Identical Pyoverdines from *Pseudomonas fluorescens* 9AW and from *Pseudomonas putida* 9BW*

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From *Pseudomonas fluorescens* 9AW and from *Pseudomonas putida* 9BW identical pyoverdine-type siderophores were isolated and their structures were elucidated by spectroscopic methods and degradation studies. These novel compounds are of interest as they contain L-threo-β-hydroxy histidine in their peptide chains, an amino acid sofar encountered in nature only rarely. The co-occurence of the same pyoverdine in different *Pseudomonas* species and its significance for the classification is discussed.

Introduction

Iron possesses two stable oxidation states (Fe^{2+} and Fe^{3+}) and the redox potential between them can be influenced strongly by complexing ligands. It plays, therefore, an important role for many redox processes in biological systems. Due to the low solubility of its various oxide hydrates the concentration of free Fe^{3+} in the soil is at best about 10^{-17} mol/l at pH values around 7. To maintain a sufficient supply of iron, soil bacteria excrete water soluble low molecular weight compounds with high complexing constants for Fe^{3+}. A structurally interesting type of these so-called siderophores is produced by the fluorescent group of the genus *Pseudomonas* referred to as pseudobactins or more commonly as pyoverdines (Budzikiewicz, 1993). Their common structural feature is a dihydroxyquinoline nucleus responsible for the yellowish-green fluorescence which gave the name to the "fluorescent" pseudomonads. It is one of the binding sites for Fe^{3+}; the other two necessary to form an octahedral complex are contained in a peptide chain attached to the quinoline chromophore. It contains 6 to 12 amino acid (both D and L). These binding sites are either two hydroxamate units derived from Orn or one hydroxamate and one α-hydroxy carboxylate. The various fluorescent *Pseudomonas* spp. or even strains produce pyoverdines differing in their peptide chains responsible for the recognition at the cell surface (Hohnadel and Meyer, 1988).

We now wish to report the isolation and structure elucidation of a pyoverdine which is remarkable in a twofold way: The α-hydroxy acid commonly encountered in pyoverdines is threo-β-hydroxy Asp while here threo-β-hydroxy His was found, an amino acid which has been sofar encountered in nature only in exochelin MN from *Mycobacterium neoaurum* (Sharman *et al.*, 1995) and in a pyoverdine from *Pseudomonas fluorescens* 244 (Hancock *et al.*, 1993), while the erythro-isomer was found in the bleomycins (Koyama

Abbreviations: Common amino acids, 3-letter code; OHHis, β-hydroxy His; c-OH-Orn, cyclo-N^5-hydroxy Orn (3-amino-1-hydroxy-piperidone-2); TAP, trifluoroacetyl (amino acid) O-isopropyl ester; GC/MS, gas chromatograph coupled with a mass spectrometer; FAB-MS, fast atom bombardement mass spectrometry; ESI, electrospray ionisation; RP-HPLC, reversed phase high performance liquid chromatography; NMR-techniques: COSY, correlation spectroscopy; NOESY, nuclear Overhauser and exchange spectroscopy; ROESY, rotating frame nuclear Overhauser and exchange spectroscopy; TOCSY, total correlated spectroscopy; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence; DSS, 2,2-dimethyl-5-silapentane-5-sulfonate; TMS, tetramethylsilane; HVEP, high voltage paper electrophoresis.

* Part LXXIII of the series "Bacterial Constituents". For part LXXII see Budzikiewicz *et al.* (1997).

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et al., 1973). Secondly, pyoverdines are highly specific regarding the recognition by the excreting *Pseudomonas* strain. The fact that the same pyoverdine could be isolated both from a *Pseudomonas fluorescens* and a *P. putida* strain rises classification problems wich will be discussed below.

Material and Methods

Instruments

Mass spectrometer: Finnigan-MAT HSQ-30 (FAB; matrix thioglycerol), Finnigan-MAT 900ST (ESI; aquous solution 5 pmol/µl); Kratos MS 25 RFA (GC/MS). Sample preparation by adsorption on Sep-Pak RP-18, removing of inorganic material with H_2O, desorption with CH_3OH/H_2O 1:1 and drying i.v.

