Taxonomic heterogeneity, as shown by siderotyping, of strains primarily identified as *Pseudomonas putida*

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One hundred and forty-four fluorescent pseudomonad strains isolated from various environments (soil, water, plant rhizosphere, hospital) and received as *Pseudomonas putida* (83 strains), *P. putida* biovar A (49 strains), *P. putida* biovar B (10 strains) and *P. putida* biovar C (2 strains), were analysed by the pyoverdine-isoelectrofocusing and pyoverdine-mediated iron uptake methods of siderotyping. Both methods demonstrated a great diversity among these strains, which could be subdivided into 35 siderovars. Some siderovars specifically included strains that have subsequently been transferred to well-defined *Pseudomonas* species, e.g. *Pseudomonas monteilii* or *Pseudomonas mosselii*, or which could be related by their siderotype to *Pseudomonas jessenii* or *Pseudomonas mandelii*. Other siderovars included strains sharing a high level of DNA-DNA relatedness (>70%), thus demonstrating that siderotyping could easily circumscribe strains at the species level. However, a group of seven strains, including the type strain, *P. putida* ATCC 12633^T, were allocated into four siderovars, despite sharing DNA-DNA relatedness values of higher than 70%. Interestingly, the strong genomic relationships between these seven strains were supported by the structural relationships among their pyoverdines, thus reflecting their phylogenetic affinities. These results strongly support the view that pyoverdine-based siderotyping could be used as a powerful tool in *Pseudomonas* taxonomy.

INTRODUCTION

Although some reports have described *Pseudomonas putida* as an opportunist human pathogen associated with clinical infections, particularly among children (Bouallegue *et al.*, 2004; Perz *et al.*, 2005), this species is usually considered as an innocuous environmental micro-organism with a significant potential for biotechnological applications due to its metabolic versatility and great environmental adaptability (Timmis, 2002). Widely distributed in soil and water, *P. putida* thrives in highly polluted environments where it may actively participate in bioremediation processes. The importance of *P. putida*, as well as other ubiquitous soil-borne pseudomonads, e.g. *Pseudomonas fluorescens* or *Pseudomonas stutzeri*, has generated many ecological and environmental studies that have required numerous attempts to identify strains and have often faced many difficulties due to the great diversity encountered within these species (Palleroni, 1993; Moore *et al.*, 1996; Anzai *et al.*, 2000).

The taxonomic heterogeneity of *P. putida* was the basis for the characterization of two biovars, A and B (Stanier *et al.*, 1966; Palleroni, 1984, 2005), and the minor biovar, C (Barrett *et al.*, 1986). While recent methods have demonstrated high efficiency in *Pseudomonas* identification, with assays specific to *Pseudomonas* at the genus level or specific to a single species (i.e. *Pseudomonas aeruginosa*, Spilker *et al.*, 2004), the present status of the fluorescent saprophytic members of the genus *Pseudomonas* is not satisfactory. In particular, there is a consensus among taxonomists that *P. fluorescens* and *P. putida* should be considered as very heterogeneous species which each need to be divided into several nomenclatures (Bossis *et al.*, 2000). Together with the pioneering work of Stanier, Palleroni and Doudoroff (Stanier *et al.*, 1966), many other studies based on biochemical characteristics (Grimont *et al.*, 1996), whole-cell protein analysis (Tesar *et al.*, 1996; Vancanneyt *et al.*, 1996), 16S rRNA gene sequence comparisons (Moore *et al.*, 1996) or involving both phenotypic and genomic typing approaches, e.g. Biolog metabolic profiling, restriction fragment length polymorphism ribotyping, PCR ribotyping, repetitive element sequence-based PCR (rep-PCR), using BOX or enterobacterial repetitive intergenic consensus

Abbreviations: bv., biovar; pHi, isoelectric pH; PVD, pyoverdine; PVD-IEF, pyoverdine-isoelectrofocusing; sv., siderovar.

(ERIC) primers (Brosch *et al.*, 1996; Dawson *et al.*, 2002), effectively concluded that great taxonomic heterogeneity was the rule within the species *P. fluorescens* and *P. putida*. The need for a detailed revision at the biochemical and molecular levels was also shown by a combined dataset of four molecular sequences (*atpD*, *carA*, *recA* and 16S rRNA gene sequences; Hilario *et al.*, 2004) and by sequence comparisons focused on *gyrB* and *rpoD* gene sequences (Yamamoto *et al.*, 2000). Interestingly, all these studies, as well as a few others specifically focused on *P. putida* (Yamamoto & Harayama, 1995, 1998), concluded that *P. putida* strains belonging to biovar B are phylogenetically closer to *P. fluorescens* strains than to strains of *P. putida* biovar A.

A great diversity also exists at the level of the pyoverdines, the main siderophores produced by the fluorescent members of the genus *Pseudomonas* (Meyer, 2000). Well known as the yellow-green fluorescent pigments which characterize these bacteria, pyoverdine molecules are mainly composed of a quinoline-based chromophore associated with a peptide chain and an acyl side chain (see Budzikiewicz, 2004 for details). The amino acid composition of the peptide chain varies greatly depending on the producing strain, while the chromophore is well conserved among strains. The acyl side chain can also vary within a limited range of dicarboxylic compounds, e.g. succinic acid and malic acid and their amides, or α -ketoglutaric acid for the most frequently distributed ones, resulting in several isoforms of an otherwise identical pyoverdine produced by a given strain (Budzikiewicz, 2004). Based on the peptide chain diversity which takes into account the number and types of amino acyl residues, with the presence of L- and D-forms arranged in linear or partially cyclic chains, close to 50 different pyoverdine structures have been recognized to date. Twelve of these structures have been isolated from strains belonging to the species *P. putida* and 22 have been isolated from *P. fluorescens* strains (Budzikiewicz, 2004).

With the development of siderotyping methods and their application to numerous strains to give an easy and fast discrimination of the pyoverdines they produce, it soon became evident that pyoverdines are not only powerful siderophores but are also efficient taxonomical markers for fluorescent pseudomonads. As a general rule, strains belonging to a given species produce an identical pyoverdine, whereas strains belonging to different species produce different pyoverdines (Meyer, 2000; Bultreys *et al.*, 2001; Meyer *et al.*, 2002; Meyer & Geoffroy, 2004). To be valid, however, this rule should also apply to well-defined species, i.e. species defined by a polyphasic taxonomic approach (Vandamme *et al.*, 1996). Effectively, within the taxonomically heterogeneous species *P. fluorescens*, it has already been well established that 28 strains belonging to biovar I are divided into 10 siderovars, thus supporting the heterogeneity of the *P. fluorescens* species and also demonstrating that biovars do not represent a valid, clear-cut taxonomy for these bacteria (Meyer *et al.*, 2002).

