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Iron Acquisition in *Rhodococcus erythropolis*: The Isolation of Mutant(s) that do not
Produce a Siderophore

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

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

by
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December 2001

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Keywords: *Rhodococcus*, Iron acquisition, Siderophore

ABSTRACT

Iron Acquisition in *Rhodococcus erythropolis*: The Isolation of Mutant(s) that do not

Produce a Siderophore

by

Jaishree M. Vellore

Rhodococcus, a soil bacterium, displays a diverse range of metabolic capabilities with a number of potential practical applications. To exploit the metabolic potential of *Rhodococcus*, their basic physiology, genetics, and especially the acquisition of essential nutrients like iron, must be understood.

R. erythropolis strain IGTS8 releases a small compound called a siderophore that scavenges ferric iron from the environment. To learn more about the genetic control of iron acquisition, mutant(s) defective in siderophore production were isolated. Mutants were generated by inserting a mutagenic plasmid, pJCS506, into the bacterial cell using electroporation. The plasmid, which cannot replicate in these bacterial cells, randomly inserts into the *R. erythropolis* genome producing mutations. The potential mutants were detected by screening on a chrome azurol S plate, which detects siderophore production. Colonies that failed to produce siderophore were tested by liquid assays. The strain N5-59 was confirmed as a non-siderophore producing mutant by liquid assays.

DEDICATION

To My Parents

ACKNOWLEDGMENTS

I am deeply indebted to Dr. Lampson, my major advisor, for his guidance and support. He took time to get me started on my project, to help me when I ran into obstacles, and to explain to me difficult concepts and procedures. Thank you! I am grateful to my committee members, Dr. Mustain and Dr. Pike, for their valuable input to my project. I am also grateful to the faculty of the Health Sciences and Biological Sciences for making my course work interesting and enjoyable. The staff members of the Department of Health Sciences were very helpful. Ralph Coffman and Robin Grindstaff helped me find chemicals and other materials that I needed and removed media from the autoclave when I was away. Nancy Coffman cheerfully helped me purchase materials needed for my research and in many other ways. I am thankful to the Department of Health Sciences for providing me with financial assistance. Dennis Kunkel Microscopy, Inc., and Lippincott Williams & Wilkins were kind to grant me permission to use photographs.

My parents have been a major influence in all my successes. I am grateful to them for the values and principles they have taught me. My husband, Bhaskar Arumugam, has been very supportive over the last two years. His discipline and meticulous nature was a positive influence. Following his advice that a major task like completing a master's thesis project can be accomplished by working consistently, one step at a time, helped me greatly in completing my thesis in a timely manner. When I was busy preparing for exams or attending late evening classes, he thoughtfully prepared warm dinners for me. Thank you, God, for blessing me with a loving, caring, and supportive husband.

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CHAPTER 1

LITERATURE REVIEW

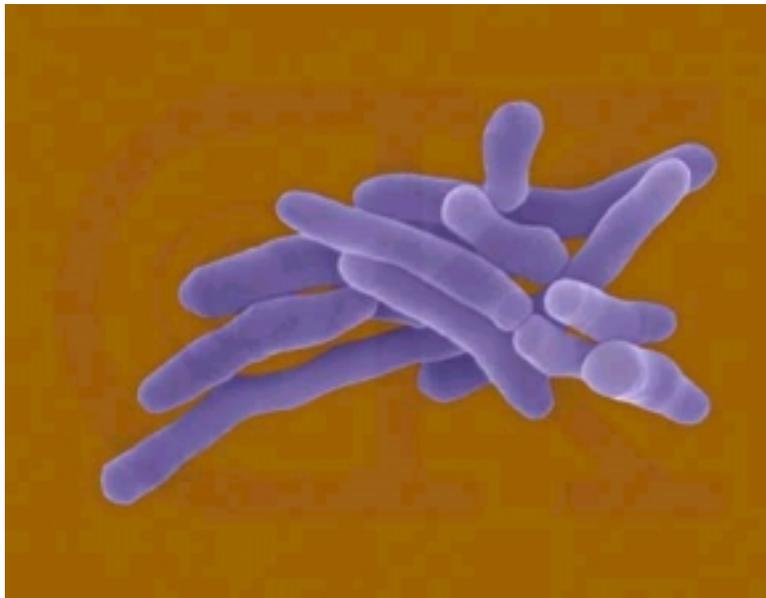
Introduction

This thesis describes my research on the iron acquisition system of *Rhodococcus erythropolis*. *R. erythropolis* is an aerobic soil bacterium that displays a diverse range of metabolic capabilities. To exploit the metabolic potential of *Rhodococcus*, their basic physiology and genetics must be understood. This potential exploitation has commercial implications for various industries. For example, *R. erythropolis* strain IGTS8's capability to desulfurize selectively petroleum-based compounds makes it a microbe of commercial importance to the chemical and biotechnology industries. Little is known about iron acquisition in *Rhodococcus*. Iron is an essential nutrient for growth of most bacteria and plays an important role in the functioning of various processes. Iron is the fourth most abundant element on the earth's crust and seems to be readily available to meet the requirements of all organisms. However, the availability of iron for biological consumption in aerobic environments is severely restricted due to the extreme insolubility of ferric iron at neutral pH. The amount of iron a cell acquires must be regulated because too much free iron in a bacterial cell is a potential poison. Hence, the acquisition and regulation of this essential nutrient, iron, plays an important role in bacteria and needs further elucidation.

Characteristics of *Rhodococcus*

The genus *Rhodococcus* includes a diverse group of microorganisms. There are currently 13 established *Rhodococcus* species, namely *R. chlorophenolicus*, *R. coprophilus*, *R. equi*, *R. fascians*, *R. erythropolis*, *R. globerulus*, *R. marinonascens*, *R. opacus*, *R. percolatus*, *R. rhodnii*, *R. rhodochrous*, *R. ruber*, and *R. zopfii* (Bell *et al.* 1998). These species of *Rhodococcus* are found in various environmental niches, such as soil, rocks, ground water, seawater, animal dung, and the gut of insects. Various members of the genus *Rhodococcus* are pathogenic for animals, plants, and humans. Rhodococci are described as aerobic, gram positive, non-motile, non-sporulating, and catalase positive microorganisms. They have a variety of growth patterns. They can form filaments with short projections, elementary branching, or, in some species, extremely branched hyphae (Figure1) (Finnerty 1992). The filaments formed by rhodococci can undergo fragmentation into rod shaped or coccoid elements. Rhodococci form colonies that are entire, smooth, and large with a diameter ranging from 2 to 4 millimeter (mm). Colonies formed by most rhodococci have a distinct red, orange, or salmon-pink pigmentation (Figure 2). The pigmentation is believed to be caused by the presence of carotenoids. Rhodococci characteristically contain mycolic acid in their cell wall and are related to other mycolic acid containing genera, namely *Corynebacterium*, *Mycobacterium*, and *Nocardia* (Bell *et al.* 1998). Mycolic acid helps to prevent dessication of the bacteria in dry environmental conditions. *Rhodococcus* cells are hydrophobic due to the aliphatic chains of mycolic acid in their cell wall.

The genus *Rhodococcus* is defined on the basis of cell wall composition. The cell walls of rhodococci have the following characteristics: (1) N-acytylglucosamine, N-glycolylmuramic acid, D- and L-alanine, D-glutamic acid and *meso*-diaminopimelic acid are present in the peptidoglycan; (2) the cell wall carbohydrates consist of arabinose and galactose; (3) cardiolipin, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylinositol mannosides are present in the phospholipids; (4) tuberculostearic acid is present along with normal, saturated and unsaturated, fatty acids; and (5) mycolic acids contain 34-52 carbon atoms (Finnerty 1992).



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Figure 1. Scanning Electron Microscopy of Rhodococci.

Rhodococci can form filaments, that can undergo fragmentation into rod shaped or coccoid elements. Magnification: x 27, 200.



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Figure 2. Culture of *Rhodococcus* Species.

Colonies are large with a diameter ranging from 2-4 mm, entire, smooth and have a distinct salmon-pink pigmentation.

The Importance of Rhodococcus

The economic significance of *Rhodococcus* species is being recognized increasingly. Rhodococci display a diverse range of metabolic capabilities and these features are present in various environments. They exhibit the ability to degrade a wide range of organic compounds. Rhodococci can degrade various chemical pollutants such as simple hydrocarbons, aromatic hydrocarbons, nitroaromatics, chlorinated polycyclic aromatics such as polychlorinated biphenyls (PCBs), and other recalcitrant toxic pollutants (Bell *et al.* 1998).

Chlorinated phenols are a class of chemicals that are recalcitrant to degradation and they exist as hazardous and persistent pollutants in soil and ground water. A new chlorophenol-degrading microbe, *R. chlorophenolicus* was isolated (Finnerty 1992). This organism was found to efficiently degrade a series of chlorinated phenols ranging from dichloro to pentachloro compounds. This species is currently not included in the list of the established *Rhodococcus* species. Though most of the research has been done on *R. chlorophenolicus*, other *Rhodococcus* strains are also capable of degrading chlorophenols. Another class of chemicals that are hazardous and persistent in the environment are polychlorinated biphenyls, chemicals that are widely used in industry due to their stability. *R. rhodochrous*, *R. globerulus*, *R. erythropolis*, and other unclassified rhodococci are known to degrade various polychlorinated biphenyls and may help in the bioremediation process. Rhodococci can degrade various recalcitrant toxic pollutants such as sulphonated azo dyes, and pesticides such as metamitron, s-triazines, and *n*-methyl carbamates (Bell *et al.* 1998). Rhodococci are ideal candidates for enhancing bioremediation of contaminated sites because of three characteristics: (1) their environmental persistence; (2) their tolerance to starvation; and (3) their frequent lack of catabolite repression. *Rhodococcus* species have the ability to produce biosurfactants and bioflocculants. A surfactant is a surface-active agent. Biosurfactants produced by rhodococci may be more effective and more efficient than the existing synthetic surfactants. They have the advantage of being less toxic and are biodegradable. A flocculant can cause flocculation of a wide variety of suspended solids. Bioflocculants

produced by rhodococci help in the removal of suspended solids in wastewater or effluent treatment (Bell *et al.* 1998).

Rhodococcus species have the potential to remove organic sulfur from fossil fuels such as coal and petroleum. Fossil fuels contain organo-sulfur compounds such as dibenzothiophene. The emission of organo-sulfur compounds from the combustion of fossil fuels is associated with the problem of acid rain. *R. erythropolis* strain IGTS8 has the metabolic capability to convert certain aromatic sulfur compounds like dibenzothiophene to 2-hydroxybiphenyl and sulfite. This microbe specifically breaks the carbon-sulfur bonds in dibenzothiophene for the removal of sulfur without degrading the aromatic ring (carbon-carbon bonds). This desulfurization does not affect the fuel value of the compound. This special property of IGTS8, known as biocatalytic desulfurization, is being developed by the biotechnology industry (Gray *et al.* 1996).

