RivR Is a Negative Regulator of Virulence Factor Expression in Group A *Streptococcus*

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The bacterial pathogen group A *Streptococcus* (GAS) causes human diseases ranging from self-limiting pharyngitis (also known as strep throat) to severely invasive necrotizing fasciitis (also known as the flesh-eating syndrome). To control virulence factor expression, GAS utilizes both protein- and RNA-based mechanisms of regulation. Here we report that the transcription factor RivR (RofA-like protein IV) negatively regulates the abundance of mRNAs encoding the hyaluronic acid capsule biosynthesis proteins (*hasABC*; ~7-fold) and the protein G-related α_2 -macroglobulin-binding protein (*grab*; ~29-fold). Our data differ significantly from those of a previous study of the RivR regulon. Given that *grab* and *hasABC* are also negatively regulated by the two-component system CovR/S (control of virulence), we tested whether RivR functions through CovR/S. A comparison of *riv* and *cov* single and double mutant strains showed that RivR requires CovR activity for *grab* and *hasABC* regulation. Analysis of the upstream region of *rivR* identified a novel promoter the deletion of which reduced *rivR* mRNA abundance by 70%. A *rivR* mutant strain had a reduced ability to adhere to human keratinocytes relative to that of the parental and complemented strains, a phenotype that was abolished upon GAS pretreatment with hyaluronidase, highlighting the importance of capsule regulation by RivR during colonization. The *rivR* mutant strain was also attenuated for virulence in a murine model of bacteremia infection. Thus, we identify RivR as an important regulator of GAS virulence and provide new insight into the regulatory networks controlling virulence factor production in this pathogen.

The bacterial pathogen group A *Streptococcus* (GAS, *Streptococcus pyogenes*) can cause distinct human diseases that vary in morbidity and mortality. Mild and self-limiting infections include GAS pharyngitis and the skin infection impetigo, while severe invasive infections include streptococcal toxic shock syndrome and necrotizing fasciitis (1). The ability of GAS to cause a wide variety of infections is in part due to its ability to coordinately regulate virulence factor expression, producing different assortments of virulence factors in response to environmental cues. Virulence factors produced by GAS include a hyaluronic acid capsule that surrounds the cell and has antiphagocytic and adhesive properties (2, 3), a series of secreted proteins such as the thrombolytic agent streptokinase (4), and a series of cell wall-anchored proteins, including protein G-related α_2 -macroglobulin-binding protein (GRAB), which binds the human protease inhibitor α_2 -macroglobulin (5).

The regulation of virulence factor production in GAS is controlled through both protein- and RNA-based mechanisms (6, 7). For example, the expression of pili on the GAS cell surface is positively regulated at the transcriptional level by the RofA transcription factor (8, 9) and negatively regulated at the posttranscriptional level by the small regulatory RNA (sRNA) FasX (fibronectin/fibrinogen binding/hemolytic activity/streptokinase regulator X) (10). Two of the best-described transcriptional regulatory systems in GAS are the Mga (multi-gene regulator in GAS) stand-alone transcription factor and the CovR/S (control of virulence R/S, also known as CsrRS) two-component system (11, 12). Mga regulates ~10% of the GAS transcriptome, including the positive regulation of genes encoding the virulence factors C5a peptidase, M protein, and streptococcal collagen-like protein A (13, 14). The CovR/S system also regulates ~10% of the GAS transcriptome but primarily serves as a negative regulator of virulence factors, including the hyaluronic acid capsule, streptolysin

O, the cysteine protease SpeB, and GRAB (15–18). The CovR/S system also negatively regulates the abundance of transcripts encoding several regulatory proteins, for example, the RivR (RofA-like protein IV) and TrxR (GAS two-component regulatory system X) regulators (19–21), and hence contributes to a series of regulatory cascades in this pathogen.

Through unknown mechanisms, the RivR transcription factor and the downstream-encoded sRNA RivX are purported to independently positively regulate the transcript levels of members of the Mga regulon (e.g., *scpA* encoding the C5a peptidase and *emm* encoding the M protein) (22). However, here we present data that challenge this view, with RivR being a negative regulator of the virulence factor-encoding genes *hasABC* and *grab* rather than a positive regulator of the Mga regulon. In addition, we show that RivR promotes GAS virulence, as evident by the decreased adherence of a *rivR* mutant strain to a human keratinocyte cell line and by the decreased lethality of a *rivR* mutant strain in a murine model of bacteremia infection.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. MGAS2221 and MGAS5005 are representative of the highly virulent M1T1 GAS clone responsible for significant morbidity and mortality since the mid-1980s in the United States, Canada, and western Europe (16). GAS bacteria were grown in Todd-Hewitt broth with 0.2% yeast extract (THY broth) at 37°C (5% CO₂). Note that MGAS2221 and all derivatives (see Table S3 in the supplemental material) had identical growth curves in THY broth (data not shown). Chloramphenicol (4 µg/ml), kanamycin (200 µg/ml), and/or spectinomycin (150 µg/ml) were added when required.

Construction of the isogenic *rivRX* mutant derivative 2221Δ*rivRX*.

