The CsrR/CsrS two-component system of group A *Streptococcus* responds to environmental Mg^{2+}

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Group A streptococci control expression of key virulence determinants via the two-component sensor/regulator system CsrR/CsrS. The membrane-bound sensor CsrS is thought to respond to previously unknown environmental signal(s) by controlling phosphorylation of its cognate regulator component CsrR. Phosphorylation of CsrR increases its affinity for binding to the promoter regions of Csr-regulated genes to repress transcription. Here we show that environmental Mg^{2+} concentration is a potent and specific stimulus for CsrR/CsrS-mediated regulation. We studied the effect of divalent cations on expression of the Csr-regulated hyaluronic acid capsule genes (*hasABC*) by measuring chloramphenicol acetyltransferase (CAT) activity in a reporter strain of group A *Streptococcus* carrying a *has* operon promoter-*cat* fusion. Addition of Mg^{2+}, but not of Ca^{2+}, Mn^{2+}, or Zn^{2+}, repressed capsule gene expression by up to 80% in a dose-dependent fashion. The decrease in capsule gene transcription was associated with a marked reduction in cell-associated capsular polysaccharide. RNA hybridization analysis demonstrated reduced expression of the Csr-regulated *hasABC* operon, streptokinase (*ska*), and streptolysin S (*sagA*) during growth in the presence of 15 mM Mg^{2+} for the wild-type strain 003CAT but not for an isogenic *csrS* mutant. We propose that Mg^{2+} binds to CsrS to induce phosphorylation of CsrR and subsequent repression of virulence gene expression. The low concentration of Mg^{2+} in extracellular body fluids predicts that the CsrR/CsrS system is maintained in the inactive state during infection, thereby allowing maximal expression of critical virulence determinants in the human host.

Pathogenic bacteria are able to adapt rapidly in the host by up-regulating expression of gene products necessary for survival in the specific environmental niche defined by the site of infection. In the human host, environments encountered by bacteria can vary widely, for example, from the external skin to mucosal surfaces of the airway or the intestine, or deeper tissues and the bloodstream. Therefore, adaptation and survival of the bacteria hinge on their ability to probe the environment and respond appropriately. Group A *Streptococcus* (GAS or *Streptococcus pyogenes*) can produce a spectrum of clinical syndromes in humans that range from superficial infection of the pharyngeal mucosa to invasive infection of deep tissues or the bloodstream. GAS elaborate a repertoire of cell-associated (capsular polysaccharide, M protein, extracellular matrix binding proteins) and secreted products (pyrogenic exotoxins, enzymes, cytotoxins) that contribute to the pathogenesis of infection (reviewed in ref. 1). Regulated expression of these products may augment the organism's survival and its virulence in specific niches within the infected host.

Expression of several GAS virulence determinants is controlled by the CsrR/CsrS two-component regulatory system (also called CovR/CovS) (2–5). First reported as a regulator of the *has* operon that directs synthesis of the hyaluronic acid capsule, the Csr system also controls expression of secreted and cell-associated GAS proteins including streptokinase (*ska*), the integrin-like protein/IgG protease, Mac or IdeS (*mac* or *ideS*), streptolysin S (*sag* operon), and streptodornase (*speMF*) (4–7). Csr-mediated regulation of cysteine protease (*speB*) has been observed in some strains, but not in others (2, 4, 5). DNA microarray analysis suggests the system influences, directly or indirectly, expression of 15% of chromosomal genes (8).

Of 13 putative two-component systems identified in the GAS genome, the CsrR/CsrS system is the best characterized to date and, together with the FasBCA system, one of only two such systems linked to virulence (9, 10). By analogy to well characterized two-component sensor/regulator bacterial systems, the predicted extracellular domain of the membrane-bound sensor component CsrS is expected to bind an extracellular ligand or to sense an environmental condition. This interaction is predicted to trigger autophosphorylation of the cytoplasmic domain of the CsrS protein. The phosphate group is then transferred on to the receiver domain of the cognate regulator CsrR. The regulator component CsrR, when phosphorylated, shows increased binding affinity for the promoters of several genes proposed to be regulated by the system, including the *has* operon promoter (3, 6, 11). Both CsrR and CsrS mutants exhibit increased transcription of Csr-regulated genes, a phenotype that indicates the system acts to repress target gene expression through its regulator component, CsrR.

A critical issue in understanding the potential function of the Csr system in virulence gene regulation *in vivo* is the identification of the environmental signal(s) sensed by CsrS. As is the case with many bacterial two-component systems, the identity of such signal(s) for the Csr system has, until now, been completely unknown. In this investigation we used a GAS reporter strain that carries a fusion of the *has* operon promoter to the gene encoding chloramphenicol acetyltransferase (*cat*) to identify the environmental signal(s) that interact with the sensor component CsrS. The results of these studies, together with experiments measuring transcription of other Csr-regulated genes, support a model in which environmental Mg^{2+} binds to CsrS to trigger the phosphorelay that results in repression of Csr-regulated genes.

