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East Tennessee State University

In partial fulfillment
of the requirements for the degree
Master of Science in Microbiology

by
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Keywords: iron transport, siderophore, *Rhizobium leguminosarum*, hydroxamate, schizokinen
ABSTRACT


by

Erin P. Storey

Iron is essential to the majority of microorganisms; it is an important cofactor in many cellular processes and enzymes. However in an aerobic environment and at biological pH, iron is primarily found as insoluble oxyhydroxides and is unavailable to microorganisms. Many bacteria have the ability to produce siderophores, low molecular weight compounds that have a high affinity for Fe$^{3+}$. Siderophores are part of a multi-component system that actively transports the iron-siderophore complex into the cytoplasm. Rhizobia are characterized by their ability to form symbiotic relationships with leguminous plants, where they can fix nitrogen for the host plant and the plant provides the bacteria with nutrients. Under iron-limiting conditions, Rhizobia are known to produce siderophores. *Rhizobium leguminosarum* IARI 917 produces one dihydroxamate-type siderophore. This siderophore has been purified and chemically characterized. Results indicate that this strain is producing schizokinen, which has not been described in a member of the Rhizobia family.
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CHAPTER 1
INTRODUCTION

Iron and Bacterial Cells

Iron is a vital element required by virtually all living organisms, including bacteria, with the exception of only a few, including Streptococcus sanguis, some Lactobacillus species, and Borrelia burgdorferi (Guiseppi and Fridovich 1982, Archibald 1983, Posey and Gherardini 2000). It is important in many cellular processes including the electron transport chain and in deoxyribonucleotide synthesis and acts as a cofactor for many enzymes, such as ribotide reductase, nitrogenase, peroxidase, catalase, and succinic dehydrogenase (Litwin and Calderwood 1993). It is the fourth most abundant element in the earth’s crust following oxygen, silicon, and aluminum. However, at biological pH and under aerobic conditions, iron is oxidized to insoluble oxyhydroxides polymers, which are unavailable to a microorganism. In fact, the maximum free ferric iron concentration in an environment is about $10^{-18}$ M, while most bacteria must maintain an internal iron concentration of around $10^{-6}$ M (Raymond et al. 2003). This iron limitation poses a serious threat to microorganisms, as they are unable to survive without adequate supplies of iron. To overcome this iron deficiency, bacteria have adapted to aerobic environments by evolving several sophisticated mechanisms for acquiring iron in almost any environment, including inside a host, in the soil, or other habitats. Siderophore-mediated iron transport systems, which will be the focus of this work, are one such mechanism used by many bacteria to obtain iron. Other mechanisms include acquisition of iron from heme or heme compounds and systems that acquire iron from
transferrin or lactoferrin, which will be discussed in more detail below. These systems are regulated by iron concentration (Lankford 1973) and are only expressed under iron-deficient conditions and suppressed under high iron concentrations. Many Gram-negative bacteria are known to produce one or more siderophores and the components for their transport. Such systems have been well studied in *Escherichia coli* (Bagg and Neilands 1987, Visca et al. 2002, Wandersman and Delepelaire 2004). Siderophores are part of a multi-component system for transporting ferric iron into a cell. Other components include a specific outer membrane receptor protein, the TonB-ExbB-ExbD protein complex in the inner membrane, a periplasmic binding protein, and an inner membrane ATP-dependent ABC-type transporter (Chakraborty et al. 2003), all of which are described in detail below. Gram-positive bacteria also secrete siderophores under iron stress, but mechanisms for their transport are not as well understood as the siderophore-mediated transport systems of Gram-negative bacteria.

*Rhizobium leguminosarum*, a member of the Rhizobia family, is a Gram-negative soil bacterium capable of inducing nitrogen-fixing nodules in leguminous plants, making this family agriculturally significant. Many members of this family are also known to produce siderophores (Carson et al. 2000), and this work will focus on the siderophore mediated iron-transport system of *R. leguminosarum* IARI 917.

**Siderophores**

The term 'siderophore' is Greek for “iron carrier” and is so named because these molecules produced by microorganisms have an extremely high affinity for ferric iron (Lankford 1973); thus, siderophores bind ferric iron and transport it into the bacterial
cell. They are low molecular weight (350-1500 Daltons) organic molecules, which can compete for ferric iron in ferric hydroxide complexes (Postle 1990). There are over 500 described siderophores (Wandersman and Delepelaire 2004) that are classified based on their chelating group specific for ferric iron. There are two main siderophore classes, the catechol-type and the hydroxamate-type. Catechol-type siderophores bind ferric iron with adjacent hydroxyls of catechol rings, and are almost always derived from 2,3-dihydroxybenzoic acid (DHBA) (Crosa and Walsh 2002). The best-studied example of a catechol-type siderophore is enterobactin (Figure 1A), which is produced by *E. coli* (O’Brien and Gibson 1970). Hydroxamate-type siderophores contain a carboxyl group attached to an adjacent nitrogen, which chelates ferric iron. An example of this type is ferrichrome (Figure 1B), a fungal siderophore produced by *Ustilago sphaerogena* (Emery 1971). Hydroxamates are generally more complex structurally and are also considered more hydrophilic in nature. In addition to these classes, a miscellaneous class of siderophores has also been established. Siderophores belonging to this class may contain both catechol and hydroxamate groups, which is the case for heterobactin, which is produced by *Rhodococcus erythropolis* (Carran et al. 2001), or other groups responsible for iron chelation. The binding capabilities vary depending on the siderophore; enterobactin has a stability constant (K_f) of 10^{52} for ferric iron, while ferrichrome exhibits a K_f of 10^{29} (Höfte 1993). Several assays have been developed to detect the presence of siderophore as well as to determine the chemical type of siderophore produced by a microorganism and these will be further discussed in chapter 2 (Arnow 1937, Atkin et al. 1970, Schwyn and Neilands 1987). Siderophores are secondary metabolites and are assembled by nonribosomal cytoplasmic peptide
synthases (Wandersman and Delepelaire 2004). Currently little is known about siderophore excretion. One 43 kDa inner membrane protein thought to be involved in enterobactin secretion, EntS, has been described and shows some homology to known export pumps, such as TetA that exports tetracycline. The gene, entS, which codes for this protein is found in the region of genes required for enterobactin synthesis and mutant strains that produce defective EntS secrete much less intact enterobactin than the wild-type (Furrer et al. 2002). Work is ongoing to better characterize the systems responsible for siderophore export.
Figure 1. Chemical structures of common siderophores: (A) enterobactin, a catechol-type siderophore and (B) ferrichrome, a hydroxamate-type siderophore
Siderophore Transport

Outer Membrane Receptor Proteins

Because of higher concentrations of iron inside the cell and an extremely low concentration of available iron outside the cell, diffusion is not an option for the transport of ferric iron into the cell. Thus, ferric siderophore complexes are actively transported across the outer membrane of Gram-negative bacteria through a specific outer membrane receptor protein (OMRP), whose genes are also induced only under iron-deficient conditions (Earhart and McIntosh 1977). The crystal structures of four outer membrane receptor proteins responsible for transporting ferric-siderophore complexes have recently been solved. These include FepA, which transports ferric enterobactin (Buchanan et al. 1999), FhuA, which transports ferrichrome (Ferguson et al. 1998), FecA, which transports ferric dicitrate (Ferguson et al. 2002), and FpvA, which transports ferric pyoverdine (Cobessi et al. 2005). Figure 2 shows the ribbon structures for the first three OMRPs mentioned. (It is important to note here that citrate is not a classical siderophore but functions as one in bacteria as it has a high affinity for ferric iron and has a specific receptor, which makes its transport across bacterial membranes possible.) The crystal structures of the receptors show remarkable similarities; they are each composed of a β-barrel, consisting of 22 anti-parallel β-strands and a globular domain, which is referred to as a “cork” or “plug” because of the way it is oriented into the β-barrel domain (Endriss and Braun 2004). The β-barrel has short periplasmic loops and longer extracellular loops, which allow entrance of the ligand (Chakraborty et al. 2003). The complete mechanism for transport through the OMRP is not understood,
but it is presumed that the TonB protein complex provides the necessary energy needed for transport via the proton motive force since no energy is available in the outer membrane of Gram-negative bacteria. Two hypotheses have been proposed for transport of ferric siderophore complexes through these receptor proteins. Either the plug domain of the OMRP rearranges upon binding, allowing passage of the complex, or the plug completely removes itself from the β-barrel region. The first is probably more logical as more than 60 hydrogen bonds would have to be broken in order for the entire plug domain to move out of the β-barrel (Braun and Braun 2002).

Figure 2. Crystal structures of three OMRPs

In addition to the four crystal structures that have been solved, more than 30 sequences for various receptors involved in iron-siderophore transport have been reported (van der Helm and Chakraborty 2001). The sequences of these proteins show little homology, except for three conserved regions, comprising approximately 20 amino acid residues. These include the lock region, which positions the plug properly in the β-barrel, the TonB box, which is thought to physically interact with the TonB protein.
complex, and a third set of conserved residues which probably allows for conformational changes that permit passage of the substrate through a channel in the OMRP (van der Helm 2004). These residues are thought to be involved in transport but not binding of the ferric siderophore complexes (Chakraborty et al. 2003). While not a conserved region, all OMRPs also appear to have a switch helix, which unwinds upon ligand binding and it seems this facilitates the interaction between the TonB protein complex and the TonB box (Endriss et al. 2003). As expected, the binding sites are variable depending on the ligand to be transported and are present in both the extracellular loops of the β-barrel and in the plug domain (Endriss and Braun 2004). Binding of specific ligand appears to cause several extracellular loops of the β-barrel to change their conformation, which closes the OMRP, possibly for prevention of diffusion of other molecules through this open channel or to prevent the substrate from leaking outside the cell (Ferguson and Deisenhoffer 2004). Once the channel is closed, it seems that the ferric siderophore complex can then slide into the periplasm by rearrangement or removal of the plug region. This phenomenon of bipartite gating is thought to be an important feature of these receptors, although it has not been observed in the crystal structures of FhuA and FepA. It should be noted, however, the crystal structure for FepA has only been solved in the absence of ligand (Buchanan et al. 1999). OMRPs are generally specific for a single siderophore but can also bind other ligands. For example, FhuA can transport the antibiotics albomycin and microcin J25, phages T1, T5, and φ80, and the toxin colicin M in addition to ferrichrome (Endriss and Braun 2004).
The TonB-ExbB-ExbD Protein Complex

As mentioned previously, the outer membrane of a Gram-negative cell cannot generate or maintain an electrochemical potential. The TonB-ExbB-ExbD protein complex is thought to couple cytoplasmic membrane energy to energy-dependent processes occurring at the outer membrane. The TonB protein complex is common to all siderophore-mediated iron transport systems of Gram-negative bacteria. TonB is also required for other non-siderophore mediated iron transport systems such as those involved in the transport of heme (Wandersman and Delepelaire 2004). This involvement was first recognized in 1971 (Wang and Newton 1971) and has been studied widely since, mainly through mutation of the genes coding for this protein and its subsequent effect on transport (Postle 1990, Braun and Braun 2002). As described in the previous section, all outer membrane proteins involved in siderophore-mediated iron transport appear to have a short sequence of conserved residues at the N-terminus termed the ‘TonB box’ located on the periplasmic side of the outer membrane (Ferguson et al. 1998). Although the interaction between OMRPs and the TonB complex is not completely understood, TonB presumably physically interacts with this region, providing the necessary energy for transport. The TonB protein is 239 residues in length and primarily exists in the inner membrane except for the carboxy terminal domain (residues 164-239), which extends into the periplasm. The crystal structure for the carboxy domain of the Ton B complex has been solved (Chang et al 2001). It has also been observed that tonB transcription is regulated by the same factors that regulate genes involved in siderophore synthesis and transport; this includes the repressor Fur and iron concentration in the surrounding environment (Postle 1990).
Periplasmic Binding Proteins

