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Characterization of 'Schizokinen'; a dihydroxamate-type siderophore produced by *Rhizobium leguminosarum* IARI 917

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Abstract

The Rhizobia comprise one of the most important groups of beneficial bacteria, which form nodules on the roots (rarely on the stems) of leguminous plants. They live within the nodules and reduce atmospheric nitrogen to ammonia, which is further assimilated by plants into required nitrogenous compounds. The Rhizobia in return obtain nutrition from the plant. Rhizobia are free-living soil bacteria and have to compete with other microorganisms for the limited available iron in the rhizosphere. In order to acquire iron Rhizobia have been shown to express siderophore-mediated iron transport systems. *Rhizobium leguminosarum* IARI 917 was investigated for its ability to produce siderophore. It was found to produce a dihydroxamate type siderophore under iron restricted conditions. The siderophore was purified and chemically characterized. The ESMS, MS/MS and NMR analysis indicate the dihydroxamate siderophore to be 'schizokinen', a siderophore reported to be produced by *Bacillus megaterium* that shares a similar structure to 'rhizobactin 1021' produced by *Sinorhizobium meliloti* 1021. This is the first report of production of schizokinen by a strain of *R. leguminosarum*, therefore it was carefully investigated to confirm that it is indeed 'schizokinen' and not a degradation product of 'rhizobactin 1021'. Since ferric–siderophore complexes are transported across the outer membrane (OM) into the periplasm via an OM receptor protein, *R. leguminosarum* IARI 917 was investigated for the presence of an OM receptor for 'ferric–schizokinen'. SDS PAGE analysis of whole cell pellet and extracted OM fractions indicate the presence of a possible iron-repressible OM receptor protein with the molecular weight (MW) of approximately 74 kDa.

Introduction

Iron is an essential element required by almost all microorganisms except for some bacteria of the genera *Lactobacillus* and *Streptococcus* (Guiseppi & Fridovich 1982; Archibald 1983). Microorganisms growing under aerobic conditions require iron for a variety of functions including reduction of ribotide precursors of DNA, synthesis of ATP via the electron transport chain, synthesis of heme and many other vital processes (Litwin & Calderwood 1993; Neilands 1995). In spite of being the fourth most abundant metal ion in the earth's crust, it is not readily available to

microorganisms due to the presence of its insoluble oxyhydroxide polymeric form under aerobic conditions at physiological pH. Under these conditions the concentration of soluble iron is about 10^{-18} M (Raymond *et al*. 2003). Many microorganisms have overcome the restricted availability of iron by evolving a variety of sophisticated iron transport systems (Wandersman & Delepelaire 2004). Many bacteria and fungi produce siderophores, low molecular weight (400–1500 Da) organic molecules that specifically chelate Fe^{3+} with very high affinity and transport it across the cell wall and cell membranes into the cell (Neilands 1995). Their transport into the cell requires energy and is assisted by the receptors and several other proteins located in the OM, periplasm and the inner membrane of bacteria (Postle 1993).

Rhizobia are free-living soil bacteria and include the genera, *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Azorhizobium* and *Mesorhizobium*. For the Rhizobia living in soil, particularly in neutral and alkaline soil (near physiological pH) competition with other microbes for limited iron is intense (Lynch *et al*. 2001). Within root nodules Rhizobia are converted into the nitrogen fixing forms known as 'bacteroids' for which the demand for iron increases since many proteins involved in N_2 fixation require iron as a cofactor (Johnston 2004). Bacteroids also have in abundance iron containing cytochromes and other electron donors. Thus to satisfy their demand for iron Rhizobia are known to express high affinity siderophore mediated iron transport systems (Johnston 2004); these transport systems have not been studied in detail. Most strains of the fast-growing Rhizobia, e.g. *Sinorhizobium* and *Rhizobium* produce at least one type of siderophore (Persmark *et al*. 1993; Dilworth *et al*. 1998). Usually siderophores are classified on the basis of the functional groups they use for chelating ferric ion (Wandersman & Delepelaire 2004). In the majority of the siderophores characterized, these functional groups are either catechols or hydroxamic acids but siderophores containing other groups such as hydroxy carboxylic acid or mixed functional groups have also been reported (Raymond & Dertz 2004). Rhizobia have been reported to produce siderophores containing catechols, carboxylates, or di- and trihydroxamates (Smith *et al*. 1985; Patel *et al*. 1988; Johnston 2004).

The majority of siderophores are amino acid conjugates, while a few are citrate based or have an unique chemical structure (O'Brien & Gibson 1970; Raymond & Dertz 2004). 'Rhizobactin', the first chemically characterized siderophore produced by a strain of *S. meliloti*, has two iron-ligating carboxyl groups (Figure 1c) (Smith *et al*. 1985). Most strains of *R. leguminosarum* produce hydroxamate containing siderophores with some exceptions that also produce catechol type siderophores (Patel *et al*.1988). For example, 'vicibactin' is a trihydroxamate and is made by most strains of *R. leguminosarum* (Figure 1a) (Dilworth *et al*. 1998). Some strains of *S. meliloti* make a chemically distinct dihydroxamate-type siderophore known as 'rhizobactin 1021' (produced by well characterized strain 1021) (Figure 1b) (Persmark *et al*. 1993). The chemical structure of rhizobactin 1021 is citrate based and is similar to aerobactin and schizokinen, the siderophores produced by *Escherichia coli* and *Bacillus megaterium*, respectively (Figure 4a) (Gibson & Magrath 1969; Mullis *et al*. 1971). The genes involved in the biosynthesis of vicibactin and rhizobactin 1021 have been identified and steps in the biosynthetic pathway have been proposed (Lynch *et al*. 2001), although a complete mechanism of their synthesis is yet to be understood.

