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Iron Acquisition in *Rhodococcus erythropolis* Strain IGTS8: Characterization of a Mutant Strain that Over Produces Siderophore.

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by
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ABSTRACT

Iron Acquisition in *Rhodococcus erythropolis* Strain IGTS8: Characterization of a Mutant Strain that Over Produces Siderophore

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Iron is an essential nutrient for most bacteria because enzymes like nitrate reductase and cytochromes use it as a cofactor. However, in most aerobic, neutral pH environments, iron is essentially insoluble and not easily available for bacteria to use. Many bacteria respond to this problem by releasing small organic compounds called siderophores that bind and effectively solubilize iron so that it can be transported into the cell for growth. The focus of this study was to learn more about the iron acquisition and especially the transport of iron by the soil bacterium *Rhodococcus erythropolis*. To fulfill this aim, mutant strains of the bacteria were screened for those that overproduce siderophore. Often, a bacterium will over produce siderophore to compensate for a defect in transport. One such mutant, R187-12, was further analyzed by cloning the region of the chromosome containing the defective gene responsible for over production of siderophore into a plasmid vector. The DNA sequence of this region was determined and analyzed for the presence of similar genes encoding transport proteins.

DEDICATION

To Mom and Dad

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INTRODUCTION

The Genus *Rhodococcus*

The genus *Rhodococcus* encompasses a group of Gram positive, aerobic, non-motile, mycolate-containing actinomycetes (Bell et al. 1998). The genus is closely related to the Mycobacteria, with both containing mycolic acids in the cell wall, although the rhodoccoci contain fewer numbers and are capable of being gram stained, unlike the mycobacteria. *Rhodococcus* cells are described as norcardioform, which is a morphologically descriptive term referring to mycelial growth with fragmentation into rod-shaped or coccoid elements. These bacteria do not form spores under adverse conditions, although the mycolic acids enable the cells to resist desiccation for a period of time. *Rhodococcus, Norcardia, Mycobacteria*, and *Corynebacteria* encompass a phylogenetic group of bacteria known as the Mycolata. This group of bacteria has a high GC content and contains a number of pathogens (Meijer and Prescott 2004). The mycolata are characterized by a unique cell envelope that contains mycolic acids linked to arabinogalactan wall polysaccharide and (glycol)lipids (Mejier and Prescott 2004). This unique cell envelope is vastly different from those of Gram positive and Gram-negative bacteria and forms a permeability layer to hydrophilic compounds. This cell wall is likely important for the survival of these bacteria under harsh environmental conditions. One species of *Rhodococcus, R. equi*, is extremely resistant to environmental stress and oxidative stress as well as low pH, likely because of the cell wall (Meijer and Prescott 2004). The rhodococci form colonies with bright pigmentation, ranging from pink to orange to red (Figure 1), although colorless variants do exist. Colonies can be

smooth, rough, mucoid, or even mycobacterial like (Goodfellow and Alderson 1977). The type species of this genus is *Rhodococcus rhodochrous*. This species forms rough, orange to red colonies on most media and can grow at temperatures ranging from 10^{o}C to 40^{o}C (Goodfellow and Alderson 1977).

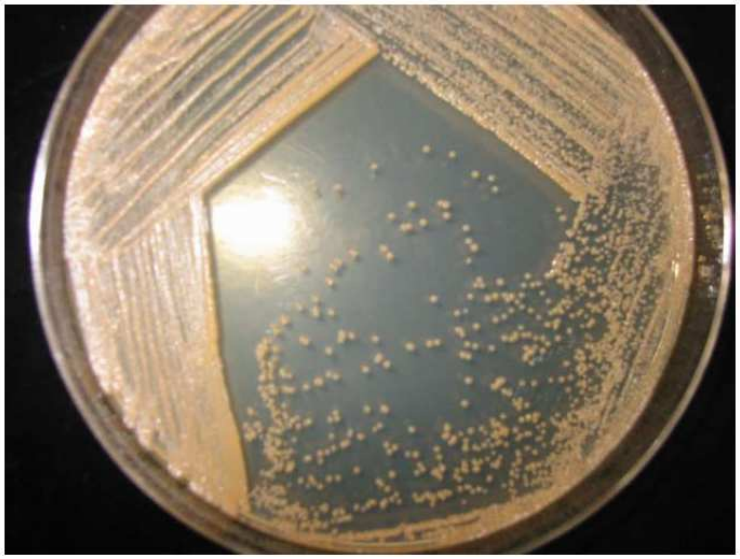


Figure 1: Characteristic *Rhodococcus erythropolis* IGTSS8 colonies on RM agar

The Rhodococci have diverse metabolic activities, enabling them to degrade a wide range of environmental pollutants as well as giving these bacteria the ability to transform and synthesize compounds that may have useful applications. In recent years, the commercial potential of *Rhodococcus* species has been increasingly recognized. Rhodococci are able to transform or degrade a wide range of chemicals, making them actually or potentially useful in environmental and industrial biotechnology. These bacteria are also able to synthesize several products including surfactants, flocculants, amides, and polymers, increasing their potential usefulness in biotechnology (Bell et al. 1998). The usefulness of these bacteria can be seen in the number of patents relating to rhodococci.

Most species of rhodococci grow optimally at 30^{o}C, although *Rhodococcus equi*, as an animal pathogen, grows optimally at 37^{o}C. Rhodococci are able to use a variety of carbon sources, including, but not limited to, glucose and lactate. The rhodococci are generally considered to be soil bacteria, although some, including *R. equi* and *R. fascians* can also be pathogenic in animals and plants, respectively. Bacteria from this genus have been isolated from a variety of sources, encompassing soils, groundwater, animal dung, insect guts, and both healthy and diseased plants and animals (Bell et al. 1998). They are found from deep sea to coastal sediments and from the Arctic to Antarctic soil samples (de Caravalho and da Fonesca 2005).

In 1998, there were 12 recognized species in the genus *Rhodococcus*. According to Bell et al (1998), the genus name of *Rhodococcus* was first used in 1891 and then redefined and revived in 1977 to accommodate the "rhodocrous" complex that comprised a number of strains of bacteria that resembled, but did not belong to the established genera *Mycobacterium, Corynebacterium* and *Norcardia* (Bell et al. 1998). Several new species of *Rhodococcus*, including *R. yunnanensis, R. kunmingensis, R. qingshengii* and *R. cercidiphylli* have been described in the literature in recent years, and there are now over 40 described species of the genus (Zhang et al. 2005, Xu et al. 2007, Wang et al. 2008, Li et al. 2008).

Although most species of Rhodococci are soil bacteria, some are considered pathogens. *R. fascians* is considered a pathogen of plants. This bacterium is capable of colonizing *Nicotiana tabacum*, making it an agriculturally important bacterium. *R. fascians* causes malformations on aerial plant parts, causing leafy galls at the axillary meristems (Cornelis et al. 2001). Besides infecting tobacco plants, *R. fascians* infects

other plants, interfering with the development and growth of a wide variety of hosts. Infection with *R. fascians* results in malformations and stunted growth. Both the nature and the location of the malformations depend on the infected host species and the infection method (Cornelis et al. 2001). Outbreaks of *R. fascians* in ornamental plant species can cause serious financial losses for gardeners and garden centers. *R. fascians* has been isolated from infected plants and was found on both the surface and epidermal cells of sweet pea seedlings, indicating this bacterium can colonize the interior of the plant (Cornelis et al. 2001).

Rhodococcus equi is primarily a pathogen of horses but can also be a cause of pneumonia in humans, particularly in those with compromised immune systems such as HIV patients. Like *Mycobacterium tuberculosis*, *R. equi* is able to infect the alveolar macrophages and survive within these immune cells. *R. equi* is an important cause of bronchopneumonia in foals up to 3 to 5 months old. Occasionally, this bacterium causes pneumonia in other farm animals such as pigs and goats but is rarely seen as a pathogen in other species unless an immunodeficiency exists. However, *R. equi* is an important cause of AIDS-associated pneumonia in HIV patients (Meijer and Prescott 2004). The pathogenicity of this species is defined by its ability to survive within the macrophages of the lungs, and the basis of its pathogenicity is its ability to multiply in and eventually destroy the alveolar macrophages. A large virulence plasmid containing a 27kb pathogenicity island that encodes for 7 related virulence associated proteins, or VAPs, is crucial to the virulence of *R. equi*. The different genes on the pathogenicity island are regulated by changes in temperature, pH, iron, and oxidative stress, all of which are environmental changes experienced by *R. equi* when inhaled in dust and ingested by the

alveolar macrophages (Meijer and Prescott 2004). A partial genome sequencing of *R. equi* has shown that a majority of genes in this bacterium are most closely related to those of *M. tuberculosis* (Meijer and Prescott 2004).

While *R. equi* in humans generally occurs in those that are immunosuppressed, infections occur rarely in healthy individuals. These infections may be associated with deep, penetrating wounds (Bell et al. 1998). Resistance to β–lactam antibiotics appears to be common (Bell et al. 1998).

The importance of the rhodococci in bioremediation is now apparent. The ability of these bacteria to degrade substituted hydrocarbons and other chemicals indicates they play a significant role in the natural degradation of compounds and in bioremediation. Bioremediation is the use of living organisms, particularly microorganisms, in the removal of contaminants from soil and water. There have been few studies of bioremediation using the rhodococci in the field; however, these limited studies show promise. One study showed increased rates of the degradation of pentachlorophenol (PCP), a biocide that is found in pressure treated wood, in sandy soil that was inoculated with *R. chlorophenolicus*, which has now been reclassified as a *Myocbacterium* (Miethling and Karlson 1996). They also found that high numbers of the organism remained in the soil for months. Yet another study showed that inoculation with *R. rubber* increased the number of hydrocarbon-oxidizing bacteria persisting in composted soil that was contaminated with crude oil (Christofi et al. 1998). This finding indicates that *R. rubber* could be potentially useful in cleaning up oil spills. A 1996 study using *R. erythropolis* reported that introducing a hydrocarbon-degrading strain of this bacterium into artificially contaminated soil resulted in an increased number of hydrocarbon

degrading bacteria as well as an increased rate of hydrocarbon degradation (Bell et al. 1998).

Polychlorinated biphenyls (PCBs) are a mixture of chemicals containing up to 209 individual chlorinated compounds. There are no known natural sources of PCBs. Until the 1970s, PCBs were manufactured in the United States. However, in 1977, after evidence arose that these compounds accumulated in the environment and could lead to harmful health effects, the manufacture of PCBs was halted in the United States. Though these compounds are no longer produced in the US, they are still present in the environment. PCBs were used as coolants and lubricants in electrical equipment because they do no easily burn and are considered to be good insulators. Older electrical equipment still contains these compounds. PCBs are released into the environment upon their disposal, and because they do not readily break down in the environment, they are present for a long time in the air, water and soil (Furukawa and Fujihara 2008). Because of their presence in the environment with potential harmful health effects, research into the degradation of PCBs has been extensive. It was shown that *R. erythropolis, R. globerulus,* and *R. rhodochrous* are able to degrade these compounds (Boyle et al. 1992; Asturias and Timmis 1993; Maeda et al. 1995; Seto et al. 1995). The ability of these species of rhodococci to degrade PCBs makes them potentially useful in bioremediation and the cleanup of these potentially harmful compounds remaining in our environment.

Biosurfactants are surface-active substances that are synthesized by living cells. These compounds can reduce surface tension, stabilize emulsions, and promote foaming. Generally, biosurfactants are nontoxic and biodegradable. The relevance of biosurfactants to the biodegradation of pollutants has 3 parts. Cellular surfactants like mycolic acids

cause the adherence of rhodococci to hydrophobic phases. Secondly, surfactants are capable of lowering the interfacial tension between the phases in a 2-phase system, thus making it easier for hydrophobic compounds to enter the microbial cells. Finally, extracellular surfactants disperse hydrophobic compounds that increase the surface area for microbial attack. It has been shown that some biosurfactants, including those produced by rhodococci, are more effective than many synthetic surfactants and are potentially less toxic and more biodegradable than the synthetic ones (Bell et al. 1998).