Once inside the periplasm, specific periplasmic binding proteins (PBP) have been identified that bind the ferric siderophore complexes. These proteins are generally synthesized at a lower level compared with PBPs that bind amino acids or sugars and have a lower affinity for ferric siderophore complexes as compared with OMRPs (Sprenzel et al. 2000). These can have a wide range of specificity, demonstrated by the fact that FhuD can bind the siderophores ferrichrome and family members, aerobactin, copragen, and rhodoturilic acid. This can be explained by a wider and shallower binding site than that of other PBPs (Wandersman and Delepelaire 2004). The crystal structure of FhuD has been solved (Clarke et al. 2000), and from this it is thought that human iron-binding proteins, transferrin and lactoferrin, evolved from bacterial PBPs as they resemble the N- and C-terminal lobes of these proteins (Braun and Braun 2002). The PBP-ferric siderophore complex provides a substrate for the next component of these iron transport systems, the ATP-dependent ABC-type transporter.
ABC-type Transporters and the Fate of Complexes upon Entering the Cytoplasm

Ferric siderophores bound to their respective PBP are transported to a specific ATP-dependent ABC-type transporter located in the inner membrane. A typical ABC-type transporter has four subunits. Two are hydrophobic and span the membrane multiple times, and two bind nucleotides and are exposed to the cytoplasm. Interaction between ligand-bound PBP and this transporter stimulates the ATPase activity of the transporter, initiating transport (Locher 2004). In the ferrichrome transport system, FhuB is a hydrophobic cytoplasmic membrane protein, and FhuC is a cytoplasmic ATPase associated with FhuB. FhuD with bound Fe$^{3+}$-siderophore complex appears to insert deeply into the FhuB channel, coming into close proximity of the binding site of FhuC ATPase. This observation suggests that the triggering of ATP hydrolysis occurs via direct contact of FhuD with FhuC, or possibly through a short FhuB linker. This is an interesting mechanism, as the other well-studied mechanisms of transport through an ABC-type transporter depend on transmembrane signaling as demonstrated by the maltose transport system (Braun and Braun 2002).

Once transport into the cytoplasm has occurred, there are two possible ways that iron is released from the siderophore. One of these is that ferric reductases reduce ferric iron to ferrous iron, which is released from the siderophore because of reduced binding affinity in the ferrous state (Wandersman and Delepelaire 2004). The second is that the siderophore is broken down, which releases the iron. Genes have been identified in the enterobactin transport system, which code for ferric enterobactin esterase (fes) that are thought to breakdown enterobactin (Brickman and McIntosh
More work needs to be done in order to fully understand these processes. A generalized mechanism for siderophore-mediated iron transport is given in Figure 3.

Figure 3. The generalized mechanism of siderophore-mediated iron transport
Regulation of Siderophore-Mediated Iron Transport Systems

Because of iron’s potential toxicity, its transport must be strictly regulated. In the majority of Gram-negative organisms, ‘Fur’ (ferric uptake regulator) is considered to be the key regulator for expression of genes involved in iron transport (Hantke 1981). Fur is a transcriptional repressor of more than 90 genes, many of which are involved in siderophore synthesis and uptake (Wexler et al. 2003). In environments where iron is abundant, the Fur protein (in the presence of ferrous iron) binds fur boxes, blocking access of RNA polymerase to the promoter region and in turn, preventing transcription of certain genes (Masse and Gottesman 2002). Fur was identified over 30 years ago (Ernst et al. 1978) and is part of the Fur superfamily, which also includes PerR, Zur, and Irr (Wexler et al. 2003). In addition to Fur, other Fe-responsive regulators can mediate iron regulation. For example, iron transport in many Gram-negative bacteria is mediated by DtxR and related family members. This repressor was identified in Corynebacterium diptheriae (Boyd et al. 1990). This protein also uses ferrous iron as a corepressor (Qian et al. 2002) but shows no sequence homology to Fur (Wexler et al. 2003).

Other Iron Acquisition Systems in Bacteria

In addition to producing one or multiple siderophore acquisition systems, bacteria have evolved other mechanisms for obtaining scarce iron from a wide range of environments. Many Gram-negative bacteria possess a system for transporting ferrous iron, termed the Feo system, which is typically only expressed under anaerobic conditions (Kammler et al. 1993). In some bacteria this system is essential, particularly
in organisms that colonize the stomach or intestine, such as *Helicobacter pylori* and *Salmonella enterica* (Wandersman and Delepelaire 2004). Another potential source for iron is from heme and heme bound to hemoglobin, haptoglobin, and hemopexin, among other heme-containing compounds. Some organisms can directly bind heme and heme compounds, which are then transported across the membrane in a TonB-dependent fashion. In *E. coli*, ChuA is the receptor for heme (ShuA in *Shigella*) and these receptors show some sequence identity with siderophore receptors (Wandersman and Stojiljkovic 2000). Similar systems have been described, including HemR in *Yersinia enterocolitica* and HmbR and HpuA in *Neisseria meningitidis*, but are not well defined (Wandersman and Stojiljkovic 2000).

Besides these systems that directly bind heme and heme compounds, Gram-negative bacteria can also produce hemophores, extracellular proteins that can acquire heme from a variety of sources. The HasA system has been described in *Serratia marcescens, Pseudomonas aeruginosa, Y. pestis*, and a few others (Letoffe et al. 1994, Letoffe et al. 1998, Rossi et al. 2001). This hemophore is apparently secreted by an ABC-type transporter and binds heme with a $K_d$ of $10^{-11}$ M. A crystal structure has been solved and binding site residues determined (Amoux et al. 1999). In addition, the OMRP, HasC has been identified in transporting this hemophore complex (Otto et al. 1998). In *E. coli*, a hemophore has recently been described, which is a hemoglobin protease. These are thought to degrade hemoglobin and then bind heme. These enzymes appear to have two heme binding sites, one with a higher affinity for heme than the other. The receptor for this system has not been identified (Otto et al. 2005). Hemophore systems have also been identified in *Haemophilus influenzae* (HxuA), which
binds heme-hemopexin and is presented to the outer membrane receptor protein, HxuC (Cope et al. 1995, Cope et al. 2001). Other aspects of these systems including transport and excretion mechanisms remain to be identified (Wandersman and Delepelaire 2004). Bacteria can also acquire iron from lactoferrin and transferrin, where iron is extracted at the cell surface by an unknown mechanism. These systems have been best characterized in *N. meningitidis* and *N. gonorrhoeae* (Cornelissen 2003).

A last putative mechanism for iron acquisition is the SitABCD system found in *Salmonella* and *Shigella* species and in enteroinvasive *E. coli*. Depending on the organism, this system is thought to transport ferric or ferrous iron (Runyan-Janecky et al. 2003). It is not certain that this system transports iron, but studies have shown it is partially regulated by Fur and available iron in the environment (Janakiraman and Slauch 2000). Additionally, it has been shown that induction of the *sit* genes can improve growth of mutants deficient in iron transport (Runyan-Janecky et al. 2003). It is interesting to note that all described mechanisms for iron transport, with the exception of the Feo system, appear to be TonB-dependent, and some known receptors that either directly bind heme or bind heme complexed with hemophores have the same conserved TonB boxes as described for OMRPs that transport ferric siderophore complexes (Wandersman and Delepelaire 2004).
Iron and Virulence

It is well established that acquisition of iron is an important adaptive response for bacterial pathogenesis, as it is needed for survival of most bacteria (Weinberg 1978). Other indications that iron can play a role in virulence are suggested by the fact that several virulence factors are iron-regulated. For example, shiga toxin (Shigella), shiga-like toxin (enterohemorrhagic E. coli), diphtheria toxin (C. diptheriae), and exotoxin A (Pseudomonas aeruginosa) are regulated by iron concentration directly or by an iron-responsive regulator (Litwin and Calderwood 1993). Additionally, the genes for hemophore expression in E. coli are located on the pColV virulence plasmid, indicating its potential role in virulence (Otto et al. 1998). Although free iron is extremely limited in the mammalian host, as intracellularly it is bound to heme, hemoglobin, ferritin, and other molecules, and extracellularly it is sequestered by the glycoproteins, transferrin and lactoferrin (Litwin and Calderwood 1993), bacteria still find ways obtain the iron needed to support their growth (see previous section). It has long been proposed that bacterial inhibition could be accomplished by blocking a pathogen’s ability to acquire iron. The earliest attempts at this form of microbial inhibition were in 1945 and were aimed at Mycobacterium tuberculosis (Snow 1970). While initially these studies were not very fruitful, later efforts have shown that this could possibly be accomplished. For example, a significant decrease in virulence was noted in uropathogenic E. coli double mutants unable to synthesize the OMRPs FepA and IutA (OMRP for aerobactin) when introduced into the mouse kidney Torres et al. 2001), because of the organisms’ decreased ability to transport iron. The possibility of using chemotherapeutic agents
that target OMRPs should be further explored. Another potential target for antimicrobial agents is siderophore biosynthesis. In a recent study, a small molecule, salicyl-AMS, inhibited siderophore biosynthesis and, subsequently, growth of *M. tuberculosis* and *Y. pestis* in vitro (Ferreras et al. 2005). It has been mentioned that some OMRPs for siderophore transport can also bind and transport antibiotics. With antibiotic resistance becoming a growing concern, this dual function could prove to be beneficial in overcoming some resistance by linking antibiotics with low diffusion rates to ferric siderophore complexes (Braun and Braun 2002).

**Other Applications of Studying Iron-transport Mechanisms**

There are many potential benefits of studying iron-acquisition systems in bacteria. Many of these have been addressed in the previous section. Other applications include development of treatment options for diseases associated with iron overload. An example of this is a treatment method that uses Desferal, which is a synthesized siderophore of the ferrichrome family (Hussain et al. 1976). It has been used to treat patients with β-thalassemia who produce defective hemoglobin and must undergo periodic blood transfusions, which leads to iron accumulation in the body. Desferal is used to chelate excess iron and has also been used in treating iron poisoning. However, this therapy is available only by injection and a strict regimen must be followed (Cao et al. 1997). More research in this area could prove to be advantageous in providing patients with iron overload disorders simpler and more effective treatments. Studying iron transport can also have agricultural implications,
which will be discussed in more detail in the following sections. These applications of studying iron-transport mechanisms, in addition to providing a better understanding of the general mechanism of iron transport, make this field an exciting and growing area of interest for research.

