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Siderotyping of fluorescent *Pseudomonas*: molecular mass determination by mass spectrometry as a powerful pyoverdine siderotyping method

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Abstract The numerous pyoverdines so far characterized as siderophores of fluorescent *Pseudomonas* could be usually differentiated one from each others by the two physico-chemical and physiological methods of siderotyping, i.e., siderophore-isoelectrofocusing and siderophore-mediated iron uptake. As shown in the present paper, the structural diversity of the peptide chain characterizing these molecules results in a very large panel of molecular masses representing 64 different values ranging from 889 to 1,764 Da for the 68 compounds included in the study, with only a few structurally different compounds presenting an identical molecular mass. Thus, the molecular mass determination of pyoverdines through mass spectrometry could be used as a powerful siderotyping method.

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Introduction

Pyoverdines, the fluorescent pigments and siderophores of the fluorescent *Pseudomonas* (Meyer 2000), present the most complex chemical structures among siderophores. The molecules are composed of a chromophore (commonly (1 *S*)-5-amino-2,3-dihydro-8,9-dihydroxy-1*H*-pyrimido-[1,2-*a*]quinoline-1-carboxylic acid), with the carboxylic group branched to a peptide chain and the amino group branched to a dicarboxylic acid (or its amide) chain, e.g., succinic acid or malic acid as the most frequent ones (Budzikiewicz 2004). The iron complexation properties of the molecule are provided by the chromophore thanks to a catecholate function, while two hydroxamate groups, partially or totally replaced by hydroxycarboxyl groups in some pyoverdines, are delivered by the peptide chain (Budzikiewicz 2004).

Since the first pyoverdine structure elucidated at the beginning of the 1980s (Teintze et al. 1981), about 50 structurally different pyoverdines have been described so far, each presenting a particular peptide chain varying by the type and number (6–12) of aminoacyl residues. Because of this huge diversity and to overcome redundancies among structural studies, rapid methods of pyoverdine differentiation were elaborated (Meyer et al. 1998; Fuchs et al.

2001). The two most practical and most used ones are (1) the analysis by isoelectrophoresis of the pyoverdine content of iron-starved bacterial culture supernatants (PVD-IEF, for pyoverdine isoelectrofocusing), and (2) the determination of bacterial specificity of the pyoverdine-mediated iron uptake. The two methods already allowed the recognition of numerous original compounds representing most of the 50 structurally defined pyoverdines presently known and about 60 additional compounds with still unknown structures (J.M. Meyer, unpublished results). For most of these compounds, the two methods of siderophore-typing (or shorter, siderotyping) resulted in a specific PVD-IEF pattern together with an iron-uptake capacity strictly limited to the producing strain, thus allowing an unambiguous pyoverdine differentiation. A few exceptions have been observed for some pyoverdines sharing an identical IEF pattern but having each a specific iron uptake behavior, or for some others presenting on the contrary strong cross-incorporation capacities with their respective producing strains, but presenting each a specific PVD-IEF pattern (Schlegel et al. 2001; Meyer et al. 2002b). Thus, the concomitant use of the two siderotyping methods allowed a clear discrimination of these pyoverdines, despite of their respective similarities.

Among the structurally known pyoverdines there are, however, a few which could not be distinguished one from the other because of identical IEF- and iron uptake behaviors. Such closely related siderotyping features are due to very small structural differences between these compounds. The few examples which have been encountered so far concern the pyoverdine of *Pseudomonas aeruginosa* strain R′ which has one Gln residue in its peptide chain (Ruangviriyachai et al. 2001), while strain *P. aeruginosa* R produces an almost identical pyoverdine, but with two Gln residues in its peptide chain (Gipp et al. 1991). Another example concerns *Pseudomonas cichorii* pyoverdine which has a Gly residue in its peptidic part replaced by a Ser residue in the *Pseudomonas syringae* pyoverdine, all the other components being strictly identical within the two compounds (Bultreys et al. 2004). These discrete changes occurring at the level of neutral aminoacyl residues do not affect the overall charges of the molecules and thus did not affect their IEF patterns. Meanwhile, the recognition of the iron complexes of these closely related pyoverdines by their corresponding outer membrane receptor proteins was apparently not affected since the iron transport properties of these compounds remained identical.

Indeed, because of these exceptions, another method of siderotyping would be of interest to easily discriminate pyoverdines with a maximal accuracy among the more than 100 pyoverdine compounds so far distinguished by siderotyping. Moreover, since pyoverdines have been seen as powerful taxonomic markers allowing an easy discrimination of fluorescent *Pseudomonas* at the species level (Meyer et al. 2002a), a supplementary siderotyping method for an accurate grouping of strains into siderovars (strains producing an identical pyoverdine) would be welcome also for strain characterization and species definition within the *Pseudomonas* genus. It is the purpose of the present work to demonstrate that the determination of the molecular mass of pyoverdines by mass spectrometry should be considered as a third powerful siderotyping method which could favorably complete the physicochemical discrimination of compounds within this important siderophore family.

