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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 gel 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 ^{55}Fe 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 *dtxR* 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 diphtheria 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 metalloregulators 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 noninvasive diseases such as impetigo and pharyngitis to invasive diseases such as streptococcal toxic shock syndrome, cellulitis,

TABLE 1. Oligonucleotide primers used in this study

Name	Sequence	Location
sia GS F	5′ GGCGCGGATCCTCAGCTCTAGTATTAACTATC 3′	P_{shr}
sia GS R	5′ GGCGCGGATTCCATTTTCAATCAACTTTCT 3′	shr
SRAL	5′ GCGTTCAGGAGGTCTAGCTC 3′	recA
SRAR	5′ CTGATGCTACTGCCATAGCAG 3′	recA
orfX-delA	5′ CCCGAATTCTGATTGAACTTCACATCTAA 3′	shr
orfX-delS	5′ CCCGAATTCAAAAGATATCAATGGTAGCT 3′	shr
ZEDHS	5′ CCCCCATGGATGACGCCTAATAAAGAAGATTACTTG 3′	mtsR
ZEDHA	5′ AAGGGCTGTGACATAAGTTGCTTAGC 3′	mtsR
mtsB F	5′ GCCAGCCATTTTGAGCTCTTTCA 3′	mtsB
dtxR-R	5′ GGGGAATTCAGCATAGCCTTTGTC 3′	mtsR
dtxR-F	5′ GGAAGAATTCTTATGGCGACAAGGAACTTG 3′	mtsR
mutF	5′ GGGAAACTCGAGCTTCCCAACAATCT 3′	SPy0444
Kan BB S	5′ AACTAGCTAGCCGGGGGATCAGCTTTTTA 3′	pBBS2
Kan BB A	5′ ATACGGAATTCCTTCCAACTGATCTGCGC 3′	pBBS2
DtxRLKOS	5′ CCCGAATTCAATTGGAGAACAAGAACCCA 3′	mtsR

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 strip iron from both hemoglobin and haptoglobin-hemoglobin complexes (14) and has also been shown to acquire ^{55}Fe from the culture media (24). A multimetal transport system (mts) involved in the transport of zinc, manganese, and iron has been identified in GAS (24, 25). An mts mutant was affected in growth under aerobic or metal-limiting conditions and was attenuated for virulence in a mouse model (25). We recently described an iron-regulated 10-gene operon, sia, which mediates acquisition of iron from hemoproteins (4). The sia locus encodes the hemoprotein receptor Shr, the heme binding protein Shp (30), 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 (60).

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 (16) and the M protein (36) and the secretion of the surface glyceraldehyde-3-phosphate-dehydrogenase (11). Recently, a Fur homolog, PerR, was found in S. pyogenes. A perR mutant exhibited constitutive resistance to peroxide stress (27) and was hypersensitive to paraquat (6, 52). PerR positively regulates sod and mtsA genes and represses mrgA, which encodes a Dps-like protein (6, 52). Mutants with mutations in perR were attenuated in murine infection models (6, 52). A putative regulatory protein (named MtsR) that belongs to the DtxR family of metallorepressors is divergently transcribed from the mts gene cluster (23). 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 (59). 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) (11). ZTH medium was analyzed for metal content by inductivity-coupled plasma–mass spectrometry 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 (NTA) (4). NTA is a metal chelator with high specificity for iron; its first-stability constants (log K_1) for Fe^{+3} and Fe^{+2} are 15.87 and 8.83, respectively (15). Since NTA has affinity for zinc, manganese, magnesium, and calcium (log K_1 of 10.45, 7.44, 5.4, and 6.41, respectively) (15), 0.55 mM of $MgCl_2$, $MnCl_2$, $CaCl_2$, and $ZnCl_2$ 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-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 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 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 by use of 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 (33) 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 (43). A 4.9-kb fragment containing the mtsR::kan allele was amplified by PCR from pBBS3Z by use of 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 (70 μg/ml). The resulting mtsR mutant strain was named 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-terminal 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 containing ampicillin (100 μg/ml). Once the cells reached an optical density at 600 nm (OD$_{600}$) 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$_6$ 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 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 rSiaA or rShr 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 *sia* operon (P$_{shr}$ 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-^{32}P]ATP by use of T4 kinase (Invitrogen). The ^{32}P-labeled DNA fragments were purified from an 8% polyacrylamide 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 20 mM Na$_2$HPO$_4$, 50 mM NaCl, 5 mM MgCl$_2$, 2 mM 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 250 μM EDTA with or without FeSO$_4$ (made fresh). To remove the metal from the purified rMtsR, the protein was incubated with 2 mM EDTA for 15 min at 4°C and then dialyzed overnight against 10% glycerol and 20 mM sodium phosphate buffer (pH 7.0). A total of 10 μl of the binding reaction mixture was loaded per well (without a loading dye) and fractionated over a 5% polyacrylamide gel containing 2.5% glycerol, 20 mM Na$_2$HPO$_4$, and 2 mM dithiothreitol. Electrophoresis was performed at 65 V at room temperature for 1.5 h using 20 mM Na$_2$HPO$_4$ buffer containing 1 mM dithiothreitol. Gels were dried at 80°C for 25 min and visualized using a phosphorimager.

