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Characterization of the Genes Involved in Biosynthesis and Transport of Schizokinen, a Siderophore Produced by *Rhizobium leguminosarum* IARI 917.

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Characterization of the Genes Involved in Biosynthesis and Transport of Schizokinen, a

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by

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ABSTRACT

Characterization of the Genes Involved in Biosynthesis and Transport of Schizokinen, a

Siderophore Produced by *Rhizobium leguminosarum* IARI 917

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David Hammond, Jr.

Iron is the 4th most abundant metal on the earth's crust and is required by most organisms as a cofactor for many enzymes; however, at physiological pH and aerobic conditions iron forms insoluble ferric oxyhydroxide polymers. Siderophores are low molecular weight compounds that scavenge ferric ions, bind with high affinity, and transport it into the cell via multicomponent transport systems. *Rhizobia* are soil dwelling organisms that form symbiotic relationships with host plants and fix atmospheric nitrogen, while the bacteria receive nutrients. *R. leguminosarum* IARI 917 produces a siderophore characterized as 'schizokinen'. In the present study, we have characterized the binding and transport kinetics of 'schizokinen' and have also attempted to identify the genes involved in its biosynthesis using mini Tn5 random mutagenesis. DNA sequence analysis of a non siderophore producing transconjugant revealed a gene involved in PAC/PAS signal transduction systems that respond to many extracellular cues.

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CHAPTER 1

INTRODUCTION

Genus of Rhizobia

The Rhizobia are a collective group of soil dwelling microorganisms known to nodulate leguminous plants. Leguminous plants are defined as herbaceous woody plants that produce seeds enclosed within a pod. Most of the plants colonized by the bacteria are agriculturally important crops including soy beans, peas, peanuts, alfalfa, and chick peas. The nodules are produced in the roots and rarely the stems of these plants. Within the nodule, the bacteria convert relatively nonreactive or inert atmospheric nitrogen (N_2) into ammonia for the plant's use for this essential nutrient for growth. The bacteria receive nutrients in the form of simple sugars produced by the plant during photosynthesis in a mutual symbiotic relationship. Nodulation requirements are different for each species of bacteria within the rhizobial group. One factor affecting nodulation is the species of the host plant because the bacteria have a narrow host range in they infect.

At present, there are many different organic fertilizers in the market containing Rhizobia species to effectively provide ammonia for the plant's consumption. Many previous studies have shown that the bacterial presence will increase the yield of the crop per acre (Carter et al., 2000; Zahran, 1999) but each inoculum must be coordinated with the crop to be nodulated effectively.

The rhizobial group are classified into five different genera or species including *Rhizobium, Mesorhizobium, Azorhizobium, Bradyrhizobium,* and *Sinorhizobium.* Within

each of these genera of bacteria, the species and strains of bacteria determine the plants

nodulated resulting in predictable sizes and shapes of nodules. Nodules formed by

Rhizobium trifolii in clover roots are small, round, or oval nodules. The cowpea

(nodulated by *Rhizobium trifolii)*, common bean (*Phaseolus vulgaris*), and soybeans

(*Rhizobium fredii, Bradyrhizobium japonicum,* and *Bradyrhizobium elkanii*) nodules are

round, large, and firmly attached to the roots. Crops such as alfalfa (*Rhizobium meliloti*),

peas (*Rhizobium leguminosarum*), and vetch (*Rhizobium leguminosarum biovar viciae*)

have long, finger-like nodules. The mechanism for this bacterium-plant host specificity

is due to the biochemical interactions between the bacteria and the plant prior to

nodulation. The biochemical interactions are discussed later in more detail.

Many rhizobial species have extremely large genomes (~8Mb) and interestingly

50% of their genome does not resemble other rhizobial species, noting the diversity of

this family (Johnston, 2004). With the recent sequencing of the genome of *Rhizobium*

leguminosarum biovar viciae strain 3841, the inclusion of many genes involved in

nutrient scavenging (Young et al., 2006) were noted. The incorporation of many

different genes involved in substrate transport for a wide range of catabolic systems using

many different nutrients (Boussau, Karlberg, Frank, Legault, & Andersson, 2004)

increases the organism's ability to survive in the nutrient deplete environment of the soil.

For example, rhizobia have ~170 ATP binding cassette (ABC) transport systems

compared to 47 in *Escherichia coli.* The ABC type transport systems enable rhizobia to

use a far greater range of nutrients present at low concentrations in soil and the plant

rhizosphere (Prell & Poole, 2006) as they are functionally similar to other genes involved

in transport systems of other organisms (Young et al.). Rhizobia are also known to

contain many plasmids (*Rhizobium leguminosarum biovar viciae* strain 3841 contains 6 plasmids, Young et al.) used for such reactions as nitrogen fixation and nutrient scavenging that are externally received via conjugation (Young et al.).

Despite the diversity seen in the group, some general characteristics are shared. Rhizobia are aerobic, mostly motile, non-sporulating, Gram negative bacilli that are classified as diazotrophs (or nitrogen fixers). Many are surrounded by a slimy, thick exopolysaccharide layer that is thought to facilitate attachment to root hairs. The optimal temperature for growth of the bacteria is 25-30°C and mannitol is the preferred carbon source (Bergerson, 1961). All of the species have the ability to produce the enzyme nitrogenase, responsible for nitrogen fixation. More detail on the nitrogen fixation and symbiosis of plants is discussed in a later section. The rhizobia are maintained on a modified media called Congo red. Rhizobia take up the Congo red dye weakly while many other microorganisms will more strongly absorb the dye. Once the dye is absorbed, the bacterial colony will appear red that helped identification of contamination of pure cultures (Kneen & Larue, 1983). The rhizobial colonies will appear white, while contaminants will appear red in color resulting from the ability or inability to uptake or absorption of the Congo red dye. A picture of a streak plate in Figure 1 shows the colony color of a pure culture of IARI 917.

Figure 1: *Rhizobium leguminosarum* IARI 917 grown on a Congo red plate (adapted from Storey, 2005).

Nodulation and Effect on Host Plant

Rhizobia are well known for converting atmospheric dinitrogen (N_2), that is chemically inert, into ammonia. The reaction is conducted by bacteriods in the nodule at 30°C at 1 atmosphere of pressure by the enzyme nitrogenase. Bacteroids are defined as functionally and metabolically different forms as compared to their active free dwelling form outside the plant host. The bacteroids are less metabolically active while most of their energy is used for fixing atmospheric nitrogen. In contrast to bacterial nitrogen fixation, industrial conversion by humans is achieved at 300-400°C at 500 atmospheres of pressure. By comparing the two reactions, it is an astonishing fact that bacteria can perform such a reaction under "normal" conditions. Nitrogenase is produced by the bacteroids in the nodule that performs the reaction of converting dinitrogen to ammonia. Nitrogenase catalyzes the six electron reduction of dinitrogen to ammonium that is associated with the reduction of $2H^+$ to H_2 that uses 16-18 molecules of ATP per mole of dinitrogen (Dixon, 2004). The enzyme is oxygen labile and is housed in an anaerobic

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environment within the aerobic cell. Oxygen is buffered between the bacterial cell and the plant via the plant protein leghemoglobin. Figure 2 shows the interaction between the bacteroid and the plant during nitrogen fixation and how various compounds are shared in the symbiotic relationship.

Before nitrogen fixation can begin, nodulation of the plant by the bacteria must occur. The nodulation process is initiated by biochemical signaling between the plant and the bacteria. To initiate the symbiosis, plants are known to produce compounds that will attract soil dwelling bacteria, including rhizobia, to their roots. Once in close contact to plant roots, other biochemical signals such as flavonoids (some example structures are in Figure 3) are produced by the plant to induce the expression of *nod* (nodulation) genes of the rhizobia located in the chromosomal DNA (Peters, Frost, & Long, 1986).
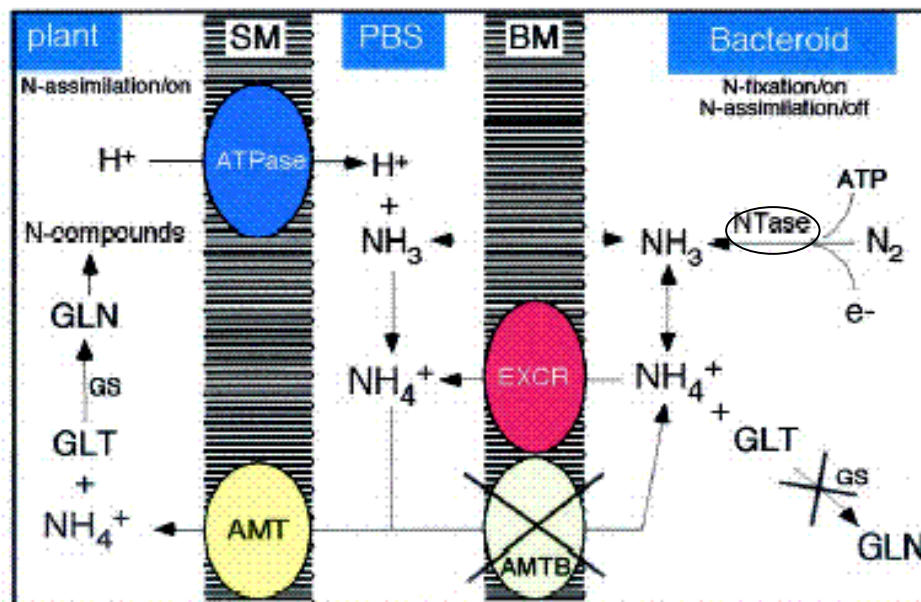
Figure 2: Interactions Between Rhizobia and the Host Plant During Nodulation. NH_3 produced by nitrogenase (Ntase) diffuses freely through the BT membrane (BM) that is then converted into NH_4^+, thatcannot be assimilated into glutamate (GLT) and glutamine (GLN) through glutamine

synthetase (GS) so it is excreted probably by an active mechanism (EXCR) into the peribacteroid space (PBS), where an ATPase present on the symbiosome membrane (SM) pumps protons and keeps a high NH_4^+/NH_3 ratio, and a transport system for NH_4^+ uptake (AMT) moves NH_4^+ to the plant cytoplasm, where it is assimilated. AMTB (NH_4^+ transporter) is not expressed by the bacteroid forcing the use of EXCR. (adapted from Patriarca, Tate, & Iaccarino, 2002).

When the biochemical signal is received by the bacteria, secretion of lipochitooligosaccharides (or nodulation factors, Nod factors) is thought to bind to specific plant receptor kinases. The nodulation factors secreted by the bacteria are host plant specific and induce many changes in plant cells and tissues. The changes include formation and deformation of root hairs, intra- and extracellular alkalinization, membrane potential depolarization, changes in ion fluxes, early nodulin gene expression, and the formation of the nodule primordia (D'Haeze & Holsters, 2002). Nodulins are molecules
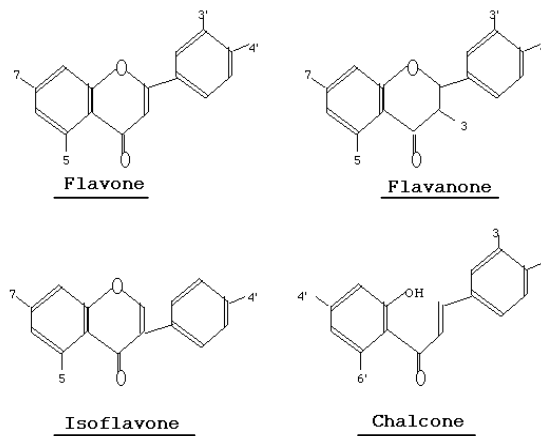
Figure 3: Structures of plant produced flavonoids (adapted from Watanabe, 2000).

involved in nodulation and primordia is a term used to explain the first developmental stage of a organ or tissue. For more detail on nod factors (structures and functions), see

the review of D'Haeze and Holsters from *Glycobiology* in 2002. The factors are effective

at even nano- to picomolar concentrations (D'Haeze & Holsters) and initiate a complex

signaling pathway involving calcium spiking in root hairs (Mitra et al., 2004). The end

result of this pathway is the curling of root hairs thus trapping the rhizobia (formation is

called a shepherd's crook). The bacteria enter the root hair through tubular structures

known as inward growing infection threads formed by the plant. The invading bacteria

are internalized into the plant cell lumen by endocytosis. The newly formed structure,

consists of bacteria differentiating into bacteroids enclosed in a plant cell membrane, is

called a symbiosome. The infection threads finally grow into the developing nodule

tissue (Gage, 2004). Development of the nodule and interaction of the plant and bacteria

can be seen in Figure 4. Figure 5 provides pictures showing nodules formed in the roots

of leguminous plants.

Once the bacteria are enclosed in the plant tissue, many metabolic changes in both

organisms are seen (Mylona, Pawlowski, & Bisseling, 1995; Zahran, 1999). Bacteriods

are less metabolically active as compared to free-living rhizobia. Research performed on

mature bacteroids compared with free-living cultures show that transcription of 342

genes was upregulated and transcription of 640 genes was downregulated (Becker et al.,

2004). Many of the downregulated genes, including the genes essential for purine

biosynthesis, DNA metabolism, and the two main sigma factors *rpoE1* and *sigA,* are

explained by the physiological state of the bacteroids that are not actively replicating

(Prell & Poole, 2006).
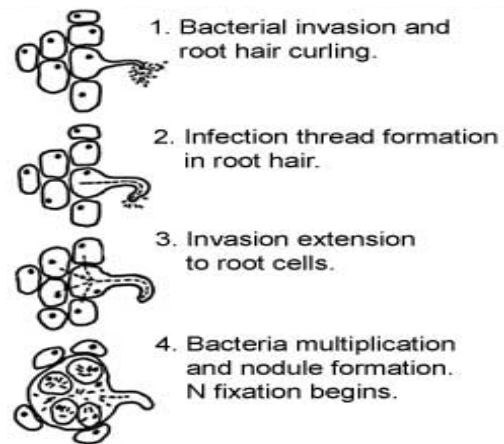
Figure 4: General schematic of nodulation of root hairs by rhizobia (adapted from Pennsylvania State University, 1986).
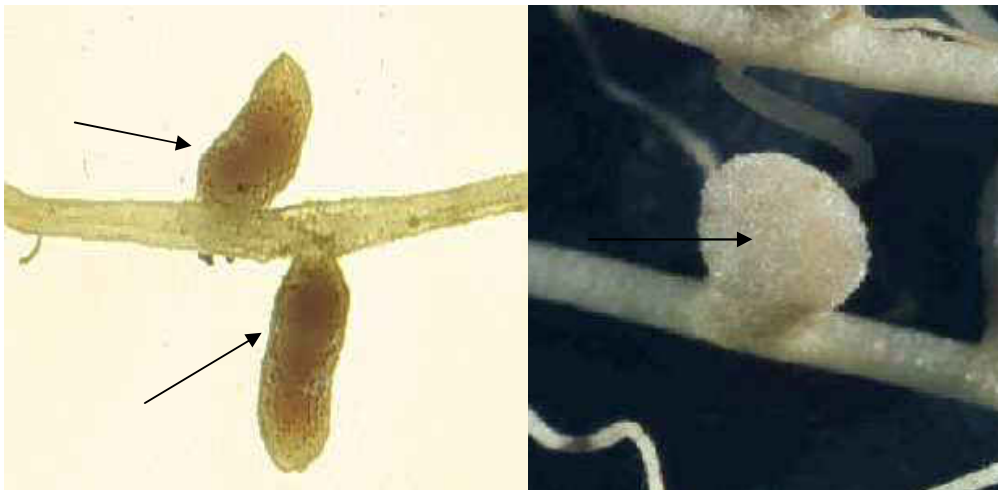
Figure 5: Nodules formed in the roots of leguminous plants (adapted from European Association for Grain Legume Research (AEP), 2008). Note the red color of the nodule indicating the presence of leghemoglobin and nitrogen fixation is occurring.

Some systems of nutrient acquisition by the invading bacteria are known, but many questions remain. The bacterial carbon storage compound polyhydroxybutyrate (PHB) accumulates in the rhizobial cells in infection threads (Lodwig et al., 2005). However, during the differentiation of bacteria to bacteroids in indeterminate nodules, the PHB granules are broken down. This fact provides an explanation of how the bacteria acquire an essential nutrient, carbon, by breaking down PHB aiding in growth in infection threads. Oxygen is another limited molecule in the nodule. Nitrogenase is oxygen labile; thus it must be enclosed in an anaerobic or microaerophilic environment. Leghemoglobin is a plant compound seen in the nodule to facilitate buffering of oxygen by binding oxygen and limiting its availability. Leghemoglobin is an iron rich protein and estimated by some to comprise up to 30% of the soluble protein in the nodule (Appleby, 1984). The reddish color seen in some nodules is due to an accumulation of leghemoglobin indicating nitrogen fixation is occurring; the red color of leghemoglobin is due to the large amount of iron present in the protein. Another nutrient in high demand in the nodule is iron. Iron is important in many redox reactions and is an important atom in the structure of many different proteins or enzymes located within the bacterial cell. Within the anaerobic nodules, ferrous iron has been suggested as the iron source for the bacteroids (Payne, 2004). Other requirements for iron and iron acquisition by bacteria are discussed in more detail in a later section.

Rhizopines are compounds (inositol derivatives) produced by bacteroids that are released from the root nodule and are catabolised to provide a source of carbon by free-living rhizobia of the same strain (Bahar et al., 1998). This could provide a competitive advantage for similar strains of bacteria that can use these compounds to nodulate the

same plant. When the bacteroids are enclosed in the nodule, they release ammonia for

the plant's consumption. The plants incorporate the ammonia into the amides (glutamine

and asparagine), into ureides, or the molecules directly enter into the Entner-Doudoroff

(ED) pathway, Pentose Phosphate Pathway (PPP), or tricarboxylic acid cycle (TCA) for

the majority of central metabolism. In turn, the plant provides carbon and energy sources

to the bacteroids in the form of dicarboxylic acids, particularly malate and succinate

(Lodwig & Poole, 2003) that feed directly into the TCA for the bacteria's central

metabolism. It has been determined that as the concentration of these dicarboxylic acids

decrease, nitrogen fixation is stimulated as the plant limits nutrients until more nitrogen is

fixed by the bacteria. Even at modest amounts the compounds are inhibitory on nitrogen

fixation (Lodwig & Poole). The controlled release of these compounds is due to the

biochemical interactions between the plant and the bacteria signaling their requirements

for nutrients. The nodulation process has been reviewed in detail by D' Haeze (2002),

Mylona et al. (1995), and Zahran, (1999).

Global and Agricultural Significance of Nitrogen Fixation

Nitrogen fixation is essential in many ways. The atmospheric content of

dinitrogen (N_2) is approximately 10^{15} tons and roughly 3×10^9 tons are converted by

nitrogen fixation back into a usable form for use in the global ecosystem, that is 60% of

production of fixed nitrogen from dinitrogen (Postgate, 1982). The vast majority of the

atmosphere is nitrogen (78%) that was released and lost from many different reactions

from the earth's surface. Global consumption or application of nitrogenous fertilizers is

on the rise, doubling in the weight sold during a 15-year period dating from 1973-1988

(Food and Agriculture Organization of the United Nations or FAO, 1990). With the world's increasing population, consumption of nitrogenous fertilizers is expected to increase even more than previously projected.

