Characterization of MtsR, a New Metal Regulator in Group A Streptococcus, Involved in Iron Acquisition and Virulence

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Group A streptococcus (GAS) is a common pathogen of the human skin and mucosal surfaces capable of producing a variety of diseases. In this study, we investigated regulation of iron uptake in GAS and the role of a putative transcriptional regulator named MtsR (for Mts repressor) with homology to the DtxR family of metal-dependent regulatory proteins. An mtsR mutant was constructed in NZ131 (M49 serotype) and analyzed. Western blot and RNA analysis showed that mtsR inactivation results in constitutive transcription of the sia (streptococcal iron acquisition) operon, which was negatively regulated by iron in the parent strain. A recombinant MtsR with C-terminal His6 tag fusion (rMtsR) was cloned and purified. Electrophoretic mobility shift assays demonstrated that rMtsR specifically binds to the sia promoter region in an iron- and manganese-dependent manner. Together, these observations indicate that MtsR directly represses the sia operon during cell growth under conditions of high metal levels. Consistent with deregulation of iron uptake, the mtsR mutant is hypersensitive to streptonigrin and hydrogen peroxide, and 55Fe uptake assays demonstrate that it accumulates 80% ± 22.5% more iron than the wild-type strain during growth in complete medium. Studies with a zebrafish infection model revealed that the mtsR mutant is attenuated for virulence in both the intramuscular and the intraperitoneal routes. In conclusion, MtsR, a new regulatory protein in GAS, controls iron homeostasis and has a role in disease production.

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 (2). 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 (28, 42, 47, 53, 64). Deregulation of iron homeostasis often leads to oxidative stress, and the disruption of dxr or fur frequently results in an increased sensitivity to hydrogen peroxide (10, 21, 40, 53, 66).

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 (3), Shiga-like toxin in Escherichia coli (7), and diptheria toxin (56) 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 (51), Mycobacterium tuberculosis (34, 53) P. aeruginosa (for a review, see reference 67), and Staphylococcus aureus (1, 21).

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 motif for DNA binding (for a review, see reference 35) and have similar modes 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 (62). Members of the Fur family of metalloregulators include Zur, which is involved in zinc homeostasis (18), and PerR, which controls peroxide resistance (37). 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 (31, 48, 63, 68). The DtxR family comprises a variety of metalresponsive regulators that use, in vivo, iron, manganese, or both as corepressors, including SirR in S. epidermidis (Fe and Mn) (20); MntR in S. aureus (Mn) (1, 22), Corynebacterium diphtheriae (Mn) (55), and Bacillus subtilis (Mn) (49); IdeR in M. tuberculosis, M. smegmatis (Fe) (10, 57), Rhodococcus erythropolis, and R. equi (Fe) (5); and TroR in Treponema pallidum (Mn and/or Zn) (19, 46).

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 invasive diseases such as impetigo and pharyngitis to invasive diseases such as streptococcal toxic shock syndrome, cellulitis,
and necrotizing fasciitis (9). Cases involving invasive GAS are rare but dangerous. In 2002, the Centers for Disease Control and Prevention reported that over 9,000 cases of invasive GAS infections occurred within the United States alone and that 14 percent of those cases ended in death (8). 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 an iron source (4, 12, 44). GAS can also lead to the nonsuppurative sequelae rheumatic fever and glomerulonephritis.

MATERIALS AND METHODS

Strains, media, and growth conditions. GAS NZ131 (M49) has been previously described (59). GAS was grown in Todd Hewitt broth (TH; Difco Laboratories), TH with 0.2% yeast extract (THY), and TH with 10 mM Tris, adjusted to pH 6.9 (ZTH) (11). ZTH medium was analyzed for metal content by inductivity-coupled plasma–mass spectrometry analysis (Laboratory for Environmen- tal Analysis, University of Georgia at Athens). This analysis demonstrated that ZTH contains about 17.5 ± 0.6 μM iron, 0.53 ± 0.2 μM of manganese, and 15.5 ± 0.2 μM of zinc, depending on the batch and manufacturer. To starve for iron, cells were grown in ZTH containing 10 mM nitrotriacetic acid (NTA) (4). NTA is a metal chelator with high specificity for iron; its first-stability constants (log Kf) for Fe3+ and Fe2+ are 15.87 and 8.83, respectively (15). Since NTA has affinity for zinc, manganese, magnesium, and calcium (log Kf of 10.45, 7.44, 5.4, and 6.41, respectively) (15), 0.55 mM of MgCl2, MnCl2, CaCl2, and ZnCl2 was added to NTA-containing media (ZTH-NTA). For the infection of zebrafish, GAS were 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-caps polycarbonate tubes at 37°C.