Rhodococcus species are capable of a variety of biotransformations (Bell *et al.* 1998). Biotransformation is the use of living organisms to cause specific minor modification of compounds. The enzymes that are responsible for this conversion can be intracellular or extracellular. The production of acrylamide from nitriles is an example where the overproduced nitrilase enzyme of *R. rhodochrous* strain J1 is exploited and used as a biocatalyst to produce more than 30,000 tons of acrylamide annually by Nitto Chemistry Industry Company Ltd. (Japan). Other biotransformations catalyzed by rhodococci include steroid modification and enantioselective synthesis (Larkin *et al.* 1998).

Other applications of rhodococci include oil prospecting, fecal detection, biosensors, and bio-leaching. Some strains of rhodococci can grow using gaseous hydrocarbons such as propane, butane, and acetylene. The detection of such strains that oxidize gaseous alkanes in soil or ground water could be used as an indicator for the presence of subterranean hydrocarbon deposits. Some species of rhodococci are used as fecal indicator organisms. For example, *R. corprophilus* can grow in animal dung. Hence, the detection of this species in waterways can be used to assess pollution by farm animal effluents. *Rhodococcus* cells and enzymes are used for the construction of biosensors. Biosensors play a role in the detection of target compounds. For example, a *Rhodococcus* strain that produces heroin esterase is being developed as a biosensor for detection of heroin. Another application of rhodococci is in the area of bio-leaching. Bio-leaching is the use of microbes to solubilize metals from low-grade ores (Bell *et al.* 1998).

Rhodococcus and Disease

The incidence of rhodococcal infections is increasingly being reported. Rhodococci can produce disease in plants, animals, and humans. The infections in humans are being reported in both immuno-compromised and non-immuno-compromised hosts.

Rhodococcus fascians is a plant pathogen that lives on the surface of the host tissue. *R. fascians* has a broad host range, causing infection in diverse plant species, including crop plants such as peas and tobacco. The production of cytokinins by these bacteria is the main virulence factor. The cytokinin causes fasciation in plants. This

disease is characterized by abnormal growth of the plant such as loss of apical dominance and growth of lateral shoots (Bell *et al.* 1998).

Rhodococcus equi is a veterinary pathogen and causes pneumonia in foals. Infection with *R. equi* is also been reported in adult horses and pigs. *R. equi* is found in the soil and in the gut of herbivores. The main route of infection in animals and humans is through the inhalation of dust (Mosser and Hondalus 1996). *R. equi* is a facultative, intracellular bacterium that mainly infects the macrophages in the lungs. *R. equi* normally infect the respiratory tract in foals and cause chronic pus filled lung abscesses (Bell *et al.* 1998). If the lesions are left untreated, the animals can die of asphyxiation.

R. equi infections are also reported in humans and are isolated with increased frequency from patients undergoing immuno-suppressive therapy, especially from patients with acquired immuno-deficiency syndrome (AIDS). The main route of *R. equi* infections in humans is through contact with livestock and manure. Inhalation of dust containing the organism is also a source of infection. *R. equi* causes pneumonia in infected persons. The symptoms that the affected individuals express are fever, cough, chest pain, pulmonary abscess, and cavitation. Bacteremia and formation of lesions in other organs can occur when the infection spreads (Mosser and Hondalus 1996).

CHAPTER 2

IRON ACQUISITION IN MICROORGANISMS

Most living organisms require iron for growth. Iron is the fourth most abundant element in the earth's crust. Although iron is abundant in the environment, it is not readily available. Iron exists either in the ferrous, Fe (II) or ferric, Fe (III) state. Iron is required to be in the ferrous state for use by microorganisms. In this reduced form, iron is soluble and can be easily taken into the living cell through diffusion. However, under aerobic conditions, ferrous iron is oxidized to ferric iron. In this ferric state, iron exists as oxy-hydroxide polymers, which are not very easily soluble (Neilands 1995). These hydroxides are very stable and are extremely insoluble (10^{-18} μM) at neutral pH (Vasil and Ochsner 1999). The availability of iron in vertebrate hosts is scarce. In vertebrates, iron after being assimilated from food passes into the blood stream, where it complexes with proteins called transferrin (Ratledge and Dover 2000). There are 3 different types of transferrins: serum transferrin, lactotransferrin, and ovotransferrin. The iron bound to the transferrin is transported to various cells. After the iron has been donated, the transferrin is recycled and reused to bind iron in the blood stream. The iron binding capacity of transferrins ensures that there is little free iron in the circulation of vertebrate hosts. Hence, the acquisition of iron, either from the environment or from vertebrate hosts, is problematic.

Microbes have evolved various mechanisms to scavenge iron from the abundant but biologically unusable sources in the environment as well as in animal hosts. Among

the various mechanisms employed, production of iron binding compounds called siderophores is the best studied. The other mechanisms that microbes have used to acquire iron are: (1) reduction of extremely insoluble forms of ferric ion to soluble forms of ferrous ion that can be used easily; (2) use of iron present in hemoglobin by the destruction of erythrocytes and hydrolysis of hemoglobin; (3) direct use of the iron stored in ferritin (Ferritin, found in animals, plants [phytoferritins] and even microorganisms [bacterioferritin], are complexes that store iron in a form that is soluble, bio-available, and non-toxic); and (4) enzymatic degradation of compounds that bind iron like transferrin (Vasil and Ochsner 1999).

The Importance Of Iron

A large number of proteins require iron for their activity, which underlines the importance of iron for living organisms. The iron that is present in proteins can exist in several different forms: heme, iron-sulfur, iron-nickel, di-iron and mononuclear iron (Andrews 1998). Iron containing proteins participate in various processes such as: (1) respiration where cytochromes, ferredoxins, and other iron-sulfur proteins play a role; (2) activation of oxygen where cytochrome oxidase plays a role; (3) degradation of hydrogen peroxide and hydroxyl radicals where heme-catalase, iron-superoxide dismutase, and peroxidase play a role; (4) amino acid and pyrimidine biosynthesis where glutamate synthase and dihydro-orotate dehydrogenase play a role; (5) the citric acid cycle where fumarase, aconitase, and succinate dehydrogenase play a role; (6) DNA synthesis where ribonucleotide reductase plays a role; (7) nitrogen fixation where nitrogenase plays a role;

(8) carbon fixation metabolism where methane monooxygenase plays a role; (9) photosynthesis where light-harvesting complexes and ferredoxin play a role; and (10) oxygen binding where globins play a role (Andrews 1998).

The concentration of iron required for optimum growth of cells is at least one micromolar (μM). Though iron is required by a majority of microorganisms, there are some exceptions like the lactic acid bacteria. Their growth is not enhanced by the addition of iron because they do not contain heme enzymes and the iron containing ribonucleotide reductase (Neilands 1995).

If a cell has too much free iron (ferrous ion), it is a potential poison. High concentration of ferrous ion can provoke the production of hydroxyl radicals as shown in the following equation, the Fenton reaction (Crichton and Charlotheaux-Wauters 1987).



This problem is alleviated with enzymes such as superoxide dismutase, catalase, and peroxidase that can degrade reactive oxygen species as shown in the following equations.



Iron toxicity is also alleviated by the presence of anti-oxidants such as glutathione and enzymes such as endonucleases that repair damages caused to DNA during redox stress (Andrews 1998).

Siderophore Mediated Iron Acquisition

A high affinity system for the uptake of iron from the external medium is present in many microorganisms. This system has three parts: (1) a siderophore that acts as a high affinity ferric-ion specific ligand that is usually released to the extracellular environment by microbes; (2) a membrane receptor for iron bound-siderophore (ferri-siderophore) complex that transports the chelated iron across the microbial membrane; and (3) an enzymatic system that is present within the cell that can release ferric ion bound to the siderophore.

Siderophores are water soluble, low molecular weight molecules that are secreted by bacteria and fungi. The term siderophore stands for “iron carriers” or “iron bearers” in Greek. There are more than 500 different types of siderophores produced by bacteria, yeasts, and fungi. Siderophores are produced and secreted only when the amount of iron is low in the growth environment. The genes involved in siderophore production regulate siderophore production based on the concentration of iron in the environment. That is, siderophore production is shut off when iron is present at sufficient concentration and vice versa. Siderophores specifically bind to ferric ion with high affinity. The binding power of the siderophore for iron has a stability constant range from 10^{22} to 10^{50} (Ratledge and Dover 2000). This range is sufficiently high for the removal of iron attached to molecules like ferritin and transferrin by siderophore, but not high enough for the removal of iron present in heme proteins. Siderophore molecules display considerable structural variation but can be classified as either hydroxamates or catechols. Structurally, siderophores are ring or semi-ring shaped structures containing oxygen atoms.

Siderophores show high affinity for ferric ion, since the oxygen atoms present can form coordination bonds with a single Fe (III) ion (Neilands 1995). The production of siderophores has been reported in aerobic and facultative anaerobic microbes, but their production has not yet been reported in strict anaerobes, lactic acid bacteria, or in higher organisms such as plants and animals. The main function of siderophores is involved in the high affinity acquisition and receptor dependent transport of ferric ion. Siderophores are also associated with growth or germination factors and virulence factors (Crichton and Charloteaux-Wauters 1987).

Not much is known about iron acquisition in *Rhodococcus*. Understanding iron acquisition in other bacterial genera provides the background for the study of iron acquisition in *Rhodococcus*. Iron acquisition and uptake is best characterized in the enteric bacterium, *Escherichia coli*. *E. coli* employs the strategy of producing siderophores for iron acquisition. *E. coli* produces 2 types of siderophores, aerobactin and enterobactin (or enterochelin). The stability constant of aerobactin for ferric ion is 10^{23} and the stability constant of enterobactin for ferric ion is 10^{52} . *E. coli* can use siderophores produced by other bacteria and fungi. For example, *E. coli* can use the siderophores ferrichrome, coprogen, rhodotorulic acid, and ferrioxamine produced by fungi, for iron acquisition (Ratledge and Dover 2000). In *E. coli*, the Fur (ferric iron uptake regulator) protein negatively regulates the genes that are involved in iron acquisition.

Once the siderophore is bound to the ferric ion, the ferri-siderophore complex has to be transported into the cell. *E. coli*, a gram negative bacteria, has an outer membrane

which has pores or porins that allow the transport of substances smaller than 600 daltons. The ferri-siderophore complex is too large (varies between 500-1000 daltons) to diffuse through the pores present in the outer membrane.

