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Characterization Of A Global Regulatory Pathway In Streptococcus Pneumoniae

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CHARACTERIZATION OF A GLOBAL REGULATORY PATHWAY IN *STREPTOCOCCUS PNEUMONIAE*

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A DISSERTATION

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MICROBIOLOGY

ABSTRACT

Streptococcus pneumoniae is a versatile organism that adapts to many different environments in the host. *S. pneumoniae* can asymptomatically colonize the nasopharynx of humans. However, dissemination of the bacterium from the nasopharynx to different locations in the body can lead to invasive diseases such as pneumonia, bacteremia, and meningitis. How *S. pneumoniae* modulates factors that are important for survival in these niches has not been well characterized. In the studies described here, we propose that *S. pneumoniae* may modulate gene expression in these niches by sensing the different glucose concentrations via carbon catabolite control protein A (CcpA).

Initially we characterized CcpA-dependent regulation of *bgaA*, a surface-associated β–galactosidase, via CcpA's binding to the promoter of the upstream *pts* operon, one of the main sugar transporters of the cell. The regulation of *bgaA* by CcpA was altered by changing the sugars in the growth medium. Furthermore, repression of *bgaA* was shown to increase with increasing glucose concentrations. We also demonstrated that NanA, a neuraminidase, was repressed by CcpA. This repression was alleviated in the absence of glucose. Both BgaA and NanA are differentially regulated in phase variants of *S. pneumoniae*. *S. pneumoniae* exhibits two phase variable phenotypes, opaque and transparent. Previous studies have shown that transparent variants have increased levels of BgaA, NanA, StrH, and PspC, but decreased capsule production. In contrast, opaque variants have increased capsule production, but decreased levels of BgaA, NanA, StrH,

and PspC. Differential expression of these phase variable factors leads to increased colonization by transparent variants, whereas opaque variants are better adapted for systemic infections. We hypothesized that CcpA may be involved in sensing environmental conditions and differentially regulating phase variable factors.

We have shown that CcpA responds to the changing metabolic state of the cell due to alterations in available glucose and up-regulates or down-regulates phase variable factors. In the nasopharynx, glucose is undetectable, which leads to decreased metabolism in the cell. CcpA responds to the decreased glucose and up-regulates factors involved in colonization, such as BgaA, NanA, and LytA, and down-regulates capsule. In contrast, in the blood where normal glucose concentrations are 0.1%, the cell is actively metabolizing the sugar leading to CcpA activation of capsule and repression of BgaA, NanA, and LytA. Other regulatory mechanisms besides CcpA are involved in glucose-mediated regulation, as shown by the fact that StrH activity responds to changing glucose concentrations but was not affected by CcpA. In conclusion, we have characterized a key regulatory pathway enabling *S. pneumoniae* adaptation to changing environments.

DEDICATION

I dedicate this dissertation to my mother, Patricia Kaufman, who gave me unconditional love and support throughout my graduate career.

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Thank you so much Janet for all your help and support throughout my time in your lab. You were not only a mentor but a great friend. Without your support, I would not havesurvived all the challenges I faced during my graduate career.

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INTRODUCTION

Historical significance of *Streptococcus pneumoniae*

In 1880, *S. pneumoniae* was independently isolated by 2 laboratories: the Sternberg laboratory at the Medical Department of the United States and the Pasteur Institute in France, although neither group published their findings until 1881 (83). Since then, *S. pneumoniae* has been utilized in many important scientific discoveries. *S. pneumoniae* was initially described as a diplococcus but has since been shown to also grow in chains. In 1884, *S. pneumoniae* was one of the first organisms differentiated as a Gram positive by the Gram stain. Griffith et al. in 1928 showed that a live non-encapsulated avirulent strain of *S. pneumoniae* could be transformed to an encapsulated virulent strain by co-inoculation with a heterologous heat-killed encapsulated variant (27). This resulted in pneumococcal infection with the previously avirulent strain that now expressed the capsule of the heat-killed strain. Over a decade later, Avery, MacLeod, and McCarty showed that DNA was the genetic material responsible for the capsule transformation observed by Griffith (7). Initial studies by MacLeod (48) and Heidelberger (31) using the capsular polysaccharide as a vaccine showed a 60% efficacy of protection. These studies led to the modern 23-valent capsule vaccine and the 7-valent protein conjugated vaccine (55, 57).

Disease

S. pneumoniae is a common component of the human respiratory tract, with up to 60% of the population being asymptomatically colonized (6). Exposure to *S. pneumoniae* occurs at an early age. A recent study in South Indian infants showed that 54% of infants were colonized within 2 months of birth (18). Dissemination of *S. pneumoniae* from the nasopharynx can lead to invasive diseases such as pneumonia, bacteremia, septicemia, and meningitis. Risk of serious infection is highest with children, the elderly, and those with chronic infections, including HIV and diabetes mellitus. It continues to be the leading cause of community-acquired pneumonia in these groups (13). More than two-thirds of the community-acquired pneumonia cases are due to *S. pneumoniae* (25). Death from community acquired pneumonia ranges from 6% to 20% of cases in Canada and the United States, respectively (37). More than 40,000 deaths per year in the United States are attributed to *S. pneumoniae*. It also causes over 50,000 cases of bacteremia and 500,000 cases of pneumonia annually (15, 16, 55). *S. pneumoniae* is the most common cause of bacterial meningitis leading to 3,300 cases a year, and results in death more frequently than any other manifestation of *S. pneumoniae* (9, 66). Drug resistance is a major problem in *S. pneumoniae* infections. Eighteen to 22 % of *S. pneumoniae* isolates are resistant to antibiotics and 24 to 38% are resistant to macrolides (24, 33).

Surface structures of Gram-positive bacteria

The surface of a Gram-positive bacterium is made up of the peptidoglycan composing the cell wall. The cell wall is decorated with covalently and noncovalently

anchored proteins, teichoic acids and polysaccharides, which are strain and species-specific (Fig. 1). Many proteins on the *S. pneumoniae* surface, such as β-galactosidase (BgaA), Neuraminidase A (NanA), and N-acetylglucosaminidase (StrH) are covalently anchored to the peptidoglycan via an enzyme called sortase (Srt) (12, 17, 93). In contrast, proteins such as pneumococcal surface protein A (PspA) and pneumococcal surface protein C (PspC) are noncovalently anchored to the lipoteichoic acid (92).

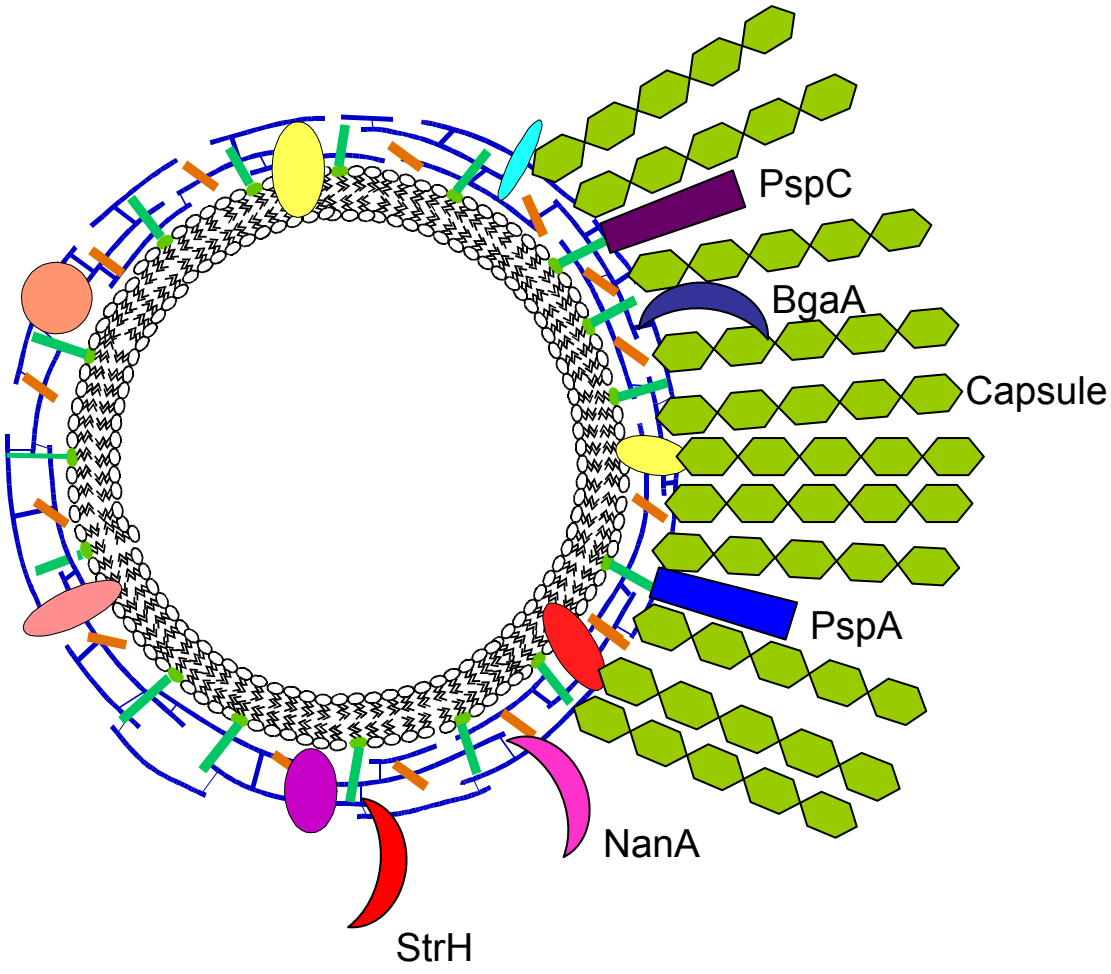


Fig. 1. Diagram of *S. pneumoniae* surface and cellular factors.

Virulence factors

There are many factors associated with *S. pneumoniae* virulence and their requirement is often niche-dependent. The capsular polysaccharide is the most important virulence factor, evidenced by the fact that non-encapsulated strains of *S. pneumoniae* are avirulent in mice and humans (27, 29). Studies by Magee and Yother showed that capsule was also important for colonization, as nonencapsulated derivatives were unable to colonize mice (49). In *S. pneumoniae* there are 91 different capsule structures (38, 60). Although there is great diversity in the capsular serotypes, only 10 serotypes cause 62 % of invasive disease (36). The capsule prevents opsonophagocytosis primarily by inhibiting the interaction of the bound complement with C3b receptors on phagocytes (1, 11).

S. pneumoniae also has many proteins that are associated with virulence. Many of these virulence factors are located on *S. pneumoniae's* surface. Some of the best characterized protein virulence factors include pneumolysin (Pln) (35, 61), NanA (50, 84), Neuraminidase B (NanB) (50), autolysin (LytA) (67, 70), pneumococcal surface protein C (PspC or CbpA) (68), and pneumococcal surface protein A (PspA) (10, 53). LytA, PspC and PspA are noncovalently linked to the choline residues on teichoic acid and lipoteichoic acid in the cell wall (68, 92). LytA is involved in degrading the peptidoglycan, causing pneumococcal cells to lyse (82), but may also be important in cell division. PspA prevents complement binding and binds to human lactoferrin to help protect against killing by lactoferrin (1, 74). Full virulence of *S. pneumoniae* also requires PspA (53). PspC has been shown to be involved in adherence and colonization (68). In contrast, pneumolysin is a secreted protein that is important for the early stages of

infection. Pneumolysin is a cholesterol-dependent pore forming toxin, which can reduce the ability of the bronchial cells to clear pneumococcal infections due to its toxic effect (65, 78).

Recent advances in technology have led to the ability to study the regulation of the genes *in vivo*. Two different studies quantified differential expression of virulence genes *in vivo*. LeMessurier et al. compared *S. pneumoniae* mRNA levels in the nasopharynx, lungs and blood relative to 16s rRNA to determine relative changes in each niche (46). They found that *nanA* was significantly up-regulated in the nasopharynx compared to the lungs or blood, and that *pspA* mRNA levels were increased in the nasopharynx relative to the other niches. *pspC* was more abundant in the nasopharynx and lung compared to the blood. In contrast, *cps2A* (capsule) levels did not seem to change significantly in any of the niches. Oggioni et al. evaluated differential gene expression of virulence factors the blood, brain, and lung compared to *in vitro* expression (58). Their study showed a significant increase in *nanA* in the brain and lung compared to the blood, whereas there was an overall increase in expression of *nanB* in all the tissues compared to *in vitro* expression. *pln* and *pspA* were up-regulated in the blood compared to the brain and lungs. They also saw very little change in capsule (*cps4A*) in all tissues evaluated. Differences in expression of *pspA* in these studies may be strain differences. The contributions of various virulence factors in *S. pneumoniae* dissemination to different tissues were also evaluated *in vivo* using real-time bioluminescent imaging in the mouse (59). This study found that NanA aids in both colonization and dissemination to the lower respiratory tract. Similar results were seen for PspC, but to a lesser extent. PspC was also

important for dissemination to the cerebrospinal fluid. In contrast, Pln and LytA were important for survival in the lung as well as the bloodstream.

Sortase-anchoring to the peptidoglycan

The model organism for studying sortase anchored proteins is *Staphylococcus aureus* (72). These proteins are secreted via a Sec-dependent mechanism that involves an N-terminal signal sequence (8, 52, 72). Initial studies determined that an LPXTG motif, hydrophobic tail, and charged tail were sufficient for cell wall anchoring (72). Further studies showed that the LPXTG motif is cleaved between the threonine and glycine and the protein is then anchored to the pentaglycine crossbridge of the peptidoglycan (56, 71). The transpeptidation reactions occur via the sortase encoded by SrtA (51). Transpeptidation initiated by cleavage of the LPXTG results in an acyl-enzyme intermediate. Nucleophilic attack by an amino group in the peptidoglycan crossbridge leads to the release of the enzyme and the anchoring of the protein to the peptidoglycan crossbridge.

BgaA. β-galactosidase activity was first characterized in *S. pneumoniae* in 1964 (32). The purified enzyme was shown to be an exoglycosidase, which was capable of cleaving a galactose at the non-reducing end of an oligosaccharide. Further studies showed that it was specific for β,1-4 linked galactose residues and could cleave galactose from glycolipids as well as oligosaccharides (45). The enzyme was also shown to be efficient at transglycosylation, being capable of removing galactose from *p*-nitrophenyl-*β*-galactose and transferring it to a disaccharide acceptor (26).

In 2000, upon partial completion of the sequencing of the TIGR4 strain (capsule type 4), *bgaA* was identified as the gene encoding the β-galactosidase activity in *S. pneumoniae* (93). In contrast to most β-galactosidases, which are approximately 1,000 amino acids, BgaA contains 2,235 amino acids. On the N-terminus of BgaA is a signal sequence, which suggested that the protein is secreted. The putative β-galactosidase activity is also located in the N-terminal portion of the protein. The C-terminal portion of the protein has no homology with any previously characterized proteins. BgaA is attached to the cell wall via sortase and an LPXTG motif located on the C-terminus. This is different than most β-galactosidases which are usually cytoplasmic. Antibodies to BgaA were identified during a study of convalescent phase serum collected from patients 26 days after diagnosis of pneumococcal infection (94). Antibodies to other surface proteins such as PspA, NanA, and StrH were also found during the course of this study. NanA and StrH are also LPXTG anchored proteins whereas PspA is a choline binding protein. BgaA, NanA, and StrH have been shown able to cleave the sugars from glycosylated host proteins (44). These sugars are galactose, sialic acid and N-acetylglucosamine, respectively (17).

Metabolic regulation

When microorganisms are exposed to multiple carbon sources, they preferentially utilize the sugar that is more rapidly metabolized (28). Mechanisms have evolved to alter gene expression based upon the nutritional source present. This regulation mechanism is referred to as catabolite repression. Catabolite repression was first described in the early 1900s in the yeast *Saccharomyces cerevisiae* (76). Since then, studies have extended to

both Gram-positive and Gram negative bacteria (for a review see (20, 81, 86). Catabolite repression involves the up-regulation of genes required for transport and metabolism of a preferred carbon source, and the down-regulation of genes required for less favorable carbon sources.

In low G+C Gram-positive bacteria the main sugar transporter is the phosphoenolpyruvate transferase system (PTS) (Fig. 2). There are 2 main components of the PTS system: 1) sugar specific components, called enzyme II (EIIs); and 2) sugar non-specific components, EI and HPr. Transport of sugars by the PTS system occurs via a phosphorelay system initiated by the transfer of a phosphate to EI via phosphoenolpyruvate. EI in turn transfers the phosphate to HPr on His-15. Sequential transfer of the phosphate then occurs from HPr to the EII portions of the system. At this point the sugar is phosphorylated by the EIIC as it is transported into the cell.

