Characterization of a Catechol-Type Siderophore and the Detection of a Possible Outer Membrane Receptor Protein from *Rhizobium leguminosarum* strain IARI 312.

Brianne Lee Clark  
*East Tennessee State University*

Follow this and additional works at: [http://dc.etsu.edu/etd](http://dc.etsu.edu/etd)

Recommended Citation  

This Thesis - Open Access is brought to you for free and open access by Digital Commons @ East Tennessee State University. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of Digital Commons @ East Tennessee State University. For more information, please contact dcadmin@etsu.edu.
Characterization of a Catechol-type Siderophore and the Detection of a Possible Outer Membrane Receptor Protein from \textit{Rhizobium leguminosarum} Strain IARI 312

A thesis presented to the faculty of the Department of Health Sciences East Tennessee State University

In partial fulfillment of the requirements for the degree Masters of Science in Biology

by

Brianne L. Clark
August 2004

Dr. Ranjan Chakraborty, Chair
Dr. Bert C. Lampson
Dr. Lee M. Pike

Keywords: \textit{Rhizobium leguminosarum}, Siderophore, Iron Acquisition, Catechol, Enterobactin
ABSTRACT

Characterization of a Catechol-type Siderophore and the Detection of an Outer Membrane Receptor Protein from *Rhizobium leguminosarum* Strain IARI 312

by

Brianne L. Clark

Many gram-negative bacteria produce and secrete siderophores under iron-deficient conditions. Siderophores are low molecular weight compounds (600-1500 Daltons), which chelate ferric iron with an extremely high affinity, and the complex is actively transported across the outer and inner membranes of gram-negative bacteria. There are two main classes of siderophores: catechol and hydroxamate. Catechol-type siderophores chelate ferric iron via hydroxyl groups, and hydroxamate-type siderophores chelate ferric iron via a carbonyl group with an adjacent nitrogen. Rhizobia fix atmospheric nitrogen symbiotically in leguminous plants using the iron-containing enzyme nitrogenase. To satisfy their iron requirements, many rhizobia are known to produce siderophores. *Rhizobium leguminosarum* Strain IARI 312 is known to infect pigeon pea plants. *R. leguminosarum* Strain IARI 312 produces both a catechol-type and a hydroxamate-type siderophore when grown under iron deficient conditions. The catechol-type siderophore has been purified and chemically characterized and is consistent with that of enterobactin.
DEDICATION

To Mom, Dad, and Chris

In Loving Memory of Sandy
ACKNOWLEDGEMENTS

I would first like to thank my committee chair and advisor, Dr. Ranjan Chakraborty, for all of his guidance and support over these last three years. I have learned a lot from you, and I appreciate all of the patience and enthusiasm you have shown. I would also like to thank Dr. Bert Lampson for all of his assistance in both the lab and in preparing my thesis manuscript. I would like to thank Dr. Lee Pike as well for all of his advice and support.

I would like to express my sincere appreciation to Mrs. Nancy Coffman for all of her help in taking care of departmental matters, as well as Mr. Ralph Coffman and Mrs. Robin Grindstaff for all of their help and company. I hope you all know how much I appreciate everything you did for me. I would like to thank my fellow graduate students, Sam Moretz and Erin Storey, for all of their help and support. I would also like to thank Dr. Ray Mohseni and Dr. James Little for all of their assistance in chemically characterizing my siderophore.

I would like to thank the College of Public and Allied Health, the Department of Health Sciences, and the Department of Biological Sciences for all of their support, both financially and academically, throughout both my graduate and undergraduate careers. I would also like to thank the faculty and staff of the Department of Health Sciences for their support as well.

Last, but not least, I want to thank my family. Mom and Dad, you know I would not be here without you and I want you to know how grateful I am for all of the love and support you have given me over the years. And to Chris, my husband, I want to thank you for being a friend and companion and teaching me so much about life and love. I look forward to what life has to offer us both and to sharing every moment with you.
## CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>2</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>3</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>4</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>8</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>9</td>
</tr>
<tr>
<td>Chapter</td>
<td></td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>11</td>
</tr>
<tr>
<td>The Importance of Iron</td>
<td>11</td>
</tr>
<tr>
<td>Siderophores</td>
<td>12</td>
</tr>
<tr>
<td>Outer Membrane Receptor Proteins</td>
<td>15</td>
</tr>
<tr>
<td>Periplasmic Binding Proteins</td>
<td>16</td>
</tr>
<tr>
<td>ATP-dependent Binding Cassette-type (ABC-type) Transporters</td>
<td>17</td>
</tr>
<tr>
<td>TonB-ExbB-ExbD Complex</td>
<td>17</td>
</tr>
<tr>
<td>Siderophore-mediated Iron Transport</td>
<td>19</td>
</tr>
<tr>
<td>Genetic Regulation of Bacterial Iron-uptake Systems</td>
<td>20</td>
</tr>
<tr>
<td>Characteristics of Rhizobia</td>
<td>23</td>
</tr>
<tr>
<td>2. MATERIALS AND METHODS</td>
<td>29</td>
</tr>
<tr>
<td>Growth and Maintenance of Bacterial Cultures</td>
<td>29</td>
</tr>
<tr>
<td>Preparation of Mannitol-Yeast Agar with Congo Red</td>
<td>30</td>
</tr>
<tr>
<td>Preparation of Inoculum</td>
<td>30</td>
</tr>
<tr>
<td>Preparation of LB Broth Medium</td>
<td>31</td>
</tr>
<tr>
<td>Siderophore Isolation</td>
<td>31</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Iron-restricted Media</td>
<td>31</td>
</tr>
<tr>
<td>Preparation of Modified Fiss Minimal Medium</td>
<td>32</td>
</tr>
<tr>
<td>Siderophore Detection Assays</td>
<td>33</td>
</tr>
<tr>
<td>Siderophore Detection</td>
<td>33</td>
</tr>
<tr>
<td>Chrome Azurol S (CAS) Agar</td>
<td>33</td>
</tr>
<tr>
<td>Arnow’s Assay for the Estimation of Catechol-type Siderophores</td>
<td>36</td>
</tr>
<tr>
<td>Atkin’s Assay for the Estimation of Hydroxamate-type Siderophores</td>
<td>37</td>
</tr>
<tr>
<td>Optimization of Growth Conditions</td>
<td>38</td>
</tr>
<tr>
<td>Growth Curve with Siderophore Production</td>
<td>38</td>
</tr>
<tr>
<td>Iron Concentration Standardization</td>
<td>39</td>
</tr>
<tr>
<td>Modified Fiss Minimal Medium Standardization</td>
<td>39</td>
</tr>
<tr>
<td>Temperature Standardization</td>
<td>39</td>
</tr>
<tr>
<td>Purification of Siderophore</td>
<td>40</td>
</tr>
<tr>
<td>Batch Cultures</td>
<td>40</td>
</tr>
<tr>
<td>XAD-2 Purification</td>
<td>40</td>
</tr>
<tr>
<td>Sephadex LH20 Purification</td>
<td>41</td>
</tr>
<tr>
<td>Chemical Characterization of Siderophore</td>
<td>42</td>
</tr>
<tr>
<td>UV Spectroscopy</td>
<td>42</td>
</tr>
<tr>
<td>NMR Spectroscopy</td>
<td>43</td>
</tr>
<tr>
<td>Analytical HPLC</td>
<td>43</td>
</tr>
<tr>
<td>Cyclic Voltammetry</td>
<td>43</td>
</tr>
<tr>
<td>ESMS (LC/MS)</td>
<td>44</td>
</tr>
<tr>
<td>Amino Acid Analysis</td>
<td>44</td>
</tr>
<tr>
<td>Identification of Possible Outer Membrane Receptor Proteins</td>
<td>45</td>
</tr>
<tr>
<td>3. RESULTS</td>
<td>51</td>
</tr>
<tr>
<td>Siderophore Detection</td>
<td>51</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>--------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Chemical Characterization of Siderophore</td>
<td>52</td>
</tr>
<tr>
<td>Standardization of Growth Conditions</td>
<td>55</td>
</tr>
<tr>
<td>Purification of Siderophore</td>
<td>63</td>
</tr>
<tr>
<td>Chemical Characterization of Siderophore</td>
<td>65</td>
</tr>
<tr>
<td>Identification of Possible Outer Membrane Receptor Proteins</td>
<td>73</td>
</tr>
<tr>
<td>4. DISCUSSION</td>
<td>76</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>83</td>
</tr>
<tr>
<td>VITA</td>
<td>89</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Optimum Concentrations of Modified Fiss Minimal Medium Components</td>
<td>61</td>
</tr>
<tr>
<td>2. Siderophore Production in Original versus Optimized Modified Fiss Minimal Medium</td>
<td>63</td>
</tr>
<tr>
<td>3. Rf Values for Amino Acids and Samples</td>
<td>72</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1.</td>
<td>Representative Siderophore Structures</td>
</tr>
<tr>
<td>2.</td>
<td>General Siderophore-mediated Iron Transport in a Gram-Negative Cell</td>
</tr>
<tr>
<td>3.</td>
<td><em>R. leguminosarum</em> Strain IARI 312 on Mannitol-Yeast Agar with Congo Red.</td>
</tr>
<tr>
<td>4.</td>
<td>Chrome Azurol S Assay</td>
</tr>
<tr>
<td>5.</td>
<td>Arnow’s Assay</td>
</tr>
<tr>
<td>6.</td>
<td>2,3-DHBA Standard Curve</td>
</tr>
<tr>
<td>7.</td>
<td>Atkin’s Assay</td>
</tr>
<tr>
<td>8.</td>
<td>Growth Curve with Siderophore Production</td>
</tr>
<tr>
<td>9.</td>
<td>Effects of Iron Concentration on Siderophore Production</td>
</tr>
<tr>
<td>10.</td>
<td>Effects of Glucose Concentration on Siderophore Production</td>
</tr>
<tr>
<td>11.</td>
<td>Effects of MgSO₄ Concentration on Siderophore Production</td>
</tr>
<tr>
<td>12.</td>
<td>Effects of MnSO₄ Concentration on Siderophore Production</td>
</tr>
<tr>
<td>13.</td>
<td>Effects of ZnCl₂ Concentration on Siderophore Production</td>
</tr>
<tr>
<td>14.</td>
<td>Effects of Temperature on Siderophore Production and Growth</td>
</tr>
<tr>
<td>15.</td>
<td>Detection of Siderophore Production in XAD-2 Eluted Fractions by Thin Layer Chromatography with a n-butanol : acetic acid : ddH₂O (12:3:5)</td>
</tr>
<tr>
<td>16.</td>
<td>UV Spectra</td>
</tr>
<tr>
<td>17.</td>
<td>NMR Spectra</td>
</tr>
<tr>
<td>18.</td>
<td>Analytical HPLC</td>
</tr>
<tr>
<td>19.</td>
<td>Cyclic Voltammetry</td>
</tr>
<tr>
<td>20.</td>
<td>ESMS of Catechol-type Siderophore</td>
</tr>
</tbody>
</table>
21. Detection of Amino Acid in Hydrolyzed Siderophore Sample Using Thin Layer Chromatography .............................................................. 71
22. SDS-PAGE of Possible Outer Membrane Receptor Proteins .................. 73
23. SDS-PAGE of Possible Outer Membrane Receptor Proteins Compared to a Known Outer Membrane Receptor Protein ........................................... 75
24. Proposed Catechol-type Siderophore Structures .................................... 81
CHAPTER 1
INTRODUCTION

The Importance of Iron

Iron is a growth-limiting factor for the majority of microorganisms (Archibald 1983). Some notable exceptions are Lactobacilli, Legionella, Neisseria, and the fungus Saccharomyces cervisiae (Neilands et al. 1987). For example, Lactobacilli have no heme enzymes and use the cobalt form of ribonucleotide reductase, and so do not require iron (Wayne & Neilands 1975). Although iron is present in abundance, it is unavailable due to its presence as insoluble iron oxyhydroxide polymers under aerobic conditions at biological pH. Ferric iron’s ($Fe^{3+}$) solubility under these conditions is $10^{-17}$ M, whereas cytoplasmic iron concentrations are approximately $10^{-7}$ M in metabolically active microbes (Ishimaru 1993). This difference in concentration illustrates that uptake by diffusion is not an option for these microbes.