Materials and methods

Bacterial strains

About 68 fluorescent *Pseudomonas* strains producing each a particular pyoverdine are described in the present study, among them 50 with pyoverdine structure fully determined and already published (see references in Table 1), 14 with pyoverdine structures partially known, most of them determined during the present study, and 4 with pyoverdine structures presently still undetermined. Of the 68 strains producing these pyoverdines, 65 were obtained from different laboratories or type culture collections where they have been more or less taxonomically characterized. Their respective PVD-IEF pattern and iron uptake specificity were determined in the present study. The three additional strains, producing pyoverdines $n = 3$, 40, and 51 in Table 1, were no more available at the time of the present study. Of the 65 workable strains, 25 were received as belonging to the *Pseudomonas fluorescens* species, 16 as belonging to the *Pseudomonas*

Table 1 Pyoverdines classified according to their respective molecular mass with their isoelectrofocusing characteristics and peptide composition

PVD N°	Strain	Species	MM	Number of isoforms and pHi values		Pyoverdine peptide chain	References
1	B10	*Pseudomonas* sp.	989	3	7.5/5.2/4.6	εLys–OHAsp–Ala–aThr–Ala–cOHOrn	Teintze et al. (1981)
2	GS43	*P. putida*	1,007	2	7.2/4.4	Lys–OHAsp–Ser–Ser–Ser–cOHOrn	This study
3	Thai	*P. putida*	1,016	nd	nd	(Ser–Dab)–Thr–Ser–AOHOrn–cOHOrn	Ruangviriyachai et al. (2004)
4	9AW	*P. fluorescens*	1,043	3	9.2/8.7/7.7	Ser–εLys–OHHis–aThr–Ser cOHOrn	Budzikiewicz et al. (1997)
5	R′	*P. aeruginosa*	1,045	2	7.3/5.2	(Ser–Dab)–FOHOrn–Gln–FOHOrn–Gly	Ruangviriyachai et al. (2001)
6	PL7	*P. fluorescens*	**1,046**	3	7.7/7.5/4.3	Ser–AOHOrn–Ala–Gly–aThr–Ala–cOHOrn	Barelmann et al. (2002)
7	Ps4a	*P. fluorescens*	**1,046**	3	9.0/8.8/7.6	Ala–Lys–Thr–Ser–AOHOrn–cOHOrn	Budzikiewicz et al. (1992)
8	BTP1/90-40	*P. putida*	1,047	2	4.2/3.9	Asp–Ala–Asp–AOHOrn–Ser–cOHOrn	Jacques et al. (1995)
9	BTP2	*P. fluorescens*	1,049	2	4.9/3.9	Ser–Val–OHAsp–Gly–Thr–Ser–cOHOrn	Ongena et al. (2001)
10	G4R	*P. putida*	1,073	2	7.6/5.4	Asp–Orn–(OHAsp–Dab)–Gly–Ser–cOHOrn	Salah-el-Din et al. (1997)
11	2908	*Pseudomonas* sp.	1,088	3	7.5/5.0/4.8	Ser–Orn–OHAsp–Ser–Ser–Ser–cOHOrn	Vossen and Taraz (1999)
12	Ps 6-10	*Pseudomonas* sp.	**1,091**	3	8.9/7.6/4.6	Ala–Orn–OHAsp–Dab–AOHOrn–Lys	Budzikiewicz et al. (2006)
13	Pa 27853	*P. aeruginosa*	**1,091**	4	8.9/8.5/7.5/7.4	Ser–FOHOrn–Orn–Gly–aThr–Ser–cOHOrn	Tappe et al. (1993)
14	96-195	*P. libanensis*	**1,091**	2	9.5/7.6	Ala–Orn–OHAsp–Ser–Orn–Ser–cOHOrn	This study
15	P. cic	*P. cichorii*	1,093*	2	5.0/4.0	εLys–OHAsp–Thr–(Thr–Gly–OHAsp–Ser)	Bultreys et al. (2004)
16	PL8	*P. fluorescens*	1,103	2	8.9/7.7	Lys–AOHOrn–Ala–Gly–aThr–Ser–cOHOrn	Barelmann et al. (2002)
17	11370	*P. putida*	1,105	3	5.2/4.5/3.9	Asp–εLys–OHAsp–Ser–Ala–Ser–cOHOrn	Budzikiewicz et al. (1999)
18	G85	*Pseudomonas* sp.	1,121	1	3.9	Ser–Lys–OHAsp–Ser–Orn–Ser–cOHOrn	This study
19	Syr 19310	*P. syringae*	1,123*	2	5.0/4.0	εLys–OH Asp–Thr–(Thr–Ser-OH Asp–Ser)	Jülich et al. (2001)
20	ATCC 39167	*P. putida*	1,134*	3	5.0/4.6/4.1	Ser–AOHOrn–Ala–Gly–(Ser–Ala–OHAsp–Thr)	Uría-Fernández et al. (2003b)
21	PL9	*P. fluorescens*	1,150*	3	5.0/4.5/4.0	Ser–AOHOrn–Ala–Gly–(Ser–Ser–OHAsp–Thr)	Uría-Fernández et al. (2003b)
22	Pfl 13525	*P. fluorescens*	1,160	3	8.7/7.3/7.1	Ser–Lys–Gly–FOHOrn–(Lys–FOHOrn–Ser)	Hohlneicher et al. (1995)
23	90-33	*P. putida*	1,164	2	5.0/3.9	Asp–Lys–Thr–OHAsp–Thr–aThr–cOHOrn	Sultana et al. (2001b)
24	Pa6 (R)	*P. aeruginosa*	1,173	2	7.3/5.2	(Ser–Dab)–FOHOrn–Gln–Gln–FOHOrn–Gly	Gipp et al. (1991)
25	G173	*P. fluorescens*	1,175	3	5.3/4.5/3.9	Ser–Ala–AOHOrn–(Orn–Asp–AOHOrn–Ser)	Uría-Fernández et al. (2003a)
26	G166	*P. fluorescens*	1,185*	4	5.4/5.0/4.7/4.5	Undetermined	This study
27	Pfl W	*P. fluorescens*	1,187	2	7.4/5.2	Ser–Dab–Gly–Ser–OHAsp–Ala–Gly–Ala–Gly–cOHOrn	Demange et al. (1990)
28	96-312	*Pseudomonas* sp.	1,190	4	8.6/8.5/7.7/7.4	Ser–Ser–FOHOrn–(Lys–FOHOrn–Lys–Ser)	Schlegel et al. (2001)
29	D47	*Pseudomonas* sp.	1,218	3	7.3/5.3/5.2	Ser–Orn–FOHOrn–(Lys–FOHOrn–Glu–Ser)	Schäfer et al. (2006)
30	D-TR133	*P. chlororaphis*	1,230*	3	7.5/5.4/4.1	Asp–FOHOrn–Lys–(Thr–Ala–Ala–FOHOrn–Ala)	Barelmann et al. (2003)
31	96-188	*Pseudomonas* sp.	1,232	3	7.3/5.3/5.2	Ser–Lys–FOHOrn–(Lys–FOHOrn–Glu–Ser)	Weber et al. (2000)
32	90-51	*P. putida*	1,234	3	7.3/7.0/5.3	Asp–εLys–OHAsp–Ser–Gly–aThr–Lys–cOHOrn	Sultana et al. (2000b)