The uptake of ferri-siderophores is carried out by specific, high affinity receptor proteins present on the surface of the outer membrane. The receptors are thought to undergo conformational changes to transport the ferri-siderophore complex into the cell. The binding of ferri-siderophores to the receptors has a dissociation constant (K_D) value of $< 0.1 \mu\text{M}$ (Braun and Killmann 1999). It is also interesting to note that these receptors in *E. coli* are sites where phages can bind. The receptor protein forms a channel across the outer membrane and these channels regulate transport of ferri-siderophores across the outer membrane. The receptor protein may have a closed channel, which undergoes conformational changes and opens for transport of ferri-siderophores (Braun and Killmann 1999).

Energy is needed for the release of the ferri-siderophore from its receptors as well as transport across the outer membrane. Energy is also required for the controlled opening and closing of the channels of the receptor proteins (Braun and Killmann 1999). However, the presence of energy generating mechanisms in the outer membrane is not known. In fact, the proton motive force generated at the cytoplasmic or inner membrane drives the transport of ferri-siderophore across the outer membrane. This process requires a Ton system. The Ton system consists of TonB, ExbB, and ExbD proteins. The TonB protein is present in the periplasm and is fastened to the cytoplasmic membrane via its N-terminus (Figure 3). The TonB protein spans the periplasm and interacts with the outer

membrane. The TonB protein interacts with two additional proteins, ExbB and ExbD, that are anchored in the cytoplasmic membrane (Guerinot 1994). Most of the ExbB protein is present in the cytoplasm and its N-terminus protrudes into the periplasm (Figure 3). The ExbD protein is present mostly in the periplasm (Figure 3). The functions of ExbB and ExbD proteins are not yet known. The TonB protein functions as an energy transducer (Ratledge and Dover 2000). The physical translocation of iron and its chelates through the outer membrane into the periplasm requires TonB. TonB couples the proton motive force generated at the cytoplasmic membrane to the outer membrane receptors, thereby bringing about active transport of iron.

Once the ferri-siderophore is delivered into the periplasm, it is bound by binding proteins that are present in the periplasm. *E. coli* is known to carry three different types of periplasmic binding proteins: FhuD, FecB, and FepB. The function of these binding proteins is to carry the ferri-siderophore to the transport proteins, FhuB and FhuC, in the cytoplasmic membrane. ATP hydrolysis drives the transport of the ferri-siderophores across the cytoplasmic membrane (Braun and Killmann 1999). It has been demonstrated that ATP hydrolysis is triggered only after the binding proteins in the periplasm are loaded with the substrate, ferri-siderophore. This ensures that ATP hydrolysis occurs only when ferri-siderophores have to be transported across the cytoplasmic membrane.

The release of iron from the intact ferri-siderophore complex occurs either by reduction or chemical breakdown of the ligand, siderophore. The release of iron from the stable ferric siderophore complexes via reduction is accomplished by ferric reductases.

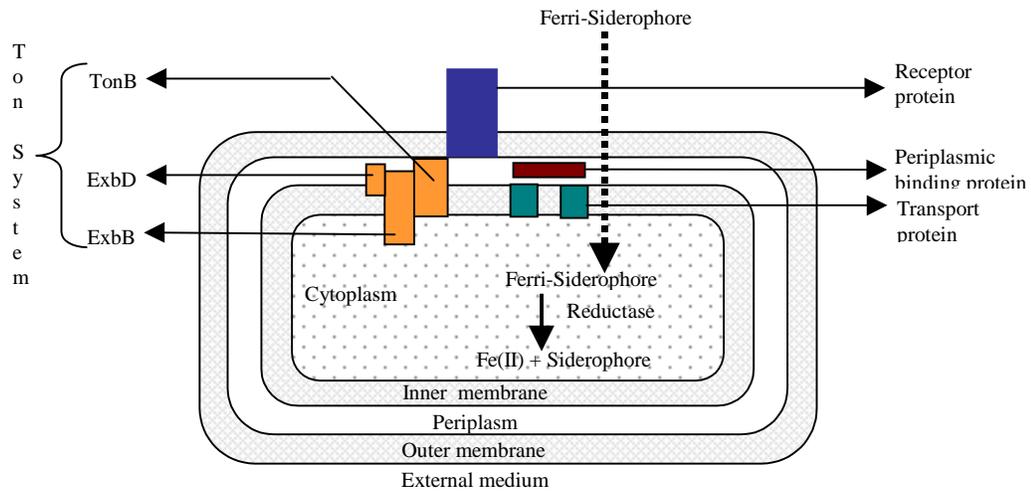


Figure 3. An Iron-Uptake System in *Escherichia coli*.

When iron is scarce, *E. coli* produces siderophores, which bind to ferric iron in the external medium. The transport of ferri-siderophore complexes across the outer membrane is carried out by the receptor protein present in the outer membrane. The receptor protein for the transport of aerobactin is IutA and for enterobactin is FepA. The proton motive force generated at the cytoplasmic membrane drives the transport of ferri-siderophore across the outer membrane by a protein complex called the Ton system. The periplasmic binding proteins bind to the ferri-siderophore complexes and deliver them to the transport proteins present in the cytoplasmic membrane. In *E. coli* the periplasmic binding protein for aerobactin is FhuD and for enterobactin is FepB. The transport proteins for aerobactin are FhuB and FhuC and for enterobactin are FepC, FepD, and FepG. ATP hydrolysis drives the transport of the ferri-siderophores across the cytoplasmic membrane. The ferric-siderophore is then reduced to ferrous-siderophore by enzymes such as ferric reductase. The siderophores may be recycled to be used again.

Ferric reductases are present in many bacteria and use NADH or NADPH as the reductant as shown in the following equation (Ratledge and Dover 2000):



The Fe (II)-siderophore complex is not very stable because siderophores have very little affinity for ferrous iron. The reduced form of iron, Fe (II), is released from the siderophore (Ratledge and Dover 2000). Chemical modification of the ligand during or after the reduction process is possible. Modification of enterobactin during the release of iron occurs by acetylation. The modified enterobactin cannot be reused as it has a decreased affinity for ferric iron. Aerobactin is not modified during the release of iron and is reused for iron transport. The second method by which the release of iron from the ferri-siderophore complex can occur is by the chemical breakdown of the ligand by the enzyme, esterase. Esterase hydrolyzes the ligand thereby releasing iron. The siderophore is destroyed during the above process. The above mechanism has been shown to be used for the release of iron from ferri-enterobactin complex (Crichton and Charloteaux-Wauters 1987).

Although the iron acquisition system is best studied in *E. coli*, *E. coli* is distantly related to *Rhodococcus*. It is perhaps more relevant to review the iron uptake mechanisms of the more closely related mycobacteria.

Mycobacteria are aerobic, acid-fast bacteria containing mycolic acids in their cell wall. Some species of mycobacteria are free living organisms in soil and water while other species such as *Mycobacterium smegmatis* are harmless saprophytes. However, some species of mycobacteria are pathogens causing diseases such as tuberculosis (*M.*

tuberculosis) and leprosy (*M. leprae*). Iron is an essential nutrient for the growth of mycobacteria and they require about 7 to 64 microgram (μg) of iron per gram of cells (De Voss *et al.* 1999). Mycobacteria produce siderophores to solve the problem of iron acquisition from the environment or host intracellular sources. Mycobacteria produce both extracellular siderophores and a cell wall associated siderophore. The extracellular siderophore plays a prominent role in the acquisition of environmental iron while the cell wall associated siderophore facilitates transport into the cell cytoplasm (De Voss *et al.* 1999). Three types of siderophores are produced by mycobacteria: mycobactins, exochelins, and carboxymycobactins.

Mycobactins are lipid soluble intracellular siderophores located adjacent to the cytoplasmic membrane of mycobacteria. They are extremely insoluble in water. Mycobactins have a distinct phenyloxazolidine ring. They are known to have high affinity for iron. The importance of mycobactins may lie in the possible role they play in the short-term storage of iron within the cell envelope before it is transferred into the cell (Ratledge and Dover 2000).

Exochelins are one of the extracellular siderophores produced by mycobacteria. Exochelins are water-soluble peptide-like siderophores. The iron chelating ability of exochelin is associated with the ornithine-derived hydroxamates (De Voss *et al.* 1999). The production of exochelins occurs only in saprophytic mycobacterial species and not in pathogenic mycobacteria. Iron from sources such as ferric hydroxide, ferric phosphate, and ferritin are solubilized by exochelins (Ratledge and Dover 2000). De Voss and co-workers (De Voss *et al.* 1999) have elucidated the structure of the major exochelin

produced by *M. smegmatis* (Exochelin MS) and one of the exochelins from *M. noecaurum* (Exochelin MN). Exochelin MS is a formylated pentapeptide consisting of 3 molecules of δ -N-hydroxyornithine, 1 β -alanine, and a threonine. The iron coordination sites are provided by hydroxamic acids obtained from the three-ornithine moieties (De Voss *et al.* 1999). Exochelin MN is a hexapeptide. It contains 2 δ -N-hydroxyornithine, a β -hydroxyhistidine, 2 β -alanines, and an ornithine. The iron coordination sites are provided by δ -N-hydroxyornithines and β -hydroxyhistidine (De Voss *et al.* 1999).

Carboxymycobactin is the other extracellular siderophore produced by mycobacteria. Carboxymycobactins are related to mycobactins except that their modified structure makes them water soluble and thereby recoverable from culture filtrates. Mycobactins have a long alkyl chain while carboxymycobactins have a shorter carboxylic chain (Ratledge and Dover 2000). Iron from a number of sources including ferritin and transferrins are solubilized by carboxymycobactins. Carboxymycobactins are produced by pathogenic mycobacteria. Experiments have indicated that there is an increase in the amount of carboxymycobactin produced as the virulence of a particular strain increases (Ratledge and Dover 2000). Hence, the production of carboxymycobactins may play a role in the pathogenicity of mycobacteria.

The transport of the ferri-siderophore complexes into the mycobacterial cell is not yet known and needs further research.

Regulation of Siderophore Production

Regulation by the Ferric Iron Uptake Regulator

The production of siderophores is highly regulated by the concentration of iron present within a cell. In *E. coli* the production of siderophores is negatively regulated by the ferric iron uptake regulator, Fur. The Fur protein is a repressor containing 148 amino acid residues with high histidine content (Braun *et al.* 1998). The term “Fur box” or “iron box” is given to the site where the Fur protein binds to the DNA. Iron, Fe (II), is required as a corepressor for the Fur protein to act as an active repressor.

