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Regulation of Virulence by a Two-Component System in Group B *Streptococcus*†

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Group B *Streptococcus* (GBS) is frequently carried in the gastrointestinal or genitourinary tract as a commensal organism, yet it has the potential to cause life-threatening infection in newborn infants, pregnant women, and individuals with chronic illness. Regulation of virulence factor expression may affect whether GBS behaves as an asymptomatic colonizer or an invasive pathogen, but little is known about how such factors are controlled in GBS. We now report the characterization of a GBS locus that encodes a two-component regulatory system similar to CsrRS (or CovRS) in *Streptococcus pyogenes*. Inactivation of *csrR*, encoding the putative response regulator, in two unrelated wild-type strains of GBS resulted in a marked increase in production of beta-hemolysin/cytolysin and a striking decrease in production of CAMP factor, an unrelated cytolytic toxin. Quantitative RNA hybridization experiments revealed that these two phenotypes were associated with a marked increase and decrease in expression of the corresponding genes, *cylE* and *cfb*, respectively. The CsrR mutant strains also displayed increased expression of *scpB* encoding C5a peptidase. Similar, but less marked, changes in gene expression were observed in CsrS (putative sensor component) mutants, evidence that CsrR and CsrS constitute a functional two-component system. Experimental infection studies in mice demonstrated reduced virulence of both CsrR and CsrS mutant strains relative to the wild type. Together, these results indicate that CsrRS regulates expression of multiple GBS virulence determinants and is likely to play an important role in GBS pathogenesis.

Group B *Streptococcus* (GBS) (or *Streptococcus agalactiae*) is an important cause of invasive infection in newborn infants, in women around the time of childbirth, and in older individuals with underlying chronic illnesses (28). Although GBS has the capacity to produce life-threatening infection in susceptible hosts, more often it behaves as a harmless commensal organism. Surveys of asymptomatic volunteers have demonstrated that GBS colonizes the vagina and/or rectum of one-third to one-half of healthy women (12). Because GBS encounters a variety of environmental conditions in the varied niches it occupies, the organism's survival in the human host may be enhanced by its ability to perceive changes in the external milieu and to adapt by altering expression of specific genes. This dynamic adaptation of GBS to the human host is likely to involve one or more regulatory systems that control expression of bacterial factors important in adhesion, nutrient acquisition, resistance to host immune effectors, and under certain circumstances, invasion of host tissues.

One mechanism for adaptation to changing environments is through two-component regulatory systems, a family of proteins that are widely distributed among many bacterial genera (20, 32). Two-component systems allow sensing of specific environmental signals through a sensor histidine kinase that is usually associated with the cell membrane. In the basic model of two-component systems, interaction of an appropriate extracellular stimulus with the sensor histidine kinase alters the phosphorylation state of its cytoplasmic domain. The prototypic sensor protein has kinase and/or phosphatase activity for a cognate regulator protein; phosphorylation (or dephosphorylation) of the regulator controls its activity as a transcriptional activator or repressor for one or more target genes. In many cases, signaling through a single two-component system results in a coordinated change in expression of multiple genes whose products play a role in adaptation to a particular environment.

Two-component regulatory systems in GBS have been described, although their characterization has been limited. Poyart et al. identified a two-component system encoded by *dltR* and *dltS*, two genes at the 3′ end of an operon that directs incorporation of D-alanine residues into lipoteichoic acid (23). Their data suggest that DltR/DltS functions to control expression of the *dlt* operon, thereby regulating the level of D-alanine esters in GBS lipoteichoic acid. Another two-component system in GBS was identified in a screen for mutants defective in fibrinogen binding. The RgfA/RgfC response regulator and histidine kinase appear to regulate expression of C5a peptidase (31). In addition to these two typical bacterial histidine kinase/phosphoregulator systems, Rajagopal et al. described a eukaryotic type serine-threonine kinase coupled to a cognate phosphatase (25). The latter system appears to regulate pyrophosphatase activity and possibly other cellular functions in GBS.

Analysis of the GBS genome of the capsular type V strain 2603 revealed the presence of genes predicted to encode at least 17 two-component systems (36). Similarly, the genome of type III GBS strain NEM316 contains 20 putative histidine

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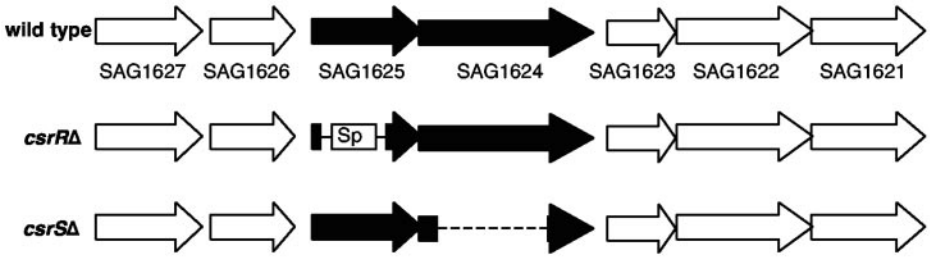

CsrR/CsrS locus in strain 2603V/R

Locus Name	Gene Symbol	Gene Length (bp)	Protein Length (aa)	Protein Mass (kDa)	Proposed Function
SAG1627	*htpX*	888	296	32.4	Heat shock protein
SAG1626	none	531	177	20.2	Conserved hypothetical protein
SAG1625	***csrR***	**687**	**229**	**26.5**	**DNA binding response regulator**
SAG1624	***csrS***	**1503**	**501**	**57.5**	**Sensor histidine kinase**
SAG1623	none	477	159	18.5	Conserved hypothetical protein
SAG1622	none	1173	391	45.4	Conserved hypothetical protein
SAG1621	*dnaI*	900	300	34.7	Primosomal protein

