Schizokinen, the Siderophore of the Plant Deleterious Bacterium *Ralstonia (Pseudomonas) solanacearum* ATCC 11696*

Herbert Budzikiewicz, Maik Münzinger, Kambiz Taraz\(^a\) and Jean-Marie Meyer\(^b\)

\(^a\) Institut für Organische Chemie der Universität zu Köln, Greinstr. 4, D-50939 Köln, Germany

\(^b\) Laboratoire de Microbiologie et de Génétique, Université Louis Pasteur, Unité de Recherche, Associée au Centre National de la Recherche Scientifique n° 1481, 28 rue Goethe, F-67000 Strasbourg, France

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*Ralstonia* (formerly *Burkholderia* or *Pseudomonas*) *solanacearum* ATCC 11696 – a plant deleterious bacterium – was shown to produce under iron limited conditions of growth an iron complexing compound which facilitated iron uptake into iron-starved cells. The structure of the siderophore was elucidated as 4-[[3-acetylhydroxyamino)-propyl]amino]-2-[2-[[3-(acetylhydroxyamino)-propyl]amino]-2-oxoethyl]-2-hydroxy-4-oxo-butanolic acid, a compound known under the trivial name of schizokinen. Its partially cyclized form, schizokinen A, could also be isolated from the *R. solanacearum* culture supernatant and was characterized.

### Introduction

The soil bacterium *Pseudomonas solanacearum* (= *Burkholderia solanacearum*, Yabuuchi et al. 1992; and *Ralstonia solanacearum*, Yabuuchi et al., 1995) is a dangerous phytopathogen which causes wilt disease of plants not only belonging to the nightshade family (*Solanaceae*) as its name would suggest. A periodical published by the Australian Centre for International Agricultural Research, the *Bacterial Wilt Newsletter* is essentially dedicated to it. The way of infection and the destruction of the host plant has been studied with respect to macromolecular virulence factors as e.g. cell wall degrading enzymes and slime forming polysaccharides (Schell et al., 1994). However, nothing seems to be known about low molecular weight substances produced by this bacterium. In view of our interest in secondary metabolites of *Pseudomonas* spp. (Budzikiewicz, 1993) we studied its siderophores (i.e., the Fe\(^{3+}\) chelating substances), and we could identify them as schizokinen (1, Fig. 1) and schizokinen A (2, Fig. 1). This can be of importance for understanding the virulence mechanism as it was shown for other phytopathogens that a functioning iron transport system is an essential prerequisite (e.g., Enard et al., 1988; Enard et al., 1991).

### Materials and Methods

*Bacteria and bacterial growth*

The investigated strain of *Ralstonia (Pseudomonas) solanacearum* ATCC 11696 was kindly provided by T. Heulin, C.N.R.S., Vandeuvre-les-Nancy, France and its identity was confirmed by the Biolog Micro-Station test (Biolog Inc., Hayward CA, USA). For siderophore production and uptake studies, *R. solanacearum* was grown at 30°C respectively in 11 and 100 ml shaker Erlen-meyer flasks containing per flask 500 (or 50) ml of a medium consisting of 5 g/l low-iron casein hydrolysate (DIFCO Bacto Casamino Acids), 1.54 g/l KH\(_2\)PO\(_4\), 3H\(_2\)O and 0.25 g/l MgSO\(_4\)-7H\(_2\)O, with final pH adjusted to 6.8 (CAA medium). The same medium treated first with 8-hydroxyquinoline for removal of contaminating Fe\(^{3+}\) (CAAQ medium; Waring and Werkman, 1942) was used for growth studies (7.5 ml of medium in 180×18 mm capped test tube). For growth inhibition tests a CAA-agar.

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**Abbreviations** (NMR): COSY, correlation spectroscopy; HMQC, 1H-detected multiple bond heteronuclear multiple quantum coherence; HMBC, 1H-detected 2D heteronuclear multiple quantum coherence.

* Part LXIX of the series “Bacterial Constituents”. For part LXVIII see Michalke et al. (1996).

Reprint requests to Prof. Dr. H. Budzikiewicz.