Table 1 continued

PVD N°	Strain	Species	MM	Number of isoforms and pHi values		Pyoverdine peptide chain	References
33	G76	*Pseudomonas* sp.	1,236	4	7.2/5.1/4.8/4.0	Ser–Ser–FOHOrn–Ser–Ser–(Lys–Ser–FOHOrn)	This study
34	HR6	*Pseudomonas* sp.	1,238	3	4.9/4.4/4.0	Asp–εLys–OHAsp–Ser–Ser–Thr–Thr–Thr–cOHOrn	This study
35	LBSA1	*Pseudomonas* sp.	1,260	2	5.1/4.6	Asp–Arg–AOHOrn–Lys–Ser–Asp–cOHOrn	This study
36	ML45	*P. thivervalensis*	1,261	3	7.6/5.2/5.0	Undetermined	This study
37	96-318	*Pseudomonas* sp.	1,263	2	8.5/7.3	Ser–Orn–FOHOrn–Ser–Ser–(Lys–FOHOrn–Ser)	Schlegel et al. (2001)
38	P.au	*P. aureofaciens*	1,277	3	7.6/5.2/4.9	Ser–AOHOrn–Gly–aThr–Thr–Gln–Gly–Ser cOHOrn	Beiderbeck et al. (1999a)
39	Pfl 1.3	*P. fluorescens*	1,285	2	9.0/7.5	Ala–Lys–Gly–Gly–OHAsp–(Gln–Dab)–Gly–Ser–cOHOrn	Georgias et al. (1999)
40	Pfl "ng"	*P. fluorescens*	1,286	nd	nd	Asn–FOHOrn–Lys–(Thr–Ala–Ala–FOHOrn–Lys)	Poppe et al. (1987)
41	CHA0	*P. fluorescens*	1,287	3	8.5/7.5/5.1	Asp–FOHOrn–Lys–(Thr–Ala–Ala–FOHOrn–Lys)	Wong-Lun-Sang et al. (1996)
42	Lille 1	*P. monteilii*	1,291	2	7.3/4.6	Asp–Lys–AcOHOrn–Ala–Ser–Ser–Gly–Ser–cOHOrn	This study
43	G153	*P. fluorescens*	1,293	3	9.0/7.5/7.4	Ser–Lys–Ala–Ser–Ser–AcOHOrn–Ser–Ser–cOHOrn	This study
44	Pfl 17400	*P. fluorescens*	1,299	2	9.2/7.6	Ala–Lys–Gly–Gly–OHAsp–(Gln–Dab)–Ser–Ala–cOHOrn	Demange et al. (1990)
45	G17	*P. fuscovaginae*	1,316	4	8.9/7.4/5.1/4.3	2 βOHAsp, 2 Gly, 2 Thr, 1 Dab, 1 Ala, 1 Lys,	This study
46	A6	*P. fluorescens*	1,318	2	8.9/7.6	Lys–AOHOrn–Gly–aThr–Thr–Gln–Gly–Ser–cOHOrn	Beiderbeck et al. (1999b)
47	PAO1	*P. aeruginosa*	1,333	2	8.8/7.0	Ser–Arg–Ser–FOHOrn–(Lys–FOHOrn–Thr–Thr)	Briskot et al. (1989)
48	G172	*P. putida*	1,335	2	4.9/4.1	Ala–Lys–Dab–OHAsp– (Thr–Gly–OHAsp–Gly–Thr–Thr)	This study
49	ATCC 12633	*P. putida*	1,336	3	4.6/4.2/4.0	Asp–εLys–OHAsp–Ser–Thr–Ala–Glu–Ser–cOHOrn	Persmark et al. (1990)
50	L1	*P. putida*	1,349	3	7.5/5.3/5.0	Asp–εLys–OHAsp–Ser–aThr–Ala–Thr–Lys–cOHOrn	Uría-Fernández et al. (2003b)
51	3b	*P. putida*	1,358	nd	nd	Asp–(AOHOrn–Dab)–Thr–Ala–Thr–Thr–Gln–cOHOrn	Budzikiewicz (2004)
52	95-275	*P. fluorescens*	1,364	3	8.5/8.4/7.3	Ser–Ser–FOHOrn–Ser–Ser–(Lys–FOHOrn–Lys–Ser)	Sultana et al. (2000a)
53	PutC	*P. putida*	1,370	4	5.0/4.6/4.2/4.0	Asp–OHbutOHOrn–Dab–Thr–Gly–Ser–Ser–OHAsp–Thr	Seinsche et al. (1993)
54	51W	*P. fluorescens*	1,375	2	7.7/5.1	Ala–Lys–Gly–Gly–OHAsp–Gln–Ser–Ala–Gly–aThr–cOHOrn	Voss et al. (1999)
55	DSM 50106	*P. fluorescens*	1,377	3	8.6/8.5/7.0	Ser–Lys–Gly–FOHOrn–Ser–Ser–Gly–(Orn–FOHOrn–Ser)	This study
56	Pf0-1	*P. fluorescens*	1,381	3	7.5/5.2/4.0	Ala–AcOHOrn–Orn–Ser–Ser–Ser–Arg–OHAsp–Thr	This study
57	Pfl18.1	*P. fluorescens*	1,391	3	8.5/7.4/7.1	Ser–Lys–Gly–FOHOrn–Ser–Ser–Gly–(Lys–FOHOrn–Ser)	Amann et al. (2000)
58	Gwose	*P. fluorescens*	1,405	2	9.2/7.6	Ser–Thr–Ser–Orn–OHAsp–(Gln–Dab)–Ser–aThr–cOHOrn	Gwose and Taraz (1992)
59	90-44	*P. putida*	1,408	4	7.1/4.8/4.3/3.9	Asp–Lys–AOHOrn–Thr–Ser–Ser–Gly–Ser–Ser–cOHOrn	Sultana et al. (2001a)
60	Lille 25	*P. rhodesiae*	1,421	4	8.2/8.0/7.1/7.0	Ser–Lys–FOHOrn–Ser–Ser–Gly–(Lys–FOHOrn–Ser–Ser)	Budzikiewicz (2004)
61	Tol	*P. tolaasii*^{T}	**1,424**	3	8.9/7.5/7.3	Ser–Lys–Ser–Ser–Thr–Ser–AcOHOrn–Thr–Ser–cOHOrn	Demange et al. (1990)
62	90-136, G168	*P. putida*	**1,424**	3	8.9/8.7/7.4	Ser–Lys–Ser–Ser–Thr–Thr–AcOHOrn–Ser–Ser–cOHOrn	This study
63	Pflii	*P. fluorescens*	1,430	2	7.6/5.1	Ala–Lys–Gly–Gly–OHAsp–Gln–Ser–Ala–Ala–Ala–Ala–cOHOrn	Mohn et al. (1990)
64	G400	*Pseudomonas* sp.	1,509	3	8.9/8.7/7.5	Undetermined	This study