FIG. 1. Schematic of the region of GBS strain 2603 chromosome that contains the *csrRS* locus (wild type) and the corresponding chromosomal regions in mutant strains 2603*csrR*Δ and 515*csrR*Δ (*csrR*Δ mutants) and in mutant strains 2603*csrS*Δ and 515*csrS*Δ (*csrS* mutants). ORF designations are according to Tettelin et al. (36). Features of the predicted proteins are indicated in the table. Sp indicates the location of the spectinomycin cassette in *csrR*Δ; the broken line indicates the internal deletion in *csrS*Δ. aa, amino acids.

kinase sensors and 21 response regulators (8). GBS has a larger number of two-component systems than does *Streptococcus pneumoniae*, *Streptococcus pyogenes*, or *Lactococcus lactis*, an observation that suggests that such systems may be especially important in adaptation of GBS to various niches in its human and animal hosts. Among the two-component systems predicted by the GBS genome sequences, one has a high degree of sequence similarity to the CsrR/CsrS (also called CovR/CovS) proteins of *S. pyogenes*. The *S. pyogenes* CsrRS system has been shown to regulate expression of several virulence determinants, including the hyaluronic acid capsule, streptolysin S, and streptokinase (7, 11, 16). Microarray transcriptional profiling studies suggest that CsrRS regulates expression, directly or indirectly, of 15% of *S. pyogenes* genes (9).

Given the importance of the CsrRS system in *S. pyogenes* virulence (6, 11, 16), we searched for orthologs of CsrR and CsrS in the GBS genome. Open reading frames (ORFs) with a high degree of similarity to both *csrR* and *csrS* were found in both the GBS 2603 and NEM316 genome sequences (see Fig. 1). In the present study, we show that inactivation of either component of the *csrR*/*csrS* locus in two independent GBS strain backgrounds resulted in striking alterations in expression of multiple virulence determinants. The results identify the GBS CsrR/CsrS system as a major global regulatory system that plays an important role in GBS pathogenesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. GBS type Ia strain 515 (38) and type V strain 2603V/R (called 2603 hereafter) (36) were used as the parent strains for mutants constructed in this report. GBS was grown in liquid culture in Todd-Hewitt broth (Difco), on Trypticase soy agar supplemented with 5% defibrinated sheep blood (PML Microbiologicals), or on Todd-Hewitt agar supplemented with antibiotics and 5% defibrinated sheep blood. *Escherichia coli* DH5α and DY330 (40) were grown in Luria-Bertani medium or on Luria-Bertani agar. When appropriate, antibiotics were added at the following concentrations: ampicillin, 100 μg/ml; spectinomycin, 100 μg/ml; or erythromycin, 1 μg/ml for GBS or 250 μg/ml for *E. coli*. GBS was grown without shaking in liquid culture. *E. coli* was grown with shaking at 37°C or at 30°C (DY330). Plasmid pGEM-T (Promega) was used for the direct cloning of PCR products; pJRS233 is a temperature-sensitive *E. coli*/gram-positive shuttle vector (21).

DNA isolation and manipulation. Plasmid DNA was isolated using either the QIAGEN midiprep or miniprep kit according to the manufacturer's recommendations. GBS chromosomal DNA was prepared as described previously (19). Restriction endonuclease digestions, DNA ligations, transformation of $CaCl_2$-competent *E. coli*, PCR, agarose gel electrophoresis, and Southern hybridizations (ECL kit; Amersham Pharmacia Biotech) were performed by standard techniques (26). GBS electrocompetent cells were prepared as described previously (2) and transformed by electroporation using Bio-Rad Gene Pulser II (Bio-Rad) as described previously (1). Oligonucleotide primers are listed in Table S1 in the supplemental material.

Construction of *csrR* mutagenesis plasmid. For construction of an internal deletion of *csrR*, primers 738 and 740 were used to amplify by PCR the first 122 bp of *csrR* and 878 bp of adjacent upstream flanking sequence using GBS strain 515 chromosomal DNA as a template. Primers 737 and 739 were used to amplify the last 118 bp of *csrR* and 879 bp of downstream flanking DNA. Primer 738 contains 18 bp of DNA that is complementary to primer 739. The two gel-purified PCR products containing complementary ends were mixed and amplified with primers 737 and 740 to create a 450-bp internal deletion of *csrR* by overlap PCR (13, 14). The 1,997-bp overlap PCR product was digested with BamHI and KpnI and ligated into BamHI/KpnI-digested pJRS233.

To improve screening efficiency, a nonpolar spectinomycin gene cassette was inserted into the internally deleted *csrR* gene within the recombinant plasmid by recombination exchange in *E. coli* strain DY330, which harbors an efficient prophage recombination system (40). Briefly, the spectinomycin cassette was PCR amplified from plasmid pDL278 with two hybrid oligonucleotide primers 799 and 800, each containing a 5′ 36-bp segment from internal sequences of the *csrR* gene and a 3′ 23- or 24-bp segment from the terminal sequences of the spectinomycin cassette (15). The recombinant pJRS233*csrR*Δ construct was transformed into strain DY330 by electroporation. After induction of the λ_{RED} recombination system at 42°C for 15 min, the DY330 strain containing pJRS233*csrR*Δ was transformed with the linear spectinomycin PCR product. Recombination occurs between the *csrR* sequences on the ends of the linear

cassette and homologous sequences on pJRS233*csrR*Δ. Clones with the desired *csrR*Δ::Sp replacement of the *csrR*Δ allele on the pJRS233 construct were selected by growth on medium containing spectinomycin and were confirmed by PCR and sequencing before the recombinant plasmid, pJRS233*csrR*Δ::Sp, was introduced into GBS strains.

