TrxR, a New CovR-Repressed Response Regulator That Activates the Mga Virulence Regulon in Group A Streptococcus

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Coordinate regulation of virulence factors by the group A streptococcus (GAS) Streptococcus pyogenes is important in this pathogen's ability to cause disease. To further elucidate the regulatory network in this human pathogen, the CovR-repressed two-component system (TCS) trxSR was chosen for further analysis based on its homology to a virulence-related TCS in Streptococcus pneumoniae. In a murine skin infection model, an insertion mutation in the response regulator gene, trxR, led to a significant reduction in lesion size, lesion severity, and lethality. Curing the trxR mutation restored virulence comparable to the wild-type strain. The trxSR operon was defined in vitro, and CovR was found to directly repress its promoter in vitro. DNA microarray analysis established that TrxR activates transcription of Mga-regulated virulence genes, which may explain the virulence attenuation of the trxR mutant. This regulation appears to occur by activation of the mga promoter, Pmga, as demonstrated by analysis of a luciferase reporter fusion. Complementation of the trxR mutant with trxR on a plasmid restored expression of Mga regulon genes and restored virulence in the mouse model to wild-type levels. TrxR is the first TCS shown to regulate Mga expression. Because it is CovR repressed, TrxR defines a new pathway by which CovR can influence Mga to affect pathogenesis in the GAS.

Bacterial pathogens often adapt to various host niches during an infection by sensing changes in their surroundings and altering their gene expression patterns. This is typically accomplished through the use of environmentally responsive regulatory networks, such as two-component signal transduction systems (TCSs), as a means of tailoring a virulence response to a specific host condition. A TCS often consists of a membrane-bound histidine kinase that senses a specific external signal and transduces it via a phosphotransfer event to modulate the activity of a cognate response regulator. The response regulator component is usually a transcriptional regulator able to affect cellular responses through direct control of a defined set of genes, or regulon. Consequently, TCSs are often important for in vivo survival of pathogens during an infection yet are not essential for laboratory growth in rich medium.

Streptococcus pyogenes (group A streptococcus, or GAS) is a medically important bacterial pathogen that elicits a variety of diseases in humans ranging from benign to life-threatening (8), including noninvasive infections (pharyngitis and impetigo) and severe invasive infections (necrotizing fasciitis and streptococcal toxic shock syndrome). As a pathogen that is capable of causing disease in such varied human tissues, GAS has evolved a number of strategies to regulate appropriate sets of virulence genes in response to different host environments during an infection (25). Unlike many prokaryotes, GAS does not appear to rely on alternative sigma factors to control gene expression as only one has been identified to date (SigX), and it is not expressed under laboratory growth conditions (29, 31). Instead, the sequenced GAS genomes reveal a large number of predicted transcriptional regulators and signal transduction molecules that likely perform the bulk of coordinate gene regulation in this pathogen. These include classical TCSs as well as proteins with no identified sensory domain, termed stand-alone response regulators (25). The three characterized stand-alone regulators include Mga, Rgg/RopB, and the RofA-like family members (3, 6, 26, 28, 33).

In the 12 available GAS genome sequences from strains of serotypes M1, M2, M3, M4, M5, M6, M12, M18 and M28, 13 TCSs have been identified (2, 4, 5, 13, 16, 40). However, the functional role of only some of these systems in GAS pathogenesis has begun to be assessed in any significant detail. The Ihk/Irr system (12) plays an important role in GAS protection from killing by polymorphonuclear leukocytes, which are part of the innate immune response (49). The FasBCAX system, which shows homology to quorum-sensing TCSs in Staphylococcus aureus and Streptococcus pneumoniae, controls genes encoding some GAS adhesins (fbp54 and mrp) and aggressins (sagA and ska) in a growth phase-dependent manner (24). The SpkR/S TCS has been shown to be important for the persistence of GAS in human saliva (38). Recently, a study looking at four additional conserved TCS systems in the M1 GAS genome (Spy0875-Spy0875,
Spy1061-Spy1062, Spy1106-Spy1107, and Spy1553-Spy1556) found that inactivation of the putative response regulator Spy0680 led to a hypervirulent phenotype in a murine skin infection model (39).