DNA manipulations. Cloning, plasmid construction, chromosomal DNA extraction, and restriction analyses were done according to standard protocols as described previously (13, 54). The primers used in this study are listed in Table 1.

Construction of plasmids and strains. (ii) 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 mtsBF and dtxR-R and cloned into the SacI and EcoRI sites of pBluescript II KS (Stratagene), resulting in a 3.8-kb fragment containing the upstream region and the first 202 bp of the mtsR coding sequence. A 1.8-kb fragment containing the kanamycin-resistant cassette was amplified by PCR from pJRS700 by use of primers Kan BB A and Kan BB S and cloned into the EcoRI and NheI sites on pBBS2, resulting in pBBS3. Plasmid pJRS700 is a derivative of pVE6037 (33) produced by the ligation of a 4.1-kb HindIII fragment of pVE6037 to a kanamycin resistance cassette coding for the adaA gene and the chloramphenicol resistance gene. The kanamycin resistance cassette (1.4 kb) was amplified by PCR from pJRS700 by use of primers Kan BB A and Kan BB S and cloned into the EcoRI and NheI sites on pBBS2, resulting in pBBS3. Plasmid pBBS700 is a derivative of pVE6037 (33) produced by the ligation of a 4.1-kb HindIII fragment of pVE6037 to a kanamycin resistance cassette coding for the adaA gene and the chloramphenicol resistance gene. The kanamycin resistance cassette (1.4 kb) was amplified by PCR from pBBS3 by use of the primers mutMF and mutTF and cloned into strain NZ131 by electroporation (Bio-Rad Gene Pulser). Allelic exchange events were selected by plating the transformants on THY with kanamycin (70 μg/ml) and four days by electroporation (Bio-Rad Gene Pulser). Allelic exchange events were selected by plating the transformants on THY with kanamycin (70 μg/ml). The resulting mtsR mutant strain was named ZE491. The chromosomal mutation in ZE491 was confirmed by PCR using the primers mutMF and dtxR-R, and cloned into the SacI and EcoRI sites of pBluescript II KS (Stratagene), resulting in a 3.8-kb fragment containing the upstream region and the first 202 bp of the mtsR coding sequence. A 1.8-kb fragment containing the kanamycin-resistant cassette was amplified by PCR from pJRS700 by use of primers Kan BB A and Kan BB S and cloned into the EcoRI and NheI sites on pBBS2, resulting in pBBS3. Plasmid pJRS700 is a derivative of pVE6037 (33) produced by the ligation of a 4.1-kb HindIII fragment of pVE6037 to a kanamycin resistance cassette coding for the adaA gene and the chloramphenicol resistance gene. The kanamycin resistance cassette (1.4 kb) was amplified by PCR from pJRS700 by use of primers Kan BB A and Kan BB S and cloned into the EcoRI and NheI sites on pBBS2, resulting in pBBS3. Plasmid pBBS700 is a derivative of pVE6037 (33) produced by the ligation of a 4.1-kb HindIII fragment of pVE6037 to a kanamycin resistance cassette coding for the adaA gene and the chloramphenicol resistance gene. The kanamycin resistance cassette (1.4 kb) was amplified by PCR from pBBS3 by use of the primers mutMF and mutTF and cloned into strain NZ131 by electroporation (Bio-Rad Gene Pulser). Allelic exchange events were selected by plating the transformants on THY with kanamycin (70 μg/ml). The resulting mtsR mutant strain was named ZE491. The chromosomal mutation in ZE491 was confirmed by PCR using the primers mutMF and DtxR.LKOS primers.

(ii) Construction of pZEDH3.1. A 654-bp fragment from the NZ131 chromo-osome 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-terminal fusion of MtsR to a His6 tag (mMtS) driven by a T7 promoter. Restriction enzyme analysis verified the orientation of the insert in the resulting clones.

