The Structure of the Pyoverdin Isolated from Various *Pseudomonas syringae* Pathovars[§]

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From seven different pathovars of *Pseudomonas syringae* representing various genetic subgroups, and one strain of *Pseudomonas viridiflava* the same pyoverdin siderophore (**1**) was isolated, probably identical with the pyoverdin whose amino acid composition (but not their sequence) had been reported before. **1** is the first pyoverdin where two of the ligands for Fe^{3+} are β-hydroxy Asp units. Its remarkably high complexing constant for Fe^{3+} at pH 5 as compared with other pyoverdins offers a definite advantage in plant infection. The structure elucidation of **1** will be described and the taxonomical implications regarding pyoverdins with different structures ascribed previously to *P. syringae* strains will be discussed.

§ Part CV of the series "Bacterial constituents". For part CIV see Hohlneicher *et al.* (2001).

Abbreviations: Common amino acids, 3-letter code; aThr, *allo*Thr; OHAsp, β-hydroxy-Asp; AcOHOrn, N^5-acetyl-N^5-hydroxy-Orn; cOHOrn, *cyclo*-N^5-hydroxy-Orn (3-amino-1-hydroxy-piperidone-2); TAP, N/O-trifluoroacetyl (amino acid) isopropyl ester; Chr, pyoverdin chromophore; Suc, succinic acid side chain; Suca, succinamide side chain; ESI, electrospray ionization; FAB, fast atom bombardment; CA, collision activation; u, mass units based on the ^{12}C scale; COSY, correlated spectroscopy; DEPT, distortionless enhancement by polarization transfer; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence; NOE, nuclear Overhauser effect; ROESY, rotating frame nuclear Overhauser and exchange spectroscopy; TOCSY, total correlation spectroscopy; WATERGATE, water suppression by gradient-tailored excitation; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; TMS, tetramethylsilane; pv, pathovar; ATCC, American Type Culture Collection; CFBP, Collection Française de bactéries phytopathogènes; LMG, Laboratorium voor Microbiologie van Ghent, Belgian Coordinated Collection of Microorganisms.

Introduction

Several *Pseudomonas* spp. are known as dangerous plant pathogens, e.g., *P. marginalis* for the bacterial soft rot of many plants (Bradbury, 1986), *P. tolaasii* infecting mushroom cultures (Munsch *et al.*, 2000; Uría Fernández *et al.*, 2001), and *P. syringae* whose so-called pathovars (abbreviated pv.) infect a large variety of plant species. For this purpose *P. syringae* can rely on a large supply of secondary metabolites, small cytotoxic compounds as, e.g., coronatine and congeners (Mitchel, 1985) as well as a variety of lipopeptides (e.g., syringomycin, Fukuchi *et al.,* 1992, or pseudomycins, Ballio *et al.*, 1994) which can facilitate the penetration of cell walls. It is astonishing that relatively little is known about its siderophores necessary for an adequate supply of iron (see below).

P. syringae belongs to the γ branch of the Proteobacteriaceae (Kersters *et al.*, 1996) and is a member of the phytopathogenic group (characterized by the absence of oxidase and Arg hydrolase) of the fluorescent *Pseudomonas* spp. in the rRNA homology group I of the Pseudomonadaceae (Palleroni, 1984), the typical siderophores of which are the pyoverdins (Budzikiewicz, 1997). The pyoverdin produced by the type species *P. syringae* pv. *syringae* ATCC 19310 (**1**, Fig. 1), whose structure elucidation will be described below, shows several peculiarities as compared with the