Rhizobia are root nodule bacteria, including the genera Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium, and Sinorhizobium, and when in a symbiotic association with their host plant, they have an added difficulty in acquiring iron from their host environment because there is such a high demand for iron by both the plant and bacterial cells due to the synthesis of several vital iron-containing proteins (Guerinot 1994, Lodwig et al. 2003). Iron-containing proteins such as nitrogenase and leghaemoglobin are required for nitrogen fixation during symbiosis between legumes and rhizobia. Leghaemoglobin may represent 25-30% of the total soluble proteins in an infected plant cell and nitrogenase can make up 10-12% of the
bacterial protein (Verma and Long 1983). Because these and other iron-containing proteins make up such a large portion of the protein mass of both cell types, the amount of iron available to bacterial symbionts is restricted. Therefore, many rhizobia, along with the majority of gram-negative bacteria, express high affinity iron transport systems to overcome iron deficiency. High affinity iron transport systems in general are made up of several components, including siderophores, outer membrane receptor proteins, periplasmic binding proteins, ATP-dependent ABC-type transporters, and the TonB-ExbB-ExbD protein complex, each vital to the success of the transport system.

**Siderophores**

Lankford coined the term siderophore in 1973 as a term to describe low molecular weight molecules that bind ferric iron with an extremely high affinity (Lankford 1973). Siderophore was derived from a Greek term meaning “iron carrier” (Ishimaru 1993). This is an appropriate term because the siderophore binds iron with an extremely high affinity and is specifically recognized by a corresponding outer membrane receptor protein, which in turn actively transports the complex into the periplasm of the cell. The molecular weights of siderophores range from approximately 600 to 1500 daltons, and because passive diffusion does not occur for molecules greater than 600 daltons, siderophores must be actively transported (Ishimaru 1993).

Many bacteria and fungi are capable of producing more than one type of siderophore or have more than one iron-uptake system to take up multiple siderophores (Neilands 1981). Siderophores are classified on the basis of the
chemical functional groups they use to chelate iron. Catecholate-type (phenolate) siderophores bind Fe$^{3+}$ using adjacent hydroxyl groups of catechol rings. Enterobactin, also known as enterochelin, is produced by a number of bacteria including *E. coli* and is the classic example of a catechol-type siderophore (Figure 1A) (O'Brien & Gibson 1970, Pollack et al. 1970). It possesses the highest known affinity for Fe$^{3+}$ with a stability constant ($K_f$) of 10$^{52}$ (Höfte 1993). Enterobactin production has been demonstrated in some nitrogen-fixing bacteria, including *Klebsiella pneumoniae* and *K. terrigena* (Höfte 1993).

Fe$^{3+}$ is chelated using nitrogen atoms of thiazoline and oxazoline rings in hydroxamate-type siderophores (Crosa and Walsh 2002). Ferrichrome is the classic hydroxamate-type siderophore (Figure 1B). It is produced by a number of fungi including *Ustilago sphaerogena*. Although produced by fungi, ferrichrome is used by a number of bacterial species with the appropriate receptor protein (Höfte 1993). Aerobactin (Figure 1C) is another hydroxamate-type siderophore that is produced by many bacteria including *E. coli* (Buyer et al. 1991).

A third class of siderophores utilizes N-hydroxy amino side chains with an oxygen atom as one of the ligands for Fe$^{3+}$. Anguibactin, produced by *Vibrio anguillarum* incorporates this functional group, but it is also a combination of all three siderophore types in that it is made up of all three functional groups, with three different methods of binding Fe$^{3+}$ (Figure 1D) (Crosa and Walsh 2002). A combination of functional groups is not uncommon to find in many siderophores (Crosa and Walsh 2002).
Figure 1  Representative Siderophore Structures. A) Enterobactin (catechol-type) B) Ferrichrome (hydroxamate-type) C) Aerobactin (hydroxamate-type) D) Anguibactin (mixed)
Outer Membrane Receptor Proteins

Outer membrane receptor proteins are located in the outer membrane of gram-negative bacteria and are responsible for recognition, binding, and transport of the iron-siderophore complex into the periplasm of the cell. There are two types of outer membrane transport proteins: passive transporters have a low substrate binding affinity, using energy provided by an existing chemical gradient; and active transporters, which include those receptor proteins involved with iron-siderophore uptake systems, with high binding affinity and energy provided by ATP (Ferguson and Deisenhofer 2004). Although the general mechanism of iron-siderophore uptake is understood, including recognition of the substrate by a specific outer membrane receptor protein, which then actively transports the iron-siderophore complex into the periplasm using energy presumably provided by the TonB complex, many specific details are still relatively unknown.

The crystal structures of several outer membrane receptor proteins have been solved, including FepA (ferric enterobactin), FhuA (ferrichrome), FecA (ferric citrate), and BtuB (vitamin B₁₂), and all of these proteins have been crystallized in both the bound and unbound forms except FepA (Furguson et al. 1998, Locher et al. 1998, Buchanan et al. 1999, Furguson et al. 2002, Chimento et al. 2003). It is known that outer membrane receptor proteins are comprised of two distinct domains, a plug region and a β-barrel region. The β-barrel of each protein, which inserts across the lipid bilayer of the outer membrane, is formed by 22 antiparallel β-strands that are
connected in the periplasm by short turns and external loops that extend above the cell surface. The β-barrel is closed completely by the plug region (Braun 2002).

The outer membrane receptor protein specifically recognizes and binds the substrate and is then known to undergo major conformational changes; however, the actual mode of transport into the cell is unknown because the typical open channel conformation has not yet been crystallized. The α-helix at the N-terminal of the plug is unwound and extends to a flexible conformation, and the N-proximal end does not conform to a fixed structure. It is, however, the N-proximal end that is believed to facilitate interaction between the outer membrane receptor protein and the TonB complex to provide the energy required for the active transport of the substrates. Genetic and biochemical tests have indicated that there is a region on the proximal end, termed the TonB box, that interacts with residue 160 of the TonB protein. Once actively transported into the periplasm, the iron-siderophore complex is bound to a periplasmic binding protein (Braun 2002).

**Periplasmic Binding Proteins**

Periplasmic binding proteins recognize and bind the iron-siderophore complex that has been actively transported by the outer membrane receptor proteins into the periplasm. Periplasmic binding proteins are a necessary component for transport of the iron-siderophore complex into the cytoplasm. They undergo a conformational change upon binding of the substrate. Periplasmic binding proteins are not specific for single metal chelates and can transport more than one type of siderophore complex.
An example of a periplasmic binding protein is FhuD, which “shuttles” all hydroxamate siderophores that have been imported by the outer membrane receptor proteins FhuA, FhuE, and IutA to a single ABC-type transporter. The crystal structures of both FhuD (ferrichrome) and BtuF (vitamin B$_{12}$) are known (Clarke 2000, Borths et al. 2002, Karpowich et al. 2003) and FepB is the periplasmic binding protein for ferric enterobactin (Chakraborty et al. 2003).

**ATP-dependent Binding Cassette-type (ABC-type) Transporters**

ABC-type transporters are located in the cytoplasmic membrane and actively transport the iron-siderophore complex that has been shuttled through the periplasm into the cytoplasm. They are considered ATP-dependent because unlike the outer membrane receptor proteins, ABC-type transporters use energy released from the hydrolysis of ATP to pump substrates against a concentration gradient into the cytoplasm. ABC-type transporters are members of a highly conserved family of active transporters known as the ABC superfamily that couple ATP-hydrolysis to a variety of physiological processes. The structures of the ABC-type transporters MsbA (lipid A) and BtuCE (vitamin B$_{12}$) are known (Locher 2002, Chang 2003).

**TonB-ExbB-ExbD Complex**

The TonB complex consists of 3 cytoplasmic membrane proteins, TonB, ExbB, and ExbD to “exploit” the proton motive force of the cytoplasmic membrane in order to provide energy to outer membrane receptor proteins for active transport of the iron-
siderophore complex. The TonB complex presumably drives import of the substrate by forming a complex with the outer membrane protein transporters and transduces energy by a mechanism that is unclear (Ferguson and Deisenhofer 2004).

TonB and ExbB are located in the cytoplasmic membrane and are anchored by their N-termini. The majority of ExbD is located in the cytoplasm, but it does span the cytoplasmic membrane three times, and its N-terminus is located in the periplasm (Braun and Killmann 1999). Binding of the iron-siderophore complex to the outer membrane receptor protein generates an allosteric signal that is transmitted through the outer membrane by the plug region of the transporter. The TonB box region of the transporter changes conformation with “loading” of the outer membrane receptor protein to allow transduction of energy (Ferguson and Deisenhofer 2004).

Two contrasting models have been generalized as the molecular basis of energy transduction from the TonB complex to the outer membrane receptor protein. The “Propeller Model”, proposed by Chang et al. in 2001, suggests that ExbB and ExbD couple the proton motive force of the cytoplasmic membrane to the torsional motion of TonB, which is similar to flagellar motion in bacteria. A loop of TonB, including residue 160, transiently interacts with the TonB box region of the outer membrane transport protein. This causes an unknown conformational change of the outer membrane receptor protein that allows active transport of the iron-siderophore complex.

The “Shuttle Model”, proposed by Letain and Postle in 1997, suggests sequential changes in the conformation of TonB are coupled with the proton motive force by ExbB and ExbD. The structural differences of energized and unenergized
TonB, as well as how the ExbB-ExbD complex energizes TonB are not fully understood. It is suggested, however, that once TonB is energized, it extends across the periplasm and transiently binds to the TonB box region of the outer membrane receptor protein. With interaction of the TonB box and TonB, TonB, which is anchored in the cytoplasmic membrane, is completely released from the cytoplasmic membrane and transduces stored potential energy to the outer membrane receptor protein for import of the iron-siderophore complex. This model could explain energy transduction, but it does not explain how TonB would reinsert itself in the cytoplasmic membrane and recomplex with ExbB and ExbD (Ferguson and Deisenhofer 2004).

**Siderophore-mediated Iron Transport**

Initially, the siderophore binds to ferric iron in the external environment. The iron-siderophore complex is then recognized by the corresponding outer membrane receptor protein. Binding of the ferric-siderophore complex induces considerable conformational changes, perhaps signaling to initiate TonB interaction. Using energy presumably provided by the TonB complex (proton motive force), the ferric-siderophore complex is actively transported into the periplasm. Once in the periplasm, the iron-siderophore complex is bound to a periplasmic binding protein that transports the complex to the ABC-type transporter in the cytoplasmic membrane, which transports the complex into the cytoplasm utilizing energy from the hydrolysis of ATP (Figure 2). Iron is released from the siderophore by either reduction via ferric reductases, or by chemical modification or breakdown of ferric siderophore complexes by acetylation and
esterases, respectively (Neilands et al. 1987). *E. coli* iron transport systems have been widely studied, but the same detail is still lacking in many other genera.