**The Rhizobia Family**

Rhizobia are a very diverse family of bacteria generally classified by their ability to nodulate leguminous plants, forming a symbiotic relationship in which the bacteria can fix atmospheric nitrogen to ammonia, a form that plants can use to assimilate nitrogenous compounds. In turn, the bacteria receive some nutrients and energy from the plant. There are five genera in the family and these include *Rhizobium*, *Mesorhizobium*, *Azorhizobium*, *Sinorhizobium*, and *Bradyrhizobium*. Different legumes are nodulated by different strains, species, or genera of Rhizobia. Rhizobial genomes are very large (~8 Mb) and 50% of their genome does not resemble other rhizobial species, which greatly contributes to the diversity seen within this family (Johnston 2004). However, some common characteristics of this family are that they are aerobic, Gram-negative bacilli. They are non-spore forming and are generally motile. Additionally, most are surrounded by a slimy capsule made of exopolysaccharide, which is thought to play a role in their attachment to root hairs. They grow optimally at 25-30°C and generally grow well on mannitol as a carbon source (Bergerson 1961). All have the ability to produce the nitrogenase enzyme complex and nod factors, which are important in symbiosis with their host plant. These organisms are very important agriculturally as they provide the major biological source of fixed nitrogen in the soil,
estimated at over $2 \times 10^{13}$ grams per year (Falkowski 1997). They have been used commercially as a soil inoculant and have shown to increase plant productivity when present (Carson et al. 2000).

Intercellular signaling plays an important role in allowing both the host plant and bacteria to perceive their external environment and turn on the expression of certain genes (Loh and Stacey 2003). Plant flavanoids secreted by roots trigger nod factor production by Rhizobia (Denarie et al. 1996). Upon recognition of these nod factors, root hairs of legumes redirect root development (Truchet et al. 1991). The bacteria invade through these root hairs and penetrate into plant-made infection threads. They undergo endocytosis into plant cortical cells where they are enclosed in a membrane called the symbiosome. The bacteria then differentiate into bacteroids. Bacteroids are intracellular forms of Rhizobia and the functional nitrogen-fixing unit of the nodule (Nadler et al. 1990). Many steps in the establishment of *Rhizobium*-legume symbiosis are unknown and recently DNA macroarrays have been used to discover 200 potential genes involved in this process (Ampe et al. 2003).

**Rhizobia and Iron**

Rhizobia must exist in diverse environments, all with varying nutrient availabilities. In their free-living form, they must compete with other bacteria in the soil for nutrients. In the rhizosphere, where root hairs are available, they must compete with their own species to gain access to a host plant (Loh and Stacey 2003). The inside of a bacteroid provides yet another environment to which these organisms must adapt. Iron is very important to their fitness in each of these environments, and iron-acquisition
systems in the Rhizobia are a fairly new field of study (Johnston 2004). Poor nodulation caused by limited iron in the soil has shown to affect many crops. This is because bacteria must divide rapidly prior to infecting a host, which requires large amounts of iron (Carson et al. 2000). Iron is also essential to development of the root nodule as some signaling factors are iron-regulated (Loh et al. 2002). In addition, iron is necessary for the process of nitrogen fixation as it is a major component of the nitrogenase enzyme complex (Loh and Stacey 2003). This complex is a heterodimer consisting of iron-containing nitrogenase reductase and nitrogenase, which is an iron-molybdenum cofactor where nitrogen reduction occurs (Johnston 2004). It has been reported that nitrogenase accounts for 10-12% of the total protein weight of a bacteroid (Verma and Long 1983). Leghemoglobin, a plant protein found in the nodules that supplies the Rhizobia with oxygen, also contains iron and accounts for approximately 30% of the total soluble protein in the nodule (Verma and Long 1983). Several other regulatory proteins found in the bacteroid also contain iron (Persmark et al. 1993). Like other bacteria, Rhizobia have great flexibility in their use of iron sources. Different rhizobial species generally make at least one siderophore, but production is much less common in some genera including Bradyrhizobium. Siderophores common to Rhizobia are vicibactin, a trihydroxamate first isolated from Rhizobium leguminosarum (Dilworth et al. 1998), rhizobactin 1021, a dihydroxamate identified in R. meliloti (Persmark et al. 1993), and rhizobactin, a carboxylate found in R. meliloti of which little is known (Smith et al. 1985). While it is not certain if Rhizobia use siderophores in the bacteroid, it has been shown that mutant R. leguminosarum deficient in siderophore production was unable to fix nitrogen in pea nodules (Stevens et al. 1999).
Rhizobia can also grow with heme as the sole source of iron and *hmu* genes have been identified in some rhizobial species that encode a putative heme receptor (Battistoni et al. 2002). Some *Bradyrhizobia* sp. reportedly secrete citrate in response to iron stress, although it is unclear how it is transported into the cell because there is no FecA receptor or homolog and *tonB* mutants are not affected in their ability to use citrate (Guerinot et al. 1990, Johnston 2004). It has even been reported that a *Rhizobium* strain can use leghemoglobin as an iron source (Noya et al. 1997). Not much is known about iron acquisition in the nodule as most studies are done on the free-living microbes, although it has been shown that some siderophores are not produced in the nodule (Lynch et al. 2001).

**Rhizobial Iron Regulation**

Rhizobia are no exception in that genes necessary for siderophore synthesis and uptake are tightly regulated and are only expressed under iron-deficient conditions. However, Fur is not thought to be the global regulator of iron-responsive genes in this family (Wexler et al. 2003). Some Rhizobia have a gene that resembles Fur (called Mur), but it does not seem to play a major role in iron-dependent gene regulation; instead, it is shown to be involved in uptake of manganese in *Rhizobium* and *Sinorhizobium* (Diaz-Mireles 2004, Platero et al. 2004). RirA is a newly discovered chromosomal protein that seems to affect the majority of iron-responsive operons in *R. leguminosarum* (Todd et al. 2002) and is responsible for regulation of rhizobactin 1021 biosynthesis and transport genes in *S. meliloti* (Viguier et al. 2005). This regulator has no similarity to Fur and is not a signature of Rhizobia, as this protein has not been found
in Bradyrhizobium. It is also not fully understood how this regulator functions, but it is not thought to bind conserved DNA sequences near iron-responsive promoters as these consensus sequences are absent in genome scans of iron-regulated genes (Johnston 2004). Another regulator that is a member of the Fur superfamily, Irr, and has only been found in Rhizobium, is shown to be a transcriptional repressor of hemB, which is involved in heme synthesis in Bradyrhizobium japonicum, and is also under the control of iron availability (Hamza et al. 1998). It is not known if and what other genes this repressor regulates. It is thought that these bacteria adapted to the many diverse environments they encounter by enlarging their genomes through lateral transfer, making this family very diverse in their iron-acquisition systems and regulation methods. What is known is that no single model can describe iron regulation in all Rhizobia (Johnston 2004).

Present Work

*R. leguminosarum* IARI 917 was obtained from the Indian Agricultural Research Institute in New Delhi, India, and was investigated for the presence of siderophore-mediated iron transport systems. It was first determined that this strain produces a hydroxamate-type siderophore under iron-deficient conditions. Media and proper growth conditions were standardized for the optimal production of this siderophore. This siderophore was then purified using manual column chromatography and high performance liquid chromatography (HPLC). The purified compound was then analyzed using various chemical methods in order to better understand the chemical nature of the siderophore. Preliminary results indicate that the siderophore being produced by this
strain is schizokinen, which has not been described as a rhizobial siderophore.

Additionally, a possible outer membrane receptor protein that is suppressed under high iron conditions has been identified using polyacrylamide gel electrophoresis (SDS-PAGE) analysis.
CHAPTER 2
MATERIALS AND METHODS

Bacterial Strain and Growth Conditions

The bacterial strain used in this study was *Rhizobium leguminosarum* IARI 917, which was obtained from the Indian Agricultural Research Institute in New Delhi, India and infects the pigeon pea plant. The culture was grown for 24–48 hours at 28°C.

**Congo Red Agar**

The culture was maintained on a modified Mannitol Yeast Agar supplemented with Congo Red dye (Jadhav and Desai 1996). This is used because Rhizobia do not generally take up this dye as readily as other organisms, which can help identify if a contaminant is present (Kneen and Larue 1983). This media has the following composition: 1% mannitol, 0.05% K$_2$HPO$_4$, 0.02% MgSO$_4$·7H$_2$O, 0.01% NaCl, 0.1% Yeast Extract (Difco), and 3% Bacto-agar (Difco). A volume of 0.25 ml of 1% Congo Red solution was added per 100 ml of media prepared. The pH of the media was brought to 6.8 with 6 M NaOH before autoclaving. Both agar plates and slants were prepared from this medium. *R. leguminosarum* IARI 917 was incubated for 48 hours at 28°C. Figure 4 shows the growth of *R. leguminosarum* IARI 917 on Congo Red agar.
Figure 4. *R. leguminosarum* IARI 917 grown on Congo Red agar.

**Glycerol Stocks**

Glycerol stock cultures of this strain were also prepared and stored at -80°C. Cultures were grown in 50 ml Luria-Bertani (LB) broth for 5-6 hours or until $\text{OD}_{600\text{nm}} = 0.5-0.6$ and 0.8 ml aliquots were added to 0.2 ml sterile 75% glycerol in 2 ml vials.

**Fiss-Glucose Minimal Media**

Because siderophores are only produced under iron-limiting conditions, Fiss-glucose minimal media (Vellore 2001) was used as an iron-restricted media. Media was prepared by dissolving 5.0 g $\text{K}_2\text{HPO}_4$ and 5.0 g L-asparagine in 954 ml $\text{H}_2\text{O}$ and pH was adjusted to 6.8. After autoclaving, 9.94mL of each of the following solutions was added to the 954 ml sterile media: 50% glucose, 0.005% ZnCl$_2$, 0.001% MnSO$_4$, and 0.4%
MgSO₄. To minimize the amount of contaminating iron, all media and components are prepared with deionized-distilled water and all glassware used for media storage and for growth of the culture were treated with concentrated HNO₃ and rinsed with ddH₂O.

**Preparation of Inoculum (Seed Culture)**

*R. leguminosarum* IARI 917 was grown in LB broth on a rotary shaker for 18-20 hours prior to inoculating iron-restricted media.

**Detection of Siderophore Production**

**Chrome Azurol S (CAS) Assay**

The CAS Assay (Schwyn and Neilands 1987) is the universal chemical assay for siderophore detection and is based on a siderophore’s high affinity for ferric iron. CAS plates are blue in color because chrome azurol S dye is complexed with ferric iron. When siderophore is present, the following reaction occurs, which releases the free dye, which is orange in color.

\[
\text{Fe}^{3+}-\text{dye (blue)} + \text{siderophore} \rightarrow \text{Fe}^{3+}\text{-siderophore} + \text{dye (orange)}
\]

*R. leguminosarum* IARI 917 was grown in Fiss-glucose minimal media containing no added iron, minimal media supplemented with 0.5 µM FeSO₄ (low iron) and minimal media supplemented with 20 µM FeSO₄ (high iron control). Cultures were grown for 24 hours on a rotary shaker and the supernatant from each was collected by centrifugation
at 13,500 rpm. Using a #2 cork borer, wells were bored into a CAS plate and 60 µl aliquots of each culture supernatant was pipetted into a separate well. Sterile media was also added to a well as a control. The plate was then incubated at room temperature. Depending on the culture, color formation may take 30 minutes to 5 hours. Formation of an orange halo around the well indicates that the culture is producing a siderophore.