TABLE 1. Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Location</th>
</tr>
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<tr>
<td>sia GS F</td>
<td>5′-GGCCGGCATTCCTTAGTGATTTACAATTACATGTC 3′</td>
<td>Pm</td>
</tr>
<tr>
<td>sia GS R</td>
<td>5′-GGCCGGATTTCAATTTCATTACATAACCTTCT 3′</td>
<td>recA</td>
</tr>
<tr>
<td>SRAL</td>
<td>5′-GGTCTAAGGATGTGTCATCT 3′</td>
<td>recA</td>
</tr>
<tr>
<td>SRAR</td>
<td>5′-CTGATGCTACTGCCATGACG 3′</td>
<td></td>
</tr>
<tr>
<td>orfX-delA</td>
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<td>sh</td>
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<tr>
<td>orfX-delB</td>
<td>5′-CCTAGATCTCAAAGATATCAATTGGACTGT 3′</td>
<td>sh</td>
</tr>
<tr>
<td>ZEDHS</td>
<td>5′-CCTACATGAGGATCCAATAAGAAAGATATTGCTG3′</td>
<td>mtsR</td>
</tr>
<tr>
<td>mtsB F</td>
<td>5′-GCCAGCCATTTTGAGCTTTCTCA 3′</td>
<td>mtsB</td>
</tr>
<tr>
<td>dtxR-R</td>
<td>5′-GGGAAATTCGACATGCTTCTTTCGTC 3′</td>
<td>mtsR</td>
</tr>
<tr>
<td>dtxR-F</td>
<td>5′-GGGAAATTCTTGGTTCAGAAATGTCATTGACG 3′</td>
<td>mtsR</td>
</tr>
<tr>
<td>mutF</td>
<td>5′-GGGAAAATGGGATCCTGCAGTTCCAACAATCT 3′</td>
<td>SP6444</td>
</tr>
<tr>
<td>Kan BB S</td>
<td>5′-AAGGGCTGTGACATAAGTTGCTTAGC 3′</td>
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<td>5′-ATACGGAGATTCCTCTCTCCACACTGATCTGGCCG 3′</td>
<td>pBBS2</td>
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<tr>
<td>DtxRLKOS</td>
<td>5′-CCCTAGCTCCCTCTCTCCTCTCGGACGATTCTTTTA 3′</td>
<td>mtsR</td>
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Expression and purification of rMtsR (MtsR-His<sub>6</sub>). BL21(DE3). E. coli cells harboring plasmid pZEDH3.1 were grown at 37°C in Luria Bertani containing ampicillin (100 μg/ml). Once the cells reached an optical density at 600 nm (OD<sub>600</sub>) of 0.6, 0.6 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the medium and the culture was incubated overnight at 20°C. Cells were then harvested and lysed by sonication, and the rMtsR was purified over a nickel column by use of a ProBond purification system (Invitrogen) according to the manufacturer’s protocol. The rMtsR was then applied to an anion exchange column (HighTrap Q5 ml column), and fractions containing the purified protein were detected on the chromatogram and examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis with anti-His<sub>6</sub> 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 that were grown in either ZTH or ZTH-NTA as described by Bates et al. (4). Total proteins were standardized on the basis of cell number, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were blocked at room temperature for 15 min using 5% skim milk in phosphate-buffered saline–Tween, rinsed, and incubated with polyclonal anti-Sia or anti-Shr antibodies for 2 h (4). After rinsing, membranes were incubated in goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Sigma). Blots were then rinsed, and the color developed.

EMSA. The electrophoretic mobility gel shift assay (EMSA) was done according to Schmitt et al. (58). A 337-bp fragment containing the upstream region of the spa operon (P<sub>spa</sub> fragment) was amplified from the NZ131 chromosome by use of siaGSF and siaGSR primers. The PCR products were purified using a Rapid PCR purification system (Marligen Bioscience Inc.) and end labeled with [γ-<sup>32</sup>P]ATP by use of T4 kinase (Invitrogen). The 32P-labeled DNA fragments were purified from an 8% polyacrylamide gel, using the Rapid PCR purification system (Marligen Bioscience Inc.) and end labeled with Protein X (VNTI-InforMax). Proteins were sequenced using ClustalW from EMBL-EBI (http://www.ebi.ac.uk/clustalw/). The sequence of MtsR was submitted to SWISS-MODEL (http://www.expasy.org/swissmod/SWISS-MODEL.html) 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 Swiss Pdb viewer 3.7 (SPV) (http://us.expasy.org/spvod). Phylogenetic trees were generated using Gene ClustalW 1.75 (http://www.genebee.msu.su/clustal/basic.html) with the parameters of slow alignment and BLOSUM protein weight matrix, without Kimura’s corrections, and displayed using the PHYLIP format. Statistical analyses were done using StatView (SAS Institute).

