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Isolation of a Siderophore Produced by Methicillin-Resistant *Staphylococcus aureus* Strain H372.

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Isolation of a Siderophore Produced by Methicillin-Resistant *Staphylococcus aureus* Strain H372

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by

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ABSTRACT

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Iron is necessary for many cellular processes such as the electron transport chain and gene regulation. However, most iron on earth is found in insoluble iron-hydroxide complexes. In addition, iron is tightly sequestered in the human body by proteins such as transferrin, making it unavailable for pathogens. In order to overcome these limitations bacteria have evolved siderophores. Siderophores are low molecular weight compounds that bind ferric iron with a high affinity. *Staphylococcus aureus* is an important human pathogen that is known to produce at least four siderophores, and these siderophores contribute to its virulence. *S. aureus* strain H372 was found to produce a siderophore that was a carboxylate type, hydrophilic, and contained ornithine. These properties were similar to the known siderophore staphyloferrin A. However, the probable molecular weight was 658, which is different from known staphylococcal siderophores.

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CHAPTER 1

INTRODUCTION

Staphylococci

Staphylococci are members of a genus of Gram positive spherical bacteria. There are 33 species in this genus most of which are part of the normal flora of humans and animals. This genus is broken down into two broad groups, the coagulase-positive staphylococci and the coagulase-negative staphylococci (CoNS). Coagulase is an enzyme that causes blood clot formation and is a factor in virulence. The CoNS are far less virulent than coagulase-positive staphylococci and mainly cause opportunistic infections in the immune-compromised (24). The primary pathogen of this genus, and the only species that is coagulase-positive, is *Staphylococcus aureus.*

Staphylococcus aureus is an important human pathogen that causes many diseases including skin infections, septic shock, and pneumonia (21). It is facultatively anaerobic, catalase positive, and coagulase positive. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a form of the bacteria that can be resistant to many antibiotics including methicillin, oxacillin, penicillin, amoxicillin, and others (16). Some MRSA strains show increased virulence as well as resistance to additional antibiotics. A majority of all staph infections are now due to MRSA instead of methicillin-susceptible *S. aureus* (MSSA) (15). There are two forms of MRSA: Healthcare-Associated MRSA (HA-MRSA) and Community-Associated MRSA (CA-MRSA).

HA-MRSA is associated with being hospitalized or having had a medical procedure within the last year (16). Nosocomial infections increase morbidity in an already immune-compromised population, so preventing transmission is becoming a goal of all hospitals. These infections can manifest as surgical site infections, skin infections, and pneumonias.

Approximately 94,000 persons a year in the United States have serious, invasive MRSA infections, and 86% of these are HA-MRSA (16). About 19,000 people a year die of MRSA (15). HA-MRSA is also genetically distinct from CA-MRSA in its antibiotic resistance genes. Strains of HA-MRSA contain type I, II, or III of the staphylococcal chromosomal cassette *mec* (SCC*mec*). This mobile genetic element is capable of carrying multiple antibiotic resistance genes across strains of *S. aureus.* Type I contains the *mec*A gene for beta-lactam resistance. Type II and type III contain genes conferring tetracycline, erythromycin, and spectinomycin resistance (50)

CA-MRSA occurs in persons who have not recently been hospitalized or had a medical procedure. CA-MRSA can be spread in gyms, schools, and other places where people have close contact. CA-MRSA often occurs in seemingly healthy people and not traditional immune-compromised populations. CA-MRSA typically manifests as skin infections such as boils. Although CA-MRSA patients typically have a better prognosis than HA-MRSA patients, 14% of invasive cases involve CA-MRSA and deaths have occurred (15). CA-MRSA has a type IV *mec* element distinct from HA-MRSA isolates. This mec element is smaller than the ones present in HA-MRSA and confers only beta-lactam antibiotic resistance. (59) Regardless of the type of MRSA, antibiotic resistance is a growing public health problem, and new approaches to treatment are needed. One area of interest is targeting iron acquisition systems of bacteria (Torres, 2006).

Iron and Bacteria

Iron is a necessary factor for growth in the vast majority of bacteria and it is used in numerous ways in the cell. The electron transport chain uses Fe (II)/Fe(III) redox pairs. Over 100 enzymes require iron cofactors such as heme groups or iron-sulfur clusters, and iron plays a role

in gene regulation (63). Actively growing bacteria generally require an intracellular concentration of 10^{-6} Molar (M) iron to perform these functions (77). For bacteria iron is paradoxically both abundant and scarce. It is the fourth most common mineral in earth's crust, but in aerobic conditions and physiological pH it is bound in insoluble iron-hydroxides, (21) causing the free iron concentration in the environment to be only 10^{-18} M. Pathogenic bacteria are also affected by the body's sequestering of iron. Iron is under a strict homeostasis in the human body that drops the free iron concentration to an amazingly low 10^{-24} M (70). This low level is achieved by hemoglobin and other proteins that sequester approximately two-thirds of the iron and ferritin, which stores another 30% of the body's iron inside cells. The remaining small percentage of iron is bound to transferrin and lactoferrin in blood and other fluids (63). This scarcity of usable iron has driven the evolution of numerous ways for bacteria to acquire iron such as using heme as an iron source, using transferrin as an iron source, and the use of siderophores to sequester ferric iron and transport it back into the bacterial cell. Siderophores are the focus of the work presented here.

Siderophores

Siderophores are low molecular weight compounds that bind ferric iron with high affinity and they are the most common method employed in iron acquisition by bacteria. The word siderophore is Greek for "iron carrier." Siderophores are produced by a wide variety of organisms including bacteria, fungi, and even some plants (63). Siderophores produced by bacteria are the most widely studied and are the focus of my research. Siderophores undergo complex processes of regulation, production, export, and uptake before the iron can be used by the bacterial cell.

Siderophores can use different types of functional groups to bind the iron, and they are classified according to what kind of functional group is used. The most common types of siderophores are hydroxamate and catechol types. Hydroxamate siderophores use a carboxyl group attached to a nitrogen to chelate the iron, and catechol siderophores use hydroxyls of catechol rings (23). Other groups include the carboxylates of which *S. aureus* has at least two kinds (29).

Iron Regulation of Siderophore Production

Fur and DtxR. Iron homeostasis is a delicate balance. If iron happens to accumulate inside the cell, the Fenton reaction creates high levels of dangerous hydroxyl radicals known as oxidative stress. The free radicals can damage DNA and proteins ultimately killing the cell (48). So not only must bacteria have siderophores or similar systems, but they must repress them at times as well. Siderophores and their associated transport proteins are only produced by the bacteria under conditions of low iron availability. Abundant iron will repress the production of siderophores, as they are not needed, and very small amounts of iron will induce production. The primary regulation of siderophore production and iron homeostasis in bacteria is performed at the transcriptional level by Ferric uptake regulator (Fur) in Gram-negative and low GC-content Gram positive bacteria and DtxR (diphtheria toxin regulator) in high GC-content Gram positives (44). Fur is a classical repressor that uses iron(II) as a corepressor (4). It binds to the promoter region of a siderophore operon (such as aerobactin) and prevents transcription as long as iron is available. When iron concentration becomes low, the repression is stopped, and transcription of siderophore biosynthesis and transport genes is allowed (4). Fur and DtxR also seem to have a role in homeostasis of other metals including zinc and manganese (44).

Fur in *S. aureus*. *Staphylococcus aureus* has 3 Fur homologues (Fur, PerR, and Zur) and one DtxR homologue (MntR) contributing to its iron regulation (47). Fur is the main regulator of iron uptake in *S. aureus* and it represses the genes for the fhuD2 hydroxamate uptake system and the siderophore transport operons sirABC and sstABCD in iron-rich conditions. PerR regulates oxidative stress resistance and iron storage. Zur is involved in zinc homeostasis. MntR regulates manganese transport (48).

PerR in *S. aureus*. While Fur is the global regulator for iron in *S. aureus*, it is *Per*R that performs most of the functions of iron homeostasis. *Per*R is self-regulated, manganese responsive, iron responsive, and can repress Fur. *Per*R controls a wide range of genes including the genes of catalase *Kat*A, the alkyl hydroperoxide reductase *ahp*CF, the bacterioferritin comigratory protein *Bcp*, the thioredoxin reductase *Trx*B, the ferritin *Ftn*, and the ferritin-like *Mrg*A (48). All of these genes are involved in reducing oxidative stress when iron is abundant and need to be repressed when iron is scarce. *Per*R has also been shown to be necessary for virulence in a mouse model, but its mechanism is unknown (48)

Besides Fur and DtxR there are other transcriptional regulators that sense the presence of iron-bound siderophores. There are 4 types: alternative sigma factors, 2-component sensory transduction systems, AraC-type regulators, and further transcriptional regulator types (63).

Alternative Sigma Factors. Sigma factors are involved in transcription of genes by assisting the binding of RNA Polymerase to promoter regions. Different sigma factors control different sets of genes. Bacteria often have a primary sigma factor for control of genes used for vegetative cells in normal conditions and alternative sigma factors to activate the genes necessary during specific situations such as heat shock, stationary phase, or starvation (40).

Some of these alternative sigma factors are used during low iron conditions that involve use of siderophore-mediated acquisition. *Escherichia coli*'s FecI sigma factor system is one of these. The outer membrane (OM) receptor FecA interacts with a ferric dicitrate siderophore and transmits a binding signal to FecR in the cytoplasmic membrane. FecR activates FecI. FecI then binds to the RNA polymerase core enzyme, directs it to the *fec*A promoter, and initiates transcription of the *fec*ABCDE genes, which control transport of the ferric dicitrate siderophore (30). There are many homologues to this system in several species including PupI-PupR-PupB regulating pseudobactin in *Pseudomonas putida* and FpvI/PvdS-FpvR-FpvA regulating pyoverdin in *P.aeruginosa* (9).

Two-Component Sensory Transduction Systems. Two-component signal transduction systems are the primary way that bacteria sense and respond to extracellular signals (82). These systems consist of a sensor histidine kinase and a response regulator substrate. After receiving the signal, the bacteria modifies its physiology in some way including gene expression, catalyzing reactions, and modifying protein-protein interactions (82). These systems have been found to regulate siderophore uptake in *P. aeruginosa*. When iron-bound enterobactin is detected in the periplasm, the PfeR-PfeS system induces the pfeA Fe-enterobactin receptor (28).

