Characterization of a Two-component System in Streptococcus pyogenes Which Is Involved in Regulation of Hyaluronic Acid Production*

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Hyaluronic acid production by group A streptococci is regulated by transcriptional control. In this study, transposon mutagenesis of an unencapsulated strain yielded an encapsulated mutant. Two genes homologous to sensors and response regulators of bacterial two-component system were identified downstream of the transposon insertion. Inactivation of the putative sensor gene, csrS, in three different unencapsulated strains yielded encapsulated mutant strains. Electrophoretic mobility shift assays determined factor(s) in a cytoplasmic extract of an unencapsulated group A streptococcal strain was binding to a double-stranded DNA fragment derived from the has operon promoter. In contrast, similarly prepared cytoplasmic extracts from a csrS deletion mutant did not shift the fragment. The putative response regulator, CsrR, was partially purified and was shown to bind the has operon promoter fragment. The affinity and specificity of CsrR for the fragment were increased significantly after incubation with acetyl phosphate. DNase I footprinting determined that the acetyl phosphate-treated CsrR was binding to key sequences in the promoter and the coding region of hasA. Therefore, a two-component system is repressing the production of hyaluronic acid in group A streptococci using a phosphorylation-dependent binding interaction between the response regulator CsrR and the promoter region of the has operon.

The Gram-positive bacteria Streptococcus pyogenes are the cause for many suppurative infections of the skin and throat, as well as the nonsuppurative sequelae of rheumatic fever and post-streptococcal glomerulonephritis. Virulence factors produced by group A streptococci (GAS) such as C5a peptidase and pyrogenic exotoxins have been shown to promote host damage while the surface-exposed M-protein and the hyaluronic acid capsule have been shown specifically to prevent complement-mediated phagocytosis. Strains of GAS which lack M-protein are more readily destroyed in the presence of phagocytes than strains with M-protein (1). Similar studies have shown that the presence of a hyaluronic capsule inhibited phagocytosis and increased the virulence of GAS during invasive infection (2–4). Increased isolation of mucoid strains of GAS from human outbreaks of rheumatic fever as well as severe invasive infections suggests that capsule production may be an important virulence factor in these diseases (5). In addition, production of hyaluronic acid by GAS is apparently important in the initial stages of colonization of the upper respiratory tract of intranasally inoculated mice (6, 7). This evidence is supported by the observation that hyaluronic acid can function as an adhesive capable of binding to C44 on keratinocytes found on the pharyngeal mucosa and skin (8).

Hyaluronic acid is a linear glycosaminoglycan that consists of repeating subunits of β1,4-linked disaccharides of glucuronic acid and acetylglucosamine. Its structure is chemically indistinguishable from hyaluronic acid found in the connective tissues of its hosts. Production of hyaluronic acid by GAS occurs only at the exponential phase of growth and is lost at the stationary phase. This loss of production has been correlated with a loss of synthase activity from membranes of GAS isolated at the stationary phase (9). The enzymes required for hyaluronic production are encoded in the has operon. These enzymes are hasA (encodes the hyaluronic acid synthase) (10–12), hasB (encodes UDP-glucose dehydrogenase) (13), and hasC (encodes UDP-glucose pyrophosphorylase) (14). Expression of the has operon is under the control of a single promoter identified upstream of hasA (11, 15). In addition, this operon is highly conserved among GAS regardless of capsule phenotype (15). As shown by Crater and van de Rijn, the simultaneous detection of the has operon mRNA transcript and hyaluronic acid during the exponential growth phase of encapsulated strains but not unencapsulated strains suggests that transcriptional mechanisms control hyaluronic acid production (15).

The expression of the M-protein as well as many of the secreted extracellular products of GAS is controlled at the transcriptional level by the trans-activator Mga (16–18). A decrease in transcription of hasA in a strain of GAS in which the gene encoding Mga ( mga) was inactivated indicated that Mga may be involved in has operon regulation (19). However, Perez-Casal et al. (17) reported no effect on hyaluronic production after inactivation of mga. Recently, Alberti et al. (20) defined strain-specific cis-acting sequences within the promoter region of hasA which affected capsule gene expression, thus implying that differences observed in capsule expression among strains of GAS are related to individual promoter struc-
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**Oligonucleotide primers used for PCR and sequencing reactions**

| Name   | Sequence (5′→3′)       | Gene<sup>source</sup> |
|--------|------------------------|-----------------------|
| D23    | TACATCGAGTCGATAGA      | hasA<sup>a</sup> (−774 to −757) |
| D24    | TCGCTGAGATTAAGGAGGTC   | hasA<sup>a</sup> (−896 to −879) |
| D48    | GGCAAATTCGAGTGGTCATG   | hasA<sup>a</sup> (+118 to +140) |
| D55    | GCCGAGCTTCTTTTAAGGAGCAACATTATT    | hasA<sup>a</sup> (−80 to −60) |
| D51    | GCGGGAGCTTCTTTTGCAGATGTCCTG    | hasA<sup>a</sup> (−879 to −860) |
| D224   | GCGGATTAACTAGTATTTTATCAGTC  | Tn916<sup>b</sup> |
| D225   | CATGGTTGAGTGCAGGAGAAGAAGCT    | Tn916 |
| D231   | GTCGAGCAGGGTTTGGTGTG     | Upstream csrS<sup>c</sup> |
| D232   | TCCGAGCATGACTTCTTTTCTCAGC | csrR |
| D233b  | CCAATGGGAGCATCAAAATGATG    | csrS<sup>c</sup> |
| D234   | GCACTGAAATACCATTACAGTACTTGT | Downstream csrS |
| D239   | TCGCCCTTACCTCTCTCAGTGT   | csrS |
| D240   | CGCGAAATCTGCTAAC          | csrR |
| D241   | CATTAGCAGAAAGAATAATTATTTGT | csrR |
| D242   | GATGCTTTATTTCTCACGAAATAAGCTATC | csrR |