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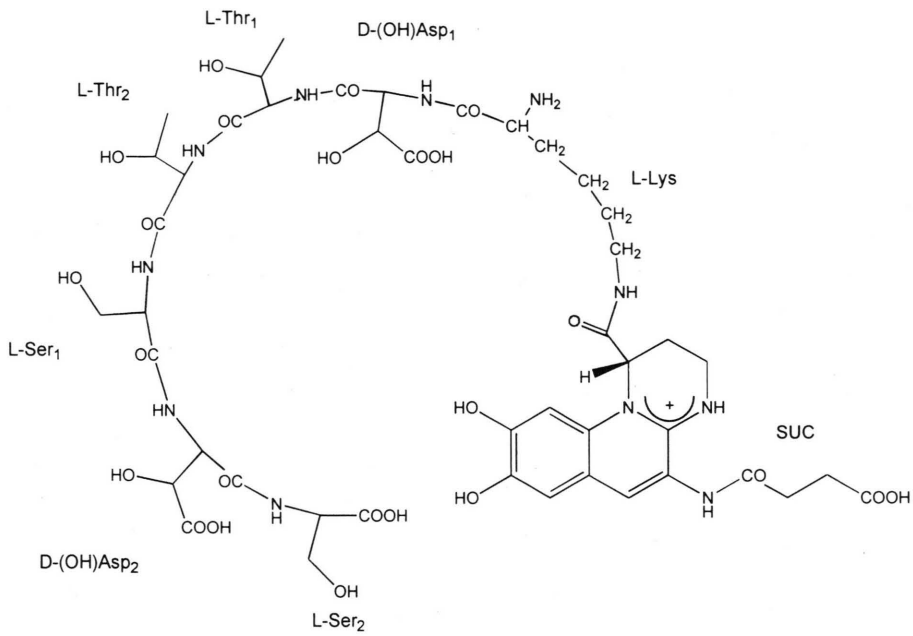

Fig. 1. Structure **1a** of the pyoverdins from *Pseudomonas syringae* strains; **1b** with $CONH_2$ (Suca) instead of COOH (Suc) in the side chain.

pyoverdins isolated from saprophytic *Pseudomonas* spp. (Kilz *et al.*,1999; Fuchs and Budzikiewicz, 2001). **1** seems to be the prevalent (if not the sole) pyoverdin produced by the many genetically differing pathovars of *P. syringae* in contrast to the large variety of pyoverdins encountered with the saprophytic fluorescent *Pseudomonas* spp.

Materials and Methods

Instruments, techniques and chemicals

Mass spectrometry: FAB Finnigan-MAT HSQ-30 (matrix thioglycerol/dithiodiethanol), ESI Finnigan-MAT 900 ST (solvent $CH_3OH/H_2O/1\%$ CH_3COOH in H_2O 50:50:1 v/v); GC/MS Incos 500 (both Finnigan-MAT, Bremen). Collision activation (CA) was effected either in the ion trap or in the octapole region before the ion trap.

NMR: DRX 300 and 500 (Bruker, Karlsruhe). Chemical shifts relative to TMS with the internal standard DSS; δ(TMS) = δ(DSS) for ^1H, δ(DSS) = −1.61 ppm for ^{13}C. Suppression of the H_2O signal by the WATERGATE puls sequence. Samples ca. 10 mg in 0.6 ml H_2O/D_2O 9:1.

UV/Vis: Lambda 7 (Perkin-Elmer, Überlingen), 1 mg in 10 ml buffer solution (0.12 mM, phosphate buffer, pH 5.0 and 7.0) with the buffer solution as reference.

Chromatography: Low pressure chromatography columns XAD-4 (Serva, Heidelberg), Biogel P-2 (Bio-Rad, Richmond CA, USA), CM-Sephadex C-25 and DEAE Sephadex A-25 and QAE Sephadex A-25 (Pharmacia, Uppsala, S), Sep-Pak RP_{18} cartridges (Waters, Milford MA, USA); HPLC Nucleosil-100 C_{18} (5 μm) and Eurosphere-100 C-18 (7 μm); GC/MS: Chirasil-L-Val (Chrompack, Frankfurt). DC Polygram Polyamid-$6UV_{254}$ (Macherey-Nagel, Düren).