Materials and Methods

Bacterial Strains and Growth Conditions. GAS strains used in this study are listed in Table 1. DLS003 is an encapsulated M-type 3 strain isolated from a patient with necrotizing fasciitis (12). Derivative strain 003CAT carries on its chromosome a fusion of a 688-bp fragment, encompassing its autologous *has* operon promoter sequences with the 5′ portion of *hasA*, to the *cat* sequence (13). GAS was grown at 37°C in Todd-Hewitt broth (Difco) supplemented with 0.5% yeast extract (THY), or on THY agar supplemented with 5% defibrinated sheep blood. For cloning experiments, *Escherichia coli* DH5α was grown in Luria–Bertani (LB) broth (Difco) or on LB agar. When appropriate, antibiotics were added at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml for *E. coli*, 300 μg/ml for

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Abbreviations: GAS, group A *Streptococcus*; CAT, chloramphenicol acetyltransferase.

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Table 1. GAS strains used in this study

GAS strain	M type	Ref./source
DLS003	3	12
003CAT (DLS003*has*$_p$-*cat*)	3	13
003*csrS*Ω	3	This study
DLS003*hasA*::ΩKm-2	3	12
87–764	3	Edward Kaplan, University of Minnesota
950802	3	Edward Kaplan
87–282	18	36
282CAT (87–282*has*$_p$-*cat*)	18	13
DLS048	1	Dennis Stevens, University of Washington

GAS; erythromycin, 200 μg/ml for *E. coli*, 1 μg/ml for GAS; chloramphenicol, 20 μg/ml for *E. coli*, 5 μg/ml for GAS.

CAT Assays. GAS cytoplasmic extracts were prepared as described (13) with slight modifications. Briefly, bacteria grown overnight on THY-blood agar were inoculated in THY broth and collected by centrifugation (1,250 × *g*; 8 min) at mid-exponential phase growth (A_{600} = 0.3–0.4) unless otherwise stated. Pellets were washed in 1 ml Tris (10 mM, pH 7.8) and resuspended in 180 μl of the same buffer, to which 20 μl (≈15 units) of mutanolysin was added. Bacteria were treated for 1 h at 37°C and then centrifuged at 18,000 × *g* for 5 min, and the supernatant carrying cytoplasmic extracts was collected. Determination of CAT activities in 3–5 μg of total protein extract was performed by using the spectrophotometric assay described by Shaw (14). To control for the effects of the different chemicals used on both mutanolysin and CAT activity, control bacterial cultures received the chemical studied immediately before collection of samples. Cytoplasmic extracts from the non-CAT expressing wild-type GAS strains DLS003 and 87-282 did not produce any background CAT activity, nor did the CAT-expressing strains 003CAT and 282CAT when chloramphenicol, the acetyl acceptor in the enzymatic reaction, was omitted from the reaction mix. Results were recorded as milliunits of CAT per mg total cell protein per min (mu/mg/min).

Measurement of GAS Cell-Associated Capsular Polysaccharide. Cell-associated hyaluronic acid was measured as described (15) with slight modifications. Briefly, 6 ml of GAS culture grown in THY to mid-exponential phase (A_{600} = 0.35–0.4) were harvested by centrifugation, washed in 10 mM Tris buffer (pH 7.5), resuspended in 300 μl of the same buffer, and mixed on a vortex mixer in an equal volume of chloroform. The hyaluronic acid content of the aqueous phase was then measured spectrophotometrically at 640 nm by using 1-ethyl-2-[3-(1-ethylnaptho-[1,2-*d*]thiazolin-2-ylidene)-2-methylpropenyl]-naptho-[1,2-d]-thiazolium bromide (Stains-All, Sigma). The values shown represent the values obtained for each strain tested minus the background values obtained by using the acapsular mutant strain DLS003*hasA*::ΩK_m−2 (12).

Measurement of Total Magnesium and Calcium Concentrations in Growth Media. Magnesium and calcium concentration measurements in THY broth were performed at the Brigham and Women's Hospital Chemistry Laboratory (Boston) by using an Olympus AU640 analyzer according to the manufacturer's instructions. Mg^{2+} bound to xylidyl blue at pH 11. 4 was measured bichromatically at 520/800 nm, whereas Ca^{2+} bound to Arsenazo III (2,2-[1,8-dihydroxy-3,6-disulphonaphthylene-2,7-bisazo]-bisbenzenearsonic acid) at pH 6.5 was measured bichromatically at 660/800 nm. To control for the accuracy of the measurements, magnesium and calcium concentration measurements in chemically defined medium were determined in parallel.

