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# Expression of the Secondary Sigma Factor $\sigma^X$ in *Streptococcus pyogenes* Is Restricted at Two Levels

Jason A. Opdyke, June R. Scott, and Charles P. Moran, Jr.*

*Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, Georgia 30322*

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**Secondary RNA polymerase sigma factors in many bacteria are responsible for regulating a vast range of processes including virulence. A protein ($\sigma^X$) in the gram-positive human pathogen *Streptococcus pyogenes* (the group A *Streptococcus* or GAS) was recently shown to function in vitro as a secondary sigma factor. We report here the isolation of a mutant in which both *sigX* genes are inactivated, show that $\sigma^X$ functions in GAS cells, and show that the amount of $\sigma^X$ is controlled at two levels. Primer extension analysis indicates that *sigX* transcription is low in GAS cells grown in Todd-Hewitt yeast broth, and immunoblot assays with a $\sigma^X$-specific polyclonal antibody demonstrate that the protein does not accumulate in these cells. To increase the level of *sigX* transcription in GAS, we constructed a strain that constitutively expresses the *sigX* gene from a heterologous promoter. Expression of *sigX* from this promoter led to transcription of the $\sigma^X$-dependent *cinA* promoter in GAS cells. We found that expression of the *sigX* gene in a *clpP* mutant strain resulted in greater accumulation of $\sigma^X$ protein, which resulted in higher levels of transcription from the $\sigma^X$-dependent promoters *cinA*, *smf*, and *cglA*. In addition, a *clpP* mutant containing *sigX* only at its wild-type loci on the chromosome generated more transcription from the $\sigma^X$-dependent *cinA* promoter than did the wild-type parental strain. Therefore, $\sigma^X$ activity in GAS is limited by low-level transcription of the *sigX* structural genes and by *clpP*, which appears to negatively regulate $\sigma^X$ accumulation.**

*Streptococcus pyogenes* (the group A *Streptococcus* [GAS]) is a gram-positive pathogen that is responsible for a wide range of diseases in people. GAS can cause localized infections of the throat or skin, such as pharyngitis and impetigo, or deep tissue infections, such as fasciitis and myositis. GAS can also cause systemic infections, including toxic shock syndrome and septicemia (for a review see reference 7). For its success as a pathogen, GAS must be able to sense and respond to the different and changing environments that it encounters during infection, and its response to these probably ultimately controls disease outcome. Thus, understanding of regulation of GAS gene expression is critical for understanding and predicting GAS disease outcome.

Currently, two global transcriptional regulators have been identified in GAS. The multiple gene activator Mga regulates transcription of several virulence genes, including the gene encoding the M protein, which is considered the major virulence factor of this pathogen (6, 18, 29, 31). Transcription of *mga* is responsive to changes in environmental conditions (4, 25). In addition, CovR represses transcription of about 15% of the GAS genome, either directly or indirectly (14). Among the operons that CovR regulates directly is the *has* operon, which contains the biosynthetic genes necessary for the production of the hyaluronic acid capsule, another important virulence factor of this organism (3, 12, 15, 22). Because CovR appears to be the response regulator of a two-component signal transduction system, it seems likely that it facilitates alterations in gene expression in the GAS as it encounters different environments.

In addition to specific transcriptional regulators whose activity may vary in response to environmental conditions, secondary RNA polymerase sigma factors play a critical role in a wide variety of bacteria in regulating gene transcription in an environmentally sensitive way. Such secondary sigma factors control a vast range of processes in different bacteria and respond to changes in environmental conditions including pH, osmotic shock, the presence of specific ions, and temperature. In *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella enterica* serovar Typhimurium, secondary sigma factors are essential for the transcription of virulence factors (10, 11, 38).

The sequence of the GAS genome indicates that it encodes the primary or housekeeping sigma factor, *sigA*, and two copies of a gene with homology to *comX* of *Streptococcus pneumoniae* (21). We have named the latter genes *sigX1* and *sigX2* because we have recently shown that their product acts in vitro as a sigma factor (27). Purified recombinant $\sigma^X$ directs RNA polymerase from GAS to specifically use promoters (e.g., *cinA* and *femB*) that contain a sequence similar to that recognized by ComX of *S. pneumoniae* (called the "cin-box") in vitro (27). However, whether GAS $\sigma^X$ can function in vivo has not been tested, and its role in GAS has not been investigated. Because two identical copies of the structural gene for $\sigma^X$ are found in all GAS strains for which data are available, it seems likely that $\sigma^X$ plays an important role in GAS biology.