For approximately 100 years, biological nitrogen fixation has been exploited by agriculture (Burris, 1994). At the present, more experimental and practical manipulation of nitrogen fixation by bacteria is being examined for their possible use as a source of nitrogen for crops. The increased use of bacteria producing nitrogen for crops could help many ecosystems by decreasing the amount of nitrogenous fertilizers used. It could reduce the use of fossil fuels, help in reforestation, and restoration of nutrient-stripped lands (Burris, 1994; Sprent & Sprent, 1990). The reason nitrogen fixation should be investigated and applied is due to its relatively benign presence. In comparison, nitrogenous fertilizers can lead to unacceptable water pollution by increasing the amount of toxic nitrates located within bodies of water. An increase in nitrogen and other nutrients contained within the fertilizer load can lead to eutrophication of lakes and rivers (Al-Sherif, 1998). Eutrophication is defined as a decrease in the productivity of an ecosystem (usually aquatic) due to an increase in chemical compounds containing nitrogen and phosphorus entering the ecosystem. The increase of these chemicals leads to an increase in plant growth and decay plus an increase in plankton growth that exhausts the oxygen supply in the water. The removal of oxygen results in the death of many fish and other animals. Also, the bacterial amount introduced can be "tailor" made or designed specifically for the species of agriculturally important plants growing while most of the applied nitrogenous fertilizer (up to 50%) ends up being leached in the soil (Sprent & Sprent, 1990). While the position of farmers is to produce the highest yield of

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crop possible, they end up wasting money, energy, and time by over using nitrogenous fertilizers that in turn lead to the destruction of habitats and contamination of drinking water.

With all of these aspects in consideration, many different studies have focused on the ability of nodulated plants to persist in areas of harsh conditions as compared to uninoculated plants. Harsh conditions are identified as being high temperature (low moisture), limited nutrients, high salt and osmotic pressure, and soil acidity or alkalinity (Zahran, 1999). According to much published data, the nodulating bacteria are versatile while the plants are fastidious. The studies show the plants are attenuated or killed by these harsh environmental parameters while the bacteria continue to grow. The studies also suggest plants can be crossbred for resistance or the ability to grow under these harsh conditions (Zahran). If the plants can grow normally (or close to normal), the bacteria can nodulate and aid in the plants' survival. This could be a great asset for many countries considering how many have one or more of these harsh conditions present at different locations. If this symbiotic fertilization system can be manipulated to increase crop production, food could be made available to many citizens of undeveloped nations as well as developed ones whose climate or environment limits crop cultivation.

Iron and Bacteria

Iron is the fourth most abundant metal ion on the earth's surface. Iron is a vital element for nearly all organisms, eukaryotic and prokaryotic, with only a few exceptions in the genera of *Lactobacillus* and *Streptococcus* (Archibald, 1983; Guiseppi, 1982). But

a problem arises at physiological pH (neutral) and aerobic conditions, ferric iron (Fe^{3+})

ions form insoluble oxyhydroxide polymers. In this state, iron is unusable for any

organism. The maximum amount of soluble ferric iron in the environment is around 10^{-18}

M while most bacteria keep an internal concentration of iron at 10^{-6} M (Raymond, Dertz,

& Kim, 2003). For pathogenic organisms, the iron concentration inside their mammalian

host is lowered to 10^{-24} M due to host iron binding proteins (Raymond et al.). The war

between the host's defense system and the invading pathogen illustrates the importance

of iron in the onset of disease (discussed more in a later section). Most bacteria and fungi

that grow in aerobic conditions at approximately neutral pH are known to produce

siderophores that can solubilize the oxyhydroxide polymers. The organisms produce

these molecules under iron limiting conditions and the molecules serve as iron scavengers

for the cells.

Other sources of iron for bacteria whether internally (in a host) or externally (in a

free-dwelling environment) includes ferrous iron, heme, and host proteins like transferrin

and lactoferrin. Ferrous ions (Fe^{2+}) are plentiful in a reduced or anaerobic environment.

This form of iron can diffuse across the outer membrane and can be directly used for the

production of iron containing proteins by bacteria or oxidized and stored as ferric iron.

But ferrous ions can also be harmful (see Fenton reaction below). Once ferrous ions

enter the outer membrane via simple diffusion, an active transporter is required to

facilitate entry through the cytoplasmic membrane. Some bacteria can actually produce

extracellular ferric reductases that reduce Fe^{3+} to Fe^{2+} and internalize this form by the

above mechanism. For methods on iron acquisition from sources containing heme

groups and host proteins see the section "Uses of Iron and Means of Iron Acquisition in Bacteria" below.

Excess iron in the body of humans is toxic due to the formation of oxidizing radicals. More recent research has discussed the role of iron in degenerative diseases such as cancer, arthritis, and circulatory disease (Neilands, 1993) and as a contributing factor to diseases like Alzheimer's, Parkinson's, amyotrophic lateral sclerosis (ALS) (Kaur et al., 2003; Youdim & Buccafesco, 2005; Zecca, Youdim, Riederer, Connor, & Crichton, 2004; Zheng, Gal, Fridkin, Weiner, & Youdim, 2005). As previously mentioned iron, when oxidized, is insoluble but in its reduced form is highly toxic. The reduced form's toxicity is due to the formation of hydroxyl radicals by the Fenton reaction. The Fenton reaction is as shown:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH$$

The Fenton reaction products are deleterious for many macromolecules such as proteins, DNA, and cellular membranes.

Requirements for Iron and Iron Acquisition by Bacteria

As discussed previously, iron homeostasis is very important for most organisms. Excess iron is toxic and too little prevents growth, so it is vital for cells to maintain a constant concentration. Excess is stored in a nonreactive form (ferritin). Bacteria store iron in a molecule called bacterioferritin. Bacterioferritin is a protein with 24 subunits that assemble to form a hollow ball-like complex. Oxygen atoms bind the iron that is then reoxygenized to di-ferric iron (insoluble). The insoluble form is then stored in the hollow center of the complex where approximately 5000 iron atoms can be stored.

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Iron is important for cells to serve as a cofactor for many different enzymes and is required for many redox reactions within the cell. These enzymes are central to many different functions including: electron transport for energy production via aerobic respiration, activation of oxygen, peroxide reduction, amino acid synthesis, nucleotide synthesis, DNA synthesis, and photosynthesis (Wandersmann & Delepelaire, 2004). Additionally, some of the signaling factors in nodule development and nitrogenase contain iron (Loh & Stacey, 2003). In some bacteroids, it is estimated that nitrogenase comprises 10%-12% of the total protein inside the cell (Verma & Long, 1983). Leghemoglobin is an iron rich plant protein that buffers oxygen levels to the bacteroids within the nodule and is estimated by some to comprise up to 30% of the soluble protein in the nodule (Appleby, 1984). The protein is postulated to serve as an iron source if necessary. Several other regulatory proteins and molecules inside the cell also contain iron. In total, there are more than 100 enzymes catalyzing reactions in primary and secondary metabolism that use iron as a cofactor (iron-sulfur clusters or heme groups) (Miethke & Marahiel, 2007). Noting how many molecules of iron are necessary for growth, it is essential for the cell to acquire iron and it is known that iron acquisition increases the virulence of pathogenic organisms (Miethke & Marahiel).

To acquire iron from the environment, many bacteria and fungi produce small organic molecules known as siderophores. Siderophore mediated iron transport is the topic of this research. As mentioned before, when a pathogenic organism invades a mammalian host some of the initial immune response is aimed at curbing the bacteria's ability to acquire iron. Two of these proteins, lactoferrin and transferrin, are released into the circulation to chelate any free iron to prevent acquisition by the pathogen.

Lactoferrin and transferrin have two sites for binding iron and deliver the atom to host cells through receptor mediated endocytosis (especially transferrin). Lactoferrin is mainly found in breast milk as well as other bodily fluids but the two proteins are similar in structure and function. Other hemoproteins, like hemoglobin, contain about two thirds of the body's available iron in a heme bound state (Miethke & Marahiel, 2007). The host defenses can counter siderophore production by some pathogens for example lipocalin 2. Lipocalin 2 is a host protein that chelates iron bound siderophore (an example is enterobactin produced by *E.coli*) and prevent its return to the pathogen. Without this iron acquisition system, the bacterial growth and the pathogenesis of this organism is limited (Flo et al., 2004). Additional results have shown lipocalin 2 can bind carboxymycobactins that could be helpful in prevention of disease against mycobacterial pathogens like *Mycobacterium tuberculosis*. With these defenses present, iron concentration in the mammalian host is limited to approximately 10^{-24}M (Raymond, Dertz, & Kim, 2003).

There are two main divisions to describe how an organism can acquire iron from the host, direct and indirect. Direct iron acquisition is seen when bacteria directly uptake the iron sequestering molecules or hemoproteins like transferrin, lactoferrin, and heme (Wandersmann & Delepelaire, 2004). The disadvantage of the direct method is that the bacteria must contain a specific receptor to internalize the specific iron containing molecules. One of the best studied species of bacteria that can use transferrin and lactoferrin directly by binding these proteins and removing the iron are the human pathogens, *Neisseria gonorrhoeae* and *Neisseria meningitides* (Guerinot, 1994).

Within the indirect method of iron acquisition, much diversity is seen. Some Gram negative organisms produce molecules called hemophores (Miethke & Marahiel, 2007; Wandersmann & Delepelaire, 2004). These molecules are produced to acquire iron from different heme sources. The iron is removed from the bound state of heme by various mechanisms. There are two systems well studied in this method of acquisition. The first of these systems is *hxu* of *Haemophilus influenzae* that uses a heme-loaded host protein hemopexin as an iron source (Cope, Yogev, Muller-Eberhard, & Hansen, 1995; Hanson, Pelzel, Latimer, Muller-Eberhard, & Hansen, 1992). This organism also has an outer membrane receptor protein for use of hemoglobin (Hickman, Morton, & Stull, 1993) in iron acquisition. Some other nonpathogenic organisms (examples include *Rhizobium*) are known to use heme compounds as sources for iron (Noya, Arias, & Fabiano, 1997). The second method is the *has* system that uses a hemophore to scavenge iron and is a more widely used system in Gram negative organisms such as *Escherichia coli* and *Serratia marcenes* for using heme for iron from many different sources (see Rossi et al., 2001 for more details on the *has* system). By far the most widely used system categorized as an indirect method for iron acquisition is based on a low molecular weight iron chelating compound termed siderophores. Siderophores scavenge the ferric ion from many different sources. Organisms produce ferric siderophore specific receptors for internalization once the ferric siderophore complex returns to the cell. With these facts known, siderophore iron acquisition is a much more versatile system for scavenging ferric ions.

<u>Introduction to Siderophores</u>

Siderophores are defined as low molecular weight (400-1500 Da) organic compounds secreted by bacteria, fungi, and plants in response to low iron conditions. Siderophores have extremely high affinity and specificity for ferric ions (Fe^{3+}). The compounds are classified on the basis of the functional groups used to bind iron. The chelators are classified into four main groups, the catecholates, hydroxamates, hydroxy acid, and mixed types. Catecholates are molecules with hydroxyl side groups of catechol rings that bind ferric ions (example is enterobactin produced by *Escherichia coli*). Hydroxamates are designed with n-hydroxy side groups with oxygen atoms that ligate to ferric ions (example is ferrichrome produced by *Ustilago maydis*). Hydroxy acid or phenolate based siderophores contain nitrogen atoms on thiazoline rings that chelate ferric ion (example is pyochelin produced by *Pseudomonas aeruginosa*). Mixed types contain some combination of the previously mentioned groups to chelate iron (example is mycobactin produced by *Mycobacterium tuberculosis*). Figure 6 illustrates the different functional groups of siderophores and also provides one or more examples of each. *E. coli*'s ability to chelate iron via an endogenously produced siderophore, enterobactin, is very well studied. Enterobactin is a model system for catecholate-type siderophores and is one of the strongest chelators known.
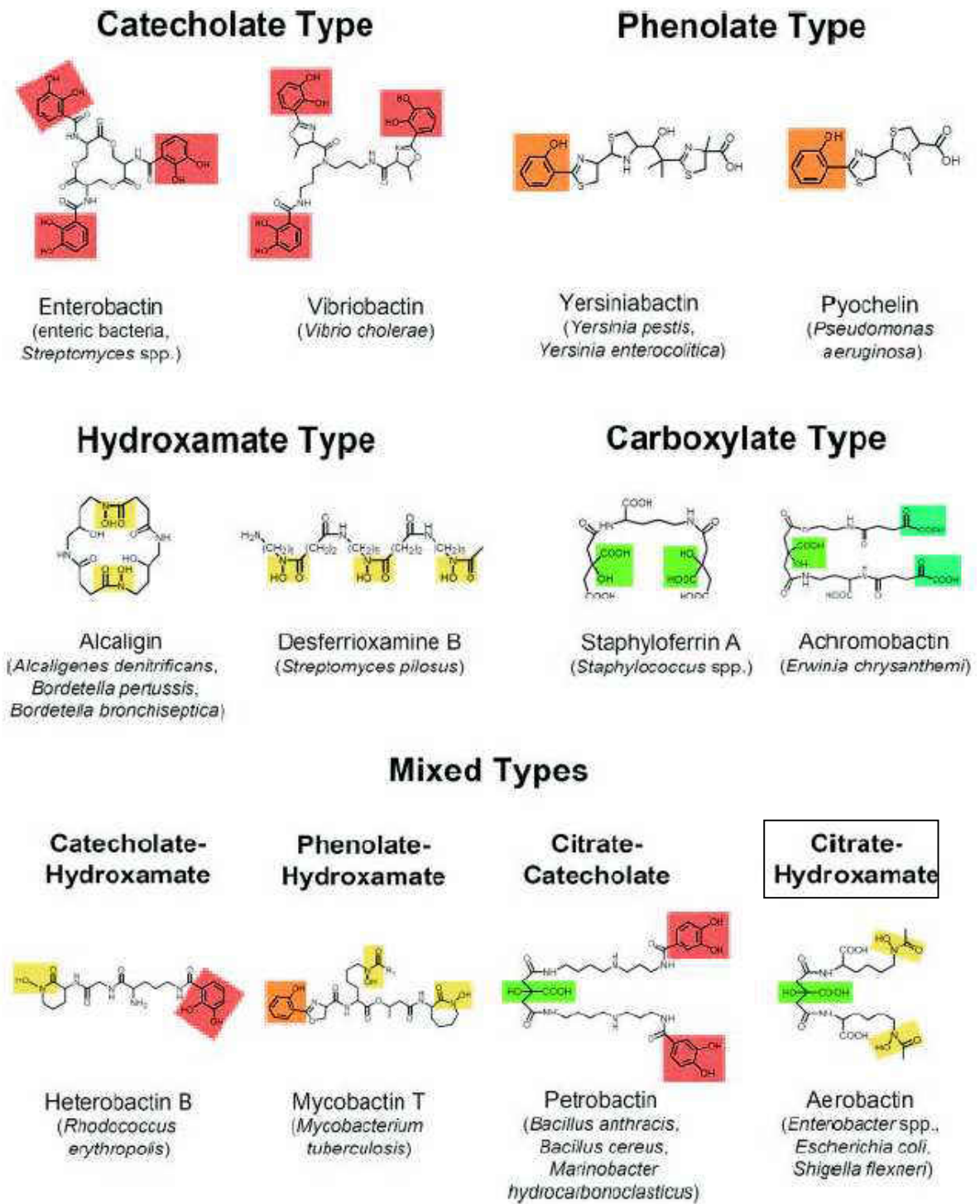
Figure 6: Structural comparison showing an example of siderophore(s) within each type based on their functional groups (adapted from Miethke & Marahiel, 2007).

Once the complex is internalized via a specific outer membrane receptor protein, it is postulated that cleavage of this complex by the bacteria is required to release the bound iron. This system relies on catalytic hydrolytic cleavage by enterobactin esterase of the ferric-enterobactin complex to release the iron intracellularly (Raymond, Dertz, & Kim, 2003). The second method to remove ferric iron bound to siderophore is by reducing it from Fe^{3+} to Fe^{2+} facilitated by ferric-chelate reductases located either intracellularly or extracellularly in a membrane-bound form. These enzymes reduce the siderophore-bound ferric ion to ferrous that is then released from the siderophore due to reduced binding affinity. The ferric reductase enzymes located in the periplasm and cytoplasm are similar in structure and function. The enzymes will use many different electron donors to reduce ferric to ferrous ion (example of electron donor is NADPH) and have a broad range of substrate specificity (Schroder, Johnson, & Vries, 2003).

Also several of these reductases are associated with the cytoplasmic membrane that can also facilitate internalization and uptake of iron. The extracellular reductases can be surface bound or secreted into the surrounding environment. These enzymes are known to reduce ferric ions from many different sources including ferritin. They resemble the periplasmic and cytoplasmic reductases by also having broad substrate specificity and require flavins for optimal activity (Schroder, Johnson, & Vries, 2003). Currently, all of the periplasmic and extracellular reductases are produced by opportunistic or obligate pathogens including *E.coli*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium* indicating this method of iron acquisition may be an important virulence factor (Barchini & Cowart, 1996). Membrane-bound ferric reductases are

found in *E.coli* and *Staphylococcus aureus* but no enzymes have been purified or characterized.

General Characteristics of Siderophores

As mentioned in the previous section, siderophores are extracellular, secreted organic molecules. Some siderophores, however, are attached to the cells and displayed to the external environment. Mycobactins are the best example of siderophores that remain tightly bound to the cell wall of Mycobacteria. Mycobactins are lipophilic molecules postulated to receive iron from other secreted siderophores, such as carboxymycobactins and exochelins, also produced by these bacteria. The transfer of the iron from exochelin or carboxymycobactin to the cell wall bound mycobactin is unknown. Aquatic organisms are known to produce membrane bound siderophores in response to the dilution of hydrophilic siderophores throughout their habitat. So to acquire iron, the aquatic bacteria surround themselves with membrane-bound siderophores with a range of hydrophobicity creating a siderophore gradient (Martinez et al., 2003).

Presently, there are more than 500 different siderophores known to be produced by bacteria and fungi. The genes for the biosynthesis and transport of the siderophore are inversely regulated by the availability of ferrous ions (mechanism discussed in a later section). Transport systems also exist for the use of exogenous siderophores. These systems allow an organism to use chelators produced by other organisms as well as their endogenous systems. *E.coli* is known to produce *fhuA,* a receptor for ferric ferrichrome complex, but itself does not produce this siderophore. Siderophores have very high

affinity and specificity for ferric iron because the molecules have oxygen atoms forming

coordination bonds with ferric ions contributing to the strength of the chelation. Some

examples of model siderophores are seen in Figure 7. Quantification methods exist to

evaluate the siderophore's ability to chelate ferric iron by enumeration of the stability

constant (K_s) ranging in values from 10^{22} to 10^{52} (Ratledge & Dover, 2000). The higher

the stability constant, the stronger the coordination bond between the siderophore's
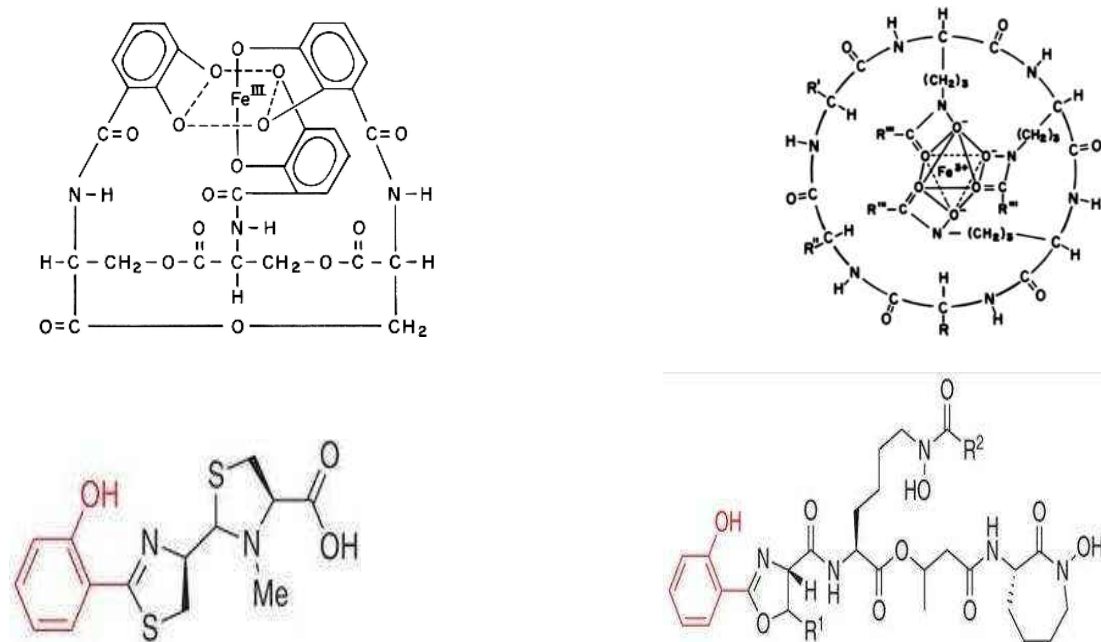
Figure 7: (Clockwise from top left) Structures of model siderophores: ferric-enterobactin

(adapted from Neilands, 1995), ferric-ferrichrome (adapted from Neilands, 1995), pyochelin

(adapted from Crosa & Walsh, 2002), and mycobactin (adapted from Crosa & Walsh, 2002).

oxygen atoms and the ferric ion. Once the siderophore binds iron, it returns to the cell for

internalization. After internalization, the ferric ion is reduced to the usable form of

ferrous iron or stored in the ferric form.

