BioMetals **15:** 1–13, 2002.

Unusual traits of the pyoverdin-mediated iron acquisition system in *Pseudomonas putida* strain BTP1

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Received 14 February 2001; accepted 14 May 2001

Key words: affinity for iron, outer membrane receptor, *Pseudomonas putida*, siderophore

Abstract

Fluorescent *Pseudomonas* species are characterized by the production of pyoverdin-type siderophores for Fe^{3+} acquisition in iron-limited environments. Since it produces a structurally specific pyoverdin, *Pseudomonas putida* strain BTP1 could represent a valuable tool in an attempt to correlate the structural features of these compounds with some specificity in their two main properties *i.e.* affinity for iron and recognition rate by other *Pseudomonas* strains. An uncommonly high affinity for iron of the pyoverdin synthetized by *P. putida* BTP1 was observed by comparing both the apparent stability constant and the decomplexation kinetic of its ferric complex with those of ferripyoverdins from other strains. On another hand, results from growth stimulation experiments and labeled ferripyoverdin uptake assays highlighted the very low recognition rate of BTP1 isopyoverdins by membrane receptors of foreign strains. By contrast, *P. putida* BTP1 was able to utilize a broad spectrum of structurally unrelated exogenous pyoverdins by means of multiple receptors that are likely constitutively expressed in its outer membrane. The unusual traits of its pyoverdin-mediated iron acquisition system should contribute to enlarge the ecological competence of *Pseudomonas putida* BTP1 in terms of colonization and persistence in the rhizosphere.

Abbreviations: CAA – Casamino acids medium; EDDHA – ethylenediaminedihydroxyphenylacetic acid; IROMP – iron-regulated outer-membrane protein; K_s – apparent stability constant of [Fe^{3+}-pyoverdin] complexes; Ps – *Pseudomonas*; (i)PVD – (iso)pyoverdin.

Introduction

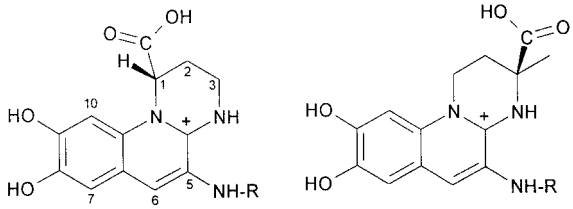
To ensure their growth in iron-limited environments, microorganisms have evolved powerful Fe^{3+}-acquisition systems based on the excretion of high-affinity iron-chelating molecules termed siderophores (Neilands 1981). Pyoverdins (or pseudobactins) are the major iron chelators produced by fluorescent species of the genus *Pseudomonas* although some strains are known to produce additional low-affinity siderophores such as pyochelin (Cox *et al.* 1981), its precursor salicylic acid (Meyer *et al.* 1992; Visca *et al.* 1993) or quinolobactin (Mossialos *et al.* 2000). Specific outer membrane receptors are concomitantly expressed under iron-limitation and facilitate the transport of iron(III)-pyoverdin complexes into the cells (Neilands 1982).

Pyoverdins from different fluorescent *Pseudomonas* strains share the same general structure: a conserved quinoline-derived chromophore (see Figure 1a) linked to a peptide chain containing two hydroxylated amino acid residues involved in Fe^{3+} coordination together with the catechol group of the chromophore (Budzikiewicz 1993). Various dicarboxylic acids or their amide forms can be attached to the 5-amino group of the quinoline ring and so several pyoverdines can be isolated after growth of one specific bacterial strain in low-iron synthetic media (Budzikiewicz

1993). In contrast, almost strain-specific variations are observed in the length and composition of the peptide part in pyoverdins (PVD) isolated so far (Kilz *et al.* 1999). Because of this structural diversity most of the fluorescent *Pseudomonas* can metabolize iron only via a very limited number of compounds produced by other strains (Bakker *et al.* 1990). Some *Pseudomonas* isolates were able to incorporate iron via so-called heterologous pyoverdins but in many cases these exogenous PVD were identical to those synthesized by the tested bacteria (Meyer *et al.* 1997; Koster *et al.* 1993). However, through the study of strains characterized by their capacity to accept a wide range of structurally unrelated pyoverdins, it was evidenced that additional specific outer membrane receptors are inducible in the presence of the corresponding heterologous siderophores (Morris *et al.* 1992; Gensberg *et al.* 1992; Koster *et al.* 1993).

In the case of soilborne strains, the high affinity for Fe^{3+} together with the high specificity of membrane receptors contribute to enlarge the nutritional role of pyoverdin-mediated iron-acquisition system to an improved ecological competence. By conferring a competitive advantage for the limited supply of essential iron traces, some root colonizing pseudomonads can also be involved in suppression of fungal soilborne plant diseases (Neilands & Leong 1986; Loper & Buyer 1991). In this context of biological control, some pyoverdins were also occasionally demonstrated to act as elicitors of systemic resistance developed by the host plant toward pathogens (Maurhofer *et al.* 1994; Leeman *et al.* 1996).

Beside its plant resistance inducing ability (Ongena *et al.* 2000), *Pseudomonas putida* strain BTP1 is also characterized by the particular structure of its pyoverdin-type siderophores that in addition to a unique peptide moiety, display a modified position of the chromophore carboxyl group on C-3 instead of C-1 (Figure 1b) as commonly observed (Jacques *et al.* 1995). So the first objective of the present study was to evaluate the influence of such structural features in BTP1 isopyoverdins on the main properties of these compounds, i.e., affinity for iron and recognition rate by foreign strains. Secondly we wanted to evaluate whether this specificity could be extended to the behavior of this strain regarding the range of heterologous ferripyoverdins that it can transport to supply with Fe^{3+}. These last results are discussed in light of the multiplicity of putative receptors estimated by analysis of outer membranes and genetic studies.



a **b**

Fig. 1. Chromophores of common pyoverdins (**a**) and of *P. putida* BTP1 isopyoverdin (**b**) as described by Jacques *et al.* (1995). The peptide moiety is bound to the carboxyl group. R, dicarboxylic acid side chain.

