The Role of the sia and siu ABC-Type Transporters in Iron Utilization and Virulence in Streptococcus pyogenes

Griselle Enid Montanez

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THE ROLE OF THE SIA AND SIU ABC-TYPE TRANSPORTERS IN IRON UTILIZATION AND VIRULENCE IN STREPTOCOCCUS PYOGENES

By
GRISELLE E. MONTAÑEZ

Under the direction of Zehava Eichenbaum

ABSTRACT

A limited understanding of iron uptake mechanisms is available for *Streptococcus pyogenes*, a hemolytic human pathogen capable of using a variety of hemoproteins in addition to ferric and ferrous iron. This study characterizes the transporters of iron-complexes *siu*ADBG (for streptococcal iron uptake) and *sia*ABC (for streptococcal iron acquisition). These ABC-type transporters are encoded by iron regulated operons and their protein products are homologous to components of heme and siderophore transporters found in both Gram-positive and Gram-negative bacteria. Mutants of the membrane permeases *siuG* and *siaB* were constructed and characterized. Mutations in both transporters demonstrated growth reduction in comparison to the parent strain when grown in complex medium containing iron in the form of hemoglobin. The addition of heme to the growth medium inhibited ferric uptake by the wild-type while the addition of protoporphyrin IX did not, suggesting that heme utilization as an iron source is
responsible for the inhibition of ferric accumulation. Inactivation of *siuG* reduced the ability of heme to inhibit ferric incorporation by the cells. Inactivation of *siaB* in addition to *siuG* had a cumulative effect, indicating that both *siu* and *sia* transporters are involved in heme utilization. We also demonstrated that purified rSiaA, the surface receptor of SiaABC, binds heme and hemoglobin *in vitro*, and we propose a mechanism of heme binding by SiaA. Studies in a zebrafish infection model revealed that the *siuG* mutant was attenuated in producing disease. While the *siaB* mutant also presented virulence attenuation, infection by this mutant was characterized by an increase in the host inflammatory response. These observations show that iron acquisition is important for *S. pyogenes* virulence. We propose that the SiaABC and SiuADBG, together with the multi-metal transporter MtsABC, are involved in iron acquisition from different iron sources present in the human body, thus contributing to the survival and pathogenesis of *S. pyogenes*.

INDEX WORDS: ABC transporter, bacteria, ferrichrome, GAS, heme, hemoglobin, hemoproteins, iron transport, *sia, siu, Streptococcus pyogenes*, virulence, zebrafish.
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GRISELLE E. MONTAÑEZ

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of
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2005
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IN IRON UTILIZATION AND VIRULENCE
IN STREPTOCOCCUS PYOGENES

by

GRISELLE E. MONTAÑEZ

Major Professor: Zehava Eichenbaum
Committee: Phang C. Tai
            Sidney Crow

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“Tu aumentas mis fuerzas/como las fuerzas de un toro,/ y viertes perfume sobre mi cabeza.” (Psalms 92:10). Thank you, Lord for your eternal Love.

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<td>ATP binding cassette</td>
</tr>
<tr>
<td>CDM</td>
<td>Chemically defined medium</td>
</tr>
<tr>
<td>c.p.m.</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>CxCDM</td>
<td>Chelex treated-chemically defined medium</td>
</tr>
<tr>
<td>GAS</td>
<td>Group A Streptococcus</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
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<td>Hp</td>
<td>Haptoglobin</td>
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<tr>
<td>i.m.</td>
<td>Intramuscular(ly)</td>
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<tr>
<td>i.p.</td>
<td>Intraperitoneal(ly)</td>
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<tr>
<td>Mb</td>
<td>Myoglobin</td>
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<tr>
<td>NTA</td>
<td>Nitrilotriacetic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RT</td>
<td>Reverse trascriptase</td>
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<tr>
<td>Sia</td>
<td>Streptococcal iron acquisition</td>
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<td>Siu</td>
<td>Streptococcal iron uptake</td>
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<td>TH</td>
<td>Todd Hewitt</td>
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<td>THY</td>
<td>TH with yeast extract</td>
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<td>THYP</td>
<td>THY with peptone</td>
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<td>ZTH</td>
<td>THY buffered with Tris-HCl</td>
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GENERAL INTRODUCTION

Streptococcus pyogenes—Description, Disease and Epidemiology

The Gram-positive Streptococcus pyogenes or Group A Streptococcus (GAS) is a frequent pathogen with a great impact on human health. This hemolytic bacterium is capable of producing common but typically mild and self-limiting infections of the nasopharynx and skin such as pharyngitis, tonsillopharyngitis, cellulitis, scarlet fever, and impetigo (Cunningham, 2000). Sometimes these infections can lead to severe immunological complications such as rheumatic fever, rheumatic heart disease, and glomerulonephritis. S. pyogenes also causes several rare but serious invasive illnesses like myositis, osteomyelitis, necrotizing fasciitis, and streptococcal toxic shock syndrome. The CDC 2003 Active Bacterial Surveillance Report for GAS determined that invasive S. pyogenes infections occurred at a rate of 3.9 per 100,000 annually, with about 11,000 cases and approximately 1,800 deaths (Prevention, 2004). Although a considerable amount of information on S. pyogenes pathogenesis is available, many questions remain on the host-bacterial interactions and no vaccine is yet available to prevent infections.

Streptococcus pyogenes Virulence Factors

Numerous bacterial products that have an integral role in the establishment of infection and the pathology of S. pyogenes have been identified. These virulence factors can be classified in three groups (although some belong to more than one category): i)
immune response evasion, ii) adherence, colonization and internalization, and iii) extracellular products (Bisno et al., 2003). Since *S. pyogenes* cannot survive phagocytosis, this bacterium uses several strategies to evade the host immune surveillance. The M protein is the most important antiphagocytic factor of *S. pyogenes*, which is classified into different serotypes based on the hypervariable regions of this surface protein. This first category of virulence factors also includes M-like proteins, such as Mtp and Enn, and the hyaluronic acid capsule, all of which protect *S. pyogenes* from phagocytosis. In addition, *S. pyogenes* produces a C5a peptidase, which contributes to the evasion of the immune response by degrading the neutrophil chemo-attractant C5a.

Several components of the second group promote streptococcal adherence to the host tissues. These include the lipoteichoic acid, various fibronectin binding proteins, and the M protein. Additionally, host cell invasion by *S. pyogenes* is facilitated by the M protein, the hyaluronic acid polymer, and protein F1. The third group of virulence factors includes secreted products that aid the spread of *S. pyogenes* throughout the tissues and elude the immune response. The DNAses A, B, C, and D, hyaluronidase, streptokinase (converts plasminogen to plasmin), streptococcal pyrogenic exotoxin B (SpeB), and the streptococcal inhibitor of complement (Sic) belong to this category. The toxins Streptolysin S and Streptolysin O are hemolysins, which damage the membrane of the erythrocyte and other cell types, also belong to the third group. Finally, *S. pyogenes* secretes several pyrogenic exotoxins (SpeA and SpeC being the most important) which function as superantigens and are associated with the production of septic shock.
Importance of Iron in Bacteria

Growth, colonization, and production of infection by bacteria are dependent on their ability to obtain essential nutrients from the surrounding environment. Iron is necessary for growth of most microorganisms. However, certain bacteria such as some Lactobacilli (Elli et al., 2000; Weinberg, 1997) and Borrelia burgdorferi (Posey & Gherardini, 2000) do not seem to need iron for growth. Iron plays a critical role in microbial metabolism and gene expression, serving as a cofactor for many proteins, including the metal-dependent transcription regulators DtxR and Fur. In addition, the metalloporphyrin heme serves as a prosthetic group for electron transport proteins (cytochromes, quinoproteins) and oxidative response enzymes (peroxidases, catalases).

The redundancy in iron uptake pathways frequently displayed by individual pathogens underscores the importance of iron to the infection process. Iron availability regulates the expression of several virulence factors. For example, the production of the diphtheria toxin in Corynebacterium diphtheriae (Boyd et al., 1990) is observed only when the bacterium grows in iron limited conditions. Also, numerous studies have shown that mutations in iron uptake genes decrease the ability of pathogenic bacteria to produce infection (Al-Tawfiq et al., 2000; Braun, 2005; Brown et al., 2001a; Dale et al., 2004; Henderson & Payne, 1994; Ratledge, 2004; Stojiljkovic et al., 1995; Torres et al., 2001). Incidentally, the human body reduces the levels of available iron in serum and secretions in response to infection, increasing host resistance to invading microorganisms (Bullen et al., 2005). This further emphasizes the importance of iron in bacterial virulence.
Iron in the Human Body

The free iron concentration of human mucosal surfaces and plasma is estimated to be in the range of $10^{-18}$ M (Bullen, 1981). This is due to the fact that the majority of the iron is found intracellularly, stored in ferritin or found in the form of heme complexes such as hemoglobin and myoglobin (Otto et al., 1992). Small amounts of hemoglobin and heme are released into the extracellular milieu by spontaneous hemolysis or due to the action of bacterial hemolysis. However, plasma hemoglobin and heme rapidly bind to the protein-carriers haptoglobin or to hemopexin and serum albumin, and are taken to the liver (Genco & Dixon, 2001; Wandersman & Stojiljkovic, 2000). The human plasma, lymphatic fluids, and secretions contain only minute amounts of inorganic iron tightly bound to the glycoproteins transferrin and lactoferrin (Wooldridge & Williams, 1993). Since the vast majority of the iron in humans and other mammals is tightly bound to host proteins, pathogenic bacteria have developed a variety of high affinity iron scavenging systems to compete for iron while colonizing the human body.

Mechanisms of Iron Uptake from the Host by Gram-Negative Pathogens

Pathogenic microorganisms utilize several strategies to obtain iron and are often able to exploit the multiple iron sources found in their host. Some microorganisms produce and bind siderophores, which are low molecular weight molecules used to capture and deliver iron to the cell via specific receptors. Their production is very common and some bacteria are able to use siderophores produced by other microorganisms. The widespread bacterial investment in the complex biosynthesis and
utilization of siderophores (Crosa & Walsh, 2002) underscores their importance to the iron acquisition process.

Beside the use of siderophores, bacteria employ high affinity receptors that recognize iron-containing proteins, such as transferrin and lactoferrin, or heme containing complexes found in the body fluids. Studies of both Gram-negative and Gram-positive bacteria have elucidated several strategies for heme capture, including the utilization of hemophores, hemoglobin proteases, and high affinity receptors for heme and heme proteins (Stojiljkovic & Perkins-Balding, 2002). The hemophore-mediated mechanism involves the secretion of proteins that specifically recognize heme-proteins, extract the heme, and carry it to a cognate surface receptor for internalization. Hemophores were first found in Serratia marcescens, but other Gram-negative bacteria also produce them (Ghigo et al., 1997; Letoffe et al., 1994; Letoffe et al., 1998). Bacteria also secrete hemolysins or proteases that cleave host hemoproteins, making heme accessible for transport via bacterial outer membrane receptors. Escherichia coli produces two extracellular proteases, Tsh and Hbp, which bind hemoglobin (Kostakioti & Stathopoulos, 2004; Otto et al., 2005). Hemolysins, which are expressed in iron-limiting conditions, have also been studied in Vibrio species (Nishina et al., 1992; Wong & Shyu, 1994). A unique group of proteases that do not share homology to a known hemoprotein protease is found in Porphyromonas gingivalis. Named gingipains, these proteases are involved in hemoglobin degradation for heme assimilation (Genco, 2004; NM et al., 2003).

The predominant mechanism of heme acquisition in Gram-negative bacteria consists of outer membrane receptors that directly bind and extract heme or iron from
host proteins and deliver it across the outer membrane (Braun et al., 1998; Braun & Killmann, 1999; Genco & Dixon, 2001; Wandersman & Stojiljkovic, 2000; Wandersman & Delepelaire, 2004; Wooldridge & Williams, 1993). Numerous outer membrane receptors for heme and heme complexes have been described (Perkins-Balding, 2004). They all share overall homology and conserved sequence motifs. These receptors have a β-barrel fold and usually recognize multiple types of hemoproteins. The mechanism by which heme is extracted and transported across the outer membrane into the periplasm is not well understood. It is known, however, that the energy for this process is provided by the TonB-ExbBD complex, which uses the proton motive force of the cytoplasmic membrane (Postle & Kadner, 2003).

The transit of heme across the cytoplasmic membrane requires the participation of ATP binding cassette (ABC) transporters (appendix A.3). These transporters are typically composed of a periplasmic binding protein, a hydrophobic membrane protein, and an ATPase (Higgins, 2001; Nikaido & Hall, 1998). Heme transporters belong to a family of ABC transporters that mediate uptake of iron complexes such as heme, siderophores, ferric citrate, and cobalamin (vitamin B₁₂) (Koster, 2001). Once in the cytoplasm, the heme may be directly incorporated into heme-proteins, or the iron can be extracted by an oxygenase homolog or heme-degrading enzyme (Schmitt, 1997b; Wegele et al., 2004; Wilks & Schmitt, 1998; Wu et al., 2005).

**Mechanisms of Iron Uptake from the Host by Gram-Positive Pathogens**

Only a few systems of iron acquisition have been described in Gram-positive bacteria. As in Gram-negatives, Gram-positive pathogens use both surface receptors and
siderophores to obtain ferric iron from the host environment. In the absence of the outer membrane, the principal machinery involved in the uptake of free or complexed iron is ABC transporters (Brown & Holden, 2002). Heme uptake seems to be mediated only by receptor-dependent pathways, as the production of hemophores has not been reported (Wandersman & Delepelaire, 2004). The HmuTUV ABC-type transporter in *C. diphtheriae* was the first heme transporter described in the Gram-positive group. It is homologous to the heme transporter HemTUV found in *Yersinia enterocolitica* (Stojiljkovic & Hantke, 1994). Heme and hemoglobin utilization mutants in *C. ulcerans* can be complemented with *C. diphtheriae hmuTUV*. HmuT, the surface associated lipoprotein, binds heme and hemoglobin *in vitro* (Drazek *et al.*, 2000; Schmitt & Drazek, 2001). An uncharacterized gene (*htaA*) located upstream of *hmuTUV* has a putative DtxR binding site in its promoter region, suggesting that it is negatively regulated by iron. It is hypothesized that *htaA* encodes for a surface or secreted protein that may be part of the *hmu* operon (Schmitt, 2004). In addition, the heme oxygenase HmuO is proposed to function as the enzyme that degrades heme once it has been transported into the cytoplasm by the HmuTUV transporter. The expression of *hmuO* in *C. diphtheriae* and *C. ulcerans* is repressed by iron and induced by heme (Schmitt, 1999).

In *S. pneumoniae* three operons encoding for ABC type transporters with homology to known iron transporters have been identified: *piuBCDA* (formerly *pit1A*), *piaABCD* (formerly *pit2A*), and *pitABCD* (Brown & Holden, 2002). Mutagenesis studies demonstrated that all three transporters are involved in iron acquisition (Brown *et al.*, 2002). A mutation in either *pia* or *piu* results in a growth defect which can be partially restored with the addition of hemoglobin but not of lactoferrin or transferrin.
Simultaneous inactivation of both transporters results in the inability to use hemoglobin. The gene products of \textit{piaABCD} probably play a predominant role in iron utilization since a \textit{pia} mutant is attenuated for virulence in animal models, whereas the \textit{piu} and \textit{pit} mutants show little or no change in virulence, depending on the route of infection (Brown \textit{et al.}, 2001a; Brown \textit{et al.}, 2002).

\textit{Staphylococcus aureus} utilizes several siderophore uptake mechanisms like the SstABCD (Morrissette \textit{et al.}, 2000), SirABC (Heinrichs \textit{et al.}, 1999), and FhuCBG (Sebulsky \textit{et al.}, 2000) systems. Recent studies show that \textit{S. aureus} can use siderophores to obtain iron from transferrin (Park \textit{et al.}, 2005). Using isotope labeled heme and transferrin, Skaar \textit{et al.} demonstrated that \textit{S. aureus} prefers heme over transferrin as an iron source. Therefore it is proposed that \textit{S. aureus} gives preference to hemoproteins because they are more abundant during the early stages of infection (Skaar \textit{et al.}, 2004). \textit{S. aureus} encodes for the HtsABC transporter (Skaar \textit{et al.}, 2004), a homolog of the \textit{C. diphtheriae} HmuTUV (Drazek \textit{et al.}, 2000) and the \textit{Y. enterocolitica} HemTUV systems (Stojiljkovic \& Hantke, 1994). Expression of HtsABC is regulated by iron via Fur and its inactivation reduces heme uptake in \textit{S. aureus}. A mutation in \textit{htsB} or \textit{htsC} reduces abscess formation in a mice animal model and decreases the number of staphylococci recovered from internal organs. A second staphylococcal transporter, IsdDEF, aided by the surface proteins IsdA, IsdB, and IsdC, is responsible for hemoprotein binding, removal of the heme molecule, and the transport of heme across the cell membrane (Mazmanian \textit{et al.}, 2003; Skaar \& Schneewind, 2004).
Iron Dependent Regulation

Whereas iron is an essential cofactor in microbial metabolism, it is also a catalyst for the synthesis of reactive oxygen species (ROS) from hydrogen peroxide, a process known as the Fenton reaction (Imlay et al., 1988). ROS are deleterious to bacteria, damaging lipids, nucleic acids and proteins. Therefore, bacteria have evolved elaborate mechanisms to capture iron for growth, while maintaining a carefully regulated iron homeostasis.

