CcpA and LacD.1 affect temporal regulation of Streptococcus pyogenes virulence genes

Colin C. Kietzman  
*Washington University School of Medicine in St. Louis*

Michael G. Caparon  
*Washington University School of Medicine in St. Louis*

Follow this and additional works at: [https://digitalcommons.wustl.edu/open_access_pubs](https://digitalcommons.wustl.edu/open_access_pubs)

**Recommended Citation**  
[https://digitalcommons.wustl.edu/open_access_pubs/2502](https://digitalcommons.wustl.edu/open_access_pubs/2502)

This Open Access Publication is brought to you for free and open access by Digital Commons@Becker. It has been accepted for inclusion in Open Access Publications by an authorized administrator of Digital Commons@Becker. For more information, please contact vanam@wustl.edu.
CcpA and LacD.1 Affect Temporal Regulation of *Streptococcus pyogenes* Virulence Genes

Colin C. Kietzman and Michael G. Caparon*

Department of Molecular Microbiology, Washington University School of Medicine, Saint Louis, Missouri 63110-1093

Received 1 July 2009/Returned for modification 17 August 2009/Accepted 13 October 2009

Production of \( \text{H}_2\text{O}_2 \) follows a growth phase-dependent pattern that mimics that of many virulence factors of *Streptococcus pyogenes*. To gain greater insight into mechanisms coupling virulence factor expression to growth phase, we investigated the molecular basis for \( \text{H}_2\text{O}_2 \) generation and its regulation. Deletion of the gene encoding lactate oxidase (*lctO*) or culture in the presence of glucose eliminated \( \text{H}_2\text{O}_2 \) production, implicating carbohydrate regulation of *lctO* as a key element of growth phase control. In examining known carbohydrate-responsive regulators, deletion of the gene encoding CcpA but not that encoding LacD.1 resulted in both derepression and an uncoupling of *lctO* transcription from its growth phase pattern. Expanding this analysis to additional virulence factors demonstrated both negative (*cfa*, encoding CAMP factor) and positive (*speB*, encoding a cysteine protease) regulation by CcpA and that CcpA mutants were highly cytotoxic for cultured macrophages. This latter property resulted from enhanced transcription of the streptolysin S biogenesis operon. Examination of CcpA-promoter interactions using a DNA pull-down assay mimicking physiological conditions showed direct binding to the promoters of *lctO* and *speB* but not those of *sagA*. CcpA but not LacD.1 mutants were attenuated in a murine model of soft-tissue infection, and analysis of gene expression in infected tissue indicated that CcpA mutants had altered expression of *lctO*, *cfa*, and *speB* but not the indirectly regulated *sagA* gene. Taken together, these data show that CcpA regulates virulence genes via at least three distinct mechanisms and that disruption of growth phase regulation alters transcriptional patterns in infected tissues.

The restriction of expression of a gene to a specific phase of the bacterial growth cycle is known as growth phase regulation and is a common feature of pathogen gene expression when examined in vitro. This mode of regulation is typically multifactorial, requiring integration of temporal cues linked directly to the growth cycle, with multiple environmental cues, including those that are characteristic of the naïve environment and those that are altered by subsequent bacterial growth (reviewed in reference 67). Of the environmental cues, the former class is composed typically of physical attributes, like oxygen tension, temperature, and specific growth substrates, while the latter class often includes depletion of nutrients and the accumulation of specific quorum-sensing molecules and other metabolites (reviewed in reference 67). It is generally assumed that growth phase-patterned pathways of gene expression observed in vitro reflect adaptations that a successful pathogen makes. However, establishing this link requires identification of specific gene regulatory elements, their hierarchical relationships, and whether the regulatory network responds in a similar pattern in vivo.

Growth phase regulation likely plays a central role in the ability of *Streptococcus pyogenes* (group A streptococcus) to cause disease. This gram-positive bacterium is the causative agent of numerous diseases of soft tissue ranging from those that are self-limiting (e.g., pharyngitis) to those that are destructive and life-threatening (e.g., necrotizing fasciitis), as well as serious postinfectious sequelae, such as rheumatic fever and acute glomerulonephritis (reviewed in reference 15). Considerable evidence has accumulated to suggest that when examined in vitro, regulation of virtually all of its recognized virulence factors involves a growth phase component. Furthermore, while changes in transcript stability do contribute to growth phase-associated changes (2), most alteration in transcript levels is controlled by regulation of transcription initiation. For example, the CovRS (CsrRS) two-component regulator and the “stand-alone” transcription regulator Mga control expression of 15% and 10%, respectively, of all chromosomal genes, including multiple important virulence-associated surface proteins and toxins (reviewed in references 33 and 43). A prominent characteristic of CovRS and Mga regulation is their growth phase-dependent pattern (33, 43). Both of these regulators also respond to specific environmental signals, including carbon dioxide (Mga [9]), Mg\(^{2+}\), and antimicrobial peptides (CovS [27, 28]). However, as has been noted (2), growth phase control for these regulators is epistatic to the specific signal, such that a temporal pattern of regulation is manifested even when a specific signal is present throughout the growth cycle. Thus, how temporal control is integrated with the processing of specific signals in *S. pyogenes* is not well understood.

Relatively more progress has been made in understanding the cues that control the timing of expression of growth phase-regulated genes. As a lactic acid bacterium, *S. pyogenes* has a relatively simple fermentative metabolism, so it is not surprising that nutritional cues have emerged as leading candidates. Examples include both CodY- and RelA-dependent and -independent pathways, which act to couple growth phase regul-
lation to the availability of amino acids as growth substrates (53, 70, 71). Similarly, several mechanisms have been described which could link carbohydrate availability to expression of the Mga regulon. These include the presence of dual phosphatases in the transferase system regulation domains in Mga that function to modulate the activity of regulatory proteins in response to sugar transport and the control of mga transcription itself by the major carbon catabolite repressor protein CcpA (reviewed in reference 33). These observations implicate nutrient availability as an important signal for controlling the timing of growth phase regulation during infection.

The link between the timing of growth phase regulation and carbohydrate availability has been demonstrated more definitively by studies that have directly compared gene expression in infected tissue with that observed during in vitro culture. Comparison of global gene expression between S. pyogenes growth during infection of muscle versus both in vitro biofilm and planktonic growth revealed that the overall pattern of in vivo gene expression most closely resembled that of planktonic culture in medium restricted for carbohydrates (14). Correlations had the highest significance when in vivo expression patterns were compared with in vitro cultures sampled during the early stationary phase of growth (14). Using the gene that encodes the SpeB cysteine protease as a model gene, conditions were identified, including growth phase, that influenced expression of speB transcription in vitro (47). Analyses of global gene expression in response to these conditions identified a set of coregulated genes whose patterns of expression were significantly correlated with that of speB in vivo, suggesting that this cohort of genes was coregulated by the same growth phase-responsive transcriptional program (47).