Isoelectrofocussing (IEF): For the analysis of pyoverdin isoforms see Meyer *et al.* (1998). The bacteria were grown under iron starvation in a CAA medium (see below). ^{59}Fe-uptake studies: For details see Geoffroy *et al.* (2001). The values given in Fig. 2 were measured after 20 min of incubation and corrected for blank values obtained in assays without bacteria.

Chemicals: Water was desalted and distilled twice in a quartz apparatus. Organic solvents were distilled over a column. Reagents were of p.a. quality. CAA-medium: 5 g low-iron casein hydrolysate (Bacto Casamino Acids, Difco, Augsburg) in 800 ml, 0.5 g $MgSO_4 \cdot 7H_2O$ in 100 ml, 0.7 g

KH_2PO_4 + 1.3 g K_2HPO_4 in 100 ml H_2O. The solutions were sterilized at 130 °C for 20 min and poured together while still hot.

Production and isolation of **1a**

Pseudomonas syringae can be grown in so-called minimal media containing only glucose, gluconate or succinate as carbon source. However, the highest pyoverdin production (ca. three times more than in minimal media was achieved after ca. 72 hrs of cultivation in a CAA medium containing ca. 1 μM/l Fe^{3+}. For the work-up of the culture medium by chromatography on a XAD-4 column see Georgias *et al.* (1999). The eluate was brought to dryness i.v. at 30 °C, redissolved in 0.02 M pyridinium acetate buffer (pH 5.0) and chromatographed on biogel P-2 with the same buffer. The same procedure was repeated by chromatography on CM-Sephadex C-25 (solvent H_2O), on DEAE Sephadex A-25 (solvent 0.02 M pyridinium acetate buffer, pH 5.0) and on QAE sephadex A-25 (same solvent). Final purification was effected by preparative HPLC on a RP-18 column with a 0.1 M CH_3COOH in H_2O/CH_3OH gradient (5 to 30% CH_3OH) and checked by analytical HPLC. Detection for all chromatographic steps at 405 nm.

For decomplexation **1a** was dissolved in 1% aqueous citric acid and extracted several times with a 5% solution of 8-hydroxyquinoline in $CHCl_3$ and than with pure $CHCl_3$. The aqueous phase was chromatographed on Biogel P-2 (solvent 0.1 M acetic acid) and brought to dryness i.v. at 30 °C, redissolved in H_2O and again brought to dryness; purity control by RP-HPLC. For qualitative and quantitative analysis of the amino acids and the determination of their configuration by GC/MS of their TAP derivatives on a chiral column and for dansyl derivatisation see Briskot *et al.* (1986) and Mohn *et al.* (1990).

Results

Siderotyping behavior of the P. syringae strains

The pyoverdin isoforms (differing in the side chain attached to the chromophore) produced during growth of *P. syringae* pv. *syringae* ATCC 19310 in a CAA medium were differentiated as two fluorescent bands with pI values of 4.5 and 3.9, respectively, when subjected to isoelectrofocusing on ampholine-containing polyacrylamide gel with a 3 to 10 pH gradient. A strictly identical pyoverdin-IEF pattern was observed for the other strains analyzed, namely *P. syringae* pv. *aptata* CFBP 1617, *P. syringae* pv. *pisi* CFBP 2105, *P. syringae* pv. *atrofaciens* CFBP 2213, *P. syringae* pv. *tabaci* CFBP 2106, *P. syringae* pv. *passiflorae* CFBP 2346, *P. syringae* pv. *oryzae* CFBP 3228 and *P. viridiflava* CFBP 2107, a strain closely related to the *P. syringae* species.