Rhizobactin 1021 produced by *S. meliloti* is transported by the receptor encoded by *rhtA*, whose product is similar to IucA, a receptor for the structurally similar siderophore, aerobactin, produced by enteric bacteria (de Lorenzo *et al*. 1986; de Lorenzo & Neilands 1986; Cuiv *et al*. 2004). Other Rhizobia including *S. meliloti* have been shown to carry multiple homologues of *fhuA* (a gene encoding a ferrichrome transporter fhuA in *E. coli*), which may be involved in the transport of hydroxamates produced by them (Yeoman *et al*. 2000). There are no homologues of *fhuBC* or D (genes encoding ABC type transporter located in the inner membrane and periplasmic binding protein respectively for the transport of ferrichrome in *E. coli*) found in *S. meliloti*, thus rhizobactin 1021 and other hydroxamates transported via FhuA homologues are further transported to the cytoplasm by some other transporter system (Johnston 2004). Similar to the siderophore-mediated transport systems in other bacteria, ferric–siderophore transport in

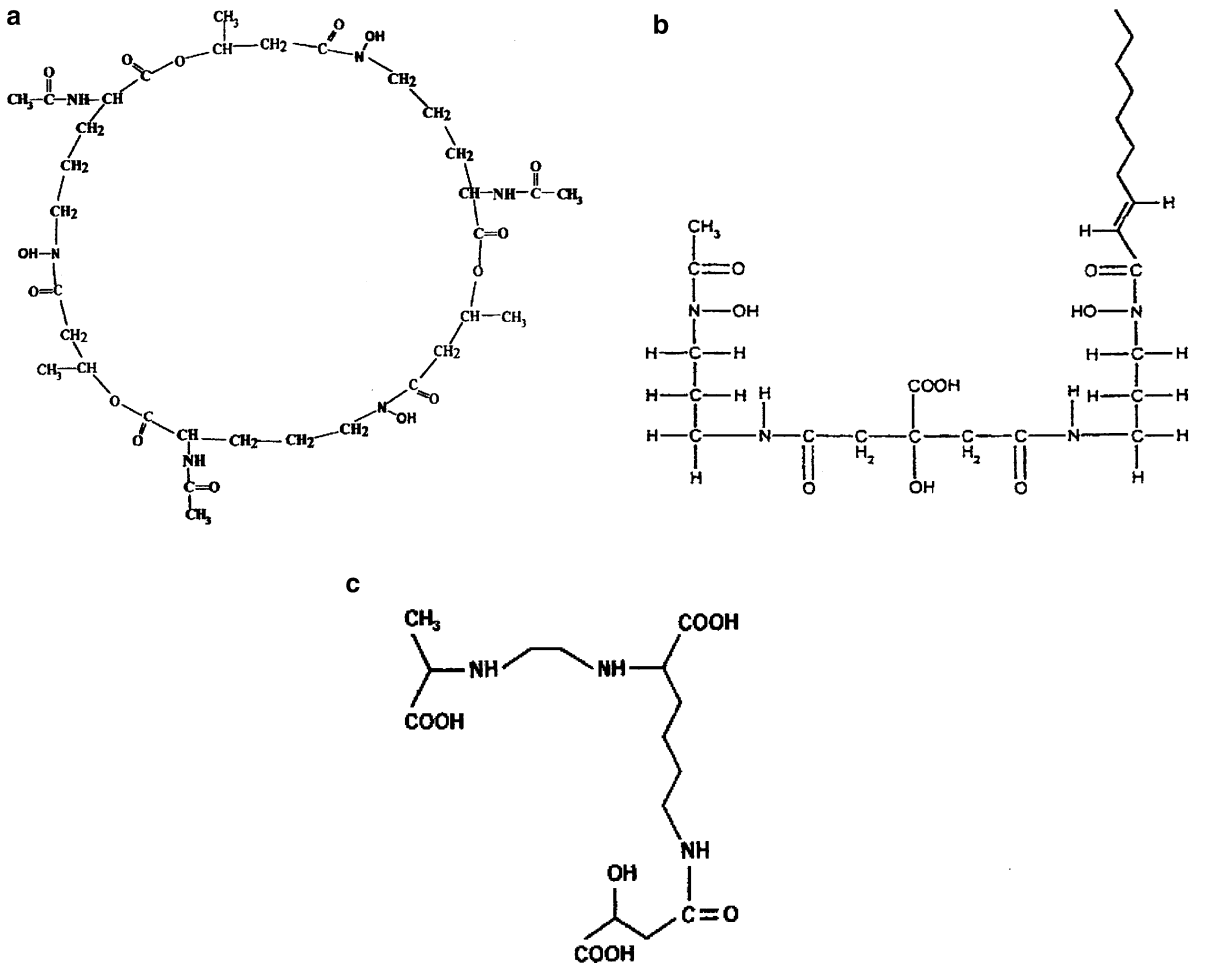


Figure 1. Structures of typical rhizobial siderophores: (a) vicibactin, (b) rhizobactin 1021 and (c) rhizobactin.

most Rhizobia is also energized by the TonB–ExbB–ExbD complex.

