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CcpA-Dependent and -Independent Control of Beta-Galactosidase Expression in *Streptococcus pneumoniae* Occurs via Regulation of an Upstream Phosphotransferase System-Encoding Operon[▽]

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A spontaneous mutant of *Streptococcus pneumoniae* strain D39 exhibiting elevated β-galactosidase activity was identified. We determined that the 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 catabolite-responsive element (*cre*) and affected the binding of CcpA (catabolite control protein A), a key regulator of many carbon metabolism genes. The *pts* operon and *bgaA* were cotranscribed, and their transcription was regulated by CcpA. Deletion of *ccpA* altered β-galactosidase activity, leading to a sevenfold increase in the parent but a fivefold decrease in the BgaA^C mutant. The resulting β-galactosidase activities were the same 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 the 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.

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 lead 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 (42, 43), 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 (46). 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 comprise 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 it has been reported not to be involved in lactose metabolism (46). It has been suggested to be involved in digestion of host cell polysaccharides, which may play a role in adherence or host cell interaction (46). King et al. showed that BgaA is important in adherence to upper-airway epithelial cells in the absence of capsule and that it could aid in the deglycosylation of human secretory component (24). The latter activity was increased due to increased transcription of *bgaA* in transparent strains, which exhibit less capsule production and more teichoic acid and colonize the host better than opaque strains (21–23). BgaA activity is specific for β,1-4 galactose linkages (25), which can be found in many glycosylated host cell proteins, as well as some *S. pneumoniae* capsule structures (6, 17, 35). BgaA is expressed in the host, as demonstrated using convalescent-phase serum (48), and disruption of *bgaA* attenuates the virulence of *S. pneumoniae* in a mouse pneumonia model (14). 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). The fundamental aspects of this system have been described primarily from studies with *Bacillus* sp. (reviewed in references 37 and 39). CcpA, a member of the LacI-GalR family, binds to catabolite-responsive elements (*cre*) located within or near promoters (40). *cre* are identified

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by the sequence TGWAANCGNTNWCA (the underlined nucleotides are involved in binding to CcpA) (18, 19, 31, 41). 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 (20). Binding of CcpA to a *cre* located upstream of the promoter is proposed to enhance transcription by allowing CcpA to interact with RNA polymerase (38). 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 (37, 39). 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 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 EIIB, which phosphorylates the sugar upon translocation into the cell. 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 the 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.

Catabolite repression in bacilli, as well as streptococci, may involve many additional levels of regulation. For multiple streptococcal species (9, 11, 16, 33, 47) and *Lactobacillus casei* (12), CcpA (also referred to as RegM [11, 33]) is not responsible for all catabolite repression. For example, in *Streptococcus mutans*, disruption of *ccpA* results in increased glucose repression of enzymes involved in carbohydrate utilization (33). In *Streptococcus gordonii*, the arginine deaminase system (ADS) involves multiple levels of regulation (9, 47). Here, regulation involves both CcpA and ArcR, which is an activator of the ADS. The two regulators share overlapping binding sequences in the promoter regions, which results in CcpA inhibition of RNA polymerase binding, as well as ArcR binding (47). The results of previous studies of *S. pneumoniae* suggested that CcpA regulates both β-galactosidase and α-glucosidase but is not involved in glucose repression of either of these enzymes (11). Further studies by Iyer et al. using a different strain of *S. pneumoniae* suggested that glucose repression of β-galactosidase was partially mediated via CcpA; however, a secondary regulator was also involved (16). In contrast, glucose repression of α-galactosidase, α-glucosidase, and β-glucosidase was not mediated by CcpA. In both studies, CcpA was shown to be important in virulence (11, 16). The results from the streptococcal systems suggest a complex regulatory network involving CcpA, as well as secondary regulators, that allows rapid adaptation to changing environments in the host.

The two previous reports regarding *S. pneumoniae* noted a role for CcpA in the repression of β-galactosidase activity, but the mechanism for repression was not known (11, 16). 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 CcpA-dependent and -independent mechanisms.

