

The Pyoverdins of *Pseudomonas syringae* and *Pseudomonas cichorii*

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Z. Naturforsch. **59c**, 613–618 (2004); received June 23, 2004

The structure elucidation of the cyclic (lactonic) forms of the pyoverdins with a succinamide side chain originally produced by the closely related species *Pseudomonas syringae* and *P. cichorii* is reported. Mass spectrometry and nuclear magnetic resonance analyses as well as the determination of the configuration of the amino acids after degradation indicate that these two pyoverdins differ only by the replacement of the first in-chain serine by glycine. The pyoverdins of *P. syringae* and *P. cichorii* and the dihydropyoverdin of *P. syringae* can be used by both species as siderophores.

Key words: *Pseudomonas syringae* and *cichorii*, Pyoverdin, Siderophore

Introduction

The fluorescent species of the genus *Pseudomonas* are characterized by the production of potent iron chelators, the so-called pyoverdins. The pyoverdins consist of three distinct structural parts, viz. a dihydroxyquinoline chromophore responsible for their fluorescence, a peptide chain comprising 6 to 12 amino acids bound to the chromophore carboxyl group, and a small dicarboxylic acid (or its monoamide) connected amidically to its NH₂-group (cf. **1**). The dicarboxylic acids are derived from the citric acid cycle (Schäfer *et al.*, 1991).

The fluorescent *Pseudomonas* species can be divided into two major groups, the arginine dihydrolase-positive saprophytes and opportunistic animal-pathogens *P. aeruginosa*, *P. fluorescens*, and *P. putida*, and the arginine dihydrolase-negative phytopathogens *P. syringae*, *P. viridiflava*, and *P. cichorii*. Concerning the phytopathogenic group, it was shown in several publications that all pathovars of *P. syringae* and of *P. viridiflava* tested

so far (Bultreys and Gheysen, 2000; Bultreys *et al.*, 2001, 2003; Jülich *et al.*, 2001) produce the same pyoverdin. The closely related species *P. cichorii* produces a structurally very similar pyoverdin (Bultreys *et al.*, 2003). In their structures, both pyoverdins differ from the many pyoverdins (Budzikiewicz, 2004) isolated from the saprophytic group in the way that they contain two β -hydroxyaspartic acid units acting as two of the ligand sites for Fe³⁺ (the pyoverdins of the saprophytic group provide either two hydroxamic acid units or one hydroxamic acid and one β -hydroxyaspartic acid unit). The presence of two β -hydroxyaspartic acid units influences *i.a.* the complexing behavior and some spectral properties (Bultreys and Gheysen, 2000; Bultreys *et al.*, 2001, 2003). For the *P. syringae* pyoverdin the structure **1b** had been proposed (Jülich *et al.*, 2001).

In several cases it had been shown (Budzikiewicz, 2004) that pyoverdins with a C-terminal free carboxyl group are actually hydrolysis products of originally produced lactones. These lactonic structures are formed by an ester bond between the C-terminal carboxyl group and an in-chain serine or threonine. A lactonic structure could also be expected for the *P. syringae* pyoverdin, since the molecular mass reported for a succinamide form of this pyoverdin by Bultreys (Bultreys *et al.*, 2001, 2003) was 18 mass units lower than that observed

Abbreviations: Common amino acids, 3-letter code; OHAsp, β -hydroxy-Asp; HPLC, high performance liquid chromatography; MS, mass spectrometry; ESI, electrospray ionization; CA, collision activation; NMR, nuclear magnetic resonance; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; EDTA, ethylenediaminetetraacetic acid; TMS, tetramethylsilane; pv, pathovar.

by Jülich (Jülich *et al.*, 2001). The structure elucidation of cyclic forms of the pyoverdins of *Pseudomonas syringae* and of that of *P. cichorii* will be reported here.

Materials and Methods

Pyoverdin production and isolation

The production of pyoverdins was carried out using the liquid-solid technique in Petri dishes with agar blocks (Bultreys and Gheysen, 2000). The unchelated purified pyoverdins of the strains *P. syringae* pv. *syringae* LMG 1247 [identical with the strain ATCC 19310 used by Jülich (Jülich *et al.*, 2001)] and *P. syringae* pv. *morsprunorum* LMG 2222 were obtained from partially purified productions stocked since 1996 and 1997, respectively.