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 pellets 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 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 5°C 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 of PCR. Primer pairs used for the PCR were primer pair SRAR and SRAL and primer pair orfX-del S and orfX-delA.

Streptonigrin and hydrogen peroxide susceptibility. Sensitivity to streptonigrin 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 streptonigrin (Sigma) in screw-cap 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 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$_{600}$.

^{55}Fe accumulation assays. Iron accumulation assays were performed essentially as previously described (25) with small modifications. Overnight cultures were used to inoculate (1:500; OD$_{600}$ of 0.02) 5 ml THY medium containing ^{55}FeCl$_3$ (Amersham Pharmacia Biotech) (0.4μCi/μl, 0.04 μM) 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. Culture samples (1 ml; OD$_{600}$ 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 ^{3}H standard. The fraction of ^{55}Fe 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 cell pellet and 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 Neely 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).

Computer analysis. GAS sequences used for alignments and primer design from the sequenced M1 strain, SF370, are available at the National Center for Biotechnology Information 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 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 Pbd viewer 3.7 (SP5) (http://us.expasy.org/sprot). Phylogenetic trees were generated using Gene Bee 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).

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 enterica* serovar Typhimurium LT2 MntR, Q8XH22; *S. aureus* Mu50 Fur, Q9R3G5; *Bordetella pertussis* Fur, Q45765; *S. enterica* serovar 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 phyologenetic 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. enterica* serovar 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 (4, 29, 30). A DtxR homologue is found in the GAS chromosome (23), and we reasoned that it regulates *sia* transcription in response to iron availability. This gene, *spy0450* in SF370 (M1), which encodes
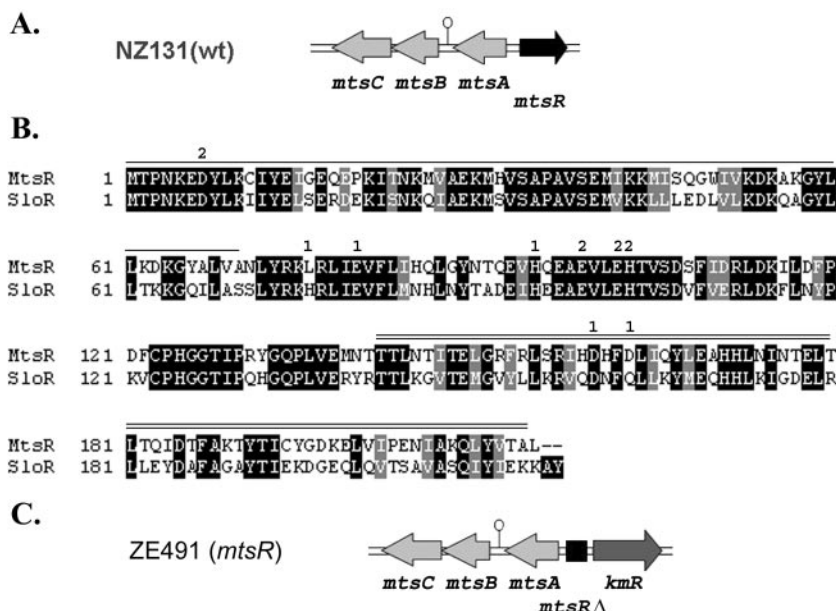
FIG. 1. The streptococcal metal transport repressor (MtsR). (A) Schematic presentation of the *mtsR* (*spy0450*) chromosomal locus in the wild-type strain NZ131. The stem-loop structures represent transcriptional terminators. The genes are represented by their names. The *mts* genes are annotated as such in the *S. pyogenes* SF370 genome, while *mtsR* is annotated as *spy0450*. (B) CLUSTALW alignment of MtsR (Spy0450) with SloR of *S. mutans*. White letters on a black background indicate identical residues; white letters on a gray background indicate similar residues. The residues overscored 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 over the white or black letters, 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 *aphA-3*, the kanamycin resistance gene, flanked by the Omega transcriptional termination signals (43); *mtsR*Δ indicates the 5′ portion of *mtsR* remaining in the ZE491 mutant.