The goals of the present study were, firstly, to analyse the diversity of strains assigned to the *P. putida* species through siderotyping and, secondly, to establish that strain grouping into siderovars is of taxonomical value for these bacteria and could result in the proposal of novel *Pseudomonas* species.

METHODS

***Pseudomonas putida* isolates and reference strains.** Most of the strains used in the present study (listed in Table 1) were obtained from several laboratories where they had been phenotypically characterized as belonging to the species *Pseudomonas putida*. For some strains, characterization was done by the use of a restricted panel of physiological and biochemical characters, allowing the recognition of strains at the biovar level (Palleroni, 1984, 2005; Bossis *et al.*, 2000). For the others, commercially available galleries and treatment of the data by numerical taxonomy were the main means of identification (Elomari *et al.*, 1994). Two phenotypic clusters analysed at the Laboratoire de Bactériologie, Faculté de Médecine, Lille, France, and further characterized by a polyphasic approach as *Pseudomonas monteilii* and *Pseudomonas mosselii* (Elomari *et al.*, 1997; Dabboussi *et al.*, 2002), were included in the present study as the majority of the isolates, which were all of medical origin, were first identified as *P. putida*. The other strains were isolated from soil, water or from the rhizospheres of various plants (Table 1).

Growth conditions. For routine cultures, strains were grown in a rich medium comprising (l^{-1}); 10 g peptone (Difco), 5 g beef extract (Difco) and 5 g NaCl. For bacterial purity control, strains were grown on plates in the same medium supplemented with 12 g agar (Difco). Iron-deficient cultures were prepared using Casamino acid (CAA) medium containing (l^{-1}); 5 g Casamino acids with low iron and chloride content (Difco), 1.18 g K_2HPO_4 and 0.25 g $MgSO_4 \cdot 7H_2O$. Strains grown on iron-deficient media were used for the production and purification of pyoverdines and for their analysis through isoelectrophoresis. For iron uptake studies, iron-deficient cells were harvested from cultures in succinate medium (Meyer *et al.*, 1997). Cultures were incubated at 25 °C for 40 h with vigorous shaking (200 r.p.m.) in autoclave-sterilized capped test tubes (180 × 18 mm) containing 7.5 ml medium or in Erlenmeyer flasks (50 or 500 ml medium in 250 ml or 1 l flasks, respectively). Strains were preserved at -80 °C as a mixture of 0.5 ml rich medium liquid culture reaching the end of the exponential phase and the same volume of sterilized glycerol/H₂O solution (50 %, v/v).

Siderotyping by isoelectrophoresis. A 1 ml sample of a 40 h CAA-culture at 25 °C of each *P. putida* isolate was harvested and centrifuged in an Eppendorf tube for 5 min at 13 000 r.p.m. A 0.4 ml sample of the supernatant was then lyophilized and the residue resuspended in 20 µl ultrapure H₂O. A 1 µl sample of the 20-fold concentrated culture supernatant containing the various pyoverdine (PVD) isoforms accumulated during bacterial growth was analysed on ampholine-containing polyacrylamide gels as described by Fuchs *et al.* (2001). The respective pyoverdine-isoelectrofocusing (PVD-IEF) patterns were visualized under UV-illumination at 365 nm. For each strain, the fluorescent PVD-bands representing the different isoforms of an otherwise identical pyoverdine (Fig. 1; Budzikiewicz, 2004) were further computer-analysed for isoelectric pH (pHi) determination using a standard mixture of pyoverdines as described by Fuchs *et al.* (2001). A schematic representation of PVD-bands by pHi-bars was then constructed, with bar thicknesses proportional to the intensity of fluorescence of the PVD-bands, as represented in Tables 2, 3 and 4. Systematically, strains presenting identical or closely related PVD-IEF patterns were subjected to a second analysis, including new cultures

Table 1. Strains used in the study

With the exception of the reference strains, all strains were primarily identified as *Pseudomonas putida* and are presently still recognized as such, except those which have been reclassified as *Pseudomonas monteilii* (Elomari *et al.*, 1997) or as *Pseudomonas mosselii* (Dabboussi *et al.*, 2002). CFBP, Collection Française de Bactéries Phytopathogènes; CFML, Collection de la Faculté de Médecine de Lille; ATCC, American type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen.

Source	Strain designation	Origin	Reference
INRA-Angers (CFBP) Gardan-Bossis (GB) collection	<i>Pseudomonas putida</i> CFBP/GB 11302, 11341, 11342, 11349, 11366, 11370, 11378, 11379, 11384, 11385, 11387, 11388, 11391, 11394, 11395, 11399	Plant rhizosphere (tomato, maize)	Bossis (1995)
INRA-Angers (CFBP) Gardan-Mattar (GM) collection	<i>Pseudomonas putida</i> CFBP/GM 10090, 10096, 11274, 11275, 11277, 11797, 11799.1, 11799.2, 11802, 11804, 11806, 11907, 11908, 11911, 11912, 11916, 11917, 12220	Plant rhizosphere	Mattar (1993)
INRA-Angers (CFBP) Gardan-Sutra (GS) collection	<i>Pseudomonas putida</i> CFBP/GS 12017, 12019, 12048, 12055, 12059, 12062, 12063, 12065, 12070, 12074, 12077, 12081, 12082, 12086, 12087, 12089, 12090, 12091	Plant rhizosphere (banana tree)	Sutra <i>et al.</i> (2000)
INRA-Dijon Lemanceau collection	<i>Pseudomonas putida</i> C-S111, C-S413, C-S215, C-LRp214, D-S1026, D-LR223, D-S824, D-LR228, D-TRP621, D-LE3216, D-S131, D-TR331, D-LRp414, D-LRp212, D-LE611, D-LRp511, D-LE414 J, D-TR1035, D-TE612, D-LE711, D-LRp921, L1	Soil or plant rhizosphere (tomato, flax)	Lemanceau <i>et al.</i> (1995); Latour <i>et al.</i> (1996)
Faculté de Médecine de Lille (CFML)	<i>Pseudomonas putida</i> CFML 90-33, 90-34, 90-40, 90-42, 90-44 to 90-52, 90-72, 90-135, 90-136, 90-138, 90-139; <i>Pseudomonas monteilii</i> CFML 90-54 to 90-63; <i>Pseudomonas mosselii</i> CFML 90-70, 90-71, 90-73 to 90-75, 90-77 to 90-80, 90-82, 90-83 ^T	Medical environment; mineral water	Elomari <i>et al.</i> (1997); Dabboussi <i>et al.</i> (2002); Verhille <i>et al.</i> (1999)
Japan collection	<i>Pseudomonas putida</i> A10L, BH, PpG7 (=ATCC 17485), K23-1, KF715, PB4	Miscellaneous	Yamamoto & Harayama (1998)
Belgium collection	<i>Pseudomonas putida</i> PGSB8508, PGSB8301, BW13M1, BW16M1, BW16S1, RW5S2, BW11P1, BW11P2, BW11M1, RW10S1, GR12-2R3	Maize, rice, banana, grass	Parret & De Mot (2000)
Miscellaneous	<i>Pseudomonas putida</i> ATCC 12633 ^T , ATCC 39167, DSM 3601, DSM 3602, MF6313, MFH2-127, PutC, BTP1, BTP16, WCS358, KT2440, 9BW, 10CW, Gwose		ATCC and DSM collections; Jacques <i>et al.</i> (1995); Shivaji <i>et al.</i> (1989); Timmis (2002)
Reference strains	<i>Pseudomonas jessenii</i> CFML 95-307 ^T ; <i>Pseudomonas mandelii</i> CFML 95-303 ^T ; <i>Pseudomonas fluorescens</i> ATCC 13525 ^T ; <i>Pseudomonas</i> sp. A214; <i>Pseudomonas</i> sp. B10		Verhille <i>et al.</i> (1999); Teintze <i>et al.</i> (1981); Buyer <i>et al.</i> (1986)