Iron-Perchlorate Assay for Detection of Hydroxamic Acids

If siderophore is detected with the CAS assay, then further assays are employed to determine what type of siderophore is being produced. The iron-perchlorate assay (Atkin et al. 1970) is a colorimetric assay used for detection and estimation of hydroxamate-type siderophores. Because this assay is done under acidic conditions, it does not detect the presence of a catechol-type siderophore, which react at alkaline pH. Culture supernatants are collected as described previously and 0.5 ml supernatant is added to 2.5 ml 5 mM Fe(ClO\textsubscript{4})\textsubscript{3} in 0.1M HClO\textsubscript{4} solution and allowed to incubate at room temperature for approximately five minutes. If a hydroxamate-type siderophore is being produced, an orange-red color will form, which varies in intensity based on how much siderophore is produced. Absorbance is measured at 480 nm, with uninoculated media mixed with reagent used as a blank.
Estimation of Siderophore Concentration

The iron-perchlorate assay is colorimetric and amount of siderophore produced by a culture can be visually estimated based on the intensity of the orange-red color formation. To better estimate the concentration of siderophore in a sample, $\text{OD}_{480\,\text{nm}}$ was measured and compared to a standard curve prepared using a known concentration of ferrichrome (Figure 5).

![Hydroxamate Standard Curve](image)

Figure 5. Hydroxamate standard curve
Arnow’s Assay for Catechol-type Siderophores

To determine whether a culture is producing a siderophore that contains catechol groups, Arnow’s method is used (Arnow 1937). This is also a colorimetric assay and can be used to estimate catechol concentration using a known catechol as a standard. The assay is performed by mixing the following in order: 1 ml culture supernatant, 1 ml 0.5 M HCl, 1 ml nitrite-molybdate reagent (prepared by dissolving 10 g sodium nitrite and 10 g sodium molybdate in 100 ml ddH₂O), and 1 ml 1 M NaOH. These are allowed to incubate for 5 minutes for the reaction to fully occur. Absorbance is measured at 500 nm with uninoculated media instead of supernatant used in the blank.

Catechol groups can be detected because they form a yellow color in nitrous acid, which turns pink-red when excess sodium hydroxide is present. A control (either a culture grown in high iron or uninoculated media) will remain colorless with the addition of reagents.

Siderophore Detection Using Thin Layer Chromatography (TLC)

Siderophore can also be detected by using thin-layer chromatography. Culture supernatant or concentrated samples of siderophore (see later sections) are spotted on Selecto Scientific 10 x 20 silica gel plates and spots are allowed to dry. The plates are then run in an n-butanol:acetic acid:dH₂O (12:3:5) solvent system until the solvent front reaches the top of the plate. Plates are then dried and sprayed with 0.1 M FeCl₃ in 0.1 N HCl. The formation of a wine-colored spot indicates a hydroxamate-type siderophore, while a dark gray spot indicates production of a catechol-type siderophore. Siderophores are separated on the basis of hydrophobicity using these plates.
Optimization of Conditions for Siderophore Production

Once the chemical type of siderophore has been determined, conditions can be optimized in order to achieve the maximum amount of siderophore production. This makes purification of siderophore a much easier process.

Optimization of Media Components

The components in a medium can have a great effect on the amount of siderophore produced by a bacterial culture. For example, it is known that many members of the Rhizobia family prefer to grow on mannitol or maltose as a carbon source (Murray and Smith 1957, Bergerson 1961). When growth is increased, siderophore production also tends to increase. Fiss-glucose minimal medium as described above was used as the base medium for determining siderophore production and type. This medium was modified to determine the effects of various components on siderophore production. Each of the four media components was tested for its effect by varying their concentration in the minimal media. Each of these was done one at a time to ensure correct results. Also, the minimal media was supplemented with either 1% mannitol or 1% maltose as an alternate carbon source. Ammonium chloride or ammonium sulfate (0.1%) was also added to the media as an extra nitrogen source, and the effects evaluated. After 32 hours, growth was measured for each culture at OD\textsubscript{600 nm} and culture supernatant was collected and the iron-perchlorate assay was used to determine the approximate amount of siderophore production in each media type.
Optimization of Iron Concentration

Iron concentration of a medium is critical to whether or not siderophore biosynthesis genes are expressed. Some bacteria produce optimum amounts of siderophore when a small amount of iron is supplemented in the media. For this reason, it was important to test the effect of varying iron concentration of the media on siderophore production. While precautions are taken to eliminate contaminating iron from the media, iron is present in the media components as contaminants, which is calculated to be approximately 2.7 µM. This amount can be chelated by supplementing the media with 0.2 mM 2’2-dipyridyl. Cultures were grown in the following concentrations: absolute 0 (2’2-dipyridyl added), 0.0 µM (only contaminating iron present), 0.25 µM, 0.5 µM, 0.75 µM, 1.0 µM, 2.0 µM, 5.0 µM, 10.0 µM, 20.0 µM, and 100.0 µM. The optimized Fiss-glucose minimal media was supplemented with FeSO₄ in order to obtain the respective concentrations of iron where necessary. After 24 hours, growth was measured for each culture at OD₆₀₀ nm and culture supernatant was collected and the iron-perchlorate assay was used to determine the approximate amount of siderophore production.

Effect of Incubation Time on Siderophore Production

Incubation time was also evaluated to determine the proper time to harvest cells for the extraction of siderophore and to determine when siderophore production begins in this organism. One liter modified Fiss-glucose minimal media in a 2.8 L flask was inoculated with 10 ml seed inoculum. At various times, ranging from 1-42 hours, 1 ml
aliquots were removed and growth was measured for each at OD$_{600}$ nm and culture supernatant was collected for siderophore estimation.

**Effect of Temperature on Siderophore Production**

While most Rhizobia grow optimally at 25-30°C, this may not be the optimal temperature for siderophore production. Five 250 ml flasks were prepared with 50 ml modified Fiss-glucose minimal media and inoculated with *R. leguminosarum* IARI 917 seed culture. The flasks were grown at different temperatures (4°C, 22°C, 28°C, 37°C, and 55°C). After 24 hours, growth was measured and siderophore production was estimated for each culture.

**Purification of Siderophore**

**Batch Cultures**

In order to obtain enough purified siderophore for chemical characterization, large volumes of culture were grown in the optimized medium. Typically, 5-6 L of medium was prepared and each liter was inoculated with 10 ml seed inoculum. The cultures were grown for 24 hours at 28°C on a rotary shaker. After incubation, the culture supernatant was collected by centrifuging at 7,000 rpm for 30 minutes. The supernatant was then acidified to pH 2.0 with 6 M HCl in order to make the siderophore less soluble in water.
XAD-2 Column Chromatography

The first step in purification of siderophore from *R. leguminosarum* IARI 917 was to pass the acidified supernatant through a 30 x 5 cm column packed with Amberlite XAD-2, which binds cyclic compounds. The column was prepared by suspending approximately 60 g XAD-2 in ddH$_2$O and letting the mixture sit at room temperature overnight so that the material can fully absorb the water. The column was then packed (approximately 20 cm) with the prepared XAD-2 and equilibrated with four bed volumes of ddH$_2$O. The acidified supernatant was then passed through the column and the flow-through was collected. Once all supernatant had been run, the column was washed with two bed volumes of ddH$_2$O. This wash was also collected in a separate bottle. The column is then eluted with approximately 250 ml methanol and ~five 50 ml fractions are collected. The first fraction is mostly water and is colorless. When the color coming out of the column turns yellow and the flow rate increases indicating only methanol is present, this is collected separately as fraction two. Fractions are collected until no color is present in the flow-through. The column is then washed with four bed volumes of methanol, followed by four bed volumes of ddH$_2$O to re-equilibrate the column.

The flow-through, ddH$_2$O wash, and all fractions collected are tested for their siderophore content using the iron-perchlorate assay. Fractions testing positive for siderophore are combined in a 250 ml round-bottom flask and are dried on a Büchi R-200 rotary evaporator, with the temperature set to 25°C. The dried sample is redissolved in 4-5 ml methanol and stored in a 15 ml polypropylene tube at –20°C till further purification.
Sephadex LH-20 Column Chromatography

Sephadex LH-20 column material separates compounds based on their hydrophobicity and is also used for gel filtration to separate compounds based on molecular weight. It is prepared by suspending 50 g LH-20 in methanol and deaerating with shaking for around 20 minutes. The material is then packed into a 50 x 1.5 cm column (packed to the top of the column) and is equilibrated with four bed volumes of methanol. The concentrated sample can then be loaded onto the column and eluted with methanol. Approximately 65 150-drop fractions are collected and are tested for their siderophore content using TLC plates. Fractions positive for siderophore are combined in a 100 ml boiling flask and evaporated to dryness using a rotary evaporator. The dried sample is then re-dissolved in ~3 ml ddH$_2$O. If the solution is cloudy, it can be syringe-filtered (0.45 µm pore size) into a 15 ml polypropylene tube. The sample can be estimated using the iron-perchlorate assay and should be stored at –20°C.
High Pressure Liquid Chromatography (HPLC)

The concentrated siderophore was further purified using a BioRad Biologic Duoflow HPLC system with a Waters 7.8 mm x 300 mm Novapak HR C$_{18}$ hydrophobic column as the stationary phase and deaerated, filtered ddH$_2$O and filtered 90% methanol as mobile phases. These were filtered using a Millipore filtration system with 0.45 µm membranes. The UV detector was set at 280 nm. The column was equilibrated with 3 bed volumes ddH$_2$O prior to use. Sample volume injected onto the column varied between 0.5 ml-1 ml. Several preliminary runs were made to determine the gradient at which the siderophore eluted from the column. Various programs were created to best separate the pure siderophore from impurities in the sample. A chromatogram was generated after each run and fractions showing peaks on chromatograms were tested for their siderophore content using TLC. After the entire sample had been run through HPLC, any fraction containing siderophore was pooled and concentrated using a rotary evaporator.
Chemical Characterization of Purified Siderophore

Once enough purified sample of siderophore had been obtained, it was used for various chemical analyses in order to chemically characterize the siderophore and possibly determine a chemical structure.

Spectral Scan Analysis

A spectral scan (300-700 nm) was done on the purified siderophore to determine whether this hydroxamate-type siderophore was a dihydroxamate or trihydroxamate (Jalal and van der Helm 1991). Samples are prepared according to the Atkin’s method, except that because a concentrated sample is used, only 20-50 µl aliquot is used and the corresponding amount of ddH$_2$O is added to bring the sample volume to 0.5 ml. At an acidic pH, ferric dihydroxamates show an absorbance maximum in the range of 500-520 nm, while trihydroxamates show an absorbance maximum in the 420-440 nm range.