Table 1 continued

PVD N°	Strain	Species	MM	Number of isoforms and pHi values	Pyoverdine peptide chain	References
65	Pfl12	*P. fluorescens*	1,520	3 7.1/4.9/4.8	Ser–Lys–Gly–FOHOrn–Ser–Ser–Gly–(Lys–FOHOrn–Glu–Ser)	Geisen et al. (1992)
66	1547	*P. fluorescens*	1,547	2 8.9/7.3	Ser–Lys–Ala–AOHOrn–Thr–Ala–Gly–Gln–Ala–Ser–Ser–cOHOrn	Ruangviriyachai et al. (2000)
67	GS37	*P. putida*	1,651	2 7.5/4.7	Undetermined	This study
68	IB3	*Pseudomonas* sp.	1,764	2 9.3/8.7	Ser–Ala–Thr–Lys–Orn–AcOHOrn–Thr–Thr–Ala–Ser–Thr–Ala–Ala–cOHOrn	This study

Usual amino acids, three letter code; *aThr allo-Thr*, *εLys* Lys linked by its ε-NH2, *AOHOrn* δN-acetyl-δN-hydroxy-ornithine, *FOHOrn* δN-formyl-δN-hydroxy-ornithine, *cOHOrn* cyclo-hydroxy-ornithine (3-amino-1-hydroxy-piperidone-2), *OHHis* threo-β-hydroxy-histidine, *OHAsp* threo-β-hydroxy-aspartic acid, *Dab* diamino-butanoic acid

Parentheses indicate cyclic structures. D-amino acids are underlined. A brocken line means that the two enantiomers have been detected among the underlined residues but with no precise affectation. Stereochemistry remains to be done for pyoverdines with no amino acid underlined

Molecular mass values in bold characters correspond to more than one pyoverdine. Those followed by an asterisk design pyoverdines with an internal cycle formed by an ester bond in their respective peptide chain. The corresponding open form could also be found in the growth medium with a molecular mass increased by 18 Da

putida species, while 4 were recognized as *P. aeruginosa* strains, and 13 as *Pseudomonas* sp. Moreover, representatives of ten other well defined *Pseudomonas* species were also concerned, namely *Pseudomonas chlororaphis*, *P. cichorii*, *Pseudomonas costantinii*, *Pseudomonas fuscovaginae*, *Pseudomonas libanensis*, *Pseudomonas monteilii*, *Pseudomonas rhodesiae*, *P. syringae*, *Pseudomonas thivervalensis*, and *Pseudomonas tolaasii*.