a putative 215-amino-acid protein (M_r, 24,814 Da; pI, 5.99), is located 5′ 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*. Despite 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 (*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 (Fig. 1C). The structure of the *mtsR::kan* mutation was confirmed by PCR analysis. Interestingly, growth analysis showed that the *mtsR*
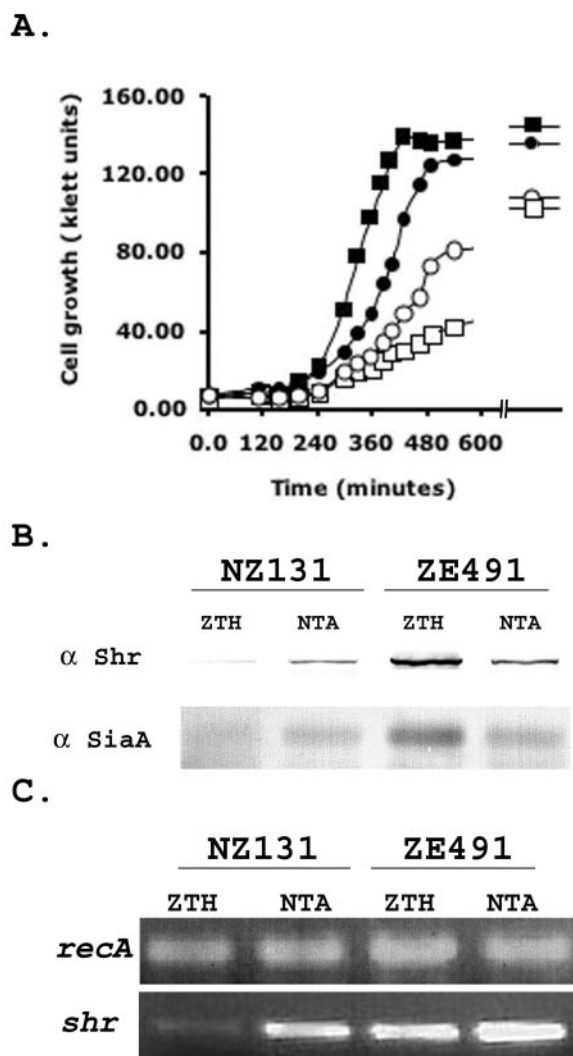
A.

B.

C.

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

increase of transcript was found in cells grown in ZTH-NTA (containing 0.55 mM of $MgCl_2$, $MnCl_2$, $CaCl_2$, and $ZnCl_2$) 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 ^{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

FIG. 3. MtsR directly binds to the promoter region of *shr*. (A) Schematic presentation of the *sia* operon's promoter/operator region (P_{shr}). 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.