AraC-type Regulators. The araC-type regulators are a class of positive transcriptional regulators in bacteria that are highly conserved and widely distributed across Gram positives, proteobacteria, and cyanobacteria. Their 3 main regulatory functions are carbon metabolism, stress response, and pathogenesis (36). AraC-type regulators can also function as intracellular siderophore sensors. These regulators can respond to the presence of siderophore before export or after uptake and this signal can help the cell "fine-tune" its siderophore synthesis (62).

Members of this class include PchR in *P. aeruginosa* regulating pyochelin, AlcR in *Bordetella pertussis* regulating alcaligin, and YbtA in *Yersinia pestis* regulating yersiniabactin (11, 31, 62).

Further transcriptional Regulators. While the majority of non-Fur iron regulators can be placed into the above categories, there are a few unique systems that have been discovered. IrgB is the only LysR-type regulator that has been found to have a role in iron regulation. It activates the enterobactin receptor IrgA in *Vibrio cholerae* but does not interact with enterobactin itself or other known siderophores (39). AngR is a non-ribosomal peptide synthase found in *Vibrio anguillarum* that can activate the genes for anguibactin synthesis, transport, and uptake. It is encoded in a virulence plasmid and mutations can cause hyperproduction of the anguibactin (87).

Post transcriptional Regulation. There are 2 general mechanisms of posttranscriptional regulation of iron homeostasis in bacteria: one involving RNAs and one involving proteins similar to those used in mammalian iron homeostasis. In some bacteria Fur-regulated antisense RNAs cause mRNA degradation of iron-related genes. Examples include RhyB in enteric bacteria and PrrFr1/PrrFr2 in *P. aeruginosa* (88). In *Bacillus subtilis*, however, the aconitase CitB loses its iron cluster during oxidative stress or iron depletion and subsequently interacts with operons that have structures similar to mammalian iron-responsive elements. This function corresponds to the mammalian protein IRP1 (2, 74).

Siderophore Biosynthesis

Non-Ribosomal Peptide Synthases. Non-ribosomal peptide synthases (NSPS) are large multienzyme complexes that assemble a large variety of products in the cell including many siderophores (41). NSPS are responsible for the synthesis of aryl-capped siderophores and many non-hydroxamate and non-catechol siderophores. The NSPS synthesis pathways of many

siderophores have been characterized in detail including enterobactin, yersiniabactin, pyoverdin, vibriobactin, and mycobactin (63).

Independent of NSPS. The majority of hydroxamate and catecholate siderophores are assembled by NSPS-independent mechanisms (63). These siderophores are often virulence factors. There are 2 steps in the typical hydroxamate synthesis: N-hydroxylation and formylation or acylation of the hydroxylated amine. The N-hydroxylation is catalyzed by flavin adenine dinucleotide (FAD)-dependent monooxygenases. One oxygen atom may be transferred to lysine, ornithine, cadaverine, putrescine, or other similar amino acids (19, 89). The acylation step (more common than formylation) is catalyzed by acyl coenzyme A transferases. Substrates are carboxy acids such as acetate, succinate, B-hydroxybutyrate, or decenoate. The final step in NSPS-independent siderophore synthesis is catalyzed by IucA and/or IucC-type siderophore synthases. These enzymes were discovered in the synthesis of aerobactin, and all known NSPS-independent pathways use at least one enzyme that is very similar to these (19).

Siderophore Export

Few siderophore export systems are known in bacteria; however, the known types fall into the categories of types of efflux pumps: the major facilitator superfamily (MFS), resistance, nodulation, cell division superfamily (RND), and the ATP-binding cassette superfamily (ABC) (63).

MFS. The MFS superfamily is a group of transporters that carry out uniport, symport, and antiport transport of many compounds including drugs, primary metabolites, neurotransmitters, and anions. They also perform both siderophore efflux and uptake (76). One of the best studied of these MFS proteins is EntS in *E. coli*, which is involved in enterobactin

export. EntS has 12 trans-membrane segments and is regulated by Fur. EntS is responsible for export across the cytoplasmic membrane (CM), but another protein, TolC, is required for transport across the OM (7, 35). EntS mutants do not export enterobactin but do release its byproducts. Because of this it is hypothesized that many other unknown proteins must be involved in export. Besides *E coli*, many other bacteria have transporters with high similarity to EntS including LbtB in *Legionella pneumophila* (3).

RND. The RND superfamily is a group of transporters that use proton antiport mechanisms to perform efflux of heavy metals, drugs, lipids, and siderophores (66). The MexA-MexB-OprM system in *Pseudomonas aeruginosa* is thought to be an RND-type transporter involved in the export of the siderophore pyoverdin (69). The Mex operon is iron-regulated and pyoverdin has a similar structure to other molecules exported by this system, but mutant data are not available to fully elucidate its role in export (54). RND transporter systems as well as MFS systems are ubiquitous across all three kingdoms of life, so it is likely that siderophore export will be found to involve many of these types of systems (63).

ABC. ABC-type transporters are involved in both uptake and efflux of siderophores and numerous other substrates. These transporters have 2 integral membrane domains and 2 cytoplasmic membrane domains for ATP binding and hydrolysis (8). The models proposed for the transport of the substrate include movement of the subunit halves (71), domain swapping (27), and a conformational change in the trans-membrane segments to allow the substrate to pass through (49, 57, 63). Some mutants of these transporters do not accumulate siderophore intracellularly, indicating that the processes of synthesis and export are linked in some way. This appears to be the case only when NRPS synthesis is employed (35, 91).

Examples of siderophores with ABC transport systems include exochelin in mycobacteria and salmochelins in *Salmonella* (55).

Siderophore Uptake

After siderophores have been synthesized, secreted, and have bound with iron, the bacteria must then bring that iron back into the cell to use it. Some bacteria have membrane-bound reductases that reduce the iron, which is then taken up as an ion, but most bacteria internalize the entire siderophore complex. The transport systems for iron-bound siderophores are well studied and diverse. Many bacteria produce more than one type of siderophore transporter because they have evolved uptake mechanisms for xenosiderophores, siderophores that are produced by other species and strains of bacteria (63).

Gram Negative. Siderophores of Gram negative bacteria have complex transport mechanisms involving an outer membrane receptor, periplasmic proteins, and an ABC transporter in the cytoplasmic membrane. Many of the Gram negative transport systems show marked similarities. FecA, FhuA, FepA, FpvA, and FptA are OM receptors found in *E. coli* or *P. aeruginosa.* All have a beta barrel structure at the C-terminus and N-terminal residues that form a plug (18). When a siderophore binds, the plug residues presumably undergo a conformational change and create a channel (17). The process of transport is mediated by the TonB complex, which presumably supplies energy. TonB is anchored in the CM, spans the periplasm, interacts with the OM receptor, and contacts 2 CM-embedded proteins, ExbB and ExbD (45). It is thought that the TonB complex tranduces proton motive force energy to the OM receptor to allow transport of the siderophore complex (45).

After transport into the periplasm, the siderophore must be further transported into the cytoplasm. In Gram negative bacteria this is accomplished with the use of periplasmic binding proteins. These proteins bind the Fe-siderophore complex and interact with transmembrane permeases to allow the complex to be channeled through the CM. Energy is supplied by ABC subunits in the cytoplasm that dimerize and undergo conformational changes when ATP binds (51).

Gram Positive. While Gram negative siderophore systems are well-studied, much less data are available concerning all aspects of Gram positive siderophores including those of *S. aureus*, the focus of this research (13). However, because transport is so tied to the OM and periplasm in Gram negatives, differences in transport have been studied and a few things are known about Gram positives. Because Gram positives lack an OM and a periplasm, siderophore complexes only need to cross the CM. Lipoproteins on the outside surface of the CM take the place of periplasmic binding proteins and OM receptors (85). Because of the lack of a periplasm, the functions of extracellular binding, transmembrane channeling, and cytoplasmic ATP hydrolysis are often fused into a single ABC transporter such as IrtA in *M. tuberculosis* (72).

Release of Iron

As mentioned previously, iron may be reduced extracellularly by ferric-chelate reductases and taken up by the cell or the iron-siderophore complex may be transported across the membrane. If the entire siderophore complex is taken up, intracellular ferric-siderophore reductases or ferric-siderophore hydrolases are used to free the iron (68). The use of reductases appears to be more common and is a non-specific adaptation of established reductase activities (43). The intact siderophore left after iron is removed could potentially be reused. The use of

hydrolases is siderophore specific and appears to be more costly for the cell due to the degradation of the siderophore (63). After iron is released into the cytoplasm, it can be used immediately or be stored in a protein such as bacterioferritin (14)

Staphylococcal Siderophores and Virulence

While siderophores are important for all bacteria, the processes of mutation and horizontal transport have endowed some bacteria with better siderophore systems than others, and this can have consequences for virulence. In *S. aureus*, greater production of siderophore (nonspecifically measured by the CAS assay) is correlated with virulence in the form of higher bacterial counts in infected mice, larger abcesses, and more inflammation (75). Strains with higher siderophore production have also been found to be more resistant to the activity of neutrophils *in vitro* (75). The uncharacterized siderophore Staphylobactin has also been shown to be an important virulence factor that separates the highly pathogenic coagulase positive from the more opportunistic coagulase-negative staphylococci (CoNS) (24). Mutants that cannot produce staphylobactin are unable to persist *in vivo* in a mouse model. This suggests that non-siderophore iron sources such as heme or transferrin may be important in the early stages of infection, and siderophores are important in later stages of infection (24).

Virulence in Other Bacteria

Siderophores are essential for virulence in a wide range of bacteria and contribute to virulence in many ways. In the case of *Bordetella pertussis* its siderophore alcaligin is important in the establishment of infection. Mutant strains that did not produce the alcaligin transporter were avirulent in a mouse model and strains with reduced expression of the transporter took several more days to colonize mice than the wild-type (12). Siderophores are also known to be

significant virulence factors in the enterobacteria. The majority of enterobacteria can produce enterobactin, some produce aerobactin, and very few produce yersiniabactin. Aerobactin and yersiniabactin are correlated with virulence. In *Klebsiella pneumoniae*, mutants that cannot make yersiniabactin have greatly reduced growth in vivo in the lungs, reduced lethality, and a reduced ability to spread systemically. Aerobactin mutants of *K. pneumoniae* have a reduced ability to cause intraperitoneal infections, showing that types of siderophores are important in determining which sites strains can colonize (53). While most siderophores are used simply to acquire iron in the establishment or persistence of the infection, some siderophores also have a role in evading the immune system. *Bacillus anthracis* produces bacillibactin and petrobactin, which are both efficient at binding iron. However, bacillobactin is easily bound by siderocalin, but petrobactin is not due to its unusual 3.4-dihydroxybenzoate structure. Thus, the production of petrobactin seems to be defensive in nature, as bacillobactin would be sufficient for iron acquisition if siderocalin were not present (1).