Construction of *csrR* deletion mutants in GBS. Allelic exchange of the internally deleted gene for the chromosomal wild-type *csrR* gene was achieved by a two-step process in which the plasmid carrying the mutant allele was first integrated into the *csrR* region by homologous recombination. Plasmid excision from the chromosome via a second recombination event at the permissive temperature (30°C) either completed the allelic exchange or reconstituted the wild-type genotype. The *csrR* deletion construct (pJRS233*csrR*Δ::Sp) was introduced into GBS strains 515 and 2603 by electroporation, and transformants were selected by growth at 30°C in the presence of erythromycin. A single erythromycin-resistant colony was used to inoculate a liquid culture supplemented with erythromycin. After overnight incubation at 30°C, the culture was diluted 10-fold with fresh broth containing erythromycin and incubated at 37°C to select organisms in which the recombinant plasmid had integrated in the *csr* locus of the GBS chromosome by homologous recombination. Dilutions of each culture were plated on medium containing erythromycin and incubated overnight; erythromycin-resistant colonies representing plasmid integrants were serially passaged twice on solid medium at 37°C. Integrant strains were serially passaged at least five times in broth at 30°C in the absence of erythromycin. Erythromycin-sensitive or spectinomycin-resistant colonies were screened for the expected deletion mutation by PCR amplification using primer pairs that flank the target gene. Approximately 5 to 50% of excisants harbored the desired deletion mutation.

Repair of the *csrR* deletion mutants. In order to repair the mutation in strains 515*csrR*Δ and 2603*csrR*Δ, we constructed a recombinant pJRS233 plasmid carrying the *csrR* wild-type allele and its flanking regions. To distinguish the repaired strain from the original wild type, a genetic marker was introduced upstream of the *csrR* locus by an overlap PCR. Primer pairs 914 and 737 were used to amplify the complete *csrR* gene, 312 bp of adjacent upstream DNA, and 879 bp adjacent downstream DNA. The 565-bp sequences from positions 313 to 878 upstream of the *csrR* start codon were amplified with primers 740 and 913 (see Table S1 in the supplemental material), the latter containing 22 bp of DNA that is complementary to primer 914 and two synonymous nucleotide replacements: T replaced C at positions 313 and 316 (see Table S1 in the supplemental material). The two amplification products were used in an overlap PCR with primers 737 and 740 to introduce the silent mutations into positions 313 and 316 upstream of the *csrR* start codon. The genetically "labeled" wild-type *csrR* construction was introduced into GBS 515*csrR*Δ and 2603*csrR*Δ strains by electroporation and allelic exchange of the wild-type gene for the chromosomal deleted *csrR* gene was achieved by a two-step process as described above. Candidate excisants (erythromycin-sensitive, spectinomycin-sensitive colonies) were screened by PCR and then confirmed by sequence analysis.

Construction of *csrS* deletion mutants. *csrS* deletion mutants were constructed as described above for the *csrR* mutants except that we did not use an antibiotic resistance marker in the internally deleted gene. Primers 830 and 861 were used to PCR amplify the first 137 bp of *csrS* and 1,000 bp of adjacent upstream flanking sequence using GBS strain 515 chromosomal DNA as a template. Primers 829 and 862 were used to amplify the last 137 bp of *csrS* and 995 bp of downstream flanking DNA. The two gel-purified PCR products containing complementary ends were mixed and amplified with primers 829 and 830 to create a 1,230-bp internal deletion of *csrS* by overlap PCR (13, 14). The 2,269-bp overlap PCR product was digested with BamHI and KpnI and ligated into BamHI/KpnI-digested pJRS233. Transformation of GBS strains 515 and 2603 with the *csrS* deletion construct and allelic exchange with the chromosomal *csrS* locus were accomplished as described above for the *csrR* mutants.

Hemolysin assay. Approximately 10^8 CFU of GBS cells were collected from liquid cultures at exponential phase, washed once with phosphate-buffered saline (PBS), and resuspended in 1 ml of PBS containing 0.2% glucose. Serial dilutions of this suspension in PBS containing 0.2% glucose were mixed with an equal volume of 1% sheep erythrocytes (RBC) in the same buffer and incubated at 37°C for 1 h. After incubation, unlysed RBC and bacteria were removed by centrifugation, and hemoglobin content of the supernatant was assessed by measuring A_{405}. The hemolytic titer of each strain was determined as the reciprocal of the greatest dilution producing 50% hemoglobin release compared with control samples in which all RBC were lysed by 1% sodium dodecyl sulfate (SDS).

RNA isolation. Total bacterial RNA was isolated using RNeasy mini kit (QIAGEN) according to the manufacturer's instructions, except that GBS cells were lysed by shaking with glass beads (39). RNA samples were treated with DNase I for 30 min at room temperature to remove any contaminating DNA. RNA concentration was adjusted to 100 ng/μl, and samples were stored at −80°C until use.

RT-PCR. For reverse transcription-PCR (RT-PCR), 20 ng of RNA was used for cDNA synthesis and subsequent PCR amplification using the Access RT-PCR kit (Promega) according to the manufacturer's recommendations. PCRs were performed with a 1 μM primer concentration in a Perkin-Elmer thermal cycler using a temperature program as follows: (i) reverse transcription (45 min at 48°C); (ii) denaturation (3 min at 94°C); and (iii) 25 cycles of PCR, with 1 cycle consisting of denaturation (30 s at 94°C), annealing (45 s at 52°C), and extension (1 min at 72°C). To exclude DNA contamination of RNA samples, replicate control assays were performed in which reverse transcriptase was omitted. The amplification product was fractionated on a 0.8% agarose gel and stained with ethidium bromide.

RNA blot hybridization. Standardized amounts of RNA (2, 0.5, 0.12, and 0.03 μg) were blotted onto nylon membranes (Hybond N+; Pharmacia) using a vacuum manifold blotter (Bio-Dot; Bio-Rad). DNA probes were generated by PCR using GBS chromosomal DNA as the template and radiolabeled with [α-^{32}P]dATP by random priming (RadPrime DNA labeling system; Invitrogen). Hybridization was performed in Church buffer at 60°C for 16 h, followed by one wash in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at room temperature and three washes in 0.5× SSC–0.1% SDS at 60°C. Membranes were exposed to X-ray film, and the intensity of individual spots was evaluated by densitometry using NIH Image, version 1.63 (http://rsb.info.nih.gov/nih-image/). To compare the relative transcription of each target gene in CsrR and CsrS mutants with that in the wild type, densitometry data were chosen from a value range that displayed a linear relationship to the $\log_{10}$ RNA concentration.