Materials and methods

Bacterial strains, growth condition and pyoverdin purification

Fluorescent *Pseudomonas* isolates of the BTPx-type were isolated from rhizospheres as previously mentioned (Jacques *et al.* 1995). The pyoverdin-deficient mutant M3 was obtained and characterized as described in Ongena *et al.* (1999). Other *Pseudomonas* strains (see Table 1) were kindly provided by Prof. H. Budzikiewicz from the University of Cologne. Bacterial isolates were maintained on King's B medium (King *et al.* 1954) at 4 °C before experimental use, and stored at −20 °C in glycerol 50% for long-term storage. For siderophore production, bacteria were grown in a 20 L-fermentor with pH maintained at 7 ± 0.5 using the semi-synthetic iron-restricted Casamino acids medium (CAA, Casamino acids (Difco) 5 g l^{-1}, $MgSO_4$ 0.25 g l^{-1}, K_2HPO_4 0.9 g l^{-1}). Methods for culturing, for determination of pyoverdin (PVD) or isopyoverdin (iPVD) concentrations and for ferric PVD and iPVD purification are detailed in Jacques *et al.* (1995). This allowed isolation of the various native or degradative forms produced by each strain and varying in their chromophore acid side chain (Budzikiewicz 1993). Unless specified, the succinic amide forms were used for growth stimulation and Fe^{3+}-PVD uptake experiments except for $iPVD_{BTP1}$ which was isolated in its glutamic acid form. The other pyoverdins used in this study and described in Table 1 were provided as pure compounds by Prof. H. Budzikiewicz.

Formation constant determination

Apparent stability constants of pure ferripyoverdin complexes were determined using EDTA as compet-

Table 1. List of pyoverdins and corresponding fluorescent *Pseudomonas* strains used in this study.

Name[a]	Peptide sequence[b]	Cycle[c]	Reference
$PVD_{C-E\ 15692}$ (ae)	Ser-Arg-Ser-Fo(OH)Orn-Lys-Fo(OH)Orn-Thr-Thr	Lys^5-Thr^8	1
$PVD_{PpC2,3}$ (p)	Asp-Bu(OH)Orn-Dab-Thr-Gly-Ser-Ser-(OH)Asp-Thr	/	2
$PVD_{Pf\ 12}$ (f)	Ser-Lys-Gly-Fo(OH)Orn-Ser-Ser-Gly-Lys-Fo(OH)Orn-Glu-Ser	Lys^8-Ser^{11}	3
$PVD_{Pp\ 1,2}$ (p)	Ser-Thr-Ser-Orn-(OH)Asp-Gln-cDab-Ser-aThr-c(OH)Orn	/	4
Azoverdin (A.m)	HSe-HSe-cDab-Ac(OH)Orn-Ser-Ac(OH)Orn[d]	/	5
PVD_{GM} (f)	Ala-Lys-Gly-Gly-(OH)Asp-Gln-Ser-Ala-Ala-Ala-Ala-c(OH)Orn	/	6
PVD_{I-III} (f)	Asn-Fo(OH)Orn-Lys-Thr-Ala-Ala-Fo(OH)Orn-Lys	Thr^4-Lys^8	7
PVD_{B10} (f)	Lys-(OH)Asp-Ala-aThr-Ala-c(OH)Orn	/	8,9
PVD_{R} (ae)	Ser-cDab-Fo(OH)Orn-Gln-Gln-Fo(OH)Orn-Gly	/	10
$PVD_{Pf\ 17400}$ (f)	Ala-Lys-Gly-Gly-(OH)Asp-Gln-cDab-Ser-Ala-c(OH)Orn	/	11
$iPVD_{BTP1}$ (p)	Asp-Ala-Asp-Ac(OH)Orn-Ser-c(OH)Orn[d]	/	12
PVD_{BTP2} (f)	Ser-Val-(OH)Asp-Gly-Ser-Thr-c(OH)Orn	/	13
PVD_{BTP7} (f)	Ser-Ser-Fo(OH)Orn-Ser-Ser-Lys-Fo(OH)Orn-Lys-Ser	Lys^6-Ser^9	14
PVD_{BTP9} (f)	5 Ser, 2 Lys, 2 Fo(OH)Orn	?	13
PVD_{BTP16} (p)	Asp-Bu(OH)Orn-Dab-(a)Thr-Gly-(L,D)Ser-(L,D)Ser-(OH)Asp-(a)Thr	/	14
PVD_{BTP14} (p)	Thr, aThr, 2 Ser, Gly, Asx, Glx, Dab, Orn	?	13

[a]PVD, pyoverdin. Indices referred to the name of the producing strain, the species is indicated in brakets with *ae, p, f* and *A.m.* for *aeruginosa, putida, fluorescens* and *Azomonas macrocytogenes* respectively. [b]Abbreviations used for uncommon amino acids: (OH)Orn, N^{δ}-hydroxy Ornithine; Ac(OH)Orn, N^{δ}-acetyl(OH)Orn; Fo(OH)Orn, N^{δ}-formyl(OH)Orn; Bu(OH)Orn, N^{δ}-β-hydroxybutyryl(OH)Orn; c(OH)Orn, cyclo-(OH)Orn (3-amino-1-hydroxy-piperidone-2); (OH)Asp, threo-β-hydroxy aspartic acid; aThr, allo-Threonine; cDab, condensation product of the γ-NH2 group of diaminobutyric acid with the amide carbonyl group of the preceding amino acid giving a tetrahydropyrimidine ring. [c]Large internal ring formed by an amide or ester bond between two residues. Numbers referred to the position of the residue in the peptide chain starting from the N-terminus (left). [d]compounds with isopyoverdin-type chromophore. References: 1, Demange *et al.* 1990a; 2, Seinsche *et al.*1993; 3, Geisen *et al.* 1992; 4, Gwose *et al.* 1992; 5, Michalke *et al.* 1996, 6, Mohn *et al.* 1990; 7, Poppe *et al.* 1987; 8, Demange *et al.* 1986; 9, Kiltz *et al.* 1999; 10, Gipp *et al.* 1991; 11, Demange *et al.* 1990b; 12, Jacques *et al.* 1995; 13, Ongena *et al.* submitted paper; 14, Ongena *et al.* 1998.

itive Fe^{3+} binding species as previously described (Meyer & Abdallah 1978). Ferripyoverdins (50 μM, $\varepsilon_{450nm} = 9600\ M^{-1}\ cm^{-1}$) were mixed with various EDTA concentrations (5, 10, 25, 50, 100 mM) in either sodium acetate (pH 4 and 5) or phosphate (pH 6 and 7) buffers and the absorbance at 450 nm was recorded after equilibration for 8 h.