DNA Techniques. Plasmid pCR2.1 is a linearized *E. coli* vector used for direct cloning of PCR products (Invitrogen); pUC19 is a high copy number cloning vector (16); pBRΩK_m−2 is a pBR322 derivative carrying the ΩK_m−2 cassette (17); and pJRS233 is a temperature sensitive *E. coli*-Gram-positive shuttle vector provided by June Scott (18). Plasmid DNA was obtained from *E. coli* by using the Qiagen miniprep or maxiprep kit according to manufacturer's instructions. GAS chromosomal DNA was prepared as described (19). Restriction endonuclease digestions, DNA ligations, transformations of chemically competent *E. coli*, and Southern hybridizations (ECL, Pharmacia) were performed by using standard protocols (20). GAS electrocompetent cells were prepared and transformed by using Gene Pulsar II (Bio-Rad) as described (13).

PCR. PCR was performed with *Taq* DNA polymerase (Life Technologies) on a GeneAmp PCR 2400 System (Perkin–Elmer) for 32 cycles at the following conditions: 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min. Primers used in PCR are shown in Table 3, which is published as supporting information on the PNAS web site, www.pnas.org.

Construction of a *csrS* Mutant. The sensor component gene *csrS* of GAS strain DLS003 was inactivated by inserting in its 5′ end the kanamycin resistance cassette ΩK_m−2. In brief, a 1.1-kb DNA fragment encompassing the 3′ end of *csrR* and the 5′ end of *csrS* was amplified from DLS003 chromosomal DNA by using primers csrR-F1 and csrS-R1 (Table 3). The amplicon was cloned into vector pCR2.1 (Invitrogen), released by *Eco*RI digestion and ligated into pUC19 to give plasmid pUC*csrS*. The ΩK_m−2 cassette obtained from vector pBRΩK_m−2 (17) by *Sma*I digestion was then inserted into the *Eco*RV site 310 bp downstream of the *csrS* start codon. The resulting *csrS*ΩK_m−2 construct was released by *Pvu*II digestion and cloned into *Sma*I-digested pJRS233 (18) to give plasmid pJR*csrS*ΩK_m−2. The fidelity of the final construct containing the 5′ end of *csrS* disrupted with ΩK_m−2 was verified by DNA sequencing. pJR*csrS*ΩK_m−2 was introduced into DLS003 by electroporation and the *csrS* mutant obtained as described (2). Replacement of the wild-type *csrS* copy with *csrS*ΩK_m−2 and loss of pJRS233 sequences was verified by Southern hybridization.

DNA Sequencing. Sequencing of plasmid DNA was performed at the Brigham and Women's Hospital Automated Sequencing and Genotyping Facility. Primers used for sequencing are listed in Table 3.

RNA Dot Hybridizations. Total bacterial RNA was isolated by using the Rneasy mini kit (Qiagen) according to manufacturer's instructions, except that bacteria were lysed by shaking with glass beads on a dental amalgamator (Patterson Brand). RNA samples were treated with 5 units of DNase I (Invitrogen) for 30 min at room temperature to remove any contaminating DNA. The final concentrations of the RNA obtained were adjusted to 100 ng/μl, and the samples were divided into aliquots and frozen at −80°C until needed. RNA was blotted onto Hybond N^+ membranes (Pharmacia) by using the Bio-Dot Microfiltration apparatus (Bio-Rad). DNA probes were generated by PCR using 003CAT chromosomal DNA as template, and were radiolabeled with [α-^{32}P]dATP by random priming using the RadPrime DNA Labeling System (Invitrogen). The primer pairs used to generate probes for *rpsL*, *recA*, *emm3*, *csrR*, *csrS*, *hasB*, *ska*, *sagBCD*, and *speB* are listed in Table 3. Hybridizations were carried out in

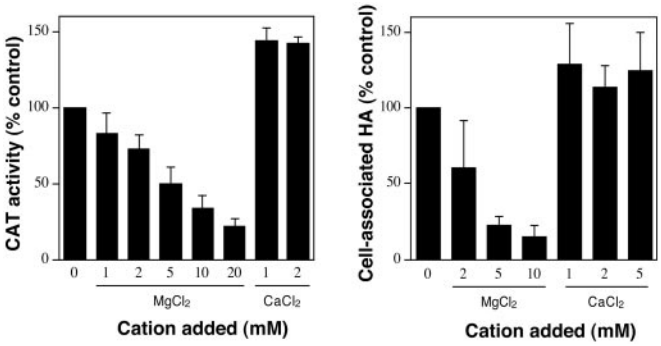

Fig. 1. Effect of Mg^{2+} or Ca^{2+} on GAS capsule gene expression. GAS strain 003CAT was grown to mid-exponential phase with or without supplemental $MgCl_2$ or $CaCl_2$. Capsule gene expression levels were measured by CAT assay (*Left*) and cell-associated hyaluronic acid was quantified (*Right*). Data are represented as percent of the values obtained in the absence of added Mg^{2+} or Ca^{2+}. Data points represent mean ± SD from at least three independent experiments.

