CcpA and LacD.1 Affect Temporal Regulation of

*S. pyogenes* Virulence Genes

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ABSTRACT

Production of H₂O₂ 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 H₂O₂ generation and its regulation. Deletion of the gene encoding lactate oxidase (*lctO*) or culture in the presence of glucose eliminated H₂O₂ 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 of 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 *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*, *speB*, but not the indirectly-regulated *sagA*. Taken together, these data show that CcpA regulates virulence genes via at least 3 distinct mechanisms and that disruption of growth phase regulation alters transcriptional patterns in infected tissues.

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INTRODUCTION

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 multi-factorial, 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 (67)). Of the environmental cues, the former class is typically composed 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 (67)). It is generally assumed that growth phase-linked patterns of gene expression observed in vitro reflect adaptations that a successful pathogen makes in response to the dynamic host milieu (reviewed in (55, 56)). 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 self-limiting (eg. pharyngitis) to those that are destructive and life-threatening (eg. necrotizing fasciitis), as well as serious post-infectious sequelae such as rheumatic fever and acute glomerulonephritis (reviewed in (15)). Considerable evidence has accumulated to suggest that regulation of virtually all of its recognized virulence factors involves a
growth phase component when examined *in vitro*. 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
by (33, 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 anti-microbial
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 regulation
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 phosphotransferase
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 (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 more definitively demonstrated 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 during infection of muscle vs. 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 co-regulated genes whose patterns of expression were significantly correlated with that of speB when compared in vivo, suggesting that this cohort of genes was co-regulated 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 (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: 1) genes negatively regulated by CcpA; 2) genes positively regulated by CcpA; 3) genes regulated indirectly
by CcpA; and 4) 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 *E. 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% 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 Erlemeyer flasks containing 10 ml of medium subjected to orbital shaking (225 rpm) at 37°C. Streptococcal 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 OD<sub>600nm</sub> did not change after 20m. Approximate final OD<sub>600nm</sub> values are noted. Where appropriate, antibiotics were added to media at the following concentrations: Kanamycin: 500 µg/ml *S. pyogenes*, 1 µg/ml *E. coli*; Erythromycin: 1 µg/ml *S. pyogenes*, 500 µg/ml *E. coli*.

DNA and computational techniques. Plasmid DNA was isolated by standard techniques and used to transform *E. coli* as described (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 allowing for 1 mismatched base pair from the consensus binding site elucidated in *B. subtilis*.

Measurement of H$_2$O$_2$ and hemolytic titer. Accumulation of H$_2$O$_2$ in culture supernatant was conducted 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 hr 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 (62). Where indicated, the SLS-specific inhibitor Trypan Blue (Sigma) and 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 where approximately 50% hemolysis was observed.

Construction of CcpA$^-$ and LctO$^-$ mutants. In-frame deletion mutations in the genes encoding CcpA (SPy_0514) and LctO (SPy_0414) were generated using allelic replacement and the PCR primers listed in Table S1. The deletion alleles were transferred to the HSC5 chromosome using the allelic replacement vector pJRS233 (19)
to create strains CKB207 (CcpA-) and CKB044 (LctO-). The presence of the correct alleles was 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 as indicated in the text, as previously described (6). RNA was subjected to reverse transcription using Superscript II (Invitrogen) as per the manufacturer’s instructions. Real-time RT-PCR analysis of cDNA samples was performed using iQ SYBR Green Supermix (Bio-Rad) using the primers listed in Table S1. Relative transcript levels were determined using the \( \Delta \Delta C_t \) method with the recA transcript as a standard as described (5). Data presented are the mean and standard error 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 (5).