Siderocalin

The majority of the human immune response to siderophores is to tightly sequester iron in proteins such as transferrin; however, there is one direct means of defense against siderophores, siderocalin. Siderocalin is a member of the lipocalin family and is a part of the innate immune system (38). Siderocalin has been shown to bind enterobactin, bacillibactin, carboxymycobactins, and parabactin (1, 46).

However, bacteria are masters at avoiding immune responses including siderocalin. Mycobacteria usually colonize intracellular compartments of macrophages, which protects their siderophores and allows them to exploit iron-transferrin uptake inside phagosomes (58). Some

bacteria that produce siderophores that can be bound by siderocalin occupy niches such as intestinal sites where they are protected from the siderocalin in the serum. And of course, many bacteria produce siderophores that cannot be bound by siderocalin (1).

Tear Lipocalin. Tear lipocalin (Tlc) is another human protein with siderophore binding capability. It is found in many secretory tissues and glands including tears. Tlc has a deeper, more hydrophobic binding pocket than siderocalin and is capable of binding a wide range of lipid products as well as siderophores (10). Tlc can bind catecholates, hydroxamates, and mixed citrate-hydroxamate siderophores including enterobactin, desferrioxamine B, coprogen, ferrichrome, and aerobactin (34). However, the strength of this binding is relatively low and is not influenced by the amount of iron carried by the siderophore. Because of this, the effect of Tlc may not be significant when binding bacterial siderophores, which can have extremely high iron affinity. It may be important in binding the less efficient fungal siderophores (34).

Siderophores as Drug Targets

Siderophores are a potential target for antibiotics because stopping the acquisition of iron can severely restrict growth. Inhibition of siderophore biosynthesis has been effective in reducing *in vitro* growth of *Mycobacterium tuberculosis* (84). A bisubstrate inhibitor was used to block the MbtA enzyme. MbtA is important in the second step of mycobactin synthesis, which is the incorporation of salicylic acid into the core scaffold of mycobactin. Inhibiting this specific step could be useful in other species that also use aryl-capped siderophores (84). Siderophore-antibiotic conjugates are also a promising potential development in treatment of infections. Siderophore-antibiotic conjugates enter the bacterial cell through the specific receptors for the siderophore, thus bypassing any efflux pumps that would normally remove the antibiotic and

foster resistance. The conjugates would also accumulate quickly in the cell due to active transport. This has been demonstrated in *E. coli* with a siderophore-carbacephalosporin conjugate (64). Mixed-ligand siderophore-antibiotic conjugates enter through multiple outer membrane receptors, possibly accumulating faster in the cell, and lowering the possibility that bacteria could gain resistance to this antibiotic delivery method (37).

S. aureus and Iron

Although *S. aureus* has an intrinsically low requirement for iron, (56) it has evolved many iron acquisition mechanisms including usage of heme as an iron source, direct acquisition of iron from transferrin, and siderophores. It is the only known organism with all three of these iron acquisition systems (67).

Heme

Heme is the preferred iron source of *S. aureus* (81). Hemoproteins such as hemoglobin and myoglobin account for 80% of the iron in the human body. While siderophores are important in persistence of infections, (24) heme usage is important in initiation of infection (81). Mutating essential elements of the heme uptake system causes severely reduced virulence in infections of *C. elegans* and murine models (81).

The main components of the heme uptake system are iron-regulated surface determinant (Isd) proteins. The system consists of many components including cell wall anchored proteins, membrane transporters, a transpeptidase, cytoplasmic heme-degrading monooxygenases, and a sortase (86). The mechanism is thought to be that *S. aureus* lyses erythrocytes to release the hemoglobin, binds hemoglobin with IsdB, removes the heme cofactor, transports the heme into the cytoplasm, and then degrades it with monooxygenases (86).

Transferrin

Although transferrin accounts for only 1% of the iron in the human body, (81) it can still be an important source of iron for *S. aureus*. *Staphylococcus aureus* is unique in this ability, as the only other known users of transferrin-bound iron are the non-siderophore producing *Hemophilus* and *Neisseria* (67). Although some surface proteins such as IsdA (a component of the heme system) can bind to transferrin, the main method of iron acquisition from transferrin is through siderophores (67). While some siderophores use proteases to liberate iron from transferrin, this does not seem to be the case in *S. aureus* (67). In some studies it has been reported that the siderophore staphyloferrin A can remove iron from transferrin (65).

Siderophores in *S. aureus*

Staphylococcus aureus is known to produce at least 4 siderophores: staphyloferrin A, staphyloferrin B, aureochelin, and staphylobactin. Staphyloferrin A and B are carboxylate type siderophores that are similar in structure. Staphylobactin is a hydroxamate type and aureochelin is a catechol type; however, their structures have not yet been published (90).

Staphyloferrin A. Staphyloferrin A was the first siderophore isolated from staphylococci. Staphyloferrin A is a carboxylate type of siderophore, consisting of one ornithine and 2 citric acid residues linked by 2 amide bonds (52) (Fig. 1). It is highly hydrophilic and has a molecular weight of 481 Daltons (Da) (22, 61). Staphyloferrin A was first isolated from *S. hyicus* DSM 20459, which was the only strain able to produce it when grown in the steel stirred tank reactor used. Other strains of staphylococci used in the Meiwes study needed a lower iron concentration to induce its production than was possible to obtain in the steel reactor (61). However, staphyloferrin A has been detected in *S. aureus* and many other species of staphylococci. The

production of Staphyloferrin A is increased up to 19 times in some strains with the addition of D-ornithine (52). While not all strains produce staphyloferrin A, and most need supplementation to do so, all 37 strains in Meiwes study were able to use staphyloferrin A, indicating that the transport system is widespread (61). Genetic information concerning the synthesis and transport of staphyloferrin A has also been elucidated. The *sfna*ABCD gene cluster codes for staphyloferrin A biosynthesis. Staphyloferrin A is synthesized independent of the non-ribosomal peptide synthase pathway (NIS) using *sfaB* and *sfa*D. *Sfa*D form a citryl-D-ornithine intermediate and *sfa*B condenses the second citric acid onto the intermediate. *Sfa*C racemases L-ornithine to the D-ornithine needed in the molecule (21). The *hts*ABC operon has been implicated in both heme transport and staphyloferrin A transport (6).

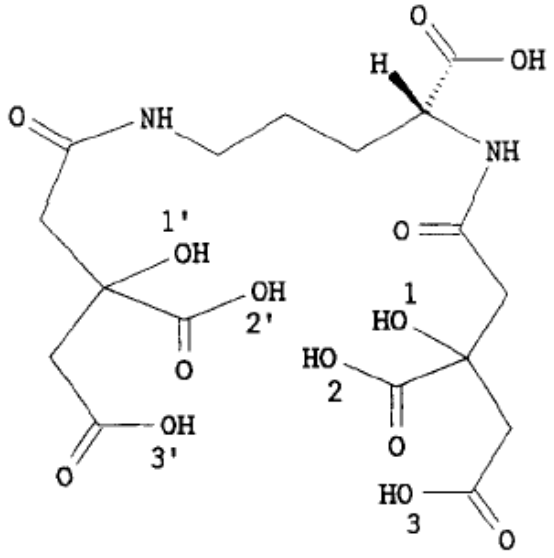

Figure 1: Structure of Staphyloferrin A (52)

Staphyloferrin B. Like staphyloferrin A, staphyloferrin B is a carboxylate type siderophore that was first isolated from *S. hyicus* DSM 20459 (61) (Fig. 2). It is highly hydrophilic and has a molecular weight of 448 Da. Staphyloferrin B is produced by a wide

variety of strains of staphylococci both pathogenic and non-pathogenic (42). The yield of staphyloferrin B is increased 2.5 times when L-2,3 diaminopropionic acid is added. (29) However, in contrast to staphyloferrin A where D-ornithine supplementation is necessary for approximately 70% strains to produce any detectable staphyloferrin A, staphyloferrin B was detectably produced without supplementation by approximately 90% of the strains used (61). The biosynthesis of staphyloferrin B is thought to be by the NIS pathway and controlled by the *sbn* operon (20). The *sbn* operon was previously associated with staphylobactin (24). The staphyloferrin B synthesis operon of *Ralstonia solacearum* is similar to the *sbn* operon in *S. aureus* (7). However, the *sbn* operon has not been reported in any coagulase-negative staphylococci (CoNS), while staphyloferrin B production has been reported (6, 29, 61). Transport of staphyloferrin B is thought to be performed by the SirABC operon (5)

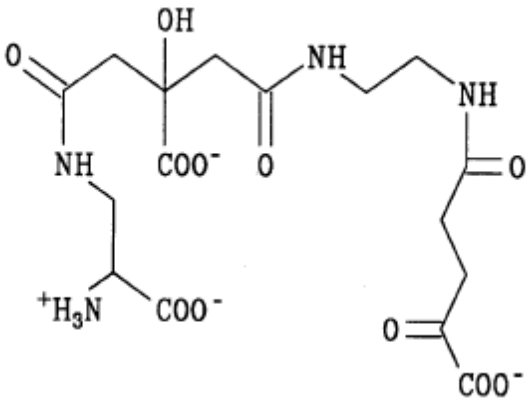

Figure 2: Structure of Staphyloferrin B (42)

Staphylobactin. Staphylobactin was discovered when researchers studied siderophores and virulence in *S. aureus*. In a mouse kidney model of infection strains that produced siderophores showed increased virulence over the coagulase-negative staphylococci (CoNS) that did not (24). An operon termed *sbn* was found to be responsible for the siderophore detected in

the study. Mutants of *sbn*E were found to be avirulent due to lack of siderophore production. Because the siderophore was not able to be isolated using the published procedures for Staphyloferrin A and B and ESI-MS results were not consistent with the staphyloferrins, the siderophore was tentatively named staphylobactin (24). Staphylobactin was extracted using a method originally designed for the extraction of ornibactin siderophores in *Burkholderia cepacia* (26, 83). Staphylobactin was later found to be a hydroxamate siderophore with citric acid residues (90). An ABC transporter for staphylobactin has been described. The *sir*ABC operon is transcribed in the opposite direction of the sbn operon, which is known to code for the biosynthetic genes of staphylobactin. *Sir*A is a lipoprotein and *sir*B and *sir*C likely code for the transmembrane domains of the transporter. Mutants of *sir*A or *sir*B are compromised in the ability to uptake staphylobactin-iron complexes but not other complexes such as ferric citrate, ferric enterobactin, or ferric hydroxamates (24).

