Corrections

MICROBIOLOGY

The authors note that on page 1699, Fig. 1 appeared incorrectly in part. In Fig. 1A, the pictures of strain MGAS5005 and \text{comp\Delta ccpA} were inadvertently derived from the same bacterial plate. This error does not affect the conclusions of the article. The corrected figure and its legend appear below.

![Fig. 1. CcpA influences streptolysin S and hyalouronic acid capsule expression. (A) Colony phenotype of strain MGAS5005, its \Delta ccpA mutant derivative strain, and \text{comp\Delta ccpA} strain on sheep blood agar plates. The \Delta ccpA strain has significantly increased hemolysis and decreased colony size compared with strain MGAS5005 and strain \text{comp\Delta ccpA}. All three images are of same magnification with size indications at right. (B) Changes in colony hemolysis and size were associated with altered \text{pel/sagA} and hasA gene transcript levels. Strain MGAS5005 and \Delta ccpA were grown in THY to early- and late-logarithmic growth phase, and \text{pel/sagA} and hasA transcript levels were measured by real-time TaqMan QRT-PCR with the \Delta ΔCt method. Values above the x axis indicate higher gene transcript levels in the \Delta ccpA strain, whereas values below the x axis indicate higher gene transcript levels in strain MGAS5005. Error bars indicate standard deviation among quadruplicate samples done on two separate occasions.](image)

NEUROSCIENCE

The authors note that due to a printer’s error, on page 4460, Fig. 2 appears incorrectly in part. The middle and bottom panels were transposed. The corrected figure and its legend appear below.

![Fig. 2. Lack of functional GABA_b receptors in orexin neurons of oxGKO mice. Representative traces that show the effects of GABA (0.6 mM), muscimol (30 \mu M), and baclofen (100 \mu M) in orexin neurons in control mice (A) and oxGKO mice (B) during current clamp recording. Drugs were applied during the periods indicated by bars.](image)
A direct link between carbohydrate utilization and virulence in the major human pathogen group A Streptococcus

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Communicated by Richard M. Krause, National Institutes of Health, Bethesda, MD, December 16, 2007 (received for review October 22, 2007)

Although central to pathogenesis, the molecular mechanisms used by microbes to regulate virulence factor production in specific environments during host–pathogen interaction are poorly defined. Several recent ex vivo and in vivo studies have found that the level of group A Streptococcus (GAS) virulence factor gene transcripts is temporally related to altered expression of genes encoding carbohydrate utilization proteins. These findings stimulated us to analyze the role in pathogenesis of catabolite control protein A (CcpA) and HPr, a GAS ortholog of a key global regulator of carbohydrate utilization in Bacillus subtilis. Inasmuch as the genomewide effects of CcpA in a human pathogen are unknown, we analyzed the transcriptome of a ΔccpA isogenic mutant strain grown in nutrient-rich medium. CcpA influences the transcript levels of many carbohydrate utilization genes and several well-characterized GAS virulence factors, including the potent cytolysin streptolysin S. Compared with the wild-type parental strain, the ΔccpA isogenic mutant strain was significantly less virulent in a mouse model of invasive infection. Moreover, the isogenic mutant strain was significantly impaired in ability to colonize the mouse oropharynx. When grown in human saliva, a nutrient-limited environment, CcpA influenced production of several key virulence factors not influenced during growth in nutrient-rich medium. Purified recombinant CcpA bound to the promoter region of the gene encoding streptolysin S. Our discovery that GAS virulence and complex carbohydrate utilization are directly linked through CcpA provides enhanced understanding of a mechanism used by a Gram-positive pathogen to modulate virulence factor production in specific environments.

ccpA | pharyngitis | regulation | streptolysin | transcriptome

Investigations in bacterial pathogenesis have suggested close links between basic metabolic processes and microbial pathogenesis (1, 2). For example, bacteria alter transcription of carbohydrate utilization genes and virulence factor production in response to changes in environmental conditions encountered during infection in humans (3, 4). Therefore, it is reasonable to speculate that pathogenic bacteria have developed molecular strategies to directly link regulation of carbohydrate utilization and virulence factor production. However, the mechanisms underlying such relationships are largely undefined.