The size of the ferric siderophore complex is too large to enter the bacterial cell through nonspecific porins (less than 400 Da to enter through porins) thus requiring a specific, dedicated transport system. To internalize the complex, a system must be in place to provide energy and facilitate the entry into the cell for use. The most common system for the active transport of such molecules is the Ton system. The Ton system presumably provides electrochemical potential in the form of the proton motive force to provide the energy for the internalization process (Guerinot, 1994). The system is composed of three inner membrane proteins known as TonB, ExbB, and ExbD. TonB spans the entire periplasmic space and is anchored to the inner membrane by its amino terminal end. TonB interacts with the outer membrane ferric-siderophore receptor protein to provide energy and facilitate internalization (Letain & Postle, 1997). ExbB and ExbD are proteins thought to be involved in the confirmation change of TonB from its inactive form to its active form. The definite functions of ExbB and ExbD are unknown, but all three proteins (ExbB, ExbD, and TonB) are required for energy transduction (Ratledge & Dover, 2000).

Siderophores of *Escherichia coli*

Siderophore mediated iron transport systems have been studied the most in *E. coli*. There are two siderophores known to be produced by *E. coli*, enterobactin and aerobactin. The stability constants for enterobactin and aerobactin are 10^{52} and 10^{23}, respectively. The biosynthesis and transport operon of aerobactin has also been seen as a virulence determinant for some clinical isolates of *E. coli* (Bagg & Neilands, 1987). It is

32

also interesting to note that *E. coli* expresses many other receptors to transport exogenous

siderophores, as shown in Figure 8.
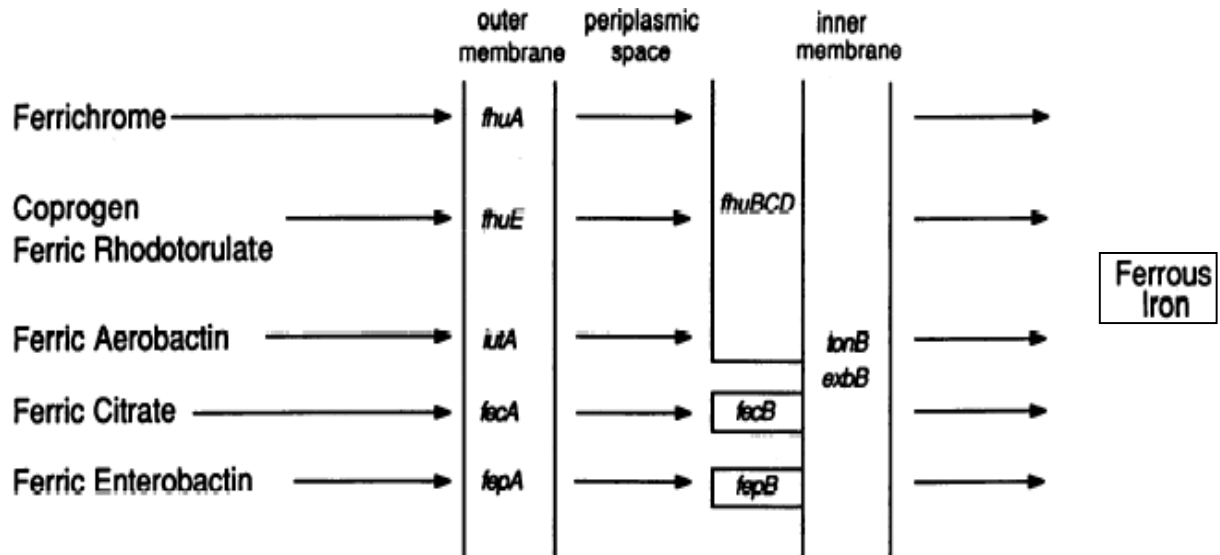
Figure 8: Siderophores used by *E. coli* (adapted from Bagg & Neiland, 1987).

Siderophores of the Rhizobia

Due to their genetic diversity, many different siderophores are known to be

produced by the rhizobia. At least one example of each functional group or class of

siderophore is produced by rhizobia. When metabolic changes occur in the bacteria

transforming into bacteroids, the expression of some siderophore systems were repressed,

and this had no effect on nitrogen fixation (Carter et al., 2002; Lynch et al., 2001; Stevens

et al., 1999; Yeoman et al., 2000). When siderophore acquisition systems are repressed in

bacteroids, iron must be attained by other means. As previously mentioned, ferrous iron

is available in the nodule, or some have suggested that there are bacteroid-specific

siderophores (Wittenberg, Wittenberg, Day, Udvardi, & Appleby, 1996) produced in the nodule.

The best studied siderophores in the rhizobia are rhizobactin 1021 and vicibactin (structures seen in Figure 9). Rhizobactin 1021 is a hydroxamate type siderophore produced by the bacterium *Sinorhizobium meliloti* 1021. Rhizobactin 1021 is similar in structure to other citrate based siderophores like aerobactin (made by some enteric bacteria) and schizokinen (produced by *Rhizobium leguminosarum* IARI 917 and *Bacillus megaterium*), but it is distinguishable due to the addition of a fatty acid or lipid group (Johnston, 2004). The fatty acid group located on rhizobactin 1021 structure is postulated to induce micelle formation (Johnston). Micelles are structures that protect the siderophore from extracellular damage.
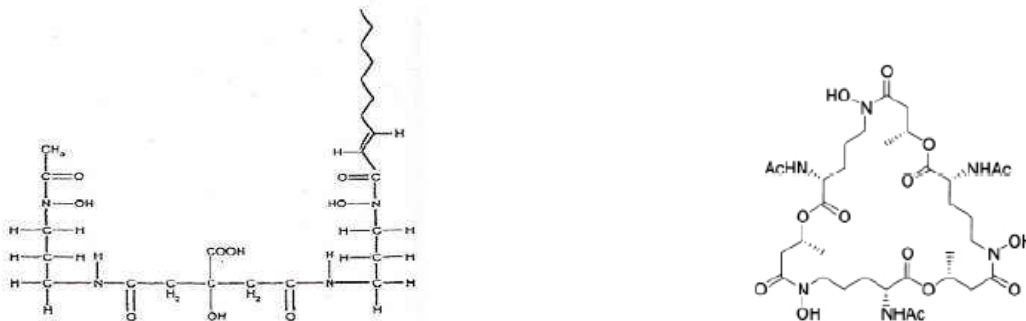
Figure 9: Structures of two siderophores seen in rhizobia. On the right is vicibactin (adapted from Carter et al., 2002) and on the left is rhizobactin 1021 (adapted from Storey, 2005).

Vicibactin is a trihydroxamate type siderophore produced by some strains of *Rhizobium leguminosarum biovar viciae*. The siderophore is a cyclic molecule comprising three groups each of D-3-hydroxybutyric acid and N^2-acetyl-N^5-hydroxy-D-ornithine linked by alternating amide and ester bonds.

Siderophores as Drug Delivery Agents or Drug Targets

      Siderophore-antibiotic conjugates are natural or synthetic siderophores conjugated with established antibiotics for specific delivery to the bacterial pathogen. The introduced complex of siderophore-antibiotic will chelate iron and return to the bacterial cell with both iron and the antibiotic. Once the complex enters the cell, the antibiotic can reach its target without being pumped out thus killing the cell or arresting the cell's growth. Figure 10 below shows some examples of how an antibiotic is conjugated to siderophore and the resulting structure that would be used in treatment. Because siderophore transport systems are high affinity, there is a faster accumulation of antibiotic in the cells that could reduce the amount of antibiotic required for effective treatments. Tests using many bacteria, including *E.coli* (Miethke & Marahiel, 2007) and *Pseudomonas aeruginosa* (Budzikiewicz, 2001)*,* have been successful in *in vitro* studies but require more rigorous testing before use in humans is possible.

      Another option is to design drugs to attack siderophore systems by disrupting or halting the siderophore biosynthetic pathways or the transport mechanism. Iron acquisition is essential for some pathogens to establish disease in humans and can increase the virulence of others. An example is *Mycobacterium tuberculosis* that has been shown to have decreased virulence and decreased ability to grow within macrophages when starved for iron (Cronje & Bornman, 2005).   Other examples of iron removal as therapy for important, deadly transmissible diseases include *Mycobacterium avium* (Gomes, Dom, Pedrosa, Boelaert, & Appelberg, 1999)*, Plasmodium falciparum* (Mabeza, Loyevsky, Gordeuk, & Weiss, 1999)*,* hepatitis C (Sartori, Andorno, Rigamonti, & Boldorini, 2001), and human immunodeficiency virus (HIV) (Georgiou et al., 2000).

The treatments include synthetic or semisynthetic molecules to scavenge any available iron from the body preventing acquisition by the pathogen. Once the complex is bound, it is filtered out of the blood by the kidneys, thus limiting iron more with the addition of the chelator along with the host's immune defenses.

The use of iron chelators is not reserved to the treatment of infectious disease. It is also being tested for the ability to treat many other noninfectious diseases. Currently, there is a synthetic iron chelator used in the treatment of hemochromatosis called Desferal®.
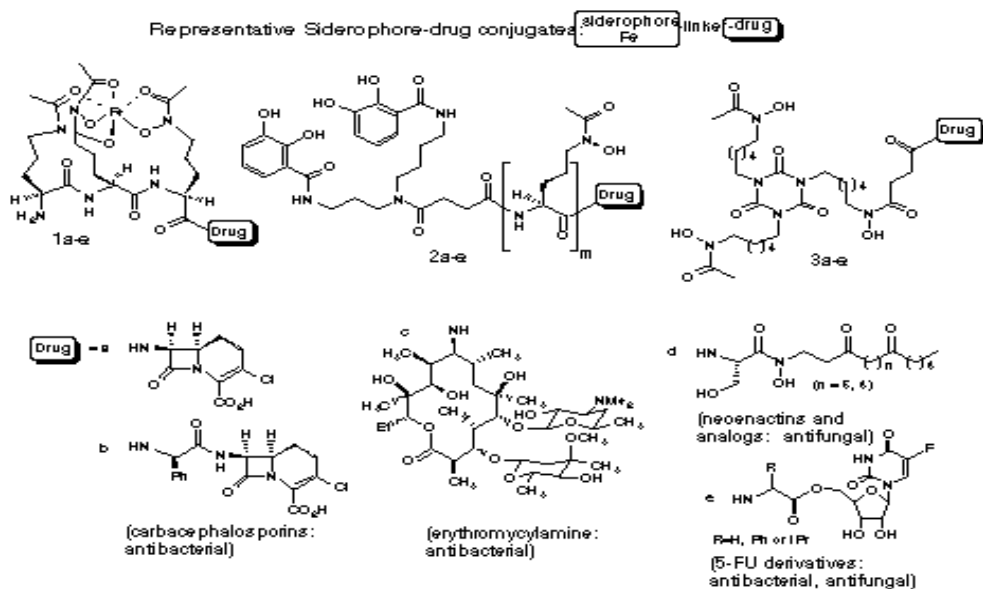
Figure 10: Structures of siderophores complexed with antibiotics for a more efficient delivery mechanism (adapted from Miller, 1995).

Desferal® is a synthetic iron chelator with a mechanism of action similar to a siderophore. Hemochromatosis is defined as an overload of iron in the blood due to excessive amounts absorbed in the body. The disease can cause many different symptoms including

impaired immune system functioning (especially macrophages), increased pathogenesis of many organisms, free radical tissue damage, organ failure, cirrhosis of the liver or liver cancer, diabetes mellitus, arthritis, and hypothyroidism (Berkow, Beers, & Fletcher, 1997). Until now, the only treatment for the disease was bloodletting. Desferal®, produced and sold by Novartis pharmaceuticals, is a synthetic iron chelator that can bind free iron. The chelator binds to free iron with the complex being filtered out of the blood by the kidneys and excreted in the urine. Other compounds used for similar treatments are summarized in Figure 11 and Figure 12. These molecules have one trait in common with siderophores in that all form stable molecules after binding to ferric iron.

Other ailments associated with increased levels of iron are neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis (ALS), multiple sclerosis, and Huntington's disease (Kaur et al., 2003; Zheng et al., 2005). These diseases are shown in new experimental data to respond to iron chelation. Two of these diseases examined in a mouse model used iron chelation as treatment are Parkinson's and Alzheimer's diseases. Results of mouse model studies have shown to delay or to prevent these diseases with iron chelation treatment (Kaur et al.; Zheng et al.). Excessive iron accumulation is seen in the brains of patients with these diseases and can lead to some of their classical symptoms. Excess of iron in the brain can lead to oxidative stress and free hydroxyl radical formation, both of damage proteins, nucleic acids, and membrane phospholipids, thus leading to cell death. When cells located in the brain are destroyed, many symptoms of the respective diseases are seen including involuntary movement, sluggish muscle movement, delayed cell signaling in the brain (affects memory and movement), and muscle rigidity. The research is mainly focusing

on chelators like ferritin and cliquinol to bind and convert the free iron into a nonreactive form (Fe^{3+}), thus preventing free radical formation without interfering with normal iron levels. Some promising results have been noted that administration of cliquinol prevents plaque formation and behavioral declines seen in Alzheimer's disease. The treatments were in a mouse model and did not disrupt normal iron levels (Cherny et al., 2001). Also, the use of cliquinol can slow or prevent the onset of Parkinson's disease by halting the free radical damage seen in patients' brains without interfering with the normal functions of iron in the central nervous system (Kaur et al.).
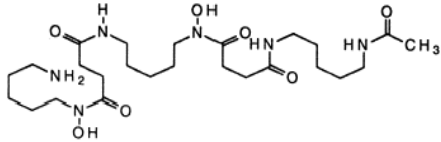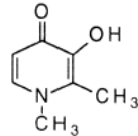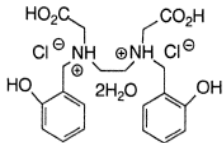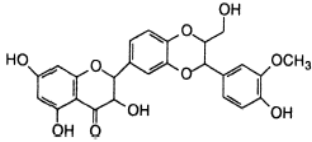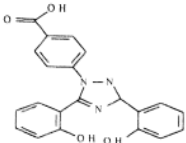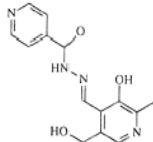
Chelator	Chelator capacity	Affinity constant	Pharmacokinetics	Membrane permeability	Application
Desferrioxamine (*DFO/desferal*)	High affinity for Fe^{3+} Forms high-stability iron-chelator complex in 1:1 ratio	10^{31} 100 mg/kg dose	Intravenous infusion: 100 mg/kg dose Peak plasma concentration: 80–130 μM Terminal half life: 3 h Subcutaneous infusion: 40 mg/kg dose Peak plasma concentration: 10 μM within 4–8 h Terminal half life: 9.8 h	Minimal and slow	Thalassaemia Sickle cell disease Malaria
Deferiprone (L1)	Chelates Fe^{3+} in 3:1 ratio Forms less stable iron-chelator complex	NA	Dose: 50 mg/kg Appear in plasma 5–10 min after ingestion Peak plasma concentration: 300 μM after 1 h Elimination half-life: 1.52 h	More easily than DFO, readily absorbed in gut	Malaria HIV infection
HBED (N,N-bis[2-hydroxy-benzyl]ethylenediamine-N,N-diacetic acid)	Forms 1:1 complex with Fe^{3+}	NA	NA	Permeates membranes when encapsulated	Thalassaemia In vitro *M. avium* infection
Silybin (3,5,7-trihydoxy-2-[3-(4-hydroxy-3-methoxy-phenil) 2-hydroxymethyl-1,4-benzodioxan-6-yl]chronan-4-one)	Chelates Fe^{3+} in 1:1 ratio	NA	NA	Carrier-mediated membrane permeable	Extrahepatic biliary obstruction Fe overload in rats
ICL670A (4-[3,5-bis(hydroxy-phenyl)-1,2,4-triazol-1-yl]-benzoic acid)	Chelates Fe^{3+} in 2:1 ratio	NA	NA	Permeates membranes	Thalassaemia (phase I trial) Malaria (in vitro)
PIH (pyridoxal isonicotinoyl hydrazone)	Chelates Fe^{2+}, quickly autoxidised to Fe^{3+}	10^{28}	NA	NA	None

Figure 11: Iron chelators used as therapy for iron overload and other diseases (Figure modified from Cronje & Bornman, 2005).

Natural compounds:
- albomycin
- ferrimycins
- danomycins
- salmycins
- microcins

Synthetic compounds:
- β-lactame conjugates
- vancomycin conjugates
- norfloxacin conjugates
- spiramycin conjugates
- erythromycylamine conjugates

anti-bacterial

- neoenactin conjugates
- nucleoside analog conjugates

anti-fungal

"Trojan Horse" antibiotics (siderophore-antibiotic conjugates)

Microbial defense/ Infection treatment

Iron overload therapy
- deferitrin
- deferiprone
- deferasirox

• DFO
-anticancer
-anti iron overload
-antimalarial

Cancer therapy
- dexrazoxane
- O-Trensox
- desferriexochelins
- desferrithiocin
- PIHs
- tachpyridine

Figure 12: Diagram of the possible roles for treatment using siderophores or siderophore-like compounds (adapted by Miethke & Marahiel, 2007). DFO stands for desferrioxamine thatis an iron chelating molecule.

General Genetic Regulation of Siderophore Systems

As previously mentioned, iron homeostasis is very important in many organisms. In environments replete with iron, siderophore systems are not required to obtain the metal thus must be repressed. The repression of these systems takes place at a

transcriptional level and prevents the loss of energy and resources used to acquire iron. When the environment is depleted of iron, siderophore systems are required and are transcribed. Regulation of both the biosynthesis and transport genes of siderophore systems can be controlled at a local level or at a global level. Local level is a term used to describe regulation that controls transcription of a few genes. While global level transcription describes regulation of many different genes simultaneously in response to environmental stimuli. Siderophore systems can be controlled at a local or global level or both. Sigma (σ) factors are a subunit of RNA polymerase that will bind to promoter regions and engage the enzyme for initiation of transcription. Sigma factors can regulate siderophore systems at a local level by turning on or off the genes involved. Other mechanisms, such as ferric uptake regulator (Fur), RirA, and DtxR, regulate genes involved in siderophore biosynthesis and transport on a global scale.

Ferric uptake regulator or Fur is a major global controller of iron response genes in many Gram negative bacteria. Fur is a negative regulator of the iron response genes involved in iron processing whether acquisition, use, or signaling. Fur becomes an active repressor when ferrous (Fe^{2+}) iron (acting as a corepressor) binds to the Fur sequence. When Fe^{2+} binds to Fur thus activating it, the complex binds tightly to a common highly conserved sequence of DNA called the Fur box (Guerinot, 1994) preventing transcription of the genes downstream usually involved in siderophore systems. When Fe^{2+} is not present or at low concentrations, Fur only binds weakly if at all to Fur boxes. If a weak or nonexistent interaction is present, genes for siderophore systems are transcribed because the repressor no longer binds to the Fur box (derepression). Fur regulation controls over 90 different genes involved in the iron response of *E. coli* (Hantke, 2001).