The best characterized TCS in GAS is the CovRS/CsrRS system that functions as a repressor of many known and putative virulence genes, including those encoding capsule synthesis (hasIBC), streptolysin S (spa4), streptokinase (skA), and streptodornase (sdrN) (7, 12, 15, 21). CovRS has been shown to regulate transcription of 15% of the GAS genome either directly or indirectly and is responsive to nonphysiological concentrations of Mg2+ and various stress conditions likely to be encountered during infection of the host (11, 15, 17, 18). CovS is required for GAS to grow under stress conditions, which it does by relieving CovR-mediated repression (9). Recently, mutations in covS have been linked to an in vivo transcriptome conversion from a localized pharyngeal profile to a more invasive profile associated with severe systemic virulence and lethality in mice (43, 50).

Several TCSs in GAS remain uncharacterized, and these may also play important roles during infection. In a previous study, we constructed mutations in the 12 nonessential S. pyogenes TCS (Spt) response regulator genes in the serotype M1 strain SF370 (34). One of these TCS response regulator mutants, originally called Spt108R (TCS-10; Spy1587-Spy1588) and here renamed GAS two-component regulatory system X or trxSR, shows homology to the hik07-r07 TCS in S. pneumoniae (20, 46), which is essential for full virulence in several models of pneumococcal infection. Furthermore, trxSR is repressed by the CovRS virulence TCS in strains of two different serotypes of GAS (10, 15). In this study, a trxR mutant of strain MGAS5005 (serotype M1; M5005_Spy_1305) was assessed using a murine model of GAS soft tissue infection, DNA microarray analysis, and real-time reverse transcription-PCR (RT-PCR), to determine its role in GAS pathogenesis.

MATERIALS AND METHODS

Bacterial strains and media. S. pyogenes (GAS) strain SF370 is the serotype M1 strain used for the initial GAS genome sequence (13, 45). MGAS5005 (covS) is a well-characterized invasive serotype M1 strain with an available genome sequence that is virulent in mice (6, 42). Escherichia coli strain DHS00x [s8881lacZAM5 (lacZYA-argF)U196 recA1 endA1 hsdR17(λ- m-, r+) supE44 thi-l gyrA relA1] was used as the host for plasmid constructions. All E. coli strains were grown in Luria-Bertani broth. GAS was cultured in Todd-Hewitt medium supplemented with 0.2% yeast extract (THY), and growth was assayed by optical density with a Klett-Summerson photometric colorimeter with the A filter. Antibiotics were used at the following concentrations: erythromycin at 500 μg/ml for E. coli and 1.0 μg/ml for GAS, spectinomycin at 100 μg/ml for both E. coli and GAS, kanamycin at 50 μg/ml for E. coli and 300 μg/ml for GAS, and ampicillin at 100 μg/ml for E. coli.

DNA manipulations. Plasmid DNA was isolated using either a Wizard Miniprep (Promega) or Midi/Maxi prep purification system (Qiagen). DNA fragments were isolated from agarose gels using a QIAquick gel extraction kit (Qiagen). GAS chromosomal DNA was isolated using a FastDNA kit and a FastPrep cell disruptor (Bio 101, Inc.). PCR for cloning was performed using Pfu Turbo high-fidelity polymerase (Strategene), and reaction mixtures were purified using a QIAquick PCR purification system (Qiagen). PCR for diagnostic assays was performed using Platinum Taq DNA polymerase (Invitrogen). DNA sequencing was performed either using an Excel II cycle sequencing kit (Epicentre) or through the automated sequencing core facility in the McDermott Center at the University of Texas Southwestern Medical Center.

Inactivation of trxR in MGAS5005. trxR (M5005_Spy_1305) was inactivated in strain MGAS5005 using the temperature-sensitive integration method as previously described (32) to produce MGAS5005::trxR. Briefly, plasmid p233-10R (34) was electroporated into MGAS5005, followed by passage at 30°C with erythromycin selection. To allow for integration of the plasmid, cells were passaged at 37°C under erythromycin selection. Integrants were screened by PCR for junctions and the absence of the wild-type trxR gene (see Table S1 in the supplemental material).

MGAS5005::trxR was cured of the plasmid inactivating trxR by passage in liquid culture twice at 37°C and then three times at 37°C in THY broth without drug selection. Restoration of each locus was verified using both PCR and DNA sequencing.