The *rivRX* genes were deleted in the MGAS2221 background by allelic exchange using the suicide vector pBBL740 via a previously described protocol (23). Briefly, 1-kb regions flanking either side of the *rivRX* genes were amplified by PCR, joined via overlap extension PCR, and cloned into pBBL740 (see Table S4 in the supplemental material). The PCR primers used are listed in Table S2. The resultant plasmid was transformed into MGAS2221, and colonies were selected on THY agar plates containing chloramphenicol (these transformants have the plasmid integrated into the chromosome). To promote excision of the plasmid from the chromosome, which can either leave the intact *rivRX* genes behind or delete *rivRX*, we serially passaged the strain in THY broth without antibiotics. Two 5-h passages, followed by a 15-h passage and two more 5-h passages, were performed (1/100 dilutions of the old culture into the new culture were performed for each of the 5-h passages, with a 1/1,000 dilution for the 15-h passage). The final culture was diluted and plated onto blood agar plates to obtain single colonies. Colonies were patched onto THY agar plates, one containing chloramphenicol and one containing no antibiotic. Strains that grew on the THY plate but not the THY plus chloramphenicol plate were analyzed by PCR and sequencing to identify whether they contained the *rivRX* genes or were deletion mutants.

Construction of the isogenic mutant derivatives 2221Δ*grab* and 2221Δ*rivRX*Δ*grab*. The *grab* gene was replaced with the streptomycin resistance cassette in strains MGAS2221 and 2221Δ*rivRX* to create strains 2221Δ*grab* and 2221Δ*rivRX*Δ*grab*, respectively. The PCR primers used to create 1-kb *grab*-flanking regions separated by the spectinomycin cassette are listed in Table S2 in the supplemental material.

Construction of the isogenic *sr195750* mutant derivative 2221Δ*sr195750*. The putative *sr195750* gene upstream of *rivR* was replaced with the omega-kanamycin cassette in strain MGAS2221 to create strain 2221Δ*sr195750*. The omega-kanamycin cassette has strong transcriptional terminators flanking either side to prevent transcription out of the cassette into neighboring genes. The PCR primers used to create 1-kb *sr195750*-flanking regions separated by the omega-kanamycin cassette are listed in Table S2 in the supplemental material.

Construction of the isogenic *rivRX* and *covR* mutant derivative 2221Δ*rivRX*Δ*covR*. A *covR* mutant of strain 2221Δ*rivRX* was created by replacing the DNA sequence encoding the CovR DNA-binding domain with a kanamycin resistance cassette. This mutation has previously been determined to be nonpolar (18). The primers used to create this strain are listed in Table S2 in the supplemental material.

Construction of the isogenic *rivRX* and *covS* mutant derivative 2221Δ*rivRX*Δ*covS*. A *covS* mutant of strain 2221Δ*rivRX* was created by deleting 2 bp from within the *covS* gene, knocking the gene out of frame. The 2-bp deletion was introduced by using a pBBL740 derivative as described above for the creation of strain 2221Δ*rivRX*. The primers used to create this strain are listed in Table S2 in the supplemental material.

Construction of the isogenic *rivR* mutant derivative JRS950Δ*rivR*. JRS950 is a previously constructed derivative of the clinical isolate MGAS5005 that has a wild-type *covS* gene but a mutant *covR* gene (22). A *rivR* mutant of JRS950 was created by replacing the promoter and the first two-thirds of *rivR* with a spectinomycin resistance cassette. The PCR primers used to create 1-kb flanking regions separated by the spectinomycin resistance cassette are listed in Table S2 in the supplemental material.

Total RNA isolation from GAS. GAS strains were grown in THY broth to mid-exponential phase, corresponding to an optical density at 600 nm (OD₆₀₀) of 0.5. One volume of GAS culture was added to 2 volumes of RNeasy Protect (Qiagen) and incubated at room temperature for 5 min before centrifugation for 10 min at 4,000 × g. The supernatant was removed, and the pellets were quick-frozen in liquid nitrogen and stored at −80°C until they were used. RNA was isolated from each GAS cell pellet by a mechanical lysis method described previously (19). Contaminating DNA was removed by using TURBO DNase-free (Life Technologies).

Expression microarray analysis. A custom Affymetrix microarray was used to monitor GAS gene expression. Each gene in the MGAS2221 genome was represented on our custom array, with 16 probe pairs for each gene. Duplicate cultures of GAS strains MGAS2221 and 2221Δ*rivRX* were grown to exponential phase at 37°C (5% CO₂) in THY broth. RNA was isolated, converted to cDNA, labeled, and hybridized to our custom array as described previously (17). Gene expression estimates were calculated by using GCOS software v1.4 (Affymetrix Inc.). Data were normalized across samples to minimize discrepancies that can arise because of experimental variables (e.g., probe preparation). Genes with average expression values below 100 were manually removed.

qRT-PCR analysis. To determine relative mRNA abundance, we used quantitative reverse transcription (qRT)-PCR with TaqMan primers and probes. In each experiment, RNA samples from triplicate cultures of the GAS strains to be analyzed were converted into cDNA and used in association with an ABI 7500 Fast System (Life Technologies). Gene transcript levels were compared by using the ΔΔC_T method (27). TaqMan primers and probes for the genes of interest and the internal control gene *proS* are shown in Table S2 in the supplemental material. Each experiment was performed in triplicate, and mean values ± standard deviations are shown.

Isolation of secreted protein fractions. Supernatant proteins from overnight or exponential-phase THY broth GAS cultures were concentrated by ethanol precipitation and resuspended in SDS-PAGE buffer at 1/20 of the original volume.