Amino Acid Analysis Using TLC

Many siderophores contain one or more amino acid residues in their chemical structure. For example, enterobactin is composed of three residues of serine and ferrichrome is built on modified ornithine residues. The purified siderophore was analyzed for its amino acid content using various methods. The pure siderophore was acid/alkaline hydrolyzed by combining it with an equal volume of 6 M HCl for an acid hydrolysis or 6 N NaOH for an alkaline hydrolysis and then autoclaving at 121°C for 6 hours. Some of each hydrolyzed sample was neutralized with 6 M NaOH or 6 M HCl
and tested also. Amino acid standards were prepared using the 20 standard amino acids and also ornithine by dissolving 1 mg of amino acid in 1 ml ddH$_2$O. Some had to be autoclaved in order to dissolve them in water or a less concentrated standard had to be prepared. Standards and hydrolyzed samples were spotted (amount varied based on concentration) on 20 x 20 silica gel plates and run using three different solvent systems. These included methanol:0.1 M ammonium acetate (60:40), acetonitrile: 0.1 M ammonium acetate (60:40), and n-propanol:ddH$_2$O (70:30) (Laskar et al. 2001).

Each plate was developed until the solvent front reached the top of the plate and was marked. For the first two solvent systems, the plates were dried and sprayed with 0.5% (w/v) ninhydrin in ethanol and incubated at 55°C for 15 minutes. For the last solvent system, the plate was sprayed immediately with 0.25% (w/v) ninhydrin in acetone and allowed to dry at room temperature. Once the plate has dried, it is sprayed with a second reagent, aqueous 0.1% n-cyanoguanidine (pH 10.5) and again allowed to dry completely. Amino acid spots now appear colored and are easily distinguished from one another. The plate can also be incubated at 100°C for 10 minutes in order for some spots to be developed.

**Amino Acid Analysis Using Analytical HPLC**

Analytical HPLC was used for identification of possible amino acids in the acid-hydrolyzed siderophore based on retention times of this sample and that of the amino acid standards tryptophan and tyrosine. These two amino acids were tested because they were the only two that were possible candidates based on amino acid analysis of hydrolyzed samples using TLC. The system used for analysis was a Waters 501 dual-
pump equipped with a Waters Tunable Absorbance Detector, which was set to 280 nm. The stationary phase used was a Waters 30 cm x 3.9 mm µ-Bondapak C\textsubscript{18} column. The mobile phase was degassed methanol:water (20:80) and the column was equilibrated with the mobile phase for 30 minutes prior to use at a flow rate of 1.00 ml/minute. The acid-hydrolyzed sample, tryptophan, and tyrosine were dissolved in methanol, with the standards at a concentration of 1.0 mg/ml and 0.24 mg/ml respectively. A 20 µl sample size was injected onto the column and each sample was run in triplicate. Methanol was also injected as a control and retention times were compared to determine amino acid composition, if any, of the acid-hydrolyzed siderophore.

**Mass Spectroscopy**

To determine a molecular weight for the purified siderophore, samples were analyzed by electrospray atmospheric pressure ionization (ESP API) in both positive and negative ion modes. Accurate mass data were obtained on a Waters LCT (orthogonal acceleration time-of-flight mass spectrometer) equipped with an Agilent 1110 autosampler, liquid chromatography, and diode array detector. Samples analyzed were in both a complexed (ferriated) form and a deferriated form. This was essential to determining a molecular weight because a difference of 56 atomic mass units (amu), the approximate atomic weight of iron, could be used to indicate the correct molecular weight of the chelating species. Purified sample was complexed with equimolar FeSO\textsubscript{4}, assuming a molecular weight of approximately 800 Da. One hundred µl of each purified sample (in ddH\textsubscript{2}O) was dissolved in 900 µl methanol and 5 µl was injected onto a 50 x 2
Varian monochrom C₁₈ column. Mobile phases were 30 ml methanol in 1 L 10 mM ammonium acetate (pump A) and methanol (pump B). Peaks generated with HPLC were analyzed using ESP API. When charged sample is injected through a charged nozzle, they are separated based on their mass per charge ratio (m/z). These fragments are then sent through a detector and molecular weight is generated based on their velocity and travel time to the detector.

Once a potential molecular weight is established, this fragment can be further analyzed using mass spectroscopy/mass spectroscopy (MS/MS). The system used for this analysis was a Waters Micromass Quattro micro™ API Tandem Quadrupole System equipped with Acquity liquid chromatograph, diode array, and autosampler. This type of mass spectroscopy allows one to select a parent ion of interest for analysis by using a magnetic quadropole, which consists of four electrodes with varying potentials arranged in a square fashion. Voltage and frequency for these electrodes can be manipulated to only allow passage of a particular ion for a second round of mass spectroscopy, so that the fragmentation pattern can be observed for that particular ion only. For these reasons, MS/MS is much cleaner than ESP API alone and can give great structural information on a compound. For this analysis, 10 µl of purified sample was dissolved in 1 ml 10 mM ammonium acetate:methanol (75:25). The sample is injected and the ion of interest can be selected for and then collided with argon gas to obtain fragments that can then be analyzed based on m/z. Siderophores are often degraded during stringent purification procedures and this technology can also be used to determine differences in degradation products as compared with intact siderophore.
**Nuclear Magnetic Resonance (NMR) Spectroscopy**

The purified siderophore was examined by $^1$H 1D NMR. For this analysis, the entire dried sample was dissolved in 0.75 ml D$_2$O. 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. Proton 1D NMR spectrum was collected with 65,560 data points and 20 ppm sweep width with $^{13}$C decoupling using GARP decoupling and a 15-sec relaxation delay. The residual proton resonance in HOD was used as the $^1$H chemical shift standard at 4.80 ppm.

**Detection of a Possible Outer Membrane Receptor Protein (OMRP)**

Ferric siderophores are transported into the cytoplasm of a bacterial cell through an outer membrane receptor protein (see Chapter 1). Like siderophores, OMRPs are only expressed under iron-limiting conditions. It was in our interest to possibly detect the presence of an outer membrane receptor protein involved in siderophore transport that was expressed only when cells were starved for iron and was not present when cultures were grown in high iron. To do this, whole cell pellets and membrane pellets were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Maniatis et al. 1982).
Preparation of Samples for SDS-PAGE

A 2 ml seed culture of *R. leguminosarum* IARI 917 was used to inoculate 50 ml of modified Fiss-glucose media with no added iron and 50 ml of Fiss-glucose media with 20 µM FeSO₄ added. Cultures were grown for 24 hours on a rotary shaker at 28°C. Cells were harvested by centrifugation at 7,000 rpm for 10 minutes and supernatant was discarded. Some of the whole cell pellet was saved and stored in an eppendorf tube at –80°C. The remaining pellet was resuspended in 10 ml 10 mM Tris buffer (pH 8.0) and sonicated in an ice bath using a large probe (5-0.7 second bursts of one minute with one minute pauses). Sonicated samples were centrifuged at 7,000 rpm for 10 minutes and the supernatant was poured into ultracentrifuge tubes. These were centrifuged at 30,000 rpm (Beckman 50.2Ti rotor) for 90 minutes and the resulting membrane pellets were stored at –20°C.

SDS-PAGE Analysis of Samples

A 10% separating gel was prepared (Table 1) and ingredients were deaerated for 10 minutes before addition of 10% ammonium persulfate solution that polymerizes the gel. The separating gel was pipetted into a gel-casting unit and was allowed to polymerize for 30 minutes. A stacking gel was prepared (Table 1) and was also deaerated 10 minutes before adding 10% ammonium persulfate. This gel was pipetted on top of the solidified separating gel and a comb was placed. This gel was allowed to polymerize for 30 minutes and then the gel was placed into an electrophoresis unit. The upper and lower chambers were filled with tank buffer.
Whole cell pellets and membrane pellets are prepared by adding an equal volume of 2x gel loading buffer (~20 µl) in an eppendorf tube. Additionally, a protein molecular weight standard was prepared by adding 2 µl BioRad SDS-PAGE broad-range molecular size marker with 8 µl of 2x gel loading buffer. All samples were then placed in a boiling water bath for five minutes. Samples were loaded onto the gel (5 µl of whole cell pellets and standard and 15 µl of membrane pellets), and the gel was run at 30 milliamps per gel for approximately one hour or until the dye front reached the bottom of the gel. The gel was placed in Coomasie Blue Stain for 30 minutes, destained, and stored in 5% acetic acid.

Table 1. Ingredients and amounts used in preparation of 10% SDS-PAGE gel.

<table>
<thead>
<tr>
<th></th>
<th>10% Separating Gel</th>
<th>Stacking Gel</th>
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<tbody>
<tr>
<td>30% Acrylamide Solution</td>
<td>6.66 ml</td>
<td>1.33 ml</td>
</tr>
<tr>
<td>Running Gel Buffer</td>
<td>5.0 ml</td>
<td>--</td>
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<tr>
<td>Stacking Gel Buffer</td>
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<td>2.50 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.2 ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>8.0 ml</td>
<td>6.1 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>10% ammonium persulfate</td>
<td>100 µl</td>
<td>50 µl</td>
</tr>
</tbody>
</table>
Extraction of Iron

When it was necessary to extract iron from a siderophore, the following procedure was used (Waring and Workman 1942). Siderophore solution and 5% (w/v) 8-quinolinol (8-hydroxyquinozoline) in chloroform were combined in a 1:3 ratio in a 60 ml separating funnel. The solution was shaken vigorously, removing air periodically, and then layers were allowed to separate completely. The chloroform layer was on the bottom and was removed. This extraction process was repeated 3-4 times by adding fresh 5% w/v 8-quinolinol in chloroform, removing the bottom layer each time. This was followed by adding (in a 1:1 ratio) chloroform to remove excess 8-quinolinol.
CHAPTER 3
RESULTS

Detection of Siderophore Production by *R. leguminosarum* IARI 917

To determine if *R. leguminosarum* IARI 917 was producing a siderophore under iron-limiting conditions, the CAS assay was used. Formation of an orange halo around a well bored into the CAS plate indicates that siderophore is present in the supernatant of cultures. This assay can also indicate the relative amount of siderophore in the supernatant based on the size of the halo formed. After incubation, both the low iron (0.5 µM FeSO₄) and the no iron (no iron added to the media) supernatants produced an orange halo on the CAS plate (Figure 6), indicating that siderophore had been produced by these cultures, which stripped the iron from the CAS dye. Uninoculated media used as a control and a high iron (20 µM FeSO₄) culture did not produce an orange halo around their respective well. These results indicated that *R. leguminosarum* IARI 917 produces a siderophore under iron-limiting conditions, which is not produced when sufficient iron is available for the organism.
Figure 6. Results of CAS assay: A) *R. leguminosarum* IARI 917- high iron supernatant, B) *R. leguminosarum* IARI 917- no added iron supernatant, C) *R. leguminosarum* IARI 917- 0.5µM FeSO₄ (low iron) supernatant, D) uninoculated media (control)
Initial Characterization of Siderophore

Once it had been indicated that *R. leguminosarum* IARI 917 was producing a siderophore, the iron-perchlorate assay and Arnow’s assay could be used to determine the chemical type of the siderophore. In the iron-perchlorate assay, the formation of an orange-red color is indicative of a hydroxamate-type siderophore. In Arnow’s assay, the formation of a red color after addition of reagents signifies the production of a catechol-type siderophore. *R. leguminosarum* IARI 917 supernatant from both a no added iron culture and a high iron (20 µM FeSO$_4$) culture were tested using both assays. Arnow’s assay was repeatedly negative for a catechol-type siderophore (results not shown). However, the no added iron supernatant of *R. leguminosarum* IARI 917 did produce a significant color change when added to the iron-perchlorate reagent, which was not observed in the high iron supernatant or in the media alone (Figure 7), indicating the presence of a hydroxamate-type siderophore.
Figure 7. Results of iron-perchlorate assay: A) uninoculated media (control), B) R. leguminosarum IARI 917- high iron supernatant, C) R. leguminosarum IARI 917- no added iron supernatant