In Bacillus subtilis, alterations in gene transcription in response to environmental carbohydrate concentrations are controlled in part by catabolite control protein A (CcpA), which binds to DNA at catabolite response element (cre) sites (5). Binding of CcpA to cre sites is enhanced by interaction of CcpA with the phosphoprotein HPr-Ser-46-P, the phosphorylation state of which in turn is affected by uptake of glucose and other readily metabolized carbohydrates by phosphotransferase (PTS) systems (6). Thus, in Bacillus spp., CcpA directly links environmental carbohydrate levels with transcriptional regulation of carbohydrate utilization genes. Most studies of CcpA have been conducted in Bacillus spp. (5, 7). Several Gram-positive pathogens encode proteins with significant homology to B. subtilis CcpA and HPr suggesting that similar molecular processes may occur in other microbes (8–11).

Group A Streptococcus (GAS) causes diverse infections in humans ranging from colonization and uncomplicated pharyngeal and skin infections to necrotizing fasciitis and toxic shock syndrome (12). The diversity in routes and manifestations suggests that GAS colonization and infection involve complex regulatory networks that are differentially regulated in distinct environments (13). In fact, recent genomewide investigations of GAS gene expression have demonstrated that GAS responds to different environments by altering the transcription of genes involved in meeting basic metabolic demands and differential transcription of genes encoding major virulence factors (14–17). These studies have resulted in a new understanding of the relationship between metabolism and virulence in GAS. Here, we report the results of studies that extend this understanding to the molecular level.

Results

Comparison of GAS Gene Transcript Levels in Saliva and a Nutrient-Rich Medium. Genomewide transcriptome analyses have suggested that differences exist in GAS gene expression during interaction with saliva and the oropharynx compared with growth in laboratory media, but no direct comparison has been done (15, 17). We used real-time TaqMan quantitative reverse transcription (QRT) PCR to test the hypothesis that GAS gene transcript levels differ significantly during growth in human saliva, a major component of innate and acquired immunity in the oropharynx, compared with growth in Todd–Hewitt broth with yeast extract (THY). We measured the transcript levels of 78 GAS genes encoding transcription regulators or proteins with either a known or putative extracellular location because of the likelihood such genes are involved in host–pathogen interaction [see supporting information (SI) Table 1]. Fifty-nine of the 78 genes had at least a twofold significantly different transcript level between the two media for at least one of the time points measured (select genes are shown in SI Fig. 7 with gene functions


The authors declare no conflict of interest.


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This article contains supporting information online at www.pnas.org/cgi/content/full/0711767105/DC1.

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Inactivation of the ccpA Gene Results in Medium-Specific Growth Defects. To test the hypothesis that CcpA directly mediates some of the observed transcript differences we created isogenic mutant strain ΔccpA from wild-type serotype M1 strain MGAS5005 (confirmatory Southern blot shown in SI Fig. 8). We genetically complemented the ΔccpA isogenic mutant strain by using a plasmid capable of replicating in GAS to make strain compΔccpA. The growth curves for the three strains in THY were superimposable (SI Table 2 and SI Fig. 9). Conversely, compared with wild-type strain MGAS5005, the ΔccpA mutant strain had a prolonged lag phase in a glucose medium and an increased (slower) doubling time in a maltose medium (Fig. 9 B and C). Moreover, the ΔccpA transcript did not reach as high a cell density or maintain as many viable colony-forming units (CFUs) in human saliva as wild-type strain MGAS5005 (Fig. 9D). Gram stain of the ΔccpA isogenic mutant strain grown in human saliva did not reveal enhanced clumping or increased chain length, two possibilities to explain the observed CFU differences (data not shown). There was no significant difference in growth between strain MGAS5005 and the compΔccpA strain in any of the media tested.

The ΔccpA Isogenic Mutant Strain Has an Altered Colony Morphology Associated with Differential Expression of pel/sagA and hasA Transcripts. The ΔccpA mutant strain had altered colony morphology and hemolytic phenotype compared with wild-type strain MGAS5005 when grown on blood agar plates (Fig. 1A). The colony diameter (1.7 ± 0.1 mm) of the ΔccpA strain was significantly less than that of wild-type strain MGAS5005 (2.6 ± 0.3 mm) or the compΔccpA strain (2.4 ± 0.4 mm, *P* < 0.001). The observed difference in colony diameter was not caused by a reduced number of bacterial cells, because there was no significant difference in CFUs within colonies of the three strains (1.45 ± 0.3 × 10⁹, 1.21 ± 0.51 × 10⁹, 1.33 ± 0.49 × 10⁹ for strain MGAS5005, the ΔccpA strain, and compΔccpA strains, respectively, *P* = 0.461). In addition, the width of the zone of β-hemolysis surrounding the ΔccpA mutant strain (1.7 ± 0.1 mm) was significantly larger than the zone around wild-type strain MGAS5005 (0.4 ± 0.1 mm) and the compΔccpA strain (0.6 mm ± 0.2 mm, *P* = 0.005).