The transcriptional regulators Fur and DtxR regulate iron uptake in response to iron availability. These proteins function mostly as repressors which, when bound to iron, have an increased affinity for a conserved sequence within the promoter region of the regulated genes, blocking their transcription (Andrews et al., 2003; Braun, 1997; Braun, 2001; Escolar et al., 1999; Hantke, 2001; Tao et al., 1994). Homologs of each of these global regulators make up two large families of metal dependent regulators found in both Gram-positive and Gram-negative bacteria.

Iron Utilization and Regulation in *Streptococcus pyogenes*

Very little is known about the role that iron plays in the physiology and virulence of *S. pyogenes*. It has been demonstrated that *S. pyogenes* requires iron for growth. Hemoglobin and other hemoproteins but not transferrin or lactoferrin support *S. pyogenes* growth in iron-depleted medium (Eichenbaum et al., 1996; Francis et al., 1985; Podbielski et al., 1999). In addition, chemical assays and bioassays have failed to detect production of siderophores by *S. pyogenes* and a survey of the genome sequence does not identify any genes encoding putative siderophore biosynthetic enzymes. Therefore, we
infer that heme-bound proteins are the primary iron source for *S. pyogenes* during infection of the human host.

The *S. pyogenes* genome encodes for a multi-metal transporter named *mtsABC* (Janulczyk *et al.*, 1999) which is involved in manganese and ferric uptake (Janulczyk *et al.*, 2003). MtsA, the surface lipoprotein, binds iron, zinc, and manganese *in vitro* (Janulczyk *et al.*, 1999). Two other transporters homologous to systems involved in the utilization of iron-complexes, *siuADBG* (Streptococcal Iron Uptake) and *siaABC* (Streptococcal Iron Acquisition) remain uncharacterized (appendix A).

PerR, a Fur homolog, was identified in *S. pyogenes*. A *perR* mutant is hyper-resistant to hydrogen peroxide and more sensitive to the superoxide anion, demonstrating that PerR is essential for resistance to oxidative stress. The transcription of the sole superoxide dismutase is significantly reduced in the *perR* mutant. This could explain the increased sensitivity of *S. pyogenes* to superoxide (Brenot *et al.*, 2005; Ricci *et al.*, 2002). In addition, MrgA, a Dps-like peroxide resistance protein, is repressed by PerR and a PerR binding site is found in its promoter region (Brenot *et al.*, 2005). PerR also contributes to iron homeostasis and virulence in *S. pyogenes*. A *perR* mutant accumulates almost 50% less iron than its parent strain and the transcript of MtsA is lower in this strain than in the wild-type (Ricci *et al.*, 2002). Inactivation of *perR* significantly attenuates *S. pyogenes* virulence in murine models (Brenot *et al.*, 2005; Ricci *et al.*, 2002).

A DtxR homolog, *mtsR*, is encoded in the genome of *S. pyogenes* as well, and like PerR, it is directly involved in iron regulation and pathogenesis. MtsR binds the promoter region of *siaABC* in an iron and manganese dependent manner. Inactivation of
mtsR results in constitutive expression of the sia operon, an increase in cellular iron, and the attenuation of S. pyogenes virulence when tested in a zebrafish infection model (Bates et al., 2005).

This dissertation explores the mechanisms of iron acquisition in S. pyogenes. Chapter 1 (Montanez et al., 2005) describes SiuADBG, an uncharacterized iron transporter. Using cell growth and iron uptake assays I established that this transporter contributes to hemoprotein utilization by S. pyogenes. I also demonstrated the importance of SiuADBG in streptococcal virulence in zebrafish. Studies on a second heme transporter, SiaABC, are presented in chapter 2 (manuscript in writing). Here I report that inactivation of a siaB mutation reduces the ability of S. pyogenes to utilize hemoglobin, but that iron transport is less affected than in a siu mutant. I also studied the effect of this mutation in the ability to cause disease in zebrafish. Inactivation of siaB resulted in a delayed immune response but caused the same mortality of fish as the wild-type. Furthermore, examination of tissue infected with the siaB mutant revealed an abnormal immune response not detected in the wild-type. Chapter 3 (Bates et al., 2003) evaluates the role of the SiaA as a receptor protein in iron transport. I cloned, expressed, and purified SiaA and found that it binds heme and hemoglobin in vitro. I also demonstrated that its expression is regulated by the availability of iron. Based on these studies we propose a model for iron acquisition in which all three ABC-type transporters of iron encoded in the S. pyogenes genome contribute to the acquisition of iron from the available iron sources, giving a survival advantage to this important pathogen.
CHAPTER I

The streptococcal iron uptake (Siu) transporter is required for iron uptake and virulence in a zebrafish infection model

INTRODUCTION

Pathogenic bacteria use a variety of high-affinity iron scavenging systems to compete for iron while colonizing the human body, as the vast majority of the iron in mammals is tightly bound to host proteins. Most of the intracellular iron is stored in ferritin or found in the form of heme complexes such as hemoglobin and myoglobin. In the body fluids hemoglobin is bound to haptoglobin and heme is carried by hemopexin or serum albumin (Genco & Dixon, 2001; Wandersman & Stojiljkovic, 2000), while ferric iron is sequestered by transferrin and lactoferrin (Wooldridge & Williams, 1993). The redundancy in iron uptake pathways frequently displayed by individual pathogens underscores their importance to the infection process. Accordingly, mutants in one or more of these pathways are often attenuated for virulence (Brown et al., 2001a; Henderson & Payne, 1994; Ratledge, 2004; Stojiljkovic et al., 1995; Torres et al., 2001). Despite its apparent role in bacterial virulence, iron acquisition is only partially understood in Gram-positive pathogens.

The acquisition of iron from host proteins has been studied in a handful of Gram-positive microbes. Some species employ surface receptors for host proteins like transferrin or lactoferrin (Hartford et al., 1993; Modun et al., 1998) or siderophores to obtain ferric iron (Coulanges et al., 1998; Courcol et al., 1997; De Voss et al., 1999; Russell et al., 1984; Sebulsky & Heinrichs, 2001). The use of heme and host hemoproteins has been demonstrated in Corynebacterium diphtheriae (Schmitt, 1997a;
Schmitt, 1999), *Staphylococcus aureus* (Mazmanian *et al.*, 2003), and several streptococci (Bates *et al.*, 2003; Brown *et al.*, 2001a; Eichenbaum *et al.*, 1996; Francis *et al.*, 1985; Podbielski *et al.*, 1999). Heme uptake in Gram-positive organisms seems to be mediated by dedicated surface receptors for heme or hemoproteins, while the production of hemophores (secreted heme-binding proteins found in several Gram-negative bacteria) has not been reported (Wandersman & Delepelaire, 2004).

The principal machinery involved in the uptake of free or complex iron in Gram-positive bacteria is ABC transporters, which consist of a substrate-binding lipoprotein, one or two membrane permease subunits, and a hydrophilic ATPase (Brown & Holden, 2002; Gilson *et al.*, 1988; Higgins, 1992; Wandersman & Stojiljkovic, 2000). Heme and siderophore transporters share significant homology and belong to a defined cluster of ABC transporters. In addition, Gram-positive pathogens carry ABC metal transporters, which are part of a separate cluster of transporters and have affinity for multiple metals (Brown & Holden, 2002; Claverys, 2001). In some of these multi-metal transporters, the metal binding receptors function as bacterial adhesins as well (Dintilhac *et al.*, 1997; Elsner *et al.*, 2002; Oligino & Fives-Taylor, 1993; Spellerberg *et al.*, 1999).

*Streptococcus pyogenes* is a hemolytic pathogen capable of producing a diverse array of skin and mucous membrane infections as well as aggressive deep tissue diseases and streptococcal toxic shock syndrome. Untreated streptococcal infections can lead to the serious complications of rheumatic fever and acute glomerulonephritis (Bisno *et al.*, 2003; Cunningham, 2000). Under laboratory conditions *S. pyogenes* can use heme and a variety of hemoproteins such as hemoglobin-haptoglobin, hemoglobin, myoglobin, heme-albumin and catalase as a source of iron, but it cannot use transferrin or lactoferrin.
S. pyogenes possesses a multi-metal transporter encoded by mts (Janulczyk et al., 1999) and two transporters from the iron complex family: sia (streptococcal iron acquisition; (Bates et al., 2003) or hts (Lei et al., 2003) and a transporter which we name here siu (streptococcal iron uptake). The siaABC genes were suggested to function as a heme transporter, and SiaA (or HtsA), the binding protein homolog, was shown to bind hemoglobin and heme (Bates et al., 2003; Lei et al., 2003). On the other hand, the mts transporter is involved in uptake of manganese and ferric iron (Janulczyk et al., 2003), where MtsA binds iron, zinc and manganese in vitro (Janulczyk et al., 1999). The ligand and the function of the siu transporter have not yet been defined. In this study, we investigated the role of the siu transporter in iron acquisition and disease production.

**METHODS**

**Bacterial strains and growth assay conditions.** Escherichia coli DH5α was used for gene cloning. E. coli cells were grown aerobically in Luria–Bertani broth. S. pyogenes NZ131 (M49 type; (Nordstrand et al., 1998) and the mutant strains were grown statically at 37 °C in Todd–Hewitt (Difco or Oxoid) broth with 0.2 % (w/v) yeast extract (THY), in Todd–Hewitt broth buffered with 10 mM Tris and adjusted to pH 6.9 prior to autoclaving (ZTH), or in ZTH medium containing 12 mM nitrilotriacetic acid (NTA). NTA is a metal chelator with high specificity for iron; its first stability constants (log K₁) for Fe³⁺ and Fe²⁺ are 15.87 and 8.83 respectively. Since NTA has affinity for zinc, manganese, calcium and magnesium (log K₁ of 10.45, 7.44, 6.41 and 5.4 respectively), 0.66 mM of
these cations was added to NTA-containing media (Eichenbaum et al., 1996). ZTH was inoculated with a THY overnight culture at a 1/300 dilution. Iron was added to ZTH-NTA in the form of 3 mM ferric chloride (FeCl₃), 0.13% (v/v) sheep’s blood (Colorado Serum), 0.13% (v/v) horse serum (Sigma) or 12 µM human hemoglobin (Sigma). Human hemoglobin was prepared as a 10 mg ml⁻¹ stock solution in phosphate-buffered saline (PBS; pH 7.4).

Todd–Hewitt broth (no yeast extract, TH) was also treated for 3 or 20 h with 5% (w/v) Chelex-100 (Bio-Rad). The pH of the resin-treated medium was adjusted to 7.65, autoclaved, and 0.5 mM CaCl₂ and 0.9 mM MgSO₄ were added before inoculation. Inductivity-coupled plasma mass spectrometry (ICP-MS) analysis (at the Laboratory for Environmental Analysis, University of Georgia at Athens) demonstrated that TH contains about 17.5 ± 6.5 µM iron, 0.53 ± 0.2 µM manganese and 15.5 ± 0.2 µM zinc, depending on the batch and manufacturer. Chelex-100 treatment for 3 h resulted in 2.7 µM iron, less than 0.18 µM manganese and about 0.3 µM zinc in the medium. Treatment with Chelex-100 for 20 h did not significantly change its iron content in comparison to 3 h of treatment.

S. pyogenes was also grown in a chemically defined medium (CDM) (Podbielski et al., 1999; van de Rijn & Kessler, 1980). CDM was also treated with 3 % (w/v) Chelex-100 for 6 h and filter-sterilized (CxCDM). ICP-MS analysis showed that CxCDM contains 1.6 µM iron and 0.43 µM zinc; the manganese concentration is below the detection level. For cell growth, CxCDM was supplemented with 33 µM MgCl₂ and 68 µM CaCl₂. All experiments done with S. pyogenes cells growing in CDM or CxCDM were inoculated using mid exponential-phase cells, which were prepared as follows: cells
cultured in ZTH medium were harvested at the exponential phase (OD$_{600}$ 0.6), washed twice with PBS, and stored in small frozen aliquots in 16% (v/v) glycerol. All glassware used for streptococcal growth was soaked for 30 min in a chromic/sulfuric acid solution (Fisher Scientific) and rinsed with double-distilled water (ddH$_2$O). When necessary, the antibiotics spectinomycin and erythromycin were used for *E. coli* at 100 µg ml$^{-1}$ and 500 µg ml$^{-1}$, respectively. For *S. pyogenes*, spectinomycin and erythromycin were used at 100 µg ml$^{-1}$ and 1 µg ml$^{-1}$, respectively. Optical density was measured with a Beckman DU640 spectrophotometer (600 nm) or with a Scienceware 800-3 Klett colorimeter (640–700 transmission filter).

**Construction of strains ZE4913, ZE4914 and ZE4915.** The primers used in this study are listed in Table 1. Mutants with insertional inactivation of the *siuG* and *siaB* genes were constructed in *S. pyogenes* NZ131 (M49 type) using primers designed according to the *S. pyogenes* SF370 genome database (Ferretti *et al.*, 2001) (NCBI and TIGR complete genome databases). All of the constructed chromosomal mutations were verified by PCR analysis. The *siuG* mutant (ZE4915) was constructed by amplifying a 2.9 kb fragment from NZ131 chromosomal DNA using primers fhuX-S and fhuX-A. The PCR fragment, which included the 3’-end of *siuB*, the entire *siuG* gene, and a region downstream of *siuG*, was digested with *Aat*II and *Sal*I and ligated into pBR322, generating the plasmid pSaAa. The *ermAM* gene (erythromycin resistance) from pFW15 (Podbielski *et al.*, 1996) was amplified using primers *erm*-S and *erm*-A and cloned into the *Eco*RI site of the *siuG* gene in pSaAa, generating pSaAaerm. A fragment containing the *siuG::ermAM* allele and flanking region was released by *Aat*II/*Sal*I digestion and electroporated into *S.*
TABLE 1.1 Oligonucleotide primer sets

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<tr>
<th>locus</th>
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<th>primer sequence</th>
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<tr>
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<td>erm-S</td>
<td>5’-GGGAATTCTAATATATATAAATA-3’</td>
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<tr>
<td></td>
<td>erm-A</td>
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<tr>
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</tr>
<tr>
<td>siaB</td>
<td>Stoj5</td>
<td>5’-GGTTGCTGTGTGGATATCTGTGAC-3’</td>
</tr>
<tr>
<td></td>
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<td>5’-GTCATGTAATACTAACACATCGTGA-3’</td>
</tr>
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<tr>
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<tr>
<td></td>
<td>204C-R</td>
<td>5’-CTGATGCTACTGCATAGCAG-3’</td>
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*Streptococcus pyogenes* NZ131. Allelic replacement clones were selected on THY agar plates containing erythromycin. A *siaG siaB* double mutant (ZE4914) was constructed by introducing a disrupted *siaB* copy in the background of ZE4915. For this purpose, a 2 kb fragment including the 3’-end of *siaA*, the entire *siaB* gene and the 5’-end of *siaC* was amplified from NZ131 chromosomal DNA using primers stoj5 and stoj6. The *siaB* PCR product was cloned into the *XmnI* site of pACYC184, producing plasmid pStoj3. The *aad9* gene (spectinomycin resistance) was amplified from pUCSpec (Husmann et al., 1997) using spc-S and spc-A primers and cloned into the *BclI* site of *siaB*, generating plasmid p5spc1. The fragment containing the *siaB::aad9* allele and flanking chromosomal regions was released by *XmnI/Stul* digestion and electroporated into the ZE4915 strain. Allelic replacement mutants were selected on THY agar containing erythromycin and spectinomycin. The construction of the *siaB* mutant (ZE4913) was the same as the construction of ZE4914, except that the *siaB::aad9* allele was introduced into the wild-type NZ131 strain.

**RNA analysis of *Streptococcus pyogenes* NZ131 grown in chemically defined medium.** *S. pyogenes* cells were used to inoculate 50 ml CxCDM and CxCDM supplemented with 20 µM FeCl$_3$ or with 33 µM MnSO$_4$ in side-arm (Klett) flasks at 37 °C. Cells were harvested at late exponential phase (60 Klett units) and RNA was isolated using RiboPure-Bacteria (Ambion), following the manufacturer’s recommendations. Reverse transcriptase (RT) reactions were done using Superscript III reverse transcriptase, following the manufacturer’s protocol (Invitrogen). Approximately 50 ng of the cDNA was used as the template for each PCR reaction (25 cycles with Taq
polymerase, Roche). Gene-specific primer sets listed in Table 1 were used in the RT and PCR reactions at a concentration of 4 µM and 30 µM, respectively.