Optimization of Conditions for Siderophore Production

Optimization of Media

Fiss-glucose minimal media (Vellore 2001) was used in the preliminary characterization of the siderophore produced by R. leguminosarum IARI 917. However, this media needed to be optimized to achieve maximum siderophore production for
purification. Each component (50% glucose, 0.005% ZnCl$_2$, 0.001% MnSO$_4$, and 0.4% MgSO$_4$) added to the media stock was varied separately to determine its effect on siderophore production. No change in any of these components had a major effect on siderophore production, so molarities of each of these components were kept the same in the minimal media. A variety of media combinations were tried to optimize siderophore production. These include Fiss-glucose minimal medium supplemented with 1% mannitol, Fiss-glucose supplemented with 1% maltose, Fiss-glucose supplemented with 0.1% (NH$_4$)$_2$SO$_4$, Fiss-glucose supplemented with 0.1% NH$_4$Cl, Fiss-glucose supplemented with 1% mannitol and 0.1% (NH$_4$)$_2$SO$_4$, Fiss-glucose supplemented with 1% mannitol and 0.1% NH$_4$Cl, Fiss-glucose supplemented with 1% maltose and 0.1% (NH$_4$)$_2$SO$_4$, and Fiss-glucose supplemented with 1% maltose and 0.1% NH$_4$Cl. Cultures were grown in 50 ml of each medium type for 24 hours at 30°C on a rotary shaker before evaluating each for its siderophore production. Figure 8 summarizes the results on siderophore production for each media type tried based on the iron-perchlorate assay for estimation. These results indicated that the addition of 1% maltose and 0.1% (NH$_4$)$_2$SO$_4$ to the original Fiss-glucose minimal media greatly increased the amount of siderophore produced. Cultures grown in this media produced almost three-fold more siderophore than that produced in Fiss-glucose minimal media.
Siderophore Production Measured with Various Media

![Siderophore Production Bar Chart]

Figure 8. Siderophore production measured with various media

Optimization of Iron Concentration

Iron concentration is probably the single most important factor in how much siderophore an organism produces, as genes for siderophore biosynthesis are under the direct control of iron concentration in the environment. The addition of limited amounts of iron in the media can increase growth of the culture, which can lead to an increased production of siderophore. However, higher concentrations of iron in the media can completely suppress production of siderophore. To determine the
concentration of iron at which *R. leguminosarum* IARI 917 produced maximum siderophore, the organism was grown in various concentrations of ferric iron, by supplementing the media with FeSO₄. In addition, *R. leguminosarum* IARI 917 was grown in the modified Fiss-glucose minimal medium supplemented with 0.2 mM 2’2-dipyridyl to chelate iron present as a contaminant in the media components (~2.7 µM). The results of growing this culture in various iron concentrations are shown in Figure 9. This graph depicts the classic biphasic relationship between siderophore production and iron concentration in the medium. In a culture where iron has been completely removed with 2’2-dipyridyl, cell growth cannot be sustained and thus the culture cannot produce any siderophore. Siderophore production is highest when a ferric iron concentration of 0.5 µM is added to the modified minimal medium. At a concentration higher than this, siderophore production decreases. It was concluded from these results and the ones obtained by varying the medium components, the optimal medium for maximum siderophore production was Fiss-glucose supplemented with 1% maltose, 0.1% (NH₄)₂SO₄, and 0.5 µM FeSO₄.
Figure 9. The effect of iron concentration on growth (red circles) and hydroxamate production (blue triangles). The peak of siderophore production for *R. leguminosarum* IARI 917 is 0.5 µM FeSO₄.

Optimization of Incubation Time for Siderophore Production

Siderophores are secondary metabolites and a given culture may begin producing siderophore at a wide range of times, with production increasing as cultures are grown for longer periods. We wanted to determine the best incubation time for maximum siderophore production by *R. leguminosarum* IARI 917. This culture was grown for a maximum of 42 hours, and both growth and siderophore production were
measured at various intervals, which enabled us to determine when the organism began producing siderophore and also the peak of siderophore production. Figure 10 shows the growth curve for *R. leguminosarum* IARI 917 along with the amount of siderophore production produced at the various incubation times measured. Results indicated that *R. leguminosarum* IARI 917 begins producing siderophore at 8 to 9 hours post-inoculation, with maximum production occurring at 24 hours.

![Figure 10](image.png)

**Figure 10.** The effects of incubation time on growth (green circles) and hydroxamate production (blue squares) for *R. leguminosarum* IARI 917
Effect of Temperature on Siderophore Production

As described previously, *R. leguminosarum* IARI 917 was grown at varying temperatures to assess its effects on siderophore production. Figure 11 shows that both growth and siderophore production are highest at 30°C.

![Graph showing the effect of temperature on growth and hydroxamate production in *R. leguminosarum* IARI 917](Image)

**Figure 11.** The effect of temperature on growth (green circles) and hydroxamate production (blue squares) in *R. leguminosarum* IARI 917

Table 2 shows the original conditions versus the optimized ones and also shows the amount of siderophore production observed in each of these conditions. The results of the improved conditions for siderophore production show the importance of optimization, as production increased approximately 3.8 times.
### Table 2. Original versus optimized media and its effects on hydroxamate production

<table>
<thead>
<tr>
<th></th>
<th>Original Conditions</th>
<th>Optimized Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Media</strong></td>
<td>Fiss-glucose</td>
<td>Fiss-glucose with 1% maltose and 0.1% (NH₄)₂SO₄</td>
</tr>
<tr>
<td><strong>Iron Concentration</strong></td>
<td>None added</td>
<td>0.5 µM</td>
</tr>
<tr>
<td><strong>Incubation Time</strong></td>
<td>24 hours</td>
<td>24 hours</td>
</tr>
<tr>
<td><strong>Temperature</strong></td>
<td>28°C</td>
<td>28°C</td>
</tr>
<tr>
<td><strong>Hydroxamate Production</strong></td>
<td>0.20 mg/ml</td>
<td>0.72 mg/ml</td>
</tr>
</tbody>
</table>

**Purification of Siderophore**

Once the growth conditions had been optimized, it was possible to produce large amounts of siderophore by growing *R. leguminosarum* IARI 917 in batch cultures. Around 4-5 liters of culture were grown under the optimized conditions and the acidified supernatant was first purified through XAD-2 column chromatography. Fraction 1 is colorless, fractions 2 and 3 are dark yellow in color and have a putrid odor, and fractions 4 and 5 are light yellow in color. These fractions are tested for their siderophore content using the iron-perchlorate assay (Atkin 1970). Fractions 2 and 3 show the highest amount of siderophore content based on intensity of the red color. Figure 12 shows the results from testing fractions collected from the XAD-2 column. In addition to testing each fraction, the ddH₂O wash was also tested to ensure that most siderophore had bound the column and was not in the wash.
Figure 12. XAD-2 fractions collected and tested for their siderophore content; A) flow-through, B) ddH$_2$O wash, C) Fraction 1, D) Fraction 2, E) Fraction 3, F) Fraction 4, G) Fraction 5

Fractions 2 and 3 collected from the XAD-2 column were combined, dried, and redissolved in 4-5 ml methanol. The concentration of the siderophore was estimated and, at this stage, usually 70-100 mg of siderophore was present in the sample. This concentrated sample was then purified using Sephadex LH-20 column chromatography. Approximately 60 fractions (3 ml) were collected and earlier fractions (18-27) had a yellow tint, while later fractions (fractions 40-55) sometimes had an orange tint. The fractions were tested for their siderophore content using TLC. The fractions that tested
positive for hydroxamate-type siderophore were generally the earlier fractions and most often had a yellow tint. Other fractions also had a yellow color, but these did not test positive for siderophore, and were most likely an impurity as were the fractions that were orange in color. Figure 13 shows the results of the TLC plates performed on fractions collected from LH-20 column (only fractions 18-29 are shown). From this particular column, fractions 20-25 were positive.

Figure 13. TLC plates tested on fractions collected from LH-20 column. Two spots characteristic of a hydroxamate-type siderophore are present in fractions 22-24 after being sprayed with ferric chloride reagent.

One thing that was noticed after running the TLC plates on these fractions was the presence of two wine-colored spots in some of the fractions (Figure 13). It was not possible to determine based on the TLC results whether the higher spot was a degradation product of the intact siderophore that could still chelate ferric iron, or if it
was an entirely different siderophore. We decided to treat these as two separate siderophores and labeled them as compound ‘H’ spot, for the higher spot shown on the TLC plate, and compound ‘L’, for the lower spot. Attempts were made to separate these spots using HPLC. Due to their chemical similarity as they are both hydroxamic acid-type siderophores, the peaks were practically inseparable on HPLC. Instead, we decided to pool fractions collected from the LH-20 column separately based on what type of siderophore they contained. Those fractions that contained both compounds ‘H’ and ‘L’ (‘mixed’) were also pooled separately and were run through the LH-20 column again to separate the two compounds.

Once enough of each compound (‘H’ and ‘L’) had been collected, they were again combined, dried, and redissolved in 2-3 ml ddH$_2$O for further purification using HPLC. If the siderophores were dissolved in methanol, then they usually did not bind the hydrophobic C$_{18}$ column because they are highly soluble in methanol. It was also determined at this point that neither was soluble in acetonitrile. The siderophores were loaded onto the HPLC column, and through many trials, better separation of peaks was obtained. In the final solvent gradient that separated peaks well, the siderophore eluted at approximately 47%-60% solvent B (90% methanol). The fractions showing peaks on the chromatogram were analyzed using TLC. Figure 14 shows both a chromatogram from a preliminary run and also one from the standardized HPLC method used to purify each type of siderophore. Peaks in the latter chromatogram are better separated from one another.

The chromatograms generated from the high and low compounds were very similar to one another and the fraction(s) that tested positive for siderophore were
usually fractions 17-19. Several runs were performed using HPLC until all sample for each compound had been purified through HPLC. These were combined separately and concentrated to approximately a 2 ml volume using a rotary evaporator. They were estimated for their siderophore content, which at this stage was anywhere from 3.0 mg/ml to 12.0 mg/ml.
Figure 14. HPLC chromatograms from a preliminary run (A) and a standardized run (B); peaks are much better isolated using the standardized run.
Chemical Characterization of Purified Siderophore(s)

Spectral Scan Analysis

A spectral scan (300-700 nm) was performed to determine whether or not each purified siderophore was a dihydroxamate (500-520 nm) or a trihydroxamate (420-440 nm) (Jalal and van der Helm 1991). This analysis was also used to determine a difference between the compounds ‘H’ and ‘L.’ Figure 15 shows the results of each spectrum. Compound ‘L’ shows an absorbance maximum at 500 nm indicating that this is a dihydroxamate-type siderophore, as they have an absorbance maximum in the range of 500-520 nm. Compound ‘H’ showed an absorbance maximum of 486 nm. The difference in the two compounds’ absorbance maximum initially led us to believe that these were two different hydroxamate-type siderophores.
Amino Acid Analysis Using TLC

As described in Chapter 2, many siderophores contain one or more amino acids in their structure. We wanted to determine if the siderophores produced by *R. leguminosarum* IARI 917 are amino acid conjugates. Three different solvent systems were used to analyze the acid-hydrolyzed and alkaline-hydrolyzed compound ‘L’, as well as neutralized samples of each of these. The alkaline-hydrolyzed and both neutralized
compound ‘L’ samples did not give good spots and were not used for analysis. The acid-hydrolyzed sample was often smeared and a distinct spot was difficult to obtain. However, the compound ‘L’ did have a positive reaction with the ninhydrin reagent, which was reacting with an amine group. While results were often inconsistent, some amino acids could be ruled out based on the distance traveled on the TLC plate and on their color after spraying with a ninhydrin reagent. From the results of running several amino acid TLC plates, the most likely candidates for an amino acid component of the compound ‘L’ were tryptophan and tyrosine, both aromatic amino acids (data not shown).