![General Siderophore-Mediated Iron Transport in a Gram-Negative Cell](image)

Figure 2  General Siderophore-Mediated Iron Transport in a Gram-Negative Cell

**Genetic Regulation of Bacterial Iron-Uptake Systems**

The Fur (ferric uptake regulation) protein or Fur-like proteins regulate iron uptake in a variety of bacterial species (Raymond et al. 2003). Since its discovery in 1981 by Hantke, Fur has been coined as the “global” iron regulator protein as it has been found to be a transcriptional repressor of more than 90 different genes in model bacteria, one of the most studied of which is from *E. coli* (Hantke 1981, Wexler et al. 2003). Many of the genes that Fur has been found to regulate are involved in the
synthesis and uptake of siderophores, are directly involved in iron nutrition, and encode adaptive responses to iron-deficient conditions (Wexler et al. 2003).

In general, iron transport is negatively regulated, but some regulation has been found to occur through positive regulation (Hantke 2001). For example, it has been demonstrated in E. coli that induction of the transcription of the ferric citrate transport genes \textit{fecABCDE} requires the binding of ferric citrate to its outer membrane receptor protein FecA (Wriedt et al. 1995). Pseudobactin BN7 and pseudobactin BN8 also up-regulates the expression of PupB in \textit{Pseudomonas putida} WCS358 (Lamont et al. 2002).

Negative regulation on the other hand, can be seen in E. coli, where, in the presence of iron, Fur (bound to ferrous iron) binds to \textit{fur} boxes, the Fur-binding site on DNA strands, and represses the transcription of all genes involved in siderophore biosynthesis and transport except \textit{tonB} and \textit{exbBD}, which are needed for other cellular process (Panina et al. 2001). The Fur protein has been found to be conserved not only in gram negative bacteria, but in gram positive bacteria as well. This is somewhat surprising given the fact that iron-acquisition systems are often located on plasmids or within transposable pathogenicity islands and are frequently horizontally transferred between cells (Panina et al. 2001).

The DtxR family is responsible for iron-responsive gene regulation in many gram positive bacteria. DtxR was identified in \textit{Corynebacterium diptheriae} as a regulator of the iron-dependent diptheria toxin (Boyd et al. 1990). DtxR is a member of the Fur superfamily, which includes PerR, an oxidative stress-response regulator, Zur, which
regulates genes involved in zinc uptake, and Irr, which is found in rhizobia and is responsible for the induction of nitrogen-fixing nodules on the roots of legumes (Wexler et al. 2003). Transcription of *irr* is moderately repressed under iron-deficient conditions, and is dependent on a protein, Fur$_{Bj}$, that is homologous to the Fur protein (Hamza et al. 1999).

Although Fur and Fur homologues were believed to be the major iron regulators, recent research involving rhizobia has indicated that Fur may not be the “global” iron regulator of *Rhizobium*. Although homologues of Fur do exist in some *Rhizobium*, their roles appear to be different than that of Fur in “model” bacteria such as *E. coli*. Wexler et al. in 2001 demonstrated that mutations in a *R. leguminosarum* gene with significant sequence similarity to *fur* of other bacteria had no detectable effect on the iron-responsive repression of two operons, *hmuPSTUV* and *orf1-tonB*. Both operons are normally expressed under low iron conditions and are required for the uptake of heme as a sole source of iron. Several other operons whose products are involved in iron uptake and whose transcription is normally lessened by high iron levels showed unaffected expression by mutations in the *R. leguminosarum fur* gene (Todd et al. 2002).

Each of the operons involved in vicibactin synthesis, a trihydroxamate siderophore produced by a variety of *Rhizobium*, is deregulated in *R. leguminosarum* with mutations in *rirA* (rhizobial iron regulator). *rirA* is a recently discovered gene whose protein product has no sequence similarity to that of Fur, but which has close homologues in other rhizobia (Wexler et al. 2003). It has been suggested that possibly
fur and rirA may work together to regulate iron in *Rhizobium* or that there may be a novel iron-responsive regulation system in *Rhizobium* that has yet to be revealed.

**Characteristics of Rhizobia**

“Rhizobia” refers to the collective group of gram-negative bacilli that live freely in the soil and are capable of symbiotic nitrogen fixation (Kimball 2004). These root nodule bacteria currently include the genera *Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium, and Sinorhizobium* (Lodwig et al. 2003). Rhizobia induce nitrogen-fixing nodules on the roots of leguminous plants, nodulating specific legumes, and differentiating within their roots to form nitrogen-fixing bacteria (Lynch et al. 2001). The symbiosis between a particular strain of rhizobia and a specific legume is mediated by a Nod factor secreted by rhizobia and transmembrane receptors on the cells on the root hairs of the legume. Different Nod factors and receptors are produced by different strains of rhizobia and different legumes, respectively. If the Nod factor and the receptor are compatible, infection can occur (Kimball 2004).

This symbiotic relationship begins with a continuous exchange of signals occurring between the legume and bacteria to coordinate the expression of both bacterial and plant genes required for effective symbiotic development. Flavanoids are produced by the roots of legumes and are among the earliest signals exchanged (Viprey et al. 2000). Flavanoids are very important in this signaling pathway because they interact with regulators of the NodD family in rhizobia, triggering the expression of nodulating genes (*nod, noe*, and *nol*). Most nodulating genes produce a family of
lipochito-oligosaccharide molecules, which are the Nod factors that are essential to bacterial entry into the legume root hairs (Viprey et al. 2000).

Poor nodulation caused by iron-deficiency affects many common agricultural crops, such as beans and peas (Matiru and Dakora 2004). Effective nodulation relies upon persistence of root nodule bacteria in the soil. The agricultural importance of *Rhizobium* demonstrates the need for more research involving all rhizobia. Rhizobia are already known to use a variety of potential sources for iron.

Unusual for non-pathogenic bacteria, haem uptake has been identified in *R. leguminosarum* and *S. meliloti* (Noya 1997), and *B. japonicum* has been shown to grow on haemoglobin and leghaemoglobin as sole iron sources (Noya 1997, Nienaber et al. 2001). Rhizobia are also known to produce a wide variety of siderophores, only a few of which have been characterized. These include anthranilate, citrate, rhizobactin and other carboxylates, rhizobactin 1021, vicibactin, and vicibactin 7101, as well as other unidentified catechols and hydroxamates (Carson et al. 2000). Both *R. leguminosarum* and *B. japonicum* have been identified as producing catechol-type siderophores, but the siderophores have not yet been characterized (Nambiar and Sivaramakrishnan 1987, Patel et al. 1988). The most commonly studied of these siderophores are the dihydroxamate rhizobactin 1021 and trihydroxamate vicibactin, both of which have known structures (Persmark et al. 1993, Dilworth et al. 1998). Many other strains of rhizobia have not been examined for siderophore production, have been labeled as CAS positive or negative, or are identified as producing catechol or hydroxamate-type siderophores (Carson et al. 2000). Rhizobia have been characterized for hydroxamate-
type siderophore production as being species specific. In general, *Sinorhizobium* produce dihydroxamates, the other fast-growing rhizobia trihydroxamates, and bradyrhizobia produces neither of these (Carson et al. 2000).

The components of vicibactin transport have been described, being members of the Fhu hydroxamate uptake family. The inner membrane transporter FhuB, a periplasmic binding protein FhuD, and an ATPase FhuC are encoded on the *fhuCDB* operon. *FhuA* encodes an outer membrane receptor protein but is not linked to *fhuCDB*. TonB presumably provides the energy required for vicibactin uptake and is also needed to import haem in *R. leguminosarum* but is not required for haemoglobin uptake (Carter et al. 2002). The genes involved in rhizobactin 1021 biosynthesis and uptake have also been identified in *S. meliloti*. The operon *RhbABCDEF* functions in biosynthesis of the siderophore, and the *rhtA* gene encodes the outer membrane receptor of rhizobactin 1021 (Lynch et al. 2001). Selected species have been studied in greater detail; however, because of the great variation observed among rhizobial species and their associated hosts, more research is still needed.

There are currently 30 species within the genus *Rhizobium*, including *R. leguminosarum* (DSMZ 2004). *R. leguminosarum* is distinguished only by its host range and is therefore subdivided into three biovars: *viciae* (peas, vetches), *trifolii* (clovers), and *phaseoli* (common beans) (Lodwig et al. 2003). The target of this research, *R. leguminosarum* Strain IARI 312 is capable of nodulating the pigeon pea plant, and would therefore be classified as a member of the *viciae* biovar. Strains of *R. leguminosarum* are specifically gram negative bacilli, 0.5-0.9 X 1.2-3.0 microns in size.
They are non-spore forming, non-pigmented, circular, convex, semi-translucent, raised, mucilaginous, and generally motile (Breed et al. 1957).

Continuing research focusing on the iron-siderophore transport systems of microorganisms is not only an important part of understanding how cells are able to acquire iron, but is also needed to better understand transport mechanisms as a whole. Although current research is providing a glimpse into how the systems may work, much is still relatively unknown, including how the transporters function, how signals are communicated, and even specifically how the TonB protein complex provides energy to the system. Siderophore transport systems are intriguing because of the applications of the system to other areas of research, including pathogenic microbiology, antibiotic and drug development, virology, and many other areas.

Siderophore-mediated iron transport systems are often associated with pathogenesis, and many outer membrane receptors that are involved in iron transport can also serve as phage-binding sites, and provide points of entry for toxic proteins and peptides. For example, the FhuA protein, which transports ferrichrome, also transports the structurally similar antibiotic albomycin, and binds the phages T1, T5, Φ80, and the colicins UC-1, colicin M and microcin 25 (Braun and Killmamm 1999). To better understand the binding and transport of antibiotics as well as phages and in order to provide more useful information to benefit drug-development, including to develop potentially new targets and improve treatment of infections, we must understand these receptor proteins and the iron transport systems to which they are linked.

Siderophores also have been shown to be useful as a drug when administered to patients combating iron-overload diseases. Iron-overload diseases, the most well-known of which is thalassemia, are a major problem in the world, affecting hundreds of thousands of people each year (Savulescu 2004). Iron overload can lead to, among
other things, organ damage and dysfunction (Franchini et al. 2004). Patients suffering from thalassemia major often must be subject to transfusions for the rest of their lives, which can lead to harmful levels of iron accumulation in the body (Ceci 2003). Although not highly publicized as an aspect of the disease, Parkinson’s disease is a disease that is affected by iron-overload (Youdim et al. 2004). Thus far, the only siderophore that is being used for iron chelation therapy disorders (hemochromatosis) is desferal (i.e. deferoxamine/deferiprone), which was introduced in 1976 (Beutler et al. 2003). However, the use of desferal does not give optimum results to many patients, very poor in others, and cannot be administered to those patients who are allergic or otherwise incompatible with the drug (Savulescu 2004). Because of the impact of iron-overload diseases, the search for other potential siderophore-type drugs should be continued to allow other options for treatment.

The primary goal of my research was to investigate the siderophores and siderophore-mediated iron transport systems of a strain of *R. leguminosarum*. It has been noted that different rhizobial species and strains produce a number of different siderophores, with much interspecies variation (Carson et al. 2000). With such a large number of siderophore-producing strains of root-nodule bacteria, which are agriculturally significant, it would be useful to know what range of siderophore types is being produced by a single species. The focus of my research is *R. leguminosarum* Strain IARI 312, which infects the pigeon pea plant. The culture was obtained from the Indian Agricultural Research Institute (IARI) in New Delhi, India. Because *R. leguminosarum* Strain IARI 312 originated from a completely different geographic region than many strains that have been studied here in the United States, we expect that it may be significantly different than strains already studied.