All these strains were then analyzed for their pyoverdin-mediated iron uptake capacity towards their respective pyoverdins and also towards the pyoverdin of the type strain *P. syringae* pv. *syringae* ATCC 19310. As shown in Fig. 2, each strain was able to use the pyoverdin of *P. syringae* pv. *syringae* ATCC 19310 as well as its own one with the same efficiency. Moreover, *P. syringae* pv. *syringae* ATCC 19310 was tested versus a collection of 34 pyoverdins of different bacterial origin (see Weber *et al.*, 2000 for a listing of the pyoverdin collection and Kilz *et al.*, 1999, for their structures) and revealed a strict specificity of recognition towards its own pyoverdin; none of the 34 foreign pyoverdins were able to mediate iron incorporation in the *P. syringae* strain (Geoffroy *et al.*, 2001). Thus, it could be expected from the siderotyping and the uptake data that the set of *P. syringae* strains and the *P. viridiflava* strain produce the same novel pyoverdin.

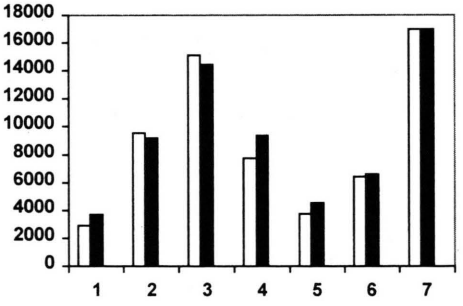

Fig. 2. Iron uptake (ordinate cpm) by *P. syringae* pv. *aptata* (1), *atrofaciens* (2), *tabaci* (3); *orycae* (4), *passiflorae* (5), *pisi* (6) and *P. viridiflava* CFBP 2107 as mediated by the respective pyoverdins (white bars) and by the pyoverdin of *P. syringae* pv. *syringae* ATCC 19310 (black bars).

Characterization of **1a** *from Pseudomonas syringae* pv. *syringae* ATCC 19310

1a gives the characteristic UV/Vis spectrum of pyoverdins (Budzikiewicz, 1997), viz. 404 nm at pH 7.0 and a split band 368 and 373 nm at pH 3.0; its Fe^{3+} complex shows the broad charge transfer bands at ca. 470 and 560 nm. The molecular mass of **1a** as determined by FAB-MS amounts to 1140 u. Amino acid analysis after total hydrolysis showed the presence of 2 D-OHAsp, L-Lys, 2 L-Ser and 2 L-Thr; in addition succinic acid di-isopropyl ester was detected. Total hydrolysis after dansylation yielded α-dansyl Lys as could be shown by DC-chromatographic analysis in comparison with authentic α- and ε-dansyl Lys. Lys is, therefore, incorporated into the pyoverdin peptide chain by its ε-amino group (cf. below the NMR discussion). The absolute configuration of C-1 of the chromophore was found to be *S* from the CD-spectrum of the 4-hydroxy chromophore obtained by hydrolysis (Michels *et al.*, 1991). The Fe^{3+} complexing constants of **1a** were determined *via* the equilibrium with the EDTA complex (Mohn *et al.*, 1990). They were found to be $1.61 \cdot 10^{25}$ at pH 7.0 and $6.03 \cdot 10^{21}$ at pH 5.0. While the pH 7.0 value agrees with the average of those determined for a series of pyoverdins (Budzikiewicz, 1997), the one at pH 5.0 is by ca. two orders of magnitude higher than the average. The less facile protonation of the two OHAsp units as compared with hydroxamic acids could be the reason.

Further information regarding the structural elements contained in **1a** can be gained from NMR data: Basis is the unambiguous identification of all ^1H- and ^{13}C-signals by a combination of homo- and heteronuclear one- and two-dimensional experiments: H, H-COSY allows to detect the ^3J-, TOCSY higher H,H-couplings. HMQC identifies ^1J-C,H, HMBC ^2J- and ^3J-coupling and allows thus to identify also quaternary C-atoms. HMBC indicates the number of H-atoms bound to a carbon. Sequence information is obtained by ROESY which correlates spatially close protons and by HMBC correlating carbonyl and amide signals of neighboring amino acids.

