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MTSR is a Dual Regulator that Controls Virulence Genes and Metabolic Functions in Addition to Metal Homeostasis in Group A Streptococcus

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MTSR IS A DUAL REGULATOR THAT CONTROLS VIRULENCE GENES AND METABOLIC FUNCTIONS IN ADDITION TO METAL HOMEOSTASIS IN GROUP A STREPTOCOCCUS

by

CHADIA TOUKOKI

Under the Direction of Zehava Eichenbaum

ABSTRACT

Group A Streptococcus (GAS) is a common pathogen of the human skin and mucosal surfaces and is capable of producing a variety of diseases. This dissertation investigates the function of a metalloregulator named MtsR in GAS physiology and disease process. An *mtsR* mutant was constructed and analyzed. Consistent with MtsR role in iron uptake regulation, the *mtsR* mutant accumulates more iron (80 ± 22.5%) than the wild type strain. Inactivation of *mtsR* results in constitutive transcription of the *sia* (Streptococcal Iron Acquisition) operon, which is negatively regulated by iron in the parent strain. We identified the promoter that controls the expression of the *sia* operon (*P* _shr_) and used it as a model to study MtsR interaction with DNA. Electrophoretic
mobility gel shift assays (EMSAs) demonstrated that MtsR binds to the shr upstream region specifically and in an iron and manganese dependent manner. DNase I footprint analysis revealed that MtsR protects a 69 bp segment in P_shr that includes 2 inverted repeats, overlapping the core promoter elements. A global transcriptional analysis determined that MtsR modulates the expression of 64 genes, of which 44 were upregulated and 20 were downregulated in the mtsR mutant. MtsR controls genes with diverse functions including immune evasion, colonization, dissemination, metal homeostasis, nucleic acid and amino acid metabolism, and protein stability. MtsR functions as a dual regulator as it binds to the promoters of the repressed genes ska, aroE, and nrdF.2, as well as the upstream region of the positively regulated genes mga, emm49, and pyrF. A 16 bp MtsR-binding consensus region was identified in all of the promoters that are directly regulated by MtsR. In conclusion, we have demonstrated that MtsR is a global regulator in GAS that controls the expression of vital virulence factors and genes involved in metal transport, virulence and metabolic pathways.

INDEX WORDS: Iron regulation, Sia operon, Metal homeostasis, Virulence, Microarray, Streptococcus pyogenes
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by

CHADIA TOUKOKI

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the College of Arts and Sciences Georgia State University 2009
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GENERAL INTRODUCTION

Bacteria must acquire all the nutrients they cannot produce themselves from the environment in order to survive and replicate. Key nutrients include metals such as iron, manganese and zinc, which serve as important cofactors for many proteins and enzymes. As part of the innate immunity the mammalian host has developed multiple strategies to withhold growth-essential metals from invading microorganisms. Bacterial pathogens in return use high affinity mechanisms to acquire and deliver metals across the cell membrane. As metals are scarce in most infection sites, bacteria also use metal availability as an environmental cue to regulate the transcription of genes that are involved in colonization and disease production. The focus of this dissertation is a metalloregulatory protein named MtsR that is used by GAS to control metal transport and virulence. This introduction provides an overview of metal availability in the human host and mechanisms used by pathogenic bacteria to obtain iron and to regulate iron uptake.

Iron and Manganese in the Human Host

Iron is essential to the vast majority of organisms as iron containing proteins play a critical role in necessary processes such as the respiratory chain (cytochromes, cytochrome oxidase), tricarboxylic acid cycle (TCA; acenitase, succinate dehydrogenase), oxidative defence systems (catalase, peroxidase, superoxide dismutase (SOD)), and nucleic acid synthesis (Jakubovics and Jenkinson 2001; Wandersman and Delepelaire 2004). The intracellular compartments store most of the 4-5 gram of iron found in the human body. The primary iron storage protein in the cell is
ferritin, which is found in high levels in the cells of the liver, spleen, skeletal muscles, and bone marrow. Ferritin has a molecular weight of 500 kDa and is comprised of 24 protein subunits that form a hollow sphere that can hold more than 4000 Fe (III) molecules as a macro-inorganic complex. A large fraction of the intracellular iron is also found in the form of heme, which is bound to heme-containing proteins such as hemoglobin, myoglobin, hemosiderin, and heme-type enzymes (Otto, Verweij-van Vught et al. 1992; Edison, Bajel et al. 2008). Hemoglobin, the most abundant metalloprotein in humans, carries more than two thirds of the total iron. Hemoglobin and heme that are released from lysed erythrocytes are bound by serum haptoglobin and hemopexin and carried to the liver for processing. Hemoglobin consists of 4 globin polypeptides (2α & 2β), each holding 4 heme prosthetic groups. In the extracellular milieu iron is found in extremely low concentration (10^{-18} M), a result of the low solubility of Fe (III) under physiological conditions and the presence of high affinity carrier proteins that sequester the free iron and shuttle it to target organs. The primary transport protein, transferrin (TF), circulates in serum and lymph and binds Fe (III) with a very high affinity. TF is composed of a single polypeptide with 2 homologous globular lobes, each with a metal binding cleft. Lactoferrin (LF) is a transferrin homolog. LF is secreted to the mucosal fluids (including breast milk) and is also found within the granules of phagocytic cells including macrophages and polymorphonuclear cells (PMNs). LF, which also has two Fe (III) binding sites, binds iron with a higher affinity than TF (Bleackley, Wong et al. 2009).

During infection, the free iron concentration in body fluid is further decreased due to the induction of an acute phase response called hypoferremia. Hypoferremia, which is triggered by the presence of pathogens or secreted toxins, is mediated by the proinflammatory cytokines interleukin-1 and tumor necrosis factor alpha (TNFα). By elevating the levels of TF production,
hypoferremia further limits the availability of iron for bacteria in the blood. LF levels are also increased during inflammation, since apo-LF is released by leukocytes during degranulation, allowing more sequestering of iron (Litwin and Calderwood 1993). Unlike iron, manganese is present in significant level in the human nasopharynx and its concentration in saliva is 36 μM. The manganese level at internal infection sites is significantly lower, and its concentration in the serum is 10^{-9} M. Manganese functions as a cofactor for a number of bacterial proteins which are involved in glycolysis, gluconeogenesis, sugar and amino acid metabolism, peptide cleavage, nucleic acid degradation, signal transduction, and oxidative stress defense. Manganese is particularly important for some lactic acid bacteria that require manganese and not iron for survival. The need for manganese may relieve the demand for iron, which is very restricted at the host environment. Manganese may also play a binary role in reducing the toxicity of reactive oxygen species, as lactic acid bacteria lack catalase but contain manganese-dependent superoxide dismutase (Litwin and Calderwood 1993; Janulczyk, Ricci et al. 2003; Johnston, Myers et al. 2004).

**Metal homeostasis in bacteria**

Metals are essential for the structural integrity of proteins, enzyme functions, metabolic processes, and for some modes of transcriptional regulation (Moore and Helmann 2005). Bacteria use various mechanisms to maintain their metallome. These include membrane-bound proteins that are involved in efflux and influx, metal reductases, soluble periplasmic and cytoplasmic shuttle proteins, and intracellular metal storage polypeptides (Pennella and Giedroc 2005). During infections, pathogenic bacteria frequently encounter high levels of toxic reactive
oxygen species (ROS) that are released by phagocytes. An excess of iron is toxic by itself and increases the potency of ROS. ROS are also formed naturally as by-products of molecular oxygen during aerobic metabolism. The main reduction products of oxygen are superoxide and hydrogen peroxide, which are only moderately reactive under physiological conditions. Fe (II), however, reacts with superoxide and hydrogen peroxide to produce robust and damaging radicals (Fenton reaction) (Andrews, Robinson et al. 2003). In order to maintain the required intracellular metal equilibrium and avoid toxicity, bacteria must coordinate metal uptake in response to metal availability and keep the intracellular metals in a non-toxic form. The withholding of iron by bacterioferritin, for example, prevents its interactions with ROS.

Metal-responsive transcription regulators are employed by bacteria to regulate the expression of genes involved in metal transport and defense (Hantke 2001). DNA binding by such regulatory proteins depends on the intracellular concentration of certain metals. Seven major families of metal-responsive regulators, which are named after the founding members, were identified in bacteria. These families of metalloregulators allow the transcriptional response to Mn, Fe, Co, Ni, Cu and Zn, the most important transitional metals in biological systems. Five of the seven sensor families use a conserved pervasive winged helix-turn-helix domain to bind DNA. The ArsR, MerR, CsoR, and CopY families function by turning “on” sequestration systems and metal efflux in response to toxic metals. Proteins from DtxR, Fur, and NikR families, on the other hand, act by down regulating metal uptake systems in the presence of metal surplus (Giedroc and Arunkumar 2007).
Iron uptake in pathogenic bacteria

Microorganisms have to compete with the host for iron in order to colonize and produce an infection. Bacterial pathogens have developed several sophisticated mechanisms to overcome the extremely low iron availability in the human body. Many pathogens produce hemolysins, which will free intracellular iron. Cell lysis typically liberates ferritin and heme-bound proteins such as hemoglobin and myoglobin. Bacteria often express high affinity receptors that directly bind to host proteins such as TF, LF, hemoglobin, and sequester Fe (III) or heme and deliver it across the bacterial membrane. Other mechanisms use secreted mediators, such as siderophores or hemophores, to obtain Fe (III) or heme from the environment or host proteins. Bacteria that use such systems use surface receptors to mediate the uptake of the loaded siderophores, or to unload the heme from hemophores and deliver it into the bacterial cell. Many pathogens can use one or more host proteins to fulfill their iron needs. Some pathogens such as Yersinia pestis use free heme as a main source of iron for growth, while other bacteria including Vibrio vulnificus exploit the hemoglobin-haptoglobin complex. Haemophilis influenzae is able to obtain iron from several host proteins including heme-hemopexin, heme-albumin, hemoglobin-haptoglobin, and hemoglobin (Litwin and Calderwood 1993).

Fur-dependent regulation in Gram-negative pathogens

Early studies in Escherichia coli identified and characterized a global metalloregulator named Fur (Ferric-uptake-regulator). More then 90 genes in E. coli are regulated by iron via Fur. The Fur regulon consists of genes that encode for biosynthesis and transport of siderophores, virulence factors, and genes implicated in iron metabolism and oxidative stress response. Fur is
a 17 kDa homodimer protein that is comprised of two domains. The amino terminal domain has a helix-turn-helix motif that is involved in DNA binding. The carboxyl terminal domain contains two metal binding sites and has shown to be essential for dimerization. Fur forms a complex with Fe (II) and subsequently binds to a 19 bp specific palindromic DNA sequence, which is found in Fur-regulated promoters (Hantke 2001). While functioning mostly as a repressor, Fur was also shown in some cases to directly activate gene expression (Lee, Bang et al. 2007). The *E. coli* Fur is the prototype for a large family of iron sensory regulatory proteins, which are found amongst many Gram positive and Gram negative bacteria, including important pathogens such as *Staphylococcus epidermidis*, *Bordetella spp.*, and *Bacillus spp.* Related families include PerR and Zur which mediate the response to oxidative stress and zinc homeostasis, respectively (Andrews, Robinson et al. 2003).

**The DtxR family**

The DtxR family of metal-dependent regulatory proteins is functionally similar to the Fur family; DNA binding by proteins from both families is allosterically activated by metals. At the structural level, however, the DtxR proteins share very low homology to proteins from the Fur group. The prototype, DtxR (Diphtheria toxin Repressor), was identified in *Corynebacterium diphteriae*. DtxR was initially referred as the DtoxR protein as it represses the tox operon, which encodes genes involved in the production and expression of the diphtheria toxin in an iron dependent manner. Proteins from the DtxR cluster consist of two domains that are linked by a flexible tether. DtxR N-terminal domain is implicated in metal binding, dimerization, and DNA recognition. The C-terminal domain contains two metal binding sites and folds into a SH3-like
structure, which is a docking domain found in eukaryotic proteins involved in signal transduction. The function of the C-terminal domain, however, has yet to be determined, although it is implicated in protein-protein interactions. DtxR-type regulators can use several metals as corepressors. However, Fe (II) was shown to be the biological relevant metal in vivo (Love, VanderSpek et al. 2003). Several related proteins such as MntR (Bacillus subtilis) use manganese as their primary regulatory metal (Kliegman, Griner et al. 2006). DtxR operator consists of a 19 bp palindromic DNA sequence that is found at regulated promoters. Two DtxR dimers bind each to the opposite face of the duplex. Since the discovery of the C. diphtheriae DtxR, many orthologs have been identified and characterized in a variety of Gram positive bacteria including Streptomyces spp., Staphylococci spp., Streptococci spp., and Mycobacterium spp. (Andrews, Robinson et al. 2003). DtxR orthologs in Streptococci species, such as SloR (Streptococcus mutans), PsaR (Streptococcus pneumonia) and ScaR (Streptococcus gordonii), are highly related and appear to diverge early from the proteins found in diphtheria and mycobacteria species (Pennella and Giedroc 2005). Some bacteria, including GAS code for both Fur and DtxR type proteins.

**DtxR in Corynebacterium species**

*C. diphtheriae* is a Gram-positive bacterium that is the causative agent of diphtheria. The diphtheria toxin is an ADP-ribosylating enzyme that kills susceptible eukaryotic cells by inhibiting protein synthesis. The toxin is encoded by the tox gene and is carried by a number of corynebacteriophage genomes. Only *C. diphtheriae* lysogenes carrying a tox+ prophage are able to produce the diphtheria toxin. DtxR mediates the iron-dependent expression of the tox gene
Schmitt, Twiddy et al. 1992; Kunkle and Schmitt 2003). The DtxR regulon in *C. diphtheriae* consists of genes encoding siderophores, high affinity iron uptake systems, and genes implicated in iron transport (Schmitt, Twiddy et al. 1992; Lee, Wang et al. 1997; Kunkle and Schmitt 2003). The DtxR regulon of the related bacterium *C. glutamicum* shows, consists of genes encoding transcriptional regulators and pathways that mediate the formation and repair of iron-sulfur clusters in addition to genes involved in iron acquisition and storage (Wennerhold and Bott 2006). The DtxR regulatory protein has become the prototypic member of a large family of metal dependent transcriptional regulators in Gram-positive bacteria (Love, VanderSpek et al. 2003).

**IdeR in Mycobacterium tuberculosis**

*M. tuberculosis* is a human pathogen of the respiratory system. Tuberculosis is initiated with the inhalation of *M. tuberculosis*-containing droplets. *M. tuberculosis* is a facultative intracellular pathogen that proliferates mainly within the mononuclear phagocytes. Like most human pathogens *M. tuberculosis* has to cause infection in order to acquire iron from its environment. Therefore, *M. tuberculosis* has developed several iron acquisition and transport systems including siderophore production and utilization systems (mycobacterin) (Rodriguez and Smith 2003). *M. tuberculosis* encodes a DtxR-like regulatory protein IdeR (Iron-Dependent Regulatory protein). The first 180 amino acids of IdeR share 90% amino acid identity with DtxR. IdeR has two metal binding sites and three functional domains; the amino terminal domain with the helix-turn-helix DNA binding motif, a dimerization domain which contains several of the amino acids that comprise of the metal binding sites (the other amino acids are located in the first 180 amino
acids of the protein) and a C-terminal domain with SH3-like fold. IdeR is essential for *M. tuberculosis* viability, as *ideR* cannot be deleted unless a second copy of the gene is present in trans in the bacteria. IdeR binds to a 19 bp inverted repeat consensus sequence in promoters of genes encoding for siderophores and iron storage. Comparing the IdeR binding consensus with the DtxR binding signature shows low homology (7 out of 19 bp) (Yellaboina, Ranjan et al. 2006). IdeR represses the transcription of genes encoding for siderophores and directly activates the expression of genes important for iron storage. Microarray analysis revealed IdeR regulates also the expression of genes encoding transcriptional regulators, enzymes that play a role in lipid metabolism, and a membrane protein involved in virulence (Rodriguez, Voskuil et al. 2002).

**ScaR in *Streptococcus gordonii***

*S. gordonii* is a commensal member of the human oral flora, a colonizer of dental plaque and one of the etiological agents of endocarditis. *S. gordonii* forms a highly structured biofilm while colonizing the tooth enamel and produces dental plaque. Studies have shown that metal ions play an essential role in biofilm formation and pathogenesis (Mitrakul, Loo et al. 2004). At least two manganese transport systems are encoded by *S. gordonii*. One transporter is encoded by the *adc* operon, which consists of four genes; *adcR, adcC, adcB*, and *adcA*. AdcR is a metal-dependent repressor protein and the three proteins (AdcC through AdcA) make up a typical ABC transporter. AdcB is a lipoprotein that functions as the ligand binding component, AdcA is a permease that delivers the ligand through the cytoplasmic membrane, and AdcC is an ATPase that provides energy for the process. The four-gene operon plays an important role in manganese homeostasis and biofilm formation (Loo, Mitrakul et al. 2003). A second manganese transporter
is encoded by the $sca\text{CBA}$ operon. The Sca transporter is a high affinity manganese acquisition system. Its expression is induced under low manganese concentration (Kolenbrander, Andersen et al. 1998). The $sca$ operon encodes an ATP-binding protein (ScaC), a membrane component (ScaB), and a lipoprotein (ScaA). $S.\text{gordonii}$ codes for a metal dependent transcription regulator ScaR, which regulates the transcription of the $sca$ operon in response to manganese availability.

In the presence of high intracellular manganese levels, ScaR binds to manganese and represses the $scaC$ promoter. ScaR primary sequence shares 26% identity with the sequence of DtxR from $C.\text{diphtheriae}$. In contrast to DtxR, ScaR regulates transcription in response to Mn (II), and not to Fe (II) or Fe (III). ScaR recognizes a 46 bp sequence region in the $scaC$ promoter region with two palindromic elements (Jakubovics, Smith et al. 2000).

**PsaR in Streptococcus pneumoniae**

*S. pneumoniae* is a Gram positive pathogenic bacterium that colonizes the human nasal mucosa. This pathogenic microorganism produces upper respiratory tract infections such as acute sinutisis and otitis media infection and can spread from the upper respiratory tract to the lungs, causing pneumonia. In some cases *S. pneumoniae* can cause invasive diseases including meningitis, bacteremia, sepsis, or brain abscess. Manganese is essential for *S. pneumoniae* growth and manganese availability is an important environmental cue for the pathogen. *S. pneumoniae* uses the manganese ABC-type transporter, encoded by the $psa\text{BCA}$ genes, to acquire this metal from the external environment. Inactivation any of the $psa$ genes leads to significant attenuation of growth and virulence incompetence in animal models for sepsis and respiratory tract and otitis media infections. Inactivation of the *S. pneumoniae* DtxR homolog, PsaR, results in
derepression of the *scaBCA* genes as well as genes that encode surface proteins, sortase enzymes, and a transcription regulator. Gene regulation by PsaR is required for full virulence and pneumonia production (Johnston, Briles et al. 2006; Hendriksen, Bootsma et al. 2009). Interestingly, while manganese is PsaR corepressor, the repressor binding to zinc has the opposite effect, and leads to the protein release from the DNA (Kloosterman, Witwicki et al. 2008).

**SloR in *Streptococcus mutans***

*S. mutans* is a Gram-positive facultative anaerobic bacterium that is commonly found in the human oral cavity and is a significant contributor to tooth decay and consequently can also cause endocarditis. The bacterium can also form biofilms, and is well adapt to oxidative stress and low pH conditions. *S. mutans* uses host dietary sucrose along with other carbon sources for cellular energy requirements. Like all Streptococci, *S. mutans* does not possess cytochromes. The bacterium generates energy using glycolysis, while releasing mixed acids as fermentation products (Paik, Brown et al. 2003). The discharge of acids contributes to pathogenesis of dental caries by *S. mutans*. Metal homeostasis, particularly iron and manganese, play an important role in the physiology and virulence in *S. mutans*. The SloR metalloregulator, a DtxR homolog, was initially identified to represses the *sloABC* operon, a manganese and iron ABC-type transporter. Global transcription analysis revealed that SloR also regulates genes that are involved in adherence, biofilm formation, genetic competence, oxidative stress tolerance, and antibiotic resistance (Rolerson, Swick et al. 2006).
Metal uptake and regulation in Group A Streptococcus

The Gram-positive bacterium GAS (S. pyogenes) is an obligate human pathogen that is an agent of a variety of non-invasive and invasive diseases. GAS is a β-hemolytic bacterium that can cause superficial infections such as pharyngitis, tonsillitis, otitis media, scarlet fever, cellulitis and impetigo. GAS infections can occasionally evolve into severe immunological complications such as acute rheumatic fever (RF), rheumatic heart disease, and glomerulonephritis. GAS can also cause severe invasive illnesses such as myositis, necrotizing fasciitis, and streptococcal toxic shock syndrome (STSS). The CDC reported in their Active Bacterial Core Surveillance Report of 2008 that invasive GAS infections occurred at a rate of 3.8 per 100,000 population, accounting for 11,500 cases and 1,500 deaths (Centers for Disease Control and Prevention. 2009. Active Bacterial Core Surveillance Report, Emerging Infections Program Network, Group A Streptococcus, provisional 2008).

