\mu\text{g ml}^{-1}$ chloramphenicol. A bank of 2,000 transconjugants was screened for mutants unable to produce pyoverdine as detected by loss of fluorescence. Pyoverdine-negative mutants were obtained in *P. putida* using the same conditions but the transconjugants were plated on CAA + 100 $\mu\text{g ml}^{-1}$ tetracycline and 25 $\mu\text{g ml}^{-1}$ chloramphenicol. To molecularly characterize the mutants the chromosomal DNA was isolated using the Puregene genomic DNA purification kit (Gentra systems), digested with *Pst*I or *Sal*I (Fermentas) and self-ligated. After transformation in *E. coli* DH5 α , the plasmid was isolated with the Invisorb spin plasmid mini two kit (Invitex—Westburg) and sequenced using primer pTnTc-F (5'-CGATGAGCGCATTGTTAGAT-3') and pTnTc-R (5'-TCCCAGTCACGACGTTGTA-3').

Construction of *pmsCEAB* in-frame deletion mutant of *P. entomophila* L48

For construction of the $\Delta pmsCEAB$ mutant the four genes were deleted in-frame of strain L48. A 1,017-bp fragment, including the first 16-bp of *pmsC* and the neighboring upstream region, was amplified by PCR with primers pmsC-AF-XbI (GTGTCTAGAGCGTACTCGCTCTCATCT) and pmsC-AR-HIII (GTGAA GCTTGAGGTCGACATCAACGATCA). A 784-bp fragment including the last 11 bp of *pmsB* and the adjacent downstream region was amplified by PCR with primers pmsB-BF-XbI (GTGTCTAGAAGAA GGCTGATCGGTAG) and pmsB-BR-EI (GTGGA ATTTCGTCACAACACTGGCTTCGACTT). The resulting upstream and downstream fragments were cut with the restriction enzymes *Hind*III and *Xba*I and with *Xba*I and *Eco*RI, respectively, and cloned by triple ligation into *Eco*RI and *Hind*III-digested pUK21. The 1,807-bp *Eco*RI–*Hind*III insert was checked by sequencing and re-cloned into the suicide plasmid pME3087. The resulting plasmid was then integrated into the chromosome of strain L48 by triparental mating using *E. coli* HB101/pME497 as the mobilizing strain, with selection for tetracycline- and chloramphenicol-resistant recombinants. Excision of the vector by a second crossing-over occurred after enrichment for tetracycline-sensitive cells (Schnider-Keel et al. 2000). The obtained mutant was verified by PCR.

In silico analyses

Gene(s)/operon(s) for pyoverdine and pseudomonine biosynthesis, transport and regulation were found by BLAST homology and by searching the *P. entomophila* L48 and *P. putida* KT2440 genome (<http://www.pseudomonas.com>). The program NRPS predictor (<http://www-ab.informatik.uni-tuebingen.de/toolbox/index.php?view=domainpred>) which uses the methods of Stachelhaus et al. (1999) and Rausch et al. (2005) was used to predict the peptide backbone of the pyoverdine.

Utilization of exogenous pyoverdines by *P. entomophila* L48 and *P. putida* KT2440

Twenty milliliter CAA agar plates containing 0.5 mg ml^{-1} EDDHA were overlaid with 5×10^6 cells of the pyoverdine-negative mutant 1E10 (of *P. entomophila*) or 5A12 (of *P. putida*), and filter-

paper disks impregnated with 6 µl of 8 mM purified pyoverdine were placed on the agar. The plates were incubated at 26°C and scored for the presence of detectable growth of the pyoverdine-negative mutant after 2 days.

Chrome azurol S assay

Siderophore production was detected by the chrome-azurol S (CAS) assay (Schwyn and Neilands 1987). On CAS-agar, siderophores remove iron from CAS, resulting in a blue to yellow–orange color change in zones surrounding the colonies.

EDDHA synthesis

Ethylenediamine bis(2-hydroxyphenyl)acetic acid (EDDHA) was synthesized according to Yunta et al. (2003). The synthesized product was verified by ¹H-NMR.