P. syringae pv. *syringae* LMG 1247 was grown either for 5 d at 10 °C in a modified glucose-asparagine medium (GASN) (Bultreys and Gheysen, 2000) containing 5 g/l glucose [ASP medium; this medium was selected in a previous study because – compared with other amino acid-containing agar media – it induced the greatest pyoverdin-related inhibitions of the growth of a yeast by two *P. syringae* strains (Bultreys and Gheysen, 2000)], or for 3 d at 28 °C in the same medium containing 10 g/l glucose. After centrifugation and filtration the culture media containing unchelated pyoverdins were passed through an octadecylsilane column, as previously described for the Fe(III)-chelated pyoverdins (Bultreys and Gheysen, 2000). The water/methanol (1:1, v/v) fractions were brought to dryness and could be stocked for several years. The two conserved fractions were combined and dissolved in water for further purification of the dominant siderophore present in both of them, as ascertained by HPLC. To the solutions of the pyoverdins FeCl₃ was added (pH 4.0) and the solvent was evaporated. The residue was then dissolved in a 50 mM NaOH/acetic acid buffer (pH 5.0) and passed through an octadecylsilane column to remove the excess of iron. The column was washed with the buffer and with water, the Fe(III)-chelated pyoverdins were desorbed with water/methanol (1:1, v/v) and the solvent was evaporated.

P. syringae pv. *morsprunorum* LMG 2222 and *P. cichorii* LMG 8401 were grown in a GASN medium for 3 d at 20 °C. The purification steps were as previously described (Bultreys and Gheysen, 2000). The main fractions containing the pyover-

dins with a succinamide side chain were selected by HPLC as described earlier (Bultreys and Gheysen, 2000; Bultreys *et al.*, 2001, 2003). After purification, the iron was removed. The ferric complexes were dissolved in 100 mM of a NaOH/phosphoric acid buffer (pH 7.0). Equal volumes of a 500 mM EDTA solution at pH 7.0 were added. The resulting mixtures (ca. pH 5.0) were passed through an octadecylsilane column to remove Fe(III)-EDTA and EDTA. The unchelated pyoverdins were desorbed with water/methanol (1:1, v/v) and brought to dryness.

Mass and NMR spectroscopy

A Finnigan-MAT 900 ST with an ESI source, solvent CH₃OH/H₂O (1:1) was used. Mass selected fragmentation by CA was conducted either in the quadrupole region in front of or in the ion trap. NMR: DRX 500 (¹H 500, ¹³C 125 MHz) (Bruker, Karlsruhe). Chemical shifts relative to TMS with the internal standard DSS; $\delta(\text{TMS}) = \delta(\text{DSS})$ for ¹H, $\delta(\text{DSS}) = -1.61$ ppm for ¹³C. The MS analyses of the pyoverdin of *P. syringae* were carried out with the pyoverdin of strain LMG 1247 and the NMR analyses with the pyoverdin of strain LMG 2222.

Amino acid analysis

1 mg of each pyoverdin was dissolved in 100 μ l of 6 N HCl; nitrogen was flushed into the tube before closure, and the tube was heated to 110 °C for 48 h. 10 μ l were brought to dryness, the residue was dissolved in 200 μ l of a pH 2.2 citrate buffer and analyzed by ion exchange chromatography (Biochrom 20 Plus).

Amino acid configurations

The amino acids were derivatized to pentafluoropropionyl isopropyl esters with the Alltech derivatisation kit (part. 18093) and separated on an Alltech Chirasil-VAL column (25 m, 0.25 mm ID, 0.16 mm film thickness, part. 13636) injector 250 °C, 4 min at 90 °C, then 4 °C/min until 200 °C. The purified lactonic forms (with a succinamide side chain) of the free pyoverdin of *P. cichorii* LMG 5484 and of the Fe(III)-chelated pyoverdin of *P. syringae* pv. *syringae* LMG 1247 (obtained from six stock samples coming from small volume productions in an ASP medium or in an ASP medium containing 10 g/l glucose) were analyzed in comparison with control amino acids.

Growth stimulation tests

They were carried out as previously described using 0.16 g dipyriddyil per liter as iron chelators (Bultreys *et al.*, 2001). The siderophores tested were the Fe(III)-chelated pyoverdins of *P. syringae* pv. *aptata* LMG 5059 and *P. cichorii* LMG 8401, as well as the Fe(III)-chelated dihydropyoverdin of *P. syringae* pv. *aptata* UPB 133; these molecules had been purified as described in previous studies (Bultreys *et al.*, 2001, 2003). The strains tested were *P. syringae* pv. *syringae* B301D, *P. syringae* pv. *tomato* LMG 5093, *P. syringae* pv. *aptata* UPB 110, *P. syringae* pv. *morsprunorum* PmC36, *P. cichorii* LMG 2162 and LMG 8401 and *P. fluorescens* LMG 5822.