It was subsequently found that this regulatory program is under the control of LacD.1 (49), an aldolase enzyme that has been adapted to function exclusively as a component of a transcriptional regulatory pathway (48). Mutational analyses suggested that LacD.1 has been adapted to function as a sensor of its substrates, which are the central intermediates of the Embden-Meyerhoff-Parnas glycolytic pathway, implicating LacD.1 as a regulator of global carbon catabolite control (48). This idea was supported by the observation that glucose and certain other carbohydrates are also signals processed by the pathway (48, 49). In most species of firmicutes, the transcriptional regulator CcpA has been recognized as the central regulator of carbon catabolite repression (reviewed in reference 72). However, mutational analyses have shown that the LacD.1 pathway functions independently of the canonical CcpA pathway (48, 49).

Taken together, the data presented above implicate carbohydrate availability as an important signal for the control of growth phase regulation in infected tissue. However, the existence of two pathways for carbon catabolite repression has complicated our understanding of how carbohydrates may regulate the timing of growth phase regulation. At issue is that like LacD.1, the CcpA pathway also monitors the flow of carbon through glycolysis (72). It is not clear why two independent pathways function to process what is essentially the same input signal. Several recent studies have confirmed the role of the S. pyogenes CcpA pathway in global regulation and have suggested that the pathway contributes to virulence (41, 68). However, how this pathway contributes to growth phase regulation, its relationship to the LacD.1 pathway, and how these pathways may interact during infection of tissue is not clear. In the present study, we extended our approach of using model genes for analysis of the CcpA pathway. Our goal was to identify and characterize several suitable model genes for analysis of the contribution of the pathway in growth phase regulation both in vitro and in infected tissue. This analysis revealed at least one model gene for each of the following categories: (i) genes negatively regulated by CcpA, (ii) genes positively regulated by CcpA, (iii) genes regulated indirectly by CcpA, and (iv) genes regulated by both LacD.1 and CcpA. Characterization of regulation of these model genes demonstrated that CcpA plays an important role in linking growth phase regulation and carbohydrate availability during infection.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The Escherichia coli strain TOP10 (Invitrogen) was used for cloning using standard molecular biology techniques. The S. pyogenes strain HSC5 (29) and various isogenic mutants (see below) were grown in Todd Hewitt broth (THYB) with 0.2% yeast extract (Difco), C medium (51), or RPMI 1640 (Lonza) supplemented with 10% fetal bovine serum (FBS) (Lonza). Routine growth was conducted using sealed culture tubes incubated under static conditions at 37°C. Where indicated, cultures were grown under conditions of enhanced aeration in 125-ml Erlenmeyer flasks containing 10 ml of medium subjected to orbital shaking (225 rpm) at 37°C. Streplococcal strains grown on solid medium containing 1.4% Bacto agar (Difco) were cultured in a sealed jar with a commercial gas generator (GasPak catalogue no. 70304; BBL). Stationary phase was defined as the point when the optical density at 600 nm (OD600) did not change after 20 min. Approximate final OD600 values are noted. When appropriate, antibiotics were added to media at the following concentrations: kanamycin, 500 μg/ml for S. pyogenes and 1 μg/ml for E. coli; and erythromycin, 1 μg/ml for S. pyogenes and 500 μg/ml for E. coli. DNA and computational techniques. Plasmid DNA was isolated by standard techniques and used to transform E. coli as described previously (10). Transformation of S. pyogenes was performed by electroporation as previously described (10). Restriction endonucleases, ligases, and polymerases were used according to the manufacturers’ recommendations. Chromosomal DNA was purified from S. pyogenes as previously described (10). The fidelity of all plasmid constructs was confirmed by DNA sequencing, which was performed by a commercial service (SeqWright, Houston, TX). All references to genomic loci are based on the genome of strain SF370 (17). Analyses of homology were conducted using BLAST (1, 21). Analysis and identification of CRE sites were carried out using Vector NTI (Invitrogen) and by allowing for one mismatched base pair from the consensus binding site elucidated in Bacillus subtilis.

Measurement of H2O2 and hemolytic titer. Measurement of H2O2 and hemolytic titer. Accumulation of H2O2 in culture supernatant was measured as previously described (20) and on solid medium modified by the addition of horseradish peroxidase (200 μg/ml; Sigma) and 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, 3 mg/ml; Sigma) following overnight culture under anaerobic conditions and exposure to ambient air for approximately 1 h at 37°C. Determination of hemolytic titers was conducted following overnight culture in RMPI 1640 with 10% FBS that was subjected to centrifugation, filtered through 0.22-μm filters (Millipore), and then assessed using defibrinated rabbit erythrocytes (Hemostat Labs, CA) as described previously (62). Where indicated, the streptolysin S (SLS)-specific inhibitor trypan blue (Sigma) and streptolysin O (SLO)-specific inhibitor cholesterol (Sigma) were added to undiluted supernatants at concentrations of 50 μg/ml and 25 μg/ml, respectively. Hemolytic titer is represented as the inverse of the greatest dilution in which approximately 50% hemolysis was observed.

Construction of CcpA− and LetO− mutants. In-frame deletion mutations in the genes encoding CcpA (SPy_0514) and LetO (SPy_0414) were generated using allelic replacement and the PCR primers listed in Table S1 in the supplemental material. The deletion alleles were transferred to the HSC5 chromosome using allelic replacement and the PCR primers listed in Table S1 in the supplemental material. The deletion alleles were confirmed by PCR.

Isolation of RNA and real-time RT-PCR. Total RNA was isolated from strains grown in the media and at the times during the growth cycle indicated in the text, as previously described (6). RNA was subjected to reverse transcription (RT) using Superscript II (Invitrogen) per the manufacturer’s instructions. Real-time PCR was performed on the isolated RNA using SYBR Green PCR Master Mix (Applied Biosystems) in a volume of 25 μl containing 400 ng of RNA, 1× of SYBR Green master mix, 1× of the PCR primers, and 200 μM of each dNTP. The PCR conditions were as follows: 1 cycle of 30 s at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 55°C, and 1 min at 72°C.
RT-PCR analysis of cDNA samples was performed using IQ SYBR green Supermix (Bio-Rad) using the primers listed in Table S1 in the supplemental material. Relative transcript levels were determined using the threshold cycle method, with the 
cmact transcript as a standard, as described previously (5). Data presented are the means and standard errors of the mean derived from triplicate determinations of samples prepared from at least two independent experiments. Differences between mean values were tested for significance using the paired Student’s t test as described previously (5).