**Ferric iron uptake assays.** Iron uptake assays were essentially performed as previously described, with small modifications (Brown *et al.*, 2001a; Janulczyk *et al.*, 2003). CxCDM was inoculated with *S. pyogenes* cells (1/250) and the cultures were grown to mid-exponential phase (35 Klett units) at 37 °C. $^{55}$FeCl$_3$ (0.2 µCi ml$^{-1}$, 7.4 kBq µl$^{-1}$; 0.02 µM) was added to 1 ml cultures and incubated at 37 °C. Culture samples (200 µl) were drawn every 30 min and washed twice with 500 µl CxCDM containing 10 mM NTA. The radioactivity associated with the cell pellet and the supernatant was measured as counts per minute (c.p.m.) for 5 min against a $^3$H standard using a Beckman LS6500 scintillation counter. The culture’s OD$_{600}$ was measured at the same time using a Beckman DU640 spectrophotometer. $^{55}$Fe incorporation for each time point was standardized for the cell quantity by dividing the c.p.m. by the culture OD$_{600}$. Competition assays with iron and manganese were performed as above except that increasing concentrations of nonradioactive FeCl$_3$ or MnSO$_4$ were provided in addition to $^{55}$FeCl$_3$. Culture samples were drawn after about 60 min, washed twice, and their radioactivity was measured. For the inhibition of ferric iron uptake, increasing concentrations of heme or protoporphyrin IX were added with the $^{55}$FeCl$_3$. Samples were taken after 60 min (OD$_{600}$ about 1), washed, and the radioactivity measured. $^{55}$Fe incorporation was defined as the fraction of c.p.m. of the pellet divided by the sum of the c.p.m. in pellet and the supernatant. Heme was prepared as a 10 mg ml$^{-1}$ stock solution of hemin chloride (Sigma) in 0.1 M NaOH pH 10. Protoporphyrin IX was prepared as a
10 mg ml\(^{-1}\) stock solution dissolved in 1:1 dimethyl formamide/methanol. As a control, a 10 mg ml\(^{-1}\) heme solution was treated with 5 % (w/v) Chelex-100 for 1 h prior to adding it to the cells to remove any unbound iron. When necessary, dilutions were prepared in ddH\(_2\)O.

**Zebrafish care and virulence assays.** Care and feeding of zebrafish (*Danio rerio*) followed published methods (Neely *et al.*, 2002; Westerfield, 1995). Streptococci were cultured overnight in THY plus 2 % (w/v) peptone (THYP) at 37 °C, diluted 1:100 the next day in THYP, and incubated at 37 °C. The cells were harvested at OD\(_{600}\) 0.3, washed once with THYP, and diluted to the appropriate concentration in fresh THYP. Injection of zebrafish followed a previously described method (Neely *et al.*, 2002). Briefly, streptococcal cells (10 µl of 10\(^5\) ml\(^{-1}\)) were aseptically injected into groups of four to six anaesthetized male breeder zebrafish (Scientific Hatcheries). Following intraperitoneal (i.p.) or intramuscular (i.m.) injection, the fish were allowed to recover in 225 ml sterilized ddH\(_2\)O supplemented with aquarium salts (Instant Ocean; Aquarium Systems) at a concentration of 60 mg l\(^{-1}\) in a 29 °C incubator. A control animal group was injected with sterile medium. Infected fish were monitored for 48 h and death recorded in intervals of 12 h. For *S. pyogenes*, the 50 % lethal dose (LD\(_{50}\)) for infection of zebrafish was determined by the method of Neely *et al.* (2002), where zebrafish were challenged over a range of 10\(^1\) – 10\(^6\) c.f.u. of each streptococcal strain.

**Zebrafish tissue analysis.** Selected whole zebrafish were fixed following euthanasia at 40 h after infection and 5 µm thick longitudinal sections of the dorsal muscle were
prepared for staining as described previously (Neely et al., 2002). Fixed samples were stained with hematoxylin and eosin and examined with an Olympus BX60 microscope equipped with a digital camera and a motorized stage.

**Statistics and data analysis.** Statistical significance was determined by using the two-sample Student t-test. The standard error of the mean (SEM) was calculated by dividing the standard deviation by the square root of \( n \).

**RESULTS AND DISCUSSION**

**The streptococcal iron uptake (siu) transporter**

Sequence analysis of the M1 SF370 *S. pyogenes* genome identified two ABC transporters that belong to the family of iron-complex transporters. One of these streptococcal systems, *sia* (streptococcal iron acquisition), was previously demonstrated to be involved in hemoglobin binding and acquisition of iron (Bates et al., 2003; Lei et al., 2003). Here we characterize the second iron-complex transporter, which is currently annotated as *fhuADB.1G* (*Spy0383-0386*) due to the homology to the corresponding genes of the *Bacillus subtilis* ferrichrome uptake system. We renamed this transporter *siu* (streptococcal iron uptake), since we found it to be involved in iron uptake. The *siu* transporter is conserved in all of the published *S. pyogenes* genomes (five complete and two unfinished genomes). It consists of four genes encoding an ATP-binding protein (*siuA*), a substrate binding protein with a lipoprotein signal (*siuD*), and two subunits of a membrane permease (*siuB* and *siuG*). The *siuABDG* genes constitute an operon that is
induced by depletion of iron and other metals (Lei et al., 2003; Smoot et al., 2001). Sequence alignments show that proteins in the Siu system have homology with siderophore uptake systems such as the *S. aureus* SirABC (Heinrichs et al., 1999) and SstABCD (Morrissey et al., 2000) and with transporters involved in heme and hemoproteins utilization such as the staphylococcal HtsABC (Skaar et al., 2004) and IsdDEF (Mazmanian et al., 2003), the pneumococcal PiaABCD and PiuBCDA (Brown et al., 2001a), and the streptococcal SiaABC (Bates et al., 2003).

**The siu transporter is involved in iron acquisition**

The iron needs of *S. pyogenes* NZ131 were investigated in various media. NZ131 grew in TH medium treated with the chelating resin Chelex-100 or in CxCDM (see Methods), demonstrating that it can proliferate in media containing only 1.6 µM iron and trace amounts of manganese and zinc. Similar observations were made in THY medium that was treated with Chelex-100 (Janulczyk et al., 2003; Ricci et al., 2002). RNA analysis showed that growth in CxCDM allows significant expression of both the *siu* and the *sia* transporters (appendix B.1) are both negatively regulated by iron (Bates et al., 2003; Lei et al., 2003; Smoot et al., 2001). The addition of 20 µM iron (but not of 20 µM manganese) repressed *siu* expression, confirming that while the iron concentration found in this medium is sufficient to support growth, it is low enough to produce an iron-stress signal. Growth of NZ131 was significantly impaired in a buffered Todd–Hewitt broth containing 12 mM NTA (ZTH-NTA) that was supplemented with a mix of the bivalent metals calcium, magnesium, manganese and zinc as described by Bates et al. (2003)
(black bar, NTA in Fig. 1.1). Similarly, 20 mM NTA was previously used to restrict the growth of a second M49 strain, CS101, in THY (Podbielski et al., 1999).

Growth of NZ131 in the NTA-containing medium was significantly enhanced by the addition of different iron sources. The addition of 3 mM ferric chloride, 0.13 % whole blood or 12 µM hemoglobin to ZTH-NTA medium enhanced cell growth up to 86 %, 76 % and 66 %, respectively, of the growth seen in ZTH. On the other hand, the addition of 0.13 % serum resulted in only a small increase of cell growth (32% of the growth seen in ZTH), indicating that the iron available for the bacterium in serum is low and growth limiting (Fig. 1.1). These observations suggest that restricted iron availability plays a major role in the restriction of *S. pyogenes* growth in the ZTH-NTA medium. It is possible that this medium limits the availability of other metals as well, since ferric chloride and hemoglobin could not completely restore growth. A mutation in *siuG*, one of the membrane permease genes, was constructed in *S. pyogenes* NZ131 by the insertional inactivation method using an *ermAM* cassette (strain ZE4915). The construction of the mutant was verified by PCR analysis. In agreement with the presence of the *siuG*::*ermAM* allele, the PCR product obtained from the *siuG* mutant was 1.2 kb larger than that obtained from the wild-type strain (appendix B.2). Growth of the *siuG* mutant (grey bars in Fig. 1.1) was compared to that of the parent strain NZ131. The addition of ferric chloride to the iron-depleted medium enhanced the growth of *siuG* mutant to a level slightly lower than that of the wild-type (70% vs 86% of that observed in ZTH). On the other hand, growth of the *siuG* mutant was significantly reduced in ZTH-NTA medium containing whole blood or hemoglobin, to only 34% and 21 respectively, of the wild-type level. The most significant effect of *siuG* inactivation was
Fig. 1.1 Growth analysis of the wild-type and the siuG mutant in iron-depleted media supplemented with various iron sources. *S. pyogenes* wild-type (NZ131, black bars) and siuG (ZE4915, grey bars) cells were grown in iron-depleted medium, ZTH-NTA (ZTH with 12 mM NTA, 0.66 mM MgCl₂, MnCl₂, CaCl₂ and ZnCl₂), or in iron-depleted medium supplemented with 3 mM ferric chloride, 0.13 % sheep’s blood, 12 µM human hemoglobin or 0.13 % horse serum. The culture’s OD₆₀₀ was determined following overnight incubation. Results are shown as percentage growth with respect to that obtained in complete medium (ZTH). Data represent the mean of at least three experiments. Error bars represent SEM; significant *P* values (< 0.05) are indicated by *. 
on the ability of the bacteria to grow in ZTH-NTA medium supplemented with serum, where only 3% of the wild-type level of growth was obtained. Since *S. pyogenes* cannot use transferrin as an iron source, the Siu transporter may contribute to the use of heme bound to serum albumin or hemopexin. While the growth phenotype of the *siuG* mutant is partial, it establishes the role of the Siu transporter in iron acquisition. Redundancy in iron uptake pathways in *S. pyogenes* is probably a major factor in the partial growth phenotype. However, it is also possible that the *siuG* mutation allows residual transporter activity (with the remaining membrane permease subunit, *siuB*), and therefore may hinder the full appreciation of this transporter’s role in iron acquisition.

**Inactivation of the *siu* transporter results in decreased Fe$^{3+}$ utilization**

The presence of NTA in the ZTH-NTA medium complicates the study of the cell’s use of ferric iron. Therefore, we investigated Fe$^{3+}$ utilization by the wild-type and the *siuG* mutant using a $^{55}$Fe$^{3+}$ uptake assay in a low-iron medium that does not contain a chelator. $^{55}$FeCl$_3$ was added to cells growing in CxCDM at the early exponential phase and incorporation by the cells was monitored every 30 min. Iron accumulation by both the wild-type and the *siuG* mutant cells increased over time (Fig. 1.2), with maximum incorporation by wild-type cells observed after about 30 min incubation. The addition of 2 µM non-radioactive iron ($^{56}$Fe$^{3+}$) or manganese ($^{54}$Mn$^{2+}$) inhibited $^{55}$Fe$^{3+}$ incorporation into NZ131 cells by 30% and 75%, respectively. Inhibition reached 62% and 80% with 6 µM iron or manganese (appendix B.3). Inhibition of Fe$^{3+}$ uptake by manganese suggests that at least some of the Fe$^{3+}$ uptake in NZ131 is mediated by a multi-metal transporter such as the *mts* transporter (PCR confirmed the presence of *mts* transcript).
Fig. 1.2 Incorporation of radioactive ferric iron by the wild-type and the siuG mutant. $^{55}$FeCl$_3$ was added to *S. pyogenes* wild-type (NZ131, ▲) and siuG (ZE4915, ●) cultures at the early exponential phase grown in CxCDM. Cells were harvested every 30 min, washed, and the radioactivity associated with the cell pellet was measured. Results are expressed as c.p.m. divided by the OD$_{600}$ of the culture. Data points represent the mean of three experiments done in duplicate; error bars represent SEM.
in the NZ131 strain; Bates et al, 2005). Similarly, manganese could compete with Fe$^{3+}$ uptake in the AP1 (M1 type) strain, in which inactivation of mts reduced accumulation of both $^{54}$Mn$^{2+}$ and $^{55}$Fe$^{3+}$ (Janulczyk et al., 2003).

Comparison between the wild-type and the siuG mutant revealed that the rate of Fe$^{3+}$ uptake was reduced in the mutant, in which maximum incorporation was observed only after 60 min incubation and reached about 70 % of that observed in the wild-type. Therefore, the siu transporter is involved in Fe$^{3+}$ uptake, although to a lesser extent than the mts transporter, inactivation of which resulted in a 90 % decrease of Fe$^{3+}$ accumulation (Janulczyk et al., 2003). Similarly to siu, contribution both to Fe$^{3+}$ uptake and to the cell’s ability to grow in iron-restricted medium containing hemoglobin was also demonstrated by the homologous piu and pia transporters in Streptococcus pneumoniae (Brown et al., 2001a).

**Heme inhibits Fe$^{3+}$ accumulation by Streptococcus pyogenes**

We studied the effect of heme present in the growth medium on Fe$^{3+}$ accumulation by *S. pyogenes* NZ131. Heme at a concentration as low as 0.75 µM inhibits the accumulation of $^{55}$Fe$^{3+}$ by 55 % as compared to the uptake in the absence of heme, and the addition of 6 µM heme results in about 76 % inhibition (Fig. 1.3a, black bars). Treatment of heme with Chelex-100 to remove free iron possibly present in the solution did not change the percentage inhibition of $^{55}$Fe$^{3+}$ uptake by heme (Fig. 1.3a, white bar). This is consistent with the proposal that it is the heme, and not free iron, that inhibits $^{55}$Fe$^{3+}$ uptake. On the other hand, the addition of protoporphyrin IX, the core structure of heme did not significantly interfere with $^{55}$Fe$^{3+}$ incorporation, even at a concentration as high as 6 µM
Fig. 1.3  Inhibition of $^{55}$Fe incorporation by heme. $^{55}$FeCl$_3$ was added to S. pyogenes cultures grown in CxCDM at the early exponential phase. (a) $^{55}$Fe accumulation by S. pyogenes wild-type (NZ131) in the presence of 6 µM Chelex treated heme (white bar), or increasing concentrations of heme (black bars), or protoporphyrin IX (grey bars). (b) $^{55}$Fe accumulation by the wild-type (NZ131, black bars), the siuG mutant (ZE4915, white bars), the siaB mutant (ZE4913, hatched bars) and the siuG siaB mutant (ZE4914, grey bars) in the presence of increasing concentrations of heme. $^{55}$Fe uptake was calculated as incorporation in the presence of heme or protoporphyrin IX as a percentage of the incorporation without the inhibitors. Fe$^{3+}$ accumulation for each strain was calculated as incorporation in the presence of heme as a percentage of the incorporation in the same strain in the absence of heme. Data represent the mean of at least four experiments; error bars represent SEM.
Since iron is important for the ability of heme to hinder ferric transport, we suggest that this inhibition is not the outcome of non-specific interference of the heme moiety, but that the use of heme as an iron source leads to repression of the Fe\(^{3+}\) uptake pathways. Heme may also compete with ferric iron for some of the transporters that contribute to Fe\(^{3+}\) uptake.

Since we found that heme utilization reduced the accumulation of \(^{55}\)Fe\(^{3+}\) by the wild-type cells, we asked whether inactivation of \(siuG\) would interfere with this phenomenon. The ability of heme to hinder \(^{55}\)Fe\(^{3+}\) uptake in the \(siuG\) mutant was tested using a range of heme concentrations. The addition of 2 and 4 µM heme resulted in about 54 % and 76 % inhibition of \(^{55}\)Fe\(^{3+}\) uptake in the wild-type strain (Fig. 1.3b, black bars). However, the ability of heme to inhibit uptake was lessened in the \(siuG\) mutant; at 2 and 4 µM heme concentration Fe\(^{3+}\) uptake by the \(siuG\) strain was reduced only by 36 % and 60 % \((P < 0.05, n = 4)\) (Fig. 1.3b, white bars). Therefore, it seems that the \(siu\) is required for effective inhibition of \(^{55}\)Fe\(^{3+}\) transport by heme.