Results

Genomic, genetic and structural analysis of pyoverdine of *P. entomophila* L48

The pyoverdine of *P. entomophila* was separated into three isoelectrophoresed bands (Fig. 1, lane 24), a

major one characterized by a pHi value of 5.0 and two others, less pronounced in intensity, at pHi of 4.3 and 4.5, respectively. Thus, pyoverdine of *P. entomophila* L48 was classified into the acidic group of pyoverdines. Therefore, to determine its pyoverdine-mediated iron uptake capacity, strain L48 was tested towards its own pyoverdine and also towards the 22 pyoverdines of different bacterial origin already recognized as belonging to that group. None among the 22 heterologous pyoverdines was able to promote efficiently iron incorporation in strain L48 as did pyoverdine of strain L48 (J. -M. Meyer, unpublished results). Therefore, it was concluded that *P. entomophila* was characterized by an original pyoverdine structurally different from each of the more than 60 different compounds presently identified (Meyer et al. 2008).

A search of the complete genome sequence of *P. entomophila* revealed 28 genes involved in pyoverdine-mediated iron acquisition, transport and regulation which are distributed across three different loci of the genome. The results are summarized in Fig. 2. As observed for other *Pseudomonas* species, including *P. aeruginosa* PAO1, *P. putida* KT2440, *P. fluorescens* Pf0-1 and *P. fluorescens* SBW25, the chromophore NRPS gene *pvsA* (PSEEN1815) is located at a separate locus (generally together with 1 enzyme involved of the modification of the peptide chain, and the sigma factor regulator PvdS) while the

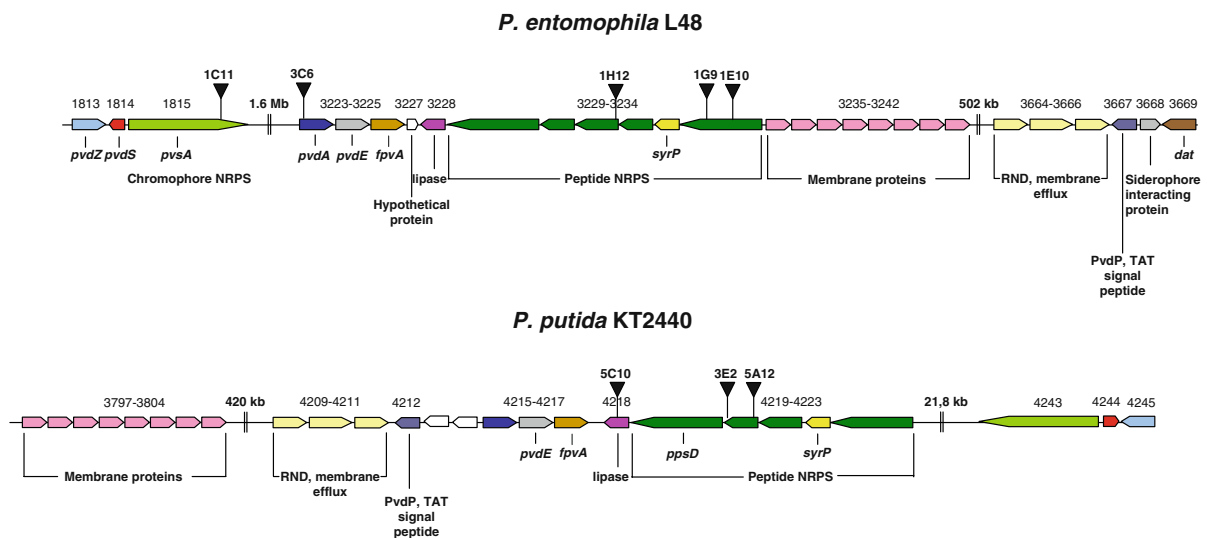


Fig. 2 The organization of the pyoverdine genes in *P. entomophila* L48 and *P. putida* KT2440. Gene names, if given, are shown beneath genes, PSEEN and PP numbers are

shown above. Tn5 insertions are indicated by a triangle. Genes are not drawn to linear scale, and double vertical lines represent intervening DNA with the size shown above

receptor gene *fpvA* and *pvdE* gene are found at the same locus as the peptide chain biosynthesis genes (Moon et al. 2008).