Biotinylated DNA pulldowns. A biotinylated DNA fragment pulldown assay was adapted from Grundling, et al. (25) as follows: biotinylated DNA fragments were generated by PCR using 5’ biotinylated primers (Invitrogen, Table S1) and HSC5 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,) were then coated with 3 µg of biotinylated DNA as per manufacturer’s instructions. A whole-cell soluble protein extract was produced as follows: 40 ml cultures of HSC5 were grown to mid-exponential phase (OD\(_{600nm} = 0.2\)) in
THYB. Cells were harvested by centrifugation (6,000 x g, 5 min, 4°C), the cell pellet 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 Complete Mini Protease Inhibitor cocktail, EDTA free (Roche). The resuspended cells were then disrupted using glass beads (G4649, Sigma) in a high-speed reciprocating shaking device (FastPrep 100, MPbiomedicals) at a setting of 6.0 for 40 sec. Debris and undisrupted cells were removed by centrifugation (14,000 x g, 5 min, 4°C) and the total protein concentration of the cleared supernatant determined using a BCA assay (Pierce/Thermo-Fisher). The protein concentration was adjusted to 1.5 mg/ml, glycerol was added to a final concentration of 20% and the solution stored at -20°C until use. For the pull-down, an aliquot of DNA-coated beads (200 µl, prepared from above) was mixed with the protein extract (1 mg) and Binding Buffer (10 mM Tris-HCl pH 8, 100 mM KCl, 3 mM MgCl₂, 20 mM EDTA, 5% glycerol, 40 µg/ml sheared Salmon Sperm DNA [Invitrogen], and 10 µg/ml BSA [Sigma]) 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 4 times with 500 µl Binding Buffer, resuspended in 30 µl of 1X 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 SDS sample buffer (1X: 50 mM Tris HCl pH 6.8, 100 mM DTT, 2% SDS (w/v), 0.1% Bromophenol Blue (w/v), 10% glycerol) was then added at 2X diluted from a 6X stock and the sample was boiled for an additional 5 min. The magnetic beads were removed with the magnetic particle separator and the
resulting sample subjected to electrophoresis using a 12% SDS-PAGE gel. Protein bands were visualized by staining with SYPRO Ruby (Molecular Probes/Invitrogen) as per the manufacturer’s instructions. Band(s) of interest were excised and then identified by MALDI-TOF 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 PBS (Cellgro) followed by gentle scraping. The macrophages were harvested by centrifugation (1000 x g, 10 min, 4°C), resuspended in RPMI 1640 with 10% FBS and plated in 6 well tissue culture plates (TPP) at 3x10^6 macrophages per well in 4 ml total medium. The macrophages were allowed to settle on the plates for 4 hr at 37°C in 5% CO_2. The various streptococcal strains were cultured overnight in THYB, diluted to an OD_600 of 0.05 in 40 ml RPMI 1640 with 10% FBS, and allowed to grow for an additional 2.5 hr. The streptococcal strains were then collected by centrifugation and resuspended in RPMI 1640 with 10% FBS to a final OD_600 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% CO_2 for the times indicated in the text. At the conclusion of the infection, the media were removed and each well washed with 2 ml cold PBS. The PBS was then removed and cells stained using a fluorescent reagent to assess viability (LIVE/DEAD for mammalian cells, Invitrogen) as per the manufacturer’s instructions. Cells were imaged using a Leica DMRE2.
fluorescence microscope and images analyzed using Openlab software (Improvision). For each tissue culture well at least 200 macrophages were examined. Data presented represent the mean and standard error 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), five to six week old female SKH1 hairless mice (Charles River Labs) were injected subcutaneously with $10^7$ colony forming units (CFU) of streptococci of the strains indicated in the text and the areas of the resulting ulcers quantitated on Day 3 post-infection as described (6, 8). 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. 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 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 H$_2$O$_2$; however, the addition of glucose both suppressed this lethality and the production of H$_2$O$_2$ (20). It has also been observed that many clinical isolates of *S. pyogenes* produce auto-intoxicating amounts of H$_2$O$_2$ upon entry into 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 H$_2$O$_2$ 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 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 H$_2$O$_2$ when subsequently exposed to air (Fig 1A). When grown aerobically in liquid culture the wild type 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 H$_2$O$_2$ in stationary phase, as compared to the millimolar amounts produced by
the wild type strain (Fig 1B). As previously observed, addition of glucose suppressed the ability of wild type bacteria to produce H$_2$O$_2$ (Fig 1B). The defect in H$_2$O$_2$ production apparent in the LctO$^{-}$ mutant was complemented by an lctO allele expressed constitutively off a plasmid (supplemental Fig 1).