When a higher concentration of heme was used, \(^{55}\)Fe\(^{3+}\) accumulation by the \(siu\) mutant was inhibited to a level similar to that observed in the wild-type cells. This suggests that an additional transporter(s) is involved in heme utilization and or is affected by heme. To test this hypothesis, a mutant in \(siaB\) (the \(sia\) membrane permease component, strain ZE4913) and a \(siuG\ siaB\) double mutant (strain ZE4914) were constructed by the insertional inactivation method using an \(aad9\) cassette. The presence of the \(siaB::aad9\) allele in both mutant strains was confirmed by PCR; as expected the \(sia\) fragment amplified from the \(siaB\) and the \(siuG\ siaB\) strains was 1.25 kb larger than that produced from the wild-type strain. To test if the \(sia\) transporter contributes to the
heme effect seen in the siuG mutant, we repeated the assay with the siaB and the siuG siaB strains. Similar to the siuG mutant, 2 µM heme did not efficiently inhibit $^{55}$Fe$^{3+}$ uptake in the siaB mutant (Fig. 1.3b, hatched bars, $P < 0.025$, n = 6), while 4 µM heme led to a decrease in Fe$^{3+}$ uptake similar to that seen in the wild-type strain. Inactivation of both siaB and siuG had a cumulative effect at 4 and 6 µM heme. In the presence of 6 µM heme, only 52 % inhibition was observed in the double mutant (Fig. 1.3b, grey bar), while about 76 – 80 % inhibition was observed in the wild-type, siuG or siaB strains. While this reduction is not striking, it is statistically significant ($P < 0.005$, n = 4) when compared to the wild-type. Based on these observations we suggest that heme utilization is partially impaired if the siu transporter is disrupted and that inactivation of the sia transporter reduces heme usage by the cell even further. Additionally, the ability of heme to reduce $^{55}$Fe$^{3+}$ uptake by 52 % in the double mutant may result from the residual activity of the siu system and the presence of other heme utilization pathways. Redundancy in heme utilization pathways has been demonstrated in several Gram-positive bacteria, such as isdDEF and htsABC in S. aureus (Mazmanian et al., 2003; Skaar et al., 2004), piaABCD and piuBCDA in S. pneumoniae (Brown et al., 2001a), hmuTUV and an uncharacterized transporter in C. diphtheriae (Drazek et al., 2000; Schmitt & Drazek, 2001).

siuG is required for virulence of Streptococcus pyogenes in zebrafish

Competitive index studies showed that piaA in S. pneumoniae is important in both a pulmonary and a systemic murine model for disease (Brown et al., 2001a), and mice immunized with recombinant PiuA and PiaA are protected against systemic
pneumococcal challenge (Brown et al., 2001b). Likewise, the *S. pyogenes mtsA* and the *S. aureus hts* mutants are attenuated in animal infection models (Janulczyk et al., 2003; Skaar et al., 2004). Using a zebrafish animal model we investigated the role of *siuG* in disease progression by *S. pyogenes*. The zebrafish immune system has many similarities to the mammalian system (Postlethwait et al., 1998; Trede et al., 2001) and numerous studies have characterized its cardiovascular components (MacRae & Fishman, 2002). Recent studies established that the zebrafish is a suitable model to investigate streptococcal infections. I.p. and i.m. injection of *S. pyogenes* HSC5 (M5 type) produced lethal infections in the fish, along with hypopigmented lesions and tissue necrosis (Miller & Neely, 2004; Neely et al., 2002). We used this model to investigate the role of iron acquisition in disease production and progression by *S. pyogenes* NZ131.

When zebrafish were injected i.m. with a range of $10^1$ – $10^6$ c.f.u. of the wild-type NZ131, the dose response was similar to that reported for HSC5 (LD$_{50}$ $10^4$ cells ml$^{-1}$) (Neely et al., 2002). I.m. injection of NZ131 also produced a hypopigmented lesion with extensive muscular necrosis. A control animal group, mock injected with sterile medium, showed no signs of distress. Forty hours after injection, infected zebrafish were fixed and longitudinal sections were prepared. Staining of the tissue revealed streptococcal cells arranged in clusters at the site of infection, as well as the appearance of some host immune cells (appendix C.1). When the fish were injected i.p. with a range of $10^1$ – $10^6$ c.f.u. of the wild-type NZ131, the LD$_{50}$ was higher ($>10^5$ cells ml$^{-1}$) than when they were injected i.m. This observation is different from the observations made with the HCS5 strain, were the LD$_{50}$ in the i.p. route was lower than that in the i.m. infection (Neely et al., 2002).
We investigated the role of the *siu* transporter in virulence by comparing the mutant strain to the parent strain when injected separately by both the i.m. and i.p. routes of infection. Groups of four to six zebrafish were challenged with $10^5$ cells ml$^{-1}$ of *S. pyogenes* wild-type and the *siuG* mutant and monitored for 2 days. I.m. injection with NZ131 resulted in only 14 % survival of the fish by 48 h (Fig. 1. 4). In the *siuG* mutant the ability to cause death of the fish was significantly reduced (88 % survival, $P < 0.0115$, n = 3). I.p. injection with $10^5$ cells ml$^{-1}$ of *S. pyogenes* wild-type resulted in about 50 % increase in animal survival as compared to the i.m. injection. Still, the *siuG* was less virulent as compared to the wild-type (data not shown). These results suggest that acquisition of iron is important for *S. pyogenes* pathogenesis in the zebrafish model and that *siuG* function has an important role *in vivo* in the establishment of infection. Iron metabolism and erythroid development in zebrafish is analogous to that of higher vertebrates; zebrafish produce heme and hemoglobin, carry out hemoglobin switching during development (Brownlie *et al.*, 2003), use transferrin receptors (Wingert *et al.*, 2004) and divalent metal transporter 1 (DMT1; Donovan *et al.*, 2002) to transport iron into and within the cell’s compartments, and employ ferroprotein 1 (Fpr1) as an intestinal and macrophage iron exporter (Donovan *et al.*, 2000). Therefore, it is likely that the iron acquisition mechanisms used by *S. pyogenes* during infection of zebrafish are relevant for iron acquisition during human infection.

In conclusion, *siuADBG* is the third ABC-type iron transporter in *S. pyogenes* shown to be involved in iron uptake. The growth assays and the reduced ability of heme to decrease ferric transport by the *siuG* mutant implicated the *siu* transporter in iron acquisition from hemoglobin, heme, blood and serum. However, additional studies are
Fig. 1.4  Zebrafish survival curve following *S. pyogenes* infection. Groups of four to six zebrafish were challenged with $10^5$ c.f.u. of *S. pyogenes* wild-type (NZ131, ▲) and the *siuG* mutant (ZE4915, ●) by i.m. injection and monitored for 2 days. Data were pooled from three to five independent experiments and presented as total percentage fish survival as a function of time.
required to determine the ligand of the \textit{siu} transporter and how it contributes to the use of hemoglobin and heme.
CHAPTER II

A *Streptococcus pyogenes siaB* mutant is mildly impaired in hemoglobin utilization and causes a severe and unusual inflammatory response in zebrafish

INTRODUCTION

Heme and heme compounds represent the largest iron reservoir in the mammalian body. Accordingly, the ability to compete for heme and to use the host hemoproteins as an iron source is prevalent among pathogenic bacteria. Heme acquisition and transport are best understood in Gram-negative pathogens, while this area is only beginning to be uncovered in Gram-positive organisms. ABC-type transporters, which are composed of a substrate binding lipoprotein, one or two membrane permease subunits, and an ATP-binding protein, appear to be the central component of heme uptake in Gram-positive bacteria, which lack the outer membrane and the associated TonB-dependent receptors found in Gram-negative bacteria.

To date, heme uptake has been described in only a few Gram-positive organisms. The *Corynebacterium diphtheriae hmuTUV* system was the first heme ABC transporter to be identified in the Gram-positive group. This *C. diphtheriae* gene cluster encodes for an ABC transporter that can complement heme utilization mutants in *Corynebacterium ulcerans*. HmuT, the substrate lipoprotein, binds heme and hemoglobin *in vitro*, and an *hmuT* mutant has reduced ability to use heme and hemoglobin. Since only a reduction rather than complete loss of hemoglobin utilization was demonstrated in the *hmuT* mutant, it is suggested that other unidentified protein(s) contribute to heme utilization in
C. diphtheriae (Drazek et al., 2000; Schmitt & Drazek, 2001). The C. diphtheriae hmuO gene was the first bacterial heme oxygenase to be identified. Similar to mammalian heme oxygenases, HmuO oxidatively cleaves heme to biliverdin with the release of carbon monoxide and iron (Schmitt, 1997a; Schmitt, 1997b; Wilks & Schmitt, 1998).

Two ABC-type transporters, piuBCDA and piaABCD, are involved in hemoglobin utilization in Streptococcus pneumoniae. While a single mutation in either transporter does not have a significant effect on iron acquisition, a mutant with disrupted genes in both transporters cannot use hemoglobin as a source of iron and demonstrated a reduced rate of ferric uptake. A mutation in piaABCD, located in a pathogenicity island in the S. pneumoniae genome, results in a strain moderately attenuated for virulence. On the other hand, a double piu pia mutant strain is significantly impaired in the ability to cause disease in animal models (Brown et al., 2001a). A third ABC-type transporter pitABCD is also involved in iron acquisition and virulence in S. pneumoniae. However, the ligand of any of the pneumococcal iron transporters has not been identified yet.

Recent observations suggest that heme acquisition and transport may involve a more complex mechanism in Staphylococcus aureus, Listeria monocytogenes, and Streptococcus pyogenes, which produce surface receptors for heme and hemoproteins in conjunction with some of their iron transporters. Such receptors may function in heme capture and relay it to the other transport components. This phenomenon may be more widely spread since computational analysis has identified putative surface receptors next to iron transporters in additional Gram-positive organisms like Clostridium perfringens and Bacillus halodurans. S. aureus for example has two heme transporters, HtsABC and IsdDEF, in addition to various transporters for the utilization of siderophores and
transferrin. HtsABC appears to be encoded in the genome as a separate cluster without any other genes transcribed with it. Its inactivation reduces the ability of \textit{S. aureus} to use heme as an iron source and reduces its virulence in mice (Skaar \textit{et al.}, 2004). On the other hand, the IsdDEF transporter is accompanied by the genes \textit{isdABC}, which are located upstream and encode surface proteins that bind heme and hemoglobin (in the case of IsdB) \textit{in vitro}. While deletion of \textit{isdA} does not significantly change growth of \textit{S. aureus} in the presence of heme, a deletion of \textit{srtB}, which anchors IsdC to the cell wall, reduces heme utilization and transport across the cell envelope (Mazmanian \textit{et al.}, 2003).

\textit{Listeria monocytogenes} encodes for a transporter homologous to iron complex ABC transporters. Located in the \textit{svpA-srtB} cluster, this transporter is flanked by a sortase (\textit{srtB}) and three surface proteins with SrtB motifs. Two of these proteins \textit{svpA} and \textit{lmo2180} share homology with the staphylococcal IsdC protein. SvpA binds heme in solution; however individual deletions in \textit{lmo2186}, \textit{svpA}, or the ABC-type transporter permease do not impair growth in iron deficient medium supplemented with heme, hemoglobin, or ferrichrome. Therefore, a second transporter(s) may be involved in the utilization of these iron sources (Newton \textit{et al.}, 2005).

The hemolytic bacterium \textit{S. pyogenes} can use heme and hemoproteins from the human host to fulfill its iron requirements for growth (Eichenbaum \textit{et al.}, 1996; Francis \textit{et al.}, 1985; Podbielski \textit{et al.}, 1999). \textit{S. pyogenes} carries three ABC-type transporters involved in iron uptake. The first, encoded by \textit{mtsABC}, mediates the uptake of multiple metals including manganese and iron (Janulczyk \textit{et al.}, 1999; Janulczyk \textit{et al.}, 2003). The other two, \textit{siuADBG} and \textit{siaABC} have high homology to heme and siderophore transporters. We recently demonstrated that an \textit{siuG} mutant cannot efficiently use
hemoglobin, exhibits reduced ferric uptake, and is significantly attenuated for virulence in the zebrafish model (Montanez et al., 2005). Similarly to the staphylococcal Isd and the listerial Svp transporters, the SiaABC transporter in \textit{S. pyogenes} is part of a large gene cluster. The \textit{siaABC} genes, which code for a substrate binding protein, a membrane permease, and an ATPase, respectively, are expressed from a 10 gene operon. Two proteins, Shr and Shp, are encoded by genes located upstream of \textit{siaA}. Shr, a large hydrophilic protein that is found in the supernatant and on the surface of \textit{S. pyogenes}, binds multiple hemoproteins. Shp, a small surface protein, binds heme. The \textit{siaDEFGH} (\textit{spy1787-1791}), located downstream of \textit{siaABC}, encode for genes with homology to cysteine transporters required for cytochrome biosynthesis. The ability of SiaA to bind hemoglobin and heme is consistent with its function as a heme transporter. The recent report by Liu and Lei (2005), which shows that Shp can remove heme from hemoglobin and delivers it to SiaA, provides strong support for this proposal and indicates that at least some of the proteins from the \textit{sia} operon are involved in a multi component heme acquisition and transport system. In this work, we used \textit{siaB} and \textit{siuG siaB} mutants to illustrate the role of the \textit{siaABC} transporter in hemoprotein utilization and its importance in \textit{S. pyogenes} pathogenesis.

**METHODS**

**Bacterial strains and growth conditions.** The \textit{S. pyogenes} strains used in this study include: the M49-type NZ131 (Nordstrand et al., 1998) and its derived mutants ZE4913 (\textit{siaB::aad9}), ZE4914 (\textit{siuG::ermAM}), and ZE4915 (\textit{siaB::aad9 siuG::ermAM};
Montanez et al., 2005). Streptococcal cells were grown statically at 37 °C in Todd-Hewitt broth with 0.2 % (w/v) yeast extract (THY) and 10 mM Tris, adjusted to pH 6.9 (ZTH). ZTH was depleted of iron by the addition of 12 mM chelating agent nitrilotriacetic acid (NTA). ZTH was supplemented with 0.66 mM MgCl₂, MnCl₂, CaCl₂, and ZnCl₂ (Eichenbaum et al., 1996). Iron was provided in the form of 10 mM ferric citrate (FeC₆H₅O₇), 0.13 % (v/v) sheep’s blood (Colorado serum), 0.13 % (v/v) horse serum (Sigma) or 12 µM human hemoglobin (Sigma). Human hemoglobin was prepared as a 10 mg ml⁻¹ stock solution in phosphate-buffered saline (PBS; pH 7) and filter-sterilized. ZTH was inoculated with a THY overnight culture at a 1/300 dilution. All glassware used for streptococcal cultures were washed by soaking for 30 min in a chromic/sulfuric acid solution (Fisher Scientific) and rinsed with double-distilled water. For S. pyogenes chloramphenicol and erythromycin were used at 1 – 2 µg ml⁻¹, and spectinomycin at 100 µg ml⁻¹. Optical density was measured with a Beckman DU640 spectrophotometer (600nm) or with a Scienceware 800-3 Klett colorimeter (640-700 nm transmission filter).

**Reverse Transcriptase (RT)-PCR analyses.** S. pyogenes cells harvested from ZTH at mid-exponential phase were inoculated into Chelex-100 (BioRad) treated chemically defined medium supplemented with 33 µM MgCl₂ and 68 µM CaCl₂ (CxCDM) (Montanez et al., 2005; van de Rijn & Kessler, 1980) in the presence or absence of 20 µM FeCl₃. Total RNA was isolated using RiboPure Bacteria (Ambion) following the manufacturer’s recommendations. All gene-specific primers sets used are listed in Table 2.1. RNA, primers (2 µM), dNTPs, and distilled deionized H₂O (ddH₂O) were mixed
**TABLE 2.1.** Oligonucleotide primer sets

<table>
<thead>
<tr>
<th>locus</th>
<th>primer</th>
<th>primer sequence</th>
</tr>
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<tbody>
<tr>
<td>recA</td>
<td>sraL</td>
<td>5’-GCGTTTCAGGAGGTCTAGCTC-3’</td>
</tr>
<tr>
<td></td>
<td>sraR</td>
<td>5’-CTGATGCTACTGCCATAGCAG-3’</td>
</tr>
<tr>
<td>siaD</td>
<td>shuorf1S</td>
<td>5’- CCCGAATTTCGTCTTTTTTAATTTATCCATTGG -3’</td>
</tr>
<tr>
<td></td>
<td>shuorf1AS</td>
<td>5’- CCCGAATTCTACTCTTTGTTATCTTTTCTTGC -3’</td>
</tr>
<tr>
<td>shr</td>
<td>204yS</td>
<td>5’- TGAAGACGTTACTATTACCG -3’</td>
</tr>
<tr>
<td></td>
<td>204yAS</td>
<td>5’- TCTACCTGATAACCACCTCCA -3’</td>
</tr>
</tbody>
</table>
according to the manufacturer’s directions and incubated as follows: 98 °C (30 sec), 65 °C (5 min), 50 °C (30 sec), 4 °C (5 min). Then, as in the manufacturer’s protocols (Invitrogen), Superscript III enzyme buffer and dithiothreitol were added and incubated at 42 °C (2 min) followed by the addition of Superscript III reverse transcriptase and final incubation of 42 °C (60 min) and 70 °C (15 min). A 10\textsuperscript{th} of the RT reaction was used as the template for each Taq (Roche) DNA polymerase PCR reaction with primers at a concentration of 30 µM in 20 cycles. PCR amplification of \textit{recA} was used as a standard to ensure equal amounts of RNA template.