Isolation of cell wall protein fractions. GAS strains were grown to the mid-exponential (OD₆₀₀ of 0.5) and stationary (OD₆₀₀ of 1.5) phases of growth in THY broth. At each growth phase, 40-ml aliquots were recovered and the cells were pelleted by centrifugation (4,000 × g for 10 min). The bacterial cell pellets were washed once with 10 ml of TE buffer before resuspension in 1 ml of TE-sucrose buffer (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 20% sucrose, protease inhibitors, 2 mg/ml lysozyme, 400 U of mutanolysin). Samples were incubated at 37°C with end-to-end rotation in 1.5-ml tubes for 2 h. Samples were centrifuged at 15,000 × g for 5 min to pellet protoplasts, and the supernatants were transferred to new 1.5-ml tubes. The centrifugation was repeated to pellet any insoluble material carried over, and the supernatant was analyzed by SDS-PAGE.

Western blot analysis. Protein samples were separated on 12% Tris-HCl gels. An affinity-purified rabbit anti-GRAB antibody was generated by Pacific Immunology Corp. from rabbits that were coimmunized with two peptide fragments of GRAB (see Fig. S1 in the supplemental material) and was used at a 1:1,000 dilution. The rabbit anti-Spd3 antibody used as a loading control has been described previously (24). After washing, a goat anti-rabbit secondary antibody (horseradish peroxidase [HRP] conjugated; used at a 1:10,000 dilution) was used, and signal was generated using the SuperSignal West Pico kit (Pierce).

Hyaluronic acid capsule assays. Assays were performed using a slightly modified version of a previously described method (25). Briefly, GAS strains were grown in THY broth to an OD₆₀₀ of 0.6. Two 5-ml aliquots of each culture were recovered, and the bacteria were pelleted by centrifugation (5 min at 4,000 × g). Bacterial pellets were resuspended in 500 µl of water, and serial dilutions were performed to ensure equivalent numbers of CFU of all of the strains. To remove the capsule from the bacteria, 400 µl of each suspension was placed in a 2-ml screw-cap tube containing 1 ml of chloroform and run in a FastPrep machine at speed 4.5 for 1 min. After cooling on ice for 1 min, the samples were reprocessed in

the FastPrep machine before centrifugation at $13,000 \times g$ for 10 min. The aqueous phase was transferred to a clean tube, and the hyaluronic acid content was determined using an enzyme-linked immunosorbent assay (ELISA) kit (Corgenix) in accordance with the manufacturer's instructions.

Far-Western blot analysis. Secreted protein samples from exponential-phase GAS cultures or 5 μg of α_2 -macroglobulin purified from human plasma (Sigma-Aldrich) were separated by 12% SDS-PAGE before transfer of the proteins to nitrocellulose membrane. Note that because of the intense signal obtained when using undiluted protein samples from GAS strains containing the *grab* complementation plasmid pGRABC, we added only 1/20 of the protein to the wells for these samples (far-Western blot analysis only; we added the same amount for the Spd3 loading control Western blot analysis). The membrane was blocked by incubation in phosphate-buffered saline (PBS)–Tween 20 with 5% milk for 1 h at room temperature. Subsequently, α_2 -macroglobulin was added to the blocking buffer to a final concentration of 4 $\mu\text{g}/\text{ml}$ and the membrane was incubated for a further hour. After the membrane was washed to ensure the removal of unbound α_2 -macroglobulin, a rabbit polyclonal IgG anti-human α_2 -macroglobulin antibody (Sigma-Aldrich) was used as the primary antibody and an HRP-conjugated goat anti-rabbit antibody was used as the secondary antibody (Pierce).

Northern blot analysis. Total RNA from exponential-phase cultures was loaded onto a 5% Tris-borate-EDTA–urea gel and separated by electrophoresis. Biotinylated RNA size standards ranging in size from 100 to 1,000 nucleotides (nt) were used to enable determination of the sizes of the transcripts detected. RNA was transferred to nylon membrane via electroblotting, UV cross-linked, and probed overnight with an *in vitro*-transcribed probe complementary to the SR195750 RNA. *In vitro*-transcribed probes were generated by using the Strip-EZ T7 kit (Life Technologies). DNA templates for *in vitro* transcription reactions were generated by PCR, with one primer containing the T7 promoter sequence (see Table S2 in the supplemental material). RNA probes were labeled with biotin prior to hybridization (BrightStar Psoralen-Biotin labeling kit; Life Technologies). Following washes, Northern blot assays were developed (BrightStar Biodetect kit; Life Technologies) and exposed to autoradiography film.

RT-PCR analysis of *sr195750* and *rivR* cotranscription. RNA was isolated from an exponential-phase culture of MGAS2221 and used in two cDNA synthesis reactions, where one of the reaction mixtures did not have any reverse transcriptase added (–RT; to be used as a control against contaminating genomic DNA [gDNA]). Isolated genomic DNA (gDNA) from MGAS2221 and the +RT and –RT cDNA synthesis reactions were each used as the template in PCRs with primers C2RIV1/2, C2RIV1/3, and C2RIV1/4 (see Table S2 in the supplemental material; see also Fig. 6A). Reaction products were separated by electrophoresis, and the gel was imaged.