Genome analysis shows "binding boxes" are absent at the promoter regions of rhizobial iron regulated genes (Johnston, 2004). The Fur sequence is present and functional but is not the only regulator of these genes in the rhizobia (Rudolph, Hennecke, & Fischer, 2006; Wexler et al., 2003). In rhizobia, Fur also functions as a regulator for manganese (Mn^{2+}). With the genetic diversity seen in the rhizobia, it is not surprising that one regulation mechanism does not encompass the whole group.

Another mechanism of siderophore regulation seen in rhizobia is RirA. RirA has no similarity to Fur and may be confined to the rhizobia and a few close relatives (Johnston, 2004). The regulator is seen in *Mesorhizobium*, *Sinorhizobium*, and other relatives including *Brucella* and *Agrobacterium* (Johnston, 2004). In this method of regulation, there is no common, highly conserved region proximal to promoters suggesting that simple binding to a DNA sequence near iron-responsive promoters is not present. Modeling of the N-terminal end of the RirA protein predicted a similar structure to another iron response regulator, DtxR, but no sequence homology is present (Johnston, 2004). Data do suggest RirA is a DNA binding metalloprotein.

DtxR regulation mechanism is similar to Fur but the proteins are structurally distinct. A structural change in DtxR is induced when bound by Fe^{2+} activating the repressor thus blocking the genes involved in siderophore acquisition. When intracellular iron levels are low, the repressor will leave the promoter region thus facilitating the transcription of the siderophore genes. The major organism that uses this regulation is *Corynebacterium diphtheriae,* the causative agent of diphtheria.

Transport of the ferric-siderophore complex into the bacterial cell is a complicated process. Loops of the outer membrane receptor extend into the external environment and facilitate ferric-siderophore binding. When this occurs according to one hypothesis, the plug region in the center of the receptor protein (see Figure 14) undergoes conformational changes that allow the complex to enter the cell (Raymond, Dertz, & Kim, 2003) in an energy dependent manner facilitated by the TonB-ExbB-ExbD proteins. After the complex has entered the outer membrane, a common method of transport is seen. First, the complex enters the periplasm where a periplasmic binding protein binds to the substrate and transports it to the inner membrane via an ABC type transporter. When this interaction takes place, ATP hydrolysis of the ABC-type transporter occurs to facilitate entry into the cytoplasm (Raymond et al.). An example of enterobactin transport via membrane transport systems is seen in Figure 13 in *E. coli*.
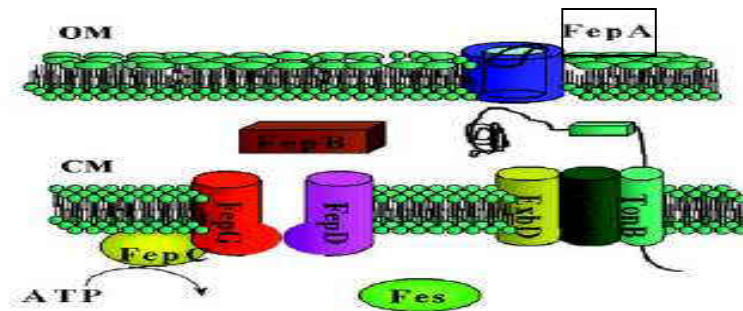
Figure 13: Diagram showing the interaction of enterobactin and its internalization mechanism from E. coli (adapted from Raymond et al.).
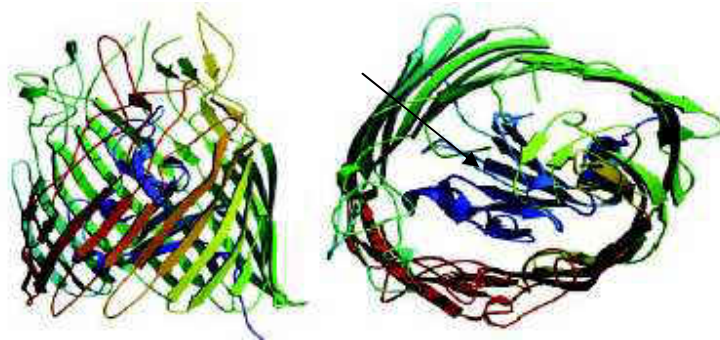
Figure 14: Crystal structure of FepA receptor (for enterobactin) with plug region intact (adapted from Raymond et al.).

Methods of Siderophore Biosynthesis

Most siderophores are synthesized by nonribosomal peptide synthetases but some are independent of this mechanism. Several siderophores are not polypeptides but contain alternating dicarboxylic acid and diamine or amino alcohol building blocks linked by amide or ester bonds (Challis, 2005). Two of the non-polypeptide siderophores are aerobactin and rhizobactin 1021. Both of these siderophores are structurally similar to schizokinen, a siderophore produced by *Rhizobium leguminosarum* IARI 917. Due to the structural similarities it is possible that they may also share similarities in their biosynthetic pathways.

Rhizobactin 1021 is a siderophore produced by the symbiont of alfalfa *Sinorhizobium meliloti* (Lynch et al., 2001). The genes involved in the biosynthesis of the siderophore are designated as *rhbABCDEF*, *rhrA* is assigned a role in regulation, and *rhtA* is assigned to transport (proposed pathway in Figure 15). The final structure of rhizobactin 1021 is identical to schizokinen except for the addition of a fatty acid chain.

Rhizobactin 1021 and its synthesis are shown in Figure 15.   The genes involved in aerobactin and rhizobactin 1021 synthesis are seen in Figure 17 for comparison purposes.

Aerobactin is a siderophore produced by some strains of *E. coli* and can be a virulence determinant (Neilands, 1995). There are five genes identified in the operon for aerobactin biosynthesis and transport designated as *iucABCD* and *iutA*. The genes designated *iucABCD* are involved in the biosynthesis of the siderophore and *iutA* gene encodes for the transporter protein (Challis, 2005). Aerobactin synthesis is also seen in many other organisms including the *Shigella* species and increases the virulence of these organisms. The genes and molecules involved in aerobactin synthesis are shown in Figure 16 to compare with rhizobactin 1021 synthesis shown in Figure 15.
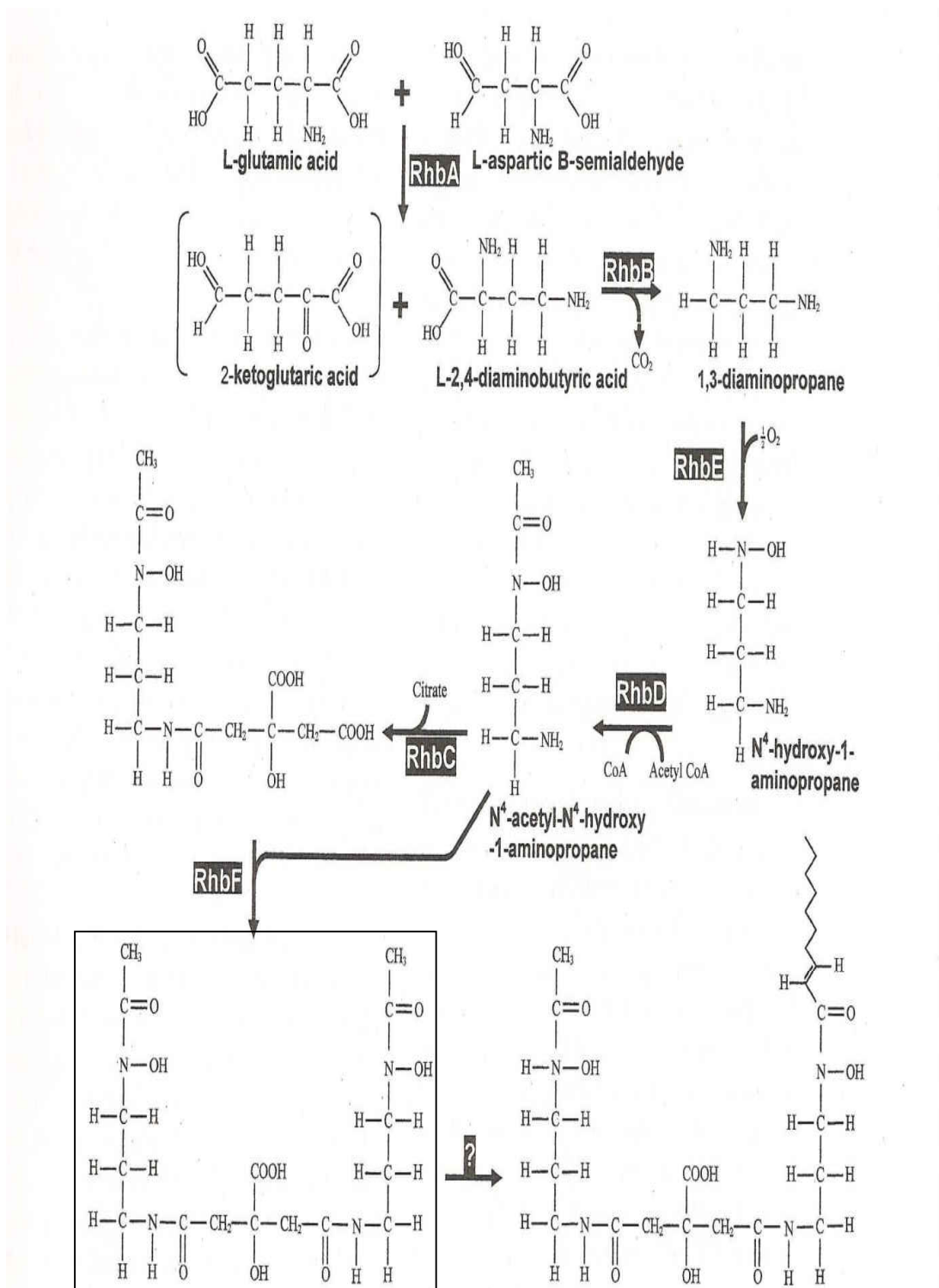
Figure 15: Proposed biosynthetic pathway for rhizobactin 1021 produced by *Sinorhizobium meliloti* (adapted from Lynch et al., 2001).
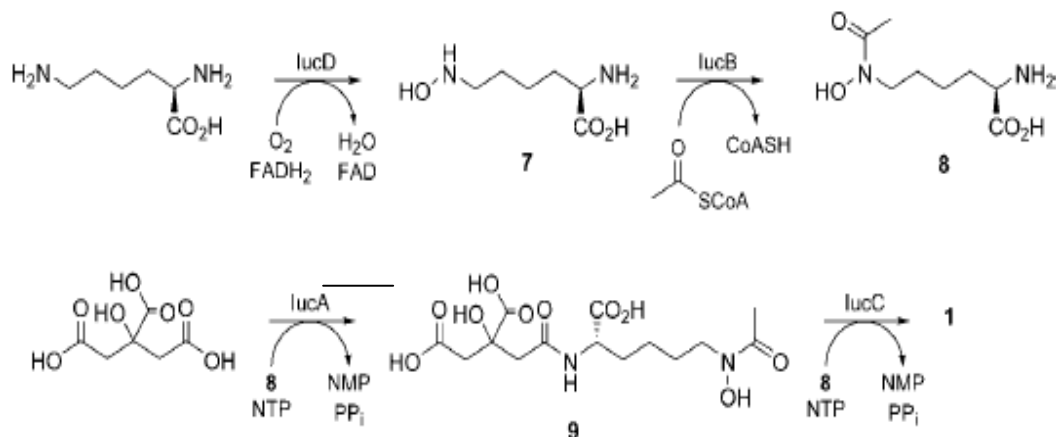
Figure 16: Proposed biosynthetic pathway of aerobactin (adapted from Challis, 2005).
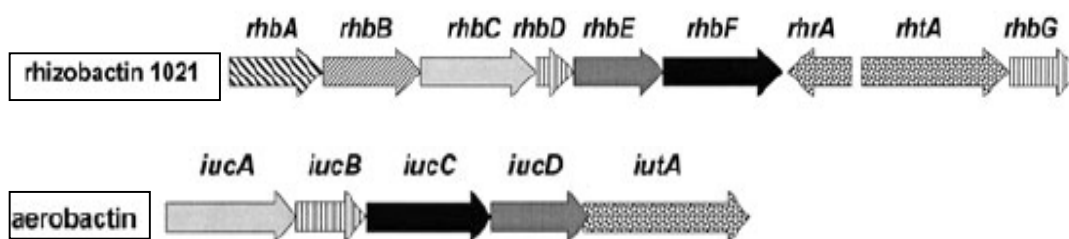
Figure 17: Genetic maps of rhizobactin 1021 and aerobactin synthesis, transport, and regulation (adapted from Challis, 2005). The shaded arrows denote similarity in functions of the gene products.

Rhizobactin 1021 and Schizokinen

      With the siderophore rhizobactin 1021 so structurally similar to schizokinen, it seems likely there would be some similarities in their biosynthetic pathways. The structure of the dihydroxamate type siderophore produced by *Rhizobium leguminosarum* IARI 917 was identified by Erin Storey in our lab in 2005 as schizokinen. Schizokinen is a siderophore produced by *Bacillus megaterium* (Mullis, Pollack, & Neilands, 1971). Given such striking structural similarities to rhizobactin 1021 (see Figure 18); the next

step is to determine if the biosynthetic pathway for the synthesis of schizokinen is similar to rhizobactin 1021 or if it is synthesized via a unique pathway.
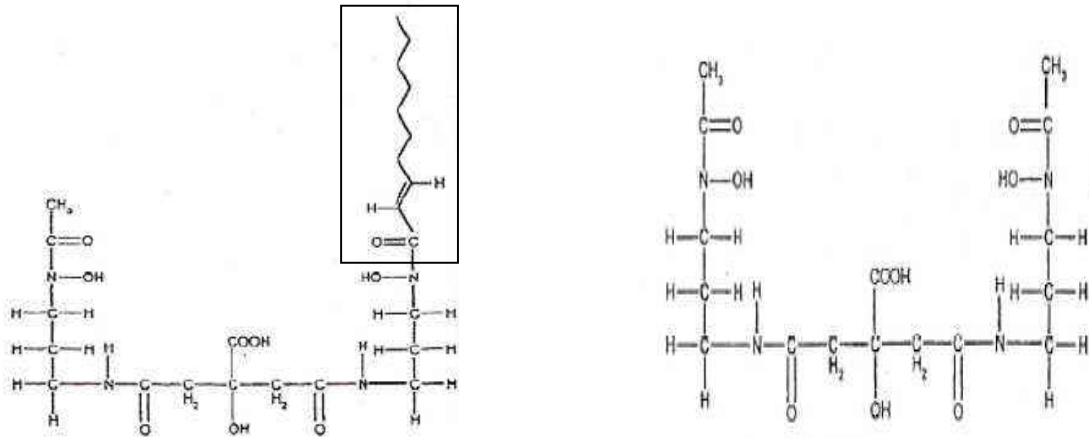
Figure 18: Structures of rhizobactin 1021 (left) and schizokinen (right) (adapted from Lynch et al., 2001; adapted from Storey, 2005).

Present Research

A previous graduate student, Erin Storey, isolated and characterized a siderophore produced by *Rhizobium leguminosarum* IARI 917 identified as schizokinen. As mentioned in the previous sections, this siderophore is not produced by any other known rhizobial species. The biosynthetic pathway or genes involved in the biosynthesis of schizokinen have not been determined. The biosynthetic operon for rhizobactin 1021 has been characterized with assignments given to the enzymes and to the genes involved in transport and could thus serve as a means of comparison between the two bacteria due to their structural similarities. Also, the kinetics of schizokinen system has not been previously reported with its ability to bind and transport iron into the bacterial cell. The present work was aimed at identifying the genes involved in siderophore production and transport of the siderophore, schizokinen, produced by *R. leguminosarum* IARI 917.

CHAPTER 2

DEVELOPMENT OF HYPOTHESES

Hypothesis 1

When the structure of the siderophore produced by *Rhizobium leguminosarum* IARI 917 was determined by Erin Storey, a strikingly similarity was seen. The siderophore produced, schizokinen, was structurally identical to another siderophore, rhizobactin 1021 produced by another member of the rhizobia group, *Sinorhizobium meliloti.* The only structural difference is the addition of a fatty acid chain to rhizobactin 1021. With the similarity seen between the two siderophores, a logical approach was to hypothesize the two siderophores may share a common genetic pathway for synthesizing the molecule. With this in mind, the first hypothesis for this research states that given the structural similarities between schizokinen and rhizobactin 1021, the two siderophores are synthesized via an identical biosynthetic pathway.

Hypothesis 2

Structural similarities between molecules do not always mean genetic similarities must be present for their biosynthesis. The two siderophores could be structurally similar without a genetic link. So the alternative hypothesis for this research is that schizokinen is synthesized by a unique or modified pathway as compared to rhizobactin 1021 despite their similar structures.

CHAPTER 3

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Rhizobium leguminosarum IARI 917 (IARI 917) was the bacterium used in this study. The culture was obtained from the Indian Agricultural Research Institute (IARI) in New Delhi, India. The organism was grown at 28-30°C for 24-48 hours. *E. coli* was grown at 37°C in Luria-Bertani (LB) broth. Other strains used in this study are listed in Table 1.

Table 1: Bacterial strains used in this study

Strain	Description	Reference or Source
E.coli strains		
C118λpir	pUT Tn5 LacZ1, Amp^R, Km^R	de Lorenzo (1990)
MM294 pRK2013	Helper of CC118 λpir, Neo^R	Alice (2005)
pPOC1	Rhizobactin 1021 regulon of *Sinorhizobium meliloti* 2011, pLAFR1 based cosmid, Tet ^{R}	Cuiv (2005)
pRK600	Helper of pPOC1, Cm^R	Cuiv (2005)
pRL27 (BW20767)^K	Hyperactive Tn5, Km^R	Welch (2005)
DH5αλpir$^+$	Insertional cloning vector	Welch (2005)
Rhizobium leguminosarum	IARI 917 wild type	Indian Agricultural Research Institute

Modified Fiss-Glucose Minimal Media

The minimal media, with no added iron, was a modified version of Fiss-glucose (Vellore, 2001) to induce siderophore production. The medium was standardized for optimal siderophore production by strain IARI 917 by Storey, Boghozian, Little, Lowman, and Chakraborty, in 2007. The medium was stored in acid treated bottles and ingredients were dissolved in deionized distilled water (ddH$_2$0) to prevent iron contamination. The culture flasks used for growth of the bacteria were also acid treated to prevent the introduction of iron. The optimized minimal medium composition was 2.5 g potassium phosphate (KH$_2$PO$_4$), 2.5 g L-asparagine, and 0.5 g of ammonium sulfate in 400 ml of ddH$_2$0. The pH of the solution was adjusted to 6.8 with 6 M NaOH. Once the pH was adjusted, the volume was adjusted by adding ddH$_2$0 to 485 ml. The medium was autoclaved and stored in an acid treated bottle. After autoclaving and cooling, more sterile ingredients were added including 10 µl of zinc sulfate (50 mg/ml), 10 µl of manganese chloride (25 mg/ml), 5 ml glucose (50%), 5 ml magnesium sulfate (0.4%), and 5 ml maltose (50%) were added to the medium (Storey, Boghozian, Little, Lowman, & Chakraborty, 2007). To make plates (except for Congo red, requires more agar), 1.5% of agar was added to the medium before autoclaving. After autoclaving and cooling, ~25 ml were dispensed into sterile Petri dishes and allowed to solidify.