Strains were preserved by deep freezing at -80°C as a bacterial suspension developed in rich medium (peptone 10 g/l, beef extract 5 g/l, and NaCl 5 g/l) and diluted by half with sterile 50% (v/v) glycerol/water solution.

Pyoverdine production and purification

Pseudomonas strains were grown in Casamino Acid (CAA) medium made of CAA with low iron and chloride content (Difco, Surrey, UK) 5 g/l, K_2HPO_4 1.18 g/l, $MgSO_4 \cdot 7H_2O$ 0.25 g/l, dispatched in 1-l Erlenmeyer flasks containing 500 ml medium. Cultures were done under vigorous shaking (200 rpm) at 25°C during 24–40 h, depending on the strains, so to reach the maximal pyoverdine production observed at the beginning of the stationary phase of growth. Bacterial cells were removed by centrifugation at 12,000 rpm during 10 mn, and the pyoverdine-containing supernatant was adjusted to pH 6.0 with 1 N HCl and passed at a speed of 2 drops/s through an Amberlite-XAD-4 column (2.5 × 20 cm^2 for 500 ml culture supernatant). The column was then washed with 100 ml distilled water and pyoverdines were eluted from the column with methanol/H_2O 50/50% (100 ml). The XAD-purified pyoverdines were obtained dried following vacuum- and lyophilization treatment.

Pyoverdine-isoelectrophoresis analysis

An already described method (Meyer et al. 1998) adapted from an earlier siderophore isoelectrofocusing study (Koedam et al. 1994) to the mini-IEF gel apparatus of Bio-Rad, Hercules, CA, USA, was used. The recommendations of the manufacturer were followed for the ampholines (pH 3–10)-containing polyacrylamid gel preparation as well as for the

electrophoresis conditions. Detection of the electrophoresed pyoverdine bands was done under UV-light at 365 nm, and their respective isoelectric pH values were determined by a computerized comparison with a mixture of pyoverdines with well known pHi values (Fuchs et al. 2001) systematically used as internal standard.

Usually, the PVD-IEF pattern characterizing each bacterial strain and reported in Table 1 by the number and respective pHi values of the pyoverdine isoform bands, was obtained by analyzing the native pyoverdines accumulated during growth in the culture supernatant. Samples of 1 µl of 20-fold concentrated CAA-culture supernatants were deposited on the IEF gel for analysis. Concentration was obtained through lyophilization. For strains with a weak production of pyoverdines (too low to reach well visible PVD bands on the UV-illuminated electrophoresed gels) PVD-IEF patterns were obtained by analyzing samples of the corresponding XAD-purified pyoverdines as 1 µl deposits of 5 mg/ml PVD solutions in distilled water.

Siderotyping-mediated iron uptake

Pyoverdines sharing an identical molecular mass were subjected to further differentiation by analyzing their respective ^{59}Fe uptake properties in cross-uptake experiments involving the pyoverdines of concern and their respective producing strains. One-point kinetics as defined previously (Meyer et al. 2002a) were used. Results were expressed as the percentage of incorporation compared to the homologous system (100%) and represented usually the middle average value of two experiments.

Molecular mass determination by mass spectrometry

Mass spectral data were obtained with a MAT 900 ST instrument providing an EB-QIT (quadrupole ion trap) geometry and equipped with an ESI II ion source (Finnigan MAT, Bremen, Germany); spray voltage 3.4–3.6 kV, capillary temperature 230°C. Samples of XAD-purified pyoverdines were dissolved in water/methanol/trifluoroacetic acid 50:50:0.1 (v/v/v). Fragmentation induced by collision activation (CA) was effected in the QIT ($\sim 2 \times 10^{-3}$ Pa He as bath gas). XAD-purified samples of pyoverdine were analyzed since the mixture of pyoverdine isoforms usually favored an easier identification of the generated molecular ions (Budzikiewicz et al. 2007a). The molecular masses of the succinyl isoforms of pyoverdines were chosen for tabulation purposes. How the corresponding molecular ions have been recognized in the mass spectrum of the siderophore mixture is detailed elsewhere (Budzikiewicz et al. 2007a).

Results

Molecular mass diversity among pyoverdines

The synthesis of pyoverdine by fluorescent *Pseudomonas* rarely results in the accumulation within the growth medium of a single molecular species. Most frequently, several major fluorescent molecules are present in the culture supernatant and can be separated in as many fluorescent bands by isoelectrophoresis (Fuchs et al. 2001). Among the 68 bacterial strains producing the pyoverdines described in Table 1, *Pseudomonas* sp. G85 was the only one strain presenting a single pyoverdine fluorescent band when its CAA-culture supernatant was analyzed by isoelectrophoresis on ampholine-containing polyacrylamid gel (Table 1; PVD $n = 18$). Three highly fluorescent bands were visualized for 30 strains, while 26 strains were characterized by two bands and eight strains by four bands. As discussed in detail elsewhere (Fuchs et al. 2001; Budzikiewicz 2004; Meyer 2007), this band-multiplicity and the resulting diversity encountered among PVD-IEF patterns are the consequence of multiple variations which could affect the different parts of the pyoverdine chemical structure, namely the chromophore, the peptide chain and, most important in the definition of the IEF-pattern, the dicarboxylic acid side chain. Within a given pyoverdine-containing culture supernatant, several different pyoverdine molecules can effectively be found. These pyoverdine isoforms share an identical chromophore and an identical peptide chain but differ one from each others by the nature of the dicarboxylic side chain, resulting in molecular mass ions with well defined mass differences, e.g., 16 Da between a succinic versus a malic isoform, 1 Da difference between the acidic and the