Based on in silico analysis of non-ribosomal peptide synthetases (NRPS) genes PSEEN3229 till PSEEN3232, and PSEEN3234 a ten amino acid long peptide backbone was predicted. To determine the structure the purified pyoverdine was subjected to mass analysis. Assuming that m/z 668.5 corresponds to $[M + H + K]^{++}$ it may lose K^+ and yield m/z 1298 being $[M + H]^+$. Quadrupole CA yields m/z 204 typical for the pyoverdine chromophore and 400 (A1 Suca-Chr-Ala) which loses NH_3/H_2O (383/382) and Suca (301). 542 is B1 (428) plus 114 Da. This can be Asn, Orn or two Gly. Ion trap CA yields the same ions and in addition 795. The mass difference 542–795 corresponds to Dab + OHHis. 795–96 yields 699. This loss is typical for OHHis. Additional ions are 777 (795– H_2O) and 620.5 (668.5–96/2). 795 + 114 corresponds to 909 (again Asn, Orn or 2 Gly). Ions of higher mass (except 1298) are of too low abundance. The sequence Ala–Thr–Ser–cOHOrn would account for the mass difference 909–1,298 (71 + 101 + 87 + 130). Through the combination of mass analysis and in silico analysis, the following ten amino acids long peptide backbone for the pyoverdine of *P. entomophila* could be determined (Suca–Chr)–Ala–Asn–Dab–OHHis–Gly–Gly–Ala–Thr–Ser–cOHOrn where Chr represents the chromophore and Suca succinamide. The abbreviation cOHOrn stands for cyclo-hydroxy-ornithine (3-amino-1-hydroxy-piperidone-2).

Five pyoverdine-negative Tn5 mutants (1C11, 1E10, 1G9, 1H12 and 3C6) were isolated; which showed a complete loss of pyoverdine fluorescence under UV light and were unable to grow on CAA supplemented with the strong iron chelator EDDHA. These mutants were still able to decolorize chrome azurol S, suggesting the production of a second siderophore. HPLC analysis of the culture supernatant showed that for all mutants no pyoverdine was detected anymore while pseudomonine was found at a level comparable to the wild type. The mutants were characterized at the molecular level and were shown to have Tn5 insertions in genes predicted to be involved in pyoverdine synthesis (see Fig. 2) namely in PSEEN3234 (1G9 and 1E10 at genome position 3491208 and 3490460, respectively), in *pvsA*

encoding the chromophore NRPS PSEEN3234 (Mossialos et al. 2002; 1C11 at genome position 1904651) and in *pvdA* coding for a putative L-ornithine N5-oxygenase (PSEEN2323; Ge and Seah 2006; 3C6 at genome position 3450500).

Genomic, genetic and structural analysis of pyoverdine of *P. putida* KT2440

A search of the genome sequence of *P. putida* KT2440 revealed 26 genes with predicted roles in pyoverdine biosynthesis, regulation and transport which are distributed across three different loci on the genome as observed for *P. entomophila*. The results are summarized in Fig. 2.

In silico pyoverdine backbone structural prediction of KT2440 by Ravel and Cornelis (2003) predicted following structure: OHAsp–Lys–OHAsp–Dab–Gly–Ser–hOrn. Comparison of the IEF profile with the reference strains showed that the IEF pattern of KT2440 was identical to the one of *P. putida* G4R. In addition both pyoverdines have the same mass (1,073). Based on these results the pyoverdine of *P. putida* KT2440 has following peptide chain: Asp–Orn–(OHAsp–Dab)–Gly–Ser–cOHOrn which fits closely, but not entirely, the peptide chain predicted from the NRPS genes.

Three Tn5 insertions caused a pyoverdine-negative phenotype, including two in the NRPS gene PP_4220 (3E2 and 5A12 at genome position 4779691 and 4783050, respectively) confirming the role of the gene in pyoverdine biosynthesis. Interestingly, one Tn5 insertion was obtained in gene PP_4218 (5C10 at genome position 4768248), a lipase/esterase family protein lying downstream of the NRPS genes. This enzyme is predicted to act on carboxylic esters. To our knowledge this is the first time that a mutant in this gene has been described. Next to the NRPS genes lies a *syrP* gene in *P. entomophila* and *P. putida* genomes. It has been recently shown that the *syrP* gene is involved in the hydroxylation reaction of the aspartate residue in NRPS encoded syringomycin E and related non ribosomal peptides (Singh et al. 2008). A *syrP* homologue is found in *P. putida* KT2440 (Fig. 2, PP_4222) and in the pyoverdine biosynthetic genes of *P. syringae* and *P. fluorescens* Pf0-1 of which both pyoverdines contain a HO-Asp residue. A homologue of the *syrP* gene is also found

in *P. entomophila* (Fig. 2, PSEEN3233) which does not contain a HO-Aps residue, but a HO-His residue, possibly this gene has in *P. entomophila* a different substrate.