Growth stimulation tests

The effect of ferripyoverdins on growth of the different *Pseudomonas* strains was tested on plates using agar-solidified (bacto agar, 12 g l^{-1}) CAA medium supplemented with 500 mg l^{-1} ethylenediaminedihydroxyphenylacetic acid (EDDHA) to ensure complexation of contaminating iron and therefore to inhibit bacterial growth unless it can use the added siderophore (Meyer *et al.* 1997). 100 μl of pure iron-complexed pyoverdins (1.5 mM, filter sterilized) were dispensed in a well made in the centre of the plates and allowed to diffuse in the gelified medium for 3 h. Plates were then homogeneously inoculated with 100 μl of a 6×10^8 cfu ml^{-1}-cell suspension from the strain to be tested. To obtain these suspensions, bacterial colonies were scrapped from 24 h-old King's B plates (King *et al.* 1954), suspended in 5 ml of sterile CAA cultures and adjusted to the desired cell concentration with sterile peptone water. Plates were incubated at 30 °C (for *P. putida* and *P. fluorescens* isolates) or 37 °C (in the case of *P. aeruginosa* strains) and scored after 10 h and 20 h. Growth stimulation was rated as follows: −, no stimulation; +, slight stimulation (light growth in the zone of diffusion of added ferripyoverdins) and ++, strong stimulation (thick growth in the diffusion zone equivalent to that observed for the homologous or producing strain).

The same principle was applied for stimulation tests in liquid CAA medium which was supplemented with 1.5 g l^{-1} EDDHA. Five microliters of the ferripyoverdin solutions were added to 5 ml-culture tubes subsequently inoculated with 20 μl of the bacterial suspension prepared as described above. Tubes were incubated under agitation at 30 °C or 37 °C depend-

ing on the strain. Bacterial development was measured after 5 h by O.D. at 540 nm.

Uptake of ^{59}Fe pyoverdins

The capacity of *Pseudomonas* strains to utilize exogenous labeled ferric pyoverdins was tested in 30 min kinetics following a method previously described by Cornélis *et al.* (1989) with minor changes. Cells were grown in 50 ml of CAA medium to an A_{540nm} value comprised between 1 and 1.2. Cells were harvested by centrifugation and resuspended in ten times-diluted CAA medium to a final A_{540nm} of 0.3. 9-ml aliquots were transferred in 100-ml flasks and incubated at 30 °C for 10 min before addition of 1 ml of the labeling mixture (5 μl of a $^{59}FeCl_3$ solution at 1.65 mCi ml^{-1}, 10 μl of a 1 mM solution of pyoverdin, and 985 μl of ten times-diluted CAA). Samples of 1 ml were taken at 0, 2, 5, 10, 20, and 30 min, filtered on 0.45 μm membranes and washed twice with 3 ml of NaCl 0.9%. Filters were dried for 16 h at 55 °C and radioactivity was measured with a Beckman LS 5000 CE-type counter.

Preparation of outer membranes

Outer membranes were prepared by the Sarkosyl solubilization method of Filip *et al.* (1973) modified as follows. The washed bacterial pellet from a 500-ml culture in CAA medium or in CAA supplemented with 100 μM $FeCl_3$ was suspended in 10 ml of the extraction buffer 5 mM Tris/HCl, 5 mM DL-dithiothreitol (DTT), 1 mM EDTA, pH 7.8. Cells were disrupted by the use of a French press (7000 Psi) and PMSF (phenylmethylsulfonyl fluoride) was added to reach a 1mM-final concentration. Unbroken cells were removed by centrifugation at 8,900 *g* for 10 min. Sarkosyl (N-laurylsarcosinate sodium salt) was added to the supernatant at a final concentration of 2% (w/v). Cell membranes were harvested by centrifugation at 48,200 *g* for 1 h, resuspended in 10 mM phosphate buffer pH 7.2 containing 10 mM EDTA and 1mM PMSF and sonicated in an ice bath with 5 × 30 s pulses. After incubation for 20 min at room temperature, the mixture was centrifuged at 48,200 *g* for 2 h. Outer membranes were resuspended in 200 μl of 40 mM phosphate buffer pH 7 containing 1% glycerol and 1 mM PMSF and stored at −20 °C before use. The protein content was determined by the method of Lowry *et al.* (1951). 15 μl of the outer membrane suspension (4.5 mg protein ml^{-1}) was mixed with an equal volume of denaturing buffer (80 mM Tris/HCl, 2% SDS, 0.0025% bromophenol blue, 0.1 M DTT, and 1% glycerol) and boiled at 100 °C for 2 min prior to electrophoresis. Electrophoresis was performed on 8–18% gradient Excel gel SDS using a Multiphor II apparatus and following the procedure described by the manufacturer (Pharmacia/LKB). Gels were stained with Coomassie Brilliant blue R-250 0.1% in methanol/water/acetic acid 45:46:9, v/v/v).

For analyses of IROMPs in bacteria grown in the presence of exogenous pyoverdins, the later were added in their ferric form to the CAA medium at a final concentration of 20 μM. Cells were harvested by centrifugation when cultures reached an optical density value at 600 nm of 1 (5×10^8 cfu ml^{-1}) and were treated as described above for outer membrane preparation.

Detection of receptor genes

The chromosomal DNA was isolated from bacteria grown in 200 ml CAA medium. Cells harvested by centrifugation (approx. 2×10^{11}) were suspended in 15 ml 50mM Tris/HCl, 10 mM EDTA, 100 μg ml^{-1} RNase A buffer, pH 7.5. This suspension was mixed with an equal volume of 0.2 M NaOH, 1% SDS for cell lysis during 20 min. 15 ml of 1.32 M potassium acetate buffer, pH 4.8 were then added. The resulting mixture was gently shaked and centrifuged at 14,000 *g* for 15 min. A half-volume of isopropanol was added to the supernatant previously filtered through a Whatman no. 1 filter paper and the mixture was centrifuged again under the same conditions. The ADN-containing pellet was resuspended in 2 ml 10 mM Tris/HCl, 1 mM EDTA buffer pH 7.5.

The primers used in the PCR are identical to those used by Koster *et al.* (1995). Each PCR reaction was performed with the following ingredients in a total volume of 50 ml buffer supplied by the manufacturer of the polymerase (Eurogentec SA, Liège, Belgium): 2 mM $MgCl_2$, 0.1 μg chromosomal DNA, 2 μg of primers, 50 μM each of dATP, dGTP, dCTP and dTTP, and 3.7×10^5 Bq of [α-^{35}S] dATP, 0.8 U of Goldstar polymerase. The PCR parameters were: 4 min at 95 °C followed by 35 cycles of denaturation for 1 min by 94 °C, primer annealing for 40 s by 42 °C and elongation for 1.5 min by 72 °C or for 7 min in the last cycle. The radioactive PCR products were separated by electrophoresis on 6% acrylamide gels with a Flowgene apparatus. Autoradiography of the dried gels (80 °C under vacuum for 1 h) was performed with an phosphorImager apparatus (Molecular Dynamics).

