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


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

Received 25 March 2003/Accepted 9 May 2003

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 $\sigma^X$ 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 $\sigma^X$ 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 $\sigma^X$ transcription in GAS, we constructed a strain that constitutively expresses the $\sigma^X$ gene from a heterologous promoter. Expression of $\sigma^X$ from this promoter led to transcription of the $\sigma^X$-dependent $cmaA$ promoter in GAS cells. We found that expression of the $\sigma^X$ 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 $cmaA$, $smf$, and $cglA$. In addition, a $clpP$ mutant containing $\sigma^X$ only at its wild-type loci on the chromosome generated more transcription from the $\sigma^X$-dependent $cmaA$ promoter than did the wild-type parental strain. Therefore, $\sigma^X$ activity in GAS is limited by low-level transcription of the $\sigma^X$ structural genes and by $clpP$, which appears to negatively regulate $\sigma^X$ accumulation.

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 a protein (called the “cin-box”) in vitro (27). 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., $cmaA$ 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 σ26 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′CACAACAGATATCAGCCATGTGCTTCTGCTCTCATGCAGA3′ and 5′CACACAGATATCAGCCATGTGCTTCTGCTCTCATGCAGA3′ and cloned into the EcoRI and BamHI restriction sites of plPTmus28 (New England Biolabs) to create plasmid pJO106. A fragment of DNA directly downstream of sigX was PCR amplified from JRS4 chromosomal DNA with primers 5′CAACATACCTAATGCATGACGGACCTAAGACCTCAGGACTGCAAGGCTATGCG3′ and 5′CAACATACCTAATGCATGACGGACCTAAGACCTCAGGACTGCAAGGCTATGCG3′ and cloned into the EcoRI and BamHI restriction sites of plPTmus28 (New England Biolabs) to create plasmid pJO112. The Δ kat86 resistance cassette of pUC4Kn (29) was removed by BamHI digestion and cloned into the BglII restriction site of pJO112 to create pJO117. The BamHI restriction fragment from pJO117 was then cloned into pRS233 (30), creating plasmid pJO118.

A DNA fragment directly downstream of sigX was PCR amplified from JRS4 chromosomal DNA with primers 5′CAACACAGATATCAGCCATGTGCTTCTGCTCTCATGCAGA3′ and 5′CAACATACCTAATGCATGACGGACCTAAGACCTCAGGACTGCAAGGCTATGCG3′ and cloned into the BglII and SpeI restriction sites of pJO106 to create pJO119. A DNA fragment of the cat86 gene was PCR amplified from plasmid pZL12 (9) with primers 5′CAACAGGATCCATCTAGGCCTCCTCATATTATAA GCAAGACCC3′ and 5′CAACACATACCTAATGCATGACGGACCTAAGACCTCAGGACTGCAAGGCTATGCG3′ and cloned into the BglII and SpeI restriction sites of pJO106 to create plasmid pJO133. The gusA gene was PCR amplified from plasmid pMLK90 (17) with primers 5′CAACACATACCTAATGCATGACGGACCTAAGACCTCAGGACTGCAAGGCTATGCG3′ and 5′CAACACATACCTAATGCATGACGGACCTAAGACCTCAGGACTGCAAGGCTATGCG3′ and cloned into the SpeI and BglII restriction sites of pJO112 to create plasmid pJO137. The BamHI restriction fragment from pJO137 was then cloned into the BamHI restriction site of pRS233 to create pJO138.

A DNA fragment directly downstream of sigX2 was PCR amplified from AM3 (37) chromosomal DNA with primers 5′CAACACAGATATCAGCCATGTGCTTCTGCTCTCATGCAGAATTTG3′ and 5′CAACACATACCTAATGCATGACGGACCTAAGACCTCAGGACTGCAAGGCTATGCG3′ and cloned into the BglII and SpeI restriction sites of pJO106 to create pJO120. A DNA fragment of the cat86 gene was PCR amplified from plasmid pZL12 (9) with primers 5′CAACACATACCTAATGCATGACGGACCTAAGACCTCAGGACTGCAAGGCTATGCG3′ and 5′CAACACATACCTAATGCATGACGGACCTAAGACCTCAGGACTGCAAGGCTATGCG3′ and cloned into the SpeI and BglII restriction sites of pJO112 to create plasmid pJO140. A DNA fragment of sigX gene was PCR amplified from AM3 chromosomal DNA with primers 5′CAACACAGATATCAGCCATGTGCTTCTGCTCTCATGCAGAATTTG3′ and 5′CAACACATACCTAATGCATGACGGACCTAAGACCTCAGGACTGCAAGGCTATGCG3′ and cloned into the BglII and SpeI restriction sites of pJO106 to create pJO120. A DNA fragment of the cat86 gene was PCR amplified from plasmid pZL12 (9) with primers 5′CAACACATACCTAATGCATGACGGACCTAAGACCTCAGGACTGCAAGGCTATGCG3′ and 5′CAACACATACCTAATGCATGACGGACCTAAGACCTCAGGACTGCAAGGCTATGCG3′ and cloned into the SpeI and BglII restriction sites of pJO112 to create plasmid pJO140.