Glucose and growth phase regulate transcription of lctO. The data presented above suggest that LctO is responsible for H$_2$O$_2$ production and the glucose-inhibited, growth phase-dependent loss of viability in aerobic conditions. To examine the basis of these latter phenomena, the transcription of lctO in the wild type strain was examined using real-time RT-PCR. Relative to early exponential phase (OD$_{600}$ 0.05, approximately 2 hr post inoculation, Fig 2A) the transcript abundance of lctO stays low through mid-exponential phase growth (OD 0.2, approximately 4 hr post inoculation, Fig 2A), followed by a large up-regulation upon the onset of stationary phase (OD$_{600}$ 1.15, approximately 6.5 hr post inoculation, Fig. 2A). In late stationary phase the transcript abundance of lctO was still relatively high (OD$_{600}$ 1.15 approximately 7.5 hr post inoculation, Fig 2A) suggesting that when cultures have reached a maximum OD$_{600}$, there is a large dynamic shift towards increased transcription of lctO that persists throughout stationary phase. To test if glucose was a signal controlling transcription of lctO, wild type 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 hundred fold (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 \textit{lctO} transcript abundance less than two fold (Aer and H₂O₂, Fig 2B).

**CcpA affects growth phase regulation of \textit{lctO} in response to carbohydrate signals.** A biochemical approach was used to identify potential growth phase and glucose responsive regulators of \textit{lctO} transcription. A biotinylated DNA probe that included the entire intergenic region between \textit{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 kD that, was not precipitated with a non-specific DNA probe, which was identified by MALDI-TOF mass spectrometry as the carbon catabolite control protein CcpA (SPy_0514; Fig 3B). As a transcriptional repressor CcpA binds to a an operator site termed a catabolite responsive element (CRE) and using the consensus site determined for \textit{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 CcpA, an observation supported by previous studies of CcpA interaction with CRE elements in \textit{S. pyogenes} (Probes A, D, E; Fig 3A, C)(68).

The contribution of CcpA to the regulation of \textit{lctO} transcription was determined by analysis of a mutant strain constructed to contain an in-frame deletion allele of \textit{ccpA}.
Two major effects were observed in the resulting CcpA−mutant. Whereas the wild type strain switches from a state of low lctO transcript abundance in exponential phase to high abundance in stationary phase, transcript levels in the CcpA−strain were similar in both exponential and stationary growth and consistent with the high level seen in stationary phase of the wild type strain (Table 1). The increase in lctO transcript abundance upon the onset of stationary phase was no longer apparent in the CcpA−strain, rather transcript abundance was relatively high and unchanged by growth phase (Table 1). Secondly, addition of glucose to the culture medium no longer caused a repressive affect on lctO transcription in the CcpA−strain, differing from the wild type strain where 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 CcpA−mutant. Therefore we conclude that growth phase and glucose regulation of lctO transcription are due to the direct repressive effects of CcpA.