Results and discussion

Specificity of iPVD$_{BTP1}$ regarding the stability constant of its ferric complex

Affinity for iron of pyoverdin-type siderophores is usually estimated from the apparent stability constants of their Fe(III)-complexes. Such complexing constants (K_s) are measured by the addition of EDTA, an auxiliary competiting species, to a ferripyoverdin solution. Iron is displaced from the complex leading to decrease in A_{450nm} since both iron-free pyoverdins and Fe^{3+}-EDTA did not absorb significantly at this wavelength. As the formation constant of the Fe^{3+}-EDTA complex at various pH values was known, the formation constant of the Fe^{3+}-pyoverdin complexes could be calculated as described (Meyer & Abdallah 1978). As previously reported for other pyoverdins (Meyer & Abdallah 1978; Torres *et al.* 1986; Persmark *et al.* 1990), K_s of Fe^{3+}-iPVD$_{BTP1}$ varied greatly with pH. Only values obtained at pH 5 and pH 7 are mentioned in Table 2 together with those calculated for other fluorescent siderophores. In general, K_s values obtained for other pyoverdins under our experimental conditions are closely similar to the one mentioned in the literature allowing a reliable comparison of this parameter from one molecule to another. The mean log K_s measured for Fe^{3+}-iPVD$_{BTP1}$ at pH 5 and pH 7 were the highest values so far mentioned.

Furthermore, decomplexation kinetic studies at pH 5 revealed that Fe^{3+} is more slowly released from ferric BTP1 isopyoverdin complexes before reaching the equilibrium at which K_s are measured. As an example, Figure 2 shows the decrease of A_{450nm} due to iron competition with EDTA 25 mM observed for iPVD$_{BTP1}$ compared with four other pyoverdins. These curves are from one representative experiment but mean values of initial decomplexation rates (Dr_i) were calculated from four repetitions: $Dr_i = -0.4 \pm 0.05\ 10^{-2}$ AU (absorbance units) min^{-1} for iPVD$_{BTP1}$, $Dr_i = -6 \pm 0.75\ 10^{-2}$ AU min^{-1} for PVD$_{BTP2}$, $Dr_i = -8 \pm 0.7\ 10^{-2}$ AU min^{-1} for PVD$_{BTP7}$, $Dr_i = -2.3 \pm 0.28\ 10^{-2}$ AU min^{-1} for PVD$_{BTP16}$ and $Dr_i = -10.1 \pm 1.5\ 10^{-2}$ AU min^{-1} for PVD$_{C2,3}$.

Such observations attest for an uncommonly high affinity for iron of the siderophore synthetized by *P. putida* BTP1 compared to other pyoverdins. Albrecht-Gary and collaborators (1994) proposed a step by step mechanism for the dissociation (and formation) of ferric pyoverdin PaA complexes in acidic conditions that is initiated by the rapid loss of the hydroxyquinoline-type bidendate coordination site. This is followed by defolding of the anchored structure around the Fe^{3+} ion with successive dissociation of middle chain and C-terminal hydroxamate binding sites. The final dissociation step involving the terminal hydroxamate coordination site is considered to be rate limiting. Based on this model, the structural change observed in the quinoline moiety of iPVD$_{BTP1}$ (Figure 1) could induce some modifications in the tridimensional structure of the entire molecule resulting in increased stability of the anchored ferric form of the BTP1 isopyoverdin. By favoring accessibility of the ornithine binding sites into the peptide chain, a somewhat altered flexibility of the molecule could influence the dissociation rate by acting on the two slower steps of the process.

Utilization rate of iPVD$_{BTP1}$ by other Pseudomonas *strains*

Several foreign *Pseudomonas* strains were tested for their ability to utilize iPVD$_{BTP1}$ by reversal of growth restriction caused by the iron-complexing agent EDDHA on both solid medium and in liquid cultures. With the exception of the producing BTP1 strain and of its pyoverdin deficient mutant M3, only strains *Pseudomonas putida* Pp C2,3 and *Pseudomonas fluorescens* GM could incorporate iPVD$_{BTP1}$ as deduced from the growth stimulation zones observed in the diffusion area of ferric siderophore (Figure 3a). Growth enhancement of strain Pp C2,3 observed after 10 h of incubation was to the same level as for the homologous strain BTP1. However, iron was incorporated via iPVD$_{BTP1}$ into GM cells with a reduced efficiency leading to only slightly visible bacterial growth after the same 10-h incubation time (Figure 3a). When plates were checked after 24 h, growth stimulation was evident for both four isolates but no significant cell development was visible with the other strains tested (data not shown). These observations were in agreement with results from liquid cultures where cell density increases only occurred with strain BTP1, its siderophore deficient M3 and strain PpC2,3 during the first 6 h of incubation in the presence of iPVD$_{BTP1}$ (Figure 3b). In these experiments, growth of strain GM was not markedly induced compared to other non-stimulated strains in the same conditions.

Due to possible iron displacement from the added heterologous siderophore by wild-type pyoverdins, growth-stimulation experiments can lead to misinter-

Table 2. Apparent stability contants determined for ferric $iPVD_{BTP1}$ and for other Fe^{3+}-pyoverdin complexes. Values in parentheses are those reported in the specified references.

Pyoverdin	Log K_{pH5}	Log K_{pH7}	Reference
PVD_{BTP1}	21.26 ± 0.24	27.14 ± 0.1	This work
PVD_{BTP2}	20.34 ± 0.22	26.18 ± 0.013	This work
PVD_{BTP16}	21.15 ± 0.17	26.42 ± 0.05	This work
PVD_{BTP7}	20.40 ± 0.14	25.95 ± 0.08	This work
$PVD_{PpC2,3}$	21.02 ± 0.07 (18.45)	25.54 ± 0.09 (25.45)	1
$PVD_{C-E15692}$	19.76 ± 0.07 (19.81)	/	2
$PVD_{Pf\ 12}$	20.02 ± 0.12 (19.76)	26.84 ± 0.05 (27)	3
$PVD_{Pp\ 1,2}$	19.74 ± 0.1 (19.76)	26.53 ± 0.08 (26.49)	4
PVD_{R}	20.93 ± 0.11 (21.13)	/	5
PVD_{GM}	(19.25)	(26.3)	6
PVD_{589A}	(19.49)	(25.65)	7
PVD_{PSS}		(26.04)	8
PVD_{Pf}	(17.44)	(24.26)	9
PVD_{Ps}	(19.54)	(26.5)	10