My research involved identification and chemical characterization of siderophores produced by *R. leguminosarum* Strain IARI 312. Culture conditions were
also optimized for both siderophore production and growth. Possible outer membrane receptor proteins were also identified for *R. leguminosarum* Strain IARI 312. Future studies with the strain could involve further chemical and three-dimensional structure determination of the siderophores and of outer membrane receptor proteins, and studies on binding and uptake kinetics of transport to understand the mechanism of siderophore-mediated iron transport in *R. leguminosarum* Strain IARI 312.
CHAPTER 2
MATERIALS AND METHODS

Growth and Maintenance of Bacterial Cultures

Rhizobium leguminosarum Strain IARI 312 was obtained from the Indian Agricultural Research Institute in New Dehli, India. It is a motile, gram negative bacillus, and forms non-pigmented, circular, convex, semi-translucent, raised, mucilaginous colonies (Figure 3). All cultures were maintained on Mannitol-Yeast Agar with Congo Red, grown for 48-72 hours at room temperature (Jadhav and Desai 1996).

Figure 3  *R. leguminosarum* Strain IARI 312 on Mannitol-Yeast Agar with Congo Red
Preparation of Mannitol-Yeast Agar with Congo Red

Combine the following per 100 ml media by stirring constantly:

- 1.0 g Mannitol
- 0.05 g K₂HPO₄
- 0.02 g MgSO₄·7H₂O
- 0.01 g NaCl
- 0.1 g Yeast extract
- 0.25 ml 1% Congo red solution

Bring the pH of the solution to 6.8 with 6 M NaOH and up to the volume using distilled water (dH₂O). Add 3.0 g agar to the solution while heating and stirring constantly until the agar has melted, then autoclave the solution. Place in a 50°C water bath and let it cool. Once cooled, the solution can either be poured into sterile plastic plates, each plate receiving approximately 20 ml, or into sterile tubes, each tube receiving approximately 3 ml. The tubes were either cooled standing up to make agar deeps, or at an angle to make agar slants. The cooled agar is pink in color.

Congo red is present in the media because in general, rhizobia do not take up Congo red, or absorb it weakly. Therefore, non-rhizobial species are darker in appearance than rhizobial species because they have taken up the Congo red dye in the medium (Kneen and LaRue 1983).

Preparation of Inoculum

For the preparation of inoculum, *R. leguminosarum* Strain IARI 312 was grown in Luria-Bertani (LB) broth medium for 16-18 hours at 27°C (Maniatis et al. 1982).
Preparation of LB Broth Medium

The following components were combined per liter of dH₂O, dissolved while stirring constantly. The pH was adjusted to 7.5 with 6.0 M NaOH and autoclaved.

10 g Tryptone
5 g Yeast Extract
10 g NaCl

Siderophore Isolation

Iron-restricted Media

Because siderophores are only produced under low iron conditions, *R. leguminosarum* Strain IARI 312 was grown in media with low concentrations of iron. Because siderophore production can vary with media composition, the modified Fiss minimal medium (Vellore 2001) was used as a basic medium and different media ingredients were varied to check their effects on growth and siderophore production.

All glasswares used to store the stock solutions of the modified Fiss minimal medium were treated with concentrated nitric acid (HNO₃) to remove trace amounts of contaminating iron. The glassware was acid-treated by adding one-third of the glassware’s volume of concentrated HNO₃. The containers were agitated and left to sit overnight. After approximately 24 hours, the acid was removed and the glassware was rinsed thoroughly with double distilled water (ddH₂O).
Preparation of Modified Fiss Minimal Medium. The following stock solutions were prepared, autoclaved (FeSO₄ was syringe filtered), and stored at 4°C:

1. Potassium phosphate (KH₂PO₄) and asparagine solution:
   A 0.524% solution of KH₂PO₄ and L-asparagine were prepared by dissolving 5 g of KH₂PO₄ and 5 g of L-asparagine in ddH₂O to make a final volume of 954 ml. The pH was adjusted to 6.8 with a solution of 6.0 M NaOH.

2. Glucose solution:
   A 50% solution of glucose was prepared by dissolving 50 g of glucose in ddH₂O to make a final volume of 100 ml.

3. Manganese sulphate (MnSO₄) solution:
   A 0.001% solution of MnSO₄ was prepared by dissolving 0.001 g MnSO₄ in ddH₂O to make a final volume of 100 ml.

4. Magnesium sulphate (MgSO₄) solution:
   A 0.4% solution of MgSO₄ was prepared by dissolving 0.4 g MgSO₄ in ddH₂O to make a final volume of 100 ml.

5. Zinc chloride (ZnCl₂) solution:
   A 0.005% solution of ZnCl₂ was prepared by dissolving 0.005 g ZnCl₂ in ddH₂O to make a final volume of 100 ml.
6. Ferrous sulfate (FeSO$_4$·7H$_2$O) solution:

A 1 mM solution of FeSO$_4$ was prepared by dissolving 0.0278 g FeSO$_4$·7H$_2$O in ddH$_2$O to make a final volume of 100 ml. The solution was sterilized using a syringe filter with a pore size of 0.45 µm.

Modified Fiss minimal medium contained: 5.03 g/L KH$_2$PO$_4$, 5.03 g/L L-asparagine, 5.0 g/L glucose, 40 mg/L MgSO$_4$, 100 µg/L MnSO$_4$, and 500 µg/L ZnCl$_2$. Iron-restricted modified Fiss minimal medium was prepared by adding 139 µg/L FeSO$_4$ to the final medium (0.5 µM). High iron modified Fiss minimal medium was prepared by adding 5.56 mg/L FeSO$_4$ to the final medium (20 µM).

Siderophore Detection Assays

Siderophore Detection

For the detection of siderophore, _R. leguminosarum_ Strain IARI 312 was grown in modified Fiss minimal medium containing 0.5 µM added iron for 24 hours on a rotary shaker at 27°C. The assays used to detect siderophore were the CAS assay, Arnow’s assay, and the Atkin’s assay.

Chrome Azurol S (CAS) Agar

The CAS plates were used to check the culture supernatant for the presence of siderophore. The CAS assay is the universal chemical assay for the detection of siderophores. It is based on the high affinity of siderophores for ferric iron, whereby
ferric iron bound to dye, is complexed and released from the dye. The blue color of the medium is due to the dye complexed with iron. When siderophore is added, the siderophore binds the ferric iron, releasing the free dye, which is orange in color.

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

Hence, the presence of siderophore is indicated by a color change from blue to orange.

The CAS plates were prepared in three separate steps.

1. Preparation of CAS Indicator Solution:

   Initially, 60.5 mg of chrome azurol S was dissolved in 50 ml of ddH₂O. 10 ml of Fe III solution (27 mg FeCl₃·6H₂O and 83.3 µL concentrated HCl in 100 ml ddH₂O) was added, along with 72.9 mg hexadecyltrimethyl ammonium bromide (HDTMA) dissolved in 40 ml ddH₂O. The HDTMA solution was added slowly while stirring, resulting in a dark blue solution (100 ml total volume), which was then autoclaved.

2. Preparation of Basal Agar Medium

   In a 250 ml flask, 3 g 3-(N-morpholino) propane sulfonic acid (MOPS) (0.1 M), 0.05 g NaCl, 0.03 g KH₂PO₄, 0.01 g ammonium chloride (NH₃Cl), and 0.05 g L-asparagine were dissolved in 83 ml ddH₂O. The pH of the solution was adjusted to 6.8 using 6 M NaOH. The total volume was brought to 88 ml using ddH₂O, and 1.5 g agar was added to the solution while stirring and heating until melted. The solution was then autoclaved.
3. Preparation of CAS Agar Plates

The autoclaved basal agar medium was cooled to 50°C in a water bath. The CAS indicator solution was also cooled to 50°C, along with a 50% solution of glucose. Once cooled, 2 ml of the 50% glucose solution was added to the basal agar medium with constant stirring, followed by 10 ml of the CAS indicator solution, which was added carefully and slowly along the walls of the flask with constant stirring, but at a speed so as not to generate any bubbles. Once mixed thoroughly, the resulting solution (100 ml) was poured into sterile plastic plates, each plate receiving approximately 25 ml of blue agar.

Under minimal iron conditions, siderophore is produced and released into the culture medium. To isolate and collect siderophore, *R. leguminosarum* Strain IARI 312 was grown in iron-restricted (0.5 µM added iron) modified Fiss minimal medium and modified Fiss minimal medium with a high concentration of iron (20 µM). After 24 hours of growth, the culture was centrifuged and the cell supernatant was separated and collected by centrifugation for 2 minutes at 13,500 rpm. Supernatant was applied to CAS plates by using a #2 cork borer to make a well on the plate. Culture supernatant was added to the well (60 µL), and the plate was incubated at room temperature to develop. A maximum of 8 hours was given for any color change to develop. If siderophore is present, an orange halo is visible. A halo should be produced from the supernatant of cultures grown in iron-restricted media, and cultures grown under high iron conditions should create no color change. In addition to using supernatant from
culture grown in high iron medium as a control, uninoculated medium is also added to a separate well to ensure the medium alone does not cause a color change.

**Arnow’s Assay for the Estimation of Catechol-type Siderophores**

If siderophore production was detected, the supernatant was further tested using Arnow’s assay for the detection of catechol-type siderophores because the CAS assay does not indicate the type of siderophore being produced. Arnow’s assay was performed by combining the following in order, mixing between each step:

1) 1 ml culture supernatant/ uninoculated medium

2) 1 ml 0.5 M HCl

   - assay is colorless

3) Nitrite-molybdate reagent

   10 g sodium nitrite + 10 g sodium molybdate dissolved in 100 ml ddH₂O

   - The presence of catechol-type siderophore is indicated by the formation of a yellow color, while the control remains colorless.

4) 1 M NaOH (4.0 g NaOH dissolved in ddH₂O to make a final volume of 100 ml)

   - The presence of catechol-type siderophore is indicated by the formation of a pink color, while the control remains colorless.

Arnow’s method is based on the fact that catechol, when combined with nitrous acid, gives a yellow color. The yellow becomes an intense orange-red in the presence of excess sodium hydroxide (Arnow 1936). Because this is a colorimetric assay, after
all components have been added, the assay is allowed to incubate at room temperature for approximately 5 minutes to allow the color to fully develop. Again, supernatant of cultures grown under high iron conditions as well as uninoculated media with reagents were used as controls. Once developed, the absorbance of the solution is measured at 500 nm, using the uninoculated modified Fiss minimal medium with no added iron and components 2-4 added as a blank. The control assays are colorless, and a positive reaction is indicated by a pink to deep red color being produced, depending on intensity (based on amount of catechol present) (Arnow 1936).

Atkin’s Assay for the Estimation of Hydroxamate-type Siderophores

The culture supernatant is further tested using Atkin’s assay for the detection of hydroxamate-type siderophores. Atkin’s assay was performed by combining 0.5 ml culture supernatant with 2.5 ml Atkin’s reagent (0.1771 g Fe(ClO₄)₃ dissolved in 100 ml ddH₂O + 1.43 ml perchloric acid). The Atkin’s assay is also a colorimetric assay, and is allowed to incubate at room temperature for approximately 5 minutes to allow the color to fully develop. Once developed, the absorbance of the solutions is measured at 480 nm, using the uninoculated modified Fiss minimal medium with reagents as a blank, along with culture supernatant grown under high iron conditions as a control. The control assays are colorless to slightly yellow in color, and a positive reaction is orange in color (intensity based on amount of siderophore present) (Atkin et al. 1970).
Optimization of Growth Conditions

In order to maximize catechol-type siderophore production, various growth conditions were tested for optimal siderophore production. A seed culture (3 ml LB in 10 ml test tube incubated on rotary shaker for 20 hours) was used as inoculum. A 1/100 volume seed was used to inoculate each flask. HNO₃-treated 50 ml flasks were used for the standardizations, containing approximately 10 ml media, and were inoculated with 100 µL seed culture.