CcpA regulates virulence factors CAMP factor 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 CcpA. 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 pathogenic lactic acid bacteria (46). Similar to lctO, cfa transcript levels increase significantly in stationary phase, and the loss of CcpA leads to constitutively high transcript abundance and a loss of growth phase dependent regulation (Table 2). This suggests that like lctO, cfa is growth phase regulated by CcpA due to
respression of cfa transcription in exponential phase followed by up-regulation due to a release of repression in stationary phase. When CcpA is absent, transcript levels of cfa are constitutively at a level similar to stationary phase transcription in the wild type strain (Table 2). The gene for SpeB (SPy_2039), the major secreted cysteine protease of group A streptococci, is preceded by a large intergenic region containing a predicted CRE (Fig 4A) and is subject to complex transcriptional regulation (47, 57). Unlike either lctO or cfa, speB transcript abundance decreased in the absence of CcpA in both phases of growth (Table 2). In the CcpA’ strain, transcription of speB was consistently 7-10 fold lower than in wild type bacteria in the same stage of growth (Table 2). However, similar to wild type bacteria, there was still a large growth phase dependent up-regulation of speB transcription in the CcpA’ strain (Table 2). Despite the significant activating affect of CcpA on speB expression these analyses revealed that CcpA does not function as a growth phase regulator of speB transcription. Thus CcpA acts as an important 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 the fragment indicated for the speB promoter (Fig 4B), CcpA was co-precipitated from lysates of the wild type strain of streptococci, but not using a non-specific 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 and a LacD.1− strain in comparison to wild type regulation. Deletion of ccpA abolished growth phase dependent regulation of lctO whereas inactivation of lacD.1 had no affect on lctO transcription compared to the wild type strain (Fig 5). If CcpA and LacD.1 function in a linear pathway both deletions should have a similar effect on lctO transcription. This suggests that CcpA and LacD.1 are at least partially independent but have opposing transcriptional effects on the speB promoter.

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 one or both of CcpA and LacD.1 activities. 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 TNFα, IL-1 and IL-6 (23). However, unlike infection with the wild type strain, after infection with the CcpA− strain for 4 hr bone marrow derived macrophages had rounded and detached from the tissue culture flask surface (not shown). At earlier time points, about one hour post infection, most bone marrow derived macrophages were viable after interaction with wild type streptococci, but significantly more macrophages had lost viability after infection with the CcpA− streptococci (Fig 6A, B). This suggested that the CcpA− strain was more cytotoxic than the wild type strain. In hemolytic assays the supernatant of the CcpA− strain had a hemolytic titer 16 fold higher than that of wild type strain (Table 3).

The two best characterized group A streptococcal hemolysins are the cholesterol-dependent cytolysin Streptolysin O (SLO) and the lantibiotic related peptide hemolysin Streptolysin S (SLS). Including the SLO specific inhibitor cholesterol in hemolytic assays did not prevent hemolysis upon incubation with supernatant from the CcpA− strain suggesting that SLO was not responsible for the increased cytotoxicity observed in macrophage infection (Table 3). Addition of the SLS specific inhibitor trypan blue to the hemolytic assay prevented hemolysis in the CcpA− supernatant samples (Table 3). This suggested that increased cytotoxicity of the CcpA− strain was due to increased SLS production. When trypan blue was added to bone marrow derived macrophages during infection with the CcpA− strain, macrophages retained viability comparable to infection with wild type streptococci (Fig 6A, B). This confirmed that increased cytotoxicity of the CcpA− mutant was due to overproduction of SLS. To test whether SLS overproduction was due to altered transcriptional regulation, transcript levels of the first two genes in the SLS biogenesis operon (sagA and sagB, SPy_0738 and SPy_0739) were tested as well as
the SLO structural gene, slo (SPy_0167). Under the conditions used for infection of macrophages both the sagA and sagB transcripts were upregulated about 25 fold in the CcpA− strain as compared to wild type bacteria (Fig 6C). This result confirms observations from previous studies that inactivation of CcpA leads to an upregulation of sag operon transcription (41, 68). As expected the slo transcript was not affected by CcpA under these conditions (Fig 6C). Since both the sagA and sagB transcripts were upregulated to the same degree it is likely that loss of CcpA regulation results in the entire sag operon being mis-regulated, 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 wild type and CcpA− strains revealed that in the wild type strain 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 lctO and cfa, is repressed by CcpA in exponential phase and loss of CcpA leads to overproduction of SLS due to a de-repression 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 pulldown assay with a DNA fragment from the sag promoter we were unable co-precipitate CcpA (Fig 6E) whereas CcpA was co-precipitated with the lctO DNA fragment using the same protein sample (Fig 6E). To validate these findings in other serotypes of group A streptococcus we repeated these DNA pulldown assays using a protein lysate from M1 strain SF370. The results from this pulldown confirm results from our wild type strain.
(supplemental Fig 2). 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 wild type 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: 1) Direct repression in response to growth phase; 2) Indirect repression in response to growth phase; and 3) Direct activation independent of growth phase. We analyzed the regulation of lctO, cfa, speB, and sagA in lesions from mice infected with wild type, 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 wild type streptococcal strains, a local necrotic lesion forms, enlarging and forming an eschar, but does not progress to invasive disease (8). At three days post injection, when lesions with wild type bacteria were maximal, lesions were measured for area, a general indicator of overall strain virulence, and total RNA was extracted from excised lesions and subject to real time RT-PCR analysis of these streptococcal genes. Infection by the CcpA− strain leads to significantly smaller lesions than in the wild type strain (Fig 7A) suggesting the CcpA− strain is attenuated in this virulence model. Analysis of the transcript levels from these lesions showed that lctO and cfa transcript levels were significantly increased in the
CcpA- strain over levels in wild type lesions and that speB levels are significantly lower than in wild type lesions (Fig 7B). This closely mirrors the results seen in vitro.