and isoelectrophoresis, but with comigration of the samples on the same gel for a better visual control of relatedness. Strains presenting identical PVD-IEF patterns were grouped in a so-called IEF-group. For clarity, Table 2 was built with IEF-groups characterized by PVD-bands with acidic pHi values (from 3.9 to 6.0), Table 3 with IEF-groups having PVD-bands at neutral pHi values (below pH 8.0) with very often at least one supplementary band at an acidic pH value, while Table 4 contained the IEF-groups showing IEF bands at neutral and alkaline pHi values.

Siderotyping by pyoverdine-mediated ⁵⁹Fe-uptake. Iron-deficient cells harvested by centrifugation from a 40 h culture in succinate medium at 25 °C were washed and resuspended just before use in an incubation medium made of succinate medium without a nitrogen source. The suspension was adjusted to an OD₆₀₀ of 0.33 and 1.8 ml aliquots were used for each assay. The label mixture containing ⁵⁹Fe-PVD complex consisted of 5 µl commercial ⁵⁹Fe³⁺ solution (iron chloride in 0.1 M HCl; specific activity, 110–925 MBq mg⁻¹ iron; Amersham, France) diluted first with 100 µl of distilled water and then mixed with 100 µl of pyoverdine-containing succinate culture supernatant. After 10 min incubation at room temperature, the final volume of label mixture was adjusted to 1 ml with incubation

medium. The uptake assay was begun by the addition of 0.2 ml label mixture to 1.8 ml cell suspension and the sample was incubated in a water bath at 30 °C with gentle shaking for 20 min. Following this, a 1 ml sample was withdrawn and rapidly filtered through a nitrocellulose filter (0.45 µm pore size), which was then washed twice with incubation medium. The filter was wrapped in aluminium foil and counted for radioactivity in a counter (Gammamatic 4000; Beckman). The remaining 1 ml bacterial suspension was directly counted to determine the total amount of radioactivity present in the assay. A control assay without cells was performed for each label mixture in order to verify the complete solubilization of the iron through PVD complexation. Each strain was tested for iron incorporation mediated by its own pyoverdine to evaluate its 100 % efficiency in iron uptake and also for iron incorporation mediated by the pyoverdine of the type strain of the group (in bold typeface in Tables 2, 3 and 4). Strains presenting an identical PVD-IEF pattern and presenting cross-incorporations at 100 ± 15 % efficiency were considered as producing identical pyoverdines and thus were grouped into siderovars, according to the definition given in Meyer *et al.* (2002). In most cases, a precise correlation was observed between PVD-IEF groups and siderovars. There were some exceptions, as described in the Results section. Therefore, a second method for

Fig. 1. Pyoverdine-isoelectrophoresis patterns of *Pseudomonas putida* strains belonging to various siderovars as shown on ampholine-containing polyacrylamide gels photographed under UV-light (365 nm). (a) Lanes: 1, KF715 (sv. 17); 2, CFML 90-44 (sv. 17); 3, GM11917 (sv. 15); 4, GM11277 (sv. 1); 5, L1 (sv. 20); 6, GM11799.1 (sv. 21); 7, GM11799.2 (sv. 21); 8, BTP16 (sv. 2); 9, GS 12065 (sv. 24); M, pyoverdine standard for pHi measurement (Fuchs *et al.*, 2001). (b) Lanes: 1, GS 12019 (sv.16); 2, GS 12048 (sv. 16); 3, GS 12062 (sv. 16); 4, GS 12081 (sv. 16); 5, GS 12090 (sv. 16); 6, GS 12055 (sv. 18); 7, GB11341 (sv. 18); 8, GB11342 (sv. 18); 9, GB11394 (sv. 23); M, pyoverdine standard as in (a).

validating the strain grouping was to test the type strains with the pyoverdines that had the same acidic, neutral or alkaline behaviours. When such heterologous uptakes occurred, they are shown in the right hand panels of Tables 2, 3 and 4 as the percentage of efficiency of iron incorporation compared with that of the homologous pyoverdine (100%). A third class of pyoverdine-mediated iron uptake experiment was to test the type strain of each siderovar against a collection of pyoverdines with well-established structures and/or species assignments (the 36 pyoverdines listed in Fuchs *et al.*, 2001). Such incorporation studies determined whether a particular pyoverdine characterizing a siderovar was a new representative or whether it corresponded to an already structurally-known pyoverdine.

DNA preparation and DNA–DNA hybridization. Chromosomal DNA was extracted, purified and labelled *in vitro* with [^3H]cytosine by nick translation as described previously (Elomari *et al.*, 1996). The procedure used for hybridization experiments (S1 nuclease-trichloroacetic acid method) has been previously described (Grimont *et al.*, 1980). The determination of T_m (melting temperature) and ΔT_m (difference between the T_m of the heteroduplex and T_m of the homoduplex) was performed as described in Crosa *et al.* (1973).