The cultures were grown in the appropriate medium for 24 hours at 27°C on a rotary shaker, and the growth and siderophore production were measured. Growth was measured by making a 10-fold dilution of the culture (0.2 ml culture + 1.8 ml ddH₂O) and measuring absorbance at 600 nm, using uninoculated media as a blank. Siderophore production was measured by performing Arnow’s assay on the culture supernatant and measuring absorbance at 500 nm as previously described.

Growth Curve with Siderophore Production Kinetics

To test the production of catechol-type siderophore over various incubation times, *R. leguminosarum* Strain IARI 312 was grown in 560 ml modified Fiss minimal medium with no added iron in a 2.8 L flask. An aliquot of 1.3 ml culture was removed at the various intervals and tested for growth and siderophore production as described earlier. Time intervals from 9 to 42 hours post-inoculation were tested.
Iron Concentration Standardization

The iron concentrations of the modified Fiss minimal medium were varied by varying the amounts of FeSO₄ added and by chelating contaminating iron in the medium by adding 2,2′-dipyridyl at varying concentrations to the medium with no added iron. The iron concentrations are based on the added iron to the modified Fiss minimal medium, though some contaminating iron is already present (~2.7 µM). The level of 0.0 µM FeSO₄ indicates no added iron in the modified Fiss minimal medium, containing only iron as contaminants in the medium ingredients. Negative levels indicate the addition of 2,2′-dipyridyl, which chelates the contaminating iron. A 0.1 M solution of 2,2′-dipyridyl (0.1562 g 2,2′-dipyridyl dissolved in 100 ml ddH₂O) was used to add 2,2′-dipyridyl to the media.

Modified Fiss Minimal Medium Standardization

To test the effect of the medium components on siderophore production, the major components of the modified Fiss minimal medium were varied to test the effect of their concentration on siderophore production. Each component was tested individually, varying only the concentration of one component at a time.

Temperature Standardization

To test the effect of temperature on siderophore production, inoculated flasks of modified Fiss minimal medium with no added iron were incubated at varying temperature, grown for 24 hours on a rotary shaker, and removed. Growth and
siderophore production were measured as discussed earlier. The temperatures tested were 4°C, 25°C, 30°C, 37°C, and 55°C.

**Purification of Siderophore**

**Batch Cultures**

With the growth conditions standardized for the optimum siderophore production, the conditions were used to grow *R. leguminosarum* Strain IARI 312 in larger volume batch cultures to obtain pure siderophore in larger quantity for chemical characterization. Batch cultures were prepared by growing *R. leguminosarum* Strain IARI 312 (10 ml seed inoculum) in 1 L of modified Fiss minimal medium with no added iron under optimum siderophore production conditions in a 2.8 L flask on a rotary shaker at 37°C for 24 hours. Total volume collected varied from 4-7 liters. After 24 hours, the cultures were removed, growth and siderophore production were measured as before, and the culture was centrifuged at 7,000 rpm for 30 minutes. The supernatant was collected and acidified to pH 2.0 using concentrated HCl.

**XAD-2 Purification**

The acidified supernatant was passed through a 50 X 20 cm column packed with Amberlite XAD-2 in ddH₂O after equilibrating the column with 4 bed-volumes of ddH₂O. XAD-2 binds cyclic compounds, so the siderophore binds to the column. Acidified supernatant makes the siderophore less soluble in water and it binds more readily to the hydrophobic material in the column. After all supernatant was passed through the
column and washed with 4 bed-volumes ddH₂O, the siderophore was eluted using approximately 250 ml methanol and collected in three fractions of approximately equal volume. Fraction 1 is fairly colorless, containing mostly water. Fraction 2 contains the majority of the collected siderophore in methanol and is bright yellow in color. Fraction 3 contains some residual siderophore, but is mostly methanol and is light yellow in color. Each fraction is tested for siderophore content using Arnow’s assay and thin layer chromatography (TLC). Arnow’s assay was performed as before, but the sample is diluted by adding 0.5 ml ddH₂O to 0.5 ml of each sample. Thin-layer chromatography is used to test approximately 5 µL of each fraction on a Selecto Scientific 5 cm X 20 cm Silica gel 60 plate. The plates are run in a chamber filled with n-butanol (360 ml): acetic acid (90 ml): ddH₂O (150 ml) in a 12:3:5 ratio. The plates were allowed to run approximately three inches above the sample line and removed. The plates were dried and then sprayed with 0.1 M FeCl₃ in 0.1 M HCl (2.7 g FeCl₃ dissolved in 100 ml 0.1 M HCl). Fractions testing positive for siderophore production were dried using a rotary evaporator. The dried siderophore was then stored at -20°C until further purification.

Sephadex LH20 Purification

The concentrated XAD-2 eluted fractions were further purified by chromatography on a 2.5 X 50 cm column packed with deaerated Sephadex LH20 in methanol. Approximately 3 ml of sample redissolved in methanol was loaded onto the column, and methanol was used as an eluting solvent. Approximately 95 fractions were collected in 7 ml volumes and tested as previously mentioned on TLC plates run in the
n-butanol : acetic acid : ddH₂O solvent system. Fractions testing positive for siderophore content were pooled, dried by rotary evaporation, and redissolved in 3 ml methanol. The amount of siderophore was estimated using Arnow’s method. The concentrated sample was then again loaded and further purified through the LH20 column to obtain as pure of a sample as possible. Fractions were collected, tested, pooled, evaporated, and stored at -20°C until used for further purification or chemical characterization.

Chemical Characterization of Siderophore

Because the majority of siderophores are amino acid conjugates, the siderophore sample was acid/alkaline hydrolyzed in order to obtain free amino acids in the sample and analyze the presence of amino acids in the sample by comparing to known standards. Therefore, 1 ml of the purified siderophore sample containing ~5 mg of siderophore was acid hydrolyzed (1 ml sample + 1 ml 6 M HCl, autoclaved for 6 hours) and alkaline hydrolyzed (1 ml sample + 1 ml 6 M NaOH, autoclaved for 6 hours). The original sample and the hydrolyzed samples were then chemically analyzed.

UV Spectroscopy

Spectra were obtained for the purified sample and for dihydroxybenzoic acid standards (2,3-DHBA, 2,4-DHBA, 2,5-DHBA, and 3,4-DHBA) by UV spectroscopy.
NMR Spectroscopy

The purified sample and the standards were also analyzed using nuclear magnetic resonance (NMR) spectroscopy to investigate the chemical structural properties. The NMR experiments were conducted on a 400 MHz JOEL NMR spectrophotometer with tetramethylsilane (TMS) as the standard. Essentially, the sample is immersed in a magnetic field and hit with radio waves. The “spinning” nucleus is irradiated with a radio frequency, the relaxation of which causes the nucleus and its magnetic field to resonate. The signal is detected by the spectrophotometer and is generated in an interpretable form of peaks. The electron density around the proton affects the position of the peak. This is known as the chemical shift.

Analytical HPLC

The purified and acid hydrolyzed sample were analyzed using analytical HPLC, along with the DHBA standards, to identify catechol component of the catechol-type siderophore based on retention times. A Waters C18 column was used.

Cyclic Voltammetry

The purified sample and the DHBA standards were analyzed using cyclic voltammetry, whereby positive and negative potentials are applied to a sample, and oxidation and reduction of the sample occurs. This is represented by visible peaks generated on a graph. The samples are subjected to repeated “cyclic” oxidations and reductions for the interpretation of data. A voltage range of −0.6 to 1.0 V was applied
to a glassy carbon electrode at a rate of 0.10 V/s with six segments. The oxidation and reduction patterns of a sample can be compared to those of known standards.

**ESMS (LC/MS)**

The purified sample was also analyzed using electron spray mass spectroscopy (ESMS) equipped with analytical HPLC (LC/MS) to determine the mass of the purified siderophore. ESMS creates gas-phase ion to analyze molecular masses of sample compounds.

**Amino Acid Analysis**

The amino acid content of the sample was analyzed using acid/alkaline hydrolyzed samples, amino acid standards, and TLC. Neutralized acid and alkaline hydrolyzed samples were tested. The neutralized samples were prepared by adding equal volume 6 M NaOH to the acid hydrolyzed sample and 6 M HCl to the alkaline hydrolyzed sample. Any precipitate formed was removed by centrifugation and stored separately. The 20 amino acid standard solutions contained 1 mg amino acid per ml of methanol.

The amino acid standards, with the DHBA standards (1 mg/ml), the purified sample, and the neutralized acid and alkaline hydrolyzed samples, were tested on a 20 X 20 cm TLC plate using two solvent systems: 1) methanol: ammonium acetate (60:40) and 2) acetonitrile: ammonium acetate (60:40). Approximately 1 µl of each sample and standard were each placed on a TLC plate and the plate was chromatographed in a
chamber filled with each solvent. The plates were allowed to run until the solvent front had traveled the entire length of the plate. The plates were allowed to dry, then sprayed with a ninhydrin reagent (0.5% in ethanol) and incubated at 55°C for 15 minutes. After the plates had developed, colored spots were visible and the Rf values of each resulting spot were measured and compared.

Identification of Possible Outer Membrane Receptor Proteins

As mentioned in the introduction, iron-siderophore complexes are transported via outer membrane receptor proteins; therefore, it was our intent to investigate the presence of possible outer membrane receptor proteins in the outer membrane fractions isolated from *R. leguminosarum* Strain IARI 312. To identify possible outer membrane receptor protein(s), the outer membrane fractions were isolated and analyzed using SDS-PAGE analysis (Laemmli 1970). *R. leguminosarum* Strain IARI 312 was grown in 50 ml of optimized Fiss minimal medium with no added iron and under high iron conditions in 250 ml flasks at 37°C for 24 hours. To compare the sizes of the possible outer membrane receptor proteins to a known outer membrane receptor protein, FepA, *E. coli* BL21(DE3) with the FepA gene in pET17b was also grown in 50 ml LB medium with 100 µg/ml ampicillin in a 250 ml flask at 37°C for 15 hours. To induce production of the FepA protein, 0.4 mM IPTG was added, and *E. coli* BL21(DE3) was allowed to incubate at 37°C for an additional 3 hours.