Murine invasive skin infection model. An overnight culture (5 ml) was used to inoculate 75 ml of THY broth and was incubated statically at 37°C until late-logarithmic phase. When plasmids were present, appropriate antibiotics were used. Bacteria were vortexed for 5 min and centrifuged for 20 min at 7,500 × g at 4°C, and the pellet was resuspended in 3 ml of saline. Approximately 2 × 107 CFU/ml, as determined by microscope counts and verified by plating for viable colonies, were used to infect mice as previously described (37). Briefly, anesthetized 6- to 7-week-old female CD-1 mice (Charles River Laboratories) were depilated for a ~2 cm2 area of their back with Nair (Carter Products, New York, NY), and 100 μl of the cell suspension (2 × 107 CFU/mouse) was injected subcutaneously. Mice were monitored twice daily and were euthanized by CO2 asphyxiation upon signs of systemic morbidity (bumping, lethargy, and hind leg paralysis). Lesion sizes were measured at 48 to 72 h postinfection. Statistical analyses were performed using the Prism program (GraphPad Software). Lesion size data were analyzed and tested for significance using an unpaired two-tailed t test. Survival data were assessed by Kaplan-Meier survival analysis and tested for significance by a log rank test.

Microarray and real-time RT-PCR validation. Microarray experiments were performed as previously described (35). Briefly, total RNA from three biological replicates was isolated from MGAS5005 and the isogenic trxR mutant strain MGAS5005::trxR containing the empty vector pRS525 at late logarithmic phase (100 Klett units) using a Triton X-100 isolation protocol (44). Both strains were grown in the presence of spectinomycin for the overnight seed cultures but not during growth for RNA isolation. DNAse I-treated RNA samples were converted to cDNA with amino allyl UTP and were Cy3 and Cy5 labeled using an amino allyl labeling kit (Ambion) to allow for dye swap experiments. Yields and incorporation rate of the labeled cDNA were determined using a Nanodrop ND-1000 (Nanodrop Technologies). Equal volumes (35.42 μl) of labeled Cy3 cDNA and Cy5 cDNA were dried under vacuum, resuspended in 23.8 μl of distilled H2O, and boiled for 5 min, followed by cooling on ice for 5 min. Hybrid buffer (5×:17 μl) (GE Healthcare) and formamide (27.2 μl) were added to the cDNA and applied to array slides under raised coverslips (Lifterslip, Inc.). Microarray slides were hybridized at 50°C overnight in slide chambers (Array It). Slides were washed twice for 10 min each in the following buffer concentrations and at the indicated temperatures: 6× SSPE–0.1% Tween 20 at 50°C, 0.8× SSPE–0.001% Tween 20 at 50°C, and 0.8× SSPE at room temperature (1× SSPE is 0.18 M NaCl, 10 mM NaH2PO4, and 1 mM EDTA, pH 7.7). Slides were scanned using a GenePix 4100A personal array scanner and GenePixPro, version 4.0.1.8 (Axon Instruments).

Data obtained from the wild-type and trxR mutant strains were compared for twofold changes in expression, ≥2.0 or ≤0.50, and were analyzed using Acuity, version 4.0.1.8 (Axon Instruments). Using ratio-based normalization, data were normalized by the ratio of the means, and samples were removed when four out of the six experiments did not show significance.

Validation of array data was carried out by real-time RT-PCR of 12 differentially regulated genes (Table 1) using the primers listed in Table S1 in the supplemental material. Correlation coefficients for the arrays were determined by plotting the log value of the array on the x axis against the log value of the real-time RT-PCR on the y axis. An equation determining the line of best fit was determined, and the resulting R2 value was calculated, which represented the fitness of the data.

Real-time RT-PCR. Briefly, total RNA was isolated as described above, and 25 ng was DNase I treated, added to a Sybr Green Master mix (Applied Biosystems) containing 5 μg of each specific real-time primer (see Table S1 in the supplemental material), and combined with 6.25 units of Multiscribe reverse transcriptase (Applied Biosystems) in a 25-μl volume for one-step real-time RT-PCR. The real-time RT-PCR experiments were completed using a Lightcycler 480 (Roche), and transcript levels were detected in the relative quantification mode. Samples were compared to wild-type gas4 transcript levels, with the levels presented as the relative expression level to the wild type.

In vitro transcription assays. To construct the Pspy1307 promoter template used in the in vitro transcription reactions, the primers Trx-Pes1-BamHI and Trx-Pes3-XhoI (see Table S1 in the supplemental material) were used to amplify by PCR a 323-bp region including the M5005_Spy_1307-bga4 (Pspy1307) pro-
TrxR regulatory circuitry and virulence in M1 GAS

**RESULTS**

Inactivation of *trxR* in M1 MGAS5005 leads to attenuation in virulence. The predicted TCS encoded by M5005_Spy_1305/...
1306 was chosen for further analysis for two reasons: (i) it shares homology with the rr07-hk07 TCS associated with virulence in S. pneumoniae, and (ii) it is repressed by the CovRS TCS in GAS (10, 15). The locus was renamed trxSR (for two-component regulatory system X) to reflect its location as the 10th TCS annotated in each of the sequenced GAS genomes.