<sup>a</sup> Streptococcal (B931) chromosomal DNA sequence.<br><sup>b</sup> Tn916 sequence, non-streptococcal DNA.<br><sup>c</sup> M1 streptococcus DNA sequence, University of Oklahoma Advanced Center for Genome Technology.

To confirm that the transposon insertion was the cause of the observed mutation, generalized transductions were conducted using the GAS phage A25 as described by Caparon and Scott (29).

### Southern Blot Analysis—Chromosomal DNA was digested with HindIII, separated on a 0.5% agarose gel, and transferred to Zeta-Probe<sup>®</sup> GT Genomic Tested Blotting Membranes (Bio-Rad) via capillary action. DNA-DNA hybridization between the DNA blotted on the membrane and the probe DNA was conducted according to the Genius System (Boehringer Mannheim) with the following modifications. After incubation with anti-digoxigenin alkaline phosphatase antibody, the membrane was washed with 1 × Post-SAAP (0.05 M Tris-HCl, pH 10, 0.1 M NaCl) for 20 min, four times, with 5-min washes with distilled water between each wash. Hybridization was detected by incubation of the membrane with disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2-5-chloro]tricyclo[3.3.1.1<sup>3,7</sup>]decane)-4-yl)phenylphosphonate (Boehringer Mannheim) for 15 min at 57 °C, and bands were visualized by exposure of the membrane to BioMax 228 MR film (Eastman Kodak) at room temperature.

### Hyaluronic Acid Detection and Quantitation—Capsule production was initially detected by India ink staining (30). Quantitation of hyaluronic acid production was conducted using a colorimetric assay described previously by Moses et al. (31) with the exception that chemically defined medium was used as the growth medium instead of THY because THY contains interfering substances.

### Analysis of Regions Flanking Tn916 Insertion—Chromosomal DNA from strains being analyzed was purified, digested with HindIII, and analyzed by Southern blotting to determine the size of the two fragments of Tn916 generated. The digested DNA was run on a 0.5% agarose gel, and the desired fragments of DNA were extracted from the gel using the Qiagen Qia-Quick system. The extracted DNA was ligated and purified, and primers specific for a site at the left end of Tn916 (D224; Table I) and the region flanking the HindIII site (D225; Table I) were used to amplify the DNA adjacent to the insertion site via PCR. The standard conditions used for PCR in these studies were as follows: 8 μl of 20 mm MgSO<sub>4</sub>, 5 μl of 10 × PCR buffer without MgSO<sub>4</sub> for pwo polymerase, 5 μl of a 10 μM stock of the oligonucleotide primers, 5 μl of the ligated DNA, 4 μl of a 10 mm stock of dNTPs, 18 μl of deionized water, and 0.26 μl of pwo polymerase. Using a DNA Thermal Cycler (Perkin Elmer Cetus), the region was amplified for 35 cycles using the following conditions: melting temperature (95 °C) for 45 s, annealing temperature (47 °C) for 30 s, and extension temperature (72 °C) for 2 min. The PCR product was sequenced at the WPU/BMC Sequencing Laboratory using the primer D224 (Table I). The sequence obtained was analyzed using the data base of the M1 streptococcus DNA sequence at the University of Oklahoma Advanced Center for Genome Technology (www.genome.ou.edu).

### Allelic Exchange—A shuttle vector for allelic replacement of a portion of the second open reading frame in the potential two-component system was generated by amplifying two regions approximately 1 kb in size, flanking the replacement site (via PCR) and ligating them into the allelic replacement vector pFW6. This PCR amplification required the oligonucleotide primers D231and D232 (Table I) for generation of the region upstream of the replacement site and D233b and D234 (Table I) for generation of the region downstream of the site. The standard conditions for PCR for these studies were used except with the following...
Transformation was transferred to 5 ml of TYPG and grown at 37 °C to

Southern analysis. The DNA for this probe was amplified using primers

chool (33). The following modifications were

The DNA fragment was amplified for 35 cycles using the following conditions:

The partially purified CsrR, the 220-bp fragment extending from −80 to +140 (strain B931) of hasA was used. To label the DNA fragment, the 220-bp fragment was digested with EcoRI, and the end was labeled with the Klenow fragment in the presence of [α-32P]dATP. Free, unlabelled radiocucleotide was removed using a G-50 spin column (5′−3′, Inc.; Boulder, CO). The radioactivity of the DNA fragment was measured in a liquid scintillation counter, and the DNA was stored at −70 °C until used.