Most of the existing knowledge about siderophore-mediated iron transport is based on research using *E. coli* as a model organism (Braun *et al.* 1998; Chakraborty *et al.* 2003; Visca *et al.* 2002). Ferric–siderophore complexes are transported via OM receptor proteins into the periplasm in a TonB dependent manner. It is known that, from the periplasm, ferric–siderophore complexes are transported via ABC-type transporters located in the inner membrane into the cytoplasm, but not much is known about their transport across the OM. Iron transport systems of Rhizobia have not been investigated thoroughly in spite of their beneficial agro-economical role. Nothing is known about the structure or mechanism of transport of the ferric–siderophores via OM receptors in Rhizobia. In the present study we have chemically characterized a dihydroxamate containing siderophore known as schizokinen from *R. leguminosarum* IARI 917. Rhizobactin 1021, a common rhizobial siderophore, differs from schizokinen only in that it contains a lipid moiety attached to it that may facilitate the formation of micelles, protecting it from extracellular damage (Persmark *et al.* 1993, Johnston 2004). An iron-repressible OM protein of 74 kDa has also been detected, which falls within the reported range for molecular weights of known ferric–siderophore OM receptor proteins (Reigh and O'Connell 1993; Diarra *et al.* 1996).

Materials and methods

Bacterial strains and media

Rhizobium leguminosarum IARI 917 was obtained from Indian Agricultural Research Institute, New Delhi, India. This culture was isolated from root nodules of pigeon pea and is maintained on modified mannitol yeast agar medium supplemented with Congo Red containing 1% mannitol, 0.05% K_2 HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% NaCl, 0.1% yeast extract and 3% agar (0.25 ml of 1% Congo Red solution was added per 100 ml of medium) (Jadhav & Desai 1996). Congo red dye is generally not taken up by Rhizobia as readily as other organisms, which helps in identification of contaminants (Kneen & Larue 1983). The culture was stored in the form of glycerol stocks in LB broth at −80 °C for long-term storage. For siderophore production, the culture was grown on modified Fiss-glucose medium (Vellore 2001), supplemented with 1.0% maltose, 0.1% $(NH_4)_2$ SO_4, and 0.5 μM $FeSO_4$ (Storey 2005).

Siderophore detection assays and estimation

The Chrome Azurol S (CAS) assay developed by Schwyn and Neilands was used for the detection of siderophore (Schwyn & Neilands 1987). Iron starved culture supernatants were tested for siderophore production by dispensing about 60 μl of supernatant into a bored well in CAS plates and incubating the plates at room temperature for 30 min–5 h. Formation of an orange halo indicated production of siderophore. To determine the chemical type of siderophore, the supernatants were tested with Arnow's method (Arnow 1937) for catechol-type and Atkin's method (Atkin *et al.* 1970) for hydroxamate-type siderophores. Both are colorimetric assays where 2,3-dihydroxybenzoic acid and ferrichrome were used as standards for catechol-type and hydroxamate-type siderophores, respectively.

Extraction and purification of siderophores

For siderophore production, the culture was grown in optimized minimal medium in 5 l volume (1 l per 2.8 l flask) at 28 °C for 24 h on a shaker. The supernatant was collected and the pH was adjusted to 7.0. Amberlite XAD-2 columns (20 × 5.0 cm) were prepared manually and equilibrated with deionized water (dH_2O). The culture supernatant was passed through the column and bound siderophores were washed with at least two bed volumes of dH_2 O and eluted into several fractions with methanol (HPLC grade, Fisher Co.). The fractions were tested for the presence of siderophore using TLC (following section). Fractions containing siderophore were dried, the residues re-dissolved in methanol, and then complexed with $FeSO_4$. They were then loaded on a 50 × 1.5 cm column packed with Sephadex LH-20 (Sigma Co), a hydrophobic and gel filtration resin. The siderophore was eluted with methanol in a number of fractions. All the fractions were checked for the presence of siderophore using thin layer chromatography (TLC). The fractions positive for siderophores were collected, flash evaporated, re-dissolved in dH_2 O and purified by HPLC (300 mm × 7.8 mm Nova Pack HR-C18 column) using 100% dH_2 O and 90% methanol as mobile phases. Purified fractions were freeze-dried and stored at −20 °C until further analysis. When necessary, the ferric–siderophore complexes were deferriated with 8-hydroxyquinoline (Waring & Workman 1942).

Thin layer chromatography (TLC)

The presence of siderophores in various fractions during the process of purification was checked by TLC using silica gel plates (Selecto Scientific) with a solvent system consisting of *n*-butanol:acetic acid: dH_2 O in 12:3:5 ratio (Atkin *et al.* 1970). At the end of the TLC run the plates were dried and developed with an iron reagent (0.1 M $FeCl_3$ in 0.1 N HCl). Hydroxamate-type siderophores form wine-colored spots while catechol-type form dark gray colored spots on TLC. For amino acid analysis, siderophores were acid hydrolyzed using 6 N HCl and autoclaved at 121 °C for 6 h. The solvent system used for the separation of amino acids was methanol: 0.1 M ammonium acetate (60:40) and plates were developed by spraying ninhydrin reagent (0.5% ninhydrin in ethanol) and incubating the plates at 55 °C for 15 min. The amino acid standards were used at 1 mg/ml concentrations.

Spectral scans of purified siderophores

The Atkin's assay used for the determination of the presence of hydroxamate type siderophores could also be used to determine the number of hydroxamate groups present in the compound. 0.5 ml of solution of purified hydroxamate-type siderophore was mixed with iron perchlorate reagent and an absorbance scan was taken in the range of 300–700 nm wavelength using a spectrophotometer (Perkin–Elmer). Compounds containing trihydroxamates showed absorbance maxima between 420 and 440 nm while dihydroxamate containing compounds showed absorbance maxima between 500 and 520 nm (Jalal & van der Helm 1991).