The β-hemolytic properties and colony morphology of GAS are determined in part by streptolysin S activity and capsule size, respectively (18, 19). Thus, we used real-time TaqMan QRT-PCR to test the hypothesis that the ΔccpA strain had higher levels of sagA transcript, the first gene in a nine-gene operon responsible for streptolysin S production, and lower transcript levels of hasA, the first gene in a three-gene operon of capsule synthesis genes. Consistent with the hypothesis, the level of sagA transcript was increased ~10-fold in the ΔccpA mutant strain compared with wild-type strain MGAS5005 (Fig. 1B). Moreover, the hasA transcript was significantly decreased in the ΔccpA mutant strain compared with its parental strain at both time points. Although not studied in strain MGAS5005, in another GAS strain the sagA gene is transcribed as part of an RNA molecule with regulatory activity known as the pleiotropic effect locus (pel) (20). Based on QRT-PCR analysis, we found no significant difference in the transcript level of pel and sagA, consistent with the hypothesis that sagA is part of the larger pel transcript in strain MGAS5005 (data not shown). There was no significant difference in pel/sagA or hasA transcript level between the wild-type and compΔccpA strains (data not shown). Therefore, we conclude that CcpA directly or indirectly influences the transcript levels of GAS genes encoding critical virulence factors.

Analysis of the CcpA Transcriptome. To test the hypothesis that CcpA influences a broader array of GAS genes, we next compared the genomewide transcriptome of wild-type strain MGAS5005 and the ΔccpA isogenic mutant strain grown in THY. After accounting for multiple comparisons, in the early-exponential growth phase the level of transcripts of ~20% of all ORFs differed between the wild-type and ΔccpA isogenic mutant strain (Fig. 2A, SI Table 3). In the late-exponential phase ~10% of all ORFs were differentially expressed.

The largest number of transcripts differentially expressed between the two isogenic strains encode proteins known to be or putatively involved in carbohydrate transport and metabolism (Fig. 2B, SI Table 3). The differentially expressed genes included ATP-binding cassette (ABC) transporters or PTS operons responsible for glucose, lactose, maltodextrin, mannose, fructose, cellobiose, lactose, galactose, tagatose, and sialic acid transport. The transcript levels of genes in all of the aforementioned operons were increased in the ΔccpA strain except for the maltodextrin operon. The decreased level of the maltodextrin
operon in the ΔccpA strain is in accord with the decreased growth of the ΔccpA strain in a maltodextrin medium (SI Fig. 9C). We also observed significant differences in transcript levels of 21 genes encoding known and putative transcriptional regulators. Six of these genes comprise three two-component (TCS) gene regulatory systems, including M5005.spy0784/5, M5005.spy1305/6, and spyR/spyS. M5005.spy0784/5 positively influences a putative mannose/fructose phosphotransferase system, whereas spyR/spyS affects carbohydrate metabolism and virulence factor production in human saliva (15, 21).

A third major category of genes affected in the ΔccpA mutant strain included those encoding proteins putatively or known to be involved in GAS virulence. As predicted from QRT-PCR data, the transcript levels of the nine-gene operon encoding streptolysin S were significantly elevated in the ΔccpA strain, whereas the entire hasABC operon involved in capsule synthesis was significantly down-regulated in the ΔccpA strain (Fig. 2C). Other virulence factors affected by ccpA inactivation included spd, which encodes an extracellular DNase, and endoS, which encodes a protein that cleaves human Ig (22, 23). No significant difference in transcript levels was observed for emm, which encodes the anti-opsonophagocytic M protein, or mga, which encodes a transcriptional regulator involved in up-regulation of several virulence factors (Fig. 2C). Taken together, we conclude that CcpA is a global regulator of carbohydrate metabolism in GAS and has important effects on regulation of genes encoding transcriptional regulators and major virulence factors.