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. IS1548 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 $\sigma^X$ pre-mixed 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 $\sigma^X$ consensus −35 sequence (TTGACA) and a near-consen-

sus −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).

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 genes in GAS.

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 c, 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 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 (data not shown).
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 cin transcript that appeared to start at an A residue positioned 6 nucleotides downstream from a ~10 promoter sequence (TAAAAT) similar to those recognized by $\sigma^X$-RNA polymerase (Fig. 3, lanes k and l). As expected for a $\sigma^X$-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 $\sigma$X 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 stability in many bacteria is ClpP, which is conserved in most clpP $\mu$S34 (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.

under conditions of overexpression of sigX, ClpP negatively regulates $\sigma^X$ by directly or indirectly controlling the level of the protein in the cell.

**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, cinX, smf, and cglX, as well as promoters Penm-PE (A), sigX-R2 (B), cinX-R (C), smf-R (D), and cglX-PE2 (E) and subjected to electrophoresis on a 6% polyacrylamide gel containing 7 M urea. The position of each transcript is indicated.
indicating that ClpP inhibits 

However, a weak 

strain or in the 

in the 

within the rRNA operon, possibly from the rRNA promoters 

unknown signal, or from a promoter far upstream of 

sigX 

conditions, they may be transcribed from a novel promoter 

genes are transcribed in this strain under some, as yet unknown 

these three Com box promoters.

The previous experiments showed that 

is sufficient to increase transcription from 

clpP mutant) (lane e), JOS21 (sigX deletion mutan 

tant) (lane f), and JOS34 (clpP mutant) (lane g) was subjected to 

primer extension analysis with primer cinA-R. DNA template pJ096 

(cinA promoter cloned in pUC19 [27]) (lanes a to d) was used for 

sequencing with radiolabeled primer cinA-X (lanes a to d). Indicated 

in the figure are the positions of the \( \sigma^X \)-dependent 

transcript was detected 

-transacting targeting protein such as RssB adds specificity 

the proteolytic complex, thereby targeting it for degradation (41, 42). The use of a 

trans-acting targeting protein is not needed by the cell (34). This process relies on a 

protein, RssB, to bind to \( \sigma^X \) and deliver it to the proteolytic 

complex, thereby repressing the expression 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 \( \sigma^X \) in 

E. coli, ComK is targeted for degradation by a trans-acting protein, MecA (40). ClpP mu 

tants 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 immu 

moblot 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 in 
duced 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 σ× activity coordinately with increased expression of virulence factors. However, as yet no direct experimental result demonstrates a role for σ× in virulence of any GAS strain.

In conclusion, we found that σ× functions in an M6 strain of GAS. However, σ× function is limited by low-level transcription of its structural genes and by clpP, which appears to negatively regulate σ× 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 σ× plays different roles in virulence, survival, and possibly competence in different GAS strains.

ACKNOWLEDGMENTS

This work was supported by NIH grant AI049013 from the National Institutes of Health. Jason Opdyke was partially supported by training grant AI07470 from the National Institutes of Health.

REFERENCES

3. Berthelot, B., and I. van de Rijn. 1999. Characterization of a two-component system in Streptococcus pyogenes virulence gene is present 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 σ× activity coordinately with increased expression of virulence factors. However, as yet no direct experimental result demonstrates a role for σ× in virulence of any GAS strain.

In conclusion, we found that σ× functions in an M6 strain of GAS. However, σ× function is limited by low-level transcription of its structural genes and by clpP, which appears to negatively regulate σ× 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 σ× plays different roles in virulence, survival, and possibly competence in different GAS strains.

ACKNOWLEDGMENTS

This work was supported by NIH grant AI049013 from the National Institutes of Health. Jason Opdyke was partially supported by training grant AI07470 from the National Institutes of Health.

REFERENCES

3. Berthelot, B., and I. van de Rijn. 1999. Characterization of a two-component system in Streptococcus pyogenes virulence gene is present 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 σ× activity coordinately with increased expression of virulence factors. However, as yet no direct experimental result demonstrates a role for σ× in virulence of any GAS strain.

In conclusion, we found that σ× functions in an M6 strain of GAS. However, σ× function is limited by low-level transcription of its structural genes and by clpP, which appears to negatively regulate σ× 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 σ× plays different roles in virulence, survival, and possibly competence in different GAS strains.

ACKNOWLEDGMENTS

This work was supported by NIH grant AI049013 from the National Institutes of Health. Jason Opdyke was partially supported by training grant AI07470 from the National Institutes of Health.

REFERENCES

3. Berthelot, B., and I. van de Rijn. 1999. Characterization of a two-component system in Streptococcus pyogenes virulence gene is present 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 σ× activity coordinately with increased expression of virulence factors. However, as yet no direct experimental result demonstrates a role for σ× in virulence of any GAS strain.

In conclusion, we found that σ× functions in an M6 strain of GAS. However, σ× function is limited by low-level transcription of its structural genes and by clpP, which appears to negatively regulate σ× 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 σ× plays different roles in virulence, survival, and possibly competence in different GAS strains.

ACKNOWLEDGMENTS

This work was supported by NIH grant AI049013 from the National Institutes of Health. Jason Opdyke was partially supported by training grant AI07470 from the National Institutes of Health.

REFERENCES