1, Seinsche *et al.* 1993; 2, Demange *et al.* 1990b; 3, Geisen *et al.* 1992; 4, Gwose *et al.* 1992; 5, Gipp *et al.* 1991; 6, Mohn *et al.* 1990; 7, Persmark *et al.* 1990; 8, Cody & Gross 1987; 9, Meyer & Abdallah 1978; 10, Torres *et al.* 1986.

pretations in determining the specificity of the iron transport machinery in one particular strain. They have to be confirmed by ^{59}Fe pyoverdin uptake assays before a reliable conclusion on the active participation of one siderophore in the iron transport of a given strain can be established. Such uptake studies confirmed that strain Pp C2,3 was the sole foreign isolate that efficiently recognized labeled ferric pyoverdins from BTP1 (Figure 3c). The very low radioactivity uptake level measured with strain GM again suggested a poor recognition of $iPVD_{BTP1}$ by this strain.

So, among twelve foreign strains tested, only *P. putida* Pp C2,3 and to a much lower extend *P. fluorescens* GM were the sole isolates able to utilize $iPVD_{BTP1}$ for iron acquisition. However, from a structural point of view, $PVD_{C2,3}$ and PVD_{GM} widely differ both in length and amino acid composition of the peptide chain compared to $iPVD_{BTP1}$ (Table 1). So it is likely that $iPVD_{BTP1}$ is transported into *Pseudomonas* Pp C2,3 cells via an other receptor than the one involved in the recognition of its own pyoverdin. The rapid and significant ^{59}Fe-$iPVD_{BTP1}$ uptake by strain Pp C2,3 suggested that an additional broad-spectrum receptor or multiple transporters are present *de novo* in the outer membrane of this strain as it was evidenced for other fluorescent pseudomonads (Morris *et al.* 1992; Koster *et al.* 1993; Raaijmakers *et al.* 1995). Supporting this hypothesis, additional cross feeding experiments with strain Pp C2,3 showed that it could utilize all the exogenous pyoverdins tested (data not shown). A similar conclusion can be drawn about *P. fluorescens* GM that recognized 85% of the heterologous pyoverdins tested (data not shown). In this case, $iPVD_{BTP1}$ could induce the expression of an additional specific receptor in the cell envelope of strain GM as previously reported in other cases (Gensberg *et al.* 1992; Morris *et al.* 1992; Koster *et al.* 1993).

As for strain Pp C2,3 and *P. fluorescens* GM, little information is available on siderophore-specific receptors present in most strains used in this study. However, results from both uptake assays and cross-feeding experiments highlighted the great specificity of $iPVD_{BTP1}$ recognition by the producing strain and suggested that whether of constitutive or inducible nature, the ferrisiderophore transport machineries of other strains are unable to supply the cells with iron via $iPVD_{BTP1}$. This is first exemplified with the strain ATCC 17400 that is able to utilize exogenous pyoverdins and structurally unrelated siderophores probably via induction of additional receptors (Hohnadel & Meyer 1988; Mossialos *et al.* 2000). In the case of strain ATCC 15692, integration of endogenous and exogenous structurally different pyoverdins seems to be mediated by a unique receptor with somewhat extended broad-spectrum characteristics (Hohnadel & Meyer 1988; Cornélis *et al.* 1989; Gensberg *et al.*

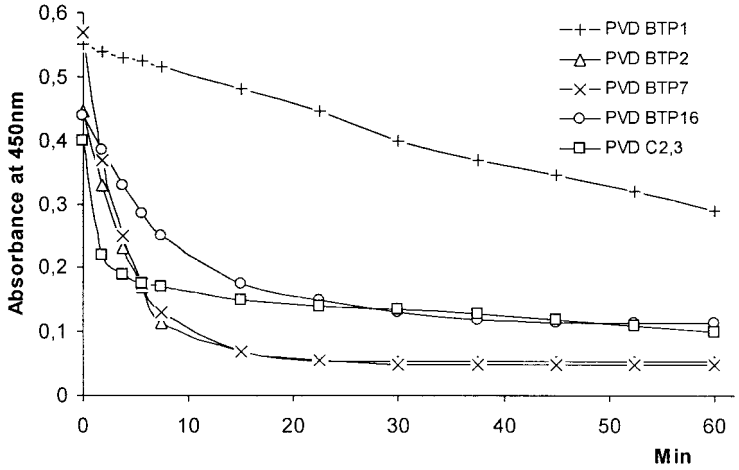


Fig. 2. Evolution of the absorbance at 450 nm observed in dissociation kinetics of various ferric pyoverdins in sodium acetate 0.1 M at pH 5 and in the presence of 25 mM EDTA. Curves are from one representative experiment.

1992; Meyer *et al.* 1999) but which is no longer able to recognize the BTP1 isopyoverdin. Modification of the peptide fixation site in $iPVD_{BTP1}$ is probably not the sole trait responsible for its high recognition specificity. Indeed, growth stimulation tests performed with azoverdin from *Azomonas macrocytogenes* that contains the same iso-chromophore, revealed that this molecule was accepted by a larger number of other strains (*i.e. P. fluorescens* ATCC 17400, *P. putida* BTP2 and P. fluorescens BTP7 in addition to strains recognizing BTP1 isopyoverdins, see Figure 3a). Another structural trait specific for $iPVD_{BTP1}$ is the presence of two Asp in the peptide chain together with Glu as chromophore side chain. These residues confer an unusually high negative global charge to this small ferripyoverdin at physiological pH. This could therefore limit interactions with recognition sites of exogenous receptors.

Study of the pyoverdin-specific transport system in P. putida *strain BTP1*

Recognition of heterologous pyoverdins

Cross-feeding experiments on solid iron-depleted medium revealed that strain BTP1 was able to utilize all the heterologous pyoverdins tested for its growth albeit with a lower efficiency in some cases as shown by a reduced bacterial cell development in the first hours of incubation compared to the one observed for the homologous system (Table 3). In general, this correlated well with results from ^{59}Fe pyoverdin uptake assays with lower incorporation rates (10% or less of radioactivity uptake after 30 min of incubation relative to the uptake observed for the homologuous system, Table 3) measured for heterologous pyoverdins that did not readily stimulate BTP1 growth (+ instead of ++) on plates after 8 h of incubation. As examples, uptake kinetics of labeled ferripyoverdins from strains Ps BTP2, Ps BTP7, Ps GM and Ps pf 17400 are shown in Figure 4A. These last experiments show that *Pseudomonas putida* BTP1 can recognize and efficiently transport six pyoverdins of foreign origin out of 12 tested.