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/liter; neopeptone, 5 g/liter; Tris, 1.25 g/liter; sodium chloride, 5 g/liter; yeast extract, 1.25 g/liter; and 0.1% of the indicated sugar source) (2). For growth on solid medium, blood agar plates (blood agar base no. 2 [Difco] supplemented with 3% defibrinated sheep blood [Colorado Serum Company]) or THY or D medium with 1.5% Bacto agar (Difco) was used. Where indicated, catalase and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were added to final concentrations of 5,000 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*. Allelic exchange was used to generate deletions in *bgaA* and *ccpA*. To construct plasmids for generating *bgaA* deletions, PCR fragments flanking *bgaA* were generated using *S. pneumoniae* constitutive β-galactosidase ($BgaA^C$) mutant chromosomal DNAs as 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, respectively (Table 2), were initially cloned separately in pCR2.1 (Invitrogen). The cloned fragments were excised from pCR2.1 by digestion with EcoRI, which is contained in the multiple cloning site of the vector, and KpnI. The latter restriction site was generated by addition of the KpnI recognition sequence to the PCR primers R-UpBgaA and F-DownBgaA, which were used in the initial amplification. The excised fragments were cloned together 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 in the absence of antibiotic selection for the plasmid. 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* by allelic exchange in these isolates was confirmed by PCR. For deletion of *bgaA* in strains that exhibited low β-galactosidase activity, the transformation mixture was plated in the absence of selection on D agar supplemented with X-Gal and catalase, where the cells exhibit more β-galactosidase activity than on THY agar. Colonies that appeared 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 microliters of the mixture was used in a PCR with the primer pair F-UpBgaA/R-DownBgaA, and the resulting products were screened for reduction in size.

The plasmid construct for deletion of *ccpA* was generated in a manner similar to that for the *bgaA* deletion construct. The primer pairs used for amplification of the flanking regions were F-UpCcpA1/R-UpCCpa2 and F-DownCcpA3/R-DownCcpA4 (Table 2). To allow selection of *ccpA* deletions, a Km resistance marker (*aphA-3*) was inserted between the upstream and downstream flanking regions using the restriction enzyme BglII, as described previously (44).

Linkage analysis. For linkage analyses, insertion-duplication was 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, and pGK547 (Table 1). Recipients transformed with the plasmids were selected by plating them on blood agar plates containing Em. Competence was induced as described previously (13), except

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Reference or source
S. pneumoniae		
D39	Type 2 parent; $BgaA^+$	4a
GK155	pGK528 × GK1000; Em^r downstream of *bgaA*	This study
GK157	pGK530 × D39; Em^r downstream of *bgaA*	This study
GK159	pGK538 xGK1000; Δ*bgaA* in GK1000	This study
GK165	pGK538 × D39; Δ*bgaA* in D39	This study
GK220	pGK579 × D39; Δ*ccpA* in D39; Km^r	This study
GK304, GK305	pGK638 × D39; Em^r between *pts* and *bgaA*	This study
GK308, GK309	pGK638 × GK1000; Em^r between *pts* and *bgaA*	This study
GK311	pGK641 × GK1000; Em^r upstream of *pts*	This study
GK313	pGK646 × D39; Km^r between *pts* and *bgaA*	This study
GK315, GK316	Independent isogenic $BgaA^C$ derivatives of D39 with constitutive β-Gal mutation; −56G→C mutation in *pts* promoter	This study
GK317, GK318	pGK646 × GK1000; Km^r between *pts* and *bgaA*	This study
GK320	pGK579 × GK315; Δ*ccpA* in GK315; Km^r	This study
GK322	pGK538 × GK315; Δ*bgaA* in GK315	This study
GK338	pGK663 × D39; *pts* insertion in D39; Em^r	This study
GK339	pGK663 × GK1000; *pts* insertion in GK1000; Em^r	This study
GK344, GK345	pGK663 × GK220; *pts* insertion and Δ*ccpA* in D39; Km^r Em^r	This study
GK346	pGK663 × 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 mutation in *pts* promoter	This study
E. coli		
BL21(AI)	F^- *ompT hsdSB*(r_B^- m_B^-) *gal dcm lon araB*::T7RNAP-*tetA*	Invitrogen
DH5αF′	F′ ϕ80d*lacZ*ΔM15Δ (*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44*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	45
pGK528	pJY4164 derivative containing PCR fragments from the amplification of GK1000 chromosomal DNA by the primer pair DBgaA3/DBgaA4; 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 DBgaA3/DBgaA4; 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 two fragments; for deletion of *ccpA*	This study
pGK638	pJY4164 derivative containing PCR fragments from the amplification of D39 chromosomal DNA by the primer pair F-UpBgaA/R-UpBgaA; for polar insertion between *pts* and *bgaA*	This study
pGK639	pET-20b::XhoI-NdeI from PCR product amplified from D39 using primer pair F-CcpAORF/R-CcpAORF; 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-BgaApro/BgaA2 with Km resistance gene *aphA-3* between two 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

that cultures were incubated only 2 to 2.5 h prior to being plated. The location of the insertion-duplication in the expected sites was 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 them on THY-catalase-Em plates supplemented with X-Gal.

β-Galactosidase assays. β-Galactosidase activity was determined as described by Miller (28). Briefly, cultures were grown to mid-exponential phase (cell density, 3×10^8 CFU/ml). A 0.2-ml aliquot of the culture was added directly to 0.8 ml Z buffer (28), and the suspension was incubated at 30°C with 0.2 ml of 4 mg/ml *o*-nitrophenol-β-D-galactopyranoside. Reactions were stopped by the addition of 0.5 ml of 1 M Na_2CO_3. Activity was calculated in Miller units, as described previously (28).