**Detection of SiaA in \textit{Streptococcus pyogenes}**. Total protein preparation was done based on the method of Pancholi and Fischetti (Pancholi & Fischetti, 1992). \textit{S. pyogenes} cells were harvested from 10 ml overnight cultures growing in ZTH or ZTH-NTA (10 mM) supplemented with 0.66 mM MgCl\textsubscript{2}, MnCl\textsubscript{2}, CaCl\textsubscript{2}, and ZnCl\textsubscript{2}. Equal amounts of cells taken from each culture were washed with saline, resuspended in PED (20 mM phosphate buffer, 5 mM EDTA, 10 mM dithiothreitol), and treated with 15 units of mutanolysin in the presence of a broad protease inhibitor for 60 min at 37°C. The lysates were then boiled for 10 min in the presence of Laemmli sample buffer (Laemmli, 1970), resolved on 10 % SDS-PAGE, and transferred to nitrocellulose membranes for immunobloting. For Western blot analysis, the membranes were incubated with a 1:10,000 dilution of polyclonal SiaA antiserum raised in rabbit (Bates \textit{et al.}, 2003), followed by 1:10,000 of anti-rabbit IgG conjugated to alkaline phosphatase (Sigma).
**Ferric iron uptake assays.** Iron uptake assays were performed as previously described (Montanez et al., 2005). $^{55}$FeCl$_3$ (0.2 µCi µl$^{-1}$, 0.02 µM; 7.4 x $10^3$ Bq) was added to cells at early exponential phase in CxCDM at 37 °C. Culture samples (200 µl) were drawn every 30 minutes and washed twice with 500 µl CxCDM containing 10 mM NTA. The radioactivity associated with the cell pellet was measured as counts per minute (c.p.m.) for 5 minutes against a $^3$H standard using a Beckman LS6500 scintillation counter. $^{55}$Fe$^{3+}$ incorporation for each time point was standardized for the cell quantity by dividing the CPM by the culture’s OD$_{600}$.

**Zebrafish care and virulence assays.** Care, feeding, and injection of zebrafish followed published methods (Montanez et al., 2005; Neely et al., 2002; Westerfield, 1995). Briefly, streptococci cells (10 µl of $10^5$ ml$^{-1}$) grown in THY with 2% peptone were aseptically injected into groups of 4-6 anesthetized male breeder zebrafish (Scientific Hatcheries). Following intraperitoneal or intramuscular injection, the fish were allowed to recover in sterilized ddH$_2$O supplemented with aquarium salts. A control animal group was injected with sterile medium. Infected fish were monitored for 48 hours and death recorded in intervals of 12 hours. The 50% lethal dose (LD$_{50}$) for infection of zebrafish was determined by challenging the zebrafish over a range of $10^1$ to $10^6$ c.f.u.

**Zebrafish tissue analysis.** Selected whole zebrafish were fixed following euthanasia at 40 hours after infection and 5 µm thick longitudinal sections of the dorsal muscle were prepared and stained as described previously (Neely et al., 2002). Fixed samples were stained with hematoxylin and eosin and examined with an Olympus BX60 microscope.
equipped with a digital camera and a motorized stage. For streptococcal identification, tissue sections were incubated with a *S. pyogenes* antiserum (Lee Laboratories, Grayson, Ga) at a 1:10 dilution, followed by detection with goat anti-rabbit antibodies conjugated to rhodamine red following manufacturer’s recommendations (Sigma).

**Statistics and data analysis.** Statistical significance was determined by using the Two-sample Student’s *t*-test. The standard error of the mean (SEM) was calculated by dividing the standard deviation by the square root of n. For *S. pyogenes*, the 50% lethal dose (LD<sub>50</sub>) for infection of zebrafish was determined by the method of Neely *et al* (2002), where zebrafish were challenged over a range of 10<sup>1</sup>-10<sup>6</sup> c.f.u. of each streptococcal strain.

**RESULTS**

**Inactivation of siaB produces changes in the iron regulated expression of the sia operon.**

To elucidate the role of the SiaABC transporter in iron uptake, a mutant in which the *siaB* gene was inactivated by a spectinomycin resistance cassette (strain ZE4913) (Montanez *et al.*, 2005) was analyzed. The effect of the *siaB* mutation on the expression from the *sia* operon was analyzed by RT-PCR. The amounts of *sia* transcript produced in cells grown in iron depleted medium (CxCDM) was compared to that provided by cells grown in iron replete (CxCDM plus FeCl₃). Amplification of *recA* was used as a control for equal amounts of mRNA. As previously reported for wild-type cells grown in a Todd-Hewitt base medium (ZTH) (Bates *et al.*, 2003), the expression from the *sia* operon is negatively
regulated by iron and transcripts of *shr* (first gene in the operon) and *siaD* (located downstream to *siaC*) were observed only in cells grown in iron depleted conditions (Fig. 2.1, compare wild-type with and without iron). In the *siaB* mutant however, transcription downstream of the spectinomycin cassette was constitutive and independent of the iron availability in the medium (Fig. 2.1, *siaD*). This indicates that inactivation of *siaB* was not polar on the downstream genes and that the expression from the non-regulated spectinomycin promoter continues into the *siaCDEFGH* genes. To our surprise, *siaB* inactivation also resulted in a significant increase of the basal expression of the genes found upstream to *siaB*. The amount of *shr* transcript produced in the *siaB* mutant in iron-depleted medium was much higher than that observed in the wild-type strain grown in the same condition (Fig. 2.1, *shr*). Additional increase in *shr* transcript was observed in cells grown in iron starvation as compared to those grown with iron, indicating that iron regulation from the *shr* promoter was not completely lost in the *siaB* mutant. A similar deregulation of expression of *sia* was observed in the *siaB* mutant grown in ZTH medium at the protein level. Western blot analysis demonstrated that while a very small amount of SiaA was produced in the wild-type cells grown in the presence of iron, the *siaB* mutant produces a higher amount of SiaA in this medium. In fact, the amount of protein was higher than the amount produced by the wild-type cells grown in iron-starved medium (Fig 2.2). Coomassie staining of total protein extract confirmed that equal amounts of total protein were loaded to the gel. In conclusion, the *siaB* mutant transcribes and probably expresses all the genes from the *sia* operon other than *siaB* in higher and less regulated levels.
Fig. 2.1 Transcription analysis of the *siaB* mutant. Total RNA from the wild-type (wt) and the *siaB* mutant (*siaB*−) grown in the presence (+) or absence (−) of FeCl₃ (Fe) was used as template in RT-PCR using primer pairs specific for *siaD* and *shr* genes. RT-PCR reaction with the recA specific primers was used as an internal control for equal amounts of RNA.
Fig. 2.2 Detection of SiaA in the siaB mutant. Western blot analysis with rSiaA antiserum. Total protein extract from whole S. pyogenes wild-type (wt) and siaB mutant (siaB) cells grown in iron replete (+Fe) or iron deplete (-Fe) medium.
*siaABC* is involved in hemoglobin utilization.

Growth of the *siaB* mutant strain was compared to that of the parent strain in rich medium (ZTH), iron depleted medium (ZTH plus 12 mM NTA, ZTH-NTA), and iron depleted medium supplemented with various iron sources (Fig. 2.3). Growth of both the wild-type and the *siaB* mutant was inhibited in the iron depleted medium (ZTH-NTA). Addition of 10 mM ferric citrate restored growth of both the wild-type and the *siaB* mutant, and no significant difference in growth between the strains was observed. The addition of hemoglobin to ZTH-NTA restored 66% of the wild-type growth in ZTH. The *siaB* mutant growth was reduced in comparison to the wild-type and reached only 45% of the growth observed in ZTH. This growth reduction, although small, is statistically significant (*P* < 0.04) and indicates that the *siaB* mutant is mildly impaired in its ability to use hemoglobin as an iron source. The addition of 0.13% whole blood or serum to ZTH-NTA medium could support the growth of both the wild-type and the *siaB* mutant to the same extent (76% vs. 70% in blood and 32% vs. 26% in serum). We previously reported that the *siuADBG* transporter is involved in hemoprotein utilization in *S. pyogenes*. To examine the contribution of *siuADBG* to the growth of the *siaB* mutant in iron-depleted medium, we investigated the ability of an *siuG siaB* double mutant (Montanez *et al.*, 2005) to use hemoproteins. Unlike the *siaB* mutant, the double mutant was significantly impaired in its ability to utilize blood and serum (35% and 2% of the growth observed in ZTH). The *siuG siaB* strain also demonstrated further reduction in growth on hemoglobin containing medium with only 33% of growth (a 50% reduction as compared to the wild-type growth in the same condition). Therefore, inactivation of
Fig. 2.3 Growth analysis of the wild-type, the siaB, and the siuG siaB mutant in iron-depleted media supplemented with various iron sources. *S. pyogenes* wild-type (NZ131, black bars), siaB (ZE4913, hatched bars), or siuG siaB (ZE4914, white bars) cells were grown in iron-depleted medium, NTA (ZTH with 12 mM NTA, 0.66 mM MgCl2, MnCl2, CaCl2, and ZnCl2), or in iron-depleted medium supplemented with 10 mM ferric citrate, 0.13% sheep’s blood, 0.13% horse serum or, 12 µM human hemoglobin. The culture’s OD$_{600}$ was determined following overnight incubation. Results are shown as percent growth with respect to that obtained in ZTH medium. Data represent the mean of at least three experiments. Error bars represent SEM; significant $P$ values (<0.05) are indicated by *.
both *sia* and *siu* transporters further impaired the ability of *S. pyogenes* to utilize heme sources.

**$^{55}\text{Fe}^{3+}$ uptake is affected in the *sia* and *siu* mutants.**

A mutation in *siuG* is associated with a decrease in ferric uptake in addition to the deficiency in hemoprotein utilization (Montanez et al., 2005). To investigate the role of the SiaABC transporter in ferric utilization, the effects of the *siaB* and *siuG* inactivation on ferric incorporation were measured. Since the presence of a strong chelator could complicate ferric uptake assays, these experiments were done in a chemically defined medium that was treated with the chelating resin Chelex-100 (CxCDM), which produces iron starvation signals that lead to enhanced expression of the *sia* (Fig. 2.1) and the *siu* genes (Montanez et al., 2005). $^{55}\text{FeCl}_3$ was added to cell cultures in their early logarithmic phase and the radioactivity of the cells was monitored over time. While the wild-type had reached maximum accumulation of $^{55}\text{Fe}^{3+}$ after 30 min of incubation, the *siaB* mutant showed a slower rate of intake, reaching maximum uptake after 60 min. However, the total $^{55}\text{Fe}^{3+}$ accumulation was comparable to that observed by the wild-type strain (Fig. 2.4). Although inactivation of *siuG* reduced iron uptake by 30 % in *S. pyogenes* (Montanez et al., 2005), knocking out *siaB* in the background of the *siuG* resulted in a level of ferric uptake that was similar to that of the wild-type (Fig. 2.4).

**The *Streptococcus pyogenes* *siaB* mutant is attenuated for virulence in zebrafish**

Neely *et al* developed a zebrafish (*Danio rerio*) animal model for virulence studies of streptococcal infections (Miller & Neely, 2004; Neely *et al.*, 2002). We previously used
Fig. 2.4. Incorporation of radioactive ferric iron by the wild-type and the *siuG* mutant. $^{55}$FeCl$_3$ was added to *S. pyogenes* wild-type (NZ131, ▲-), *siaB* (ZE4913, ■-), and *siuG siaB* (ZE4914, ●-) cultures grown in CxCDM at the early exponential phase. Cells were harvested every 30 min, washed, and the radioactivity associated with the cell pellet was measured. To standardize for the cell number in each sample the results are expressed as c.p.m. divided by the OD$_{600}$ of the culture. Data points represent the mean of three experiments done in duplicate; error bars represent SEM.
this model to investigate the role of iron acquisition in disease production and progression by *S. pyogenes* NZ131. Intramuscular (i.m.) injection of *S. pyogenes* NZ131 produces a myositis lethal infection, killing 86 % of the fish by 48 hours (Fig. 2.5a), while intraperitoneal (i.p.) injection of $10^5$ c.f.u of *S. pyogenes* NZ131 results in 43 % death. Streptococcal infected fish present a hypopigmented lesion and extensive tissue necrosis. Staining of necrotic muscle tissue sections from fish infected with *S. pyogenes* NZ131, revealed that the bacteria form large clusters spread throughout the necrotic area (appendix C.1). A similar cell arrangement was also detected when zebrafish were injected with the M14-type *S. pyogenes* strain HSC5 (Neely *et al.*, 2002).

We compared infection produced by the *siaB* and *siuG siaB* mutant strains to that produced by the parent strain following i.m. or i.p. injections. The kinetics of fish death in response to i.m. injection with the *siaB* or the *siuG siaB* mutant was different from that observed in response to injection with the wild-type strain. While at 36 hours only 57 % of the fish injected with NZ131 survived, all of the fish injected with the *siaB* mutant or with the *siuG siaB* mutant survived ($P < 0.0185$). However, 48 hours post infection the survival of the fish injected with the *siaB* mutant was reduced to that of fish injected with the wild-type strain (13 % and 14 %, respectively; Fig 2.5a). This indicates that it took longer time for the *siaB* mutant to produce a lethal infection than the time required by the wild-type strain. On the other hand, a significant higher fish survival was observed in fish injected with the double mutant (69 %). Therefore, the *siaB* mutant was moderately attenuated for virulence and the *siuG siaB* was significantly impaired in its ability to inflict fish mortality. Fish survival following i.p. infection with the *siaB* or the *siuG siaB* mutant was somewhat higher than the survival rate of the fish injected with the wild-type
Fig. 2.5 Zebrafish survival curve following *Streptococcus pyogenes* infection. Groups of four to six zebrafish were challenged with $10^5$ c.f.u. of *S. pyogenes* wild-type (NZ131, ▲-), *siaB* (ZE4913, ■-), and *siuG siaB* (ZE4914, ●-) by intramuscular (a) or intraperitoneal (b) injection and monitored for 2 days. Data were pooled from three to five independent experiments and presented as total percent fish survival as a function of time.
strain (Fig. 2.5b). However, while that appears to be the trend for both mutants, the difference in fish survival was not statistically significant when injected i.p. with $10^5$ c.f.u. Therefore, the route of injection appears to play a significant role in the outcome.

**Infection of zebrafish with the siaB mutant leads to a more severe lesion and increased neutrophil migration**

While the siaB mutant appeared attenuated for the first 36 hours, it eventually produced a lethal infection. In addition, fish infected with the siaB mutant developed aggressive lesions with severe necrosis and often developed a swollen and bloody appearance of the mouth and gills. Microscopic examination of tissue sections stained with hematoxylin and eosin revealed a very large number of neutrophils at the necrotic site in fish injected with the siaB mutant (Fig. 2.6). This massive infiltration of inflammatory cells was not seen in the lesion caused by the wild-type strain. The absence of a significant inflammatory response during infection was previously reported for zebrafish injected with *S. pyogenes* HSC5 (Neely *et al.*, 2002). Staining of the tissue with specific anti-streptococcal antibodies revealed the presence of large clusters of bacteria in fish infected with the wild-type strain or the siaB mutant (Fig 2.7). However, in tissue infected with the siaB mutant the neutrophils were found in large amounts and in the proximity of the bacterial cells. Only a few neutrophils were seen in tissue infected with NZ131 and thus appeared to be spread throughout the necrotic site. Therefore, infections with the siaB mutant resulted in an unusually aggressive inflammatory response that is missing in infections produced by the wild-type strains.
Fig 2.6  Histopathological examination of zebrafish muscle infected with *Streptococcus pyogenes*. Longitudinal sections of 3 zebrafish infected with the *S. pyogenes* wild-type NZ131 (upper panels) or the siaB mutant (lower panels) were prepared and stained with hematoxylin and eosin viewed by light microscopy at 100X magnification.
Fig 2.7 Identification of *Streptococcus pyogenes* and inflammatory cells in zebrafish infected tissue. Longitudinal sections of zebrafish infected with the *siaB* mutant were prepared and stained with a *S. pyogenes* antiserum and viewed with a rhodamine filter (a) or with a rhodamine filter under light microscopy (b) at 100X magnification. Arrows indicate *S. pyogenes* cells and arrowheads point to visible neutrophils in the proximity of the bacteria.
DISCUSSION

*S. pyogenes* is a medically important pathogen capable of colonizing different types of human tissue and is able to produce a diverse array of infections, ranging from self-limited infections such as pharyngitis and impetigo to aggressive, invasive infections such as necrotizing fasciitis and streptococcal toxic shock syndrome. This hemolytic bacterium carries two ABC-type transporters from the iron complex family. We recently demonstrated that one of these transporters, named *siuADBG*, contributes both to hemoprotein and to ferric utilization in *S. pyogenes* (Montanez et al., 2005). The second transporter, named *siaABC* (or *htsABC*), is part of a 10 gene operon and it is preceded by two surface proteins, a hemoprotein receptor named *shr*, and a heme binding protein called *shp*. The *siaDEFGH* genes are found downstream of the *siaABC* transporter. Work done by others and ourselves suggested that the *siaABC* transporter functions in heme uptake. In this study, we analyzed a mutant in *siaB*, which codes for a membrane permease, and a double mutant, in which both *siaB* and a membrane permease subunit, *siuG*, were inactivated.