Aureochelin. Aureochelin is a phenolate-catecholate siderophore with a molecular weight of 577 Da (22). Its structure is currently unknown. Aureochelin was extracted using an ethyl acetate extraction method originally used for catechol siderophores of *E. coli* (73). Although no transport protein for aureochelin has yet been found, 2 iron repressible proteins of 120 and 88 Da were found to correlate with aureochelin production in high and low siderophore producing strains. This relationship was variable, however, prompting the authors to speculate that plasmids may be involved. These iron repressible proteins are also antigenic, as evidenced by immunoblotting using serum from rabbits injected with *S. aureus* and human serum from septicemia patients. Healthy donor serum showed no reaction to the proteins (22).

Hydroxamate Uptake System in *S. aureus*

S. aureus had been shown to be able to use the exogenous siderophores ferrichrome, aerobactin, desferal, (77, 78) and actinoferrin (90). The hydroxamate uptake system consists of the *fhu*CBG operon and proteins *fhu*D1 and *fhu*D2. These show homology to the uptake system of *Bacillus subtilis* (78). *Fhu*C codes for an ATP-binding protein, while *fhu*B and *fhuG* are highly hydrophobic and membrane-bound (13). This operon is unusual due to it containing only the membrane spanning and ATPase functions of a classical traffic ATPase but no receptor. *Fhu*D1 and *fhu*D2 are genes outside of the *fhu*CBG operon that were found to code for lipoprotein receptors (78). *Fhu*D2 is involved in the transport of ferrichrome, ferrioxamine B, aerobactin, and coprogen. *Fhu*D1 only transported ferrichrome and ferrioxamine B in this study. It is unclear why these receptors have overlapping substrate range or why they are separate from the overall *fhu* operon (78).

*Fhu*D2's structure and transport properties have been well-studied. Mutagenesis studies showed several residues critical for binding and transport of iron-hydroxamates. Specifically, Tyr-191, Trp-197, and Glu-202 are necessary for ligand binding (79). Iron-hydroxamate transport was impaired by mutagenesis in residues Glu-97 and Glu-231. Residues for mutagenesis were chosen based on conserved regions seen in other Gram-positive bacteria such as *Clostridium acetobutylicum*, *Streptococcus pyogenes*, *Bacillus halodurans,* and *Bacillus subtilis.* *Fhu*D2 was not shown to undergo a conformational change upon binding, making it significantly different than other known transport systems (79).

FhuD1 is a receptor that is similar in structure and sequence to fhuD2 albeit with a narrower siderophore range (78). In contrast to the highly conserved *fhu*CBG and *fhu*D2, *fhu*D1

is not present in all *S.aureus* strains and its location is variable. FhuD1 binds ferrichrome, desferal, and coprogen with a slightly lower affinity than fhuD2 and does not bind aerobactin at all. However, binding affinity did not correlate with growth promotion. Knockout mutants of *Fhu*D2 were still not able to uptake coprogen or aerobactin, and they required high amounts of ferrichrome and desferal to grow. It is hypothesized that *fhu*D1 is a duplication of *fhu*D2 and it is unknown if there are conditions where its presence confers a selective advantage (80). The entire *fhu* system is regulated by Fur (80).

Present Work

The purpose of this study was to determine if strains of *S. aureus* produced siderophores and what siderophore(s) they produced. Strains of *S. aureus* were obtained from Dr. Sanjay Shukla at the Marshfield Clinic in Wisconsin. These strains were screened for siderophore production and all were positive. Strain H372 was chosen for further purification due to its high siderophore production. Growth conditions were optimized for siderophore production, and manual column chromatography and high pressure liquid chromatography (HPLC) were used to purify the siderophore for chemical characterization.

CHAPTER 2

MATERIALS AND METHODS

Bacterial Strains and Characteristics

Samples of MRSA (methicillin-resistant *S. aureus*) were received on blood plates from Dr. Sanjay Shukla at the Marshfield Clinic in Wisconsin. Antibiotic resistance information was also sent with the cultures, as seen in Table 1. The strain used in this study was H372.

Table 1: Marshfield clinic Heathcare-associated (HA-MRSA) Strains. Received November 13, 2007.

HA-MRSA	Antibiotic Profile						
Strain	Cip[1]	Ery[2]	Cli[3]	Tet[4]	Sxt[5]	Gen[6]	Rif[7]
004	R[a]	R	R	S[b]	R	N[c]	N
006	R	R	R	S	S	N	N
008	R	R	R	S	R	N	N
009	R	R	R	S	S	N	N
012	R	R	R	S	S	N	N
015	R	R	R	S	S	N	N
025	R	R	R	S	R	N	N
027	R	R	R	S	S	N	N
032	R	R	R	S	S	N	N
151	R	R	R	S	R	N	N
161	R	R	R	R	S	N	N
162	R	R	R	R	S	R	R
167	R	R	R	R	S	R	R
168	R	R	R	R	S	R	R
174	R	R	R	R	S	R	R
175	R	R	R	R	S	R	R
176	R	R	R	R	S	R	R
181	R	R	R	I[d]	R	N	N
182	R	R	R	R	R	R	S
183	R	R	R	R	S	R	R
251	R	S	S	S	S	N	N
273	R	R	S	S	S	N	N
282	R	S	S	S	S	N	N

Table 1 (Continued)

323	R	R	R	S	S	S	R
328	R	R	R	S	S	S	S
330	R	R	R	R	R	R	S
331	R	R	S	S	S	S	S
333	R	R	R	S	S	S	S
344	R	R	R	S	S	S	S
346	R	R	S	S	S	S	S
347	R	R	R	S	S	S	S
350	R	R	S	S	S	S	S
354	R	R	R	R	R	R	R
370	R	R	R	S	S	S	S
372	R	R	S	S	S	S	S
377	R	R	S	S	S	S	S
388	R	R	R	S	S	S	S
413	R	R	R	S	S	S	S
418	R	R	S	S	S	S	S
421	R	R	S	S	S	S	S

1. Ciprofloxacin 2. Erythromycin 3. Clindamycin 4. Tetracycline 5. Sulfamethoxazole 6.Gentamycin 7.Rifampin

a. Resistant b.Susceptible c.Not tested d.Intermediate

Glycerol Stocks

Glycerol stocks of all strains were prepared by growing the bacteria in Luria-Bertani (LB) broth for 3 to 4 hours (until OD was 0.5) and adding 0.8 mL of culture to 0.2 mL of sterile 75% glycerol. These were stored at -80 C.

Hemolysis

Hemolysis was measured by spotting bacterial cultures onto blood agar plates and observing the clearing of blood cells on the media. Beta hemolysis was noted as clear halos of lysed cells around the inoculum.

Heme Testing

Testing for the usage of hemin was performed by observing a growth halo around a paper disc impregnated with 10 µl hemin. Three milliliters of nutrient agar with 300 µm dipyridyl was mixed with 100 µl of a 24-hour culture of MRSA and poured into a small plate. The hemin disc was placed in the middle of the plate. After 24 hours the halo of growth around the disc was measured.

Growth and Siderophore Production

Growth Conditions

Strains were grown in LB Broth with 500 µm dipyridyl. The cultures were grown at 37 C on a shaker for 24 hours. The cultures were then centrifuged at 10,000 rpm for 15 minutes and the supernatant collected.

Determination of Siderophore Production

Siderophore production was determined by the use of CAS media. Chrom Azurol S is a dye that appears blue when complexed with ferric iron. When the iron is removed, the CAS turns yellow. This is a universal assay for the presence of siderophores, although it gives no chemical or structural information.

Supernatant or purified samples were added to wells cut in CAS agar plates and the formation of yellow halos indicated the presence of siderophore. Depending on the concentration of siderophore and the temperature halo formation could occur in as little as 30 minutes or take up to 4 hours.

Purification

For purification of the siderophore a large volume of culture was needed. Batch cultures of 4-5 liters were grown in LB broth with 500 μm dipyridyl for 24 hours at 37 C on a rotary shaker. The seed inoculum was 10 mL of culture, and 1 mL of erythromycin (concentration 1 mg per 1 mL) was added to prevent contamination. After incubation the cultures were centrifuged at 10,000 rpm for 15 minutes, and the supernatant was collected. To reduce the siderophore's solubility in water the supernatant was acidified to pH 2.00.

Manual Column Chromatography

Amberlite XAD-2. XAD-2 binds cyclic compounds, and it was the first step in purification. The column was prepared by suspending approximately 100 g of the resin in ddH_20, and allowing it to sit and expand overnight. The XAD mixture was deaerated, packed into the column, and equilibrated with ddH_20. The acidified supernatant was passed through the column, and the filtrate was collected. The filtrate was checked on CAS media to ascertain if the siderophore was bound in the column. After all the supernatant and several bed volumes of ddH_20 are passed through, methanol is used to elute the substances bound in the column. Approximately 50 fractions are collected using a fraction collector. The fractions are checked on CAS media to determine siderophore presence. The positive fractions are pooled and concentrated by evaporation.

Sephadex LH-20. Sephadex LH-20 separates molecules based on both size and hydrophobicity. The column was prepared by adding 20 g of the sephadex LH-20 to ddH_20, deaerating it, and packing it in the column. The column was equilibrated with ddH_20, and the sample was loaded. The column was run with ddH_20 and fractions were collected using a

fraction collector. The fractions were tested with CAS media, and those testing positive were pooled and concentrated.

HPLC

HPLC was performed using a Watters 7.8 mm by 300 mm C-18 column with 0.1% Trifluoroacetic acid (TFA) as solvent A and 0.1% TFA/Acetonitrile as solvent B. Both solvents were filtered and deaerated before use. A program was created that equilibrated the column with 15 mL of solvent A, then slowly added solvent B in a gradient until it reached 100% over the span of 60 minutes, then finished with 10 mL of solvent B. The sample was injected (generally 0.5 mL) and the program was started. The fractions were tested on CAS for siderophore activity. The positive results were used to refine the gradient until it was determined that the siderophore eluted at 23% of solvent B. The samples from each of these runs were collected and concentrated by evaporation. When only one peak on the chromatogram was evident, the sample was considered pure.