Transcription analysis. Total RNA was isolated from a 50-ml culture grown in THY using a hot-acid phenol extraction, as described previously (10). 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 μl 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 20× SSC (1× 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

TABLE 2. Oligonucleotide primers used in this study

ORF and primer[a]	Sequence[b]	Position[c]
spr0562–0565		
F-spr0562	[d]TTGATTGAAAGGGTTAGTATTGAC	11–34
F-PTSpro2	CTAGCTTCCTAGTTTACTCTTTG	266–288
R-PTSpro3	CAAAGAGTAAACTAGGAAGCTAG	288–266
R-spr0562	[d]CTATATGAAACCGTTGTCAATTAC	429–406
R-PTSpro	[d]CTAATACCATAAGTTTTCCCTTC	483–461
F-PTS3	[d]GGAATTTCTAGGAAAGGACTTGC	647–669
R-PTS2	[d]CGATAACTGGGATACCTGGTTC	1201–1180
F-UpBgaA	GCTACAGCAGCTATCGTTCTTG	1818–1839
R-UpBgaA	[e]TATTTTGCTTTTGCTGCGTACTC	2815–2793
F-bgaApro	[e]TGCGCTCCTATAAAATATAAACTC	2818–2840
BgaA2	CTACGATACCAAAGTAAGAGCT	5329–5308
F-DownBgaA	[e]GTGCAGGATTAGTAGTTACTAAAG	9767–9790
DBgaA3	GTCCTAAATAGAAGATAAAGAG	9854–9875
spr0566–0579		
DBgaA4	GAACGAACGCTATCAAAACTTGAAAGC	183–157
R-DownBgaA	CAGTTCCTTCTTACCACAAGACC	762–741
spr1806–1817		
F-UpCcpA1	[d]ACATATGCTGGTCCTCTACCAG	5336–5357
R-UpCcpA2	[f]GAAAAAATCAGGGAATCGAGAAG	6370–6348
F-CcpAORF	[g]TTTACGTTTTCGTGTTGAG	6374–6392
R-CcpAORF	[h]ATGTGTGAGATAGAAAGG	7411–7394
F-DownCcpA3	[f]TCTTTTACAAGTAGAGGTACTGATTG	7412–7437
R-DownCcpA4	[d]CATCCAACGGAAGTGCAAGTTC	8451–8433

[a] Primers are from the R6 genome. Open reading frame (ORF) designations are given for the regions containing the groups of primers. Forward and reverse primers are represented by F and R, respectively.

[b] Sequences are from the complete R6 genome (15).

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

[d] Primer also contains an EcoRI restriction site and additional nucleotides at the 5′ end (GGAATTCC; restriction site underlined).

[e] Primer also contains a KpnI restriction site (GGTACC) at the 5′ end.

[f] Primer also contains a BglII restriction site and additional nucleotides at the 5′ end (GAAGATCT; restriction site underlined).

[g] Primer also contains an XhoI restriction site and additional nucleotides at the 5′ end (CCGCTCGAG; restriction site underlined).

[h] Primer also contains an NdeI restriction site and additional nucleotides at the 5′ end (GGAATTCCATATG; restriction site underlined).

cross-linked and then prehybridized for 3 h at 42°C in high-sodium dodecyl sulfate (SDS) hybridization buffer (7% SDS, 50% formamide, 5× SSC, 2% blocking reagent [Roche], 50 mM sodium phosphate, and 0.1% *N*-laurylsarcosine). The 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 nonspecifically bound probe, the membranes were washed twice with 2× SSC containing 0.1% SDS for 15 min at room temperature and then twice with 0.5× SSC containing 0.1% SDS for 15 min at 65°C. The blots were developed using the Anti-DIG-AP Fab fragments (Roche) and the 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 (13).

Electrophoretic mobility shift assays. To clone *ccpA*, the open reading frame was PCR amplified with primers that contained restriction enzyme sites for XhoI and NdeI on the 5′ ends of the forward and reverse primers, respectively (F-CcpAORF/R-CcpAORF) (Table 2). After NdeI and XhoI 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 nondenaturing conditions in wash/extraction buffer using a cobalt resin (Clontech) to affinity purify the His-tagged protein, as described by the manufacturer. Protein eluted from the cobalt column was dialyzed overnight at 4°C in 1× phosphate-buffered saline (342.5 mM NaCl, 6.75 mM KCl, 13.5 mM Na_2HPO_4, and 4.5 mM KH_2PO_4) containing 10% glycerol. The purified protein was stored at −80°C. 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°C (19). After incubation, samples in bromophenol blue loading buffer were separated by electrophoresis using 5% acrylamide gels in 1× Tris-borate-EDTA buffer, pH 8.0 (10.8 g Tris-HCl, 5.55 g boric acid, 0.74 g EDTA) that had been prerun in 1× Tris-borate-EDTA. 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) (24). Analysis of a D39 stock culture in our laboratory that was originally obtained more than 20 years ago (26) revealed the presence of both β-galactosidase-positive and apparent β-galactosidase-negative isolates, as determined by plating them in the presence of the chromogenic substrate X-Gal to identify blue and white colonies, respectively. Following growth in THY liquid medium to a density of $\sim 3 \times 10^8$ CFU/ml, the 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 the wild type and are therefore referred to as D39.

