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

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Z. Naturforsch. **52 c,** 496–503 (1997); received April 3/May 23, 1997

Ralstonia solanacearum, Pseudomonas solanacearum, Schizokinen, Siderophore

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-butanoic 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).

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

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

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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., Vandœuvre-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 1 l and 100 ml shaken Erlenmeyer 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 $K_2HPO_4 \cdot 3H_2O$ and 0.25 g/l $MgSO_4 \cdot 7H_2O$, 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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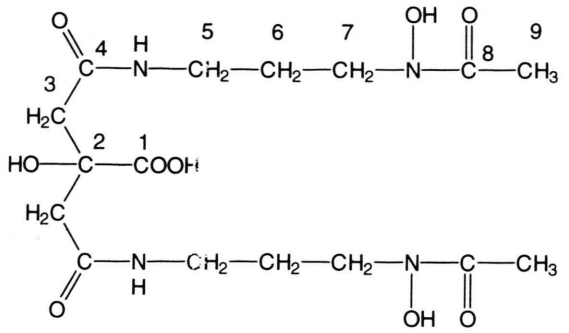

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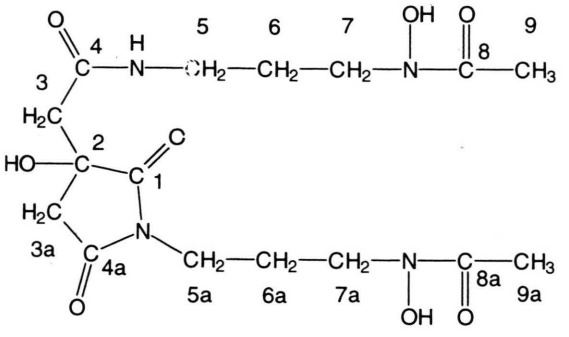

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Fig. 1. Structures of schizokinen (**1**) and schizokinen A (**2**).

medium was prepared from 5 g/l casein hydrolysate, 13 g/l agar-agar (MERCK, Darmstadt, D), 3.3 g/l $K_2HPO_4 \cdot 3H_2O$, 2.6 g/l KH_2PO_4 and 0.25 g/l $MgSO_4 \cdot 7H_2O$ 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 glas beads. Three discs of filter paper (∅ 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 (Hohlneicher *et al.*, 1995) and the third one sterile H_2O. 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 5×50 cm column loaded with XAD-4 resin (Serva, Heidelberg, D) activated by shaking with 1 l CH_3OH and subsequent washing with 3 l H_2O. Polar substances such as salts were removed with 2 l H_2O, and the organic material was eluted with 1 l CH_3OH/H_2O 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 5×21 cm) with 0.1 N CH_3COOH. An orange fraction was collected and concentrated i.v. to ca. 5 ml. According to TLC (silicagel, CH_3OH/H_2O 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 2.6×30 cm). One fraction could be eluted with H_2O, 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 $CHCl_3$ 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 H_2O.

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 ^{59}Fe-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 ^{59}Fe-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 ornibactin 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 desferriferrioxamine 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 $(C_2H_5)_3N$ in tetrahydrofuran/H_2O 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 Fe^{3+} 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 $FeCl_3$ 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 Fe^{3+} in the culture medium induced a strong increase in cell yield. Maximal growth after 24 hrs was observed with 5 to 10 μM Fe^{3+}. 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 Fe^{3+} (5 μl of 2M $FeCl_3$ 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 Fe^{3+} in the CAAQ medium, and no production at all for Fe^{3+} concentrations $\geq$ 5 μM. Growth in a CAA

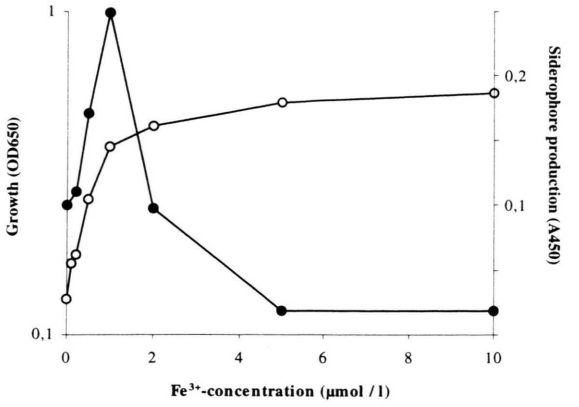

Fig. 2. Growth of *Ralstonia solanacearum* ATCC 11696 and siderophore production as a function of the growth medium iron concentration. Growth (O) in a CAAQ medium supplemented with various amounts of a sterile $FeCl_3$ solution was followed turbidimetrically at 650 nm (OD650). To stationary cultures (7.5 ml, stationary phase reached after 24–40 hrs, depending on the iron-concentration) 5 µl of a 2 M $FeCl_3$ solution were added and the siderophore content (●) was determined by measuring the optical density of the centrifuged culture supernatants at 450 nm (OD450).