Telefax: +49-221-4705057.
medium was prepared from 5 g/l casein hydrolysate, 13 g/l agar-agar (MERCK, Darmstadt, D), 3.3 g/l K$_2$HPO$_4$·3H$_2$O, 2.6 g/l KH$_2$PO$_4$ and 0.25 g/l MgSO$_4$·7H$_2$O with resulting pH 6.8. Phosphate buffer, MgSO$_4$ solution and CAA-agar mixture were sterilized separately in an autoclave, hot combined and poured into sterile Petri dishes which were kept sterile under UV-light until use. Bacteria were grown in the same medium without agar. For comparison purposes the schizokinen producing strain *Bacillus megaterium* ATCC 19213 (obtained directly from the American Type Culture Collection) was grown in the liquid growth medium defined by Mullis et al. (1971), with the exception that sucrose was replaced by glucose. For iron uptake experiments 0.5 µl of a $^{59}$Fe preparation (Amersham, Les Ulis, F) containing 12.7 µg/ml Fe with an activity of 0.1 mCi/ml were added per ml medium containing 0.12 mg cell material (dry weight).

**Growth inhibition experiments**

CAA-agar plates were inoculated with 50 µl of the stationary bacterium culture (after 48 hrs of growth) which was distributed equally by shaking with glass beads. Three discs of filter paper (Ø 4 mm) were placed on the cultures, 2 of them containing 20 µl of a solution of either the free or the ferri-pyoverdine (succinic acid side chain) of *Pseudomonas fluorescens* ATCC 13525 (Hohln-eicher et al., 1995) and the third one sterile H$_2$O. The plates were incubated for 20 hrs at 37°C. All plates were covered evenly with small circular bacterial colonies except for inhibition zones around the pyoverdine impregnated paper discs (see Table I). Pyoverdine fluorescens was checked with 366 nm UV irradiation.

**Isolation of the siderophores**

During the growth of *R. solanacearum* in the CAA medium an increase of the pH was observed. When after 2 to 3 days it reached 8.1, 20 ml/l of a 5% ferric citrate solution were added and the pH brought to 5.8 with 6 N HCl. The bacterial cells were removed by tangential filtration and 0.2 g/l NaN$_3$ was added to the filtrate to inhibit further bacterial growth. The filtrate of 5 l culture medium was adsorbed on a 5x50 cm column loaded with XAD-4 resin (Serva, Heidelberg, D) activated by shaking with 11 CH$_3$OH and subsequent washing with 31 H$_2$O. Polar substances such as salts were removed with 21 H$_2$O, and the organic material was eluted with 11 CH$_3$OH/H$_2$O 1:1 (v/v). The eluate was concentrated i.v. to ca. 5 ml. This concentrate was then chromatographed on Bio-Gel P-2 (Bio-Rad, Hercules, USA) (column 5x21 cm) with 0.1 N CH$_3$COOH. An orange fraction was collected and concentrated i.v. to ca. 5 ml. According to TLC (silicagel, CH$_3$OH/H$_2$O 7:3 v/v) and to high voltage paper electrophoresis at pH 6.9 the fraction contained i.a. two orange substances, one of them uncharged and the other with one negative charge. Preparative separation was possible by anion exchange chromatography on DEAE Sephadex A-25 (Pharmacia, Uppsala, S) equilibrated with NaCl (column 2.6x30 cm). One fraction could be eluted with H$_2$O, the second one which...
was adsorbed on the top of the column could be desorbed with a 0.1 molar NaCl solution. The first fraction was re-chromatographed on CM Sephadex C-25, the second one was freed from NaCl by chromatography on Bio-Gel P-2. Evaporation to dryness yielded dark-red solids (5 and 13 mg, resp.) which were pure as judged by TLC.

Schizokinen from *B. megaterium* ATCC 19213 was purified according to Mullis *et al.* (1971) as well as by the method described above for the *R. solanacearum* siderophore. The Chrome-Azurol-S (CAS) assay of Schwyn and Neilands (1978) was used for the detection of siderophores in growth supernatants.