NMR: Bruker AM 300 (^1H 300, ^{13}C 75.5 MHz), Bruker DRX 500 (^1H 500, ^{13}C 125 MHz). Chemical shifts are given relative to TMS with the internal standard DSS silapentane-5- using the relations δ(TMS) = δ(DSS) for ^1H and δ(TMS) = δ(DSS) – 1.61 ppm for ^{13}C. Samples: 12 mg desferri-**1a** were twice dissolved in 0.5 ml D_2O and brought to dryness i.v. at 30 °C. The dry substances were redissolved in 0.6 ml phosphate buffer (D_2O/H_2O 1:9 v/v; pH 4.3).

UV/Vis: Perkin-Elmer Lambda 7 and Hitachi 200; 1 mg substance in 20 ml 0.1 M phosphate buffer.

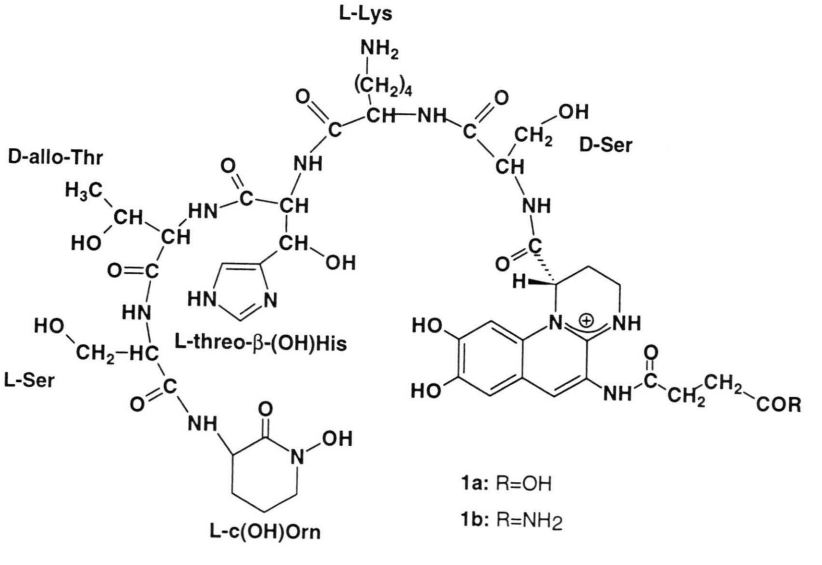
Chromatography: HPLC Knauer, column Nucleosil 100-C_{18}, 5 µm, Kromasil 100-C_4 (5mµ) and Nucleodex-β-OH (Macherey & Nagel, Düren); GC Carlo Erba HRGC 4160, FID detector, column Chirasil-L-Val (Chrompack, Middelburg, NL); column chromatography CM-Sephadex C-25 (Pharmacia, Uppsala, S), XAD-4 (Serva, Heidelberg), BioGel P2 (Bio-Rad, Richmond, USA), Sep-Pak RP-18 (Water, Milford, GB).

HVEP: Camag HVE system 60600, paper MN 261 (Camag, Muttenz, CH). The samples were dissolved in citrate/HCl buffer (Merck, Darmstadt) (pH 4.0) or in 0.5 M phosphate buffer (pH 6.9), standard desferal and glucose.

Isoelectrofocussing electrophoresis (IEF): Bio-Rad model 111 (Ivry sur Seine, F) using a Mini IEF cell with 125x65x0.4 mm polyacrylamide (5%) gel containing ampholine Byolyte 3/10 following the procedure recommended by the manufacturer.