To begin to understand the role of $\sigma^X$ in GAS, we deleted both copies of the $\sigma^X$ structural gene and found that *sigX* is dispensable for growth under standard laboratory conditions. Moreover, we found that the level of *sigX* transcription is very low when the GAS is grown under these conditions. Therefore, we expressed the *sigX* gene in GAS from a heterologous promoter to study its transcriptional effects. We show that expression of *sigX* in GAS leads to transcription from three different

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30322. Phone: (404) 727-5969. Fax: (404) 727-3659. E-mail: moran@microbio.emory.edu.

promoters containing Cin box sequences. Furthermore, we have identified ClpP as a negative regulator of the $\sigma^X$ protein.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** All GAS strains are derivatives of the M6 serotype strain JRS4 (35) and were grown in Todd-Hewitt medium supplemented with 0.2% yeast extract (THY).

A fragment of DNA directly upstream of the *sigX* gene (this sequence is identical for *sigX1* and *sigX2*) was PCR amplified from JRS4 chromosomal DNA with primers 5′CACACAGAATTCACGCTGCTCTTGTCTCTATCGACA3′ and 5′CACACAGGATCCGTTGGAAATCAATCGCAGAG3′ and cloned into the *Eco*RI and *Bam*HI restriction sites of pLitmus28 (New England Biolabs) to created plasmid pJO106. A fragment of DNA directly downstream of *sigX1* was PCR amplified from JRS4 chromosomal DNA with primers 5′CACACAAGAT CTCACAGCAGTTGTAAGCAAGACC3′ and 5′CACACAACTAGTGGATC CGGTCATACCTTATGTAAGTCGC3′ and cloned into the *Bgl*II and *Spe*I restriction sites of pJO106 to create pJO112. The Ω kanamycin resistance cassette of pUC4ΩKan (29) was removed by *Bam*HI digestion and cloned into the *Bgl*II restriction site of pJO112 to create pJO117. The *Bam*HI restriction fragment from pJO117 was then cloned into pJRS233 (30), creating plasmid pJO118.

A DNA fragment directly downstream of *sigX2* was PCR amplified from JRS4 chromosomal DNA with primers 5′CACACAAGATCTCACAGCAGTTGTAA GCAAGACC3′ and 5′CACACAACTAGTGGATCCCTCATTGATACGCCC AATCA3′ and cloned into the *Bgl*II and *Spe*I restriction sites of pJO106 to create pJO119. A DNA fragment of the *cat86* gene was PCR amplified from plasmid pLZ12 (9) with primers 5′CACACAGGATCCAGTTCAACAAACGA AAATTG3′ and 5′CAGCGGGATCCCATCTAGGCCTCCTCATATTATAA AAGCCAGTC3′ and cloned into the *Bgl*II restriction site of pJO119 to create plasmid pJO133. The *gusA* gene was PCR amplified from plasmid pMLK99 (17) with primers 5′ACACACATGCATCGACGGTATCGATAAGCTTG3′ and 5′ ACACACATGCATAAGCTTCCCCACCGAGGCTGTAGC3′ and cloned into the *Nsi*I restriction site of pJO133 to create pJO138. The *Bam*HI restriction fragment from pJO139 was then cloned into the *Bam*HI restriction site of pJRS233 to create pJO140.

A DNA fragment directly downstream of *sigX2* was PCR amplified from AM3 (37) chromosomal DNA with primers 5′CACACAAGATCTCACAGCAGTTG TAAGCAAGACC3′ and 5′CACACAACTAGTGGATCCGTCCACTGAAAT CAGTTGATG3′ and cloned into the *Bgl*II and *Spe*I restriction sites of pJO106 to create pJO120. A DNA fragment of the *cat86* gene was PCR amplified from plasmid pLZ12 (9) with primers 5′CACACAGGATCCAGTTCAACAAACGA AAATTG3′ and 5′CAGCGGGATCCCATCTAGGCCTCCTCATATTATAA AAGCCAGTC3′ and cloned into the *Bgl*II restriction site of pJO120 to create plasmid pJO137. The *Bam*HI restriction fragment from pJO137 was then cloned into the *Bam*HI restriction site of pJRS233 to create pJO141.

pJO118 and pJO140 were then used to delete *sigX1* and *sigX2*, respectively, in the M6 strain JRS4. pJO118 and pJO141 were used to delete *sigX1* and *sigX2*, respectively, in M3 strain AM3. Plasmids pJO118, pJO140, and pJO141 were introduced into GAS cells by electroporation, and cells were plated on THY agar with 200 μg of kanamycin/ml or 2.5 μg of chloramphenicol/ml and grown at 30°C, which is a permissive temperature for the replication of these three plasmids. Single colonies were picked, inoculated into THY broth, and grown overnight at 37°C, which is nonpermissive for replication of these plasmids. Cells were then plated for single colonies on THY agar plates with 200 μg of kanamycin/ml or 2.5 μg of chloramphenicol/ml and grown at 37°C. Resulting colonies contained the plasmid integrated into the chromosome by homologous recombination. Individual recombinants were screened for the loss of the plasmid by their sensitivity to 0.5 μg of erythromycin/ml while maintaining kanamycin or chloramphenicol resistance on THY agar plates at 37°C. The resulting *sigX* double deletion strain in M6 was named JOS21, and the *sigX* double deletion strain in M3 was named JOS24.