MOPS Minimal Media

MOPS minimal media (lacking iron) was used for the binding and transport assays in *E. coli* KDF-541 (Neidhardt, Bloch, & Smith, 1974). The components of MOPS minimal media were prepared individually and filter sterilized. All of the stock

solutions were stored at 4°C. The final medium was made by combining individual sterile stock solutions in an acid treated bottle. The composition of the medium can be found in Neidhardt et al.

Luria-Bertani (LB) Complex Media

Luria-Bertani (LB) broth (Sambrook, Fritsch, & Maniatis, 1989) was a complex media used for optimal growth of the *E. coli* strains. All *E. coli* strains are grown at 37° C for 24 hours. LB broth contained 5.0 g of NaCl, 5.0 g tryptone, and 2.5 g yeast extract dissolved in 500 ml distilled water (dH_20). Once it was dissolved fully, the pH was adjusted to 7.5 before autoclaving.

Tryptone-Yeast (TY) Complex Media

Tryptone-Yeast (TY) broth was used to cultivate IARI 917 wild type and transconjugant strains (Beringer, 1974) for optimal growth. The composition of TY broth was 5 g tryptone, 3 g of yeast extract, and 0.9 g of calcium chloride dihydrate per liter of dH_20.

Congo Red Media

Congo red media was used for the maintenance of IARI 917 because the Rhizobia in general take up the dye (Kneen & Larue, 1983) weakly from the medium appearing as white, glistening colonies. Rhizobial colonies appear shiny due to the production of an exopolysaccharide layer. Because most other organisms readily absorb the dye, contaminant colonies appear red. The composition of Congo red agar was 4 g of

mannitol, 0.2 g of potassium phosphate (KH$_2$PO$_4$), 0.08 g magnesium sulfate heptahydrate, 0.04 g NaCl, and 0.4 g of yeast extract dissolved in 500 ml ddH$_2$0. The pH was adjusted to 6.8 and 1 ml of a 1% Congo red dye solution was added to the medium. Lastly, 12 g of agar were added to prepare plating media, then it was autoclaved.

Antibiotic Concentrations

Antibiotics, if required, were added to the media at the following concentrations unless otherwise indicated: kanamycin 50 µg/ml, carbenicillin or ampicillin 100 µg/ml, tetracycline 10 µg/ml, rifampicin 100 µg/ml, and chloramphenicol 20 µg/ml.

Glycerol Stock Cultures

The glycerol stocks were made with the addition of 0.2 ml of 75% glycerol to 2 ml vials and sterilized. Bacterial cultures were grown in complex media until the optical density at 600 nm (OD$_{600}$) wavelength equaled 0.5-0.8 indicating exponential growth. After this OD$_{600}$ was achieved, 0.8 ml of the culture was placed into the 2 ml vial that contained the sterile glycerol. The vial was vortexed to coat the bacterial cells with glycerol to prevent ice crystal rupture of the cells and stored at -80°C.

Seed Inoculum

The seed cultures were grown in complex media to obtain maximum growth. A seed inoculum of IARI 917 was grown on a rotary shaker for 18-20 hours at 30°C. The overnight culture, if used to seed a larger minimal media, must be washed to prevent residual iron from entering the restricted environment. To wash the cells, the seed culture

was placed into a 2 ml tube and centrifuged for 4 minutes at 13,000 rpm to harvest the cells. The supernatant was discarded and the pellet was resuspended in 2 ml of fresh iron free media. The wash step was repeated two additional times to ensure removal of residual iron. The pellet was resuspended a final time in a fresh 2 ml of iron-free Fiss-glucose media. For inoculating 50 ml of media, 0.5 ml of the resuspended cells was added (or 1% of total media volume). The 50 ml culture was grown with shaking for 15-18 hours at 30°C for IARI 917. Because *E.coli* grow faster, the 50 ml culture of iron restricted medium was only allowed to grow 3-4 hours on a rotary shaker at 37°C. The cultures were grown to an OD_{600} of 0.5-0.8 to obtain cultures in the exponential growth phase.

Chrom Azurol Sulphonate (CAS) Plate Assay

The chrom azurol sulphonate (CAS) assay, described by Schwynn and Neilands in 1987, is a generalized test used to detect the presence of siderophores with no discretion of the functional groups the molecule may contain. The assay is based on a complex formed when the chrom azurol sulphonate dye is chelated to ferric iron producing a dark blue color. When the organism produces siderophores, the siderophore strips the ferric ions from the CAS dye producing a color change from blue to orange. The CAS assay was preformed by first growing IARI 917 in low iron Fiss-glucose as previously described to induce siderophore production. The bacterial cells were harvested (centrifuged at 13,000 rpm for 4 minutes) and the supernatant was used for the siderophore assays. A #2 cork borer was flame sterilized and used to form wells in the CAS agar to that60 µl of culture supernatant was added. The reaction was allowed to

proceed at room temperature for 24 hours for maximum visualization of the color change. Another method used to determine siderophore production was inoculating the bacteria onto a CAS plate.

Preparation of CAS medium consists of two parts, beginning with the preparation of the indicator solution. The indicator solution for CAS plates was composed of two solutions, solution A and solution B. Solution A was made by dissolving 60.5 mg of chrom azurol S in 50 ml of ddH_2O. A 10 ml volume of iron III solution (27 mg iron chloride hexahydrate in 83.3 μl of 12 M HCl in 100 ml ddH_2O) was added to solution A producing a final volume of 60 ml. Next, solution B was made by dissolving 72.9 mg of hexadecyltrimethyl ammonium bromide (HDTMA) in 40ml of ddH_2O. After both solutions A and B were dissolved, solution B was slowly added to a stirring solution A to yield 100 ml of CAS indicator solution. The indicator solution was autoclaved and stored in an acid treated bottle.

The second part of composing CAS plates was compiling the basal agar medium. The medium was made by dissolving the following in 415 ml ddH_2O: 15 g of 3-(N-morpholino) propane sulfonic acid (MOPS), 0.25g of NaCl, 0.15 g potassium phosphate, 0.05 g of ammonium chloride, and 2.5 g of L-asparagine. Once the components were dissolved, the pH was adjusted to 6.8 with 5 M NaOH. The final volume was adjusted to 440 ml with the addition of ddH_2O. 7.5 g of agar was added to the medium then autoclaved with a stir bar left in the flask.

After sterilization of the indicator solution and the basal media, the solutions were cooled for 1-1.5 hours in a 50°C water bath. During the cooling process, a bottle containing 50% dextrose (filter sterilized) was also placed in the water bath to warm to

the same temperature. After the agar and indicator solution has cooled, the basal medium was placed on a stir plate. Ten ml of the 50% dextrose solution was added while the basal medium was slowly stirred to prevent bubble formation. After the addition of dextrose, 50 ml of CAS indicator solution was added down the glass walls of the flask at a rate of 0.5 ml per second to prevent precipitation of the dye. After the addition of dextrose and indicator solution, ~25 ml of the final CAS agar medium was dispensed into each sterile Petri dish and allowed to solidify.

Arnow's Assay for Catechol-Type Siderophores

Once the presence of siderophore was detected by the CAS assay, other more specific assays were performed to determine the molecule's functional group. One specific assay used was Arnow's assay (Arnow, 1936) that detects the presence of catechol functional groups. The assay is based on the reaction of catechol functional groups with nitrous acid yielding a yellow color. The yellow color produced becomes an intense red wine color in the presence of excessive amounts of sodium hydroxide (Arnow). The test is a colorimetric assay using culture supernatant from a low iron culture, thus the more intense the color formed the higher the concentration of the catechol functional group as compared to high iron culture supernatant.

The assay was performed by adding the following reagents in order: 1 ml of culture supernatant, 1 ml of 0.5 M HCl, 1 ml nitrite-molybdate reagent (made by dissolving 10 g sodium nitrite and 10 g sodium molybdate in 100 ml ddH2O), and 1ml of 1 M NaOH. The mixture was incubated at room temperature for 5 minutes to allow the reaction to proceed. The absorbance was taken using a spectrophotometer at 500 nm

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wavelength using uninoculated medium as a blank. Any OD_{500} reading above 0.03 for this assay was considered positive for the presence of catechol functional groups.

Atkin's Assay for Hydroxamate-Type Siderophores

Another specific test used to chemically characterize siderophores was the Atkin's assay to detect the presence of hydroxamate functional groups (Atkin, Neilands, & Phaff, 1970). Atkin's assay was performed by combining 0.5 ml of low iron culture supernatant with 2.5 ml Atkin's reagent (0.1771 g Fe $(ClO_4)_3$ in 100 ml ddH$_2$O and 1.43 ml perchloric acid). The blank used in this assay was uninoculated media replacing culture supernatant. After the reagents were mixed, the tube was incubated at room temperature for 5 minutes. Once the reaction had taken place, a pink color indicated a positive reaction for the presence of hydroxamate functional groups. Following the 5 minute incubation, the absorbance was measured at 480 nm wavelength in a spectrophotometer. An absorbance reading above 0.03 was considered a positive reaction for the presence of hydroxamate functional groups in the siderophore.

Methods for Testing Hypothesis 1

Isolation of Plasmid DNA

Plasmid DNA used in this study was isolated using the protocol provided by the QIAprep spin miniprep kit® from Qiagen® or the alkaline lysis method described by Sambrook, Fritsch, and Maniatis in 1989. The method was performed according to the manufacturer's protocol and the alkaline lysis (Sambrook et al.) method was modified as described below.

Extraction of plasmid DNA from *E. coli* harboring the pPOC1 plasmid began

with growing the bacteria in 2 ml of LB broth with appropriate antibiotic. After

overnight incubation, the 2 ml culture was harvested by centrifugation at 13,000 rpm for

4 minutes. The supernatant was discarded and the cells were resuspended in 0.5 ml of 50

mM Tris[hydroxymethyl]aminomethane adjusted to pH 7.8 with HCl (Tris-HCl),

centrifuged at 13,000 rpm for 4 minutes, and the supernatant was discarded. The cells

were resuspended in 100 μl of solution I (50 mM glucose, 10 mM

ethylenediaminetetraacetate or EDTA, 25 mM Tris-HCl, pH 8.0) containing 2 mg/ml of

fresh lysozyme and incubated at room temperature for 15 minutes. Upon completion of

this step, the solution became cloudy then 200 μl of freshly made solution II (0.2 M

NaOH, 1% sodium dodecyl sulfate or SDS) was added. The tube was mixed gently by

inverting with hands several times and incubated at room temperature for no more than 5

minutes. After this step, the solution became clear as lysis of the bacterial cell had

occurred. Following cell lysis, 150 μl of solution III (5 M potassium acetate, 17.4 M

acetate, pH 6.0) was added, mixed gently with hands several times, and incubated on ice

for 15 minutes. After incubation, the tube was centrifuged for 10 minutes at 13,000 rpm

to pellet the precipitates of proteins, chromosomal DNA, and other cell metabolites

released upon lysis leaving the plasmid DNA in the clear supernatant. Next, the clear

supernatant was transferred to a new 1.5 ml centrifuge tube and 1 ml of ice cold 100%

ethanol was added. The tube was mixed by inverting then incubated on ice for 5 minutes.

The mixture was centrifuged at 13,000 rpm for 10 minutes to pellet the DNA then all

ethanol was aspirated off using a Pasteur pipette. The DNA pellet was redissolved in 400

μl of solution IV (0.1 M sodium acetate, 50 mM Tris-HCl, pH 7.5) and vortexed until

completely dissolved. Once the pellet was dissolved, 1 ml of ice cold 100% ethanol was added, mixed by inverting, and incubated on ice for 5 minutes. The tubes were centrifuged for 10 minutes at 13,000 rpm and all ethanol was aspirated off. The tubes were air dried for 5 minutes and the DNA pellet was dissolved in 30 μl of 0.1x TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) with RNase (20 mg/ml). Plasmid DNA was stored at -20°C.

Isolation of Genomic DNA

Two methods were used to isolate genomic DNA from IARI 917 that were the alkaline lysis method (Sambrook et al., 1989) and QIAamp DNA mini kit® from Qiagen®. The SDS lysis method was performed according to Sambrook et al. with some modifications to the protocol as described below. The QIAamp DNA mini kit® from Qiagen® isolation was performed according to the manufacturer's protocol.

The bacterial culture used for chromosomal DNA isolation was either from a fresh streaked plate or from a liquid overnight 2 ml culture. If cells were taken from a plate, two or three loopfuls were removed from the plate and placed into a 1.5 ml centrifuge tube containing 500 μl of 50 mM Tris pH 8.0 that was vortexed to wash the cells. The tube was centrifuged for 45 seconds at 13,000 rpm to pellet the cells and the supernatant was discarded. The cells were resuspended in 350 μl of TES buffer (50 mM Tris, 1 mM EDTA, 25 mM sucrose) and 50 μl of fresh lysozyme (100 mg/ml in TES buffer) was added to partially degrade the cell wall. The mixture was incubated for 30 minutes at 37°C in a water bath and vortexed to disperse sample every 10 minutes. After incubation, 20 μl of a 20% SDS solution was added along with 7.5 μl proteinase K (20

mg/ml). The mixture was incubated for 30 minutes at 37°C in a water bath and vortexed

to disperse the sample every 10 minutes. After the second incubation, the tube was

placed on ice and 400 μl of TE saturated phenol pH 8.0 was added to remove proteins

and other cellular material. The tube was mixed gently then centrifuged for 5 minutes at

13,000 rpm to separate the mixture into two layers. The top layer of the mixture was

retrieved with a pipette with care taken not to disturb the interface and placed it into a

fresh 1.5 ml tube on ice. To the aqueous phase or top layer, 400 μl of chloroform:

isoamyl alcohol (24:1) was added to remove trace amounts of phenol. The tube was

centrifuged at 13,000 rpm for 5 minutes to separate the mixture into two layers. The top

layer again was taken and placed into another centrifuge tube on ice. The chloroform:

isoamyl alcohol (24:1) step was repeated to ensure removal of all phenol. After the

second extraction with chloroform:isoamyl alcohol, the aqueous layer was removed and

placed into another 1.5 ml centrifuge tube on ice containing 1 ml ice cold 100% ethanol.

Upon addition of ice cold ethanol, the chromosomal DNA was precipitated out of

solution by inverting the tube several times. The precipitated DNA was spooled onto a

pipette tip and transferred to another 1.5 ml tube containing 400 μl of 70% ethanol to

rinse the molecule by inverting the tube several times. After rinsing, the tube was

centrifuged for 25 seconds at 13,000 rpm to pellet the DNA and all ethanol was removed

with a Pasteur pipette. The tube was air dried to remove all traces of ethanol then the

DNA pellet was rehydrated in 50-75 μl of 0.1x TE plus RNase. The DNA solution was

refrigerated overnight to allow time for rehydration and chromosomal DNA was stored at

4°C.

Estimation and Size Determination of DNA Samples

Agarose gels used for size determination and estimation of DNA concentration were composed of 0.75% agarose in 1X TAE buffer (40 mM Tris, 20 mM Acetic acid, 1 mM EDTA, pH 7.6). DNA ladders were used as controls to estimate the size and concentration of DNA. The ladder routinely used in this research was HindIII digested λ DNA. The DNA samples used were mixed with 6X loading dye at a ratio of 5:1 unless noted otherwise. After electrophoresis, the gel was stained in 100 ml of ethidium bromide solution (0.5 μg/mL) for 15-20 minutes in the dark. The gel was destained in water for 10-15 minutes with slow agitation.

Design Primers for Amplification of Specific Genes via Polymerase Chain Reaction

The primers used in this study (see Table 2 and Table 3) were designed using the Primer3 program available online (http://fokker.wi.mit.edu/primer3/input.htm). The names of the primers indicated the gene being amplified and the position of the primer in relation to the base pair number in the target gene. For example, rhbC4795 is a primer at base pair 4795 in the rhizobactin 1021 cosmid for the rhbC gene. Or another method of naming included the name of the target gene and the base pair number where the primer begins to bind to the target gene. Given the two primers and their binding positions, it was quick to know the size of the desired PCR product, so the second method of naming was used on all genes except rhbC.

Table 2: Primers used in this study, designed with Primer3 program online using known DNA sequence (Lynch et al., 2001). The numbers represent the position of the primer in the denoted gene.

Primer Name	**Primer Sequence 5' to 3'**
rhbA21	CCG AAC GTC ATC GAA AAT AT
rhbA1373	AGA GCG CCA GAG ACC TGA T
rhbB21	ATT CAG AAC TCC ACC GAC CA
rhbB1478	GCA TGA TGG GTC TCC AGT TC
rhbC4795	TAT CAA GCT TGC GAT GAG GTC TCT TTC GTC
rhbC5736	AGT TCT GCA GCC CTG ATT GTC ACG TAG
rhbD2	GAC ACT GCC TTG GCC TAT TC
rhbD562	CC GGT AGC AGA ACA TCA GT
rhbE1	ATG ACG GAT TTC GAT CTG G
rhbE302	ACC GAA CTT CTG GAA CGA TG
rhbF6	CAT GCA TCA TGA TCC GCT AC
rhbF1775	ACC AGT GGA TTG TTC ACC A
rhrA49	CGG CTC CTA AAG GTT CGA TT
rhrA766	CCG TCT ATT TCC CCA GAA CA
rhtA107	TGA CGA GCC TGG AAG AAA TC
rhtA2236	AA CCT TTG TCA GCG AGA CC

Table 3: Primers used for sequencing (tpnRL27-1 and 2 are from Larsen, Wilson, Guss, &

Metcalf, 2002).

Primer Name	Primer Sequence (5' to 3')
tpnRL27-1	AAC AAG CCA GGG ATG TAA CG
tpnRL27-2	CAG CAA CAC CTT CTT CAC GA
tpnRL27-3	CAG CAA GAC AGG AAC GAC AA
tpnRL27-4	AAC CCT TAC CGC CTG AAA GTC

Preparation of Probe DNA using Digoxygenin-11-dUTP (DIG) Labeling

Each primer was dissolved in distilled water to a final stock concentration of 200

μM. The stock solution was diluted to 20 μM for further use and denoted as working

stock solutions. A primer was designed for each gene in the rhizobactin 1021 operon

individually for amplification and isolation of the target gene. PCR was used to amplify

the target gene using the inward directed primers with bacterial DNA serving as a

template. The whole bacterial cells were prepared by first adding an isolated colony from

a fresh streak plate into 10 μl of ddH_2O and were mixed thoroughly to prevent clumps.

PCR was performed by adding 37 μl of ddH_2O, 1 μl DNA or bacterial cells, 1.25 μl of

each primer at 20 μM, 1 μl of dNTP mix, 5 μl of 10X Taq buffer (without magnesium

chloride), 3 μl of magnesium chloride at 25 μM, and 0.5 μl Taq polymerase. The tube

was placed in a thermocycler at appropriate cycle according to manufacturer's

instructions. The cycle included 1 cycle 95°C for 2 minutes, 1 cycle at 95°C for 1

minute, cycles at 50°C for 2 minutes, and 2 minute cycles at 72°C (cycle called

KNNAMED). After the reaction was complete, agarose gel electrophoresis was employed to visualize the DNA.

Once the gel was electrophoresed, the DNA was isolated from the agarose by using the GENECLEAN turbo elution kit® from Qbiogene Inc® according to the manufacturer's protocol. The kit removes agarose from the sample leaving the purified DNA. To initiate the digoxygenin-11-dUTP (DIG) labeling reaction, 10 μl of the target gene was mixed with 3 μl of sterile ddH$_2$O in a 0.5 ml or 1.5 ml tube. The DNA was heat denatured by placing the tube in a 95°C water bath for 10 minutes to separate the double stranded DNA into single strands. Following heat denaturation, the tube was quickly cooled in a -10°C bath containing a sodium chloride and ice slurry to prevent recoiling of the helix. The temperature was monitored with a thermometer to maintain -10°C. The quick cooled tube was centrifuged at 13,000 rpm for 15 seconds to pool the DNA sample and placed on ice. The following components were added in order to the tube: 2 μl of 10x EcoPol buffer, 2 μl of random heximer primers [pd (N)$_6$ sodium salts], 2 μl of 10x DIG DNA mix, and 1.5 μl of Klenow fragment. The reaction mixture was incubated at 37°C for 20 hours to facilitate the labeling reaction.