Production, Isolation and Derivatisation of the Pyoverdines

Pseudomonas fluorescens 9AW or *Pseudomonas putida* 9BW, both isolated at the Schirmacher Oasis, Antarctica (Shivaji *et al.*, 1989) were grown for 72 hrs. in a medium consisting of 4 g succinic acid, 6 g K_2HPO_4, 3 g KH_2PO_4, 1 g $(NH_4)_2SO_4$ and 0.2 g $MgSO_4.7H_2O$ in 1 l H_2O (pH adjusted to 6.5 with 10% KOH). After removal of the cell material by centrifugation the culture medium was adjusted to



pH 6.0 and passed through a XAD-4 resin column. The pyoverdine-containing fraction was subsequently eluted with CH_3OH/H_2O 1:1 (v/v) and lyophilized. To 180 mg of the lyophilized material dissolved in 25 ml H_2O (bidest.) 3 ml of a 3% solution of Fe(III)citrate in H_2O were added and H_2O was removed i.v. at 30 °C. The dry residue was redissolved in 2 ml 0.1 M CH_3COOH (pH 2.7) and chromatographed on BioGel P-2 with 0.1 M CH_3COOH. From the fractions showing an absorption at 405 nm CH_3COOH was removed i.v. at 30 °C by adding several times H_2O, evaporation to dryness and subsequent drying at 0.1 torr. The fractions containing the ferri-pyoverdines were dissolved in 1 ml 0.2 M pyridinium acetate buffer (pH 5.0) and chromatographed on CM-Sephadex C-25 with 0.2 M pyridinium acetate buffer. Chromatography was repeated and from the brown fractions the buffer was removed i.v. at 30 °C by adding several times H_2O and bringing to dryness. For the structure elucidation described below the pyoverdines from *Pseudomonas fluorescens* 9AW were investigated in detail. Depending on the time needed for the work-up varying amounts of ferri-**1a** and -**1b** could be obtained (**1a** is a hydrolysis product of **1b**; cf. Schäfer *et al.*, 1991), yield together ca. 17 mg. Ferri-**1b** was transformed into ferri-**1a** by letting stand an aquous solution (pH 9.0) for 10 days at room temperature (Geisen *et al.*, 1992). Decomplexation was achieved by dissolving the ferri-**1a** in 1% aquous citric acid and shaking several times with a 5% solution of 8-hydroxyquinoline in $CHCl_3$ (Briskot *et al.*, 1986). The free pyoverdines were purfied by chromatography on Bio-Gel P2 with 0.1 M acetic acid. For qualitative and quantitative analysis of the amino acids, determination of their configuration and dansyl derivatisation of free amino groups see Briskot *et al.* (1986) and Mohn *et al.* (1990).

For the identification of OHHis 5 mg **1a** were hydrolized with 6 M HCl at 110 °C for 21 hrs, the hydrolysate was adsorbed on a Sep-Pak RP-18 cartridge and the amino acids were eluted with H_2O. The eluate was brought to dryness i.v., three times redissolved in H_2O and finally dried for 30 min at 0.1 torr. After dansylation (see above) the purified dansylated amino acids were chromatographed on Kromasil using a gradient CH_3OH/ 20 mM CH_3COOH going from 20 to 100% CH_3OH v/v (detection at 254 nm). Comparison with authentic material (Weber, 1997) and co-injection identified the isolated product as dansyl-threo-OHHis. Subsequent chromatography on Nucleodex using a gradient CH_3OH/50 mM CH_3COONH_4 buffer (pH 6.1) going from 10 to 100% CH_3OH v/v (detection at 254 nm) and comparison with authentic material including co-injection established the L-configuration.

To establish the position of D- and L-Ser in the molecule **1a** was hydrolyzed for 30 min. The hydrolysate was adsorbed on a Sep-Pak RP-18 cartridge which retains the fractions containing parts of the peptide chain bound to the chromophore while peptides and amino acids not bound to the chromophore can be eluted with 0.1 M CH_3COOH. The retained material was desorbed with CH_3OH/H_2O 8:2 v/v. The chromophore-containing petides were separated by chromatography on Bio-Gel P2 with 0.1 M CH_3COOH (detection at 254 nm). The molecular masses of the various fractions were determined by ESI-MS; the one whose mass corresponded to chromophore plus Ser was hydrolyzed for 21 hrs. In this way D-Ser could be identified after TAP-derivatization and chromatography on a chiral column (see above).