Mouse virulence studies. All animal studies were performed in accordance with institutional and federal animal care and use guidelines. Mouse virulence studies were performed using 6- to 8-week-old female CD1 mice. Briefly, 10-fold dilutions of a broth culture of GBS grown to exponential phase were administered by intraperitoneal injection to groups of six mice in an injection volume of 1 ml. Mortality was monitored for 5 days after challenge. The 50% lethal dose (LD_{50}) was calculated for each strain on the basis of results of at least two independent experiments including a total of at least 60 mice for each strain. LD_{50} estimates and confidence intervals were derived from logistic regression models for the probability of death conditional on dose and strain (37). Confidence intervals were established using the delta method (37). *P* values for the hypothesis of common LD_{50}s for pairs of strains were derived from standard likelihood ratio tests for logistic regression.

Measurement of cell-associated capsular polysaccharide. Capsular polysaccharide was released from GBS cells by treatment with mutanolysin, and the amount of type Ia (strain 515 and 515*csrR*Δ) or type V (strain 2603 and 2603*csrR*Δ) was quantified by competition enzyme-linked immunosorbent assay (ELISA) as described previously (4).

RESULTS

Identification of CsrRS orthologs in the GBS genome. Recent completion of genome sequences for two serotypes of GBS revealed the presence of at least 17 putative two-component regulatory systems (8, 36). Genes predicted to encode one such system displayed striking similarity to CsrRS, a two-component system in *S. pyogenes* known to control expression of several virulence determinants. Comparative genome hybridization experiments demonstrated that the putative GBS orthologs of *csrR*/*csrS* were present in all 19 GBS strains examined, including representatives of each of the known capsular serotypes (36; H. Tettelin, personal communication). In this report, we have retained the *csrR*/*csrS* designation used in the annotation of the GBS type V genome (36). This nomenclature respects the precedent established by the earlier publication and is consistent with the high degree of similarity between the predicted GBS proteins and CsrR/CsrS of *S. pyogenes* (see below). Like *csrRS* in *S. pyogenes*, the first gene in the GBS locus encodes a presumed transcriptional regulator of 229 amino acids (compared to 228 amino acids in *S. pyogenes)* (Fig. 1). The GBS protein includes in its amino terminus a consen-

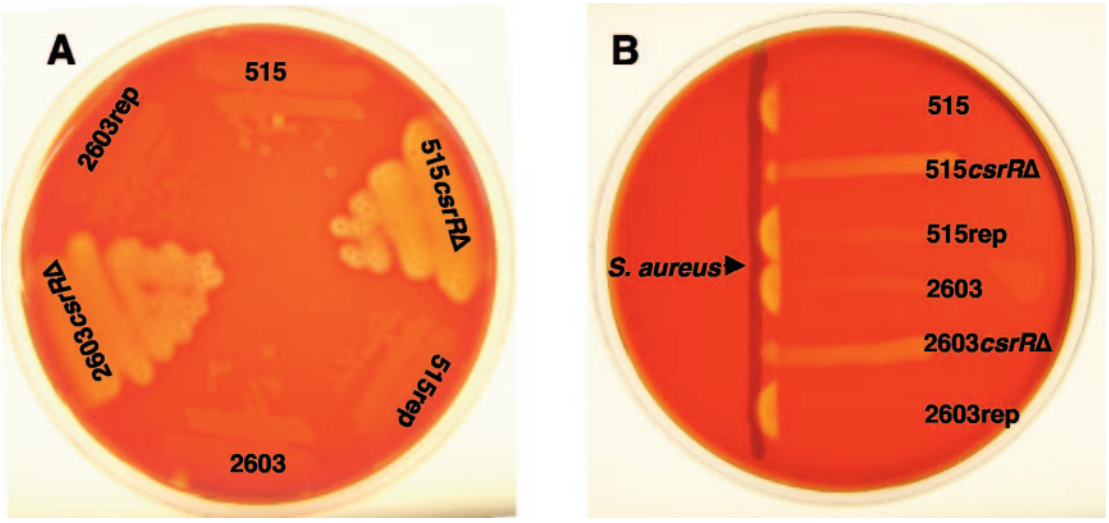

FIG. 2. Increased hemolysis and reduced CAMP factor activity in CsrR mutant strains. Blood agar plates were inoculated with wild-type GBS strains 515 and 2603, CsrR mutant strains 515*csrR*Δ and 2603*csrR*Δ, and repaired mutant strains 515*csrR*Δrep and 2603*csrR*Δrep (labeled 515rep and 2603rep in the figure, respectively). (A) Blood agar plate demonstrating increased zones of beta-hemolysis surrounding colonies of CsrR mutant strains 515*csrR*Δ and 2603*csrR*Δ. (B) CAMP test demonstrating reduced synergistic hemolysis at the intersection of streaks of CsrR mutant strains 515*csrR*Δ and 2603*csrR*Δ with a perpendicular streak of beta-lysin-producing *S. aureus*. The increased level of hemolysis by the mutant strains is also evident along the GBS streaks.

sus sequence motif (Asp10, Asp53, Lys102) characteristic of the receiver domain of response regulators (34). The GBS CsrR protein is strikingly similar to the orthologous protein in *S. pyogenes* with 83% amino acid identity and 91% similarity.

A second ORF, designated *csrS*, overlaps 11 nucleotides of the 3′ terminus of the GBS *csrR* locus and is transcribed in the same orientation. The GBS *csrS* gene encodes a predicted protein of 501 amino acids with features of a sensor histidine kinase. These features include, in the amino-terminal half of the protein, hydrophobic regions consistent with membrane-spanning domains that flank a predicted extracellular domain and a conserved histidine residue (His278) that, in other characterized systems, represents the site of autophosphorylation (33). The degree of similarity of the GBS CsrS protein to its *S. pyogenes* ortholog, 50% identity and 72% similarity, is greater than to any other protein in the databases but is lower than the degree of similarity between the respective GBS and *S. pyogenes* CsrR proteins. The predicted extracellular domain of the GBS CsrS protein is even less similar to *S. pyogenes*—29% identity and 59% similarity, an observation that suggests the GBS CsrS may respond to different environmental signals than the *S. pyogenes* system. Flanking the GBS *csrRS* locus in type V strain 2603 are ORFs predicted to encode proteins of unknown function (Fig. 1). Homologs of the flanking genes are also found in the same relative positions in *S. pyogenes*, but they are not transcriptionally linked to *csrRS* in that species (16).