Church buffer at 60°C for 16 h followed by one wash in 1× SSC − 0.1% SDS at room temperature and three washes in 0.5× SSC − 0.1% SDS at 60°C (20). Negative controls consisted of 2 μg total *E. coli* RNA; positive controls carried 0.3 ng of unlabeled denatured probe DNA in addition to 2 μg total *E. coli* RNA.

Results

GAS Capsule Gene Expression Is Repressed at High Mg^{2+} Concentrations. In an effort to identify the signal(s) that regulate virulence gene expression in GAS through the two-component system CsrR/CsrS, we studied the effect of divalent cations on expression of the *csr*-regulated capsule gene operon (*hasABC*). Divalent cations such as Mg^{2+} and Ca^{2+} are required for fundamental biochemical reactions in bacteria, but also function as environmental stimuli to which two-component systems of certain pathogenic species respond by altering expression of genes involved in virulence (21–23). Capsule gene expression levels in GAS were quantified by measuring CAT activity in cell extracts of strain 003CAT that carries on its chromosome a fusion of its autologous *has* operon promoter to the gene encoding chloramphenicol acetyltransferase (13). To determine whether the divalent cation concentration influenced capsule gene expression, we compared the CAT activity in extracts of 003CAT grown in unsupplemented broth with that from the same strain grown in broth supplemented with additional amounts of one of four divalent cations: Mg^{2+}, Ca^{2+}, Mn^{2+}, or Zn^{2+}. During growth in THY broth, GAS capsule gene expression is maximal at mid-exponential phase after which it declines to a very low level in stationary phase. Maximum CAT activity consistent with high capsule gene expression was observed in cell extracts of 003CAT obtained at mid-exponential phase growth (see below). However, when 003CAT was grown in broth supplemented with $MgCl_2$ (or $MgSO_4$), capsule gene expression was reduced in a dose-dependent fashion. CAT activities for bacteria grown in 5 and 20 mM additional $MgCl_2$ were reduced by 50% and 78%, respectively, compared with those for bacteria grown in THY broth without additional $MgCl_2$ (Fig. 1). Growth rate, viability, and final cell density reached at stationary phase remained unaffected. Capsule gene expression did not increase when the Mg^{2+} concentration in THY broth was lowered by the addition of EDTA. Instead, capsule gene expression decreased, as reflected by a 3-fold reduction in CAT activity in extracts of bacteria grown in THY-0.5 mM EDTA. This reduction in capsule gene expression was not attributable to Mg^{2+} depletion, however, because wild-type CAT activity was restored during growth in THY-0.5 mM EDTA supplemented with 0.5 mM $CaCl_2$ (data not shown). Therefore, capsule gene expression levels were maximal in THY broth without additional Mg^{2+}. The total Mg^{2+} concentration in THY broth was measured at 0.97 ± 0.02 mM, a level that is similar to the physiological Mg^{2+} concentrations encountered by GAS in human serum and extracellular body fluids (0.7–1 mM) (24).

The reduced CAT activity of 003CAT at high $MgCl_2$ concentrations correlated with reduced capsule production: the amount of cell-associated capsular polysaccharide measured on cells grown in 5 and 10 mM additional $MgCl_2$ was 22% and 15%, respectively, of the amount on cells grown in THY broth without additional $MgCl_2$ (Fig. 1). Repression of capsule expression was also evident during growth of 003CAT on THY-blood agar supplemented with $MgCl_2$. Colonies on medium supplemented with 10 mM $MgCl_2$ were small and dry, an appearance indistinguishable from that of colonies of an isogenic acapsular mutant strain DLS003*hasA*::ΩK_m−2 (12).

Down-regulation of capsule gene expression was specific to Mg^{2+} as no repression was evident when bacteria were grown in THY broth supplemented with 5 mM or 10 mM additional $CaCl_2$. On the contrary, capsule gene expression increased by ≈40% when an additional 1 or 2 mM $CaCl_2$ was provided (Fig. 1). Because the total concentration of calcium in unsupplemented THY broth was measured at 0.36 ± 0.02 mM, addition of 1 mM $CaCl_2$ produced a total Ca^{2+} concentration near the physiological range found in human serum and extracellular body fluids (1–1.3 mM) (25). Addition of Mn^{2+} up to 0.5 mM or Zn^{2+} up to 0.1 mM did not alter capsule gene expression levels or the amount of cell-associated hyaluronic acid. Addition of either of the two cations at higher concentrations reduced bacterial viability as determined by counts of colony-forming units, growth rates, and final cell densities reached in culture at stationary phase.