The physiological state of the cell determines the phosphorylation state of HPr. When rapidly metabolized sugars are present, HPr is predominately phosphorylated on the His-15 by phosphoenolpyruvate. Rapid metabolism leads to a build up of glycolytic intermediates, such as fructose 1,6 bisphosphate, which activate HPr kinase (HPrK) to phosphorylate HPr on the Ser-46. HPrK/P is a bifunctional enzyme, such that its activity is based upon substrates present in the cell. A build up of glycolytic intermediates activates the kinase activity of HPrK/P, which phosphorylates HPr on Ser-46 making it a poor substrate for the PTS transport system. In *Lactococcus lactis*, a 600-fold decrease in EI phosphorylation of HPr was observed when HPr was phosphorylated on Ser46 conformation (21, 79) . Therefore, HPr-Ser46 results in the down-regulation of PTS transporters and a decrease in sugar transport (22). In a feedback loop, the decrease of

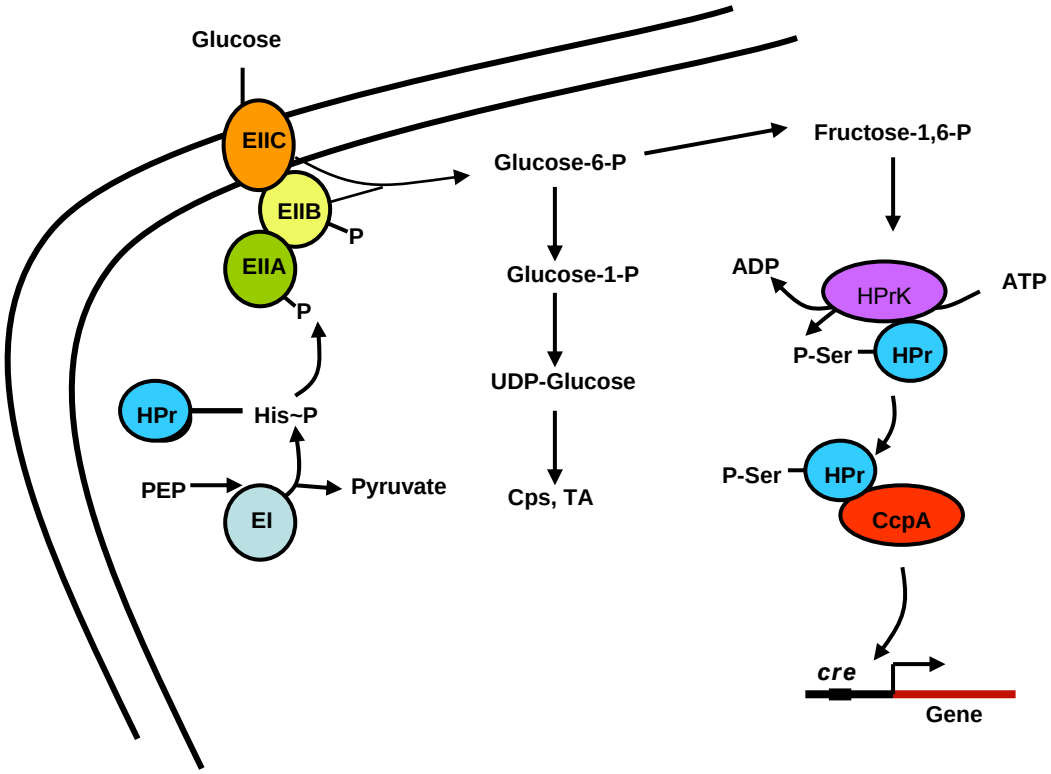


Fig. 2. Schematic representation of catabolite repression and how it integrates with other cellular processes.

glycolytic intermediates and increase of inorganic phosphate results in the activation of the phosphatase activity of HPrK/P, leading to the dephosphorylation of HPr, once again making it a substrate for the PTS transport system.

When HPr is phosphorylated on the Ser-46 residue, it is involved in a global regulatory pathway, which involves the transcriptional regulator carbon catabolite protein A (CcpA). When the high level of glycolytic intermediates causes phosphorylation of HPr on Ser46, it then interacts with CcpA. This complex acts as either a transcriptional repressor or activator. In most cases, the CcpA-HPr complex binds to a consensus

palindromic sequence called the catabolite response element (*cre*), which is located within the promoter or near the regulated gene. In cases when the *cre* is located upstream of the promoter, CcpA acts as an activator the gene expression (85). When the *cre* is located in the promoter region or in the open reading frame, CcpA acts as a repressor due to inhibition of RNA polymerase binding or initiation (41).

The consensus sequence for the *cre* is TGWAANCGNTNWCA, where nucleotides involved in binding to CcpA are underlined (39, 40, 73, 87). Recently a CcpA-DNA complex was crystallized confirming many of the previous findings of CcpA-DNA interactions, but also enhancing the understanding of the CcpA-DNA complex (73). Two of the most conserved bases are C8 and G9, which interact with Leucine-55 of CcpA. This interaction is important for minor groove expansion for intercalation of CcpA within the DNA.

Phase variation

S. pneumoniae is exposed to many different environments in the host. One way in which *S. pneumoniae* adapts to these niches is through phase variation (89). Phase variation in *S. pneumoniae* has been characterized by the differential regulation of virulence factors. The 2 phase variants characterized in *S. pneumoniae* are optimized for survival in different environmental niches, a strategy that has also been shown for other microorganisms, such as *Neisseria* and *Haemophilus* (34, 80, 88-90). The transparent variant has been shown to colonize mice at a higher level than the opaque variant (89). Moreover, within 7 days after nasopharyngeal infection, the opaque colonies had reverted to transparent colonies in the nasopharynx (89). Later studies showed that capsule was

decreased in transparent colonies, whereas teichoic acid was increased (42). Increased capsule production in the opaque variant correlated with an increase in virulence in the sepsis model of infection (42). PspA was also shown to be increased in the opaque variant.

In a more recent study, microarray analysis was used to compare gene expression in opaque versus transparent variants. Eleven of the 24 *orfs* with significant differences between the 2 phenotypes were involved in sugar metabolism (43). Two of these *orfs*, whose expression was increased in transparent variants, encode the surface-associated sugar hydrolases, NanA and BgaA. These proteins were later shown to be involved in adherence to human epithelial cells in the absence of capsule (44). A third surface-associated sugar hydrolase, StrH, was later shown to be differentially regulated in phase variants. These results suggested that these enzymes may be involved in colonization (44). *In vitro* studies with the phase variants have shown differences in adherence efficiency. Transparent variants are efficient at adhering to immobilized galactose, N-acetylgalactosamine, and N-acetylglucosamine, but are unable to bind to glucose (19). Although opaque variants have similar binding abilities, they are less efficient at binding N-acetylglucosamine. The mechanism by which the variants switch has not been identified.

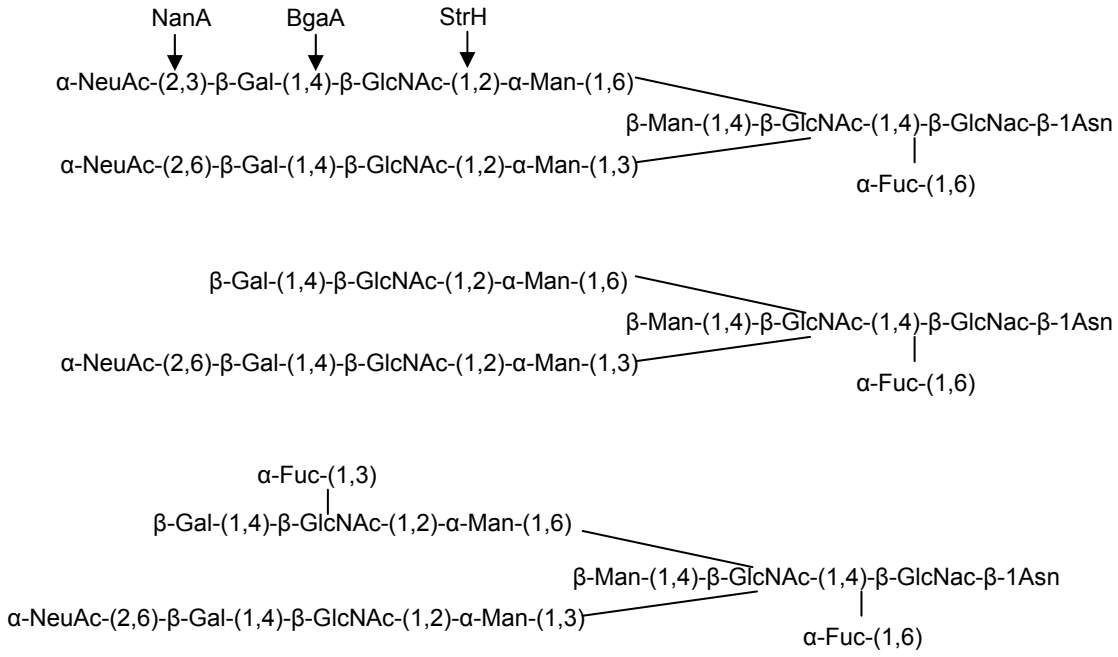
Glycosylated host cell proteins

Lactoferrin. Lactoferrin is an 80 kDa glycoprotein that was first isolated from humans in 1960 (Fig. 3A). It has been isolated from mucosal fluids such as saliva and tears, and is also found in polymorphonuclear leukocytes. Early studies by Arnold et al.

(4, 5) demonstrated that lactoferrin is one of the primary defenses against pathogens. It was shown to be bactericidal for *Streptococcus mutans* and *Vibrio cholerae*, although this was not the case for *Escherichia coli* (4, 5). It has also been shown to destabilize the outer membrane of Gram-negative organisms (2). In a limited number of organisms, the bactericidal effect of lactoferrin can be blocked by cations such as iron, calcium, and magnesium (3, 23). Results of recent studies in *S. pneumoniae* by Mirza et al. have shown that the effects of lactoferrin are diverse and vary between strains (54). Nevertheless, all strains show some degree of susceptibility to killing by lactoferrin (74). PspA was shown to protect *S. pneumonia*e from this killing.

Mucins. Mucins are glycoproteins that are the main component of mucus and an important part of the host defenses at the mucosal surface (Fig. 3B). They are either secreted or membrane bound and are found in respiratory, gastrointestinal, and reproductive tracts. Seventy to 80% of the weight of mucin is composed of sugars, which are linked to the peptide via O-glycosidic linkages. The main sugar components of mucin are fucose, galactose, N-acetylglucosamine, N-acetylgalactosamine, and sialic acid (14, 69). Respiratory mucins may also contain a small amount of mannose and sulfate (75). The sugars that decorate mucins are the same ones that provide attachment sites for many bacteria. Binding of the bacteria to mucins causes them to become trapped in the mucus layer, allowing clearance by the mucociliary defense (63, 64). However, many bacteria are able to exploit mucin for survival. *Clostridium septicum*, which is a pathogen of the gastrointestinal tract, was shown to increase mucin degradative enzymes such as neuraminidase, β-galactosidase, and N-acetyl-β-glucosaminidase upon exposure to

A.



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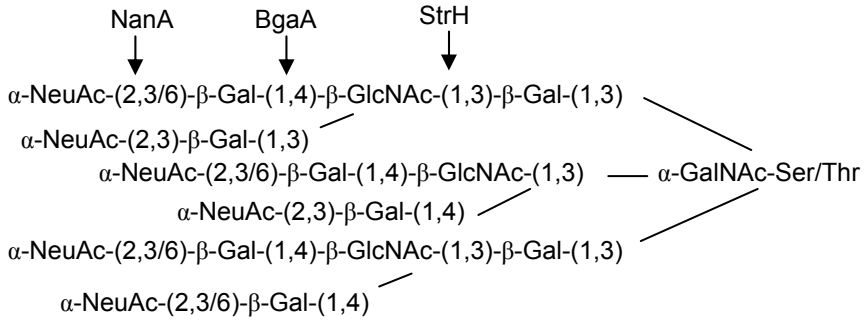

Fig 3. Schematic diagrams of glycosylated host proteins. (A) Glycosylation structures of human milk lactoferrin (77). (B) Potential glycosylation structure of mucin. In both (A) and (B), arrows designate potential cleavage sites of *S. pneumoniae* enzymes.

mucin. These enzymes, however, were repressed by a preferred carbon source such as glucose (47). Further analysis showed that *C. septicum* was able to utilize N-acetylglucosamine, N-acetylgalactosamine, and galactose released from the mucin.

Aims of the Studies

Due to the versatility of *S. pneumoniae*, it can survive in many different niches. Rapid adaptation to changing environments is imperative for survival in the host. The focus of the studies in the dissertation was to characterize a global regulatory pathway involving the integration of metabolism and virulence. We identified a spontaneous mutant that exhibited constitutive *bgaA* expression and β-galactosidase activity. Extensive studies were done to characterize the regulation of BgaA by CcpA via an upstream *pts* promoter as well as to identify the role of BgaA in virulence. BgaA is in a family of surface exoglycosidases that are LPXTG-anchored proteins on the surface of *S. pneumoniae*. We therefore determined the role of CcpA in the regulation of other surface glycosidases, namely NanA and StrH. Since these factors are differentially regulated during phase variation, we also determined the role of CcpA in the regulation of capsule, PspA and PspC by CcpA. We hypothesize that CcpA is a key regulator in the adaptation of *S. pneumoniae* to different environments via the metabolic activity of the cell.

CCPA-DEPENDENT AND -INDEPENDENT CONTROL OF BETA-GALACTOSIDASE EXPRESSION IN *STREPTOCOCCUS PNEUMONIAE* OCCURS VIA REGULATION OF AN UPSTREAM PTS-ENCODING OPERON

GREER E. KAUFMAN AND JANET YOTHER

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ABSTRACT

A spontaneous mutant of *Streptococcus pneumoniae* strain D39 exhibiting elevated β-galactosidase activity was identified. We determined that β-galactosidase activity was due to BgaA, a surface protein in *S. pneumoniae*, and that the expression of *bgaA* was regulated. Transcription analyses demonstrated expression of *bgaA* in the constitutive β-galactosidase ($BgaA^C$) mutant but not in the parent. β-galactosidase expression was induced in the parent under specific growth conditions, however the levels did not reach those of the $BgaA^C$ mutant. We localized the mutation resulting in the $BgaA^C$ phenotype to a region upstream of *bgaA* and in the promoter of a phosphoenolpyruvate-dependent phosphotransferase system (PTS) operon. The mutation was in a *cre* and affected binding of CcpA (catabolite control protein A), a key regulator of many carbon metabolism genes. The *pts* operon and *bgaA* were co-transcribed, and their transcription was regulated by CcpA. Deletion of *ccpA* altered β-galactosidase activity, leading to a 7-fold increase in the parent but a 5-fold decrease in the $BgaA^C$ mutant. The resulting β-galactosidase activities were equalized in the two strains, suggesting the presence of a second repressor. The presence of glucose in the growth medium resulted in *pts-bgaA* repression by both CcpA and the second repressor, with the latter being important in responding to glucose concentration. Expression of β-galactosidase is important for *S. pneumoniae* adherence during colonization of the nasopharynx, a site normally devoid of glucose. CcpA and environmental glucose concentrations thus appear to play important roles in the regulation of a niche-specific virulence factor.

INTRODUCTION

Streptococcus pneumoniae is a low-GC gram positive pathogen. It is a major cause of death worldwide, primarily due to invasive diseases such as pneumonia and bacteremia (1). It is also a frequent cause of many other infections, including meningitis, otitis media and sinusitis. Sixty percent of the adult population may be asymptomatically colonized in the nasopharynx by *S. pneumoniae* (4). Adaptation to different environmental niches in the host and changes in the expression of many virulence factors leads to the progression from colonization to invasive disease. Environmental sensing leading to changes in virulence gene expression has been shown for many important human pathogens (39, 40), although the exact mechanisms involved in *S. pneumoniae* are not well characterized.

The present studies were initiated when a spontaneous mutant of *S. pneumoniae* that exhibited constitutive β-galactosidase activity was identified. *S. pneumoniae* has a 6,704 bp gene, *bgaA*, which encodes the 2,235 amino acid β-galactosidase (43). The protein is surface exposed and anchored to the cell wall via sortase-mediated cleavage at the LPXTG motif. The N-terminus contains a putative signal sequence, consistent with the protein being exported. Typical β-galactosidases are approximately 1,000 amino acids and are cytoplasmic proteins. In BgaA, 365 residues located in the N-terminal half of the protein have homology to the *Escherichia coli* and *Streptococcus thermophilus* β-galactosidases. The remainder of the protein has no homology to other described proteins.

The function of BgaA has not been well characterized, but has been reported not to be involved in lactose metabolism (43). It has been suggested to be involved in digestion of host cell polysaccharides, which may play a role in adherence or host cell interaction (43). King et al. showed that BgaA is important in adherence to upper airway epithelial cells in the absence of capsule and could aid in the deglycosylation of human secretory component (22). The latter activity was increased due to increased transcription of *bgaA* in transparent strains, which exhibit less capsule production, more teichoic acid and colonize the host better than opaque strains (19-21). BgaA activity is specific for β,1-4 galactose linkages (23), which can be found in many glycosylated host cell proteins as well as some *S. pneumoniae* capsule structures (6, 15, 32). BgaA is expressed in the host, as demonstrated using convalescent-phase serum (44), and disruption of *bgaA* attenuates virulence of *S. pneumoniae* in a mouse pneumonia model (12). These data suggest that BgaA is important for *S. pneumoniae* adaptation and survival in the host.