In the absence of iron or when the concentration of iron is low in the cell, the Fur protein does not bind to the operator DNA due to weak affinity (Guerinot 1994). However, at higher concentrations of iron, the Fur protein binds tightly to the operator DNA and thereby represses the expression of the genes that encode the proteins involved in iron acquisition, i.e., siderophores (Braun and Killmann 1999). The Fur protein is known to regulate genes involved in toxin production, like the shiga toxin from *Shigella dysenteriae* and exotoxin A from *Pseudomonas aeruginosa* (Litwin and Calderwood 1993).

Regulation by the Diphtheria Toxin Repressor

Diphtheria toxin is produced by toxigenic strains of *Corynebacterium diphtheriae*. *C. diphtheriae* can cause the fatal respiratory disease, diphtheria.. The major virulence factor of *C. diphtheriae* is the production of diphtheria toxin. The diphtheria toxin

repressor (DtxR) is an iron-activated regulatory protein that represses the expression of the *tox* gene for diphtheria toxin. The DtxR repressor also controls the production of siderophore in an iron-sensitive manner.

Only strains of *C. diphtheriae* that are infected and lysogenized by corynebacteriophage carrying the structural gene *tox* for the toxin can cause diphtheria. Expression of diphtheria toxin by the *tox* gene is regulated negatively by the activity of the iron-dependent DtxR, which is encoded on the chromosome of the host bacterium (Love and Murphy 2000). Expression of the *tox* gene in *C. diphtheriae*, and thereby diphtheria toxin, occurs only when iron in the growth medium is depleted. Under iron sufficient conditions there is repression of diphtheria toxin production.

The repressor DtxR is converted into an active repressor, $(\text{Fe}\cdot\text{DtxR})_2$, in the presence of the co-repressor, Fe (II). Co-repressors act by combining with the repressor which causes a conformational change in the repressor to form an active complex. The repressor is active only when cells are growing in iron sufficient growth conditions because under these conditions intracellular levels of the co-repressor, Fe (II), will be high. The active repressor binds to the *tox* operator and blocks transcription by preventing mRNA synthesis of the *tox* gene (Figure 4). Two active repressors bind to the *tox* operator and repress *tox* expression. The *tox* operator is a 27 base pair inverted repeat nucleotide sequence (palindromic sequence) located upstream from the *tox* gene (Tao *et al.* 1994). Under iron-limiting conditions, the active repressor that is bound to the *tox* operator dissociates and the *tox* gene is expressed producing toxin. A similar operator system also controls the genes required for the production of siderophores.

In conclusion, the production of the virulence factor diphtheria toxin and the production of siderophores that are used for high affinity uptake of iron are coordinately regulated by the activity of the iron dependent repressor, DtxR, and repressed under high iron growth conditions (Tai *et al.* 1990).

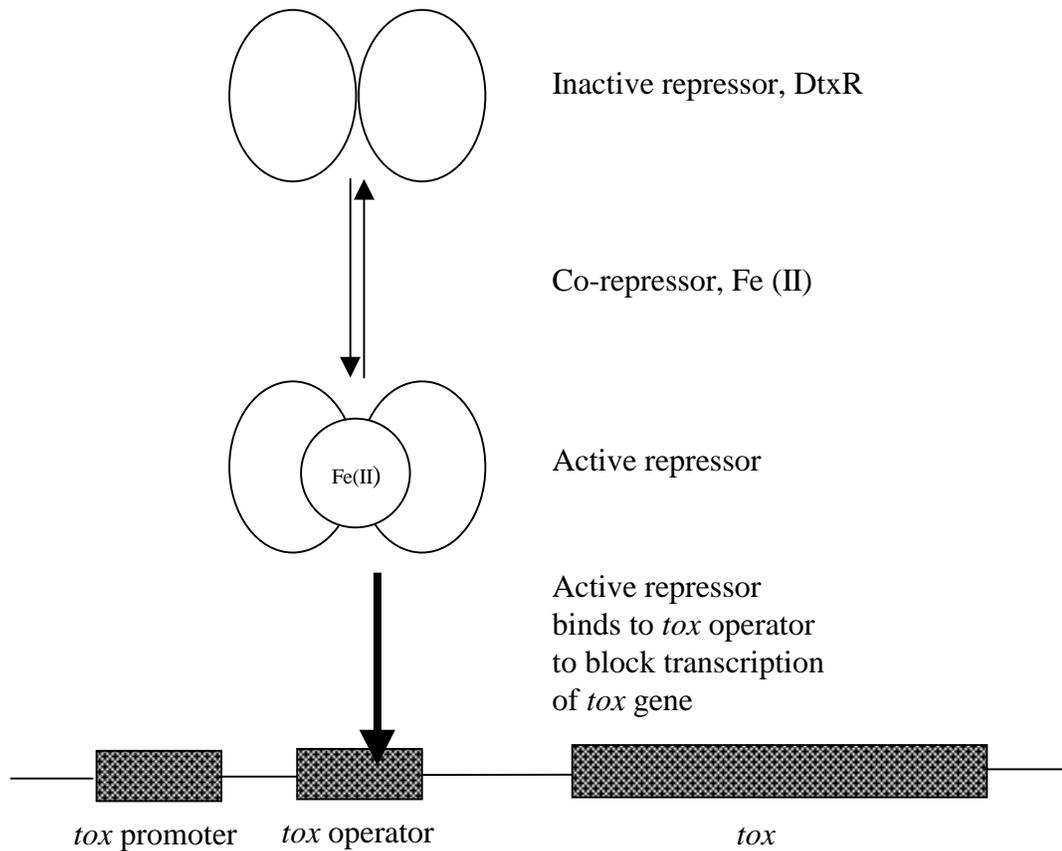


Figure 4. Regulation of Diphtheria Toxin Expression.

Expression of diphtheria toxin, by the *tox* gene is negatively regulated by the activity of the iron-dependent diphtheria toxin repressor (DtxR). Fe (II) acts as co-repressor along with DtxR. Under iron-sufficient and thus elevated levels of Fe (II), the inactive repressor is converted to active repressor, which then binds to the *tox* operator and represses *tox* expression. Binding of the repressor at the operator inhibits initiation of transcription of the *tox* genes.

CHAPTER 3

HYPOTHESIS

A gene *ridR* was unexpectedly discovered in an IGTS8 genomic expression library. The *ridR* gene produces a protein RidR. The RidR (*Rhodococcus* iron dependent regulator) protein was found to be similar to a known protein DtxR (diphtheria toxin repressor). The DtxR protein is produced by *C. diphtheriae* and is a repressor of toxin and siderophore production in *C. diphtheriae* (Love and Murphy 2000). There is 72% amino-acid identity between the two proteins, RidR and DtxR (Lampson *et al.* 2000).

Based on the amino acid identity of RidR and DtxR proteins, it is hypothesized that there is functional similarity between RidR and DtxR proteins. Specifically, it is hypothesized that *R. erythropolis* strain IGTS8 produces a siderophore type system to acquire iron. Further, it is hypothesized that the above siderophore production is regulated by the RidR protein.

Indeed, during the course of this project, 2 important papers were published which confirm part of this hypothesis. Boland and Meijer (2000) have reported that an *ideR* (iron dependent regulator) gene is present in *R. erythropolis* and *R. equi*. The *ideR* gene is thought to be common to the *Rhodococcus* genus. The IdeR protein of *R. equi* was determined to have a molecular weight of 25,619 and to contain 230 amino acids. The IdeR protein of *R. equi* was found to be 75% identical to the IdeR protein of *Mycobacterium tuberculosis* and 57% identical to the DtxR protein (IdeR homologue) of *C. diphtheriae*. The DNA-binding regions of the IdeR protein of *R. equi* contain a helix-

turn-helix motif. The 2 α -helices of the helix-turn-helix motif were shown to be identical to the IdeR homologues of *M. tuberculosis* and *C. diphtheriae*. The IdeR protein of *R. equi* was used in place of DtxR (IdeR homologue in *C. diphtheriae*) and it was found that the IdeR protein of *R. equi* could repress transcription of the *tox* (diphtheria toxin) gene of *C. diphtheriae*. This indicates that the DtxR and IdeR proteins recognize and bind to the same DNA sequence. Based on the above evidence, it was suggested that the IdeR protein of *R. equi* is very similar to the DtxR protein of *C. diphtheriae* (Boland and Meijer 2000).

The *ideR* gene is thought to be common to the *Rhodococcus* genus. It is hypothesized that the *ridR* gene discovered by Lampson *et al.* (2000), from *R. erythropolis* is probably identical to the *ideR* gene reported by Boland and Meijer (2000).

In the second paper, the structure of a novel type of siderophore called heterobactins, isolated from *R. erythropolis* strain IGTS8, was recently reported (Carrano *et al.* 2001). Heterobactins were produced by *R. erythropolis* IGTS8 when cultured on low iron, glucose containing medium. These heterobactins can be used for iron acquisition by other bacteria that do not produce heterobactins. There are 2 types of heterobactins, heterobactin A and heterobactin B. Heterobactins consist of a tri-peptide with the amino acids, L-Orn-Gly-D-Orn. The structure of heterobactins, has been determined (Carrano *et al.* 2001) and is shown in Figure 5. These siderophores were given the name heterobactins based on their dual nature in receptor recognition. The hydroxamate transport system of *E. coli* recognizes one part of the molecule and the

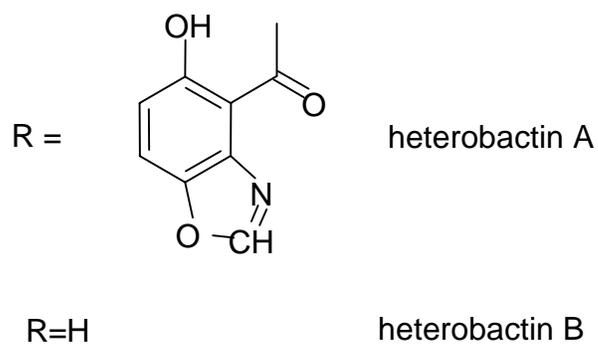
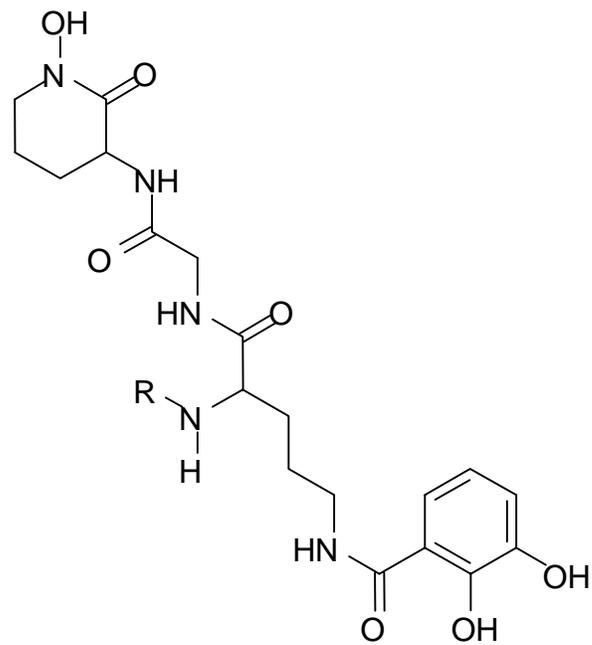


Figure 5. Structure of Heterobactin A and B.