Biotinylated DNA pulldowns. A biotinylated DNA fragment pull-down assay was adapted from the method of Grundling et al. (25) as follows: biotinylated DNA fragments were generated by PCR using 5’ biotinylated primers (Invitrogen) (see Table S1 in the supplemental material) and HSCS genomic DNA. Unincorporated primers were removed by gel purification of PCR products using a commercial reagent (QIAquick gel extraction kit; Qiagen). A 100-μl aliquot of a solution of streptavidin-coated magnetic beads (Dynabeads M-270 streptavidin; Invitrogen) was then coated with 3 μg of biotinylated DNA per the manufacturer’s instructions. A whole-cell soluble protein extract was produced as follows: 40-ml cultures of HSC5 were grown to mid-exponential phase (OD600 of 0.2) in THYB. Cells were harvested by centrifugation (5,000 × g, 5 min, 4°C), and the cell pellet was washed twice with 5 ml of lysis buffer (10 mM Tris-HCl, pH 7.5; 50 mM NaCl) and then resuspended in 1 ml of lysis buffer with EDTA-free complete mini protease inhibitor cocktail (Roche). The resuspended cells were then disrupted using glass beads (G4649; Sigma) in a high-speed reciprocating shaking device (FastPrep 100; MBI) at a setting of 6.0 for 40 s. Debris and undisrupted cells were removed by centrifugation (14,000 × g, 5 min, 4°C), and the total protein concentration of the cleared supernatant was determined using a bicinchoninic acid assay (Pierce/Thermo-Fisher). The protein concentration was diluted to 1 mg/ml, glycerol was added to a final concentration of 20%, and the solution was stored at −20°C until use. For the pulldown, an aliquot of DNA-coated beads (200 μl, prepared as described above) was mixed with the protein extract (1 mg) and binding buffer (10 mM Tris-HCl [pH 8.0], 100 mM KCl, 3 mM MgCl2, 20 mM EDTA, 5% glycerol, 40 μg/ml sheared salmon sperm DNA [Invitrogen], and 10 μg/ml bovine serum albumin [Sigma]) and added to a total volume of 1 ml. Following a 30-min incubation at room temperature with gentle mixing on a rotating mixer, the beads were collected using a magnetic particle separator (catalog number K1585; Invitrogen) for 1 min at room temperature, washed four times with 500 μl binding buffer, resuspended in 30 μl of 1× DNase I buffer (Invitrogen), boiled for 5 min, and then allowed to slowly cool to room temperature. The DNA probes were then removed by incubation with DNase I (2 μl, AMP grade; Invitrogen) for 10 min. Protein sodium dodecyl sulfate (SDS) sample buffer (1×, 50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 2% [wt/vol] SDS, 0.1% [wt/vol] bromphenol blue, 10% glycerol) was then added in a 2× dilution from a 6× stock, and the sample was boiled for an additional 5 min. The magnetic beads were removed from the magnetic particle separator, and the resulting sample was subjected to electrophoresis using a 12% SDS-polyacrylamide gel electrophoresis gel. Protein bands were visualized by staining with Sypro ruby (Molecular Probes/Invitrogen) per the manufacturer’s instructions. The band(s) of interest were excised and then identified by matrix-assisted laser desorption ionization-time of flight (mass spectrometry) by the Proteomics Core at Washington University (http://proteomics.wustl.edu/sitemap). Preparation and infection of mouse bone marrow-derived macrophages. Bone marrow-derived macrophages were prepared as previously described (11). Following 8 days of culture in RPMI 1640 with 20% FBS and 30% L-cell-preconditioned medium, macrophages were harvested by incubation in ice-cold phosphate-buffered saline (PBS) (Cellgro) and then gently scraped. The macrophages were harvested by centrifugation (1,000 × g, 10 min, 4°C), resuspended in RPMI 1640 with 10% FBS, and plated in six-well tissue culture plates (TPP) at 3 × 106 macrophages per well in 4 ml total medium. The macrophages were allowed to settle on the plates for 4 h at 37°C in 5% CO2. The various streptococcal strains were cultured overnight in THYB, diluted to an OD600 of 0.05 in 40 ml RPMI 1640 with 10% FBS, and allowed to grow for an additional 2.5 h. The streptococcal strains were then collected by centrifugation and resuspended in RPMI 1640 with 10% FBS to a final OD600 of 0.87. An aliquot of the bacterial suspension (150 μl) was added to each macrophage-containing well and incubated at 37°C in 5% CO2 for the times indicated in the text. At the conclusion of the infection, the media were removed and each well was washed with 2 ml cold PBS. The PBS was then removed, and cells were stained using a fluorescent reagent to assess viability (Live/Dead for mammalian cells; Invitrogen) per the manufacturer’s instructions. Cells were imaged using a Leica DMIRE2 fluorescence microscope, and images were analyzed using OpenLab software (Improvision). For each tissue culture well, at least 200 macrophages were examined. The data presented represent the means and standard errors of the mean derived from triplicate determinations. Differences between mean values were tested for significance with the paired Student’s t test.

Subcutaneous infection of mice. As previously described (6, 8), 5- to 6-week-old female SKH1 hairless mice (Charles River Labs) were injected subcutaneously with 107 CFU of streptococci of the strains indicated in the text, and the areas of the resulting ulcers were quantitated on day 3 postinfection as described previously (6b). Briefly, images of resulting lesions were analyzed using MetaMorph (Improvision) image analysis software to determine the area contained by the irregular border of each ulcer. The data presented are representative of at least two independent experiments conducted with 10 mice in each experimental group. Differences in the areas of the resulting ulcers were tested for significance by the Mann-Whitney U test (22). RNA was harvested from selected ulcers and subjected to analysis using real-time RT-PCR to assess expression of various streptococcal genes using the primers listed in Table S1 in the supplemental material and the methods described previously (7).