After incubation, the tube was centrifuge at 13,000 rpm for 15 seconds to pool the reaction mixture. The total volume was adjusted to 100 μl with sterile ddH$_2$0. Next, 50 μl of TE saturated phenol (pH 8.0) and 50 μl of chloroform (results in a 1:1 ratio) was added to the mixture (Sambrook et al., 1989). The mixture was hand mixed, centrifuged for 5 minutes to separate the aqueous phase from the organic phase at 13,000 rpm, and the top layer (aqueous) was transferred to another 1.5 ml tube. The top layer underwent chloroform: isoamyl alcohol (24:1) extraction with the addition of 90 μl of the solution.

After 90 μl of chloroform:isoamyl alcohol (24:1) was added, the mixture was hand mixed, centrifuged for 5 minutes to separate the two layers at 13,000 rpm, and the top layer (aqueous) was drawn out and placed into another 1.5 ml tube. This step was repeated to ensure all phenol and proteins were removed from the sample. The final step to recover the labeled probe DNA was to add 10 μl of 10 μM ammonium acetate and 220 μl of 100% ice cold ethanol to precipitate the DNA. The mixture was chilled at -80°C for 30 minutes. After incubation, the tube was centrifuged at 13,000 rpm for 25 minutes in a cold centrifuge to pellet the DNA and the supernatant was removed by a Pasteur pipette. The pellet was air dried to remove traces of ethanol and finally resuspended in 20 μl of 0.1x TE buffer. The recovered probe DNA was stored at -20°C.

Once the DNA had been recovered, an estimation of the concentration was performed to determine the amount of probe present. A serial dilution was set up using a DIG labeling kit from Roche®. The kit contained DNA detection buffer and control DNA used to compare to the unknown probe. The comparison of the unknown DNA to the known DNA provided a standard for the estimation of DNA concentration of the newly made probe. A serial dilution was set up with 5 tubes labeled A-E. Tube A contained 8 μl of DNA detection buffer and 2 μl of control DNA. From tube A, 2 μl was taken and added to tube B containing 18 μl of DNA detection buffer. From tube B, 2 μl was taken and added to tube C that contained 18 μl of DNA detection buffer. This was continued until all tubes had been serially diluted. The same diluting procedure performed was repeated for the experimental DNA. Once all dilutions were made, a small piece of Maximum Strength Nytran Plus® membrane was soaked in water and air dried on filter paper. After the membrane dried, sections were labeled on the membrane

with a pencil to denote each samples position. Once the sections were labeled, 1 μl of each tube A-E (control and experimental) was spotted onto the membrane in its corresponding area. The DNA was fixed to the membrane by baking at 80°C *in vacuo* for 30 minutes.

Once the DNA was fixed to the membrane, detection and estimation was the next step. The membrane was first incubated in washing buffer (0.1 M maleic acid, 0.15 M NaCl) at room temperature for 5 minutes with slow shaking. The wash buffer was discarded and replaced with blocking solution (dilute stock blocking reagent 1:10 in maleic acid or 20 g blocking reagent and 200 ml of maleic acid buffer) and was incubated at room temperature for 5 minutes with slow shaking. While this was incubating, a dilution of anti-DIG alkaline phosphatase antibody in blocking solution was made by adding 3 μl of antibody to 15 ml of blocking solution (1: 5,000). Once the incubation was completed, the blocking solution was discarded and replaced with the diluted antibody for 15 minutes at room temperature with slow shaking. The diluted antibody was discarded and the membrane was washed twice (100 ml per wash) in washing buffer for 15 minutes per wash at room temperature with slow shaking. While the second wash was performed, a color solution was made with the addition of 200 μl of NBT-BCIP stock solution (provided in the DIG labeling kit) to 10 ml of detection buffer (0.1 M Tris-HCl, 0.1 M NaCl). The color solution was made fresh each time and stored protected from light. After the second incubation in washing buffer, the used solution was discarded and the membrane was incubated in detection buffer for 2 minutes at room temperature with slow shaking. The detection buffer was discarded and replaced with the color solution. The membrane, when incubated in the color solution, was placed in the

dark and color development occurred within 20-60 minutes. After incubation, the color solution was discarded and 1x TE buffer was added to stop the reaction. When the reaction had occurred, the membrane was air dried on filter paper. The dried membrane was analyzed by matching the intensity of color development of the experimental probe DNA with the control DNA providing an estimation of the recovered probe DNA. The membrane was dried on blotting paper and stored between two blotting papers wrapped in aluminum foil at room temperature.

Preparation of Chromosomal DNA for Southern Transfer

Once the DIG-labeled probe DNA was made, the next step was to probe chromosomal DNA from IARI 917 for determining the homology to the probe. In Southern transfers, the probe only binds to sequence with 75%-80% homology with binding detected in the same manner as the probe estimation. Before probing occurred, the chromosomal DNA was digested with restriction enzymes that did not cut within each specific gene to be probed ensuring that digestion would not fragment the target gene. The chromosomal DNA of bacteria is large thus detection of one gene would be difficult so the DNA must first be digested into smaller fragments for hybridization to occur. Chromosomal DNA was restriction digested with PstI and HindIII because they do not cut within the rhbC gene. This gene was chosen because it codes for an enzyme that attaches citric acid groups to the siderophore that rhizobactin 1021 and schizokinen both contain. On this basis, the thought was the rhbC gene may be shared due to the siderophore's structural similarities.

The digested chromosomal DNA was electrophoresed on a 0.75% agarose gel

overnight (~16 hours) at 12-13 volts to separate all fragments. The gel was stained with 100 ml of ethidium bromide solution (0.5 μg/ml) for 15-20 minutes in the dark. The ethidium bromide solution was discarded and the gel was destained in 200 ml of water for 10 minutes with slow shaking. The bottom left corner and the top of the gel (leaving well bridges intact) were cut to ensure correct orientation of the gel and its lanes during transfer. The trimmed gel was used as a template to label the positions of the wells on the transfer membrane. The gel was photographed with a ruler along side to aid in analysis after the transfer has occurred. Once the gel was photographed, the DNA was prepared for transfer to the nylon membrane. The gel was rinsed with water between the removal of one solution and the addition of the next in the following preparation. The gel was soaked in 0.25 N HCl for 5 minutes with slow shaking at room temperature. After incubation in HCl, the solution was discarded and replaced with denaturing buffer (0.5 M NaOH, 1.5 M NaCl) and the gel was incubated in denaturing buffer for 30 minutes with slow shaking at room temperature. Next, the denaturing buffer was removed and the gel was soaked in neutralizing buffer (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.0) with slow shaking for 30 minutes. The neutralizing buffer was discarded then the gel was soaked in 20x SSC transfer buffer (3 M NaCl, 0.3 M sodium citrate, pH 7.0) for 30 minutes with slow shaking.

Southern Transfer of DNA

After the incubations, the gel and the DNA were prepared for transfer to the nylon membrane. Schleicher and Schuell TurboBlotter® rapid downward transfer system was used for the transfer of DNA. A Nytran® SuPer Charge nylon membrane, receiver of the

transferred DNA, was cut to 0.5 cm larger than the gel. To begin the transfer of DNA, the membrane was soaked in 20x SSC transfer buffer for 5 minutes at room temperature with slow shaking. While the membrane was soaking, the stack tray of the turbo blotter unit was placed on a level surface and 20 sheets of dry GB004 (thick) blotting paper were placed in the stack tray. To this stack, 4 sheets of dry GB002 (thin) blotting paper were placed on top. On the stack of 24 dry papers, one GB002 (thin) blotting paper was pre wetted in 20X SSC then placed on the top. The nylon membrane was removed after the 5 minute incubation period and placed on the pre wetted GB002 blotting paper. The membrane and the stack of blotting papers were covered with Saran® wrap containing an open window over the nylon membrane. The gel was placed on top of the nylon membrane preventing the formation of air bubbles between the two. The wells and the bottom left corner of the gel were marked on the membrane with a pencil to denote their positions. A small amount of 20x SSC was pipetted on top of the gel and three sheets of GB002 blotting paper pre wetted in 20x SSC were placed on top of the gel. The turbo blotter buffer tray was placed on top of the stack tray and filled with 20x SSC transfer buffer. The transfer of DNA began by placing the center of a 20x SSC pre soaked wick across the transfer stack with the ends of the wick in the 20x SSC buffer located in the buffer tray. A light weight object was placed on the center of the wick to keep it in contact with the transfer stack to prevent drying out during transfer. The assembled unit and the transfer stack was allowed to transfer overnight for 16-18 hours at room temperature.

After the overnight transfer, the tray was disassembled and the gel was removed with tweezers. The gel was back stained in the ethidium bromide solution to determine if

the DNA transferred properly. The membrane was removed and washed with 2x SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0) for 5 minutes with slow shaking at room temperature. After the 5 minute incubation, the membrane was air dried on a dry sheet of blotting paper. The dry membrane was baked between two dry blotting papers at 80°C *in vacuo* for 1 hour. The membrane was stored between the two blotting papers wrapped in aluminum foil at room temperature.

Hybridization of Southern Blots

After the DNA was baked to the nylon membrane, hybridization was the next step. Hybridization solution (50% formamide, 5X SSPE, 5X Denhardt's solution, 10% SDS) was prepared fresh before each reaction with the composition of 10 ml of formamide, 5 ml of 20x SSPE (3 M NaCl, 230 mM sodium phosphate, 20 mM EDTA, pH 7.4), 2 ml 50x Dehardt's solution, 2 ml water, and 1 ml of 10% SDS. The 50X Denhardt's solution was made by preparing 1 g/20 ml dH_2O solutions of polyvinylpyrrolodone and Ficoll. The Ficoll and polyvinylpyrrolodone solutions were autoclaved separately then mixed together. Sterile bovine serum albumin (1 g in 7.5 ml dH_2O) and 2 ml of sterile 0.25 M EDTA were added to the combined Ficoll/ polyvinylpyrrolodone mixture and sterile water was used to bring the volume of the 50X Denhardt's solution to 100 ml. The hybridization solution was divided into two tubes, with one tube containing 12 ml used for prehybridization and the second containing 8 ml used for hybridization. Both were warmed to 42°C before use. The membrane was pre wetted in a small volume of 2X SSC then placed into a hybridization tube pre warmed to 42°C. Prehybridization solution was added to the hybridization tube and allowed to

prehybridize at 42°C for 1-2 hours with rotation in the Fisher Biotech® hybridization incubator. Once the incubation was complete, the prehybridization solution was discarded and replaced with 8 ml of hybridization solution supplemented with 18 μl of heat denatured DIG-labeled probe DNA. Heat denatured DNA was achieved by incubation in a 95°C water bath for 5 minutes, then quick cooled in a -10°C sodium chloride and ice slurry. The membrane was incubated in the hybridization solution supplemented with DIG-labeled probe DNA for 18 hours at 42°C with rotation.

When hybridization was completed, posthybridization washes were required. The hybridization solution plus probe DNA was kept and stored at -20°C and could be reused for further blotting reactions. The membrane was washed first with an incubation in wash solution A (2x SSC, 0.1% SDS) for 5 minutes at room temperature with shaking. Wash solution A was discarded and replaced with wash solution B (0.1x SSC, 0.1% SDS). The membrane was washed twice with wash solution B for 5 minutes at 55°C with shaking. Finally, the membrane was rinsed briefly with 1x TBS (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) and developed according to hybridization development protocol as previously described.

<u>Methods for Testing Hypothesis 2</u>

<u>Determination of Minimal Inhibitory Concentration and Isolation of Spontaneous Antibiotic Resistant Mutants</u>

Before conjugation could be performed, it was essential to determine a minimal inhibitory antibiotic concentration (MIC) of each bacterium. MIC determination was performed to identify the minimal amount of antibiotic required to inhibit the bacteria.

Conjugation was a method employed in this research to transfer plasmid DNA from one organism (donor) to another (recipient). The recipient bacteria containing transferred plasmid DNA were then called transconjugants. In this research, the plasmid transferred to IARI 917 contains a mobile DNA element called a transposon. The transpositional element moves from the transferred plasmid DNA and integrates into the chromosomal DNA of IARI 917, thus interrupting certain genes. The transconjugants were screened for their ability to either over produce or not produce siderophore compared to the wild type strain due to the random insertion of the transposon.

To determine the MIC of each bacterium (Clinical and Laboratories Standards Institute, 2005), 2 fold serial dilutions were set up with LB broth containing different concentrations of antibiotics. To standardize the bacterial inoculum, 4 or 5 colonies from a fresh streak plate were suspended into 1 ml of LB broth to match the 0.5 McFarland standards for turbidity that represents 10^8 colony forming units per ml (CFU/ml). A portion of this standardized suspension was diluted 1:100 to 10^6 CFU/ml with LB broth. When 1 ml of this diluted inoculum was added to each tube containing 1 ml of the antibiotic diluted in 1 ml of LB broth, the final inoculum was 5×10^5 CFU/ml. Each tube for the 2 fold serial dilution had a decrease in half of the concentration of antibiotic present. A growth control was set up with 2 ml of LB broth without antibiotic. The bacteria were incubated at appropriate temperatures for 16-20 hours before determining MIC. The tubes were aligned in such a way that comparison of all could be performed. The MIC was determined as the lowest concentration of antibiotic that effectively inhibited the growth of the organism. The inhibitory antibiotic concentration was used

during further experiments to effectively kill the donor and helper strains (if required) while leaving only the recipient strain.

Once an MIC was defined for the donor and helper strains, a spontaneous antibiotic resistant mutant of IARI 917 (recipient) to the rifampicin was isolated. These spontaneous resistant recipients were used in conjugation for proper selection. The transferred plasmid contained a kanamycin resistance marker and a second antibiotic was required to inhibit the donor and helper while the recipient was resistant to both antibiotics. The method used to isolate spontaneous antibiotic resistant mutants was the gradient plate method (Szybalski & Bryson, 1952).

Gradient plates are large culture dishes made by pouring two wedges of agar. The wedges were poured one at a time by elevating the plate on an object to form an agar wedge. The bottom wedge (55 ml of media) contained no antibiotic and was solidified with one side elevated thus forming a wedge. After the bottom wedge solidified, another 55 ml of media containing antibiotic was added to the existing wedge resulting in a second wedge. When the second wedge was poured, the plates were not elevated to allow the formation of a level agar surface. The top wedge has a higher concentration of antibiotic at its thicker portion that thins as the two wedges meet. Bacterial cultures grown fresh as seed cultures overnight were spread out on a gradient plate using a sterile glass spreader. The plates were incubated for 48-72 hours at 30°C. The spontaneous antibiotic resistant mutants were harvested and their respective MICs were determined as previously described.

Triparental Conjugation and Transposon Induced Random Mutagenesis

Once an antibiotic was determined to inhibit the donor bacteria and not affect the recipient strain, the next step was introduction of a plasmid containing the transposon. The transposon element, Tn5, was harbored in a plasmid maintained in *E. coli* strain cc118λ. This strain must have a helper strain, *E. coli* pRK2013, for conjugation to occur. The two *E. coli* strains plus the recipient, IARI 917, were required for the triparental conjugation. Once the plasmid was transferred, expression of the transposon occurs in the recipient. The expression results in the random insertion of transposon DNA into the chromosome of IARI 917 thus interrupting genes.

The triparental conjugation experiment was started by growing the three strains in 10 ml of LB broth or TY broth plus antibiotic if necessary. The cultures were incubated overnight at appropriate temperatures with shaking. After incubation, the cultures were centrifuged for 10 minutes at 10,000 rpm and the supernatant was discarded. Each of the cell pellets was thoroughly resuspended in 100 μl of 0.85% NaCl and the entire 100 μl of each strain were mixed together. After the cells were mixed, 150-200 μl of the mixture was pipetted onto a 0.22 μm sterile cellulose nitrate membrane filter placed on the surface of a prewarmed LB plate. The plates were incubated filter up at appropriate temperature overnight to facilitate conjugation. After incubation, the filter was removed from the plate and placed into a tube containing 1 ml of 0.85% NaCl used to wash off the cells from the surface of the membrane. Once the cells were removed, 150 μl of the re-suspended cell mixture was spread onto LB plates containing kanamycin along with rifampicin that inhibits the donor strains. The plates were incubated at 30°C for 24-48 hours and the colonies present were now called transconjugants. All colonies were

picked from the transformation plates and patched onto another for maintenance. The patch plate was called a master plate as it contained 52 transconjugants arranged in a grid pattern aided by a template for orientation. The master plates were used for maintaining the large numbers of transconjugants.

Biparental Conjugation

The process of biparental conjugation was performed to the same protocol as triparental conjugation described above. The only exception was the strain used, pRL27, has the ability to transfer the plasmid to a recipient without the need for a helper strain. The transposon, mini Tn5, contained a high frequency mobile element that transposes at a higher frequency than Tn5. The transconjugants were isolated and inoculated onto a master plate for growth and were further characterized.

Screen for Non-Siderophore Transconjugants

Once many master plates were patched, characterization of each organism's ability to produce siderophore was required before further analysis. The transconjugants were first patched onto a minimal media plate (Fiss-Glucose) with antibiotics to starve the bacteria for iron to induced siderophore production. Once the bacteria had grown sufficiently, the colonies were patched onto CAS agar to determine the transconjugants' ability to produce siderophore. During each screening of the transconjugants, a wild type IARI 917 colony was also transferred for comparison to determine if the transconjugants overproduced or underproduced siderophore. Another method used to characterize siderophore production was growth in a liquid minimal media with antibiotics. IARI 917

was used for comparison throughout the characterization of the transconjugant's ability to produce siderophore. Minimal broth cultures were grown and pelleted while the supernatant was saved. A sterile #2 cork borer was used to create wells in a CAS plate to that70 μl of supernatant was added. The presence of siderophore resulted in the production of an orange halo around the well. The remaining supernatant was used to perform the Atkin's and Arnow's assays as previously described. These three assays characterized the transconjugants' ability to produce siderophore as compared to the wild type strain. The transconjugants were described as siderophore nonproducers and overproducers as compared to the wild type.

Preparation of DH5αλpir$^+$Competent Cells for Cloning

The preparation of DH5αλpir$^+$competent cells (calcium competent for heat shock transformation) was carried out using established protocol (Ausubel et al., 1994). The prepared competent cells were stored at -80°C.

Determination of Interrupted Sequence by Transposon Insertion

The next step to determine the sequence of DNA interrupted by the transposon insertion was to isolate transconjugant chromosomal DNA. The isolated chromosomal DNA was digested with BamHI restriction enzyme or any enzyme that doesn't cut within the transposon sequence. When the DNA was digested, the many fragments produced were separated on an agarose gel then subjected to Southern blotting. The plasmid, pRL27, was DIG-labeled and served as a hybridization probe against each transconjugant's digested chromosomal DNA to detect the presence of the transposon.

The digested DNA of 7 different nonsiderophore producing transconjugants were separated on an agarose gel and transferred via Southern blotting as previously described. After the blots were completed, the transconjugants with the transposon insertion were used for further analysis.

Once the transposon's insertion was detected, the transconjugants' digested chromosomal DNA was religated to form a plasmid. The newly formed plasmid contained transposon sequence with flanking chromosomal DNA of the transconjugant. The newly formed plasmid was transformed into *E. coli* strain DH5$\alpha\lambda$pir$^+$ by the heat shock method for propagation of the DNA (Ausubel et al., 1994). The plasmid DNA was extracted and purified. When the DNA was determined to be pure and concentrated, the plasmid was sent for sequencing using primers denoted in Table 3. The primers were designed to sequence outwardly from the ends of the transposon sequence into the flanking chromosomal DNA of the transconjugants to determine what gene or open reading frame may have been interrupted by the transposon insertion. The sequence was subjected to BLAST searches to determine the protein product of the DNA code. Next, the product was analyzed to determine if it had any role in siderophore regulation, biosynthesis, or transport by comparison to known proteins.