**Decomplexation**

The ferric siderophores were dissolved each in 3 ml 1% citric acid and shaken with 1 ml portions of a 5% solution of 8-hydroxyquinoline in CHCl₃ until the organic phase remained colorless. The aqueous phase was brought to dryness i.v. and the free siderophores were chromatographed on Sephadex G-15 (column 2.6×25 cm) with H₂O.

**Spectroscopy**

NMR: Bruker AM 300 (Bruker, Karlsruhe, D); mass spectra: Finnigan MAT HSQ30 (Finnigan MAT, Bremen, D); UV/Vis: Perkin-Elmer Lambda 7; IR: Perkin-Elmer FT-IR 1600 (Perkin-Elmer, Überlingen, D).

**Other methods**

Siderophore-mediated growth stimulation tests using a plate bioassay, uptake studies using ⁵⁹Fe-siderophore iron complexes, isolation of *R. solanacearum* outer membranes and the analysis of their protein pattern on Na dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis were done as described before (Cornelis *et al.*, 1989). For the purification of the siderophores used in ⁵⁹Fe-uptake experiments see: Pyoverdines from *Pseudomonas aeruginosa* ATCC 15692, ATCC 27853 and Pa6, *Ps. fluorescens* ATCC 13525, ATCC 17400 and CCM 2798, of pyochelin, cepabactin and orni-bactin from *Burkholderia cepacia* ATCC 25416 and of enterobactin from *Escherichia coli* (Hohnadel and Meyer, 1988; Cornelis *et al.*, 1989, Meyer, 1992; Meyer *et al.*, 1995), of desferrireroxamine E from *Ps. stutzeri* ATCC 17588 (Meyer and Abdallah, 1980). Desferrioxamine B (Desferal) and salicylic acid are commercial samples. Aerobactin, coprogen, ferricrocin, ferrirubin, and ferrichrom A were kindly provided by Prof. G. Winkelmann (Univ. Tübingen).

**Synthesis**

Schizokinen (1) was synthesized by a combination of literature procedures: Mono-N-benzyloxy-1,3-diaminopropane ditosylate was prepared according to Lee and Miller (1983), but starting from 3-bromopropylamine HBr (Fluka, Buchs, CH) which was transformed into its Boc derivative (treatment with (Boc)₂O and (C₂H₅)₃N in tetrahydrofuran/H₂O 1/1 for 3 hrs at room temp.) rather than from 3-aminopropanol-1. The synthesis of 1 then followed the procedure of Milewska *et al.* (1987).

**Results**

**Bacterial growth and siderophore production**

Growth of *R. solanacearum* as a function of the Fe³⁺ concentration of the growth medium was studied in a CAAQ medium. Iron was added to the CAAQ medium in form of a sterile 20 mM FeCl₃ solution. As shown in Fig. 2, *R. solanacearum* responded to a severe iron starvation with almost no growth occurring in CAAQ medium even after 72 hrs of incubation. A concentration of 1 μM Fe³⁺ in the culture medium induced a strong increase in cell yield. Maximal growth after 24 hrs was observed with 5 to 10 μM Fe³⁺. Only growth supernatants from cultures with iron concentration between 0 and 2 μM gave a positive CAS test (Schwyn and Neilands, 1987) and developed a faint yellow-orange color following the addition of an excess of Fe³⁺ (5 μl of 2 μM FeCl₃ commercial solution per test tube containing 7.5 ml culture). The amount of iron-complexing compound(s) produced during the culture was estimated by measuring the optical density at 450 nm of the iron-supplemented centrifuged growth supernatants. The curve obtained (Fig. 2) shows that the synthesis of the iron-complexing material was under iron control with a maximal production for 1 μM Fe³⁺ in the CAAQ medium, and no production at all for Fe³⁺ concentrations ≥ 5 μM. Growth in a CAA
medium gave rise to about the same amount of iron-complexing compound(s).