Construction and characterization of CsrR mutants. To investigate the function of the CsrRS system in GBS, a *csrR* mutant was developed in two independent strain backgrounds, GBS type Ia strain 515 and type V strain 2603. We replaced a 450-bp segment of the *csrR* gene with a spectinomycin cassette by allelic exchange mutagenesis. Candidate mutants (erythromycin-sensitive, spectinomycin-resistant colonies) were screened by PCR to confirm the expected chromosomal gene replacement with the mutant *csrR* allele. The genotype of *csrR* mutants was further confirmed by Southern hybridization analysis of PstI-digested chromosomal DNA using the *csrR* sequence as probe (not shown). The mutagenesis strategy made use of an antibiotic cassette for gene replacement with the expectation that transcription of downstream genes would not be affected. RT-PCR analysis confirmed that expression of mRNA from the downstream ORFs (*csrS* and Sag1623) was preserved in 515*csrR*Δ and 2603*csrR*Δ (not shown). Growth curves revealed slightly slower growth of the *csrR* mutant strain 515*csrR*Δ compared to wild-type strain 515, whereas growth curves for 2603*csrR*Δ and wild-type strain 2603 did not differ significantly (not shown). Because certain GBS loci may have a greater effect on growth in the presence of plasma or serum (29), we performed growth curves of both wild-type and mutant strains in 90% human plasma and found similar results to those in broth alone (not shown).

Effect of CsrR inactivation on hemolytic activity. An immediately obvious phenotype of the *csrR* mutants in both strain backgrounds was a marked increase in the zone of beta-hemolysis surrounding the colonies on blood agar plates (Fig. 2A). This observation suggested that inactivation of CsrR resulted in increased production of the GBS beta-hemolysin/cytolysin. To test this hypothesis, hemolysin production by wild-type strains 2603 and 515 and their *csrR* mutants was quantified using a modified version of the method of Marchlewicz and Duncan (17). The hemolytic titers for mutant strains 515*csrR*Δ and 2603*csrR*Δ were 32- to 64-fold higher than those of their respective parent strains (data not shown). These data confirmed that the increased hemolytic zone around *csrR* mutant colonies reflected a marked increase in beta-hemolysin production. Previous studies have noted an association between beta-hemolytic activity of GBS strains and production of orange pigment, and the *cyl* gene cluster appears to be required for both (24, 30). Consistent with those findings, we observed

an obvious increase in orange pigment in cultures of 515*csrR*Δ and 2603*csrR*Δ relative to the wild-type strains (not shown).

Effect of CsrR inactivation on CAMP factor activity. As part of the phenotypic characterization of mutant strains 515*csrR*Δ and 2603*csrR*Δ, we tested for production of CAMP factor. CAMP factor is a 25-kDa secreted protein of GBS that augments the hemolytic activity of *Staphylococcus aureus* sphingomyelinase; it is routinely assayed by streaking an indicator strain of *S. aureus* perpendicular to a streak of GBS (3, 35). CAMP factor production results in a characteristic arrowhead-shaped widening of the zone of hemolysis where the two streaks intersect. CAMP testing of 515*csrR*Δ and 2603*csrR*Δ revealed a marked decrease in CAMP activity compared to the respective wild-type strains (Fig. 2B). Thus, inactivation of the *csrR* locus appears to result in opposite effects on beta-hemolysin and CAMP factor production—a marked increase in hemolysin and a marked decrease in CAMP activity. Both phenotypes are apparent on the CAMP test plate in Fig. 2B.

Repair of the *csrR* mutation by replacement with the wild-type allele. To confirm that the increase in hemolytic activity and decrease in CAMP factor were due, directly or indirectly, to the *csrR* mutation, we repaired the mutation by replacing the mutant *csrR* allele with a copy of the wild-type gene. We used this approach rather than plasmid complementation for two reasons: (ii) to ensure that regulation of expression of the *csrR* locus by *cis*-acting chromosomal elements would not be altered and (ii) to ensure stable retention of the complementing allele during experimental infection experiments (see below). So that the repaired strain could be distinguished from the original wild-type strain, we first introduced two silent mutations in the cloned DNA sequence that included the complete wild-type *csrR* gene before performing the gene replacement. The marked wild-type allele was then substituted for the mutant *csrR* allele in both strains 515*csrR*Δ and 2603*csrR*Δ. Gene replacement in both strains was confirmed by DNA sequence analysis of the chromosomal *csrR* locus in the two repaired strains, designated 515*csrR*rep and 2603*csrR*rep. Both repaired strains exhibited growth rates, hemolysis, and CAMP factor activity indistinguishable from those of the original wild type (Fig. 2). These results provided further evidence that the observed changes in hemolysis and CAMP factor were a result of the *csrR* mutation.

Analysis of *cylE* and *cfb* transcription. The GBS CsrRS system is presumed to function through the effects of the CsrR protein on target gene transcription. Accordingly, we investigated whether the observed changes in hemolysin and CAMP activity in *csrR* mutants reflected altered transcription of the genes encoding these products. RT-PCR using primers specific for *cylE*, the presumed structural gene for beta-hemolysin/cytolysin (24), revealed increased *cylE* message in both 515*csrR*Δ and 2603*csrR*Δ strains compared to their respective wild-type parent strains (data not shown). RT-PCR with primers specific for *cfb* encoding CAMP factor (22, 27) demonstrated a marked reduction in *cfb* message in the mutant strains (data not shown). This qualitative analysis of gene expression confirmed that increased (hemolysin) or decreased (CAMP factor) virulence factor production in the *csrR* mutants correlated with increased or decreased transcription of the *cylE* or *cfb* gene, respectively.