The ^1H- and ^{13}C-data are collected in Tables I and II. They correspond to those observed with other pyoverdins (Budzikiewicz, 1997). The following ones deserve a comment: The shift values of the β-H atoms of the Ser and aThr units indicate that the hydroxyl groups are not esterified, otherwise they would be observed about 0.5–1.0 ppm downfield (Budzikiewicz, 1997). The dansylation experiment had shown that Lys has a free α-amino group and is, therefore, bound amidically by its ε-amino group to the carboxyl group of the chromophore. The signals of the Lys chain can be identified in the TOCSY and in the COSY spectrum, that of the ε-NH shows correlations with the δ- and the ε-CH_2 groups. The low shift value of the ε-NH indicates a linkage to the chromophore carboxyl group. Sequence relevant

Table I. ^1H-NMR data of **1a** (H_2O/D_2O 9:1, pH 4.5, 25 °C)

Chr	1	2	3	4-NH^+	5-NH	6	7	10
	5.60	2.43 2.67	3.38 3.72	8.70	9.95	7.91	7.14	7.05
peptide chain	NH	α	ß	γ	δ	ε	NH_2	
Lys	9.08	4.13	1.88	1.31	1.57	3.32	[a]	
OHAsp[1]	8.68	4.99	4.61					
Thr[1]	8.30	4.56	4.39	1.22				
Thr[2]	8.26	4.45	4.30	1.22				
Ser[1]	8.45	4.55	3.86					
OHAsp[2]	8.38	4.95	4.69					
Ser[2]	8.09	4.47	3.90					
Suc	2′	3′						
	2.88	2.80						

[a] Not visible due to fast exchange.

Table II. ^{13}C-NMR data of **1a** (conditions as for Table I)

Chr	CO	1	2	3	4a	5	6	6a	7	8	9	10	10a
	170.4	58.4	23.2	36.5	150.5	118.8	139.9	116.0	115.0	144.7	152.4	101.5	132.8
pept.chain	CO		α		ß	γ		δ		ε	CO'		
Lys	171.0		54.5		31.7	22.4		28.9		40.5			
OHAsp^1	173.0		57.8		72.9						177.6		
Thr^1	173.3		60.1		68.2	20.2							
Thr^2	173.0		60.1		68.3	20.2							
Ser^1	172.9		57.1		62.4								
OHAsp^2	171.9		57.7		72.4						177.2		
Ser^2	176.0		57.7		63.0								
Suc	1'		2'		3'	4'							
	177.0		31.7		30.8	179.1							

ROESY and HMBC cross peaks are indicated in Fig. 3.

The structure deduced from NMR data is confirmed by mass spectrometric fragmentation by CA (Fuchs and Budzikiewicz, 2001). The most important fragment ions of the peptide chain upon CA of $[M+H]^+$ or of $[M+2H]^{2+}$ are the N-terminal so-called B-ions ($R'NH\text{-}CHR\text{-}CO^+$) (Roepstorff and Fohlman, 1984). The whole B-series accompanied by $[B - H_2O]^+$-ions can be observed. Several C-terminal Y"-ions ($NH_3{}^+CHRCOR"$) complete the pattern (Table III). Direct mass spectrometric analysis of the XAD extract shows the presence mainly of **1b** with a succinamide (Suca) side chain in agreement with the earlier observation that the succinic acid (Suc) side chain is formed by hydrolysis in the culture medium (Schäfer *et al.*, 1991) and during work-up. Due to the presence of Suca instead of Suc the masses of the B-ions are 1u lower than those given in Table III for **1a**.