TABLE II

Hyaluronic acid production by transposon mutant, and allelic exchange trans

E. coli, BL21 (DE3)(pLysS) (pET-11a-Has), and grown to an OD600

-32P-labeled radiocucleotide was added to the reaction buffer (50 mM Tris-HCl, pH 8, 10 mM MgCl2, 5 mM dithioerythritol) for 75 min at 37 °C and then analyzed using EMSA as described above. The final volume for each reaction was 6 μl prior to the addition of 4 μl of the EMSA mixture buffer (detailed above).

DNase I Footprinting of the has Operon Promoter—To footprint the promoter of the has operon with the partially purified CsrR, the 220-bp fragment extending from −80 to +140 (strain B931) of hasA was used. To label the DNA fragment, the 220-bp fragment was digested with EcoRI, and the end was labeled with the Klenow fragment in the presence of [α-32P]dATP. Free, unlabelled radiocucleotide was removed using a G-50 spin column (5′−3′, Inc.). The DNA fragment was purified by phenol chloroform extraction and concentrated via ethanol precipitation. To ensure purity, the DNA fragment was electrophoresed on a 5% acrylamide gel, excised, and eluted from the acrylamide in a solution containing 1× EMSA buffer, 2 μg of poly(dA-dT), and the radiolabeled DNA fragment. The binding reactions were conducted using the same conditions as described for EMSA. After incubation, 50 μl of a solution containing 10 mM MgCl2 and 5 mM CaCl2 was added to each reaction. A 1:100 dilution of RQ1 RNase-Free DNase (Promega) was added to each tube and incubated for 2 min at 37 °C. After 2 min, 100 μl of a stop solution (1% SDS, 20 mM MgCl2, 2 mM EDTA, pH 8, 4 μg/μl salmon sperm DNA) was immediately added to each tube and mixed. Finally, each sample was purified via phenol chloroform extraction and concentrated via ethanol precipitation. After drying, the DNA was suspended in a 1:1 dilution of the stop solution from the Sequenase 2.0 kit (Amersham Pharmacia Biotech). The gel was transferred to and dried upon 3MM Whatman paper and subjected to autoradiography using BioMax 228 MR film.

RESULTS

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changes: melting temperature (95 °C) for 1 min, annealing temperature (52 °C) for 1 min, and extension temperature (72 °C) for 1 min; 35 cycles. The upstream and downstream fragments were cloned into the MCSII and MCSIII sites of pFW6, respectively, and electroeluted into streptococci using a method described previously (32). Transformants were electroporated into electrocompetent E. coli with spectinomycin 10 μg/ml. Using a fragment specific for the region targeted for allelic exchange within csrS, double and single crossovers were confirmed by Southern analysis. The DNA for this probe was amplified using primers D239 and D240 (Table I). Standard conditions for PCR were used except for the following changes: annealing temperature (60 °C) for 1 min and extension temperature (72 °C) for 1 min; 35 cycles.

Electrophoretic Mobility Shift Assays (EMSA)—Cytoplasmic extracts from strains being analyzed were prepared (11) and assayed for the ability to bind to the promoter region of the has operon using a protocol described previously by Chodosh (33). The following modifications were made.

reaction mixture and were incubated for 15 min at 15 °C. Next, the DNA-protein complexes were separated by electrophoresis through a 5% polyacrylamide gel in TBE (90 mM Tris, 90 mM boric acid, and 2 mM EDTA) buffer. Gels were run for 40 min at 200 volts (constant voltage, 4 °C) and transferred to and dried upon 3MM Whatman paper (80 °C). Finally, the dried gel was subjected to autoradiography using BioMax 228 MR film.

The radiolabeled DNA fragment was generated using the primers D48 and D55 (Table I). The PCR conditions for generating this DNA fragment were as follows: 9 μl of 20 mM MgSO4, 9 μl of 10 × PCR buffer without MgSO4, for puc0 polymerase; 3 μl of a 10 μM stock of the oligonucleotide primers; 1 μl of individual 10 μM stocks of dTTP, dCTP, and dGTP; 0.5 μl of a 10 μM stock of dATP; 1 μl of a 1:10 dilution of B931 chromosomal DNA; 25 μl of deionized water; and 2 μl of [α-32P]dATP (3,000 Ci mmol−1, 10 μCi μl−1; ICN, Costa Mesa, CA). The DNA fragment was amplified for 35 cycles using the following conditions: melting temperature (95 °C) for 30 s, annealing temperature (53 °C) for 35 s, and extension temperature (72 °C) for 25 s. After completion of amplification free unlabeled radiocucleotide was removed using a G-50 spin column (5′−3′, Inc.; Boulder, CO). The radioactivity of the DNA fragment was measured in a liquid scintillation counter, and the DNA was stored at −70 °C until used.

T7 Overexpression of csrR in E. coli—To clone csrR into the expression vector pT7-11a, csrR was amplified from the plasmid D241 to incorporate an NdeI site at the 5′-end of csrR and the primer D242 to incorporate a BamHI at the 3′-end. The standard PCR conditions were used with the following changes: annealing temperature (58 °C) for 1 min and extension temperature (72 °C) for 55 s. The 696-bp PCR product was subjected to T4 polynucleotide kinase to phosphorylate the ends to prepare them for ligation into the EcoRI-digested pBluescript plasmid. The phosphorylated plasmid was treated with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside by digestion with NdeI and BamHI and was then ligated into NdeI/BamHI-digested pET-7a-csrR. Finally, the pET7a-csrR plasmid was transformed into the E. coli strain BL21 (DE3)(pLyS8) to overexpress the protein using the T7 promoter expression system (25). Plasmid pET-11a was used as a negative control. After the plasmids were transformed into E. coli, a microcolony from each transformation was transferred to 5 ml of TYPG and grown at 37 °C to an OD600 = 0.7. A 1-ml sample of this culture was removed to represent a preinduction culture, while the remaining culture was induced with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside. This culture was induced for 3 additional h, and a 1-ml sample was taken. The pre- and postinduction samples were sedimented at 13,000 × g for 10 s, and the pellet was suspended in 150 μl of TE. A nonreducing sample buffer was added to the samples and boiled for 10 min in a water bath. Aliquots of each sample were removed and separated on a 10% SDS-polyacrylamide gel via electrophoresis (28) to confirm expression of CsrR.