Characterization

Atkin's Test

After determining siderophore production the next step is to determine siderophore type. A positive Atkin's Test indicates a hydroxamate siderophore. Culture supernatant (0.5 mL) is added to 2.5 mL of iron-perchlorate reagent (5 mM $Fe(ClO_4)_3$ in 0.1M $HClO_4$) and absorbance is measured after 5 minutes of incubation at room temperature. The formation of a red color indicates the presence of hydroxamic acids. Absorbance is measured at 480 nm with sterile media as the blank.

Arnow's Test

The Arnow's test detects catechol siderophores. A mixture of 1mL supernantant, 1 mL 0.5 M HCl, 1 mL of 1M NaOH, and 1 mL of Nitrite-molybdenate reagent (10 g sodium nitrite and 10g sodium molybdate in 100 mL ddH_20) is used. After 5 minutes of incubation at room temperature, a red color will form if catechol groups are present. Absorbance is measured at 500nm with sterile media as a blank.

Amino acid Analysis

Siderophores are often conjugates of amino acids. For example both Staphyloferrin A and B contain ornithine. For this reason amino acid analysis was performed. It was necessary to hydrolyze the sample for amino acid analysis. The sample was hydrolyzed by adding an equal volume of 6M HCl and autoclaving at 121 C for 6 hours. Amino acid standards were prepared by adding 1 mg/mL of the amino acid to ddh_20. Standards of all 20 protein-building amino acids plus ornithine were made. The neutralized, hydrolyzed sample and the amino acids standards were spotted on TLC plates (approximately 5µL each) and a solvent system of n-propanol/ddH_20 in a 70:30 ratio was used to develop the TLC plate,

ESMS

The post-HPLC purified samples were divided into 2 vials and one was complexed with iron. These were given to the Analytical Dividion of Eastman Chemical for electrospray mass spectrometry (ESMS) analysis. A Varian Monochrom 3 column (C18 50 x 2.0 mm, PN A040005oX020) was used with solvent A being deionized water with 260 mg ammonium acetate and solvent B was methanol. The sample was dissolved in approximately 200 µl of

methanol, and 5 µl was injected at a temperature of 35 C. The ESMS was performed at 10, +25, -25, +75, and -75 volts.

NMR

The sample was also analyzed with 1D 1H Nuclear Magnetic Resonance (NMR). The sample was dissolved in 1mL of methanol-d4. A JEOL Eclipse 600 MHz NMR spectrometer with a 5mm OD NMR tube was used for the analysis.

CHAPTER 3

RESULTS

The Marshfield Clinic in Wisconsin initially sent 40 strains of HA-MRSA to our lab. All of these strains (listed in Table 1) were screened for siderophore production with the CAS assay, and all were positive. However, the amount of siderophore production varied as evidenced by differing sizes and brightness of the halos on the CAS plate. Strain H372 had one of the largest and brightest halos, so it was chosen for further study.

S. aureus Strain H372 Characteristics

In the course of researching the siderophore produced by strain H372, many characteristics of this strain were observed. These are summarized in Table 2 and discussed further below.

Table 2: Characteristics of *S. aureus* H372

S. aureus Strain H372	
Siderophore Production	CAS diameter 14mm
Hemolysis	Beta
Arnow's test	Negative
Atkins' test	Negative
Heme	12mm halo of growth
Antibiotic Profile	
Ciprofloxacin	R
Erythromycin	R
Clindamycin	S
Tetracycline	S
Sulfamethoxazole	S
Gentamycin	S
Rifampin	R

Siderophore Production by *S. aureus* Strain H372

The CAS assay was used to determine if strain H372 produced a siderophore. The bacteria were grown in LB broth with 500 μm dipyridyl for 24 hours and centrifuged. The

supernatant was then placed into wells on the CAS plate, and a yellow halo indicated siderophore production (Figure 3). The size and relative brightness indicated the amount and iron-chelating strength of the siderophore. Strain H372 did not produce siderophore in LB broth, TMS media, or Congo Red but did produce siderophore in LB broth with dipyridyl to sequester the ferrous iron. The optimum amount of dipyridyl and incubation time for siderophore production were determined to be 24 hours in LB broth with 500 µm dipyridyl. These results are summarized in Tables 3 and 4.

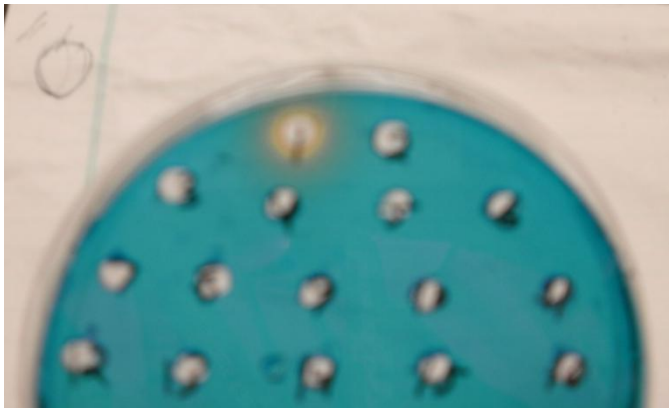

Figure 3: An example of an H372 halo on CAS media

Table 3: Growth rates and siderophore production in different concentrations of LB Broth + Dipyridyl.

Media	Growth at 24 hr (OD at 600 nm)	Siderophore Production on CAS
LB Broth	2.05	No halo
LB Broth + 250 µm dipyridyl	1.78	10mm halo (light)
LB Broth + 500 µm dipyridyl	1.61	14mm halo (bright)
LB Broth + 750 µm dipyridyl	1.49	14mm halo (medium)
LB Broth +1000 µm dipyridyl	1.30	12mm halo (medium)

Table 4: Growth and siderophore production of H372 at different times in LB Broth + 500 μm dipyridyl.

Time	Growth (OD at 600 nm)	Siderophore Production on CAS
12 hours	1.23	9mm halo (Very light)
18 hours	1.37	10 mm halo (medium)
24 hours	1.64	14mm halo (bright)
30 hours	1.58	14mm halo (bright)
36 hours	1.53	12 mm halo (medium)
48 hours	1.47	12mm halo (medium)

Hemolysis

Hemolysis correlates with virulence, so H372 was inoculated on a blood agar plate. Strain H372 was beta-hemolytic, meaning that it fully cleared the red blood cells around it on the plate (Figure 4).

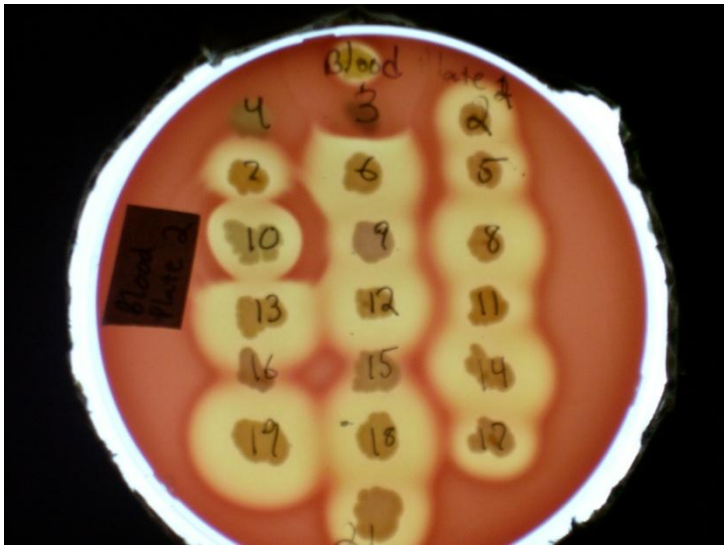

Figure 4: Examples of hemolysis on a blood agar plate

Heme Usage

Heme is the preferred source of iron for *S. aureus* (24), so the heme usage of strain H372 was determined experimentally. Strain H372 was inoculated onto agar containing dipyridyl, and a disc impregnated with hemin was placed on the agar. A ring of growth around the hemin disc

indicated usage of heme as an iron source. The diameter of the ring in H372 was 12 mm, indicating that it did have the ability to use heme.

Siderophore Purification

After the growth conditions for the optimum production of siderophore were determined, H372 was grown in large batch cultures of 4-5 L for purification. After checking for siderophore production on CAS, the broth was centrifuged. The supernatant was then acidified to pH 2.00 to reduce the solubility of the siderophore for chromatography using an XAD-2 column.

Manual Column Chromatography

Amberlite XAD-2. The XAD-2 column was the first column used for purification. The acidified supernatant was run through the column, and then the column was washed with ddH_20. The filtrate and H_20 wash were checked for siderophore on CAS to make sure that the siderophore had bound in the column. If the CAS assay was negative, elution of the column with methanol followed. Fifty fractions of approximately 3mL each were collected. Every other fraction was tested on CAS media for siderophore activity, and those testing positive were pooled and concentrated by evaporation for further purification. Depending on the flow rate of the column, the positive fractions were generally in the range of fractions 15-30 and had a brownish-red color.

Sephadex LH-20. The positive samples were further purified using an LH-20 column. The evaporated, post-XAD sample was dissolved in pH 2.00 ddH_20 and loaded on the LH-20 column. The column was run with ddh_20 as the solvent and approximately 40 fractions were taken. These fractions were checked on CAS for siderophore activity, and the positive fractions

were pooled and evaporated. Depending on flow rate the positive fractions were generally in the range of fractions 15-25, and had a reddish color.

HPLC

After manual column chromatography the sample was further purified by high pressure liquid chromatography (HPLC). A Watters C-18 column was used, and an appropriate solvent system had to be found. A water/methanol system was attempted first, but the sample was highly hydrophilic so it did not work. Then a system of 0.1% trifluoroacetic acid (TFA) as solvent A and 0.1% TFA/acetonitrile as solvent B was used, with success. The HPLC was run with this solvent system over a gradient of 1-100% solvent B, and the fractions collected and tested on CAS. Two fractions tested positive, and based on the HPLC profile it was somewhere between 20-40% solvent B. Subsequent HPLC runs were made, further refining the gradient until it was determined that the siderophore eluted at 23% solvent B. This percentage of solvent B correlated to a specific peak on the HPLC profile (Figure 5) Further HPLC runs were made with fresh samples using the refined gradient until only a single peak was evident on the HPLC profile, indicating the elution of a possibly pure compound. The fraction corresponding to the peak was collected, tested on CAS, dried, and saved for chemical and structural characterization experiments.