One way in which many low GC gram-positive bacteria, including *S. pneumoniae*, adapt to changing growth conditions is through catabolite repression mediated by catabolite control protein A (CcpA). CcpA, a member of the LacI-GalR family, binds to catabolite responsive elements (*cre*) located within or near promoters (37). *cre* are identified by the sequence TGWAANCGNTNWCA (underlined nucleotides are involved with binding to CcpA) (16, 17, 29, 38). If the *cre* is located within the promoter region or open reading frame, binding of CcpA inhibits RNA polymerase interaction with the promoter or its progression through the DNA, thereby repressing transcription (18). Binding of CcpA to a *cre* located upstream of the promoter is proposed to enhance transcription by allowing CcpA to interact with RNA polymerase

(35). CcpA binding to *cre* is enhanced by the binding of Ser46-phosphorylated HPr to CcpA. HPr is a component of the phosphoenolpyruvate-dependent phosphotransferase system (PTS), which is also regulated by CcpA (reviewed in (34, 36)). The PTS is the main uptake system for sugars in many bacteria. It involves a series of enzymatic reactions that are responsible for the coupling of carbohydrate uptake and phosphorylation. HPr is phosphorylated on His-15 by transfer of the phosphoryl group from phosphoenolpyruvate (PEP) via Enzyme I (EI), which is an event that is not sugar-specific. The sugar-specific components of the pathway are designated Enzyme II (EII), which can be composed of multiple proteins or a single protein with specific EII domains. The phosphate is transferred from HPr (His-15) to the sugar-specific enzymes IIA (EIIA) and then IIB (EIIB), which phosphorylates the sugar upon translocation into the cell. Enzyme IIC (EIIC) makes up the channel involved in translocating the sugar across the membrane. When the concentrations of cellular glycolytic intermediates such as fructose-1,6-bisphosphate are increased, HPr kinase phosphorylates HPr on the Ser-46 residue. Upon Ser-46 phosphorylation, HPr is not a good substrate for EI in the PTS pathway but can interact with CcpA to enhance its binding to *cre*. When a preferred PTS sugar, such as glucose, is present, CcpA represses transcription of other PTS transporters. *bgaA* is located downstream of a PTS operon, and putative *cre* are located upstream of both *bgaA* and the PTS operon.

Two previous reports have noted a role for CcpA in the repression of β-galactosidase activity in *S. pneumoniae*, but the mechanism for repression was not known (10, 14). In this study, we identified a mutant exhibiting constitutive β-galactosidase activity. Characterization of this mutant allowed us to determine that CcpA controls β-

galactosidase activity through regulation of the PTS-encoding operon located upstream of *bgaA*. We also show that glucose regulates *bgaA* expression through both a CcpA-dependent and independent mechanism.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are shown in Table 1. For growth in liquid medium, cells were inoculated into either THY (Todd-Hewitt broth supplemented with 0.5 % yeast extract, Difco) or D-medium (tryptone 10 g/L, neopeptone 5 g/L, Tris 1.25 g/L, sodium chloride 5 g/L, yeast extract 1.25 g/L, and 0.1% of the indicated sugar source) (2). For growth on solid medium, blood agar plates (BAP, Blood agar base no. 2 [Difco] supplemented with 3% defibrinated sheep blood [Colorado Serum Company]) or THY with 1.5% Bacto-agar (Difco) were used. Where indicated, catalase and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) were added to final concentrations of 5000 U (Worthington Biochemicals) and 0.25 mg/ml, respectively. Where applicable, media were supplemented with erythromycin (Em) or kanamycin (Km) at concentrations of 0.3 µg/ml and 250 µg/ml, respectively. *E. coli* strains were maintained in L-broth or on L-agar supplemented as needed with ampicillin (Ap), Km, or Em at concentrations of 50, 50, and 300 µg/ml, respectively.

Deletion of *bgaA* and *ccpA*. To construct *bgaA* deletions, PCR fragments flanking *bgaA* were generated using *S. pneumoniae* $BgaA^C$ chromosomal DNAs as

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Reference or source
S. pneumoniae		
D39	Type 2 parent; $BgaA^+$	
GK155	pGK528 x GK1000, Em^r downstream of *bgaA*	This study
GK157	pGK530 x D39, Em^r downstream of *bgaA*	This study
GK159	pGK538 xGK1000; Δ*bgaA* in GK1000	This study
GK165	pGK538 x D39; Δ*bgaA* in D39	This study
GK220	pGK579 x D39; Δ*ccpA* in D39, Km^r	This study
GK304, GK305	pGK638 x D39, Em^r between *pts* and *bgaA*	This study
GK308, GK309	pGK638 x GK1000, Em^r between *pts* and *bgaA*	This study
GK311	pGK641 x GK1000, Em^r upstream of *pts*	This study
GK313	pGK646 x D39, Km^r between *pts* and *bgaA*	This study
GK315, GK316	Independent isogenic derivatives of D39 with constitutive β-gal mutation; -56G→C mutation in *pts* promoter	This study
GK317, GK318	pGK646 x GK1000, Km^r between *pts* and *bgaA*	This study
GK320	pGK579 x GK315; Δ*ccpA* in GK315, Km^r	This study
GK322	pGK538 x GK315; Δ*bgaA* in GK315	This study
GK338	pGK663 x D39; *pts* insertion in D39, Em^r	This study
GK339	pGK663 x GK1000; *pts* insertion in GK1000, Em^r	This study
GK344, GK345	pGK663 x GK220; *pts* insertion and Δ*ccpA* in D39, Km^r, Em^r	This study
GK346	pGK663 x GK320; *pts* insertion and Δ*ccpA* in GK1000, Km^r, Em^r	This study
GK1000	Spontaneous $BgaA^C$ derivative of D39; -56G→C mutation in *pts* promoter	This study
GK1001	Spontaneous $BgaA^C$ derivative of D39; -57C→T	This study

	mutation in *pts* promoter	
E. coli		
BL21(AI)™	*F- ompT hsdSB(rB-mB-)gal dcm lon araB::T7RNAP-tetA*	Invitrogen
DH5αF'	F'Φ80d*lacZ*ΔM15Δ (*lacZYA-argF)*U169 *deoR recA1 endA1 hsdR17*($r_k^-m_k^+$) *phoA* sup*E44*λ^- *thi-1 gyrA96 relA1*	Life Technologies
GK639	BL21(AI)™, pGK639, CcpA-His Ap^R	This study
Plasmids		
pCR2.1	PCR Cloning vector, Ap^R, Km^r	Invitrogen
pET20b	T7 RNAP expression vector, Ap^R	Promega
pJY4164	Lacks origin of replication for *S. pneumoniae*, Em^R	(42)
pGK528	pJY4164 derivative containing PCR fragments from the amplification of GK1000 chromosomal DNA by the primer pair D-BgaA3/D-BgaA4; for linkage of Em upstream of *bgaA*	This study
pGK530	pJY4164 derivative containing PCR fragments from the amplification of D39 chromosomal DNA by the primer pair D-BgaA3/D-BgaA4; for linkage of Em downstream of *bgaA*	This study
pGK538	pJY4164 derivative containing PCR fragments from the amplification of GK1000 DNA by the primer pairs F-UpbgaA/R-UpbgaA and F-downbgaA/R-DownbgaA; for deletion of *bgaA*	This study
pGK579	pJY4164 derivative containing PCR fragments from the amplification of GK1000 DNA by the primer pairs F-UpCcpA1/R-UpCcpA2 and F-downCcpA3/R-DownCcpA4 with Km-resistance gene, *aphA-3*, between 2 fragments; for deletion of *ccpA*	This study
pGK638	pJY4164 derivative containing PCR fragments from the	This study

	amplification of D39 chromosomal DNA by the primer pair F-UpbgaA/R-UpbgaA; for polar insertion between *pts* and *bgaA*	
pGK639	pET-20b::*Nde*I-*Xho*I from PCR product amplified from D39 using primer pair CcpA-NdeI/*CcpA*XhoI; C-terminal His-tagged CcpA	This study
pGK641	pJY4164 derivative containing PCR fragments from the amplification of GK1000 chromosomal DNA by the primer F-spr0562/R-spr0562; for linkage of Em downstream of *bgaA*	This study
pGK646	pJY4164 derivative containing PCR fragments from the amplification of D39 chromosomal DNA by the primer pair F-UpbgaA/R-UpbgaA and F-bgaAproKpnI/BgaA2 with Km-resistance gene, *aphA-3*, between 2 fragments: used to insert Km-resistance between *pts* and *bgaA*	This study
pGK663	pJY4164 derivative containing PCR fragments from the amplification of D39 chromosomal DNA by the primer pair F-PTS3/R-PTS2; for polar insertion in *pts* operon	This study

templates. Chromosomal DNA was extracted using Qiagen genomic-tips, as described by the manufacturer (Qiagen). The upstream and downstream regions obtained using primer pairs F-UpBgaA/R-UpBgaA and F-DownBgaA/R-DownBgaA (Table 2) were initially cloned in pCR2.1 (Invitrogen). The fragments were excised from pCR2.1 by digestion with *Eco*RI, which is contained in the multiple cloning site of the vector, and *Kpn*I, which was added to the PCR product. The excised fragments were cloned together

Table 2. Oligonucleotide primers used is this study

Primer[a]	Sequence[b]	Position[c]
From *S. pneumoniae* R6 section 51 of 184 of the complete genome (spr0562-0565)		
F-spr0562	TTGATTGAAAGGGTTAGTATTGAC	11-34
F-PTSpro2	CTAGCTTCCTAGTTTACTCTTTG	266-288
R-PTSpro3	CAAAGAGTAAACTAGGAAGCTAG	288-266
R-spr0562	CTATATGAAACCGTTGTCAATTAC	429-406
R-PTSpro	CTAATACCATAAGTTTTCCCTTC	483-461
F-PTS3	GGAATTTCTAGGAAAGGACTTGC	647-669
R-PTS2	CGATAACTGGGATACCTGGTTC	1201-1180
F-UpBgaA	GCAGCTATCGTTCTTGTCGGTGTGTTG	1824-1850
R-UpBgaA	TATTTTGCTTTTGCTGCGTACTC	2815-2793
F-bgaAproKpnI	TGCGCTCCTATAAAATATAAACTC	2818-2840
BgaA2	CTACGATACCAAAGTAAGAGCT	5329-5308
F-DownBgaA	GAGTGCAGGATTAGTAGTTACTAAAG	9765-9790
D-BgaA3	GTCCTAAATAGAAGATAAAGAG	9854-9875
From *S. pneumoniae* R6 section 52 of 184 of the complete genome (spr0566-0579)		
D-BgaA4	GAACGAACGCTATCAAAACTTGAAAGC	183-157
R-DownBgaA	CAGTTCCTTCTTACCACAAGACC	762-741
From *S. pneumoniae* R6 section 161 of 184 of the complete genome (spr1806-1817)		
F-UpCcpA1	ACATATGCTGGTCCTCTACCAG	5336-5357
R-UpCcpA2	GAAAAAATCAGGGAATCGAGAAG	6370-6348
F-CcpAORF-XhoI	TTTACGTTTTCGTGTTGAG	6374-6392
F-CcpAORF-NdeI	ATGTGTGAGATAGAAAGG	7411-7394
F-DownCcpA3	TCTTTTACAAGTAGAGGTACTGATTG	7412-7437
R-DownCcpA4	CATCCAACGGAAGTGCAAGTTC	8451-8433

[a] Forward and reverse primers are represented by F and R, respectively.
[b] sequences are from the complete R6 genome (13)

[c] nucleotide positions for primers are listed in the forward or reverse orientation, as necessary

in the *S. pneumoniae* suicide vector pJY4164. The deletion plasmid (pGK538) was maintained in DH5αF' and the appropriate construction was confirmed by sequencing. pGK538 was transformed into competent *S. pneumoniae*, and the transformation mixture was plated on THY agar plates. For deletion of *bgaA* in strains constitutive for β-galactosidase activity, colonies were screened on plates supplemented with X-gal and catalase to identify white isolates, indicative of loss of β-galactosidase expression. Deletion of *bgaA* in these isolates was confirmed by PCR. For deletion of *bgaA* in strains that exhibited low β-galactosidase activity, the transformation mixture was plated on D-agar supplemented with X-gal and catalase, where the cells exhibit more β-galactosidase activity than on THY-agar. Colonies that were reduced in β-galactosidase activity were patched onto D-agar plates supplemented with X-gal and catalase. They were screened for deletion of *bgaA* by colony PCR in which 10 patches were suspended in 300 µl of water and then boiled for 10 min. Three µl of the mixture was used in a PCR reaction with primer pair F-UpBgaA/R-DownBgaA and the resulting products were screened for reduction in size.

The construct for deletion of *ccpA* was generated in a similar manner as the *bgaA* deletion construct. Primer pairs used for amplification of the flanking regions were: F-UpCcpA1/R-UpCCpa2, F-DownCcpA3/R-DownCcpA4, Table 2). For selection of *ccpA* deletions, a Km resistance marker (*aphA-3*) was inserted between the upstream and downstream flanking regions using restriction enzyme *Kpn*I, as described in (41).

Linkage analysis. For linkage analyses, insertion-duplications were used to place an Em-resistance marker either 3 kb upstream, 0.5 kb downstream, or 15 kb downstream of *bgaA*. Target DNA fragments were cloned into pJY4164 to generate plasmids pGK641, pGK530, pGK547, respectively (Table 1). Recipients transformed with the plasmids were selected by plating on BAP containing Em. Competence was induced as described in (11) except that cultures were incubated only 2-2.5 h prior to plating. Location of the insertion-duplications in the expected sites were confirmed by PCR. For linkage analyses, either intact or restriction-digested chromosomal DNA from the marked strains was transformed into recipient strains. Em-resistant isolates were selected on THY agar plates supplemented with Em and catalase. Em-resistant transformants were screened for the β-galactosidase phenotype by plating on THY-catalase-Em plates supplemented with X-gal.

β-galactosidase assays. β-galactosidase activity was determined as described by Miller (26). Briefly, cultures were grown to mid-exponential phase (cell density of 3 x 10^8 CFU/ml). A 0.2 ml aliquot of the culture was added directly to 0.8 ml Z-buffer and the suspension was incubated at 30°C with 0.2 ml of 4 mg/ml *o*-nitrophenol-β-D-galactopyranoside (ONPG). Reactions were stopped by the addition of 0.5 ml of 1 M Na_2CO_3. Activity was calculated as Miller units, as described (26).

Transcription analysis. Total RNA was isolated from a 50 ml culture grown in THY using a hot acid phenol extraction, as described previously (9). RNA concentrations were determined using UV spectrophotometry. Transcript levels were determined using slot-blotting (3). Briefly, samples were diluted to 3 and 0.5 µg per 30 µl for each probe and denatured for 15 min at 65°C in 90 ul of denaturing solution 500 µl

formamide, 162 µl 12.3 M [37%] formaldehyde, and 100 µl MOPS [3-(*N*-morpholino)-propanesulfonic acid] buffer [0.2 M MOPS, (pH 7.0), 0.5 M sodium acetate, and 0.01 M EDTA]. Then, 240 µl of cold 20x SSC (1x SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) was added to each sample. Denatured samples were spotted on nylon membranes which were UV crosslinked and then prehybridized for 3 h at 42°C in high SDS hybridization buffer (7% SDS, 50% formamide, 5x SSC, 2% blocking Reagent [Roche], 50 mM sodium phosphate, and 0.1% *N*-laurylsarcosine). Membranes were incubated overnight with denatured digoxigenin (DIG)-labeled PCR probes made with primers BgaA1/BgaA2 and F-PTS3/R-PTS2 (Table 2), which were added directly to the membranes in the high SDS hybridization buffer. To remove non-specifically bound probe, membranes were washed twice with 2x SSC containing 0.1% SDS for 15 min at room temperature, and then twice with 0.5x SSC containing 0.1% SDS for 15 min at 65°C. The blots were developed using the Anti-DIG-AP Fab fragments (Roche) and Phototope™-Star Detection Kit for Nucleic Acids (New England BioLabs). The relative levels of transcript were determined by densitometry using ImageJ software (http://rsb.info.nih.gov/ij). A *pspA* probe was used as an internal control to ensure equal loading (11).