**CcpA Contributes to GAS Virulence and Ability to Colonize the Mouse Oropharynx.** In light of our findings that CcpA affects the transcript levels of multiple GAS virulence factors, we next tested the hypothesis that the ΔccpA isogenic mutant strain was less virulent for mice than for the wild-type parental strain. Consistent with the hypothesis, significantly more mice inoculated i.p. with wild-type strain MGAS5005 died than with the ΔccpA isogenic mutant strain (ΔccpA strain: P < 0.001; Fig. 3A). Similarly, the compΔccpA strain was significantly more virulent than the ΔccpA strain (P < 0.001) and as virulent as strain MGAS5005 (P = 0.072).

Next, we tested the hypothesis that ccpA contributes to the ability of GAS to colonize the mouse oropharynx. As hypothesized, after intranasal inoculation, the percentage of mice colonized over time with strain MGAS5005 was significantly greater than the ΔccpA mutant strain (P < 0.001; Fig. 3B). Similarly, the average number of CFUs recovered from mice inoculated with the ΔccpA mutant strain was significantly greater than with the ΔccpA mutant strain (P < 0.001; data not shown). The compΔccpA strain was recovered at significantly higher CFUs and from more mice compared with the ΔccpA strain (P < 0.01 for both). Taken together, these data indicate that CcpA contributes to GAS virulence and ability to colonize the mouse oropharynx.

**CcpA Affects the Transcript Levels of GAS Virulence Factors During Growth in Human Saliva.** We next sought to determine whether, under glucose-limiting conditions, CcpA influences the transcript levels of genes encoding virulence factors not influenced by CcpA in glucose-rich conditions (e.g., THY). To this end, we tested the transcript levels of several GAS virulence factors in strain MGAS5005 and the ΔccpA strain during growth in human saliva, a glucose-poor medium. The transcript levels of speB (encoding a cysteine protease), mac (encoding an Ig-degrading enzyme), and spd3 (encoding a DNase) were significantly higher in strain MGAS5005 than in the ΔccpA strain during growth in human saliva (Fig. 4). These data demonstrate that CcpA is needed for GAS to respond to human saliva by increasing transcript levels of virulence factors shown to affect the ability of GAS to persist in human saliva (speB) and cause pharyngitis in a non-human primate model (spd3) (23, 24).

**Recombinant GAS CcpA Binds to the Streptolysin S Promoter Region.** When complexed with its co-effector HPr-Ser-46-P, CcpA functions by binding to cognate DNA sites (cre) in the promoter...
region or within target genes in *B. subtilis* (25, 26). A bioinformatic analysis of the genome of strain MGAS5005 identified 37 sites that contain a *B. subtilis* consensus cre sequence (TIWANCGNTNWCA; see SI Text and SI Table 4). To elucidate whether CcpA exerts its regulatory effect on virulence factor production by direct DNA interaction, we analyzed the binding of purified recombinant GAS CcpA (SI Fig. 10) to DNA sequences in the promoter region of three different genes by fluorescence polarization: (i) *pelsagA*, a virulence gene whose expression was repressed by CcpA in our transcriptome analysis; (ii) *lctO*, which contains a consensus cre sequence and was differentially transcribed between strain MGAS5005 and the Δ*ccpA* mutant strain and thus served as a positive control; and (iii) *ftsX*, a gene that does not contain a consensus cre sequence, was not differentially expressed in the transcriptome analysis, and therefore was chosen as a negative control (SI Table 5). GAS CcpA bound specifically to the *pelsagA* DNA (Fig. 5). The binding affinity was specific (Kd = 950 ± 98 nM, assuming a 100% active CcpA) and enhanced ~65-fold by the presence of 50 μM HPr-Ser-46-P (Kd = 14.5 ± 2.2 nM), further supporting the notion that CcpA binds to the *pelsagA* promoter in vivo and controls its transcription. Nonphosphorylated HPr did not increase the CcpA–DNA interaction (data not shown). Similar results were found for *lctO*, and, as expected, specific binding was not observed for the promoter region of *ftsX* (SI Fig. 11). Thus, binding of CcpA to the promoter region of *pelsagA* demonstrates a direct mechanism of CcpA regulation of a key GAS virulence factor.

Fig. 3. Inactivation of CcpA significantly decreases GAS virulence. (A) Invasive disease model. Twenty-five adult outbred CD-1 mice per group were inoculated i.p. with 1 × 10⁸ CFU of the indicated strains. Percent survival is graphed with P values for Kaplan–Meier survival analysis. (B) Oropharyngeal colonization model. Adult outbred CD-1 mice (35 per group) were inoculated intranasally with 1 × 10⁶ CFU of the indicated strains. Mice oropharynges were swabbed daily. Percentage of mice with GAS isolated by day with P values shown for repeated measures analysis.