The rhodococci are also useful in the chemical industry. One example is acrylamide that is used in coagulators, soil conditioners, and in stock additives for paper treatment and paper sizing and for adhesives, paints and petroleum recovering agents (Yamada and Kobayashi 1996). The Nitto Chemical Industry Company uses *Rhodococcus rhodochrous* J1 to produce around 30,000 tons of acrylamide annually using the nitrile hydratase (NHase) enzyme produced by this strain. This particular strain produces 2 types of nitrile converting enzymes, nitrilase and NHase, depending on the inducer used. Through studies, it was determined that the NHase was optimal for acrylamide production by the company (Yamada and Kobayashi 1996). The production of acrylamide from acrylonitrile is an example of the industrial application of a nitrile hydrolase from a bacterial strain, *R. rhodocrhous* J1 (de Carvalho and da Fonseca 2005).

Another potentially important use of the rhodococci is its ability to degrade the carbon-sulfur bonds in fossil fuels while leaving the carbon-carbon bonds intact. The removal of sulfur from coal and petroleum has been suggested as a way of preventing sulfur emissions caused by combustion and reducing the associated problems such as acid rain, as well as increasing fuel value. However, the removal of organic sulfur from fossil

fuels is difficult. Several groups have researched the potential of different *Rhodococcus* species in the desulfurization of fossil fuels. The selectivity of breaking carbon-sulfur bonds while leaving carbon-carbon bonds unaffected seems to be unique to the rhodococci (Bell et al. 1998). *R. erythropolis* IGTS8 is able to release inorganic sulfur. It has been shown that with this method of desulfurization of fossil fuels, the fuel calorific value of the crude oil is maintained. This is due to the release of sulfur without the degradation of the aromatic ring of carbons (de Carvalho and da Fonseca 2005). Continuing studies with this bacterium may prove to be useful in the biodesulfurization of fossil fuels, particularly as older methods of desulfurization are quickly becoming obsolete and there is increased interest in getting the most out of crude oil.

The unique metabolic activities of the rhodococci may also be of use in detecting illicit drugs such as cocaine and heroin. Recently, a cocaine esterase was discovered in *Rhodococcus* sp. strain MB1. This species is capable of using cocaine as a sole source of carbon and nitrogen for growth. It was isolated from the soil surrounding the source of cocaine, *Erythroxylum coca*. Cocaine is naturally present in the leaves of this plant (Bresler et al. 2000). The cocaine esterase discovered in this bacterium is capable of initiating the degradation of cocaine. The cocaine is first hydrolyzed to ecgonine methyl ester and benzoate and then these compounds were further metabolized by the bacterium (Bresler et al. 2000). Previously, it was shown that another species of *Rhodococcus*, strain H1, was capable of producing a heroin esterase. This bacterium is capable of using heroin as a sole source of carbon as well as its sole energy source. The heroin esterase of *Rhodococcus* sp strain H1 was shown to catalyze the hyrolysis of both of the acetylester groups of heroin, yielding morphine (Cameron et al. 1994). Researchers have shown that

enzymes initiating heroin metabolism can be used in conjunction with bacterial luciferase to detect nanogram quantities of heroin. Similar research is being conducted to detect small quantities of cocaine (Bresler et al. 2000). The ability of these species of *Rhodococccus* to use cocaine and heroin as their sole carbon sources emphasizes the incredibly diverse metabolic activities of this genus.

Currently, the genome of only 1 species of *Rhodococcus* has been sequenced, *Rhodococcus* sp RHA1. This bacterium was isolated from soil contaminated with lindane and is known for its ability to degrade polychlorinated biphenyls. It has one of the largest bacterial genomes sequenced to date at 9,702,737 base pairs with a GC content of 67% (McLeod et al. 2006). The chromosome of *Rhodococcus* sp strain RHA1 is arranged into a linear chromosome and 3 linear plasmids. The sequence of this bacterium is most similar to norcardial and mycobacterial strains (McLeod et al. 2006). Using 16S RNA sequencing, it has been demonstrated that *Rhodococcus* sp strain RHA1 is closely related to *R. opacus* (McLeod et al. 2006).

Getting Iron for Growth

Iron is an essential element for nearly all microorganisms. Iron is required for amino acid and pyrimidine synthesis as well as in enzymes involved in the tricarboxylic acid cycle (TCA cycle), DNA synthesis, superoxide, photosynthesis, and nitrogen fixation (De Voss et al. 1999, Carronodo 2003). Although iron is vitally important for cellular viability, bacteria generally require only trace amounts of the element.

Although iron is one of the most abundant elements in the Earth's crust, it is often difficult to obtain for microorganisms. The bacterium's capacity to acquire iron from the environment is difficult, as most iron is not soluble in the presence of oxygen and at

neutral pH. In an environmental setting, iron is found in insoluble oxy-hydroxides. In the human host, iron is tightly bound in a complex with high affinity iron-binding proteins. To subvert this, pathogenic bacteria have evolved strategies enabling them to compete for iron in the host (Rodriguez 2006). Iron is found in 2 common forms: Ferric iron (FeIII) and ferrous iron (FeII). Ferric iron is plentiful but highly insoluble, while ferrous iron is soluble and can freely diffuse across the cellular membrane but can produce toxic oxygen radicals when combined with the products of normal aerobic respiration. Under environmental conditions, ferrous iron is oxidized to form ferric iron. Ferric iron has an extremely low solubility under environmental conditions, found at a concentration of around 10^{-18} M . Within the bacterial cell, iron concentrations are found at around 10^{-3} to 10^{-5} M (Carronodo 2003). Ferric iron is unable to freely diffuse across the cell membrane because it is largely insoluble. Ferrous iron is able to diffuse across the cell membrane, but this does not generally occur as the ferrous iron is readily oxidized to ferric iron under environmental conditions.

Iron acquisition in bacteria must be controlled, as too much iron is toxic to the cell. While iron is required by almost all bacteria for the activity of enzymes involved in vital cell functions, excess iron can harm the cell. Iron participates in reactions that generate toxic oxygen radicals, known as the Fenton reaction. Ferrous iron complexes can act as electron reducing agents and ferric iron complexes can act as electron oxidizing agents (Pierre and Fontecave 1999). These iron complexes can react with oxygen or reduced oxygen producing highly reactive iron-oxo species. A ferric complex can only catalyze the formation of hydroxyl radicals if 2 conditions are simultaneously met: the complex is reducible into its ferrous state and the ferrous complex has a redox

potential allowing the Fenton reaction ($FeII + H_2O_2 \rightarrow FeIII + OH^- + OH$). This reaction leads to the partial reduction of oxygen into hydroxyl radicals. These radicals are damaging to many macromolecules. Therefore, the bacterial cell must protect against damage by these toxic hydroxyl radicals (Wandersman and Delepelaire 2004)

Thus, regulation of iron level is essential to the survival of the bacterial cell. Regulation of iron levels includes sensing cellular concentrations of iron and responding by modifying iron uptake, storage of excess iron for times of iron starvation, and possibly the efflux of iron (Rodriguez 2006). Regulation can also take the form of binding iron in complexes such as lactoferrin and transferrin or binding the iron into protoporphyrin rings in hemoproteins as well as iron storage inside of ferritins. Because of the extremely tight iron homeostasis, there is virtually no free iron in a living bacterial cell (Wandersman and Delepelaire 2004).

Iron Acquisition Systems

In addition to ferrous and ferric iron as iron sources, pathogenic bacteria can use heme as an iron source. In mammalian hosts, the majority of iron is sequestered. Much of this iron is found in hemoglobin, a protein found in erythrocytes. Host cells also contain a storage form of iron known as ferritin. Any extracellular iron in the host is bound by transferrin and lactoferrin, high-affinity iron binding proteins (Mills and Payne 1995). Pathogens have several mechanisms to acquire this iron. One method of iron acquisition is for the bacteria to obtain iron directly from host iron sources. Ferrous iron can directly diffuse across the cellular membrane. Some pathogenic bacteria use specific receptors to obtain iron from transferrin and lactoferrin. Transferrin is generally found in the serum and functions both in iron transport as well as protecting the host from iron toxicity.

Lactoferrin is generally found in the lymph and mucosal secretions and protects the host by functioning as an iron chelator. Both lactoferrin and transferrin have an extremely high affinity for FeIII and a much lower affinity for FeII. *Neisseria meningitidis* and *N. gonorrhoeae* have transferrin and/or lactoferrin iron uptake systems. In Gram negative bacteria, iron is extracted from siderophore-iron complexes at the cell surface by a mechanism that is not well understood, and then the iron is transported via an ABC transporter. Gram positive bacteria also use transferrin but by less well characterized means (Wandersman and Delepelaire 2004). Heme proteins also provide an iron source for some pathogens. Heme is an iron protoporphyrin molecule present in many enzymes. Because of its high toxicity, heme is rarely found in a free form. While micromolar concentrations of iron are required for bacteria, only nanomolar concentrations of heme are required. Hemoglobin is found in red blood cells and functions as an oxygen transporter. Some bacteria contain heme-hemoglobin uptake systems. Some receptors such as HemR of *Yersinia enterocolitica* recognize both heme and hemoglobin. Other receptors such as HmbR of *N. meningitidis* are more specific for hemoglobin (Wandersman and Delepelaire 2004). Some bacteria are known to possess heme transport systems and a number of bacteria are able to survive in media with heme as the sole iron source (Mills and Payne 1995).

A common system for acquiring iron is through the use of siderophores. Bacteria and fungi produce siderophores in response to iron starvation conditions. These molecules are low molecular weight iron-chelating molecules that sequester ferric iron from the environment, enabling microorganisms to use this iron. Siderophores are nonribosomally synthesized and contain a functional group that binds the iron. The

majority of siderophores contain 1 or more of the following functional groups: hydroxamate, catecholate, hyroxy acid, or citric acid. A siderophore containing multiple functional groups is considered a mixed ligand siderophore (Mills and Payne 1995). Siderophores bind to iron with an extremely high affinity. Genes that control the synthesis of siderophores are often found clustered with the genes for siderophore uptake (Wandersman and Delepelaire 2004).

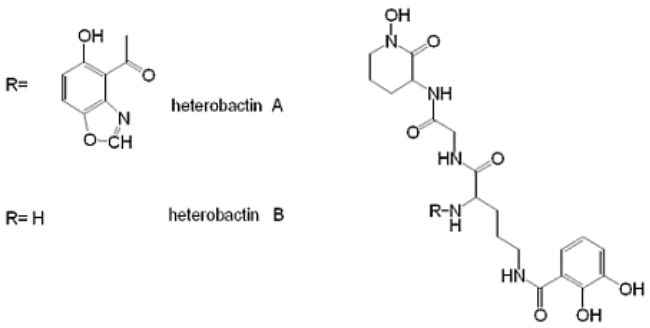
Under low iron conditions regulatory proteins such as IdeR stimulate the production of siderophores. Once synthesized, the siderophore is released into the environment surrounding the cell. It creates a siderophore-iron complex when it comes into contact with ferric iron, for which the siderophore has a high affinity, the complex then returns to the cell. The siderophore-iron complex is first transported across the outer membrane (in a Gram negative bacteria like *Escherichia coli*) via a specific receptor and then utilizes an ATP-binding-cassette, or ABC type transporter, to cross the inner membrane. Once the siderophore-iron complex is inside the cell, the iron is converted to ferrous iron via a ferric reductase which reduces the ferric iron to ferrous iron, for which the siderophore has a much lower affinity. This releases the iron from the siderophore, and the siderophore is then free to exit the cell and sequester additional iron. Another possible method of release within the cell is the degradation of the siderophore, which also releases the ferrous iron within the cell. Once released from the siderophore, excess iron can be stored in structures such as bacterioferritins. Bacterioferritins are large, complex structures composed of approximately 24 individual protein subunits. These subunits form a ball like structure with a hollow center. The main function of a ferritin within a cell is to store iron in the ferric form. A secondary function may be to detoxify

iron so as to protect against oxygen radicals. The bacterioferritin can store around 4500 iron ions (Carronodo 2003). Oxygen atoms in the bacteriferritin reoxidize ferrous iron to ferric iron, allowing for the storage of ferric iron within the bacterioferritin.