Once compound ‘H’ had been purified, it was first tested on TLC plates against only acid-hydrolyzed compound ‘L’, tryptophan and tyrosine to determine if these exhibited the same amino acid profiles. Figure 16 shows the results of this plate, which indicated that both samples showed a spot that was very close to both tryptophan and tyrosine and strongly resembled one another. This led us to believe that, although the spectral scans were slightly different, these two compounds collected separately were in fact the same siderophore, as it would be highly unlikely that both were different and contained one of these two amino acids because these amino acids have not yet been described in a hydroxamate-type siderophore.
Amino Acid TLC plate ran in acetonitrile: 0.1 M ammonium acetate (60:40);
A) compound ‘H,’ B) tryptophan, C) tyrosine, D) compound ‘L’

Amino Acid Analysis Using Analytical HPLC

To further confirm the presence of tryptophan or tyrosine as an amino acid component of acid-hydrolyzed compound ‘L’, we analyzed the retention times of the acid hydrolyzed sample, along with amino acid standards of tryptophan and tyrosine. Because all were dissolved in methanol, we also determined the retention time for methanol as a control. Figure 17 shows the results from this analysis using analytical HPLC. Methanol showed a small peak at 3.14 minutes. Tyrosine had a retention time of 3.40 minutes and tryptophan showed a peak at 4.46 minutes. There is also a smaller
peak at 3.14 minutes in the tryptophan spectrum most likely due to the methanol solvent. Acid-hydrolyzed compound ‘L’ had a retention time of 4.42 minutes, very closely matching the retention time observed for tryptophan. A peak at 3.08 can also be seen in the acid-hydrolyzed sample, which could be from the solvent or from another component in the sample. The sizes of the peaks observed are indicative of the concentration of the sample. From these results, tyrosine could be ruled out as a component of the siderophore and tryptophan was a likely component based on the retention times obtained.

Figure 17. Spectra obtained from analytical HPLC analysis: A) methanol (control), B) tryptophan, C) tyrosine, D) acid-hydrolyzed ‘L’ spot
Mass Spectroscopy of Compound ‘L’ and Compound ‘H’

We wanted to further chemically characterize the siderophore produced by *R. leguminosarum* IARI 917, confirm the presence of tryptophan, and also confirm that these siderophores were in fact the same and one was a degraded product of the intact siderophore. We sent four different samples for mass spectroscopy: compound ‘L’ in a deferriated form, compound ‘L’ in an iron-complexed form, compound ‘H’ in a deferriated form, and compound ‘H’ in a iron-complexed form. We had previously sent a ‘mixed’ sample for mass spectroscopy before we separated the two, but the results were inconclusive because we had no idea about the molecular weight of this siderophore and it was hard to determine a correct molecular weight based on the many peaks obtained in the mass spectrum. By evaluating each sample in an iron-complexed form and in a non-complexed form, we had a reference for comparing the two spectra obtained for both compounds ‘H’ and ‘L’. In other words, we could look for a difference of 53 ([Fe$^{3+}$ – 3H$^+$]) due to these being lost as ferric iron is bound to the siderophore) between masses identified in the non-complexed and complexed form for each spot analyzed. Figure 18 shows the spectrum generated for the compound ‘L’ in both the complexed and non-complexed form. A major peak was observed with a *m/z* of 421 in the non-complexed form. In the complexed form a *m/z* of 474 was observed which was assumed to be the mass plus ferric iron. Figure 19 shows the two spectra from both forms of compound ‘H’. A large peak with a *m/z* of 403 was observed in the non-complexed sample and a peak with a *m/z* of 456 was noted in the complexed sample. Because the samples are charged and contain an extra proton (H$^+$), the correct mass
could be generated by subtracting one from the $m/z$ value obtained from each spectrum. Table 3 summarizes the masses generated using ESP API.

Figure 18. ESP API spectra for compound ‘L’: (A) deferriated form, (B) Fe$^{3+}$-complexed form; important peaks are indicated with arrows.
Figure 19. ESP API spectra for compound ‘H’: (A) deferriated form, (B) Fe$^{3+}$-complexed form; important peaks are indicated with arrows.

Table 3. Molecular weights generated using ESP API for compounds ‘L’ and ‘H’

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mass</th>
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<tbody>
<tr>
<td>1. Non-complexed compound ‘L’</td>
<td>420</td>
</tr>
<tr>
<td>2. Iron-complexed compound ‘L’</td>
<td>473</td>
</tr>
<tr>
<td>3. Non-complexed compound ‘H’</td>
<td>402</td>
</tr>
<tr>
<td>4. Iron-complexed compound ‘H’</td>
<td>455</td>
</tr>
</tbody>
</table>
It was important to determine whether these two compounds were the same siderophore or not. From the masses obtained it was noticed that there was a difference of 18, the molecular weight of water, between the two masses (420 and 402). This indicated that the two compounds ('L' and 'H') were the same, and that the compound 'H' was a dehydrated form of compound ‘L’, which was considered to be the intact siderophore. However, this must be confirmed because it is possible that these are still two structurally different siderophores with a close molecular weight. This was accomplished using MS/MS as described in Chapter 2. With this type of mass spectroscopy a particular ion species can be selected and only its fragments analyzed. This was performed only on the samples that were not complexed with iron. With compound 'L', only a \( m/z \) of 421 was allowed to be fragmented and passed through to the detector. Its fragmentation pattern is shown in Figure 20. In the same manner, only a \( m/z \) of 403 was analyzed for its fragmentation pattern (Figure 21). If these samples are the same siderophore then it is expected that their fragmentation patterns should be very similar. By analyzing these two spectra it is very clear that the fragmentation patterns are virtually exact. For example, some of the major peaks in each spectrum have been highlighted in Figures 19 and 20, and both show fragments of exactly the same size. This is concrete evidence that these are exactly the same in structure and that the difference between the compounds ‘H’ and ‘L’ is that compound ‘H’ is a dehydrated form. From here on, these will be referred to as the intact siderophore and the dehydrated form.
Figure 20. MS/MS spectrum generated for compound 'L', the intact siderophore produced by *R. leguminosarum* IARI 917
Figure 21. MS/MS spectrum generated for compound 'H', the dehydrated form of the siderophore produced by *R. leguminosarum* IARI 917; comparison with Figure 20 reveals the exact same fragmentation pattern for the two compounds.
Besides confirming that *R. leguminosarum* IARI 917 is producing only one dihydroxamate-type siderophore, we also wanted to learn something about the chemical structure of this siderophore, including if tryptophan was actually a component of this siderophore. By analyzing the fragments generated through MS/MS, one can determine some structural information based on the fragment sizes. Tryptophan has a molecular weight of 204 Da, which was not observed as a fragment in the spectra. Also, a molecular weight of 420 for the intact siderophore seemed low to contain an amino acid such as tryptophan. While it was puzzling why some of our previous results indicated that tryptophan was a likely component, the results from mass spectroscopy proved otherwise.

One known citrate-based dihydroxamate siderophore with a molecular weight of 420 called schizokinen (Mullis et al. 1971). To better determine if this siderophore is the one being produced by *R. leguminosarum* IARI 917, we compared the structure of schizokinen with the fragmentation pattern generated using MS/MS (Figure 22). Citrate has a molecular weight of 192, which is a fragment observed in the spectrum. Also, the peak with *m/z* 361 is probably due to an acetiline (CH$_3$OH) group being removed from the intact siderophore upon fragmentation.
Figure 22. MS/MS spectrum of the intact siderophore compared with the structure of schizokinen; an arrow indicates a peak at 193, the molecular weight of citrate (plus H⁺)
NMR Results

Resonances in the proton NMR spectrum (Figure 23) for this isolate were assigned based upon multiplicities, chemical shifts and comparison with the literature (Mullis et al. 1971, Persmark et al. 1993). These chemical shift assignments and multiplicities are in excellent agreement with literature assignments for Schizokinen (Table 4).

Figure 23. Proton NMR spectrum of the purified siderophore
Detection of a Possible OMRP Using SDS/PAGE Analysis

SDS/PAGE analysis was performed on whole cell pellets and membrane pellets of cultures grown in a no-added iron medium and a high iron medium to detect a possible OMRP involved in siderophore transport. This protein should be expressed in the no added iron pellets and repressed in high iron conditions. The molecular weights for most described OMRPs are in the range of 70-80 kilodaltons (kDa), which is the region of focus in the gel. The SDS/PAGE gel (Figure 23) shows the presence of a band in this molecular weight range that is only present in the no added iron cultures, indicating that it is regulated by the amount of iron in the medium. This protein is likely involved in siderophore transport, but should be confirmed using radioactively labeled ferric iron or through western blotting.
Figure 24. SDS-PAGE analysis of whole and membrane pellets: A) molecular weight standard, B) *R. leguminosarum* IARI 917 no added iron whole cell pellet, C) *R. leguminosarum* IARI 917 high iron whole cell pellet, D) *R. leguminosarum* IARI 917 no added iron membrane pellet, E) *R. leguminosarum* IARI 917 high iron membrane pellet
CHAPTER 4
DISCUSSION

Many gram-negative organisms are known to secrete siderophores under iron-limiting conditions, either in the environment or in an animal host in the case of pathogens. Siderophore-mediated iron transport has been studied in many organisms, but most of the research has been done using the iron transport systems of *E. coli*. These systems have not been as well studied in the family Rhizobia. It is known that many Rhizobia produce at least one type of siderophore and the most common ones are rhizobactin (Smith et al. 1985), rhizobactin 1021 (Persmark et al. 1993), and vicibactin (Dilworth 1998). It has been found that the family can also use anthranilate and citrate for iron uptake (Rioux et al. 1986, Guerinot et al. 1990). Because of the agricultural importance of these organisms and the fact that poor nodulation linked to iron-deficient soil affects many crops, there is a need to study the siderophore-mediated iron transport systems of this family in greater detail. Additionally, while the general mechanism of siderophore transport in Rhizobia is similar to that of other organisms, there are major differences in how their transport is regulated (Johnston 2004).