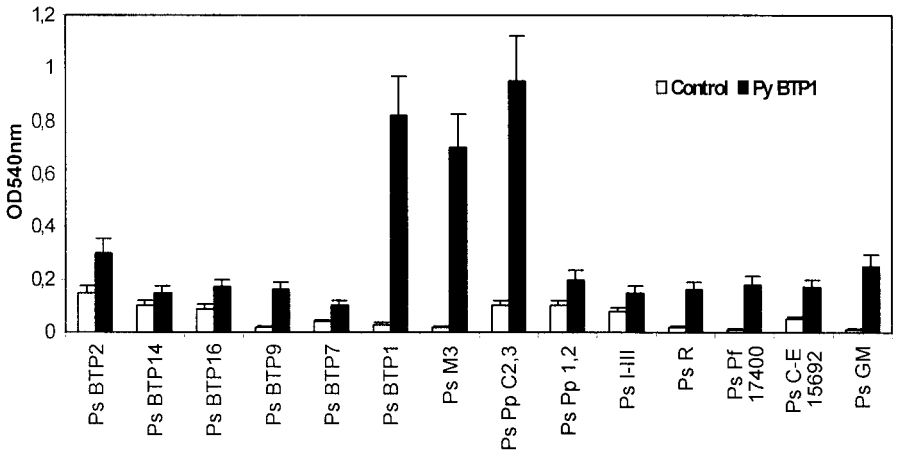
In order to assess whether the efficiently recognized exogenous pyoverdins can be transported via the receptor specific for $iPVD_{BTP1}$, competitive iron uptake studies were performed by incubating BTP1 in the presence of unlabeled ferric PVD_{BTP2} and ferric PVD_{BTP7} before addition of ^{59}Fe-labeled endogenous pyoverdin at the same concentration. In such conditions, uptake of ^{59}Fe-$iPVD_{BTP1}$ by BTP1 cells decreased respectively by 74% and 53% compared to the homologous system without pre-incubation. This reduced uptake level was interpreted as a competition for the same receptor in *P. putida* BTP1 between the homologous and the heterologous pyoverdins. These results suggest the presence of a broad-spectrum receptor responsible not only for $iPVD_{BTP1}$-mediated iron transport but also able to accept several structurally unrelated heterologous pyoverdins.

As stated above, interactions between pyoverdins and membrane receptors are usually considered as strain-specific. However, some *Pseudomonas* strains are able to utilize several exogenous siderophores (Hohnadel et al 1989; Leong *et al.* 1991; Weber

a

Azoverdin	++	-	-	-	+	++	++	++	-	-	-	++	-	++
$iPVD_{BTP1}$	-	-	-	-	-	++	++	++	-	-	-	-	-	+

b



c

^{59}Fe $iPVD_{BTP1}$ uptake (%)														
	2	1	nd	nd	2	100	66	23	nd	nd	nd	3	3	5

Fig. 3. Utilization rates of isopyoverdin BTP1 by various fluorescent pseudomonads strains estimated with 3 different methods. **a**, Effect of $iPVD_{BTP1}$ and azoverdin on growth of different *Pseudomonas* strains on solid EDDHA-supplemented succinate medium observed after 10 h of incubation. ++, strong stimulation as observed for the producing strain; +, slight stimulation; −, no stimulation. **b**, Growth-stimulation effect of $iPVD_{BTP1}$ added in liquid EDDHA-supplemented succinate medium estimated by increase of $O.D._{540nm}$ in the first 6 h following inoculation. Controls are cultures realized in the same conditions without addition of ferripyoverdins. Error bars are percentage values calculated from general variability in cellular densities observed in three different experiments. **c**, Uptake of ^{59}Fe $iPVD_{BTP1}$ expressed as the percentage of the uptake observed for the homologous system. Radioactivity incorporated into cells was measured after a 30 min-incubation time. Data are representative of to separate experiments.

et al. 2000; Amann *et al.* 2000; Mirleau *et al.* 2000) but none was described to efficiently transport about half of the pyoverdins tested as observed for strain BTP1. In most cases, positive cross-feeding reactions are observed when the pyoverdins of foreign origin are structurally very similar to the one synthesized by the strain tested. In the cases of different pyoverdins, the presence of a cyclopeptidic substructure or other 'key-sequences' as well as the length of the linear part of the peptide chain are structural features that were suggested to be essential for an efficient pyoverdin/receptor recognition (Georgias *et al.* 1999; Meyer *et al.* 1999; Weber *et al.* 2000). In this study, the comparison of the $iPVD_{BTP1}$ structure with those of exogenous pyoverdins that are rapidly incorporated by *Pseudomonas putida* BTP1 (Table 1) shows that peptide sequences are very different. Although the length of PVD_{BTP2} is similar to $iPVD_{BTP1}$, amino acid compositions and sequences of the two molecules widely differ with only small neutral residues in PVD_{BTP2} by contrast with the presence of two Asp among the four amino acids not involved in Fe^{3+} fixation in $iPVD_{BTP1}$. Moreover, positive cross-feeding results with PVD_{BTP7}, $PVD_{D,E}$, $PVD_{Pf17400}$ and PVD_{GM} showed that the main receptor of strain BTP1 can also facilitate the transport of pyoverdins with longer peptide chains that contain an internal cycle or not. Efficient transport of structurally unrelated pyoverdins from different strains was already demonstrated for other pseudomonads (see the above mentioned refer-

Table 3. Utilization of heterologous pyoverdins by strain *P. putida* BTP1 and by its siderophore negative mutant M3 estimated by growth stimulation tests (for both isolates) and by ^{59}Fe pyoverdin uptake (for BTP1 only) as described in Figure 2.