The plasmid pJO162 was constructed to constitutively express the *sigX* gene. The *veg* promoter (26) was PCR amplified from *Bacillus subtilis* MB24 (28) chromosomal DNA with primers 5′GTCCAATTAACAGTTGAAAAC3′ and 5′CACCTCACTACATTTATTG3′. The *sigX* gene was PCR amplified with primers 5′AATGTAGTGAGGTGAAAGGAGACTCAAAATGTCG3′ and 5′ GATTTACCCCGAATTCCTTATAGG3′. The *veg* promoter and the *sigX* gene were then fused together by PCR with primers 5′CACAAAGCTTCTGCATA GGAGAGCTATGCG3′ and 5′CACACAGAATTCTTACAACTGCTGTGCA AATTCCTT3′ and cloned into the *Eco*RI and *Hin*dIII restriction sites of plasmid pLZ12-Spec (1). pJO162 was introduced into GAS cells by electroporation, and transformants were grown on THY agar plates containing 100 μg of spectinomycin/ml.

An internal fragment of the *clpP* gene was PCR amplified using *Pfu* DNA polymerase (Stratagene) from JRS4 chromosomal DNA with primers 5′CACA GGATCCCCTGTTGTTATTGAACAAAC3′ and 5′CACAGGATCCTGCTG CGATAGCCATATCCG3′ and blunt end cloned into the *Eco*RV restriction site of pSK-erm, creating pJO164. pSK-erm contains an erythromycin resistance cassette from Tn*1545* (39) cloned to replace the *bla* gene of pBluescript SK (Stratagene). pJO164 was introduced into GAS cells by electroporation, and transformants resulting in the insertional inactivation of the *clpP* gene by pJO164 were selected on plates containing 0.5 μg of erythromycin/ml. A transformant was picked and named JOS34. Southern blot analysis was used to confirm the mutation in *clpP*.

**RNA purification.** Bacteria were grown at 37°C in THY broth to an optical density at 600 nm ($OD_{600}$) of 0.7. Cells were treated with 2 mg of lysozyme/ml and 1% sodium dodecyl sulfate and then boiled to lyse the bacteria. RNA was harvested as described in the work of Macdonald et al. by $CsCl_2$ purification and resuspended in a final volume of 400 μl of $H_2O$ to a final concentration of ~3.0 μg/μl (24).

**Primer extension reactions.** Primers cinA-R (5′CCGACAGAAATTGAGCA TTGG3′), smf-R (5′TTTGGTAGTCAAGAATATTGAGAATG3′), sigX-R2 (5′GTCATCTCTATCCCACAATTG3′), and cglA-PE2 (5′GATCATATTGAT CTGCTCTTGGC3′) were end labeled with [γ-$^{32}$P]ATP as described in the T4 polynucleotide kinase user manual (Promega). Then, 1.5 pmol of labeled primer was added to 20 μg of total RNA along with annealing buffer to final concentrations of 200 mM KCl and 20 mM Tris (pH 8.3) in a final volume of 10 μl. The RNA and primer were heated to 80°C for 5 min and then slowly cooled to 42°C. Elongation buffer was added to a final concentration of 100 mM Tris (pH 8.3)–10 mM $MgCl_2$–10 mM dithiothreitol in a final volume of 20 μl. Two hundred units of avian myeloblastosis virus reverse transcriptase (Promega) was added, and the reaction mixture was incubated at 42°C for 30 min. The primer extension product was ethanol precipitated, resuspended in 5 μl of formamide sequencing loading buffer, and subjected to electrophoresis in a 6% polyacrylamide gel containing 7 M urea alongside a sequencing reaction ladder generated with primer cinA-R or sigX-R2 and template pJO96 or pJO162.

**Immunoblotting.** Cultures were grown in THY broth at 37°C to an $OD_{600}$ of 0.7, harvested by centrifugation, and resuspended in 1 ml of phosphate-buffered saline, pH 7.0. Samples were then added to lysing matrix (Applied Biosystems) and lysed in a Fast Prep cell disruptor (Savant). To obtain the soluble protein fraction, samples were centrifuged at 13,000 × *g*, and the supernatant was saved. Twenty micrograms of total protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotting was done as described in the work of Ozin et al. (28). The blot was reacted with a 1:5,000 dilution of rabbit anti-$\sigma^X$ antibody raised against recombinant $\sigma^X$ purified from *E. coli* (27) and detected with anti-rabbit–horseradish peroxidase by enhanced chemiluminescence.