Electrophoretic mobility shift assays (EMSA). To clone *ccpA*, the open reading frame was PCR amplified with primers that contained restriction enzyme sites for *Xho*I and *Nde*I on the 5' ends of the forward and reverse primers, respectively (F-CcpAORF-XhoI/R-CcpAORF-NdeI, Table 2). After *Nde*I and *Xho*I digestion, the PCR product was ligated into pET20b to generate a C-terminal His tag, and the ligation mixture was transformed into BL21-AI (Invitrogen). To induce *ccpA* expression,

arabinose (2% final concentration) was added to a 50 ml culture at late exponential growth phase. CcpA was purified under non-denaturing conditions in wash/extraction buffer using a cobalt resin (Clontech) to affinity purify the His-tagged protein, as described by the manufacturer. For cellular lysate preparation, D39 was grown to mid-exponential phase in THY and concentrated 10-fold. The cells were lysed by incubation at 37°C in 0.01 M Tris (pH 8) containing 0.5% sodium-deoxycholate with protease and nuclease inhibitors. The promoter regions of the *pts* operons (positions – 206 to +10) were PCR-amplified from the indicated strains using primers F-PTSpro2/R-PTSpro (Table 2). The purified PCR products were DIG-labeled as described by the manufacturer (Roche, DIG gel shift kit). Labeled probes (0.8 ng) were incubated with increasing concentrations of purified CcpA in binding buffer (10 mM Tris-HCl pH 7.4, 1 mM dithiothreitol, 1 mM EDTA, 50 mM KCl, 5% glycerol, 50 µg/ml bovine serum albumin, 0.05% Nonidet P-40) for 20 min at 37^{o}C (17). After incubation, samples in bromophenol blue loading buffer were separated by electrophoresis using 5%-acrylamide gels in 1x Tris-borate-EDTA (TBE) buffer pH 8.0 (10.8 g Tris-HCl, 5.55 g Boric Acid, 0.74 g EDTA) that had been pre-run in 1x TBE. The samples were electroblotted from the gels onto Zeta-Probe positively charged nylon membranes (Bio-Rad). The membranes were UV cross-linked (Stratagene) and developed using chemiluminescence, as described for the DIG gel shift kit (Roche). Chemiluminescent images were viewed using an EpiChemi^{3} DarkRoom (UVP).

RESULTS

Isolation of β-galactosidase-constitutive mutants. Previous studies of the *S. pneumoniae* capsule serotype 2 strain D39 demonstrated a low level of *bgaA*-encoded β-galactosidase activity (~5 Miller units) (22). Analysis of a D39 stock culture in our laboratory that was originally obtained more than 20 years ago (24) revealed the presence of both β-galactosidase-positive and apparent β-galactosidase-negative isolates, as determined by plating in the presence of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) to identify blue and white colonies, respectively. Following growth in THY liquid medium to a density of ~3 x 10^8 CFU/ml, β-galactosidase activity of the white isolates was 2 to 5 Miller units whereas that of the blue isolates was 60 to 75 Miller units. Deletion of *bgaA* in the white and blue isolates demonstrated that BgaA was responsible for the activities observed in both. As described below, the elevated β-galactosidase activity was found to be due to constitutive expression of *bgaA*, therefore these isolates are referred to as $BgaA^C$. The isolates producing low levels of β-galactosidase are considered to be wild-type and are therefore referred to as D39.

We initially chose a single $BgaA^C$ isolate (GK1000) for further characterization. Both D39 and the constitutive strain exhibited similar growth rates in THY. Restriction digests of chromosomal DNAs with *Hin*dIII yielded identical patterns for the two strains (data not shown). The size and monoclonal antibody reactivities of the surface protein PspA, which is size and antigenically variable among strains (7), were also identical (data not shown). These results indicated that the $BgaA^C$ strain was a D39 derivative. RNA

analyses demonstrated elevated transcription of *bgaA* in the BgaA^C mutant (see below), but no mutations were evident in the *bgaA* promoter region (data not shown). We therefore undertook linkage analyses to localize the mutation(s) responsible for the altered β-galactosidase levels. To determine if the mutation was located in *bgaA*, we introduced the D39 *bgaA* chromosomal region into the BgaA^C derivative GK159, in which *bgaA* had been deleted. The ability to isolate BgaA^C transformants with this cross would indicate that the mutation leading to this phenotype was not located in *bgaA*. For linkage analyses, an erythromycin (Em) marker was inserted 475 bp downstream of *bgaA* in D39, and chromosomal DNA from this strain (GK157) was used to transform GK159. Among the Em-resistant transformants, 11% exhibited the BgaA^C phenotype, as determined by blue-white colony screening on X-Gal plates. We next introduced the same Em insertion downstream of *bgaA* in the original BgaA^C mutant (GK1000) and transformed DNA from this strain (GK155) into D39. Here, 25% of the Em-resistant transformants exhibited the BgaA^C phenotype, indicating that the mutation was closely linked to *bgaA*. Further linkage analyses utilized derivatives of the BgaA^C strain that contained Em markers located at various sites around *bgaA*. Following transformation of D39 with DNA from the BgaA^C derivative GK311 that contained the Em marker 3 kb upstream of *bgaA*, 89% of the Em-resistant transformants exhibited the BgaA^C phenotype. Using restriction enzyme-digested DNA from GK311 in further transformations of D39, the mutation was localized to a 4 kb region (Fig. 1). Transformation of D39 with PCR products derived from this region showed that the mutation was in a region located immediately upstream of *bgaA* and containing genes encoding PTS enzymes. Sequence analyses identified a single point mutation located

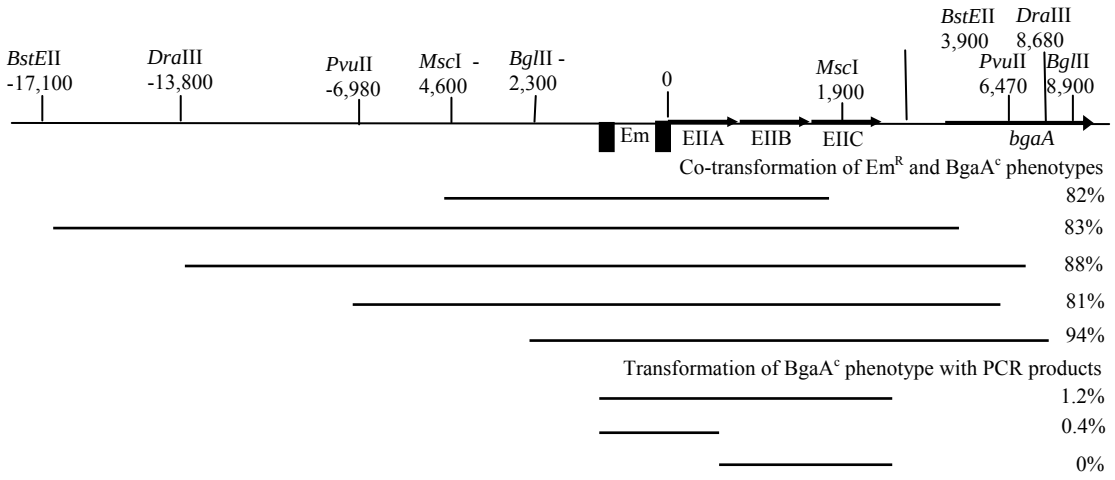

FIG. 1 Mapping of mutation leading to BgaA^C phenotype. Restriction fragments from the BgaA^C strain GK311 were transformed to D39. Em^R transformants were screened for the blue colony BgaA^C phenotype by plating in the presence of X-gal. Co-transformation of the BgaA^C phenotype with Em^R represents close linkage to the antibiotic marker. In transformations of D39 with PCR products, the transformation mixtures were plated in the presence of X-gal and the percentage of blue colonies was determined. Transformation of the BgaA^C phenotype with the PCR fragments indicates that the mutation is located in the specific fragment. EIIA, EIIB, and EIIC encode PTS enzymes for sugar transport.

between the -35 and -10 sequences of the putative promoter for the *pts* operon. This region contains a putative *cre* (Fig. 2A). Sequence analyses of the *pts* promoter regions from ten additional BgaA^C isolates purified from the primordial stock demonstrated that six of the isolates contained the same G to C transversion mutation found in GK1000, while the remaining four isolates contained a C to T transition mutation (GK1001) of the nucleotide immediately upstream of this position (Fig. 2A). Thus two independent mutants appear to have arisen during the passage of the culture leading to the stock. The mutations appear to be rare, however, as we have not subsequently observed transition from the white-to-blue phenotype, or vice-versa.

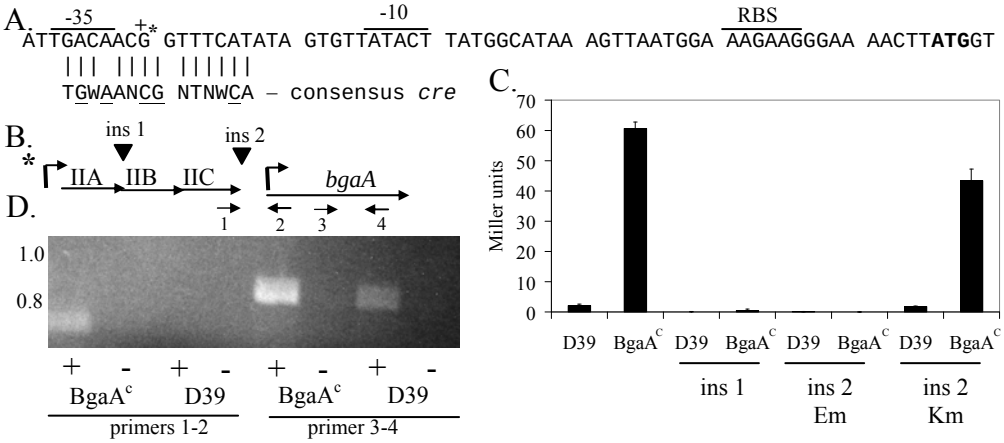

FIG. 2. Transcription of the *pts-bgaA* region. (A) *pts* promoter region. * , point mutation (G→C) in the original BgaA^C mutant GK1000 and derivatives; +, point mutation (C→T) in a second mutant, GK1001, and derivatives. *cre* consensus sequence is aligned below the *pts* promoter. Underlined amino acids are important in CcpA binding (29, 38). N, any base, W, adenine or thymine. Lines above the sequence indicate the -35, -10, and ribosome binding site (RBS). The translation start codon is bolded. (B) Insertions and primers used to characterize *bgaA* expression. Arrows and numbers beneath the figure indicate primers used in RT-PCR analyses in panel C. Asterisk indicates the location of the *pts* promoter point mutation in the BgaA^C strain. Insertions are indicated by arrows above the *pts* region. (C) β-galactosidase activity of insertion mutants. Locations of insertions 1 and 2 are indicated in panel B. Insertion 2-Em is polar on downstream genes. Insertion 2-Km is non-polar. Results are the means (± standard error) of 3 replicates. (D) RT-PCR analyses of D39 and the BgaA^C strain, GK1000. + and – indicate the presence or absence, respectively, of reverse transcriptase in the reaction. Numbers below the figure represent the primer pairs used in the reaction, as shown in panel B. Molecular size (kb) is indicated on the left.

To confirm that the G to C mutation was responsible for the BgaA^C phenotype in GK1000, an isogenic derivative (GK315) was obtained by transforming D39 with a 1 kb PCR product amplified from GK1000 and encompassing the mutation. Approximately 0.4% of the colonies on X-Gal plates were blue, indicating introduction of the BgaA^C phenotype. Sequence analysis of the 1 kb region in the recipients confirmed that the only mutation occurred in the *pts* promoter. Subsequent studies were performed with the isogenic derivative GK315, except where stated.

Co-transcription of *pts* and *bgaA*. To determine whether the constitutive transcription of *bgaA* in the BgaA^C mutant GK315 was due to a *cis* effect of the mutation in the *pts* promoter, we analyzed the effect of insertion-duplication mutations in and around the *pts* operon. Polar insertion mutations either in the *pts* operon (Fig. 2B, insertion 1) or between the *pts* operon and *bgaA* (Fig. 2B, insertion 2 - Em) eliminated β-galactosidase activity (Fig. 2C). However, when a non-polar kanamycin-resistance marker lacking transcription termination sequences was inserted between the *pts* operon and *bgaA*, β-galactosidase activities were unchanged from the D39 and BgaA^C parents (Fig. 2B and 2C, insertion 2 – Km). RT-PCR analysis confirmed that *bgaA* and the upstream *pts* operon were located on the same transcript (Fig. 2D), and RNA slot blotting demonstrated that the insertion in the *pts* operon eliminated the *bgaA* transcript (data not shown). The region between the *pts* operon and *bgaA* does not contain any apparent transcription termination sequences, but a near consensus promoter is located 14 nt upstream of the putative BgaA start codon and a Box element is located between *pts* and *bgaA*. Box elements in *S. pneumoniae* have been predicted to contain secondary structure but their function is unknown (25). Deletion of the Box element in D39 had no effect on

β-galactosidase activity (data not shown). Thus, under the conditions examined, the *bgaA* promoter was not utilized, and the BgaA^C phenotype resulted from co-transcription of *bgaA* with the *pts* genes from the *pts* promoter.

CcpA binding to the *pts* promoter. Because the point mutations affecting *bgaA* expression were located in a putative *cre* overlapping the -35 region of the *pts* promoter, we used gel shift assays to examine the interaction of CcpA with this region amplified from D39 and the BgaA^C mutants, GK1000 and GK1001, containing the two different point mutations. Efficient binding of CcpA to the D39 *pts* promoter was observed, but very little binding occurred with the BgaA^C mutants (Fig. 3). With the D39 *pts* promoter, binding was shown with CcpA protein levels as low as 3.75 ng whereas levels as high as 50 ng were not sufficient to bind the BgaA^C *pts* promoters (data not shown). This result suggested that CcpA binding normally represses *pts* and *bgaA* expression, and that the mutations led to a decreased affinity of CcpA for the *cre*. We did not detect binding of CcpA to the *bgaA* promoter region although a potential *cre* was located in this region (data not shown).

Alteration of *pts-bgaA* expression and β-galactosidase activity by deletion of *ccpA*. Since the transcriptional regulator, CcpA, binds to the *pts* promoter and co-regulates *bgaA*, we examined the effect of the deletion of *ccpA* on *bgaA* and *pts* transcription. In D39, the deletion resulted in an increase in both transcript levels, whereas in the BgaA^C mutant decreases occurred for both (Fig 4A). The result was an

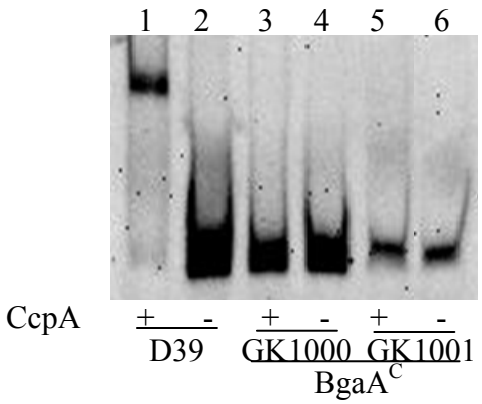

FIG. 3. CcpA binding to *pts* promoter. Electrophoretic mobility shift assays were used to determine if the point mutation identified in BgaA^C resulted in altered binding of CcpA. Purified CcpA (15 ng) incubated with DIG-labeled D39 or BgaA^C *pts* promoter DNAs was electrophoresed on a 5% non-dentaturing acrylamide gel. The labeled DNA was detected by chemiluminescence.

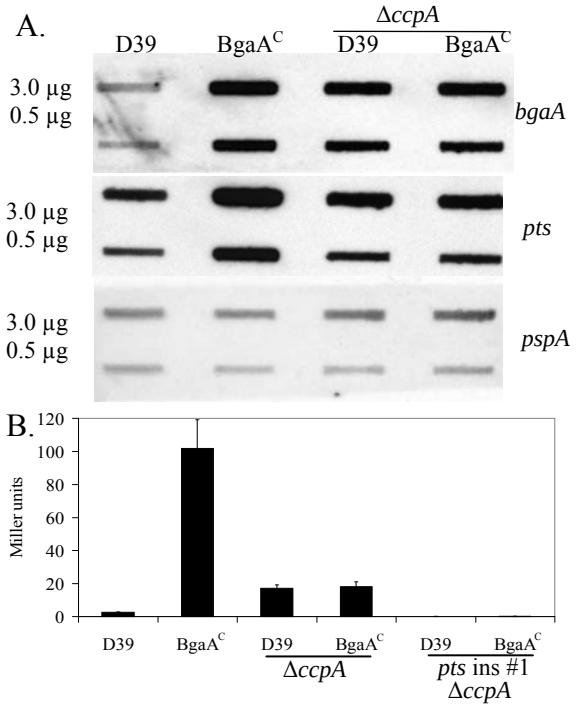

FIG. 4. (A) Effect of *ccpA* deletion on transcription and β-galactosidase activity. Slot blots containing the amount of RNA indicated on the left were probed with internal sequences from genes indicated on the right. BgaA^C is GK1000. *pspA* was used as a loading control. (B) β-galactosidase activity. Cultures were grown to mid-exponential phase in THY. Results are the means (± standard error) of 3 replicates.

equalization of the respective transcript levels in the two strains. We also examined levels of β-galactosidase activity in the *ccpA* deletion strains. The result was similar to the transcriptional analysis, such that the levels of β-galactosidase activity equalized for the D39 and BgaA^{C} *ccpA* mutants (Fig 4B, Δ*ccpA*). To confirm that the β-galactosidase activity and *bgaA* expression in the *ccpA* deletions were not due to expression from the promoter immediately upstream of *bgaA*, *pts* was disrupted in the deletion strains. As shown in Fig 4B, β-galactosidase activity was lost in these strains (*pts* ins #1, Δ*ccpA*). The results of these experiments suggested that there was a second repressor of *pts* and *bgaA* expression.