The cells were collected by centrifuging the cultures at 7000 rpm for 10 minutes. The supernatants were poured off and the cell pellets were suspended in 10
ml Tris buffer (50 mM Tris/Sodium azide). The suspended cells were sonicated, which exposes the cells to high frequency sound waves to agitate and break the cells. The cells were exposed to five-1 minute exposures at 7 pulses/second, by exposing for 1 minute, then placing the cells on ice for 1 minute, and repeating until the suspended cells had been sonicated for a total of 5 minutes. This was repeated for each sample. The sonicated cells were then placed in a 10 ml centrifuge tube and centrifuged for 10 minutes at 8000 rpm to remove cell debris. The supernatant was then poured into a fresh ultracentrifuge tube and ultracentrifuged for 1.5 hours at 30,000 rpm to pellet membrane fractions. The supernatant present after ultracentrifugation was removed and discarded. The pellets containing the outer membrane fraction were suspended in an equal volume loading buffer (~20 µl), and the whole cell pellets (collected prior to sonication) were suspended in an equal volume loading buffer as well (~30 µl) to analyze the whole cell extract. To prepare the protein standard, 2 µl SDS-PAGE broad range protein size marker (BIO-RAD, approximately 200kDa, 116 kDa, 97.4 kDa, 66 kDa, 45 kDa, 31 kDa) was suspended in 8 µl loading buffer. The suspended samples and protein size markers were placed in a floating rack and boiled for 5 minutes, cooled, and were ready for loading onto the SDS-PAGE gel. The gel components are listed below:

1) 30% Bis-Acrylamide

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>58.4 g</td>
</tr>
<tr>
<td>Bis</td>
<td>1.6 g</td>
</tr>
<tr>
<td>dH₂O</td>
<td>to 200 ml</td>
</tr>
</tbody>
</table>
2) Running gel buffer
   Tris 36.3 g
   dH₂O to 200 ml
   - adjust pH to 8.8 with HCl

3) Stacking gel buffer
   Tris 3.0 g
   dH₂O to 50 ml
   - adjust pH to 6.8 with HCl

4) 10% sodium dodecyl sulfate (SDS)
   SDS 10 g
   dH₂O to 100 ml

5) 10% ammonium persulfate
   ammonium persulfate 1 g
   dH₂O to 10 ml

6) 2X loading buffer
   Tris (3 above) 2.5 ml
   SDS (4 above) 4.0 ml
   Glycerol 2.0 ml
   2-mercaptoethanol 1.0 ml
   Bromophenol blue 2.0 mg
   dH₂O 10.0 ml

7) Tank buffer
   Tris 12.0 g
   Glycine 57.6 g
   SDS 4.0 g
   dH₂O to 4 L

8) Stain stock
   Coomassie blue R-250 2.0 g
   dH₂O to 200 ml
   - stir and filter
9) Stain
   Coomassie blue R-250  62.5 ml (8 above)
   Methanol  250 ml
   Acetic acid  50 ml
   dH₂O  to 500 ml
10) Destaining solution
    Methanol  500 ml
    Acetic acid  100 ml
    dH₂O  to 1 L

The gel was prepared by combining all of the following, except the ammonium persulfate.

<table>
<thead>
<tr>
<th></th>
<th>Separating Gel</th>
<th>Stacking Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Bis-Acrylamide</td>
<td>4.0 ml</td>
<td>0.532 ml</td>
</tr>
<tr>
<td>Running gel buffer</td>
<td>3.0 ml</td>
<td>------</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td>------</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.12 ml</td>
<td>40 µl</td>
</tr>
<tr>
<td>dH₂O</td>
<td>4.8 ml</td>
<td>2.44 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>4 µl</td>
<td>2 µl</td>
</tr>
<tr>
<td>Ammonium persulfate</td>
<td>60 µl</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

The solutions were deaerated for 10 minutes, the ammonium persulfate was added and gently mixed, and the gel was cast. The separating gel is cast first, allowed to solidify, then the stacking gel is cast on top, placing the comb in position before the gel solidifies. Once the gel had set, the casting tray and gel were placed into the electrophoresis chamber, the chamber was filled with tank buffer, and the samples were loaded onto the gel.
The following amounts of each sample were loaded:

*R. leguminosarum* IARI 312 only:

- Protein size markers: 5 µl
- IARI 312 low iron cell pellet: 5 µl
- IARI 312 high iron cell pellet: 5 µl
- IARI 312 low iron OM pellet: 5 µl
- IARI 312 high iron OM pellet: 5 µl
- IARI 312 low iron OM pellet: 10 µl
- IARI 312 high iron OM pellet: 10 µl
- IARI 312 low iron OM pellet: 15 µl
- IARI 312 high iron OM pellet: 15 µl

*R. leguminosarum* IARI 312 with *E. coli* BL21(DE3) with FepA in pET17B:

- Protein size markers: 5 µl
- IARI 312 low iron cell pellet: 5 µl
- *E. coli* cell pellet: 5 µl
- IARI 312 low iron OM fraction: 5 µl
- *E. coli* OM fraction: 5 µl
- IARI 312 high iron OM fraction: 10 µl
- IARI 312 low iron OM fraction: 10 µl
- *E. coli* OM fraction: 10 µl

The gel was run at 30 mAmp per gel until the bromophenol blue dye front reached the bottom of the gel. The gel was carefully removed from the casting tray and placed into a tray for staining. The gel was stained by pouring staining solution over the gel until it was covered. The tray was placed on a desktop rotary shaker and
agitated for 15-20 minutes at a low speed until the gel was completely stained. The staining solution was poured off and destaining solution was added to cover the gel, which was again agitated until the gel was destained and protein bands were visible. The gel was stored in 5% acetic acid.
CHAPTER 3
RESULTS

Siderophore Detection

For the initial detection of siderophore, *R. leguminosarum* Strain IARI 312 was grown in modified Fiss minimal medium (Vellore 2001) under low iron conditions because siderophores are produced only under iron-restricted conditions. High iron-media conditions repress the siderophore-mediated iron uptake system, and, therefore, there is no siderophore production. *R. leguminosarum* Strain IARI 312 was grown in modified Fiss minimal medium with no added iron, low iron-modified Fiss minimal medium (0.5 µM), and high iron-modified Fiss minimal medium (20 µM) for 24 hours at 27°C. Supernatant was collected by centrifugation and 60 µl was added to the appropriate well on the CAS plates. Uninoculated modified Fiss minimal medium with no added iron was used as a control. The plate was allowed to incubate at room temperature for up to 8 hours.

Both the high iron supernatant and media control wells did not show a color change around the well, which is expected because no siderophore should have been produced. A color change in the form of an orange halo did result around the wells of both the no iron and low iron supernatants, indicating siderophore is produced under iron-restricting conditions (Figure 4).
Chemical Characterization of Siderophore

Once it was confirmed that the organism produced a siderophore, the next step was to determine the chemical type of the siderophore produced by *R. leguminosarum* Strain IARI 312. Arnow’s assay for the detection of catechol-type siderophores and Atkin’s assay for the detection of hydroxamate-type siderophores were each used to test iron-restricted culture supernatants for the detection of the type of siderophore produced.

Both no and low iron supernatants of *R. leguminosarum* Strain IARI 312 were tested using the Arnow’s assay, along with high iron supernatant and uninoculated
media controls. Both the high iron supernatant and uninoculated media controls showed no color change and remained colorless. The no and low iron supernatants developed a pink/red color, measured at 500 nm, indicating the production of a catechol-type siderophore.

![Figure 5 Arnow's Assay. A) uninoculated medium B) no iron supernatant (0.0 µM) C) low iron supernatant (0.5 µM) D) high iron supernatant (20 µM)](image)

Color intensity is indicative of the amount of catechol-type siderophore produced, but to more accurately estimate concentration, a standard curve was generated using known concentrations of 2,3-DHBA using the Arnow’s assay. All future measurements of catechol-type siderophore production were carried out using Arnow’s assay and compared to the 2,3-DHBA standard curve.
The no iron supernatant of *R. leguminosarum* Strain IARI 312 was also tested using the Atkin's assay, along with high iron supernatant and uninoculated media controls. Both the high iron supernatant and uninoculated media controls showed no color change, remaining colorless. The no iron supernatant developed an orange color, measured at 480 nm, indicating the production of a hydroxamate-type siderophore (Figure 7).
Figure 7  Atkin’s Assay. A) uninoculated medium B) no iron supernatant (0.0 µM) C) high iron supernatant (20 µM)

Testing supernatants using the Arnow’s and Atkin’s assays showed that *R. leguminosarum* Strain IARI 312 produces both a catechol and a hydroxamate-type siderophore.

**Standardization of Growth Conditions**

In order to purify the siderophores and characterize them, the growth conditions need to be optimized for the maximum production of each type of siderophore. It is important to obtain siderophore in larger quantity because the purification steps usually led to loss of considerable amounts of sample. Because the production of a catechol-type siderophore is much more uncommon in rhizobial species than hydroxamate-type siderophores, the remainder of this research focuses on the catechol-type siderophore (Carson et al. 2000). To optimize growth conditions for maximum production of the
catechol-type siderophore, the incubation time, iron concentration of the medium, the concentration of the components of the modified Fiss minimal medium (glucose, MgSO$_4$, MnSO$_4$, ZnCl$_2$), and temperature were examined separately.

To test the effect of incubation time on siderophore production, we looked at growth and catechol-type siderophore production over a period of 42 hours incubation time. It was important to know the kinetics of siderophore production. Monitoring siderophore production during the growth curve experiment helped us to know the time of beginning (onset) as well as the peak of siderophore production. *R. leguminosarum* Strain IARI 312 was grown in modified Fiss minimal medium with no added iron and supernatant was collected at various times. The growth was measured at 600 nm wavelength using the culture, and the supernatant was tested using Arnow’s assay to estimate catechol-type siderophore concentration. The results indicate that optimum siderophore production is achieved after 24 hours of growth, while the organism entered stationary phase at 24 hours (Figure 8).

![Figure 8](image_url)

Figure 8  Growth Curve With Siderophore Production. Growth (maroon) is shown on the left y-axis, and siderophore production (teal) is on the right y-axis.
As mentioned in the introduction, the genes controlling the iron-siderophore transport system are regulated by the concentration of iron in the medium (Panina et al. 2001). To examine the effect of the concentration of ferric iron on the catechol-type siderophore production, the culture was grown in the presence of varying concentrations of ferric iron (FeSO₄) in the medium. It is known that siderophore expression is repressed under high iron conditions, but growth of the organism does require some iron in the medium (Neilands et al. 1987). Thus, the relationship is biphasic as depicted in Figure 9. *R. leguminosarum* Strain IARI 312 was grown in modified Fiss minimal medium with varying concentration of iron added, along with 2,2'-dipyridyl to bind contaminating Fe²⁺. Cultures were grown for 24 hours at 27°C, growth was measured, supernatants were collected, and siderophore concentration was estimated using Arnow’s assay.

Figure 9 shows the classic biphasic relationship between siderophore production and iron concentration in the medium. With all contaminating iron removed from the medium (~2.7 µM) with the help of 0.5 mM 2,2'-dipyridyl, both growth and siderophore production were virtually undetectable because no iron was present to support growth of the bacteria and siderophore production. With no added iron in the media (only trace iron present, which is approximately 2.7 µM) siderophore production is at its highest. As additional iron is added to the media, siderophore production decreases as the system begins to be repressed by higher iron concentrations; with 2 µM added iron, siderophore production has been completely repressed, and growth continues to increase, since higher levels of iron support better growth.
To examine the effects of varying the concentrations of media components, the organism was grown on a range of concentrations; growth and siderophore production were measured. The concentrations of glucose, MgSO$_4$, MnSO$_4$, and ZnCl$_2$ were each varied independently of the others, while the other components not being standardized were maintained at normal modified Fiss minimal medium concentrations. The resulting graphs of each of the media component standardizations are shown in Figures 10-13, and an optimum concentration for catechol-type siderophore production and for growth can be seen with each component.
Figure 10  Effects of Glucose Concentration on Siderophore Production. Growth (maroon) is shown on the left y-axis, and siderophore production (teal) is on the right y-axis.

Figure 11  Effects of MgSO₄ Concentration on Siderophore Production. Growth (maroon) is shown on the left y-axis, and siderophore production (teal) is on the right y-axis.
Figure 12  Effects of MnSO₄ Concentration on Siderophore Production. Growth (maroon) is shown on the left y-axis, and siderophore production (teal) is on the right y-axis.

Figure 13  Effects of ZnCl₂ Concentration on Siderophore Production. Growth (maroon) is shown on the left y-axis, and siderophore production (teal) is on the right y-axis.
The optimum concentrations obtained for each component for the maximum production of siderophore are shown in Table 1.