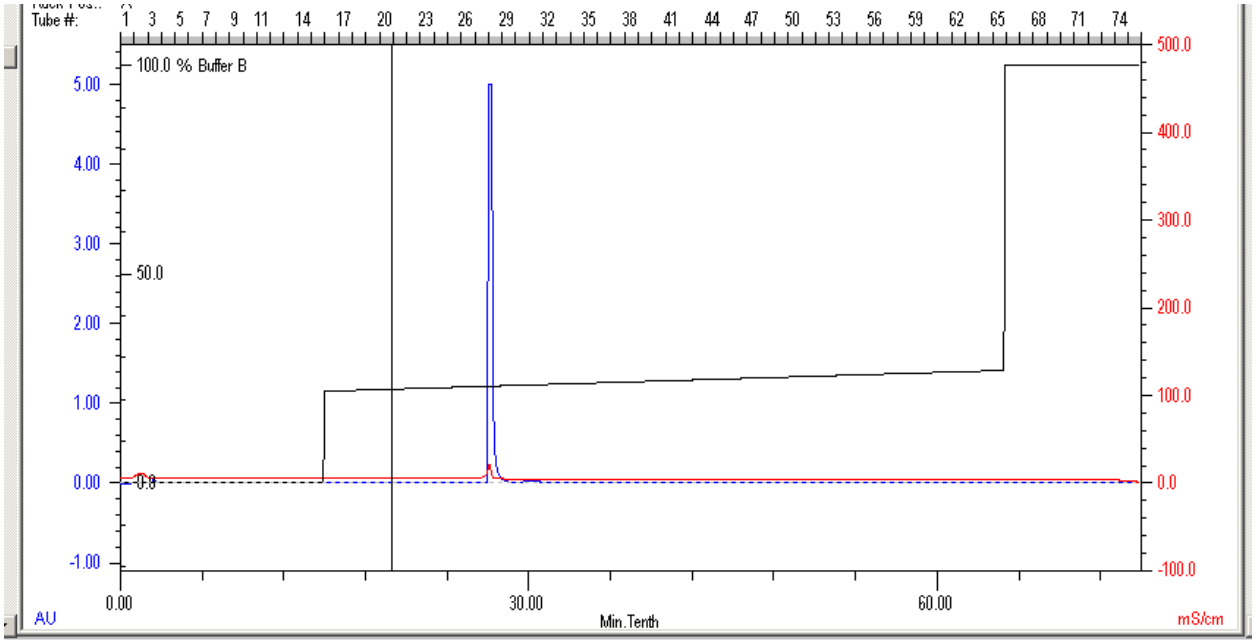

Figure 5: HPLC profile of H372 showing a single peak, indicating a pure siderophore

Siderophore Characterization

Atkin's and Arnow's Tests

After determining that H372 produced a siderophore, the next step was to characterize the siderophore. The Arnow's test was performed to determine if catechol species were present, and the Atkins' test was performed to determine if hydroxamate species were present. Both tests indicate a positive result if the sample turns red or has a spectrophotomer reading of 0.003 or greater. The Arnow's test was negative with no color change and a reading of -0.008. The Atkin's test was negative with no color change and a reading of 0.001. The negative results of these tests indicated that the siderophore did not belong to the common hydroxamate or catechol types. If a siderophore is not a hydroxamate or catechol type, the default category is carboxylate.

Amino Acid Analysis

Many siderophores are conjugates of amino acids, so amino acid analysis was performed on the sample to determine the amino acids present. The sample was acid hydrolyzed and spotted on a TLC plate alongside amino acid standards. The TLC plate was developed in solvent and sprayed with ninhydrin reagent to reveal the spots. The hydrolyzed sample showed the presence of 3 products indicated by 3 spots (Figure 6). Spot 1 had the same Rf value as ornithine. Spot 2 had the closest Rf value to tryptophan but was close to phenylalanine and isoleucine as well (Figure 7). Spot 3 had a higher Rf value than any of the amino acids possibly indicating some sort of degradative product that reacted with the ninhydrin but not an amino acid.

Figure 6: Amino Acid Analysis with the sample and ornithine

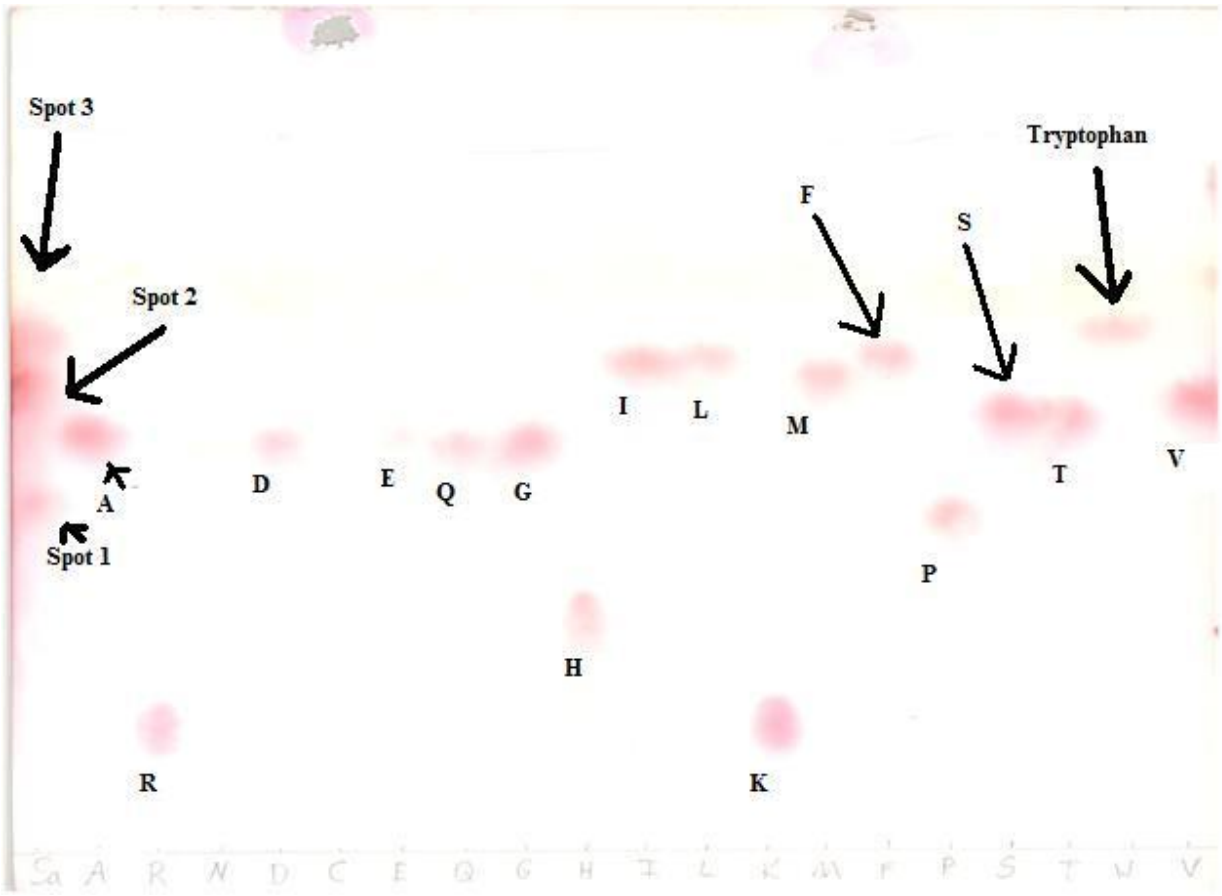

Figure 7: Amino Acid Analysis with the sample and 20 amino acids

A-alanine, R-arginine, N-asparagine, D-aspartic acid, C-cysteine, E-glutamic acid, Q-glutamine, G-glycine, H-histidine, I-isoleucine, L-leucine, K-lysine, M-methionine, F-phenylalanine, P-proline, S-serine, T-threonine, W-tryptophan, V-valine

ESMS

Electron spray mass spectrometry (ESMS) was performed by the Analytical Division of Eastman Chemical for the molecular weight and possible structural elucidation of the sample. A regular sample and an iron-complexed sample were provided. Unfortunately, the iron-complexed sample did not yield any useful data. The reason for this is unknown.

Figure 8 shows an HPLC profile of the sample. While the HPLC performed at the ETSU lab showed a single peak, this HPLC showed 14 peaks. This difference may be explained by the use of a different solvent system. The peak with the largest area under the curve corresponds to the major product, and this is peak 8. The assumption is that because the purification process was designed to isolate a siderophore, the major product will indeed be the siderophore; however, it is possible that is not the case. Because the mixture was complex, we did not get useful structural information to confirm the likelihood of a siderophore.

After HPLC the fraction corresponding to each peak was analyzed by ESMS for molecular weight data. The ESMS was perfomed at 10v, -25v, +25v, -75v, and 75v (Figures 8-13). The 10v setting's function is to ascertain an accurate mass as the molecules will not break apart at that voltage. The mass at the 10v setting appears to be 681 kDa (Figure 9). However, the mass at the 25v setting is 658 (Figure 10). This difference is likely explained by a sodium adduct. Sodium adducts are common in ESMS analysis and they add 23 kDa to the molecular weight. A sodium adduct is also present in the 75v data (Figure 12). The 25v and 75v settings can be used for structural information as fragmentation of the molecule can occur. Some fragmentation did occur at +75v as the peak at 681 kDa (molecular weight + sodium) is smaller and a new, high peak is seen at 245 kDa.