Hundreds of siderophores have been isolated and purified. Enterobactin, also known as enterochelin, is a catecholate type siderophore produced by *Escherichia coli*. This is one of the best studied siderophores. Enterobactin shows a very high affinity for iron (Ratledge and Dover 2000). Another well studied siderophore is a cell associated siderophore, mycobactin, produced by the mycobacteria.

It was discovered that *R. erythropolis* produces and releases a mixed ligand siderophore, Heterobactin A and the structurally similar Heterobactin B, Figure 2A (Carrano et al. 2001). The Heterobactins contain both catecholate and hydroxamate groups. The Heterobactins are synthesized and secreted in response to iron poor conditions. Once bound to FeIII, the iron-siderophore complex returns to the *Rhodococccus* cell. Recently, it was discovered that *R. rhodochrous* produces a siderophore named Rhodobactin, Figure 2B. Rhodobactin is a mixed ligand siderophore containing 1 hydroxamate and 2 catecholate groups (Dhungana et al. 2006). A third species, *R. equi*, is known to produce a catecholate-type siderophore, although this siderophore has not yet been characterized (Miranda-CasoLuego et al. 2008).

(A)



(B)

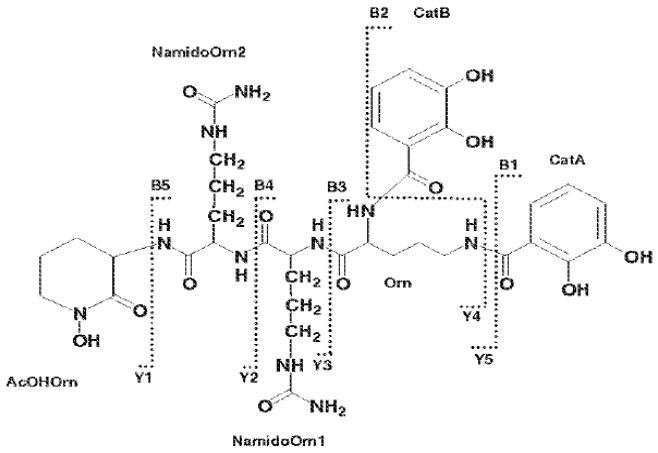


Figure 2: Siderophores of *R. erythropolis*. (A) Heterobactin A and B of *Rhodococcus* erythropolis IGTS8 (Carrano et al. 2001) . (B) Rhodobactin produced by *R. rhodochrous* (Dhungana et al. 2007).

These are true siderophores in that they relieve iron-limited growth in the producing strain. However, in some bacteria, even though they do not produce a particular siderophore, they are still able to transport siderophores produced by other organisms. Often, an organism will have a transport system for transporting siderophores produced by other organisms. For example, *Escherichia coli* transports Heterobactin A and Heterobactin B while *Arthrobacter flavescens* is able to transport Heterobactin B, but neither synthesizes these siderophores (Carrano et al. 2001)

Iron Uptake in Mycobacteria

Iron uptake in the mycobacteria is thought to be similar to that of the rhodococci. Therefore, an examination of iron acquisition and uptake in the mycobacteria should be explored in order to explain that of the rhodococci. Genes involved in iron acquisition are regulated by both iron rich and iron poor conditions. Under iron poor conditions, both siderophore biosynthetic genes and transporter genes are upregulated (Rodriguez and Smith 2003). Iron overload is toxic for bacterial cells. Therefore, many of the genes involved in the assimilation of iron and in iron acquisition are only expressed under iron poor conditions. Once the intracellular iron concentration rises, these genes are no longer expressed (Wandersman and Delepelaire 2004).

Regulation by Ferric Iron Uptake Regulator

The ferric uptake regulator, Fur protein, was first identified in *Salmonella* and *E. coli* unable to repress siderophore receptors under iron rich conditions (Rodriguez and Smith 2003). Fur regulates iron acquisition in many different bacteria. Fur homologues can repress the expression of genes that are involved in iron acquisition when intracellular iron concentration increases, halting iron acquisition (Bereswill et al. 2000). Fur acts as a transcriptional repressor when bound to ferrous iron. This Fur-iron complex binds to a particular sequence, the Fur box, in iron regulated promoter regions and then inhibits the transcription of genes downstream (Rodriguez and Smith 2003)

Regulation by IdeR and DtxR

Iron metabolism can also be regulated through the iron dependent regulator (IdeR), a 230-amino acid protein not related to Fur (Rodriguez 2006). IdeR functions as a

homodimer. Each monomer has three functional domains with two metal binding sites. The N-terminal region contains the DNA binding domain and the dimerization domain contains most of the metal-binding residues. A third domain is located in the C-terminal region (Rodriguez 2006). A IdeR homolog, the diphtheria toxin repressor (DtxR) of *Corynybacterium diptheriae* is found in some actinomycetes. DtxR acts in the same way as Fur and IdeR (Figure 3).

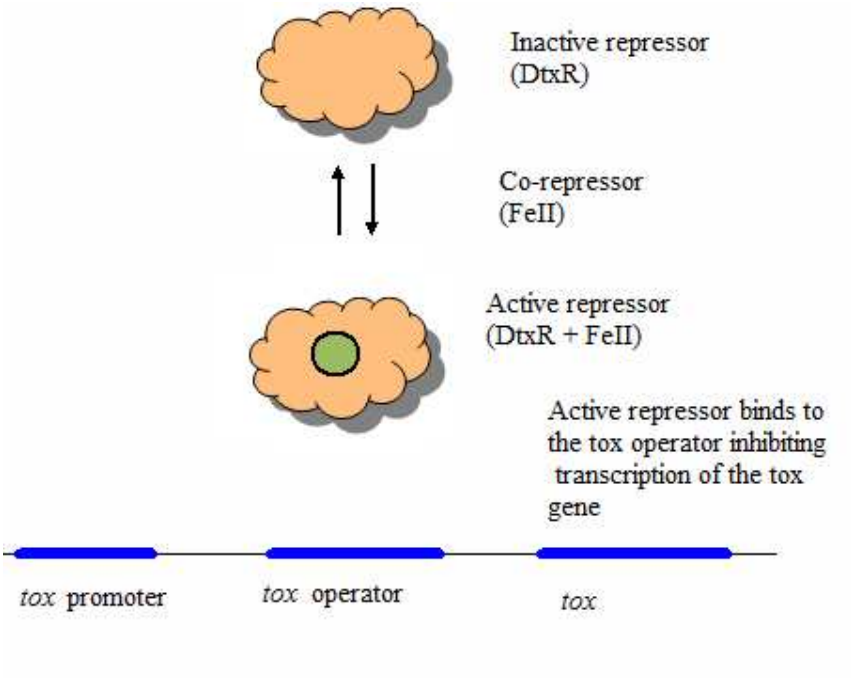


Figure 3: Regulation of the Diphtheria toxin repressor by DtxR

The mycobacteria produce 2 siderophores: the cell associated mycobactin and the secreted exochelin. When iron is present in the environment, the iron binds to the regulatory protein, IdeR. This complex then binds to the promoter region of the biosynthetic genes for the siderophores and acts as a repressor, stopping the synthesis of siderophore products. However, under iron limited conditions, iron does not bind to IdeR and, therefore, IdeR does not act to repress synthesis of siderophores.

Transport of Siderophores

ATP binding cassette transport systems (ABC transport systems) are widespread among living organisms. These transport systems are ATP dependent and couple the hydrolysis of ATP with the translocation of a solute across a biological membrane. In the case of siderophores, the hydrolysis of ATP is coupled with the translocation of the siderophore molecule plus ferric iron across the bacterial membrane. ABC transport systems are characterized by a common modular organization and 2 sequence motifs that constitute a nucleotide binding fold. ABC transport systems are involved not only in the uptake of nutrients but also in processes such as signal transduction, drug resistance, protein secretion, antigen presentation, bacterial pathogenesis, and sporulation. Defective ABC transport systems are also the cause of some human inheritable diseases such as cystic fibrosis (Schneider and Hunke 1997).

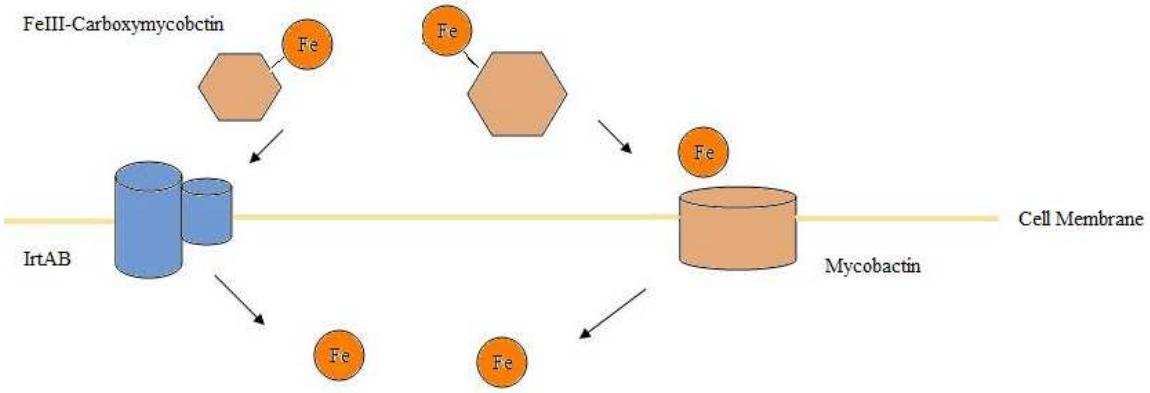
The mycobacteria contain a specific ABC transporter for the export of siderophores. *Mycobacterium tuberculosis* contains 2 ABC transporters, IrtA and IrtB. It is believed that IrtA functions in the export of carboxymycobactin from the bacteria. IrtB functions as an ABC transporter in conjunction with the protein Rv2895c. IrtB-Rv2895c acts as a 2-component importer of the iron-carboxymactin complex (Farhana et al. 2008). Both IrtA and IrtB have the general characteristics of an ABC transporter. They are localized in the membrane and are upregulated by IdeR iron stress. Rv2895c facilitates the import of the iron-siderophore complex by specifically interacting with IrtB via a permease domain in Rv2895c (Farhana et al. 2008).

Iron uptake is tightly controlled, often by employing active transporter systems to maintain homeostatis, especially under hostile conditions. IrtA is believed to export the siderophores to prevent a buildup of siderophores in the cell. This could lead to the death of the cell either through the buildup of siderophores that leads to cell lysis or the binding of carboxymycobactin to iron inside of the cell. IrtB then imports iron-siderophore complexes. The 2 proteins function together to maintain a delicate balance between the import and export of siderophore to prevent their deleterious effect on the mycobacterial cells (Figure 4B) (Farhana et al. 2008).

Another proposed method of action by the ABC transporter of *M. tuberculosis* states that IrtA and IrtB, together known as IrtAB, work together to transport ferrated carboxymycobactin into the bacterial cell (Figure 4A) (Rodriguez and Smith 2006).

Because the mycobacteria produce both a membrane bound siderophore as well as a secreted siderophore, there has been interest as to why the bacteria produce 2 types of siderophores. Carboxymycobactin, or exochelin, is able to remove iron from host iron binding proteins such as transferrin (Gobin and Horwitz 1996). The current theory is that the membrane-bound mycobactin accepts iron from the secreted carboxymycobactin. The mycobactin is not essential for cell growth and survival, but it may increase the efficiency of iron transport into the cell by functioning as a cellular receptor for the FeIII that is captured by the carboxymycobactin. The mycobactin could then serve as an ionophore for the transport of the FeIII across the lipid envelope. It has also been suggested that the membrane-bound mycobactin might serve as a temporary iron storage molecule (Rodriguez 2006).