We initially chose a single $BgaA^C$ isolate (GK1000) for further characterization. D39 and the constitutive strain exhibited similar growth rates in THY. Restriction digests of chromosomal DNAs with HindIII 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 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)

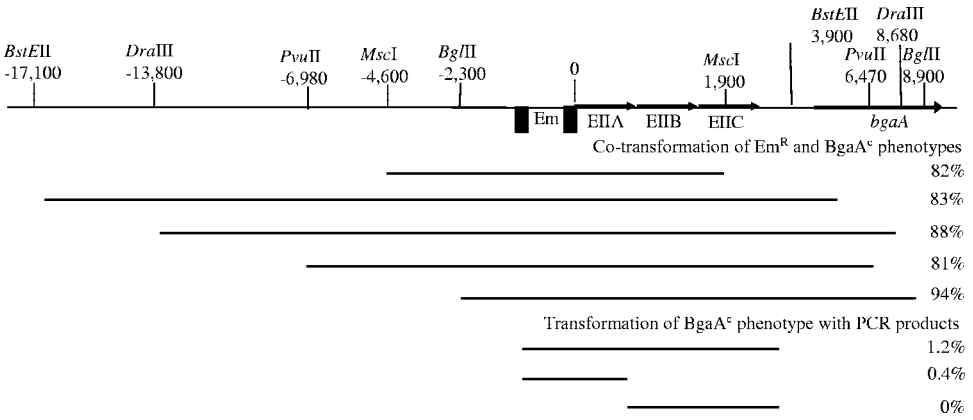

FIG. 1. Mapping of mutation leading to the BgaA^C phenotype. Restriction fragments from the BgaA^C strain GK311 were transformed to D39. Em^r transformants were screened for the blue colony BgaA^C phenotype by plating them in the presence of X-Gal. Cotransformation of the BgaA^C phenotype with Em^r represents 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.

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, which 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. These enzymes have 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). Sequence analyses identified a single point mutation located 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 10 additional BgaA^C isolates purified from the primordial stock demonstrated that 6 of the isolates contained the same G-to-C transversion mutation found in GK1000, while the remaining 4 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

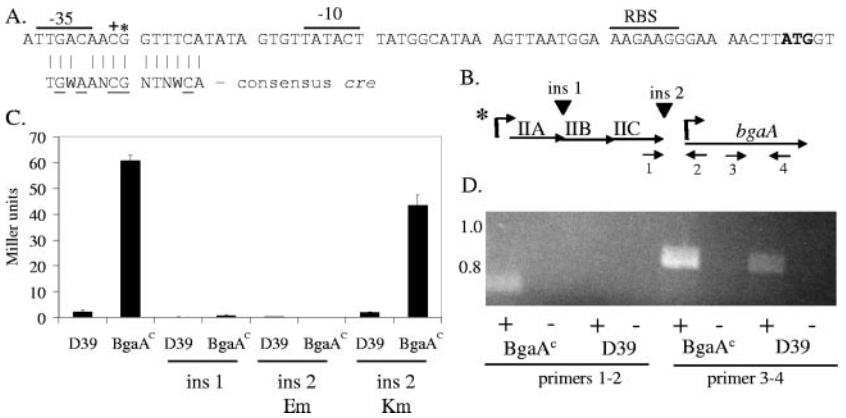

FIG. 2. Transcription of the *pts-bgaA* region. (A) *pts* promoter region. *, point mutation (G→C) in the original BgaA^C mutant GK1000 and derivatives; +, point mutation (C→T) in a second mutant, GK1001, and derivatives. The *cre* consensus sequence is aligned below the *pts* promoter. Underlined amino acids are important in CcpA binding (31, 41). N, any base, W, adenine or thymine. The lines above the sequence indicate the −35, −10, and ribosome binding site (RBS). The translation start codon is in boldface. (B) Insertions and primers used to characterize *bgaA* expression. Arrows and numbers beneath the figure indicate primers used in RT-PCR analyses in panel D. The asterisk indicates the location of the *pts* promoter point mutation in the BgaA^C strain. Insertions (ins) are indicated by arrowheads above the *pts* region. (C) β-Galactosidase activities of insertion mutants. The locations of insertions 1 and 2 are indicated in panel B. Insertion 2, Em, is polar on downstream genes. Insertion 2, Km, is nonpolar. The results are the means (plus standard error) of three 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. The 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.