## RESULTS

**Disruption of *sigX1* and *sigX2*.** There are two identical copies of the *sigX* gene at unlinked regions of the chromosome of the GAS strains for which the genome sequence is available (M types 1, 3, and 18) (2, 13, 36). In addition, Southern blot analysis showed that the M6 strain JRS4, used in our studies, also contains two copies of *sigX* (M. J. Federle and J. R. Scott, unpublished data). In all of these strains, both copies of the *sigX* genes reside directly downstream of 6 kb of identical sequence encoding an rRNA operon (Fig. 1). The conserved sequence at the *sigX* loci terminates approximately 30 bp downstream from the *sigX* coding sequence.

Homologous recombination was used to sequentially delete each copy of the *sigX* gene in an M6 and an M3 strain (Fig. 2). The *sigX1* gene was replaced with *aphA3*, conferring kanamycin resistance, and *sigX2* was replaced with a fragment containing *cat86*, which confers chloramphenicol resistance (Fig. 2). The resulting strains were confirmed by Southern blot analysis to have both copies of the *sigX* gene deleted (data not shown). The growth rates of the parent and the double *sigX* mutant at 37°C in THY broth for both the M6 strain and the M3 strain showed no obvious difference (data not shown).

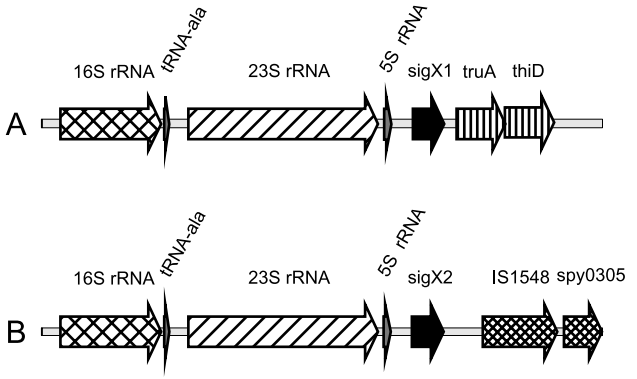


FIG. 1. Genetic organization of the *sigX1* and *sigX2* loci. Shown are 9-kb segments of GAS DNA surrounding *sigX1* (A) and *sigX2* (B) from M1 strain SF370. The two *sigX* genes are represented by black arrows. Six kilobases of sequence upstream of *sigX* are conserved for each copy of the two genes and contain two of the six rRNA operons. The sequence downstream from each copy of *sigX* is unique at each locus. IS*1548* is present in all three sequenced strains but not in JRS4.

To examine $\sigma^X$ accumulation in these cultures, we used a $\sigma^X$-specific polyclonal antibody raised against recombinant $\sigma^X$ purified from *E. coli* (27) in Western immunoblotting assays. The sensitivity of this antibody was tested by performing blotting assays on serial dilutions of purified recombinant $\sigma^X$ premixed with GAS protein extracts. Although 0.5 ng of recombinant $\sigma^X$ was detected on the immunoblots (data not shown), we did not detect $\sigma^X$ in cell extracts from the GAS cultures (data not shown). Therefore, there were fewer than 75 molecules of $\sigma^X$ per cell in the JRS4 strain.

**Expression of *sigX* from a heterologous promoter leads to expression of $\sigma^X$-dependent genes in GAS.** Since there was little expression of *sigX* during growth of GAS in THY broth, we expressed the *sigX* gene from a heterologous promoter in GAS. The *sigX* gene was cloned downstream from the *B. subtilis veg* promoter to create pJO162 (see Materials and Methods), a multicopy plasmid that replicates in GAS. The *veg* promoter was expected to be active in GAS because it contains a $\sigma^A$ consensus −35 sequence (TTGACA) and a near-consensus −10 sequence (TACAAT) with 17 bp between the two elements (26). To assess *sigX* transcription from this promoter, primer extension analysis was used. RNA was isolated late in exponential phase ($OD_{600}$ of 0.7) and 2 h after the beginning of the stationary phase. A *sigX*-specific transcript was detected from strain JRS4/pJO162 but not from JRS4 without the *sigX* expression plasmid (Fig. 3, lanes e and f). No effect of growth phase on the amount of *sigX* transcript was detected (data not shown). Confirmation that the product produced originated from the *veg* promoter at a G residue 5 nucleotides downstream from the −10 sequence was obtained by comparison of the *sigX* product size with the sequence of pJO162 generated with the identical primer used in the primer extension reaction (Fig. 3, lane f).