Edman-degradation of **1a** (cf. Tarr, 1977). To 1 mg **1a** dissolved in 20 ml H_2O/pyridine 1:1 (v/v) 10 ml of a 10% solution of phenylisothiocyanate in pyridine was added. The solution was degassed with N_2 for 10 sec and heated to 37 °C for 1 hr. The reaction mixture was extracted twice with 30 ml hexane/ethyl acetate 10:1 (v/v) each and 4 times with hexane/ethyl acetate 1:1 (v/v). After removal of the solvents from the aquous phase i.v. the residue was dried at 0.1 torr over P_4O_{10} for 1 hr. The residue dissolved in 10 ml waterfree CF_3COOH was degassed wirth N_2 for 10 sec and maintained at 37 °C for 30 min. The solvent was removed i.v., the residue was kept over solid NaOH i.v. for 30 min., then dissolved in 20 ml pyridine/H_2O 1:2 (v/v) and extracted 3 times with 30 ml hexane/ethyl acetate 1:1 (v/v). The aquous phase was brought to dryness i.v. The residue was than dansylated and subsequently hydrolyzed (cf. above).

Results

Characterization of **1a** *from Pseudomonas fluorescens 9AW*

The UV/Vis-spectra of **1a** (λ nm, E $cm^2 \cdot mmol^{-1}$; pH 7.0: 399, 15511; 227, 30771; pH 3.0: 374, 6244;

362, 6516; 245, 8826; 218, 19904) and of ferri-**1a** (pH 7.0: 401, 15386; 262, 12821; 231 32701; plus broad charge-transfer bands at about 470 (465, 3618) and 560 nm (546, 1950)) are typical for pyoverdines (Budzikiewicz, 1993). The molecular mass as determined by FAB- and ESI-MS was 1043 u. After total hydrolysis the following amino acids could be identified: L-threo-OHHis (by HPLC), L-Lys, L-OH-Orn, D-Ser, L-Ser, D-alloThr (by GC as TAP derivatives). The electrophoretic mobility of **1a** (cf. Poppe *et al.*, 1987) showed +1 charge at pH 6.9 and +2 charges at pH 4.0. The chromophore and Lys are protonated at pH 6.9, the succinate side chain provides −1 charge (Σ +1). The additional positive charge at pH 4.0 comes from the imidazole ring of OHHis ($pK_{S,1}$ 3.68, $pK_{S,2}$ 5.29; Mooberry *et al.*, 1980).

To determine which NH_2-group (α or ε) is free in **1a** ferri-**1a** was dansylated and hydrolyzed. ε-Dansylamino-Lys could be identified by HPLC using authentic comparison material. Other dansylated amino acids (esp. α-dansylamino-Lys) were not detected. This result was confirmed by the failure of an Edman degradation. Especially after dansylation and hydrolysis no dansyl-OHHis (or any other dansylated amino acid) could be detected. It follows that no cleavage of the molecule had occurred and hence no free α-amino group was present. Note that no ε-dansylamino-Lys could be found either; apparently the ε-amino group had reacted with phenylisothiocyanate.

From partial hydrolysis a fragment containing the chromophore and Ser could be isolated; subsequent hydrolysis gave D-Ser as identified after TAP-derivatization by GC on a chiral column. Thus the N-terminal amino acid of the of the peptide chain bound to the chromophore is D-Ser.

Sequence determination by NMR

For a detailled discussion of the NMR-techniques see Evans (1995). H,H-COSY shows ^{3}J-coupling of H-C-C-H while ^{4}J- and ^{5}J-coupling within one amino acid residue can be detected by TOCSY. The single amino acids can be identified by these techniques corroborated by shift values in comparison with literature data. Direct (^{1}J) C,H-connections can be determined by HMQC, ^{2}J- and ^{3}J-C,H-coupling by HMBC. Peptide sequencing is possible by ROESY which by resorting to Nuclear Overhauser Effects allows a correlation between an NH-proton and spacially close α- and β-H of the preceding amino acid (-C**H**-C**H**-CO-N**H**-).