The structure of heterobactin A and B has been determined by Carrano *et al.* (2001).

catecholate receptor of *E. coli* recognizes the other part of the molecule. A detailed study was conducted to find out which catecholate (Flu, Cir, or FepA) and hydroxamate (FhuA or FhuE) receptors were used by heterobactins to enter *E. coli* cells. It was found that heterobactin A recognizes the Cir receptor and heterobactin B recognizes the FhuE receptor to enter *E. coli* cells. The iron binding centers in heterobactin A and B have not yet been elucidated.

When *R. erythropolis* IGTS8 was cultured in succinate containing medium, heterobactin A and B were detected only in small amounts (Carrano *et al.* 2001). However, 2 other low molecular weight iron-binding compounds, dihydroxybenzoic acid and 1-carboxy-2-hydroxybenzoxazole, were detected. Heterobactin A and B were not secreted by another strain of *R. erythropolis* (DSM 43066^T) and a strain of *R. rhodochrous* (DSM 43241). However, the above strains secreted two different iron-binding compounds. One of the compounds was determined to be salicylic acid while the structure of the other compound has not yet been determined (Carrano *et al.* 2001). Salicylic acid, which has a low binding constant for iron, is not thought to function as siderophores.

R. erythropolis is considered not to be a homogenous group as heterobactins were produced in some strains (IGTS8) and not in others (DSM 43066). Siderophores having different structures may be produced within the same species of *erythropolis* (Carrano *et al.* 2001).

Research Proposal

The purpose of this research project is to test the hypothesis that the recently discovered RidR (IdeR) protein from *R. erythropolis* is functionally similar to DtxR and is involved in the regulation of siderophore production. This will be shown by generating mutant strains of *R. erythropolis*, which are defective in the regulation and production of siderophore. For example, a mutant strain defective in the regulation of siderophore production would be expected to have a defect in the *ridR (ideR)* gene. This can be shown, by restoring the mutant strain to the wild type phenotype with the wild type *ridR* gene.

CHAPTER 4

MATERIALS AND METHODS

Isolation of Plasmid (pJCS506) DNA

E. coli cells containing the plasmid pJCS506 were selected by growing them in Luria-Bertani (LB) broth (LB broth contains 10 g tryptone, 5 g yeast extract, and 5 g sodium chloride per liter of water) containing 50 micrograms/milliliter ($\mu\text{g/ml}$) of ampicillin at an optimum temperature of 37 degree celsius ($^{\circ}\text{C}$) with shaking overnight. The plasmid possesses the ampicillin resistance gene, *ampR*.

The plasmid pJCS506 was isolated and purified using the QIA prep spin mini prep kit protocol. Agarose gel electrophoresis was carried out to ensure that the plasmid was pure.

Preparation of Electro-Competent Cells of *Rhodococcus*

A seed culture of *R. erythropolis* strain IGTS8 was prepared by inoculating a small colony of the culture into 2-ml of rich media (RM) broth. (RM contains 10 g glucose, 8 g nutrient broth, 0.5 g yeast extract, and 15 g agar per liter of water.) The seed culture was incubated with shaking at 30 $^{\circ}\text{C}$ for 24 hours. A flask containing 50 ml RM broth was inoculated with 1 ml of the overnight grown seed culture. An initial optical density at 600 nm (OD_{600}) of the culture was noted. The culture was then incubated at 30 $^{\circ}\text{C}$ with shaking till an OD_{600} reading of 2-4 was observed (approximately 24 hours). To ensure that the culture was not contaminated a loop full of the culture was streaked out on

a RM agar plate and kept for incubation at 30 °C. The remaining culture was transferred to a centrifuge bottle and centrifuged in a large rotor (Sorvall super speed RC2-B) at 4°C and 5,000-6,000 revolutions per minute (rpm) for 7 minutes. The supernatant was discarded and the cells were re-suspended in 30 ml of ice cold 10 % glycerol using a pipette. It is important to note that while re-suspension was being carried out, the cells were placed on ice. The centrifugation procedure was repeated at the same conditions and the supernatant was discarded. The cells were re-suspended in 15 ml ice cold 10% glycerol. The cell suspension was then transferred to an oak-ridge centrifuge tube and the centrifugation step was repeated at the same conditions. The supernatant was discarded and the cells were re-suspended in 2.5 ml ice cold 10% glycerol. Centrifugation was repeated at the same conditions and the supernatant was discarded. The pellet of cells was re-suspended in 600 µl ice cold 10% glycerol using a small eppendroff pipette. Care was taken to ensure that the cells were re-suspended without generating bubbles. Finally, 100 µl aliquots of the above re-suspended cells were transferred into small microfuge tubes and the electro-competent *Rhodococcus* cells were frozen at -70°C for long term storage.

Electroporation of *Rhodococcus* Cells

Electro-competent cells of *Rhodococcus* were placed on ice and allowed to thaw. Three sterile electroporation cuvettes were placed on ice to chill. The plasmid (pJCS506 and pTOX) DNAs were thawed and placed on ice; 2 µl of the pJCS506 DNA was transferred to a 1.5 ml-microfuge tube and placed on ice. To this, 100 µl of electrocompetent cells of *Rhodococcus* was added and mixed gently without generating

any air bubbles. In another microfuge tube, electrocompetent *Rhodococcus* cells only (100 µl) were placed to serve as a negative control. To check the electroporation technique, a second control was set up. For the second control, 2 µl of pTOX DNA and 100 µl of electrocompetent cells of *Rhodococcus* were added to a microfuge tube and mixed without generating air bubbles. The above mixture in the microfuge tubes was transferred to the correspondingly labeled electrocuvettes. The mixture was transferred with the help of Pasteur pipettes, allowing the mixture to run along the walls of the cuvettes until the bottom surface of the cuvette was covered with the mixture. While transferring the mixture, the cuvettes were kept on ice and care was taken to not create air bubbles.

Electroporation was carried out using the electroporator (Eppendorf model 2510) at 2500 volts. The time constant value was noted. A time constant value of 3 to 5 is required for efficient electroporation of *Rhodococcus*. After electroporation, 400 µl of rich medium broth was added into the cuvettes. Then, using a sterile Pasteur pipette, the mixtures in the cuvettes were transferred into correspondingly labeled sterile test tubes and incubated at 30 °C with shaking for 4 to 5 hours. After the incubation period was completed, 100 µl of mixture (cells and RM broth) was plated using the spread plate technique on RM agar containing the corresponding antibiotic. RM agar plates containing 75 µg/ml of kanamycin were used to select *Rhodococcus* cells transformed with plasmid pJCS506. The negative control was also plated on RM agar plates containing 75 µg/ml of kanamycin. The *Rhodococcus* cells transformed with the plasmid pTOX were selected on

RM agar plates containing 30 µg/ml of chloramphenicol. The plates were incubated for 4 days at 30 °C. The number of individual colonies obtained per plate was recorded.

Transformation of *Rhodococcus erythropolis* Strain IGTS8 Cells

Transformation of electrocompetent IGTS8 cells was carried out with the insertional plasmid pJCS506 by electroporation. IGTS8 cells transformed with the plasmid were selected by plating on RM agar containing 75 µg/ml of kanamycin. The kanamycin resistant colonies growing on RM agar plates were screened for mutant(s) that could not produce siderophore on chrome azurol S (CAS) plates. Mutant(s) that have a defect in the gene or genes involved in siderophore synthesis can be recognized by a lack of halo or color change around the colony. The size of the IGTS8 genome is estimated to be about 6 to 8 million base pairs (Mb) (Pisabarro *et al.* 1998) and the average size of a single gene is 1.3 kilo base pairs (Kb). Therefore, screening of approximately 4,500 to 9,000 transformants will be required to identify the mutant.

Preparation of Chrome Azurol S Assay Medium

Chrome azurol S plates were prepared in 3 steps: (1) Preparation of CAS indicator solution; (2) Preparation of basal agar medium; and (3) Preparation of CAS agar plates.

Step 1: Preparation of CAS Indicator Solution

To prepare 100 ml of CAS indicator solution, 60.5 mg of chrome azurol S was dissolved in 50 ml of distilled water. To the above solution, 10 ml of iron III solution was added. (Iron III solution was prepared by adding 27 mg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 83.3 μl of concentrated HCl in 100 ml of distilled water.) To the above solution, 72.9mg of hexadecyltrimethyl ammonium bromide (HDTMA) dissolved in 40 ml of water was added slowly along with stirring. The resultant dark blue solution was autoclaved.

Step 2: Preparation of Basal Agar Medium

To prepare 100 ml of basal agar medium, 3 g of 3-(N-morpholino) propane sulfonic acid (MOPS), 0.05 g of sodium chloride, 0.03 g of potassium phosphate, 0.01 g of ammonium chloride, 0.5 g of L-asparagine were dissolved in 83 ml of distilled water. The pH was adjusted to 6.8 by adding a solution of sodium hydroxide. The total volume of the mixture was brought up to 88 ml with distilled water. Finally, 1.5 g of agar was added to the above solution accompanied by stirring. The resultant solution was autoclaved.

Step 3: Preparation of CAS Agar Plates

To prepare CAS agar plates, the autoclaved basal agar medium was cooled to 50°C in a water bath. CAS indicator solution and a 50% glucose solution were also placed in the 50°C water bath. After the basal agar was cooled, 2 ml of the 50% glucose solution was added to the basal agar medium along with stirring. Then, 10 ml of the CAS

indicator solution was added very slowly along the glass walls, with enough agitation to achieve mixing without generation of bubbles. Blue agar was poured aseptically into sterile plastic disposable plates. Each plate received about 25 ml of the blue agar.

Liquid Assays for the Detection of Siderophore

A liquid assay was carried out to confirm the potential mutant(s) isolated from the initial screening on a CAS plate. Two types of liquid assays were carried out to confirm the mutant(s), referred to as Liquid Assay I and Liquid Assay II.