Transcript levels of sagA were not significantly different (less than two-fold change) between CcpA- and wild type lesions (Fig 7B).

Lesions caused by the LacD.1- strain were not significantly different from those caused by the wild type strain (Fig 7C). In culture conditions, repression through LacD.1 results in a several hundred-fold reduction in speB transcript abundance (49). In samples from mouse lesions the transcription of the speB gene was only 3 fold higher in LacD.1- samples than in wild type 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 (environmental) or intrinsic (metabolic, signaling molecules) cues to regulators controlling the abundance of virulence factor transcripts. Uncoupling of virulence gene regulation from growth phase through the disruption of signaling pathways or specific regulators can affect pathogen fitness in host tissues (43, 69). The variety of signals and regulators as well as the unique virulence factor repertoires amongst related pathogens necessitates the identification and characterization of specific regulators and signals to better understand how the pathogenesis of important infectious diseases is controlled by cues linked to growth phase.

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 with 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 amongst 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 *S. aureus*, *S. pneumoniae* and *E. 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 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 *L. 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 *ccpA* 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 in 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 H$_2$O$_2$ generation. In *S. pneumoniae* the ability to produce H$_2$O$_2$ can serve as a virulence trait or more likely as a colonization factor by both slowing the clearance of bacteria through the inhibition of ciliary beating and by the bacteriostatic and bacteriocidal activities of H$_2$O$_2$ on other upper respiratory tract bacteria (32, 59, 61). That H$_2$O$_2$ 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, cofactors and an easily metabolized organic carbon source for growth. During times of low metabolite availability the ability to produce H$_2$O$_2$ 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 may help to avoid the self-lethal accumulation of H$_2$O$_2$ in the environment.

Co-regulation of virulence and metabolic genes in group A streptococci has been previously demonstrated for other regulators. RopB, an *rgg* family regulator controlling the activation of transcription of the *speB* gene, has affects on amino acid 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 affects 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 not 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 for pH and chloride repressive signals on 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 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 change in regulatory pattern or magnitude of regulation from that observed *in vitro*. In addition it is difficult to study the microenvironments or differential expression among populations of bacteria within an infection. Our data suggest that direct
or indirect regulation by CcpA can behave distinctly in infected tissues, perhaps due to
the actions of multiple pathways at indirectly regulated promoters. Taken together this
supports a model where 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 best
been studied in *B. subtilis* where 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 on CcpA regulation in pathogenesis have generally used
genomic-based techniques and focused on the role of CcpA as a transcriptional repressor,
as described in 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 over-expression of SLS due to increased
transcript abundance of the *sag* operon. However we have shown here that SLS over-
expression in the absence of CcpA does not follow a pattern in tissue similar to other
directly repressed genes. In contrast to other genes directly repressed by CcpA and within
infected tissues the loss of CcpA has almost no affect on *sagA* transcription. This may
explain why in the murine subcutaneous lesion model, as opposed to infection of
macrophages where we observed hypervirulence, the CcpA mutant shows a significant
loss of virulence. Whereas in previous studies much focus on the link between CcpA and virulence focused on sagA and SLS biogenesis, here our findings suggest that SLS is likely only to have a minor contribution to differences in GAS virulence when analyzing CcpA strains in a subcutaneous lesion model of infection.