To generate a large preparation of the overexpressed CsrR, 125 ml of TYPG was inoculated with 1 ml of a culture (OD600 = 0.7) containing BL21 (DE3)(pLyS8)(pET-11a-csrR or pET-11a) and grown to an OD600 of 0.8 with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside. The induced culture was grown for 3 h postinduction, and then the cells were sedimented at 13,000 × g for 10 min. The cells were suspended in 10 ml of the following buffer: 0.05 mM NaH/KH2 phosphate buffer, pH 6.9, 0.15 μM NaCl, 10 mM MgCl2, 5 mM dithiothreitol, and 10% glycerol. Cells were lysed with a French press (12–14,000 p.s.i.), and the membranes were sedimented at 30,000 × g. The supernatant was

TABLE II

Hyaluronic acid production

Strain

Hyaluronic acid bound

Total produced* µg/ml

B931

<10
<br>

B931ΔcsrS

50 ± 3
<br>

B931ΔEnTn916

87 ± 10
<br>

D47I ΔcsrS

10 ± 0.7
<br>

D47ΔcsrS

10 ± 0.7
<br>

GTS760

<10
<br>

GTS760ΔcsrS

10 ± 2
<br>

* Bound plus secreted.
<br>

S. E. of the mean.

| Strain | Hyaluronic acid bound | Total produced * µg/ml |
|--------|----------------------|-----------------------|
| B931   | <10                  |                       |
| B931ΔcsrS | 50 ± 3               |                       |
| B931ΔEnTn916 | 87 ± 10              |                       |
| D47I ΔcsrS | 10 ± 0.7             |                       |
| D47ΔcsrS | 10 ± 0.7             |                       |
| GTS760 | <10                  |                       |
| GTS760ΔcsrS | 10 ± 2               |                       |

* Bound plus secreted.

S. E. of the mean.
Tn916 transposon mutagenesis. Using the enterococcal strain CG110 as the donor and a streptomycin-resistant derivative of GAS strain B931 as the recipient, the conjugative transposon Tn916 was transferred via filter matings. Of the resulting transconjugants, one transconjugant (B931EnTn916) was encapsulated and was isolated for further study. This mutant excluded the capsule stain India ink, and this exclusion was lost in the presence of the enzyme hyaluronidase. Unencapsulated transconjugants were also analyzed and did not exclude India ink. To confirm that the capsule produced by the transconjugant B931EnTn916 was hyaluronic acid, the hyaluronic acid content of both the parent strain and the mutant strain was examined. The actual amount of a cell-associated total (bound plus secreted) hyaluronic acid was determined by Southern analysis, and the values are presented in Table II. As shown, mutant strain B931EnTn916 produced hyaluronic acid during the exponential phase of growth (61 fg/cfu) compared with the parent strain B931 (<10 fg/cfu).

Analysis of Tn916 Insertion in B931En—To determine the number and location of Tn916 insertions in the chromosome of strain B931EnTn916, Southern analysis was employed using a digoxigenin-11-dUTP-labeled probe derived from the plasmid pAM620 which contains Tn916. The results indicated that a single insertion of Tn916 was present in the genome of B931EnTn916. Two bands were detected (14 and 8 kb) with strain B931EnTn916, but no bands were detected from strain B931. To link the Tn916 insertion to the encapsulated phenotype observed with the mutant, transduction using the GAS bacteriophage A25 was utilized. Lysates from this infection were then used to infect the unencapsulated parent strain (B931) in order to transduce the insertion into its genome. Transductants were selected for resistance to tetracycline and screened for the production of hyaluronic acid. The frequency of transduction was $4 \times 10^{-8}$ plaque-forming units/ml, and all transductants were encapsulated. Southern analysis showed that the transductants had the bands of the same size as those of the original B931EnTn916. These results confirmed that the Tn916 insertion directly affected the synthesis of hyaluronic acid in strain B931.