Tissue culture adherence assay. Our tissue culture adherence assay was performed as previously described (10). Briefly, the human keratinocyte cell line HaCaT was cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 2 mM L-glutamine and 10% fetal calf serum. For adherence assays, cells were resuspended at a concentration of approximately $1 \times 10^6/\text{ml}$ in DMEM and 900 μl was seeded into each well of a 12-well tissue culture plate, which was then incubated for 24 h at 37°C with 5% CO₂. GAS bacteria were prepared by growing each strain to an OD₆₀₀ of 0.5 before pelleting of the bacteria from 1 ml of culture and resuspension of the pellet in 1 ml of PBS. Aliquots (100 μl) of bacterial suspension were added to separate wells of the 12-well tissue culture plate (three wells per strain) containing lawns of HaCaT cells. After 1 min of incubation at 37°C (5% CO₂), the liquid was removed from the wells and the wells were washed five times to remove nonadherent GAS. Following washing, 1 ml of PBS containing 1% saponin was added to each well and the plate was incubated for 10 min at room temperature to allow for lysis of the HaCaT cells. GAS bacteria were recovered from each well, and the adherent bacteria were enumerated following

plating on blood agar plates. The average number of bacteria recovered per ml from three independent wells was determined for each GAS strain, and the percentage of adhering bacteria versus the number of bacteria in the initial inoculum was calculated. The experiment was performed in triplicate, and means $\pm$ standard deviations are shown.

To identify the contribution of the hyaluronic acid capsule to GAS adhesion, we first pretreated each GAS strain, after it had reached an OD₆₀₀ of 0.4, in 450 μl of PBS containing hyaluronidase (10 mg/ml). Reaction mixtures were incubated at 37°C for 15 min before the cells were washed once with PBS and then each strain was resuspended in 1 ml of PBS. The resuspended cells were used in our standard HaCaT cell assay as described above.

Mouse model of bacteremia infection. The study protocol was approved by the Institutional Animal Care and Use Committee of the Methodist Hospital Research Institute. Each GAS strain was grown to the mid-exponential phase of growth in THY broth and washed twice with PBS, and then 250 μl of a suspension of 6×10^7 CFU/ml in PBS was used to infect 10 female CD-1 mice intraperitoneally. Mouse survival was monitored over time. The experiment was performed twice, giving a total of 20 mice per GAS strain. Statistical significance was tested by using the log-rank test.

Microarray data accession number. The expression microarray data have been deposited at the Gene Expression Omnibus database at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/geo>) under accession number GSE36646.

RESULTS

RivR and/or RivX regulate *grab* and *has* mRNA levels, but not the *Mga* regulon, in clinical isolate MGAS2221. Previous analysis of the RivRX regulon was performed in a *covR* mutant background of a serotype M1T1 strain (22). To facilitate analysis of the RivRX regulon in a wild-type background, we created strain 2221 Δ rivRX, an isogenic *rivRX* mutant of parental M1T1 strain MGAS2221. Exponential-phase cultures of MGAS2221 and 2221 Δ rivRX were compared by expression microarray analysis. A total of 25 genes were expressed differentially at least 2-fold by the strains (see Table S1 in the supplemental material; Fig. 1A). Surprisingly, the genes we identified as being RivRX regulated differed significantly from those in the previously published study (22), despite the use of similar growth conditions. The differing data sets were not a consequence of the *covR* mutant background used previously, as our RivR regulon data were unchanged, even after the introduction of the same *covR* mutation into our strains (data not shown). Thus, RivRX does not significantly regulate the *Mga* regulon in the representative M1T1 GAS isolate MGAS2221. Of the 25 genes differentially regulated in MGAS2221 and 2221 Δ rivRX, four encode virulence factors: *scpC* (encodes the chemokine protease SpyCEP) (26), *sclA* (encodes streptococcal collagen-like protein A) (27), *speB* (encodes the cysteine protease) (28), and *grab* (encodes the protein G-related α_2 -macroglobulin-binding protein) (5) (Fig. 1A). In addition, while the abundance of *hasABC* mRNAs (transcribed from the hyaluronic acid capsule biosynthesis genes) (29) did not differ between the two strains at the 2-fold level, it did differ when a 1.5-fold cutoff level was used (Fig. 1A).

To verify the expression microarray data, we used qRT-PCR. Exponential-phase cultures of the parental, *rivRX* mutant, and complemented mutant strains were analyzed. The complemented mutant strain was constructed by introducing plasmid pRivRX, which contains wild-type *rivRX* genes, into strain 2221 Δ rivRX. The data confirmed that RivR and/or RivX regulate the transcript levels of *grab* and *hasA* (29-fold and 7-fold higher levels following

FIG 1 Deletion of *rivRX* alters the abundance of transcripts for the virulence-factor encoding genes *grab* and *hasABC* but not those from the Mga regulon. (A) Expression microarray analysis of transcriptional differences between parental strain MGAS2221 and isogenic mutant derivative 2221 Δ *rivRX*. Each data point (diamond) represents an individual gene. Selected genes are highlighted (blue diamonds) and labeled. Note that the large difference in *rivR* levels is a consequence of the deletion of this gene from strain 2221 Δ *rivRX* and also that the *rivX* gene was not included in the microarray. (B) Verification of expression microarray data by qRT-PCR analysis. The transcript levels of selected genes were determined by qRT-PCR in strains MGAS2221 (plus the empty vector), 2221 Δ *rivRX* (plus the empty vector), and 2221 Δ *rivRX* (plus pRivRX, a plasmid that encodes wild-type *rivRX* genes). Strains were assayed at the exponential phase (OD₆₀₀ of 0.5) of growth. Data are presented as *n*-fold transcript levels relative to MGAS2221 (plus the empty vector). The asterisk highlights the lack of a detectable *rivR* transcript in strain 2221 Δ *rivRX* containing the empty vector. The experiment was performed in triplicate, and mean values $\pm$ standard deviations are shown. Dashed lines highlight 2-fold increases or decreases in transcript levels.

rivRX mutation, respectively; Fig. 1B) but not the transcript levels of the Mga regulon (e.g., *emm*, *mga*). The decreased abundance of *grab* and *hasA* mRNAs in the complemented strain relative to the wild type is hypothesized to be due to increased RivR expression (the *rivR* mRNA level is 10-fold higher in the complemented strain; Fig. 1B), with this being a consequence of the multicopy nature of the complementing plasmid. Note that qRT-PCR analysis, which is more quantitative than microarray analysis, did not reveal any difference in the *scpC*, *sclA*, or *speB* transcript levels (Fig. 1B).