RESULTS AND DISCUSSION

Strains grouping according to PVD-IEF patterns and pyoverdine-mediated iron uptake

As illustrated in Fig. 1, the PVD-IEF patterns of *P. putida* isolates showed great heterogeneity. Among the nine strains analysed in Fig. 1a, at least six different patterns could be easily identified, as seen in lanes 1, 3, 4, 6, 8 and 9. The differences were due to the number of PVD-bands (corresponding to different PVD-isoforms; Fuchs *et al.*, 2001), with some strains apparently producing a unique PVD-isoform (e.g. lanes 4 and 8), while most strains showed three bands and a few others showed two bands (e.g. lanes 1 and 2). The IEF patterns could also vary according to the fluorescence intensity of the PVD-bands, which reflected the concentration of isoforms present in the culture supernatants. As seen by direct observation, the PVD-patterns of lanes 1 and 2 looked strictly identical, as did those of lanes 6 and 7 except that the intensity of the lower band was brighter in lane 7 compared with lane 6. Thus, electrophoresis analysis suggested the grouping of strains KF715 and CFML 90-44, corresponding to lanes 1 and 2, while the grouping of strains GB11799.1 and GB11799.2 (lanes 6 and 7) was performed with some degree of uncertainty. To determine whether or not the pyoverdines of strains GB11799.1 and GB11799.2 were identical, iron uptake studies were performed using iron-starved cells of both strains and their respective pyoverdines liganded to ^{59}Fe . Both strains were able to incorporate and cross-incorporate labelled iron at 100% efficiency (data not shown). On this basis, therefore, they were grouped in the same siderovar. The necessity of using the siderophore-mediated iron incorporation method as an adjunct to the IEF method is even better illustrated in Fig. 1b, where eight strains (lanes 1–8) presented apparently identical PVD-IEF profiles. However, cross-incorporation studies clearly demonstrated that the eight strains were divided into two subgroups. The first five strains (lanes 1–5) cross-reacted efficiently with each other, as did the three other strains (lanes 6–8), but there was no cross-reactivity detectable between strains belonging to the two subgroups (Table 3). Thus, although presenting very close if not identical PVD-IEF patterns, these strains should synthesize structurally different pyoverdines and consequently they were assigned to two different siderovars (sv. 16 and sv. 18 in Table 3). Most of the other PVD-IEF groups, however, presented sufficient IEF-pattern differences from each other to allow correct strain groupings. These groupings were also supported by uptake studies, as shown in Tables 2, 3 and 4. Only three isolates, CFML 90-59, CFML 90-60 and CFML 90-61 of siderovar 14 in Table 3 were defective in pyoverdine synthesis and thus could not be analysed by isoelectrofocusing. Their grouping, at least for two of them (isolates CFML 90-59 and CFML 90-61), could, however, be determined by iron incorporation studies. Only the pyoverdine characterizing siderovar 14 was used by the two strains (Table 3). Strain CFML 90-60

Table 2. *Pseudomonas putida* strains producing acidic pyoverdines and grouped into siderovars according to PVD-IEF profiles and PVD-mediated iron uptake specificity

Some non-*P. putida* reference strains have been included. They are indicated by brackets. *P. fl.*, *P. fluorescens*; *P. j.*, *P. jessenii*; *P. m.*, *P. mandelii*; *P. sp.*, *Pseudomonas* sp. Biovar assignment, if known, is indicated by the letter code A, B or C. PVD-pHi values of the PVD-isoform bands separated by isoelectrophoresis are indicated as bars with thickness proportional to the intensity of fluorescence demonstrated under UV-light illumination (see Fig. 1).

sv. no.	Strain	bv.	PVD-pHi values					Iron uptake as mediated by PVD of sv. no.*												
			4.0	4.5	5.0	5.5	6.0	1	2	3	4	5	6	7	8	9	10	11	12	13
1	BH	B																		
1	GB 11370	B																		
1	GB 11384	A																		
1	GB 11385	A																		
1	GM 11907	A																		
1	GM 11275	A																		
1	GM 11908	A																		
1	GM 10096	A																		
1	GM 11911	A																		
1	GM 11277	A																		
1	GM 11804	A																		
1	GS 12077																			
1	GS 12074																			
2	CFML 90-42																			
2	K23.1	A																		
2	PB4	A																		
2	PutC																			
2	BTP16																			
2	GM 11806	A																		
2	GM 11802																			
3	BTP1																			
3	CFML 90-40																			
3	CFML 90-52																			
3	PpG7	A																		
4	CFML 90-33																			
4	CFML 90-34																			
4	CFML 90-139																			
4	DSM 3601																			
5	ATCC 39167																			
5	DSM 3602																			
5	[<i>P. sp.</i> A214]																			
6	GB 11391	A																		
6	GB 11302	A																		
7	GM 10090	A																		
7	GM 11916	A																		
8	ATCC 12633 ^T																			
9	GB 11387	B																		
10	GB 11399	A																		
11	GB 11378	A																		
12	PGSB8508																			
13	GR12-2R3																			

*Values are expressed in percentage of incorporation compared to the homologous system (100 %). For clarity, values below 10 % (threshold limit) are replaced by a minus sign, while values of 100 ± 15 % are represented by a black square for the homologous uptakes. Values are middle means of at least two independent experiments.

Table 3. *Pseudomonas putida* strains producing neutral pyoverdines and grouped into siderovars according to PVD-IEF profiles and PVD-mediated iron uptake specificity.

Some non-*P. putida* reference strains have been included. They are indicated by brackets. P. fl., *P. fluorescens*; P. j., *P. jessenii*; P. m., *P. mandelii*; P. sp., *Pseudomonas* sp. The CFML strains of siderovar 14 are now recognized as *Pseudomonas monteilii* (Elomari *et al.*, 1997). Biovar assignment, if known, is indicated by the letter code A, B or C. PVD-pHi values of the PVD-isoform bands separated by isoelectrophoresis are indicated as bars with thickness proportional to the intensity of fluorescence demonstrated under UV-light illumination (see Fig. 1).

sv. no.	Strain	bv.	PVD-pHi value							Iron uptake as mediated by PVD of sv. no.*														
			4.5	5.0	5.5	6.0	6.5	7.0	7.5	14	15	16	17	18	19	20	21	22	23	24	25			
14	CFML 90-54	A B																						
14	CFML 90-55																							
14	CFML 90-56																							
14	CFML 90-57																							
14	CFML 90-58																							
14	CFML 90-59		(PVD-)																					
14	CFML 90-60		(PVD-)																					
14	CFML 90-61		(PVD-)																					
14	CFML 90-62																							
14	CFML 90-63																							
14	GS 12087																							
14	GS 12086																							
14	GS 12063																							
14	GS 12089																							
14	GS 12017																							
14	GM 11912		A																					
14	GM 11797		B																					
15	GS 12091	A																						
15	CFML 90-45																							
15	CFML 90-48																							
15	CFML 90-49																							
15	CFML 90-50																							
15	CFML 90-51																							
15	GM 11917																							
16	GS 12019																							
16	GS 12048																							
16	GS 12062																							
16	GS 12081																							
16	GS 12090																							
16	RW 10S1																							
17	KF 715																							
17	CFML 90-44																							
17	CFML 90-46																							
17	CFML 90-47																							
17	CFML 90-72																							
18	GS 12055	A A																						
18	GB 11341																							
18	GB 11342																							
18	[P. sp. B10]																							
19	BW 13M1																							
19	BW 16S1																							
19	BW 11P1																							
19	BW 11P2																							
20	L1																							
20	WC S358																							