Concentration Dependent Binding of ^{55}Fe-Schizokinen

The method to detect ferric siderophore binding to a specific receptor was set up using a radioactive isotope of iron, iron-55 or ^{55}Fe bound to purified schizokinen (^{55}Fe-SK). The binding assay was performed by diluting different concentrations of ^{55}Fe -SK ranging from 5 nM to 200 nM in Fiss-Glucose minimal media or 0.1 M MOPS, pH 7.4.

Each concentration was done in triplicate and the three readings were averaged to get a better representation of the data and to increase the accuracy of the assay. A lithium chloride solution of 0.9% was made and stored at 4°C. A seed culture was inoculated and incubated overnight at appropriate temperature (30°C for rhizobia and 37°C for *E. coli)* with shaking in LB or TY broth with antibiotics if necessary.

The next day after incubation of the seed culture, the seed was subcultured into 50 ml of Fiss-Glucose minimal media in a 1:100 ratio for IARI 917 wild type and transconjugant 3-17. The Rhizobial cultures were allowed to grow for 13-16 hours until the OD_{600} reached 0.5-0.9 at 30°C with shaking. The *E. coli* strain KDF-541 seed culture was sub cultured into MOPS minimal media and was incubated for 3-4 hours at 37°C with shaking.

After the optical densities were achieved, the culture was placed on ice for 1 hour prior to performing the binding assay. While the culture was on ice, 27-30 nitrocellulose membranes were placed in a small Petri dish with ddH_2O. The membranes were left to soak for 30 minutes prior to use. All the experimental equipment was kept on ice during binding assays to prevent the transport of bound Fe^{55}-SK but still allowed binding of ferric siderophore complex to the specific outer membrane receptor.

After incubation, the assay began by placing tubes in racks with four concentrations with six tubes per concentration were done at a time. Three of the tubes per concentration contained 1 ml of diluted [55]Fe-SK while the other three tubes contained 100 µl of culture. The two tubes were mixed together and incubated on ice for 5 minutes to facilitate binding to the bacterial cell. While the tubes were incubating, a vacuum manifold was set up with one membrane filter per well with 12 wells in total. After

incubation was completed, the contents of each tube were vacuum filtered to separate the cells from the reaction mixture. The membrane filter was washed with 20 ml of ice cold 0.9% lithium chloride to remove all nonspecifically bound or excess [55]Fe-SK. The membranes were moved to plastic scintillation vials and 10 ml of scintillation fluid was added. This process was repeated for each concentration and the membranes were incubated in scintillation fluid at room temperature for at least 24 hours before counting.

Concentration Dependent Transport of [55]Fe-Schizokinen

Preparation for concentration dependent transport of [55]Fe-SK was similar to concentration dependent binding. A two ml seed culture was inoculated and grown overnight at appropriate temperature with shaking for IARI 917 wild type, transconjugant 3-17, and *E.coli* KDF-541. The two ml seeds of rhizobial cultures were used to subculture a 50 ml Fiss-Glucose minimal media that was grown for 13-16 hours or until OD_{600} reaches 0.5-0.9. The *E. coli* strain KDF-541 seed culture was subcultured into MOPS minimal media and was allowed to grow for 3-4 hours until the OD_{600} was reached. In contrast to the binding assay, the transport experiments must be performed at the organism's optimal growth temperatures to facilitate the transport of the [55]Fe-SK complexes into the cell. The transport experiment was performed with the same concentrations as the binding assay in triplicate and was then averaged.

For the transport assay, 48-50 membranes were soaked in ddH_2O for 30 minutes prior to use. Each concentration had 12 tubes; three tubes contained 100 µl of cells and three tubes contained the desired concentration of [55]Fe-SK. The tubes with the culture and the diluted [55]Fe-SK were mixed together and incubated for 2 minutes. The same

concentration was also performed for three minutes. While the tubes were incubated, a vacuum manifold was set up with one membrane filter per well with 12 wells in total. After the incubation time was completed, a 100 fold excess of purified schizokinen was added to stop the transport. The contents of each tube was quickly poured into corresponding well and vacuumed to remove all excess liquid. The membrane filter was washed with 20 ml of ice cold 0.9% lithium chloride to remove all nonspecifically bound or excess ^{55}Fe-SK.

The membranes were moved to plastic scintillation vials to that10 ml of scintillation fluid was added. This process was repeated for each concentration and the membranes were incubated in scintillation fluid at room temperature for at least 24 hours prior to counting. Once the vials were counted, averages were taken for each time observed. The 3 minute average was subtracted from the 2 minute average to yield the transport of ^{55}Fe-SK per minute.

CHAPTER 4

RESULTS AND DISCUSSION

Hypothesis 1

When the siderophore produced by *Rhizobium leguminosarum* IARI 917 was

isolated and chemically characterized (Storey, Boghozian, Little, Lowman, &

Chakraborty, 2007) as schizokinen, many questions arose. What genes are involved in

the biosynthesis and transport of schizokinen, a siderophore previously not described in

rhizobia? With no previous research describing the genetic pathway or the mechanism of

iron binding and transport by schizokinen, this was a difficult task to undertake. When

the structure was identified, more questions were presented. With a striking structural

similarity to another siderophore, rhizobactin 1021, could these molecules be synthesized

via a common genetic pathway? Or could the molecules be synthesized via a completely

new and different pathway? Because schizokinen is very similar in structure to

rhizobactin 1021, I hypothesized that the two siderophores are synthesized via an

identical biosynthetic pathway.

Amplification of rhb Genes via PCR

Primers previously reported by Lynch et al. (2001) were ordered through the

Microbiology Core Facility located within the James H. Quillen College of Medicine.

The primers were used to amplify each gene within the rhizobactin 1021 synthesis and

transport operon via PCR using a positive control plasmid, pPOC1. pPOC1 was used as

a template because it contains all the genes (rhbA-F, rhtA, and rhrA) required for the

synthesis, regulation, and transport of rhizobactin 1021 (Lynch et al.). A test reaction

was also performed using IARI 917 chromosomal DNA as a template. PCR is a stringent

experiment because primers must be exactly complementary to the DNA sequence. If a

single base pair in the template are not complementary to the primer sequence, annealing

will not take place thus there will be no amplification. Amplified DNA (PCR products)

products were visualized by electrophoresis. The amplified corresponding DNA(s) to

known gene(s) with known sizes described in the literature (Lynch et al.) were purified

from the agarose gel. λ DNA digested with HindIII (λ/HindIII) was used as a size and

concentration marker.

The results of the PCR show the presence of all rhb genes within the pPOC1

plasmid as expected (Table 4) and it was used in further analysis as the positive control.

Use of plasmid DNA as a positive control was technically not correct when comparing to

chromosomal DNA. When the genes were used as probes in Southern blotting, 75%-

80% homology was not detected. This implies that the origin of the gene probes was not

essential to the experiment. The rhb genes amplified in the pPOC1 were of the size noted

in the literature while amplification, using IARI 917 chromosomal DNA as a template,

produced no traceable product (data not shown). The results indicate the possibility of no

similarity in the operons for rhizobactin 1021 and schizokinen regulation, biosynthesis,

and transport. Another interpretation could be the flanks of the genes were different thus

annealing of the primers to the template DNA was not attainable.

Table 4: Rhizobactin 1021 genes and their base pair content

Lane	Gene	bp content
A	rhbA	1352
B	rhbB	1457
C	rhbC	941
D	rhbD	540
E	rhbE	1301
F	rhbF	1769
G	rhtA	2129
H	rhrA	717

Results of Southern Blot

Once all the genes were amplified and electrophoresed from pPOC1, the bands were purified individually and DIG labeled to make probe DNA. The probes were hybridized to chromosomal DNA under conditions to detect sequence with 75%-80% homology to it located within IARI 917 chromosomal DNA. The hybridization of the probe to homologous DNA was visualized via a color reaction on a nylon membrane. The digested chromosomal DNA of IARI 917 was electrophoresed, along with a positive control, slowly to facilitate proper separation of the fragments before Southern transfer. The nylon membrane, containing the transferred DNA, was probed to determine homology. The results (Figure 19 and 20 below) detected no binding to any of the rhb genes (rhbA-F, rhrA, and rhtA) in the IARI 917 chromosomal DNA. The interpretation

of these results was that the genes for regulation, biosynthesis, and transport of schizokinen and rhizobactin 1021 do not share at least 75%-80% homology. The genes in the two operons could share less than 70%, homology that may not be detected by the Southern blot method. This experiment shows the two pathways are not highly conserved despite the products' structural similarities. Hypothesis 1 stated that the two operons for the production of the entire siderophore systems, both IARI 917 and rhizobactin 1021, were identical or highly homologous. The results of Southern blot (Figure 19 and Figure 20) and PCR experiments performed indicated that this was not the case.
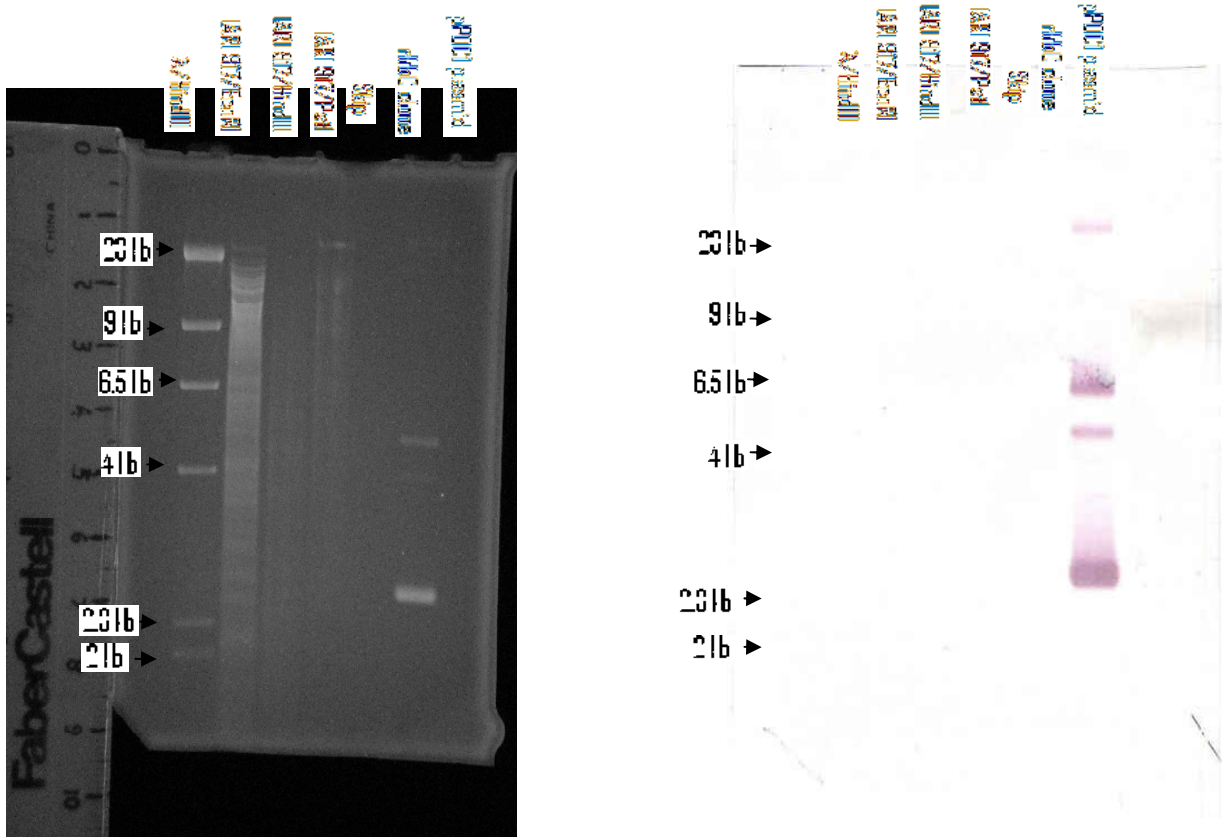
Figure 19: Agarose gel electrophoresis and Southern blot of IARI 917 chromosomal DNA using the DIG-labeled rhbC gene as a probe.

A   B   C   D   E   F   G
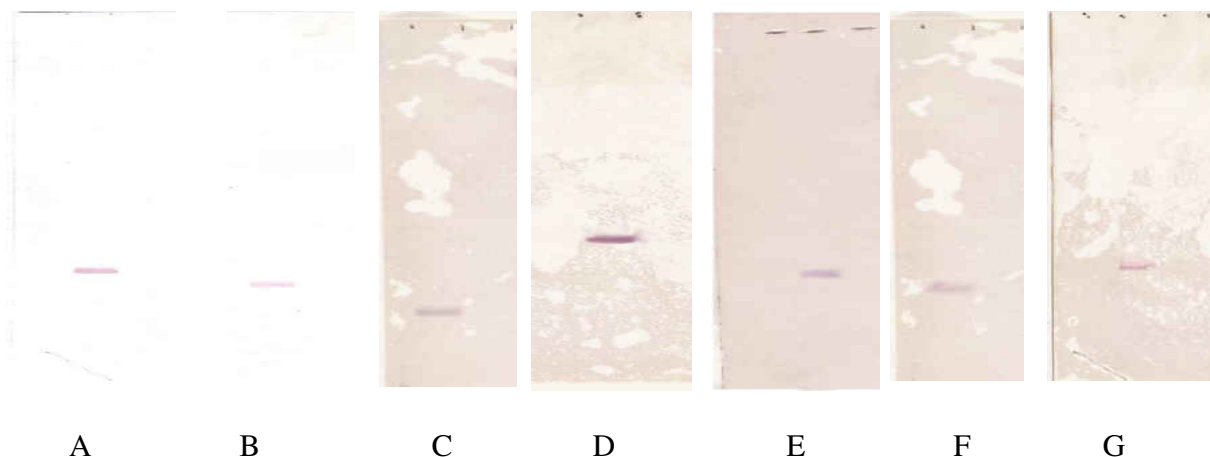
Figure 20: Southern blot analysis of digested chromosomal DNA from IARI 917 wild type with the genes involved in rhizobactin 1021 biosynthesis, transport, and regulation. The binding detected on each membrane were the respective probe's positive control DNA. The digested chromosomal DNA of IARI 917 was located adjacent to the positive control (no binding detected). (A) Probe was DIG-labeled rhbA gene. (B) Probe was DIG-labeled rhbB gene. (C) Probe was DIG-labeled rhbD gene. (D) Probe was DIG-labeled rhbE gene. (E) Probe was DIG-labeled rhbF gene. (F) Probe was DIG-labeled rhrA (regulation) gene. (G) Probe was DIG-labeled rhtA (transport) gene.

Hypothesis 2

Once it was determined the operons of schizokinen and rhizobactin 1021 biosynthesis are not homologous, we decided to use random mutagenesis that employs transposon insertions for the detection of the genes involved in the biosynthesis and transport of schizokinen. Transposon induced random mutagenesis was used to create mutant strains of IARI 917 to aid in the identification of genes responsible for siderophore regulation, biosynthesis, or transport. Mutagenesis is a tool used to remove the functionality of unknown gene(s) producing a desirable phenotype. The mutagenesis

experiment was performed by introduction of a plasmid containing a transposase gene via conjugation. The donor organism contains the plasmid and the recipient will receive the DNA after transfer. When the plasmid was introduced and expressed in the recipient organism, random insertions of the transposon created interruptions or mutations in the recipient DNA. The transconjugants were screened to determine their phenotype and were classified as nonproducing or overproducing siderophore transconjugants as compared to the wild type strain. The chromosomal DNA of the transconjugant was then isolated, restriction digested, religated, cloned, and sequenced for the identification of the gene(s) involved. All of these methods were employed to test the second hypothesis that states that schizokinen is produced by a unique or modified pathway as rhizobactin 1021 despite their similar structures.

Isolation of Spontaneous Antibiotic Resistant Mutants and MIC Determination

Before mutagenesis could occur, other steps must be completed to ensure proper selection of mutant strains. Antibiotic cassettes are routinely used to select for desired bacteria while excluding others, and this method was used for selection of mutants. The first step in this process was to identify an antibiotic capable of inhibiting the growth of the donor strains. Conjugation is an experiment that uses a donor strain that transfers plasmid DNA to a recipient. The recipient, following conjugation, is transformed with the addition of the new exogenous DNA (i.e. transconjugants). Many genes can also be transferred in this process that could provide an advantage for the recipient organism over their wild type competitors.

Streptomycin was the first antibiotic chosen to eliminate the donor and helper strains in triparental conjugation. The strains used in conjugation (CC118λpir, pRK2013, and IARI 917) were grown in the presence of 2-fold serially diluted streptomycin and the strains grew in the presence of concentrations up to 37.5 μg/ml. Spontaneous resistant mutants, isolated using the gradient plate method, of IARI 917 were able to grow in the presence of streptomycin up to a concentration of 150 μg/ml. The plates used for selection of transconjugants were TY with streptomycin at a concentration of 150 μg/ml and kanamycin at a concentration of 50 μg/ml. The streptomycin was used to inhibit the donor and helper strains (CC118λpir and pRK2013), while kanamycin was used to select for the transconjugants of IARI 917 with the transposon insertion. The resulting transconjugants were resistant to both antibiotics. A positive control was setup using *E.coli* DH5α that was also selected for spontaneous resistance to streptomycin at the same concentrations.

Results of Conjugation

After multiple attempts at triparental conjugation, only 28 transconjugants were isolated. The positive control reaction resulted in only 58 colonies indicating triparental conjugation method was not very effective in production of transconjugants for this research. The plasmid containing Tn5 transposes at a low frequency creating a low number of transconjugants. Another method with a higher transposing frequency must be found to create more transconjugants. Biparental conjugation using a Tn5 derivative, mini Tn5, was employed that transposes at a higher frequency.

After the triparental experiment resulted in such low numbers of colonies, biparental conjugation was used to create more transconjugants. The plasmid harboring a mini Tn5 transposon, pRL27, was received from Tim Welch of the United States Department of Agriculture (Larsen et al., 2002). The Tn5 derivative was constructed by Larsen et al. to transpose at a high frequency thus producing more transconjugants per conjugation experiment. The mini Tn5 transposon was created to transpose at a higher frequency (~1000 fold greater than wild type Tn5) and theoretically would create more transconjugants (Larsen et al.). The genes encoded by the plasmid are important to understanding the mechanism of transposition. The oriT gene facilitates the transfer of the plasmid via conjugation. The tetAp gene is a promoter from the plasmid RP4 controlling the expression of the tnp gene coding for the transposase enzyme. The special origin of replication, oriR6K, allowed for cloning of transposon insertions. The aph gene provided kanamycin resistance.

Another strain, *E.coli* DH5αλpir$^+$, was used for the insertional cloning of the resulting transconjugant chromosomal DNA digested with BamHI. The increased efficiency of the mini Tn5 biparental conjugation system was apparent after the first conjugation experiment. The results can be seen in Table 5. There were 1687 transconjugant colonies isolated from biparental conjugation. The characterization of siderophore production by the transconjugants as compared to the wild type was the next step to be performed.