RivR, but not RivX, regulates *grab* and *hasA* transcript levels. Whether the RivR protein, the RivX sRNA, or both were negative regulators of *grab* and *hasA* mRNA abundance was investigated through the creation of 2221 Δ *rivRX* derivatives containing plasmids harboring one or both *riv* genes. A plasmid containing the *rivR* gene (pRivR), but not a plasmid containing the *rivX* gene

FIG 2 RivR, but not RivX, regulates *grab* and *hasA* transcript abundance. qRT-PCR analysis of MGAS2221 (plus the empty vector) and 2221 Δ *rivRX* derivatives containing the empty vector (+ vector), a RivR-expressing plasmid (+ pRivR), a RivX-expressing plasmid (+ pRivX), a RivX-expressing plasmid (+ pRivRX), or a mutant version of pRivRX in which the *rivR* gene has a 4-bp frameshifting insertion (+ pRivR*X). The transcript levels of *grab*, *hasA*, and the internal control gene *proS* were measured.

(pRivX), was able to complement strain 2221 Δ *rivRX* (Fig. 2). Similarly, while a plasmid containing both *rivR* and *rivX* was able to complement strain 2221 Δ *rivRX* (pRivRX), a derivative of this plasmid in which a 4-bp insert was introduced into *rivR* to create a frameshift mutation (pRivR*X) could not complement. The inability of the *rivX*-containing plasmids to complement was not due to the absence of *rivX* transcription (see Fig. S4 in the supplemental material). Our data are consistent with the idea that the RivR protein, and not the RivX sRNA, is the regulatory molecule responsible for the negative regulation of *grab* and *hasA* mRNA abundance.

RivR negatively regulates production of GRAB and binding of α_2 -macroglobulin. To determine whether the observed mRNA level regulation of *grab* by RivR results in a difference at the protein level, we used Western blot analysis. We generated *grab* mutant derivatives of parental strain MGAS2221 and *rivRX* mutant strain 2221 Δ *rivRX* (strains 2221 Δ *grab* and 2221 Δ *rivRX* Δ *grab*, respectively) to facilitate analysis of GRAB expression. Plasmid pGRABC, a medium-copy-number plasmid containing a wild-type *grab* gene, was created to enable complementation of the *grab* mutant strains. Finally, we generated anti-GRAB antibodies by using rabbits immunized with two peptide fragments of GRAB (see Fig. S1 in the supplemental material). As GRAB is a cell wall-anchored protein, we initially compared cell wall protein fractions from our strains; however, these samples failed to generate any reactivity with our anti-GRAB antibody (data not shown). We then analyzed secreted protein fractions, with which we observed reactivity with our antibody and identified significant upregulation of GRAB expression by strain 2221 Δ *rivRX* relative to MGAS2221 (Fig. 3). Plasmid pRivR complemented strain 2221 Δ *rivRX*, reducing GRAB expression to undetectable levels, while plasmid pGRABC endowed all of the strains with high-level GRAB expression. The repression of GRAB expression by RivR decreased the ability of GAS to bind the human protease inhibitor α_2 -macroglobulin (see Fig. S5 in the supplemental material).

RivR negatively regulates production of the hyaluronic acid capsule. Whether the observed mRNA level regulation of *hasABC* by RivR leads to a difference in the level of hyaluronic acid capsule was assessed by ELISA. Hyaluronic acid extracts from cultures of

FIG 3 RivR negatively regulates GRAB expression. Western blot analysis of secreted GAS protein fractions using an anti-GRAB antibody (test) and an anti-Spd3 antibody (loading control). The protein that reacted to the anti-GRAB antibody was 34 kDa, a size that matches a previous Western blot study of recombinant GRAB protein (40). (*) Note that because of the very high level of GRAB expression from the pGRABC plasmid, the protein samples from strains containing this plasmid were diluted 1:100 relative to the other samples for the GRAB Western blot analysis but were not diluted for the Spd3 Western blot analysis.

the parental, mutant, and complemented mutant strains were compared against a standard curve, and the data are presented as percentages of the capsule expression of the parental strain. The *rivRX* mutant strain 2221Δ*rivRX* produced approximately 8-fold greater capsule levels than the parental and complemented mutant strains (Fig. 4).

The RivR-mediated regulation of *hasA* and *grab* requires the CovR/S system. In addition to RivR repressing the abundance of *grab* and *hasA* mRNAs, their abundance is also repressed by the CovR/S two-component regulatory system (11, 16). Furthermore, CovR/S also represses *rivR* transcription 3-fold (20). Given this coregulation, we set out to test whether the RivR-mediated regulation of *grab* and *hasA* mRNAs is dependent on or independent of CovR/S. To investigate this issue, we analyzed a series of GAS strains, including previously described *covR* and *covS* mutant derivatives of MGAS2221 (19), and the newly constructed derivatives 2221Δ*rivRX*Δ*covR* and 2221Δ*rivRX*Δ*covS* (see Table S3 in the supplemental material). Comparison of *covR* and *rivRX* single and double mutant strains revealed that CovR is epistatic to RivR (Fig. 5A), that is, that RivR regulatory activity is dependent upon

FIG 4 RivR negatively regulates capsule production. Chloroform-extracted hyaluronic acid capsule fractions of the indicated GAS strains were compared by ELISA against a standard curve of known hyaluronic acid concentrations. The experiment was performed in triplicate, and mean values ± standard deviations are shown. Significance was tested by Student's *t* test. WT, wild type.