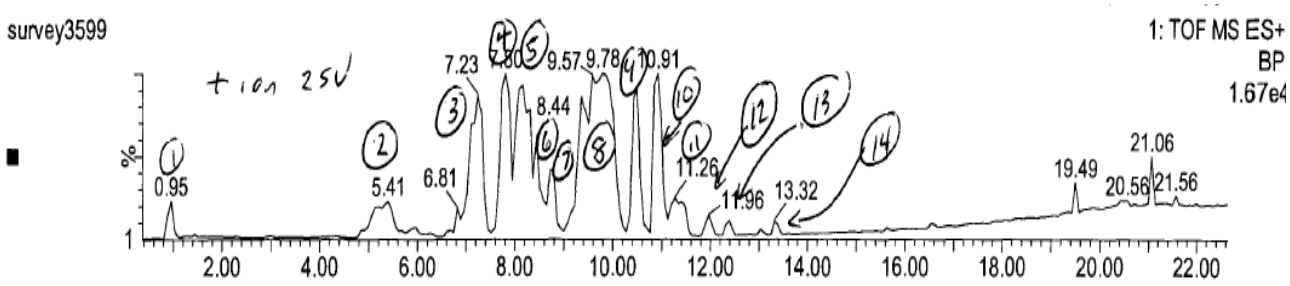

Figure 8: Eastman Chemical HPLC data for strain H372 showing 14 peaks

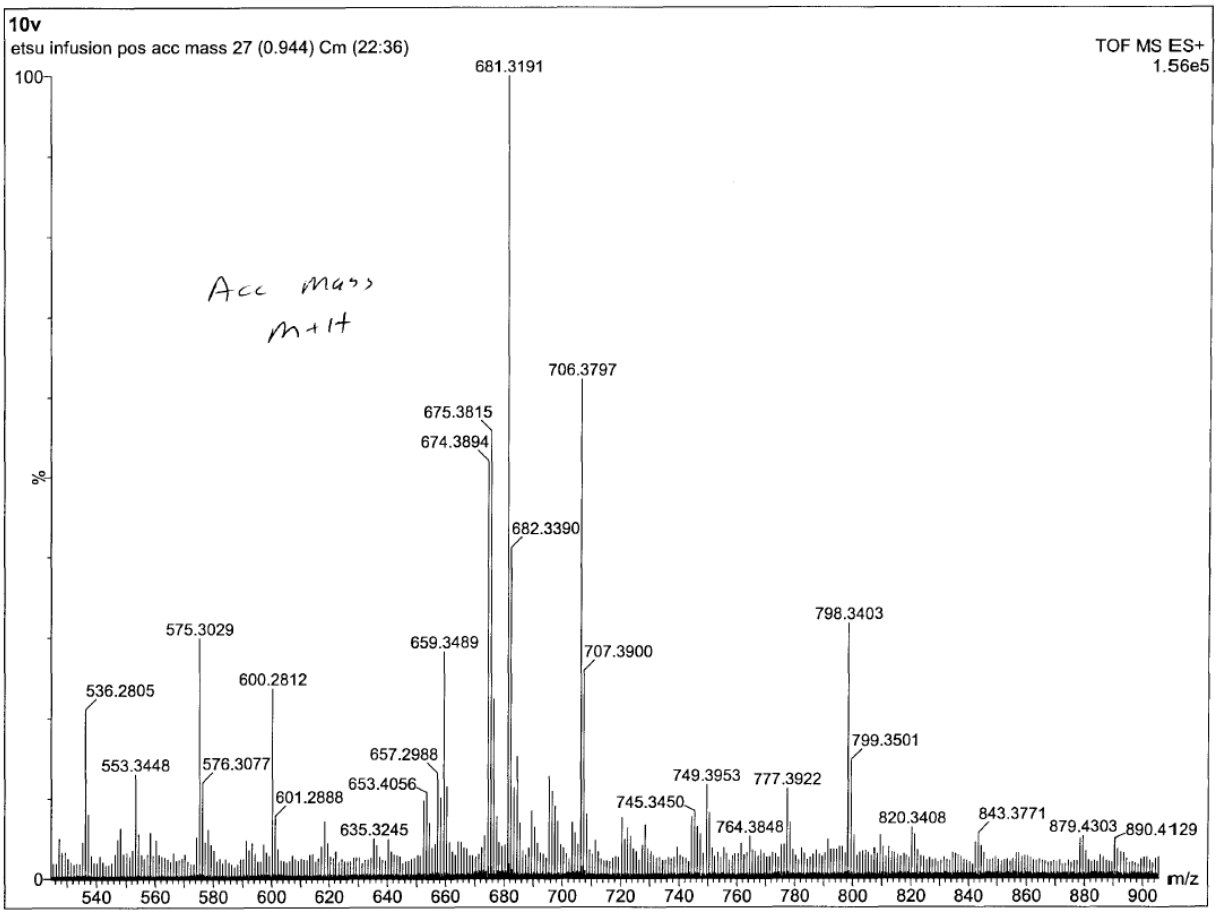

Figure 9: 10v accurate mass data for H372 sample peak 8

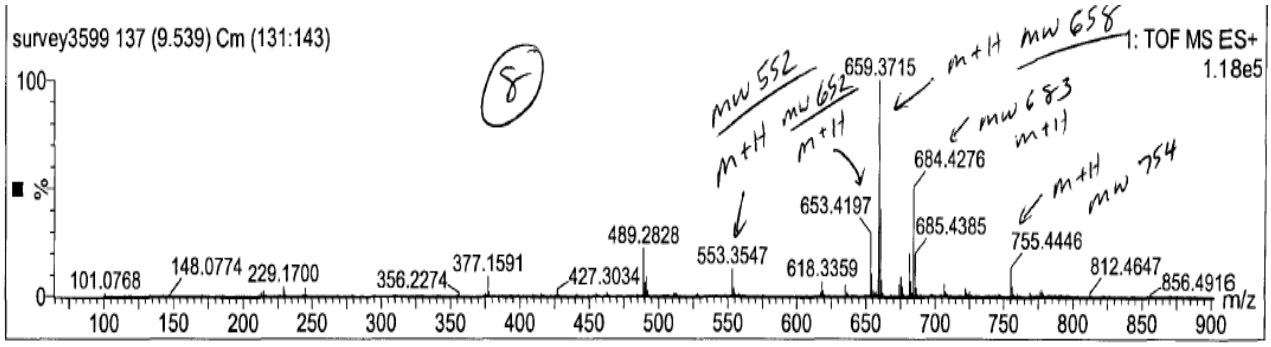

Figure 10: ESMS data at +25v

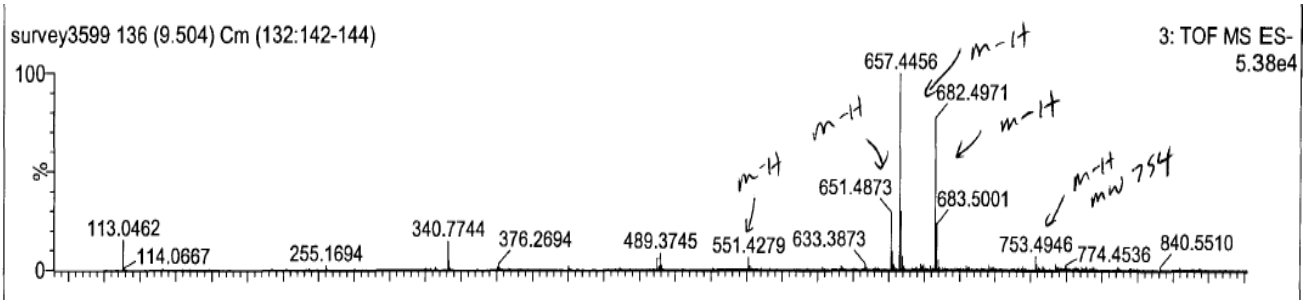

Figure 11:ESMS data at -25v

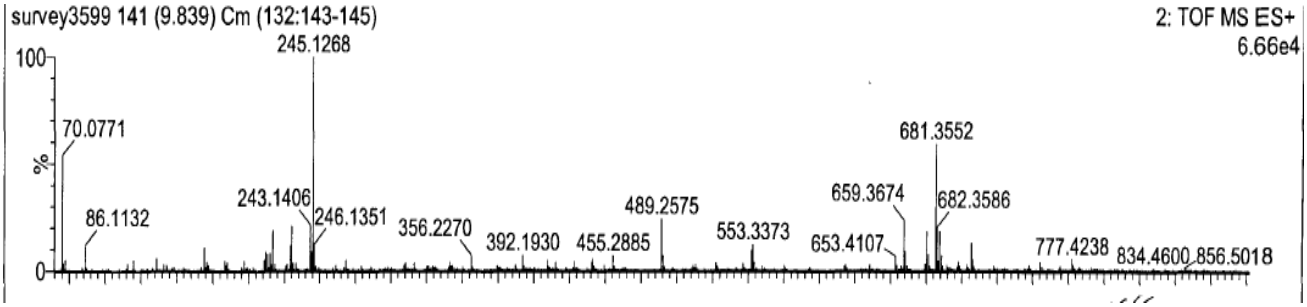

Figure 12: ESMS data at +75v

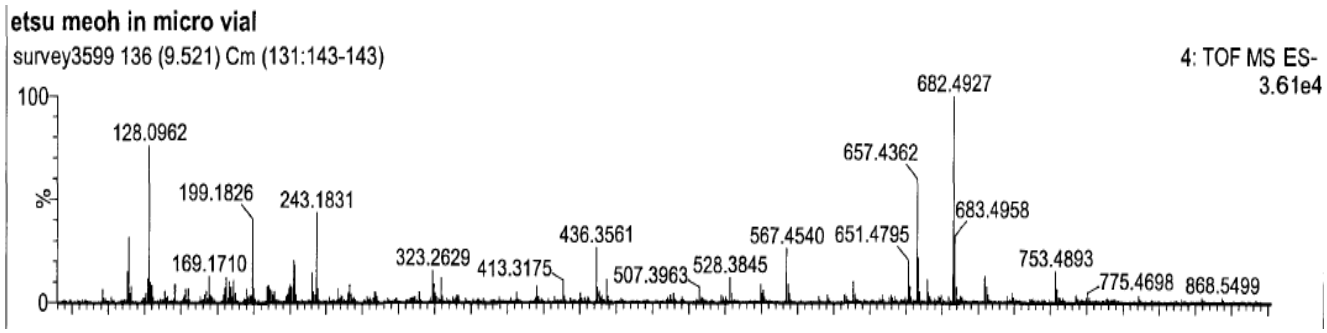

Figure 13: ESMS data at -75v

Based on the molecular weight and the combination of the elements carbon, hydrogen, oxygen, and nitrogen, the ESMS machine produced possible molecular formulas for the major product of the sample (Table 5). The formulas were then entered into the SciFinder database to see if they corresponded to any known siderophores. No corresponding siderophores were found. This could indicate a completely new siderophore or that the major product is not a siderophore.