Genomic, genetic and structural analysis of pseudomonine

A CAS-positive fraction was purified from the pyoverdine-negative mutant 1E10 of *P. entomophila* using the same methodology as for the medium scale pyoverdine purification. The EI mass spectrum is directly comparable to the data published in Mercado-Blanco et al. (2001) confirming that the siderophore purified is pseudomonine. The molecular ion is observed at m/z 330, and fragment ions are observed at m/z 82, 95, 110, 121, 127, 138, and 204 (data not shown).

A genome search identified fifteen ORFs which are involved in the synthesis and transport of pseudomonine in *P. entomophila* L48 (Fig. 3). The same gene cluster (except for one ORF) consisting out of highly similar genes with identical organization was present in the pseudomonine producer *P. fluorescens* WCS374 (Fig. 3).

Of these 15 ORFs only *pmsCEAB* has been studied in *P. fluorescens* WCS374 (Mercado-Blanco et al. 2001). It has been shown in that study that the *pmsCEAB* genes are co-transcribed and that the expression is iron-regulated. In addition it was demonstrated that salicylic acid (SA) biosynthesis was encoded by *pmsCEAB*. PmsC is a putative isochorismate synthetase which catalyzes the conversion of chorismate to isochorismate and PmsB is a putative isochorismate-pyruvate lyase which catalyzes the cleavage of isochorismate to give SA and pyruvate. The constructed $\Delta pmsCEAB$ mutant of

P. entomophila showed a complete loss of pseudomonine production as verified by HPLC (data not shown) confirming that the genes are involved in pseudomonine production. Mercado-Blanco et al. (2001) showed that deletions affecting *pmsC* diminished SA production, whereas deletion of *pmsB* resulted in a complete loss.

The third ORF *pmsE* is annotated as a putative 2,3-dihydroxybenzoate-AMP ligase and shows strong similarities with 2,3-dihydroxybenzoate-AMP ligases from different species. This enzyme activates the carboxylate group of 2,3-dihydroxybenzoic acid (2,3-DHB) via an ATP-dependent PPi-exchange reaction. Since SA (2-hydroxybenzoic acid) and not 2,3-dihydroxybenzoic acid is a moiety of pseudomonine it is assumed that the actual substrate of PmsE is not 2,3-DHB but the structurally very similar SA. This would also explain why a putative isochorismate hydrolase, which catalyzes the hydrolysis of isochorismate to 2,3-dihydroxybenzoic acid, the substrate of 2,3-dihydroxybenzoate-AMP ligase, is not present in the operon. In addition, BlastX analysis with *pmsB* of *P. entomophila* showed that *pmsB* was detected in *Pseudomonas* sp. producing the SA containing siderophore pyochelin [PchB of *P. fluorescens* CHA0 (57% identity), *P. fluorescens* 5 (38% identity), *P. aeruginosa* spp. (58% identity)] and *Paracoccus denitrificans*, which also produces a SA containing siderophore, parabactin, but not in the genomes of for example *Acinetobacter baumannii* sp., which produces the 2,3-DHB containing siderophore acinetobactin.

The region also contained a putative pyridoxal-dependent histidine decarboxylase (HDC) gene *pmsA*. HDC catalyzes decarboxylation of histidine to histamine, the second moiety of pseudomonine. Histamine is also a precursor of the siderophore

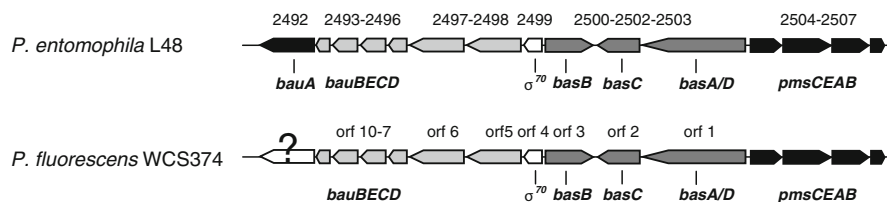


Fig. 3 Comparison of pseudomonine biosynthetic and transport gene clusters of *P. entomophila* L48 and *P. fluorescens* WCS374. Gene names, if given, are shown beneath genes,

gene numbers are shown above. Homologous genes are shown in the same color. Genes are not represented according to linear scale

anguibactin produced by *Vibrio anguillarum*, and it was found that the gene, which shows high similarities to PmsA of *P. entomophila*, was essential for its biosynthesis (Tolmasky et al. 1995).