FIG 5 RivR functions through both CovR-dependent and CovR-independent pathways. (A) qRT-PCR analysis of the contributions of CovR, CovS, and RivR to the regulation of *grab* and *hasA* transcript levels. Exponential-phase cultures of the indicated GAS strains were analyzed. The top graph shows the *grab* data, the middle graph shows the *hasA* data, and the bottom graph shows the *rivR* data. The experiment was performed in triplicate, and mean values ± standard deviations are shown. Asterisks highlight the lack of detectable *rivR* transcripts in the noncomplemented *rivR* mutant strains. WT, wild type. (B) Model showing the interconnectivity among CovR, CovS, and RivR regulatory activities. Green arrows represent positive regulation. Red T's represent negative regulation.

the presence of a functioning CovR protein. As expected, given our previous finding that CovS inhibits the CovR-mediated repression of *grab* mRNA transcription (19), lower *grab* transcript levels were observed for strain 2221Δ*covS* (5-fold decrease) than for the parental strain (Fig. 5A). Figure 5B highlights our current

FIG 6 Most transcripts originating from a newly discovered promoter upstream of *rivR* are prematurely terminated. (A) Visualization of SR195750 signal intensity relative to the *rivR* gene. The *rivR* gene is represented by an arrow showing the direction of transcription. Vertical lines represent signal intensities from microarray probes tiled within intergenic regions as previously described (30). Vertical lines that extend downward indicate that transcription is occurring right to left. Note the high signal intensity observed for SR195750 and the very low signal intensity observed for *rivX*. P1 and P2 refer to the locations of the two previously described *rivR* promoters (22). P3 refers to the location of the newly described promoter identified in this study. Solid triangles numbered 1 through 4 represent the relative locations of the PCR primers used in panel C. (B) Northern blot analysis of RNAs from strain MGAS2221 containing the empty vector and from strain 2221ΔSR195750 containing the empty vector, pSR195750, or pSR195750.*rivR*. The Northern blot was probed with an SR195750-specific probe. The locations and sizes of an RNA ladder are displayed to the right of the Northern blot. The locations of the SR195750 transcript and the putative cotranscribed SR195750.*rivR* transcript are indicated. (C) RT-PCR confirming the transcriptional coupling of SR195750 and *rivR*. MGAS2221 gDNA and cDNA from reaction mixtures containing (+RT) or not containing (−RT) reverse transcriptase were used as the templates in three PCRs. The relative locations of the primers used (numbered 1 through 4) are shown in panel A. The samples without reverse transcriptase served as a control against gDNA contamination. (D) Analysis of the contribution of the *sr195750* promoter to *rivR*, *grab*, and *mga* transcript levels via qRT-PCR analysis. The experiment was performed in triplicate, and mean values $\pm$ standard deviations are shown.

understanding of the interplay between the CovRS and RivR regulators with respect to *grab* and *hasA* mRNA levels.

A newly discovered promoter, which primarily generates a 140-nt RNA, contributes to *rivR* transcription. Previously, we identified a highly transcribed candidate sRNA-encoding gene upstream of *rivR* which we named *sr195750* (Fig. 6A) (30). Given that *sr195750* and *rivR* are in the same orientation, we reasoned that some *sr195750* transcripts may read through into *rivR*. To investigate this, we performed a Northern blot analysis using an SR195750-specific probe to identify whether transcripts could be detected that also include *rivR*. A single band with the expected size of SR195750 (~140 nt) was observed for strain MGAS2221 containing the empty vector and also for strain 2221Δ*sr195750* (an *sr195750* mutant derivative of MGAS2221) containing SR195750-encoding plasmid pSR195750 (Fig. 6B). However, bands of ~140 nt and >1,500 nt were observed for 2221ΔSR195750 transformed with pSR195750.*rivR* (which contains both *sr195750* and *rivR*; Fig. 6B). We postulate that the >1,500-nt transcript contains both *sr195750* and *rivR*.

To confirm that some SR195750 transcripts include *rivR*, we performed RT-PCR. A single forward primer embedded within *rivR* (primer 1 in Fig. 6A) and a series of three reverse primers located at increasing distances from the two known *rivR* promoters (primers 2 to 4 in Fig. 6A) were used. PCR products of the expected sizes were obtained for all three PCRs by using cDNA

synthesized from MGAS2221 RNA (Fig. 6C). Thus, some SR195750 transcripts are cotranscribed with *rivR*. To identify the relative contribution of the *sr195750* promoter to *rivR* transcription, we compared *rivR* mRNA levels in MGAS2221 and 2221ΔSR195750 and identified a 3-fold reduction following *sr195750* mutation (Fig. 6D). Thus, most *rivR* transcripts originate from the *sr195750* promoter.