(A)



(B)

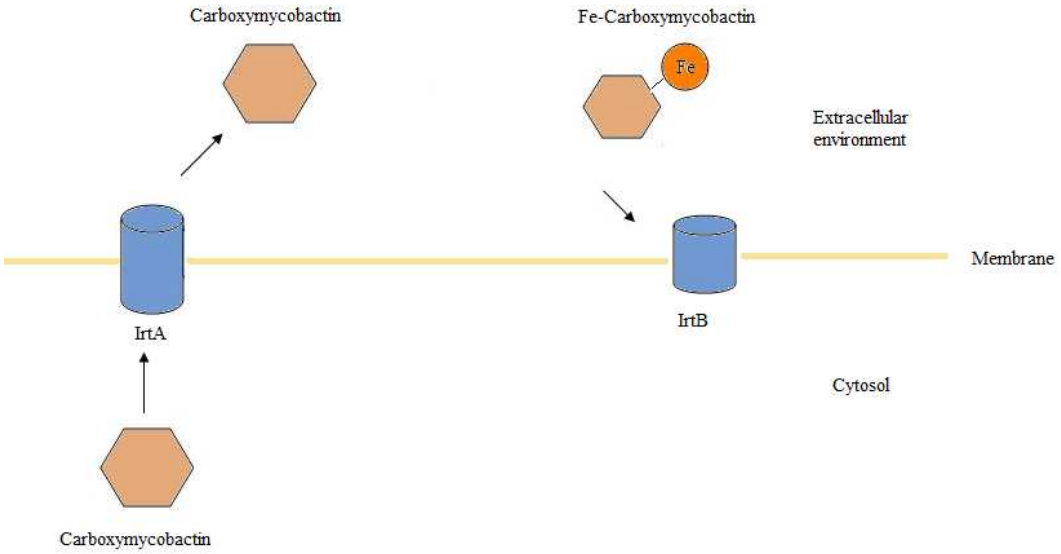


Figure 4: Mechanism of ABC Transporter in *Mycobacterium tuberculosis*. (A) Iron transport via ABC transporter, IrtAB in *Mycobacterium tuberculosis* adapted from Rodriguez and Smith, 2006. (B) Iron transport via an ABC transporter in *M. tuberculosis* adapted from Farhana et al, 2008.

CHAPTER 2
HYPOTHESIS

Recently, Jaishree Vellore isolated a mutant strain of IGTS8 that she designated R187-12. R187-12 appears to over produce the siderophore heterobactin. This mutant was created via insertional mutagenesis with a plasmid suicide vector known as pJCS506 (Vellore 2001). R187-12 is a potential tool to identify a gene(s) from IGTS8 that is responsible for either the uptake of ferri-siderophore complexes or regulate the production of siderophore. My research hypothesis is as follows:

1. Mutant bacteria that over produce siderophore often do so to compensate for a defect in the uptake of this compound; therefore, I hypothesize that the mutant strain R187-12 represents a defect in the gene(s), probably a transport protein, responsible for the uptake of iron-siderophore complexes.
2. R187-12 chromosomal DNA flanking the insertional mutagen, pJCS506, can be used to identify wild type (IGTS8) DNA corresponding to DNA that is interrupted by the insertional mutation during generation of the mutant, R187-12.

The aim of this research is to test the preceding hypotheses and to characterize the mutant R187-12. The characterization of R187-12 will be accomplished through Southern blotting and DNA sequencing

CHAPTER 3
MATERIALS AND METHODS

Bacterial Strains, Plasmids and Growth Conditions

Rhodococcus erythropolis IGTS8 cells were grown on Rich Media (RM) (10 grams (g) glucose, 8g nutrient broth, and 0.5g yeast extract per liter of water) or Lauria-Bertani (LB) (10 g tryptone, 5g yeast extract, and 5g sodium chloride per liter of distilled water) at 30^{o}C for 3 days. Liquid cultures were grown under the same conditions with shaking. Plasmid DNA (pTNR) was obtained from the National Institute of Advanced Industrial Sciences and Technology (AIST), Japan. Mutants arising from either pJCS506 or pTNR were maintained on LB + Kanamycin or RM + Kanamycin (75μg/ml Kanamycin). The bacterial strains and plasmids used in this study are listed in Table 1.

Table 1: Bacterial Strains and Plasmids Used in This Study

Bacterial strains and plasmids	Description	Source
pJCS506	*Amp^{r}, Kan^{r}, E. coli* propogated *R. erythropolis* suicide vector	Schneider (1999)
pUC18	*Amp^{r}, E. coli* vector containing *lacZα* gene	Yanisch-Perron et al. (1985)
pTNR	*Rhodococcus* insertional mutagen containing IS1415, *E. coli* ori, and *Kan^{r}*	Sallam (2006)
R. erythropolis R187-12	*Km^{r}* siderophore overproducing mutant of IGTS8	Vellore, 2001
R. erythropolis R187-5	*Km^{r}* siderophore overproducing mutant of IGTS8	Vellore, 2001
R. erythropolis L51-10	*Km^{r}* siderophore overproducing mutant of IGTS8	This study
R. erythropolis IGTS8	Wild type	Kilbane and Jackowski (1992)

Preparation of Competent Cells

The method for the preparation of electro-competent *Rhodococcus* cells is the same as that described by Vellore (MS thesis, 2001).

Isolation of Plasmid DNA

Plasmid DNA was isolated using either the QIAprep kit (for sequencing) or the following method:

A 10ml culture of transformed *E. coli* was grown overnight in LB with the addition of the required antibiotic. The following day, the culture was transferred to an oak ridge centrifuge tube and centrifuged for 7 minutes at 4^{o}C at maximum speed. The cell pellet was washed in 1ml of STE buffer (0.01M NaCl, 10mM Tris pH 8.0, 1mM EDTA). The washed cells were transferred to a 1.5ml microfuge tube and centrifuged for 45 seconds at 13000 rpm at 4^{o}C. All liquid was aspirated. The cell pellet was then resuspended in 200µl of solution I + fresh lysozyme (2mg/ml). The cells were left in this solution for 10 minutes at room temperature. Then, 400µl of freshly made solution II (1N NaOH, 10% SDS) was added to the tubes and mixed thoroughly by inverting the tube many times. Care was taken not to expose DNA to this solution for more than 5 minutes. Next, 300µl of Solution III (5M Potassium Acetate) was added and the tube was inverted several times until a white flocculant formed. The tubes were then left to sit on ice for 10 to 15 minutes. The tubes were then centrifuged at 13000 rpm for 15 minutes. Half of the clear supernatant (approximately 450 µl) was transferred to a 1.5ml microfuge tube and the other half of the supernatant was transferred to a second 1.5ml microfuge tube. The supernatant was then phenol:chloroform extracted by adding 200 µl of TE saturated

phenol and 200 µl of chloroform:isoamyl alcohol (24:1). The tubes were then centrifuged for 5 minutes at maximum speed. The top layer was collected to a clean 1.5ml microfuge tube and chloroform extracted with 400 µl of chloroform:isoamyl alcohol twice and centrifuged 2 minutes each time. Following the second chloroform extraction, the top layer was transferred to a labeled microfuge tube and 1ml of cold ethanol was added. The tube was cooled on ice for 5 minutes and centrifuged for 10 minutes at maximum speed. All ethanol was aspirated and the DNA pellet was washed with 400 µl of 70% ethanol. The DNA was centrifuged at maximum speed for 1 to 2 minutes and all ethanol was aspirated and the DNA pellet was allowed to briefly air dry. The DNA pellet was then redissolved in 30 µl/tube of 0.1TE + RNase.

Transformation by Electroporation of *Rhodococcus* Cells

The method for transformation of *Rhodococcus* cells using electroporation is the same as that described by Vellore (MS thesis, 2001). Transformed cells were transferred to sterile test tubes and incubated along with shaking at 30^{o}C for 4 to 5 hours. Following incubation, dilutions (100µl undiluted, 75µl undiluted, 50µl undiluted, 1:10 dilutions, 1:100 dilutions, and 1:1,000 dilutions) of the transformed cells were plated onto LB + Kanamycin (200µg/mL Kanamycin was used to select for *Rhodococcus* cells transformed with pTNR). The control transformations with pJCS506 or no plasmid were plated in the same way. Plates were incubated at 30^{o}C for 3-4 days and the number of resulting colonies was noted.

Fiss Minimal Medium for *Rhodococcus*

Fiss minimal medium used for *Rhodococcus* was prepared using the method described by Vellore (MS thesis 2001).

Preparation of Chrome Azurol S (CAS) Assay Medium

The method for the preparation of Chrome Azurol S (CAS) assay medium is the same as that described by Vellore (MS thesis 2001).

Detection of Siderophore Production via CAS Assay

Following the transformation of the *Rhodococcus* cells, mutants were replica plated on both LB + Kanamycin and Fiss Minimal Media + Kanamycin (75 μg/ml) using numbered grids to indicate the location of each mutant strain. Sterile toothpicks were used to pick colonies from transformant plates to LB and Fiss minimal plates. Colonies that were able to grow on the Fiss Minimal Media after 3 to 4 days were then plated onto CAS agar plates, using the same numerical grid system. A small, round spot of cells was placed in the center of each grid square. The CAS plates were incubated at 30^{o}C for 3 to 4 days to allow for the detection of siderophore production.

Isolation of Chromosomal DNA

Chromosomal DNA was isolated from *Rhodococcus* cells by the following method. First, a 2ml LB + Kanamycin (75 μg/ml) seed culture was grown in a 30^{o}C water bath overnight with shaking. The following day, a 10ml LB + Kanamycin broth was inoculated with 150μl of the overnight grown seed culture and grown under the same conditions. The following day, 6μg of ampicillin was added to the late log phase culture and allowed to incubate at 30^{o}C with shaking for 3 hours. The culture was then centrifuged in an oak ridge tube for 10 minutes at 4^{o}C. The cell pellet was rinsed with 2ml of 1X TE buffer and centrifuged again under the same conditions. The pellet was then resuspended in 1ml of TES + lysozyme (100mg lysozyme/ml) and split into two

1.5ml microfuge tubes. The cells were incubated in the lyzozyme solution for 3 hours at 37°C with periodic agitation. Then, 20µl of 20% SDS, 160 µl 3M NaCl and 10 µl RNase were added and mixed by gently inverting the tube many times to avoid shearing the DNA. The tubes were then incubated at 55°C for 15 minutes. Following the time period, 7.5µl of Proteinase K was added and gently mixed by inverting the tube and incubated at 55°C for 15 minutes. Following the incubations, 400-500µl of phenol was added to each tube and mixed. The tubes were then centrifuged at maximum speed for 8 minutes. The top aqueous layer was removed to a new tube and 400-500µl of chloroform:isoamyl alcohol was added and gently mixed and the tube was centrifuged for 2 to 3 minutes. The top aqueous layer was removed to a new tube and a second chloroform extraction was performed. The top aqueous layer was removed and transferred to a new tube and approximately 1ml of ice cold ethanol was added to each tube. The tubes were gently inverted many times to precipitate the chromosomal DNA. The chromosomal DNA was spooled on the end of a pipette tip, then transferred to 400µl of 70% ethanol and briefly centrifuged for 1 to 2 minutes. The ethanol was then aspirated and the chromosomal DNA briefly air dried. The chromosomal DNA was then resuspended in 50 to 100 µl of 0.1TE + RNase and stored at 4°C overnight to re-hydrate. The next day, a sample of the chromosomal DNA was loaded onto a 0.75% agarose gel to ensure the quality of the chromosomal DNA.

Gel Electrophoresis and Electro-Elution

The method for gel electrophoresis and gel electro-elution is the same as that described by Moretz (MS thesis, 2003).

Southern Blotting

Preparation and Transfer of DNA

The method for the preparation of DNA and the transfer of DNA to be used for Southern Blotting was the same as that described by Moretz (2003).

