



## **S, S-rhizoferrin (*enantio*-rhizoferrin) – a siderophore of *Ralstonia* (*Pseudomonas*) *pickettii* DSM 6297 – the optical antipode of R, R-rhizoferrin isolated from fungi**

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### **Abstract**

From the culture medium of *Ralstonia* (formerly *Burkholderia* or *Pseudomonas*) *pickettii* DSM 6297 grown under iron-limited conditions an iron complexing compound (siderophore) could be isolated. The structure of the isolated polycarboxylate siderophore was determined by spectroscopic methods as *S,S*-rhizoferrin, the enantiomer of *R,R*-rhizoferrin produced by fungi (Zygomycetes). Transport experiments with radiolabelled iron using *S,S*- and *R,R*-rhizoferrin showed no differences in the bacterial *Ralstonia* strain, while transport of *R,R*-rhizoferrin was superior in the producing fungal *Rhizopus* strain, suggesting stereoselective recognition in the fungus.

### **Introduction**

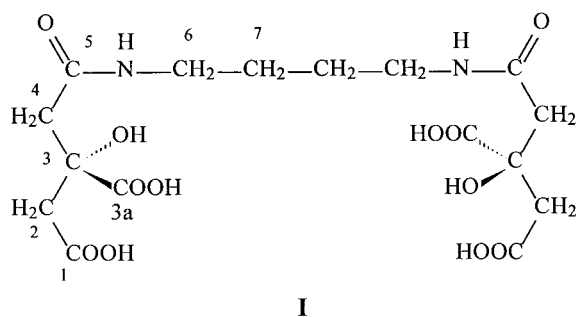
Many microorganisms when grown under iron deficient conditions produce Fe(III)-chelating compounds (so-called siderophores) to make available otherwise insoluble ferric oxide hydrates present in the soil, or organic-bound iron when affecting living organisms (for review see: Drechsel & Winkelmann 1997). In the course of investigations of the siderophores of the bacterial genus *Pseudomonas sensu lato* (Budzikiewicz 1997) the plant deleterious *Ralstonia* (*Pseudomonas*) *solanacearum* (Budzikiewicz *et al.* 1997) was found to produce the citrate-derived schizokinen. *Ralstonia solanacearum* was among the seven species of the *Pseudomonas* RNA homology group II which were separated under the generic name *Burkholderia*, but subsequently combined with *Pseudomonas pickettii* and *Alcaligenes eutrophus* as *genus novum Ralstonia* (Yabuuchi *et al.* 1995). In contrast to the plant pathogen *R. solanacearum*, *R. pickettii* is an opportunistic human pathogen responsible for occasional

nosocomial infections (e.g., Fernandez *et al.* 1996). It seemed of interest to see whether the close relationship of the two species is also reflected in the nature of their siderophores. The siderophore of *R. pickettii* could be identified as *S,S*-rhizoferrin (*enantio*-rhizoferrin) **I**, the enantiomer of *R,R*-rhizoferrin obtained from fungi of the zygomycetes.

### **Materials and methods**

#### *Strain and growth conditions*

The strain *Ralstonia pickettii* DSM 6297 was obtained from DSMZ, Braunschweig. The growth medium contained per liter 5 g low-iron casein hydrolysate (DIFCO Bacto Casamino Acids), 0.25 g MgSO<sub>4</sub> · 7H<sub>2</sub>O, 2 g K<sub>2</sub>HPO<sub>4</sub> · 3H<sub>2</sub>O, 1.6 g KH<sub>2</sub>PO<sub>4</sub> (pH 6.8) and 0.5 µmol Fe<sup>3+</sup> added as 5% Fe(III) citrate solution (all chemicals from Merck, Darmstadt). The bacterial pellet was suspended in sterile medium. From this suspension 250 ml medium in



**I**  
Scheme 1.

500 ml Erlenmeyer flasks with indentations were inoculated and shaken for 48 h at 26 °C; the pH went up to 7.5 and was subsequently adjusted to 7.0 with 6 N HCl. This culture was stored in a refrigerator at 5 °C. For the main culture 6 indentated fermenters (volume 3 l) containing each 1.33 l medium were inoculated with ca 10 drops of the storage culture and shaken for 60 h at 26 °C. The pH went up to 8.1 and was subsequently adjusted with 6 N HCl to 7.0.