Assessment of virulence factor expression by RNA blot hybridization. As a more quantitative means of comparing relative expression levels, we performed RNA blot hybridization experiments using radiolabeled DNA probes specific to the same two target genes. Like the RT-PCR results, the RNA hybridization experiments demonstrated an 18- to 50-fold increase in transcription of *cylE* in both 515*csrR*Δ and 2603*csrR*Δ strains relative to that in the respective wild-type strains (Fig. 3). A decrease in transcription of similar magnitude was observed for *cfb* in the *csrR* mutant strains. Control hybridizations done in parallel showed little or no difference between mutant and wild-type strains in transcript levels of *recA* encoding a DNA repair enzyme or the ribosomal protein gene, *rpsL*.

To investigate whether other virulence factors or surface components were regulated by the CsrRS system, we performed similar RNA hybridization experiments using probes for several additional genes. We found that expression of C5a peptidase (*scpB*) was increased 11- to 34-fold in strains 515*csrR*Δ and 2603*csrR*Δ relative to the wild-type strains (Fig. 3). We also noted in the mutant strains a small (approximately twofold) increase in expression of transcript for surface proteins Rib (expressed in strain 2603) and Alp1 (expressed in strain 515) (Fig. 3). We found no significant difference in expression of Sip (both strains) or laminin binding protein (*lmb*, both strains) (Table 1).

We also did not detect a significant effect of the *csrR* mutation on expression of the biosynthetic operon that directs synthesis of the capsular polysaccharide (as reflected by expression of the initiator glycosyltransferase encoded by *cpsE)* (Table 1). Because inactivation of CsrRS in *S. pyogenes* has a marked effect on capsular polysaccharide production, we assessed not only *cps* gene expression but also capsular polysaccharide production by strains 515*csrR*Δ and 2603*csrR*Δ relative to the wild-type strains. We found no significant difference between the mutant and wild-type strains in the amount of cell-associated type Ia (515*csrR*Δ and 515) or type V (2603*csrR*Δ and 2603) polysaccharide in ELISA inhibition assays (data not shown).

Construction and characterization of CsrS mutants. The high degree of similarity of the GBS *csrS* locus with the *csrS* gene of *S. pyogenes* strongly suggested that the GBS gene encodes a functional sensor protein that interacts with the GBS CsrR protein. To test that idea, we inactivated the *csrS* locus in GBS strains 515 and 2603 using a strategy similar to that described above for inactivation of the *csrR* gene. Both mutant strains, 515*csrS*Δ and 2603*csrS*Δ, showed increased hemolysis and decreased CAMP factor activity compared to the wild type. The results of RNA hybridization experiments using probes for *cylE*, *cfb*, and *scpB* were similar to those observed for strains 515*csrR*Δ and 2603*csrR*Δ, but the changes in gene expression for all three genes were less extreme in the *csrS* mutants than in the *csrR* mutants (Fig. 4 and Table 2). The more extreme phenotype associated with inactivation of *csrR* compared to *csrS* is not unexpected, since complete loss of the presumed transcriptional regulator CsrR may have a greater effect on target gene transcription than does loss of the sensor that modulates the phosphorylation state of CsrR. That mutation of either gene of the GBS *csrRS* locus produced similar changes in target gene expression provides compelling evi-