^{1}H- and ^{13}C-measurements and peptide sequence

The ^{1}H- and ^{13}C-data are assembled in Tables I and II. Those of the chromophore and of the succinic acid side chain correspond to the ones observed for other pyoverdines (Budzikiewicz, 1993). From the TOCSY spectrum the signals of two Ser can be identified. The shifts of the β-CH_2-groups (~4 ppm) indicate that the OH-groups are not esterified (esterifications results in downfield shift of ~0.5 ppm). The low-field resonance of one of the NH-protons (9.7 ppm) is in agreement with the direct connection with the chromophore-

Table I. ^{1}H-NMR data of **1a** (pH 4.3).

Suc	2′	3′								
	2.86	2.79								
Chr	HN	1	2a	2b	3a	3b	4 HN^+	6	7	10
	10.04	5.75	2.66	2.76	3.42	3.75	8.87	7.86	7.00	7.11
	HN	α	β	γ	δ	ε	H_2N	H2′	H4′	
Ser	9.70	4.40	4.02							
Lys	8.51	4.41	1.55	1.10	1.45	2.66	7.54			
			1.75			2.76				
(OH)His	8.63	4.79	5.25					8.59	7.34	
aThr	8.46	4.36	4.14	1.19						
Ser′	8.65	4.50	3.93							
c(OH)Orn	8.58	4.51	1.83	2.00	3.64					
			2.03	2.03	3.70					

Table II. ^{13}C-NMR data of **1a** (pH 4.3).

Suc	CO (1′)	2′	3′	COOH (4′)			
	177.5	31.9	31.6	180.0			
Chr	CO	1	2	3	4a	5	
	171.1	57.3	22.6	35.9	150.1	118.4	
	6	6a	7	8	9	10	10a
	139.5	115.4	114.6	144.2	152.0	100.9	132.7
	CO	α	β	γ/His 2′	δ/His 4′	ε	
Ser	172.2	57.6	61.6				
Lys	174.4	54.0	30.7	22.6	26.5	39.7	
(OH)His	170.5	58.2	65.6	134.6	117.0		
aThr	171.8	59.9	67.4	19.1			
Ser′	172.5	56.4	61.8				
c(OH)Orn	167.1	51.1	27.3	20.6	52.3		

COOH (see above). From the shift of the β-CH of aThr (4.14 ppm) in the same way as for Ser esterification of the OH-group can be excluded. Also the values for the ε-CH_2-group of Lys agree with a free NH_2-group and exclude an amide bond in accordance with the degradation data. For the imidazol ring of OHHis two aromatic singlets are observed. The C-terminal N-hydroxy-cyclo-Orn shows the typical signals for this system (Mohn *et al.*, 1990). Since all amide NH could be identified within the amino acid residues constituing the peptide chain (see Table I) sequence information could be obtained from the ROESY spectra as depicted in Fig. 1. In this way the partial structures L-Ser-D-aThr-D-OHHis-L-Lys and D-Ser-chromophore-succinic acid can be derived. L-cOHOrn can only be the C-terminus of the peptide chain. Hence the complete squence of **1a** amounts to L-cOHOrn-L-Ser-D-aThr-D-OHHis-L-Lys-D-Ser-chromophore-succinic acid.

Mass spectrometric evidence

In the ESI-MS spectrum of **1a** after collision induced fragmentation either in the skimmer region or in the ion trap several sequence-characteristic ions could be observed arising from cleavages at the peptide bonds. They are summarized in Fig. 2 and confirm the conclusions derived from the NMR data.