corresponding amide forms, and so on (see Fuchs et al. 2001). Thus, a precise molecular mass comparison of molecular ions detected in the mass spectrum of the isoform mixture of a given pyoverdine, and the study of the respective CA spectra, allow an unambiguous recognition of the succinyl isoform-corresponding ion of any pyoverdine so far investigated and its precise molecular mass determination (Budzikiewicz et al. 2007a). Such values were collected directly from the literature for the 50 published pyoverdine structures (references in Table 1), while they were experimentally determined for the 18 compounds under investigation in the present study. Data are given in Table 1. The 68 pyoverdine molecules with identical chromophore and identical succinyl side chain but differing each by their respective peptide chains represented a total of 64 different molecular mass values ranging from 989 Da for the *Pseudomonas* sp. B10 pyoverdine to 1,764 Da for the pyoverdine IB3 which, according to its CA spectrum, is composed of 14 amino acyl residues in its peptide chain. It thus represents a record in peptide length and molecular mass among all pyoverdines so far investigated.

As discussed in detail elsewhere (Budzikiewicz et al. 2007a), a few pyoverdines (marked by an asterisk in Table 1) have the peculiarity to be present in culture supernatants as cyclic molecules with an internal ester (depsipeptidic) ring in their peptide chain or as the corresponding hydrolized open forms which thus present a molecular mass 18 Da higher compared to the cyclic forms. The molecular masses of the cyclic forms are given in Table 1. It can be verified that none of the other pyoverdines in Table 1 presents a molecular mass corresponding to an open form of these peculiar type of pyoverdines (respectively, 1,111, 1,141, 1,152, 1,168, 1,203, and 1,248 Da).

Siderotyping analysis of pyoverdines with identical molecular masses

Among the 68 pyoverdines of Table 1, a few, however, presented an identical molecular mass value although being different in structure (Table 1), i.e., the pyoverdines of *P. fluorescens* PL7 and *P. fluorescens* Ps4a, both at 1,046 Da (PVDs $n = 6$ and 7), the pyoverdines of the three strains *Pseudomonas* sp. PS6-10, *P. aeruginosa* ATCC 27853, and *P. libanensis* CFML 96-195 at 1,091 Da (PVDs $n = 12$, 13, and 14), and the ones of the *P. tolaasii* type-strain and *P. putida* CFML 90-136 with 1,424 Da as molecular mass for the respective succinyl pyoverdine isoforms (PVDs $n = 61$ and 62). Among the 18 unknown compounds investigated in the present study, one, i.e., the pyoverdine of strain G168, also reached an identical 1,424 Da value. Moreover, its CA spectrum concluded to an identical peptidic part as the pyoverdine of strain CFML 90-136. Therefore, the IEF-patterns of the two compounds were compared and from the close identity they presented (see Fig. 1, lanes 8 and 9), it was concluded that strains CFML 90-136 and G168 were producing identical pyoverdines, a conclusion which was also conforted by cross-incorporations as shown in Table 2. Pyoverdine of *P. tolaasii*, although its closely related but different structure compared to PVD(90-136) (Table 1, PVD $n = 61$), presented also a strong cross-incorporation (Table 2) but was differentiable by its slightly different PVD-IEF pattern from PVD(90-136) or PVD(G168) (one fluorescent band less, Fig. 1, lane 7; Table 1). The other pyoverdines which were not differentiated by their molecular masses, i.e., PVD(PL7) and PVD(Ps4a) or PVD(27853), PVD(96-195), and PVD(PS6-10), could be differentiated one from each other by their

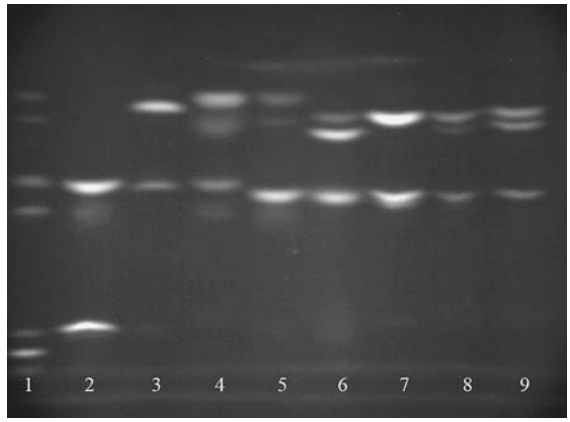

Fig. 1 Isoelectrofocusing patterns of pyoverdines produced by strains *P. fluorescens* PL7 (lane 2), *P. fluorescens* Ps4a (lane 3), *Pseudomonas* sp. PS6-10 (lane 4), *P. libanensis* CFML 96-195 (lane 5), *P. aeruginosa* ATCC 27853 (lane 6), *P. tolaasii* (lane 7), *P. putida* G168 (lane 8), *P. putida* CFML 90-136 (lane 9). Lane 1 corresponds to a standard of purified pyoverdine isoforms with well known pHi values (Fuchs et al. 2001)