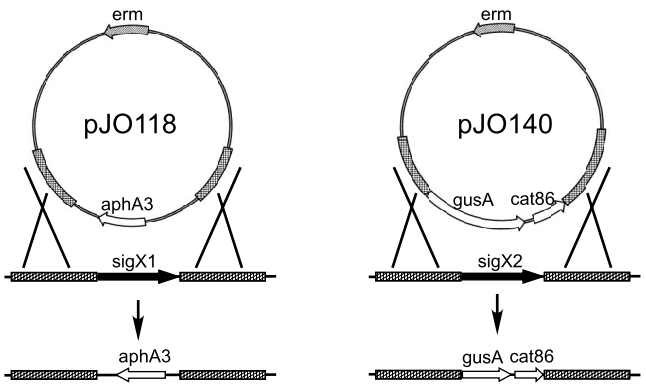


FIG. 2. Deletion of the *sigX1* and *sigX2* genes. Plasmid pJO118 was used to delete *sigX1*, and plasmid pJO140 was used to delete *sigX2*. Shaded regions indicate sequences upstream and downstream from *sigX1* and *sigX2* that were cloned into pJO118 and pJO140 to target homologous recombination with the GAS chromosome.

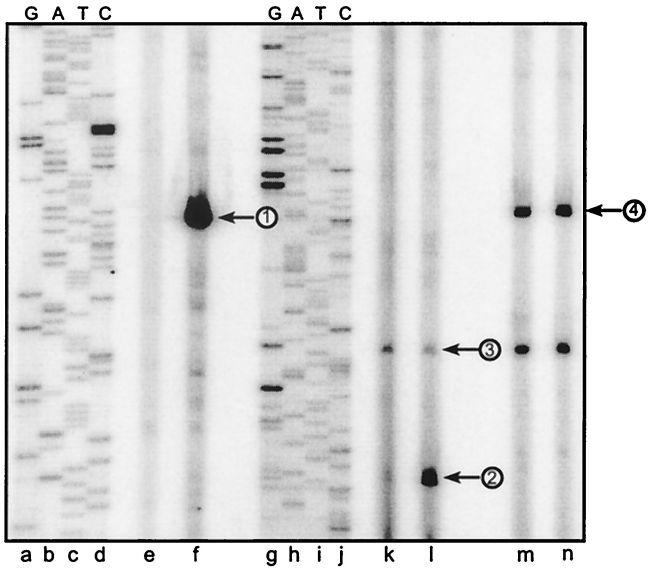


FIG. 3. Primer extension analysis of transcription products in GAS expressing *sigX* from the *veg* promoter. Shown is an autoradiograph of radiolabeled primer extension products subjected to electrophoresis on a 6% polyacrylamide gel containing 7 M urea. Twenty micrograms of GAS RNA from strains JRS4 (wild type) (lanes e, k, and m) and JRS4/pJO162 (*sigX* expression plasmid) (lanes f, l, and n) was subjected to primer extension analysis with primers sigX-R2 (lanes e and f), cinA-R (lanes k and l), and Pemm-PE (lanes m and n). DNA template pJO162 (lanes a to d) or pJO96 (*cinA* promoter cloned in pUC19 [27]) (lanes g to j) was used for sequencing with radiolabeled primers sigX-R2 (lanes a to d) or cinA-R (lanes g to j). Arrows show the positions of the *sigX* transcript originating from pJO162 (1), the $\sigma^X$-dependent *cinA* product (2), the $\sigma^A$-dependent *cinA* product (3), and the $\sigma^X$-independent *emm* product (4).

Purified $\sigma^X$ directs the utilization of a promoter located upstream from the GAS *cinA* gene by GAS core RNA polymerase in in vitro transcription reactions (27). However, it is not known whether $\sigma^X$ functions within GAS cells. Therefore, we tested whether expression of *sigX* on the expression plasmid in GAS strain JRS4/pJO162 would result in production of a transcript from the $\sigma^X$-dependent *cinA* promoter. Primer extension reactions demonstrated that strain JRS4 grown in THY broth to late exponential phase produced little $\sigma^X$-dependent transcript from the *cinA* promoter (Fig. 3, lane k). However, when *sigX* was expressed in GAS from the *veg* promoter, a significant amount of $\sigma^X$-dependent *cinA* transcript

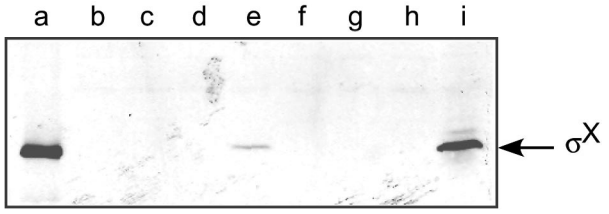


FIG. 4. Immunoblot analysis of $\sigma^X$ in a *clpP* mutant. Soluble fractions (lanes b to e) and whole-cell fractions (lanes f to i) of strains JRS4 (lanes b and f), JRS4/pJO162 (*sigX* expression plasmid) (lanes c and g), JOS34 (*clpP* mutant) (lanes d and h), and JOS34/pJO162 (lanes e and i) were subjected to electrophoresis on a sodium dodecyl sulfate–15% polyacrylamide gel and immunoblotted with anti-$\sigma^X$ antiserum. Five nanograms of purified recombinant $\sigma^X$ protein (lane a) ran at the predicted molecular mass of 19.6 kDa. The position of the $\sigma^X$ protein is indicated.

was detected (Fig. 3, lane l). We mapped the 5′ end of this transcript to an A residue that was shown previously to be the start point of $\sigma^X$-dependent transcription from this promoter in vitro (Fig. 3, lane l). As noted previously, this transcription start point is 8 nucleotides downstream from the Cin box-like sequence.