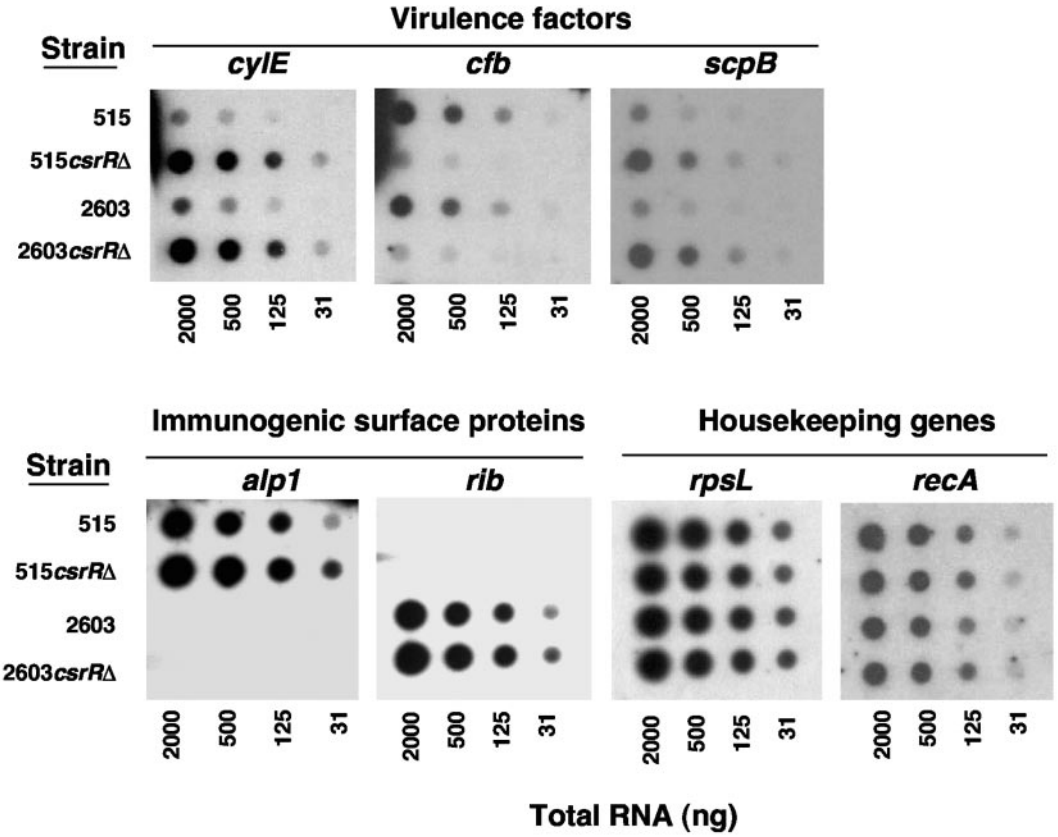

FIG. 3. Regulation of virulence factor gene expression in CsrR mutant strains. Autoradiography of RNA blot hybridizations demonstrates increased *cylE* (beta-hemolysin) and *scpB* (C5a peptidase) transcripts, reduced *cfb* (CAMP factor)transcript, and slightly increased *alp1* (alpha-like protein 1) and *rib* (protein Rib) transcripts in CsrR mutant strains 515*csrR*Δ and/or 2603*csrR*Δ. Indicated amounts of RNA were blotted onto nylon membranes, which were then hybridized with ^{32}P-labeled DNA probes from the indicated genes. No significant difference was observed between wild-type and mutant strains in expression of control genes *recA* and *rpsL*. Each blot is representative of two to four independent experiments.

dence that the locus encodes a functional two-component system.

Effect of CsrRS inactivation on GBS virulence. The experiments summarized above indicate that the GBS CsrRS system regulates expression of at least three virulence determinants. It seems likely that such regulation of virulence factor expression enhances the fitness of GBS during infection. In order to examine the potential contribution of CsrRS-mediated gene regulation to GBS pathogenesis, we compared relative virulence of the CsrR mutants with the wild-type strains from which they were derived. Groups of adult mice were challenged by intraperitoneal injection with various doses of GBS, and survival was monitored over 5 days. These studies demonstrated significantly reduced virulence of both 515*csrR*Δ and 2603*csrR*Δ strains compared to their respective wild-type parent strains (Table 3). The results were particularly striking for strain 515*csrR*Δ: wild-type strain 515 was highly virulent in this model system with a LD_{50} of 423 CFU, whereas the LD_{50} for 515*csrR*Δ was more than 500-fold higher. The LD_{50} of the repaired strain, 515*csrR*rep, was 890 CFU, a value not significantly different from that for wild-type strain 515 ($P = 0.41$). That replacement of the mutant allele with the wild-type *csrR* gene in strain 515*csrR*rep restored wild-type virulence confirmed that the striking attenuation of 515*csrR*Δ was due to inactivation of *csrR* and not to an unrelated second mutation. Wild-type strain 2603 was considerably less virulent than strain 515, but even in this relatively avirulent background, inactivation of *csrR* resulted in a 20-fold increase in LD_{50} compared to the wild-type strain (Table 3). These data provide in vivo evidence that the CsrRS system plays an important role in pathogenesis of GBS infection.

It was also of interest to evaluate the relative effect on

TABLE 1. Effects of *csrR* mutation on expression of GBS virulence factors and immunogenic surface proteins

Product or function	Gene	Expression in CsrR mutant(s)
Virulence factors		
Hemolysin/cytolysin	*cylE*	Increased
C5a peptidase	*scpB*	Increased
CAMP factor	*cfb*	Reduced
Laminin binding protein	*lmb*	No significant change
Capsular polysaccharide	*cpsE*	No significant change
Immunogenic surface proteins		
Protein Rib (strain 2603)	*rib*	Slightly increased
Alpha-like protein 1 (strain 515)	*alp1*	Slightly increased
Protein Sip	*sip*	No significant change

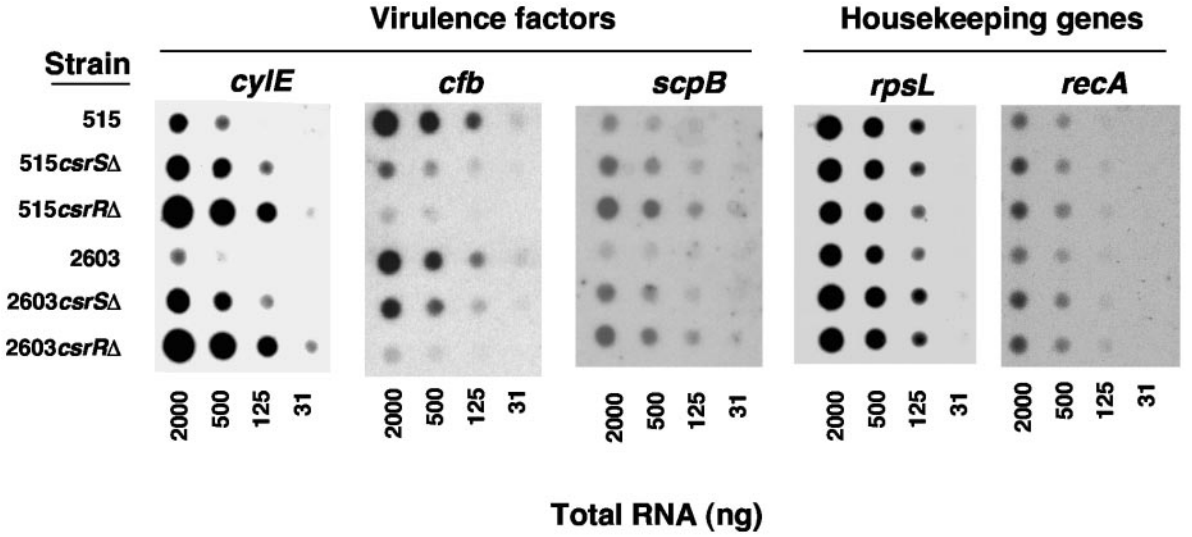

FIG. 4. Regulation of virulence factor gene expression in CsrS mutant strains. Compared to results for the corresponding CsrR mutant strains, hybridizations of RNA from CsrS mutant strains 515Δ*csrS* and 2603Δ*csrS* demonstrate similar, but less marked, increases in *cylE* (beta-hemolysin) and *scpB* (C5a peptidase) transcripts and a less marked reduction in *cfb* (CAMP factor) transcript. Hybridizations were performed as described in the legend to Fig. 3. No significant difference was observed between wild-type and mutant strains in expression of control genes *recA* and *rpsL*. Each blot is representative of two to four independent experiments.