Mass spectrometry analyses of purified siderophores

Samples of partially purified siderophores were analyzed by positive and negative ion electronspray mass spectrometry (ESI MS), employing a Waters Quattro Micro triple quadrupole mass spectrometer (Milford). Methanol solutions (15–30 μl) of the samples were dissolved in 1 ml of 50:50 acetontrile:water containing 4 mM ammonium acetate. The resulting solutions were infused into the ESI MS source using the following settings: source temperature 130 °C; desolvation temperature 400 °C; capillary voltage 2 kV; syringe pump flow 25 μl/min; cone voltage 15 volts; desolvation gas flow 750 l/h. The mass spectrometry/mass spectrometry (MS/MS) analyses employed 3×10^{-3} mbar of argon as the collision gas. The MS/MS spectra were obtained with voltages between 19 and 25 volts.

The accurate mass positive ion ESI MS data was obtained on a Waters LCT TOF mass spectrometer (Milford). The samples were infused into the instrument with the following settings: source temperature 90 °C; desolvation temperature 250 °C; capillary voltage 3 kV; syringe pump flow 15 μl/min; cone voltage varied; desolvation gas flow 750 l/h; Resolution 5000, Np multiplier (dead time correction) 0.7; Rf lens 100 volts. Methanol solutions ($\sim$10 μl) of the samples were dissolved in 1 ml of 50:50 acetontrile:water containing 4 mM ammonium acetate. About 10 μg of propylene glycol (PPG) was added to the mixture as an internal standard for accurate mass determinations.

Concentrations of analytes and calibrants were carefully adjusted to minimize dead-time effects caused by peak intensities that are too large to be corrected by the dead-time computer algorithm. Approximately 10 scans at a cone voltage of 16 (molecular ions obtained for siderophores and ammonium adducts for PPG oligomers) were averaged with 10 scans at 50–75 cone volts (fragment ion series at low mass for propylene glycol such as m/z 117, 175, etc.). The summed spectrum was corrected for dead time, centroided, and calibrated against a PPG calibration table to four significant figures. The accurate mass values for the siderophores were then determined from the calibrated file.

NMR analysis of purified siderophores

The purified siderophore was examined by ^{1}H and ^{13}C 1D NMR as well as HMQC and HMBC 2D NMR. For these spectra, the entire sample of siderophore was dissolved in 0.75 ml D_2O. The NMR spectra were collected in a 5-mm OD NMR tube on a Bruker Model DRX-500 NMR spectrometer operating at ambient probe temperature. A proton 1D NMR spectrum was collected with 65,536 data points and 20 ppm sweep width with ^{13}C decoupling using GARP decoupling and a 15-sec relaxation delay. The ^{13}C 1D NMR spectrum was collected with 131,072 data points and 200 ppm sweep width with WALTZ16 composite pulse ^{1}H decoupling and an 8-sec relaxation delay. The residual proton resonance in HOD was used as the ^{1}H chemical shift standard at 4.80 ppm. Protonated DMSO (5 μl) was added as the ^{13}C NMR chemical shift standard at 39.44 ppm. For the 2D NMR spectra, the HMQC and HMBC spectra were collected with 2048 points per row and 256 rows and a 2-sec relaxation delay.

SDS PAGE analyses for the detection of iron-repressible protein in OM

Cells were grown overnight in the presence and absence of iron in 50 ml minimal media (Storey 2005). After removal of 100 μl of culture for whole cell collection, the rest of the cells were fractionated to isolate OMs using previously described methods (Reigh & O'Connell 1993). The intact

cells and the membrane fractions from the cells grown under iron supplemented and depleted conditions were analyzed using SDS PAGE.

Results and discussion

Many species belonging to Rhizobia, including those in the genera *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Azorhizobium* and *Mesorhizobium* have been reported to produce one or more types of siderophores (Dilworth *et al.* 1998; Johnston 2004). The majority of the species have been known to produce hydroxamate containing siderophores while very few contain catechol as the iron chelating group (Carson *et al.* 2000). *R. leguminosarum* IARI 917 was found to produce a dihydroxamate-type of siderophore under iron restricted conditions. The TLC analysis of the partially purified siderophore showed two distinct spots, which led us to believe there were two possible hydroxamate-type siderophores being produced (data not shown). We were able to separate these two compounds using a Sephadex LH-20 column and purified them further through reversed phase HPLC; the analysis of the purified compounds indicated that the fast moving spot on TLC was actually the dehydrated form of the intact siderophore. The siderophore was chemically characterized to be schizokinen, a siderophore previously reported to be produced by *B. megaterium* (Byers *et al.* 1967). The chemical structure of schizokinen is similar to that of rhizobactin 1021, a dihydroxamate siderophore produced by *S. meliloti* 1021 (Persmark *et al.* 1993). There is an addition of a single lipid moiety, decenoic acid to schizokinen to form rhizobactin 1021; this also makes rhizobactin 1021 an asymmetric molecule (Persmark *et al.* 1993; Lynch *et al.* 2001). It is believed that an addition of a lipid moiety perhaps helps siderophores to form micelles, which may provide protection against degradative cleavages (Martinez *et al.* 2000). Without a lipid moiety schizokinen still functions fully as a siderophore; complexing and transporting iron in *R. leguminosarum* IARI 917.