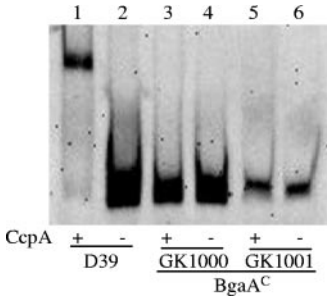

FIG. 3. CcpA binding to the *pts* promoter. Electrophoretic mobility shift assays were used to determine if the point mutation identified in BgaA^C resulted in altered binding of CcpA. Purified CcpA (15 ng) incubated with DIG-labeled D39 or BgaA^C *pts* promoter DNAs was electrophoresed on a 5% nondenaturing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

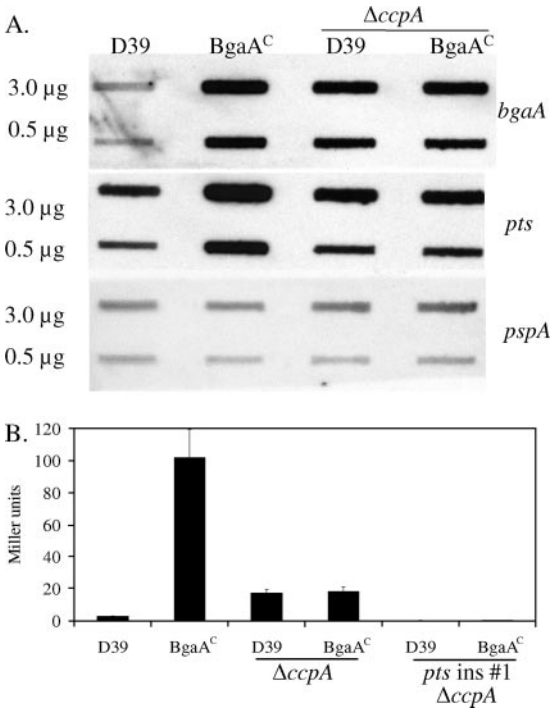

FIG. 4. (A) Effects of *ccpA* deletion on transcription and β-galactosidase activity. Slot blots containing the amounts of RNA indicated on the left were probed with internal sequences from the genes indicated on the right. BgaA^C is GK1000. *pspA* was used as a loading control. (B) β-Galactosidase activity.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

the culture leading to the stock. The mutations appear to be rare, however, as we have not subsequently observed white-to-blue phenotype transition or vice versa.

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.

Cotranscription 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 nonpolar Km 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 C, 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 nucleotides 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 (27). 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 cotranscription 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 coregulates *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). As a result, the respective transcript levels in the two strains were the same. We also examined levels of β-galactosidase activity in the *ccpA* deletion strains. The result was similar to the transcriptional analysis, so that the levels of β-galactosidase activity were the same for the D39 and BgaA^C *ccpA* mutants (Fig. 4B, Δ*ccpA*). To confirm that the β-galactosidase activity and *bgaA* expression in the

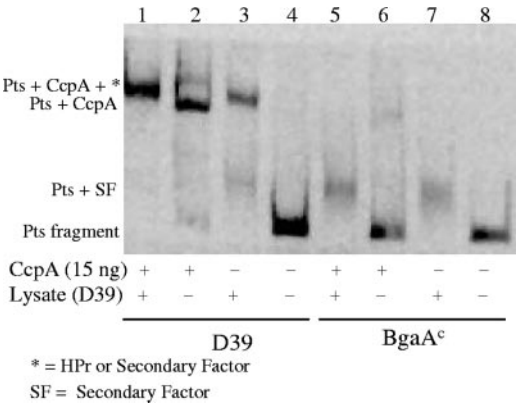

FIG. 5. Binding of a second repressor to the *pts* promoter. Electorphoretic mobility shift assays were used to determine if the second repressor of *pts-bgaA* was a DNA binding protein. Purified CcpA and total cellular lysates from D39 incubated with DIG-labeled D39 and BgaA^C (GK1000) *pts* promoter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

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* insertion 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 to 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 plus 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.

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 cotranscribed, 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 semidefined 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, similar levels of repression were observed with both strains, and they were elevated over that observed for the CcpA$^+$ D39 (Fig. 6B). These 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.