RNA methods. (i) RNA preparation. GAS was harvested at the logarithmic growth phase by being poured over frozen Tris buffer (100 mM Tris [pH 6.8], 2 mM EDTA) containing 0.06% sodium azide. Total RNA was prepared from the cell pellet as described by MacDonald et al. (32). RNA was pelleted by sedimentation through 5.7 M CsCl. RNA pellets were allowed to dry before suspending them in RNAsecure (Ambion), and contaminating genomic DNA was removed by DNase using DNA-Free (Ambion) per the 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) and 1 μg of DNA 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 5°C increments. Reactions were terminated by heat inactivation of the enzyme. SRAR and ORX-daIA primers were used in the generation of cDNA, and 1/20 of the reaction was used as a template for 25 cycles of PCR. Primer pairs used for the PCR were primer pair SRAR and SRAL and primer pair ORX-daI and ORX-daIA.

Streptogrin and hydrogen peroxide susceptibility. Sensitivity to streptogrin was determined as described by Bates et al. (4) with the exception of the use of THY as the growth medium. Briefly, cells from glycerol stocks were used to inoculate 5 ml of THY in either the presence or the absence of 0.36 μM streptogrin (Sigma) in screw-cap tubes. Growth was measured by the OD<sub>600</sub> after overnight growth and expressed as a percentage of growth in the presence of the drug compared to growth without the drug. For testing the sensitivity to hydrogen peroxide, overnight cultures of NZ131 and ZE491 grown in THY were used to inoculate 5 ml THY containing 1 mM hydrogen peroxide (Fisher) and allowed to grow overnight. Cell growth was determined after overnight incubation by measuring the OD<sub>600</sub>.

55Fe accumulation assays. Iron accumulation assays were performed essentially as previously described (25) with small modifications. Overnight cultures were used to inoculate (1:500; OD<sub>600</sub> of 0.02) 5 ml THY medium containing 55FeCl<sub>3</sub> (Amersham Pharmacia Biotech) (0.4 μCi/ml, 0.04 μM) and incubated at 37°C. Since incorporation of 55Fe by the cells following an overnight growth was low, a second passage (from an overnight culture) (1:500) of the 55Fe medium was used. Culture samples (1 ml; OD<sub>600</sub> of 0.9) were then drawn in duplicates, supernatants were collected, and the cells were washed twice with saline solution containing 10 mM NTA, resuspended in 0.2 ml saline solution, 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 for 5 min against a 1 H standard. The fraction of 55Fe associated with the bacterial cells was calculated by dividing the counts per minute of the cell pellet by the sum of the counts per minute of the culture supernatant and was expressed as a percentage.

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 by Neeley et al. (38). Five groups of experiments with from 5 to 10 fish per group were used per strain per injection route (totaling 33 fish per strain per injection route). Fish were monitored for 48 h postinfection. Zebrafish care and feeding was done as previously described (38).

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 (4, 29, 30). A DtxR homologue is found in the GAS chromosome (3), and we reasoned that it regulates sia transcription in response to iron availability. This gene, spv0450 in SF370 (M1), which encodes...
a putative 215-amino-acid protein (Mr, 24,814 Da; pI, 5.99), is located 5'/H11032 proximal to the mtsABC operon and is transcribed in the opposite direction (Fig. 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 (23). A BLAST analysis showed that the predicted amino acid sequence of MtsR is highly conserved among the publicly available sequenced strains of GAS (demonstrating 97 to 99% amino acid identity) and that it shares homology with the large group of transcriptional regulators from the DtxR family. The highest homology (60 to 50% amino acid identity) was found between MtsR and proteins or putative open reading frames from pathogenic streptococci, including S. mutans (SloR/Dlg; these appear to be the same protein identified in different strains of S. mutans [accession no. NP_720655]) (Fig. 1B), S. gordonii (ScaR; accession no. AAF25184), and S. pneumoniae (NP_359073).