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 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 reaction mixtures (Fig. 4A). These observations suggest that MtsR requires iron for DNA binding.

To investigate the specificity of rMtsR binding to P_{shr} 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 P_{shr} 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 P_{shr} 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 P_{shr} 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.

FIG. 4. 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 from the left, [^{32}P]P_{shr} fragment only. Lane 2, [^{32}P]P_{shr} fragment but with 7.5 ng of purified MtsR-His$_6$. Lane 3, the same as in lane 2 but with 250 µM EDTA. Lane 4, the same as in lane 3 but with 25 µM FeSO$_4$. Lane 5, the same as in lane 3 but with 75 µM FeSO$_4$. Lane 6, the same as in lane 3 but with 50 µM FeSO$_4$. Lane 7, the same as in lane 3 but with 100 µM FeSO$_4$. Lane 8, the same as in lane 2 but with a 10-fold increase in the level of unlabeled (cold) P_{shr} fragment. Lane 9, the same as in lane 2 but with a 10-fold increase in the level of unlabeled *recA* fragment. Lane 10, [^{32}P]P_{shr} fragment and 100 ng of SiaA-His$_6$ (4). B. Iron restores binding of EDTA-treated rMtsR to DNA. Lane 1, [^{32}P]P_{shr} fragment only. Lane 2, the same as in lane 1 but with 10 ng of rMtsR pretreated with EDTA. Lane 3, the same as in lane 1 but with 10 ng of untreated rMtsR. Lane 4 the same as in lane 2 but with 25 µM FeSO$_4$. Lane 5, the same as in lane 2 but with 75 µM FeSO$_4$. Lane 6, the same as in lane 2 but with 150 µM FeSO$_4$. C. Manganese restores binding of EDTA-treated rMtsR to DNA. Lane 1, [^{32}P]P_{shr} fragment only. Lane 2, the same as in lane 1 but with 10 ng of rMtsR pretreated with EDTA. Lanes 3 and 4, the same as in lane 2 but with 25 and 50 µM MnCl$_2$, respectively.
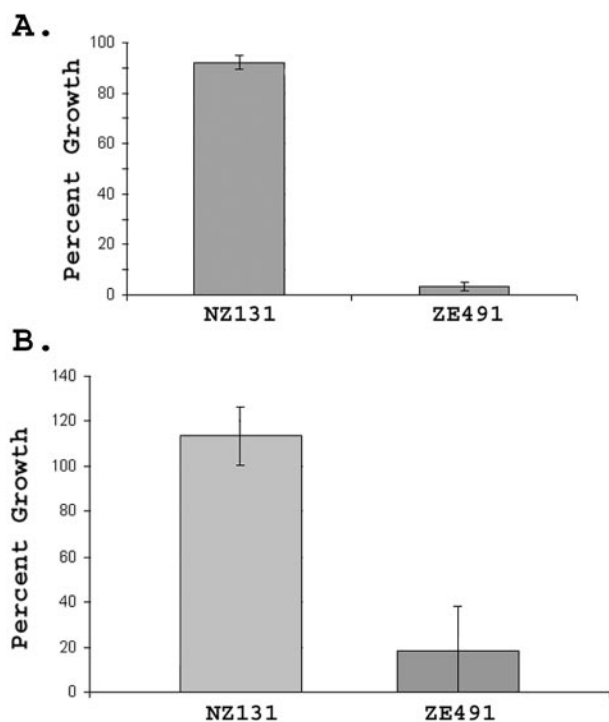
FIG. 5. MtsR inactivation results in hypersensitivity to streptonigrin and hydrogen peroxide. (A) Sensitivity of the wild-type (NZ131) and *mtsR* mutant (ZE491) to streptonigrin. Bacteria were inoculated into fresh THY medium containing 0.36 μM streptonigrin. The culture optical density (OD_{600}) was determined after overnight growth ($\sim$20 h). The data are presented as the ratio of the OD_{600} obtained in overnight cultures grown in THY containing the drug to that obtained in THY alone over the same time period. (B) Sensitivity of NZ131 and ZE491 to hydrogen peroxide. Bacteria were inoculated into fresh THY containing 1 mM hydrogen peroxide, and overnight growth was determined and presented as described for panel A. In both panel A and panel B, error bars represent the standard deviation of the mean ($n = 3$).