<table>
<thead>
<tr>
<th>Component</th>
<th>Optimum Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>55 mM</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.77 mM</td>
</tr>
<tr>
<td>MnSO₄</td>
<td>1.1 µM</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>8.7 µM</td>
</tr>
</tbody>
</table>

Table 1 Optimum Concentrations of Modified Fiss Minimal Medium Components

The organism was grown in the standardized medium for 24 hours to test the effect of temperature on siderophore production. It is known that *Rhizobium* generally has an optimum growth temperature of 25-28°C. However, after growing the cultures in the standardized Fiss minimal medium with no added iron for 24 hours at various temperatures (4°C, 25°C, 30°C, 37°C, 55°C), we found that the growth of *R. leguminosarum* Strain IARI 312 was best at 25°C, but siderophore production was highest at 37°C (Figure 14).
Figure 14  Effects of Temperature on Siderophore Production and Growth. Growth (maroon) is shown on the left y-axis, and siderophore production (teal) is on the right y-axis.

With growth conditions optimized, the new growth conditions resulted in an increase in catechol-type siderophore production. Table 2 shows siderophore production both under the initial growth conditions and the new optimized growth conditions.
Table 2  Siderophore Production in Original versus Optimized Modified Fiss Minimal Medium

Purification of Siderophore

It was possible to produce large amounts of siderophore once the growth conditions were optimized for maximum siderophore production. In order to purify siderophore from *R. leguminosarum* Strain IARI 312, the culture was grown in large volume batch cultures. Cultures were grown under optimized media conditions for a total volume of 4-7 liters. The cultures were centrifuged and the supernatant was collected and acidified to pH 2 using concentrated HCl. Acidifying the supernatant helps the siderophore to bind more readily to the hydrophobic XAD-2 column by decreasing the solubility of the siderophore in water. The acidified supernatant was then passed through the column, washed with 1 L ddH₂O, and the siderophore was eluted in ~250 ml methanol. The siderophore was collected in three fractions, and each fraction was tested on a TLC plate (n-butanol: acetic acid: ddH₂O solvent system) to confirm the elution of siderophore from the column. TLC plates were developed with 0.1 M FeCl₃ in 0.1 M HCl, which shows a distinct purple-grey colored spot for catechol-type siderophore production, and a wine-colored spot is visible if a hydroxamate-type
siderophore is present. The TLC plate shown in Figure 15 shows the production of both a catechol-type and a hydroxamate-type siderophore by *R. leguminosarum* Strain IARI 312, and their spots compared to the spots generated from samples of 2,3-DHBA and ferrichrome, a hydroxamate-type siderophore.

Figure 15 Detection of Siderophore Production in XAD-2 Eluted Fractions by Thin Layer Chromatography with a n-butanol : acetic acid : ddH₂O (12:3:5). A) *R. leguminosarum* Strain IARI 312 fraction 2 showing both a catechol (A1) and a hydroxamate-type siderophore (A2) B) 2,3-DHBA C) Ferrichrome

Since the XAD-2 column binds cyclic compounds, the siderophores being usually cyclic compounds bind to the XAD-2 column. Both catechol-type and hydroxamate-type siderophores are eluted together from the column. In order to separate these siderophores and to remove other cyclic compounds that might have been eluted, further purification was necessary.
The XAD-2 eluted sample was further purified using a Sephadex LH20 column; however, to achieve better separation, only small amounts of sample can be loaded onto the column (2-4 ml), and so the fractions must be evaporated to dryness and redissolved in small amounts of methanol. The XAD-2 fractions containing siderophore (usually fractions 2 and 3) were dried using a rotary evaporator. Once dry, 5 ml methanol was added to the flask to redissolve the siderophore. This concentrated sample was loaded onto the Sephadex LH20 column. Using methanol as the eluting solvent, the sample was loaded and passed through the column, collecting approximately 95, 7ml fractions.

The fractions were checked for siderophore content using TLC as described earlier. Typically the hydroxamate-type siderophore was eluted earlier and appeared among fractions 5-25, and the catechol-type siderophore was eluted later and appeared within fractions 65-90. The fractions testing positive for each siderophore type were pooled (catechol and hydroxamate separately) and evaporated to dryness. Each sample was then redissolved in ~5 ml methanol and reloaded on the Sephadex LH20 column for further purification. The samples were repeated on the column to ensure separation of the siderophores and to obtain as pure of a sample as possible. The same steps were followed as described for the first round of purification through the Sephadex LH20 column. The sample thus purified twice through the Sephadex LH20 column was used for chemical characterization. A portion of the final purified sample was acid or alkaline hydrolyzed by adding 1 ml 6 M HCl or NaOH, respectively, to 1 ml of purified sample. The resulting solution was then autoclaved for 6 hours.

**Chemical Characterization of Siderophore**

In order to chemically characterize the catechol-type siderophore, both the hydrolyzed and intact purified samples were analyzed. The intact purified sample was
chemically analyzed using UV spectroscopy, NMR spectroscopy, analytical HPLC, and cyclic voltammetry, and ESMS. The hydrolyzed samples were analyzed using TLC.

The first method used to identify the chemical components of the catechol-type siderophore produced by *R. leguminosarum* Strain IARI 312 was a UV spectral scan. The UV spectrum of the sample compound was compared to the spectral scans of several DHBA standards because the catechol portion of the siderophore can have varying arrangements of the hydroxyl groups on the benzene ring of dihydroxybenzoic acid. The results are shown in Figure 16.

![UV Spectra](image)

**Figure 16** UV Spectra. A) Purified sample B) 2,3-DHBA C) 2,4-DHBA D) 2,5-DHBA E) 3,5-DHBA

The UV spectra show the similarity of the UV scan of the sample to the UV scan of 2,3-DHBA, which is consistent with the presence of 2,3-DHBA in the sample.

To further confirm the presence of 2,3-DHBA in the sample, NMR spectroscopy was performed. The broad hydroxyl peak around 8 and the set of peaks for the
benzene ring around 7 in the 2,3-DHBA standard correspond to similar peaks in the sample (Figure 17). This pattern similarity is also consistent with the presence of 2,3-DHBA in the sample.

![NMR Spectra](image)

Figure 17  NMR Spectra. A) 2,3-DHBA and B) purified sample

To further confirm the presence of 2,3-DHBA as a component of the catechol-type siderophore produced by *R. leguminosarum* Strain IARI 312, analytical HPLC was performed. The peak for 2,3-DHBA occurred at 2.9 minutes, and a similar peak was seen in the purified sample at 3.00 minutes (Figure 18). This again is consistent with the presence of 2,3-DHBA in the sample siderophore. Additional peaks visible in the sample are due to impurities and other siderophore components, including degraded products and amino acids.
Figure 18  Analytical HPLC. Retention times of A) purified sample B) 2,3-DHBA C) 2,4-DHBA D) 2,5-DHBA E) 3,4-DHBA

Cyclic voltammetry was also used to confirm the presence of 2,3-DHBA. Four consecutive cycles of positive and negative potentials were applied to both the sample and 2,3-DHBA. The graph shown in Figure 19 illustrates the similarity in both the appearance of the oxidation peaks of both the 2,3-DHBA standard and the sample and their similar potentials. The sample shows its peaks due to oxidation products. Catechols are commonly oxidized to quinones, which are not easily reduced; therefore, there is less sample after each run that can be oxidized, which is also evident in the
graph. This can also be seen where there is no reduction peak of the sample. The similarity of the cyclic voltammetry results further confirms the presence of 2,3-DHBA in the siderophore produced by *R. leguminosarum* Strain IARI 312.

Figure 19  Cyclic Voltammetry. A) 2,3-DHBA and B) purified sample

Electron Spray Mass Spectroscopy (ESMS) was also performed on the purified sample. The resulting spectroscopy is shown in Figure 20. Molecules with molecular weights of 669, 687, and 701 are indicated by the ESMS spectrum in the sample, which are similar to the known molecular weights of intact enterobactin, hydrolyzed enterobactin, and methylated enterobactin (Berner et al. 1991). The significance of these masses will be discussed in the “Discussion” section.
Figure 20 ESMS of Catechol-type Siderophore
The chemical characterization methods used thus far have focused on supporting 2,3-DHBA as a component of the siderophore; however, siderophores are typically conjugates of 2,3-DHBA and amino acids. In order to analyze the amino acid content, the samples were hydrolyzed to break amide bonds. The first method used was to run the acid and alkaline hydrolyzed sample, along with known amino acid standards, on TLC plates in two solvent systems: 1) methanol: ammonium acetate (60:40), 2) acetonitrile: ammonium acetate (60:40). Figure 21 shows the spot patterns of the amino acid standards and the samples in each respective solvent system, and their spot patterns. Table 3 lists the \( R_f \) values for the amino acid standards and all samples in each system, as well as the abbreviation for each sample shown in Figure 21.

![Figure 21 Detection of Amino Acid in Hydrolyzed Siderophore Sample Using Thin Layer Chromatography. Amino acid and sample spots on TLC plates in A) methanol: ammonium acetate (solvent 1) and B) acetonitrile: ammonium acetate (solvent 2)](image-url)
The results indicate the possible presence of tyrosine using solvent system 1. However, the range of $R_f$ values for the amino acids is very small, and many other
amino acids are also close to the sample R_f value. Solvent system 2 indicates the possible presence of serine or valine. The relevance of the serine being present will be discussed in the “Discussion” chapter.

**Identification of Possible Outer Membrane Receptor Proteins**

We wanted to identify the possible outer membrane receptor proteins involved in ferric-siderophore transport of *R. leguminosarum* IARI 312. The SDS-PAGE results of the whole cell pellet and outer membrane fractions are shown below for both the no iron and high iron cultures (Figure 22). Two bands are visible in all no iron samples with molecular weights of approximately 78 kDa and 80 kDa, and the bands are absent in the high iron samples. This indicates repression of these proteins under high iron conditions, which is consistent with the behavior of the iron-regulated genes of iron transport systems.

![Figure 22 SDS-PAGE of Possible Outer Membrane Receptor Proteins. A 7% SDS-PAGE of cell pellets and outer membranes of *R. leguminosarum* IARI 312 grown in no and high iron optimized Fiss minimal medium. A) mol. wt. marker B) no iron cell pellet C) high iron cell pellet D) no iron OM- 5 µl E) high iron OM- 5 µl F) no iron OM- 10 µl G) high iron OM- 10 µl H) no iron OM- 15 µl I) high iron OM- 15 µl](image)
The SDS-PAGE results of the whole cell pellet and outer membrane fractions examining similarity to the FepA protein are shown for both \textit{R. leguminosarum} Strain IARI 312 and \textit{E. coli} BL21(DE3) (Figure 23). Two bands are visible in all no iron samples with molecular weights of approximately 78 kDa and 80 kDa, and the bands are absent in the high iron samples. This indicates repression of these proteins under high iron conditions, which is consistent with the behavior of the iron-regulated genes of iron transport systems. The results indicate that the 80 kDa band observed in \textit{R. leguminosarum} Strain IARI 312 coincides with the 80 kDa FepA band, an outer membrane receptor for ferric enterobactin expressed in \textit{E. coli} BL21(DE3) with the FepA gene in pET17b. The significance of the FepA band will be discussed in the “Discussion” chapter.
Figure 23  SDS-PAGE of Possible Outer Membrane Receptor Proteins Compared to a Known Outer Membrane Receptor Protein. A 7% SDS-PAGE of cell pellets and outer membranes of *R. leguminosarum* Strain IARI 312, grown in low and high iron optimized Fiss minimal medium, and *E. coli* BL21(DE3) with FepA in pET17b. (A) Mol. wt. marker (B) IARI 312 low iron cell pellet (C) *E. coli* BL21(DE3) cell pellet (D) IARI 312 low iron OM fraction (E) *E. coli* BL21(DE3) OM fraction (F) IARI 312 high iron OM fraction (G) IARI 312 low iron OM fraction (H) *E. coli* BL21(DE3) OM fraction
Iron is a growth-limiting factor for the majority of microorganisms. It is present in abundance but is unavailable due to its presence as insoluble iron oxyhydroxide polymers under aerobic conditions at biological pH. Many gram-negative bacteria express high affinity iron transport systems to overcome iron deficiency, including members of rhizobia. Rhizobia induce N$_2$-fixing nodules on the roots of leguminous plants. The plant produces leghaemoglobin and components of respiration, both of which contain iron. Because of the great demand for iron as a result of competition with its host plant, and largely due to the iron content of the bacterial nitrogenase complex, bacteria synthesize and secrete siderophores to overcome iron deficiency.