Sequence Analysis of Regions Flanking the Tn916 Insertion—To characterize the sequences flanking the Tn916 insertion in B931EnTn916, PCR was employed using oligonucleotide primers derived from the known sequence of Tn916. As determined by Southern analysis, HindIII digestion of the chromosomal DNA from strain B931EnTn916 yielded two fragments (14 and 8 kb) which contained the arms of Tn916 and a portion of the DNA flanking the insertion. To isolate the fragments for sequencing, they were separated on an agarose gel, eluted, and religated to form closed, circular DNA. Next, PCR amplification of the ligated DNA from the large fragment (14 kb) yielded a 1.6-kb fragment of chromosomal DNA flanking the Tn916 insertion. Finally, the fragment yielded approximately 400 bp of DNA sequence that was analyzed using the data base of the M1 streptococcus DNA sequence at the University of Oklahoma Advanced Center for Genome Technology. The sequence submitted matched exactly with a portion of the M1 streptococcus sequence, including a portion of an unidentified open reading frame. Upon further analysis, as shown in Fig. 1, two open reading frames (684 and 1,500 bp) were identified 220 bp downstream of the Tn916 insertion site using the sequencing analysis software GCG. No open reading frames were observed upstream of the insertion site after scanning 800 bp upstream. The two open reading frames were 5 bp apart and showed homology to genes found in two-component regulatory systems. Since the completion of this work, Levin and Wessels (36) also identified these two open reading frames and named them csrR and csrS for capsule synthesis regulator and Sensor component, respectively.

Allelic Exchange to Generate a Deletion Mutant of csrS—Because the Tn916 inserted 220 bp upstream of the two open reading frames, it was uncertain if csrR and csrS encoded products that were involved in the regulation of hyaluronic acid synthesis. To analyze the genes in this potential two-component system, allelic exchange was employed to disrupt csrS (the second gene of the two-component system, which has homology to sensors from other two-component systems). A 1-kb portion of the 5’-end of csrS was replaced with the gene for spectinomycin resistance using allelic exchange with a nonreplicating suicide vector (pFW6ΔcsrS) which contained two 1-kb portions flanking the csrS deletion site (Fig. 1A). As shown, the region upstream of the area targeted for allelic exchange included all of csrR and a portion of the region upstream, whereas the region downstream included the last third of csrS and extended approximately 500 bp 3’.
The plasmid pFW6ΔcsrS was electroporated into unencapsulated strains of GAS (B931, D471, and GT8760), and mutants were selected for spectinomycin resistance and screened for hyaluronic acid production. One encapsulated colony was isolated from strains B931, D471, and GT8760. However, there were approximately 20 unencapsulated transformants isolated for every one encapsulated. Because the possibility existed that a single crossover event could occur via integration of the entire plasmid into the chromosome without disrupting either csrR or csrS, genomic DNA (digested with HindIII) from all transformants (encapsulated and unencapsulated) was analyzed via Southern analysis using a digoxigenin-11-dUTP-labeled DNA fragment containing the csrS region that was inactivated. All unencapsulated transformants produced a signal on a Southern blot, indicating that a single crossover event had occurred and that the region had not been exchanged (data not shown). However, the encapsulated transformants yielded no signal, indicating that the targeted region was replaced successfully with the spectinomycin resistance gene (25, lanes 2, 4, and 6). Chromosomal DNA from the wild type strains tested displayed the same band observed in the unencapsulated transformants (Fig. 1B, lanes 1, 3, and 5). In addition, PCR analysis using primers specific for the region being deleted in csrS yielded product with all unencapsulated transformants but not with the encapsulated transformants (data not shown).

The amount of hyaluronic acid produced by all of the transformants was assayed and is shown in Table II. As shown, the null mutants B931ΔcsrS, D471ΔcsrS, and GT8760ΔcsrS produced hyaluronic acid during exponential phase of growth (50, 101, and 12 fg/cfu; respectively) compared with the parent strains B931, D471, and GT8760 (<10 fg/cfu). When total hyaluronic acid produced was measured (Table II), the mutant strains B931ΔcsrS, D471ΔcsrS, and GT8760ΔcsrS produced 87, 176, and 10 μg/ml, whereas the parent strains all produced <10 μg/ml. These results, therefore, indicated that the product of csrS is involved in regulation of the synthesis of hyaluronic acid in GAS.

As shown in Table II, there is approximately a 10–20-fold difference between the amount of hyaluronic acid produced by D471ΔcsrS and GT8760ΔcsrS. This difference suggested that there are additional factors involved in regulating capsule besides those encoded by the csr locus. Because it was suggested recently by Alberti et al. (20) that there are cis-acting elements that determine the strength of the has operon promoter, the hypothesis was formed that the difference observed in capsule production in the csrS-inactivated strains may be caused by variations in the has promoters of these strains. To test this hypothesis, the promoter regions from the csrS-inactivated strains tested as well as other GAS strains were sequenced and analyzed. As shown in Fig. 2, the cis-elements (boldfaced with asterisks above) identified by Alberti et al. (20) in the strong promoter of an M-type 18 (highly encapsulated) were found in unencapsulated strains of GAS. As shown, strain B931 had the same cis-elements of the M18 promoter with the exception of an additional cytosine at position +4. In addition, D471 does not have the M18 cis-elements (Fig. 2), but when csrS is inactivated in D471, a significant amount of hyaluronic acid is produced by this strain (Table II). Together these data suggest that there are variations in the has operon promoters of GAS which do not correlate with the variations in capsule production observed in the csrS-inactivated strains.