GAS requires iron for growth and can obtain it from hemoproteins using a number of sophisticated high affinity iron scavenging systems to compete for iron while colonizing the human host (Litwin and Calderwood 1993). GAS acquires iron from the host proteins ferritin, hemin, hemoglobin, and myoglobin (Eichenbaum, Muller et al. 1996). Current research describes three ABC-type iron transport systems in GAS named siaABC (htsABC), mtsABC, and siuABDG (ftsABCD). The sia system acquires iron from hemoglobin, myoglobin, heme-albumin, and hemoglobin-haptoglobin (Bates, Montanez et al. 2003; Lei, Liu et al. 2003). The mts acquisition system is involved in manganese and iron uptake (Janulczyk, Ricci et al. 2003; Hanks, Liu et al. 2006) and the siu system acquires iron from hemoglobin and ferrichrome (Hanks, Liu et al. 2005; Montanez, Neely et al. 2005). GAS encodes a Fur-like metalloregulator named PerR, which is involved in the response of GAS to oxidative stress. PerR regulon
includes genes involved in sugar utilization and transport as well as metal homeostasis, and oxygen defense (Ricci, Janulczyk et al. 2002; Brenot, King et al. 2005; Gryllos, Grifantini et al. 2008; Weston, Brenot et al. 2009). In this dissertation we identify a second metalloregulator named MtsR and characterize its function and role in GAS physiology and disease production.
CHAPTER I CHARACTERIZATION OF MTSR, A NEW METAL REGULATOR IN GROUP A STREPTOCOCCUS, INVOLVED IN IRON ACQUISITION AND VIRULENCE

INTRODUCTION

Iron acquisition is a delicate balancing act in bacteria. While iron is important for a wide range of metabolic functions from DNA synthesis and repair to the electron transport chain, excess iron uptake can be deleterious to the cell due to the involvement of iron in the generation of oxygen radicals by the Fenton reaction. Iron homeostasis in bacteria is typically maintained by metal-dependent transcription regulators belonging to the Fur or the DtxR family (Andrews, Robinson et al. 2003). Fur and DtxR regulate the production of iron storage proteins and of multiple iron acquisition systems including siderophore biosynthesis machinery and transporters for iron complexes and inorganic iron (Panina, Mironov et al. 2001; Qian, Lee et al. 2002; Rodriguez, Voskuil et al. 2002; Thompson, Beliaev et al. 2002; Kunkle and Schmitt 2003). Deregulation of iron homeostasis often leads to oxidative stress, and the disruption of dtxR or fur frequently results in an increased sensitivity to hydrogen peroxide (Touati, Jacques et al. 1995; Dussurget, Rodriguez et al. 1996; Horsburgh, Ingham et al. 2001; Oram, Avdalovic et al. 2002; Rodriguez, Voskuil et al. 2002).

In pathogenic bacteria, Fur and DtxR not only play a role in iron acquisition and oxidative stress, they often control the expression of virulence factors as well. For example, the expression of toxins such as exotoxin A in Pseudomonas aeruginosa (Barton, Johnson et al. 1996), Shiga-like toxin in Escherichia coli (Calderwood and Mekalanos 1987) and diphtheria toxin (Schmitt and
Holmes 1991) are under the control of Fur or DtxR proteins. Mutations in either protein family may be lethal or result in attenuated virulence in bacterial pathogens such as *Listeria monocytogenes* (Almengor and McIver 2004), *Mycobacterium tuberculosis* (Manabe, Saviola et al. 1999; Rodriguez, Voskuil et al. 2002) *P. aeruginosa* (for review see (Vasil and Ochsner 1999)), and *Staphylococcus aureus* (Horsburgh, Ingham et al. 2001; Ando, Manabe et al. 2003).

While Fur- and DtxR-like proteins are very different from each other in primary sequence and DNA binding sites, members of both families contain a winged-helix-turn-helix motif for DNA binding (for review see (Martinez-Hackert and Stock 1997)) and have a similar mode of action. Metal binding to both types of regulators results in the homodimer binding at or near promoter regions, leading to gene repression. Protein fusions between Fur and the λ phage repressor demonstrated that the amino terminus of Fur is responsible for DNA binding, and the carboxy terminus is involved in dimerization (Stojiljkovic and Hantke 1995). Members of the Fur family of metalloregulators include Zur, which is involved in zinc homeostasis (Hantke 2001), and PerR, which controls peroxide resistance (Mongkolsuk and Helmann 2002). Both PerR and Zur can be found in a variety of Gram-positive as well as Gram-negative bacteria.

DtxR functions as a homodimer, and each monomer contains an N-terminal DNA binding domain, a central metal-binding and dimerization domain, and a flexible domain with an SH3-like fold. The SH3-like domain is thought to contribute to the metal-mediated activation of DtxR, as it carries two of the ligands in the ancillary binding site, and contains residues that modulate the behavior of the site (Tao and Murphy 1993; Wang, Schmitt et al. 1994; Qiu, Verlinde et al. 1995; Love, VanderSpek et al. 2003). The DtxR family comprises a variety of metalloregulators that use, in vivo, iron, manganese, or both as co-repressors, including SirR in *S. epidermidis* (Fe and Mn) (Hill, Cockayne et al. 1998); MntR in *S. aureus* (Mn) (Horsburgh, Wharton et al. 2002;
Ando, Manabe et al. 2003), Corynebacterium diphtheriae (Mn) (Schmitt 2002), and Bacillus subtilis (Mn) (Al-Tawfiq, Fortney et al. 2000); IdeR in M. tuberculosis, M. smegmatis (Fe), (Qiu, Verlinde et al. 1995; Dussurg et, Rodriguez et al. 1996), Rhodococcus erythropolis, and R. equi (Fe) (Boland and Meijer 2000); and TroR in Treponema pallidum (Mn and/or Zn) (Posey, Hardham et al. 1999; Hazlett, Rusnak et al. 2003).

The Gram-positive bacterium Streptococcus pyogenes, or Group A Streptococcus (GAS), is an obligate human pathogen. GAS causes a wide range of maladies in humans from noninvasive diseases such as impetigo and pharyngitis, to invasive diseases such as streptococcal toxic shock syndrome, cellulitis and necrotizing fasciitis (Cunningham 2000). Cases involving invasive GAS are rare but dangerous. In 2002, the CDC reported that over 9000 cases of invasive GAS infections occurred within the United States alone and 14 percent of those cases ended in death (Kotloff, Corretti et al. 2004). Infections by GAS can also lead to the nonsuppurative sequelae rheumatic fever and glomerulonephritis.

GAS has been shown to require iron for growth and can use heme, hemoglobin, ferritin, myoglobin, and catalase but not transferrin or lactoferrin as iron sources (Eichenbaum, Green et al. 1996; Elsner, Kreikemeyer et al. 2002; Bates, Montanez et al. 2003). GAS can strip iron from both hemoglobin and haptoglobin-hemoglobin complexes (Francis, Booth et al. 1985) and has also been shown to acquire $^{55}$Fe from the culture media (Janulczyk, Pallon et al. 1999). A multi-metal transport system (mts) involved in the transport of zinc, manganese and iron has been identified in GAS (Janulczyk, Pallon et al. 1999; Janulczyk, Ricci et al. 2003). An mts mutant was affected in growth under aerobic or metal limiting conditions, and was attenuated for virulence in a mouse model (Janulczyk, Ricci et al. 2003). We recently described an iron-regulated 10-gene operon, sia, which mediates acquisition of iron from hemoproteins (Bates,
Montanez et al. 2003). The *sia* locus encodes the hemoprotein receptor Shr, the heme binding protein Shp (Lei, Smoot et al. 2002), and an ABC transport system belonging to the iron complex family. Inactivation of the *sia* locus results in reduced iron uptake and decreased hemoglobin binding. A third transporter for iron complexes (*spy0386-0383* in the SF370 database) is encoded by GAS (Graham, Smoot et al. 2001).

Very little is known about the mechanisms involved in regulating and maintaining iron-homeostasis in GAS. Iron has been shown to influence the production of a variety of GAS products such as streptolysin S (Griffiths and McClain 1988), the M protein (McIver, Heath et al. 1995), and the secretion of the surface glyceraldehyde-3-phosphate-dehydrogenase (Eichenbaum, Green et al. 1996). Recently a Fur homolog, PerR, was found in *S. pyogenes*. A *perR* mutant exhibited constitutive resistance to peroxide stress (Drazek, Hammack et al. 2000), and was hypersensitive to paraquat (Ricci, Janulczyk et al. 2002; Brenot, King et al. 2005). PerR positively regulates *sod* and *mtsA* genes and represses *mrgA*, which encodes a Dps-like protein (Ricci, Janulczyk et al. 2002; Brenot, King et al. 2005). Mutants in *perR* were attenuated in murine infection models (Ricci, Janulczyk et al. 2002; Brenot, King et al. 2005). A putative regulatory protein (named MtsR) that belongs to the DtxR family of metalloregressors is divergently transcribed from the *mts* gene cluster (Jakubovics, Smith et al. 2000). In this study, we have begun to characterize the role of MtsR in GAS, and demonstrate that it regulates iron uptake genes and is required for full virulence in a zebrafish infection model.
MATERIALS AND METHODS

Strains, media, and growth conditions. GAS NZ131 (M49) has been previously described (Simon and Ferretti 1991). GAS was grown in Todd Hewitt broth (TH; Difco Laboratories), TH with 0.2% Yeast Extract (THY), and THY with 10 mM Tris, adjusted to pH 6.9 (ZTH) (Eichenbaum, Green et al. 1996). ZTH medium was analyzed for metal content by inductivity coupled plasma mass spectrometry (ICP-MS) analysis (Laboratory for Environmental Analysis, University of Georgia at Athens). This analysis demonstrated that ZTH contains about 17.5±6.5 µM iron, 0.53±0.2 µM of manganese, and 15.5±0.2 µM zinc, depending on the batch and manufacturer. To starve for iron, cells were grown in ZTH containing 10 mM nitrilotriacetic acid (Bates, Montanez et al. 2003). NTA is a metal chelator with high specificity for iron; its first stability constants (log K₁) for Fe⁺³ and Fe⁺² are 15.87 and 8.83 respectively (Furia and Chemical Rubber Company. 1972). Since NTA has affinity for zinc, manganese, magnesium, and calcium (log K₁ of 10.45, 7.44, 5.4, and 6.41 respectively, (Furia and Chemical Rubber Company. 1972)), 0.55mM of MgCl₂, MnCl₂, CaCl₂, and ZnCl₂ was added to NTA-containing media (ZTH-NTA). For the infection of zebrafish, GAS was grown in THY supplemented with 2% proteose peptone (THY+P, Difco). In all cases, GAS was grown statically in acid-washed Klett flasks or screw cap polypropylene tubes at 37°C.

DNA manipulations. Cloning, plasmid construction, chromosomal DNA extraction, and restriction analyses were done according to standard protocols as described (Sambrook, Fritsch et al. 1989; Eichenbaum and Scott 1997). The primers used in this study are listed in Table 1.
<table>
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<th>Name</th>
<th>Sequence</th>
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<td>5’ cccgaatcttcaagcttctagctgctttaa 3’</td>
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</table>
Construction of plasmids and strains. (i) Construction of the mutant strain ZE491. To generate an mtsR mutant strain, a three step cloning approach was used resulting in an mtsR allele with an internal deletion and an insertion of a kanamycin resistant cassette. A 1.9 kb fragment containing the upstream region and up to the first 202 bp of the mtsR coding sequence was amplified by PCR from the NZ131 chromosome with the primers mtsB-F and dtxR-R, and cloned into the SacI and EcoRI sites of pBluescript II KS (Stratagene) resulting in plasmid pBBS-1. A 2.1 kb PCR fragment covering the last 67 bp of mtsR and the upstream sequence was amplified from the chromosome, using the primers dtxR-F and mutTF, and cloned into the EcoRI and AvaI sites in pBBS-1 resulting in plasmid pBBS-2. The kanamycin resistance cassette (1.4 kb) was amplified by PCR from pJRS700 using primers Kan BB A and Kan BB S and cloned into the EcoRI and NheI sites on pBBS-2 resulting in pBBS3Z. Plasmid pJRS700 is a derivative of pVE6037 (Maguin, Duwat et al. 1992) produced by the ligation of a 4.1 kb HindIII fragment of pVE6037 to a kanamycin resistance cassette coding for the aphA-3 gene flanked by the termination signals of the Omega insertion sequence (Perez-Casal, Caparon et al. 1991). A 4.9 kb fragment containing the mtsR::kan allele was amplified by PCR from pBBS3Z using the primers mutTF and mtsB S, and introduced to strain NZ131 by electroporation (Bio-Rad Gene Pulser). Allelic exchange events were selected by plating the transformants on THY with kanamycin (70µg/ml). The resulting mtsR mutant strain was named as ZE491. The chromosomal mutation in ZE491 was confirmed by PCR using the mutTF and DtxRLKOS primers.

(ii) Construction of pZEDH3.1. A 654 bp fragment from the NZ131 chromosome containing the mtsR coding sequence was amplified with the primers ZEDHS and ZEDHA and cloned into the NcoI site of pIVEX2.3 (Roche). The resulting construct, pZEDH3.1 contains a C-terminus
fusion of MtsR to a His$_6$ tag (rMtsR) driven by a T7 promoter. Restriction enzyme analysis verified the orientation of the insert in the resulting clones.

**Expression and Purification of rMtsR (MtsR-His$_6$).** BL21-DE3 *E. coli* cells harboring plasmid pZEDH3.1 were grown at 37°C in Luria Bertani (LB) containing ampicillin (100 μg/ml). Once the cells reached an OD$_{600}$ of 0.6, 0.6 mM IPTG was added to the medium and the culture was incubated overnight at 20°C. Cells were then harvested, lysed by sonication, and the rMtsR was purified over a nickel column using the ProBond purification system (Invitrogen) according the manufacturer’s protocol. The rMtsR was then applied to an anion exchange column (HighTrap Q5ml column) and fractions containing the purified protein were detected on the chromatogram and examined by SDS-PAGE and Western blot analysis with anti His$_6$ antibodies (Invitrogen). The purified rMtsR was quantified by the Bradford assay (Bio-Rad).

**Detection of Streptococcal proteins.** Total proteins were prepared from cells in their logarithmic phase of growth, grown in either ZTH or ZTH-NTA as described by Bates et al (Bates, Montanez et al. 2003). Total proteins were standardized based on cell number, separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked at room temperature for 15 minutes using 5% skim milk in PBS – TWEEN, rinsed, and incubated with polyclonal rSiaA or rShr antibodies for 2 hours (Bates, Montanez et al. 2003). After rinsing, membranes were incubated in goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma). Blots were then rinsed, and the color developed.
**Electrophoretic Mobility Gel Shift Assay (EMSA).** The EMSA was done according to Schmitt et al (Schmitt, Twiddy et al. 1992). A 337 bp fragment containing the upstream region of the *sia* operon (Pshr fragment) was amplified from the NZ131 chromosome using siaGSF and siaGSR primers. The PCR products were purified using the Rapid PCR Purification System (Marligen Bioscience Inc) and end labeled with $[^{32}\text{P}]$ ATP using T4 Kinase (Invitrogen). The $^{32}$P-labeled DNA fragments were purified from an 8% polacrylamide gel, using the Rapid PCR Purification System kit. In the DNA binding assay, increasing concentrations of rMtsR were incubated with approximately 0.5 pmol labeled DNA fragment for 10 min at room temperature in a 24 µl reaction buffer containing 20mM Na$_2$HPO$_4$, 50mM NaCl, 5mM MgCl$_2$, 2mM dithiothreitol, 0.4 mg/ml Bovine serum albumin, 0.2 mg/ml sheared salmon sperm DNA (Ambion), and 9.6% glycerol. In some cases the binding assays were done in the presence of 250µM EDTA with or without FeSO$_4$ (made fresh). To remove the metal from the purified rMtsR, the protein was incubated with 2mM EDTA for 15 min at 4°C, and then dialyzed overnight against 10% glycerol and 20mM sodium phosphate buffer pH 7.0. Ten µl of the binding reactions were loaded per well (without a loading dye) and fractionated over 5% polyacrylamide gel containing 2.5% glycerol, 20mM Na$_2$HPO$_4$, and 2mM dithiothreitol. Electrophoresis was performed at 65 volts at room temperature for 1.5 hours using 20mM Na$_2$HPO$_4$ buffer containing 1mM dithiothreitol. Gels were dried at 80°C for 25 minutes and visualized using a phosphoimager.

**RNA methods. (i) RNA preparation.** GAS was harvested at the logarithmic growth phase by pouring over frozen Tris buffer (100 mM Tris pH 6.8, 2mM EDTA) containing 0.06% sodium azide. Total RNA was prepared from the cell pellets as described in MacDonald et al
RNA was pelleted by sedimentation through 5.7M CsCl. RNA pellets were allowed to dry before suspending them in RNasequr (Ambion), and contaminating genomic DNA was removed by DNase using DNA-Free (Ambion) per manufacturer’s instructions. RNA was quantified spectrophotometrically, and its integrity was examined by agarose gel electrophoresis. The absence of DNA contamination was verified by PCR.

(ii) RT-PCR. cDNA was generated with Superscript III reverse-transcriptase (RT, Invitrogen) using 1µg of RNA according to the manufacturer’s specification except for the following: gene specific primers were used and after denaturation, primers were allowed to anneal by cooling in 5ºC increments. Reactions were terminated by heat inactivation of the enzyme. SRAR and orfX-delA primers were used in the generation of cDNA, and 1/20 of the reaction was used as a template for 25 cycles PCR. Primer pairs used for the PCR were SRAR, and SRAL, orfX-del S and orfX-delA.

Streptonigrin and hydrogen peroxide susceptibility. Sensitivity to streptonigrin was determined as described in Bates et al (Bates, Montanez et al. 2003) with the exception of using THY as the growth media. Briefly, cells from glycerol stocks were used to inoculate 5ml of THY in either the presence or absence of 0.36µM streptonigrin (Sigma) in screw capped tubes. Growth was measured by the OD$_{600}$ after overnight growth and expressed as a percentage of growth in the presence of the drug compared to growth without. For testing the sensitivity to hydrogen peroxide, overnight cultures of NZ131 and ZE491 grown in THY were used to inoculate 5ml
THY containing 1mM hydrogen peroxide (Fisher) and allowed to grow overnight. Cell growth was determined after overnight incubation by measuring the OD$_{600}$.

55Fe accumulation assays. Iron accumulation assays were performed essentially as previously described (Janulczyk, Ricci et al. 2003) with small modifications. Overnight cultures were used to inoculate (1:500, OD$_{600}$ of 0.02) 5ml THY medium containing $^{55}$FeCl$_3$ (0.4μCi/μl, 0.04μM, Amersham Pharmacia Biotech) and incubated at 37°C. Since incorporation of $^{55}$Fe by the cells following an overnight growth was low, a second passage (from an overnight culture, 1:500, OD$_{600}$ of 0.02) in THY-$^{55}$Fe medium was used. 1ml culture samples (OD$_{600}$ of 0.9) were then drawn in duplicates, supernatants were collected, and the cells were washed twice with Saline containing 10 mM NTA, resuspended in 0.2ml Saline, and mixed with 5 ml of Ready Safe scintillation cocktail (Beckman). The collected culture supernatants were similarly mixed with scintillation cocktail. Radioactivity was then measured as counts per minute (CPM) for 5 minutes against a $^3$H standard. The fraction of $^{55}$Fe associated with the bacterial cells was calculated by dividing the CPM of the cell pellet by the sum of the CPM of the cell pellet and the CPM of the culture supernatant, and was expressed in percent.

Infection of Zebrafish with NZ131 and ZE491. GAS were grown in THY+P to logarithmic phase, harvested, washed and injected into zebrafish (Danio rerio) either intraperitoneally or intramuscularly as described in Neely et al (Neely, Pfeifer et al. 2002). Five groups of experiments ranging from 5 to 10 fish were used per strain per injection route (totaling 33 fish
per strain per injection route). Fish were monitored for 48 hours post infection. Zebrafish care and feeding was done as previously described (Neely, Pfeifer et al. 2002).