The pyoverdine isolated from *Pseudomonas putida* 9BW has the same molecular mass as the one obtained from *P. fluorescens* 9AW as determined by FAB-MS, the same aminoacid composi-

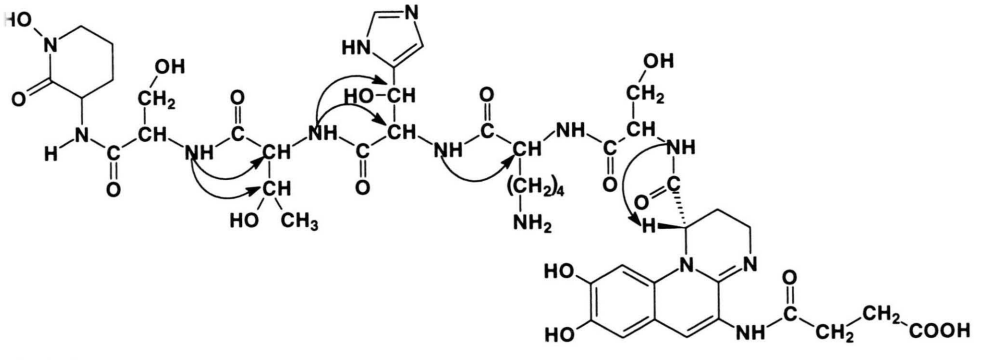


Fig. 1. Sequence specific NOE cross signals for **1a**.

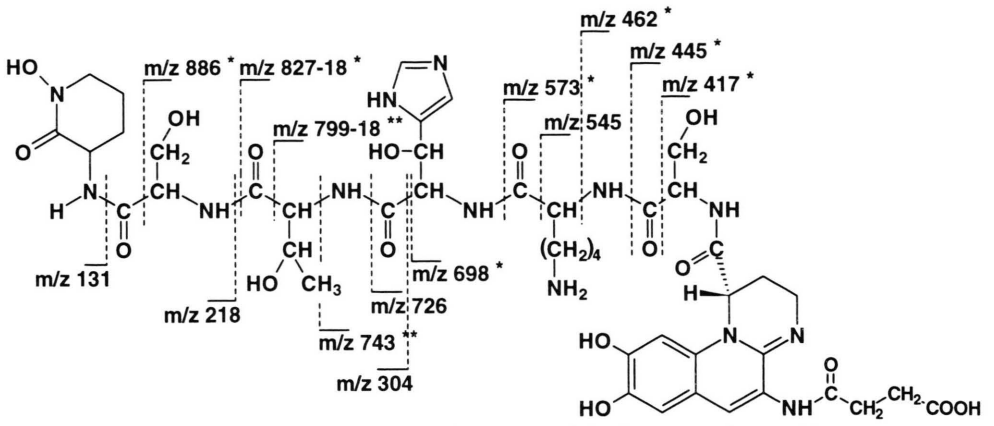


Fig. 2. Characteristic ions in the ESI mass spectrum of **1a** (mass numbers without marks: sequence ions observed only by skimmer collision activated decomposition (skimmer CA); with *: observed by skimmer CA and in the ion trap; with **: observed only in the ion trap).

tion as shown by NMR and chemical degradation including the determination of the D- and L-configurations. ESI-MS confirmed the same aminoacid sequence. Isoelectrofocussing gave identical patterns (one major band at $pH_i = 9.1$ and two minor ones at $pH_i = 8.80$ and 7.80). Thus it can be concluded that *P. fluorescens* 9AW and *P. putida* 9BW produce identical pyoverdines. Cross-^{59}Fe-pyoverdine uptake and pyoverdine receptor analysis (Meyer *et al.*, 1997) confirmed that the two strains also have identical pyoverdine-mediated iron uptake systems.

Discussion

Hancock and coworkers (Wang *et al.*, 1990; Hancock *et al.*, 1993; Hancock and Reeder, 1993; Hancock, 1994) reported a pyoverdine from *Pseudomonas fluorescens* 244 (pyoverdine Pf244) which is probably identical with **1a**: The sequence and the stereochemistry of the amino acids of the peptide chain are the same (the position of D- and L-Ser had not been determined). The only difference is that in the preliminary report (Wang *et al.*, 1990) a linkage between a Ser carboxyl group and the ε- rather than the α-amino group of Lys is proposed "as (NMR) studies on the Ga-chelated material suggested", but no data were given and the claim was not repeated (and substantiated) in the subsequent publications.