Liquid Assay I

The culture supernatant of the control (wild type) and mutant colonies, grown without iron and with high iron added to the medium were recovered by centrifugation. Fiss-minimal medium containing low (or no) iron and high iron was used to culture the colonies (see below, preparation of Fiss minimal medium for *Rhodococcus*). The culture supernatant was added to wells made in a CAS plate and incubated for 1 hour. The presence of a color change or halo around the well after the incubation period indicates siderophore production. Un-inoculated culture broth was added to the center well as a control.

Liquid Assay II

The culture supernatant of the control (wild type) and mutant colonies, grown without iron and with high iron added to the medium, were recovered by centrifugation.

The culture supernatant was filtered and the pH was adjusted to around 7.5. The absorbance at 420 nm (A_{420}) of the above culture supernatant was recorded. A 10% solution of ferric chloride (10% in ethanol) was added drop-wise very slowly, with constant stirring, to the culture supernatant. A distinct cranberry red color change in the supernatant indicates the presence of siderophores. This color change occurs due to the chelation of ferric iron by the siderophore. Ferric chloride solution was added until the culture supernatant becomes slightly cloudy. The culture supernatant was stirred for 1 additional hour. The culture supernatant was then centrifuged at 11,000 rpm for 15 minutes. The above culture supernatant was then filtered under vacuum through a Whatman filter paper to clear the supernatant of any precipitate. The absorbance at 420 nm (A_{420}) of the above filtered supernatant was recorded. The net absorbance at 420 nm was calculated by subtracting the initial A_{420} reading (A_{420} reading obtained before addition of 10 % ferric chloride solution to the culture supernatant) from the final A_{420} reading (A_{420} reading obtained after the addition of 10% ferric chloride solution to the culture supernatant). The net absorbance values are shown in Table 1.

Culture Medium for *Rhodococcus*

The glassware used to store the various stock solutions was treated with concentrated nitric acid to remove any trace amounts of contaminating iron. The procedure for acid treating the glassware is described next. One-fourth volume of the glassware was filled with concentrated nitric acid. The acid was allowed to remain in the

glassware overnight with occasional stirring. After the time period, the acid was removed and the glassware was thoroughly rinsed many times with distilled water.

Preparation of Fiss Minimal Medium for *Rhodococcus*

The stock solutions required are:

1. Potassium phosphate (KH_2PO_4) + asparagine solution:

Five grams of KH_2PO_4 and five grams of L-asparagine were dissolved in 954 ml of double distilled water with constant stirring in an acid treated flask or bottle. The pH was adjusted to 6.8 with a solution of 1 N potassium hydroxide. The above solution was autoclaved and stored in the refrigerator as stock.

2. Glucose solution: 50 g per 100 ml of solution (50%)
3. Zinc chloride solution: 0.005 g per 100 ml of solution (0.005%)
4. Manganese chloride solution: 0.001 g per 100 ml of solution (0.001%)
5. Magnesium sulfate solution: 0.4 g per 100 ml of solution (0.4%)
6. Ferrous sulfate solution: 1 millimolar

To 100 ml of double distilled water, 0.0278 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was added and dissolved with constant stirring. The above solution was sterilized by passing it through the membrane of a syringe filter with 0.45 micrometer (μm) pore size and stored in the refrigerator in a sterilized bottle.

The materials required for the preparation of low/no iron culture broth for *Rhodococcus* are:

1. Potassium phosphate + asparagine solution 96 ml

- | | |
|--|------------|
| 2. Glucose solution (50%) | 1 ml |
| 3. Zinc chloride solution (0.005%) | 1 ml |
| 4. Manganese chloride solution (0.001%) | 1 ml |
| 5. Magnesium sulfate solution (0.4%) | 1 ml |
| 6. Ferrous sulfate solution (1 millimolar) | 75 μ l |

The above solutions were mixed according to the mentioned volumes using aseptic techniques for the preparation of low iron medium for *Rhodococcus*. The preparation of no iron broth for *Rhodococcus* is the same as the above method except that the ferrous sulfate solution (75 μ l) is not added.

The materials required for the preparation of high iron culture broth for *Rhodococcus* are:

- | | |
|--|-------|
| 1. Potassium phosphate + asparagine solution | 96 ml |
| 2. Glucose solution (50%) | 1 ml |
| 3. Zinc chloride solution (0.005%) | 1 ml |
| 4. Manganese chloride solution (0.001%) | 1 ml |
| 5. Magnesium sulfate solution (0.4%) | 1 ml |
| 6. Ferrous sulfate solution (1 millimolar) | 2 ml |

The above solutions were mixed according to the mentioned volumes using aseptic techniques for the preparation of high iron broth for *Rhodococcus*.

CHAPTER 5

RESULTS AND DISCUSSION

Generation of Mutant Strains

Random mutations in the genome of *R. erythropolis* strain IGTS8 were generated using an insertional mutagen. The plasmid pJCS506 was used as an insertional mutagen (Schneider *et al.* 1999). The plasmid pJCS506 is 8390 base pairs long, carries a selectable kanamycin marker, and contains an *E. coli* origin of replication. The map of the plasmid pJCS506 is shown in Figure 6.

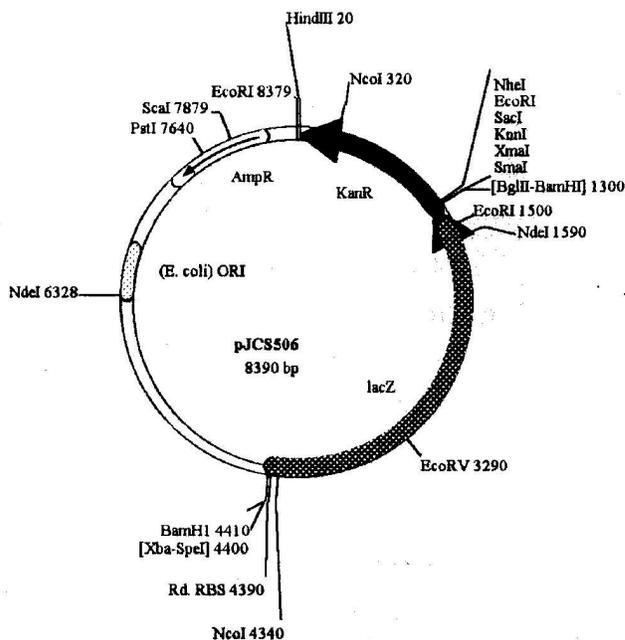


Figure 6. Map of the Plasmid-pJCS506.

The plasmid pJCS506 is 8390 base pairs long, carries a selectable kanamycin resistance marker and contains an *E. coli* origin of replication (Schneider *et al.* 1999).

An insertional mutagen inserts a piece of DNA into a gene, interrupting the gene's reading frame, thereby, blocking its function. Electroporation was used to transform electro-competent cells of *R. erythropolis* with the insertional mutagenic plasmid, pJCS506. The plasmid, which cannot replicate in *Rhodococcus* cells, randomly inserts into the *R. erythropolis* genome producing mutations. Integration of the plasmid into the *Rhodococcus* chromosome occurs by an illegitimate recombination event. For unknown reasons, this type of recombination occurs very frequently in *Rhodococcus* species (Desomer *et al.* 1991).

Transformants were selected by plating them on RM agar plates containing 75 µg/ml of kanamycin. The addition of the antibiotic kanamycin ensures that only the cells transformed, and thereby, containing the mutagenic plasmid, are selected. A total of 7,500 transformants or mutated strains were generated and screened.

Auxotrophic Mutants

The presence of auxotrophic mutants demonstrates that mutations are being generated in *R. erythropolis* genome using the plasmid pJCS506 as an insertional mutagen (Figure 7). Auxotrophic mutant(s) lack the ability to synthesize an essential nutrient such as an amino acid and in order to grow they must obtain the nutrient from the environment. A rich medium is a complex medium that contains the various essential nutrients and can support the growth of auxotrophic mutants where as a minimal medium contains only the basic salts and lacks the essential nutrients and therefore cannot support

the growth of auxotrophic mutants. Out of 250 transformants screened, 5 auxotrophic mutants were observed.

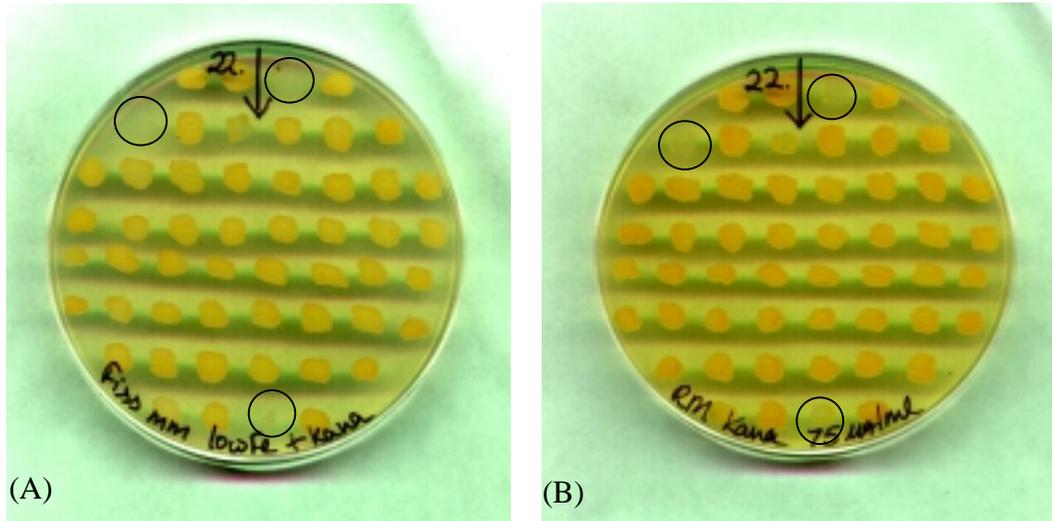


Figure 7. Auxotrophic Mutants.

Auxotrophic mutants (shown circled) do not show growth on a minimal growth medium (A) plate whereas they show growth on a rich medium plate (B).

Screening for Colony Transformants Not Producing Siderophore

The 7,500 transformants on RM agar plates were screened on CAS assay plates to detect colonies that do not longer produce a siderophore. The CAS test is a chemical assay for the detection of siderophore production (Schwyn and Neilands 1987). A non-siderophore producing mutant(s) was recognized on the CAS assay plate by a lack of halo or color change around the colony (Figure 8).