We also observed a second *cinA* transcript that appeared to start at an A residue positioned 6 nucleotides downstream from a −10 promoter sequence (TAAAAT) similar to those recognized by $\sigma^A$-RNA polymerase (Fig. 3, lanes k and l). As expected for a $\sigma^A$-dependent transcript, the appearance of this transcript was not dependent upon the presence of the $\sigma^X$ expression plasmid. Primer extension reactions from the $\sigma^X$-independent *emm* gene showed equivalent transcripts, indicating that equivalent amounts of RNA from the two strains had been used in each reaction (Fig. 3, lanes m and n).

**$\sigma^X$ accumulates in a *clpP* mutant.** The amount of $\sigma^X$ protein in the GAS *sigX* expression strain was examined by immunoblotting with the $\sigma^X$-specific antiserum. Although we found that JRS4/pJO162 contained a significant amount of a *sigX* transcript (Fig. 3, lane l), no $\sigma^X$ protein was detected in this strain with our antibody (Fig. 4, lanes c and g). As described above, this assay would detect as few as 75 molecules of $\sigma^X$ per cell. This suggested the possibility that $\sigma^X$ is unstable in the GAS cell.

One of the major proteases affecting cytoplasmic protein stability in many bacteria is ClpP, which is conserved in most gram-negative and gram-positive bacteria (32). Inactivation of *clpP* in *S. pneumoniae* results in an extended period of competence, consistent with the idea that it affects the amount of ComX in this organism (33). To test whether the ClpP protease of GAS plays a role in controlling $\sigma^X$ protein level, we generated a *clpP* mutant in JRS4 (strain JOS34) and in JRS4/pJO162 (strain JOS34/pJO162). These strains were then tested for the presence of the $\sigma^X$ protein by Western blotting with $\sigma^X$ antiserum. No $\sigma^X$ protein was detected in the *clpP* mutant (strain JOS34) as previously seen for JRS4 and JRS4/pJO162 (Fig. 4, lanes b, c, d, f, g, and h). However, a strain that contained both the *sigX* expression plasmid and the *clpP* mutation produced enough $\sigma^X$ protein to be detected easily (Fig. 4, lanes e and i). Some $\sigma^X$ protein was detected in the soluble fraction of the cell lysate; however, most accumulated in the insoluble fraction (Fig. 4, lanes e and i). This suggests that, under conditions of overexpression of *sigX*, ClpP negatively regulates $\sigma^X$ by directly or indirectly controlling the level of the protein in the cell.

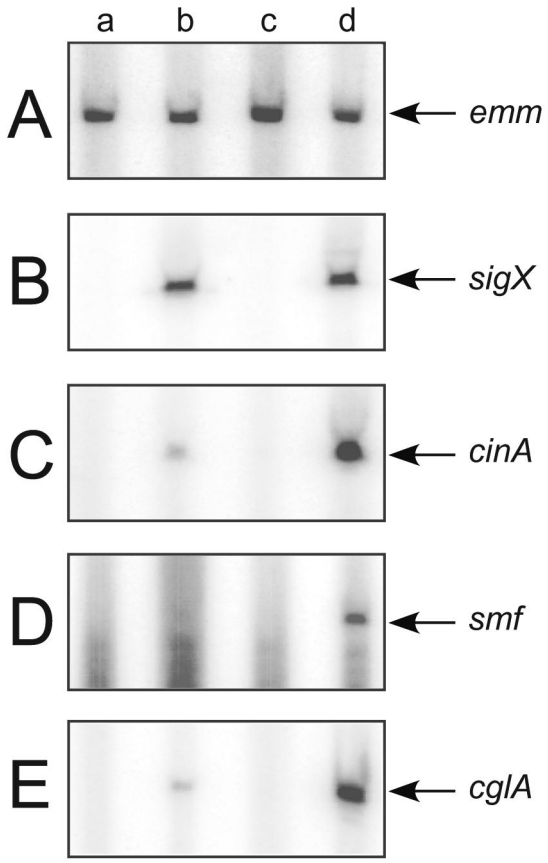


FIG. 5. Transcription products in the *clpP* mutant. Equal amounts of RNA from strains JRS4 (lane a), JRS4/pJO162 (*sigX* expression plasmid) (lane b), JOS34 (*clpP* mutant) (lane c), and JOS34/pJO162 (lane d) were subjected to primer extension analysis with primers Pemm-PE (A), sigX-R2 (B), cinA-R (C), smf-R (D), and cglA-PE2 (E) and subjected to electrophoresis on a 6% polyacrylamide gel containing 7 M urea. The position of each transcript is indicated.