Results and Discussion

Some differences in the data published by different groups concerning the pyoverdin of *P. syringae* (Bultreys *et al.*, 2001, 2003; Jülich *et al.*, 2001) prompted a further investigation of the pyoverdin studied by Bultreys (Bultreys and Gheysen, 2000; Bultreys *et al.*, 2001, 2003), which had not yet been fully characterized chemically. Samples purified by chromatography whose purity was carefully assessed by HPLC were investigated by mass spectrometry (electrospray ionization and decomposition of the $[M + 2H]^{2+}$ ion by collision activation). The mass spectra showed that all so-called B-ions obtained by cleavage of the amide bonds (Roeps-

torff and Fohlman, 1984) – starting from the second threonine – occurred with masses 18 u lower than those obtained for **1b** (Table I). This indicates that a lactone ring is formed with the second threonine (**1a**) (Fig. 1). Under CA conditions a lactone ring is opened by an elimination process transforming threonine into dehydroalanine (Voßen *et al.*, 2000), hence the observed mass shifts (Fuchs and Budzikiewicz, 2001). This conclusion is confirmed by ¹H NMR analysis of **1a**. The signal of the CH-group of the second threonine (5.30 ppm) is shifted downfield by 0.9 ppm relative to that of the first threonine (4.39 ppm), the expected effect for esterification of the hydroxyl group (Budzikiewicz, 2004). The MS analyses also indicated the presence of a succinamide residue bound to the chromophore in the dominant pyoverdin. This pyoverdin, with a cyclic peptide chain, has a molecular mass of 1122 u, as shown before (Bultreys *et al.*, 2001). The Fe³⁺ chelate would then have a molecular mass of 1175 u, as previously shown for the pyoverdin of all the pathovars of *P. syringae* investigated so far by Bultreys (Bultreys *et al.*, 2003). The production of pyoverdins in culture media containing asparagine and variable amounts of glucose were suitable to obtain prevalingly this originally produced form of the pyoverdins of *P. syringae* (Bultreys and Gheysen, unpublished). When dried this form was stable during prolonged conservation at –20 °C. However, the ester bond of the lactonic structure can be easily hydrolyzed (Budzikiewicz, 2004) and hydrolysis of the succinamide side chain to give a succinic acid is also a well known process (Schäfer *et al.*, 1991). Consequently, different cul-

Table I. Sequence characteristic ions of **1a** (lactone form) and **1b** (hydrolyzed form) (succinamide side chain) obtained after CA of $[M+2H]^{2+}$ (m/z 547).

Amino acid	1a	1b	1a	1b
	B ₀			357 ^a
ε-Lys	B ₁	A ₁ 457 ^{a,b}		457 ^{a,b}
OHAsp	B ₂	616 ^c		616 ^c
Thr	B ₃	717 ^c		717 ^c
Thr–H ₂ O	B ₄	800 ^c	Y'' ₄	407 ^c
Thr				818 ^c
Ser	B ₅	887 ^{c,d,e}	Y'' ₃	324 ^c 324 ^c
OHAsp	B ₆	1018 ^{c,d,e}	Y'' ₂	237 ^c 237 ^c
Ser			Y'' ₁	
$[M+2H]^{2+}$	562	571		

^a Observed in the quadrupole spectrum.

^b A₁ ion is missing (typical for ε-Lys).

^c Loss of H₂O observed.

^d A+H₂O ion is observed.

^e Doubly charged ions of $[B_5-H_2O]$ (m/z 435) and $[B_6-H_2O]$ (m/z 500.5) are observed.

^f A doubly charged ion of B₆ (m/z 518.5) is observed.

Table II. Sequence characteristic ions of **2** (succinamide side chain) obtained after CA of $[M+2H]^{2+}$ (m/z 547).

Amino acids	B-ions		Y''-ions	
	B ₀	357 ^a		
ε-Lys	B ₁	A ₁ 457 ^{a,b}		
OHAsp	B ₂	616 ^c		
Thr	B ₃	717 ^c		
Thr–H ₂ O	B ₄	800 ^c	Y'' ₄	377 ^c
Gly	B ₅	857 ^{c,d,e}	Y'' ₃	294 ^c
OHAsp	B ₆	988 ^{c,d,e}	Y'' ₂	237 ^c
Ser			Y'' ₁	

^a Observed in the quadrupole spectrum.