Binding of the second repressor to the *pts* promoter. Total cellular lysates from D39 were used in gel shift assays to detect binding of the second repressor to the *pts* promoter region. Incubation of the D39 *pts* promoter in the presence of either lysate or lysate plus purified CcpA yielded a band slightly higher than that observed with the purified CcpA alone (Fig. 5, lanes 1-3). The increased size of the protein-DNA complex may have been due to the presence of HPr in the cellular lysate, which would interact with CcpA in binding to the *pts* promoter, or to the binding of both CcpA and the second repressor. In the absence of purified CcpA, a smaller and less intense band was also observed following incubation of the D39 *pts* promoter with cellular lysate alone (Fig. 5, lane 3, PTS + SF). A more intense band of this same size was present in reactions with the BgaA^{C} *pts* promoter using lysate or lysate plus purified CcpA (Fig. 5, lanes 5 and 7). In these experiments, weak binding of purified CcpA to the BgaA^{C} *pts* promoter was observed in the absence of lysate (Fig. 5, lane 6). These results suggested that the D39 *pts*

promoter had a higher affinity for CcpA than for the second repressor, but the BgaA^C mutant *pts* promoter had a higher affinity for the second repressor.

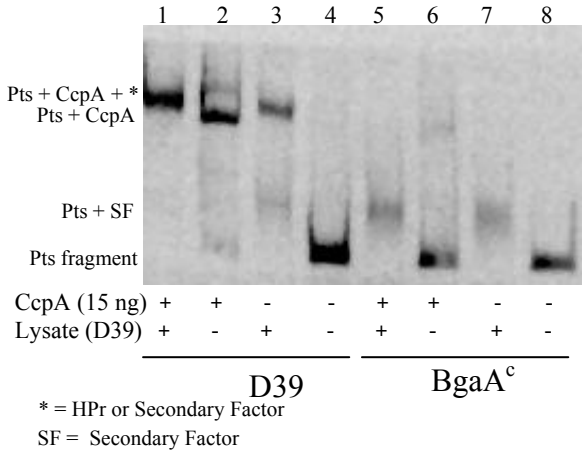

FIG. 5. Binding of a second repressor to *pts* promoter. Electorphoretic mobility shift assays were used to determine if the second repressor of *pts-bgaA* was a DNA binding protein. Purified CcpA and total cellular lysates from D39 incubated with DIG-labeled D39 and BgaA^C (GK1000) *pts* promoter DNA were processed as in Fig. 3.

Regulation of β-galactosidase activity by carbon source. The regulation of *pts* and *bgaA* by CcpA led us to examine conditions that might affect expression of these genes. Since both were co-transcribed, we utilized β-galactosidase activity from BgaA as a measure of expression. The standard growth medium (THY) used in the prior studies contained 0.2% glucose. By using a semi-defined growth medium and altering the sugar source, we found that glucose mediated repression of *pts-bgaA* expression. For D39 but not the BgaA^C mutant, repression was observed with increasing glucose concentrations (Fig. 6A). When *ccpA* was deleted, equal levels of repression were observed with both strains and these were elevated over that observed for the CcpA$^+$ D39 (Fig. 6B). These

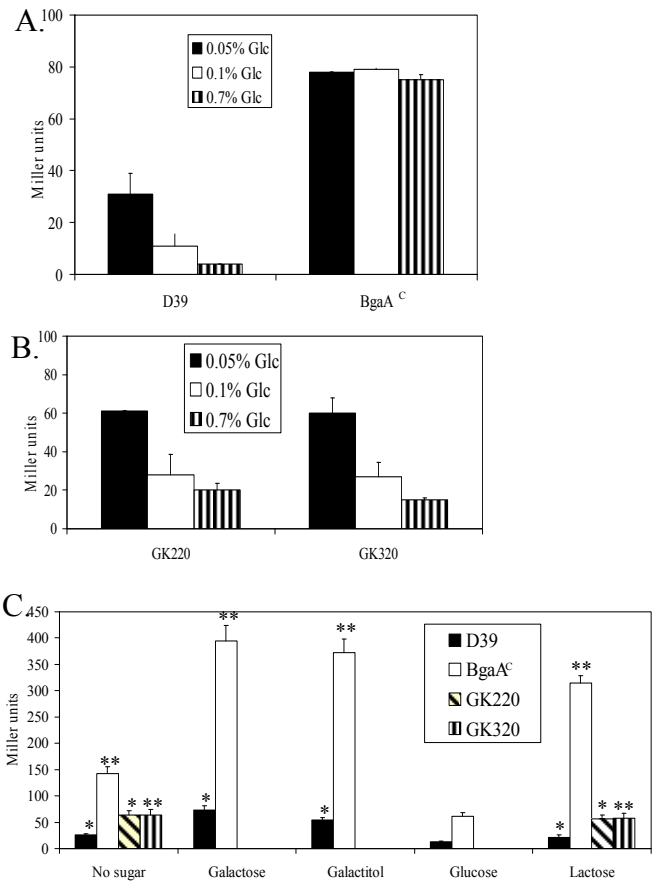

FIG. 6. β-galactosidase activity during growth in different sugars. (A) β-galactosidase activity of D39 and BgaA^C in increasing glucose. (B) β-galactosidase activity of *ccpA* deletion mutants of D39 (GK220) and BgaA^C (GK320). For both A and B, strains were grown to mid-exponential growth phase in D-medium supplemented with 0.1% glucose and then diluted 1/12 into D-medium supplemented with the indicated glucose concentration. β-galactosidase assays were performed with mid-exponential phase cultures. Results are the means (± standard error) of 3 replicates. (C) β-galactosidase activity in various PTS sugars. Strains were grown to mid-exponential growth phase in D-medium with the indicated sugar (0.1%) and then diluted 1/12 into D-medium containing the same sugar. For the no sugar culture, strains were first grown in D-medium containing 0.1% glucose and then diluted 1/12 into D-medium lacking a sugar. When either no sugar or galactitol was added to the growth medium, very little growth was observed for either D39 or the BgaA^C mutant (GK315). For these cultures, β-galactosidase assays were performed after growth ceased (after glucose was exhausted for the no sugar culture). For all others, β-galactosidase was determined at mid-exponential phase. Results are the means (± standard error) of 3 replicates. *, $P < 0.05$ compared to D39 grown in glucose. **, $P < 0.05$ compared to GK315 grown in glucose.

results suggested that glucose repression of the *pts-bgaA* operon involved both CcpA and the second repressor, and that repression with increasing glucose concentration was due primarily to the second repressor and not CcpA. The fact that repression occurred in the $BgaA^C$ mutant when *ccpA* was deleted again suggested that weak binding of CcpA to the mutant *cre* affected binding of the second repressor to its recognition sequence in the promoter region.

The PTS operon upstream of *bgaA* has homology to putative fructose and galactitol transporters in *Clostridium acetobutylicum* and *Streptococcus agalactiae* (~38% identity, ~65 % positives), and to a putative galactose transporter from *Streptococcus pyogenes* (41% identity, 63% positives). We therefore examined β-galactosidase activity during growth in these sugars and in lactose, as uptake of these sugars is mediated by PEP-dependent phosphotransferase systems. As shown in Fig. 6C for both D39 and the $BgaA^C$ mutant, high levels of β-galactosidase activity were seen when no sugar was added, and activity increased during growth with galactose, galactitol, or lactose. β-galactosidase activity for D39 never reached the level of the $BgaA^C$ mutant. As shown for glucose in Fig. 6B and lactose in Fig. 6C, deletion of *ccpA* resulted in equalization of the D39 and $BgaA^C$ β-galactosidase levels due to increased activity in D39 and decreased activity in the $BgaA^C$ mutant. For the *ccpA* deletions, β-galactosidase activity was the same during culture with lactose or no sugar, indicating that the obesrved activity in these mutants was the result of a lack of repression by glucose and not induction by lactose (Fig. 6C).

During growth of D39 in glucose or lactose, we observed increased β-galactosidase activity in early stationary phase growth (approximate 10- and 17-fold

increases, respectively, over mid-exponential phase), as previously reported for growth in glucose (22). β-galactosidase activity in the $BgaA^C$ mutant remained essentially constant throughout growth in glucose but increased approximately 2-fold in lactose upon entering stationary phase.

Effects of *pts* mutation on growth. We examined the effect of an insertion mutation in the D39 *pts* on growth in glucose and galactose, as the PTS transporter had homology with a putative galactose transporter in *S. pyogenes* and was co-transcribed with β-galactosidase, an enzyme that cleaves terminal galactose residues. The mutation affected growth in both sugars, and the mutant appeared unable to initiate growth in the presence of galactose only (Fig. 7). Growth was also slowed in rich medium (THY; data not shown). Similar results were obtained for the $BgaA^C$ constitutive mutant GK315 and its *pts* insertion mutant (GK339) in glucose, galactose, and THY (data not shown). In contrast to this PTS, several of the other PTS in D39 do not contain all of the components for sugar transport (13, 33). Therefore, this PTS may be important for the uptake of many sugars, possibly explaining the growth defects of the *pts* insertion mutants.

β-galactosidase activity in other strains. A wide variation in β-galactosidase activity was seen among other *S. pneumoniae* strains (Fig. 8). NCTC7466 is another D39 isolate that is separated by many years from our D39 (24). It exhibited the same level of β-galactosidase activity and the same promoter region sequence as our strain (by comparison with the available genome sequence (13)). From the genome sequence of TIGR4 (33) and sequence analyses of the capsule type 2 strains BG12738, BG12758, ASL-940, and SP-81, we found that all had *pts* promoter and *cre* regions identical to that of D39 (shown in Fig. 2A). The high levels of β-galactosidase activity in some strains,

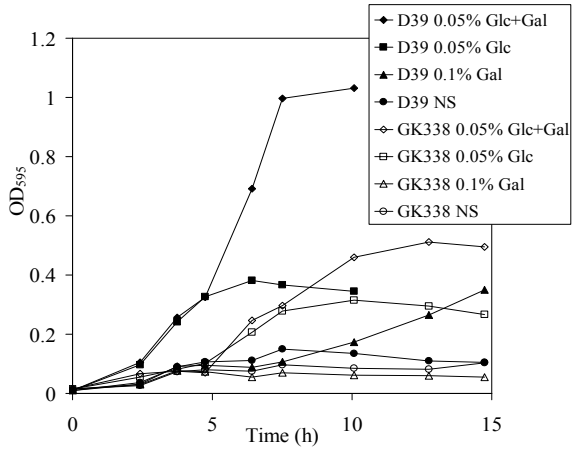

FIG. 7. Growth of D39 and its *pts* insertion mutant (GK338). Strains were grown in D-medium with the indicated sugar source. Gal, 0.1% galactose; NS, no sugar. The data presented are representative of multiple experiments.

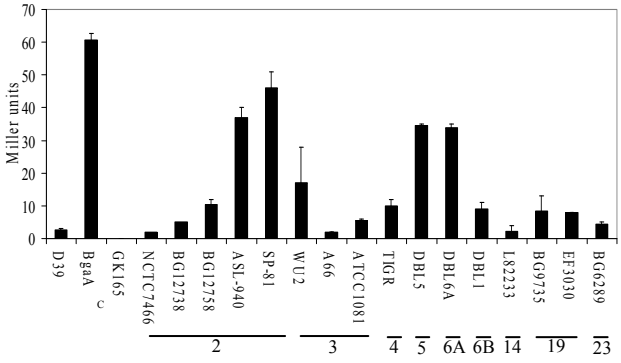

FIG. 8. β-galactosidase activity of other *S. pneumoniae* strains. Strains were grown to mid-exponential phase in THY. Results are the means (± standard error) of 3 replicates. Capsular serotype is listed below the strain name.

such as ASL-940 and SP-81, may therefore reflect the influence of other factors, possibly including the second repressor involved in β-galactosidase regulation, that vary between strains.

DISCUSSION

We have shown that a point mutation in the promoter region of the *pts* operon upstream of *bgaA* leads to constitutive β-galactosidase activity. The point mutation is located between the -35 and -10 sequences of the *pts* promoter in a *cre*. The G to C transversion mutation in the $BgaA^C$ mutants, GK1000 and GK315, altered the ability of CcpA to bind the *cre* and repress expression of the *pts* operon and *bgaA*. This residue, as well as the C immediately upstream and corresponding to the point mutation in GK1001, are universally conserved in *cre* (27, 38). Both residues are critical for interaction with CcpA, as the conserved Leu55 in CcpA intercalates between them to effect minor groove expansion (29). Binding of CcpA to the *cre* can inhibit transcription by inhibiting the binding of RNA polymerase (18). We found that the point mutations in the $BgaA^C$ mutants decreased the affinity of CcpA for the *cre*. These alterations apparently resulted in a reduced ability to inhibit RNA polymerase binding thereby leading to constitutive transcription of the *pts* operon and *bgaA*.

The *pts* operon and *bgaA* are contained on the same transcript, as demonstrated by RT-PCR and the elimination of β-galactosidase activity by the insertion of a terminator between the *pts* operon and *bgaA*. CcpA thus represses β-galactosidase expression by binding to the promoter region of the upstream *pts* operon. A second repressor was

revealed when *ccpA* was deleted in the D39 parent and the BgaA^{C} mutant strains. In the BgaA^{C} mutant, weak binding of CcpA appeared to reduce binding of the second repressor, leading to high levels of *pts-bgaA* expression and β-galactosidase activity. When *ccpA* was deleted, the second repressor was able to bind, thereby reducing *pts-bgaA* expression. Repression in D39 may occur by simultaneous binding of both CcpA and the second repressor or the two repressors may bind independently. As with the mutant strain, deletion of *ccpA* in D39 allowed binding of only the second repressor, which did not repress *pts-bgaA* expression to the level observed when CcpA was present. Since the second repressor could bind to both the parent and mutated *pts* promoter regions, it must not recognize the same sequence as CcpA. It does, however, bind in the same region as CcpA, as shown by the fact that deletion of *ccpA* allowed repression of *bgaA* expression in the BgaA^{C} mutant, which was identical to that observed with the parent.

Our results demonstrate that glucose-mediated repression of *bgaA* occurs through both CcpA and the second repressor. CcpA-mediated repression of β-galactosidase activity in the presence of glucose was also noted using the *S. pneumoniae* TIGR4 strain (14). In contrast, it was concluded that glucose was not involved in CcpA-mediated repression of β-galactosidase activity in *S. pneumoniae* D39 (10), the same strain we used in the present studies. The discrepancy appears to relate to the effect of the second repressor, which may have obscured the effect of glucose in the earlier studies (10). When *ccpA* is deleted, binding of the second repressor still allows some glucose-mediated repression to be observed. The effect of the second repressor was also seen during culture with other sugars. During growth in lactose, β-galactosidase activity of

the D39 *ccpA* deletion mutant was elevated over that of D39 and was similar to that observed for the *ccpA* deletion mutants in the absence of added sugar. However, the highest β-galactosidase activity was observed with the $BgaA^C$ mutant grown in lactose, galactose, or galactitol. This activity appears to result from both ineffective CcpA repression (due to the mutation in the *cre*) and ineffective repression by the second repressor (due to reduced accessibility to its binding site as a result of weakly bound CcpA). The difference in β-galactosidase levels between the $BgaA^C$ mutant and the Δ*ccpA* mutants grown in lactose therefore appears to reflect the activity of the second repressor. Regulation of β-galactosidase expression may therefore involve multiple levels of repression, including that relating to the presence of glucose, and the activities of CcpA and the second repressor. Full alleviation of repression may occur when neither CcpA nor the second repressor is active, such as when glucose is absent and another PTS sugar is present. To date, we have not identified the second repressor but have shown that it is not the *S. pneumoniae* homologue of the global transcriptional regulator CodY that is found in many Gram-positive bacteria and which, in *Bacillus subtilis*, binds near CcpA and has an additive effect (30, 31) (unpublished data).

CcpA-mediated catabolite repression allows Gram-positive bacteria to utilize the most efficient carbon source available for growth through a hierarchy in which expression of PTS transporters involved in the utilization of less effective sources is repressed (8). Glucose is usually at the top of this hierarchy, and its ability to repress *pts-bgaA* expression fits well with the niches occupied by *S. pneumoniae* and the functions proposed for BgaA. *S. pneumoniae* is a normal and frequent colonizer of the nasopharyngeal cavity (4), a site where there is little to no glucose (28). Glucose levels

are similarly low in healthy lungs (5) but higher levels occur during infection (28) and in the bloodstream. BgaA has previously been shown to cleave human glycoproteins and to be important in adherence by *S. pneumoniae* (22). In glucose-deficient sites, repression of *pts-bgaA* by CcpA and the second repressor would be relieved, resulting in high-level expression of β-galactosidase and the PTS. Cleavage of galactose from host glycoproteins by BgaA, and its transport into the cell by the PTS would allow *S. pneumoniae* to not only adhere but also to persist. Elevated glucose concentrations in the lung or other sites of dissemination would lead to repression of *pts-bgaA* expression by CcpA and the second repressor. CcpA, and possibly the second repressor, may therefore play central roles in regulating the transition from colonization to systemic infection.