**Computer analysis.** GAS sequence used for alignments and primer design from the sequenced M1 strain, SF370, available at the NCBI microbial genome project (Accession No. NC_002737). The sequences were analyzed and manipulated using Vector NTI (InforMax). Protein sequences were aligned using ClustalW from EMBL-EBI (http://www.ebi.ac.uk/clustalw/). The sequence of MtsR was submitted to RASMOLL-MODEL (http://www.umass.edu/microbio/rasmol/) for molecular modeling. The search yielded two proteins with enough similarity to fold MtsR, DtxR (1c0wB) and IdeR (1fx7(A-C)). The predicted model was visualized using the Swiss Pbd viewer 3.7 (SP5) (http://us.expasy.org/spdbv). Phylogenetic trees were generated using the Gene Bee ClustalW 1.75 (http://www.genebee.msu.su/clustal/basic.html) with the following parameters: Slow alignment, BLOSUM Protein weight matrix, without Kimura’s corrections, and displayed using the PHYLIP format. Statistical analyses were done using StatView (SAS Institute).

The following accession numbers were used for the metalloregulators: *S. pyogenes* MtsR, NP_268746; *S. gordonii* ScaR, AAF25184; *T. pallidum* TroR, AAC45729; *C. diphtheriae* DtxR, A35968; *M. tuberculosis* H37Rv IdeR, NP_217227; *S. mutans* UA159 SloR/Dlg, NP_720655; *S. epidermidis* ATCC 12228 SirR, NP_763963; *B. subtilis* Fur, P54574; *Pasteurella multocida* PM25 Fur, AAD01812; *Salmonella typhimurium* LT2 MntR, Q8XH22; *S. aureus* Mu50 Fur, Q9R3G5; *Bordetella pertussis* Fur, Q45765; *S. typhimurium* LT2 Fur, NP_459678; *Haemophilus ducreyi* Fur, P71333; *Yersinia pestis* Fur, P33086; *P. aeruginosa* Fur, Q03456; *B. subtilis* PerR, P71086; *R. equi* IdeR, AAG16749; *S. aureus* MntR, AAL50775; *B. subtilis* MntR, P54512; *E.
coli Fur, P06975; *H. influenzae* Fur, P44561; *S. pyogenes* PerR, NP_268566; *M. smegmatis* IdeR, AAA86056; *E. coli* MntR, Q8X7U4, *Synechococcus elongatus* PCC 7942 Fur, Q55244; *C. jejuni* Fur, P48796.

The accession numbers for the proteins comprising the RecA phylogenetic tree are as follows: *S. pyogenes*, NP_270040; *S. mutans*, NP_722374; *T. pallidum*, NP_219129; *S. aureus*, AAK15276; *C. diphtheriae*, CAE49978; *M. tuberculosis*, CAA15533; *B. subtilis*, P16971; *S. epidermidis*, NP_764518; *H. ducreyi*, NP_872986; *Y. pestis*, P37858; *P. aeruginosa*, NP_25 2307; *E. coli*, P03017; *B. pertussis*, NP_881173; *H. influenzae*, NP_438757; *M. smegmatis*, CAA67597; *S. typhimurium*, NP_461750; *P. multocida*, CAA67699; *C. jejuni*, NP_282800; *S. elongatus*, ZP_00163563.
RESULTS

The Streptococcal Metal Transport Repressor (MtsR). While iron serves as a regulatory cue affecting protein production and secretion in GAS, the mechanisms involved in iron regulation have not been characterized. It was previously shown that the expression from the Streptococcal Iron Acquisition (sia) operon was induced by iron and metal depletion (Lei, Smoot et al. 2002; Bates, Montanez et al. 2003; Epstein 2003). A DtxR homologue is found in the GAS chromosome (Jakubovics, Smith et al. 2000), and we reasoned it regulates sia transcription in response to iron availability. This gene, spy0450 in SF370 (M1), which encodes a putative 215 amino acid protein (Mr 24,814 Daltons, pI 5.99), is located 5’ proximal to the mtsABC operon and is transcribed in the opposite direction (Figure 1A). The location of spy0450 and its similarity to other metalloregulatory proteins suggest it functions as the mts repressor, and therefore it was labeled as MtsR in a phylogenetic tree that examined the relationships among homologues of the metallorepressor, ScaR (Jakubovics, Smith et al. 2000). A BLAST analysis showed the predicted amino acid sequence of MtsR is highly conserved among the publicly available sequenced strains of GAS (demonstrating 97-99 % amino acid identity), and that it shares homology with the large group of transcriptional regulators from the DtxR family. The highest homology (60-50% amino acid identity) was found between MtsR and proteins or putative ORFs from pathogenic Streptococci, including S. mutans (SloR/Dlg, these appear to be the same protein identified in different strains of S. mutans, accession # NP_720655, Figure 1B), S. gordonii (ScaR, accession # AAF25184), and S. pneumoniae (NP_359073).
Figure 1. The Streptococcal Metal Transport Repressor (MtsR).

(A) Schematic presentation of the mtsR (spy0450) chromosomal locus in the wild type strain NZ131. The stem-loop structures represent transcriptional terminators. The genes are represented by their names. The mts genes are annotated as such in the S. pyogenes SF370 genome, while mtsR is annotated as spy0450. (B) CLUSTALW alignment of MtsR (Spy0450) with SloR of S. mutans. The dark shade indicates the identical residues and the gray points at the similar residues. The over scored residues with a single line indicate the helix-turn-helix domain and those with a double line indicate the FeoA domain. Ancillary metal binding residues (Metal site 1) are indicated by the number 1, and the primary metalloregulatory residues (Metal site 2) are indicated by the number 2. (C) Schematic presentation of the mtsR mutation in ZE491 strain. KmR represents the kanamycin resistance gene, aphA-3, flanked by the Omega transcriptional termination signals (Perez-Casal, Caparon et al. 1991), mtsRΔ indicates the 5’ portion of mtsR remaining in the ZE491 mutant. (work was performed by C.S. Bates (Bates, Toukoki et al. 2005)).
The amino acid sequence of MtsR is 25% identical and 66% similar to DtxR of *C. diphtheriae*. Despite the relatively low amino acid identity, the helix-turn-helix DNA binding domain and the metal-binding and dimerization domains of DtxR are conserved in MtsR. Two of the four residues (M10 and C102) that comprise the primary metalloregulatory site in DtxR are replaced with D7 and E100 respectively in MtsR. Identical metalloregulatory sites are found in other DtxR homologues that are responsive to iron, manganese, or both including SloR / Dlg (Figure 1B), SirR, and ScaR (Hill, Cockayne et al. 1998; Spatafora, Moore et al. 2001; Guedon and Helmann 2003; Paik, Brown et al. 2003). A search for conserved protein domains (conserved domain database at NCBI.nlm.nih) identified a FeoA domain (pfam04023.6) in the C-terminus of MtsR (Figure 1B). FeoA is a small protein from *E. coli* that may be involved in uptake of ferrous iron (Kammler, Schon et al. 1993); a short amino acid sequence from FeoA protein is conserved in the carboxy termini of most DtxR-like proteins.

When molecular modeling of MtsR was performed, IdeR was chosen by the RASMOL- program as a template for MtsR protein folding (Figure 2). The resulting model included most of the MtsR polypeptide and exhibited the typical winged helix-turn-helix structure of IdeR and other DtxR-like proteins. The metal binding sites in the MtsR model were in positions similar to those found in DtxR and IdeR. A deviation from the DtxR fold was the absence of the SH3-like fold in MtsR. Based on the sequence and structural analyses we hypothesized that MtsR is a metal-dependent transcriptional regulator involved in the control of iron uptake and possibly other functions in GAS. In this study, we demonstrate that MtsR mediates the metal-dependent regulation of genes found at a distance from its chromosomal locus.
Figure 2. Predicted folding of MtsR by RASMOL-MODEL based on IdeR.

Submitting the primary sequence of MtsR to RASMOL MODEL resulted in the above model based on the homology of MtsR to IdeR. Only MtsR is presented above with the predicted secondary structure.
MtsR regulates the expression of the Streptococcal Iron Acquisition (sia) operon. To test the role of mtsR in GAS, a mutant (ZE491) carrying a truncated mtsR allele with an inserted kanamycin resistance gene (mtsR::kmR) was constructed in NZ131 (M49) by allelic replacement (Figure 1C). The structure of the mtsR::kan mutation was confirmed by PCR analysis. Interestingly, growth analysis showed that the mtsR mutant (ZE491) grows more slowly in complete medium (ZTH) than the wild type strain (NZ131). On the other hand, it grows more rapidly than the wild type in the ZTH-NTA medium, indicating it is more resistant to iron depletion (Figure 3A). A similar growth defect in iron-rich medium was recently made for a dtxR mutant in C. diphtheriae (Oram, Avdalovic et al. 2002).

The sia locus is a 10-gene operon, which codes for a hemoprotein receptor (shr), a heme binding protein (shp), and the iron transporter siaABC (Lei, Smoot et al. 2002; Bates, Montanez et al. 2003). It was earlier reported that sia expression was induced by iron restriction. A 3-4 fold increase of transcript was found in cells grown in ZTH-NTA (containing 0.55mM of MgCl₂, MnCl₂, CaCl₂, and ZnCl₂) in comparison to cells grown in ZTH, which contains 17.5±6.45 μM Fe, 0.53±0.2 μM Mn, and 15.5±0.2 μM Zn, or in ZTH-NTA supplemented with 8 μM hemoglobin (Bates, Montanez et al. 2003). To test whether MtsR is involved in the regulated expression of the sia operon, the production of Shr and SiaA proteins by the mtsR mutant was compared to that of the wild type strain in ZTH medium and ZTH-NTA (Eichenbaum, Green et al. 1996; Bates, Montanez et al. 2003). Western blot analysis demonstrated that like in strain SF370 (M1), both Shr and SiaA production was repressed during growth of NZ131 (M49) strain in ZTH. However, inactivation of mtsR (ZE491) resulted in significantly higher levels of both proteins in cells grown in high iron concentration (ZTH, Figure 3B). This observation supports
the suggestion that MtsR negatively regulates $sia$ expression in the presence of iron. A reduced production of both Shr and SiaA was observed in the $mtrR$ mutant in ZTH-NTA in comparison to ZHT, indicating that the regulation of the $sia$ operon is complex, and may involve MtsR-independent regulatory mechanisms.
A.

![Growth curve graph](image)

B.

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Figure 3. MtsR controls the expression of the sia operon.

GAS cells were used to inoculate complete (ZTH, black symbols) or iron-limiting (NTA, empty symbols) medium, incubated at 37°C, and cell growth was monitored over time. Culture samples were taken in the exponential growth phase, and total proteins and RNA were prepared. (A) Growth curves. Cell growth is expressed as Klett units. Squares indicate the wild type NZ131, and circles represent the mtsR mutant (ZE491) (B) Western blot analysis of Shr and SiaA proteins. Proteins, standardized based on cell number, were separated by SDS-PAGE and were reacted with rabbit antibody to Shr (top) or SiaA (C) RT-PCR analysis of sia genes. cDNA synthesized from 1µg of total RNA using gene specific primers was amplified by PCR and separated on an agarose gel. PCR reactions are shown for the housekeeping gene recA and the shr gene. (work was performed by C.S. Bates (Bates, Toukoki et al. 2005)).
RNA was isolated from exponentially growing cells in ZTH or in ZTH-NTA medium and semi-quantitative RT-PCR analysis was used to determine if MtsR regulation of the *sia* operon is at the transcriptional level. The housekeeping gene *recA* was used as an internal control in RT-PCR, and similar levels of amplification confirmed that the RNA quantity used as a template in each RT reaction was equal. Unlike the *recA* product, the amount of amplicon corresponding to the *shr* gene varied depending on the RNA samples. As can be seen in Figure 3C, a very low amount of *shr* product is obtained when the RNA was isolated from the wild type cells (NZ131) grown in complete medium (ZTH). Consistent with induction of the *sia* expression by iron depletion, a high level of *shr* amplicon is seen with RNA isolated from cells grown in iron-restricted (ZTH-NTA) medium. In contrast, a high level of RT-PCR product was observed in the *mtsR* mutant (ZE491) regardless of the iron availability in the medium. Identical observations were made for *spy1791* (the fifth gene in the *sia* operon, data not shown), indicating that inactivation of *mtsR* resulted in deregulation of the *sia* transcription. The decrease of Shr and SiaA protein level found in the *mtsR* mutant when grown in ZTH-NTA in comparison to ZTH (Figure 3B) is not associated with a similar decrease of *sia* transcript level (Figure 3C). This observation implies that protein translation or stability rather than transcription is reduced in the *mtsR* mutant when grown in iron depleted medium in comparison to medium rich with iron.

A C-terminal fusion of MtsR to a His-tag (rMtsR) was expressed and purified as described in the Materials and Methods section. A protein band of the expected size (~25 kDa) was observed upon SDS-PAGE without significant contaminating bands.
Figure 4. MtsR directly binds to the promoter region of shr.

(A) Schematic presentation of the sia operon’s promoter/operator region (Pshr). The positions of the putative -35 and -10 regions of shr, the sia operon’s first gene are indicated. (B). The EMSA was done with the $^{32}$P-labeled promoter fragment and purified MtsR-His protein in a range of concentrations from 0 to 22.5 ng as indicated above the lanes. The DNA-protein complexes C1 and C2 are indicated.
Subsequent Western blot analysis using anti His-tag antibodies confirmed the production and purification to homogeneity of the recombinant GAS protein (data not shown). Binding of rMtsR to the promoter region of shr (the first gene in the sia operon) was investigated using an EMSA. A $^{32}$P end-labeled fragment generated by PCR covering the upstream sequence of shr up to the first ATG codon ($P_{shr}$ fragment) was incubated with increasing amounts of purified rMtsR. A DNA fragment that migrates slower than the “free” DNA ($P_{shr}$-fragment in buffer) was obtained in the presence of 2.5 ng of rMtsR, indicating the formation of a protein-DNA complex (C1 in Figure 4B). A complete shift of the labeled DNA was observed with about 12.5 ng of rMtsR. In the presence of 15 ng of protein a second slow-migrating band begun to form; this complex (C2 in Figure 3B) was the only one observed with 22.5 ng of rMtsR. Together these results suggest that MtsR directly binds to the promoter region of the sia operon potentially forming two separate complexes.

To test for the metal requirements of MtsR, DNA binding was investigated in the presence of increasing amounts of the metal chelator EDTA. The presence of 250 μM EDTA in reactions containing 7.5 ng of rMtsR inhibited the binding of rMtsR to DNA. Binding was restored when iron was added to the reaction. While some binding was seen with 50 μM of ferrous sulfate, complete recovery of DNA binding was observed with the addition of 100 μM of iron to the EDTA-containing reactions (Figure 5A). These observations suggest that MtsR requires iron for DNA binding.
Figure 5. MtsR binding to the sia promoter region is specific and metal dependent.

A. Binding of the purified rMtsR to Pshr-fragment. All EMSAs were done with the $^{32}$P-labeled promoter fragment. Additional components of the different binding reactions are indicated above the lanes. Lane 1, $^{32}$P-Pshr-fragment only. Lane 2, $^{32}$P-Pshr-fragment and 7.5 ng of purified MtsR-His6. Lane 3, the same as in lane 2 and 250 μM EDTA. Lane 4, the same as in lane 3 and 25 μM FeSO₄. Lane 5, the same as in lane 3 and 75 μM FeSO₄. Lane 6, the same as in lane 3 and 50 μM FeSO₄. Lane 7, the same as in lane 3 and 100 μM FeSO₄. Lane 8, the same as in lane 2 and 10-fold of unlabeled (cold) Pshr fragment. Lane 9, the same as in lane 2 and 10-fold of unlabeled recA-fragment. Lane 10, $^{32}$P-Pshr-fragment and 100 ng of SiaA-His6 (Bates, Montanez et al. 2003).

B. Iron restores binding of EDTA-treated rMtsR to DNA. Lane 1, $^{32}$P-Pshr-fragment only. Lane 2, the same as in lane 1 and 10 ng of rMtsR pretreated with EDTA. Lane 3, the same as in lane 1 and 10 ng of untreated rMtsR. Lane 4 the same as in lane 2 and 25 μM FeSO₄. Lane 5, the same as in lane 2 and 75 μM FeSO₄. Lane 6, the same as in lane 2 and 150 μM FeSO₄.

C. Manganese restores binding of EDTA-treated rMtsR to DNA. Lane 1, $^{32}$P-Pshr-fragment only. Lane 2, the same as in lane 1 and 10 ng of rMtsR pretreated with EDTA. Lane 3 and 4, the same as in lane 2 and 25 or 50 μM MnCl₂ respectively.
To investigate the specificity of rMtsR binding to P_{shr} fragment we used a competition assay with specific and nonspecific unlabeled DNA (Figure 5A). These experiments showed that unlabeled fragment competes with MtsR binding to the labeled DNA, and only “free” DNA was observed in the presence of excess of the unlabeled fragment. Conversely, a control fragment corresponding to the streptococcal recA gene was unable to inhibit MtsR binding to the P_{shr} fragment even in the presence of 10-fold excess. These results show that MtsR DNA-binding is specific for the upstream region of shr. Recombinant metalloregulators fused to a His-tag were previously used in DNA binding assays (Schmitt 2002; Ando, Manabe et al. 2003). Nevertheless, we investigated the ability of rSiaA that contains a His tag in its carboxy terminus (Bates, Montanez et al. 2003) to bind to the shr promoter fragment, and no binding was observed when surplus amounts of rSiaA (100ng) were used instead of the rMtsR.

The ability of rMtsR to bind the sia promoter fragment in the absence of additional iron suggests that this protein is purified from E. coli in the metal-bound form. To investigate this hypothesis, rMtsR was treated with EDTA, and the chelator was subsequently removed by dialysis. EMSA done with the EDTA treated rMtsR demonstrated the protein lost most of its binding to the P_{shr}-fragment (lane 2 in Figure 5B and 5C). Binding of rMtsR to DNA was restored with 75 μM of ferrous sulfate (lane 5 in Figure 4B). As many of MtsR homologues respond to multiple metals, the ability of manganese to allow rMtsR binding to P_{shr}-fragment was investigated. Interestingly, 25 μM manganese was sufficient to restore rMtsR binding (lane 3 in Figure 5C). In summary, the DNA binding analyses show that MtsR functions as a metal-dependent regulatory protein that directly binds to the sia promoter region and represses its transcription during cell growth in metal rich medium.
**MtsR is involved in control of iron homeostasis in GAS.** If MtsR functions as a repressor of iron uptake, its inactivation is expected to increase the cellular iron content. To test this hypothesis we compared ZE491 sensitivity to streptonigrin with that of the NZ131 parent strain. The antibiotic streptonigrin interacts with ferrous iron to produce reactive oxygen species; *in vivo* its toxicity is directly proportional to the size of the intracellular iron-pool (White and Yeowell 1982). The growth of NZ131 and ZE491 cells in THY medium containing increasing amounts of streptonigrin (0-4.32 μM) was investigated. Growth inhibition as a function of streptonigrin concentration is seen with the wild type cells, reaching about 98% inhibition in medium containing 8 μM streptonigrin. In contrast, the growth inhibitory effect of streptonigrin on the *mtsR* mutant was more dramatic. Figure 6A shows that only 2.8 ± 1.5% survival is observed when ZE491 is grown in THY containing 0.36 μM streptonigrin, a concentration that supports 92.1 ± 3.6% survival of NZ131.

Deregulation of iron uptake is expected to impose oxidative stress on the cells due to the role of iron in the Fenton reaction (Touati 2000). Analysis of cell growth in THY medium containing an increasing concentration of hydrogen peroxide (0-3 mM) indicates that the *mtsR* mutant is hypersensitive to hydrogen peroxide. While the NZ131 cells can tolerate up to 3.4 mM hydrogen peroxide in the growth medium without demonstrating significant growth changes, ZE491 cells are rapidly killed with a much lower concentration of hydrogen peroxide. As can be seen in Figure 6B, in medium containing 1 mM hydrogen peroxide, ZE491 culture reached only 18.5 ± 19.9% of the growth observed on THY alone, while NZ131 growth is not affected (113.7 ± 13.1% of the growth observed on THY). The sensitivity to oxidative stress observed in
Figure 6. MtsR inactivation results in hypersensitivity to streptonigrin and hydrogen.