Table 5: Results of biparental conjugation

Biparental Conjugation Samples	Transconjugant Colonies
DH5α (mock conjugation, negative control)	0
pRL27 (mock conjugation, negative control)	50
DH5α Rif^r, pRL27 conjugation (positive control)	>2,000 (to many to count)
pRL27, IARI 917 Rif^r #1 conjugation	322
pRL27, IARI 917 Rif^r #1 conjugation	235
pRL27, IARI 917 Rif^r #1 conjugation	326
pRL27, IARI 917 Rif^r #1 conjugation	253
pRL27, IARI 917 Rif^r #1 conjugation	256

Screen of Transconjugants for Ability to Produce Siderophores

The transconjugants were patched onto a minimal media plate to starve them of iron thus induce siderophore production. The transconjugants were then transferred to CAS agar to determine their ability to produce siderophore. The transconjugants were also grown in a minimal broth to induce siderophore production. The broth cultures were harvested by centrifugation and the supernatant was added to wells bored into the CAS agar. The halo produced was compared to the wild type to characterize the transconjugants' ability (or inability) to synthesize and secrete siderophore. Some of the results are seen in Figures 21 and Table 6. Transconjugants defective in siderophore production were used for further analysis in this research.

Figure 21: CAS agar plate with bored wells containing culture supernatant. (A) Wells A-H contain various supernatants from nonproducing siderophore transconjugants grown in the absence of iron. The center well contains supernatant from IARI 917 wild type culture grown in broth in the absence of iron for comparison.

Table 6: Results of conjugation and CAS screening of transconjugants

Number from conjugation	Number screened with CAS	Large halo colonies (Sid$^+$)	No halo colonies (Sid$^-$)
1687	~624	76	60

Determination of Interrupted DNA Sequence by Transposon Insertion

Once the transconjugants were characterized based on their ability or inability to produce siderophore, the chromosomal DNA was probed to ensure the integration of the transposon. The next step was to determine what gene was interrupted or affected by the transposon insertion. Siderophore overproducing transconjugants (Sid$^+$) were thought to have interruptions in genes or open reading frames involving the repressor mechanism of

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the siderophore system, transport of siderophore, retrieval of ferric ions from the siderophore, or enzymes involved in intermediate steps that are critical for internalization of the complex. Siderophore nonproducing transconjugants (Sid⁻) were hypothesized to have interruptions in the biosynthetic genes, assembly of the siderophore, signal transduction involved in expression of siderophore biosynthetic genes, or exportation of the siderophore molecule. As in any pathway within a bacterial cell, some intermediate steps that are essential but unidentified could also be affected thus producing the mutant's phenotype.

After ensuring the insertion of the transposon into the chromosome of transconjugants via Southern blotting using pRL27 as the probe DNA (data not shown), the digested chromosomal DNA of the transconjugants were treated with T4 DNA ligase. The chromosomal DNA was digested with either BamHI or PstI restriction enzyme that does not cut within the transposon sequence. The T4 DNA ligase treatment religated the fragmented chromosomal DNA of the transconjugants forming self replicating plasmids. The newly formed plasmids containing an origin of replication provided by the transposon and fragmented genomic DNA were transformed into *E.coli* DH5αλpir⁺ where subcloning and propagation could occur. The plasmid DNA of a nonproducing mutant, called Mutant 3-17, was isolated and was subjected to sequencing to identify interrupted genes by comparison to a protein sequence database (GenBank) using BlastX algorithm (Altschul, Gish, Miller, Myers, & Lipman, 1990) with comparison to known proteins as shown in Figure 30.

The first sequencing reaction was performed with the primers indicated in the literature to sequence outwardly into the interrupted chromosomal DNA of the

transconjugant (Sid⁻, Mutant 3-17). The results did not highly match significantly with any known protein and the results were very random. So to increase confidence in matching to known proteins, new primers were designed to facilitate another sequencing reaction (primer walking). The second sequencing reaction was performed with primers (Table 3) tpnRL27-3 and tpnRL27-4. The primers were designed to extend the sequence already received further could in turn help with matching to known proteins. Primers tpnRL27-3 and tpnRL27-4 followed tpnRL27-1 and tpnRL27-2 respectively as shown in Figure 31. The entire sequencing reaction was then subjected to a BLAST search.

With such a large amount of amino acid matches to a known protein (Figure 22), the interrupted DNA seems to code for a domain of a signal transduction molecule. The open reading frame interrupted was sequence by outward directed primers extending from the end of the transposon sequence (Figure 23). PAS/PAC is a system of signal transduction that responds to external cues for bacteria to respond to environmental stimuli, to maintain the proper gene expression, and to control homeostasis (Crosa, 1997; Etzkorn et al., 2008; Hoch, 2000; Hoch & Silhavy, 1995; Schmitt, 1999; Taylor & Zhulin, 1999). The proper gene expression is vital for internalizing nutrients that are available in the bacteria's immediate environment. *E.coli* and *Bacillus subtilis* genomic DNA contain over 30 two-component regulatory systems responding to different stimuli and controlling the expression of many genes (Etzkorn et al.; Fabret, Feher, & Hoch, 1999; Mizuno, 1997; Taylor & Zhulin). Two-component regulatory systems consist of a transmembrane sensor protein that responds to an inducer signal by modifying the phosphorylated state of a second component, usually a transcriptional regulator, whose affinity for the promoters it regulates is controlled by phosphorylation (Crosa, 1997;

Wosten, Kox, Chamnongpol, Soncini, & Groisman, 2000). PAS/PAC domains are an

example of a two component regulatory system with a function similar to the one just

described. With such a large amount of energy and cellular metabolites required for

expression of siderophore-mediated iron transport systems, the genes involved are

controlled in a negative (repressed, high Fe conditions) or in a positive (derepressed, low

Fe conditions) manner depending on the physiological and external environments

(Crosa). When iron limiting conditions are seen, the external cue of low iron conditions

is transduced into the cytosol and positively regulates gene expression of siderophore-

mediated iron transport systems. Ferric ions have been noted to bind directly to a

periplasmic region of a signal transduction molecule, PmrB in *Salmonella* species,

providing the first example of a signal transduction system that responds to extracellular

Fe^{3+} (Wosten, Kox, Chamnongpol, Soncini, & Groisman, 2000) controlling gene

expression. Siderophore molecules have been noted to be a signal inducing molecule in

Pseudomonas species, *E.coli,* and *Vibrio anguillarium* (Crosa) that can induce expression

of uptake systems via two component regulatory systems. To test this method of

induction by schizokinen, binding and transport assays with a radioactively labeled iron-

schizokinen complex (^{55}Fe-SK) were employed. These methods should provide an

indication if schizokinen could induce the expression of the dedicated outer membrane

receptor protein because the mutant used for analysis was unable to produce siderophore

under iron limiting conditions. With ^{55}Fe-SK present, the environment could stimulate

siderophore binding and uptake.

```
Qu.    FAITDHGPDMEDAPHHWHF  I NRIWPRVDGGILQIKTSSGAETSAFIERVADISQH
       +A +  HG +++    A          I   R          GILQIKTSSGAETSAFIERVADISQH
Sbjct  WASSSHGAEIQN AQSWSAT    IRQR-----GPA--GILQIKTSSGAETSAFIERVADISQH


Qu.    MAALALEQEKSRQHIEQLIQFDPMTGLPNRNNLHNYLDDLVDKAVSPVVYLIGVDHIQDV
       MAALALEQEKSRQHIEQLIQFDPMTGLPNRNNLHNYLDDLVDKAVSPVVYLIGVDHIQDV
Sbjct  MAALALEQEKSRQHIEQLIQFDPMTGLPNRNNLHNYLDDLVDKAVSPVVYLIGVDHIQDV


Qu.    IDSLGYAWADQALLEVVNRFREKLKPDQYLCRIEGTQFVLVSLENDVSNITQIADELRNV
       IDSLGYAWADQALLEVVNRFREKLKPDQYLCRIEGTQFVLVSLENDVSNITQIADELRNV
Sbjct  IDSLGYAWADQALLEVVNRFREKLKPDQYLCRIEGTQFVLVSLENDVSNITQIADELRNV


Qu.    VSKPIMIDDKPFPLTLSIGISYDLSKNRDYLLSTAHNAMDYIRKNGGNGWQFFSPAMNEM
       VSKPIMIDDKPFPLTLSIGISYDL KNRDYLLSTAHNAMDYIRKNGGNGWQFFSPAMNEM
Sbjct  VSKPIMIDDKPFPLTLSIGISYDLGKNRDYLLSTAHNAMDYIRKNGGNGWQFFSPAMNEM


Qu.    VKERWFRS- A----ERSDSIP--LKLV--TA---QIFAE
       VKER +   A  E +      LKLV         QIFAE
Sbjct  VKERLVLG ALK-E AISNNQLKLV--YQP- QIFAE
```

Figure 22: BLAST alignment showing homology between the sequence of a nonproducing

mutant (query) and a sensory kinase domain from *E. coli* (subject). The matched protein product

was a PAS/PAC domain (sensory kinase) from *E. coli* with identities = 228/271 (84%) and

positives = 236/271 (87%). The boxed regions indicate amino acid matches of the query and
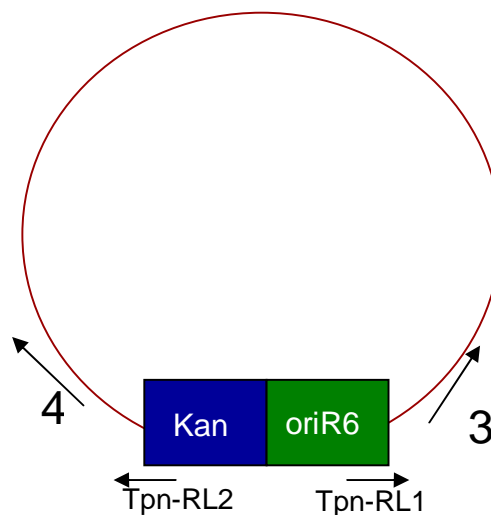
subject sequence.

Figure 23: Diagram of primer walking to illustrate how the open reading frame was sequenced.

The primers used for the first sequencing reaction were Tpn-RL2 and Tpn-RL1 and they anneal to the ends of the transposon thus sequencing into the interrupted chromosomal DNA of a transconjugant. Mutant used here for sequencing was Mutant 3-17.

Results of Concentration Dependent Binding of ^{55}Fe -Schizokinen

The binding assay was employed to answer two questions: did the transposon insertion affect the ability of the mutant to efficiently induce expression of the outer membrane receptor protein (OMRP) dedicated to siderophore internalization and also, what is the dissociation constant (K_D) of the wild type receptor that has not been determined? The OMRP faces the external environment and the loops of the protein facilitate the binding of the ferric-siderophore complex. The low temperatures maintained during the experiment prevented internalization of the complex and binding was detected by using a radioactive isotope of iron, ^{55}Fe. The isotope was bound to purified, iron free schizokinen (^{55}Fe-SK) and applied to iron starved cultures. The binding capacity of the cultures is based on the number of receptors present on the cells' surface. Theoretically, once all receptors are bound with the ^{55}Fe-SK complexes a plateau will be seen in the graphical representation of the data indicating saturation of all OMRPs.

The binding capability of the OMRP for schizokinen has not been previously defined, so it was important to evaluate this ability. The experiment was setup using a sid$^-$ mutant, mut 3-17, as a comparison to determine if the mutation present affected the organism's ability to bind the siderophore. Also, an *E.coli* strain KDF-541 was used to determine if a dedicated transporter was required for binding of the siderophore. KDF-

541 is a modified strain containing no iron transport system, so in theory the strain should not bind the siderophore (negative control).

The graph in Figure 24 shows a decrease in the binding ability of Mutant 3-17 as compared to the wild type strain. The transposon interruption, if in a transduction molecule, could prevent proper induction of a specific OMRP resulting in a decreased ability to bind the Fe^{55}-SK complex as compared to the wild type strain. With a decrease in induction present, less OMRPs could contribute to the decreased ability of mutant 3-17 (Sid$^-$) to bind the ^{55}Fe-SK complex properly. The dissociation complex or K_D is the concentration of ligand that occupies half of the receptors at equilibrium. In relation to siderophore receptors, the smaller the K_D value the higher the affinity of the receptor to the ligand. The approximate K_D of IARI 917 receptor for ^{55}Fe-SK is 54 nM. The relatively high value indicates the system is low affinity when compared to the OMRP, FepA in *E.coli*, whose K_D is in the range of 0.1-1 nM when tested under the same experimental conditions. The K_D of FepA is considered a very high affinity system.
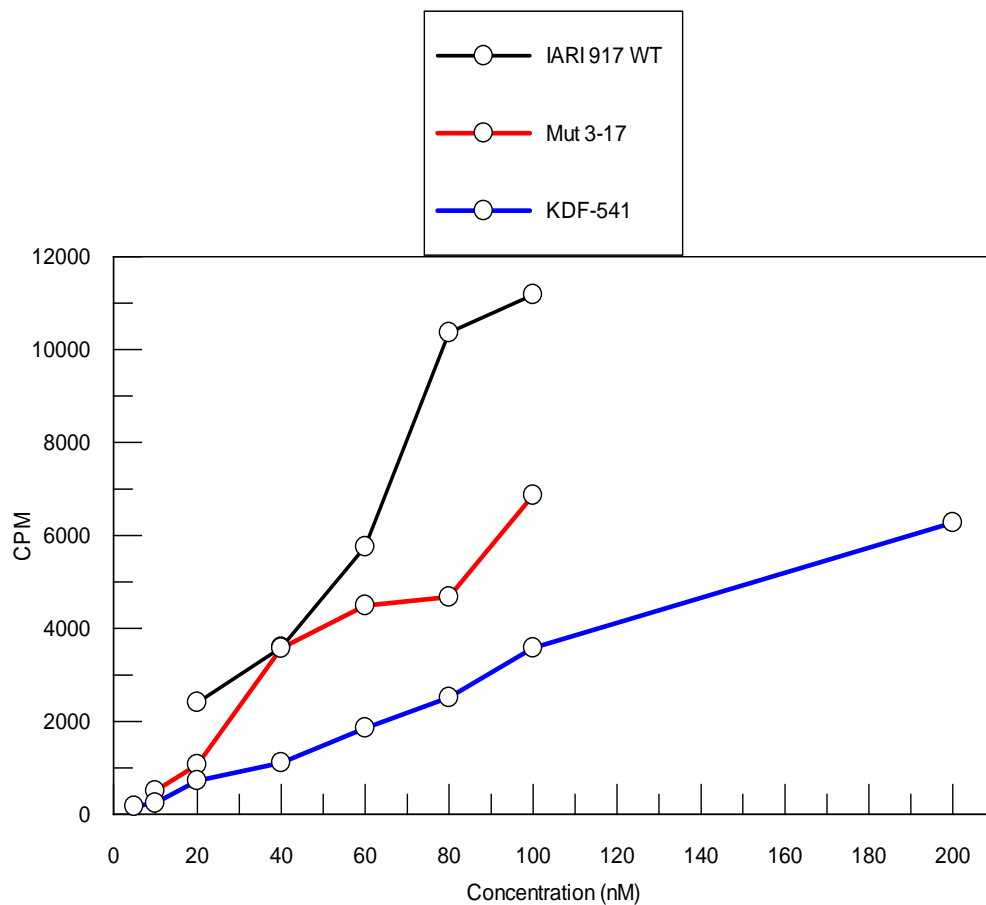
Figure 24: Concentration dependent binding of ^{55}Fe-SK by IARI 917 wild type, Mutant 3-17 (Sid$^-$), and KDF-541.

Results of Concentration Dependent Transport of ^{55}Fe -Schizokinen

The transport assay was performed to answer two questions: what is the K_m value of the OMRP for schizokinen and did the transposon insertion affect the transport of the Mutant 3-17 (Sid$^-$)? Similar to the binding of ^{55}Fe-SK, the transport of the complex has not been described in IARI 917. The transport assay is setup similar to the binding but optimal growth (30°C for rhizobia and 37°C for *E. coli)* conditions were present to facilitate internalization. If the bacteria were incubated at optimal growth conditions, internalization of the ferric-siderophore complex should occur as normal.

The graph in Figure 25 for the transport of ^{55}Fe-SK indicates the transposon insertion strongly affected the mutant's ability to transport siderophore. The K_M value is a kinetic parameter used to characterize an enzyme and is defined as a concentration of substrate that permits half the maximum velocity or rate of the reaction. The K_M value of IARI 917 wild type is approximately 48 nM that is also low when compared to FepA in *E.coli* whose K_M is in the range of 0.1-1 nM. The comparison of the two kinetic values are valid because both were determined using the same experimental conditions. The insertion could have affected a signal transduction molecule thus the signal is not received by the cell that iron limiting conditions are present. If the signal is not processed correctly by the cell, the proper gene expression will not be seen.
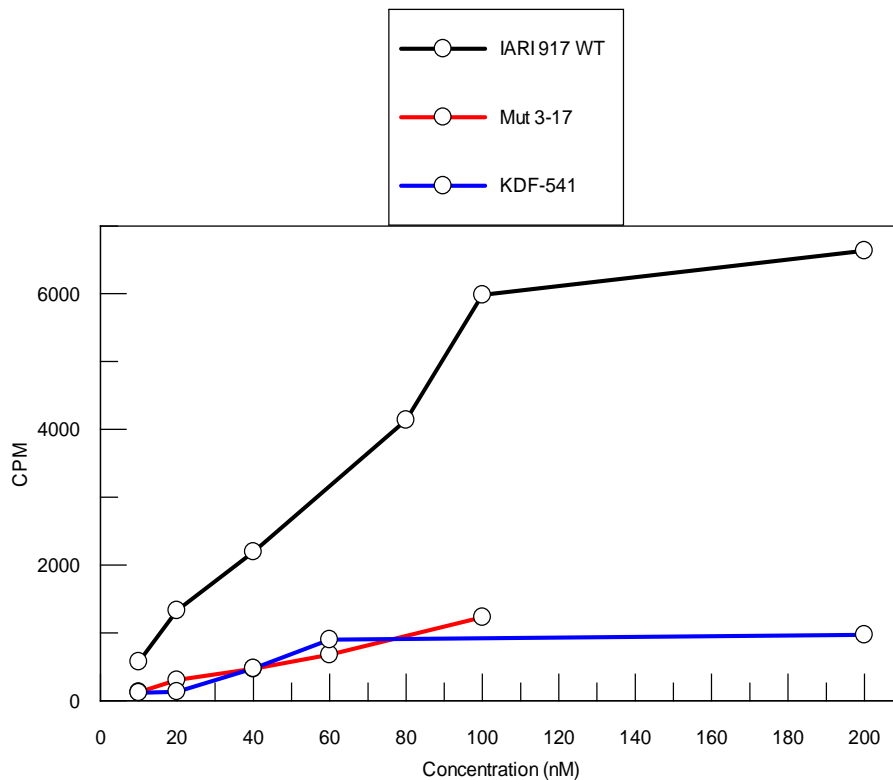

Figure 25: Concentration dependent transport of ^{55}Fe-SK by IARI 917 wild type, Mutant 3-17 (Sid⁻), and KDF-541.

Conclusion

Siderophores are produced by a number of microorganisms under iron limiting conditions. Most research focuses on pathogenic bacteria that produce siderophore in the host environment is known to increase an organism's virulence (Fischbach, Lin, Liu, & Walsh, 2006; Flo et al., 2004; Miethke & Marahiel, 2007; Ratledge & Dover, 2000). Most siderophore production and transport research has focused on *E.coli* as a model organism with minimal research devoted to rhizobial production of siderophore. With the agricultural significance of the rhizobial group, more research should focus on the physiology and metabolism of these important organisms. Nitrogen fixation by rhizobial root nodulation has been known to increase the overall health and yield of many agriculturally important crops. The presence of these organisms also increases the overall fertility and productivity of the soil with a relatively benign presence.

The focus of this research was to identify gene(s) located in the operon for the production and transport of schizokinen. A gene was identified with a high percentage of similarity to a signal transduction molecule in *E.coli*. The interrupted gene had an effect on the binding and the transport of ^{55}Fe-schizokinen. Some explanations for the mutation were discussed previously but more research should be performed to determine the exact mechanism of this interruption and its relevance to schizokinen biosynthesis or transport. Also, more research should focus on elucidating the complete biosynthesis, regulation, and transport operon of schizokinen in *Rhizobium leguminosarum* IARI 917.

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