The siderophore function of the iron-complexing compound(s) produced by *R. solanacearum* grown under iron deficiency was confirmed by iron uptake studies using $^{59}$Fe bound to the purified siderophore. Authentic schizokinen from *B. megaterium* ATCC 19213 and synthetic schizokinen were also tested. As shown in Fig. 3, in each case the incorporation of iron into the cells was induced with the same rate. Similar results were obtained regarding the iron uptake by *Bacillus megaterium*, the cells of which exhibit a higher capacity for iron uptake than the *R. solanacearum* cells. Iron incorporation was observed neither with iron-saturated cells grown in a CAA medium containing 100 μM FeCl₃ nor with iron-starved *R. solanacearum* cells in assays where the schizokinen was replaced by the Fe-complexes of pyochelin, cepabactin or ornibactin, siderophores produced by taxonomically related *Burkholderia cepacia* strains (Meyer et al., 1995). These compounds as well as other siderophores, viz. pyoverdines, salicylic acid, enterobactin, aerobactin, and the desferri-forms of ferrioxamines, coprogen, ferricrocin, ferrirubin and ferrichrome A were tested without success for iron supply in a plate bioassay (Cornelis et al., 1989), suggesting that *R. solanacearum* possessed siderophore-mediated iron uptake abilities only for schizokinen, the only one promoting bacterial growth in the plate bioassay (data not shown). That schizokinen is the only siderophore used by *R. solanacearum* is supported by the observation that just one potent ferri-siderophore receptor was induced when the bacteria were grown under iron deficiency. The comparison of the outer membrane protein patterns of iron-starved and iron-fed cells (grown in CAA and 100 μM iron supplemented CAA medium, resp.), as shown by electrophoresis on SDS-polyacrylamide gel of SDS-denatured outer membrane material (Coomasie Blue tinction of the proteins) clearly showed that a unique iron-regulated outer membrane protein was specifically produced by the iron-starved cells. Its apparent molecular mass of 65 kDa, as deduced from gel electrophoresis with protein standards, as well as the iron-regulated expression,
strongly suggests its role as ferri-schizokinen receptor.

The growth of *R. solanacearum* is, however, inhibited by the desferri-pyoverdine of *Pseudomonas fluorescens* ATCC 13525. As can be seen from Table I inhibition zones are encountered around the filter paper discs containing at least a 3 mM solution. The typical pyoverdine fluorescence (irradiation at 366 nm) disappears at lower pyoverdine concentrations where no growth inhibition is observed. Contact with the growth-medium rather than degradation by *R. solanacearum* is responsible for the disappearance of the fluorescence as could be shown by blind experiments without bacteria. The ferri-pyoverdine did not inhibit *R. solanacearum*, regardless of the concentration used. This excludes effects such as antibiotic activities rather than an iron starvation of *R. solanacearum*.

**Schizokinen (1)**

4-[[3-acetylhydroxyamino]-propyl]amino]-2-[2-[[3-(acetylhydroxyamino)-propyl]amino]-2-oxoethyl]-2-hydroxy-4-oxo-butanoic acid (CAS No. 35418-52-1). The FAB mass spectrum shows an \([M+H]^+\) ion at \(m/z\) 421. The \(^1\)H- and \(^{13}\)C-NMR data of 1 confirmed by H,H- and C,H-COSY-measurements are assembled in Tables II/III since in the literature only rather incomplete data can be found (Mullis et al., 1971; Simpson and Neilands, 1976; Plowman et al., 1984; Milewska et al., 1987). They are in agreement with the proposed structure. 1 forms a 1:1 complex with Fe\(^{3+}\) using the two hydroxamic acid units and the free hydroxyl plus carboxyl group of the citric acid part as three two-dentate ligands to accommodate the octahedral metal ion (Goldman et al., 1983; Plowman et al., 1984). The complex has, therefore, one negative charge (\(-4 H^+ + 1 Fe^{3+}\)). Its VIS-spectrum shows at pH 5.5 an absorption maximum at \(-400\) nm due to its charge transfer band, shifted to smaller wavelengths with decreasing pH (Byers et al., 1967). The isolated 1 proved to be identical (mass spectrum, NMR, UV) with a synthetic sample. Schizokinen-mediated \(^{59}\)Fe-uptake experiments demonstrated an identical effect for natural and synthetic schizokinen both in *R. solanacearum* ATCC 11696 and in *B. megaterium* ATCC 19213.