After preliminary RNA studies conducted by Heath et al. (27) in which a similar Tn916 insertion upstream of the csr locus led to an increase in has operon mRNA production and capsule production over wild type levels. Because csrR has homology to genes encoding DNA-binding proteins, a radiolabeled double-stranded DNA fragment derived from the promoter region of the has operon (11) was used in EMSA assays to determine if a binding activity could be obtained from crude cytoplasmic extracts derived from the GAS strains tested in this study. Analysis of cytoplasmic extracts derived from an exponential phase culture of strain B931 demonstrated an increase in the binding of the radiolabeled DNA fragment with increasing amounts of crude extract (Fig. 3, lanes 2–6). In contrast, similarly prepared crude extracts from the B931ΔcsrS knockout mutant did not show any binding activity (Fig. 3, lanes 7–11). However, it was uncertain if the binding activity observed with strain B931 was a result of CsrR (the product of the first gene of the two-component system) binding or another protein controlled by the csr locus.

To test the hypothesis that CsrR was binding to the DNA fragment, csrR was cloned into an expression vector and overexpressed in E. coli. Cytoplasmic extracts from E. coli with and without the overexpressed CsrR were analyzed using SDS-polyacrylamide gel electrophoresis. A band at approximately 25 kDa was identified in extracts with CsrR but not in the control extracts lacking CsrR (data not shown). The predicted size for CsrR is 26 kDa. The CsrR was partially purified with ammo-

| Strain (M-Type) | Capsule | Sequence (-35 to +11) |
|----------------|---------|----------------------|
| GT8760(T49)    | -       | GTTGGAGTTAATCCAATTTAT TTATAGTAGAATTGAGGTCCCTGCTC |
| B915(T49)     | +       | ATGAGTTAATCCAATTTAT TTATAGTAGAATTGAGGTCCCTGCTC |
| D471(T6)      | -       | GTTGGAGTTAATCCAATTTAT TTATAGTAGAATTGAGGTCCCTGCTC |
| S43(T6)       | +       | GTTGGAGTTAATCCAATTTAT TTATAGTAGAATTGAGGTCCCTGCTC |
| WF51(T18)     | +       | GTTGGAGTTAATCCAATTTAT TTATAGTAGAATTGAGGTCCCTGCTC |
| M18           | +       | GTTGGAGTTAATCCAATTTAT TTATAGTAGAATTGAGGTCCCTGCTC |
| B931(T2)      | -       | GTTGGAGTTAATCCAATTTAT TTATAGTAGAATTGAGGTCCCTGCTC |
| 5-19(T3)      | +       | GTTGGAGTTAATCCAATTTAT TTATAGTAGAATTGAGGTCCCTGCTC |
| M3            | +       | GTTGGAGTTAATCCAATTTAT TTATAGTAGAATTGAGGTCCCTGCTC |
nium sulfate fractionation and analyzed using EMSA. The partially purified CsrR increasingly bound the has promoter DNA fragment with increasing amounts of protein (data not shown). In comparison, the similarly prepared control extracts from E. coli without CsrR did not hinder the mobility of the DNA fragment (data not shown). Therefore, CsrR was binding close to or within the promoter region of the has operon.

Treatment of CsrR with Acetyl Phosphate—Once it was determined that CsrR was binding directly to the promoter region of the has operon, the question of why strain B931csrS lost its activity was addressed. As seen in other two-component systems (39), the phosphorylation of the response regulator is important in determining its functionality. To determine if phosphorylation of CsrR would increase its affinity and/or specificity for the radiolabeled DNA fragment, CsrR was treated with acetyl phosphate and then analyzed immediately for binding activity using an EMSA. After treatment with acetyl phosphate, the binding activity of the partially purified CsrR increased 50–100-fold over the activity obtained from the same amount of CsrR without treatment with acetyl phosphate (Fig. 4, lanes 2–5). A gel shift was observed with non-acetyl phosphate-treated extracts only after the minimal amount of extract needed for gel shifts with acetyl phosphate was increased more than 25-fold (Fig. 4, lane 9).

Competition Assays to Determine Specificity of CsrR—To test if the binding reaction was specific, a competition assay was conducted using increasing amounts of nonradioactive specific (original has promoter DNA fragment) and nonspecific competitor (a DNA fragment derived from -700 bp upstream of the promoter). Acetyl phosphate-treated extracts were used in the competition studies. As seen in Fig. 5 (lanes 3 and 4), with increasing amounts of the specific competitor, the binding activity of acetyl phosphate-treated CsrR is lost compared with the control. In comparison, the nonspecific competitor (Fig. 5, lanes 5 and 6) retained the binding activity of the control fraction without any competitor (Fig. 5, lane 2). These results indicate that the acetyl phosphate-treated CsrR is binding specifically to the promoter region of the has operon. Without acetyl phosphate, CsrR did not demonstrate a loss in gel shift activity after the specific competitor was added, indicating that the specificity of CsrR binding to the has operon promoter is dependent on treatment with acetyl phosphate (data not shown).

DNase I Footprinting to Identify the Binding Site of CsrR—To locate where the acetyl phosphate-treated CsrR (CsrR-P) was binding in the promoter region of the has operon, DNase I footprinting was employed. Using an end-labeled radiolabeled DNA fragment derived from the noncoding strand of the has operon promoter, footprinting indicated that CsrR treated with acetyl phosphate protected several regions including the -10, transcription start, and a portion of the coding region of the NH₂ terminus of hasA (Fig. 6A, lane 5). In addition, areas of hypersensitivity were observed flanking portions of these protected sites as well as the ~35 site (lane 5). These regions are shown schematically in Fig. 6B. CsrR not treated with acetyl phosphate did not footprint (Fig. 6A, lane 4), even at levels high enough to cause a gel shift (data not shown). Control extracts from E. coli did not protect any regions regardless of the presence of acetyl phosphate (Fig. 6A, lanes 2 and 3).