The CAS assay is based on the removal of ferric iron from an intensely pigmented complex by a competing ligand such as a siderophore. When a siderophore forms a complex with the ferric ion, the release of the free dye is indicated by a color change from blue to yellow (Schwyn and Neilands 1987). The following equation explains the principle:

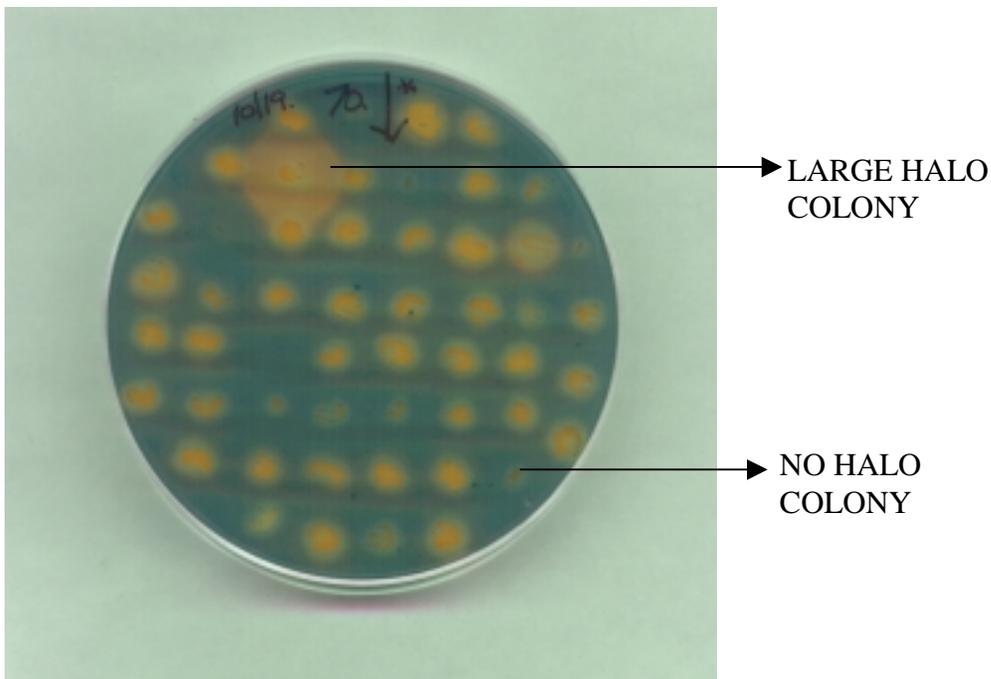
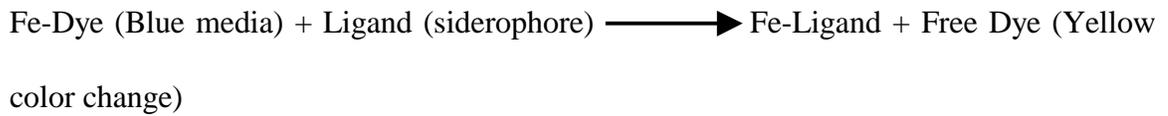


Figure 8. Chrome Azurol S Assay.

The CAS plate is used to detect siderophore production. The presence of a halo around the colony indicates siderophore production. Colonies that do not produce a siderophore and colonies that produce unregulated large amounts of siderophore can be detected on the CAS plate.

The CAS assay is highly sensitive and can detect as little as 0.002 micromoles (μmol) of siderophore (Schwyn and Neilands 1987). The CAS assay can also be used to identify mutants. Biosynthetic and regulatory mutants can be detected with the CAS assay plate. Biosynthetic mutants arise when there is a defect in the gene(s) involved in siderophore production and can be recognized on the CAS plates by a lack of halo or color change around the colony. These biosynthetic mutants should be able to survive on their low affinity iron uptake systems. Regulatory mutants should produce a large halo around the colony on CAS plates under both low and high iron conditions (Schwyn and Neilands 1987).

The transformants were picked from the RM plates using a sterile toothpick and inoculated onto the surface of the CAS plate and the leftover inoculum on the same toothpick was used to inoculate the surface of a Fiss minimal plate containing low amounts of iron. Transformants plated on the Fiss minimal plates were used as a stock culture of the transformant. A particular transformant was inoculated at the same numbered position on the CAS and Fiss minimal plate. The plates were incubated at 30 °C for 4 days before they were checked for lack of siderophore production. Most of the transformants produced a siderophore, thereby indicating that the insertional mutagen has mutated a gene that is not of interest to the present work or did not produce a mutation at all. However, 416 transformants did not show a detectable halo around the colony on CAS assay plate, indicating that they may not produce a siderophore. A secondary screening for the lack of siderophore production was carried out on the CAS assay plate with the 416 strains isolated from the initial screening. Most of these strains (about 350-

375) produced a siderophore or did not show good growth and hence were not tested further by the liquid assays. About 32 strains did not produce a siderophore in the secondary screening though they showed good growth and were tested further by the liquid assays.

Results of Liquid Assays

Results of Liquid Assay I

Liquid Assay I was performed to test the potential mutant strains for lack of siderophore production. Strain N5-59 was chosen because colonies of this strain showed good growth and produced little or no halo around the colony on CAS assay plates. The strain N5-59 and the control (wild type) strain were inoculated into a Fiss minimal broth containing no added iron or high amounts of iron (20 micromolar) and grown until an optical density greater than 5 was obtained. Culture supernatants from the strain N5-59 and the control were collected and 50 μ l was loaded into separate wells formed in the CAS agar plate.

The control (wild type) strain that was cultured without iron added to the Fiss minimal broth showed a halo or color change around the well indicating that it produced a siderophore under iron starvation conditions. The control strain that was cultured in high iron Fiss minimal broth did not show a halo or color change around the well, indicating that it did not produce a siderophore when iron is in sufficient supply (Figure 9). This is consistent as siderophores are produced only when the amount of iron is low in

the growth medium. Strain N5-59 when grown without iron or with high iron added to the Fiss minimal broth did not show the presence of a halo or color change around the well containing the corresponding culture supernatant. This result indicates that the strain N5-59 did not produce a siderophore (Figure 9).

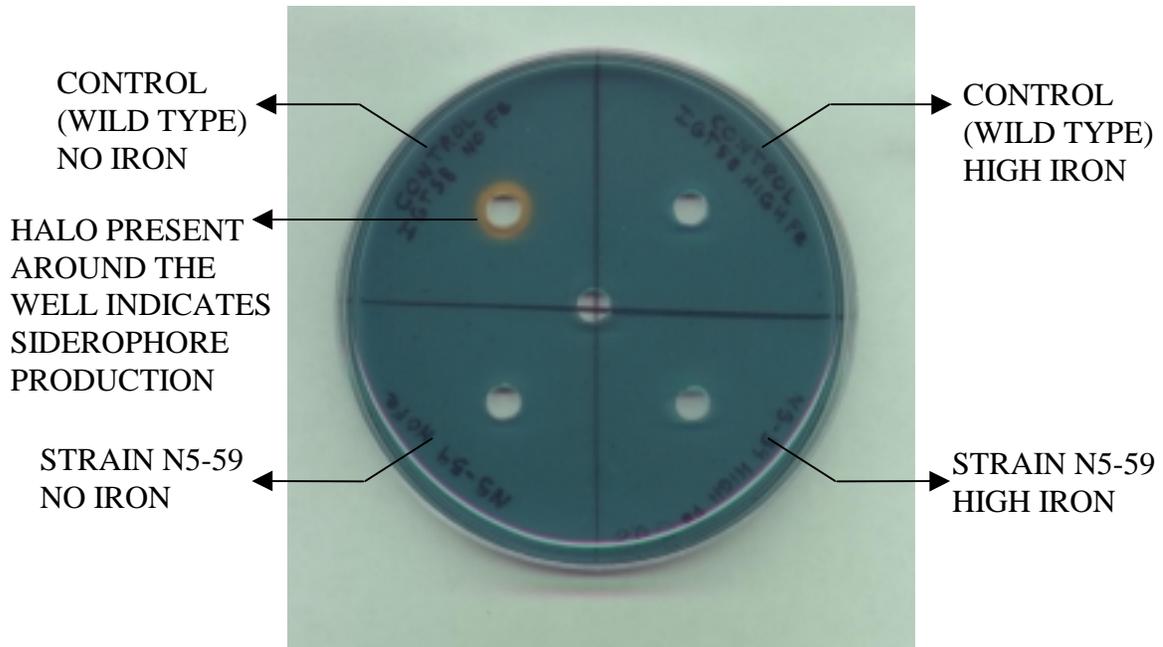


Figure 9. Liquid Assay I.

The culture supernatants of the control and mutant (N5-59) strains that were grown with and without iron added to the medium were recovered. The culture supernatants were added to wells made in a chrome azurol S assay plate and incubated for one hour. The presence of a halo or a color change around the well indicates siderophore production. Uninoculated culture broth was added to the center well.

Results of Liquid Assay II

Liquid Assay II was performed to confirm the results obtained by Liquid Assay I. The strain N5-59 and the control (wild type) strain were inoculated into a Fiss minimal broth containing no added iron or high amounts of iron (20 micromolar) and grown until an optical density greater than 5 was obtained. Culture supernatant from the strain N5-59 and the control strain were collected, filtered, and pH adjusted to around 7.5. A 10% solution of ferric chloride was added drop-wise to the culture supernatant along with constant stirring.

The control (wild type) strain that was cultured with high amounts of iron added to the Fiss minimal broth did not produce any color change upon ferric chloride addition to the culture supernatant (Figure 10). However, the control wild type strain that was cultured without iron added to the Fiss minimal broth produced a cranberry red color change upon ferric chloride addition to the culture supernatant. This indicates the presence of a siderophore, which binds the ferric ions released by addition of ferric chloride. This is consistent as siderophores are produced only under low iron growth conditions. The strain N5-59 that was cultured without iron or with high iron added to the Fiss minimal broth did not produce any color change upon ferric chloride addition to the culture supernatant (Figure 10). This indicates that the strain N5-59 does not produce a siderophore and is likely a mutant of *R. erythropolis* that is defective in some step required for the synthesis of a siderophore. Summary of the results of the liquid assays is shown in Table 1. The color change observed in liquid assay II was quantified by calculating the net absorbance at 420 nm (Table 1).

(A) CONTROL (WILD TYPE)



(B) STRAIN N5-59

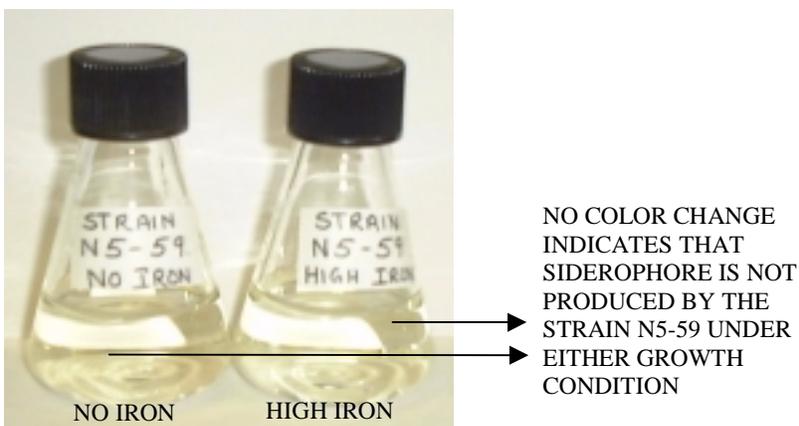


Figure 10. Liquid Assay II.