Table 3. cont.

sv. no.	Strain	bv.	PVD-pHi value							Iron uptake as mediated by PVD of sv. no.*											
			4.5	5.0	5.5	6.0	6.5	7.0	7.5	14	15	16	17	18	19	20	21	22	23	24	25
21	GM 11799.1	A								—	—	—	—	—	—	—		51	—	—	—
21	GM 11799.2	A								—	—	—	—	—	—	—			—	—	—
22	GB 11388	B								—	—	—	—	—	—	—	57		—	—	—
23	GB 11394	B								—	—	—	—	—	—	—	—			—	—
24	GS 12065									—	—	—	—	—	—	—	17	—	14		—
25	KT 2440									—	—	—	—	—	—	—	—	—	—		

*See footnote for Table 2.

did not incorporate any of the pyoverdine-iron complexes and therefore could not be assigned to a siderotype.

Finally, based on both isoelectrophoresis and PVD-mediated iron incorporation studies, a total of 35 siderovars was identified among the 144 *P. putida* strains. They are illustrated schematically in Tables 2, 3 and 4 on the basis of their acid, neutral and basic type pyoverdines, respectively. Within each table, siderovars are arranged in descending order according to the number of strains classified within each siderovars. For siderovars with more than one strain, a siderovar type strain, marked in bold typeface in Tables 2, 3 and 4, was chosen within each siderovar for pyoverdine purification and pyoverdine-mediated iron incorporation studies.

Siderovars with acidic pyoverdines

Forty *P. putida* strains were characterized by acidic PVD-IEF profiles, with bands usually ranging between pHi 3.9 and 5.3 (Table 2). The pHi values, as well as the number of PVD-isoform bands, allowed the identification of 13 IEF-groups, with seven presenting three isoform bands, two having two bands and four being characterized by a unique pyoverdine band. Cross-incorporation studies correlated perfectly with the IEF-grouping. Within each group, strains were able to incorporate the ^{59}Fe -pyoverdine of the type strain at around 100% efficiency, as indicated by black squares in the right panel of Table 2. Six siderovars were represented by only one strain, among them sv. 8 which included the type strain of *P. putida* ATCC 12633^T. The distribution of the other siderovars ranged from 13 strains for sv. 1 to two strains for sv. 6 and sv. 7. The structures of the pyoverdines characterized by siderovars 1, 2, 3, 4, 5 and 8 are known and are shown in Table 5. When comparing the amino acid composition of their respective peptidic parts, no strong similarity could be seen, except that five of the pyoverdines are characterized by an Asp residue as the N-terminal amino acid branched to the chromophore. Another peculiarity concerns the pyoverdines of sv. 3 and sv. 4, which are in fact isopyoverdines, i.e. pyoverdines with the peptide chain branched at the level of the C3 instead of the C1 of the chromophore (Budzikiewicz, 2004). The

absence of pronounced structural correlations between these six pyoverdines is in agreement with their usually high level of specificity, as determined by heterologous pyoverdine-mediated iron uptake studies. As shown in Table 2, the pyoverdine characterizing sv. 3 was the most specific, since none of the strains from the other siderovars were able to use it in iron transport. Concerning strain specificity, heterologous uptake studies showed that some strains were highly specific, e.g. the strains of sv. 1 and sv. 8, which were able to use only their own pyoverdine for iron transport. The other strains were able to use several acidic pyoverdines. Strain PutC of sv. 2 was able to use the largest number of foreign pyoverdines, being able to use seven with very high efficiency. Most of the structures of the pyoverdines with efficient heterologous uptake are presently unknown. Therefore, it cannot yet be decided whether the heterologous uptake is due to structural similarities between pyoverdines or to the presence of supplementary pyoverdine-receptors in these strains. Heterologous uptake studies involving a collection of structurally known pyoverdines (described in Fuchs *et al.*, 2001) have concluded that each of the siderovars is characterized by an original pyoverdine; none of the pyoverdines of foreign origin belonging to the collection were able to mediate iron incorporation in these strains. One exception, however, concerned the pyoverdine of *Pseudomonas* sp. A214 which was used at 100% efficiency (data not shown) by the two *P. putida* strains belonging to sv. 5. As a control of pyoverdine identity, the two *P. putida* isolates of sv. 5 and *Pseudomonas* sp. A214, as shown in Table 2, middle panel, presented effectively an identical PVD-IEF pattern. It is noteworthy that PVD(A214) is the only one of these acidic pyoverdines in which the peptide chain does not begin with an Asp residue (Table 5).

Siderovars with neutral pyoverdines

Fifty strains of *P. putida* presenting PVD-IEF patterns with one to three isoform bands with acidic pHi values and one or two with neutral pHi values within the range of pH 4.0 to pH 7.5 were grouped in 12 siderovars (Table 3) according to their isoelectrophoretic and incorporation

Table 4. *Pseudomonas putida* strains producing basic pyoverdines and grouped into siderovars according to PVD-IEF profiles and PVD-mediated iron uptake specificity.

Some non-*P. putida* reference strains have been included. They are indicated by brackets. *P. fl.*, *P. fluorescens*; *P. j.*, *P. jessenii*; *P. m.*, *P. mandelii*; *P. sp.*, *Pseudomonas* sp. The CFML strains of siderovar 27 are now recognized as *Pseudomonas mosselii*. Biovar assignation, if known, is indicated by the letter code A, B or C. PVD-pHi values of the PVD-isoform bands separated by isoelectrophoresis are indicated as bars with thickness proportional to the intensity of fluorescence demonstrated under UV-light illumination (see Fig. 1).