The *sia* operon is negatively regulated by iron via a metal dependent repressor named MtsR (Bates et al., 2005). Expression analysis of the *sia* operon in the *siaB* mutant demonstrated that the insertion of the spectinomycin resistance cassette into *siaB* leads to constitutive expression of the *siaB* downstream genes. This change in the transcription profile probably resulted from the non-regulated promoter of the spectinomycin resistance gene. We were surprised to find however, that the *siaB* mutant
exhibited an increased expression of the genes found upstream to *siaB* in medium containing iron. This increase in *sia* basal transcription was accompanied by an elevated production of SiaA and probably Shr proteins, indicating that the SiaB protein was not required for the translation or stability of Shr and SiaA. Inactivation of *siaB* may produce iron starvation signals in the cell, despite the presence of iron in the medium. Alternatively, the constitutive expression of the genes downstream to the *sia* transporter, which include a putative cysteine exporter (*siaDE*) and an ABC type transporter (*siaFGH*) of an unknown substrate, may produce the signals responsible for the changes in the transcription of the entire operon.

Growth analysis of the *siaB* mutant revealed that this mutant was moderately impaired in hemoglobin utilization. This observation is consistent with our previous finding that SiaA, the binding protein, can bind hemoglobin and further supports the role of the *sia*ABC system in heme transport. Surprisingly, inactivation of *siaB* did not result in a significant difference from the wild-type in the utilization of blood or serum. We previously demonstrated that Shr binds multiple hemoproteins including hemoglobin, myoglobin and heme-serum albumin, and Lei *et al* recently showed that Shp binds heme and delivers it to SiaA (Liu & Lei, 2005). Since at least some, if not all, of the proteins encoded by the *sia* operon are produced in the *siaB* mutant, it is possible that they still contribute to blood and serum utilization in the absence of *siaB*, perhaps via interaction with other transporters. Therefore, the moderate phenotype resulting from *siaB* inactivation may be an under representation of the full function of the *sia* operon.

Unlike the *siaB* mutant, the *siaB siuG* double mutant was impaired in the use of both blood and serum and demonstrated a greater deficiency in hemoglobin utilization.
As we previously reported, the *siuG* mutant demonstrates a defect in the use of serum, blood, and hemoglobin which is more significant than that resulting from the *siaB* inactivation. Therefore, while our observations suggest that the *sia* and the *siu* transporters both contribute to the use of host hemoproteins to fulfill the bacterium’s iron requirement, at least under the conditions used in this investigation the *siu* system has a more pronounced role in hemoglobin utilization.

Inactivation of *siaB* did not affect the growth of *S. pyogenes* in iron depleted medium supplemented with ferric citrate. In addition, unlike the *siuG* mutant, no change in the total accumulation of $^{55}\text{Fe}^{3+}$ in comparison to the wild-type strain was found in the *siaB* mutant, although a lower rate of ferric uptake by the *siaB* mutant was observed during the logarithmic phase of growth. Unexpectedly, although the *siuG* mutant demonstrates about 30% reduction in ferric accumulation, ferric uptake by the *siuG siaB* mutant was similar to that of the wild-type cells. Since the MtsABC transporter plays an important role in ferric and manganese transport in *S. pyogenes* (Janulczyk et al., 2003), it is possible that the inactivation of both the *sia* and the *siu* transporters results in a compensatory effect on the *mts* expression or activity which allowed ferric uptake at the wild-type level.

A correlation between the maintenance of proper iron homeostasis and the ability to produce disease has been established in several pathogenic bacteria (Al-Tawfiq et al., 2000; Brown et al., 2001a; Dale et al., 2004; Henderson & Payne, 1994; Ratledge, 2004; Stojiljkovic et al., 1995; Torres et al., 2001). In *S. pyogenes*, inactivation of the *siuG* gene produced an attenuated strain in the zebrafish infection model (Montanez et al., 2005). We conducted studies to determine the effect of *siaB* inactivation on virulence
using the same model system. Unlike the siuG mutant, the siaB demonstrated a slower progression of the disease, and appeared attenuated only for 36 hours post infection. However, in time siaB-injected fish resulted in fish mortality comparable to that produced by the wild-type. On the other hand, the siaB siuG mutant was attenuated and the majority of the injected fish survived. Therefore, the siuG and the siaB siuG mutant strains, which had a more significant deficiency in host hemoprotein utilization than the siaB mutant, were also more severely impaired in disease production in vivo. This observation is consistent with the notion that the ability to compete with the host for iron during infection is important for the ability of S. pyogenes to produce infection.

While infection produced by S. iniae in zebrafish resulted in a significant inflammatory response, the absence of an inflammatory response when infected with S. pyogenes was noticed. Similar to infection with NZ131, the fish injected i.m. with S. pyogenes HSC5 did not produce a significant inflammatory response (Neely et al., 2002). Therefore, the large amount of inflammatory cells found in zebrafish tissue infected by the siaB mutant was unexpected. A copious amount of neutrophils located in the proximity, but seldom mixed with streptococcal cells, were observed in tissue sections infected with the siaB mutant. During acute inflammation, neutrophils arrive at the site of damage, responding to chemotactic factors like C5a and cytokines, which diffuse from the infected area. Recently, it was reported that heme is proinflammatory. It enhances the production of adhesive molecules, promoting an increase in vascular permeability, and increasing the expression of chemoattractants such as MCP-1 (Lyoumi et al., 1999; Wagener et al., 2003). In fact, heme-oxygenase antagonizes heme-induced inflammation and was proposed for therapeutic use to reduce the inflammatory response (Wagener et
al., 2001). The $siaB$ mutant overexpresses the surface receptors Shr, SiaA and possibly Shp. Therefore it is likely that this mutant is accumulating heme on the surface in larger than normal amounts. As Shr is found both on the surface and in the culture supernatant it may lead to heme accumulation in some distance from the bacteria. These changes on the surface and the vicinity of the bacteria may result in activation of the inflammatory cascade and may explain the massive infiltration of host inflammatory cells. The enhanced inflammation and accumulation of neutrophils may also contribute to and aggravate tissue injury, leading to more serious lesions.

In summary, the SiaABC transporter contributes to the utilization of heme from hemoproteins as an iron source and contributes to the virulence of $S. pyogenes$ in zebrafish. Inactivation of the permease $siaB$ results in deregulation of the $sia$ operon components, which may contribute, through heme binding, to an exaggerated inflammatory response atypical of $S. pyogenes$ in zebrafish.
INTRODUCTION

Acquisition of iron-complexes such as siderophores, ferric dicitrate, and heme in Gram-negative bacteria usually begins with the recognition of the substrate by a specific outer membrane receptor that with the help of TonB, delivers the iron compound to the periplasm. In the case of heme, a hemophore may be needed in some systems to unload the heme from its host carrier and to transfer it to the cognate receptor molecule; while in other systems the outer membrane receptor itself is able to carry out this function. Examination of the crystal structures of the Escherichia coli siderophore uptake proteins FhuA and FepA, and the receptor for ferric dicitrate FecA, reveal striking similarities. The structure of these outer membrane receptors consists of two domains. The first is a 22-strand β-barrel domain that spans the membrane and is extended with several short periplasmic loops and with a few long extracellular loops. The second domain is an N-terminal globular domain that is covalently bonded to the first strand of the barrel. This domain is nested inside the barrel and acts as a plug for the channel formed by the barrel cavity. Substrate binding leads to a conformational change in the plug domain, which in turn allows unwinding of the switch helix and interaction with TonB. TonB uses the proton motive force to cause a second conformational change of the outer membrane receptor which results in the transport of iron complexes through a transient channel into the periplasm. A periplasmic binding protein (PBP) then binds and delivers the iron complex to a cognate ABC-type transporter located on the cytoplasmic membrane, for transport into the cell’s cytoplasm. While the structure of outer membrane receptors
involved in heme transport was not resolved, their significant sequence homology to siderophore and ferric dicitrate receptors suggests that they assume a similar fold and that they function in the same manner.

In the absence of the outer membrane in Gram-positive bacteria, ABC-type transporters are exposed to the extracellular milieu and directly involved in the recognition and uptake of iron complexes including heme. Therefore, surface binding proteins in Gram-positives share sequence homology with PBPs but act as functional analogs of the outer membrane receptors for iron complexes. A few surface receptors of heme have been characterized in Gram-positive bacteria (Crosa, 2004), but no crystal structure has been resolved yet. For example, HmuT, the binding receptor for the HmuTUV transporter in *Corynebacterium diphtheriae*, binds heme and hemoglobin (Drazek *et al.*, 2000). In some bacteria additional surface proteins that bind heme and hemoproteins are encoded near to and are produced with ABC-type transporters. The current working model suggests that these proteins may function as accessory compounds in heme acquisition and transport. Computational analysis identified common sequence motives (named NEAT for near iron transporters) in some of these proteins that are found only in Gram-positive bacteria (Smoot *et al.*, 2001). Both IsdA and IsdB in *Staphylococcus aureus* for example have NEAT motifs and were found to bind heme and hemoglobin (Skaar & Schneewind, 2004).

Treatment of *Streptococcus pyogenes* cells with proteinase K reduces hemoglobin and heme-serum albumin binding to the cell’s surface (Bates *et al.*, 2003) indicating that proteins are involved in *S. pyogenes* binding to hemoproteins. The *sia* operon encodes three protein receptors that directly bind heme or hemoproteins. Shr is a large
hydrophilic protein that shares similarities with eukaryotic receptors such as Toll and G-protein-dependent receptors. Sequence analysis of Shr revealed two NEAT motifs, a domain with leucine rich repeats, and a previously described (Reidl & Mekalanos, 1996) heme binding motif. A recombinant Shr protein directly binds hemoglobin, myoglobin, heme-serum albumin, and the hemoglobin-haptoglobin complex but not apo-haptoglobin \textit{in vitro} (Bates \textit{et al.}, 2003). Shp, encoded by the second \textit{sia} gene, is associated with heme in a 1:1 stoichiometry when purified from \textit{E. coli} (Lei \textit{et al.}, 2002). SiaA (HtsA), the lipoprotein receptor of the \textit{sia} ABC-type transporter, shares sequence homology with the heme/hemoglobin binding proteins HmuT and IsdA from \textit{C. diphtheriae} and \textit{S. aureus}, respectively.

In our studies we began the characterization of SiaA as a hemoprotein surface receptor in \textit{S. pyogenes}. We demonstrated that SiaA expression on the streptococcal surface increases in iron starvation conditions and showed that it is able to bind heme and hemoglobin \textit{in vitro}. We published these findings (Bates \textit{et al.}, 2003) and propose here a model of heme binding by SiaA.

**METHODS**

**Bacterial strains and growth assay conditions**

\textit{S. pyogenes} was grown statically at 37 °C in Todd-Hewitt broth with 0.2 % (w/v) yeast extract (THY) and 10 mM Tris, adjusted to pH 6.9 (ZTH). ZTH was starved for iron by the addition of 10 mM chelating agent nitrilotriacetic acid (NTA). ZTH was supplemented with 0.66 mM MgCl\textsubscript{2}, MnCl\textsubscript{2}, CaCl\textsubscript{2}, and ZnCl\textsubscript{2} (Eichenbaum \textit{et al.}, 1996).
Iron was supplemented as 4 µg of human hemoglobin (Sigma). Human hemoglobin was prepared as a 10 mg ml\(^{-1}\) stock solution in phosphate-buffered saline (PBS; pH 7) and filter-sterilized. The \textit{E. coli} strains DH5\(\alpha\), and Top10 (Invitrogen) were used for cloning and protein expression. \textit{E. coli} cells were grown in Luria-Bertani broth. When necessary, the antibiotic ampicillin was used at 100 µg ml\(^{-1}\), chloramphenicol at 15 µg ml\(^{-1}\). For \textit{S. pyogenes} chloramphenicol and spectinomycin were used at 1 – 2 µg ml\(^{-1}\) and at 100 µg ml\(^{-1}\), respectively. Optical density was measured with a Beckman DU640 spectrophotometer (600nm).

**Construction of plasmids.** Cloning, DNA extraction, restriction analysis, and DNA transformation were performed by published methods (Eichenbaum & Scott, 1997; Perez-Casal \textit{et al.}, 1991; Sambrook, 1989). The primers used in the present study are listed in Table 3.1. (i) **Construction of SiaA expression vector (pSiaA-His).** \textit{siaA} was amplified from the chromosome of \textit{S. pyogenes} SF370 without its leader peptide by using the primers ShuAF and ShuAR and cloned into \textit{XhoI} and \textit{EcoRI} sites of pBAD/His A (Invitrogen). The resulting plasmid, pSiaA-His, expresses the rSiaA (SiaA with an N-terminal fusion to the His\(_6\)-Xpress epitope tag) from the arabinose-regulated promoter, PBAD (Guzman \textit{et al.}, 1995). An enterokinase site exists downstream of the Xpress epitope, which facilitates the removal of the tag from the SiaA protein after purification. (ii) **Construction of SiaA expression vector under the P23 promoter (pCPSiaA).** A fragment that carries the \textit{siaA} gene was amplified with primers CP-ShuA-S and CP-ShuA-A and cloned into the \textit{BamHI} and \textit{PstI} sites of pOri23 (Que \textit{et al.}, 2000).
**TABLE 3.1.** Oligonucleotide primer sets

<table>
<thead>
<tr>
<th>locus</th>
<th>primer</th>
<th>primer sequence</th>
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<tbody>
<tr>
<td>siaA</td>
<td>ShuAF</td>
<td>5’- CCCCAACTCGAGGTGAATCAGCACCCTAAAAC -3’</td>
</tr>
<tr>
<td></td>
<td>ShuAR</td>
<td>5’- CCCCTTGAATTCTTACGGATGATCTCCCACG -3’</td>
</tr>
<tr>
<td>siaA</td>
<td>CP-ShuA-S</td>
<td>5’- CCCGGATCCCCCGTCTAAAAAAAGAC-3’</td>
</tr>
<tr>
<td></td>
<td>CP-ShuA-A</td>
<td>5’- CCCCTGCAGGTAAATCTTGATGCTG-3’</td>
</tr>
</tbody>
</table>
resulting plasmid pCPSiaA expresses the native SiaA protein from the lactococcal P23 promoter.

**Purification of rSiaA.** rSiaA was purified from Top10/pSiaA-His cells by using the HisTrap Kit (Amersham), according to the manufacturer’s instructions. Expression was induced with 0.02 % arabinose for 4 h. Cells were lysed by cycles of sonication, freezing, and thawing in the presence of the Complete Mini Protease Inhibitor Cocktail (Roche), according to manufacturer’s instructions. After elution, rSiaA was concentrated by a Centricon spin column (Millipore, 10,000-molecular-weight cutoff) and quantified by Bradford assay (BioRad). When digested with enterokinase, 20 µg of rSiaA were incubated with 1 U of EnterokinaseMax (Invitrogen) in 50 µl of EkMax buffer for 16 h at 37 °C.

**Antiserum preparation.** The purified protein (rSiaA) was emulsified with Freund complete adjuvant (Sigma) and injected into New Zealand female rabbits (Myrtle's Rabbitry). The rabbits were immunized with two booster injections in Freund’s incomplete adjuvant at 3 to 4 weeks after each injection. Serum was collected 1 week after each booster. This protocol was approved by the Georgia State University Institutional Animal Care and Use Committee.

**SDS-PAGE, Western blotting, and hemoprotein-binding assays.** Proteins were prepared in Laemmli sample buffer (Laemmli, 1970), separated by sodium dodecyl sulfate–10 % polyacrylamide gel electrophoresis (SDS–10 % PAGE), and stained with
Coomassie brilliant blue. For Western blot analysis (Burnette, 1981), proteins were transferred to a nitrocellulose membrane and reacted with the rSiaA antiserum (1:10,000) or anti-Xpress antibodies (Invitrogen, 1:15,000). For hemoproteins binding assays, 3 µg of SiaA and 3 µg human transferrin (Sigma) were suspended in a sample buffer (65 mM Tris-HCl [pH 6.8], 10 % glycerol, 0.03 % bromophenol blue), fractionated at 4 °C by SDS-PAGE without boiling (Francis & Becker, 1984), and transferred to a nitrocellulose membrane. Membranes were blocked for 2 h with 5 % skim milk, rinsed, and reacted with 6 µg of biotinylated hemoglobin, myoglobin, fibrinogen, haptoglobin-hemoglobin (Sigma), or a 1:200 dilution of streptavidin conjugated to alkaline phosphatase (streptavidin-AP) for 1 h. Binding was visualized with streptavidin-AP (Roche).