Random Primed Labeling of Probe DNA with Dioxygenin-11-dUTP (DIG)

DNA that was to be used as a probe for Southern hybridization was first digested with restriction endonucleases to linearize the plasmid DNA, then ethanol precipitated and resuspended in 16µl of distilled water. The labeling reaction was begun by taking 13µl of the digested plasmid DNA and placing it in a sterile 1.5ml microcentrifuge tube. The DNA was denatured by boiling in a hot water bath at 95^{o}C for 10 minutes and then quick cooling the denatured DNA in ice with NaCl for 5 minutes. The tube was then briefly centrifuged to pool all droplets. Following centrifugation, the DNA was placed on ice and the following components were added: 2µl 10X EcoPol buffer (New England Biolabs); 2µl random heximer primers [pd(N)$_6$ sodium salt]; 2µl 10X DIG DNA labeling mix and 1.5µl Klenow fragment. The mixture was incubated overnight at 37^{o}C. The following day, the mixture was briefly centrifuged at maximum speed to pool all droplets, and the volume was brought up to 100µl total volume with distilled water. The mixture was then phenol:chloroform (1:1) (90µl) extracted once and chloroform extracted once with 90µl chloroform:isoamyl alcohol (24:1). The DIG labeled probe DNA was recovered by adding 1/10 the total volume of 10M ammonium acetate and 2 volumes of ice cold 100% ethanol. The mixture was then cooled at -80^{o}C for at least 30 minutes and then centrifuged at maximum speed for 20 minutes. The supernatant was

discarded and the DNA briefly air dried. The DNA was then resuspended in 20µl of 0.1X TE buffer.

Hybridization

The method for the hybridization of Southern transfers was the same as that described by Moretz (2003).

Preparation of Dephosphorylated Vector Plasmid

Vector plasmid, pUC18, was prepared for ligation with insert DNA by phosphatase treating in the following manner. The vector plasmid was digested with the desired restriction endonuclease, Pst1, and then phenol:chloroform extracted and ethanol precipitated as previously described. The vector plasmid was then resuspended in a small volume of 0.1 TE. Approximately 1-2µg of DNA was phosphatase treated by adding 5µl of CIP dephosphorylation buffer (or 10X NEB #3, New England Biolabs) to the plasmid DNA, and water to bring the total volume to 48µl. The appropriate amount (1µl) of calf intestinal phosphatase (CIP) was then added and the mixture was incubated for 30 minutes at 37^{o}C. A second aliquot of CIP was then added and the incubation continued for another 30 minutes at 37^{o}C. To inactive the CIP at the end of the incubation period, 0.5% SDS and 5mM EDTA, pH 8.0 were added and thoroughly mixed. Proteinase K was added to a final concentration of 100µg/ml and the mixture was incubated for 30 minutes at 56^{o}C. The reaction was then cooled to room temperature and purified by twice extracting with phenol:chloroform (1:1) and twice extracting with chloforom:isoamyl alcohol (24:1). The phosphatase treated DNA was then ethanol

precipitated by bringing the total volume to 100µl with sterile distilled water and then adding 1/10 of the total volume with 10M ammonium acetate and 2 volumes of ice cold 100% ethanol. The mixture was cooled at -20^{o}C overnight. The following day, the mixture was centrifuged at maximum speed for 20 minutes. The supernatant was discarded and the DNA was resuspended in 25µl of sterile distilled water.

Plasmid Genomic Library

To create a plasmid genomic library, fractions of digested chromosomal DNA containing the insertional mutagen obtained through electro-elution were ligated into phosphatase treated vector plasmid. Control ligations of phosphatase treated vector and nonphosphatase treated vector as well as ligations both with and without insert DNA were preformed in order to determine the quality of the vector plasmid as well as the number of background colonies to be expected upon transformation. Following ligation, the ligated DNAs were transformed into electro-competent *E. coli* cells. Dilutions (100µl undiluted, 75µl undiluted, 50µl undiluted, 1:10, dilutions and 1:100 dilutions) of the transformation were plated onto LB + Kanamycin (50µg/ml) or LB + Ampicillin (50µg/ml) plates and incubated overnight at 37^{o}C. Colonies of phosphatase treated and untreated ligations were then counted to determine whether the phosphatase treatment was adequate. Ligations with and without insert DNA were also counted to determine the number of background colonies. Colonies resulting from ligation with insert DNA and phosphatase treatment of the vector plasmid were then grown in liquid culture for plasmid preparation as previously described. Following plasmid preparation, the plasmids were digested with Pst1 restriction endonuclease and run on a 0.75% agarose

gel. Plasmids containing an insert the size of the insert DNA, which was determined by the size of the hybridizing band on the Southern blot (approximately 9kb) were then grown in liquid culture and used for plasmid isolation using the QIAprep system. The purified DNA was used for sequencing.

Colony Hybridization

Fixation of colony DNA and colony hybridization were conducted using the method described by Moretz (2003).

DNA Sequencing

DNA sequencing was performed at the ETSU Molecular Biology Core Facility. Custom made primers were ordered from Integrated DNA Technologies (IDT) and diluted to a concentration of 3μM. A 10 to 15μl sample of primer as well as a 15μl sample of the desired plasmid DNA (concentration of DNA= 100ng/μl or greater) were sent to the Molecular Biology Core Facility for sequencing. DNA sequencing at the ETSU Molecular Biology Core Facility was conducted using the Beckman Coulter GenomeLab™ Dye Terminator Cycle Sequencer. Following sequencing, the DNA sequence was checked using the Chromas program to ensure the quality of the DNA sequence. The sequence data was then subjected to a nucleotide BLAST search using the nucleotide collection database. The resulting homologies were then noted. New primers were developed using Primer3 (http://fokker.wi.mit.edu/primer3/input.htm). This was done by subjecting the DNA sequence to a search using the Primer3 program choosing only right primers (reading from 5' to 3' on opposite strand) or left primers for sequencing in the opposite direction. As new sequence data were received, overlaps were

found and the sequence data spliced together. The spliced DNA sequence was subjected to nucleotide Blast searches as before. The DNA sequence was also translated into a protein sequence using NEB cutter V2.0 and open reading frames (ORFs) subjected to a protein Blast search to determine protein homology.

CHAPTER 4

RESULTS AND DISCUSSION

Generation of Mutant Strains Using pTNR

Mutations were produced using the plasmid pTNR containing the IS element IS1415, as an insertional mutagen, with the Kan^r marker region inserting into the genomic DNA. The insertion sequence (IS) element of the plasmid encodes for the transposase gene, which is necessary for the movement of the element from one site to another. This gene allows the movement of the DNA segment carrying the kanamycin resistance marker from IR1 to IR2 (Figure 5). The IS element does not contain any genetic material other than that required for the transposase. Once mobilized, the transposon containing the kanamycin resistance marker is able to insert into the chromosomal DNA of *Rhodococcus*. This transposon is only able to insert a single time because it does not carry the transposase genes and is unable to replicate in the *Rhodococcus* genome. However, because the transposing sequence of DNA also carries an *E. coli* origin of replication region (ori) the transposon is able to replicate in *E. coli* cells, enabling the region of *Rhodococcus* DNA containing the transposon to be cloned in *E. coli*.

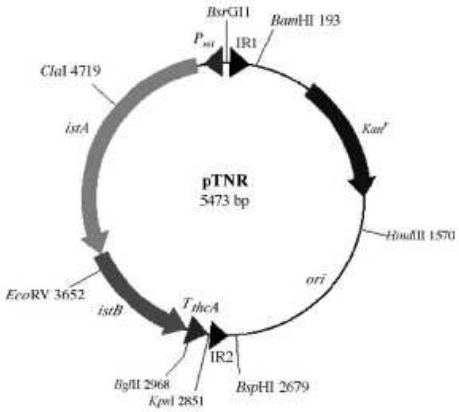


Figure 5: Map of pTNR plasmid (Sallam, 2006)

Insertional mutagens insert a piece of DNA into a gene, thereby interrupting the gene's reading frame and inactivating the gene's function. Electrotransformation was used to transform electro-competent *Rhodococcus erythropolis* cells with the plasmid pTNR carrying the IS1415 and kanamycin resistance marker (Figure 6).

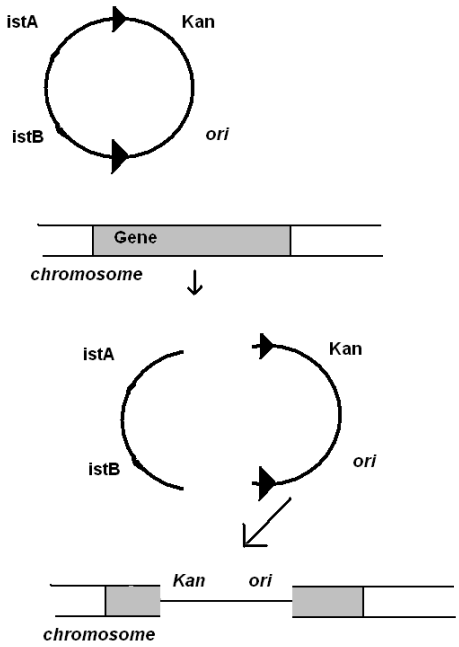


Figure 6: Mutagenesis with IS1415 of pTNR. The transposon randomly inserts into genomic DNA, interrupting chromosomal genes

Transformants resulting from pTNR were selected by plating them to LB agar plates containing 200 μg/ml of kanamycin. The addition of kanamycin ensured that only cells transformed with pTNR and containing the mutagenic kanamycin resistance marker are selected. A total of 2,331 transformants were generated and screened (Table 2).

Table 2: Mutants Generated by the Insertional Mutagen pTNR

Mutant Type	**Total Kan^r transformants**	**Auxotrophic**	**Pigment mutant**	**No Halo**	**Large halo**
Number of Transformants	2,331	5	4	7	3

Auxotrophic Mutants

The presence of auxotrophic mutants indicates that mutations are being generated within the genome of *Rhodococcus erythropolis*. Auxotrophic mutants lack the ability to synthesize an essential nutrient such as an amino acid on a minimal growth medium. In order to grow, the auxotrophic mutants must obtain these nutrients from an enriched medium. A minimal medium contains only the basic salts and glucose as a carbon source but lacks other essential nutrients and cannot support the growth of auxotrophic mutants. However, a rich medium is a complex medium that contains the essential nutrients that a cell requires and can support the growth of auxotrophic mutants. Mutants that are able to grow on the LB agar, but unable to grow on the minimal media agar are said to be auxotrophic mutants. From a screen of 2,331 kanamycin resistance pTNR transformants, 5 auxotrophic mutants were detected. Vellore (2001) detected 5 auxotrophic mutants from a screen of only 250 pJCS506 transformants.

Screening for Mutants Defective in Siderophore Production

The 2,331 transformants on LB agar plates were screened on CAS agar plates to detect colonies that either did not produce siderophores or colonies that overproduced siderophores. The CAS assay is a color-chemical assay for the detection of siderophore production (Schwyn and Neilands 1987). Non siderophore producing mutants were identified by a lack of a halo or color change surrounding the colony while over-producing mutants were identified by an oversized halo surrounding the colony (Figure 7).

The CAS assay is based on the removal of ferric iron from a pigmented complex by a competing ligand such as a siderophore. In the CAS agar plate, the ferric iron is initially bound to a dye, resulting in a bright blue color. When the siderophore is released from the cell it binds to the ferric iron, resulting in free dye, which results in a yellow halo in areas where siderophores are produced (Figure 7). Therefore, when a siderophore binds with the ferric iron, the release of the dye is indicated by a color change from blue to yellow (Schwyn and Neilands 1987). The following equation explains the reaction between siderophores and the CAS assay:

Fe-Dye (Blue media) + Ligand (siderophore) → Fe-Ligand + Free dye (Yellow color change)

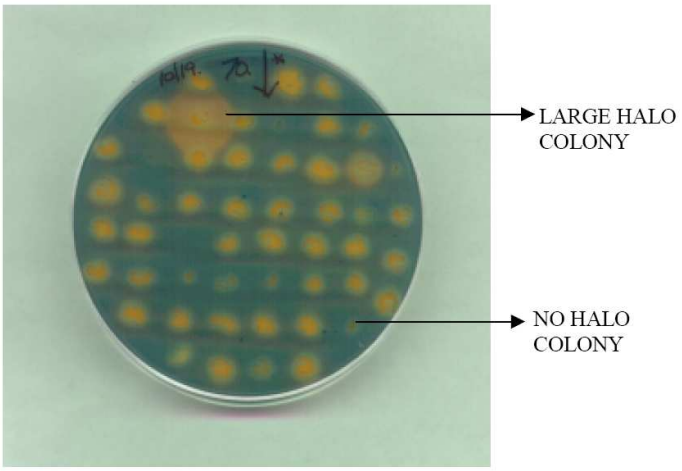


Figure 7: Chrome Azurol S plate used to screen *Rhodococcus* mutants for over-production of siderophore (large halo) or no production (no halo)

Characterization of L51-10: Large Halo Mutant

Of the 2,331 transformants generated using the insertional mutagen pTNR, 1 large halo mutant, designated L51-10 was investigated. This mutant produced a large halo on CAS assay plates, but Southern Blot analysis with pTNR as a probe was not successful in indicating a hybridizing band in the mutant. Other mutants generated from the insertional mutagen, pTNR, were studied and produced hybridizing bands on Southern blot analysis, but attempts at self-ligating these mutants was unsuccessful and no plasmid clones containing *Rhodococcus* DNA were recovered.