Our method of examining interactions with the sagA promoter uses the wild type sequence from the sag locus and is non-biased 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 1-2 copies per cell. Using this technique we observed direct CcpA binding to DNA in the lctO 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 using 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 in 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. From the earlier studies in B. subtilis 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 lctO 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 this suggests a similar specificity of CcpA to DNA containing CRE sites in group A streptococci akin to
the specificity described in *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 DNA probe by 100:1. It has previously been shown that under conditions where 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 supports 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 where in infected tissue another regulatory pathway is epistatic to CcpA leading to the regulation of the *sag* operon differing 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 has 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 \emph{in vitro} is active in virulence; furthermore, our data suggests
that integration of multiple signals is important in controlling both the activities of CcpA
and LacD.1 possibly preventing the full and antagonistic results from each pathway on
\emph{speB} transcription. Defining the signals and downstream response of the CcpA and
LacD.1 pathways \emph{in vitro} will lead to an understanding of how pathogenic bacteria
integrate multiple sensory pathways in virulence.

\textbf{Acknowledgements}

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National Institutes of Health
FIGURE LEGENDS

FIG. 1. Endogenous self-lethal H\textsubscript{2}O\textsubscript{2} production is dependent on LctO. (A) Wild Type (WT) and LctO\textsuperscript{-} strains were grown on H\textsubscript{2}O\textsubscript{2} indicator solid medium as described. Production of H\textsubscript{2}O\textsubscript{2} is indicated by colorimetric change in the medium to purple. (B) WT and LctO\textsuperscript{-} strains were grown in ThyB or ThyB supplemented with 0.2% glucose (+ gluc) aerobically. After growth overnight supernatants were assayed for H\textsubscript{2}O\textsubscript{2} concentration and data are presented as mean and standard error of two independent experiments carried out in triplicate. Asterisks denote values below limit of detection (approx. 10\textmu M). (C) Strains were grown as in B, samples taken at indicated times and appropriate dilutions plated on solid ThyB medium to count viable colony forming units. The asterisk indicates a value of less than 100 CFUs/mL of culture. Strains: WT = HSC5, LctO\textsuperscript{-} = CKB044.

FIG. 2. Transcription of lctO is regulated by growth phase and glucose concentration. (A) Wild type (WT) bacteria were grown in C medium + 0.2% glucose anaerobically to determine transcription of lctO during growth in broth culture. Growth of streptococci was measured by OD\textsubscript{600}. Total RNA was harvested at the indicated times and lctO transcript abundance was measured by real-time RT-PCR. Data are presented as the ratio of transcript abundance at the indicated time points compared to transcript levels at the first time point (2 hrs) represent the mean and standard deviation from a representative experiment carried out with three samples per time point, each analyzed in triplicate. (B) WT bacteria were grown in varied glucose, aeration and oxidative stress to test affects on lctO transcription. WT bacteria were grown in C medium supplemented
with the indicated amount of glucose, or C medium + 0.2% glucose shaking in ambient air (Air) or with 100 µM H₂O₂ (H₂O₂) added 1 hour prior to harvesting. Total RNA was harvested in early exponential phase (OD₆₀₀nm 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 – glu for glucose analysis and compared to C medium + 1% glucose for Air and H₂O₂) and represent the mean and standard deviation derived from three samples, each analyzed in triplicate, compared to C medium without glucose (- glu), or for Air and H₂O₂, compared to C medium + 0.2% glucose. Strain: WT = HSC5.

**FIG. 3. CcpA 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 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 (B) Biotinylated DNA probes were used to precipitate proteins from a lysate of wild type. The lctO probe A and a control non-specific fragment (NS) were created by PCR using HSC5 genomic DNA as template and the primer pair CK195/CK196 (Table S1). As indicated by the arrow, an lctO-specific band was identified as CcpA. The location of molecular weight standards is indicated. (C)
Biotinylated DNA probes spanning the \textit{lctO} locus were used to examine the binding of CcpA to the promoter of \textit{lctO}. Proteins were precipitated as in (B) with the DNA probes indicated in (A). The band identified as CcpA in (B) is labeled with an arrow. The location of molecular weight standards is indicated. Strain: WT = HSC5.