To assess its role in GAS virulence, a trxR mutant strain was analyzed in a murine model of streptococcal soft tissue infection. Although we had previously constructed an insertional inactivation of trxR (called spt10R) in the serotype M1 strain SF370 (34), strain SF370 does not demonstrate virulence in the mouse model of infection. Therefore, the mutagenic plasmid that was used to construct the mutation in SF370, p233-10R, was used to inactivate trxR in the serotype M1 strain MGAS5005, which has been used extensively for GAS virulence studies in mice (39, 41). The resulting trxR mutant MGAS5005.trxR exhibited growth kinetics comparable to the parent MGAS5005 in rich (THY) medium (data not shown).

MGAS5005 and MGAS5005.trxR cultures were grown to late logarithmic phase and injected subcu- taneously into the haunches of female CD-1 mice (see Materials and Methods). The progression of disease was assessed by monitoring both lesion severity and survival across a 7-day period. Mice infected with the trxR mutant exhibited significantly reduced lesion sizes compared to those infected with MGAS5005 at 48 h postinfection (Fig. 1A), and this correlated with a less purulent and less ulcerative lesion (Fig. 1B). In addition, mice infected with the trxR mutant showed a significant increase in survival across the 7-day period compared to mice infected with the parental MGAS5005 (Fig. 1C). Thus, the trxR mutation in MGAS5005 led to an attenuation of virulence as scored by lesion progression and lethality in a murine model of soft tissue infection.

To determine if a spontaneous mutation elsewhere in the chromosome was responsible for the observed attenuation in virulence, a wild-type strain was regenerated from the mutant. Since trxR inactivation results from insertion into the GAS chromosome of a plasmid unable to replicate at 37°C, several passages at a temperature permissive for plasmid replication allowed isolation of a cured MGAS5005.trxRc strain that had lost the mutation and possessed a restored wild-type trxR locus.

As a control for the effects of laboratory passage on virulence, a parental MGAS5005 strain was subjected to a comparable number of passages to generate MGAS5005*. In the mouse model, the cured TrxRc strain produced lesions of statistically similar size to those produced by the mock-passaged strain MGAS5005* (Fig. 1D) as well as to an unpassaged MGAS5005 strain (data not shown). Infection with the trxR mutant produced smaller lesions than infection with either wild-type strain even when the dose used for the mutant was more than twice that used for the wild-type strains. Therefore, the trxR mutation, and not an unknown spontaneous mutation elsewhere in the genome, was responsible for the reduced virulence observed in these studies.
The **trxSR operon**. Inspection of the MGAS5005 genome showed four tightly linked open reading frames (M5005_Spy_1307, \( \text{trxS} \), \( \text{trxR} \), and \( \text{bgaA} \)) separated by very little intergenic space, suggesting that they may be part of an operon (Fig. 2A). The comparable M1 SF370 genomic region is identical to MGAS5005 except for a 2-bp intergenic insertion between \( \text{trxS} - \text{trxR} \) and a 197-bp intergenic insertion between M5005_Spy_1307 (SPy1583 in SF370) and \( \text{trxS} \) (data not shown). The 347-bp region located upstream of M5005_Spy_1307 contains a potential Rho-independent terminator followed by a putative promoter, based on homology to the *E. coli* consensus (Fig. 2A). To learn about transcriptional linkage of \( \text{trxR} \) with these genes in the GAS chromosome, primer pairs were designed to amplify their intergenic regions (Fig. 2A). RT-PCR analysis of cDNA clearly demonstrated that some transcripts include M5005_Spy_1307, \( \text{trxS} \), and \( \text{trxR} \) (data not shown). Although a transcript that contains \( \text{trxR} \) and \( \text{bgaA} \) could be detected by RT-PCR (data not shown), the \( \text{trxR} \) mutation did not confer a polar effect on \( \text{bgaA} \) transcription (Table 1), suggesting that \( \text{bgaA} \) can be transcribed independently of \( \text{trxR} \) in M1 strain MGAS5005. Immediately downstream of \( \text{bgaA} \) in the GAS chromosome is \( \text{aroE} \) (M5005_Spy_1303), encoding a putative shikimate 5'-dehydrogenase, whose promoter (\( \text{P}_{\text{aroE}} \)) was mapped by primer extension in the M1 strain SF370 (Fig. 2A; data not shown). Thus, \( \text{trxR} \) appears to be part of an operon composed of 5005_Spy_1307, or \( \text{trxSR} \).