Generation of Mutants using pJCS506

Large halo producing mutants, R187-12 and R187-5, using pJCS506 were obtained from a previous study (Vellore 2001). Random mutations in the genome of *R. erythropolis* strain IGTS8 were generated using this plasmid as an insertional mutagen. The entire plasmid appears to insert into the *Rhodococcus* chromosome at random locations via illegitimate recombination (Figure 9) (Desomer et al. 1991). The pJCS506

plasmid is 8390bp in length and has a kanamycin resistance marker as well as an *E. coli* origin of replication (Figure 8).

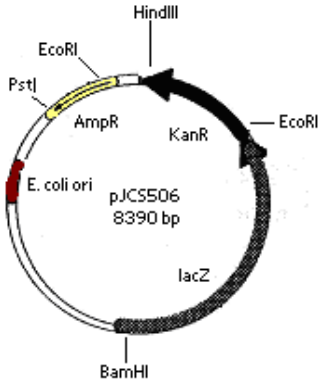


Figure 8: Map of pJCS506 plasmid adapted from Schneider, 1999

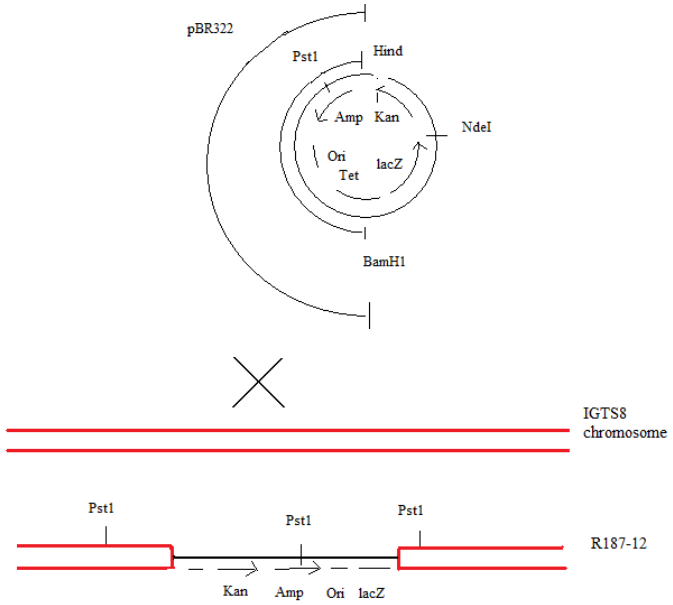


Figure 9: Recombination of pJCS506 into *R. erythropolis* chromosome.

Characterization of R187-5: Large Halo Mutant

The mutant R187-5 that over produces siderophore, created by Jaishree Vellore using the insertional mutagen, pJCS506, was investigated. It was determined through Southern blot analysis that R187-5 produced hybridizing bands when hybridized with DIG labeled pJCS506. However, these bands were not of the expected size and Southern blot analysis indicated that R187-5 cut with various restriction enzymes was producing hybridizing bands that were either not of the expected size or 2 hybridizing bands, instead of a single band, as would be expected with this system (Data not shown). Ligation into a plasmid vector of a single hybridizing band of R187-5 cut with PstI was attempted, but no kanamycin resistant transformant colonies were recovered.

Identification of the Gene(s) Responsible for Siderophore Defect in R187-12

Chromosomal DNA was isolated from the R187-12 mutant and digested with various restriction enzymes. The DNA samples were then loaded onto a 0.75% agarose gel and run at 18 volts overnight (Figure 10A). The DNA was then transferred from the agarose gel to a nylon membrane and prepared for Southern blotting. DIG labeled pJCS506 was used as a hybridization probe in order to identify fragments of the chromosome containing the insertional mutagen, pJCS506 (Figure 10B). The Southern blot indicated that R187-12 cut with PstI contained a hybridizing fragment of approximately 10kb, R187-12 cut with BamHI contained 2 hybridizing fragments of approximately 9kb and less than 2kb. R187-12 cut with EcoRI showed 2 hybridizing fragments of approximately 6.5kb and 2.3kb.

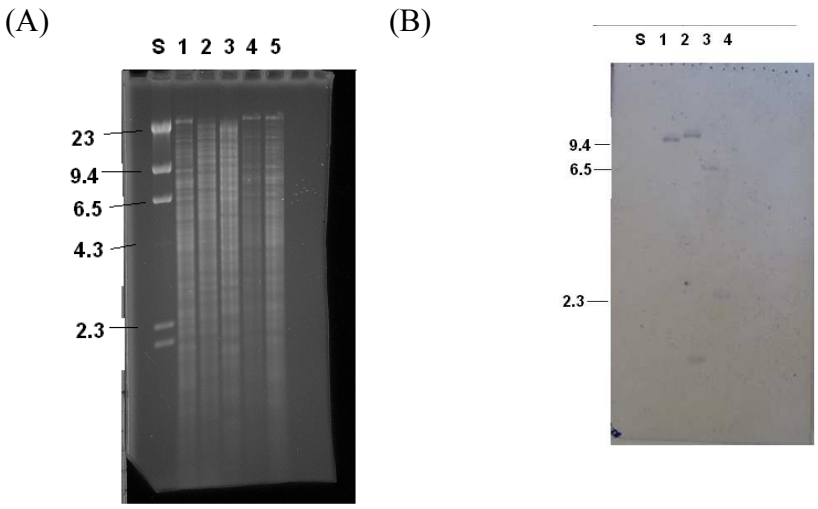


Figure 10: Gel Electrophoresis of R187-12 chromosomal restriction digests. Southern hybridiziation of R187-12 chromosome cut with the following restriction enzymes: lane 1 contains R187-12/PstI, lane 2 contains R187-12/BamHI, lane 3 contains R187-12/EcoRI, lane 4 contains R187-5/PstI and lane 5 contains IGTS8/PstI (as a negative control) were electrophoresed on an agarose gel (A) and transferred to a nylon membrane where they were hybridized with DIG labeled pJCS506 (B).

In order to recover the chromosomal fragment containing pJCS506, a gel was loaded with R187-12 cut with PstI and run overnight. The gel was then cut in 1cm fractions and electro-eluted to recover purified DNA fractions of different sizes. The recovered fractions (fractions 1 through 4) were then loaded onto a 0.75% agarose gel (Figure 11A). The gel was then transferred to a nylon membrane and prepared for Southern blotting to confirm which size fragment contained the insertional mutagen. The membrane was hybridized using DIG labeled pJCS506 as a probe DNA. The Southern blot indicated that the hybridizing fragment containing the insertional mutagen, pJCS506 was located in fraction 2 with a size range from 6.5 to 9.4 Kb (Figure 11B).

(A) (B)

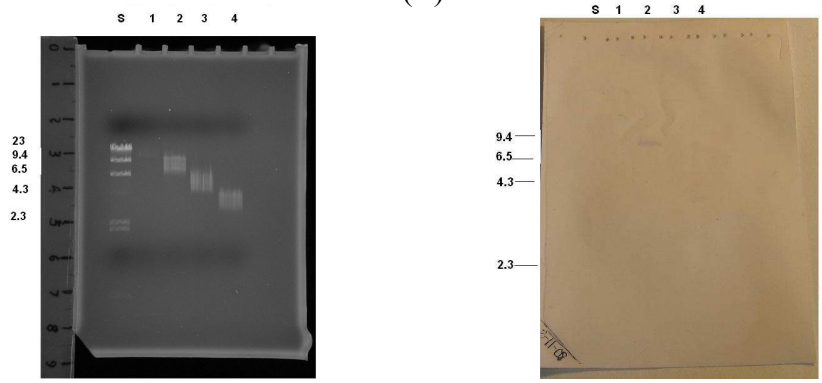


Figure 11: Southern blot analysis of R187-12/PstI gel fractions. (A) Agarose gel electrophoresis of R187-12/Pst1 fractions and (B) Southern blot of gel fractions from A using DIG labeled pJCS506 probe. Lane S contains λ/*Hind*III, lane 1 contains R187-12/PstI fraction 1, lane 2 contains R187-12/PstI fraction 2, lane 3 contains R187-12/PstI fraction 3, lane 4 contains R187-12/PstI fraction 4.

Phosphatase Treatment of pUC18 and Ligation

Vector plasmid, pUC18 cut with PstI was dephosphorylated with CIP to remove the 5' phosphate groups, thus reducing the chance that pUC18 would recircularize instead of ligating with the insert DNA. It was determined that the phosphatase treated pUC18 vector plasmid had very little background. The chromosomal DNA fraction containing the insertional mutagen from R187-12 cut with PstI, fraction 2, was then ligated into the dephosphorylated vector plasmid, pUC18 (Figure 12). Following ligation, the plasmid was transformed into electro-competent DH5α *E. coli* cells and grown on LB + Kanamycin (50μg/ml).

Of the 10 kanamycin resistant colonies screened, all appeared to contain inserts of around 9kb in size, indicating these plasmids contained the chromosome fraction with part of the insertional mutagen, pJCS506.

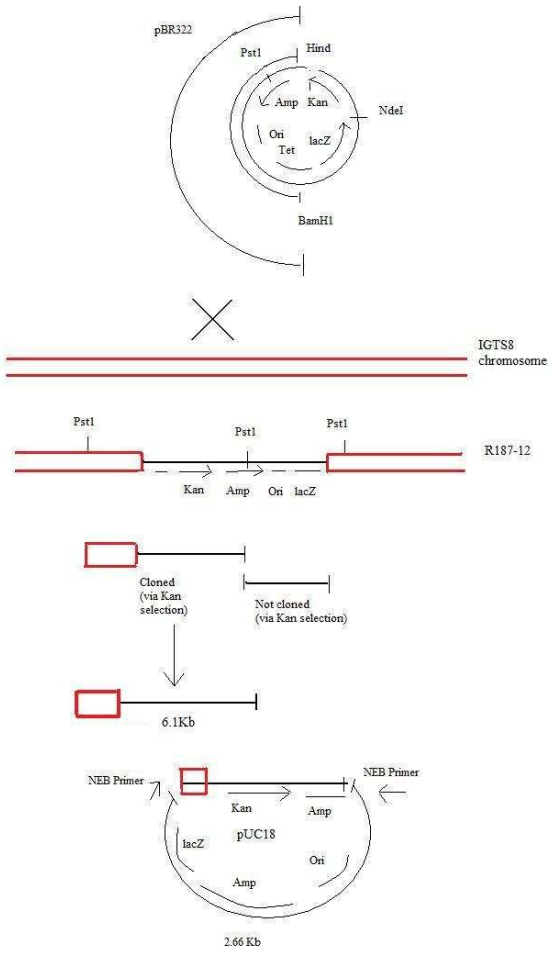


Figure 12: Postulated map of R187-12 flanking DNA adjacent to the pJCS506 insertion

DNA Sequence Analysis

DNA sequence analysis of flanking DNA adjacent to the pJCS506 insertion was accomplished through primer walking. DNA was sequenced and new primers were designed. This process of primer walking was repeated for each DNA sequencing reaction. The primers used for DNA sequencing are shown in Figure 13.