**Accumulation of $\sigma^X$ leads to increased transcription of $\sigma^X$-dependent genes.** To assess the effect of $\sigma^X$ accumulation in a *clpP* mutant, we used primer extension analysis. We assayed transcription from three different cin-box promoters, *cinA*, *smf*, and *cglA*, as well as promoters $P_{veg}$-*sigX* and *emm*, which do not contain cin-boxes, in GAS strains JRS4, JRS4/pJO162, JOS34, and JOS34/pJO162. The *emm* gene, used as a $\sigma^X$-independent control, displayed equivalent amounts of transcript from all four strains, indicating that equal amounts of mRNA were used as template in each reaction (Fig. 5A, lanes a to d). Primer extension analysis also showed that the *clpP* mutation had no effect on *sigX* transcription from the expression plasmid (Fig. 5B, lanes b and d). As expected, the two strains constitutively expressing *sigX*, JRS4/pJO162 and JOS34/pJO162, produced more *cinA*, *smf*, and *cglA* transcript than the other two strains (Fig. 5B to D, lanes a to d). In addition, strain JOS34/pJO162 produced significantly more $\sigma^X$-dependent transcript from *cinA*, *smf*, and *cglA* than did JRS4/ppJO162 (Fig. 5C to E, lanes b and d). These data indicate that increas-

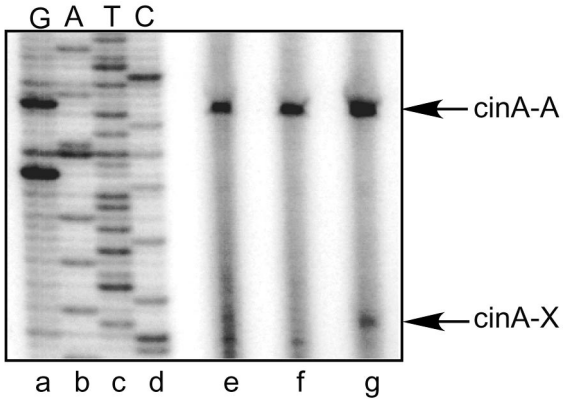


FIG. 6. Primer extension analysis of *cinA* transcription in a *clpP* mutant. RNA from strains JRS4 (lane e), JOS21 (*sigX* deletion mutant) (lane f), and JOS34 (*clpP* mutant) (lane g) was subjected to primer extension analysis with primer cinA-R. DNA template pJO96 (*cinA* promoter cloned in pUC19 [27]) (lanes a to d) was used for sequencing with radiolabeled primer cinA-R (lanes a to d). Indicated in the figure are the positions of the $\sigma^X$-dependent *cinA* product (cinA-X) and the $\sigma^A$-dependent *cinA* product (cinA-A).

ing the effective concentration of $\sigma^X$ protein in the cell by mutation of *clpP* is sufficient to increase transcription from these three Com box promoters.

**ClpP controls the activity of $\sigma^X$ expressed from its native promoter.** The previous experiments showed that *clpP* affects the amount of $\sigma^X$ protein and $\sigma^X$ transcriptional activity when *sigX* is overexpressed from a strong promoter on a multicopy plasmid. To assess the effect of ClpP on $\sigma^X$ activity when *sigX* is expressed from its wild-type locus on the chromosome, we used primer extension reactions to detect *cinA* transcripts. $\sigma^X$-dependent *cinA* transcript was not detected in the wild-type strain or in the *sigX* mutant strain (Fig. 6, lanes e and f). However, a weak $\sigma^X$-dependent *cinA* transcript was detected in the *clpP* mutant strain (Fig. 6, lane g). These experiments indicate that ClpP inhibits $\sigma^X$ activity when *sigX* is expressed from its normal chromosomal positions under its own promoter.

## DISCUSSION

An important result of this work is that $\sigma^X$ directs transcription of at least three genes when expressed in GAS. However, the amount of $\sigma^X$ in GAS is limited at two levels. The *sigX* genes are not highly transcribed in JRS4 cultured in THY medium. It is unknown whether the *sigX* genes in this strain are transcribed more actively under other conditions. Analysis of the DNA upstream from the *sigX* open reading frame yields no candidate sequences resembling a classical $\sigma^A$-dependent promoter that could direct *sigX* expression. Therefore, if the *sigX* genes are transcribed in this strain under some, as yet unknown conditions, they may be transcribed from a novel promoter located directly upstream from *sigX* that is activated by an unknown signal, or from a promoter far upstream of *sigX* within the rRNA operon, possibly from the rRNA promoters themselves.