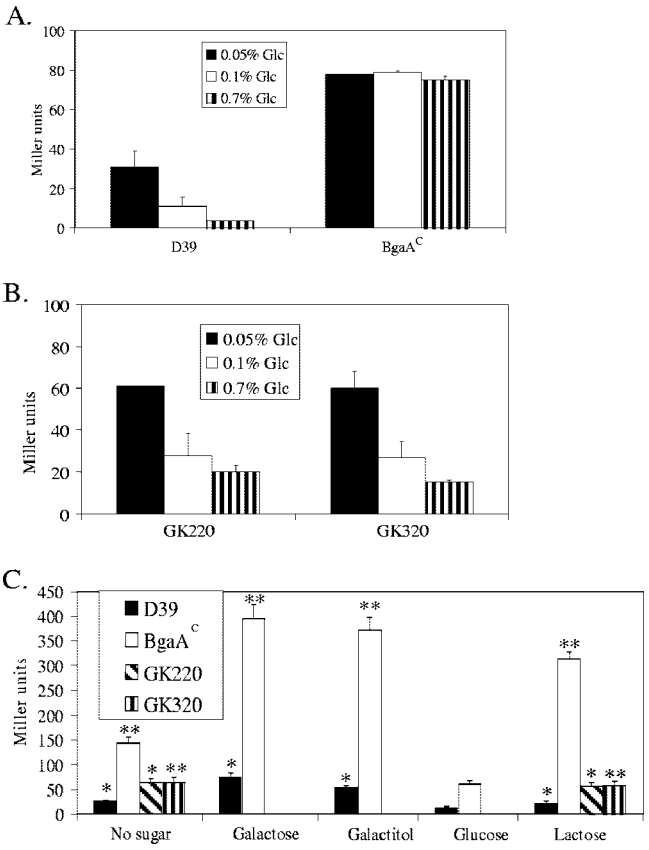

FIG. 6. β-Galactosidase activities during growth in different sugars. (A) β-Galactosidase activities of D39 and BgaA^C (GK315) in increasing glucose. (B) β-Galactosidase activities of *ccpA* deletion mutants of D39 (GK220) and BgaA^C (GK320). For both panels A and B, strains were grown to mid-exponential growth phase in D medium supplemented with 0.1% glucose and then diluted 1/12 in D medium supplemented with the indicated glucose concentration. β-Galactosidase assays were performed with mid-exponential phase cultures. The results are the means (plus standard errors) of three replicates. (C) β-Galactosidase activities 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 in 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. 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. The results are the means (plus standard errors) of three replicates. *, $P < 0.05$ compared to D39 grown in glucose. **, $P < 0.05$ compared to BgaA^C grown in glucose.

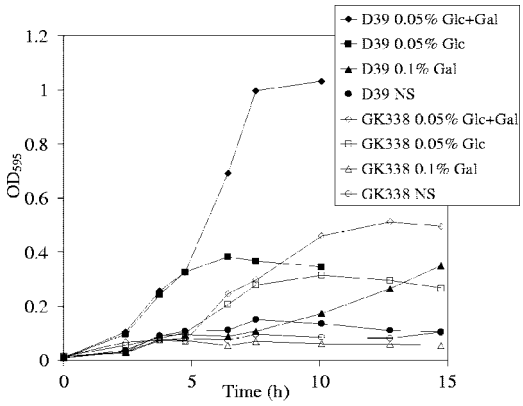

FIG. 7. Growth of D39 and its *pts* insertion mutant (GK338). Strains were grown in D medium with the indicated sugar source. Gal, 0.1% galactose; NS, no sugar; OD_{595}, optical density at 595 nm. The data presented are representative of multiple experiments.

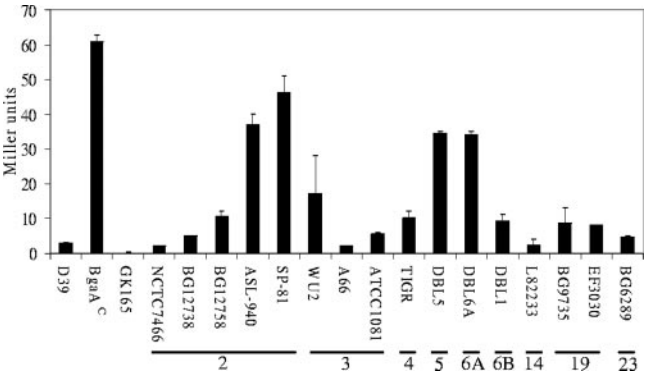

FIG. 8. β-Galactosidase activities of other *S. pneumoniae* strains. The strains were grown to mid-exponential phase in THY. The results are the means (plus standard errors) of three replicates. The capsular serotype is shown below the strain name.