Table 2 Pyoverdine-mediated iron cross-incorporations between three groups of strains producing pyoverdines with identical molecular masses

Strains	Molecular mass	Iron uptake mediated by pyoverdine of strain							
		PL7	SB8.3	PS 6.10	Pa27853	96–195	Ptol	90–136	G168
P. fluorescens PL7	1,046	100	2						
P. fluorescens SB8.3	1,046	6	100						
Pseudomonas sp. PS 6.10	1,091			100	3	5			
P. aeruginosa ATCC 27853	1,091			3	100	5			
P. libanensis CFML 96-195	1,091			1	1	100			
P. tolaasii	1,424						100	116	127
P. putida CFML 90-136	1,424						89	100	106
P. putida G168	1,424						84	90	100

Pyoverdine-mediated iron incorporations were done according to the one-point kinetics as described in Meyer et al. (2002a), with cells grown in CAA medium and iron-label mix made with pyoverdine-containing CAA culture supernatants. Values are expressed as the percentage of incorporation compared to the homologous system (100%). Values are the middle average of at least duplicate experiments

Pa Pseudomonas aeruginosa, *Ptol Pseudomonas tolaasii*, *CFML* Collection de la Faculté de Médecine de Lille, *ATCC* American type culture collection

respective PVD-IEF patterns (Table 1), as well as by their strict specificity in iron uptake capacity as shown in Table 2.

Supplementary structural informations reached by mass spectrometry

Together with the molecular mass determination of pyoverdines, reached by the detection of $(M + H^+)$ ions and their respective *m/z* values determination, a detailed analysis of the CA spectrum of a given pyoverdine allows in most cases supplementary informations on the structure of the molecule. For instance, the presence of particular *m/z* peaks is informative concerning the nature of the chromophore (Budzikiewicz et al. 2007b) and allows, therefore, the differentiation between pyoverdines (peptide chain attached on the C1 of the chromophore) and isopyoverdines (peptide chain attached to the C3 of the chromophore). Also, specific *m/z* values are relevant of the presence within the peptide chain of particular amino acyl residues, e.g., Orn, cOHOrn, Lys or Arg. Moreover, the differences in *m/z* values between two adjacent peaks usually reveal the nature of the last amino acid present in the relevant fragment. A series of B-ions (according to the CA fragment nomenclature of Roepstorff and Fohlmann 1984) allows to determine the N-terminal sequence, a series of Y″-ions the C-terminal one. Table 3 summarizes mass spectrometry data obtained on 18 pyoverdines for which a fairly complete structure could be consequently suggested as indicated in Table 1. Four pyoverdines (n = 26, 36, 64 and 67 in Table 1) presented CA spectra from which only limited structural information could be obtained, but their respective $[M + H^+]$ ions, and therefore their molecular masses, could be determined.

Discussion

Mass spectrometry has become a very powerful method for structural studies concerning the important siderophore family that are the pyoverdines of the fluorescent *Pseudomonas* (Fuchs and Budzikiewicz 2000, 2001a, b). In particular, it offers under favorable circumstances an easy and elegant way to determine molecular masses and to predict at least in part the primary structure of the peptidic part of pyoverdine molecules. As shown in the present study, regarding the huge diversity developed at the peptidic level of these compounds, most of them can be specifically defined by their respective molecular mass. The few exceptions to this general rule concerned pyoverdines which can easily be

Table 3 Mass spectrometry data allowing a peptide chain structure proposal for most of the new pyoverdines as described in Table 1