(A) Sensitivity of the wild type (NZ131) and mtsR mutant (ZE491) to streptonigrin. Bacteria were inoculated into fresh THY medium containing 0.36μM streptonigrin. The culture optical density (OD$_{600}$) was determined after overnight growth (~20 hours). The data are presented as the ratio of the OD$_{600}$ obtained in overnight cultures grown in THY containing the drug to that obtained in THY alone over the same time-period. (B) Sensitivity of NZ131 and ZE491 to hydrogen peroxide. Bacteria were inoculated into fresh THY containing 1mM hydrogen peroxide and overnight growth was determined and presented as in (A). In both (A) and (B), error bars represent the standard deviation of the mean (n=3). (work was performed by C.S. Bates (Bates, Toukoki et al. 2005)).
the *mtsR* mutant is consistent with that of iron overload; parallel results were obtained for a *dtxR* mutant in *C. diphtheriae* (Oram, Avdalovic et al. 2002).

The observations described above suggest that MtsR represses the expression of genes involved in iron transport during cell growth in complete medium, and that the inactivation of MtsR leads to an increase in intracellular iron pools. To further investigate the role of MtsR in GAS physiology we compared iron uptake by the *mtsR* mutant to that of the isogenic wild type strain. As the role of MtsR is to repress iron uptake in complete medium, we assayed for $^{55}$Fe accumulation by cells grown in THY medium containing $^{55}$FeCl$_3$. These experiments showed that while the fraction of the added $^{55}$Fe accumulated by the cells was low (1.98±0.47 % of total input for NZ131) there were significant differences between the strains. The *mtsR* mutant (ZE491) accumulated 80±22.5% more iron then the wild type strain (P≤ 0.02, n=6). These observations are consistent with the results of the streptonigrin and hydrogen peroxide sensitivity assays, and show that the loss of MtsR interferes with the maintenance of iron homeostasis in GAS.
GAS cells harvested at the logarithmic growth phase were injected into groups of zebrafish (*Danio rerio*, 5×10⁵ cells per fish) either intramuscularly (IM) or intraperitoneally (IP) as previously described (Neely, Pfeifer et al. 2002). The average number of dead fish 48 hours post injection are shown for each infection route; error bars represent the standard error of the mean (n=5). (work was performed by C.S. Bates (Bates, Toukoki et al. 2005)).
MtsR is required for GAS virulence in the zebrafish infection model. To investigate the role of MtsR in vivo, we used the recently described zebrafish (Danio rerio) infection model (Neely, Pfeifer et al. 2002). As was observed with HSC5 (M14) GAS strain, infection of zebrafish with NZ131 is lethal both in the intramuscular and intraperitoneal infection routes (Figure 7). When 5X10⁵ cells of NZ131 were injected intramuscularly 63 ± 7% (SEM) of the infected fish died within 36-48 hours. Intraperitoneal injection of the wild type strain resulted in 80±12.65% death within the same time frame. ZE491 however was significantly attenuated in both types of infection modes; only 15.5 ± 7.1 (P≤0.01, n = 5) death is observed in the intramuscular injection, and death from intraperitoneal infection was reduced to 44 ± 17.2 (P≤0.022, n = 5). These observations suggest that functional MtsR is required for full GAS virulence, although the mtsR mutant appears more attenuated in the intramuscular model.
DISCUSSION

Iron withholding by the human host is a challenge for GAS, as the bacterium requires iron for optimal growth. At the same time, maintaining iron homeostasis is important for the bacterial physiology as well. Iron overload is toxic and increases bacterial sensitivity to the reactive oxygen species encountered by GAS during an infection. Therefore, like other bacterial pathogens, GAS needs to modify iron uptake in response to changes of iron availability in the environment. To address the conundrum of iron homeostasis, the genome of GAS (SF370) has at least three high affinity transport systems for iron and heme, and it also carries two types of metal-responsive regulators, *perR* and *mtsR* (*spy0450* in the SF370 chromosome). Previous studies demonstrated that PerR is a Fur homologue that regulates the GAS response to oxidative stress (Drazek, Hammack et al. 2000; Ricci, Janulczyk et al. 2002; Brenot, King et al. 2005). In this manuscript, we demonstrated that MtsR is a DtxR homologue with an important role in iron homeostasis and virulence in GAS.

The expression from the *sia* operon was repressed by iron in Chelex treated THY (Lei, Smoot et al. 2002) or by hemoglobin in ZTH-NTA medium (Bates, Montanez et al. 2003). In this study, a significant increase in *sia* proteins and transcript is seen in WT GAS cells grown in ZTH-NTA when compared to cells grown in ZTH. High constitutive transcription of the *sia* genes is observed in the *mtsR* mutant in ZTH suggesting that MtsR represses *sia* expression in cells growing in complete medium. The induction of *sia* transcription despite the presence of manganese and zinc suggests that limited iron availability in ZTH-NTA is responsible for this effect. The possibility that the restriction of other metals also contributes to the enhanced expression of *sia* genes in ZTH-NTA medium cannot be disregarded.
It is interesting that the production of Shr and SiaA proteins was decreased in the mutant cells, when grown in ZTH-NTA in comparison to cells grown in ZTH. This observation demonstrates that the regulation of the sia operon is not simple, and it suggests that an MtsR-independent iron-regulation of sia exists as well. The second metalloregulator in GAS, PerR, may be involved in this phenomenon. PerR played a positive role in the expression of the mts and sod genes (Ricci, Janulczyk et al. 2002) and repressed the transcription of mrgR (peroxide resistance (Brenot, King et al. 2005). In addition, MtsR may have both negative and positive roles in sia expression depending on the iron availability in the medium. In either case, the effect on sia expression appears to be at the translation or stability level rather than on the sia transcriptional level as RT-PCR analysis demonstrated that the amount of sia mRNA in the mts mutant was high in both ZTH and ZTH-NTA in comparison to the WT strain.

Electrophoretic mobility shift assays demonstrated that MtsR directly binds to the sia promoter region, producing two DNA-protein complexes. This indicates that the shr promoter region may contain two MtsR binding sites. The first complex (C1) is formed with a lower protein concentration and the second complex (C2) requires a higher protein concentration and does not appear before most of the DNA is shifted to C1, MtsR may have a higher affinity to one of the binding sites. MtsR binding to this DNA fragment was specific, as non-relevant DNA could not compete with the binding.

The presence of EDTA inhibited binding of the purified rMtsR to DNA, suggesting that MtsR requires metal for activity, and that the recombinant protein was purified in the metal bound form. The addition of iron to the EDTA containing reactions restored rMtsR DNA binding, further supporting these assertions. Pretreatment of rMtsR with EDTA and subsequent dialysis prevented most of the DNA binding, demonstrating it is indeed a metal-dependent protein. Both
Iron and manganese promoted rMtsR binding to the P_{sia} fragment. The responsiveness to both metals indicates that sia expression may be dependent on the availability of manganese and possibly other metals in addition to iron. This is consistent with a previous observation that siaA transcription in a metal depleted medium was higher than that in medium depleted only with iron (Epstein 2003) (siaA is named htsA in this publication). A lower concentration of manganese than iron (25 μM and 75 μM respectively) was sufficient to restore rMtsR DNA binding. The difference in the affinity of MtsR for iron and manganese may reflect the differences in the intracellular concentration of these metals. E. coli cells contain fivefold more iron than manganese (Posey and Gherardini 2000). This however, may not be the case for all bacteria, as S. suis, which apparently does need iron for growth has significantly lower iron content than that found in E. coli cells (Niven and Ekins 2001).

The sia operon encodes the siaABC transporter, which shares high homology with transporters of siderophores and heme. We reported that inactivation of the sia operon led to increased resistance to streptonigrin suggesting a significant reduction in iron uptake (Bates, Montanez et al. 2003). Using Western and RT-PCR analysis we demonstrated here that MtsR represses the transcription of the sia operon in cells grown in a medium rich in iron. The streptonigrin hypersensitivity exhibited by the mtsR mutant is an indirect indicator of iron-uptake deregulation in cells growing in complete medium. This is further supported by the iron uptake experiments, which showed that the mtsR mutant accumulates about 80% more 55Fe than the wild type strain during growth in high iron medium. The low incorporation of 55Fe observed in these experiments is likely to result from the fact that the cells were growing in the complex THY medium, which is rich with iron. Similarly low uptake of iron was previously reported for GAS grown in THY (Janulczyk, Ricci et al. 2003). GAS contains at least two other transporters in addition to
SiaABC, which could contribute to the excess of iron uptake observed in the *mtsR* mutant. The *mts* system that is adjacent to *mtsR* mediates the uptake of iron and manganese (Janulczyk, Pallon et al. 1999; Janulczyk, Ricci et al. 2003), its deregulation as a result of MtsR inactivation, is very likely to lead to an increase in iron uptake by GAS.

As was seen in a *dtxR* mutant in *C. diphtheriae* (Oram, Avdalovic et al. 2002), inactivation of the *mtsR* gene led to hypersensitivity to hydrogen peroxide as well. Since iron amplifies the toxicity of hydrogen peroxide (White and Yeowell 1982; Ratledge and Dover 2000; Bates, Montanez et al. 2003), this phenotype may result from elevated intracellular iron levels in the *mtsR* mutant. The increased sensitivity to hydrogen peroxide may also suggest that *mtsR* has a role in regulating the defense of GAS to oxidative stress. Increased sensitivity to hydrogen peroxide that was accompanied by an altered response to oxidative stress was found in several mutants in DtxR-like proteins including the *ideR* mutant in *M. smegmatis*. The loss of *ideR* in *M. smegmatis* led to a reduction in the expression of the catalase-peroxidase (*katG*) and superoxide dismutase (*sodA*) genes (Dussurget, Rodriguez et al. 1996). Interestingly, an *ideR* mutant in *M. tuberculosis* that is also hypersensitive to hydrogen peroxide did not demonstrate any changes in the expression of *katG* or *sodA* genes; instead, it exhibited a reduction in the transcription of a bacterioferritin homologue (*bfrA*), which is likely to be involved in intracellular iron storage (Rodriguez, Voskuil et al. 2002). The resistance to peroxide stress in GAS, which is catalase deficient, is only partially understood. However, it was reported that in vitro, MrgA, a Dps-like protein, contributed to the defense against oxidative stress, while AhpC, an alkyl hydroperoxide reductase, and GpoA, a glutathione peroxidase, did not seem to play a major role (Drazek, Hammack et al. 2000; Brenot, King et al. 2005). Since regulation of iron uptake and peroxide
stress is frequently connected in bacteria (Ratledge and Dover 2000), the possible interplay between MtsR and PerR, the two metal regulators found in GAS, merits an investigation.

Phylogenetic analysis of metal-responsive regulators from the Fur and DtxR families (putative ORFs found the database were excluded) demonstrates as expected, that the Fur and Fur-like proteins comprise a class of metalloregulators that is apart from the DtxR family. In GAS, PerR is in the in the Fur group and closely related to the PerR of B. subtilis, while MtsR is placed in the DtxR group. Inspection of the DtxR family reveals that the DtxR-like proteins from Gram-positive bacteria diverged early from the MntR proteins found in E. coli and S. typhimurium. Furthermore, the DtxR proteins are split into two sub clusters. The C. diphtheriae DtxR protein and IdeR proteins from Mycobacterium species comprise one cluster and proteins such as SloR / Dlg, ScaR, SirR, and MntR from pathogenic Streptococci or Staphylococci are in a second cluster. The GAS MtsR protein belongs to the second cluster with highest similarity to the streptococcal homologues SloR / Dlg and ScaR. Curiously, this cluster also includes TroR of the spirochete T. pallidum, while the MntR from B. subtilis is found on a branch that separated earlier from both clusters described above. Proteins from the first cluster (i.e. DtxR and IdeR) are responsive in vivo only to iron in their native host. Proteins from the second cluster are more diverse and respond to manganese, (ScaR and MntR), manganese and / or zinc (TroR), iron (SirR), or both manganese and iron (SloR / Dlg and MtsR) (Posey, Hardham et al. 1999; Al-Tawfiq, Fortney et al. 2000; Jakubovics, Smith et al. 2000; Spatafora, Moore et al. 2001; Horsburgh, Wharton et al. 2002; Schmitt 2002; Ando, Manabe et al. 2003; Guedon and Helmann 2003; Hazlett, Rusnak et al. 2003; Paik, Brown et al. 2003).
Figure 8. Phylogenetic tree of Fur and DtxR homologs.

(A). Phylogenetic analysis of Fur and DtxR metalloregulators. (B). Phylogenetic analysis of the corresponding RecA proteins from the organisms used in A. Trees were generated using the ClustalW 1.75 as described in the Materials and Methods. Phylograms with weighted branch
lengths are shown. *S. gordonii* and *R. equi* RecA proteins are not included as their sequence is incomplete or not available. Accession numbers for all proteins in A and B is listed in the Materials and Methods section. (figure was adapted from Bates *et al* (Bates, Toukoki et al. 2005)).
Experiments with the zebrafish infection model demonstrate that the *mtsR* mutant is attenuated for virulence in both the intramuscular and the intraperitoneal routes of infection. The disruption of the cellular iron steady state levels in the *mtsR* mutant may have led to the reduced virulence, perhaps due to increased sensitivity to reactive oxygen species. It is also likely that MtsR affects the intracellular steady state of other metals in GAS, and like other metal-dependent regulators, it controls the expression of additional genes involved in functions other than metal transport. Deregulation of such genes may render the bacterium less virulent. The loss of *mtsR* has a more dramatic effect in the intramuscular route of infection. This may be due to the different physiological conditions that are typical of these different micro-environments. In summary, this work began to characterize the MtsR protein, a new player in the regulatory network in GAS. We showed that MtsR is a metal dependent repressor of the *sia* iron transporter, with a role in iron homeostasis and virulence.
Chapter II MTSR is a Dual Regulator that Controls Virulence Genes and Metabolic Functions in Addition to Metal Homeostasis in Group A Streptococcus

Introduction

The group A streptococcus (GAS, Streptococcus pyogenes) is an obligate human pathogen that produces various infections of the upper respiratory tract, cutaneous, and subcutaneous tissue. Superficial and benign infections, such as pharyngitis and impetigo, are the most frequent outcome of GAS infections. In rare instances GAS disseminates in the human body and produces a number of aggressive diseases and systemic manifestations, such as necrotizing fasciitis, myositis, osteomyelitis, and streptococcal toxic shock syndrome (Cunningham 2000; Cunningham 2008). GAS versatility is based on a wide and sometimes redundant repertoire of virulence factors that contribute to the different infection stages, types, and pathology (Almengor, Kinkel et al. 2007; Olsen, Shelburne et al. 2009). Bacterial adherence, for example, is mediated by binding to the host cells or the extracellular matrix using an array of adhesins including pili, protein F1 (PrtF1/Sfb1), M protein, the hyaluronic acid capsule, and more than a dozen ECM-binding proteins (Kreikemeyer, Klenk et al. 2004; Talay 2005; Abbot, Smith et al. 2007; Manetti, Zingaretti et al. 2007). To avoid and manipulate the host immune defenses, GAS employs key surface components such as the antiphagocytic M protein, the hyaluronic acid capsule, and the C5a peptidase (ScpA). Several secreted products that also contribute to GAS immune evasion include the streptococcal inhibitor of complement (Sic), the IgG-peptidase Mac1 (IdeS), the extracellular proteases SpeB and SpyCEP (ScpC), and the DNases that degrade
the DNA-mediated traps produced by neutrophils (Nizet 2007; Olsen, Shelburne et al. 2009). Among the determinants that constitute toxicity, tissue damage, and dissemination are the pyrogenic exotoxins, the hemolysins SLS and SLO, the proteases SpeB and SpyCEP (SpcC), the secreted phospholipase Sla, and the plasminogen-activator streptokinase (Ska) (Almengor, Kinkel et al. 2007; Olsen, Shelburne et al. 2009).

GAS colonization and survival within different niches in the human host relies on its ability to acquire energy sources and essential nutrients, such as metals, which can be scarce during infection. At least two transport systems for acquisition of heme, the most abundant iron form in mammals, are employed by GAS. The Sia (Hts) system is a transporter that works in conjunction with the hemoprotein receptor, Shr, and the heme-binding protein, Shp, to obtain and deliver heme (Bates, Montanez et al. 2003; Nygaard, Blouin et al. 2006; Zhu, Liu et al. 2008). The second machinery involved in hemoprotein utilization is a transporter called Siu (Fts), which was also reported to mediate uptake of ferric-ferrichrome (Hanks, Liu et al. 2005; Montanez, Neely et al. 2005). Free iron and manganese cations are taken up by GAS using a multi-metal transporter called Mts (Janulczyk, Ricci et al. 2003). Inactivation of shr, siuG, or the mts transporter leads to virulence attenuation (Janulczyk, Ricci et al. 2003; Montanez, Neely et al. 2005; Fisher, Huang et al. 2008), demonstrating that iron acquisition is imperative for disease production by GAS. The importance of zinc (Zn) for GAS growth was only recently established (Weston, Brenot et al. 2009). Lsp is a ligand binding-protein that mediates Zn uptake in GAS. Mutations in the Zn(II) binding pocket of Lsp result in decreased virulence, illustrating the significant role Zn homeostasis has on the infection process in GAS (Weston, Brenot et al. 2009).
GAS adaptation to the human environment relies on complex and cross-interacting regulatory circuits, which interpret diverse environmental signals and produce a dynamic expression profile that is adjusted to the nature of the infection, its stage, and site (Almengor, Kinkel et al. 2007). A common regulatory theme used by GAS is the two-component system (TCS), in which a sensor kinase phosphorylates a cognate response regulator (RR) in response to environmental signals. Phosphorylation of the RR changes its DNA-binding properties and, consequently, the transcription of target genes. About 13 TCSs are encoded by GAS including the CovR/S (CsrR/CsrS, (Federle, McIver et al. 1999), which is among the better-understood and important systems. CovR/S negatively regulates about 15% of the GAS transcriptome including many virulence factors such as SpeB, Ska, Sic, and SpyCEP (Graham, Smoot et al. 2002). The CovR/S system is required for GAS growth under stress conditions in vitro (Dalton and Scott 2004), and is suggested to regulate the transition from mucosal infection to invasive disease in vivo (Beres, Richter et al. 2006). In addition to TCSs, GAS also uses several RRs without known cognate sensor kinase; these encompass Mga, the RofA-like protein family, and Rgg/Rop (McIver 2009). Mga is a key stand-alone RR in GAS, affecting about 10% of GAS genome during exponential growth. Mga directly activates a number of important virulence factors; most of them are anchored to the surface and are involved in adherence, invasion, and immune evasion (including the M and M-like proteins, Fba, Sic, and ScpA). In addition, Mga influences sugar utilization and fatty acid metabolism (Ribardo and McIver 2006). Mga is activated by CO₂, temperature, iron, and metabolized sugars. Since Mga coordinates the expression of factors that are believed to play an important role in the early stages of infection, it has been suggested that Mga may be required for GAS colonization of niches in the body that support rapid growth (Hondorp and McIver 2007; McIver 2009).
In addition to RR-s, other types of transcriptional regulators are employed by GAS to coordinate gene expression. PerR belongs to a family of redox-sensing metalloproteins, in which oxidative conditions stimulate the oxidation of selected histidine residues and the subsequent release of the regulator from the DNA (Drazek, Hammack et al. 2000). GAS uses PerR to control oxidative-stress response, metal homeostasis, and sugar utilization (Brenot, King et al. 2005; Gryllos, Grifantini et al. 2008). PerR mutants are more sensitive to phagocytic killing and are less virulent in several infection models (Ricci, Janulczyk et al. 2002; Brenot, King et al. 2005; Gryllos, Grifantini et al. 2008). MtsR is a second metal-dependent regulatory protein found in GAS. MtsR belongs to the DtxR family of metalloproteins, which bind to DNA in the presence of metals. The *mtsR* gene is located upstream and divergently transcribed from the *mts* locus. Previous studies demonstrated that MtsR directly represses the *sia* and the *mts* transporters and affects the expression of *nrdFEI* and *ahpCA* genes, which are involved in nucleotide metabolism and oxidative stress, respectively (Bates, Toukoki et al. 2005; Beres, Richter et al. 2006; Hanks, Liu et al. 2006). A null *mtsR* mutant has elevated level of intracellular iron, is hypersensitive to streptonigrin and hydrogen peroxide, and is attenuated for virulence in a zebrafish infection model (Bates, Toukoki et al. 2005). A comparative genome analysis of GAS clinical isolates revealed that strains containing naturally occurring truncations in *mtsR* are significantly underrepresented among necrotizing fasciitis cases (Beres, Richter et al. 2006). MtsR contribution to pathogenesis is significant and suggests that it is a valuable component in the regulatory pathways used by GAS. In this study we used genomic microarray analysis to identify the MtsR regulon and carried out a detailed investigation of the interactions of MtsR with target promoters.
RESULTS

*The regulatory elements of* $P_{shr}$, *the sia operon promoter*

MtsR directly represses the expression from the *sia* and the *mts* operons in a metal dependent manner (Bates, Toukoki et al. 2005; Hanks, Liu et al. 2006). The MtsR operator, however, has not been identified to date and none of the promoters it regulates were previously characterized. To obtain a better understanding of gene regulation by MtsR, we examined the interactions of MtsR with the promoter that controls expression from the *sia* operon. We first used 5’Race to determine the transcription start site(s) in the 360 bp region upstream of *shr*, the first gene in the *sia* operon. Since separate initiation sites may be used in the presence or the absence of MtsR, the analysis was performed with RNA from both the wild type and the isogenic *mtsR* mutant strains. RNA was prepared from GAS cells grown in metal complete medium. The 5’Race produced a single PCR product (320bp) from both the wild type and the *mtsR* mutant strains (Fig. 9A). Sequence analysis identified an A residue found 52 bp upstream of the *shr* coding region as the transcription start site to (+1, Figure 9B). A repeat analysis with different *shr* specific primers resulted in an independent confirmation of the same transcription initiation site. Therefore, a single promoter appears to control the expression from the *sia* operon ($P_{shr}$). The region upstream of $P_{shr}$ start site contains a putative promoter with a perfect -35 (TTGACA) hexamer, a putative -10 (TAGATT) box, and a 17 base spacer (Fig. 9B).
Figure 9. The regulatory region of shr.