Csr-Regulated Genes Are Repressed by High Mg^{2+} Concentrations in Strain 003CAT. To investigate whether expression of other Csr-regulated genes was repressed at high Mg^{2+} concentrations, we studied expression not only of the hyaluronic acid synthesis genes (*hasABC*), but also of streptokinase (*ska*) during exponential phase and streptolysin S (*sagA*) during stationary phase. Expression of both Csr components, CsrS and CsrR, was also monitored at both growth phases. In addition, the effect of Mg^{2+} on expression of M-type 3 protein (*emm3*) that is regulated by a Csr-independent mechanism was studied (4, 5, 26). GAS strain 003CAT was grown in THY broth with or without supplemental $MgCl_2$ (15 mM), and total RNA was collected at mid-exponential (A_{600} = 0.3) and stationary (A_{600} = 0.8) phase. Four-fold serial dilutions of total RNA were blotted onto nylon membranes and hybridized to a radiolabeled DNA probe specific for each gene or operon of interest. The probe for the capsule gene cluster was to the middle gene (*hasB*) of the *has* operon (27). The probe for streptolysin S was to the gene cluster *sagBCD* located downstream of the streptolysin S structural gene (*sagA*) in the *sagABCDEFGHI* operon. Streptolysin S activity requires expression of the genes downstream of *sagA*; therefore, transcript levels for *sagBCD* reflect streptolysin activity more accurately than *sagA* transcript itself (28). As a control, the RNA was also probed for two housekeeping genes, *rpsL* and *recA*, neither of which is known to be regulated by Csr. As shown in Fig. 2, expression of *rpsL* and *recA* was not altered during either exponential or stationary phase in the presence of 15 mM additional $MgCl_2$. By contrast, growth in supplemental Mg^{2+} was associated with dramatic repression of the Csr-regulated capsule and streptokinase genes at exponential phase and of streptolysin S at stationary phase. Based on the relative intensity of the hybridization signals, the amount of mRNA produced under high Mg^{2+} conditions was estimated to be reduced 4- to 16-fold for each of the three genes compared with that from cells grown in unsupplemented broth. As expected, expression of streptoki-

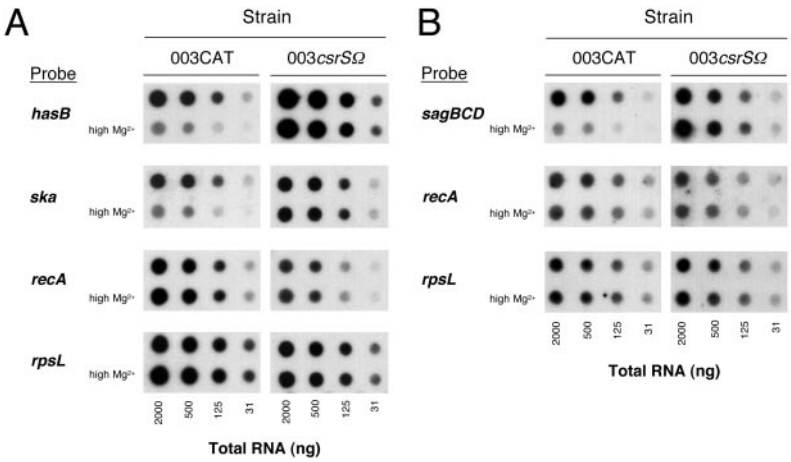

Fig. 2. Effect of Mg^{2+} on expression of Csr-regulated genes. Dot blots of total GAS RNA were hybridized to radiolabeled DNA probes specific to the indicated genes, then exposed to x-ray film. GAS wild-type strain 003CAT and the isogenic CsrS mutant strain 003*csrS*Ω were grown in unsupplemented THY or in THY supplemented with 15 mM $MgCl_2$ (high Mg^{2+}). (*A*) RNA from GAS collected at exponential phase (A_{600} = 0.3). (*B*) RNA collected at stationary phase (A_{600} = 0.8).

nase and capsular polysaccharide was maximal at exponential phase and very low or absent in stationary phase, whereas the converse was true for streptolysin S. The finding that Mg^{2+} repressed not only capsule gene expression, but also expression of streptokinase and streptolysin S suggests strongly that the effect was mediated by the Csr system.

Conflicting data have been reported regarding Csr regulation of *speB* and of the *csrRS* locus itself. Whereas *speB* expression is repressed by Csr in some strains, it appears not to be affected by Csr in others, including DLS003 (2, 4). CsrR was previously reported to repress its own expression, as increased *csrRS* transcript levels were observed in a *csrR* mutant (4). However, a subsequent study indicated that phosphorylated CsrR did not bind to its own promoter and therefore did not repress its own expression directly (6). In the present investigation, we found the transcription levels of *speB* and of the *csrRS* operon, as well as expression of the Csr-independent *emm3*, remained unaffected at high Mg^{2+} concentrations (data not shown).