Table 5: ESMS elemental composition report (first 12 results, ranked by similarity to sample)

Accurate Mass Search for Possible Formulae Sorted by PPM:

Elemental Composition Report

Single Mass Analysis

Tolerance = 25.0 PPM / DBE: min = -2.0, max = 50.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

2618 formula(e) evaluated with 69 results within limits (up to 100 closest results for each mass)

Elements Used:

C: 0-100 H: 0-200 N: 0-20 O: 0-20 Na: 1-1

Minimum:				-2.0		
Maximum:		5.0	25.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
681.3182	681.3184	-0.2	-0.3	8.5	1923.8	C27 H46 N8 O11 Na
	681.3179	0.3	0.4	26.5	1099.3	C40 H38 N10 Na
	681.3189	-0.7	-1.0	1.5	11516.8	C12 H42 N20 O12 Na
	681.3192	-1.0	-1.5	20.5	1100.1	C43 H46 O6 Na
	681.3170	1.2	1.8	3.5	2654.5	C26 H50 N4 O15 Na
	681.3170	1.2	1.8	14.5	2776.6	C24 H38 N18 O5 Na
	681.3197	-1.5	-2.2	13.5	1420.0	C28 H42 N12 O7 Na
	681.3165	1.7	2.5	21.5	684.9	C39 H42 N6 O4 Na
	681.3205	-2.3	-3.4	25.5	1682.4	C44 H42 N4 O2 Na
	681.3157	2.5	3.7	9.5	3497.6	C23 H42 N14 O9 Na
	681.3157	2.5	3.7	-1.5	3634.9	C25 H54 O19 Na
	681.3210	-2.8	-4.1	18.5	1128.1	C29 H38 N16 O3 Na

NMR

Nuclear magnetic resonance (NMR) was also performed by the Analytical Division at Eastman Chemical. The NMR also shows a very complex mixture in the sample (Figure 14). Due to the number of peaks it is difficult to glean useful information from this NMR result. The only definitive bonds that can be observed are CH_2 bonds (circled in green) and an aromatic ring (circled in purple). CH_2 bonds are extremely common, so they would be expected in almost any NMR analysis. The source of the aromatic rings is unknown.

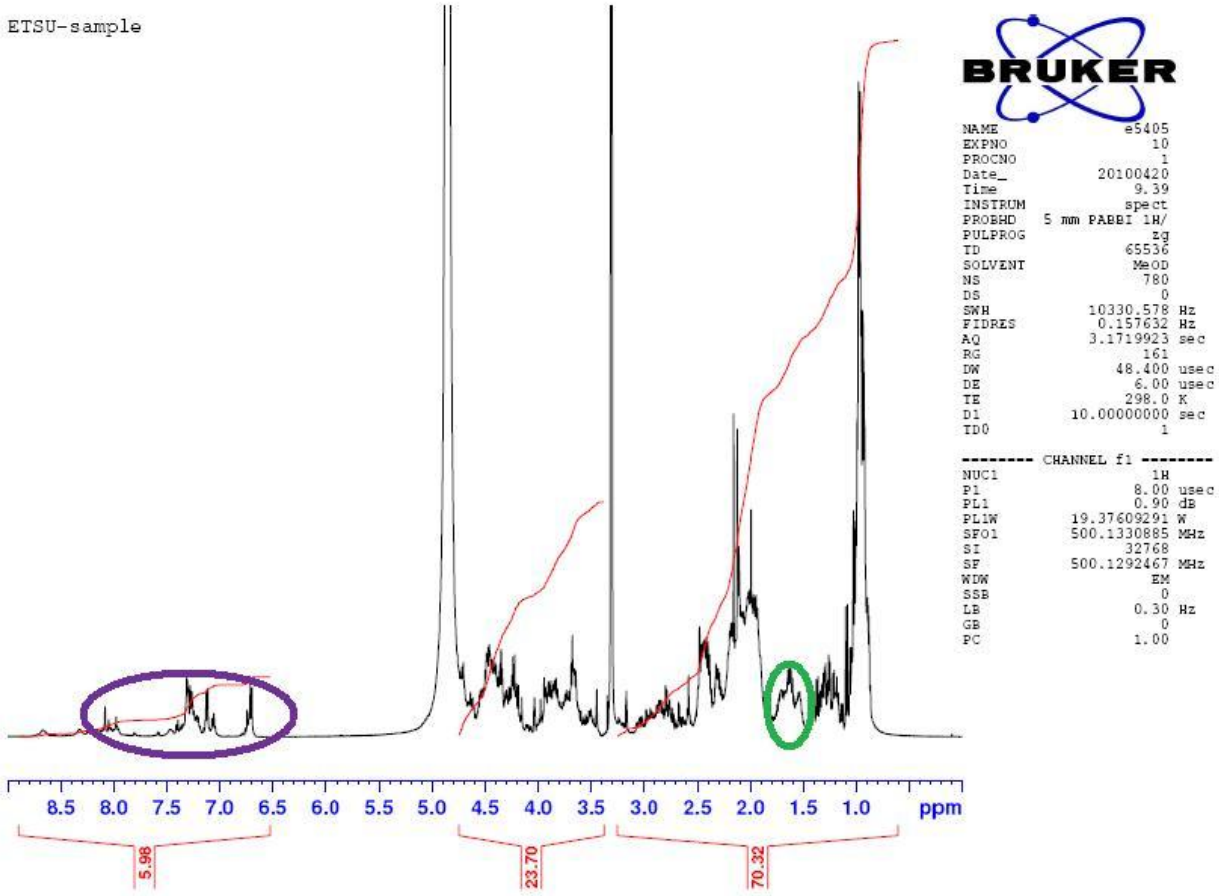

Figure 14: NMR Data. CH_2 bonds circled in green, Aromatic ring bonds circled in purple.

CHAPTER 4

DISCUSSION

The vast majority of organisms need iron to survive, and most bacteria produce siderophores for this purpose. Siderophores are known to be important in the pathogenesis of bacteria including *S. aureus* (24). However, most siderophore research focuses on Gram negative bacteria. Much less is known about siderophores produced by Gram-positive bacteria including siderophores of staphylococci. Staphylococci produce 4 known siderophores: staphyloferrin A, staphyloferrin B, staphylobactin, and aureochelin. Only the staphyloferrins have been fully characterized; the structures of staphylobactin and aureochelin remain unknown (22, 29, 42). Due to the importance of siderophores to virulence, there is a need to know more about the siderophores of *S. aureus.*

The purpose of this research was to identify and characterize siderophores in *S. aureus,* particularly in antibiotic-resistant strains. Because iron acquisition could be a drug target, it is important to learn more about these systems in *S. aureus.* In the United States alone 94,000 people a year contract invasive MRSA infections and 19,000 people die of MRSA annually (16). In our lab numerous strains of MRSA were tested for siderophore activity, and those possessing the greatest siderophore activity were selected for further study. Of those strain H372 was selected due to its strong production. After selecting this strain siderophore production needed to be optimized and the siderophore characterized.

Due to the intrinsically low iron requirement of *S. aureus* (56), it was difficult to find a growth medium with sufficient iron restriction. The medium that best supported growth and siderophore production was LB Broth with 500μm dipyridyl to sequester iron. Minimal medias

such as Fiss, TMS, Congo Red, and MMW were tried, but either growth or siderophore production were compromised.

After optimization of production purification methods were followed based on the preliminary chemical characterization of the siderophore. The Arnow's test for catechol siderophores and the Atkin's test for hydroxamate siderophores were performed. The siderophore from H372 tested negative, indicating it is neither a catechol nor hydroxamate type. Based on the data we presume it is a carboxylate type siderophore, although there is no definitive test for this type. This finding was consistent with the fact that staphyloferrin A and B are both carboxylate siderophores. Based on the presumption that it is a carboxylate siderophore, we tried to use the published procedures for the purification of the staphyloferrins.

After determining the optimum conditions and type of siderophore, large batch cultures were needed for purification. Purification involved 3 chromatographic processes. First, the acidified supernatant was passed through an amberlite XAD-2 column that binds cyclic molecules. The CAS-positive fractions from the XAD-2 were then pooled and concentrated. Second, the concentrated CAS positive material was passed through a sephadex LH-20 column that separates molecules according to size and hydrophobicity. The CAS positive fractions from the Sephadex LH-20 were then pooled and concentrated. Third, the concentrated CAS positive sample from the sephdex LH-20 column was analyzed with HPLC using a Waters C-18 column. The CAS assay was used to follow the siderophore throughout the process. The first HPLC solvent system tried was a water/methanol system; however, the siderophore was highly hydrophilic and eluted in the water immediately. This hydrophilicity is consistent with staphyloferrin A, so the system was changed to the TFA/TFA-acetonitrile that was used during the purification of staphyloferrin A (52). The high hydrophilicity of the siderophore is also why

thin-layer chromatography (TLC) of the siderophore was never satisfactory. The silica gel plates and solvent systems used for TLC were too hydrophobic and the hydrophilic siderophore samples did not migrate up the plate. The new solvent system for HPLC worked, and it was determined that the siderophore corresponded to a specific peak on the HPLC profile that eluted at 23% solvent B. This HPLC profile was significantly different than staphyloferrin A, which elutes at between 4-10% of solvent B using the same solvent system (52). When this single peak was evident on the HPLC profile, the sample was considered pure.

Siderophores are usually conjugates of amino acids, so amino acid analysis was performed on the sample. The sample was acid-hydrolyzed, spotted on TLC plates, and compared to the 20 amino acids and ornithine. The sample showed 3 spots on TLC. The lowest spot, designated as spot 1, had the Rf value of ornithine. Spot 2 had the closest Rf value to tryptophan, although it was close to phenylalanine and isoleucine as well. Spot 3 was higher than all the Rf values for the amino acids, so it is presumably a degradative product that reacted with the ninhydrin reagent. The presence of ornithine was intriguing because both staphyloferrin A and B contain ornithine.

The HPLC purified samples were analyzed by ESMS and NMR to attempt to discern structural information and molecular weight. Both an iron-complexed and a non iron-complexed sample were analyzed. Unfortunately, the iron-complexed sample did not yield any useful data for unknown reasons. Although the ESMS profile shows multiple peaks, the probable molecular weight based on the major peak is 658, which is significantly different than staphyloferrin A at 481 Da (61), staphyloferrin B at 448 Da (42), staphylobactin at 822 Da (24), or aureochelin at 577 Da (22). The molecular weight data suggest that this siderophore may not be any of the known staphylococcal siderophores. The ESMS data were also analyzed using a molecular mass

database for known compounds but no matches were found, even when specifically searching for siderophores.

The NMR data suggested the presence of CH_2 bonds and an aromatic ring. The CH_2 bonds are too common to assist in identification of the structure of the siderophore.

The final conclusion of this research is that *S. aureus* strain H372 produces a hydrophilic siderophore that contains ornithine and has a probable molecular weight of 658. The siderophore could be novel or it could be that one of the staphyloferrins is present and bound to other molecules increasing its molecular weight.

Future studies for this project include further structural elucidation of this siderophore. Although the siderophore appeared pure on HPLC, the complex profiles of the ESMS and NMR indicate either that the purity needs to be improved or that the siderophore is degrading during the process of purification or storage after purification. The identification and structural determination of this siderophore can be helpful in the study of its regulation, biosynthesis, and transport.

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VITA

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