A. Determination of shr transcription start site by 5’RACE. A schematic representation of shr upstream region and an agarose gel with the 5’RACE products are shown. The analysis was done with RNA isolated from the wild type (WT) and the mtsR mutant (mtsR-) isogenic strains. M stands for DNA size marker. B. An overview of shr regulatory elements. The transcription start site (+1), the -10 and -35 promoter elements, the ribosomal binding site (RBS), and Shr start codon are indicated in bold. The DNA region protected from DNase I digestion by MtsR is indicated by the solid lines above and below the coding and template strands. The IR1 and IR2 inverted repeats are marked. C. Sequence alignment of MtsR and ScaR footprints (Jakubovics, Smith et al. 2000).
To elucidate how MtsR interacts with and regulates $P_{shr}$ we constructed and investigated several transcriptional fusions to the $luc$ reporter gene. The expression from plasmid pCHT21, in which the entire intergenic region upstream of $shr$ is fused to the $luc$ gene (Fig. 10A and Table 2), was 2.6 fold higher in the $mtsR$ mutant than it was in the wild type strain. On the other hand, transcriptional fusions to 2 shorter fragments derived from the region upstream of the identified promoter (pCHT22 and pCHT23, Fig. 10A and Table 2) did not produce any luciferase activity, confirming the absence of additional promoters. We used site directed mutagenesis to study the importance of selected residues in the putative -10 hexamer (TAGATT). Changing the thymines at positions -7 and -8 to guanines (pCHT32, Fig. 10B and Table 2) resulted in loss of the promoter activity in both strains, suggesting that the TAGATT sequence is a functional TATA box.
A. Schematic representations of the transcriptional fusions to the *luc* reporter gene used to study the *shr* regulatory elements. The plasmid (construct) names, the fragment from *shr* upstream sequence that is carried by each construct (thin line), the *shr* transcription start site (+1), and the *luc* gene (gray arrow) are shown. B. Mutational analysis of *shr* promoter elements. The sequence of the wild type *shr* promoter with its -35 and -10 elements (bold) and the transcription start site (+1) found in the transcriptional fusion carried by plasmid pCHT21 are shown. The different mutations introduced in *shr* promoter and the names of the plasmids that carry them are indicated. Plasmid pCHT31 carries a transcriptional fusion of the entire *shr* upstream region (1-360 bp) with the G.-14 to A mutation.

**Figure 10. Analysis of *shr* promoter.**
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype</th>
<th>WT</th>
<th>mtsR</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCHT21</td>
<td>P&lt;sub&gt;shr&lt;/sub&gt; (1-360)</td>
<td>871±255</td>
<td>2,267±411</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>pCHT22</td>
<td>P&lt;sub&gt;shr&lt;/sub&gt; (1-175)</td>
<td>11±18</td>
<td>13±24</td>
<td>0.805</td>
</tr>
<tr>
<td>pCHT23</td>
<td>P&lt;sub&gt;shr&lt;/sub&gt; (1-126)</td>
<td>24±30</td>
<td>16±29</td>
<td>0.134</td>
</tr>
<tr>
<td>pCHT31</td>
<td>pCHT21 with G&lt;sub&gt;-14&lt;/sub&gt; to A</td>
<td>5,406±499</td>
<td>21,691±3087</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>pCHT32</td>
<td>pCHT21 with T&lt;sub&gt;-7&lt;/sub&gt;T&lt;sub&gt;-8&lt;/sub&gt; to GG</td>
<td>35±19</td>
<td>22±16</td>
<td>0.191</td>
</tr>
<tr>
<td>pCHT33</td>
<td>pCHT21 T&lt;sub&gt;-15&lt;/sub&gt; to G</td>
<td>36±16</td>
<td>22±17</td>
<td>0.042</td>
</tr>
</tbody>
</table>

A schematic presentation of the luc-fusions carried by each plasmid is shown in figure 10A. The positions of mutated residues are given in relation to the shr transcription start site shown in figure 10B. Luciferase activity expressed by each strain was determined as described in Material and Methods. Each data point represents the average and the standard deviation derived from at least 3 independent experiments done in quadruplicates. The significance of the difference in luciferase activity between the wild type and the mtsR strain was examined by Student’s t test.
Sequence inspection revealed the presence of a TG motif at the -14 and -15 positions respectively, which may function as an extended -10 element (Voskuil and Chambliss 1998; Mitchell, Zheng et al. 2003). The role of the TG residues in P_{shr} was tested by site directed mutagenesis. Replacing the thymine at position -15 with guanine (pCHT33, Fig. 10B) dramatically decreased the promoter activity (Table 2) demonstrating that T_{-15} is critical for P_{shr} function. Changing the guanine at position -14 to adenine resulted in a 6-fold increase in the promoter activity in the wild type strain (pCHT31, Fig. 10B). This observation shows the residue identity in the -14 position is also important for the function of P_{shr}. The promoter activity from pCHT31 was 4 fold higher in the mtsR mutant strain in comparison to the wild type strain, indicating that the G_{-14} to A mutation increases the strength of P_{shr} but does not prevent the repressor from binding to DNA.

\textit{Mapping MtsR binding to P_{shr}}

MtsR was previously shown to bind to a DNA fragment containing the entire upstream region of shr (Bates, Toukoki et al. 2005). We used EMSA with 3 fragments encompassing the shr upstream sequence to locate the MtsR operator (Fig. 11A). A DNA shift was generated with a fragment that contained the first 89 bp upstream of shr coding region (F1); this fragment includes the promoter identified by the 5’Race analysis. MtsR bound specifically and with high affinity (Kd of 7.9 x 10^{-10}) as indicated by the competition experiments done in the presence of unlabeled specific DNA (positive control) or P_{gyrA} fragment (non specific negative control). MtsR did not bind the other fragments (F2 and F3), which contain the DNA region upstream to the first 89 bp, even in the presence of higher MtsR concentrations (Fig. 11B).
Figure 11. MtsR binding to shr promoter region.

A. Schematic representation of shr promoter region. The thin solid lines represent the 3 DNA fragments used in the EMSAs: F1 (89 bp), F2 (159 bp), and F3 (160 bp). The DNA fragment used in the DNase I footprinting analysis (F4, 193 bp) is indicated by a thick solid line. B. Labeled DNA fragments (50 pmol) from shr promoter were incubated with increasing concentration of rMtsR, and the formation of DNA protein complex was analyzed by EMSA. Some reactions (indicated by the + sign) were done in the presence of 10-fold access of unlabeled DNA fragment (F1) or unlabeled DNA fragment carrying the promoter region of the gyrA gene (P_{gyrA}).
DNase I footprinting experiments done with a fragment (F4, Fig. 11A) that covers the first 193 bp upstream of shr coding sequence yielded an extensive protection region that includes P_{shr} sequence (Fig. 12). Experiments done with a larger DNA fragment revealed that MtsR did not protect any region upstream of the identified shr promoter (data not shown). MtsR binding to DNA was not symmetrical, resulting in a partial overlap between the footprints on the two strands. On the coding strand, the repressor protected a 62 bp region, starting at the -37 position. MtsR footprint on the template strand was larger (69 bp, staring at the -14 position) and the protected residues were not found on a continuous segment (Fig. 9B and Fig. 12). Several residues on both strands were hypersensitive to DNase I; most noticeable was the thymine residue at position +36 on the template strand. The 10 bp immediately upstream of T_{+36}, were resistant to DNase I digestion even in the absence of MtsR. The DNase I resistant region is part of an inverted repeat (IR2 in Fig. 9B). A second inverted repeat is also found within the MtsR-protected region upstream of IR2, which overlaps the -10 box of P_{shr} (IR1 in Fig. 9B). The presence of 2 inverted repeats within the DNA binding region of the shr promoter is reminiscent of the DNA region recognized by ScaR, a closely related metalloregulator from S. gordonii (Jakubovics, Smith et al. 2000; Bates, Toukoki et al. 2005). Examination of the footprints of MtsR and ScaR (in P_{shr} and P_{scaC} respectively) revealed that significant sequence conservation is limited to the first inverted repeat in both DNA binding regions (IR1, Fig. 9C).
Figure 12. DNase I footprinting of MtsR complex with shr promoter.

DNA fragments (1 μM) were incubated with DNase I in the presence of increasing concentration of purified rMtsR protein. The 5’end of the non-template strand was radiolabeled on the coding strand panel, and the DNA was incubated with 0, 8, 16, 24, 32, 40, 48, 56, and 64 μM MtsR. The 5’end of the template strand was labeled on the template strand panel, and the DNA was incubated with 0, 8, 16, 24, 32, 40, and 48 μM MtsR. The vertical lines specify the 62 bp (on the coding strand) and 69 bp (on the template strand) regions protected by MtsR. The transcription start site (+1) and the -10 and -35 promoter elements are indicated on both strands.
Analysis of the MtsR regulon

Bacterial pathogens often use metallopressors such as Fur and DtxR to coordinate the expression of genes involved in metal uptake and homeostasis as well as genes that are necessary for the production of infection and pathology. The full spectrum of genes that are regulated by the GAS DtxR-like regulatory protein MtsR has not been previously investigated. To determine the boundaries of MtsR regulation, we compared the transcriptome of a null mtsR mutant (Bates, Toukoki et al. 2005) with that of an isogenic wild type strain. Total RNA was isolated from GAS cells grown in iron complete medium and analyzed using a 70-mer oligonucleotide microarray (representing GAS genomes M1, M3, and M18) (Ribardo and McIver 2006). This analysis revealed that the expression of 64 GAS genes was changed in response to mtsR deletion, demonstrating that MtsR has an extensive regulatory role in GAS. Out of the 64 MtsR-controlled genes, the transcription of 44 genes was elevated 2-35 fold in the mutant strain in comparison to the wt strain (Table 3). In addition, the expression of 20 genes was down regulated 2-19 fold in the absence of MtsR (Table 4). The array analysis was validated by quantitative RT-PCR (qRT-PCR) on 9 differentially regulated genes (Tables 3 and 4), showing a strong correlation, with an $R^2$ value of 0.943 (Fig. 13).
Figure 13. Comparison of the microarray data with the qRT-PCR results.

Log values for microarray versus the log values from qRT-PCR. The solid line indicates the correlation between microarray and qRT-PCR data of 9 tested genes ($R^2 = 0.9437$).
<table>
<thead>
<tr>
<th>Spy#</th>
<th>Annotation (NCBI or TIGR)</th>
<th>Gene</th>
<th>Array Mean± SD</th>
<th>RT Mean± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spy49_0247</td>
<td>Putative D-alanyl-D-alanine carboxypeptidase</td>
<td>pbp7</td>
<td>2.021 ± 0.212</td>
<td>nt</td>
</tr>
<tr>
<td>Spy49_0339</td>
<td>Ribonucleotide reductase of class Ib (aerobic), beta subunit</td>
<td>nrdF.2</td>
<td>8.850 ± 0.391</td>
<td>24.810 ± 3.295</td>
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<tr>
<td>Spy49_0340</td>
<td>Ribonucleotide reductase protein</td>
<td>nrdI</td>
<td>8.599 ± 0.393</td>
<td>nt</td>
</tr>
<tr>
<td>Spy49_0341</td>
<td>Ribonucleotide reductase of class Ib (aerobic), alpha subunit</td>
<td>nrdE.2</td>
<td>7.214 ± 0.299</td>
<td>nt</td>
</tr>
<tr>
<td>Spy49_0450</td>
<td>Shikimate 5-dehydrogenase</td>
<td>aroE</td>
<td>35.234 ± 0.495</td>
<td>90.703 ± 8.543</td>
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<tr>
<td>Spy49_0451</td>
<td>Hypothetical protein</td>
<td></td>
<td>32.150 ± 0.543</td>
<td>nt</td>
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<tr>
<td>Spy49_0453</td>
<td>Nitroreductase family protein</td>
<td></td>
<td>27.556 ± 0.593</td>
<td>nt</td>
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<td>Spy49_0454</td>
<td>Hypothetical protein</td>
<td></td>
<td>27.416 ± 0.577</td>
<td>nt</td>
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<tr>
<td>Spy49_0455</td>
<td>Archaeal S-adenosylmethionine synthetase; Ado-Met</td>
<td>metK1</td>
<td>32.917 ± 0.588</td>
<td>73.420 ± 12.157</td>
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<tr>
<td>Spy49_0456</td>
<td>Hypothetical protein</td>
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<td>30.087 ± 0.558</td>
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<tr>
<td>Spy49_0457</td>
<td>Glycosyl transferase domain protein</td>
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<td>31.299 ± 0.570</td>
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<td>Spy49_0458</td>
<td>Hypothetical protein</td>
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<td>21.573 ± 0.454</td>
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<td>Spy49_0459</td>
<td>UDP-glucose 6-dehydrogenase</td>
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<td>17.517 ± 0.411</td>
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<td>Spy49_0460</td>
<td>Putative macrolide-efflux protein</td>
<td>mefE</td>
<td>14.832 ± 0.380</td>
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<tr>
<td>Spy49_0578</td>
<td>LPXTG-motif cell wall anchor domain protein, endonuclease/exonuclease/phosphatase family</td>
<td></td>
<td>2.247 ± 0.120</td>
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<tr>
<td>Spy49_0661</td>
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<td>Conserved hypothetical protein</td>
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<td>Spy49_1038c</td>
<td>Hypothetical protein</td>
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<td>2.056 ± 0.198</td>
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<td>Spy49_1145c</td>
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<td>3.122 ± 0.247</td>
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<tr>
<td>Spy49_1146c</td>
<td>Putative heavy metal-transporting ATPase, E1-E2 family</td>
<td>cadA/pmTA(^c)</td>
<td>5.078 ± 0.228</td>
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<tr>
<td>Spy49_1159c</td>
<td>Hypothetical protein</td>
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<td>3.643 ± 0.057</td>
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<tr>
<td>Spy49_1159c</td>
<td>Putative phosphoglucomutase/phosphomannomutases</td>
<td></td>
<td>4.136 ± 0.169</td>
<td>nt</td>
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<tr>
<td>Spy49_1332c</td>
<td>Putative copper-transporting ATPase</td>
<td>copA</td>
<td>2.498 ± 0.423</td>
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<tr>
<td>Spy49_1374c</td>
<td>Chaperone dnaK protein</td>
<td>dnaK</td>
<td>2.512 ± 0.162</td>
<td>nt</td>
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<tr>
<td>Spy49_1375c</td>
<td>Heat shock protein, Hsp-70 cofactor</td>
<td>grpE</td>
<td>2.464 ± 0.144</td>
<td>nt</td>
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<tr>
<td>Spy49_1376c</td>
<td>Heat-inducible transcription repressor HrcA</td>
<td>hrcA</td>
<td>2.808 ± 0.174</td>
<td>nt</td>
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<tr>
<td>Gene ID</td>
<td>Description</td>
<td>Annotation</td>
<td>Protein Ratio</td>
<td>Transmission</td>
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<td>--------------</td>
<td>------------------------------------------------------------------------------</td>
<td>------------</td>
<td>---------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Spy49_1395c</td>
<td>Putative ABC-type cobalt transporter, ATPase component</td>
<td>siaH&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.274 ± 0.121</td>
<td>nt</td>
</tr>
<tr>
<td>Spy49_1396c</td>
<td>Putative ABC-type cobalt transporter, permease component</td>
<td>siaG&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.376 ± 0.070</td>
<td>nt</td>
</tr>
<tr>
<td>Spy49_1397c</td>
<td>Putative ABC-type cobalt transporter, permease component</td>
<td>siaE&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.887 ± 0.212</td>
<td>nt</td>
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<td>Spy49_1398c</td>
<td>Transport ATP-binding protein hemoprotein receptors locus</td>
<td>siaF&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.164 ± 0.124</td>
<td>nt</td>
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<tr>
<td>Spy49_1400c</td>
<td>Transport ATP-binding protein hemoprotein receptors locus</td>
<td>siaE&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.085 ± 0.197</td>
<td>nt</td>
</tr>
<tr>
<td>Spy49_1401c</td>
<td>ABC transporter, ATP-binding protein, streptococcal iron acquisition locus</td>
<td>siaC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.186 ± 0.235</td>
<td>nt</td>
</tr>
<tr>
<td>Spy49_1402c</td>
<td>ABC transporter, membrane permease, streptococcal iron acquisition locus</td>
<td>siaB&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.696 ± 0.194</td>
<td>nt</td>
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<tr>
<td>Spy49_1403c</td>
<td>ABC transporter, binding protein, streptococcal iron acquisition locus</td>
<td>siaA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.050 ± 0.153</td>
<td>nt</td>
</tr>
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<td>Hemoprotein receptor, streptococcal iron acquisition locus</td>
<td>shr&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>7.913 ± 0.993</td>
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<tr>
<td>Spy49_1630</td>
<td>Streptokinase precursor</td>
<td>ska</td>
<td>3.231 ± 0.292</td>
<td>3.125 ± 0.185</td>
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<td>Spy49_1660</td>
<td>Dipeptide-binding protein, periplasmic substrate binding component</td>
<td>dppA</td>
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<td>Spy49_1707c</td>
<td>Hypothetical protein (putative translation initiation inhibitor)</td>
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<td>2.222 ± 0.122</td>
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<tr>
<td>Spy49_1752c</td>
<td>Putative arsenate reductase</td>
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<td>Spy49_1782</td>
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<td>4.420 ± 0.109</td>
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<tr>
<td>Spy49_1796c</td>
<td>Hypothetical protein</td>
<td>mrs1&lt;sup&gt;e&lt;/sup&gt;</td>
<td>19.093 ± 0.438</td>
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<td></td>
<td>Hypothetical protein</td>
<td>mrs2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>21.558 ± 0.360</td>
<td>nt</td>
</tr>
</tbody>
</table>

nt: not tested.

a. The gene id used by NCBI data base for NZ131strain.
b. The annotation used by NCBI or TIGR (parentheses).
c. Gene annotation as described by Brenot et al (Brenot, Weston et al. 2007).
e. MtsR-regulated small ORF.
<table>
<thead>
<tr>
<th>Spy#</th>
<th>Annotation (NCBI or TIGR)</th>
<th>Gene</th>
<th>Array Mean± SD</th>
<th>RT Mean± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spy49_0249</td>
<td>Oligopeptide transporter, periplasmic oligopeptide-binding protein</td>
<td>oppA</td>
<td>0.488 ± 0.338</td>
<td>nt</td>
</tr>
<tr>
<td>Spy49_0375</td>
<td>UDP-N-acetylglucosamine pyrophosphorylase</td>
<td>glmU</td>
<td>0.259 ± 0.158</td>
<td>nt</td>
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<tr>
<td>Spy49_0376</td>
<td>MutT/nudix family protein/ADP-ribose pyrophosphatase</td>
<td></td>
<td>0.211 ± 0.206</td>
<td>nt</td>
</tr>
<tr>
<td>Spy49_0377</td>
<td>Hypothetical protein</td>
<td></td>
<td>0.184 ± 0.223</td>
<td>nt</td>
</tr>
<tr>
<td>Spy49_0378</td>
<td>MTA/SAH nucleosidase</td>
<td>pfs</td>
<td>0.133 ± 0.210</td>
<td>nt</td>
</tr>
<tr>
<td>Spy49_0380c</td>
<td>Streptococcal metal-dependent transcriptional repressor, MtsR</td>
<td>mtsRc</td>
<td>0.187 ± 0.146</td>
<td>0.010 ± 0.002</td>
</tr>
<tr>
<td>Spy49_0650</td>
<td>Pyrimidine operon regulatory protein</td>
<td>pyrR</td>
<td>0.391 ± 0.149</td>
<td>nt</td>
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<tr>
<td>Spy49_0651</td>
<td>Putative uracil permease</td>
<td>pyrP</td>
<td>0.278 ± 0.295</td>
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<tr>
<td>Spy49_0652</td>
<td>Aspartate carbamoyltransferase</td>
<td>pyrB</td>
<td>0.277 ± 0.325</td>
<td>nt</td>
</tr>
<tr>
<td>Spy49_0653</td>
<td>Carbamoyl-phosphate synthase, small subunit</td>
<td>carA</td>
<td>0.267 ± 0.291</td>
<td>nt</td>
</tr>
<tr>
<td>Spy49_0654</td>
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<td>carB</td>
<td>0.237 ± 0.387</td>
<td>nt</td>
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<tr>
<td>Spy49_0712</td>
<td>Orotidine 5’-phosphate decarboxylase</td>
<td>pyrF</td>
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<td>Oropate phosphoribosyltransferase</td>
<td>pyrE</td>
<td>0.407 ± 0.311</td>
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<td>Spy49_1024</td>
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<td>Spy49_1449c</td>
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<td>Spy49_1671c</td>
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<td>emm49</td>
<td>0.110 ± 0.337</td>
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<tr>
<td>Spy49_1673c</td>
<td>Trans-acting positive regulator of M protein:mga-2 allele</td>
<td>mga</td>
<td>0.448 ± 0.119</td>
<td>0.250 ± 0.050</td>
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</tbody>
</table>

nt: not tested.

a and b the same as in Table 3.

c. Gene annotation as described by Eichenbaum et al (Bates, Toukoki et al. 2005).
quite complex and contains genes that participate in a variety of cellular roles including regulation. Genes that are activated by MtsR contribute to nucleotide metabolism, transport, cell envelope and regulation. MtsR-repressed genes cover an overlapping and wider spectrum of functions, which also include protein synthesis and fate, and the biosynthesis of amino acids. Most of the MtsR-controlled genes are found in clusters that are likely to be transcribed into polycystronic RNA molecules (Table 3 and 4).