Extraction and purification of schizokinen

Since schizokinen is structurally similar to rhizobactin 1021 and has not been reported to be produced by a strain of *Rhizobium*, it was necessary to take precautions to insure that what was produced was not a degradation product of rhizobactin 1021. These precautions included purification of siderophore as an iron complex at the pH of 7.0 throughout the extraction (the same conditions reported to be used for the purification of rhizobactin 1021) (Persmark *et al.* 1993). The evidence of the absence of rhizobactin 1021 in the partially purified siderophore extracts from *R. leguminosarum* IARI 917 also came from the observation that the siderophore was insoluble in acetonitrile and acetonitrile extracts did not show the presence of any other siderophore. Rhizobactin 1021, being amphiphilic in nature, is reported to be highly soluble in acetonitrile (Persmark *et al.* 1993). Further ESI MS analyses of both the partially purified and purified siderophores confirmed the absence of rhizobactin 1021.

UV spectroscopic analysis

The hydroxamate-type siderophores can be further classified into two groups, trihydroxamates or dihydroxamates. The spectral scans using purified siderophore and iron-perchlorate assay revealed that the siderophore produced by IARI 917 was a dihydroxamate-type siderophore, which showed a peak at 500 nm (Figure 2) while trihydroxamates have absorbance maxima between 420 and 440 nm (Jalal & van der Helm 1991).

ESI MS analyses of the partially purified siderophores

The partially purified deferriated samples were analyzed by mass spectrometry to determine the

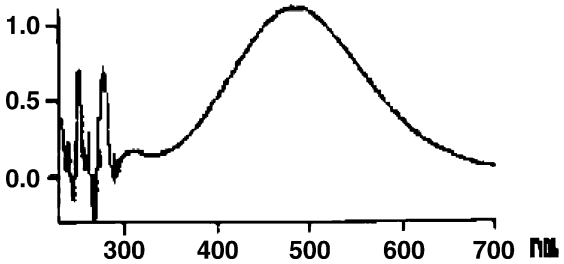


Figure 2. UV spectral scan of the siderophore produced by *R. leguminosarum* IARI 917.

possible presence of intact rhizobactin or its degradative byproducts. The previously reported (Persmark *et al*. 1993) FAB MS (fast atom bombardment mass spectrometry) analyses of rhizobactin 1021 samples showed ions at *m*/*z* 531 (M + H) and 513 (M + H), respectively, for the intact siderophore (530 Da.) and its 5-membered cyclic imide byproduct (512 Da.). The mass spectra of the partially purified and deferriated siderophore sample did not yield either of these ions (Figure 3), thus indicating the absence of either rhizobactin 1021 or its imide byproduct in our sample.

ESI MS was shown to be very successful in the characterization of siderophores (Kilz *et al*. 1999; Gledhill 2001; Spasojevic *et al*. 2001). ESI MS primarily yields molecular weight information if ions are extracted in the atmospheric pressure chemical ionization source to minimize 'in-source collisionally induced dissociation' reactions (Gabelica & De Pauw 2005). The partially purified and deferriated sample of the intact siderophore was analyzed by positive ion ESI MS and yielded ions at *m*/*z* 421 and 443, respectively, for the protonated (M + H) and sodiated species (M + Na) consistent with a molecular weight of 420 Da. This was confirmed by negative ion ESI MS spectrum which yielded an anion at *m*/*z* 419 for the deprotonated (M−H) species. This molecular weight and other analytical data indicated that the siderophore produced by *R. leguminosarum* IARI 917 is schizokinen (Figure 4a).

Two additional mass spectrometry experiments were performed to support this proposed identification. Accurate mass positive ion ESI MS data yielded ions at *m*/*z* 421.1909 and 443.1730 for the protonated and sodiated species, respectively. These values are consistent with those expected for the protonated (*m*/*z* 421.1935, −2.6 ppm error) and sodiated (*m*/*z* 443.1754, −2.4 ppm error) ions of schizokinen. Positive ion ESI MS/MS experiments were used to probe sub structural information for the protonated molecular ion, *m*/*z* 421. The MS/MS spectrum of the component, which is shown in Figure 5, is consistent with that expected for schizokinen.

Another component which was thought to be a siderophore was also analyzed by MS. The component yielded ions at *m*/*z* 403 and 401 for the M + H and M−H ions, respectively, in positive and negative ion ESI MS. This molecular weight (402 Da) is consistent with the dehydrated form of