virulence of inactivating the sensor component, CsrS, compared to that of the regulator, CsrR. Because the virulence phenotype of the *csrR* mutation was more pronounced in strain 515 than in strain 2603, we chose to make this comparison in the 515 background. Strain 515*csrS*Δ, while substantially less virulent than the parent strain 515, was slightly more virulent than 515*csrR*Δ in the mouse model of systemic GBS infection. Experiments that compared directly the mouse virulence of the two mutant strains demonstrated a difference of approximately 10-fold in LD_{50}s: 4.59×10^5 for 515*csrR*Δ and 4.95×10^4 for 515*csrS*Δ. The relative virulence of the mutants parallels the pattern of effects on virulence gene expression associated with inactivation of CsrS versus CsrR. However, because the mouse model does not recapitulate all aspects of human infection, it is possible that inactivation of CsrS might have a more profound effect on virulence during natural infection.

DISCUSSION

A survey of the two recently completed GBS genomes revealed a relatively large number of two-component regulatory systems, including one that displayed a particularly high degree of similarity to CsrRS, a multigene regulatory system in *S. pyogenes*. Results of the present investigation provide clear evidence that an orthologous CsrRS system in GBS controls expression of at least three GBS products implicated in virulence. To our knowledge, this is the first description of a two-component system shown to regulate expression of multiple GBS virulence factors and to affect virulence during experimental infection.

Whereas the *S. pyogenes* system represses expression of all the major virulence factors it is known to control, the results of the present study indicate evidence of both up- and down-regulation of different virulence determinants by GBS CsrRS. These results imply that the GBS CsrRS system has divergent effects on different target genes or that it has regulatory activity on another regulator that, in turn, acts on one or more regulated structural genes. Interaction of the CsrRS system with other regulators could be part of a regulatory cascade or network in which multiple elements link an environmental stimulus with a series of downstream responses. The possibility of one or more intermediate regulators could explain the observed up (CAMP factor)- and down (beta-hemolysin and C5a

TABLE 2. Fold change in transcription of regulated virulence genes in CsrR and CsrS mutants relative to wild-type GBS strain 515 or 2603

Mutant strains	Fold change in transcription of virulence gene[a]		
	cylE	*cfb*	*scpB*
515*csrR*Δ	18 (7.2–28)	0.018 (0.016–0.019)	11 (5.6–16)
515*csrS*Δ	5.1 (3.9–6.3)	0.16 (0.13–0.19)	3.8 (3.1–4.5)
2603*csrR*Δ	50 (46–53)	0.038 (0.022–0.062)	34 (14–54)
2603*csrS*Δ	8.8 (6.3–11)	0.38 (0.18–0.60)	7.1 (3.1–11)

[a] Data are expressed as a ratio of the amount of transcript detected in the mutant strain divided by that detected in the corresponding wild type. Values are means from at least two independent experiments. Values in parentheses are the ranges.

TABLE 3. Virulence of CsrR mutant and wild-type strains in mice after intraperitoneal challenge

Strain	LD_{50} (95% confidence interval)	*P* value[a]
515 (wild type)	4.23×10^2 (2.19×10^2–8.13×10^2)	
515*csrR*Δ	2.42×10^5 (1.29×10^5–4.57×10^5)	<0.0001
515*csrR*rep	8.90×10^2 (4.07×10^2–1.91×10^3)	0.41
2603 (wild type)	3.22×10^5 (8.32×10^4–1.23×10^6)	
2603*csrR*Δ	6.57×10^6 (1.48×10^6–2.57×10^7)	0.005

[a] *P* value comparing the value for the mutant strain to the value for the wild-type strain.

peptidase)-regulatory effects on different target genes. Certain well-characterized systems involve both activation and repression of target genes by a single response regulator, for example OmpR in *E. coli* and BvgA in *Bordetella pertussis* (5, 32). By contrast, the *S. pyogenes* CsrR protein appears to act primarily or exclusively as a repressor by binding to promoter regions upstream of regulated genes (7, 9, 18). While it is possible that the GBS CsrR protein regulates target promoters directly but with opposite effects, it seems at least equally likely that one or more of the regulated genes might be controlled by another (CsrR-regulated) regulatory system. The more complex pattern of regulation may explain why GBS CsrR mutants were attenuated in experimental infection, while *S. pyogenes* CsrR mutants were more virulent than the wild type (11, 16).

The repertoire of regulated genes is also different in the two species—both CAMP factor and C5a peptidase are also produced by *S. pyogenes*, but in contrast to GBS, their expression is not significantly altered by inactivation of *csrR* (9). Conversely, CsrR mutants in *S. pyogenes* display increased expression of the *has* operon that directs synthesis of the hyaluronic acid capsule, whereas expression of the capsular polysaccharide biosynthesis operon in GBS appears not to be controlled by the CsrRS system. Therefore, although CsrRS appears to be an important multigene regulator for both *S. pyogenes* and GBS, the GBS CsrRS system differs significantly with respect to its dual roles as an activator and repressor of virulence factor expression, the repertoire of regulated genes, and its overall role in pathogenesis.

An important but still unanswered question is identification of the environmental signal(s) to which the GBS CsrRS system responds. Extracellular magnesium concentration was recently shown to signal through the *S. pyogenes* CsrRS system, presumably by binding to the extracellular domain of the CsrS protein (10). However, since the extracellular domain of the GBS CsrS protein has only limited similarity to that of the *S. pyogenes* protein, it is possible, and perhaps likely, that the extracellular ligand(s) for the GBS system is distinct from that for *S. pyogenes*. The fact that the CsrRS system controls expression of several products implicated in infection suggests that the system may serve as a means for GBS to perceive the local host environment and adapt to it by altering expression of multiple factors that modulate bacterium-host interactions. Further investigation of this novel regulatory system is warranted to identify the environmental signals that interact with CsrS and to characterize further the role of the CsrRS system in pathogenesis of GBS infection.

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