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METABOLIC REGULATION OF PHASE VARIABLE FACTORS IN *STREPTOCOCCUS PNEUMONIAE*

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ABSTRACT

Streptococcus pneumoniae exhibits spontaneous phase variation. The regulatory mechanism for this change has not been well characterized. We have shown that the phase variable factors BgaA (β-galactosidase), NanA (Neuraminidase), StrH (N-acetylglucosaminidase), LytA (autolysin), PspA (pneumococcal surface protein A), and the capsular polysaccharide are in part regulated by CcpA (carbon catabolite protein A), a key regulator of carbon regulation. These factors also exhibit altered expression with changes in glucose concentration, and this response is regulated by CcpA. In contrast, we were unable to detect any response of PspC (pneumococcal surface protein C) to glucose changes or *ccpA* deletion. These results suggest that CcpA is an important regulator that may aid in the adaptation of *S. pneumoniae* to changing environments in the host.

INTRODUCTION

Streptococcus pneumoniae is an important human pathogen responsible for causing pneumonia, bacteremia, meningitis, and otitis media primarily in young children, the elderly, and immunocompromised people. A majority of the human population is asymptomatically colonized with *S. pneumoniae* (3). Aspiration of the bacterium leads to the development of pneumonia and can lead to sepsis as well as bacteremia. As with most pathogens, *S. pneumoniae* is exposed to a variety of environmental conditions. Adaptation to these conditions is imperative for survival of the pathogen. The

mechanisms by which *S. pneumoniae* can transition and survive in various niches in the host has not been well characterized.

Phase variation in *S. pneumoniae* is postulated to play an important role in survival to host niches. Transparent variants colonize the nasopharynx at a higher level than opaque variants. Transparent variants have decreased capsule production, but increased levels of teichoic acid and surface proteins such as Neuraminidase A (NanA), β-galactosidase (BgaA), N-acetylglucosaminidase (StrH), and pneumococcal surface protein C (PspC). NanA, BgaA, and StrH are surface-associated enzymes involved in deglycosylating host proteins (15), and NanA, BgaA, and PspC increase adherence (15, 22). These surface proteins aid in colonization (13-15). In contrast, opaque variants are adapted for systemic infections and exhibit increased amounts of capsule and PspA (a choline-binding protein important in virulence) and decreased amounts of the adhesins noted above. In *in vitro* studies, opaque and transparent variants have been shown to bind differently to sugars. Transparent variants efficiently bind to galactose, N-acetylgalactosamine, and N-acetylglucosamine, but are unable to bind glucose (7). Opaque variants have similar binding abilities, but they are less efficient at binding N-acetylglucosamine. Phase variation between the two phenotypes occurs at a frequency of approximately 10^{-6} *in vitro*, with variability among strains, but the mechanisms involved in the switching are not understood (28).

Two different studies recently quantified differential expression of virulence genes *in vivo*. LeMessurier et al. compared *S. pneumoniae* D39 mRNA levels in the nasopharynx, lungs and blood to 16s rRNA and determined the relative changes in each niche (16). They found that *nanA* and *pspA* mRNA levels were increased in the

nasopharynx relative to the other niches. *pspC* was more abundant in the nasopharynx and lung compared to the blood. In contrast, *cps2A* (capsule) levels did not seem to change significantly in any of the niches. Oggioni et al. evaluated differential gene expression of *S. pneumoniae* TIGR4 virulence factors in the blood, brain, and lung compared to *in vitro* expression (19). Their study showed a significant increase in *nanA* in the brain and lung compared to the blood. *pspA* was up-regulated in the blood compared to the brain and lungs. Both studies saw very little change in capsule (*cps2A* or *cps4A* transcripts) regardless of site. Differences in the expression of *pspA* in these studies may be due to strain variation.

NanA and BgaA have also been shown to play a role in adherence to epithelial cells (15). The capsular polysaccharide, which is essential for virulence inhibits adherence in vitro, and strains which are nonencapsulated adhere significantly better than encapsulated strains (15). Colonization studies have shown that full capsule production is not required for efficient colonization. Magee and Yother showed that strains with an 80% reduction in capsule could colonize as well as the parent strain (17). These results suggest that capsule is potentially down-regulated during colonization in the nasopharynx. However, capsule is required for *S. pneumoniae* to cause pneumonia and to survive in the blood (17), therefore, its production must be up-regulated upon or before dissemination from the nasopharynx.

The differential regulation of factors important in colonization and systemic virulence suggests that a global regulator might be important for sensing the various niches and controlling the expression of virulence factors. The global regulator carbon catabolite protein A (CcpA) has been implicated in the regulation of virulence factors in

S. pneumoniae (9, 11) as well as adaptation to different environmental conditions (Kaufman and Yother, submitted). CcpA is an important transcriptional regulator that becomes activated upon interaction with HPr that is phosphorylated on Ser-46 (reviewed in (25, 27). HPr has multiple functions, and when phosphorylated on His-15, it is involved in sugar transport. This differential phosphorylation is controlled by the metabolic activity of the cell. When the cell is involved in active metabolism and there is a build up of glycolytic intermediates, such as fructose 1,6 bisphosphate, HPr kinase (HPrK) is activated and phosphorylates HPr on the Ser-46. At this point, HPr binds to CcpA, which enhances the binding of CcpA to catabolite responsive elements (*cre*) located within or near the promoter regions of regulated genes. CcpA binding decreases transcription of these genes if the *cre* is locate within the promoter or open reading frame (12). In contrast, transcription is enhanced by CcpA binding to a *cre* upstream of the promoter (26). As glycolytic intermediates decrease and inorganic phosphate increases, HPr-Ser is dephosphorylated by HPrK and phosphorylated on His-15. It then becomes involved in the phosphorelay cascade to transport sugars into the cell.

A spontaneous mutant exhibiting constitutive β-galactosidase activity was previously identified by our laboratory (Kaufman and Yother, submitted). Using this mutant, we characterized the CcpA-dependent co-regulation of *pts-bgaA*. We identified a point mutation located in the *cre* in the promoter of the *pts* operon upstream of *bgaA*, which resulted in constitutive β-galactosidase activity. This increased activity was due to decreased binding of CcpA to the mutated *cre*. Furthermore, we found in the absence of *ccpA* a secondary regulator of *bgaA* resulted in glucose repression in both wild-type and

the constitutive mutant. However, when *ccpA* was present glucose repression was only observed in the wild-type strain not in the constitutive β-galactosidase mutant.

In these studies we show that many phase variable factors in *S. pneumoniae* are regulated, at least in part by CcpA and glucose concentration. We show that PspA, BgaA, StrH, and NanA are regulated by both glucose and CcpA. Also increasing glucose concentrations resulted in increased capsule production, but this effect is abolished in a *ccpA* deletion.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. For growth in liquid medium, cells were inoculated into either THY (Todd-Hewitt broth supplemented with 0.5 % yeast extract) or D-medium (tryptone 10 g/L, neopeptone 5 g/L, Tris 1.25 g/L, sodium chloride 5 g/L, yeast extract 1.25 g/L, and 0.1% of the indicated sugar source) (1) (Table 1). For growth on solid medium, blood agar plates (BAP, Blood agar base no. 2 [Difco] supplemented with 3% defibrinated sheep blood [Colorado Serum Company]) or THY with 1.5% Bacto-agar (Difco) were used. Where indicated, catalase and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) were added to final concentrations of 5000 U (Worthington Biochemicals) and 5 mg per plate, respectively. Where applicable, media were supplemented with erythromycin (Em) or kanamycin (Km) at concentrations of 0.3 µg/ml and 250 µg/ml, respectively. *E. coli* strains were maintained in L-broth or on L-agar supplemented as needed with ampicillin (Ap), Km, or Em at concentrations of 50 µg/ml, 50 µg/ml, and 300 µg/ml, respectively.

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant properties	Reference or source
S. pneumoniae		
AM1000	Δ*cps2A-H*, type 2 Cps^-	(17)
D39	Type 2 parent; $BgaA^+$	
GK165	pGK538 x D39; Δ*bgaA* in D39	Kaufman and Yother, Submitted
GK315	Isogenic mutant of D39 with constitutive β-gal mutation; G→C mutation in *pts* promoter	Kaufman and Yother, Submitted
GK220	pGK579 x D39; Δ*ccpA* in D39, Kan^r	Kaufman and Yother, submitted
GK1000	$BgaA^C$ derivative of D39; -56G→C mutation in *pts* promoter	Kaufman and Yother, submitted
E. coli		
DH5αF'	F'Φ80d*lacZ*ΔM15Δ (*lacZYA-argF)*U169 *deoR recA1 endA1 hsdR17*($r_k^-m_k^+$) *phoA* sup*E44*λ^- *thi-1 gyrA96 relA1*	Life Technologies
Plasmids		
pJY4164	Lacks origin of replication for *S. pneumoniae*, Em^R	(30)
pCR2.1	PCR Cloning vector, Ap^R, Kan^R	Invitrogen
pGK538	pJY4164 derivative containing PCR fragments from the amplification of GK1000 DNA by the primer pairs F-UpbgaA/R-UpbgaA and F-downbgaA/R-DownbgaA: used to delete *bgaA*	Kaufman and Yother, submitted
pGK579	pJY4164 derivative containing PCR fragments from the amplification of GK1000 DNA by the primer pairs F-UpCcpA1/R-UpCcpA2 and F-downCcpA3/R-DownCcpA4 with Km-resistance gene, *aph-3*, between 2 fragments: used to delete *ccpA*	Kaufman and Yother, submitted

β-galactosidase assays. β-galactosidase activity was determined as described by Miller (18). Cultures were grown to mid-exponential phase (cell density of ~3 x 10^8 CFU/ml). A 0.2 ml aliquot of the culture was added directly to 0.8 ml Z-buffer and the suspension was incubated at 30°C with 0.2 ml of 4 mg/ml *o*-nitrophenol-β-D-galactopyranoside (ONPG). Reactions were stopped by the addition of 0.5 ml of 1 M Na_2CO_3. Activity was calculated as Miller units, as described (18).

Transcription analysis. Total RNA was isolated from a 50 ml culture grown in THY using a hot acid phenol extraction, as described previously (8). RNA concentrations were determined using UV spectrophotometry. Transcript levels were determined using slot-blotting (2). Briefly, samples were diluted to 3 and 0.5 μg per 30 μl for each probe and denatured for 15 min at 65°C in 90 μl of denaturing solution made up of 500 μl formamide, 162 μl 12.3 M (37%) formaldehyde, and 100 μl MOPS [3-(*N*-morpholino)-propanesulfonic acid] buffer (0.2 M MOPS [pH 7.0], 0.5 M sodium acetate, and 0.01 M EDTA). Then, 240 μl of cold 20x SSC (1x SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) was added to each sample. Denatured samples were spotted on nylon membranes which were UV crosslinked and then prehybridized for 3 h at 42°C in high SDS hybridization buffer (7% SDS, 50% formamide, 5x SSC, 2% blocking Reagent (Roche), 50 mM sodium phosphate, and 0.1% *N*-laurylsarcosine). Membranes were incubated overnight with denatured digoxigenin-labeled PCR probes made with primers MTC1/MTC2 (Table 2), which were added directly to the membranes in the high SDS hybridization buffer. To remove non-specifically bound probe, membranes were washed

twice with 2x SSC containing 0.1% SDS for 5 min at room temperature, and then twice with 0.5x SSC containing 0.1% SDS for 15 min at 65°C. The blots were developed using

Table 2. Oligonucleotide primers used is this study.

Primer[a]	Sequence[b]	Position[c]
From *S. pneumoniae* R6 section 51 of 184 of the complete genome (spr0562-0565)		
F-UpBgaA	GCAGCTATCGTTCTTGTCGGTGTGTTG	1824-1850
R-UpBgaA	TATTTTGCTTTTGCTGCGTACTC	2815-2793
F-DownBgaA	GAGTGCAGGATTAGTAGTTACTAAAG	9765-9790
From *S. pneumoniae* R6 section 52 of 184 of the complete genome (spr0566-0579)		
R-DownBgaA	CAGTTCCTTCTTACCACAAGACC	762-741
From *S. pneumoniae* R6 section 138 of 184 of the complete genome (spr1534-1543)		
MTC-2	AAACTGTTTCCCATCCGAACC	5388-5409
MTC-1	CAGTGGTATTTGGAACGTCTCCTG	5873-5851
From *S. pneumoniae* R6 section 161 of 184 of the complete genome (spr1806-1817)		
F-UpCcpA1	ACATATGCTGGTCCTCTACCAG	5336-5357
R-UpCcpA2	GAAAAAATCAGGGAATCGAGAAG	6370-6348
F-DownCcpA3	TCTTTTACAAGTAGAGGTACTGATTG	7412-7437
R-DownCcpA4	CATCCAACGGAAGTGCAAGTTC	8451-8433

[a] Forward and reverse primers are represented by F and R, respectively.
[b] sequences are from the complete R6 genome (10)
[c] nucleotide positions for primers are listed in the forward or reverse orientation, as necessary

the Anti-Digoxigenin-AP Fab fragments (Roche) and Phototope™-Star Detection Kit for Nucleic Acids (New England BioLabs). The relative levels of transcript were determined by densitometry using ImageJ software (http://rsb.info.nih.gov/ij).

Neuraminidase activity. A fluorometric assay for neuraminidase activity was adapted by Berry et al (5). Briefly, cultures were grown to mid-exponential growth (cell density of ~3 x 10^8 CFU/ml) and concentrated 10-fold. Cells were lysed using 0.5% sodium deoxcholate. 10 µl of lyses sample was added to 10 µl of 0.35% (wt/vole) 2'-(4-methylumbellifery)-α-D-N-acetylneuraminic acid (MUAN) (Sigma Chemicals) and incubated at 37°C for 5 min. The reaction was stopped by addition of 100 µl of 1M Na_2CO_3. Samples were read in a 96-well format with an excitation wavelength of 355 nm and an emission wavelength of 460 nm using Victor2 (Perkin Elmer). The Δ*nanA* was kindly given to us by David Briles.

N-acetylglucosaminidase activity. N-acetylglucosaminidase assay was adapted from King et al (15). Briefly, cultures were grown to mid-exponential growth (cell density of ~3 x 10^8 CFU/ml) in either THY or D-medium supplemented with various concentrations of glucose. 0.5 ml of culture was added to 0.4 ml of 10 mM 4-Nitrophenyl N-acetyl-b-D-glucosamide (Sigma). Reaction mixture was incubated for 1 h at 37°C with rotation. To stop the reaction, 0.6 ml of 1M Na_2CO_3 was added to the reaction. The absorbance of a 0.2 ml aliquot of the reaction was read at 400 nm for the pNp substrate and 550 nm to measure cellular debris. N-acetylglucosaminidase activity was determined by the following equation ((405nm sample -405 nm blank)-(1.75*550 nm))/ (volume x 595 nm).

Apolactoferrin cleavage. Cells were grown in THY to a cell density of 10^8. Lactoferrin isolated from human milk (Sigma) was added directly to the culture medium at a concentration of 10 µg/ml and then incubated at 37°C for a specified time. To terminate the reaction, 0.75 mM PMSF (phenylmethanesulphonylfluoride) was added to

the reaction. An aliquot (7µl) of the sample was added to Laemmli loading dye and placed at -80°C. To check size changes in lactoferrin, samples were electrophoreses and monitored for changes in migration of the protein. Samples were boiled for 5 min and loaded on a 10% Bis-Tris SDS-PAGE (Invitrogen). In initial experiments, proteins were transferred to nitrocellulose membranes and developed with a poly-clonal lactoferrin antibody (Sigma) to determine that the protein visualized by Coomassie staining was lactoferrin. Upon confirmation in the remainder of experiments, the protein shifts were visualized by Coomassie staining.

Capsular polysaccharide analyses. Indirect enzyme-link immunosorbent assays (ELISAs) were performed as previously described (4, 17). In brief, triplicate cultures were grown to a density of 3 x 10^8 CFU/ml and 3 ml of each culture was centrifuged at 20,000 x g for 10 minutes. Culture supernatants were collected and filter sterilized with 0.45 µm filter (Corning). The cellular pellet was resuspended in 3 ml of 1 x PBS. 200ul of the resuspended pellet was used to measure OD to normalize samples. The samples were incubated in at 56°C for 25 min to heat kill the cells. Samples were then normalized to OD_{595} and wells of a microtitre coated overnight at 4°C. Capsule levels in samples were measured by the binding of a polyclonal rabbit anti-type antiserum that was adsorbed to the nonencapsulated mutant, AM1000, prior to use. The plate was developed using a secondary goat anti-rabbit Ig conjugated to streptavidin-alkaline phosphatase followed by incubation with 1 mg/ml *p*-nitrophenolphosphate in glycine buffer (0.1 M glycine, 1 mM $MgCl_2$, 0.1 M $ZnCl_2$, pH 10.4). Absorbance was measured at 405 nm.