*MtsR repressed genes*

The MtsR–repressed regulon included 4 metal transport systems (Table 3). Consistent with our previous findings (Bates, Toukoki et al. 2005), 2-6 fold induction of the 10-gene *sia* operon (Spy49_1405c-1395c) was observed in the MtsR− background. Five-fold increase in transcription of the PerR-regulated metal transporter *pmtA* (Spy49_1146c) was also observed in the *mtsR* mutant. *pmtA* encodes a P-type (E1-E2 type) ATPase; enzymes from this superfamily typically mediate cation transport, but the nature and the direction of flux varies between systems, however. In GAS, the *pmtA* transporter contributes to peroxide resistance and was suggested to mediate metal ion export (Brenot, Weston et al. 2007). A moderate increase in the transcription of a second putative heavy metal transporter (*copA*) from the P-type ATPase family was observed as well (2.5 fold, Spy49_1332c). In contrast to the findings in *mtsR* mutants in M1 and M3 serotypes (Beres, Richter et al. 2006; Hanks, Liu et al. 2006) the transcriptome analysis revealed that inactivation of *mtsR* in NZ131 (M49 background) did not affect *mts* transcription.
Figure 14. Microarray analysis of MtsR-regulated genes.

Repressed (striped bars) and activated (black bars) genes were graphed according to their category role assignment (TIGR). Categories with genes representing less than 5% of the total regulated genes have been omitted for simplification.
RT-PCR analysis confirmed the microarray findings that no change in \textit{mtsA} transcripts was observed in the \textit{mtsR} mutant strain (data not shown). Similarly, the MtsR-repression of \textit{ahpC} transcription that was reported in the M3 strain, MGAS9887 (Beres, Richter et al. 2006), was not seen in this study. Therefore, like other global regulators in GAS, MtsR demonstrates some strain-dependent variation of its regulon.

A cluster of 10 genes that starts with the \textit{aroE} gene (Spy49\_0450-0460, Figure 15A) demonstrated the highest increase in gene expression in the absence of MtsR (35 fold, Table 3). \textit{aroE} codes for a putative shikimate 5-dehydrogenase, which catalyzes the conversion of 3-dehydroshikimate to shikimate. The shikimate pathway links carbohydrate metabolism to the biosynthesis of aromatic amino acids and metabolites through seven enzymatic steps (Herrmann and Weaver 1999). \textit{In silico} analysis suggests that GAS carries a second \textit{aroE} gene (\textit{aroE.1}, Spy49\_1225c) and all of the enzymes that catalyze the metabolic steps from phosphoenolpyruvate and erythrose 4-phosphate to chorismate, the common precursor for tryptophan, tyrosine, and phenylalanine (soz00400 pathway for NZ131 in the KEGG database, (Okuda, Yamada et al. 2008)). The genes encoding this pathway, however, are scattered in several chromosomal loci, and \textit{aroE} appears to be the only gene from the shikimate pathway that is regulated by MtsR. In addition to the \textit{aroE} gene, the locus also includes a homologue of \textit{metK} (encoding S-adenosyllmethionine synthetase) that is involved in methionine and selenoamino acid metabolism, a putative efflux protein (\textit{mefE}) and seven additional orfs encoding hypothetical proteins (Fig. 15A). The close proximity of the 10 genes in the \textit{aroE} cluster and the similar increase in their expression in the mutant strain suggests that they make up an operon that is repressed by MtsR. A high (7-9 fold) increase in transcript abundance in response to \textit{mtsR}
Figure 15. MtsR-repressed genes.

A. Schematic representation of the aroE genomic locus in GAS. The genes and the ORF number in NZ131 strains are indicated. The genomic loci of the streptokinase (ska) and the ribonucleotide reductase genes (nrdFIE) in GAS were previously described (Frank, Steiner et al. 1995; Beres, Richter et al. 2006). B. MtsR binding to DNA fragments carrying the promoter regions of ska (407 bp), nrdF.2 (313 bp), and aroE (246 bp). The formation of DNA-protein complex was analyzed by EMSA as described in figure 11B.
inactivation was also seen in the *nrdFI.2E.2* operon (Spy49_0339-0341, Table 3). MtsR control of this operon in MGAS315 strain was previously reported (Beres, Richter et al. 2006). The *nrdE.2IF.2* genes encode for ribonucleotide reductases (RNR), which play an important role in the metabolism of deoxyribonucleotides, and for a flavodoxin (Roca, Torrents et al. 2008). The *dnaK*-operon, which encodes the *hrcA* repressor and the heat shock chaperones *grpE* and *dnaK* (Roca, Torrents et al. 2008), was up regulated 2.5-2.8 fold in the *mtsR* mutant (Spy49_1376c-1374c, Table 3). This observation suggests that the heat shock response in GAS is linked to metal availability. Among the individual genes that were down regulated by MtsR, the observed 3-fold increase in *ska* expression in the *mtsR* mutant is significant. The *ska* gene codes for the enzyme streptokinase, which converts plasminogen to plasmin, an active serine protease. Plasmin degrades fibrin matrix and is a key enzyme in the host fibrinolytic system. Multiple experimental and epidemiological data suggest that Ska is an important virulence factor in GAS (Olsen, Shelburne et al. 2009).

MtsR binding to the promoter region of several negatively regulated genes was investigated by EMSA analysis. DNA fragments derived from the upstream regions of *aroE, nrdF.2* and *ska* were incubated with increasing concentration of purified MtsR and complex formation was examined following electrophoresis (Fig. 15B). Slow migrating DNA bands, produced in a manner that was dependent on MtsR concentration, were observed with all of the tested promoter fragments. The repressor affinity for the *nrdF.2* promoter was the highest; a protein DNA complex was formed with the lowest MtsR concentration, exhibiting a dissociation constant of $1.9 \times 10^{-10}$. The calculated dissociation constants for MtsR binding to *ska* and *aroE* were $7.5 \times 10^{-10}$ and $1.9 \times 10^{-9}$, respectively. In each one of the examined promoters, the specific cold DNA efficiently competed with complex formation while the PgyrA fragment did not. These
observations demonstrate that MtsR directly represses the expression from the *aroE, nrdF.2* and *ska* genes by binding specifically to their regulatory region.

**MtsR activated genes**

Out of the 20 genes that were positively regulated by MtsR, 7 genes are involved in pyrimidine metabolism (Fig. 16A and Table 4). These genes are organized in two putative operons. The first, which begins with the *pyrR* gene, encodes 5 genes (Spy49_0650-0654, Fig. 16A) and is down regulated 3-4 fold in the *mtsR* mutant. The second cluster carries the *pyrFE* genes (Spy49_0712-0713, Fig. 16A) and demonstrates about 2-fold decrease in expression in the absence of MtsR. The transcription from a 4-gene cluster with unknown function was also reduced 4-5 fold in the absence of MtsR (Spy49_0375-0378). Orfs from this cluster code for enzymes involved in the metabolism of peptidoglycan, purines, and amino groups. In addition to regulating metabolic functions, MtsR positively affected the expression of two major virulence genes: the global transcriptional activator *mga* and the *emm49* genes (Spy49_1673c and Spy49_1671c respectively, Table 4). The surface M-protein is a key virulence factor that protects GAS from phagocytosis and mediates adherence and invasion. Mga is encoded upstream of the *emm* gene in the GAS chromosome. In addition to activating *emm* transcription, Mga also controls the expression of multiple surface-associated and secreted products that are important for host colonization and immune evasion (Ribardo and McIver 2006).
Figure 16. MtsR-activated genes.

A. Schematic representation of the *pyrR* (Spy49_0650-0654) and *pyrFE* loci in GAS. The genes and the ORF number in NZ131 strains are indicated. (B) MtsR binding to DNA fragments carrying the promoter region of *mga* (405 bp) and *pyrF* (346 bp). The formation of DNA-protein complex was analyzed by EMSA as described in Figure 11B. (C) MtsR binding to the promoter region of *emm*. MtsR binding to the promoter region of *emm* genes from NZ131 (*emm49*), MGAS5005 (*emm1*), and JRS4 (*emm6*) was examined by EMSA as described in figure 11B.
To determine if the positive effect of MtsR on transcription is direct, we tested MtsR binding to the promoter region of mga and pyrF (Fig. 16B). MtsR/DNA complexes were observed with DNA fragments that contained the upstream region of both genes. The highest affinity was found with the mga promoter, which showed dissociation constant of $6 \times 10^{-10}$. The dissociation constant of pyrF was $1.8 \times 10^{-10}$. MtsR binding to all of the tested promoters was specific, as evidenced by the ability of cold promoter fragment to effectively compete with complex formation and the failure of the gyrA promoter region to prevent complex formation. The reduction in emm49 expression in the mtsR mutant was higher than that observed for mga transcript, suggesting that MtsR may affect emm49 in both mga-dependent and mga-independent manner. We therefore tested the binding of MtsR to emm49 promoter (Fig. 16C); as with the mga and pyrF genes, MtsR bound to emm49 promoter specifically and with high affinity (Kd of $1 \times 10^{-9}$). GAS strains are classified into class I and II based on a conserved region in M-protein carboxy-terminus. NZ131 (M type 49), the strain used in this study, belongs to class II. Since significant difference in regulatory circuits are often found between the two classes of GAS we tested MtsR binding to the emm promoter in two strains from class I (MGAS5005, M type 1, and JRS4, M type 6). EMSA analysis demonstrated that MtsR bound to the promoter region of both emm1 and emm6 (Fig. 16C). These observations are consistent with the reported down regulation of emm6 in JRS4 cells grown in iron depleted medium (McIver, Heath et al. 1995) and suggest that MtsR directly controls emm expression in response to iron availability. This is the first identification of genes that require MtsR for full expression.

Comparison of the protected region in $P_{shr}$ to the upstream region of the genes that are directly regulated by MtsR, failed to identify sequence homology that spans a 62 bp region. However, a shorter (16 bp) sequence signature containing a palindrome could be found in one or two copies
in the regulatory region of each of the examined GAS genes (Fig. 17). While the putative MtsR box is not highly conserved among GAS promoters, this region that is derived from the IR1 in P_{shr} is similar to the palindrome identified by Kitten et al as the potential operator of MtsR orthologs from several streptococci (Kitten, Munro et al. 2000).
Figure 17. Proposed DNA binding motif for MtsR.

A. Alignment of the DNA segments from MtsR regulated promoters that contain the putative MtsR recognition box suggested by Kitten et al (Kitten, Munro et al. 2000). B. MtsR sequence signature. The sequence motif was produced by Sequence Logo (http://weblogo.berkeley.edu/logo.cgi) using the sequence alignment shown in panel A. The relative frequency of the residues at each position is reflected in the letter size. The y-axis indicates the information content measured in bits of the motif.
DISCUSSION

Bacteria manage metal uptake and resistance mechanisms using transcriptional regulators that sense and modify gene expression in response to the nature and availability of metals in their surroundings. Out of the 7 families of metal-dependent regulatory proteins used by bacteria, 3 groups (Fur, NikR, and DtxR) evolved to shut down transport in response to metal availability (Giedroc and Arunkumar 2007). With only a few exceptions (such as PsaR’s response to zinc (Kloosterman, Witwicki et al. 2008)), metal ions serve as allosteric co-repressors of regulators from these families. In accordance with the typical functions of DtxR-like proteins, MtsR was previously found to mediate the metal-dependent repression of two loci involved in metal acquisition in GAS: the sia operon, which allows hemoprotein utilization and heme uptake (shr, shp, and siaABC (Lei, Smoot et al. 2002) ((Bates, Toukoki et al. 2005) and possibly cobalt transport (siaFGH, Table 3), and the mtsABC genes, which codes for an iron and manganese transporter (Hanks, Liu et al. 2006). MtsR binding sites and recognition of promoter elements were not investigated previously, and its full regulatory scope has not been determined. In this study, we characterized MtsR promoter recognition and used global transcriptional analysis to elucidate its role in GAS physiology and virulence.

MtsR function and interactions with DNA

We have determined that a single promoter controls the expression of shr (the first gene in the sia operon). Sequence analysis around the +1 position of shr gene identified a putative -35 and an extended -10 promoter elements. The loss of promoter activity in pCHT32 (T-7T-8 to GG mutation, Table 2) supports the suggestion that the TAGATT sequence functions as P_shr -10
A subset of *B. subtilis* promoters possess a TRTG motif (R = purine) upstream of the -10 sequence. The TRTG motif in *B. subtilis* contributes to promoter activity by stabilizing the Open Complex that is formed during transcription initiation (Rodriguez, Voskuil et al. 2002). The role of the TG sequence in GAS promoters has not been investigated; our findings that both mutations in the -14 and the -15 positions had a significant effect on Pshr activity demonstrate that the identity of the residues in these positions can be very important for GAS promoters. T_{-15} to G transversion, which reduces DNA melting, eliminated promoter activity (pCHT33, Table 2). G_{-14} to A transition, which increases DNA melting, amplified Pshr strength (pCHT31, Table 2). These observations support the suggestion that the residues in these positions affect the stability of transcription initiation Open Complex in GAS promoters as they do in *B. subtilis*.

MtsR interaction with Pshr involves a 62 bp region starting upstream of the -35 element and continues to the residue at position +23. Thus, MtsR may operate as a repressor by causing steric hindrance for the RNA polymerase through binding to core promoter elements as well as creating a roadblock by binding to sequence downstream of the +1 site (van Hijum, Medema et al. 2009). The MtsR 62 bp DNA-binding region is significantly larger then that of DtxR, which protects a 27 bp region of the target DNA and uses a 9-bp palindrome pair to recognize cognate promoters (Schiering, Tao et al. 1994) (Schmitt and Holmes 1994). A large (46 bp) DNase I protection region containing 2 inverted repeats was also reported for the related protein ScaR while binding to the *scaC* promoter region (Jakubovics, Smith et al. 2000). However, significant homology between ScaR operator in P_{scaC} and MtsR operator in P_{shr} is limited to a shorter DNA segment that includes a single inverted repeat (Fig. 9C).

MtsR directly represses the *nrdF.2, aroE,* and *ska* genes, by binding to their upstream region specifically and with high affinity (Fig. 15B, Table 3). We have also found that MtsR binds to
the upstream region of pyrF, mga, and emm genes (Fig. 16B & C), which are down regulated in the mtsR background (Table 4). Therefore, MtsR can exert either a negative or positive effect on gene expression via direct binding to DNA. This is the first demonstration that MtsR functions as a dual transcriptional regulator in GAS. Similar observations were made for the orthologous regulators IdeR (Mycobacterium tuberculosis, (Gold, Rodriguez et al. 2001)) and DtxR (Corynebacterium glutamicum, (Brune, Werner et al. 2006)). The mechanism involved remains unexplored however, and additional studies are needed to determine how MtsR and its related proteins promote transcription activation.

Analysis of the sequence in the promoter region of GAS genes that are directly repressed by MtsR did not reveal significant conservation beyond a 16 bp sequence motif. Sequence signature of MtsR is AT rich and consists of 5 bp palindrome with a 6 bp spacer (Fig. 17). Noticeably, only a few positions (5/16) in the identified signature are highly conserved among the identified MtsR boxes (≥ 89%), suggesting that MtsR interactions with DNA may include recognition of structural properties in addition to primary sequence. The observation that out of the 62 bp MtsR-binding site in Pshr, only a 16 bp fragment appears to be somewhat conserved in other MtsR-regulated promoter is intriguing as well. It seems possible that MtsR binds initially to the DNA through its sequence signature and than polymerizes along the DNA by a more promiscuous step. The MtsR-binding sequence motif found in this study is similar to established and predicted operators of several metal responsive homologues including SloR of S. mutans and PsaR from S. pneumoniae (Kitten, Munro et al. 2000; Rolerson, Swick et al. 2006; Kloosterman, Witwicki et al. 2008) and is part of S. gordonii SeaR’s binding region (Jakubovics, Smith et al. 2000). Similar to the MtsR box, only a few positions in the PsaR operators are highly conserved (Kloosterman, Witwicki et al. 2008), and SloR autoregulation is mediated by binding to a DNA
region that is missing the SloR consensus (Rolerson, Swick et al. 2006). Therefore, lower requirements for primary sequence conservation in the DNA binding region may be shared by other Streptococcal DtxR orthologs. Phylogenetic analysis shows that DtxR orthologs in *Streptococcus* species such as MtsR, SloR, PsaR, and ScaR are closely related to each other and diverged early from metalloregulators found in species of *Corynebacterium* and *Mycobacterium* (Jakubovics, Smith et al. 2000; Bates, Toukoki et al. 2005). Therefore, the similarity in DNA recognition shared by the streptococcal metalloregulators is consistent with the amino acid homology they share.

*MtsR regulon and its implication for GAS physiology and virulence*

Preceding reports suggesting that MtsR has a significant role in GAS virulence prompted the determination of the complete regulatory scope of MtsR and the mechanisms that exert its functions in GAS. Comparing the transcriptome of the wild type strain to that of an isogenic *mtsR* mutant yielded 3 major observations: 1) MtsR is a global regulator in GAS which modulates the expression of 64 different transcripts. 2) MtsR transcriptional control expands beyond balancing metal uptake and includes additional metabolic functions as well as central virulence factors. 3) MtsR is an important player in the regulatory circuit that controls metal homeostasis and resistance in GAS.

A significant fraction of the MtsR-affected transcripts encode for hypothetical proteins with unknown function (Fig. 14), an observation that interferes with our ability to fully decipher MtsR contribution to GAS physiology and pathogenesis. The *aroE* locus, for example, consists of 10 orfs, none of which were previously investigated, and several of them lack homology with
known proteins (Table 3, Spy49_0450-0460). The function of the \textit{aroE} gene cluster is of particular interest because of its large upregulation (35 fold) in the absence of MtsR. MtsR did not regulate the second \textit{aroE} gene (\textit{aroE}.1, Spy49_1225c) in GAS nor did it affect the expression of any of the other enzymes from the shikimate pathway. This suggests that the \textit{aroE} cluster (Spy49_0450-0460) is involved in a function separate from the biosynthesis of aromatic amino acids and metabolites. Since the \textit{aroE} (Spy49_0450-0460) locus encodes a hypothetical export system (\textit{mefE}) and putative enzymes such as glycosyl transferase and UDP-glycose 6-dehydrogenase, it seems possible that it mediates the production and secretion of polysaccharides. Further investigations, however, are needed to examine the role of this operon in GAS biology.