#### Isolation of rhizoferrin (I)

The culture medium was freed from cell material by tangential filtration. Unpolar substances were removed by chromatography on XAD-2 resin (Serva, Heidelberg) which did not retain **I** and inorganic material. The eluate was concentrated i.v. to ca. 0.5 l, subsequently rechromatographed on Bio-Gel P-2 (Bio-Rad, Hercules, USA) with H<sub>2</sub>O as eluent (column 6 × 18 cm, detection at 214 nm). The Chrome-Azurol-S (CAS) assay (Schwyn & Neilands 1987) was used for detection of the colorless siderophore. CAS-positive fractions were collected and further purification was achieved by anion exchange chromatography on QAE-Sephadex A25 (Pharmacia, Uppsala, S). **I** is adsorbed and soluble material was removed with H<sub>2</sub>O as eluent. Subsequent elution with a H<sub>2</sub>O/NaCl gradient increased to 0.2 mol/l and then isocratic desorbs **I** (column 2.5 × 14 cm, detection at 214 nm); the CAS-positive fraction was collected. NaCl was finally removed by chromatography on Sephadex G10 with H<sub>2</sub>O as eluent (column 2.5 × 33 cm, detection at 214 nm). The CAS-positive fraction was lyophilized and a white crystalline powder was obtained. Yield ca 200 mg/l culture.

#### Spectroscopy

NMR: Bruker DRX 500 (Bruker, Karlsruhe) 7.0 mg **I** in 0.6 ml H<sub>2</sub>O/D<sub>2</sub>O 9:1 (v/v), 298K. 500 MHz for <sup>1</sup>H, 125 MHz for <sup>13</sup>C. Internal standard 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). For <sup>1</sup>H δ(TMS) = δ(DSS), for <sup>13</sup>C δ(TMS) = δ(DSS) – 1.61 ppm. Broadband-decoupling for the <sup>13</sup>C data acquisition. The H<sub>2</sub>O signal was suppressed by the WATERGATE puls sequence.

Mass spectrometry: Finnigan-MAT HSQ30 (Finnigan MAT, Bremen) with a FAB-gun (Ion-Tech Ltd., Teddington, GB), FAB-gas Xe.

Circular dichroism (CD) spectra: CD spectra were recorded on a Jasco 720 CD spectrometer (Japan Spectroscopic Company Ltd, Tokyo, Japan) equipped with IBM compatible PC for data aquisition and processing. Recording conditions and samples were as indicated in the figure legend.

#### Uptake studies

Kinetics of rhizoferrin-mediated iron transport in cells of *R. pickettii* were performed according to Cornelis *et al.* (1989) with the exception that cultures and incubation during transport studies were done in casamino acids medium instead of succinate medium, in which *R. pickettii* grows very poorly. HPLC-purified *S,S*-rhizoferrin originated from *R. pickettii* (this work) and HPLC-purified *R,R*-rhizoferrin isolated from *Cunninghamella elegans* (Drechsel *et al.* 1995) were used for the comparative uptake studies.

Time-dependent uptake kinetics in fungi were performed with germinated (6 h) spores of *Rhizopus arrhizus* (syn *R. oryzae*) in asparagine salts medium as described by Thieken & Winkelmann (1992) and Carrano *et al.* (1996). Uptake of radio-labelled rhizoferrin (<sup>59</sup>Fe and/or <sup>55</sup>Fe) was followed with time using iron complexes of *S,S*-rhioferrin and *R,R*-rhizoferrin. The radioactivity taken up with time was calculated as nmoles iron/mg dry weight.

## Results

#### Spectroscopic evidence

FAB-mass spectrometry revealed an [M+H]<sup>+</sup>-ion at *m/z* 437 and an [M+Na]<sup>+</sup>-ion at *m/z* 459 indicating a molecular mass of 436u.

<sup>1</sup>H- and <sup>13</sup>C-NMR (Tables 1 and 2) shows the typical pattern of a citric acid derivative with non-

Table 1.  $^1\text{H}$ -NMR-data of **I**

Signal	$\delta$ (ppm); multiplicity ( $^2J$ (Hz))	Integral
2	2.74;d/2.59;d (16.1)	2
4	2.73;d/2.57;d (14.6)	2
6	3.18;m	2
7	1.50;m	2
NH	7.81;t	1

Multiplicities: d: doublet, t: triplet, m: multiplet.

Table 2.  $^{13}\text{C}$ -NMR-data of **I**

Signal	$\delta$ (ppm)
1	178.7
2	45.9
3	76.3
3a	180.9
4	45.5
5	173.3
6	40.2
7	26.9

The assignments of the signals were supported by C,H-HMQC and C,H-HMBC-data.

equivalent carboxyl ends: 2 isolated  $\text{CH}_2$ -groups with diastereotopic protons forming AB-systems, 1 quaternary C and 3 C belonging to carboxyl groups. A broadened NH-triplet coupling with the  $\text{CH}_2$ -group at 3.18 ppm and the CO-group at 173.3 ppm demonstrates the presence of a CO-NH- $\text{CH}_2$  unit, the  $\text{CH}_2$ -group of which in turn couples with the  $\text{CH}_2$ -group at 1.50 ppm. These signals comprise one half of **I**, therefore, the other half of the molecule must be magnetically identical. The symmetry of the molecule is confirmed by a non-decoupled C,H-HMBC spectrum: The signal of the  $\text{CH}_2$ -group at 1.50 ppm (C-7) shows  $^1J$ -coupling as well as  $^2J$ -coupling with C-6 and with C-7', its analogous neighbor.