To identify the promoter upstream of gene M5005_Spy_1307, in vitro transcription experiments were performed utilizing the plasmid template pEU7094, which contains a region of DNA from -268 to +55 relative to the M5005_Spy_1307 start codon. This region is identical to that in the M1 strain SF370 background. When linearized with XhoI or KpnI, pEU7094 produced transcripts of 193 or 253 nucleotides, respectively. This corresponds to a start of transcription for the operon (\( \text{P}_{\text{Spy1307}} \)) 132 bases upstream of M5005_Spy_1307 (Fig. 2A and B).

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**FIG. 2.** CovR-repressed operon containing \( \text{trxSR} \). (A) Schematic of \( \text{trxSR} \) genomic region in M1 MGAS5005 GAS (top) including putative open reading frames and demonstrated \( \text{P}_{\text{Spy1307}} \) and \( \text{P}_{\text{aroE}} \) promoters (arrows above line). Inverted triangle represents mutation in \( \text{trxR} \), while dashed lines designate RT-PCR products demonstrating linkage of genes. Putative operon promoter (\( \text{P}_{\text{Spy1307}} \)) sequences upstream of M5005_Spy_1307 are shown (below) with –10 and –35 sequences (solid bars) and start of transcription (black arrow) indicated. A consensus CovR binding site is also shown (darkened box). (B) In vitro transcription analysis of \( \text{P}_{\text{Spy1307}} \). The start of transcription was validated using pEU7094 template linearized with XhoI or KpnI. Products corresponded to a start of transcription 132 bases upstream of the start of M5005_Spy_1307 (SPy1589 in SF370) as indicated in panel A. An RNA size marker is shown to the left. (C) In vitro transcription analysis of \( \text{P}_{\text{Spy1307}} \) following incubation with increasing amounts (\( \mu \text{M} \)) of CovR (left) or CovR-P (right). Transcription of the kanamycin resistance gene promoter \( \text{P}_{\text{aphA3}} \) was included in each reaction as an internal control. Specific transcripts are indicated by arrows and an RNA size marker is shown to the left.
CovR represses the trxSR operon directly. The CovR response regulator has been shown to repress trxSR transcription in two different microarray studies using different GAS strains (10, 15). At several target promoters, CovR binds to conserved ATTARA motifs or TTA repeats to mediate direct repression of transcription (7). The presence of a consensus CovR binding site immediately downstream of $P_{Spy1307}$ suggests that CovR might directly repress its transcription (Fig. 2A). To evaluate this, in vitro transcription studies were performed using purified CovR. Increasing amounts of CovR or CovR–$\sim P$ were incubated with the KpnI-digested pEU7094 template DNA prior to addition of RNA polymerase (Fig. 2C). In addition, an internal control for transcription was provided by including in the template mixture the plasmid pJRS462 (19) linearized with XcmI, which cuts 510 bp downstream from the kanamycin resistance gene promoter $P_{aphA4}$. Increasing concentrations of either CovR or CovR–$\sim P$ resulted in a reduction in $P_{Spy1307}$$^\sim$ specific transcript levels, with approximately twofold less CovR–$\sim P$ than CovR required to show a 50% reduction in transcripts (Fig. 2C). These results demonstrate that CovR directly represses transcription initiation or elongation at $P_{Spy1307}$ to regulate an operon that includes M5005_Spy_1307, the trxSR TCS, and possibly the β-galactosidase gene bgaA.

**Definition of the TrxR regulon in GAS.** Since inactivation of the TCS response regulator gene $trxR$ leads to attenuation of virulence in mice, the global transcription profile of the MGAS5005 trxR mutant compared to its wild-type parent was assessed using a GAS 70-mer oligonucleotide microarray (35). Total RNA was isolated from the $trxR$ mutant and wild-type strains at late logarithmic phase in three biological replicates and used to generate labeled cDNA for hybridization. Data obtained from the wild-type and $trxR$ mutant strains were compared (mutant values/wild-type values) for changes in expression. A relatively small number of transcripts (29 total) were affected in late exponential phase by inactivation of $trxR$. Since expression of the gene downstream of $trxR$, $bgaA$, was not altered in the $trxR$ mutant (Fig. 2A), the mutation does not appear to have caused a polar effect. Transcripts that were significantly reduced in the mutant (Table 1), suggesting that they were TrxR activated, represent the known members of the Mga virulence regulon (35). These included emm1 (37-fold), sic (26-fold), fba (9.3-fold), scpA (6.8-fold), sclA (4.7-fold), and gmr (2.9-fold). Although the difference in transcript levels for $mga$ between the $trxR$ mutant and wild type were just outside the significant range (1.9-fold), this might be the result of the low steady-state levels of $mga$ transcript produced in GAS (Table 1). There was no evidence from the array analysis that TrxR regulated its own expression.