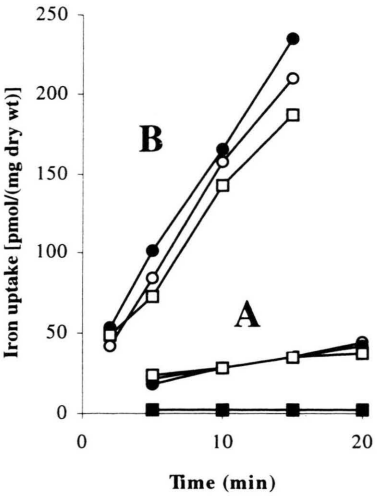

Fig. 3. ^{59}Fe uptake in iron-starved *Ralstonia solanacearum* ATCC 11696 (**A**, average values of 3 experiments, except for synthetic schizokinen and for the not active siderophores (see text) where only one experiment was performed) and *Bacillus megaterium* ATCC 19213 (**B**) mediated by schizokinen from *R. solanacearum* ATCC 11696 (○), schizokinen from *B. megaterium* ATCC 19213 (●), and synthetic schizokinen (□). No iron uptake in *R. solanacearum* was mediated by pyochelin, cepabactin or ornibactin for iron starved cells or by schizokinen for cells grown in iron rich medium (■). At the onset of all experiments the concentration of labeled iron was 0.9 nmol/mg cells (dry weight).

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 $FeCl_3$ 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 bioasssay (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-denaturated outer membrane material (Coosmassie 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 bee 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*.

Table II. ^{1}H-NMR-data (300 MHz). Chemical shifts of schizokinen and schizokinen A (D_2O; 25 °C). Signal-numbers refer to those in structures **1** and **2**, respectively.

Signal	δ (Schizokinen) [ppm]	δ (Schizokinen A) [ppm]
3 (3a)[a]	2,65;d / 2,78;d	2,91;d / 3,01;d (2,87;d / 3,14;d)
5 (5a)	3,23;t	3,20;t (3,60;t)
6 (6a)	1,85;m	1,83;m (1,97;m)
7 (7a)	3,69;t	3,69;t (3,70;t)
9 (9a)[b]	2,15;s	2,17;s (2,17;s)

Multiplicities: s: singlet, d: doublet, t: triplet, m: multiplet.
Internal standard DSS (2,2dimethyl-2-silapentan-5-sulfonate); δ(TMS) = δ (DSS).
The assignments of the signals were supported by H,H-COSY-data.
[a] The two protons are – due to the prochiral center of the molecule – magnetically not equivalent and form therefore an A,B-system with a ^{2}J-coupling-constant of 15 Hz.
[b] The singlets show a 1 shoulder due to the *cis-trans*-conformeres of the acetyl-hydroxamic acid.

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 accomodate 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 wave lengths 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-

Pyoverdine conc.	10 µM	100 µM	1 mM	2 mM	3 mM	4 mM	5 mM	10 mM
Inhibition	–	–	–	*	+	+	+	+
Inhibition zone	–	–	–	–	1.7	1.7	1.8	2.2
Fluorescence	–	–	**	+	+	+	+	+

* The colonies around the pyoverdine impregnated paper discs were smaller than the ones farther away.
** Very weak.

Table I. Growth inhibition of *Ralstonia solanacearum* by the pyoverdine of *Pseudomonas fluorescens* ATCC 13525.

Table III. ^{13}C-NMR-data (75,4 MHz). Chemical shifts of schizokinen and schizokinen A (D_2O, 25 °C). Signal-numbers refer to those in structures **1** and **2**, respectively.

Signal	δ (Schizokinen) [ppm]	δ (Schizokinen A) [ppm]
1	179,4	181,4
2	75,9	73,8
3 (3a)	45,7	43,2 (42,7)
4 (4a)	173,1	171,8 (178,5)
5 (5a)	37,8	37,4 (37,7)
6 (6a)	26,8	26,6 (25,1)
7 (7a)	46,7	46,5 (46,5)
8 (8a)	175,0	175,2 (175,2)
9 (9a)	20,5	20,5 (20,5)

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.

imide system. Since it lacks the free carboxyl group of **1** it forms a neutral Fe^{3+} complex (- $3H^+$ + Fe^{3+}).

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 Guaiquil, 1994). The data reported for the growth inhibition experiments assembled in Table I clearly show that the pyoverdine of *Ps. fluorescencs* 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

We wish to thank T. Heulin, C. N. R.S. Vandœvre-Les-Nancy for providing the *Ralstonia* strain, Prof. G. Winkelmann, Univ. Tübingen, for putting the siderophores mentioned in the text at our disposal, and Deutsche Forschungsgemeinschaft, Fonds der Chemischen Industrie and the European Community (Grant No. BIO4-CT95–0176) for financial assistance. It is also acknowledged gratefully that Dr. M. J. Milewska, Univ. Gdánsk, provided details on the synthesis of **1**, and that Dr. R. Reisbrodt, Robert Koch-Institut, Wernigerode, performed the Biolog© identification test.

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