Thus the *pmsCEAB* genes give probably raise to 2 precursors of pseudomonine, an activated SA, and histamine. Upstream of *pmsCEAB* two NRPS genes, *basB* and *basAD*, were annotated (PSEEN2500 and PSEEN2503, respectively). The conserved amino acid sequences for the cyclization (Cy) and adenylation domain (A) were identified in BasAD. The NRPS predictor predicted the amino acid threonine as the substrate for the A domain which fits together with the Thr in the molecular structure of pseudomonine. In BasB only one condensation (C) domain (C2) and a peptidyl carrier domain were identified. Both NRPS are probably responsible for the adenylation and cyclization of the amino acid threonine (probably by *basAD*) and the incorporation of the activated SA and the histamine giving raise to pseudomonine. A chain-terminating TE domain was not found in the NRPS genes. But probably termination occurs by the transfer of the siderophore chain to histamine to make an amide linkage, catalyzed by a C-terminal condensation domain rather than TE domains. This has been described for anguibactin (cosubstrate amine is histamine) and vibriobactin (cosubstrate amine is norspermidine; Crosa and Walsh 2002).

Between those two NRPS genes a putative L-lysine 6-monooxygenase (NADPH-dependent; *basC*, PSEEN2502) was annotated. The role of BasC is not clear. It is similar to BasC homologues of siderophore biosynthesis gene clusters of *A. baumannii* sp. (68–69% identity), *Vibrio* sp. (58–59% identity) and *A. salmonicida* subsp. *salmonicida* (60% identity). BasC is predicted to be involved in the oxidation of histamine, yielding N-hydroxyhistamine, one of the constituents of acinetobactin. Similarly AngU a putative monooxygenase, is also predicted to be involved in N-hydroxyhistamine production of anguibactin (Crosa and Walsh 2002). But in contrast to acinetobactin and anguibactin histamine and not N-hydroxyhistamine is a constituent of pseudomonine (Fig. 4). Therefore BasC must have a different function in *P. entomophila*.

Upstream of the biosynthesis genes putative transport genes *bauBCDE* were annotated (PSEEN 2497 and PSEEN2498).