RESULTS

Lactate oxidase is responsible for the glucose-repressible loss of viability in stationary phase. Mutants of S. pyogenes deficient in NADH oxidase (Nox, SPy_1150) produced self-lethal levels of H2O2; however, the addition of glucose both suppressed this lethality and the production of H2O2 (20). It has also been observed that many clinical isolates of S. pyogenes produce auto-intoxicating amounts of H2O2 upon entry into the stationary phase of growth when grown under aerobic conditions (63, 66). These data suggest that peroxide production is both glucose and growth phase regulated. Biochemical analyses of S. pyogenes have suggested that H2O2 is produced by an enzymatic activity consistent with a lactate oxidase (66). Examination of the prototypic S. pyogenes M1 genome revealed a single gene (lctO, SPy_0414) with significant homology to the characterized lactate oxidase gene of Streptococcus iniae (19). This locus is also present in 13/13 sequenced S. pyogenes strains and the wild-type (WT) strains used previously to study Nox (20). An in-frame deletion of lctO was constructed in one of these latter strains (HSC5), and the resulting LctO− mutant (CKB044) was found to produce colonies of normal size compared to HSC5 (WT) when grown anaerobically; however, these colonies failed to produce H2O2 when subsequently exposed to air (Fig. 1A). When grown anaerobically in liquid culture, the WT strain rapidly lost viability upon onset of stationary phase, though loss of viability could be suppressed by addition of glucose (Fig. 1C). In contrast, the LctO− strain was protected from a loss of viability in a glucose-independent manner (Fig. 1C) that correlated with failure to produce any detectable amounts of H2O2 in stationary phase, compared to the millimolar amounts produced by the WT strain (Fig. 1B). As previously observed, addition of glucose suppressed the ability of WT bacteria to produce H2O2 (Fig. 1B). The defect in H2O2 production apparent in the LctO− mutant was complemented by an lctO allele expressed constitutively off a plasmid (see Fig. S1 in the supplemental material).

Glucose and growth phase regulate transcription of lctO. The data presented above suggest that LctO is responsible for H2O2 production and the glucose-inhibited, growth phase-dependent loss of viability in aerobic conditions. To examine the basis of the latter phenomenon, the transcription of lctO in the WT strain was examined using real-time RT-PCR. Relative to early exponential phase (OD600 of 0.05, approximately 2 h postinoculation), the transcript abundance of lctO stays low through mid-exponential-phase growth (OD600 of 0.2, approx-
imately 4 h postinoculation) and then undergoes a large up-regulation upon the onset of stationary phase (OD of 1.15, approximately 6.5 h postinoculation) (Fig. 2A). In late stationary phase the transcript abundance of *lctO* was still relatively high (OD of 1.15 approximately 7.5 h postinoculation) (Fig. 2A), suggesting that when cultures have reached a maximum OD, there is a large dynamic shift toward increased transcription of *lctO* that persists throughout stationary phase. To test if glucose was a signal controlling transcription of *lctO*, WT streptococci were grown in a carbohydrate-poor medium (*C* medium). The addition of glucose in exponential phase resulted in a dose-dependent and saturable repressive effect on *lctO* transcript abundance (Fig. 2B). At low levels of glucose the transcription was relatively high, while the addition of 0.1% or greater amounts of glucose repressed *lctO* transcript abundance several-hundredfold (Fig. 2B). Further experiments showed that growth while shaking in ambient air (to increase O₂ tension) or exposure to oxidative stress in the form of added H₂O₂ changed *lctO* transcript abundance less than two-fold (Fig. 2B, Air and H₂O₂).

**FIG. 1.** Endogenous self-lethal H₂O₂ production is dependent on *LctO*. (A) WT and *LctO* strains were grown on H₂O₂ indicator solid medium as described in the text. Production of H₂O₂ is indicated by colorimetric change to purple in the medium. (B) WT and *LctO* strains were grown in ThyB or ThyB supplemented with 0.2% glucose (+ glu) aerobically. After growth overnight, supernatants were assayed for H₂O₂ concentration, and data are presented as the means and standard errors of results of two independent experiments carried out in triplicate. Asterisks denote values below the limit of detection (approximately 10 μM). (C) Strains were grown as described in the legend for panel B, samples were taken at indicated times, and appropriate dilutions were plated on solid ThyB medium to count viable CFU. The asterisk indicates a value of less than 100 CFU/ml of culture. Strains: WT, HSC5; *LctO*, CKB044.

**FIG. 2.** Transcription of *lctO* is regulated by growth phase and glucose concentration. (A) WT bacteria were grown in C medium plus 0.2% glucose anaerobically to determine transcription of *lctO* during growth in broth culture. Growth of streptococci was measured by OD. Total RNA was harvested at the indicated times, and *lctO* transcript abundance was measured by real-time RT-PCR. Data are presented as the ratios of transcript abundance at the indicated time points compared to transcript levels at the first time point (2 h) and represent the means and standard deviations from a representative experiment carried out with three samples per time point, each analyzed in triplicate. (B) WT bacteria were grown with varied glucose, aeration, and oxidative stress to test the effects on *lctO* transcription. WT bacteria were grown in C medium supplemented with the indicated amount of glucose, with C medium plus 0.2% glucose shaking in ambient air (Air), or with 100 μM H₂O₂ (H₂O₂) added 1 h prior to harvesting. Total RNA was harvested in early exponential phase (OD of 0.1) and *lctO* transcript abundance determined by real-time RT-PCR. Data are presented as the ratios of transcript abundance as noted (compared to C medium plus glucose for glucose analysis and compared to C medium plus 1% glucose for Air and H₂O₂) and represent the means and standard deviations derived from three samples, each analyzed in triplicate, compared to C medium without glucose (~glu), or for Air and H₂O₂, compared to C medium plus 0.2% glucose. WT strain, HSC5.
CepA binds to a conserved CRE site in the lctO promoter. (A) Diagram of the lctO locus and DNA probes generated by PCR for biotinylated DNA probe precipitations. Positions of DNA segments are shown as relative to the predicted start of translation of the lctO gene, with the A of the ATG start codon considered +1. Predicted CRE sites are indicated with black rectangles, and sequences of corresponding CRE sites are indicated above each site. Probes were created by PCR using HSC5 genomic DNA as a template and the primer pair CK195/CK196 (see Table S1 in the supplemental material). As indicated by the arrow, an CRE site is indicated with black rectangles, and sequences of corresponding CRE sites are indicated above each site. Probes were created by PCR using HSC5 genomic DNA as a template and the primer pair CK195/CK196 (see Table S1 in the supplemental material). (B) Biotinylated DNA probes were used to precipitate proteins from a lysate of the WT. The lctO probe A and a control non-specific fragment (NS) were created by PCR using HSC5 genomic DNA as a template and the following primer combinations: A, CK192/CK194; B, CK192/CK303; C, CK304/CK305; D, CK304/CK194; E, CK306/CK194; F, CK307/CK194. Primer sequences are listed in Table S1 in the supplemental material. (C) Biotinylated DNA probes spanning the locus and DNA probes generated by PCR for creating specific fragment (NS) were created by PCR using HSC5 genomic DNA as a template and the following primer combinations: A, CK192/CK194; B, CK192/CK303; C, CK304/CK305; D, CK304/CK194; E, CK306/CK194; F, CK307/CK194. Primer sequences are listed in Table S1 in the supplemental material.