The culture supernatant of the control (A) and mutant, N5-59, (B) strains, that were grown with and without iron added to the medium, were recovered, filtered and the pH adjusted to around 7.5. A 10% solution of ferric chloride was added to the supernatant. A cranberry red color change in the supernatant indicates the presence of siderophores.

Table 1. Summary of Results of Liquid Assays.

	CONTROL (WILD TYPE)		NON-SIDEROPHORE PRODUCING STRAIN (N5-59)	
GROWTH MEDIUM	NO IRON	HIGH IRON	NO IRON	HIGH IRON
LIQUID ASSAY I	HALO/COLOR CHANGE ^a	NO HALO/COLOR CHANGE ^b	NO HALO/COLOR CHANGE ^c	NO HALO/COLOR CHANGE
LIQUID ASSAY II	CRANBERRY RED COLOR CHANGE	NO COLOR CHANGE	NO COLOR CHANGE	NO COLOR CHANGE
NET ABSORBANCE ^d AT 420nm	2.665	0.412	0.229	0.153

^aThe control (wild type) colonies when grown without iron added to the medium produced siderophore as indicated by color change.

^bThe control (wild type) colonies when grown with high iron added to the medium did not produce siderophore as indicated by no color change.

^cThe strain N5-59 when grown without, as well as, with high iron did not produce siderophore as indicated by no color change.

^dnet absorbance at 420 nm = (Absorbance after addition of ferric chloride to the supernatant) — (Absorbance before addition of ferric chloride to the supernatant) in liquid assay II.

Large-Halo Producing Colonies

In addition to colonies which produce little or no halo on CAS plates, there were a number of colonies which produced very large halos (larger than wild type controls) even when the CAS agar plates were supplemented with iron.

An additional total of 2000 transformants were screened on CAS assay plate containing ferrous sulfate at a concentration of 10 μ M. A total of 19 large halo-producing colonies were observed out of the 2000 colonies screened. A list of the large halo colonies is shown in the Table 2.

Table 2. Large-Halo Producing Strains.

Strain	Halo size on CAS^a	Character in Liquid Assay I
R 150-26	Not Determined	Like Wild Type Strain ^b
R 151-40	Not Determined	Like Wild Type Strain
R152-25	Not Determined	Like Wild Type Strain
R152-39	Not Determined	Like Wild Type Strain
R153-7	7mm	Like Wild Type Strain
R154-9	9mm	Like Wild Type Strain
R155-50	8mm	Like Wild Type Strain
R156-19	6mm	Like Wild Type Strain
R158-5	13mm	Like Wild Type Strain
R159-46	8mm	Like Wild Type Strain
R161-6	10mm	Like Wild Type Strain
R161-20	10mm	Like Wild Type Strain
R168-40	7mm	Like Wild Type Strain
R170-23	5mm	Like Wild Type Strain
R178-40	7mm	Like Wild Type Strain
R182-37	6mm	Like Wild Type Strain
R185-18	9mm	Like Wild Type Strain
R187-37	12mm	Like Wild Type Strain
R187-12	10mm	Like Wild Type Strain
IGTS8	3mm	Wild Type Strain

^aDiameter of the halo size on CAS assay plate containing 10 μ M of ferrous sulfate.

^bThe large-halo producing strains were assayed for siderophore production under low and high iron growth conditions in liquid assay I. Each strain, however was indistinguishable from the wild type control strain in the liquid assay I.

To test whether these colonies are producing siderophore under high iron growth conditions, thereby showing that there is a defect in the regulation of siderophore production, Liquid Assay I was carried out. In order to carry out Liquid Assay I the colonies were required to be cultured in Fiss minimal medium containing low and high iron. Most of the large halo colonies showed problems with respect to growth in the liquid medium. The cultures of the large halo producing colonies had a very low optical density at 600 nm (OD_{600}), in the range of 0.200 to 0.400 after 3 days of growth, while the wild type IGTS8 strain cultures showed good growth with an OD_{600} of approximately 8.00 after 3 days of growth. This slow growth may be due to mutational defect in an essential growth-promoting gene or due to auxotrophic mutations. To overcome this growth problem, the large halo producing colonies were cultured in Fiss minimal medium containing low and high amounts of iron (50 ml) that was supplemented with 3% casamino acids (1 ml). The colonies previously showing growth problems now showed excellent growth with an OD of 9.00 to 10.00 within 2 days of growth.

Results of Liquid Assay I on Large-Halo Producing Colonies

Though the colonies produced a large halo in the initial screening on CAS assay plates containing 10 μ M of ferrous sulfate, they behaved like wild type strains in the Liquid Assay I (Table 2). Like wild type strains, the large halo producing colonies produced siderophore when cultured in low iron growth conditions and did not produce siderophore under high iron growth conditions. This behavior of the large halo producing colonies is unusual. Strains that have defects in their regulation usually produce

unregulated amounts of siderophore both under low and high iron growth conditions. It is not clear at this point why a colony that produces a large halo indicating that there is an unregulated amount of siderophore being produced in the initial CAS screening assay behaves like wild type strains in the liquid assay (see below, Discussion).

Discussion

A non-siderophore producing strain, N5-59, was isolated by generating a mutation in the *R. erythropolis* genome using an insertional mutagen, pJCS506. Screening for the non-siderophore producing colonies was carried out on a color indicator assay plate. This strain, N5-59, was confirmed to be a non-siderophore producing mutant by the liquid assays.

A non-siderophore producing mutant such as N5-59 may be due to a defect or mutation in the gene(s) involved in siderophore synthesis or in the transport mechanism that releases siderophore to the external medium. The screening assay (CAS assay) that was used to detect the non-siderophore producing mutant cannot distinguish between these two possibilities.

To rule out that there is a defect in the release of siderophore to the external medium a future experiment that could be done is proposed. If there is a defect in the release of siderophore to the external medium, the cells should be loaded with siderophore that cannot be released. The mutant strain, N5-59, can be cultured in low iron conditions, which favors siderophore production. The cell membranes can then be damaged or permeabilized via chemical treatment using detergents such as sodium

dodecyl sulfate. This should break the cells and release the cell contents. The culture supernatant of the cells treated with the detergent should show a positive color change in the liquid assays, if there is a defect in only the release of siderophore to the external medium. However, if the mutant strain, N5-59 has a defect in the synthesis of siderophore, the liquid assays performed after the disruption of cell membranes should not give a color change.

The isolation of mutant(s) of *R. erythropolis* that have defects in their siderophore production can be used for future research. A non-siderophore producing strain like N5-59 will aid in the purification and structural characterization of the siderophore produced by *R. erythropolis* strain IGTS8. Whether the non-siderophore producing mutant N5-59 has a defect in the synthesis of siderophore or in the release of siderophore does not affect the use of the mutant as a control in the purification of siderophore. The non-siderophore producing mutant can also be used in identification of the gene(s) involved in the synthesis of the siderophore. The ability of a gene library from the wild type strain to restore the mutant N5-59 to siderophore production should help to identify the gene(s) involved in the synthesis of siderophore.

The biosynthesis of the siderophore, mycobactin produced by mycobacteria is known (De Voss *et al.* 1999). The biosynthesis of mycobactins requires a cluster of seven genes, *mbtA* to *mbtG*. These genes encode proteins, which are very similar to the non-ribosomal peptide synthases (NRPSs). The *mbt* gene cluster was shown to be involved in siderophore synthesis by constructing a mutant strain (Δ *mbtB*::*hyg*) where portion of the *mbtB* gene was substituted with a hygromycin resistant DNA fragment, through

homologous recombinations. This mutant strain could not produce siderophore, thereby showing that the *mbt* gene cluster was essential for the biosynthesis of mycobactin. The biosynthesis of another siderophore, exochelin from *M. smegmatis* is thought to require a cluster of 3 genes. One of these genes encodes a protein that is similar to phosphoribosylglycineamide formyltransferases and the other 2 proteins encode NRPSs. Hence, there are several genes that are involved in the biosynthesis of siderophores in mycobacteria. This indicates that there are multiple target sites for generating mutant strain(s) that do not produce siderophore. The iron acquisition system of rhodococci may be related to that of mycobacteria. Hence, there may also be multiple target sites in *R. erythropolis* for generating mutant strains that do not produce siderophore. The number of final potential candidate's (32) that was obtained during the screening process emphasizes this point.

The large halo producing strains obtained from the CAS assay did not produce large amounts of siderophore under low and high iron growth conditions as was expected in liquid assay I. They behaved like wild type strains producing siderophore under low iron growth conditions and repressing siderophore production under high iron conditions. The reason for the difference in siderophore production on the CAS assay and liquid assay is not clear at present. There may be some ingredient in the CAS assay media that causes the excess siderophore production or release from the cell. Two different strategies may be employed at this stage. The concentration of ferrous sulfate used in the CAS assay plates that is used for screening can be increased from 10 μM to at least 50 μM or even higher. This increase in the amount of iron will ensure complete repression of

siderophore production. Hence, the detection of colonies producing large halo's during screening may be potential mutant strains that have a defect in the regulation of siderophore production. Also, increasing the concentration of ferrous sulfate should drastically reduce the number of colonies producing siderophore in excess. Another strategy that can be employed is to screen transformants grown in a liquid CAS assay rather than on CAS plates.

R. erythropolis is an important microorganism in environmental and industrial biotechnology. A vast array of practical applications is being discovered for this bacterium, but some of the basic concepts of iron acquisition by rhodococci are unknown. The production of novel siderophores by rhodococci has many potential practical applications such as using the chelating property of siderophores for iron and using them for treatment of iron overload. Another application is in the removal of other toxic metals from human tissues. Siderophores can form stable complexes with metals such as aluminium, copper (II), and molybdenum. Siderophores produced by rhodococci can also be exploited to bind and immobilize environmental pollutants such as toxic metals and radioactive compounds.

The production of siderophores is correlated with the virulence of many pathogenic microbes. For example, strains of *E. coli* that are known to cause disseminating infection were later found to produce the siderophore, aerobactin (Neilands 1995). The siderophore produced by rhodococci may also play a similar role.

The primary purpose of this research is to increase knowledge of the iron acquisition system of rhodococci.

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