MtsR is 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 cells in THY medium containing increasing amounts of streptonigrin (0 to 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 5A shows that only 2.8% $\pm$ 1.5% survival is observed when ZE491 is grown in THY containing 0.36 μM streptonigrin, a concentration that supports 92.1% $\pm$ 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 (65). Analysis of cell growth in THY medium containing an increasing concentration of hydrogen peroxide (0 to 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 Fig. 5B, in medium containing 1 mM hydrogen peroxide, ZE491 culture reached only 18.5% $\pm$ 19.9% of the growth observed on THY alone, while NZ131 growth is not affected (113.7% $\pm$ 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 *dtxR* 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 ^{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% $\pm$ 0.47% of total input for NZ131) there were significant differences between the strains. The *mtsR* mutant (ZE491) accumulated 80% $\pm$ 22.5% more iron then the wild-type strain ($P \leq 0.02$; $n = 6$). 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.

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 5×10^5 cells of NZ131 were injected intramuscularly 63% $\pm$ 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% $\pm$ 12.65% death within the same time frame. ZE491, however, was significantly attenuated in both types of infection modes; only 15.5% $\pm$ 7.1% ($P \leq 0.01$; $n = 5$) death was observed in the intramuscular injection, and death from intraperitoneal infection was reduced to 44% $\pm$ 17.2% ($P \leq 0.022$; $n = 5$). 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
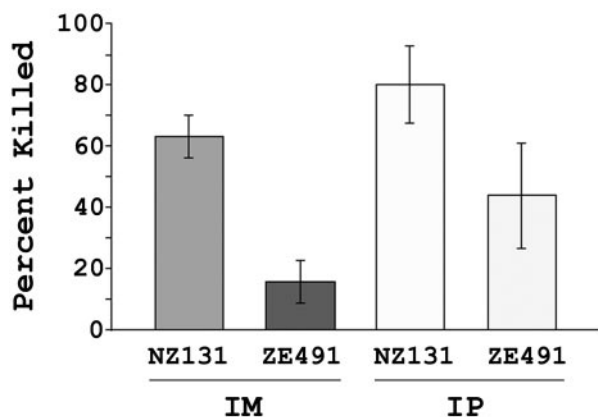
FIG. 6. ZE491 is attenuated in the zebrafish infection model. GAS cells harvested at the logarithmic growth phase were injected into groups of zebrafish (*Danio rerio*) (5×10^5 cells per fish) either intramuscularly (IM) or intraperitoneally (IP) as previously described (38). The average number of dead fish at 48 h postinjection is shown for each infection route; error bars represent the standard error of the mean ($n = 5$).