CepA affects growth phase regulation of lctO in response to carbohydrate signals. A biochemical approach was used to identify potential growth phase and glucose-responsive regulators of lctO transcription. A biotinylated DNA probe that included the entire intergenic region between lctO and an upstream open reading frame (Fig. 3A, probe A) was incubated with whole-cell lysate prepared from mid-logarithmic cultures; the probe was then recovered by precipitation with streptavidin-coated beads. This analysis consistently revealed a protein band of approximately 37 kDa that was not precipitated with a nonspecific DNA probe, which was identified by matrix-assisted laser desorption ionization–time of flight (mass spectrometry) as the carbon catabolite control protein CepA (SPy_0514) (Fig. 3B). As a transcriptional repressor, CepA binds to an operator site termed a catabolite responsive element (CRE), and using the consensus site determined for Bacillus subtilis (73), it was possible to identify two potential CRE sites in the region encompassed by probe A (CRE-1, CRE-2) (Fig. 3A). However, analysis of additional probes that contained various sections of the intergenic region indicated that only those probes containing CRE-1 were capable of co-precipitating CepA, an observation supported by previous studies of CepA interaction with CRE elements in S. pyogenes (Fig. 3A and C, probes A, D, and E) (68).

The contribution of CepA to the regulation of lctO transcription was determined by analysis of a mutant strain constructed to contain an in-frame deletion allele of ccpA (CKB206). Two major effects were observed with the resulting CepA- mutant. Whereas the WT strain switched from a state of low lctO transcript abundance in exponential phase to high abundance in stationary phase, transcript levels in the CepA- strain were similar in both exponential and stationary growth and consistent with the high level seen in stationary phase of the WT strain (Table 1). The increase in lctO transcript abundance upon the onset of stationary phase was no longer apparent in the CepA- strain; rather, transcript abundance was relatively high and unchanged by growth phase (Table 1). Second, addition of glucose to the culture medium no longer caused a repressive effect on lctO transcription in the CepA- strain, differing from the WT strain for which transcript abundance was down over 100-fold in the presence of excess glucose (Table 1). Deletion of ccpA led to a loss of repression of lctO transcription and constitutively high transcript levels in the CepA- mutant. Therefore, we conclude that growth phase and glucose regulation of lctO transcription are due to the direct repressive effects of CepA.

CepA regulates virulence factors CAMP and SpeB in growth phase-dependent and -independent manners, respectively. Using the consensus CRE sequence from B. subtilis, we identified two additional genes in group A streptococci likely to be regulated by CepA. The gene for CAMP factor, cfa (SPy_1273), has a consensus CRE sequence in the promoter region (Fig. 4A) and is a predicted virulence factor found in many patho-

![Diagram of the lctO locus and DNA probes generated by PCR for biotinylated DNA probe precipitations.](image)

**FIG. 3.** CepA binds to a conserved CRE site in the lctO promoter. (A) Diagram of the lctO locus and DNA probes generated by PCR for biotinylated DNA probe precipitations. Positions of DNA segments are shown as relative to the predicted start of translation of the lctO gene, with the A of the ATG start codon considered +1. Predicted CRE sites are indicated with black rectangles, and sequences of corresponding CRE sites are indicated above each site. Probes were created by PCR using HSC5 genomic DNA as a template and the following primer combinations: A, CK192/CK194; B, CK192/CK303; C, CK304/CK305; D, CK304/CK194; E, CK306/CK194; F, CK307/CK194. Primer sequences are listed in Table S1 in the supplemental material. (B) Biotinylated DNA probes were used to precipitate proteins from a lysate of the WT. The lctO probe A and a control non-specific fragment (NS) were created by PCR using HSC5 genomic DNA as a template and the following primer combinations: A, CK192/CK194; B, CK192/CK303; C, CK304/CK305; D, CK304/CK194; E, CK306/CK194; F, CK307/CK194. Primer sequences are listed in Table S1 in the supplemental material. (C) Biotinylated DNA probes spanning the locus and DNA probes generated by PCR for creating specific fragment (NS) were created by PCR using HSC5 genomic DNA as a template and the following primer combinations: A, CK192/CK194; B, CK192/CK303; C, CK304/CK305; D, CK304/CK194; E, CK306/CK194; F, CK307/CK194. Primer sequences are listed in Table S1 in the supplemental material.

### Table 1. Growth phase and glucose-induced changes in transcript abundance of lctO are CepA dependent

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relative expression&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Stationary&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.0081 ± 0.0002</td>
<td>425.8 ± 79.1</td>
</tr>
<tr>
<td>CepA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>407.5 ± 60.6</td>
<td>388.0 ± 53.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are derived from real-time RT-PCR analysis of total cellular RNA and normalized to WT in exponential phase in unsupplemented C medium (−Glucose).

<sup>b</sup> Exponential OD<sub>600nm</sub> = 0.1; stationary OD<sub>600nm</sub> = 1.1, in C medium plus glucose. Growth in C medium: unsupplemented (−Glucose) or 0.1% (wt/vol) glucose (+Glucose).

<sup>c</sup> WT, HSC5; CepA<sup>c</sup>, CKB206.
Disruption of the CcpA regulatory pathway renders bacteria more cytotoxic, revealing an indirect mechanism of CcpA regulation of streptolysin S. Presumably pathogenic bacteria sense and respond to many host-derived signals during infection. We hypothesized that signals encountered during pathogenesis could control either CcpA or LacD.1 activity or both. To test this hypothesis, we infected murine bone marrow-derived macrophages with various streptococcal strains to examine changes in host cell response. Previous studies have shown that interactions with group A streptococci cause macrophages to upregulate many cytokines, such as tumor necrosis factor alpha, interleukin-1, and interleukin-6 (23). However, unlike infection with the WT strain, after infection with the CcpA− strain for 4 h, bone marrow-derived macrophages had regulator of virulence genes but functions with at least two distinct mechanisms: direct repression in response to growth phase and activation of transcription independent of growth phase.