The occurence of L-threo-β-hydroxy His in **1a** is of interest as this rare amino acid had not been encountered before in any one of the over 30 known pyoverdines. But of greater importance is the fact that **1a** is produced both by a *P. fluorescens* and a *P. putida* strain.

Pseudomonas species of the fluorescent group can be subdivided into strains ("siderovars") which produce pyoverdines differing in their peptide chains and which are recognized for the most part only by the producing strain. Thus, 3 siderovars of *P. aeruginosa* are well established (Meyer *et al.*, 1997) and for *P. fluorescens* the complete structures of 10 pyoverdines and partial ones for 10 more are reported in the literature (cf. Budzikiewicz, 1993). Occasionally, enhanced bacterial growth has been observed upon addition of a "foreign" pyoverdine to a culture, as for various *P. putida* and *P. fluorescens* strains (Jacques *et al.*, 1995). Both, induction of an appropriate receptor protein (Koster *et al.*, 1993) and iron exchange between the Fe(III) complex of the added and the newly formed own pyoverdine (Gipp, 1987) has been invoked as an explanation. It is more remarkable when pseudomonads classified as different species produce the same pyoverdine. The only unequivocal example sofar is *P. fluorescens* ATCC 1352 and *P. chlororaphis* ATCC 9446 (Hohlneicher *et al.*, 1995). In this paper we report the production of **1a** both by *P. fluorescens* 9AW and *P. putida* 9BW (Shivaji *et al.*, 1989).

P. fluorescens (Palleroni, 1984; Palleroni, 1992 Elomari *et al.*, 1996) is a collective species which originally had been subdivided into 7 biovar

(A-G) from which A, B, C, F and G (G containing miscellaneous strains) were retained while D an E were separated as *P. chlororaphis* and *P. aureofaciens* mainly upon the production of phenazine pigments, viz. the green chlororaphin (a charge-transfer complex between phenazine-1-carboxamide and its 5,10-dihydro derivative) and the orange phenazine-1-carboxylic acid. More recently the two biovars D and E were recombined as *P. chlororaphis* based on nutritional and DNA similarity studies (Johnson and Palleroni, 1989). The differing nutritional patterns of the various biovars are less clear cut. *P. fluorescens* ATCC 13525 and *P. chlororaphis* ATCC 9446 may well be borderline species which would explain the formation of identical pyoverdines.

The main distinguishing feature for *P. putida* is the lack of gelatinase. The species is subdivided into the biovars A and B. Biovar B (to which *P. putida* 9BW apparently belongs, Shivaji *et al.*, 1989) "is phenotypically closer to *P. fluorescens* than to typical *P. putida* (biovar A)" (Palleroni, 1992, p. 3077). The present results confirm this conclusion; should other strains of *P. putida* (biovar B) also produce pyoverdines typical for *P. fluorescens* strains a reclassification should be considered.

In any case "in [Palleroni's (1992, p. 3095)] opinion, differentiation of *P. aeruginosa* from all other fluorescent organisms is sharp, but distinction among the remaining fluorescent organisms ... is not as clear cut." The formation of fluorescent pigments particularly in iron-deficient media has been taken sofar as a general characteristic for all species belonging to the fluorescent group, but an identification as pyoverdines and a consideration of their structural differences has not been taken into account. As more than 30 different pyoverdines are known today they should be considered as indicators for the classification of *Pseudomonas* strains of the fluorescent group which could be more reliable than nutritional patterns or the formation or absence of phenazines (from the published Tables, Palleroni 1984 and 1992, it can be seen that characteristic features as nutritional characteristics are rarely observed for 100% of the investigated strains).

Acknowledgement.

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