Pyoverdins[a]	BTP1		M3		Radioactivity uptake by BTP1 (%)
	8 h	16 h	8 h	16 h	
PVD_C	+	+	−	−	9
PVD_D	++	++	−	+	66
PVD_E	++	++	+	++	26
$PVD_{Pp\ C2,3}$	+	++	+	++	5
$PVD_{Pf\ 12}$	++	++	++	++	nd
$PVD_{Pp\ 1,2}$	+	++	+	++	nd
Azoverdin	++	++	++	++	7
PVD_{GM}	++	++	++	++	20
PVD_I	+	++	−	−	10[b]
PVD_{II}	++	++	+	+	10[b]
PVD_{B10}	++	++	++	++	nd
PVD_R	+	++	+	++	nd
$PVD_{Pf\ 17400}$	++	++	++	++	23
$iPVD_{BTP1}$	++	++	++	++	100
PVD_{BTP2}	++	++	++	++	25
PVD_{BTP7}	++	++	+	++	45
PVD_{BTP16}	++	++	++	++	nd
PVD_{BTP14}	+	++	+	++	5

[a]The three different forms of pyoverdins produced by *P. aeruginosa* ATCC 15692 (PVD_{C-E}) and by *P. fluorescens* I-III (PVD_{I-II}) varying in the nature of the chromophore acid side chain were tested independently. PVD_C, α-ketoglutaric acid; PVD_D, succinic acid; PVD_E, succinic amide; PVD_I, α-ketoglutaric acid; PVD_{II}, succinic acid. [b]Mean value measured for radioactivity uptake of a mixture of PVD_I and PVD_{II}.
nd, not determined.

ences) but as far as we know, the example of BTP1 is unique regarding the structural heterogeneity of the exogenous pyoverdins that this strain can accept.

Interestingly, individual pyoverdins from a particular strain, that only differ by the nature of the dicarboxylic acid substituent of the chromophore, were differentially accepted by BTP1 and its siderophore negative mutant M3 (Table 3 and Figure 4b, 4c for detailed kinetics of radioactivity uptakes). Among the siderophores produced by *P. aeruginosa* ATCC 15692, PVD_C (α-ketoglutaric acid side chain) was poorly or not utilized by BTP1 and M3 respectively while the succinic amide form PVD_E was incorporated by the two isolates. Although growth stimulation was evident for the wild-type, the mutant M3 displayed a reduced ability at utilizing the succinic acid PVD_D form. Similar results were obtained from tests with PVD_{I-II} produced by *P. fluorescens*, the α-ketoglutarate form being less efficiently accepted by BTP1 and its derivative M3 (growth stimulation on solid medium, Table 3). The specific behavior of M3 compared to the wild type BTP1 suggests that a mutation could affect a receptor site particularly sensitive to the nature of the chromophore side chain in BTP1. In our study, such a differential recognition of pyoverdin isoforms with various chromophore side chains was specific to strain BTP1. This phenomenon was not observed when PVD_C, PVD_D and PVD_E (or PVD_I and PVD_{II}) were tested individually for their utilization by 9 other foreign *Pseudomonas* strains that incorporated the different isoforms with the same efficiency (data not shown). To our knowledge, this is the first report highlighting the influence of the acid side chain of the chromophore on the pyoverdin recognition process. However, little information is available in the literature since, in most cases, cross-feeding experiments were done by testing either semi-purified solutions of ferripyoverdins containing a mixture of the differ-

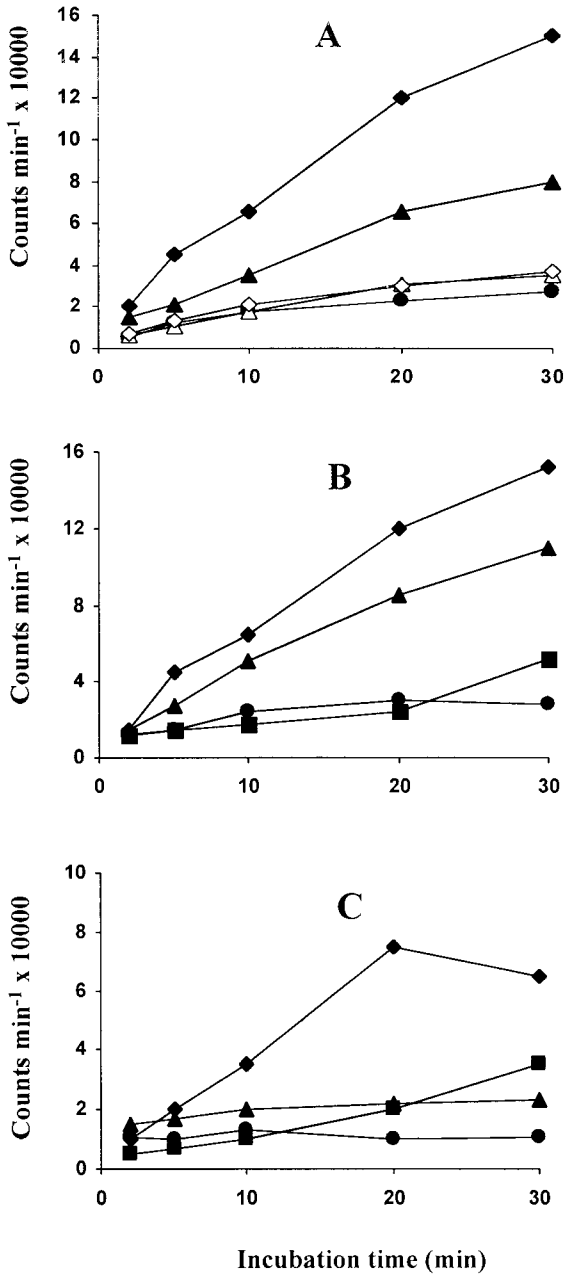


Fig. 4. ^{59}Fe iron uptake in *P. putida* BTP1 (A and B) or in its pyo^{-} derivative M3 (C) mediated by iPVD$_{BTP1}$ (♦), PVD$_{BTP2}$ (◊), PVD$_{Pf17400}$ (•), PVD$_{BTP7}$ (▲), PVD$_{GM}$ (Δ) in A or by iPVD$_{BTP1}$ (♦) and the three isoforms PVD$_{C}$ (•), PVD$_{D}$ (▲), PVD$_{E}$ (■) in B and C. Data are representative of two separate experiments.

ent isoforms or only the isoform mainly produced. This specific trait of BTP1 could be related to the fact that this bacterium does synthesize only one pyoverdin isoform (Glu as acid side chain, Ongena 1996) while several isoforms usually co-occur in cultures of other strains. The iPVD$_{BTP1}$ receptor could have thus concomitantly evolved so as it preferentially accepts molecules bearing one particular chromophore lateral chain and displays a reduced or very limited affinity for pyoverdins with acidic side chain not detected in BTP1 culture medium.