CcpA binds the speB promoter. Since regulation of speB differed from our model gene, lctO, we examined whether CcpA bound directly to the speB promoter. Both in the control lctO fragment used earlier (Fig. 3A, probe A) and in the fragment indicated for the speB promoter (Fig. 4B), CcpA was coprecipitated from lysates of the WT strain of streptococci but not by using a nonspecific DNA probe (Fig. 4B). This suggests that direct interaction of CcpA within a promoter region can result in either activation or repression of transcription.

LacD.1 and CcpA do not both regulate lctO. Complicating the models of speB transcriptional regulation is the recent discovery of a novel carbohydrate sensing regulator, the adapted tagatose aldolase LacD.1 from group A streptococci that also controls transcriptional regulation of speB (49). In contrast to activation by CcpA, the LacD.1 pathway represses speB transcription. Since the mechanism of LacD.1-mediated repression of speB is largely unknown, it was possible that CcpA and LacD.1 acted in a linear pathway (49). To test this hypothesis, we examined the transcription of lctO in the CcpA− strain and a LacD.1− strain in comparison to WT regulation. Deletion of cpa abolished growth phase-dependent regulation of lctO, whereas inactivation of lacD.1 had no effect on lctO transcription compared to the WT strain (Fig. 5). If CcpA and LacD.1 function in a linear pathway, both deletions should have similar effects on lctO transcription. This suggests that CcpA and LacD.1 are at least partially independent but have opposing transcriptional effects on the speB promoter.

Table 2. CcpA affects virulence factor transcript abundance in both growth phase-dependent and -independent manners

<table>
<thead>
<tr>
<th>Gene</th>
<th>Exponentialb</th>
<th>Stationary</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WTc</td>
<td>CcpA−</td>
</tr>
<tr>
<td>cfa</td>
<td>6.76 ± 3.19</td>
<td>7.00 ± 3.15</td>
</tr>
<tr>
<td>speB</td>
<td>0.14 ± 0.03</td>
<td>6975 ± 1294</td>
</tr>
</tbody>
</table>

*Values derived from real-time RT-PCR analysis and normalized to these of the WT in exponential phase for each gene.

a Strains were grown in C medium plus glucose (0.1% [wt/vol]). Exponential OD600 of 0.1; stationary OD600 of 1.1.

b WT, HSC5; CcpA−, CKB206.
regulated to the same degree, it is likely that loss of CcpA regulation results in the entire sag operon being misregulated, not just the pel transcript, which is largely comprised of the intergenic and sagA regions (45).

Examination of the growth phase regulation of transcript abundance in the WT and CcpA \(^{-}\) strains revealed that in the WT strain the sagA transcript is upregulated in stationary phase, whereas in the CcpA \(^{-}\) strain this upregulation is not apparent (Fig. 6D). Taken with the previous findings, this suggests that the sag operon, like \(\text{letO}\) and \(\text{cfa}\), is repressed by CcpA in exponential phase and loss of CcpA leads to overproduction of SLS due to a derepression of transcription. Analysis of the sag promoter revealed a poorly conserved CRE sequence located upstream of the start of translation of sagA (Fig. 6E). This CRE site was not found in our earlier searches due to several mutations known to abolish CcpA binding in \(B.\ subtilis\) (38, 39). Using the biotinylated DNA pull-down assay with a DNA fragment from the sag promoter, we were unable to coprecipitate CcpA (Fig. 6E), whereas CcpA was coprecipitated with the \(\text{letO}\) DNA fragment using the same protein sample (Fig. 6E). To validate these findings in other serotypes of group A streptococcus, we repeated these DNA pull-down assays using a protein lysate from M1 strain SF370. The results from this pulldown confirm results from our WT strain (see Fig. S2 in the supplemental material). This suggests that regulation of the sag operon by CcpA occurs through an indirect mechanism. Due to the epistatic overproduction of SLS we were unable to determine if host responses by macrophages were altered by CcpA compared to responses to the WT strain.

**Regulation by LacD.1 and CcpA in infected tissue is distinct and closely resembles growth-phase regulation of CcpA in vitro.** Previous experiments have shown that CcpA functions as an important regulator of virulence genes, acting by at least three different mechanisms: (i) direct repression in response to growth phase, (ii) indirect repression in response to growth phase, and (iii) direct activation independent of growth phase. We analyzed the regulation of \(\text{letO}\), \(\text{cfa}\), \(\text{speB}\), and sagA in lesions from mice infected with WT, CcpA \(^{-}\), and LacD.1 \(^{-}\} strains in a subcutaneous infection model to better understand which mechanisms of regulation observed in vitro were similar to patterns of regulation observed during tissue infection. Briefly, immuno competent hairless mice were injected subcutaneously with \(10^7\) streptococci. In this model, as with many WT streptococcal strains, a local necrotic lesion forms, enlarging and forming an eschar, but does not progress to invasive disease (8). At 3 days postinfection, when lesions with WT bacteria were maximal, lesions were measured for area, a general indicator of overall strain virulence, and total RNA was extracted from excised lesions and subjected to real-time RT-PCR analysis of these streptococcal genes. Infection by the CcpA \(^{-}\} strain leads to significantly smaller lesions than those from the WT strain (Fig. 7A), suggesting that the CcpA \(^{-}\} strain is attenuated in this virulence model. Analysis of the transcript levels from these lesions showed that \(\text{letO}\) and \(\text{cfa}\) transcript levels were significantly increased in the CcpA \(^{-}\} strain compared to levels from WT lesions and that \(\text{speB}\) levels are significantly lower than those from WT lesions (Fig. 7B). This closely mirrors the results seen in vitro. Transcript levels of sagA were not significantly different (less than twofold change) between CcpA \(^{-}\} and WT lesions (Fig. 7B).
Lesions caused by the LacD.1/H11002 strain were not significantly different from those caused by the WT strain (Fig. 7C). In culture conditions, repression through LacD.1 results in a several-hundredfold reduction in speB transcript abundance (49). In samples from mouse lesions, the transcription of the speB gene in LacD.1/H11002 samples was only threefold higher than that in WT bacteria from skin lesions (Fig. 7D). This suggests that although certain in vitro conditions lead to LacD.1 activation, these same signals are not active in mouse lesions at this time point.