RivR enhances GAS adherence to a human keratinocyte cell line. Enhanced capsule expression can mask GAS cell surface proteins and provide protection against antigen-specific antibodies (31). We therefore hypothesized that the enhanced capsule expression observed following *rivR* mutation (Fig. 4) might mask cell surface adhesins, leading to a reduced ability to adhere to host cells. To test our hypothesis, we used cell culture to compare the binding of the parental, *rivRX* mutant, and complemented mutant strains to a human keratinocyte cell line. GAS strains were added to lawns of HaCaT cells, nonadherent bacteria were removed by washing, and the percentage of the inoculum that bound was determined. As hypothesized, the *rivR* mutant strain bound the HaCaT cells at a significantly lower level than the parental or complemented mutant strain (Fig. 7A). To confirm that the decreased adherence of the *rivR* mutant strain was a consequence of the enhanced level of capsule produced by this strain, we repeated our experiments by using GAS bacteria that were pre-treated with hyaluronidase to remove the capsule. Hyaluronidase-

FIG 7 RivR enhances GAS adherence by reducing capsule production. (A) The indicated strains were added to lawns of HaCaT keratinocyte cells present in 12-well plates. The wells were washed extensively to remove nonadherent GAS before the recovery of adherent GAS cells and determination of the percentage of the GAS inoculum that bound. The experiment was performed in triplicate, and mean values $\pm$ standard deviations are shown. *P* values were generated by using Student's *t* test. Strain 2221ΔPIL was described previously (Z. Liu et al., submitted for publication), and this pilus mutant strain was used as a negative control for adherence. (B) Same experimental outline as in panel A, with the exception that GAS bacteria were pretreated with hyaluronidase, to remove the hyaluronic acid capsule, prior to their addition to HaCaT cells. WT, wild type.

treated strains, in contrast to the untreated strains, showed no relative differences in adherence in our assay (Fig. 7B). Thus, our data are consistent with RivR enhancing GAS adherence by modulating the level of hyaluronic acid capsule to reduce the masking of cell surface adhesins. It should be noted that hyaluronidase-treated GAS bound HaCaT cells at a rate 3-fold higher than that of nontreated GAS (compare Fig. 7A and B), indicating that even wild-type levels of capsule have a negative effect on adherence.

RivR contributes to GAS virulence in a murine model of bacteremia infection. Whether RivR contributes to the ability of GAS to cause invasive disease was tested by comparing the parental, *rivRX* mutant, and complemented mutant strains in a mouse model of bacteremia infection. Mice were infected intraperitoneally with 1.5×10^7 CFU of GAS, and survival was monitored over time. The *rivRX* mutant strain had a significantly attenuated ability to cause lethal infections relative to those of the parental and complemented mutant strains (Fig. 8). Thus, in addition to adherence, RivR regulates GAS invasive disease potential.

FIG 8 RivR contributes to GAS virulence in a murine bacteremia model of infection. Groups of 10 mice were infected intraperitoneally with 1×10^7 CFU of parental strain MGAS2221 (plus the vector; wild type [WT], squares), isogenic *rivRX* mutant 2221ΔrivRX (plus the vector; 2221ΔrivRX, diamonds), or complemented (COMP) mutant 2221ΔrivRX (plus pRivR, triangles). Mouse survival was monitored over time. The experiment was performed in duplicate, and the combined data set (20 mice per GAS strain) is graphed. The statistical significance of differences was determined by the log-rank test, *v.* versus.

DISCUSSION

Virulence factors enhance the ability of bacterial pathogens to circumvent the host immune response and cause disease. In part to reduce the generation of antibodies against individual virulence factors, which could promote pathogen opsonophagocytosis, as well as inhibit virulence factor activity, the expression of virulence factors is tightly regulated in a temporally and spatially specific manner. Here we show that the RivR protein is a negative transcriptional regulator of the protease inhibitor-binding protein GRAB and of the hyaluronic acid capsule in the human pathogen GAS. The contribution of this regulatory protein to GAS virulence is highlighted by the attenuated virulence of a *rivRX* mutant strain following intraperitoneal infection in mice (Fig. 8). Importantly, while our mouse data align with those of a previous study that utilized a subcutaneous mouse infection model (22), the genes identified as being RivRX regulated differ between the two studies. Both studies used the same growth conditions and GAS strain serotype (M1T1). A difference between the two studies is that while our transcriptome comparison was between wild-type strain MGAS2221 and a *rivRX* mutant derivative (Fig. 1), the study of Roberts and Scott compared a derivative of strain MGAS5005 that harbored both *covR* and *rivRX* mutations and contained either the empty vector or a plasmid that highly transcribed *rivRX*. We do not believe that the *covR* mutation accounts for why the previous study identified significant upregulation of the Mga regulon by RivRX (e.g., a 19-fold increase in *scpA* mRNA and a 93-fold increase in *emm* mRNA) (22), as we identified no change in the Mga regulon in strain 2221ΔrivRXΔcovR (+vector) relative to that in strain 2221ΔrivRXΔcovR (+pRivRX; data not shown). However, the *covR* mutation would explain why the regulation of GRAB and capsule expression by RivR was not identified previously, as regulation of the *grab* and *hasABC* genes by RivR requires CovR (Fig. 5). To determine whether the RivR regulons really do differ between MGAS5005 (i.e., Mga regulon genes via the study of Roberts and Scott) and MGAS2221 (i.e., *hasA* and *grab* genes via this study), we created a *rivR* mutant of JRS950,

which is the same *covR* mutant of MGAS5005 used in the study of Roberts and Scott (22). We transformed our double mutant strain, JRS950 Δ *rivR*, with the empty vector or the pRivR complementation plasmid and compared these strains to the JRS950 parental strain containing the empty vector. The *mga*, *emm*, and *scpA* transcript levels did not differ between our strains (see Fig. S6 in the supplemental material). We also observed no difference in the *grab* and *hasA* mRNA levels; however, this finding is explained by the fact that JRS950 is a *covR* mutant and hence the *grab* and *hasA* mRNAs are already fully derepressed. Thus, our data are consistent with RivR regulation of *grab* and *hasA* mRNA abundance in M1T1 GAS (or at least the representative isolates MGAS2221 and MGAS5005), with no effect on the transcript levels of members of the Mga regulon. With respect to the candidate sRNA RivX, we cannot confirm or disprove that this sRNA regulates Mga regulon genes in MGAS5005, as our JRS950 Δ *rivR* mutant retained the *rivX* gene. However, RivX has no detectable regulatory activity in strain MGAS2221 (Fig. 2), at least under the conditions tested.