**Heme binding assay.** *In vitro* protein binding of heme was detected by a previously described method of heme staining (Mazoy & Lemos, 1996). Briefly, 2 µg of SiaA were incubated overnight with 0.06 µg of hemin chloride (Sigma). After incubation, SiaA was resolved by non-denaturing PAGE at 4 °C, followed by heme staining with the chromogenic substrate 3,3'-dimethoxybenzidine (DMB).

**Detection of SiaA in Streptococcus pyogenes.** Total protein preparation was done based on the method of Pancholi and Fischetti (Pancholi & Fischetti, 1992). *S. pyogenes* NZ131 cells were harvested from 5 ml overnight (14 hrs) cultures growing in ZTH, ZTH-NTA (12 mM) or ZTH-NTA supplemented with 4 µg of human hemoglobin. Each sample was washed with saline, resuspended in PED (20 mM phosphate buffer, 5 mM EDTA, 10 mM dithiothreitol), and treated with the muralytic enzyme lysin for 1 h at
37°C to dissolve the cell wall. The lysates were then boiled for 10 min in the presence of SDS, resolved on 10% SDS-PAGE, and transferred to nitrocellulose membranes for immunoblotting. Membranes were incubated with a 1:10,000 dilution of rSiaA antiserum followed by 1:10,000 anti-rabbit IgG conjugated to alkaline phosphatase (Sigma).

SiaA expression was also detected by using a Fluorescence Activated Cell Sorter (FACS). *S. pyogenes* cells harvested from ZTH at mid-exponential phase were inoculated into Chelex-100 (BioRad) treated chemically defined medium supplemented with 33 µM MgCl<sub>2</sub> and 68 µM CaCl<sub>2</sub> (CxCDM) in the presence or absence of 0.5 µM FeCl<sub>3</sub>. *S. pyogenes* cells grown in this medium produce iron starvation signals (Montanez et al., 2005). The cells were harvested at early exponential phase, washed twice with PBS and adjusted to a total volume of 1 ml at OD<sub>600</sub> 0.8. SiaA antiserum (50 µl) were added for 30 min at 4 °C with mild shaking. The samples were washed twice with PBS, followed by the addition of 20 µl of anti-rabbit IgG conjugated to fluorescein-isothiocyanate (IgG-FITC; Sigma) for 30 min at 4 °C with mild shaking. After washing twice with PBS, the cell associated fluorescence was read with a FACScaliber (Becton Dickinson). IgG-FITC only, SiaA antiserum only, or rabbit polyclonal GFP antiserum (Invitrogen) were used as controls for nonspecific binding.

**RESULTS**

**SiaA is a heme and hemoglobin binding protein.**

In contrast to Gram-negative bacteria, the binding proteins in Gram-positives are extracellular and are presumed to be anchored to the cell membrane via a lipid portion
that is covalently attached to the protein. These lipoproteins have a characteristic \( LX_1X_2C \) motif (where \( X_1 \) is typically A, S, V, Q or T and \( X_2 \) is typically G or A) at the C-terminal end of their signal sequence (Sutcliffe & Russell, 1995) recognized by signal peptidase II. The N-terminus of SiaA has the characteristics of a lipoprotein signal (17-LVAC). Western blot analysis of cell fractions of \( S. pyogenes \) confirmed that SiaA is associated with the membrane (Bates et al., 2003). In addition, SiaA contains conserved domains that correspond to the group of periplasmic binding proteins involved in the transport of iron complexes such as siderophores, heme, and vitamin \( B_{12} \) (Tam & Saier, 1993).

Bacteria encounter multiple heme sources in the human host, as heme is bound to a variety of carrier proteins. The ability to bind and utilize hemoproteins involves specific recognition by surface receptors. Most receptors have been found to bind only heme and/or hemoglobin, while proteins like BhuR from \( Bordetella pertussis \) (Murphy et al., 2002), HmuR from \( Porphyromonas gingivalis \) (Olczak et al., 2001), and the streptococcal Shr bind multiple hemoproteins. As SiaA is part of a heme acquisition operon, we tested to determine if it is a surface anchored protein, which can recognize heme and/or hemoproteins.

To determine the cell location and binding specificity of SiaA we constructed an expression vector that facilitates the purification of SiaA from \( E. coli \). A leaderless SiaA was cloned for expression in \( E. coli \) under the control of PBAD, the arabinose promoter (Guzman et al., 1995) (Fig. 3.1a). An N-terminal fusion of SiaA to the His\(_6\)Xpress epitope tag allows purification by nickel affinity and Western blot detection by anti-Xpress antibodies. The presence of an enterokinase recognition site immediately
Fig. 3.1 Construction of SiaA expression vector. (a) Diagram of the pSiaA-His plasmid. Primers ShuAF and ShuAR were use to amplify siaA from S. pyogenes SF370 and the resulting product was cloned into the XhoI and EcoRI sites of pBAD-His (Invitrogen). (b) Schematic representation of rSiaA. The black area represents the 6X histidine flag, the gray area represents the Xpress epitope (Invitrogen), and EK represents the site of recognition for enterokinase enzymatic cleavage.
downstream of the epitope provided a means for the removal of the tag (Fig. 3.1b). The recombinant protein (rSiaA) was expressed and purified from *E. coli* Top 10 extracts. A protein band in the predicted size of 35 kDa (SiaA with the addition of the epitope) that reacted with the anti-Xpress antibodies was observed, confirming the production and purification of rSiaA (W in Fig.3.2).

Using a 3,3′-dimethoxybenzidine (DMB) staining method we found that SiaA binds to heme *in vitro* (Heme in Fig. 3.2). A solid-phase binding assay with purified rSiaA fixed to a membrane demonstrated that it could also bind biotinylated hemoglobin. Transferrin was included as a control, as was biotinylated fibrinogen. Human transferrin did not bind hemoglobin and rSiaA did not bind biotinylated fibrinogen or streptavidin alone (Fig 3.2). This finding indicates that rSiaA specifically binds hemoglobin and does not recognize the biotin group nor does it bind proteins nonspecifically. *S. pyogenes* growth in iron depleted medium is supported by the addition of ferritin, hemin, hemoglobin, myoglobin, catalase (Eichenbaum *et al.*, 1996), and heme-serum albumin (Bates *et al.*, 2003). It was found that Shr can bind myoglobin, heme-serum albumin, and the hemoglobin-haptoglobin as well as hemoglobin. Therefore, we tested the ability of rSiaA to bind other hemoproteins besides hemoglobin. Immobilized rSiaA was incubated with 6 µg of biotin labeled myoglobin or haptoglobin-hemoglobin, followed by the colorimetric detection assay described above. Unlike Shr, rSiaA did not bind these two proteins (Fig 3.2).

Histidine residues are common heme axial ligands in hemoproteins like hemoglobin and myoglobin. Histidine residues conserved among heme/hemoproteins receptors were demonstrated to function in heme transport in the *Yersinia enterocolitica*
**Fig. 3.2 rSiaA binding to hemoproteins.** Purified rSiaA (A) and human transferrin (T) were fractionated by SDS-PAGE under seminative conditions. A Coomassie blue-stained gel (C), Western blot analysis with anti-Xpress antibodies (W), and binding blots to biotinylated hemoglobin (Hb), myoglobin (Mb), fibrinogen (Fb), haptoglobin-hemoglobin (Hp:Hb), or streptavidin (St)-conjugated alkaline phosphatase are presented. DMB staining of heme after SiaA binding and resolution on PAGE (Heme). M represents the molecular weight marker with the numbers to the left for the kDa values.
HemR receptor (Bracken et al., 1999); thus, we were concerned that the His<sub>6</sub>-Xpress epitope tag could have contributed to hemoglobin binding. The tag was removed by enterokinase digest as indicated by the production of a protein band smaller than rSiaA that did not react with the anti-Xpress antibodies (Xp in Fig. 3.3). A less-abundant protein band representing intact rSiaA was also observed, indicating that the digest was not complete. Increasing the enterokinase concentration or the incubation time resulted in complete digest. Subsequent hemoglobin-binding assays showed that both rSiaA and rSiaA without the epitope bound hemoglobin, demonstrating that the binding ability of rSiaA is inherent to the native SiaA protein (Hb in Fig. 3.3).

**SiaA expression in Streptococcus pyogenes increases in iron depleted media.**

The transcription of the *sia* operon increases when cells are grown in low iron media. This effect can be reversed by the addition of hemoglobin (Bates et al., 2003). To demonstrate that the production of the SiaA protein is indeed iron regulated, we analyzed the presence of SiaA in cells grown in the absence or presence of iron. Total protein content was extracted from *S. pyogenes* grown in ZTH, ZTH-NTA or ZTH-NTA supplemented with 4 µg of human hemoglobin and resolved on SDS-PAGE, followed by blotting to a nitrocellulose membrane and Western blot. SiaA was detected in all samples and as expected an increase in the amount of SiaA was found in cells cultured in iron depleted media. This observation confirms that SiaA production is induced in low iron conditions (Fig. 3.4) as expected for proteins involved in iron uptake. To substantiate our results, we use a Fluorescence Activated Cell Sorter (FACS) to semi-quantitate the iron dependent expression of SiaA on the streptococcal cell surface was. Equal amounts of *S.
Fig. 3.3 SiaA binding to hemoglobin after removal of the histidine-Xpress tag. Purified rSiaA incubated with enterokinase was fractionated by SDS-PAGE under seminative conditions. A Coomassie blue-stained gel (C) shows partial enterokinase digest of rSiaA. A binding blot to biotinylated hemoglobin (Hb) and Western blot analysis with anti-Xpress antibodies (Xp) and with rSiaA antiserum (S) are presented. M represents the molecular weight marker.
Fig. 3.4 Detection of SiaA in *Streptococcus pyogenes* protein extracts. Western blot analysis with rSiaA antiserum. Total protein extract from whole *S. pyogenes* NZ131 cells grown in iron replete (+Fe), iron deplete (-Fe), or iron-deplete medium supplemented with hemoglobin (Hb). Total protein extract from whole *S. pyogenes* JRS4/pCPSiaA cells expressing the native SiaA from the lactococcal P23 promoter (C) was used as a positive control.
pyogenes cells grown in CxCDM or CxCDM plus FeCl₃ media were incubated with rSiaA antiserum followed by anti-rabbit IgG antibodies conjugated (FITC). The cells grown in iron depleted media had a 10 fold increase in fluorescence as compared to those grown in the presence of iron. Incubation with anti-GFP did not result in an increase in fluorescence signal, demonstrating that the amount of signal was directly related to the amount of SiaA expressed on the cell surface and not to non-specific IgG binding (data not shown).

**DISCUSSION**

Our understanding of the structure and function of receptors for iron complexes in Gram-positive organisms is very limited. In Gram-negatives, outer membrane receptors typically recognize hemoproteins, and then extract and transport the heme moiety into the periplasm where it is delivered to the PBP of an ABC-type transporter. In Gram-positive bacteria, with the absence of the outer membrane compartment, the PBP homologs also play a role in the detection of hemoproteins and the acquisition of heme from these host carriers. This task may take place through direct interactions with the hemoproteins with or without the help of accessory proteins found on the bacterial cell surface. SiaA is the periplasmic binding homolog of the siaABC heme transporter in *S. pyogenes*. The mature protein is bound to the cell membrane by a lipoprotein motif in its N-terminus. The iron-dependent regulation of the *sia* operon is mediated by the metallorepresor MtsR, which binds to the *shr* promoter in the presence of iron. In accordance with this, the SiaA expression is significantly enhanced in cells grown in an iron depleted medium in
comparison to cells grown in high iron medium. When the iron concentration decreases in the medium, the result is an increase in the number of surface receptors, which augments the ability of the bacteria to bind the scarce sources of iron compounds found in their environment. The increase in SiaA expression suggests that the utilization of heme is important to the metabolism of \textit{S. pyogenes} and that the components of the SiaABC system are an integral part of the mechanisms of streptococcal iron acquisition.

The specificity and affinity of a receptor is also important to exploit the sources of iron available to the bacterium. We found that purified rSiaA can bind heme and human hemoglobin \textit{in vivo}, while it does not have the capability to bind myoglobin or haptoglobin-hemoglobin complex. Shr, a surface protein of the \textit{sia} operon is known to bind multiple hemoproteins and recent studies demonstrated that apo-Shp can acquire heme from hemoglobin and deliver it to apo-SiaA by forming a direct complex \textit{in vitro} (Liu & Lei, 2005). We propose that these receptors act in coordination with SiaA to sequester heme from different types of hemoproteins, providing a survival advantage for \textit{S. pyogenes} to colonize and persist in different environments and compensating for the absence of the outer membrane receptor which carries out this task in Gram-negative bacteria.

SiaA shares sequence homology with the members of the TroA superfamily. TroA and TroA-like proteins are metal receptors with a unique folding and ligand binding mechanism (Claverys, 2001; Tam & Saier, 1993). They are comprised of two globular subdomains connected by a single helix and can bind the metal ion in the cleft between these domains. The receptor binds and releases its substrate in a so called Venus flytrap mechanism, closing and opening the binding domain (Quiocho & Ledvina, 1996).
In addition, these proteins sometimes have a histidine-rich motif which is involved in metal binding. BtuF, the closest SiaA homolog in this group, functions as the receptor for the ABC-type transporter of vitamin B12 (cobalamin) (Karpowich et al., 2003). In collaboration with Dr. Dabney Dixon at Georgia State University, the crystal structures of the *E. coli* BtuF and the FhuD (a ferrichrome PBP) were used to predict the folding of SiaA. In heme binding proteins like cytochromes, hemoglobin and myoglobin, a pair of axial ligands holds the heme moiety in its binding pocket. The predicted SiaA model revealed that a combination of His229, Met79, Tyr63 or Tyr179 makes the possible axial ligand pair that binds heme in SiaA. A series of mutant SiaA was constructed with amino acid substitutions of the predicted axial ligands. The His229-Ala and Met79-Ala substitution mutants produced a lower optical spectrum than that of the wild-type SiaA when incubated with heme (unpublished data). This suggests that these amino acids may indeed act as the axial ligand pair.

MntC, the Mn$^{2+}$ binding protein in the cyanobacterium *Synechocytis*, is also a homolog of the TroA family. It was recently found that the disulfide bond of two cysteine residues located in close proximity in MntC affects the affinity of the protein for Mn$^{2+}$ (Rukhman et al., 2005). Interestingly, the predicted model of SiaA shows two cysteine residues (Cys47 and Cys58) located close to each other. It seems possible that the reduction and oxidation states of SiaA produce open or closed states for binding and releasing of heme. Additional studies are required to understand the dynamic motions that affect SiaA heme binding.
CONCLUDING REMARKS

Impact of bacterial iron acquisition studies

In the late 1980’s a worldwide increase in the incidence of streptococcal invasive infections, (Chuang et al., 2005) and of rheumatic fever, a post infection complications, occurred (Veasy et al., 2004). The frequency of these *Streptococcus pyogenes* associated illnesses remained constant since their resurgence. Epidemiology studies suggest that highly virulent *S. pyogenes* strains emerged from stains carried by pharyngeal infections in the last two decades (Kiska et al., 1997). The acquisition of prophages by some of the strains has contributed to their genetic diversity and virulence (Beres et al., 2004). It also became apparent that, *S. pyogenes*, which was considered until recently an extracellular pathogen, is able to invade host cells (reviewed in Chhatwal & McMillan, 2005), a property that may facilitate its persistence in asymptomatic patients. The frequency of *S. pyogenes* strains resistant to multiple antibiotics has also increased considerably in the last two decades, making the treatment of infections by such strains more difficult (Baquero, 1997; Mazzulli, 2005). Recent research has identified the M protein and other surface proteins as promising candidates for immune targets (McMillan & Chhatwal, 2005), but no successful vaccine has been developed yet. Hence, an improved understanding of the pathogenesis of this organism is needed in order to find new antibiotics, therapeutic treatments, and vaccine candidates. Since iron is essential for many of the cellular functions, the assimilation of iron from the host is an important factor in the course of a bacterial infection. For that reason, a better comprehension of
the molecular mechanisms of iron acquisition by *S. pyogenes* will contribute to the quest for vaccines and for therapeutic treatments of the diseases it causes.