The MS- and NMR-data prove that the *Ralstonia pickettii* siderophore is rhizoferrin (Drechsel *et al.* 1991; Shenker *et al.* 1995). The CD spectra (Figure 1) of the free ligand and of the iron complex (Table 3) gave the surprising result that **I** is the *S,S*-enantiomer of the fungal rhizoferrin (Drechsel *et al.* 1992).

The stereochemical configuration of the isolated bacterial rhizoferrin (free ligand) was analyzed by CD measurements at two different pH values (pH 4.8 and 7.4) at a concentration of 0.835 mM (0.5 cm cell length) in the range of 190–550 nm with 0.2 nm reso-

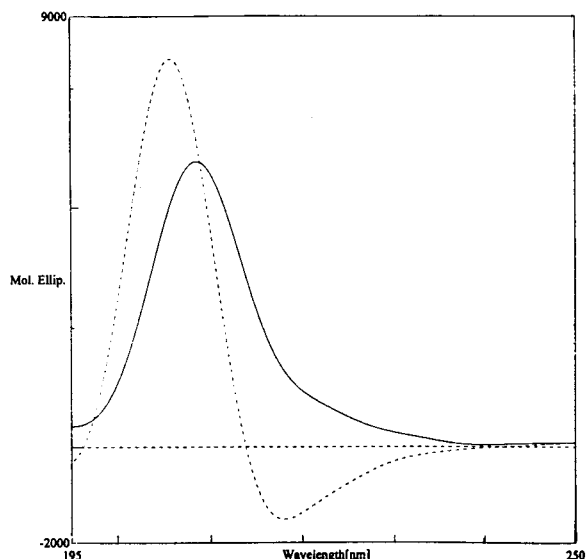


Figure 1. Circular dichroism (CD) spectra of isolated *S,S*-rhizoferrin from *R. pickettii*. Spectra were recorded on a Jasco CD spectrometer in bidistilled water. Solutions of desferri-rhizoferrin ( $8.35 \times 10^{-4}$  M, room temperature, quartz cuvette 0.5 cm) were adjusted with NaOH to pH 4.1 (or pH 7.4, dotted line).

Table 3. CD-data of **I**

Desferri, pH 4.8	Desferri, pH 7.4
max. $\theta_M = 5915$ (209 nm)	max. $\theta_M = 8020$ (206 nm) min. $\theta_M = -1600$ (218 nm)
Ferri, 1:1, pH 4.1	Ferri, 1:1, pH 5.8
min. $\theta_M = -4170$ (245 nm) shoulder $\theta_M = 150$ (275 nm) max. $\theta_M = 3040$ (320 nm) min. $\theta_M = -790$ (369 nm)	min. $\theta_M = -5240$ (243 nm) shoulder $\theta_M = 2020$ (270 nm) max. $\theta_M = 6920$ (316 nm) min. $\theta_M = -3280$ (368 nm)

Conc. (ligand) =  $8.35 \cdot 10^{-4}$  M in water, unbuffered.

lution. As shown in Table 3, the CD spectrum at pH 4.8 revealed a single maximum at 209 nm ( $\theta_M = 5915$ ) and the spectrum at pH 7.4 showed a maximum at 206 ( $\theta_M = 8020$ ) and a minimum at 218 nm ( $\theta_M = -1600$ ) indicating the presence of an *S,S* configuration. A comparison with the configuration of the fungal *R,R*-rhizoferrin (Drechsel *et al.* 1992) clearly confirms the presence of *enantio*-rhizoferrin as the product of *R. pickettii*.

The results of the transport properties of the bacterial *S,S*-rhizoferrin are shown in Figure 2. Due to the fact that two enantiomeric forms of rhizoferrin exist, it was of interest to study the transport properties of