Sv. no.	Strain	bv.	PVD-pHi value					Iron uptake as mediated by PVD of sv. no.*									
			7.5	8.0	8.5	9.0	9.5	26	27	28	29	30	31	32	33	34	35
26	D-S1026	A															
26	D-LR223	A															
26	D-S824	A															
26	D-LR228	A															
26	D-TRP621	A															
26	D-LE3216	A															
26	D-S131	A															
26	D-TR331	A															
26	D-LRp414	A															
26	D-LRp212	A															
26	D-LE611	A															
26	D-LRp511	A															
26	D-LE414J	A															
26	D-TR1035	A															
26	D-TE612	A															
26	D-LE711	A															
26	D-LRp 921	A															
26	GB 11379	A							—	—	—	—	—	—	—	—	—
26	GM 11274	A															
26	9B W																
26	[<i>P. j.</i> CFML 95-307]																
27	CFML 90-70																
27	CFML 90-71																
27	CFML 90-73																
27	CFML 90-74																
27	CFML 90-75																
27	CFML 90-77																
27	CFML 90-78																
27	CFM L 90-79																
27	CFM L 90-80																
27	CFM L 90-82																
27	CFM L 90-83								—		—	—	—	—	—	26	—
27	MF 6313	C															
27	MF H2-127	C															
27	RW 5S2																
27	BW 11M 1																
28	C-S111	A															
28	C-S413	A															
28	C-S215	A															
28	C-LRp214	A															
28	PGSB 8301																
28	GS 12082								—	—		—	—	—	88	—	35
28	[<i>P. m.</i> CFML 95-303]																
29	A10L	B															
29	GB 11349	B							—	—	—		—	—	—	—	—
29	GB 11395	B															
29	10CW																
29	[<i>P. fl.</i> ATCC 13525]																

Table 4. cont.

Sv. no.	Strain	bv.	PVD-pHi value					Iron uptake as mediated by PVD of sv. no.*									
			7.5	8.0	8.5	9.0	9.5	26	27	28	29	30	31	32	33	34	35
30	CFML 90-135																
30	CFML 90-136							—	—	—	—		—	—	—	—	—
30	CFML 90-138																
31	GS 12070	A															
	Gwose							—	—	—	—	—		—	—	—	—
32	GB 11366	B						—	—	98	—	—	—		—	—	44
33	BW16M1							—	—	—	—	—	—	—		—	—
34	GM 12220							12	—	10	—	—	—	88	—		28
35	GM 12059							—	—	—	—	—	—	25	—	72	

*See footnote for Table 2.

characteristics. The corresponding pyoverdines were highly specific in iron transport in addition to their producing strains, as only a very few heterologous uptakes were observed (Table 3, right panel). Among the six structurally known pyoverdines (Table 5, pyoverdines of siderovars 15, 17, 18, 20, 23 and 25), one corresponded to an isopyoverdine [PVD(CFML 90-44) of sv. 17], whereas three had Asp as the N-terminal amino acid of their respective peptide chain. Siderovar 14, the most important siderovar as determined by the number of strains (17), was formed by strains that originated from three different collections. Interestingly, the CFML strains of sv. 14, first identified as isolates of *P. putida*, have been since recognized as representatives of a novel species, *Pseudomonas monteilii* (Elomari *et al.*, 1997). Thus, the pyoverdine of sv. 14 was assigned to *Pseudomonas monteilii* and consequently the other strains of sv. 14 could be expected to also belong to this species. Heterologous uptakes involving the collection of structurally known pyoverdines showed that 10 of the 12 neutral pyoverdines were highly specific to their respective producing strains, while pyoverdines produced by strains of sv. 18 and sv. 23 corresponded to the pyoverdines of *Pseudomonas* sp. B10 (Teintze *et al.*, 1981) and *P. fluorescens* PL7 (Barelmann *et al.*, 2002), respectively.

Siderovars with alkaline pyoverdines

According to PVD-IEF patterns and cross-uptakes, 10 siderovars were obtained from 54 strains of *P. putida* presenting alkaline profiles for their respective pyoverdines, i.e. in most cases (Table 4, middle panel), one or two isoform-bands at pHi values between 7.0 and 8.0 and one or two isoform-bands at pHi values higher than 8.0. The predominant siderovar, sv. 26, containing 21 strains originating from five collections, was characterized, thanks to PVD-IEF and heterologous uptake studies, by the

production of a pyoverdine identical to the one already described for *P. fluorescens* 9AW and for *P. putida* 9BW (Budzikiewicz *et al.*, 1997). Furthermore, this pyoverdine was later associated with the species *P. jessenii* (Meyer *et al.*, 2002). The second siderovar in size (sv. 27, 15 strains originating from three collections) was for similar reasons correlated with *P. mosselii*, while the third (sv. 28) corresponded to the siderotype of *P. mandelii* (Meyer *et al.*, 2002). Two other siderovars, sv. 29 and sv. 34, were shown to correspond to pyoverdines characterizing the type strains of *P. fluorescens* and *P. chlororaphis* for sv. 29 and *P. fluorescens* strain PL8 for sv. 34 (Hohlneicher *et al.*, 1995; Barelmann *et al.*, 2002). In contrast to the situation found for the two other pyoverdine groups, none of the structurally known pyoverdines produced by these strains started their NH₂-terminal peptidic part with an Asp residue. Finally, as for the neutral pyoverdines, a high specificity of recognition was usually the rule between strains and their respective pyoverdines, as can be deduced from the low number of cross-incorporations detailed in Table 4, right panel.

Correlations between siderovars and bacterial species

Among *P. putida* isolates, some have been reclassified into novel species, namely *P. monteilii* (Elomari *et al.*, 1997) and *P. mosselii* (Dabboussi *et al.*, 2002). The DNA-DNA hybridization values between the strains used in the present study and these two species are reported in Table 6 (strains CFML 90-60^T and CFML 90-83^T for *P. monteilii* and *P. mosselii*, respectively). Additional hybridization data obtained with the DNAs of strains ATCC 12633^T (representative of phenon IIb, ribotype G), CFML 90-48 (phenon IIb, ribotype E), CFML 90-136 (phenon IId, ribotype B) and CFML 90-34 (phenon IIa, ribotype A) are also reported. DNA from strains belonging to these

Table 5. Pyoverdine structure and species correlation characterizing some of the *Pseudomonas putida* siderovars

Dab, Diaminobutyric acid; OHAsp, *threo*-b-hydroxy-Asp; OHHis, *threo*-b-hydroxy-His; OHOrn, *d*N-hydroxy-Orn, AOHOOrn, *d*N-acetyl-OHOOrn; BOHOOrn, *d*N-butyryl-OHOOrn; FOHOOrn, *d*N-formyl-OHOOrn; cOHOOrn, *cyclo*-OHOOrn (3-amino-1-hydroxy-piperidone-2); aThr, *allo*-Thr. D-amino acids are underlined. Half of the serine residues in PVD(ATCC 12633^T) and PVD(CFML 90-44) are of the D-form, but with enantiomer positions not determined (Budzikiewicz, 2004).