DISCUSSION

For many pathogens, virulence factor expression is coupled to growth phase by the transduction of extrinsic (environment-

<table>
<thead>
<tr>
<th>Strain</th>
<th>RPMI(^b)</th>
<th>ThyB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>Chol</td>
</tr>
<tr>
<td>WT</td>
<td>2</td>
<td>32</td>
</tr>
<tr>
<td>CcpA(^c)</td>
<td>2</td>
<td>16</td>
</tr>
</tbody>
</table>

\(\text{a} \) Values represent the reciprocal of the dilution of the supernatant in which 50% lysis was observed. NL, no lysis.

\(\text{b} \) Strains were grown in RPMI1640 with 10% FBS or ThyB to an OD\(_{600}\) of 0.1. Supernatant was sterile filtered and used in hemolytic assay. Cholesterol (25 mg/ml) or trypan blue (50 mg/ml) was added as noted.

\(\text{c} \) WT, HSC5; CcpA\(^c\), CKB206.
In this study we have characterized CcpA as a growth phase-dependent regulator of metabolic and virulence-associated genes and shown that growth phase-dependent regulation through LacD.1 and CcpA are distinguishable in mouse tissue. We have shown that CcpA can regulate transcription with three different mechanisms: direct repression, indirect repression, and direct activation. Our data suggest that the mechanism of CcpA-dependent regulation as observed in vitro has implications for regulation of target genes in infected tissues, thereby validating the study of in vitro signaling for understanding in vivo virulence responses. Furthermore, our results demonstrate a correlation between an altered pattern of virulence gene regulation in tissues and the attenuation of virulence, suggesting that growth phase-associated signals sensed through CcpA are important in pathogenesis.

Growth phase regulation of virulence factor transcription is found among many low-G+C gram-positive pathogens. Conserved regulators such as CodY, quorum-sensing systems, and alternative sigma factors coordinate complex transcriptional programs upon the onset of stationary phase in *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Enterococcus faecalis* (60, 69). However, while group A streptococci share the CodY response (53), they appear to lack a conserved quorum-sensing system (the signaling peptide SilCR is present in a minority of clinical isolates though enriched in invasive isolates [3, 4, 31]) and possess only one alternate sigma factor, which is not implicated in pathogenesis (58). Through studies of identified virulence factor regulators, it has been shown that CovR/CovS, Mga, RopB, LacD.1, and the vfr locus make significant contributions to the growth phase regulation of the *ska, emm*, and *speB* genes (16, 49, 51, 52, 54). However, mutations in CovR/CovS and LacD.1 do not completely uncouple their target genes from growth phase regulation, and the transcriptional activators RopB and Mga and the vfr locus are themselves growth phase regulated, suggesting that the sensing of growth phase signals lies upstream of these regulators. Previous studies of CcpA in growth phase-dependent regulation established that for many largely catabolic genes in *B. subtilis* and *Lactococcus lactis*, the disruption of CcpA uncoupled the accumulation of transcript from growth phase (50, 75). Since CcpA has been demonstrated to be a catabolite-sensitive regulator in *B. subtilis*, the signal controlling growth phase is most likely the depletion of glucose sensed through interaction of CcpA with a serine-phosphorylated form of the Hpr protein (34).

Inactivation of *cchA* affects virulence and virulence gene expression in several gram-positive pathogens, including *S. aureus* and many pathogenic streptococci (18, 35, 36, 41, 65, 68). Thus, studies of growth phase regulation through CcpA would likely reveal how regulation observed during infection relates to important signals and pathways elucidated in vitro. Indeed, our findings show that certain aspects of growth phase-dependent regulation observed with culture are similarly manifested in infected tissue. This is the case not only for virulence factor genes, such as *cfa* and *speB*, but also for *lctO*, the gene responsible for *H₂O₂* generation. In *S. pneumoniae*, the ability to produce *H₂O₂* can serve as a virulence trait or more likely as a colonization factor both by slowing the clearance of bacteria through the inhibition of ciliary beating and by the bacteriostatic and bactericidal activities of *H₂O₂* with other upper respiratory tract bacteria (32, 59, 61). That *H₂O₂* production functions as a colonization factor in a related bacterium may explain why *lctO* is regulated in *S. pyogenes*. Like many lactic acid bacteria, group A streptococci are polyauxotrophic, requiring a ready supply of amino acids and cofactors and an easily metabolized organic carbon source for growth. During times of low metabolite availability, the ability to produce *H₂O₂* may inhibit the growth of competing bacteria in the nasopharynx, thereby allowing streptococci to scavenge nutrients, while limiting direct competition. In contrast, in times of high nutrient availability, the repression of *lctO* production standing in vivo virulence responses.
may help to avoid the self-lethal accumulation of H$_2$O$_2$ in the environment.

Coregulation of virulence and metabolic genes in group A streptococcal has been previously demonstrated for other regulators. RopB, an rgg family regulator controlling the activation of transcription of the speB gene, has effects on amino acid metabolism (12, 13). The discovery and characterization of LacD.1 as a regulator of metabolism (12, 13). The discovery and characterization of LacD.1 as a regulator of speB transcription established a new regulator capable of linking nutritional status to transcription of virulence genes (49). Inactivation of LacD.1 coupled with overexpression of RopB led to the partial uncoupling of speB transcription from growth phase, suggesting a role for LacD.1 as an inhibitor of RopB activity until the onset of stationary phase (49). Our results show that CcpA regulates speB transcription as well, adding a third metabolic sensor. Unlike LacD.1, CcpA appears to activate transcription in a growth phase-independent manner. Activation of transcription is a described function for CcpA in the case of acetate kinase in B. subtilis and many genes in L. lactis as assessed by transcriptome analysis (26, 75). Unique to speB, CcpA-dependent activation is opposed by the inhibitory effects of LacD.1, possibly in response to the same signal. This paradox can be partially explained by measurement of transcript levels in infected tissues. Whereas transcription of speB in lesions by CcpA closely resembles the pattern and magnitude observed in culture, regulation through LacD.1 in lesions follows a similar pattern but does not have a similar magnitude as observed in culture. A possible mechanism for this observation is that both LacD.1 and CcpA are known to respond to multiple signals. LacD.1 was first discovered as a regulator of pH and chloride repressive signals in SpeB production (49), though the mechanism for sensing and regulation in response to these signals is unknown. CcpA has been shown to interact not only with the serine-phosphorylated form of Hpr but also with the metabolite signals glucose-6-phosphate and fructose-1,6-bisphosphate (24) and the cofactor NADP (40). Binding of CcpA to target DNA is not dependent on any of these binding partners; rather, complexing of CcpA with Hpr Ser-P, glucose-6-phosphate, fructose-1,6-bisphosphate, and NADP affect the affinity of CcpA-CRE interactions through conformational changes in the CcpA dimer (64). Input from any of these alternate signals to either the LacD.1 or CcpA regulatory pathways may lead to a change in regulatory pattern or magnitude of regulation from that observed in vitro. In addition, it is difficult to study the microenvironments or differential expressions among populations of bacteria within an infection. Our data suggest that direct or indirect regulation by CcpA can be distinct in infected tissues, perhaps due to the actions of multiple pathways at indirectly regulated promoters. Taken together, these data support a model in which the integration of multiple signals within a regulatory pathway and organization of multiple pathways at specific promoters is important for coordination of a virulence response and pathogen fitness within host tissues.