**FIG. 4.** CcpA binds predicted CRE sites upstream of CcpA regulated virulence genes. (A) Diagram of the \textit{cfa} and \textit{speB} loci. Predicted CRE sites are indicated with black rectangles; sequences of corresponding CRE sites are indicated above each site. Positions of the DNA features are relative to the start of translation of the \textit{cfa} and \textit{speB} genes respectively with the A of the ATG start codon considered (+1). The \textit{speB} DNA probe is indicated by the line below the \textit{speB} locus and was generated by PCR using HSC5 genomic DNA as template with the primer pair CK393/CK396 (see Table S1). (B) Precipitation of proteins from wild type interacting with the \textit{speB} promoter region. DNA probes (\textit{lctO} probe A (Fig 3A), \textit{speB} as indicated in (A), NS – nonspecific (Fig 3B)). The protein band corresponding to CcpA is indicated with an arrow. The position of molecular weight standards is indicated. Strain: WT = HSC5.

**FIG. 5.** Unlike CcpA, LacD.1 does not regulate \textit{lctO}. Wild type (WT), CcpA\(^{-}\) and LacD.1\(^{-}\) strains were grown in C medium with 0.1% glucose until early exponential phase (OD\textsubscript{600nm} 0.1) or until stationary phase (maximal OD\textsubscript{600nm} of approx 1.1 was attained). Total RNA was isolated from these cultures and used for real-time RT-PCR analysis of the \textit{lctO} transcript. Data are presented as the ratios of transcript abundance in stationary phase to that in exponential phase for each strain and represent the mean and standard
deviations derived from three samples each analyzed in triplicate. Strains: WT = HSC5, CcpA- = CKB207, LacD.1' = JL151 (49).

FIG. 6. SLS is overproduced in a CcpA- strain due to indirect regulation. (A) Bone marrow-derived macrophages (BMM) were infected with wild type (WT) and CcpA- for 1hr then assayed for viability. Viable cells fluoresce green due to calcein AM staining; non-viable cells fluoresce red due to increased permeability of cellular membranes to ethidium homodimer. The Streptolysin S (SLS) specific inhibitor Trypan Blue (50 µg/ml) was added as indicated. (B) Quantitation of ethidium positive (dead) BMMs from infections carried out in (A). At least 200 BMMs were counted in duplicate experiments. Data represents mean and standard deviation from two experiments done in triplicate; differences were analyzed for significance using the Student’s t-test. (C) Analysis of cytolysin transcript abundance in WT and CcpA-. Bacteria were grown as for infection of BMMs. Total RNA was harvested and subjected to real-time RT-PCR analysis for the sagA, sagB, and slo transcript. Data are presented as the ratios of transcript abundance in CcpA- compared to that in WT bacteria for each indicated gene and represent the mean and standard deviations derived from two experiments with three samples in each experimental group, each analyzed in triplicate. (D) Growth phase regulation of sagA in WT and CcpA-. Strains were grown in C medium + 0.1% glucose until early exponential phase (OD600 0.1) or stationary phase (OD600 approximately 1.1). Total RNA was isolated and used for real-time RT-PCR analysis of sagA transcript abundance as in (D) (E) Diagram of the sagA locus. Positions of DNA features are shown as relative to the predicted start of translation of the sagA gene with the A of the ATG start codon.
considered (+1). A putative CRE site identified in previous studies (41, 68) is indicated by a black rectangle. The sequence of the putative CRE site is indicated; bold and underlined base pairs differ from the conserved CRE sequence determined from *B. subtilis* (39). The *sagA* DNA probe indicated below the diagram was generated by PCR using HSC5 genomic DNA as template and the primer pair CK405/CK371 (see Table S1 for primer sequence). (F) Precipitation of proteins interacting with the *sagA* locus. Biotinylated DNA precipitation of proteins from a WT protein sample (*lctO* probe A (Fig 3A), *sagA* as indicated in (E), NS – nonspecific (Fig 3B)) was carried out as before, and the protein band corresponding to the CcpA band identified previously is indicated with an arrow. The position of molecular weight standards is indicated. Strains: WT = HSC5, CcpA – CKB207.