sv. no.	sv. type strain	PVD-peptide structure*	Identity with PVD-type	Species affiliation of the PVD-type	Reference
1	GB 11370	Asp-Lys-OHAsp-Ser-Ala-Ser-cOHOOrn	G176 (=GB 11370)	<i>P. putida</i>	Budzikiewicz <i>et al.</i> (1999)
2	PutC	Asp-BOHOOrn-Dab-Thr-Gly-Ser-Ser-OHAsp-aThr	PutC	<i>P. putida</i>	Seinsche <i>et al.</i> (1993)
3	CFML 90-40	Asp-Ala-Asp-AOHOOrn-Ser-cOHOOrn	BTP1 (=CFML 90-40)	<i>P. putida</i>	Jacques <i>et al.</i> (1995)
4	CFML 90-33	Asp-Lys-Thr-OHAsp-Thr-aThr-cOHOOrn	CFML 90-33	<i>P. putida</i>	Sultana <i>et al.</i> (2001b)
5	DSM 3602	Ser-AOHOOrn-Ala-Gly (Ser-Ala-OHAsp-Thr)	A214	<i>Pseudomonas</i> sp.	Uria-Fernandez <i>et al.</i> (2003)
8	ATCC 12633 ^T	Asp-Lys-OHAsp-Ser-Thr-Ala-Glu-Ser-cOHOOrn	ATCC 12633 ^T	<i>P. putida</i>	Persmark <i>et al.</i> (1990)
15	CFML 90-51	Asp-Lys-OHAsp-Ser-Gly aThr-Lys-cOHOOrn	CFML 90-51	<i>P. putida</i>	Sultana <i>et al.</i> (2000)
17	CFML 90-44	Asp-Lys-AOHOOrn-Thr-Ser-Ser-Gly Ser-Ser-cOHOOrn	CFML 90-44	<i>P. putida</i>	Sultana <i>et al.</i> (2001a)
18	GB 11341	Lys-OHAsp-Ala-aThr-Ala-cOHOOrn	B10	<i>Pseudomonas</i> sp.	Teintze <i>et al.</i> (1981)
20	L1	Asp-Lys-OHAsp-Ser-aThr-Ala-Thr-Lys-cOHOOrn	L1	<i>P. putida</i>	Uria-Fernandez <i>et al.</i> (2003)
23	GB 11394	Ser-AOHOOrn-Ala-Gly aThr-Ala-cOHOOrn	PL7	<i>P. fluorescens</i> bv. V	Barelmann <i>et al.</i> (2002)
25	KT2440	OHAsp-Lys-OHAsp-Dab-Gly Ser-cOHOOrn*	KT2440	<i>P. putida</i>	Ravel & Cornelis (2003)
26	GB 11379	Ser-Lys-OHHis-aThr-Ser-cOHOOrn	9AW	<i>P. fluorescens</i> / <i>P. jessenii</i>	Budzikiewicz <i>et al.</i> (1997)
28	GS 12082	Ala-Lys-Thr-Ser-AOHOOrn-cOHOOrn	SB8.3	<i>P. fluorescens</i> / <i>P. mandelii</i>	Budzikiewicz <i>et al.</i> (1992)
29	GB 11349	Ser-Lys-Gly FOHOOrn-c(Lys-FOHOOrn-Ser)	ATCC 13525	<i>P. fluorescens</i> bv. I	Hohlneicher <i>et al.</i> (1995)
31	Gwose	Ser-Thr-Ser-Orn-OHAsp-(Gln-Dab)-Ser-aThr-cOHOOrn	Gwose	<i>P. putida</i>	Gwose & Taraz (1992)
34	GM 12220	Lys-AOHOOrn-Ala-Gly aThr-Ser-cOHOOrn	PL8	<i>P. fluorescens</i> bv. V	Barelmann <i>et al.</i> (2002)

*Hypothetical structure without amino acid stereochemistry based on *in silico* studies of genome sequences in *P. putida* KT2440 (Ravel & Cornelis, 2003).

phenotypic groups was also hybridized against the labelled DNA of these six strains. As shown in Table 6, the strain groupings obtained by siderotyping correlated perfectly for a large majority of strains, with a clustering based on phenotypic features or DNA–DNA hybridization. Phenons IIc and IV, corresponding to the *P. monteilii* and *P. mosselii* species, respectively, formed well-circumscribed DNA–DNA relatedness groups and also formed well-characterized siderovars as shown in Tables 3 and 4 (sv. 14 and sv. 27). The same conclusion was reached for the three strains of phenon IIb, ribotype E, which formed a separate DNA–DNA hybridization group and produced an identical pyoverdine (sv. 15). Moreover, the two separate phenons

IIa and IIc, which would be expected to correspond to very closely related strains according to their DNA–DNA hybridization values (Table 6) were, nevertheless, distinguished as two separate siderovars by siderotyping. The ATCC 12633^T hybridization group, however, included seven strains that were characterized by four different siderovars (sv. 8 and sv. 15 for one strain each, sv. 17 for two strains and sv. 3 for three strains). Thus, while many clusters and species each corresponded to a specific siderovar, the species *Pseudomonas putida sensu stricto* should presently be seen as a group of strains characterized by at least four siderovars, with the sv. 15 siderotype being shared with another closely related taxonomic group. Such siderovar

Table 6. Correlations between siderotyping and DNA–DNA hybridization for strains grouped according to phenotyping and ribotyping

Values in bold type show the grouping of strains in species according to the DNA–DNA hybridization method (values between 70 and 100%). Phenotyping and ribotyping are according to Elomari *et al.* (1994) and Dabboussi *et al.* (2002).