Discussion

Hyaluronic acid production by GAS is under the control of transcriptional mechanisms as shown by Crater and van de Rijn (15). The goal of the experiments in this report was to identify one or more of the control mechanisms. Transposon mutagenesis of an unencapsulated strain of GAS yielded an encapsulated mutant producing hyaluronic acid (Table I). Confirmation of the transposon causing the capsule phenotype was obtained by transduction of the mutation into a fresh background of the parent unencapsulated GAS strain. Sequence analysis confirmed that there were two genes flanking the insertion site of the transposon which were homologous to genes encoding two-component regulatory systems (Fig. 1). These two genes, csrR and csrS, are identical to those identified in an abstract by Heath et al. (37) and recently by Levin and Wessels (36). In their studies, Tn916 mutagenesis and allelic exchange were used to inactivate the entire csr locus or csrR alone in order to generate a hyaluronic acid-producing mutant from a single strain of GAS. In this report, three encapsulated GAS mutants were generated from three different unencapsulated strains by inactivation of the gene for the putative sensor (csrS). In addition, EMSA showed that crude cytoplasmic extracts from an unencapsulated strain of GAS were able to bind a DNA fragment derived from the promoter region of the has operon. In contrast, it was shown that crude cytoplasmic extracts from a strain of GAS in which csrS was inactivated did not bind the fragment. Partially purified CsrR, the putative response regulator, was assayed using the same EMSA, and it was determined that CsrR was capable of binding the promoter fragment. The affinity and specificity for this fragment increased after CsrR was treated with the phospho-donor acetyl phosphate. DNase I footprinting of the binding site revealed that CsrR treated with acetyl phosphate was binding to the -10 transcription start site and portions of the coding region of hasA. Therefore, this report demonstrates that the regulation of hyaluronic acid production is dependent on both genes encoded in the csr locus and that CsrR will bind to key regions of

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**Fig. 3. EMSA of the hasA promoter and cytoplasmic extracts from strain B931.** A radiolabeled 220-bp DNA fragment derived from the hasA promoter (−80 to +140) was reacted with increasing amounts of cytoplasmic extracts from an exponential phase culture of B931 (lanes 2–6) or B931csrS (lanes 7–11). The amount of extract contained in each of the lanes is as follows: lane 1, 0 μg; lanes 2 and 7, 2 μg; lanes 3 and 8, 5 μg; lanes 4 and 9, 10 μg; lanes 5 and 10, 15 μg; lanes 6 and 11, 30 μg.

**Fig. 4. EMSA of CsrR binding to the hasA promoter with or without treatment with acetyl phosphate.** Increasing amounts of the partially purified CsrR were incubated in the presence (lanes 2–5) or absence (lanes 6–9) of acetyl phosphate and analyzed via an EMSA using the 220-bp hasA promoter DNA fragment. The amount of CsrR contained in each of the lanes is as follows: lane 1, 0 μg; lanes 2 and 6, 0.2 μg; lanes 3 and 7, 0.9 μg; lanes 4 and 8, 2.5 μg; lanes 5 and 9, 5.5 μg. The concentration of acetyl phosphate used per reaction is 32 mm.

**Fig. 5. EMSA of CsrR, treated with acetyl phosphate, binding to the sequence of the hasA promoter in the presence of a specific or nonspecific competitor.** Each reaction contains 0.3 μg of the partially purified CsrR treated with acetyl phosphate. The ratio of cold competitor to the radiolabeled 220-bp hasA promoter fragment in each lane is as follows: lane 1, no protein or competitor; lane 2, protein only; lane 3, 50:1 specific competitor; lane 4, 100:1 specific competitor; lane 5, 50:1 nonspecific competitor; lane 6, 100:1 nonspecific competitor.
the has operon promoter after it is treated with a phospho-donor such as acetyl phosphate or perhaps phosphorylated by CsrS.

The requirement for bacteria to adapt to rapidly changing environments has led to the evolution of efficient regulatory systems that utilize signals from the environment and from within the bacteria to modulate the expression of specific gene products. These regulatory systems often require a minimum of two components, a sensor and a response regulator (39). In most examples studied, the sensor possesses a histidine kinase that contains conserved histidine residues that are phosphorylated after autophosphorylation in the presence of ATP (39). Because most sensors are membrane-bound, transferring the phosphate to a response regulator present in the cytoplasm is required for transmission of the signal received (40). Once a conserved amino acid within the response regulator is phosphorylated, the response regulator will undergo a conformational change that affects its ability to bind regulatory regions of the targeted genes. Depending on the function and phosphorylation state of the response regulator, genes are either repressed or activated once the regulatory regions are bound.

As shown in Fig. 1, the data demonstrate that the transposon Tn916 inserted upstream of the putative -10 and -35 sites upstream of csrR and csrS in the encapsulated mutant GAS strain B931EnTn916. As described in this report, the inactivation of csrS alone led to hyaluronic acid production in three different strains of GAS (Table II). Recently, other evidence has been reported which demonstrates the presence of Tn916 upstream of the two csr genes or that the inactivation of csrR alone results in a loss of the mRNA encoding the two genes and an increase in the production of the has operon mRNA (36, 37). Therefore, one can conclude that both genes, csrR and csrS, are important in regulating capsule gene expression. Because the COOH terminus of csrS shows high homology with histidine kinases and its inactivation alone leads to hyaluronic acid production, a functional CsrS may regulate hyaluronic acid production by phosphorylating CsrR and/or other proteins involved in regulation of hyaluronic acid production.