Expression of Csr-Regulated Genes Is Not Repressed by High Mg^{2+} Concentrations in a *csrS* Mutant. If the hypothesis is correct that Mg^{2+} down-regulates gene expression by signaling through the CsrR/CsrS system, Csr-regulated genes that are repressed by Mg^{2+} in the wild-type strain 003CAT should be unaffected by high Mg^{2+} in a mutant strain that lacks the CsrS sensor. To test this prediction, we constructed an isogenic sensor component mutant (003*csrS*Ω) by inserting the ΩK_m−2 cassette (17) within the coding sequence of *csrS* near the 5′ end of the gene. In contrast to wild-type strain 003CAT, the sensor component mutant strain showed no decrease in production of capsular polysaccharide at Mg^{2+} concentrations up to 10 mM (Fig. 3). In further experiments, we examined the expression of Csr-regulated genes in 003*csrS*Ω grown in the presence or absence of 15 mM $MgCl_2$. As shown previously for *csr* mutants, expression of *hasABC*, *ska*, and *sagABCDEFGHI* during growth in unsupplemented medium was increased relative to that for wild-type strain 003CAT. However, in contrast to the results obtained with 003CAT, growth of the *csrS* mutant in 15 mM Mg^{2+} had no effect on expression of the Csr-regulated genes, *hasABC*, *ska*, or *sagABCDEFGHI* (Fig. 2). The fact that inactivation of *csrS* abrogated Mg^{2+} regulation provides strong evidence that Mg^{2+} signals repression of Csr-regulated gene expression through its interaction with the membrane-bound sensor protein, CsrS.

Kinetics of Mg^{2+}-Mediated Capsule Gene Repression. To study the kinetics of transcriptional regulation in response to a change in Mg^{2+} concentration, we grew duplicate cultures of 003CAT to early exponential phase (A_{600} = 0.15) at which point one culture was exposed to 15 mM $MgCl_2$. Sequential bacterial samples were collected from both Mg^{2+}-treated and untreated cultures and CAT activity was determined at each time point. As shown in Fig. 4, the rate of growth was similar with or without supplemental $MgCl_2$. CAT activity for bacteria grown in THY with no additional Mg^{2+} increased from early exponential phase to reach a maximum 90 min later and then dropped sharply at early stationary phase, a pattern that is also reflected in the amount of cell-associated hyaluronic acid produced (Fig. 4). In contrast, CAT activity in bacterial samples collected after addition of $MgCl_2$ remained relatively unchanged for the first 15 min and then declined over time. At 60 min and 90 min after addition of $MgCl_2$, CAT activities were reduced by 71% and 75%, respectively, compared with those recorded at the same time points for

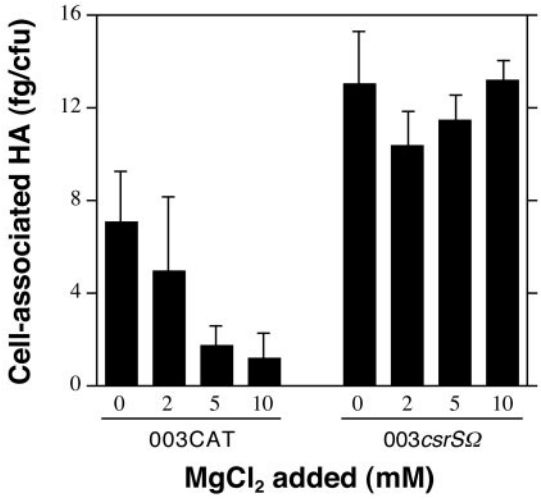

Fig. 3. Effect of Mg^{2+} on capsular polysaccharide production by wild-type strain 003CAT and the isogenic CsrS mutant strain, 003*csrS*Ω. Data represent the amount of cell-associated hyaluronic acid produced by GAS cells grown to mid-exponential phase in THY containing the indicated concentrations of supplemental $MgCl_2$. Values represent mean ± SD of at least three independent experiments.

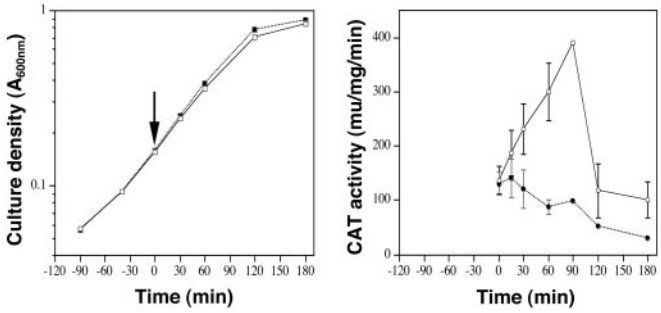

Fig. 4. Kinetics of Mg^{2+}-mediated capsule gene repression in GAS. Duplicate cultures of strain 003CAT were grown in THY to early exponential phase (arrow, Time 0), then one culture was supplemented with 15 mM $MgCl_2$ (filled symbols) and the other was not (open symbols). Growth curves for the two cultures were similar (*Left*). CAT activity, reflecting capsule gene expression, is shown for GAS cell extracts of the two cultures collected at the indicated time points (*Right*). Data points represent mean ± range for two independent experiments.

bacteria grown without additional $MgCl_2$. These results indicate that repression of Csr-regulated gene transcription occurs within minutes of a change in environmental Mg^{2+}.