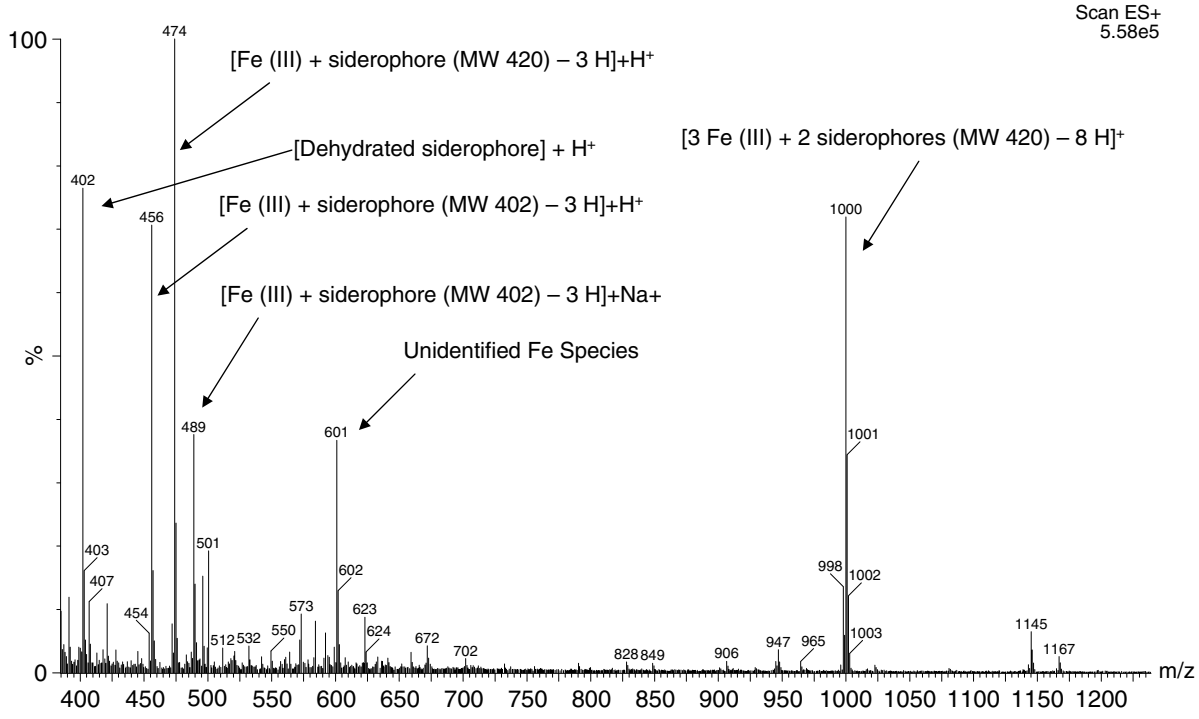


Figure 3. ESI MS spectrum of purified iron-complexed siderophore.

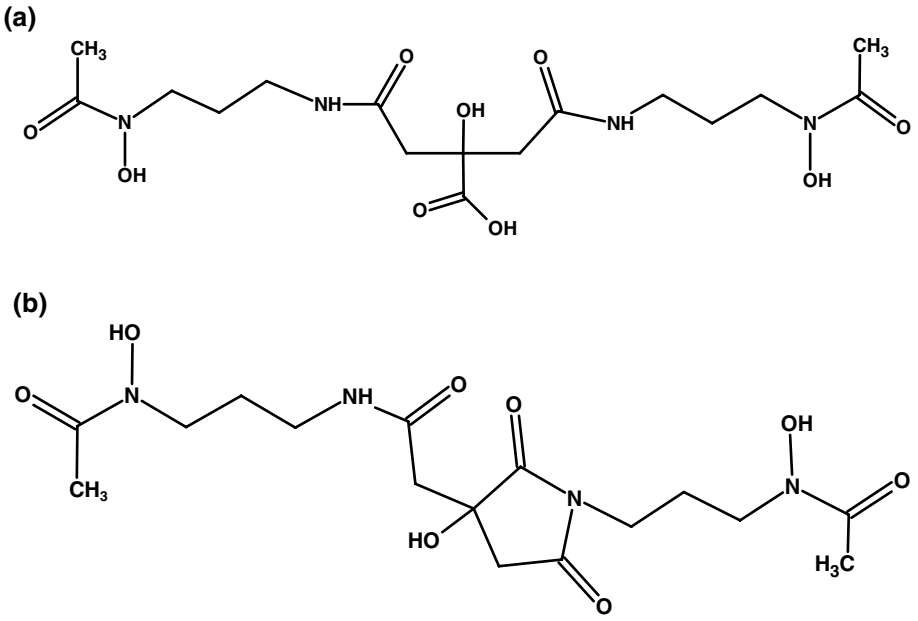


Figure 4. (a) Schizokinen and (b) Schizokinen A.