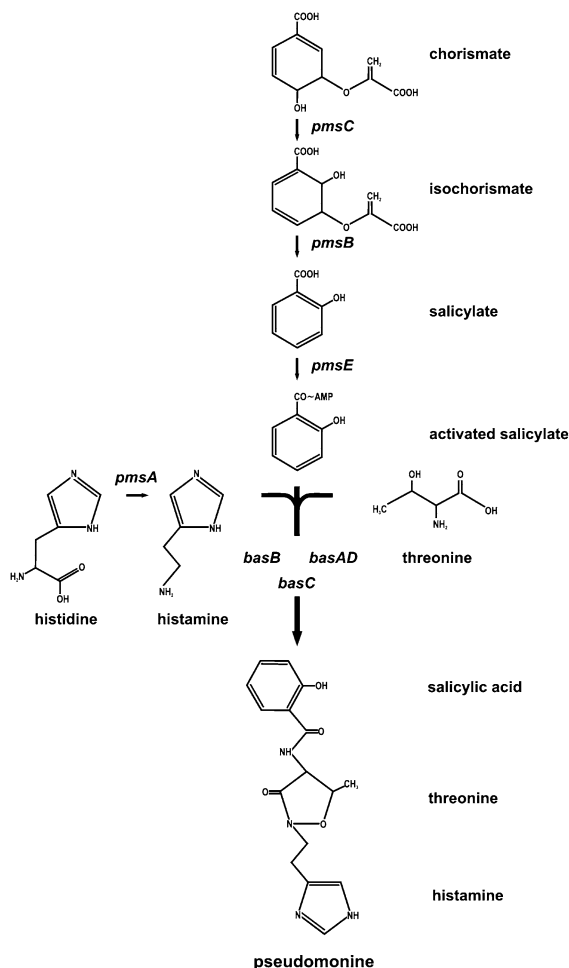


Fig. 4 The proposed pathway for the biosynthesis of pseudomonine

Utilization of exogenous pyoverdines by *P. entomophila* and *P. putida* KT2440

To estimate the diversity of exogenous pyoverdines that may be utilized by *P. entomophila* and *P. putida*, pyoverdines were purified from 24 different *Pseudomonas* strains and isolates, which had been shown to each produce a unique siderotype profile (Fig. 1; Table 2). These strains represented 11 environmental isolates collected from the Woluwe River (Brussels, Belgium), 7 type strains, and 6 strains from different origins. The pyoverdine produced by *P. fluorescens* 5, 6 Woluwe and 2 type strains (*P. brenneri* LMG 23068^T and *P. kilonensis* LMG 21624^T) could be identified based on an identical mass and IEF profile as the relevant reference strain (Table 2).

Table 3 Growth stimulation of pyoverdine-negative mutant of *P. entomophila* L48 and *P. putida* KT2440 with 24 different pyoverdines

Pyoverdine	Pyoverdine type	Growth stimulation of pyoverdine-negative mutant 1C11 of <i>P. entomophila</i>	Growth stimulation of pyoverdine-negative mutant 5C10 of <i>P. putida</i> KT2440)
W15Aug26	Type I	+	–
W15Aug23	Type II	+	–
W15Aug23	Type III	+	–
W2Dec36A	Type 1	+	–
W2Aug1	Type 2	+	–
<i>P. putida</i> KT2440	Type 3	–	+
<i>P. libanensis</i> LMG 21606 ^T	Type 5	–	–
<i>P. fluorescens</i> 5	Type 6	+	–
W15Feb38	Type 7	+	–
W2Aug36	Type 9	+	±
W2Feb31B	Type 11	–	–
W15Ap2	Type 13	+	–
<i>P. fluorescens</i> Pf0-1	Type 14	–	–
<i>P. syringae</i> subsp. <i>syringae</i> LMG 1247 ^T	Type 15	–	+
<i>P. fluorescens</i> 17400	Type 16	–	–
W2Dec33	Type 20	+	–
W2Jun14	Type 22	+	–
<i>P. brassicacearum</i> LMG 21623 ^T	Type 23	–	–
<i>P. kilonensis</i> LMG 21624 ^T	Type 32	+	–
<i>P. agarici</i> LMG 2112 ^T	Type 35	+	±
<i>P. vancouverensis</i> LMG 20222 ^T	Type 36	+	–
<i>P. brenneri</i> LMG 23068 ^T	Type 37	+	–
<i>P. fluorescens</i> LMG 14562	Type 39	–	–
<i>P. entomophila</i> L48	Type 41	+	–

The pyoverdines were supplied to a pyoverdine-deficient mutant strain (1E10) of *P. entomophila* that had been overlaid as a dilute suspension onto CAA medium containing EDDHA. Growth was clearly restored by provision of some of the purified pyoverdines, including the cognate pyoverdine (type 41; Tables 2, 3) and the three *P. aeruginosa* types (type I, II and III; Table 3) of *P. aeruginosa* isolates from the Woluwe (Pirnay et al. 2005). The pyoverdine of eight strains (*P. putida* KT2440, *P. libanensis* LMG 21606^T, W2Feb31B, *P. fluorescens* Pf0-1, *P. syringae* subsp. *syringae* LMG 1247^T, *P. fluorescens* ATCC 17400, *P. brassicacearum* LMG 21623^T and *P. fluorescens* LMG 14562) led only to a very weak but reproducible growth stimulation. This growth stimulation was considered as background

and recorded as negative. Thus, it is apparent that *P. entomophila* is able to utilize a very large range (16 out of 24 pyoverdines) of exogenous pyoverdines produced by other pseudomonads.