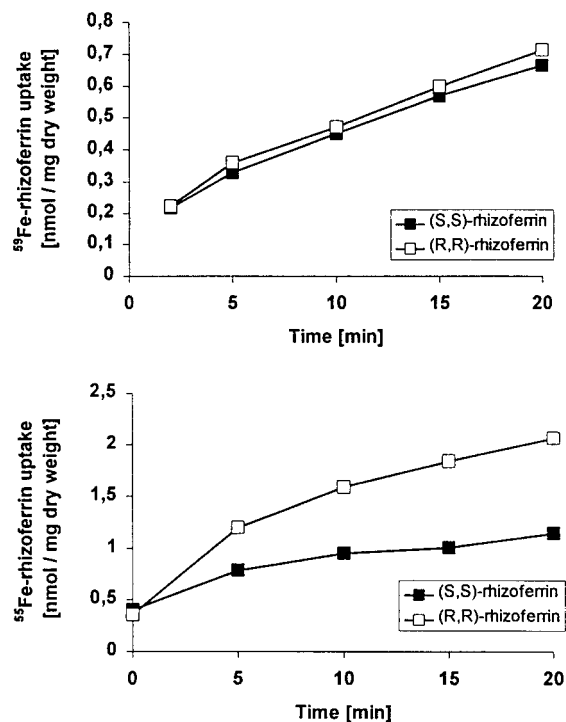


Figure 2. Time-dependent uptake of radio-labeled ( $^{55}\text{Fe}/^{59}\text{Fe}$ ) ferric rhizoferrin using bacterial *S,S*-rhizoferrin and fungal *R,R*-rhizoferrin in the producing strains (upper: *Ralstonia pickettii*; lower *Rhizopus arrhizus*). The radioactivity taken up was calculated as nmoles per mg dry weight.

both rhizoferrins in the two different producing organisms. Thus, we analyzed the time dependent iron uptake in *R. pickettii* (DSM 6297) and in *R. arrhizus* using radiolabelled ( $^{55}\text{Fe}$  and  $^{59}\text{Fe}$ ) *S,S*-rhizoferrin and *R,R*-rhizoferrin. As shown in Figure 2, iron transport mediated by *S,S*- and *R,R*-rhizoferrin proceeded in a very similar way when studied in the bacterium (*R. pickettii*). However in the fungus *R. arrhizus* the siderophore activity of *R,R*-rhizoferrin was superior (ca. 2 $\times$ ) compared to *S,S*-rhizoferrin. Also the total amount of iron uptake was twice as high in the fungal strain which might reflect the higher iron requirement of the eukaryotic organism.

## Discussion

The achiral C-3 of citric acid becomes chiral when only one of its carboxyl groups is substituted. The *R,R*-configured rhizoferrin has been found to be produced by members of several families of the Zygomycetes (Thieken & Winkelmann 1992), but so far

not by bacteria. It is not uncommon that fungi and bacteria produce identical compounds, especially when their structure is relatively simple. Also the formation of optical antipodes is known as, e.g., amongst amino acids or carbohydrates. That enantiomers are built by combining three structural subunits seems, however, to be a special case.

It was shown earlier that ferric *R,R*-rhizoferrin can be utilized not only by the producing zygomycetous fungi, but also by a non-producing bacterium *Morganella morganii* (Thieken & Winkelmann 1993; Kühn *et al.* 1996). The ferric *R,R*-rhizoferrin transport in fungi has been characterized by the so-called shuttle mechanism, that is migration of the ferric siderophore through the cell wall and release of iron inside the cell (Carrano *et al.* 1996).

The differences in the stereochemistry of the bacterial and fungal rhizoferrins are surprising and have raised the question whether enantiomeric recognition by the transport systems exist. It was shown earlier by comparing the transport of *enantio*-( $\Delta$ -*cis*) ferrichrome with the natural ( $\Delta$ -*cis*) ferrichrome in ferrichrome-producing fungi like *Neurospora* and *Penicillium* (Winkelmann 1979) and by comparing the fungal with the bacterial transport in *E. coli* (Winkelmann & Braun 1981), that the fungal system is highly stereoselective. The present finding that *R,R*-rhizoferrin shows twice as high iron uptake rates in the fungus than the *S,S*-rhizoferrin, confirms our earlier findings of stereoselective recognition of siderophores in fungi.

The production of enantiomeric compounds in fungi and bacteria also raises questions regarding its biosynthesis which due to the different chirality must have different biosynthetic pathways. *Ralstonia pickettii* produces a citrate siderophore as does *R. solanacearum*, and it uses 1,4-diaminobutane (putrescine) which seems to be the typical polyamine for the *Ralstonia* group (Busse & Auling 1988). However, there are differences between the siderophores of the two species. Rhizoferrin binds  $\text{Fe}^{3+}$  by the non-substituted parts of the two citric acid units. Schizokinen, the siderophore of *R. solanacearum*, contains only one citric acid molecule, both the 1- and 3-carboxyl group of which carry a diamine substituent (1-amino-3-(acetyl-hydroxy-amino)-propane. The ligand sites for  $\text{Fe}^{3+}$  are the two hydroxamic acid elements and the  $\alpha$ -hydroxy-2-carboxyl group of the citric acid part. Surprisingly 1,3-diaminopropane is not found amongst the polyamines of *R. solanacearum* (Busse & Auling 1988). Thus, the common feature of

the two *Ralstonia* species is the formation of citrate siderophores, containing aliphatic diamines.

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