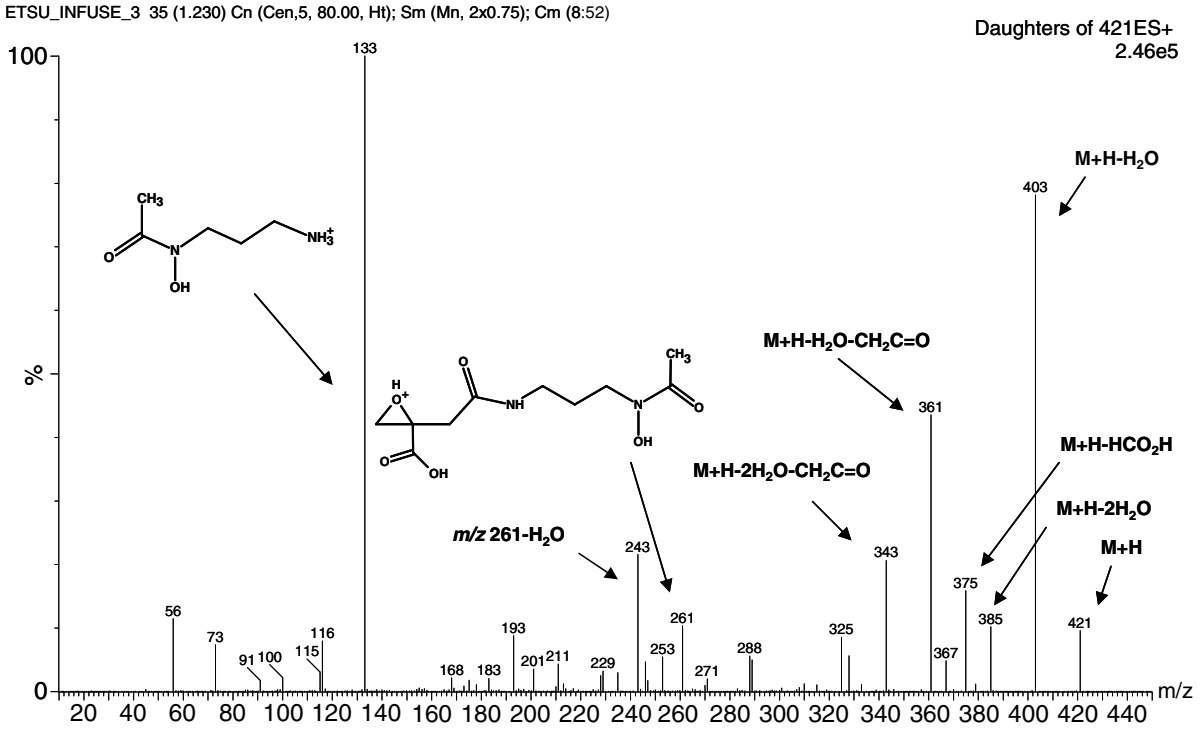


Figure 5. Positive ion ESI MS/MS spectrum of deferriated siderophore from *R. leguminosarum* IARI 917.

schizokinen. This 5-membered cyclic imide is commonly referred to as schizokinen A (Figure 4b) (Lee & Miller 1983; Hu & Boyer, 1995).

The ions found in the positive ion ESI MS/MS spectrum of this dehydrated component are essentially identical to those noted in the intact siderophore with molecular weight of 420. The major differences were the relative ion intensities. For example, the component with molecular weight of 420 (see Figure 3) has a base peak of m/z 133, but the base peak in the dehydrated species is m/z 193 (data not included here). The intact and the dehydrated siderophores were also analyzed as their iron complexes by ESI MS. The iron complexed spectrum of the intact siderophore is noted in Figure 3. The presence of iron in a species in the mass spectrum is easily discerned (McLafferty & Tureček 1993) due to the presence of several naturally occurring isotopes of iron (5.8% ^{54}Fe, 91.72% ^{56}Fe, 2.20% ^{57}Fe, and 0.28% ^{58}Fe) which yield very distinct isotopic patterns. For example, Figure 6 compares the observed measured isotopic abundance for the complex of [3 Fe(III) + 2 siderophores (MW 420) − 8H] at m/z of 1000 to that of a computer generated pattern for the species.

The positive ion ESI MS analysis of the iron complexed dehydrated siderophore (data not shown here) indicated that it still had the capacity to chelate Fe. It showed ions at m/z 456 and 858, respectively, for [Fe(III) + siderophore (MW 402) − 2H] and [Fe (III) + 2 siderophores (MW 402) − 2H].

NMR analysis of purified intact siderophore

Purified intact siderophore was analyzed by NMR spectroscopy to further confirm the structure of schizokinen. Structural characterization, accomplished using 1D and 2D NMR techniques, enabled

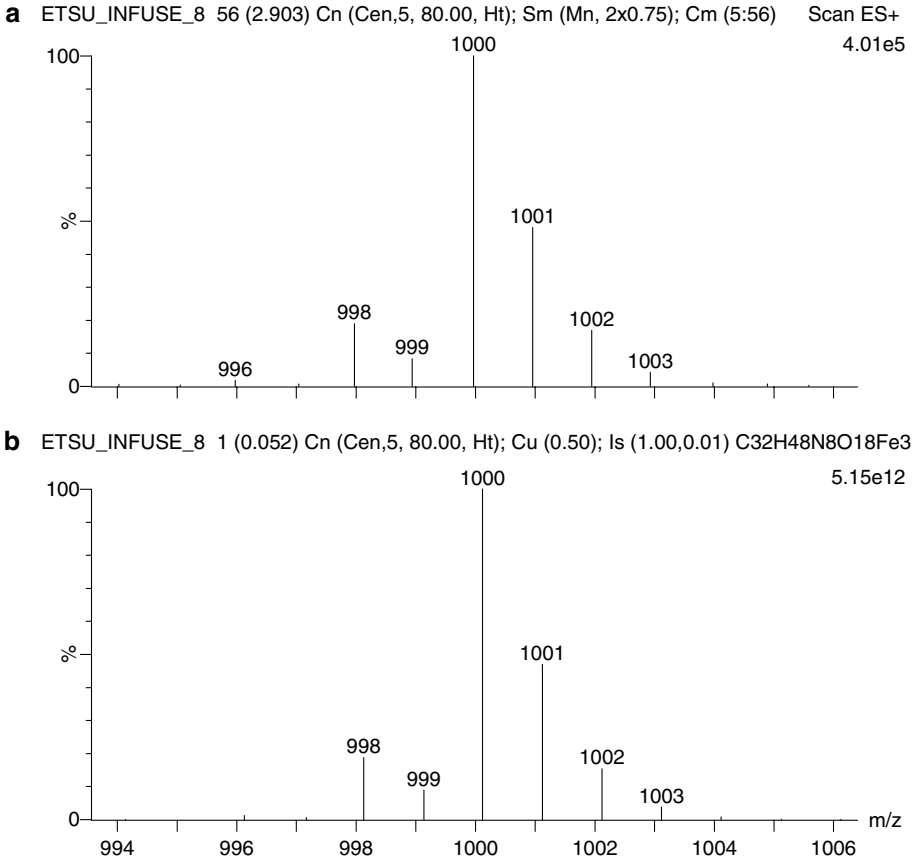


Figure 6. Comparison of (a) observed to (b) computer generated isotopic abundance ESI MS for complex of [3 Fe(III) + 2 siderophores (MW 420) − 8H].