Because the PTS operon upstream of *bgaA* has homology to putative galactose, galactitol, and fructose transporters, we next examined β-galactosidase activities during growth in these sugars and in lactose, as their uptake is mediated by phosphoenolpyruvate-dependent PTSs. 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, and, for $BgaA^C$, 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 identical β-galactosidase levels for D39 and the $BgaA^C$ mutant due to increased activity in the former and decreased activity in the latter. For the *ccpA* deletions, β-galactosidase activities were the same during culture with lactose or no sugar, indicating that the observed 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 the early stationary phase growth (approximate 10- and 17-fold increases, respectively, over mid-exponential phase), as previously reported for growth in glucose (24). β-Galactosidase activity in the $BgaA^C$ mutant remained essentially constant throughout growth in glucose but increased approximately twofold 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 cotranscribed 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 PTSs in D39 do not contain all of the components for sugar transport (15, 36). Therefore, this PTS may be important for the uptake of many sugars, possibly explaining the growth defects of the *pts* insertion mutants.

β-Galactosidase activities in other strains. A wide variation in β-galactosidase activities was seen among other *S. pneumoniae* strains (Fig. 8). NCTC7466 is another D39 isolate that is separated by many years from our D39 (26). 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) (15). From the genome sequence of TIGR4 (36) 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, 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* (29, 41). Both residues are critical for interaction with CcpA, as the conserved Leu55 in CcpA intercalates between them to effect minor groove expansion (31). Binding of CcpA to the *cre* can inhibit transcription by inhibiting the binding of RNA polymerase (20). 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. The possibility of a second repressor was suggested in studies with the TIGR4 *S. pneumoniae* strain (16), although we do not know whether the two repressors are the same. In the *S. gordonii* ADS, the binding sequences for CcpA and the second regulator, ArcR, overlap (47), possibly similar to what we observed. The ArcR binding sequence is not, however, present in the region containing the binding site for the *S. pneumoniae* second repressor (unpublished observations).

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 (16). In contrast, it was concluded that glucose was not involved in CcpA-mediated repression of β-galactosidase activity in *S. pneumoniae* D39 (11), 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 (11). 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, the 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 (32, 34) (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 (30). Glucose levels are similarly low in healthy lungs (5), but higher levels occur during infection (30) and in the bloodstream. BgaA has previously been shown to cleave human glycoproteins and to be important in adherence by *S. pneumoniae* (24). 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* not only to 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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REFERENCES

1. **Advisory Committee on Immunization Practice.** 1997. Prevention of pneumococcal disease. Morb. Mortal. Wkly. Rep. **46:**1–24.
2. **Alloing, G., C. Granadel, D. A. Morrison, and J. P. Claverys.** 1996. Competence pheromone, oligopeptide permease, and induction of competence in *Streptococcus pneumoniae*. Mol. Microbiol. **21:**471–478.
3. **Alwine, J. C., D. J. Kemp, and G. R. Stark.** 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. Proc. Natl. Acad. Sci. USA **74:**5350–5354.
4. **Austrian, R.** 1986. Some aspects of the pneumococcal carrier state. J. Antimicrob. Chemother. **18**(Suppl. A)**:**35–45.

4a. **Avery, O. T., C. M. MacLeod, and M. McCarty.** 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. Induction of transformation by a deoxyribonucleic acid fraction isolated from pneumococcus type III. J. Exp. Med. **79:**137–158.

5. **Baker, E. H., N. Clark, A. L. Brennan, D. A. Fisher, K. M. Gyi, M. E. Hodson, B. J. Philips, D. L. Baines, and D. M. Wood.** 2007. Hyperglycaemia and cystic fibrosis alter respiratory fluid glucose concentrations estimated by breath condensate analysis. J. Appl. Physiol. **102:**1969–1975.
6. **Bentley, S. D., D. M. Aanensen, A. Mavroidi, D. Saunders, E. Rabbinowitsch, M. Collins, K. Donohoe, D. Harris, L. Murphy, M. A. Quail, G. Samuel, I. C. Skovsted, M. S. Kaltoft, B. Barrell, P. R. Reeves, J. Parkhill, and B. G. Spratt.** 2006. Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes. PLoS Genet. **2:**262–269.
7. **Crain, M. J., W. D. Waltman II, J. S. Turner, J. Yother, D. F. Talkington, L. S. McDaniel, B. M. Gray, and D. E. Briles.** 1990. Pneumococcal surface protein A (PspA) is serologically highly variable and is expressed by all clinically important capsular serotypes of *Streptococcus pneumoniae*. Infect. Immun. **58:**3293–3299.