**Homology analysis suggests that the SiuADBG and SiaABC systems are examples of different modes of iron acquisition**

The presence of multiple iron transporters within individual bacteria is very common. Since iron is important for the metabolism of bacteria, selective pressure and evolution resulted in the development of redundant pathways to acquire iron. *S. pyogenes* has three ABC-type transporters for iron: a transporter of ferric iron and other metals, MtsABC, and two high affinity transporters of iron complexes, SiuADBG and SiaABC. The SiaABC and SiuADBG are similar to each other and homologous to transporters of iron complexes found in both Gram-positive and Gram-negative bacteria. However, significant differences in their homology ratings and genetic organization are found (Tables 4.1 and 4.2). SiuD, the binding protein of the Siu system, is similar to the *S. aureus* heme receptor HtsA and to the siderophore binding proteins SirA and SstD. But it shares the highest similarity (56-58 %) with other, yet uncharacterized, binding proteins of *S. aureus* (SAV2284, SAV0803, and SAV1038), *Bacillus anthracis* (BA5629) and *Listeria monocytogenes* (lmo1959). In contrast, the SiaABC binding protein SiaA is similar (64-66 %) to the heme receptor IsdE from *S. aureus*, to the *L. monocytogenes* ABC-type receptor of the *svpA-srtB* cluster, and to a second uncharacterized *B. anthracis* (BA4786) receptor. The membrane permease and ATPase subunits SiaB and SiaC also share homology to the components of the staphylococcal IsdDEF and the listerial Svp transporters; SiaB has 45% identity and 63% similarity to IsdF, and SiaC has 48% identity and 69% similarity to IsdD. Additional characteristics that separate the Siu from
### TABLE 4.1 Homology of SiaABC to iron transporters

<table>
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<tr>
<th><strong>a Sia Protein</strong></th>
<th><strong>b Homolog Accession #</strong></th>
<th><strong>Name, function, and substrate</strong></th>
<th><strong>c Identity/Similarity (%)</strong></th>
<th><strong>Organism (strain)</strong></th>
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*a* Spy number corresponds to the *S. pyogenes* TIGR notation  
*b* Accession number corresponds to The Institute for Genomic Research (TIGR) accession number.  
*c* Percent homology as calculated by TIGR
### TABLE 4.2 Homology of SiuADBG to Gram-positive iron transporters

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\( ^a \) Spy number corresponds to the S. pyogenes TIGR notation
\( ^b \) Accession number corresponds to The Institute for Genomic Research (TIGR) accession number.
\( ^c \) Percent homology as calculated by TIGR
the Sia system is that the *siuABDG* is encoded as an operon with no other genes involved in iron acquisition found in its flanking regions. On the other hand, the *siaABC* genes, like the staphylococcal *isdDEF* and the listerial ABC transporter located in the *svp-srtB* cluster, is located in the genome next to additional genes encoding for accessory proteins of heme utilization. The *siaABC* genes are co-transcribed with the Shr and Shp surface receptors, putative components of cysteine transport (*siaDE*), and an ABC type transporter (*siaFGH*) of an unknown substrate. The similarities in amino acid sequence and structural genomic organization of SiaABC to the *S. aureus* IsdEFG and the listerial Svp transporters leads us to believe that they may also share a similar mechanism of recognition and utilization of heme. Therefore the *sia* operon, which was the first heme transporter to be characterized within the multi-component heme acquisition systems, appears to be a prototype for this type of heme utilization.

The role of SiuABDG, SiaABC, and MtsABC in iron acquisition in *Streptococcus pyogenes*

Iron responsive regulators use iron starvation signals, to optimize the expression of iron uptake systems, as well as that of toxins, proteases, adhesions, internalins, and other virulence determinants that increase the bacteria’s ability to survive in the host environment. The expression of the *siaABC* operon is regulated by the metal binding regulator MtsR in an iron dependent manner (Bates *et al.*, 2005). MtsR probably regulates the multi-metal transporter *mtsABC* as well, since a potential binding box was identified in the *mts* promoter region. The expression of *siuABDG* is also iron dependent, however no studies which investigate its regulation have been reported. Sequence analysis identified a region upstream of *siuA* with sequence homology to the consensus
Fur binding box, suggesting that PerR (the streptococcal homolog to Fur) rather than MtsR is involved in the iron regulation of *siuADBG*. It is then likely that the regulation of *siuADBG* and *siaABC* is more complex and that these transporters are produced in response to different sets of environmental signals, as part of different regulons.

I have shown through mutational analysis that the *siuADBG* and *siaABC* transporters are involved in the utilization of heme and hemoproteins. A single mutation in *siuG*, one of the permease units of the *siu* transporter, results in a reduction of the utilization of blood, serum, or heme in iron depleted media. The *siaB* mutant, on the other hand, demonstrated a significant reduction of growth only in the presence of hemoglobin as an iron source, but it is not as impaired in hemoglobin use as the *siuG* mutant. In addition, the *siaB* mutant does not show growth reduction in iron depleted medium supplemented with blood or serum. Inactivating *siuG* and *siaB* in the same background augments the growth phenotype seen in the *siuG* mutant. The uptake of $^{55}$Fe is also impaired in the *siuG* mutant, while the rate of incorporation by *siaB* cells is only reduced. The total amount of ferric incorporated by this mutant was similar to that observed in the wild-type strain. The presence of heme in the growth medium inhibits $^{55}$Fe incorporation by *S. pyogenes*, suggesting that the bacterial need for iron is satisfied by the utilization of heme and therefore, they take up less ferric iron. Heme could not efficiently inhibit ferric uptake in the *siaB*, *siuG* or the *siuG siaB* mutants, indicating that the activity of both transporters contributes to heme utilization.

We propose that MtsABC, SiaABC and SiuADBG work together to acquire iron from different sources (figure 4.1). Shr binds heme, hemoglobin and other hemoproteins to the cell surface. The removal of heme from these proteins may be mediated by Shr,
Fig. 4.1 Iron acquisition transporters in *Streptococcus pyogenes*. Schematic representation of the role of SiuADBG, SiaABC, and MtsABC in iron acquisition from heme and hemoproteins. The purple bar represents the peptidoglycan cell wall and the black lines represents the cell membrane.
and similar to Shp, which can strip off heme from hemoglobin, it may relay the heme to SiaA. It is possible, however, that Shr, Shp, and SiaA work in concert to remove heme from hemoproteins and therefore they may function as a multi-component receptor, similar to the bipartite hemoglobin receptor of *Neisseria meningitides* (Lewis *et al.*, 1997). It is not clear at this point if the heme is subsequently delivered into the cytoplasm or if the iron is removed and then transported by the permease. Shr is located on the cell surface and is also secreted in substantial quantities to the environment. Bates, *et al* (Bates, 2004) suggested that Shr could act as a hemophore to sequester heme found distant from the cell’s surface.

A mutant in both *siuG* and *siaB* genes is capable of incorporating as much $^{55}$Fe as the wild-type strain. This does not seem to be consistent with the observation that a mutation in *siuG* results in considerable reduction of uptake. The multi-metal transporter *mts*ABC plays a major role in metal utilization in *S. pyogenes*. It is possible that inactivating both SiaABC and SiuADBG transporters results in a compensatory effect which restores iron uptake through the MtsABC transporter. Alternatively, other iron acquisition genes may be upregulated in this mutant. In addition, inactivation of *siaB* and of *siuG* did not affect the production of the other components in these transporters. Therefore, these mutants still produce functional SiaA, Shr, Shp, and SiuD proteins, which are capable of binding and possibly extracting iron from heme. These surface proteins may make more iron available for the MtsABC system or other transporter(s) leading to a net increase in ferric transport. The similarity of the SiaABC and SiuADBG components suggests that cross talk between the different components of these
transporters may take place at the cell’s surface; such interaction may allow the acquisition of heme and/or iron as well.

SiuD was recently reported to bind ferrichrome \textit{in vitro}. Work by us and others (Liu & Lei, 2005) demonstrated that inactivation of \textit{siiuG} significantly reduces iron incorporation from ferrichrome by the streptococcal cell (appendix D.1). Siderophores can be found in the human body in niches which are inhabited by mixed microflora. Although \textit{S. pyogenes} does not make siderophores or encodes for siderophore producing genes, these findings suggests that SiuADBG may also participate in iron acquisition from siderophores produced by other microbes.

\textbf{The functional role of SiaDEFGH in iron acquisition}

The mechanisms of heme binding and release by bacterial receptors are not completely understood and crystal structures of such receptors in Gram-positives have not been reported. Analysis of the SiaA folding model revealed two cysteine residues in close proximity. The reduction of these residues and disulfide bonding formation may produce a conformational change in SiaA that allows heme binding. Disulfide bond formation is necessary for proper heme ligation during cytochrome \textit{bd} assembly in \textit{E. coli}, in which CydDC participates to maintain the redox homeostasis of the periplasm (Georgiou \textit{et al.}, 1987; Poole \textit{et al.}, 1989; Poole \textit{et al.}, 1993). CydDC are members of an exporter of glutathione (Pittman \textit{et al.}, 2005) and, with less affinity, of cysteine (Pittman \textit{et al.}, 2002), both important reducing agents in the periplasm. It is known that glutathione is imported by Gram-positives via an active transport mechanism that is yet to be characterized (Pittman \textit{et al.}, 2002). The SiaDEFGH proteins, which are encoded
by genes (spy1787-1791) located downstream of siaABC, are part of the sia operon. SiaD and SiaE are the subunits of a putative ABC-type transporter homologous to the E. coli CydDC. Therefore, it is probable that the export of a reducing agent by SiaDEFGH during iron starvation increases the reducing environment on the cell’s surface and produces a conformational change in SiaA to bind heme.

An enhanced expression of heme binding proteins on the streptococcal cell surface may increase neutrophil migration during infection

Both, access to iron and iron-dependent regulation of bacterial virulence determinants are important for a bacterial pathogen, especially in the case of invasive infections where it will encounter different niches as the disease progresses. I used the zebrafish animal model to determine the role of the SiuADBG and the SiaABC transporters in virulence of S. pyogenes. I found that inactivation of either siuG and siaB attenuates S. pyogenes virulence when injected into the peritoneum of zebrafish. The siuG mutant is also attenuated in the myosistis model, supporting that the acquisition of iron is important for successful establishment of infection. On the other hand, a mutation in siaB results in a time delayed aggressive myosistis that was characterized by the massive recruitment of neutrophils to the site of infection. These findings highlight the importance of both SiuADBG and SiaABC as iron transporters and virulence determinants of S. pyogenes.

S. pyogenes infections in zebrafish typically lack the inflammatory response, which involves the recruitment of neutrophils via chemoattractants released by the host cells. Analysis of tissue infected by the siaB mutant revealed an increase in neutrophils that was not observed in infection with the wild-type strain. The concentration of heme,
which has proinflammatory properties (Fig. 4.2), may increase during myositis due to hemolysis. The released heme probably binds to the overexpressed surface receptors (like Shr, SiaA and Shp) in the siaB mutant. It is possible that this excess of heme in the vicinity of the bacteria induces an abnormal neutrophil migration that results in excessive tissue damage and produces the sudden increase in fish mortality (Fig. 4.3).

**Significance of understanding iron transport in bacteria**

Understanding the mechanisms of iron uptake may be helpful to find new ways to treat *S. pyogenes* infections. One approach may be the designing of drugs which mimics a specific substrate. This type of drug would act as a Trojan horse by binding to a receptor and inducing its transport into the bacterial cell, where it can antagonize its metabolism. Although antibiotics are sufficient in most cases to resolve an infection produced by *S. pyogenes*, it is important to prevent associated autoimmune diseases like rheumatic fever and rheumatic heart disease. For this reason, the development of a vaccine against *S. pyogenes* is also necessary. Surface receptors are appropriate candidates as vaccine components because of their immunogenic nature and homology conservation within a group. For example, sera of from human patients of *S. pyogenes* infections presented antibodies against Shp (Lei et al., 2002), SiuD, and SiaA (Lei et al., 2004). SiaA and SiuD are homologous to surface receptors of important pathogens like *Bacillus anthracis*. Therefore, an understanding of their structure and function could be used as model for vaccine development.

The studies presented here substantiate our hypothesis that the *S. pyogenes* SiuADBG and SiaABC ABC-type transporters are involved in iron acquisition from
**Fig. 4.2** Heme is a pro-inflammatory agent. Schematic representation of the role of heme during inflammation. The presence of heme induces the overexpression of adhesion molecules in the blood vessels. Neutrophils are captured (I), rolled (II), and adhered (III) by the adhesion molecules. After vascular permeabilization, the neutrophils migrate to the infected area following taxis towards a chemoattractant like heme.
Fig. 4.3 The heme binding proteins on the siaB mutant may increase the heme concentration, enhancing the inflammatory response. Schematic representation of the proposed model of neutrophil migration during zebrafish infection by the siaB mutant. Heme is released at the site of infection as a result of the hemolytic activity of S. pyogenes. The overexpression of the heme surface binding proteins SiaA, Shp, and Shr accumulate heme on the siaB mutant surface, increasing the heme concentration. Neutrophils migrate due to the chemoattractant properties of heme.
hemoproteins and that their function is directly related to the pathogenesis of this important pathogen. This work contributes to the understanding of the mechanisms of iron transport in Gram-positive bacteria and to their knowledge of host-bacteria interactions that take place during infectious diseases.
BIBLIOGRAPHY


Donovan, A., Brownlie, A., Dorschner, M. O. & other authors (2002). The zebrafish mutant gene chardonnay (cdy) encodes divalent metal transporter 1 (DMT1). *Blood* 100, 4655-4659.


A.1 The *sia* operon. The *sia* operon consists of 10 genes flanked by transcription terminators (represented by stem-loops). The bended arrow shows the location of the *shr* promoter region. A single *siaB* mutant in *S. pyogenes* NZ131 was constructed by insertion inactivation using an *aad9* cassette (spectinomycinR).
A.2 The *siu* operon. The *siu* locus consists of four genes encoding for an ATP binding protein (*SiuA*), a substrate binding protein (*SiuD*), and two membrane permeases (*SiuBG*). A Fur-like box is found upstream of *siuA*. A single *sitG* mutant in *S. pyogenes* NZ131 was constructed by insertion inactivation using an *ermAM* cassette. Insertion inactivation of *siuG* in the background of the *siaB* mutant produced a double mutant (*siaB/siuG*).
A.3 ABC-type iron transporters in Gram-positive vs. Gram-negative bacteria. In Gram-negative bacteria a surface receptor in the outer membrane (OM) recognizes and binds an iron-binding protein (lactoferrin is shown here as an example). The extracted iron is transported to the periplasm with the energy provided by TonB, ExbB, and ExbD. In the periplasm, iron is bound by a periplasmic binding protein (PBP), which is part of an ABC-type transporter located in the inner membrane (IM). The iron is incorporated into the cell through a permease with the energy provided by an ATPase. In Gram-positive bacteria a similar mechanism is found, where the ABC-type binding protein is bound to the cell membrane (M), exposed to the environment, and is a homolog of the PBP found in Gram-negative bacteria.
B.1 RT-PCR analysis of RNA from *S. pyogenes* wild-type grown in CxCDM. Total RNA from cells grown in the presence (+) or absence (-) of 20 μM FeSO₄ (Fe) or 33 μM MnSO₄ (Mn) were used in RT-PCR. Reactions were done with the *siuG* specific primers (upper panel) or with the *recA* specific primers (lower panel).
B.2 Construction and analysis of *siu* and *sia* mutants. Schematic representation of (a) the *siuG*:ermAM allele (ZE4915 strain) and (b) the *siaB*:aad9 allele (ZE4914 strain). ZE4914 is a double mutant containing both the *siuG* and *siaB* mutations. The long arrows indicate the putative promoter regions and the stem-loop structures represent transcriptional terminators. The genes are represented by their name or number (Spy #) annotation in the *S. pyogenes* SF370 genome. (c) PCR analysis done with chromosomal DNA from the wild-type (WT), ZE4914 (G), and ZE4915 (GB) strains. The primers used to generate the *siaB* and *siuG* products are represented by the short arrows in A & B. “N” stands for negative control.
B.3 Dose-dependent experiments of $^{55}$Fe uptake by NZ131 in the presence of MnSO$_4$ and FeCl$_3$ as competing salts. *S. pyogenes* NZ131 wild-type (wt) cells were grown to early log phase in CxCDM. $^{55}$FeCl$_3$ (0.2µCi/ul, 0.02µM) was added to 1ml of culture with increasing concentrations of cold FeCl$_3$ (-●-) or MnSO$_4$ (-▲-). Radioactivity was measured after 60 minutes as counts per minute (CPM) for 5 minutes against a $^3$H standard. Data represents an average of 2 sets of duplicates. Results are shown as percent CPM incorporated as compared to control. Error bars represent the SEM.
C.1 The zebrafish infection model. (b) Histopathological examination of *S. pyogenes* infection in zebrafish. Following intramuscular injection as described above, zebrafish were fixed entirely, and longitudinal sections were prepared and stained with hematoxylin and eosin. Arrows indicate streptococcal clusters (blue) and host erythrocytes (green). 100X magnification.
Fig. D.1 Ferrichrome uptake by the wild-type and the *siuG* mutant. *S. pyogenes* wild-type (NZ131, triangles) and *siuG* (ZE4915; circles) cells were grown to early log phase in CxCDM. 0.02µM $^{55}$Fe-Ferrichrome (FC; 0.2µCi/µl) was added to cells in the absence (closed symbols) or presence (open symbols) of 5µM DNP. Cells were incubated at 37°C and culture samples, taken every 15 minutes, were washed and measured for radioactivity. Results are shown as percent of $^{55}$Fe-ferrichrome incorporated out of total input. Data represent an average of 3 sets of experiments done in duplicates; error bars represent the SEM.