**FIG. 7.** Regulation in infected tissue through CcpA, but not LacD.1, resembles regulation in vitro. SKH1 hairless mice were inoculated subcutaneously with WT and CcpA – (A) or LacD.1 – (B) and areas of the resulting lesions were determined at day 3 post-injection. Data shown are lesion areas from individual mice from a representative experiment. Differences between groups were tested for significance using the Mann-Whitney U test and *P* values are reported in the figure. (C) SKH1 hairless mice were infected as above. On day 3 post injection the resulting skin lesions were excised, total RNA isolated and subjected to real-time RT-PCR analysis of the indicated transcripts. Data presented are ratios of transcript abundance in mutant strains compared to WT and represent the mean and standard deviation derived from two experiments in which three
mice for each bacterial strain were analyzed. Strains: WT = HSC5, CcpA- = CKB207, LacD.1- = JL151.

REFERENCES


Figure 1
Figure 4
Figure 6
Figure 7
TABLE 1: Growth phase and glucose induced changes in transcript abundance of *lctO* is CcpA dependent

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relative Expression(^a)</th>
<th>Exponential(^b)</th>
<th>Stationary(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>- Glucose(^c)</td>
<td>+ Glucose(^c)</td>
</tr>
<tr>
<td>WT(^d)</td>
<td>1</td>
<td>0.0081 ± 0.002</td>
<td>425.8 ± 79.1</td>
</tr>
<tr>
<td>CcpA(^d)</td>
<td>407.5 ± 60.6</td>
<td>388.0 ± 53.6</td>
<td>448.5 ± 143.3</td>
</tr>
</tbody>
</table>

\(^a\) Values are derived from real-time RT-PCR analysis of total cellular RNA and normalized to WT in Exponential phase (- Glucose).
\(^b\) Exponential = OD\(_{600nm}\) 0.1; Stationary = OD\(_{600nm}\) 1.1 in C medium + Glucose.
\(^c\) Growth in C medium: Unsupplemented (- Glucose) or 0.1% w/v glucose (+ Glucose).
\(^d\) WT = HSC5, CcpA = CKB207.
TABLE 2: CcpA affects virulence factor transcript abundance in both growth phase-dependent and -independent manners

<table>
<thead>
<tr>
<th>Gene</th>
<th>Exponential</th>
<th>Stationary</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>WT</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>

a Values derived from real-time RT-PCR analysis and are normalized to WT in Exponential phase for each gene.
b Strains were grown in C medium + glucose (0.1% w/v); Exponential = OD<sub>600nm</sub> of 0.1, Stationary = OD<sub>600nm</sub> of 1.1.
c WT = HSC5, CcpA<sup>-</sup> = CKB207.
### TABLE 3: Inactivation of CcpA leads to increased trypan blue inhibitable hemolysis

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hemolytic Titer &lt;sup&gt;a&lt;/sup&gt;</th>
<th>RPMI&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ThyB&lt;sup&gt;b&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>None + Chol + Trypan</td>
<td>None + Chol + Trypan</td>
<td></td>
</tr>
<tr>
<td>WT&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2 + 2 NL 4 NL 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CcpA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>32 + 16 NL 8 2 NL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Values represent the reciprocal of the dilution of the supernatant where 50% lysis is observed. NL, no lysis.

<sup>b</sup> Strains were grown in RPMI1640 with 10% FBS or ThyB to OD<sub>600nm</sub> 0.1. Supernatant was sterile filtered and used in hemolytic assay. Cholesterol (25 µg/ml) or trypan blue (50 µg/ml) was added as noted.

<sup>c</sup> WT = HSC5, CcpA<sup>-</sup> = CKB207.