In addition to activating some genes, TrxR also represses transcription of genes in GAS. There were 21 transcripts that showed a significant increase in the mutant, indicating repression by TrxR either directly or indirectly (Table 1). The TrxR-repressed genes encode proteins involved in various processes including general metabolism, such as amino acid biosynthesis ($aro$) and DNA replication ($polA$), as well as in different types of stress response (dnaJ, $clpL$), and the transport of the osmoprotectant glycine-betaine $apu$. Thus, TrxR appears to activate the Mga regulon as well as repress other apparently unrelated genes.

To validate the microarray results, 12 genes representing the range of possible changes (increase, decrease, and no effect) were selected and analyzed by real-time RT-PCR (Table 1). Overall, the real-time RT-PCR results confirmed the microarray data, with a correlation coefficient ($R^2$) calculated to be 0.892.

**Complementation with trxR restores expression of the Mga regulon.** To assess the role of $trxR$ in the reduced expression of Mga-regulated genes, a complementation assay was performed (see Materials and Methods). The wild-type $trxR$ gene was cloned under the control of the constitutive GAS $rpsL$ promoter (P$\text{rpsL}$) on the replicating plasmid pKSM612 (P$\text{rpsL-}$trxR). Real-time RT-PCR was performed on total RNA isolated at late exponential phase from MGAS5005 with an empty vector and compared to the MGAS5005 trxR mutant containing either an empty vector or the complementing plasmid pKSM612 (see Materials and Methods). The level of $trxR$ transcript was significantly higher in the complemented strain (MGAS5005 trxR/pKSM612) than in the wild-type MGAS5005 (Fig. 3, $trxR$), suggesting that multiple copies of $rpsL$ are likely to be stronger than a single copy of the $trxR$ promoter. For each Mga-regulated gene tested, the complementing plasmid restored the transcript level of the $trxR$ mutant to wild-type levels ($emm1$, sic, fba, scpA, and sclA) (Fig. 3). This demonstrates that $trxR$ alone is capable of complementing the defect in Mga regulon expression caused by inactivation of $trxR$ in MGAS5005.

**trxR activates Pmga.** Although the $trxR$ mutant had little effect on $mga$ transcription at the time chosen for the array and real-time analyses, it seemed possible that TrxR might regulate $mga$ expression at other times during growth. To address this, the firefly luciferase reporter gene was fused to the $mga$ pro-
wild-type background reduced the difference with the 
trxR activity in the wild-type strain (MGAS5005), its Mga-inactivated mutant (MGAS5005.trxR) strains 
mutant (MGAS5005.trxR/pKSM721) were 
growth. Relative to the wild-type strain, the levels of P
mga activity in the wild-type strain (MGAS5005), its Mga-inactivated mu-
the stand-alone regulator Mga activates a defined set 
activates at the late logarithmic time point used in the 
array assay. At this time, a drop in Pmga activity in the 
background reduced the difference with the trxR mu-
mutant background (Fig. 4 and Table 1). From these data, it 
appears that TrxR activates the Mga regulon through regulation of Pmga.

The virulence defect in the TrxR mutant is complemented by 
**wild-type trxR**. Since expression of trxR in trans restored the 
trxR mutant's defect in Mga regulon gene expression (Fig. 3), we 
investigated the effects of complementation on the viru-
ience attenuation observed for the trxR mutant (Fig. 1). The 
complemented mutant strain was compared to the parental 
MGAS5005 and the isogenic trxR mutant containing an empty 
vector in the mouse model of soft tissue infection. Both for 
lesion severity and for lethality, expression of trxR in trans 
restored virulence to a level at or above that of the wild-type 
parent strain (Fig. 5A and B). From this, it appears that the 
CovR-repressed trxR gene contributes to virulence of the M1 
strain MGAS5005 in a soft tissue model of infection, likely 
through activation of the Mga regulon.

**DISCUSSION**

This study was initiated to assess the importance of the 
uncharacterized trxSR TCS (MS005_Spy_1306/1305) during 
streptococcal disease. A trxR mutant was found to be both 
attenuated for virulence in a murine model of GAS soft tissue 
infection in vivo and defective in expression of the Mga viru-
ience regulon in vitro. Because this work showed that trxSR was 
found to be part of a CovR-repressed operon, it reveals a new 
pathway by which important global regulatory networks 
can influence each other to affect pathogenesis in the GAS.