In the same way **1a** was isolated from *Pseudomonas syringae* pv. *atrofaciens* CFBP 2213 and characterized by degradation, NMR and MS as described above. The XAD extracts obtained from *P. syringae* pv. *tabaci* CFBP 2106, pv. *passiflorae* CFBP 2346, pv. *oryzae* CFBP 3228, pv. *aptata* CFBP 1617, pv. *pisi* CFBP 2105 and *P. viridiflava* CFBP 2107 were subjected directly to mass spec-

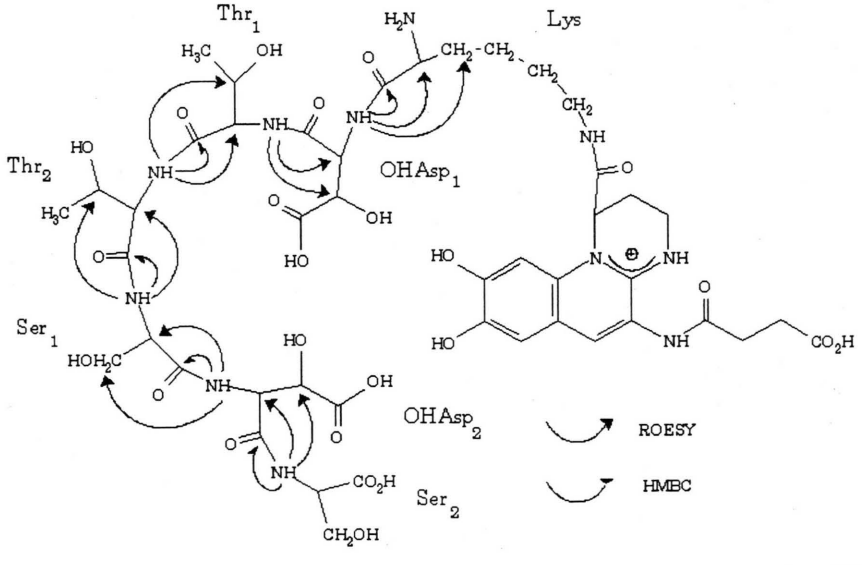

Fig. 3. Sequence relevant cross peaks for **1a** derived from ROESY (full arrows) and HMBC (half arrows) experiments.

Table III. Observed sequence specific fragment ions of **1a** after collision activation in the ion trap.

Fragment	n	Ion B	Ion Y"	n
Suc-Chr	0	358[a]		
ε-Lys	1	486		7
OHAsp	2	617		6
aThr	3	718		5
aThr	4	819	425	4
Ser	3	906	324	5
OHAsp	2	1037	237	2
Ser	7			1

[a] Detected after activation in the octapole region

trometric analysis. Fragmentation induced by CA proved the presence of same amino acid sequence in every case.

Discussion

The pyoverdin **1** shows some peculiarities. It belongs to the smallest representatives with only seven amino acids in the peptide chain (so far six amino acids is the minimal number encountered) and it shows uncommonly little variability in the D/L-pattern of the amino acids. Among the over 50 pyoverdins where complete or fairly complete structures were established it is only the third example with an ε-Lys link of the peptide chain to the chromophore (cf. Teintze *et al.,* 1981; Leong *et al.,* 1991). More important, it is the first example where the two ligand sites in the peptide chain are both OHAsp. Their lower pKa as compared with hydroxamic acids found as complexing sites in other pyoverdins may be responsible for the unexpectedly high complexing constant at pH 5.0 (ca. two orders of magnitude higher than observed for other pyoverdins) which could give *P. syringae* a selectional advantage in its habitat.