Our aim was to identify the siderophore-mediated iron transport system and its components in *Rhizobium leguminosarum* IARI 917 and further study the mechanism of iron transport as well as the genetic regulation of transport in this organism. This strain was found to produce a hydroxamate-type siderophore under iron-deficient conditions. This was determined through both the iron-perchlorate assay described by Atkin et al. and through TLC (Atkin et al. 1970). Growth conditions were then optimized in order to
achieve maximum siderophore production. Conditions that were optimized were media 
components, iron concentration in the media, incubation time, and temperature. It has 
long been known that members of the Rhizobia family prefer mannitol or maltose as a 
carbon source (Murray and Smith 1957, Bergerson 1961). Variations on the original 
Fiss-glucose minimal medium (Vellore 2001) included these sugars, various nitrogen 
sources, and variations on the four Fiss components added to the media. After trying 
several media combinations, it was determined that Fiss-glucose minimal media 
supplemented with 1% maltose and 0.1% (NH₄)₂SO₄ had the greatest effect on 
hydroxamate production by R. leguminosarum IARI 917. Cultures grown in this medium 
produced almost four times more siderophore than the cultures grown in the original 
medium. Iron concentration of a medium is also extremely important to how much 
siderophore a culture will produce, as siderophore production is directly regulated by 
iron concentration (Lankford 1973). We have shown that hydroxamate production by 
this culture is best when the media is supplemented with 0.5 µM FeSO₄, and that 
siderophore production decreases when this concentration is exceeded, and is 
completely repressed at 5 µM. This is consistent with the optimum iron concentration 
for siderophore production of other Rhizobia, which studies have found to be less than 
one µM (Guerinot et al. 1990, Carson et al. 2000).

Siderophore production was measured at various intervals over a 42-hour period 
to determine the onset of siderophore production and the peak of production. This 
strain begins producing siderophore at 8 to 9 hours post inoculation and reaches its 
peak siderophore production at 23 to 24 hours. Additionally, it was determined that the 
culture grew best and produced maximum siderophore at 30°C, which is typical for this
family (Murray and Smith 1957). It has been reported that a bacterial culture can often
grow equally well at two temperatures, but siderophore production may be much less at
the higher temperature (Garibaldi 1972, Greiger and Kluger 1977). This is the case for
*R. leguminosarum* IARI 917, as growth is actually a little better at 37°C than 24°C,
although the culture grown at 24°C produces almost twice as much siderophore. After
all growth conditions had been standardized for *R. leguminosarum* IARI 917,
siderophore production increased approximately 3.8 times over that produced at the
original conditions.

Batch cultures of *R. leguminosarum* IARI 917 were grown to isolate enough
siderophore for chemical analyses. Purification of the hydroxamate-type siderophore
included passing acidified supernatant through an XAD-2 column, followed by passing
the concentrated siderophore through a Sephadex LH-20 hydrophobic column and
finally through HPLC on a C\textsubscript{18} hydrophobic column. After each stage of purification,
siderophore content was estimated to determine how much siderophore was lost at
each step. We determined that less siderophore is lost when it is purified in an iron-
complexed form, most likely because it is more stable in this form, which has also been
noted for other hydroxamate-type siderophores like rhizobactin 1021 (Persmark et al.
1993).

Following purification through the Sephadex LH-20 column, two distinct spots
characteristic of a hydroxamate-type siderophore were observed. Often siderophores
are degraded during purification but can still bind ferric iron and will give a spot on TLC
plates. However, it was impossible to tell if this was the case or if two chemically
distinct siderophores were being produced by *R. leguminosarum* IARI 917. The two
spots seen on the TLC plates were purified separately and will be referred to as compound ‘L’ and compound ‘H’. Both were subjected to various chemical analyses to determine their chemical structure. Spectral scans (300-700 nm) can indicate whether a dihydroxamate or a trihydroxamate-type siderophore is present (Jalal and van der Helm 1991). Spectral scans of the purified siderophore isolated from \textit{R. leguminosarum} IARI 917 indicated that both were dihydroxamate-type siderophores, but their absorbance maximum were different, leading us to initially believe these were structurally different compounds.

Siderophores are often conjugates of amino acids. For example, enterobactin is synthesized from three L-serine molecules (O'Brien and Gibson 1970) and aerobactin, a dihydroxamate-type siderophore first identified in \textit{Enterobacter aerogenes}, is a conjugate of L-lysine (Gibson and McGrath 1969). Both compound ‘L’ and compound ‘H’ were acid-hydrolyzed to determine if any amino acid moiety was present in their structures. Compound ‘L’ was initially used to eliminate the possibility of several amino acids as possible components of its structure. After several analyses using three different solvent systems, all amino acids could be eliminated except the aromatic amino acids, tryptophan, and tyrosine. Analytical HPLC revealed that compound ‘L’ could possibly be an amino acid conjugate of tryptophan based on retention time and eliminated tyrosine as a potential candidate. Further analysis on compound ‘H’ using TLC gave the first indication that the two compounds were the same, as both traveled the same distance on the TLC plates and were visually similar in appearance.

ESP API followed by MS/MS was performed to determine a molecular weight of the compounds and also to give concrete evidence to whether these were the same
compound. Based on the fragmentation patterns of the MS/MS spectra for each compound, it was confirmed that they were the same and that compound ‘H’ was a dehydrated form of the intact siderophore. The molecular weight matched that of a known citrate-based dihydroxamate-type siderophore, schizokinen. MS/MS analysis confirmed that the siderophore produced by \textit{R. leguminosarum} IARI 917 was also citrate-based. NMR analysis confirmed that schizokinen was the siderophore being produced by \textit{R. leguminosarum} IARI 917, which has not yet been described in a member of the Rhizobia family.

“Schizokinens,” which is Greek for “growth stimulating,” were first described as endogenous factors that functioned to maintain exponential division, in the Gram-positive bacterium \textit{Bacillus megaterium} (Lankford et al. 1966). A year later, it was found that this endogenous factor was a hydroxamic acid that had the ability to chelate ferric iron and seemed to fit the current definition of the time for a siderophore (Byers et al. 1967). The structure for schizokinen was later determined (Mullis et al. 1971), and it was designated a citrate-based dihydroxamate siderophore (Figure 24). It has been suggested that schizokinen chelates ferric iron with two $\alpha$-hydroxamate groups and the $\alpha$-hydroxycarboxylate group of citrate (Figure 25) (Goldman et al. 1983).
Since then, this siderophore has also been isolated from the cyanobacteria *Anabaena* (Simpson and Neilands 1976). One study also indicated that this siderophore was isolated from the plant pathogen *Ralstonia* (formerly *Psuedomonas*) *solanacearum* (Budzikiewicz et al. 1997). However, recent studies on the same strain and other *R. solanacearum* strains contradicted this report and revealed that the organism was producing staphyloferrin B and not schizokinen (Bhatt and Denny 2004). It is interesting to note that schizokinen is almost identical to rhizobactin 1021, a citrate-based dihydroxamate isolated from *Sinorhizobium meliloti* 1021 (Persmark et al. 1993), except that rhizobactin 1021 has an additional fatty acid chain, decenoic acid, in its structure (Figure 25). For this reason, great care was taken to eliminate the possibility that *R. leguminosarum* IARI 917 was producing rhizobactin 1021, a common rhizobial siderophore that was losing this fatty acid chain at some point during purification. We followed the exact purification conditions as described for rhizobactin 1021, including purifying this siderophore as a ferric-complex, which usually prevents degradation of the siderophore.
siderophore (Persmark et al. 1993), and at no point saw any evidence that decenoic acid was being lost from the siderophore. Also, it would be virtually impossible that the mass spectroscopy data would not have some remainder of this fatty acid chain or intact rhizobactin 1021. There were no peaks observed in the several mass spectra that indicated a peak at either 531, the molecular weight of rhizobactin 1021, or at 110, the molecular weight of decenoic acid. The siderophore isolated from *R. leguminosarum* IARI 917 was insoluble in acetonitrile, which is not the case for the highly amphiphilic rhizobactin 1021. All evidence in this work points to schizokinen production by this rhizobial strain, which is a unique finding.
Figure 26. Structures of schizokinen (A) and rhizobactin 1021 (B)
Due to the instability of schizokinen, care was taken to avoid degradation. The primary forms of this siderophore were the hydrated and the dehydrated form (molecular weight 402 Da), but both seem to have the ability to chelate ferric iron. The dried form of the purified siderophore was yellow in color. It was observed that temperatures above 42°C increased dehydration of the siderophore. Instead of using a rotary evaporator, freeze-drying was found to be a better technique to prevent dehydration. Also, purification should proceed as rapidly as possible and the siderophore should be stored at -20°C. It is still unknown why the acid-hydrolyzed siderophore has a positive reaction with ninhydrin, although there is not an amino acid present in the structure. Other reports have indicated the same results for this siderophore (Akers 1983) but have not concluded why this occurs.

We have also identified a possible outer membrane receptor protein (OMRP) involved in the transport of schizokinen in *R. leguminosarum* IARI 917. This possible receptor is produced only under iron-deficient conditions and falls into the molecular weight range (70-90 kDa) described for other iron-regulated outer membrane proteins in Rhizobia (Reigh and O'Connell 1993). To date, there are no reports of a specific OMRP associated with transporting schizokinen. A 72 kDa iron-repressible protein RhtA, has been identified in *S. meliloti* that is responsible transporting ferric rhizobactin 1021 (Lynch et al. 2001). Subsequent work revealed that schizokinen could be transported in *E.coli* through this transporter and also through the ferric aerobactin transporter, IutA, with the help of *fhuCDB*, genes involved in the transport of many hydroxamates (Cuiv et al. 2004). Because aerobactin, rhizobactin 1021, and
schizokinen are so similar in structure, it is understandable that these siderophores can share receptors. Studies on schizokinen transport in *Anabaena* sp. indicate that aerobactin could be transported into this culture using the schizokinen transport system, but only at a much higher concentration of aerobactin (Goldman et al. 1983). Surprisingly, a similar study in *B. megaterium* revealed very contrasting results. Aerobactin was poorly transported into this organism, and excess aerobactin actually inhibited its growth (Haydon et al. 1973). It will be interesting to determine if a novel OMRP functions in transporting this siderophore. Additionally, the genes involved in synthesis of schizokinen have not been characterized. Genes encoding biosynthetic, transport, and regulatory functions for rhizobactin 1021 have been identified, and four biosynthesis genes are homologous to four biosynthesis genes for aerobactin (Lynch et al. 2001). It seems logical that genes involved in synthesis of schizokinen are also similar, but this remains unknown. Some work has also been done concerning the transport kinetics of this siderophore, particularly in *Anabaena*, which indicated the apparent $K_m$ for ferric schizokinen uptake is 0.04 µM (Lammers and Sanders-Loehr 1982), which is similar to the $K_m$ reported for uptake of other siderophores (Wang and Newton 1971).

Future studies for this project include characterizing the biosynthetic genes involved in schizokinen production and comparing them with the biosynthetic genes for rhizobactin 1021. It would also be of interest to characterize the outer membrane receptor protein involved in the transport of ferric schizokinen and compare it with the receptor for rhizobactin 1021, RhtA. Although some studies on the transport kinetics of
ferric schizokinen have been done, further work could be done to better classify this iron-transport system in *R. leguminosarum* because schizokinen production has never been reported in a member of the Rhizobia family.
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