Regulon analysis demonstrated that MtsR has a significant role in the metabolism of nucleic acids. MtsR was required for the full expression of the \textit{pyrR} and \textit{pyrFE} operons (Table 4, Spy49_0650-0654 and Spy49_0712-0713 respectively), which include a uracil permease (\textit{pyrP}) and genes involved in the pyrimidine biosynthesis (soz00240 pathway in KEGG data base). In addition, MtsR strongly repressed the expression of the \textit{nrdE}.2\textit{IF}.2 genes (Spy49_0339-0341, Table 3), which encode a ribonucleotide reductase complex (NrdE.2F.2) that allows the reduction of ribonucleotides into deoxyribonucleotides, the precursors for DNA synthesis. The GAS genome includes two types of RNRs: a strictly anaerobic, Class III enzyme (\textit{nrdDG}) and two aerobic RNRs from Class Ib (\textit{nrdHEF} and \textit{nrdF}.2\textit{IE}.2 operons). Unlike the NrdEF enzyme complex, NrdE.2F.2 proteins require the flavodoxin NrdI to function. In addition, NrdF.2 is missing 3 of the 6 conserved residues that ligate the iron center in the \(\beta\) subunit of the enzyme, and, therefore, has lower iron content than NrdF. Both Class Ib enzyme complexes are expressed independently in GAS (Roca, Torrents et al. 2008). This study demonstrated however, that MtsR represses the transcription of only the \textit{nrdF}.2\textit{IE}.2 cluster, suggesting that transcription
of this operon will be induced under low metal conditions. The reason for the redundancy in RNRs belonging to the same class is not clear, but *nrdF.2IE.2* regulation by MtsR implies that this RNR complex is more adept at functioning under low iron conditions. PerR also negatively regulates the *nrdF.2IE.2* operon (Gryllos, Grifantini et al. 2008), indicating that oxidative stress will promote *nrdF.2IE.2* induction. RNRS have a central role in regulating DNA synthesis and in keeping the ratio of DNA content to cell mass constant during growth. Therefore, it is possible that under unfavorable growth conditions (such as iron depletion or oxidative stress) NrdF.2E.2 is the RNR of choice in GAS.

In addition to controlling nucleic acid metabolism, MtsR negatively regulates the expression of genes involved in peptide uptake, amino acid metabolism, and protein synthesis (Fig. 14 and Table 3). A moderate increase in the expression of the molecular chaperone *dnaK* and its co-chaperone *grpE* was seen in the *mtsR* mutant (~2.5 fold, Table 3). DnaK and its two co-chaperones DnaJ and GrpE are involved in multiple aspects of protein fate including protein folding and assembly and disassembly of protein complexes. Like in other Gram-positive bacteria, the *hrcA* and *grpE* genes precede *dnaK* in the GAS genome. Consistent with an operon structure, all 3 genes (*hrcA*, *grpE* and *dnaK*) were upregulated in the *mtsR* mutant to a similar extent. The *dnaJ* gene, which is found downstream of *dnaK*, seems to be independently transcribed, as it was not affected by *mtsR* inactivation. HrcA is a negative transcription regulator that controls the expression of *dnaK* and *groES* operons, though a significant induction of *dnaK* is still observed following heat shock in a *hrcA* background in GAS (Woodbury and Haldenwang 2003). Our finding that MtsR represses the *dnaK* operon implicates it as the mediator of the *hrcA*-independent response of *dnaK* to heat shock. This suggestion is in
agreement with the report that *mtsR* transcription is significantly down regulated at high temperature (Graham, Smoot et al. 2001).

An important observation made in this study is that MtsR is involved in the regulation of key virulence factors in GAS. MtsR directly represses the gene encoding streptokinase (*ska*), which converts the proenzyme plasminogen to the broad-spectrum protease, plasmin. GAS streptokinase is an important virulence factor that contributes to bacterial dissemination and tissue damage, and is implicated in glomerulonephritis pathology and the coagulopathy seen during GAS invasive infections (Nordstrand, Norgren et al. 1998; Olsen, Shelburne et al. 2009). The mechanisms that control *ska* expression during infection are not fully understood. However, it was previously demonstrated that the *fasABCX* TCS system activates *ska* transcription while the RR CovR directly represses it (Kreikemeyer, Boyle et al. 2001; Churchward, Bates et al. 2009). Since the CovRS system responds to several stress cues including iron limitation by alleviating CovR-mediated gene repression (Froehlich, Bates et al. 2009), iron depletion is expected to ease *ska* repression by MtsR and CovR in a wild type strain. Inactivation of MtsR creates a regulatory conundrum, though; the mutation in *mtsR*, which relieves *ska* repression, also leads to elevated intracellular iron content (Bates, Toukoki et al. 2005), a condition that may foster repression of *ska* by CovR. Thus additional investigations are needed to determine the outcome of *mtsR* inactivation on *ska* expression *in vivo*. GAS streptokinase is specific for human plasminogen and its production is not expected to change the outcome of infection in animal models. Therefore the attenuation in virulence demonstrated by the *mtsR* mutant in a zebrafish model is likely to result from the loss of MtsR control of other genes important for GAS virulence or fitness.
In addition to repressing *ska*, MtsR positively affects the expression of the key virulence factors *mga* and *emm* (2 and 11 fold decrease respectively, Table 4). Mga is a central regulator in GAS that activates the transcription of multiple GAS virulence genes. These are typically surface components that mediate adherence, internalization and/or immune evasion, including the M and M-like proteins, C5a, Sic, and several fibronectin proteins (Hondorp and McIver 2007). It is interesting that MtsR positively regulates *emm* transcription directly in addition to the effect it has on *mga* expression. Our finding that MtsR binds to *emm* promoters from several M serotypes (M49, M1, M6) suggests that it is not a strain-specific phenomenon. MtsR activation of *emm* transcription may allow the fine-tuning of *emm* expression. The activation of *mga* and *emm* transcription by MtsR is consistent with the observation that *emm* transcription decreases in response to iron depletion in the M type 6 strain JRS4 (McIver, Heath et al. 1995), and indicates that MtsR mediates this response. No change in expression of Mga-regulated genes other than *emm* was observed in the *mtsR* mutant strain. Therefore, MtsR effect on *mga* transcription may not be sufficient to shape the expression of the entire Mga regulon. The Mga regulon is highly expressed during acute pharyngitis (Virtaneva, Graham et al. 2003). While iron concentration in secretions is very low, manganese availability in saliva is high (36 μM versus 20 nM in blood and plasma (Chicharro, Serrano et al. 1999)). The high concentration of manganese in saliva is likely to promote MtsR binding to *mga* and *emm* promoters in vivo contributing to elevated expression of *emm* during pharyngitis.

The inverse direction in which MtsR influences the transcription of *ska* or *mga* and *emm* is intriguing. Plasminogen availability at GAS infection site is affected by M protein: M-protein/fibrinogen complexes that are formed on GAS surface elicit vascular damage and, therefore, lead to plasma leakage near the infection site. GAS binds soluble plasminogen or
plasmin by GAPDH and other surface components (Walker, McArthur et al. 2005). Iron availability seems to play an important role in controlling the events mentioned above: iron limitation down regulates emm expression (McIver, Heath et al. 1995), leads to the release of GAPDH from the cell surface (Eichenbaum, Green et al. 1996), and supports increased ska transcription ((Froehlich, Bates et al. 2009) and this study). Therefore, while colonizing the nasopharynx GAS may benefit from promoting vascular leakage and the presence of surface bound plasminogen, deep tissue infections may be supported by the presence of active plasmin in the GAS extracellular environment. This study suggests that MtsR influences the cell-bound plasminogen/free plasmin cycle by mediating a reverse affect on the transcription of emm and ska in response to metal availability. This hypothesis is in accordance with the observation that MtsR is required for full virulence of GAS in invasive disease, as clinical isolates with null mutation in MtsR were less likely to produce necrotizing fasciitis (Beres, Richter et al. 2006).

Analysis of the MtsR regulon demonstrated that in addition to repressing the expression from the mts and the sia loci MtsR negatively controls the P-type cation transporters copA and pmtA (Table 3 and Fig. 18). The function of copA was not previously investigated in GAS. Sequence
Figure 18. Comparison of MtsR and PerR regulons.

The diagram shows GAS genes that are common to both the MtsR and PerR regulons, as well as genes from each regulon that are involved in metal homeostasis (indicated in red) and resistance to oxidative stress. The putative ligand of each system is provided in parentheses. Asterisks indicate a strain-specific regulation. Gene activation is indicated by arrowheads, and repression is shown by blunt ends. Direct regulation is shown with solid lines and indirect (or unknown) regulation is indicated by dashed lines. The genes displayed in the figure are a compilation of work done in this study and others (Ricci, Janulczyk et al. 2002; Brenot, King et al. 2005; Beres, Richter et al. 2006; Brenot, Weston et al. 2007; Gryllos, Grifantini et al. 2008).
homology suggests that CopA is involved in the transport of copper or other heavy metals. CopA has a Heavy Metal Associated domain (HMA, PFAM accession number PF00403) in its amino terminus and shares significant homology (57% identity) with a copper efflux ATPase from *S. mutans* (Vats and Lee 2001). The ligand and the transport direction mediated by PmtA are unknown as well. However, since PmtA promotes resistance to peroxide stress and zinc toxicity it is suggested to carry out metal efflux (Brenot, Weston et al. 2007). Overexpression of pmtA, which is directly repressed by PerR, leads to derepression of the adcR regulon, which includes a number of zinc and manganese transporters (Brenot, Weston et al. 2007). The finding that pmtA is also part of the MtsR regulon is intriguing, as no induction of the AdcR-repressed genes was observed in the mtsR mutant (Table 3). It is possible that the modest upregulation of pmtA in the mtsR mutant is not sufficient to generate the intracellular signal that is required to induce the AdcR regulon. Alternatively, activities of the other transporters that are induced in the mtsR mutant counteract the AdcR-derepression signal generated by the PmtA transporter. In either case, it is likely that PmtA links the MtsR regulon to that of AdcR, at least under some growth conditions. Thus, MtsR represses the expression of a number of transporters, which in turn mediate the traffic across the cell membrane of free and complex iron, manganese, and possibly cobalt, copper and other metals.

In addition to PmtA, the overlap between MtsR and PerR regulons includes 5 other transporters and enzymes (Fig. 18). PerR and MtsR negatively regulate the expression of 4 out of the 6 common genes, and, therefore, their maximum expression may be seen when GAS is experiencing both oxidative stress and metal starvation. Examination of both MtsR and PerR regulons reveal that many GAS genes predicted to contribute to GAS metal homeostasis are regulated by either MtsR or PerR, suggesting that both regulators are key components in
determining GAS metallome. Additional GAS regulators, though, are also involved to metal homeostasis. Mga has a positive affect on the expression of the iron complex transporter *siuADBG* (Ribardo and McIver 2006), which is regulated by neither MtsR nor PerR. And as mentioned above, the CovRS TCS system mediates the iron-dependent repression of *mtsA* (at least in the M type 6 strains JRS4 (Froehlich, Bates et al. 2009)). In summary, this work demonstrates that MtsR is a dual regulator that shapes the expression of large number of genes in GAS. Like the related orthologues SloR and PsaR (Johnston, Briles et al. 2006; Rolerson, Swick et al. 2006; Dunning, McCall et al. 2008; Hendriksen, Bootsma et al. 2009), the scope of MtsR regulation expands beyond metal homeostasis and includes functions that directly contribute to colonization and disease production.
MATERIALS AND METHODS

Strains, media, and growth conditions

*Escherichia coli* (E. coli) DH5α and BL21 (DE3) were used for cloning and gene expression. The GAS strains used in this study were NZ131, an M type 49 (Simon and Ferretti 1991) and the *mtsR* isogenic mutant ZE491 (Bates, Toukoki et al. 2005). *E. coli* cells were grown aerobically in Luria Bertani (LB) medium at 37ºC. GAS cells were grown statically at 37ºC in Chemically Defined Medium (CDM; SAFC Biosciences) as described in Montanez *et al* 2005 (Montanez, Neely *et al*. 2005) or in Todd Hewitt Broth with 0.2% (w/v) yeast extract (THYB; BD laboratories). When necessary, 100 μg/ml spectinomycin, 70 or 300 μg/ml kanamycin (for *E.coli* and GAS, respectively) was added to the medium.

DNA manipulations

Chromosomal and plasmid DNA extraction and DNA manipulations, including restriction digest, cloning, and DNA transformation into *E. coli* or GAS, were done according to the manufacturer’s recommendations and with standard protocols as previously described (Sambrook, Fritsch *et al*. 1989; Dahan-Grobgeld, Livneh *et al*. 1998). PCR for cloning was performed using the High Fidelity AccuTaq LA DNA Polymerase (Sigma). PCR products were purified with the QIAquick PCR Purification Kit (Qiagen). DNA sequencing was performed using the SequiTherm Excel II DNA sequencing kit (Epicentre) or by GSU’s core facility. End labeling with [γ-32P] ATP was performed using T4 Polynucleotide Kinase (Invitrogen or Epicentre). Site directed mutagenesis was done using the QuikChange II XL Site-Directed
Mutagenesis Kit (Stratagene). The oligonucleotide primers used in this study are listed in Table 5. Table 6 lists and describes the plasmids used in this work.

Expression and purification of recombinant MtsR (His₆-MtsR)

Recombinant MtsR (rMtsR, with a C-terminal fusion to His₆ tag) was purified from *E. coli* according to Bates *et al.* (Bates, Toukoki et al. 2005) with small modifications. Briefly, BL21 (DE3) cells harboring plasmid pZEDH3.1 were grown at 37°C in LB containing ampicillin (100 μg/ml). Protein expression was induced at OD₆₀₀=0.6 by IPTG (isopropyl-β-D-thiogalactopyranoside; Promega). The cells were harvested, resuspended in phosphate buffer (50 mM NaPO₄, 0.5 M NaCl, pH 8.0), and lysed by sonication. Proteins were precipitated with 1% (w/v) streptomycin sulphate and 40% (w/v) ammonium sulphate. The protein pellet was resuspended in binding buffer (50 mM NaPO₄ pH 7.4, 0.5 NaCl, and 20 mM imidazole) and purified over a nickel affinity column (HisTrap HP column; GE Lifesciences). The purified protein was examined by SDS-PAGE and western blot analysis with anti-MtsR antibodies (Fisher, Huang et al. 2008). Protein concentration was determined by Bradford assay (Biorad).

Electrophoretic mobility shift assays (EMSAs)

EMSAs were done as described in Bates *et al.* (Bates, Toukoki et al. 2005) with small modifications. Briefly, DNA fragments were amplified from the NZ131 chromosome, end labeled with [γ⁻³²P] ATP and purified using the Quick Spin Columns (TE) for radiolabeled DNA (Roche). For the DNA shift assays, increasing concentrations of rMtsR (in 20 mM KPO₄, pH
7.0) were incubated with approximately 50 pmol labeled DNA fragment for 15 minutes at room temperature in a reaction buffer containing 20 mM NaPO₄, 50 mM NaCl, 5 mM MgCl₂, 2 mM dithiothreitol, 0.4 mg/ml bovine serum albumin, 0.2 mg/ml sheared salmon sperm DNA (Ambion), and 9.6% (v/v) glycerol. The reaction mixture was fractionated over a 5% (v/v) polyacrylamide gel containing 2.5% (v/v) glycerol, 20 mM NaPO₄, and 2 mM dithiothreitol. Dissociation constants (Kd) were calculated using ImageQuantTL 7.0 (GE Healthcare). The density of the free DNA fragment was determined and plotted as a function of MtsR concentrations. Linear regression was used to determine the Kd value (MtsR concentration at which half of the DNA is shifted).

**DNase I protection assay**

DNase I footprint assays were performed as previously described (Gao, Gusa et al. 2005) with small modifications. Briefly, a 193 bp fragment containing shr promoter region was amplified from pCHT19 using the [γ-³²P] labeled primers ZE227 and ZE228. The PCR fragments were purified from an 8% (v/v) polyacrylamide gel. Increasing concentrations of rMtsR (in 20 mM KPO₄) were added to a DNA binding buffer containing 1 µM DNA, 33 mM Tris-acetate pH 8.0, 150 µg/ml BSA, 10 mM MgAc and 0.5 mM dithiothreitol. The reactions were incubated for 15 minutes at room temperature before the addition of 2 ng/µl DNase I and subsequently incubated at 37°C for 50 seconds. DNase was inactivated with pellet paint (Novagen) and DNase I stopbuffer (80% (v/v) EtOH and 22.5 mM NH₄OAc). The DNA was precipitated at -80°C, dried at room temperature, and resuspended in Stop>Loading buffer (Epicentre). The reactions were run for approximately 1.5 hour on a 6% (v/v) sequence gel.
Determination of the transcription start site in \( P_{shr} \)

The transcription initiation site was determined using the 5’Race method (5’Race System for Rapid Amplification of cDNA Ends, Invitrogen). Briefly, RNA was isolated from GAS cells harvested at the mid-logarithmic phase. The first strand cDNA was synthesized using the \( shr \) specific primer ZE224. Homopolymeric tails were added to the 3’ ends of the cDNA, which was then used as a template in a PCR reaction with the Abridged Anchor Primer (Invitrogen) and the nested \( shr \) primer ZE225. A second PCR reaction was done using the UAP primer (Invitrogen) and the \( shr \)-specific primer ZE226. The product was cloned into a TOPO vector (pCR8/GW/TOPO TA Cloning Kit, Invitrogen) producing plasmid pCHT12 and pCHT13 (for NZ131 and ZE491 respectively). The 5’Race procedure was repeated using the \( shr \) primer ZE225 to produce the cDNA and the \( shr \) primers ZE226 and ZE234 for the nested PCR reactions (producing plasmids pCHT25 & pCHT26 for NZ131 and ZE491 respectively). The sequence of the cloned fragments was analyzed to determine the transcription start site.

Construction of \( P_{shr} \)-luc transcriptional fusions

All of the transcriptional fusions used in this study are described in Table 5. Shr promoter fusions were constructed by amplifying DNA fragments from NZ131 chromosomal DNA and cloning them into pKSM720 (Kinkel and McIver 2008), which carries a promoterless luc gene. For plasmid pCHT21, the upstream region of \( shr \) gene (360bp) was amplified with ZE204 and ZE211 primers, digested with BglII and XhoI, and ligated to the BglII/XhoI fragment of pKSM720. The same method was used to construct pCHT22, which carries a 175 bp fragment from the \( shr \) upstream region amplified with ZE205 and ZE211 primers. Plasmid pCHT23
harboring a 126 bp fragment from the shr upstream region was cloned the same way, only using primers ZE206 and ZE211. Plasmid pCHT31, containing shr-luc promoter fusion with a mutation at G-14 of Pshr was constructed using pCHT30 as a template in a PCR reaction with ZE293 and ZE307 primers. The resulting PCR product, containing the promoter fragment, was digested and cloned into BglII and XhoI sites in pKSM720. Plasmid pCHT32, carrying shr-luc promoter fusion with mutations in T-7 and T-8 of Pshr was constructed using the same method only with the primers ZE310 and ZE311. Plasmid pCHT33, carrying shr-luc promoter fusion with a mutation at T-15 in Pshr was generated the same way only with ZE312 and ZE313 primers. Plasmid pCHT19 which encodes an shr-phoZ transcriptional fusion, was generated by amplifying the region from the 3’ end of isp to shr gene using ZE166 and ZE167 primers, and cloned into the HindIII site in pCW1 (Bates, Montanez et al. 2003).

**Luciferase Reporter Assay**

Luciferase activity in GAS strains harboring luc transcriptional fusions was done as previously described in Leday T.V. et al (Leday, Gold et al. 2008). Briefly, culture samples were harvested at OD$_{600}$ = 0.5 and the cell pellets were resuspended in Luciferase Cell Culture Lysis buffer. Britelite plus reagent (Perkin Elmer) was added to the cells in a microtiter plate and luminescence was measured using the 1420 Multilabel Counter Victor $^3$V (Perkin Elmer).
Microarray and real-time RT-PCR validation

Microarray experiments were performed as previously described (Leday, Gold et al. 2008). Briefly, total RNA from 3 biological replicates was isolated from NZ131 and the isogenic MtsR- mutant strain ZE491 at mid-logarithmic phase (90 Klett units) using the Ambion RNA purification kit. 18 µg of RNA was treated with DNase I and analyzed for quality on formaldehyde-agarose gel. RNA samples were converted to cDNA with an amino-allyl UTP and were labeled with both Cy3 and Cy5 using the Amino Allyl cDNA Labelling Kit (Ambion) to allow for dye-swap experiments. Yield and incorporation of dye was determined using a Nanodrop ND-1000 (Nanodrop Technologies). Equal volumes (35.42 µl) of labeled Cy5 cDNA and Cy3 cDNA were dried under vacuum, resuspended in 23.8 µl of dH2O and boiled for 5 min followed by cooling on ice for 5 min. 5XHyb Buffer (GE Healthcare, 17 µl) and formamide (27.2 µl) was added to the cDNA and applied to array slides under raised cover slip (Lifterslip, Inc). Microarray slides were hybridized at 50°C overnight in slide chambers (Array It). Slides were washed twice for 10 min each in the following buffer concentrations and temperatures: 6X SSPE/0.01% Tween-20 at 50°C, 0.8X SSPE/0.001% Tween-20 at 50°C, and 0.8X SSPE at RT. Slides were scanned using a Genepix 4100A personal array scanner and GenePixPro 6.0 software (Axon Instruments).