High Mg^{2+} Concentrations Repress Capsular Polysaccharide Expression in Diverse GAS Isolates. Because the *csr* system is highly conserved among GAS strains, we reasoned that Mg^{2+}-dependent repression of capsule synthesis should be demonstrable in other GAS strains in addition to 003CAT. To test this hypothesis, we examined capsule production during growth in THY supplemented with 10–20 mM $MgCl_2$ in a series of GAS isolates of various M protein types. We tested capsule gene expression in strain 282CAT, derived from the heavily encapsulated strain 87-282 (M type 18), that also carries a fusion of its autologous *has* operon promoter to *cat* (13). CAT activity in 282CAT extracts from exponential phase cells grown in THY were 2- to 3-fold higher than those for 003CAT extracts, consistent with the higher amounts of cell-associated hyaluronic acid produced by the former strain (13) (see below). Similar to the results obtained for M type 3 strain 003CAT, capsule gene expression in 282CAT decreased dramatically during growth in broth supplemented with $MgCl_2$. Somewhat higher concentrations of $MgCl_2$ were required to achieve maximum repression of capsule gene expression in 282CAT compared with 003CAT: 20 mM $MgCl_2$ was required for a 3-fold decrease in CAT activity for 282CAT, whereas 10 mM $MgCl_2$ produced a similar decrease in the less encapsulated strain 003CAT. Because the *has* operon is transcribed more actively in 282CAT than in 003CAT, it is possible that higher $MgCl_2$ concentrations are necessary to produce sufficient amounts of phosphorylated CsrR for maximal repression of gene expression in 282CAT. Reduced capsule gene expression by 282CAT during growth in high Mg^{2+} correlated with markedly reduced production of cell-associated hyaluronic acid (Table 2). Similar Mg^{2+}-dependent repression of capsule synthesis was also observed for three additional GAS strains of M types 1 and 3 (Table 2).

Table 2. Cell-associated hyaluronic acid expression by wild-type GAS strains grown in THY with or without additional Mg^{2+}

Strain	M type	Cell-associated HA (fg/cfu) THY	THY + $MgCl_2$	% Reduction
003CAT	3	7.0 ± 0.7	0.97 ± 0.2	86
87-764	3	22 ± 1.3	7.8 ± 4.3	64
950802	3	2.6 ± 1.5	0	100
DLS048	1	4.0 ± 0.7	0.82 ± 0.3	80
282CAT	18	200 ± 21	58 ± 14	71

cfu, colony-forming unit.

Discussion

The evidence presented here suggests that environmental magnesium regulates virulence gene expression by GAS through the CsrR/CsrS two-component system. The fact that high Mg^{2+} concentrations stimulated repression of Csr-dependent virulence genes in the wild-type GAS strain but not in an isogenic CsrS mutant points to a direct interaction of Mg^{2+} with the putative sensor component protein. The interaction is likely to involve the extracellular domain of CsrS in a fashion analogous to that described for the periplasmic domain of the PhoQ Mg^{2+} sensor of *Salmonella enterica* serovar Typhimurium (21, 29). Both CsrS and PhoQ belong to the EnvZ family of sensor kinases, and CsrS is similar to PhoQ in several ways: the two sensor proteins are of similar size; each has two predicted membrane spanning domains near the N terminus; both have a predicted extracellular or periplasmic domain of similar size (146 and 151 aa for PhoQ and CsrS, respectively). The two proteins share 43% similarity over 300 aa by BLASTP analysis (30). Within the predicted extracellular domains of the two proteins, there is particularly striking similarity in acidic residues, which have been implicated in cation binding. Ten of 20 acidic amino acids within the predicted extracellular domain of CsrS are similar or identical in PhoQ (Fig. 5, which is published as supporting information on the PNAS web site). PhoQ of *E. coli* has been proposed to bind Mg^{2+} on its periplasmic domain via a cluster of seven amino acids, six of which are acidic (31). Such a cluster is also found in the PhoQ periplasmic domain of *S. enterica* serovar Typhimurium, but not in some other PhoQ homologues that are also able to respond to Mg^{2+} (23, 32). Thus, a specific motif of acidic residues required for Mg^{2+} binding has not been defined.