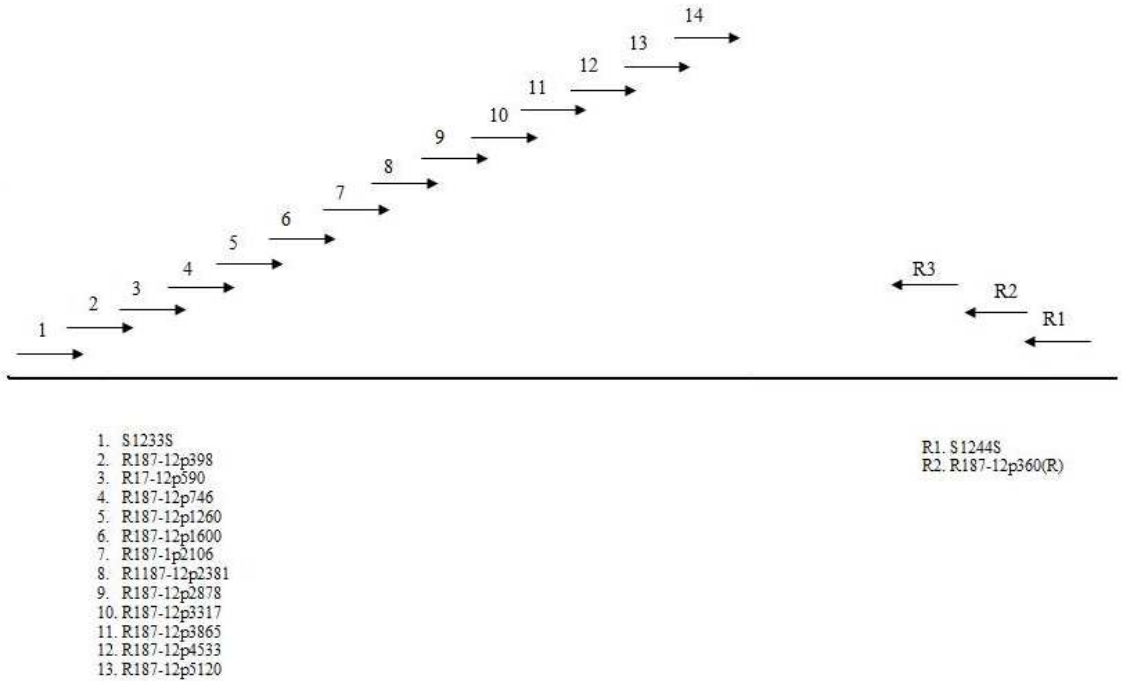


Figure 13: Primer walking of R187-12 flanking DNA to determine the DNA sequence

Once DNA sequences were received and spliced into the previous sequence reads, the DNA sequence was translated to look for large open reading frames (ORFs) using NCBI ORF finder. Translation of the DNA sequence revealed 3 large open reading frames of 300 amino acids or more. These ORFs were investigated and subjected to protein BLAST searches to determine homology to known protein sequences and to determine if these ORFs contained significant homology to known transport proteins. The results of the protein translation are shown in Figure 14.

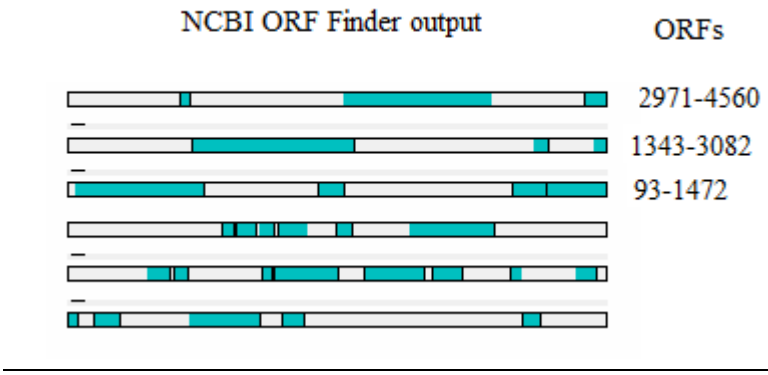


Figure 14: Open Reading Frames (ORFs) from NCBI ORF Finder

Analysis revealed 3 large ORFs of 300 amino acids or larger, designated ORF 93-1472, ORF 1343-3082, and ORF 2971-4560. Analysis of these ORFs indicated homology with a larger protein of *Rhodococcus* RHA1. This protein, designated ro00141, is a nonribosomal peptide synthetase. This gene, ro00141, is a cytoplasmic nonribosomal peptide synthetase with functions similar to EntF, involved in enterobactin synthesis. This gene is nearly 9,000 amino acids in size (or nearly 27Kb in size), an extremely large gene. However, it is known that *Rhodococcus* sp. RHA1 produces 24 nonribosomal peptide synthetases, with 6 of those being larger than 25kb in size (McLeod et al. 2006). Matches to this protein occurred for ORFs 93-1472, ORF 1343-3082, and ORF 2971-4560. However, each ORF match was close in sequence (Figure 15). It appears that the DNA sequence of R187-12 is similar to the end of the nonribosomal peptide synthetase gene of *Rhodococcus* sp RHA1.

```
Query  1     MVIVTTDIYGGAELASLIRREHVTHAFVTTAALATVDPAGLDEFRHVVVGGETLPPHLVG  60
             MVI    +YGGAELA LIR EHVTHAFVTTAAL+TVDP GLDEF+HVV GGE  PP LV
Sbjct  7245  MVIAPPTVYGGAELAELIRSEHVTHAFVTTAALSTVDPTGLDEFQHVVPGGEACPPELVT  7304

Query  61    LWAPGREFVNAYGPTETTVMADISEPMTVGAPITIGGPIRGVHEMILDSRLQPVPVGAPG  120
              WAPGR+  NAYGPTETTVMA+IS+PMTVG PIT+GGPIRGV E++LDSRLQPVPVG PG
Sbjct  7305  RWAPGRQLSNAYGPTETTVMANISDPMTVGDPITLGGPIRGVGELVLDSRLQPVPVGVPG  7364

Query  121   ELYIAGIGLARGYHRRPGLTSERFVADPFGKPGDRMYRTGDIVSWRSDHTIEYVGRSDFQ  180
             ELYI G GLARGYHRRP L+S+RFVA+PFG+ G+RMYRTGDIV WR+DHT+EYVGRSDFQ
Sbjct  7365  ELYITGAGLARGYHRRPELSSQRFVANPFGEAGERMYRTGDIVRWRADHTVEYVGRSDFQ  7424

Query  181   VKIRGFRIELGEVDAQITAMASVTNCVTLGVDGPAGATVLASYLTVEEGSTLTGAEITAH  240
             VKIRGFRIELGE+D +I     V+   TLGV GP+G TVL +YL       +  AE+TAH
Sbjct  7425  VKIRGFRIELGEIDNEIATFPGVSFAATLGVPGPSGDTVLVTYLLPNTAGGVDPAELTAH  7484

Query  241   LAGRVPSHMVPASIMIVDELPRTAVGKLDRKALPTPEFTGTGVEFPGARHQPRTSD  296
             L+ R+P+HMVP S+M +DE+P T VGKLDR ALP PEF  T  EF     QP T+D
Sbjct  7485  LSTRLPAHMVPTSVMFLDEIPLTPVGKLDRSALPAPEFHVTTTEF-----QPPTND  7535
```

Figure 15: Amino acid alignment between R187-12 and ro00141 of RHA1, ORF 93-1472.

Conclusions

DNA and protein analysis indicates that the DNA sequence of R187-12 contains genes involved in the synthesis of secondary metabolites, likely siderophore biosynthesis. Both DNA and protein analysis reveal similarities with nonribosomal peptide synthetases of *Rhodococcus* sp RHA1. Although similarities with the ro00141 gene of *Rhodococcus* sp RHA1 are homologous to the very end of this very large ORF, this does indicate homology with this protein. This gene encodes for a nonribosomal peptide synthetase, indicating it is involved in the biosynthesis of a secondary metabolite. In the case of *Rhodococcus erythropolis* R187-12, this is likely to be a siderophore. The ro00141 gene likely only extends to 6kb of the R187-12 DNA (Figure 15). If the plasmid junction is at approximately 7.5 to 8kb, as predicted by the size of the hybridizing band on Southern blot analysis, there could be another gene before the plasmid junction, potentially a transport protein. If the transport gene is interrupted by the insertional mutagen,

pJCS506, this would cause the inactivation of the transport protein, thereby inactivating the function of this protein.

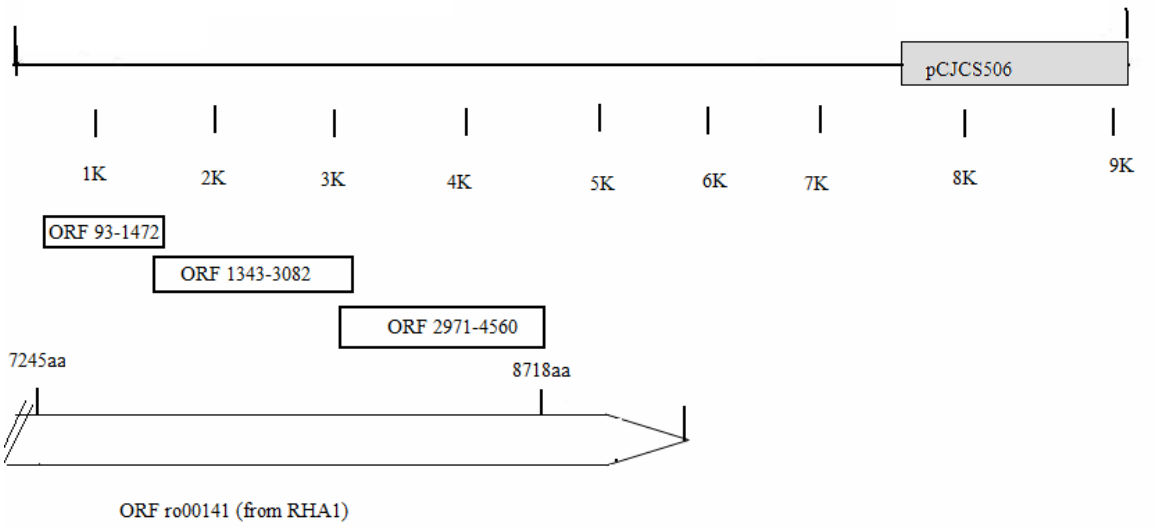


Figure 16: Schematic of R187-12 DNA with ORFs homologous with ro00141 of *Rhodococcus* RHA1.

In the RHA1 genome, there are 2 small hypothetical proteins, designated ro00142 and ro00143 immediately following ro00141. This could indicate a potential transport protein and could be indicative of a siderophore transport protein in *R. erythropolis* R187-12 immediately following the currently sequenced DNA. The hypothetical proteins designated ro00142 and ro00143 in RHA1 are cytoplasmic. The first, ro00142, is believed to have electron transport chain activity, while the activity of ro00143 is unknown. As biosynthetic genes are usually found clustered with transport proteins, there is a possibility that a small transport protein exists here.

However, DNA sequencing has not yet reached the plasmid junction. Reverse primers at the junction could not be designed because the plasmid, pJCS506, inserts into the chromosome through illegitimate recombination. Therefore, the location of recombination cross-over with the chromosome is unknown in the plasmid DNA and thus

a primer cannot be designed from this area. Protein analysis also reveals conserved domains that are involved in biosynthesis. However, continued DNA sequencing until the plasmid junction is reached may reveal more information about the mutation in R187-12 and possibly homologies with genes involved in siderophore transport. Another method of determining whether a transport protein is present in this DNA sample is to do an SDS-PAGE comparing the mutant R187-12 and wild-type IGTS8 under both low and high iron conditions. SDS PAGE separates the proteins based on molecular size. By analyzing both the wild type and R187-12 under high and low iron conditions, any differences in protein expression would be shown. Under low iron conditions, genes involved in siderophore transport and biosynthesis should be expressed in the wild type. By comparing the proteins expressed in the wild type to the proteins expressed in R187-12, this could show a difference in membrane proteins expressed in the 2 samples and could aid in determining whether a transport protein exists in the R187-12 strain. It is also possible that the insertional mutagen interrupted a regulatory region of the biosynthetic gene, causing the over production of the siderophore. Future studies should focus on the completion of the DNA sequencing as well as investigating other over-producing mutants of *Rhodococcus erythropolis* IGTS8 in order to better understand iron acquisition in this bacteria.