Western Blot Analysis. For the analysis of LytA, PspA, PspC, cells were harvested at and OD_{595} of 0.5 (~3 x 10^8 CFU/ml) and concentrated 1:50. Cells were

normalized to cell OD_{595} after concentration and boiled in SDS-PAGE loading buffer for 10 min. Proteins were separated by SDS-10% polyacrylamide gel electrophoresis. For all blots, 8-12.5 µl of sample was used. The proteins were then transferred onto nitrocellulose membranes and blocked for 1 h at room temperature in 5 % nonfat dried milk in PBST (PBS with 0.05% Tween 20). For detection blots were washed 2 times for 5 min with PBST and then incubated for 45 min with the primary antisera diluted in PBST (1:1,300 LytA, 1:2,500 PspC, 1:10 PspA). Then the blots were washed 2 times with PBST for 5 min and incubated with either goat α-rabbit or goat α-mouse Ig conjugated to streptavidin-alkaline phosphatase (Southern Biotechnology Associates) diluted 1: 2,500. The membranes were washed twice for 5 min in PBST and developed by using 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium (0.25 and 0.05 mg/ml, respectively) in 1 M Tris (pH 8.8). SeeBlue Plus2 prestained molecular-weight marker (Invitrogen) was used to determine the protein molecular weights

RESULTS

Effects of *ccpA* deletion on exoglycosidases. In a previous study, we showed that CcpA co-regulated *pts* and *bgaA* expression via binding to the *pts* promoter (Kaufman and Yother, submitted). The presence of glucose in the growth medium resulted in CcpA-mediated repression, however a second regulator that responded to the level of glucose was also involved. To determine whether there is similar regulation of other surface-localized exoglycosidases, we examined their expression in the *S. pneumoniae* strain D39 and its *ccpA* deletion derivative, GK220. As shown in Fig. 1,

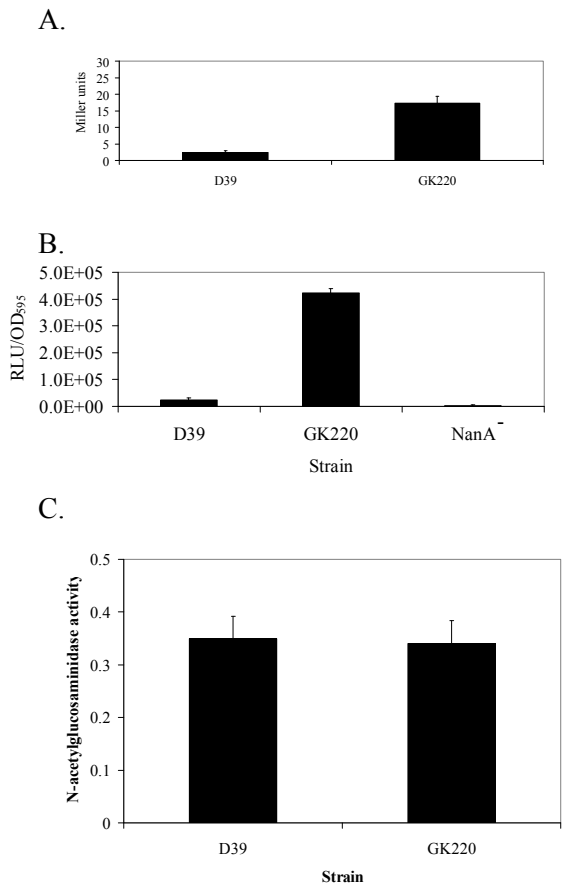


Fig. 1. Exoglycosidase activity in THY. (A) β-galactosidase activity of D39 and the *ccpA* deletion strain (GK220) when grown in THY. (B) Neuraminidase activity in THY with D39 and *ccpA* deletions. (C) N-acetylglucosaminidase activity in THY comparing D39 to *ccpA* deletion. Results are the means (± standard error) of 3 replicates.

deletion of *ccpA* resulted in increased β-galactosidase (BgaA) and neuraminidase (NanA) activities but did not affect N-acetylglucosamine (SrtH) activity when grown in THY. For both the parent and the *ccpA* mutant, BgaA and NanA activities were increased during culture in the absence of a sugar in the growth medium (Fig. 2A and 2B). Increasing concentrations of glucose resulted in increased repression of BgaA activity in both the parent and *ccpA* deletion strains, reflecting the activity of the second repressor (Fig. 2A) (Kaufman and Yother, submitted). Glucose concentration did not affect NanA activity, indicating that the second repressor does not regulate *nanA* (Fig. 2B). Although not affected by deletion of *ccpA* in THY, SrtH activity was decreased with increasing glucose concentration (Fig. 2C). The increase in StrH activity in D39 when incubated in the presence of no sugar was lost in the *ccpA* deletion, indicating that *ccpA* is an activator or StrH activity (Fig. 2C). At the low glucose concentration (0.1%), there was no difference in SrtH activity between D39 and the *ccpA* deletion. However, at the higher glucose concentration (1.4%), deletion of *ccpA* resulted in greater repression of SrtH activity compared to D39.

As shown previously for *bgaA* (Kaufman and Yother, submitted), CcpA-mediated repression of *nanA* occurred at the transcriptional level (Fig. 3). A putative *cre* was identified in the open reading frame of *nanA*, consistent with the observed repression. A putative *cre* was identified upstream of the promoter region of *srtH*, consistent with activation of StrH activity.

Because NanA and BgaA have been shown to cleave glycosylated host cell proteins, we hypothesized that cleavage of lactoferrin would be increased with the *ccpA* mutant. This protein contains fucose (1.1 M), galactose (2.2 M), mannose (3.0 M), N-

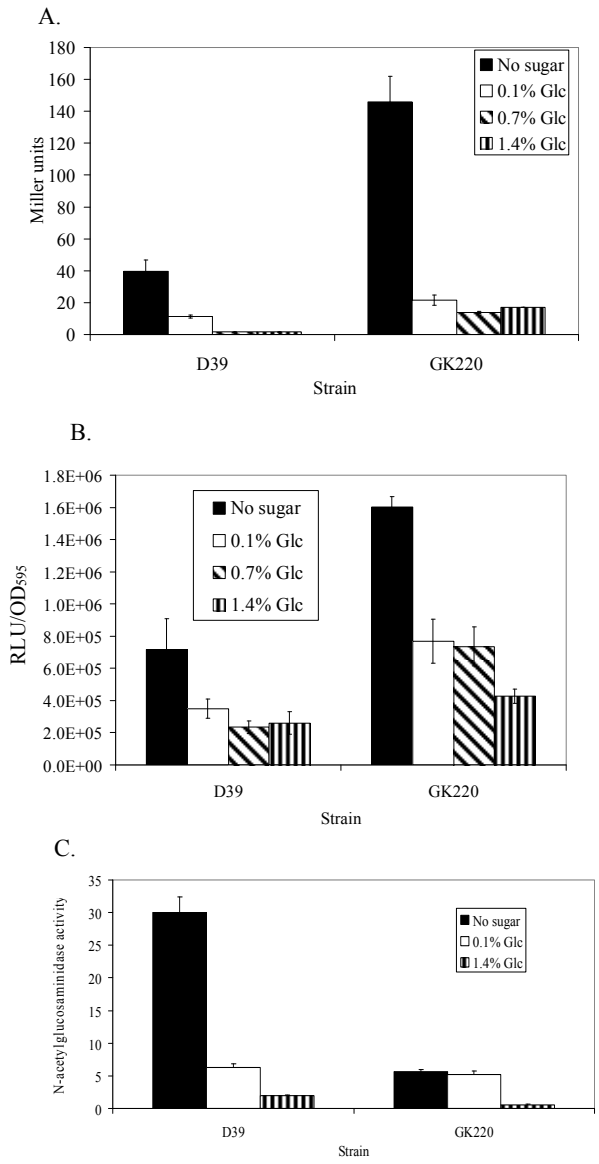


Fig. 2. Exoglycosidase activity in D with increasing glucose concentrations. (A) β-galactosidase activity of D39 and the *ccpA* deletion strain (GK220) when grown in increasing glucose (0% to 1.4%). (B) Neuraminidase activity in D medium with increasing glucose with D39 and *ccpA* deletions. (C) N-acetylglucosaminidase activity with increasing concentrations of glucose comparing D39 to *ccpA* deletion. Results are the means (± standard error) of 3 replicates.

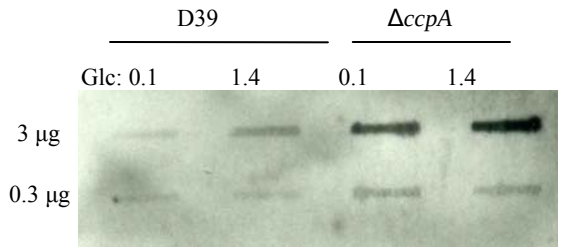


Fig 3. Effect of *ccpA* deletion on *nanA* transcript. Slot blots containing the amount of RNA indicated on the left were probed with the internal sequences from *nanA*.

acetylglucosamine (4.1 M), and sialic acid (2.0) (24). In a time course experiment using human milk lactoferrin, we observed cleavage by 1 h with the parent D39, whereas cleavage was evident with the *ccpA* mutant GK220 by 30 min (Fig. 4A, lane 4 and Fig. 4B, lane 9, respectively). This result was similar to that observed with the constitutive β-galactosidase mutant GK315 (Fig. 4A, lane 9 and Fig. 4B, lane 3). This mutant exhibits 5-fold more β-galactosidase activity than the *ccpA* mutant GK220 (Kaufman and Yother, submitted), but its NanA activity is the same as the parent D39 (i.e., ~10-fold less than GK220; Fig. 1B, data not shown).

Effects of *ccpA* deletion on choline binding proteins. The choline binding proteins PspA, PspC, and LytA have been observed to phase vary, with PspC and LytA increased in transparent variants and PspA increased in opaque variants. Deletion of *ccpA* resulted in reduced levels of PspA under both high and low glucose concentrations (Fig. 5A). PspA levels were also reduced by increasing glucose concentration, suggesting a second level of regulation. For LytA, protein levels were reduced by increasing glucose concentration, and this response was lost in the *ccpA* deletion mutant (Fig. 5B). PspC levels were not affected by glucose concentration or deletion of *ccpA* (Fig. 5C).

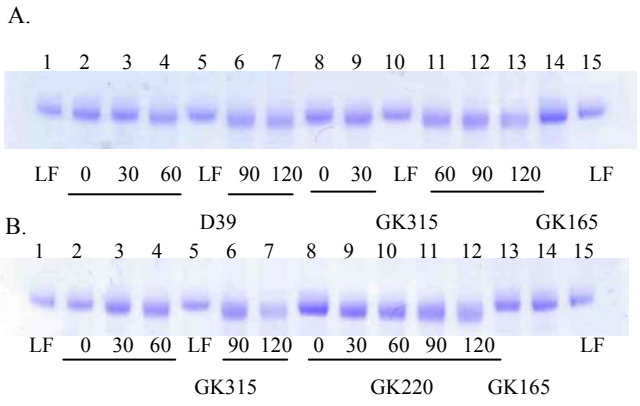


Fig. 4. Lactoferrin cleavage comparing D39 to BgaA^{C} and *ccpA* deletion. Strains were grown to mid-exponential growth phase and then incubated for the indicated time. (A) Comparing lactoferrin cleavage of D39 and GK315. (B) Comparing lactoferrin cleavage of GK315 and GK220.

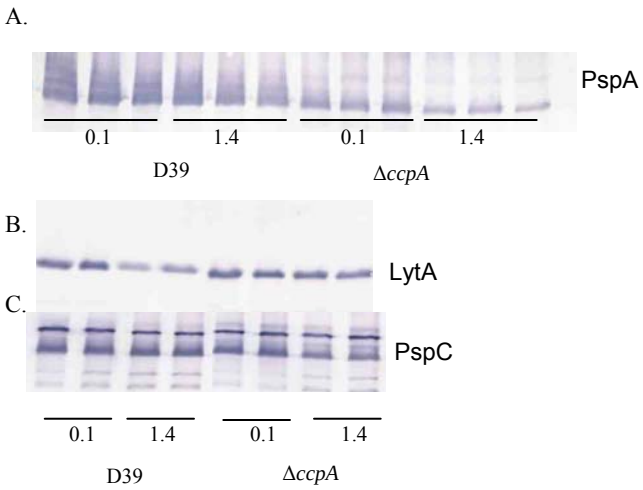


Fig. 5. Effects of *ccpA* deletion and increasing glucose concentrations on choline binding proteins. Western blot analysis was used to determine the protein levels of (A) PspA, (B) LytA and (C) PspC in *ccpA* deletions and increasing glucose concentrations.

CcpA thus appears to enhance PspA expression and reduce LytA expression. This is consistent with the putative *cre* identified upstream of the *pspA* promoter and within the LytA open reading frame, respectively.

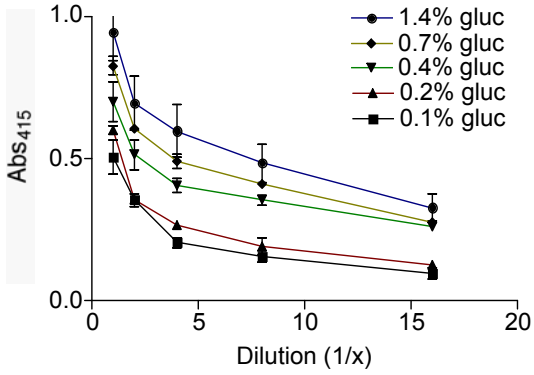
Capsule production in *ccpA* deletions. In a previous study of *ccpA* deletions in *S. pneumoniae* D39, a small colony phenotype was noted, and it was suggested to be the result of decreased capsule production (9). Our CcpA mutants also exhibited small colonies, however this phenotype was due to a severe growth defect, as noted for *ccpA* deletions in the TIGR4 strain (11). On blood agar plates, approximately 15 hour longer incubations were required for full growth of our D39 *ccpA* mutant as compared to the parent D39. Quantification of the number of cells per colony demonstrated 10^6 and 10^4 CFU/colony for the parent and mutant strains, respectively.

Since CcpA is involved in adaptation to glucose, we hypothesized that glucose modulation via CcpA might be a signal to alter capsule production. In the parent D39, increasing levels of capsule were observed with increasing levels of glucose (Fig. 6A). In *ccpA* deletions, however, the capsule response to glucose was lost (Fig. 6B). Therefore, CcpA is involved in enhancing the production of capsule in response to changing glucose.

DISCUSSION

S. pneumoniae encounters many different environments in the host. One characterized difference is the alteration in glucose levels. In the nasopharynx, glucose is typically undetectable in the secretions of a healthy individual (21), whereas normal

A.



B.

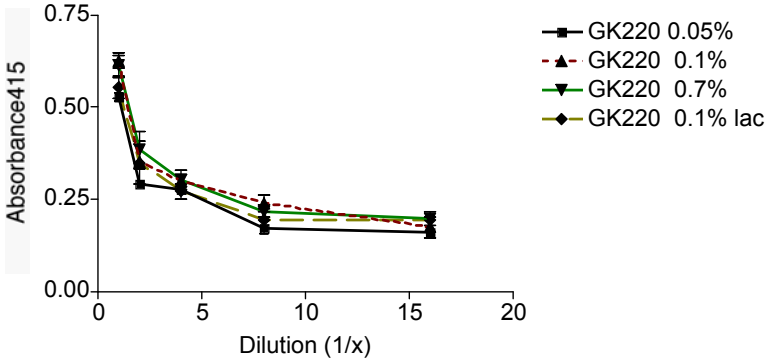


Fig. 6. Capsule ELISA with increasing concentrations of glucose. Indirect ELISA for the surface associated capsule production of D39 and *ccpA* mutant were grown in D-medium with increasing concentrations of glucose. Results are the means (± standard error) of 3 replicates and representative of multiple experiments.

blood glucose concentrations are up to 5.5 mM (0.1%). Studies with healthy volunteers have shown that glucose is not measurable in the nasal secretions until the blood glucose levels reach 7 mM (29). However in individuals with diabetes mellitus, the glucose concentration in nasal secretions is approximately 4 mM, suggesting that their blood glucose levels were higher than 7 mM. Interestingly, individuals with diabetes mellitus have a higher rate of infection with *S. pneumoniae* than healthy individuals. Glucose concentrations in nasal secretions have also been shown to increase to levels of 1 mM upon viral infection (21). Glucose may therefore be an important environmental signal for *S. pneumoniae* in both previously healthy individuals and those with underlying conditions.

In this study we have shown that many phase variable factors in *S. pneumoniae* respond to the changes in glucose concentration in the medium. This response is primarily due to regulation by CcpA. Previous studies have shown that BgaA, NanA, StrH, capsule, and choline-binding proteins, such as PspA, PspC, and LytA are differentially regulated in phase variants (13-15). Differential regulation of these phase variable factors aid in the survival in various niches. Therefore, CcpA may be important in the differential regulation of these phase variable factors in response to environmental stimuli.