Similarly, a growth stimulation assay was carried out with a pyoverdine-negative mutant 5A12 of *P. putida* KT2440. In contrast to *P. entomophila*, growth of a pyoverdine-negative *P. putida* KT2440 mutant was only well stimulated by its cognate pyoverdine and the pyoverdine of *P. syringae* subsp. *syringae* LMG 1247^T. Surprisingly these two pyoverdines do not show a large overlap in structure. Pyoverdine of isolate W2Aug36 and *P. agarici* LMG 2112^T were only able to stimulate growth very weakly. For all the other pyoverdines no growth stimulation was visible. It was observed that the

pyoverdine-negative mutant of *P. putida* KT2440 does not grow well in iron limiting conditions and that the strain is sensitive for iron stress while giving a very weak or negative CAS reaction (S. Matthijs, unpublished results). To overcome this iron stress sensitivity the assay with the pyoverdine-negative mutant of *P. putida* was repeated on a lower concentration of EDDHA (0.25 mg ml^{-1}) and for the overlay a 5 times higher number of cells was overlaid on the CAA + EDDHA plate, but with no significant effect on the outcome.

Despite the relatedness between both species, we can conclude that there is no overlap between the pyoverdines which are able to stimulate their respective growth. These two strains were also not able to utilize each other cognate pyoverdine. This observation is supported by the genome searches which did not identify a receptor with significant similarity to the *fpvA* receptor (PSEEN2325) of *P. entomophila* in *P. putida* KT2440 and vice versa.

Discussion

Pseudomonas entomophila L48 is an entomopathogenic bacterium which kills upon ingestion *Drosophila melanogaster* as well as insects from different orders (Vodovar et al. 2006). A genomic search revealed the putative pyoverdine biosynthesis, transport and regulation gene clusters, consisting of in total 28 genes which are distributed across three different loci. Inactivation of L-ornithine N5-oxygenase and two NRPS genes, all predicted to be involved in pyoverdine biosynthesis, by Tn5 mutagenesis resulted in complete loss of pyoverdine production confirming their predicted role. In silico and structural analysis of the pyoverdine showed that *P. entomophila* produces a structurally new pyoverdine with a peptide backbone of ten amino acids. Comparison of the IEF profile of this pyoverdine with the siderotypes of a diverse collection of 450 *Pseudomonas* strains (S. Matthijs, unpublished results) showed that this siderotype is unique, and has only been observed in *P. entomophila* L48.

In addition to pyoverdine, the secondary siderophore pseudomonine, which has been previously identified in *P. fluorescens* AH2 isolated from spoiled Nile perch from Lake Victoria (Africa) (Anthoni et al. 1995) and the plant growth-promoting

rhizobacterium *P. fluorescens* WCS374 (Mercado-Blanco et al. 2001), was identified in the supernatant of a pyoverdine-negative *P. entomophila* mutant. Construction of a *pmsCEAB* mutant in strain L48 resulted in a loss of pseudomonine production confirming the role of the genes in pseudomonine biosynthesis. Since at the time that pseudomonine was identified only the *pmsCEAB* genes were sequenced and analysed, the genome was searched for other pseudomonine biosynthesis genes surrounding *pmsCEAB* and 15 ORFs were identified in total. A putative role could be assigned to all the ORFs, except for *basC*. Pseudomonine is assembled from a salicylic acid moiety, which is synthesised by the gene products of *pmsCB* and activated by *pmsE*. The second constituent, histamine, is synthesized by the gene product of *pmsA*. After the condensation of threonine to SA, histamine is condensed to and the pseudomonine is released. A putative extracytoplasmic sigma factor (ECF σ) and transport genes could also be identified.

The pyoverdine of *P. putida* KT2440 was also identified. It is identical to the pyoverdine produced by another *P. putida* strain G4R. In contrast to the pyoverdine of *P. entomophila*, it is not unique and has been found in several *Pseudomonas* species in our collection (S. Matthijs, unpublished results). The pyoverdine clusters have also been identified and Tn5 mutants confirmed the role of two genes in pyoverdine biosynthesis. If an alternative iron uptake system to pyoverdine exists at all, its siderophore should have a rather low affinity for iron since only a very weak CAS decolouration activity could be observed.

Heterologous pyoverdine growth stimulation assays demonstrate that *P. entomophila*, in contrast to *P. putida* is able to utilize a high variety of structurally distinct pyoverdines produced by other *Pseudomonas* species. Based on this study it is clear that *P. entomophila* has the tools to compete efficiently for ferric iron. It would be interesting to verify the importance of iron stress during the infection of *Drosophila melanogaster*.

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