BIBLIOGRAPHY

Asturias, JA and Timmis KN. 1993. Three different 2,3-dihydroxybiphenyl-1,2-dioxygenase genes in the Gram-positive polychlorinated biphenyl-degrading bacterium *Rhodococcus globerulus* P6. J. Bacteriol. 175**:** 4631-40.

Bell, KS, Philip JC, Aw DWJ and Christofi N. 1998. The Genus *Rhodococcus*. J. Appl. Microbiol. 85: 195-210.

Bereswill, S, Greiner S, van Vliet AHM, Waidner B, Fassbinder F, Schiltz E, Kusters JG, and Kist M. 2000. Regulation of ferritin medited cytoplasmic iron storage by the ferric uptake regulator homolog (Fur) of *Helicobacter pylori*. J. Bacterio. 182: 5948-53.

Boland, CA. and Meijer WG. 2000. The iron dependent regulatory protein IdeR (DtxR) of *Rhodococcus equi*. FEMS Microbiol. Letters. 191**:** 1-5.

Boyle, AW, Silvin CJ, Hassett JP, Nakas JP, and Tanenbaum SW. 1992. Bacterial PCB degradation. Biodegration. 3: 285-98.

Bresler, MM., Rosser SL, Basran A, and Bruce NC. 2000. Gene cloning and nucleotide sequencing and properties of a cocaine esterase from *Rhodococcus* sp. strain MB1. Appl. Environ.l Microbiol. 66: 904-908.

Cameron, GWW, Jordan KN, Holt PJ, Baker PB, Lowe CR, and Bruce NC. 1994. Identification of a heroin esterase in *Rhodococcus* sp strain H1. Appl. Environ. Microbiol. 60: 3881-83.

Carrano, CJ, Jordan M, Drechsel H, Schmid DG, and Winkelmann G. 2001. Heterobactins: A new class of siderophores from *Rhodococcus erythropolis* IGTS8 containing both hydroxamate and catecholate donor groups. Biometals. 14: 119-25.

Carronodo, MA. 2003. Ferritins, iron uptake and storage from the bacterioferritin viewpoint. EMBO J. 22: 1959-68.

Christofi N, Ivshina IB, Kuyukina MS, and Philip JC. 1998. Biological treatment of crude oil contaminated soil in Russia. In *Contaminated Land and Groundwater: future Directions*. Ed. Lerner, D.N. and Walton, NR.G. pp45-51. Engineering Geology Special Publication 14. London: Geological Society.

Cornelis, K, Ritsema Nijsse TJ, Holsters M, Goenthals K, and Jaziri M. 2001. The plant pathogen *Rhodococcus fascians* colonizes the exterior and interior of the aerial parts of plants. Mol. Plant Microbe Interact. 14: 599-608

De Carvalho, CCC, and da Fonseca MMR. 2005. The remarkable *Rhodococcus erythropolis*. Appl. Microbiol. Biotechnol. 67: 715-26.

Denome, SA. and Young KD. 1995. Identification and activity of two insertion sequence elements in Rhodococcus sp. strain IGTS8. Gene. 161: 33-38.

Desomer J, Crespi M, and Montagu M. 1991. Illegitimate integration of non-replicative vectors in the genome of *Rhodococcus fascians* upon electrotransformation as an insertional mutagenesis system. Mol. Microbiol. 5(9): 2115-24.

DeVoss JJ, Rutter K, Schroeder BG, and Barry III CE. 1999. Iron acquisition and metabolism by Mycobacteria. J. Bacteriol. 181(15): 4443-51.

Dhungana SR, Michalczyk R, Boukhalfa H, Lack JG, Koppisch AT, Fairlee JM, Johnson MT, Ruggiero CE, John SG, Cox MM, et al. 2007. Purification and characterization of rhodobactin: a mixed ligand siderophore from *Rhodococcus rhodochrous* strain OFS. Biometals. 20: 853-67.

Farhana A, Kumar S, Rathore SS, Ghosh PC, Ehtesham NZ, Tyagi AK, Hasnain SE. 2008. Mechanistic insights into a novel exporter-importer system of *Mycobacterium tuberculosis* unravel its role in trafficking of iron. PLoS ONE. **3(5):** e2087.doi:10.1371/journal.pone.0002087.

.Fujimori DG, Hrvatin S, Neumann CS, Strieker M, Marahiel MA, Walsh CT. 2007. Cloning and characterization of the biosynthetic gene cluster for kutznerides. PNAS**.** 104: 16498-503.

Furukawa K, Fujihara H. 2008. Microbial degradation of polychlorinated biphenyls: Biochemical and molecular features. J. Biosci. Bioeng. 105: 433-49.

Gobin J, Horwitz MA. 1996. Exochelins of *Mycobacterium tuberculosis* remove eron from human iron-binding proteins and donate iron to mycobactins in the *M. tuberculosis* cell wall. J. Exp. Med. 183**:** 1527-32.

Goodfellow M, Alderson G. 1977. The Actinomycete-genus *Rhodococcus*: A Home for the 'rhodochrous' complex. J. Gen. Microbiol. 100: 99-122.

Hughes KL, Sulaiman I. 1987. The Ecology of *Rhodococcus equi* and physiochemical influences on growth. Vet. Microbiol. 14: 241-50.

Ichiyama S, Shimokatara K, Tsukamura M. 1989. Carotenoid pigments of genus *Rhodococcus*. Microbiol. Immunol. 33: 503-08.

Kilbane,JJ, Jackowski K. 1992. Biodesulfurization of water soluble coal derived material by *Rhodococcus rhodochrous* IGTS8. Biotechnol. Bioeng. 40: 1107-14.

Li G-Q, Li S-S, Zhang ML, Wang J, Zhu L, Liang FL, Liu RL, Ma T. 2008. Genetic rearrangement strategy for optimizing the dibenzothiopene biodesulfurization pathway in *Rhodococcus erythropolis*. Appl. Environ. Microbiol. 74: 971-76.

Lichtinger T, Reiss G, Benz R. 2000. Biochemical identification and biophysical characterization of a channel forming protein from *Rhodococcus erythropolis*. J. Bacteriol. 182: 764-70.

Maeda M, Chung SY, Song E, Kudo T. 1995. Multiple genes encoding 2,3-dihydroxybiphenyl 1,2-dioxygenase in the gram positive polychlorinated biphenyl-degrading bacterium *Rhodococcus erythropolis* TA421, isolated from a termite ecosystem. Appl. Environ. Biol**. 61:** 549-55.

McLeod MP, Warren RL, Hsiao WWL, Araki N, Myhre M, Fernandes C, Miyazawa D, Wong W, Lillquist AL, Wang D, et al. 2006. The complete genome of *Rhodococcus* sp. RHA1 provides insights into a catabolic powerhouse. PNAS. 103: 15582-87.

Meijer WG, Prescott JF. 2004. *Rhodococcus equi*. Vet. Res. 35: 383-96.

Miethling R, Karlson U. 1996. Accelerated mineralization of pentachlorophenol in soil upon inoculation with *Mycobacterium chlorophenolicum* PCP-1 and *Sphingomonas chlorophenolica* RA2. Appl. Environ. Microbiol. 62: 4361-66.

Mills M, Payne SM. 1995. Genetics and regulation of heme iron transport in *Shigella dysenteriae* and detection of an Analogous System in *Escherichia coli* O157:H7. J. Bacteriol. 177: 3004-09.

Miranda-CasoLuego R, Prescott JF, Vazquez-Boland JA, Meijer WG. 2008. The intracellular pathogen *Rhodococcus equi* produces a catecholate siderophore required for saprophytic growth. J. Bacteriol. 190: 1631-37.

Moretz SE. 2003. Iron acquisition in *Rhodococcus erythropolis* strain IGTS8: Characterization of a mutant that does not produce a siderophore. [thesis] East Tennessee State University, Johnson City, TN.

Neilands JB. 1993. Perspectives in Biochemistry and Biophysics. Arch. Biochem. Biophys. 302**:** 1-3.

Pierre JL, Fontecave M. 1999. Iron and activated oxygen species in biology: The basic chemistry. BioMetals. 12: 195-99.

Ratledge C, Dover LG. 2000. Iron metabolism in pathogenic bacteria. Ann. Rev. Microbiol 54: 881-941.

Rodriguez, GM. 2006. Control of iron metabolism in *Mycobacterium tuberculosis*. Trends Microbiol. 14(7): 320-27.

Rodriguez GM Smith I. 2006. Identification of an ABC Transporter Required for Iron Acquisition and Virulence in *Mycobacterium tuberculosis*. J. Bacteriol. 188: 424-30.

Rodriguez GM, Smith I. 2003. Mechanisms of iron regulation in mycobacteria: role in physiology and virulence. Mol. Microbiol. 47(6): 1485-94.

Sallam KI, Mitani Y, Tamura T. 2006. Construction of random transposition mutagenesis system in *Rhodococcus erythropolis* using IS*1415*. J. Biotechnol. 121: 13-22.

Sallam KI, Tamura N, Tamura T. 2007. A multipurpose transposon-based vector system mediates protein expression in *Rhodococcus erythropolis*. Gene. 386: 173-82.

Schneider E, Hunke S. 1997. ATP-binding-cassette (ABC) transport systems: Functional and structural aspects of the ATP-hydrolyzing subunits/domains. FEMS Microbiol. Rev. 22: 1-20

Schneider JC, Lantz SE, Rivera R, Wilganowski N, Pienkos PT. 1999. Random insertion of suicide vector DNA as a tool for strain improvement in the biodesulfurization biocatalyst *Rhodococcus erythropolis* strain IGTS8. Abstract, 039. 99th General meeting of the American Society for Microbiology.

Schwyn B, Neilands JB. 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. 160**:** 47-56.

Seto M, Kimbara K, Shimura M, Hatta T, Fukuda M, Yado K. 1995. A Novel transformation of polychlorinated biphenyls by *Rhodococcus* sp strain RHA1. Appl. Environ. Microbiol. 61**:** 3353-58.

Vellore JM. 2001. Iron Acquisition in *Rhodococcus erythropolis* strain IGTS8: Isolation of a non-siderophore producing mutant. [thesis], East Tennessee State University, Johnson City, TN.

Wandersman C, Delepelaire P. 2004. Bacterial iron sources: from siderophores to hemophores. Annu. Rev. Microbiol. 58: 611-47.

Wang, Y-X, Wang HB, Zhang YQ, Xu LH, Jiang CL, Li WJ. 2008. *Rhodococcus kunmingensis* sp. Nov., an actinobacterium isolated from a rhizosphere soil. International. J. Syst. Evol. Microbiol. 58**:** 1467-71.

Warhurst AM, Fewson CA. 1994. Biotransformations catalyzed by the genus *Rhodococcus*. Crit Rev. Biotechnol. 14: 29-73.

Winkelmann G. 2002. Microbial siderophore-mediated transport. BioMetals. 30: 691-96.

Xu, J-L., He J, Wang ZC, Wang K, Li WJ, Tang SK, Li SP. 2007. *Rhodococcus qingshengii* sp. nov., a carbendazim degrading bacterium. Int.J. Sys. Evol. Microbiol. 57: 2754-57.

Yamada H, Kobayashi M. 1996. Nitrile Hydratase and its Application to Industrial Production of Acrylamide. Biosci. Biotechnol. Biochem. 60: 1391-1400.

Yanisch-Perron C, Vieira J, Messing J. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13 mp18 and pUC19 vectors. Gene. 33: 103-19.

Zhang, Y-Q, Li WJ, Kroppenstedt RM, Kim CJ, Chen GZ, Park DJ, Xu LH, Jiang CL. 2005. *Rhodococcus yunnanensis* sp. Nov., a mesophilic actinobacterium isolated from forest soil. Int. J. Sys. Evol. Microbiol**. 55:** 1133-37.

Zhu, W, Arceneaux JE, Beggs ML, Byers BR, Eisenach KD, Lundrigan MD. (1998). Exochelin genes in *Mycobacterium smegmatis*: identification of an ABC transporter and two non-ribosomal peptide synthetase genes. 29(2): 629-39.

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