The mechanism by which CcpA regulates transcription of target genes has been best studied with B. subtilis, in which the CcpA protein has been shown to interact with well-conserved DNA sites in promoter regions, leading to either repression or activation of transcription (34). Studies of CcpA regulation in pathogenesis have generally used genomic-based techniques and focused on the role of CcpA as a transcriptional repressor, as described for regulation of the starch utilization locus of B. subtilis (30). Considerably less is known about the roles and biochemistry of CcpA as an indirect regulator and activator of transcription. What is known about the biochemistry and DNA binding capabilities of CcpA in B. subtilis appears to hold true for group A streptococci and related streptococcal pathogens. In group A streptococcal virulence, two previous studies have suggested that the sag locus is repressed directly by CcpA (41, 68). Our data agree, in that inactivation of CcpA can lead to an overexpression of SLS due to increased transcript abundance of the sag operon. However, we have shown here that SLS overexpression in the absence of CcpA does not follow a pattern in tissue similar to that of other directly repressed genes. In contrast to other genes directly repressed by CcpA and within infected tissues, the loss of CcpA has almost no effect on sagA transcription. This may explain why in the murine subcutaneous lesion model, as opposed to infection of macrophages in which we observed hypervirulence, the CcpA mutant shows a significant loss of virulence. Whereas in previous studies many of the results for the link between CcpA and virulence focused on sagA and SLS biogenesis, here our findings suggest that SLS is likely to have only a minor contribution to differences in GAS virulence levels in CcpA— strains in a subcutaneous lesion model of infection.

Our method of examining interactions with the sagA promoter uses the WT sequence from the sag locus and is nonbiased, in that CcpA is precipitated from a pool of total cellular proteins at protein concentrations less than cellular levels and target DNA concentrations of approximately one to two copies per cell. Using this technique we observed direct CcpA binding to DNA in the letO and speB promoters, both containing conserved CRE sites, but not to the sagA promoter. In the previous studies of CcpA in streptococcal virulence, direct regulation of the sag operon was observed by use of differing techniques for measurement of binding, which may offer an explanation for these contrasting results. In Shelburne et al. (68), the DNA probe representing the sag operon promoter utilized a CRE site corresponding to the canonical CRE site deduced from studies of B. subtilis (39, 68). The putative CRE site in the sagA promoter differs from the idealized CRE from B. subtilis in several base pairs, including a crucial C-G dyad at the axis of symmetry. It is known from the earlier studies of B. subtilis that this C-G dyad is necessary for CcpA binding; a mutation in either residue leads to a loss of CcpA regulation of the downstream gene (38, 39). It is notable that a poorly conserved putative CRE site in the letO promoter was also unable to precipitate CcpA. This putative CRE site, termed CRE2, differs at two positions compared to the consensus CRE sequence from B. subtilis and importantly lacks the C-G dyad at the axis of symmetry of the canonical CRE site. Taken together, these findings suggest a specificity of CcpA to DNA containing CRE sites in group A streptococci akin to the specificity described for B. subtilis and may explain why CcpA was unable to be precipitated by the putative CRE site in front of sagA. In Kinkel et al. (41), direct binding of CcpA to the sagA promoter was observed using electrophoretic mobility shift assays to demonstrate protein-DNA complexes (41). Under the conditions used, CcpA was in excess of the DNA probe by 100:1. It has previously been shown that under conditions in
which protein is in high excess of target DNA, CcpA can bind to DNA without CRE sites. Furthermore, analysis of CcpA from *L. lactis* has shown that once CcpA/DNA complexes have been formed, it is difficult to displace CcpA from bound DNA with competing CRE containing unlabeled probe (42). This suggests the importance of the conditions and concentrations under which CcpA is observed to bind DNA since protein to target ratios can affect the specificity of CcpA binding. Our data support indirect regulation of the *sag* operon; however, it does not rule out the possibility of extremely weak direct interactions. Indirect regulation of the *sag* operon may suggest a model in which, in infected tissue, another regulatory pathway is epistatic to CcpA, leading to a regulation of the *sag* operon that differs from patterns observed for genes directly regulated by CcpA.

Interruption of growth phase-dependent regulators has a large impact on virulence and virulence gene regulation in group A streptococci. Inactivation of the CovR (CsrR) gene leads to a marked increase in virulence in mice (44), while inactivation of CcpA leads to attenuation of virulence (68). Due to the importance of these and similar pathways to virulence, defining environmental signals and downstream effects of signaling in vitro is likely to increase our understanding of how pathogens sense signals and adapt while causing an infection. The in vitro signals for CovR/CovS activation include Mg$^{2+}$ and cationic antimicrobial peptide concentration (27, 28); little is known about the role of either Mg$^{2+}$ or cathelicidins in growth phase regulation; however, metal homeostasis and adaptation to innate immune responses have been shown to be important in infected tissues (37, 74). CcpA and LacD.1 have been shown to respond to carbohydrate availability (30, 49), an important signal in growth phase regulation. Here we have compared regulation in culture to regulation in infected tissues, showing that the CcpA pathway as defined in vitro is active in virulence; furthermore, our data suggest that integration of multiple signals is important in controlling the activities of both CcpA and LacD.1, possibly preventing the full and antagonistic results of each pathway from affecting speB transcription. Defining the signals and downstream response of the CcpA and LacD.1 pathways in vitro will lead to an understanding of how pathogenic bacteria integrate multiple sensory pathways in virulence.

**ACKNOWLEDGMENT**

This work was supported by Public Health Service Grant AI070759 from the National Institutes of Health.

**REFERENCES**