Compared with the binding activity of the wild type strain B931, the inability of cytoplasmic extracts derived from exponentially grown cells of strain B931ΔcsrS to bind the DNA fragment derived from the has promoter suggests that CsrS is required for a protein(s) to bind to this region (Fig. 3). This loss of binding activity along with the increases in hyaluronic acid production suggests that CsrS may control the activity of a repressor. Because csrR had high homology to other genes for response regulators in the OmpR subfamily of response regulators which bind to specific regions within targeted promoters, CsrR was partially purified and assayed for its ability to bind to the hasA promoter fragment. This report demonstrates that CsrR is capable of binding the promoter region of hasA (Fig. 4). In addition, a significant increase in the affinity and specificity of CsrR for key regions within and around the promoter of hasA was observed after CsrR was treated with the phospho-donor acetyl phosphate. Although it was not demonstrated in this report, this increase in binding activity after treatment of CsrR with acetyl phosphate indicates that CsrR was phosphorylated and may have caused conformational changes in CsrR which increase its binding activity. This hypothesized dependence of CsrR to be phosphorylated in order for it to bind to the has operon promoter is supported by the loss of gel shift activity observed in the CsrS deletion mutant (Fig. 3). However, it was not
determined if the loss of binding activity in the CsrS deletion mutant was caused by a loss of the phosphorylation of CsrR. Nevertheless, taken together, this evidence strongly suggests that a phosphorylated CsrR can repress the has operon (Figs. 4–6). Therefore, CsrS may phosphorylate CsrR in vivo when the appropriate signals are received allowing for CsrR-P, to bind to the promoter region of hasA.

Although the binding of CsrR to the promoter region of hasA does not directly demonstrate that CsrR is repressing the transcription of the has operon, the binding sites of CsrR within the promoter of hasA suggest that this protein could affect the binding of RNA polymerase. As shown in Fig. 6, protection of regions within the −10, around the transcription start site, and portions of the coding region as well as the observation of areas of hypersensitivity at the −35 site demonstrates that multiple sites could be bound by CsrR. Whether or not these sites are bound all at once by CsrR with varying amounts of CsrR is unknown. In addition, the number of sites bound by the protein as well as the observation that increased amounts of this protein directly decreased the mobility of the promoter fragment in the EMSA suggests that complexes consisting of multimers of CsrR are binding the promoter. This additive property of the binding interaction indicates that the level of expression could be tightly regulated allowing for varying degrees of expression of the has operon.

The transcriptional control of the has operon by the csr two-component system may be influenced by many factors. First, the overall strength of the hasA promoter for each strain studied may be variable. As shown in Table II, strain D47(lacS) showed a two-fold increase over the amount of hyaluronic acid produced by strain B931(lacS) and a 10–20-fold increase over the amount of hyaluronic acid produced by GT8760(lacS). However, as shown in Fig. 2, these variations in capsule production could not be linked to differences in cis-acting elements identified previously by Alberti et al. (20) to influence capsule production. In addition, Alberti et al. (20) stated in their report that their evidence suggested that trans-acting factors were likely influencing the activity of the has promoters. Another factor involved is the signal received by the sensor to regulate the production of hyaluronic acid. The possibility exists that strains of GAS may experience conditions in vivo promoting the need for the expression of the hyaluronic acid capsule which are not found in vitro. Levin and Wessels (36) demonstrated that the strains in which the csr locus or csrR alone was inactivated were more virulent and less easily phagocytized in a murine model than its parent strain. Although they admitted that they could not rule out the possibility the products of the csr locus were regulating other virulence factors besides hyaluronic acid, their evidence certainly suggests that the factors affecting this locus are relevant to the overall virulence of GAS.

The ability for acetyl phosphate to increase the binding activity of CsrR is similar to studies involving other response regulators such as PhoB of E. coli (35). Although the Kd for the binding of CsrR to the promoter of hasA is not presently known, this report demonstrates that an increase in affinity was observed (Fig. 4). An increase in specificity for the hasA promoter fragment was also observed after CsrR was treated with acetyl phosphate. This specificity was not observed with the non-acetyl phosphate-treated CsrR, indicating that phosphorylation may be essential for specific binding. In the case of PhoB, the presence of acetyl phosphate increased the affinity of the protein for its binding site dramatically. For PhoB, the Kd for the phosphorylated PhoB showed a 10-fold increase in affinity over the non-phosphorylated protein (35). The phosphorylation of PhoB by acetyl phosphate leads to dimerization that allows for it to bind more tightly to a consensus sequence (pho-box) found in the promoters it activates (35). Other than the sequences outlined in this report, no knowledge of the binding sites of CsrR is presently known. In addition, the mechanism by which acetyl phosphate transfers a phosphate group to the response regulator as well as whether or not acetyl phosphate is relevant to the signals affecting CsrS is unknown. Structure analysis of a fully purified CsrR and its binding sites should address questions regarding the conformational changes, if any, which occur after phosphorylation and their affect on binding affinity and the activity of the has operon promoter.

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