The 56 pathovars of *P. syringae* and related species were studied by DNA-DNA hybridization and divided in eight genomic species. Genomospecies *1* (*syringae*), *2 (savastanoi), 3* (*tomato*) and *4* (*porri*) clustered 49 of the 56 pathovars and related species (Gardan *et al.*, 1999). As it was shown above, *P. syringae* pv. *syringae* ATCC 19310, the type species, and pv. *atrofaciens* CFBP 2213 produce the same pyoverdin **1**. From six other pathovars belonging to different subclusters according to the above classification, viz. *P. syringae* pv. *tabaci* CFBP 2106, pv. *passiflorae* CFBP 2346, pv. *oryzae* CFBP 3228, pv. *aptata* CFBP 1617, pv. *pisi* CFBP 2105 and *P. viridiflava* CFBP 2107 their pyoverdins were isolated. They gave the same MS fragmentation pattern after CA as **1a** (see above), the same IEF pattern and uptake of the ferri-pyoverdin produced by the type strain with the same rate as their own ones. This indicates identical structures. Bultreys and Gheysen (2000) had analyzed the amino acid composition of the pyoverdins isolated from *P. syringae* pv. *syringae*, pv. *aptata*, pv. *morsprunorum*, pv. *tomato* and from *P. viridiflava* LMG 2352. It is identical with that of **1**. The same amino acid composition had also been found by Cody and Gross (1987) for the pyoverdin they had isolated from a *P syringae* pv. *syringae* strain. Although both groups did not determine the stereochemistry and the sequence of the amino acids, the amino acid composition is so unique (see above) that it is safe to assume, that in all cases identical pyoverdins were obtained. A more detailled discussion of the microbiological and classificational aspects involving all the *P. syringae* pathovars and other related species will be published elsewhere (Geoffroy *et al.,* 2001).

The strains investigated by us and by the Belgian group (Bultreys and Gheysen, 2000) were selected to cover a wide variety of *P. syringae* strains belonging to the four major genomospecies. They apparently all produce the same pyoverdin **1**. It is therefore astonishing that in the literature three other structures were reported for pyoverdins from supposedly *P. syringae* strains:

(1) Torres *et al.* (1986) had isolated from a not further classified *P. syringae* strain a pyoverdin for which they reported the amino acid composition 3 Ser, 3 Thr, Lys and OHOrn. Probably 2 OHOrn are present to provide two binding sites in the peptide chain (due to partial decomposition during hydrolysis the Orn values are generally too low), but when adding up the masses of the components one reaches a molecular mass much higher than the one determined by the authors from their elemental analysis. Provided the calculated molecular mass is correct within reasonable limits, the number of Ser and Thr must be too high.

(2) For the pyoverdin of *P. aptata* 4a the structure Suca-Chr-D-Ala-L-Lys-L-Thr-D-Ser-L-AcO-HOrn-L-cOHOrn was established (Budzikiewicz *et al.*, 1992). Originally, the strain was classified as *P. aptata* because it caused lesions on sugar beet leaves. However, a phenotypic analysis of the

strain revealed that it was oxidase positive and that it did not respond positively to a phytopathogenicity test done on tobacco leaves, as the *P. syringae* strains do (Geoffroy *et al.*, 2001). Therefore, it could be concluded that the so-called "*P. aptata* 4a" isolate has been misidentified and does not belong to the *P. syringae* group. Interestingly, the same amino acid sequence (the stereochemistry of the amino acids had not been determined) was found for the pyoverdin of *P. fluorescens* SB8–3, a saprophytic pseudomonad (Demange *et al.*, 1986 and unpublished results from this laboratory).

(3) For the pyoverdin of *P. aptata* 3b (identified in the same way as *P. aptata* 4a as causing lesions on sugar beet leaves; its belonging to the *P. syringae* species remains therefore doubtful) the amino acid sequence L-Asp-[L-AcOHOrn-Dab]-Thr-D-Ala-Thr-Thr-Gln-L-cOHOrn (2 D-Thr, 1 L-aThr; the amino acid analysis had been effected with the accompanying ferribactin having a Glu side chain giving D- and L-Glu, but since the ferribactin Glu side chains have so far always been found to be L, Gln in the peptide chain should be D) was established (Tappe, 1991). [AcOHOrn-Dab] indicates the condensation product between Orn and Dab giving a tetrahydropyrimidine ring (Demange *et al.*, 1990; Filsak *et al.*, 1994).

These three structures have little in common and differ grossly from that of **1**. A correct identification of the investigated strains may at least be questioned.

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