Poor nodulation caused by iron-deficiency affects many common agricultural crops, such as beans and peas. Effective nodulation relies upon persistence of root nodule bacteria in the soil. Root nodule bacteria vary widely in siderophore production and type of siderophore produced. Much is known about *E. coli* and *Pseudomonas* iron transport, while the components of iron transport systems of much of the genus *Rhizobium* lack the same detail.

The hydroxamate-type siderophores vicibactin and rhizobactin 1021 are the most characterized of siderophores produced by rhizobia, and also the most common (Carson et al. 2000). However, other types of siderophores produced by rhizobia include the carboxylate rhizobactin, vicibactin 7101, citrate, anthranilate, and other unidentified catechol and hydroxamate-type siderophores (Carson et al. 2000). They
are also capable of utilizing haem, haemoglobin, and leghaemoglobin as sources of iron (Noya et al. 1997, Nienaber et al. 2001). Many rhizobial strains have not even been evaluated for siderophore production, are identified as CAS positive or negative, or the siderophore is only identified as being catechol or hydroxamate (Carson et al. 2000). The biosynthesis and uptake systems of rhizobactin 1021 and vicibactin have also been studied in greater detail, but both are hydroxamate-type siderophores, and the same attention has not been given to other siderophores produced by rhizobia.

The agricultural importance of rhizobia demonstrates the need for understanding its iron transport systems. Select species have been studied in greater detail (e.g., *S. meliloti*), but because of the variety of species and their associated hosts within *Rhizobium*, we decided to further investigate the iron transport systems of rhizobia.

The focus of this thesis is *R. leguminosarum* Strain IARI 312, obtained from the Indian Agricultural Research Institute in New Dehli, India. Being a previously unstudied strain of *Rhizobium*, our initial goal was to investigate the siderophore-producing capabilities of the *R. leguminosarum* Strain IARI 312. Initial detection of siderophore production was confirmed using the CAS assay, which demonstrated the production of siderophore under iron-deficient conditions, with repression under high iron conditions. In order to chemically characterize the siderophore being produced, we used Arnow’s and Atkin’s methods for detecting catechol-type and hydroxamate-type siderophores, respectively. The results of the assays indicated that both a catechol-type and a hydroxamate-type siderophore were being produced under iron-deficient conditions by *R. leguminosarum* Strain IARI 312.
The detection of both a hydroxamate-type siderophore and a catechol-type siderophore produced by a strain of *R. leguminosarum* was interesting because catechol-type siderophores are much more uncommon in rhizobial species than hydroxamate-type siderophores (Carson et al. 2000). For this reason, our primary focus was to purify and characterize the catechol-type siderophore produced by *R. leguminosarum* Strain IARI 312. Because purification procedures lead to loss of a significant amount of sample, the growth conditions of the strain were first explored to provide conditions for optimum siderophore production.

The modified Fiss minimal medium composition, temperature, and incubation time were tested to obtain optimum catechol-type siderophore production. The resulting optimized growth conditions included an increase in the concentrations of some media components, no iron added to the media, and incubation at 37°C for 24 hours, resulting in a nearly four-fold increase of catechol-type siderophore production relative to the original growth conditions. The most surprising change in growth conditions was the increase in the incubation temperature from 27°C to 37°C, because the organism is a soil bacterium. However, only siderophore production is increased at 37°C, growth of the strain is still best at 27°C.

The optimized growth conditions were utilized to grow *R. leguminosarum* Strain IARI 312 in large volume batch cultures. Supernatant collected was acidified and purified using a XAD-2 column, which binds cyclic compounds. Because siderophores are cyclic compounds by nature, the column should bind any siderophore produced. Siderophore content of the collected fractions were confirmed using TLC, as described earlier, and Arnow's assay. The sample was then further purified using a hydrophobic Sephadex LH20 column, which separates compounds based on their hydrophobicity, with methanol as an eluting solvent. Catechol-type siderophores are highly
hydrophobic, should bind with a higher affinity than compounds with low hydrophobicity, and so were eluted in the latter fractions. Hydroxamate-type siderophores are fairly hydrophilic, binding with less affinity, and were eluted in earlier fractions. This purification method allowed the separation of the catechol-type siderophore and the hydroxamate-type siderophore produced by *R. leguminosarum* Strain IARI 312, as well as other cyclic impurities that might have present. The collected fractions were tested for siderophore content using TLC.

In order to minimize contaminating cyclic compounds, the collected fractions testing positive for catechol-type siderophore were dried by rotary evaporation, redissolved in methanol, and again purified using the LH20 column. The fractions testing positive for catechol-type siderophore were again dried and stored at -20°C until chemically characterized. The siderophore was stored in a dry state because catechol-type siderophores are highly unstable and are easily oxidized.

The purified catechol-type siderophore was chemically characterized using a number of methods. UV spectroscopy compared the UV absorbance spectra of the sample to those of 2,3-DHBA, 2,4-DHBA, 2,5-DHBA, and 3,4-DHBA standards, NMR spectroscopy showed the functional groups of the sample, analytical HPLC gave retention times of the sample compared with the standards, and cyclic voltammetry was performed to analyze the oxidation and reduction potential of the sample compared to the standards. All analyses detected 2,3-DHBA as a component of the siderophore produced by *R. leguminosarum* Strain IARI 312. Amino acid analysis of the hydrolyzed sample yielded a number of possible amino acids as conjugates of the siderophore, including serine.

The catechol-type siderophore produced by *R. leguminosarum* Strain IARI 312 seemed similar to enterobactin, a trimer of 2,3-dihydroxybenzoylserine, composed of both 2,3-DHBA and serine. To investigate the mass of the catechol-type siderophore,
ESMS spectroscopy was performed. The ESMS instrument was equipped with analytical HPLC (LC/MS), which separated the sample into pure compounds, and each peak was analyzed separately using ESMS. The data were analyzed using the structural program MS Interpreter, distributed by the National Institute of Standard and Technology (NIST), which yielded the structures drawn by ChemSketch, distributed by Advanced Chemistry Development (ACD).

The molecular masses of fragments obtained in ESMS were consistent with the previously reported ESMS analysis of enterobactin, a catechol-type siderophore produced by *E. coli* (Berner et al. 1991). The structural results of the sample are shown in Figure 24, which shows the structures of intact enterobactin, with a molecular weight of 669, hydrolyzed enterobactin, with a molecular weight of 687, and methylated enterobactin, with a molecular weight of 701, consistent with the production of enterobactin by *R. leguminosarum* Strain IARI 312. Enterobactin production is also supported by the identification of serine as one of the possible amino acid conjugates using TLC because enterobactin is composed of 2,3-DHBA and serine (tris-(N-(2,3-dihydroxybenzoyl)serine)) (Ehmann et al. 1999). The ESMS spectra shown in figure 20 illustrates that methylated enterobactin is the predominant product in the sample. This is not surprising given the purification of the sample using methanol.

The detection of enterobactin production by a strain of rhizobia is not as surprising as one might expect. Rhizobia are already known to express an outer membrane receptor FhuA homologue (Yeoman et al. 2000). FhuA is the outer membrane receptor protein for ferrichrome, a hydroxamate-type siderophore produced by fungi, yet is commonly found in *E. coli* and other *Enterobacteriaceae* (Crosa and Walsh 2002). In addition, enterobactin has been shown to be produced by plant-associated bacteria, including *Enterobacter cloacae* (Loper & Henkels 1999) and other nitrogen-fixing bacteria including *Klebsiella pneumoniae* (Höfte 1993).
Figure 24  Proposed Catechol-type Siderophore Structures. A) intact enterobactin B) hydrolyzed enterobactin C) methylated enterobactin

Because ferric siderophores are transported across the outer membrane with the help of outer membrane receptor proteins, it was of interest to possibly identify outer membrane receptor proteins produced by *R. leguminosarum* Strain IARI 312. SDS-PAGE analysis of the outer membrane fractions from the organism grown under iron-deficient conditions clearly indicates the expression of two distinct outer membrane proteins of approximately 78 kDa and 80 kDa, which were completely repressed under
high iron conditions. It is very likely that these proteins are involved in the siderophore transport because the molecular weights of these proteins are similar to the outer membrane ferric siderophore receptor proteins reported so far and are repressed under high iron conditions. The outer membrane fraction of *R. leguminosarum* Strain IARI 312 was also compared with the outer membrane fraction of *E. coli* BL21(DE3) with the FepA gene in pET17b using SDS-PAGE analysis to confirm the possibility of a FepA homologue. The results indicate that the 80 kDa band observed in *R. leguminosarum* Strain IARI 312 coincides with the FepA band, an outer membrane receptor for ferric enterobactin expressed in *E. coli* BL21(DE3). Thus, *R. leguminosarum* Strain IARI 312 may be expressing a FepA homologue, although this should be confirmed with western blot analysis using a monoclonal antibody against FepA.

Future studies on *R. leguminosarum* Strain IARI 312 could include purification and identification of the outer membrane receptor proteins, as well as characterization of the hydroxamate-type siderophore. Because this is the first report of enterobactin production in rhizobia, it would be of interest to know what type of hydroxamate siderophore is produced by this strain. Also, it is important to confirm the identity of this organism as *Rhizobium*. In addition, iron uptake studies using radiolabeled siderophore will be useful to characterize the kinetics of transport.


VITA

BRIANNE L. CLARK

Personal Data: Date of Birth: August 14, 1979
Place of Birth: Columbus, Georgia
Marital Status: Married

Education: Bearden High School, Knoxville, Tennessee
East Tennessee State University, Johnson City, Tennessee;
Health Sciences, B.S., 2001
East Tennessee State University, Johnson City, Tennessee;
Biology, M.S., 2003

Professional Experience: Graduate Assistant, East Tennessee State University, College of
Public and Allied Health, 2001 – 2004

Publications: Moretz, S.E., Clark, B.L., and Lampson, B. C. (2002) Siderophore
production by selected species within the genus
Rhodococcus. Abstract, 213. 18th Annual Student Research
Forum, East Tennessee State University, Johnson City, TN.

Clark, B.E., Storey, E.P., Mohseni, R., and Chakraborty, R.N.
(2003) Characterization of a catechol-type siderphore and the
detection of an outer membrane receptor protein from
Rhizobium leguminosarum RL 312. Abstract, 103th Annual
General Meeting for the American Society for Microbiology.

Clark, B.E., Storey, E.P., Mohseni, R., Little, J., and Chakraborty,
R.N. (2004) Characterization of hydroxamate and enterobactin-
like siderophore production by Rhizobium leguminosarum RL
312. Abstract, 104th Annual General Meeting for the American
Society for Microbiology.

89
Honors and Awards: ASM member 2001-2003