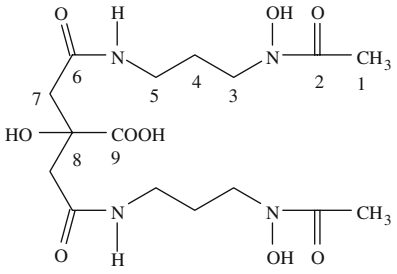
proton and C-13 NMR chemical shift assignments shown in Table 1. The proton NMR chemical shift assignments are in excellent agreement with the literature (Mullis *et al.* 1971; Persmark *et al.* 1993) for schizokinen while the C-13 NMR chemical shifts have not been presented previously.

Thus our spectral data confirm beyond doubt the production of the dihydroxamate-type siderophore schizokinen by *R. leguminosarum* IARI 917. We did get a positive ninhydrin reaction with indication of the presence of tryptophan on the basis of TLC and analytical HPLC analysis (data not presented here), although schizokinen does not contain any amino acids. Similar observations were also made by others (Akers 1983), but further investigation is needed to analyze the basis for the positive ninhydrin reaction.

Detection of a possible schizokinen OM receptor

Siderophores are excreted out of the cell where they selectively bind ferric ions with high affinity and transport them back across the membranes into the cell. The energy dependent transport is mediated via a specific receptor protein located in the OM of gram negative bacteria (Wandersman & Delepelaire 2004). Over 30 such OM receptor proteins involved in ferric–siderophore transport are known and crystal structures of four of them from enteric bacteria have been solved (Ferguson *et al.* 1998; Buchanan *et al.* 1999; Ferguson *et al.* 2002; Cobessi *et al.* 2005). All of them share a similar structure (van der Helm 2004; Cobessi *et al.* 2005). The components involved in the transport of ferric–schizokinen have not yet been characterized. Therefore it was of interest to identify the presence of a possible outer membrane receptor. It will be interesting to investigate the crystal structure of one of the rhizobial ferric–siderophore OM receptor proteins as rhizobial iron transport systems differ markedly from enteric systems. For example, rhizobial iron transport systems have greater flexibility in their use of iron sources and not all rhizobia follow some paradigms of iron-responsive gene regulations (Johnston 2004). The expression of the gene encoding the OM receptor is also regulated by the

Table 1. ^{1}H and ^{13}C NMR chemical shift assignments for schizokinen dissolved in D_2O.



Atom	^{1}H, ppm		^{13}C, ppm
	This work[a]	Lit.[b,c,d]	This work[a]
1 CH_3	2.11	2.12	20.00
2 C=O			174.62
3 CH_2	3.63	3.68	46.20
4 CH_2	1.81	1.83	26.28
5 CH_2	3.19	3.23	37.28
6 C=O			172.70
7 CH_2	2.72, 2.59	2.67	45.26
8 C			75.48
9 COOH			178.94

[a]In 0.75 ml D_2O with 5 ml protonated DMSO added as internal chemical shift standard for ^{13}C at 39.44 ppm in a 5-mm OD NMR tube on a Bruker Model DRX-500 NMR spectrometer operating at ambient probe temperature. Residual proton resonance of HOD was set to 4.80 ppm as the chemical reference for ^{1}H.[b]In D_2O as per references (c) and (d).[c]Persmark *et al.* 1993,[d]Mullis *et al.* 1971.

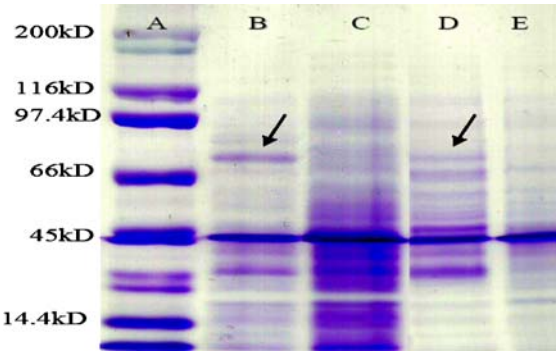


Figure 7. SDS/PAGE analysis of whole cell and membrane pellets: (a) molecular weight standard, (b) whole cells grown under low iron concentration, (c) whole cells grown under high iron, (d) membrane fraction from cells grown under low iron concentrations and (e) membrane fractions from cells grown under high iron concentration.

iron concentration in the environment in the same way as the siderophore biosynthetic genes.

The cells were grown in high (>20 μM) and low (0.5 μM) iron supplemented media to identify a possible OM receptor in *R. leguminosarum* IARI 917. The samples prepared from whole cell and isolated OM pellets from both the high and low iron cultures were analyzed using SDS PAGE. The proteins involved in iron transport should be repressed under high iron and expressed under low iron conditions. SDS PAGE analysis of these cell fractions (Figure 7) clearly indicated the expression of an OM protein under low iron conditions both in the cell and OM pellet. This protein has a MW of approximately 74 kDa, which is similar to the molecular weight of OM receptor proteins previously reported in other bacteria (Reigh & O'Connell 1993; Diarra *et al*. 1996). Vicibactin, a trihydroxamate siderophore (Figure 1a) produced by *R. leguminosarum* strain 8401 (pRL1JI), is thought to be transported by a fhuA homolog (Stevens *et al*. 1999; Yeoman *et al*. 2000). Western blot analysis using antibodies against fhuA did not show the presence of any fhuA homolog in the OM fractions of *R. leguminosarum* IARI 917 (data not included here). We plan to further characterize the outer membrane protein possibly involved in 'schizokinen' transport.

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