Detection of receptor genes and analysis of IROMPs

Altogether, our results suggested that additional transport systems are active in BTP1 and M3 to facilitate incorporation of some exogenous pyoverdins. A first evidence for such a multiplicity raised from genetic studies. Oligonucleotides corresponding to conserved domains previously identified in four pyoverdin receptors (Koster *et al.* 1995) were designed and used to amplify related sequences in chromosomal DNA of strain BTP1 by PCR. Analysis of the amplified radioactive products by electrophoresis allowed the detection of 10 distinct bands (Figure 5) within the expected size range (642 bp to 466 bp). Most of these bands were also visible in the electrophoretic profile from the pyoverdin deficient mutant M3 (data not shown). Although not all the fragments have to represent ferric siderophore receptor genes, this result gives an indication about the multiplicity of such genes in strain BTP1. Indeed, a positive correlation between the number of amplification products and the ability of some strains to utilize a large number of exogenous pyoverdins was previously established (Koster *et al.* 1995).

The multiplicity of iron-repressed proteins (IROMP) in outer membrane preparations from BTP1 was analyzed by SDS-PAGE. Growth of *P. putida* BTP1 under iron-limited conditions led to the expression of one major IROMP with a calculated size of 100 kDa and three additional protein bands in the range 85–115 kDa (Figure 6). These IROMPs probably correspond to receptors specific for pyoverdins since (i) they are close to the size range of known pyoverdin transporters (Marrug *et al.* 1989; Poole *et al.* 1991; Bitter *et al.* 1991) and (ii) it is obvious that BTP1 does not produce other siderophores than pyoverdins to supply with its iron needs. This last hypothesis is first supported by the fact that strain BTP1 does not produce salicylic acid or pyochelin in iron-limited conditions (Ongena M., unpublished results). Moreover we previ-

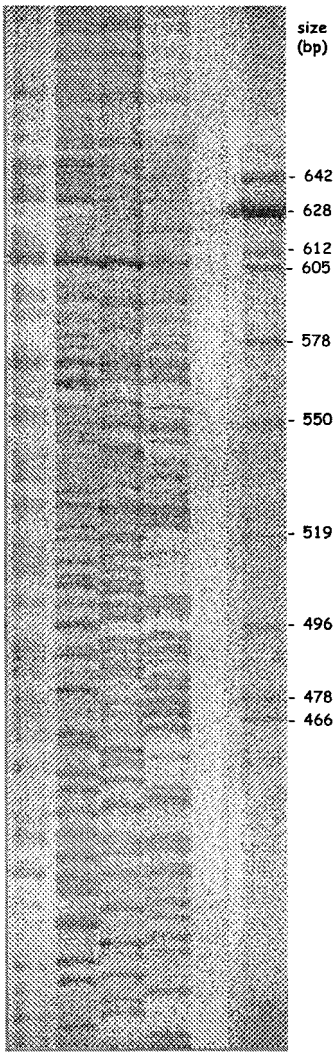


Fig. 5. Autoradiography of a 6% polyacrylamide gel obtained for the analysis of PCR-amplified products from chromosomal DNA of *P. putida* BTP1. On the left the sequence ladder is shown which was used to determine the size of the fragments mentioned on the right.

ously showed (Ongena *et al.* 1999) that the pyoverdin deficient mutant M3 was no longer able to synthesize any molecule with iron-complexing activity since it was originally selected for its inability to discolor Chromazurol S reagent (Schwyn & Neilands 1987).

On another hand, analysis of outer membrane protein content from BTP1 cells grown in the presence of PVD_C and PVD_{BTP14}, two pyoverdins with reduced uptake, showed IROMP electrophoretic patterns similar to the one observed in the absence of exogenous siderophores (data not shown). This suggested that no additional pyoverdin receptor is inducible in BTP1 and

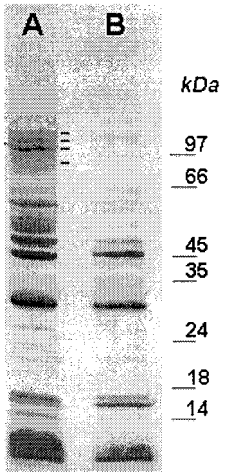


Fig. 6. SDS-PAGE on a 8–18% gradient gel of outer membrane proteins prepared from *P. putida* BTP1 cells grown under iron-limited (lane A) and excess-iron (lane B) conditions. The positions of molecular mass standards are indicated on the right.

so that the ability of the strain to utilize a broad spectrum of exogenous pyoverdins is primarily governed by the multiplicity of receptors that are constitutively expressed in its outer membrane. Individual receptors recognizing structurally distinct pyoverdins were previously identified in other fluorescent pseudomonads such as *P. aeruginosa* ATCC 15692 (Hohnadel & Meyer 1988; Cornelis *et al.* 1989; Meyer *et al.* 1997, 1999), *P. fluorescens* M114 (Morris *et al.* 1992) or *P. putida* WCS358 (Leong *et al.* 1991; Koster *et al.* 1993) although incorporation of some heterologous pyoverdins also depended on the induction of novel specific transport proteins in the last two strains.

In conclusion, our results reinforced the notion that the utilization of a particular pyoverdin by foreign *Pseudomonas* strains depends to a large extent on the multiplicity and specificity of siderophore receptors present in the membrane of these isolates. However, through the example of pyoverdins produced by *P. putida* BTP1, we showed that such recognition rate could also be markedly influenced by the structure of the exogenous pyoverdin. Although it remains to be confirmed by computational thermodynamic studies, it is likely that modification in the chromophore of $iPVD_{BTP1}$ can somewhat influence the tri-dimensional structure of the molecule since it changed the peptide fixation site. This trait may act synergistically with the highly negative ionized state (at physiological pH) of this short pyoverdin to provide a very specific behavior to the molecule regarding its two main properties, i.e., affinity for ferric iron and recognition by membrane

receptors of foreign *Pseudomonas* strains. The peculiar structure of its pyoverdin together with its ability to utilize a large variety of pyoverdins could confer a competitive advantage to *P. putida* BTP1 for Fe^{3+} acquisition in iron-limited environments and therefore contribute to enlarge its ecological competence in terms of colonization and persistence in the rhizosphere as recently demonstrated for other strains by Loper & Henkels (1999) and Mirleau *et al.* (2000). In this context, such specificities could partially compensate for the lack of antibiotic synthesis by this strain (Ongena *et al.* 1999).

Acknowledgements

M.O. is Scientific Research Worker of the Fonds National de la Recherche Scientifique in Belgium. The authors thank Prof. H. Budzikiewicz from the University of Cologne for critical reading of the manuscript.

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