**Discussion**

Although CcpA orthologs have been investigated in other Gram-positive organisms, before this study the CcpA transcriptome had not been determined in a human pathogen (8, 9, 27, 28). A key discovery was that CcpA influenced the transcript levels of several GAS virulence factors, including the potent cytolsin streptolysin S, the extracellular DNase Spd, and the Ig-degrading EndoS. In *Bacillus* spp., transport of glucose or other readily metabolized carbohydrates through the PTS system leads to phosphorylation of the phosphocarrier protein HPr at residue Ser-46, resulting in HPr-Ser-46-P (6). HPr-Ser-46-P serves as a
Bacterial Strains and Culture Media. Serotype M1 strain MGAS5005 is genetically representative of the clone responsible for most contemporary (post-1987) human infections; its genome has been sequenced (30). The ΔccpA isogenic mutant strain was created from parental serotype M1 strain MGAS5005 by nonpolar insertional mutagenesis. We used pDC123, a plasmid capable of replicating in GAS, to genetically complement the isogenic mutant strain was created from parental serotype M1 strain MGAS5005 by nonpolar insertional mutagenesis. We used pDC123, a plasmid capable of replicating in GAS, to genetically complement the isogenic mutant strain to colonize the oropharynx. Thus, our data demonstrate the GAS CcpA directly represses production of virulence factors, such as streptolysin S, under nutrient-rich conditions and augments production of other virulence factors, such as SpeB, under nutrient-limited conditions, thereby providing a key mechanism by which GAS responds to changing environments (Fig. 6).

Human pathogenic microbes differentially regulate production of key virulence factors in vivo, a hallmark of pathogen–host interaction. We have discovered that the major human pathogen GAS modulates virulence factor production required for survival and infectivity by a CcpA-mediated pathway. Given the highly conserved nature of CcpA, similar genomewide studies of other Gram-positive pathogens may yield enhanced understanding of links between basic metabolic processes and pathogenesis.

Materials and Methods

Mouse Virulence Studies. Mouse experiments were performed according to protocols approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. For the invasive disease model, 25 female outbred CD-1 Swiss mice (Harlan–Sprague–Dawley) were injected i.p. with 10^7 CFU of GAS (32). The throat of each mouse was swabbed before inoculation and then daily thereafter.

RNA Isolation, TaqMan Transcript Level Analysis, and Expression Microarray Analysis. RNA was purified from a minimum of four replicate cultures by using an RNeasy kit (Qiagen). The concentration and quality of RNA were assessed with an Agilent 2100 Bioanalyzer and analysis of the A260/A280 ratio. Select gene transcript level was performed with TaqMan real-time QRT-PCR (primers and probes listed in SI Table 6). A custom-made Affymetrix GeneChip that contains 100% of the ORFs of strain MGAS5005 was used for expression microarray (transcriptome) studies. Principal component analysis (PCA) indicated that the data were of high quality and that the two time points provided distinct information regarding GAS gene transcripts differentially expressed between these two strains (SI Fig. 12). To compare gene transcript levels between the wild-type and mutant strain, a two-sample t test (unequal variance) was applied, followed by a false discovery rate correction (Q < 0.05) to account for multiple testing. Genes were considered differentially transcribed if the t test had a corrected P value of <0.05 and the difference in mean gene transcript level was at least 2-fold. For further information on assessment of transcript levels see SI Text.

Mouse Virulence Studies. Mouse experiments were performed according to protocols approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. For the invasive disease model, 25 female outbred CD-1 Swiss mice (Harlan–Sprague–Dawley) were injected i.p. with 2.5 × 10^6 CFU of GAS CFU (31). Throat colonization studies were performed by inoculating 35 mice in each group with 1 × 10^7 CFU of GAS (32). The throat of each mouse was swabbed before inoculation and then daily thereafter.
Purification and Binding Characteristics of CcpA. CcpA was purified to homogeneity from Escherichia coli (SI Fig. 10, SI Text). Fluorescence anisotropy was used to determine the binding characteristics of CcpA to 5'-fluorescein-labeled oligonucleotides in the presence and absence of 50 μM HPr-Ser46-P (33, 34; SI Text).