The amino acid sequence of MtsR is 25% identical and 66% similar to those of DtxR of C. diphtheriae. Although the relatively low percentage of 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 (Fig. 1B), SirR, and ScaR (17, 20, 41, 61). A search for conserved protein domains (conserved domain database at NCBI.nlm.nih.gov/Structure/cdd/wrpsb.cgi) identified a FeoA domain (pfam04023.6) in the C terminus of MtsR (Fig. 1B). FeoA is a small protein from E. coli that may be involved in uptake of ferrous iron (26); 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, DtxR and IdeR were chosen by the SWISS-MODEL program as a template for MtsR protein folding (data not shown). The resulting model included most of the MtsR polypeptide and exhibited the typical winged helix-turn-helix structure of DtxR and 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. On the basis of 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.

MtsR regulates the expression of the streptococcal iron acquisition (via) operon. To test the role of mtsR in GAS, a mutant (ZE491) carrying a truncated mtsR allele with an inserted kanamycin resistance gene (mtsR::kanR) was constructed in NZ131 (M49) by allelic replacement (Fig. 1C). The structure of the mtsR::kan mutation was confirmed by PCR analysis. Interestingly, growth analysis showed that the mtsR
The expression was induced by iron restriction. A three- to fourfold increase of transcript was found in cells grown in ZTH-NTA (containing 0.55 mM 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 (4). 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 by the wild-type strain in ZTH medium and ZTH-NTA (4, 11). Western blot analysis demonstrated that as with strain SF370 (M1), both Shr production and SiaA production were 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 concentrations (ZTH) (Fig. 2B). This observation supports the suggestion that MtsR negatively regulates sia expression in the presence of iron. Reduced production of both Shr and SiaA was observed in the mtsR mutant in ZTH-NTA in comparison to ZHT results, indicating that the regulation of the sia operon is complex and may involve MtsR-independent regulatory mechanisms.

RNA was isolated from exponentially growing cells in ZTH or in ZTH-NTA medium, and semiquantitative RT-PCR analysis was used to determine whether 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 quantities used as a template in all RT reactions were equal. Unlike the recA product, the amount of amplicon corresponding to the shr gene differed depending on the RNA samples. As can be seen in Fig. 2C, 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 levels found in the mtsR mutant when grown in ZTH-NTA in comparison to ZTH (Fig. 2B) is not associated with a similar decrease of sia transcript level (Fig. 2C). 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 Materials and Methods. A protein band of the expected size (∼25 kDa) was observed upon SDS-PAGE without significant contaminating bands. 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 electrophoretic mobility shift assay (EMSA). A 35P-end-labeled fragment generated by PCR covering the upstream sequence of shr up to the first ATG codon (Pshr fragment) was incubated with increasing amounts of purified rMtsR. A DNA fragment that migrates slower than the “free” DNA (Pshr fragment in buffer) was

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

**FIG. 2.** 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 and 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 panel) or SiaA (bottom panel). (C) RT-PCR analysis of sia genes. cDNA synthesized from 1 μg of total RNA by use of gene-specific primers was amplified by PCR and separated on an agarose gel. PCRs are shown for the housekeeping gene recA (top panel) and the shr gene (bottom panel).

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 that it is more resistant to iron depletion (Fig. 2A). A similar growth defect in iron-rich medium was recently made for a dtxR mutant in *C. diphtheriae* (40).

The sia locus is a 10-gene operon that codes for a hemoprotein receptor (shr), a heme binding protein (shp), and the iron transporter siaABC (4, 30). It was earlier reported that sia expression was induced by iron restriction. A three- to fourfold
obtained in the presence of 2.5 ng of rMtsR, indicating the formation of a protein-DNA complex (C1 in Fig. 3B). 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 began to form; this complex (C2 in Fig. 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 reaction mixtures containing 7.5 ng of rMtsR inhibited the binding of MtsR 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 reaction mixtures (Fig. 4A). These observations suggest that MtsR requires iron for DNA binding.

To investigate the specificity of rMtsR binding to Pshr fragment, we used a competition assay with specific and nonspecific unlabeled DNA (Fig. 4A). 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 Pshr fragment even in the presence of a 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 (1, 55). Nevertheless, we investigated the ability of rSiaA, which contains a His tag in its carboxy terminus (4), to bind to the shr promoter fragment, and no binding was observed when surplus amounts of rSiaA (100 ng) 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 that the protein lost most of its binding to the Pshr fragment (second lane from the left in Fig. 4B and C). Binding of rMtsR to DNA was restored with 75 μM of ferrous sulfate (fifth lane in Fig. 4B). As many of MtsR homologues respond to multiple metals, the ability of manganese to allow rMtsR binding to Pshr fragment was investigated. Interestingly, 25 μM manganese was sufficient to restore rMtsR binding (third lane in Fig. 4C). 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 (69). The growth of NZ131 and ZE491 to hydrogen peroxide. Bacteria were inoculated into fresh THY containing 1 mM hydrogen peroxide, and overnight growth was determined and presented as described for panel A. In both panel A and panel B, error bars represent the standard deviation of the mean (n = 3).