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 *mts* 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 P_{shr} 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 *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 then 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 streptonigrin, 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 streptonigrin 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 ^{55}Fe than the wild-type strain during growth in high-iron medium. The low incorporation of ^{55}Fe 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
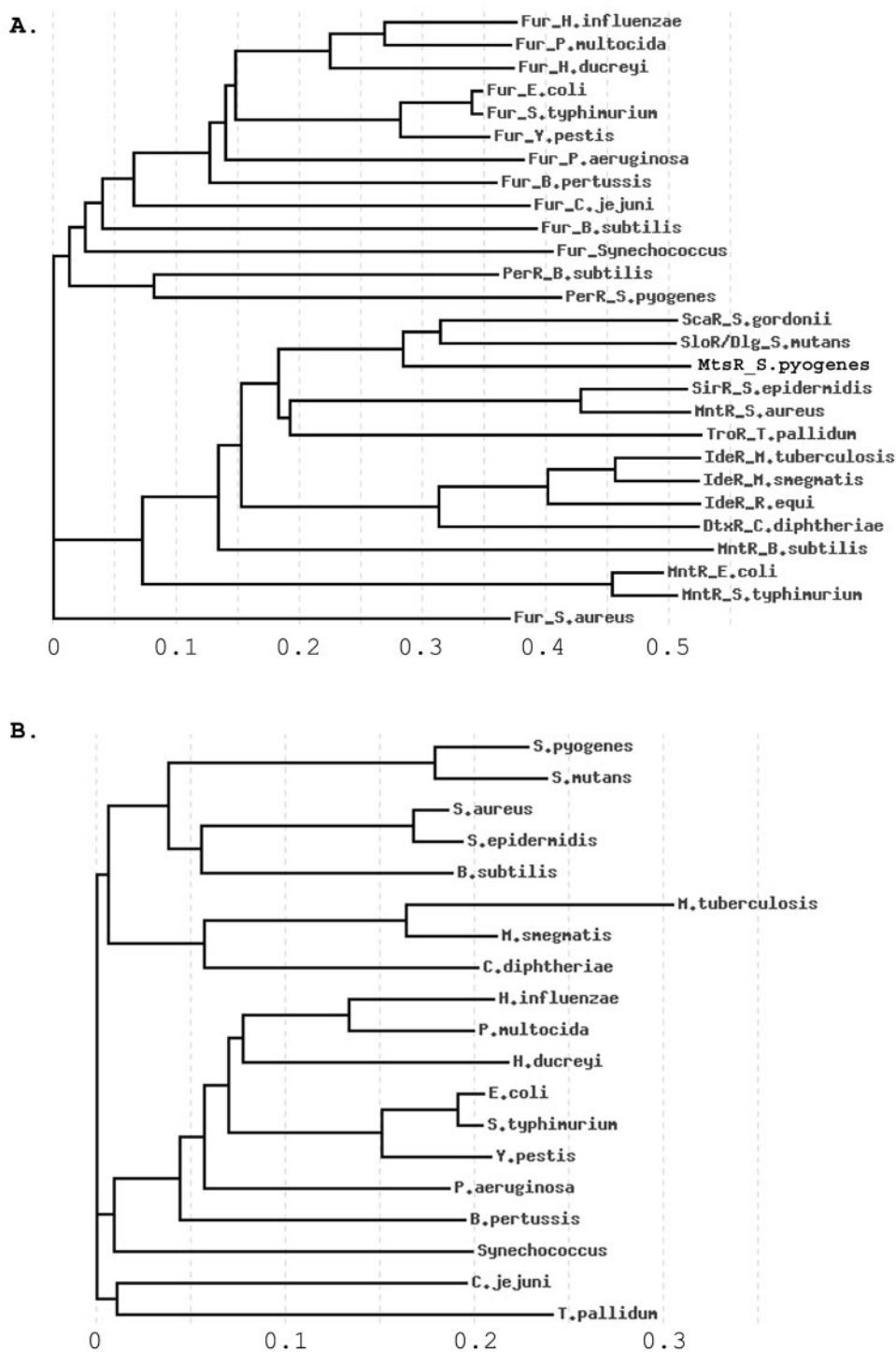
FIG. 7. Phylogenetic tree of Fur and DtxR homologs. A. Phylogenetic analysis of Fur and DtxR metalloregulators. B. Phylogenetic analysis of the corresponding RecA proteins from the organisms used in panel A. Trees were generated using ClustalW 1.75 software as described in Materials and Methods. Phylograms with weighted branch lengths are shown. *S. gordonii* and *R. equi* RecA proteins are not included, as their sequences are incomplete or not available. Accession numbers for all proteins in panels A and B are listed in Materials and Methods.

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 ox-

idative 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 in 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/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) (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. 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 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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