Pyoverdine	Dicarboxylic chain	Chromophore peak *m/z*[b]	Presence of specific *m/z* from[c]	*m/z* value and relevant terminal amino acid of pyoverdine fragments designated according to Roepstorff nomenclature[a]									
				A1	B1	B2	B3	B4	B5	B6	B7	B8	B9
GS43	Suca[d]	204	cOHOrn	457 Lys		616 OHAsp[e]	703 Ser	790 Ser	877 Ser				
96-195	Suca[d,f]	204	cOHOrn + Orn	400 Ala		542 Orn	673 OHAsp[e]	760 Ser	874 Orn				
G85	Suca[d,f]	204	cOHOrn + Orn	416 Ser		572 Lys	703 OHAsp	790 Ser	904 Orn				
G166g	Suca	204			485 Lys	567 AOHOrn	587						
G76[h]	Kgl	204/270		383 Ser	411 Ser	498 Ser	656 FOHOrn	743 Ser	830 Ser	958[i] Lys			
HR6	Suca[d]	204	cOHOrn		472 Asp	ε-Lys	731 OHAsp[e]	818 Ser	905 Ser	1006 Thr	1107 Thr	1208 Thr	
LBSA1	Suca[d]	204	cOHOrn + Arg		472 Asp	628 Arg	800 AOHOrn	928 Lys	1015 Ser	1130 Asp			
ML45	Suca[d]	204		400 Ala									
Lille 1[h]	Kgl	230	cOHOrn		439 Asp	567 Lys	739 AOHOrn	810 Ala	897 Ser	984 Ser	1041 Gly		
G153	Suca[d,f]	204	cOHOrn	416 Ser		572 Lys	643 Ala	730 Ser	817 Ser	989 AOHOrn	1076 Ser	1163 Ser	
G172	Suca[d]	204		400 Ala		556 Lys	656 Dab	787 OHAsp[e]	888 Thr	954 Gly	1076 OHAsp[e]	1133 Gly	1234[j] Thr
DSM 50106[h]	Kgl	204/270		383 Ser	411	538 Lys	596 Gly	754 FOHOrn	841 Ser	928 Ser	985 Gly	1099[k] Orn	
Pfl0-1[l]	Suca[d]	204	Orn + Arg	400 Ala		600 AOHOrn	714 Orn	801 Ser	888 Ser	975 Ser	1131 Arg	1262 OHAsp	
90-136/G168	Suca[d]	204	cOHOrn	416 Ser		572 Lys	659 Ser	746 Ser	847 Thr	948 Thr	1120 AOHOrn	1207 Ser	1294 Ser
G400	Suca[d]	204	cOHOrn	416 Ser		531 Ser							
GS37	Kgl	230	Orn		501 Asp								
IB3	Suca[d]	204	cOHOrn + Orn	416 Ser		515 Ala	616 Thr	744 Lys	858 Orn	1030 AOHOrn	1131 Thr	1232 Thr	1303 Ala
Pyoverdine	Dicarboxylic chain	Chromophore peak *m/z*[b]	Presence of specific *m/z* from[c]	*m/z* value and relevant terminal amino acid of pyoverdine fragments designated according to Roepstorff nomenclature[a]									
				B10	B11	Y2″	Y3″	Y4″	Y5″	Y6″	Y7″	Y8″	Y9″
GS43	Suca[d]	204	cOHOrn			218 Ser							
96-195	Suca[d,f]	204	cOHOrn + Orn			218 Ser							
G85	Suca[d,f]	204	cOHOrn + Orn			218 Ser							
G166g	Suca	204											
G76[h]	Kgl	204/270					374	461					
HR6	Suca[d]	204	cOHOrn			232 Thr	333 Thr	434 Thr				867	
LBSA1	Suca[d]	204	cOHOrn + Arg							789			
ML45	Suca[d]	204											
Lille 1[h]	Kgl	230	cOHOrn			218 Ser						820	
G153	Suca[d,f]	204	cOHOrn			218 Ser	305 Ser						
G172	Suca[d]	204									680	780	
DSM 50106[h]	Kgl	204/270						417	504	591	749	806	934

Table 3 continued

Pyoverdine	Dicarboxylic chain	Chromophore peak *m/z*[b]	Presence of specific *m/z* from[c]	*m/z* value and relevant terminal amino acid of pyoverdine fragments designated according to Roepstorff nomenclature[a]									
				B10	B11	Y2″	Y3″	Y4″	Y5″	Y6″	Y7″	Y8″	Y9″
Pfl0-1[l]	Suca[d]	204	Orn + Arg										
90-136/G168	Suca[d]	204	cOHOrn										
G400	Suca[d]	204	cOHOrn										
GS37	Kgl	230	Orn										
IB3	Suca[d]	204	cOHOrn + Orn	1390 Ser	1491 Thr	202 Ala	277 Ala					734	906

[a] See Roepstorff and Fohlmann (1984)

[b] Characteristic chromophore fragments are *m/z* 204 for pyoverdines with any side chain except Kgl where *m/z* 270 is of high abundance while *m/z* 204 is of low abundance, and *m/z* 230 for isopyoverdines (Budzikiewicz et al. 2007b)

[c] *m/z* values of 86/131 from cOHOrn, 70/115 from Orn or 70/157 from Arg

[d] Losses of NH_3, H_2O, and Suca from A1

[e] Loss of 74 Da observed

[f] An isoform with Kgl shows appropriate mass shifts

[g] The identification of the fragment ions is not certain

[h] Ions after loss of H_2O and CO_2 from the Kgl residue

[i] Followed by *m/z* 1045 corresponding to B6 + Ser (bound to Lys in the internal cyclic structure)

[j] The mass difference between B9 and the [M + H] ion (129 Da) would suggest dehydrobutyric acid (Dhb) as last amino acid, but since B + H_2O ions formed by OH-transfer are common, an ester bond between the N-terminal and an in-chain Thr is more likely (elimination yielding an in-chain Dhb)

[k] Followed by *m/z* 186 corresponding to B8 + Ser (bound to Orn in the internal cyclic structure)

[l] Genome analysis suggested Ala–OHOrn–Lys–Ser–Ser–Ser–Arg–OHAsp–Thr (Ravel and Cornelis 2003)

differentiated by the other siderotyping procedures. One exception, however, concerned the pyoverdine of strain G168. Interestingly, mass spectrometric molecular mass determination and CA analysis of this pyoverdine were determinant in its final identity with PVD(90-136). Thus, mass spectrometry was useful in such a case to recognize structural identity of two compounds, as it was also useful in proving structural differences between compounds not well differentiated by the two classic siderotyping methods, as described above for PVD(R) and PVD(R′), or PVD(cichorii) and PVD(syringae).

Since, as shown in the present study, PVD-IEF and/or PVD-mediated iron uptake allowed the differentiation of the few pyoverdines sharing identical molecular masses, it can be concluded that the three methods are complementary and that it is advisable to use them concomitantly for reaching an accurate discrimination of pyoverdines and their producing strains through siderotyping.

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