Although there are striking parallels between CsrR/CsrS and the PhoP/PhoQ system, there are also important differences that may reflect the specific adaptive functions of these regulatory systems for pathogens that employ different strategies for survival within their animal or human hosts. In *S. enterica* serovar Typhimurium, binding of Mg^{2+} to PhoQ promotes dephosphorylation of PhoP, which renders it inactive as a transcriptional activator of PhoP-induced genes (23). By contrast, binding of Mg^{2+} to CsrS results in repression of CsrR-regulated genes, presumably by increasing phosphorylation of CsrR. So, although the net result in both cases is repression of virulence gene expression by high Mg^{2+}, repression is achieved through opposite effects on phosphorylation of the respective regulator proteins. Another important difference between the two systems is the range of magnesium concentrations to which they respond: maximal repression of PhoP-induced genes in *Salmonella* occurs during growth in Mg^{2+} concentrations of ≈1 mM or greater, whereas the Csr system is fully active in repressing gene expression only at Mg^{2+} concentrations ≥10 mM. It has been suggested that responsiveness of the PhoP/PhoQ system is optimized for survival of *Salmonella* within the phagosome where Mg^{2+} concentrations are likely to be in the micromolar range (33). Although GAS may enter a variety of eukaryotic cells, they fail to proliferate intracellularly and their intracellular survival is limited (15, 34). The Mg^{2+} concentration at which the Csr-regulated genes are maximally expressed (≈1 mM) is approximately that of human extracellular fluids, as might be expected for an extracellular pathogen. Furthermore, whereas one of the functions of the PhoP/PhoQ system is induction of expression of two magnesium importer systems (MgtA, MgtCB) to permit *Salmonella* survival in very low Mg^{2+} concentrations, no such system for scavenging magnesium has been identified in GAS. Rather, the CsrR/CsrS system appears

to be optimized to ensure maximum expression of virulence factors under conditions encountered by GAS in mucosal secretions and extracellular fluids and not under conditions of higher Mg^{2+} concentrations such as the natural environment outside the body.

Expression of the GAS genes that direct synthesis of capsular polysaccharide, streptokinase, and streptolysin S was strikingly repressed in the presence of high Mg^{2+} concentrations. However, expression of the two Csr components in the same conditions was not affected. Although some studies have suggested autoregulation of the *csr* system, the CsrR protein appears not to interact directly with the *csr* operon promoter (6). The apparent lack of Mg^{2+} regulation of the *csr* locus itself suggests that the repressive activity of the system depends primarily on the phosphorylation state of CsrS which governs phosphorylation of CsrR, rather than on the relative abundance of the CsrS and CsrR proteins. Repression of capsule gene expression in GAS subjected to high Mg^{2+} concentrations was evident within minutes. Such rapid repression is consistent with the proposed model of Mg^{2+} interacting with CsrS already present on the cell membrane and initiation of the phosphorelay cascade. Magnesium is abundant in the natural environment and may signal GAS to adapt for survival in the external environment. Signaling through the Csr system may permit GAS to rapidly down-regulate production of host-specific virulence factors and divert energy to alternative metabolic pathways important for survival outside the human host.

The *csr* system response appears to be specific to Mg^{2+}, because other divalent cations tested did not affect capsule gene expression at nontoxic concentrations. Whereas high Mg^{2+} resulted in down-regulation of Csr-regulated genes, we observed a modest increase in capsule gene expression as the Ca^{2+} concentration was increased to a level similar to those found in human extracellular fluids. It remains to be determined whether the GAS response to Ca^{2+} is Csr-dependent. If so, both Mg^{2+} and Ca^{2+} concentrations encountered by GAS in the human host are ideal for maximum Csr-regulated gene expression.

Although the experiments presented here were performed *in vitro*, Mg^{2+}-dependent regulation of virulence gene expression by GAS is expected to operate also *in vivo* during infection. Expression of Csr-regulated virulence genes was maximal, hence, the *csr* system was minimally induced, at $\approx$1 mM Mg^{2+}, a concentration that is similar to the physiologic Mg^{2+} levels in body fluids such as mucosal secretions and the blood (24). A previous study demonstrated that expression of the Csr-regulated *has* operon is rapidly induced after introduction of GAS into the mouse peritoneum or the baboon pharynx (35). Although the precise stimulus for up-regulation of capsule gene expression was not defined, the results are consistent with those of the current investigation. In recent experiments, we also observed Mg^{2+}-dependent repression of capsule gene expression in GAS grown in 10% or 50% human plasma (data not shown). Thus, it appears unlikely that other factor(s) in plasma inhibit the down-regulatory effects of high magnesium on Csr-regulated genes. Rather, the low concentrations of Mg^{2+} at both deep tissue and mucosal sites ensure that CsrR will remain in the unphosphorylated state that has low affinity for Csr-regulated promoters, thereby permitting high-level expression of the *has* operon and other Csr-regulated virulence genes.

Identification of magnesium as a specific environmental stimulus that controls Csr-mediated regulation of GAS virulence determinants expands our understanding of the complex interactions between GAS and the human host. Successful adaptation of a pathogen requires dynamic modulation of metabolic activities and expression of critical virulence determinants in a manner that optimizes bacterial survival in the various environmental conditions the organism confronts. Our data implicate environmental Mg^{2+} as a stimulus that signals GAS to up-regulate expression of Csr-regulated virulence factors under conditions encountered during human infection. To our knowledge, this is the first specific signal demonstrated to interact with any two-component regulatory system in this important human pathogen.

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