The *sigX* genes may be more actively transcribed in other GAS strains. *S. pneumoniae* and *Streptococcus mutans* contain $\sigma^X$ homologs, ComX, that are essential for competence (21, 23). Although most GAS strains are not known to become competent for DNA uptake, an M14 GAS strain was found to be receptive for genetic exchange (16). It is unknown whether this process is transformation of competent cells by DNA; however, this genetic exchange required the *sil* locus (streptococcal invasion locus), which exhibits striking similarities to the ComDE two-component system that is required for competence development in *S. pneumoniae* (16). If this *sil* locus-dependent genetic exchange in GAS is similar to competence development in *S. pneumoniae*, it probably requires $\sigma^X$, which would serve a role homologous to that of ComX in *S. pneumoniae*. The *sil* locus was also shown to be essential for virulence in a mouse model of GAS infection (16). Therefore, if $\sigma^X$ expression is activated by *sil*, $\sigma^X$ may play a role in the virulence of this strain. While *sil* may be important for *sigX* expression in the M14 strain, multiple strains of GAS, including the M6 strain used in this study, do not contain the *sil* locus. Therefore, there may be strain-specific alternative mechanisms for regulating *sigX*.

ClpP limits the activity of $\sigma^X$, since disruption of *clpP* caused greater accumulation and activity of $\sigma^X$. Negative regulation of global transcriptional regulators by proteolysis is common among bacteria. ClpP is known to control the protein levels of transcriptional regulators in both *E. coli* and *B. subtilis*. The stationary-phase sigma factor, $\sigma^S$, in *E. coli* is specifically degraded by the ClpP protease during logarithmic growth when $\sigma^S$ is not needed by the cell (34). This process relies on a protein, RssB, to bind to $\sigma^S$ and deliver it to the proteolytic complex, thereby targeting it for degradation (41, 42). The use of a *trans*-acting targeting protein such as RssB adds specificity to the proteolytic process while not affecting the degradation of other proteins by ClpP (42). ClpP from *B. subtilis* also degrades a global regulator of transcription, ComK (40). ComK is a transcriptional activator that controls competence induction in this organism. Similar to $\sigma^S$ in *E. coli*, ComK is targeted for degradation by a *trans*-acting protein, MecA (40). ClpP mutants in *S. pneumoniae* were shown to have an extended state of competence (33). One explanation for such an effect is that ClpP degrades the $\sigma^X$ homolog, ComX, in wild-type *S. pneumoniae* and thus limits the duration of competence. Our immunoblot assays using a $\sigma^X$-specific polyclonal antibody showed increased accumulation of $\sigma^X$ protein when the *clpP* gene was inactivated. Most likely ClpP in GAS also functions by directly degrading $\sigma^X$, although this has not yet been demonstrated. Degradation of $\sigma^X$ by ClpP in GAS is a mechanism that would allow for $\sigma^X$ activity to be negatively regulated in an irreversible fashion. Moreover $\sigma^X$ activity may be induced by cellular conditions that reduce ClpP-dependent degradation of $\sigma^X$.

The amount of ClpP in both *E. coli* and *B. subtilis* is controlled at transcription. Transcription of *clpP* in *E. coli* is induced during heat shock by the secondary sigma factor $\sigma^{32}$ (19). Gram-positive bacteria do not contain a $\sigma^{32}$ homolog, and instead a transcriptional repressor, CtsR, negatively regulates *clpP* transcription in *B. subtilis* (8). ClpP degrades CtsR under stress conditions, which results in loss of repression of *clpP* (20). Consensus binding sites have been identified for CtsR in *B. subtilis*, and these binding sites have been shown to be conserved upstream of the *clpP* gene for many gram-posi-

tive organisms (8). A homolog of the *ctsR* gene is present in GAS, and the CtsR binding sites are present at the *clpP* promoter, suggesting the possibility of a similar mode of regulation (5, 8). The *ctsR* gene is negatively regulated in GAS by CovR, a global regulator of many genes including some needed for virulence (14). When CovR is inactive, CtsR would be produced in GAS cells, and this would lead to down-regulation of *clpP*. This in turn would increase $\sigma^X$ activity coordinately with increased expression of virulence factors. However, as yet no direct experimental result demonstrates a role for $\sigma^X$ in virulence of any GAS strain.

In conclusion, we found that $\sigma^X$ functions in an M6 strain of GAS. However, $\sigma^X$ function is limited by low-level transcription of its structural genes and by *clpP*, which appears to negatively regulate $\sigma^X$ accumulation. Although the *sigX* genes are conserved among several GAS strains, the mechanisms regulating their transcription may be strain specific. It is important to determine whether $\sigma^X$ plays different roles in virulence, survival, and possibly competence in different GAS strains.

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