^b A B₁ ion is missing (typical for ε-Lys).

^c Loss of H₂O observed.

^d A+H₂O ion is observed.

^e Doubly charged ions of $[B_5-H_2O]$ (m/z 420) and $[B_6-H_2O]$ (m/z 485.5) are observed.

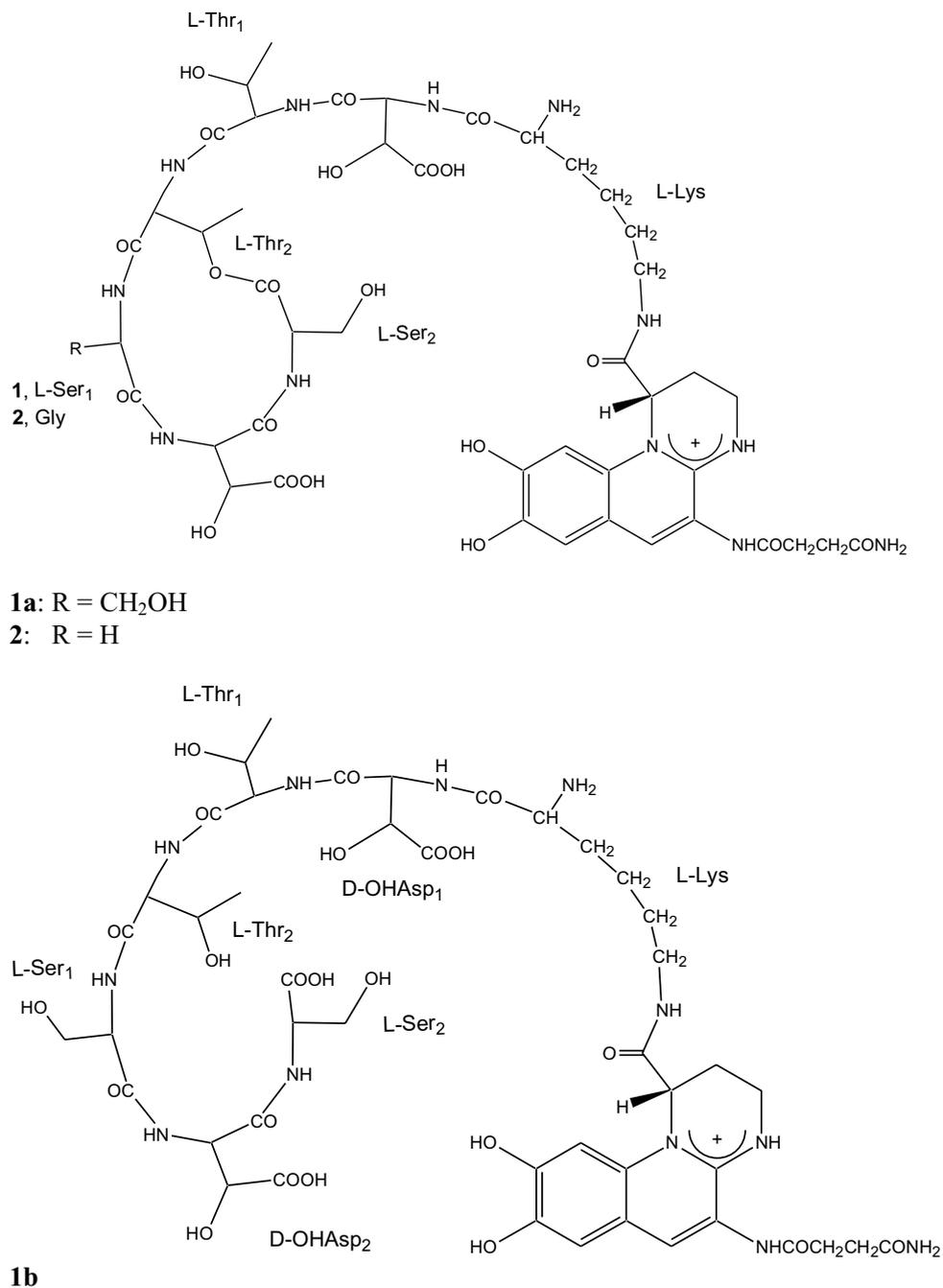


Fig. 1. Structures of the pyoverdins from *Pseudomonas syringae* (**1a**, **1b**) and *P. cichorii* (**2**).