The finding that strain 2221 Δ *rivRX* had reduced virulence in the murine model of bacteremia infection was somewhat unexpected, given that this strain expresses increased capsule and GRAB protein, two virulence factors that contribute to the ability of GAS to cause invasive disease (2, 5, 28, 32). One possible explanation for this disparity is that RivR regulates additional target genes *in vivo*. It is also possible that the high level of GRAB protein secreted by strain 2221 Δ *rivR* (Fig. 3) sequesters available α_2 -macroglobulin, preventing cell wall-anchored GRAB from retaining α_2 -macroglobulin at the GAS cell surface, leading to enhanced proteolysis. Future transcriptome and immunohistochemistry analyses using *in vivo* samples may shed further light on the mechanisms by which *rivR* mutation negatively affects GAS invasive disease.

The GAS hyaluronic acid capsule has antiphagocytic, adhesive, and signaling properties (2, 3, 33). The tight regulation of capsule production by the RivR and CovR/S regulatory systems highlights an important role for the modulation of capsule expression during infection. Our data indicate that the reduced adherence of strain 2221 Δ *rivRX* to a keratinocyte cell line relative to that of the parental strain is due to the enhanced capsule of strain 2221 Δ *rivRX* masking adhesins on the GAS cell surface (Fig. 7A). That pretreatment with hyaluronidase increased the adherence of the parental and complemented mutant strains 3-fold, in addition to abolishing the decreased-adherence phenotype of the mutant strain, implies that wild-type levels of capsule have a negative effect on adherence (Fig. 7B). Thus, it appears that capsule regulation is a tradeoff between promotion of adherence (low capsule levels) and promotion of resistance to the innate immune system (high capsule levels).

We identified a new promoter upstream of *rivR* from which approximately 70% of *rivR* transcripts originate (Fig. 6). Interestingly, transcripts from this promoter commonly terminated before entry into the *rivR* gene, generating a highly abundant 140-nt RNA that we previously named SR195750 (30). In unpublished work, we tested the hypothesis that SR195750 functions as an sRNA with intrinsic regulatory activity. Using microarray analysis, we found that no genes were differentially expressed between isogenic *rivRX* and *sr195750.rivRX* mutant strains (unpublished data). Thus, at least under the conditions tested, SR195750 has no regulatory activity and hence does not function as an sRNA. Whether SR195750 has sRNA activity under different growth con-

ditions or whether it has activity against RivR or RivX (something that would have been missed in our array analysis) warrants further investigation.

RivR negatively regulates *grab* and *has* mRNA abundance (Fig. 2). Whether this regulation is direct is unknown, although no obvious candidate RivR-binding sites were identified by sequence comparisons of the *grab* and *hasA* promoter regions (unpublished data). CovR negatively regulates *has* and *rivR* transcription directly (20, 34); however, it is unknown whether the regulation of *grab* by CovR is direct or indirect. We used qRT-PCR analysis to identify the relative contributions of *rivR*, *covR*, and *covS* to the regulation of *grab* and *hasA* (Fig. 5). Our data were consistent with RivR requiring an intact *covR* gene for activity. The requirement of a functional *covR* gene is not due to enhancement of *covR* transcription by RivR, as a *rivRX* mutant strain and a RivR-overexpressing strain have levels of *covR* mRNA similar to that of the parental strain (see Fig. S2 in the supplemental material).

RivR (502 amino acids) and Mga (529 amino acids) have 49% amino acid sequence similarity along their lengths and have similar domain structures (see Fig. S3 in the supplemental material) (14). Conserved proteins can also be found in other pathogenic bacteria, including the AtxA anthrax toxin regulator in *Bacillus anthracis* (35). While both Mga and AtxA positively regulate >100 genes in their respective genomes (13, 36, 37), RivR regulates a smaller number of genes and its regulation is negative rather than positive. The different regulatory activities of RivR and Mga/AtxA cannot be explained by any obvious sequence or domain differences. The RivR, Mga, and AtxA proteins all contain two putative phosphotransferase system (PTS) regulatory domains (PRDs). PTSs regulate carbohydrate transport and metabolism, and one mechanism by which this is achieved is the phosphorylation of PRD-containing proteins (38). Thus, it has been proposed that virulence factor regulation by Mga and AtxA may be linked to carbohydrate metabolism (14, 39), and we propose that virulence factor regulation by RivR may be similarly controlled. As different carbohydrate sources are available at distinct anatomic sites, RivR may regulate virulence factor production in a disease-specific manner.

In summation, the important virulence factors regulated by RivR, coupled with the attenuated virulence of a *rivR* mutant strain, provide a strong impetus for the further study of this regulatory protein, including whether RivR activity could be targeted as a novel therapeutic strategy. Our data provide new insights into the regulatory cascades that promote GAS virulence and influence virulence factor production.

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