Data obtained from the wild type and mtsR mutant strains were compared for 2-fold changes in expression, ≥ 2.0 or ≤ 0.50, and were analyzed using Acuity 4.0 software (Axon Instruments). Using a ratio-based normalization, data was normalized by the ratio of the means (635/532) and samples were removed when 4 out of the 6 experiments did not show significance. Array validation was carried out by real-time RT-PCR using 9 differentially regulated genes using primers in Table S1. Correlation coefficients for the arrays were determined by plotting the log
value of the array on the X-axis to the log value of the real-time RT-PCR on the Y axis. An equation determining the line of best fit was determined, and the resulting $R^2$ value was calculated to be 0.943 (Figure 13), which represented the fitness of the data.

Real-time RT-PCR

Briefly, total RNA was isolated from each strain using the TritonX-100 isolation protocol (Sung, Khan et al. 2003) and 25 ng was DNase I-treated, added to a SYBR Green Master mix (Applied Biosystems) containing 5 µg of each specific real-time primer (Table 5), and combined with 6.25 units of Multiscribe reverse transcriptase (Applied Biosystems) in a 25 µl volume for a one step real-time RT-PCR reaction. The real-time RT-PCR experiments were completed using a Lightcycler 480 (Roche) and transcript levels were detected in the relative quantification mode. Samples were compared to WT gyrA transcript levels, with the levels presented representing ratios of WT/experimental.

In silico analysis

The online directory Kyoto Encyclopedia of Genes and Genomes (KEGG) (Okuda, Yamada et al. 2008) was used for the analysis of metabolic pathways and orfs from GAS. LALIGN program {Huang, 1991 #20} was used for sequence analysis. The WebLogo software (http://weblogo.berkeley.edu/) (Crooks, Hon et al. 2004) was used to generate MtsR-binding sequence signature.
Table 5. Oligonucleotide primers used in Chapter II.

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<td>metK.1 M49 RT-R</td>
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<tr>
<td>pyrF M49 RT-L</td>
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<td></td>
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<tr>
<td>pyrF M49 RT-R</td>
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</tr>
<tr>
<td>gyRA M4 RT-L</td>
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<td></td>
</tr>
<tr>
<td>gyRA M4 RT-R</td>
<td>CACACCGAGTTCATTCATCC</td>
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</table>
Table 6. Plasmids used in Chapter II.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pZEDH3.1</td>
<td><em>E.coli</em> expression vector carrying <em>mtsR</em> under the control of the T7 RNA polymerase</td>
<td>(Bates, Toukoki et al. 2005)</td>
</tr>
<tr>
<td>pKSM720</td>
<td><em>E.coli/GAS</em> shuttle vector with the firefly luciferase (<em>luc</em>) gene and ribosomal binding site</td>
<td>(Kinkel and McIver 2008)</td>
</tr>
<tr>
<td>pCR8/GW/TOPO</td>
<td><em>E.coli</em> TOPO PCR cloning vector</td>
<td>Invitrogen</td>
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<tr>
<td>pCHT12</td>
<td><em>E.coli</em> TOPO vector carrying the DNA fragment produced by the 5’RACE protocol using NZ131 RNA as a template</td>
<td>This study</td>
</tr>
<tr>
<td>pCHT13</td>
<td><em>E.coli</em> TOPO vector carrying the DNA fragment produced by the 5’RACE protocol using ZE491 RNA as a template</td>
<td>This study</td>
</tr>
<tr>
<td>pCHT19</td>
<td><em>E.coli</em> TOPO vector with a fusion of 3’<em>isp-Pshr</em> upstream region to the <em>phoZ</em> reporter gene</td>
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</tr>
<tr>
<td>pCHT21(^a)</td>
<td><em>E.coli/GAS</em> shuttle vector with a fusion of <em>shr</em> upstream region (bp 1-360) to the <em>luc</em> gene</td>
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</tr>
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<td>pCHT22(^a)</td>
<td>The same as in pCHT21, only with a fusion of a fragment (bp 1-175) from <em>shr</em> upstream region to the <em>luc</em> gene</td>
<td>This study</td>
</tr>
<tr>
<td>pCHT23(^a)</td>
<td>The same as in pCHT21, only with a fusion of a fragment (bp 1-126) from <em>shr</em> upstream region to the <em>luc</em> gene</td>
<td>This study</td>
</tr>
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<td>pCHT25</td>
<td><em>E.coli</em> TOPO vector carrying the DNA fragment produced by the 5’RACE protocol using NZ131 RNA as a template</td>
<td>This study</td>
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<td>pCHT26</td>
<td><em>E.coli</em> TOPO vector carrying the DNA fragment produced by the 5’RACE protocol using ZE491 RNA as a template</td>
<td>This study</td>
</tr>
<tr>
<td>pCHT27(^a)</td>
<td>The same as in pCHT19 only with an <em>E.coli/GAS</em> shuttle vector</td>
<td>This study</td>
</tr>
<tr>
<td>pCHT30(^a,b)</td>
<td>Same as pCHT27, only with G(_{14}) to A mutation</td>
<td>This study</td>
</tr>
<tr>
<td>pCHT31(^a,b)</td>
<td>Same as pCHT21, only with G(_{14}) to A mutation</td>
<td>This study</td>
</tr>
<tr>
<td>pCHT32(^a,b)</td>
<td>Same as pCHT21, only with T(_{7,8}) to GG mutations</td>
<td>This study</td>
</tr>
<tr>
<td>pCHT33(^a,b)</td>
<td>Same as pCHT21, only with T(_{15}) to G mutation</td>
<td>This study</td>
</tr>
</tbody>
</table>

\(^a\). A schematic presentation of the *luc*-fusions carried by this plasmid is shown in figure 10A.

\(^b\). The mutated residue position is given in relation to the *shr* transcription start site shown in figure 10B.
GENERAL DISCUSSION

The human pathogen GAS requires manganese and iron to support fundamental cellular processes. Free iron in the fluids of the human body is very limiting ($\leq 10^{-18}$ M) as the vast majority of iron is bound to host proteins. On the other hand, manganese availability varies at different body sites. Free Mn is available in micromolar concentrations in nasopharynx secretion and saliva (36 $\mu$M), while it is found in nanomolar concentrations in serum (Johnston, Briles et al. 2006). A number of iron-scavenging systems are used by GAS to compete for iron while colonizing the human host. This machinery allows the hemolytic streptococcus to acquire iron from human iron storage and carrier proteins such as ferritin, hemoglobin, myoglobin, and hemin (Litwin and Calderwood 1993; Eichenbaum, Muller et al. 1996). Current research describes three ABC-type iron transport systems in GAS named siaABC ($htsABC$), mtsABC, and siuADB ($ftsABCD$), which mediate the transport of free or complexed iron cross the cytoplasmic membrane (Bates, Montanez et al. 2003; Janulczyk, Ricci et al. 2003; Lei, Liu et al. 2003; Hanks, Liu et al. 2005; Montanez, Neely et al. 2005). To balance the need for iron with its toxic effects, bacteria regulate iron uptake and mechanisms to avoid oxidative stress according to iron availability, using regulators from the Fur and DtxR families. In this thesis we identify and characterize in GAS a DtxR homolog named MtsR. We demonstrate that MtsR requires iron or manganese to bind DNA, and investigate its interactions with targeted promoters, including that of the sia operon ($P_{sia}$). We also illustrate in this dissertation that MtsR has a global regulatory scope, which is required for the disease production and pathology of GAS.
MtsR, the DtxR ortholog of GAS

Bates et al reported that the sia gene cluster is a 10-gene operon that mediates heme uptake and is negatively regulated by iron (Bates, Montanez et al. 2003). The use of Fur and DtxR proteins to regulate iron uptake is a widely spread theme among bacteria; we hypothesized that a similar repressor enables the iron responsive regulation of the sia operon. Moreover, we suggested that the sia operon is only a part of an iron sensitive transcriptome, which shapes the metallome composition in GAS as well as important metabolic functions and the profile of virulence genes.

GAS encodes a Fur ortholog, PerR, which was shown to mediate peroxide resistance. We identified in GAS genome 5’proximal to the mtsABC operon a dtxR ortholog, which is transcribed divergently to the mtsABC genes (Figure 1). The genes and the genetic organization of the mts cluster in GAS are similar to those of homologous operons found in several Streptococci, where a DtxR ortholog acts as the repressor of cognate metal transporter encoded nearby. The sca, slo, and psa loci in the chromosomes of S. gordonii, S. mutans, and S. pneumoniae respectively. For example, include the ABC metal transporters scaABC, sloCBA, and psaBCA and the DtxR-like repressors, scaR, sloR, and psaR respectively. Because of this similarity the DtxR ortholog in GAS was named MtsR (for mts repressor).

MtsR amino acid sequence shares high homology with SloR (55% identity) and ScaR (52% identity). The primary metal binding site in DtxR consists of His76, Glu83, His98, GLu170, and Gln173 and is essential for DNA binding (Spiering, Ringe et al. 2003). Alignment of MtsR with the DtxR orthologs ScaR (S. gordonii), SloR (S. mutans), PsaR (S. pneumoniae), SirR (Staphylococcus epidermitis), and TroR (Treponema denticola) revealed that the primary metal binding site, consists of Asp7, Glu99, Glu102, and His103, is fully conserved in these metalloregulators. The ancillary metal binding site, consisting of His76, Glu80, Cys123, His125,
and Asp160, is highly conserved among the related metalloproteins. However, MtsR has a leucine residue at position 76, while the other proteins have a histidine residue at this position (Stoll, Draper et al. 2009).

ScaR from \textit{S.gordonii} is the only one of the streptococcal metalloproteins that has been characterized structurally. Each of the two ScaR subunits binds two manganese ions, and like DtxR, two dimers of the ScaR repressor bind the \textit{scaC} promoter. In addition to manganese, other metals including Cd, Co, and Ni, were shown to induce DNA binding by ScaR. While each of the DtxR subunits binds two metal ions (one in the primary and one in the ancillary metal binding sites), ScaR crystal structure revealed that ScaR does not contain an occupied ancillary metal site. Zn or Cd atoms apparently bind to ScaR at a separate site that is labeled as “secondary”. This metal binding position is located 5 Å away from the predicted ancillary metal binding site. Therefore, ScaR does not contain a true ancillary metal binding site. These findings suggest the presence of a new DtxR-subgroup of regulatory proteins may be activated by a mechanism that is different from that of DtxR and more related homologs (Stoll, Draper et al. 2009). We are currently collaborating with D'Aquino (Brandeis, Boston) to learn how MtsR is activated by metals.

\textbf{MtsR binding to DNA}

The \textit{mtsR} gene was cloned with a carboxy-terminal His\textsubscript{6} tag, expressed, and purified. EMSAs demonstrated that MtsR binds specifically to a fragment containing the \textit{sia} promoter region in an iron- and manganese-dependent manner (Figure 5). 5’Race identified a single transcription start site in the 360 bp upstream region \textit{shr}, first gene in the \textit{sia} operon (Figure 9A & B). Reporter
fusions and site directed mutagenesis confirmed the location of the identified -10 and -35 elements of \( P_{shr} \) (Figure 10 and Table 2). MtsR binds only to the first 89 bp upstream of \( shr \). DNase I footprint analysis demonstrated MtsR binding to \( P_{shr} \) fragment was not symmetrical. On the coding strand the regulator protected a 62 bp region, while on the template strand the protected area was larger (69 bp). MtsR interaction with the DNA on the coding strand starts at the T residue at position -37 in \( P_{shr} \) and continues on the template strand downstream beyond the ribosomal binding site (Figure 9 & 12).

We also performed DNase I footprint analysis on a fragment containing the \( mtsA-mtsR \) promoter and this analysis revealed that MtsR protects a 40 bp region on the coding strand, but does not protect on the template strand (see Appendix C, Figure 21). Aligning the 62 bp MtsR protection in the \( shr \) promoter with the 40 bp protection in \( mtsA-mtsR \) promoter generates a potential 16 bp MtsR binding motif; ATTAAGTTxAGTTAAT (Figure 17). This MtsR sequence signature can be found in all the other promoters that are directly regulated by MtsR including \( aroE, ska, pyrF, mga, \) and \( emm \). Although the 16 bp signature is not highly conserved in all of the promoters, it consists of a high AT rich palindrome (Figure 17). In the negatively regulated genes, the MtsR binding motif overlaps the core promoter elements (-35 and -10). Therefore, like with many repressors, MtsR DNA binding may interfere with RNA polymerase and progress along the DNA. In positively regulated genes MtsR binding motif is found either downstream of upstream of the promoter elements. The different location of the MtsR potential binding sites and the low demand for primary sequence conservation suggests that MtsR may function as a nucleoid associate protein (NAP), such as H-NS or HU. NAPs are small basic proteins that bind preferentially to AT-rich sequence or DNA with intrinsic curvature and thereby suppress gene expression. H-NS in pathogenic bacteria regulates genes that are involved in bacterial adaptation.
to changes in environment and are referred to as “common transcription silencers”. Like H-NS, MtsR may recognize the conserved residues in its sequence signature as well as certain DNA configuration that results from the highly AT rich palindrome. This sequence may generate a curved DNA structure that is recognized by MtsR, and may be followed by lateral continuation along the DNA (Shin, Song et al. 2005). MtsR binding to DNA can either result in either repression or activation of gene transcription. Additional investigations are needed to determine if MtsR functions as a “true” activator and directly interacts with the RNA polymerase. Alternatively, MtsR binding to DNA may counteract the affect of other repressors and therefore its positive effect on transcription may be indirect.

**Iron and GAS virulence**

GAS occupies many different sites within the human body during infection. A strong fit of GAS to the infection location requires it to sense the environment and adjust its transcriptome accordingly. Pathogenic bacteria use a variety of environmental factors including metal availability, pH, osmolarity, temperature, and amino acid concentration as regulatory cues. Since iron is very limited in the human host, iron availability is also used as a signal by pathogens to adapt their transcriptional profile. It is not surprising, therefore, that the expression of toxins and hemolysins is regulated by iron in many pathogens including *C. diphtheriae*, *Pseudomonas aeruginosa*, and species of *Vibrio* and *Yersinia* (Litwin and Calderwood 1993). Relatively little is known in GAS about the role of iron accessibility on the expression of virulence determinants, other then the previous reports that the transcription of the
antiphagocytic M protein (\textit{emm}) in GAS is positively regulated by iron (McIver, Heath et al. 1995).

Several investigations demonstrated that iron homeostasis is needed for full GAS virulence. Inactivation of the \textit{siu} and \textit{mts} transporters resulted in attenuation of virulence in infection models (Janulczyk, Ricci et al. 2003; Montanez, Neely et al. 2005). An intact \textit{mtsR} gene is required for GAS pathogenesis in a \textit{Danio rerio} zebrafish infection model and humans (Figure 7 and (Beres, Richter et al. 2006)). Our studies reveal that MtsR regulates the expression of several metal transporters. The MtsR repressed transporters \textit{siaABC}, \textit{mtsABC} and \textit{siaFGH} mediate the uptake of heme, iron, manganese and possibly cobalt. On the other hand the P-type transporter PmtA and CopA may allow the evacuation of excess metals from the bacterial cells (Figure 18). MtsR is therefore, an important factor, which shapes the expression of genes involved in metal flux and defense. We demonstrated that \textit{mtsR} inactivation result in higher level of intracellular iron. This observation is likely to be an underestimation of MtsR total affect on GAS metallome. Alteration in the intracellular metal composition and levels is likely to make GAS more sensitive to oxidative stress, as demonstrated by the observed sensitivity to the streptonigrin and hydrogen peroxide \textit{in vitro} (Figure 6). \textit{In vivo}, the changes in GAS metallome may render the bacterium more sensitive to ROS it encounters during the infection process.

**The regulation of the \textit{mts} gene cluster**

In our study, RT-PCR performed using RNA isolated from \textit{mtsR} mutant and wild type strains grown in iron rich medium show no difference in \textit{mtsA} transcript levels. However, a small
increase in *mtsR* transcript level was observed in the *mtsR* mutant compared to the wild type strain (see Appendix D, Figure 22). These observations suggest that in the M type 49 strain (NZ131), MtsR regulates only its own expression and not the expression of the other *mts* genes. The regulation of the *mts* genes was previously found to vary among GAS strains. A deletion in the *mtsR* gene in MGAS5005 (M type 1) eliminated the observed metal dependent repression of *mtsA* expression (Hanks, Liu et al. 2006). On the other hand, inactivation of the *perR* in AP1 strain (M1 serotype) resulted in a decreased transcription level of *mtsA*, suggesting that *mtsA* is repressed by PerR (Ricci, Janulczyk et al. 2002). *In silico* analysis identified a potential PerR binding site in the *mtsA-mtsR* promoter region, which shares 73% identity with the PerR consensus-binding box (see Appendix D, Figure 22C). Clearly, more experiments are required to illustrate the regulatory mechanisms that control the expression of the *mts* genes in the M49 serotype (NZ131).

**MtsR and PerR cross talk**

**MtsR regulon**

We used global transcriptional analysis to define the *mtsR* regulon. Microarray analysis identified 64 genes of which expression was changed in the *mtsR* mutant strain compared to the wild type strain (Table 3 and 4). The expression of 44 genes was increased in the *mtsR* mutant (Table 3). This increase demonstrates that MtsR negatively regulates multiple genes involved in
transport, as well as those involved in metabolism of amino acids and nucleic acids, proteins fate, and energy (Figure 14). The expression of 20 genes was reduced in the mtsR mutant (Table 4). MtsR affects the expression of 3 major virulence factors. The transcription of the streptokinase (ska) is repressed by MtsR, while the expression of the global regulator mga and the antiphagocytic emm49 is positively regulated (Table 3 and 4). Therefore, MtsR contribution to GAS infection process expands beyond adaptation of GAS metabolism and adjustment of its transport machinery and includes genes that are directly required for the production of disease and pathology.
REFERENCES


APPENDICES
APPENDIX A

Figure 19. Construction of pCHT10.

Primers ZE38 and ZE39 were used to amplify \textit{mtsR} gene from the \textit{S.pyogenes} genome (M49) and cloned into EcoRI and KpnI restriction sites of pT7-FLAG-MAT (Sigma) resulting into pCHT10.
APPENDIX B

Figure 20. SDS-PAGE and Western Blot analysis of native MtsR.

Purified native MtsR was fractionated by SDS-PAGE. (A) A Coomassie blue stained gel shows the purified native MtsR runs at a size of 25 kDa. (B) Western blot analysis of native MtsR using α-MtsR antibodies.
APPENDIX C

Figure 21. DNase I footprinting of MtsR complex with mtsA-mtsR promoter.

(A) DNA fragments (1 μM) were incubated with DNase I in the presence of increasing concentration of purified MtsR protein. In the Coding strand panel the 5’end of the non-template strand is radiolabeled, and the DNA was incubated with 0, 8, 16, 24, 32, 40, 48, and 56 μM MtsR. (B) In the template strand panel the 5’end of the template strand is labeled and the DNA was incubated with 0, 8, 16, 24, 32, 40, 48, and 56 μM MtsR. The vertical lines specify the 40 bp (on the coding strand) region protected by MtsR. (C) The sequence of the wild type mtsA-mtsR promoter with putative -35 and -10 elements, and RBS (bold) are shown. The mtsA’ and mtsR’ start codons are labeled and indicated by an arrow (bold). The DNA region protected from
DNase I digest by MtsR is indicated by the overscored line, the identified putative PerR-box is boxed in the color grey.
APPENDIX D

Figure 22. RT-PCR analysis of mtsA and mtsR transcript in the wildtype and mtsR mutant strain.

M, 1 kb DNA marker. Lane 1, recA transcript in wildtype. Lane 2, recA transcript in mtsR mutant. Lane 3, mtsA transcript in wildtype (801bp). Lane 4, mtsA transcript in mtsR mutant (801bp). Lane 5, mtsR transcript in wildtype (190bp). Lane 6, mtsR transcript in mtsR mutant (190bp).
## APPENDIX E

Table 7. Oligonucleotide primers used in the Appendices.

<table>
<thead>
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<th>Name</th>
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