**Schizokinen A (2)**

N,1-bis[[3-(acetylhydroxyamino)-propyl]-3-hydroxy-2,5-dioxo-3-pyrrolidine acetamide (CAS No. 83948-77-0). The FAB mass spectrum shows an \([M+H]^+\) ion at \(m/z\) 403. The \(^1\)H- and \(^{13}\)C-NMR data confirmed by H,H- and C,H-COSY measurements are assembled in Tables II/III. It was suggested (Persmark et al., 1993) that 1 is actually the genuine bacterial metabolite which in the culture medium partially cyclizes to the more stable succin-
Table III. 13C-NMR-data (75.4 MHz). Chemical shifts of schizokinen and schizokinen A (D2O, 25 °C). Signal-numbers refer to those in structures 1 and 2, respectively.

<table>
<thead>
<tr>
<th>Signal</th>
<th>δ (Schizokinen) [ppm]</th>
<th>δ (Schizokinen A) [ppm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>179.4</td>
<td>181.4</td>
</tr>
<tr>
<td>2</td>
<td>75.9</td>
<td>73.8</td>
</tr>
<tr>
<td>3 (3a)</td>
<td>45.7</td>
<td>43.2 (42.7)</td>
</tr>
<tr>
<td>4 (4a)</td>
<td>173.1</td>
<td>171.8 (178.5)</td>
</tr>
<tr>
<td>5 (5a)</td>
<td>37.8</td>
<td>37.4 (37.7)</td>
</tr>
<tr>
<td>6 (6a)</td>
<td>26.8</td>
<td>26.6 (25.1)</td>
</tr>
<tr>
<td>7 (7a)</td>
<td>46.7</td>
<td>46.5 (46.5)</td>
</tr>
<tr>
<td>8 (8a)</td>
<td>175.0</td>
<td>175.2 (175.2)</td>
</tr>
<tr>
<td>9 (9a)</td>
<td>20.5</td>
<td>20.5 (20.5)</td>
</tr>
</tbody>
</table>

The assignments of the signals were supported by C,H-HMQC- and C,H-HMBC-data. Internal standard DSS; δ(TMS) = δ(DSS) − 1.61.

Discussion

Schizokinen (named after its bacterial cell division promoting ability) was first observed in (Lankford et al., 1966) and isolated from the culture medium of Bacillus megaterium (Byers et al., 1967) and subsequently from Anabaena sp. (Cyanobacteria, bluegreen algae) (Simpson et al., 1975). Its structure was elucidated by Mullis et al. (1971). It is a member of a group of siderophores derived from citric acid which differ only in the nature of the diamines forming the two hydroxamic acid complexing sites (for a compilation see Ghosh and Miller, 1993).

There are scattered reports in the literature on an antagonism of other Pseudomonas spp. against Ps. solanacearum: 2-Keto-D-gluconic acid secreted from the strain B5 of Ps. cepacia inhibits the growth of Ps. solanacearum in vitro and in vivo (Aoki et al., 1991). It was also reported (Gallardo et al., 1989; Gnanamanickam, 1990) that Ps. fluorescens (another soil bacterium) acts as an antagonist. The formation of a bacteriocin (fluocin BC8) or the antibiotic activity of phenazine-1-carboxylic acid or a compound related to it (Gurusiddaiah et al., 1986; see, however, Brisbane et al., 1987; Taraz et al., 1990) was invoked for this effect. An alternative explanation could be that the iron complexing constant of the R. solanacearum siderophore schizokinen (cf. Harris et al., 1990) is much lower than that of the pyoverdine of Ps. fluorescens (Mohn et al., 1990). The lack of iron caused by the competition of Ps. fluorescens will necessarily inhibit the growth of R. solanacearum (cf. also Ciampi and Guaquil, 1994). The data reported for the growth inhibition experiments assembled in Table I clearly show that the pyoverdine of Ps. fluorescens ATCC 13525 has this effect. The isolation of schizokinen from this species may be of interest even in another way: Siderophores related to schizokinen were coupled with antibiotics (Ghosh and Miller, 1993) using the siderophores as carriers into the cell (Trojan Horse strategy). The identification of schizokinen as the siderophore of Ps. solanacearum may thus provide a means for an effective combat against this plant deleterious bacterium.

Acknowledgements

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