Strain	Phenon and ribotype	sv.	% DNA–DNA hybridization with labelled DNA of strain:					Species correlation	
			CFML 90-60 ^T	CFML 90-83 ^T	ATCC 12633 ^T	CFML 90-48	CFML 90-136		CFML 90-34
CFML 90-54	IIc/C	14	94*		23	39	25	37	<i>P. monteilii</i>
CFML 90-55	IIc/C	14	86*		46	56	20	52	
CFML 90-56	IIc/D	14	99*		50	44	27	39	
CFML 90-57	IIc/C	14	86*		30	47	29	54	
CFML 90-58	IIc/C	14	95*		45	38	32	41	
CFML 90-59	IIc/C	14	91*		55	25	22	29	
CFML 90-60 ^T	IIc/C	14	100*	49*	37	46	30	42	
CFML 90-61	IIc/C	14	93*		55	56	46	34	
CFML 90-62	IIc/C	14	94*		56	58	56	43	
CFML 90-63	IIc/D	14	87*		49	54	52	53	
CFML 90-83 ^T	IV	27		100*	38	41	21	32	<i>P. mosselii</i>
CFML 90-74	IV	27		88*	35	36	40	38	
CFML 90-78	IV	27		84*	46	42	45	43	
CFML 90-70	IV	27		81*	42	35	23	54	
CFML 90-80	IV	27		80*	46	34	49	46	
CFML 90-82	IV	27		79*	33	14	47	35	
CFML 90-75	IV	27		77*	41	40	40	26	
CFML 90-71	IV	27		75*	55	36	25	47	
CFML 90-77	IV	27		74*	44	37	23	38	
CFML 90-73	IV	27		73*	40	25	49	41	
CFML 90-79	IV	27		73*	39	39		40	
ATCC 12633 ^T	IIb/E	8	40	40	100	60	30	20	<i>P. putida</i> (<i>sensu stricto</i>)
ATCC 17485	IIb/E	3			97	55	33	25	
CFML 90-40	IIb/G	3	35	29	97	55	32	50	
CFML 90-52	IIb/G	3	50	25	73	51	53	51	
CFML 90-46	IIb/E	17	54	22	84	64	55	49	
CFML 90-47	IIb/E	17	37	26	79	48	28	54	
CFML 90-49	IIb/E	15	50	19	84	67	48	38	
CFML 90-48	IIb/E	15	45		56	100	36	47	Not defined
CFML 90-50	IIb/E	15	35		57	92	56	56	
CFML 90-51	IIb/E	15	36		60	92	30	46	
CFML 90-135	IIId/B	30	44		42	40	83	65 (5.4 °C)	Not defined
CFML 90-136	IIId/B	30	42		44	44	100	68 (5.3 °C)	
CFML 90-138	IIId/B	30	40		56	47	77	68 (5.2 °C)	
CFML 90-33	IIa/A	4	42		45	35	89	82	Not defined
CFML 90-34	IIa/A	4	40		36	43	68 (1.6 °C)	100	
CFML 90-139	IIa/A	4	49		42	37	87	86	

*Hybridization values are taken from Elomari *et al.* (1997) and Dabboussi *et al.* (2002).

multiplicity within one species, although not common, has already been described for *Pseudomonas aeruginosa* (Meyer *et al.*, 1997) and for *Pseudomonas lini* (Delorme *et al.*, 2002).

On the other hand, strains belonging to different species but sharing identical pyoverdines have also been described (Hohlneicher *et al.*, 1995).

Fig. 2. Structural phylogeny among the pyoverdines of strains belonging to the species *Pseudomonas putida sensu stricto*. For explanation of the abbreviations, see the footnote for Table 5.

Structural features of the *P. putida* pyoverdines and their relationship with taxonomy

Of the 35 pyoverdines identified within the collection of *P. putida* strains, 16 are already structurally known. Their respective peptide chains are described in Table 5. In addition, the KT2440 pyoverdine, with a peptidic part deduced from a genomic analysis rather than a chemical analysis (Ravel & Cornelis, 2003), has been included in Table 5. From a comparison of these structures, several major features can be identified:

(i) with the exception of the pyoverdine produced by *P. putida* strain Gwose of sv. 31, only pyoverdines produced by strains of *P. putida* (nine strains marked in bold type in Table 5) present an aspartyl residue [hydroxy-aspartyl for PVD(KT2440)] as the N-terminal amino acid directly linked to the chromophore;

(ii) pronounced structural similarities exist between many pyoverdines of *P. putida* with, for instance, six pyoverdines (of sv. 1, 4, 8, 15, 17, 20) have a Lys following Asp and among them, four (sv. 1, 8, 15, 20) present the same Asp-Lys-OHAsp-Ser sequence that begins the peptidic part containing 7 to 9 amino acid residues, depending on the strain;

(iii) when focusing on the pyoverdines that characterize the four siderovars of the species *P. putida sensu stricto*, namely PVD(CFML 90-40) of sv. 3, PVD(ATCC 12633^T) of sv. 8, PVD(CFML 90-51) of sv. 15 and PVD(CFML 90-44) of sv. 17, structural relationships between the four compounds can be established by a dichotomic tree based on the primary sequences of the different peptide chains as illustrated in Fig. 2. As the biosynthesis of pyoverdines implies amino-acyl specific domains within multi-functional peptide synthetases (Ravel & Cornelis, 2003), it can be assumed that strains producing structurally related pyoverdines should contain common DNA sequences corresponding to common amino-acyl specific domains and thus such strains should be phylogenetically related. Thus, siderovar multiplicity within the species *P. putida sensu stricto* could be seen as the result of gene evolution particularly affecting the peptide synthetase genes involved in pyoverdine biosynthesis;

(iv) the other pyoverdines that do not begin with Asp as the N-terminal amino acid, but with Ala for sv. 28, Lys for

sv. 18 and sv. 34, or D-Ser for four siderovars (sv. 5, 23, 26, 29), actually corresponded to compounds first described as produced by strains of *P. fluorescens* (sv. 23, 26, 28, 29, 34) or by strains with no defined species attribution (sv. 5 and sv. 18) (Table 5). Among these siderovars, three (sv. 23, 29 and 34) are formed exclusively by strains identified as *P. putida* biovar B. The other siderovars (sv. 5, 18, 26, 28) contain many strains belonging (when known) to biovar A, among them a majority of strains sharing the same siderotype as *P. jessenii* (sv. 26) or *P. mandelii* (sv. 28) (Verhille *et al.*, 1999).

Thus, siderotyping allows the recognition of a major core of siderovars, i.e. the Asp-pyoverdine group, corresponding to taxa with strong phylogenetic relationships and with some of them even included within the same taxon, i.e. the species *P. putida sensu stricto*. Other siderovars corresponded to clusters which remain to be defined as novel species according to phenotyping, ribotyping and DNA–DNA hybridization, e.g. phenons IIa/A (sv. 4), IIb/B (sv. 30) and IIb/E with strains CFML 90-48, 90-50 and 90-51 (sv. 15). In addition, a second group, including siderovars correlating to more distantly related species such as *P. jessenii* and *P. mandelii* and also including siderovars closely related to the species *P. fluorescens*, was recognized among the collection of strains analysed. In the majority of cases, the *P. putida* isolates received as biovar A fell in siderovars which characterized the first group and part of the second group, e.g. sv. 26 and sv. 28 corresponding to *P. jessenii* and *P. mandelii*. In contrast, *P. putida* biovar B strains preferentially contributed to the siderovars of the second group related to the *P. fluorescens* lineage, a correlation that is in full agreement with the numerous taxonomical studies that state that *P. putida* biovar B strains are phylogenetically closer to *P. fluorescens* than to *P. putida* biovar A strains (Stanier *et al.*, 1966; Yamamoto & Harayama, 1998; Yamamoto *et al.*, 2000; Hilario *et al.*, 2004).

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