ture conditions and experimental procedures probably explain that derived forms of the original pyoverdine **1a** were obtained and studied by Jülich (Jülich *et al.*, 2001), as the hydrolyzed form both

with a succinic acid and a succinamide side chain (**1b**). This explains the differences in the published molecular masses and structures for this pyoverdine (Bultreys *et al.*, 2001, 2003; Jülich *et al.*, 2001).

pyoverdins of the strains *Pseudomonas* sp. A214 and 7SR1 which differ by the replacement of one serine by alanine (Uría Fernández *et al.*, 2003). In both cases, the exchange of one small neutral amino acid does not influence the isoelectrofocusing patterns obtained with each couple of strains in siderotyping analyses (Bultreys *et al.*, 2003; Uría Fernández *et al.*, 2003).

Some non-fluorescent strains of *P. syringae* pv. *aptata* as UPB 110 and UPB 133 were shown to produce only the dihydropyoverdin corresponding to the pyoverdin common in the whole species *P. syringae*. These strains and the non-fluorescent strain *P. syringae* pv. *morsprunorum* PmC36, which is apparently unable to produce these siderophores, were shown to be able to use both siderophores (Bultreys and Gheysen, 2000; Bultreys

et al., 2001). In this study, the growth stimulation tests on dipyrindyl-containing medium indicated the use of the pyoverdin and dihydropyoverdin of *P. syringae* by *P. cichorii* and the use of the pyoverdin of *P. cichorii* by the fluorescent and non-fluorescent strains of *P. syringae*. On the other hand, *P. fluorescens* LMG 5822 was unable to use the three siderophores. The results indicate that in spite of the small difference in the peptide chain of the pyoverdin of *P. cichorii*, there is still sufficient affinity between this peptide chain and the membrane receptor of the pyoverdin of *P. syringae*, and conversely. Similar reciprocal incorporations were also observed by the strains *Pseudomonas* sp. A214 and 7SR1 of their closely related pyoverdins (Uría Fernández *et al.*, 2003).

- Budzikiewicz, H. (2004), Siderophores of Pseudomonadaceae *sensu stricto* (fluorescent and non-fluorescent *Pseudomonas* spp.). In: Progress in the Chemistry of Organic Natural Products (Herz W., Falk H., and Kirby G. W., eds.). Springer, Wien.
- Bultreys A. and Gheysen I. (2000), Production and comparison of peptide siderophores from strains of distantly related pathovars of *Pseudomonas syringae* and *Pseudomonas viridiflava* LMG 2352. Appl. Environ. Microbiol. **66**, 325–331.
- Bultreys A., Gheysen I., Maraite H., and de Hoffman E. (2001), Characterization of fluorescent and nonfluorescent peptide siderophores produced by *Pseudomonas syringae* strains and their potential use in strain identification. Appl. Environ. Microbiol. **67**, 1718–1727.
- Bultreys A., Gheysen I., Wathelet B., Maraite H., and de Hoffman E. (2003), High-performance liquid chromatography analyses of pyoverdin siderophores differentiate among phytopathogenic fluorescent *Pseudomonas* species. Appl. Environ. Microbiol. **69**, 1143–1153.
- Fuchs R. and Budzikiewicz H. (2001), Structural studies of pyoverdins by mass spectrometry. Curr. Org. Chem. **5**, 265–288.
- Jülich M., Taraz K., Budzikiewicz H., Geoffroy V., Meyer J.-M., and Gardan L. (2001), The structure of the pyoverdin isolated from *Pseudomonas syringae* pathovars. Z. Naturforsch. **56c**, 687–694.
- Roepstorff P. and Fohlman J. (1984), Proposal of a common nomenclature for sequence ions in mass spectra of peptides. Biomed. Mass Spectrom. **11**, 601.
- Schäfer H., Taraz K., and Budzikiewicz H. (1991), Zur Genese der amidisch an den Chromophor von Pyoverdinen gebundenen Dicarbonsäuren. Z. Naturforsch. **46c**, 398–406.
- Uría Fernández D., Geoffroy V., Schäfer M., Meyer J.-M., and Budzikiewicz H. (2003), Structure revision of several pyoverdins produced by plant-growth promoting and plant-deleterious *Pseudomonas* species. Monatsh. Chem. **134**, 1421–1431.
- Voßen W., Fuchs R., Taraz K., and Budzikiewicz H. (2000), Can the peptide chain of a pyoverdin be bound by an ester bond to the chromophore? The old problem of pseudobactin 7SR1. Z. Naturforsch. **55c**, 153–164.