**The response regulator TrxR activates the Mga virulence regulon.** The stand-alone regulator Mga activates a defined set of “core” virulence genes encoding factors important for early 
stages of colonization and resistance to the host immune re-
response (22). Although the Mga regulon is induced by growth 
conditions such as logarithmic growth and elevated iron or 
CO₂ levels, only a few regulatory networks have been linked to 
mga regulation. The stationary phase-associated stand-alone 
regulators Rgg/RopB, RofA, and Nra are known to repress 
mga expression, either directly or indirectly, providing a mech-
anism for shutting down the Mga regulon. In addition, RivR 
(Ralp4) appears to work with the Mga protein to directly 
enhance Mga activation of its target gene promoters, possibly 
through protein-protein interactions, and RivX, a small RNA, 
acts at the mga promoter to activate transcription of Mga 
regulon genes (36). This work adds TrxSR to the list of regu-
lators interacting with the Mga regulon. TrxSR represents a

**FIG. 4.** Pmga activity is regulated by TrxR. Wild-type (MGAS5005), 
mga mutant (KSM165-L5005), and trxR mutant (MGAS5005.trxR) strains 
containing the Pmga-luc luciferase reporter plasmid pKSM721 were 
grown in THY medium under spectinomycin selection. Upon reaching 30 
Klett units, 500-μl samples were taken every 30 min across growth 
(dashed lines) and assayed for luciferase production, expressed as relative 
luciferase units (solid lines).

**FIG. 5.** In vivo complementation of the trxR mutant attenuation. (A) Comparison of lesion sizes in mice infected with wild-type MGAS5005 
with empty vector, the trxR mutant MGAS5005.trxR with empty vector, or the trxR mutant complemented with pKSM612 (pTrxR; PrpsL-trxR). 
Animals (n = 10) were inoculated subcutaneously with various numbers of CFU, as indicated, and lesion sizes were measured (mm²) at 72 h 
following infection. Some mice died prior to the 72-h measurement. P values were determined using an unpaired two-tailed t test. (B) Survival plot 
comparing mice inoculated with the strains above over a 7-day period following infection. A Kaplan-Meier survival analysis and log rank test were 
used to determine significance.
new example of a TCS that influences expression of the Mga regulon in GAS.

Although the trxSR coding regions of SF370 and MGAS5005 are identical and TrxR represses the Mga regulon in MGAS5005, trxR was not identified as necessary for expression of the Mga-regulated gene emm in a previous mutational analysis of the 12 nonessential TCS response regulators in SF370 (34). Additional real-time RT-PCR and Northern studies of the M1 strain SF370 trxR mutant confirmed these results for several other Mga-regulated genes (data not shown). However, although both strain SF370 and MGAS5005 belong to the M1 serotype, SF370 is genetically distinct from the serotype M1 strains associated with recent severe human infections (1, 42). Although MGAS5005 possesses a mutation in covS that is associated with invasive disease potential in mice (42) (see below), both MGAS5005 and the trxR mutant were isogenic for the mutant allele in this study. Thus, the differences in the genetic background between SF370 and MGAS5005 may result in the different observed effects of TrxR on the expression of Mga-regulated genes. Since the M1 strain SF370 does not cause significant soft tissue damage in mice, the effect of TrxR on virulence cannot be assessed using this strain. However, it is possible that the differences in virulence of these strains in this murine model may result from the same genetic differences that cause the observed effects of TrxR on the Mga regulon. Studies are needed to investigate the role of trxSR in Mga regulation and virulence in a broader range of GAS strains representing the major serotypes currently involved in streptococcal disease.

Although trxR is part of an operon, the complementation results indicate that it is the only gene in this operon necessary for activation of Mga-regulated virulence genes. The luciferase reporter experiment suggests that TrxR likely acts through the mga promoter, like RivX (Fig. 3 and 4 and Table 1) and unlike RivR that acts through the Mga protein (36). TrxR is predicted to have an AraC-like DNA-binding domain with similarity to the YesN family of response regulators from Bacillus subtilis. Whether transcriptional regulation involves direct binding of TrxR at Pmga or whether the regulation is indirect through another TrxR-regulated gene(s) still needs to be addressed. The only TrxR-regulated gene identified in this array analysis with a predicted function in transcriptional regulation is mga; therefore, if TrxR acts indirectly on Pmga, this would require a downstream regulatory factor influenced by Trx that was not detected in this study.