FIG. 5. MtsR inactivation results in hypersensitivity to streptonigrin. (A) Sensitivity of the wild-type (NZ131) and mtsR mutant (ZE491) to streptonigrin. Bacteria were inoculated into fresh THY medium containing 0.36 μM streptonigrin. The culture optical density (OD600) was determined after overnight growth (~20 h). The data are presented as the ratio of the OD600 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 1 mM hydrogen peroxide, and overnight growth was determined and presented as described for panel A. In both panel A and panel B, error bars represent the standard deviation of the mean (n = 3).

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 (38). As was observed with the HSC5 (M14) GAS strain, infection of zebrafish with NZ131 is lethal both in the intramuscular and intraperitoneal infection routes (Fig. 6). When 5 × 10^7 cells of NZ131 were injected intramuscularly 63% ± 7% (standard error of the mean) of the infected fish died within 36 to 48 h. 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 was observed in the intramuscular infection, and death from intraperitoneal infection was reduced to 44% ± 17.2% (P ≤ 0.02; 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 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 Fig. 5B, 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 the mtsR mutant is consistent with that of iron overload; parallel results were obtained for a dsr mutant in C. diphtheriae (40).

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 by the isogenic wild-type strain. As the role of MtsR is to repress iron uptake in complete medium, we assayed for [55Fe] accumulation by cells grown in THY medium containing [55FeCl₃]. These experiments showed that while the fraction of the added [55Fe] 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 than 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.
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 (6, 27, 52). In this paper, we have 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 (30) or by hemoglobin in ZTH-NTA medium (4). In this study, a significant increase in sia proteins and transcript is seen in wild-type GAS cells grown in ZTH-NTA 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 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 (52) and repressed the transcription of mrgR (peroxide resistance) (6). 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 at the sia transcriptional level, as RT-PCR analysis demonstrated that the amount of sia mRNA in the mtsR mutant was large in both ZTH and ZTH-NTA in comparison to the wild-type 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. Since 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 nonrelevant 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 reaction mixtures restored rMtsR DNA binding, further supporting these assertions. Pretreatment of rMtsR with EDTA and subsequent dialysis prevented most of the DNA binding, demonstrating that it is indeed a metal-dependent protein. Both iron and manganese promoted rMtsR binding to the Pshr 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 respect to iron (29) (siaA is named htaA 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 (45). 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 (39).

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 streptomycin, suggesting a significant reduction in iron uptake (4). Using Western and RT-PCR analysis we have demonstrated here that MtsR represses the transcription of the sia operon in cells grown in a medium rich in iron. The streptomycin hypersensitivity exhibited by the mtsR mutant is an indirect indicator of iron-uptake deregulation in cells growing in complete medium. This idea 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 (25). 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 (24, 25); 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 study using a dtxR mutation in C. diphtheriae (40), inactivation of the mtsR gene led to hypersensitivity to hydrogen peroxide as well. Since iron amplifies the toxicity of hydrogen peroxide (4, 50, 69), 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 in response to oxidative stress. Increased sensitivity to hydrogen peroxide that was accompanied by an altered response to oxidative stress was found in several mutations resulting in DtxR-like proteins, including the ideR mutation 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 (10). Interestingly, an ideR mutant of M. tuber-
culosis 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 (53). 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 (6, 27). Since regulation of iron uptake and peroxide stress are frequently connected in bacteria (50), 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 open reading frames found the database were excluded) demonstrates, as expected, that the Fur and Fur-like proteins comprise a class of metalloregulators that is separate from the DtxR family (Fig. 7). 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. enterica serovar Typhimurium. Furthermore, the DtxR proteins are split into two subclusters. The C. diphtheriae DtxR protein and IdeR proteins from Mycobacterium species comprise one cluster, and proteins such as SloR/Dig, 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/Dig 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/Dig and MtsR) (1, 17, 19, 22, 23, 41, 46, 49, 55, 61).

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. Derepression 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 microenvironments. 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.

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