BgaA, NanA, and StrH activity increased significantly when the bacteria were cultured in the absence of glucose. Although BgaA activity decreased as the concentration of glucose increased, glucose repression was not observed for neuraminidase activity. In contrast, StrH activity decreased with increasing glucose

concentrations similar to BgaA, but at low glucose concentrations (0.1%) there was no difference in SrtH activity between D39 and the *ccpA* mutant. This result suggests a different mechanism for regulation of BgaA and NanA compared to StrH. Previous studies have shown that BgaA and NanA aid in adherence whereas StrH was not necessary for adherence to epithelial cells (15). Therefore, due to the differences in function, their regulatory mechanisms may be different.

The surface-associated exoglycosidases are important for the cleavage of sugars from host glycoproteins (15). The elevated levels of exoglycosidases in the *ccpA* and *bgaA* mutants resulted in increased cleavage of lactoferrin. In other studies, we have shown that D39 is able to grow with mucin as a sole carbon source (unpublished data). Mucin is primarily composed of fucose, galactose, N-acetylglucosamine, and N-acetylgalactosamine with terminal sialic acid or sulfates (6, 23). Enhanced expression of glycosidases in the nasopharynx may therefore be important in allowing *S. pneumoniae* to both adhere and persist in an environment devoid of glucose.

We propose that in the low glucose environment of the nasopharynx, repression of exoglycosidases and LytA by CcpA and other glucose-dependent repressors is relieved. Cleavage of sugars from host glycoproteins by the exoglycosidases allows adherence and persistence of the organism, and may further up-regulate expression of the exoglycosidases (Kaufman and Yother, submitted). Under increased glucose concentrations, CcpA and other glucose-dependent regulators repress expression of these proteins and allow enhanced production of capsule. PspA appears to be important in both colonization and systemic infections (16, 19, 20), and its expression appeared to be controlled oppositely by CcpA and glucose concentration. The expression of PspC is

enhanced in the nasopharynx as compared to blood, but its expression does not appear to be regulated by CcpA or glucose.

In conclusion, we have shown that CcpA and metabolic regulation are important points of control in the expression of multiple factors shown to be associated with the transition between the transparent and opaque and phase variants in *S. pneumoniae*. Glucose may be one of the important stimuli for the differential regulation of phase variable factors. These results suggest that CcpA is not only an important regulator of metabolic genes but also is an important regulator of many virulence factors that are involved in the adaptation to various host niches.

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SUMMARY AND CONCLUSIONS

S. pneumoniae is exposed to many different niches in the host. When we initiated these studies, phase variation had been shown to be important in the differential expression of virulence factors in the host, but the mechanism for phase variation had not yet been characterized (42, 43). However, recent studies have shown differential regulation of pneumococcal virulence factors *in vivo*, which correlated with the phase variation studies, but the mechanisms by which *S. pneumoniae* adapts to the changing environments in the host is still unknown (46, 58). An understanding of how *S. pneumoniae* senses the various niches and alters virulence factors based on the environment would significantly aid in the understanding of pneumococcal pathogenesis.

CcpA is an important transcriptional regulator. CcpA is regulated based upon the metabolic state of the cell via the phosphorylation state of HPr (reviewed in (79). Previous studies using microarray analyses comparing phase variants have shown that 46% of the differentially regulated genes involve metabolism (43). Therefore, it is likely that CcpA is a key regulator of factors involved in adaptation to environmental niches. In these studies, we have shown the differential regulation of BgaA, NanA, StrH, LytA, PspA, and capsule by glucose concentrations and *ccpA* deletion. We propose that one of the key ways that *S. pneumoniae* is able to sense environments is through glucose concentration (i.e. metabolic state of the cell). In the nasopharynx, glucose concentrations are typically undetectable (62, 91), whereas in the blood the glucose levels for a normal

individual are 1 mg/ml. Therefore via the metabolic state of the cell, *S. pneumoniae* senses the presence of glucose and differentially regulates genes involved in survival in a particular niche.

These studies were initiated when we identified a mutant that exhibited constitutive β-galactosidase activity. The constitutive β-galactosidase activity was due to BgaA. Prior to our study, very little was known about the regulation of *bgaA*. Studies originating in 1964 associated β-galactosidase activity with pneumococcus (32). In those studies, β-galactosidase was purified from the supernatants of pneumococcal cultures. It was not until the partial sequence of the genome was made available that the gene encoding this enzyme was identified (93). Results from these studies showed that *bgaA* was responsible for the endogenous β-galactosidase activity. They also showed that BgaA was a surface associated protein that contained an LPXTG motif followed by a hydrophobic region and charged tail, so it was proposed to be a sortase-anchored protein (72). Further studies showed that disruption of *bgaA* resulted in attenuation of virulence in the pneumonia model (30). What is interesting about *S. pneumoniae's* β-galactosidase is that it is located on the bacterial surface. Typically, β-galactosidases are involved in lactose metabolism in the cytoplasm. For *S. pneumoniae*, however, BgaA is not involved lactose metabolism, but aids in virulence. Therefore, when we identified the constitutive β-galactosidase mutant, designated $BgaA^C$, we hypothesized that BgaA was involved in a global regulatory pathway that integrates metabolism and virulence. We set out to characterize the regulation of BgaA in hopes of elucidating this pathway.

We identified 2 independent point mutations in the promoter region of the *pts* operon upstream of *bgaA*, which led to constitutive β-galactosidase activity. Further

studies enabled us to elucidate the regulation of *bgaA* via the binding of CcpA to the *pts* promoter region containing the point mutations. The point mutations were located in a *cre* that overlapped the -35 region of the *pts* promoter. Binding of CcpA to the *cre* normally inhibits RNA polymerase binding leading to loss of transcription. Further studies showed that *pts* and *bgaA* were on the same transcript, which explained how point mutations in the *pts* promoter upstream led to constitutive β-galactosidase activity. The point mutations decreased the affinity of CcpA for the *cre* binding site, which resulted in loss of transcriptional regulation of *pts-bgaA* by CcpA.

Deletion of *ccpA* resulted in an equalization of *pts-bgaA* transcripts and β-galactosidase activity for D39 and the BgaA^{C} mutant. Deletion of *ccpA* in D39 resulted in increased β-galactosidase activity and *pts-bgaA* transcription. In contrast, in the BgaA^{C} mutant, deletion of *ccpA* had the opposite effect occurred. The levels of β-galactosidase activity and *pts-bgaA* transcript decreased to the level of D39 with a *ccpA* deletion. Disruption of the *pts* operon in the *ccpA* deletion strains abolished β-galactosidase activity in these strains. These results confirmed that CcpA regulated *pts-bgaA*, but the fact that β-galactosidase activity increased in D39 but decreased in BgaA^{C} suggested that a secondary factor was regulating *pts-bgaA* expression. When CcpA was present in the BgaA^{C} mutant, regulation by the second repressor was inhibited by weak binding of CcpA to the mutant *cre*. We were able to show binding of the secondary factor to the *pts* promoter in both D39 and the BgaA^{C} mutant, however, the band shift by CcpA interacting with D39 *pts* promoter was more prominent. This result would suggest that CcpA binding has a higher affinity than the secondary regulator for the D39 *pts* promoter. Because the secondary factor can regulate both the D39 and the BgaA^{C} mutant *pts-bgaA*

in the absence of CcpA, and we observe binding to both *pts* promoters, the point mutation in the *cre* does not affect the binding of the secondary factor.

Since CcpA is regulated by the metabolic state of the cell, we next looked at the affect of increasing glucose concentrations on β-galactosidase activity. In D39, increasing glucose levels in the growth medium resulted in decreased β-galactosidase activity, although there was a maximum level of repression by glucose, such that repression at 0.7% and 1.4% glucose was similar. Also, in the absence of any sugar, we saw a significant increase in β-galactosidase activity. In the $BgaA^C$ mutant, there was no repression of β-galactosidase activity as glucose increased. In the *ccpA* deletions for D39 and the $BgaA^C$ mutant, we saw a similar trend as was observed in the transcriptional levels. Deletion of *ccpA* in D39 resulted in increased β-galactosidase activity, and glucose repression was still observed. In the $BgaA^C$ *ccpA* deletion mutant, β-galactosidase activity decreased to the level of the D39 *ccpA* mutant. Interestingly, glucose repression was observed in the *ccpA* deletion in the $BgaA^C$ mutant when it was not observed in the parent $BgaA^C$ strain. This result would further suggest a secondary regulator of BgaA that is responsible for glucose repression.

Because the *pts* upstream of *bgaA* has homology with fructose and galactitol transporters of *Clostridium acetobutylicum* and *Streptococcus agalactiae* and a galactose transporter in *Streptococcus pyogenes*, we evaluated the induction of the *pts-bgaA* by these PTS sugars and lactose by monitoring changes in β-galactosidase activity. Induction of β-galactosidase activity was observed in both D39 and $BgaA^C$ in the absence of sugar added, as well as in the presence of galactose, galactitol, and lactose. A significant increase in the β-galactosidase activity was shown in the presence of these

sugars compared to no sugar, which suggested that these sugars have an inducing affect on β-galactosidase activity. This result suggests that there is partial alleviation of repression by the secondary factor by these sugars. Also, β-galactosidase activity in D39 did not induce to the level of $BgaA^C$ under any conditions which suggests partial repression by CcpA and/or the secondary factor in these conditions.

Previous studies by King et al. have shown the NanA, BgaA, and StrH are involved in the deglycosylation of host cell proteins (44). These exoglycosidases are anchored to the cell wall via sortase. Therefore, due to the similar functions, we hypothesized that NanA and StrH may also be regulated by CcpA. Neuraminidase activity was significantly increased with the deletion of *ccpA*, but there was no response to increasing glucose concentration in either D39 or the *ccpA* deletion. The level of neuraminidase activity exhibited by D39 in the absence of sugar was similar to the level observed with the *ccpA* deletion in the presence of glucose. However, in the absence of sugar, the *ccpA* deletion mutant showed a significant increase in neuraminidase activity. The lack of repression with increasing glucose concentrations of neuraminidase activity suggested that the secondary regulator of BgaA does not regulated NanA. Similar CcpA regulation was not observed for the N-acetylglucosaminidase activity of StrH. D39 and its *ccpA* deletion mutant exhibited similar activities at 0.1% glucose but not at high glucose or in the absence of sugar. The *ccpA* deletion mutant had significantly less N-acetylglucosaminidase activity at 0% and 1.4% glucose than D39. NanA and BgaA have been shown to play a role in adherence of *S. pneumoniae* to epithelial cells, but this was not the case for StrH (44). The difference in functions could explain the differential regulation of BgaA, NanA, and SrtH by CcpA.

Since, BgaA, NanA, and StrH cleave glycosylated host cell proteins, we studied our various mutants for their ability to cleave the glycosylated host cell protein, lactoferrin. We found that deletion of *bgaA* resulted in a complete loss of lactoferrin cleavage suggesting that either deglycosylation is required for full lactoferrin cleavage or that BgaA. The *ccpA* deletion in D39, with increased β-galactosidase and neuraminidase activity, resulted in more efficient cleavage of lactoferrin than D39. Cleavage was similar to $BgaA^{C}$ which had 5-fold more β-galactosidase activity than the *ccpA* deletion.

Therefore, BgaA and NanA are regulated by CcpA, which have been shown to be differentially regulated in phase variants (43, 44). Microarray studies comparing phase variants have shown 46% of differentially expressed genes were in metabolic genes, further suggesting CcpA as a key regulator of phase variable factors. Therefore, we studied other phase variable factors such as PspA, PspC, LytA, and capsule. In these studies we found that PspC was not regulated by CcpA or changes in glucose. *In vivo* studies have shown differential regulation in various niches in the host, therefore, PspC must be regulated by other factors. Furthermore, we found that deletion of *ccpA* resulted in an overall decrease of PspA in cells grown in 0.1% and 1.4% glucose. Growth in a high level of glucose (1.4%) resulted in a 50% decrease of PspA in the *ccpA* deletion strain but not in D39. This result would suggest that CcpA enhances *pspA* expression and regulates its transcription in response to changing glucose. We also evaluated the level of LytA in the *ccpA* deletion. LytA levels decreased 40% when glucose increased from 0.1% to 1.4%, however this response was lost when *ccpA* was deleted. This result would suggest that CcpA regulates LytA in response to changing environments.

Finally, we examined the change in levels of capsular polysaccharide resulting from increasing glucose concentrations and deletion of *ccpA.* Many studies suggest a regulation of capsule based upon niche (i.e., in the nasopharynx) since full capsule production is not necessary and may be a hindrance for adhesion (44, 49). Our studies showed that as glucose concentrations increased, capsular polysaccharide production increased. This result would suggest that capsule levels are decreased in the nasopharynx where glucose is undetectable (62), but increased in the blood, where normal levels are 1 mg/ml (0.1%). The response to glucose was lost when *ccpA* was deleted, suggesting that CcpA regulates capsule either directly or indirectly, and that CcpA aids in adaptation to changing environments.

The co-regulation of *bgaA* with a galactose *pts* suggests that galactose cleaved by BgaA may be transported by this PTS. We have shown that *S. pneumoniae* is not able to utilize galactose as efficiently as glucose. The fact that many glycosylated host cell proteins contain galactose would suggest that in environments when glucose is limited, *S. pneumoniae* scavenges sugars from these proteins to survive. However, rapid growth is not required in low glucose environments, therefore, galactose should be sufficient for survival. *S. pneumoniae* also has other surface-associated enzymes involved in scavenging sugars such as NanA and StrH, which may suggest that in combination all of these enzymes are utilized to persist in these environments. Perhaps as a byproduct of sugar cleavage, NanA and BgaA also aid in adherence. Previous studies have shown that transparent strains adhere more efficiently to galactose, N-acetylgalactosamine, and N-acetylglucosamine (19). These studies showed that free galactose, N-acetylgalactosamine, and N-acetylglucosamine, could also inhibit adherence (19). Why

would *S. pneumoniae* have enzymes located on its surface to cleave important residues for adherence and why would the activity of these enzymes be up-regulated in transparent strains if they were not beneficial? It seems likely that these enzymes cleave sugars that are transported into the cell via the PTS for growth benefit. This cleavage also aids in adherence to the host cells by exposing sugars that enhance adherence. This is particularly true with NanA. Many glycosylated host cell proteins have terminal sialic acid residues to protect against cleavage. However, *S. pneumoniae* can cleave these residues and expose more preferred sugars for adherence.

Many studies have shown that surface exposed factors are important for adherence, but in many cases these studies have to be done in a nonencapsulated strain due to the fact that capsule inhibits adherence. *In vivo*, however, we know that these factors play a role in colonization. It is assumed that capsule is decreased in environments in which *S. pneumoniae* adheres. CcpA-mediated co-regulation of capsule with multiple adherence factors in response to glucose suggests that capsule is decreased *in vivo* in low glucose environments thereby exposing adherence factors whose expression is up-regulated. Therefore, upon exposure to low glucose environments, such as the nasopharynx, the metabolism of the cell slows down. The phosphorylation state of HPr state of signals CcpA to repress expression of capsule and activate expression of NanA, BgaA, and LytA. This glucose deprived environment also signals to up-regulate StrH via a different mechanism. The response by CcpA thus allows the adherence of *S. pneumoniae* to the mucosal surface.

We propose that dissemination of *S. pneumoniae* to other niches occurs via this glucose-regulated pathway. We propose this model for infection, during the adaptation

process some of the bacteria do not down-regulate their capsule rapidly enough in response to the loss of glucose which leads to dissemination of a few bacteria. This elicits a response from the body to the infection, which results in and increases the glucose concentration in the nasopharyngeal cavity (62). The increase in glucose could also be triggered by a secondary viral infection (62). Increased glucose leads to increased metabolism of *S. pneumoniae,* which results in an up-regulation of capsule and down-regulation of adherence factor via CcpA signaling. This signaling results in the dissemination of *S. pneumoniae* to other niches in the host, potentially leading to infection. The glucose signaling may also account for why individuals with diabetes mellitus have a higher incidence of morbidity and mortality with pneumococcal infections. Individuals with diabetes mellitus have a consistently higher level of glucose in their nasopharyngeal cavity (62). This effect may lead to dissemination at a higher rate.

Overall we have characterized a key regulatory pathway for *S. pneumoniae*. Rapid adaptation to changing environments in the host is important for the survival of the bacterium. Therefore, these studies show that one way in which *S. pneumoniae* senses the various niches is through glucose concentrations via HPr/CcpA regulatory pathway. In low glucose environments, factors aiding in colonization, such as BgaA, NanA, StrH, LytA are up-regulated and capsule is down-regulated. However, upon dissemination to other niches with higher glucose concentrations, the colonization factors are down-regulated and capsular polysaccharide is up-regulated to prevent against opsonophagocytosis. Clearly other regulatory mechanisms exist, as PspC, for example, was not regulated by glucose or CcpA. Due to the fact that CcpA regulates so many of the phase variable factors, we suggest that it is very important for survival of *S.*

pneumoniae and may play a critical role in the transition from colonization to dissemination.

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