Since Mga-regulated genes encode established virulence factors and since mutations in mga lead to reduced virulence in various animal models of GAS infection (22), the reduction in Mga regulon expression observed in the trxR mutant is the most probable reason for its attenuation. However, this does not rule out that other TrxR-regulated genes may also play a role in pathogenesis. Genes in the TrxR regulon exhibit a range of different functions in GAS, including translation (rbfA and rplT), transcription (nuA), replication (polA), transport (lacE, secA, and opuAA-opuABc), and stress (clpL). Although the trxR mutant did not show an altered growth phenotype in rich THY medium in vitro, it is possible that growth in vivo would reveal an effect. Binding studies of promoters for regulated genes using purified recombinant TrxR are needed to identify direct targets of this new TCS response regulator.

**trxR is part of a CovR-repressed virulence operon.** The arrangement of M5005_Spy_1307 and trxSR as part of an operon in GAS is intriguing. It is typical in bacteria for the response regulator and sensor kinase TCS genes to be cotranscribed, with the operon often being autoregulated by the same TCS. However, it is less common for the TCS genes to be cotranscribed with other genes. When this does occur, it usually indicates that the genes encode proteins with activities that are functionally linked. In the agr system of S. aureus, the genes encoding the TCS AgrAC, required for sensing the agr autoinducing peptide are in the same transcript as the genes for AgrDB, involved in synthesis and secretion of the same peptide (30). The GAS fasBCAX operon, an Agr ortholog, encodes a TCS with two potential sensor kinases (FasBC), a response regulator (FasA), and a regulatory RNA (FasX) involved in a common regulatory unit (24). Whether the TCS TrxSR and M5005_Spy_1307, a conserved membrane protein of unknown function, perform related functions is unclear at this time. Since the trxR mutation did not show polarity on expression of the downstream β-galactosidase gene bgaA, it does not appear that bgaA is part of the trxSR operon in the M1 strain MGAS5005. Mutational studies are under way using a nonpolar mutation in M5005_Spy_1307 to determine whether it is functionally linked to TrxR in GAS.

The results presented above demonstrate that the trxSR operon is directly repressed by the response regulator CovR (Fig. 3). Therefore, CovR downregulates a TCS activator of virulence and the Mga regulon in the serotype M1 strain MGAS5005, leading to a repression of both activities when CovR is active. CovRS is pivotal in a global regulatory network that negatively regulates expression of several virulence factor genes as well as 15% of the GAS genome in response to signals such as stress (10, 15, 18). CovR is inactivated under stress conditions, relieving CovR-mediated repression of virulence genes and genes important for survival under stress conditions (11). Thus, the repression of the trxSR operon by CovR may indicate a role for Trx-regulated genes in the response to stress in addition to its virulence phenotype. However, because MGAS5005 is a covS mutant (42), there may be an additional level of control that would not be observed using this background. Studies are needed to investigate TrxRS regulation in an MGAS5005 strain repaired for the covS mutation. Given that the TrxS sensor kinase probably responds to a distinct signal from CovS, the integration of multiple signals into the pathway will likely lead to a complex pattern of regulation.

CovR has now been found to repress three different activators of the Mga virulence regulon: the TCS response regulator TrxR, the stand-alone regulator RivR, and the small RNA RivX (Fig. 6). This suggests that the expression of CovR and these regulators should be inversely correlated. This is supported by a longitudinal analysis of GAS pharyngitis in cynomolgus macaques (48), where peak expression of Mga-regulated genes in the serotype M1 strain MGAS5005 was observed during the acute phase of infection when levels of GAS growth were high and CovR was not expressed. Subsequent increased covR expression correlated with a dramatic reduction of Mga regulon expression and viable GAS in the posterior pharynx. In contrast to these in vivo studies, in vitro microarray analysis of MGAS5005 did not reveal mga and Mga-regulated genes as being significantly CovR repressed. This may reflect the com-
plex in vivo environments found in nonhuman primates and mice that are not found during in vitro growth in rich medium.

**Interactions between TCS and stand-alone regulators in GAS.** Based on the results presented here, we can begin to model the complex interactions that occur between different regulatory pathways in GAS that influence disease progression (Fig. 6). The CovRS TCS plays a central role in this process by negatively regulating important virulence genes (ska, has, and sog) as well as several TCS regulatory loci linked to virulence, in response to stress and possibly other signals. CovR also represses expression of rivR and rivX, encoding a stand-alone regulator and small RNA, respectively, that enhance transcriptional activation by an external signal for the regulatory pathways shown. Known or predicted interactions between these regulatory networks will help to better elucidate their contribution to pathogenesis and the complex interplay that occurs in vivo during GAS disease.

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