8. **Deutscher, J., C. Francke, and P. W. Postma.** 2006. How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. Microbiol. Mol. Biol Rev. **70:**939–1031.
9. **Dong, Y., Y. Y. Chen, and R. A. Burne.** 2004. Control of expression of the arginine deiminase operon of *Streptococcus gordonii* by CcpA and Flp. J. Bacteriol. **186:**2511–2514.
10. **Georgellis, D., S. Arvidson, and A. von Gabain.** 1992. Decay of *ompA* mRNA and processing of 9S RNA are immediately affected by shifts in growth rate, but in opposite manners. J. Bacteriol. **174:**5382–5390.
11. **Giammarinaro, P., and J. C. Paton.** 2002. Role of RegM, a homologue of the catabolite repressor protein CcpA, in the virulence of *Streptococcus pneumoniae*. Infect. Immun. **70:**5454–5461.
12. **Gosalbes, M. J., V. Monedero, C. A. Alpert, and G. Perez-Martinez.** 1997. Establishing a model to study the regulation of the lactose operon in *Lactobacillus casei*. FEMS Microbiol. Lett. **148:**83–89.
13. **Hardy, G. G., M. J. Caimano, and J. Yother.** 2000. Capsule biosynthesis and basic metabolism in *Streptococcus pneumoniae* are linked through the cellular phosphoglucomutase. J. Bacteriol. **182:**1854–1863.
14. **Hava, D. L., and A. Camilli.** 2002. Large-scale identification of serotype 4 *Streptococcus pneumoniae* virulence factors. Mol. Microbiol. **45:**1389–1406.
15. **Hoskins, J., W. E. Alborn, Jr., J. Arnold, L. C. Blaszczak, S. Burgett, B. S. DeHoff, S. T. Estrem, L. Fritz, D. J. Fu, W. Fuller, C. Geringer, R. Gilmour, J. S. Glass, H. Khoja, A. R. Kraft, R. E. Lagace, D. J. LeBlanc, L. N. Lee, E. J. Lefkowitz, J. Lu, P. Matsushima, S. M. McAhren, M. McHenney, K. McLeaster, C. W. Mundy, T. I. Nicas, F. H. Norris, M. O'Gara, R. B. Peery, G. T. Robertson, P. Rockey, P. M. Sun, M. E. Winkler, Y. Yang, M. Young-Bellido, G. Zhao, C. A. Zook, R. H. Baltz, S. R. Jaskunas, P. R. Rosteck, Jr., P. L. Skatrud, and J. I. Glass.** 2001. Genome of the bacterium *Streptococcus pneumoniae* strain R6. J. Bacteriol. **183:**5709–5717.
16. **Iyer, R., N. S. Baliga, and A. Camilli.** 2005. Catabolite control protein A (CcpA) contributes to virulence and regulation of sugar metabolism in *Streptococcus pneumoniae*. J. Bacteriol. **187:**8340–8349.
17. **Kamerling, J. P.** 2000. Pneumococcal polysaccharides: a chemical view, p. 81–114. *In* A. Tomasz (ed.), *Streptococcus pneumoniae*: molecular biology and mechanisms of disease. Mary Ann. Liebert, Inc., Larchmont, NY.
18. **Kim, J. H., and G. H. Chambliss.** 1997. Contacts between *Bacillus subtilis* catabolite regulatory protein CcpA and *amyO* target site. Nucleic Acids Res. **25:**3490–3496.
19. **Kim, J. H., Z. T. Guvener, J. Y. Cho, K. C. Chung, and G. H. Chambliss.** 1995. Specificity of DNA binding activity of the *Bacillus subtilis* catabolite control protein CcpA. J. Bacteriol. **177:**5129–5134.
20. **Kim, J. H., Y. K. Yang, and G. H. Chambliss.** 2005. Evidence that *Bacillus* catabolite control protein CcpA interacts with RNA polymerase to inhibit transcription. Mol. Microbiol. **56:**155–162.
21. **Kim, J. O., S. Romero-Steiner, U. B. Sorensen, J. Blom, M. Carvalho, S. Barnard, G. Carlone, and J. N. Weiser.** 1999. Relationship between cell surface carbohydrates and intrastrain variation on opsonophagocytosis of *Streptococcus pneumoniae*. Infect. Immun. **67:**2327–2333.
22. **Kim, J. O., and J. N. Weiser.** 1998. Association of intrastrain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of *Streptococcus pneumoniae*. J. Infect. Dis. **177:**368–377.
23. **King, S. J., K. R. Hippe, J. M. Gould, D. Bae, S. Peterson, R. T. Cline, C. Fasching, E. N. Janoff, and J. N. Weiser.** 2004. Phase variable desialylation of host proteins that bind to *Streptococcus pneumoniae* in vivo and protect the airway. Mol. Microbiol. **54:**159–171.
24. **King, S. J., K. R. Hippe, and J. N. Weiser.** 2006. Deglycosylation of human glycoconjugates by the sequential activities of exoglycosidases expressed by *Streptococcus pneumoniae*. Mol. Microbiol. **59:**961–974.
25. **Kojima, K., M. Iwamori, S. Takasaki, K. Kubushiro, S. Nozawa, R. Iizuka, and Y. Nagai.** 1987. Diplococcal beta-galactosidase with a specificity reacting to beta 1-4 linkage but not to beta 1-3 linkage as a useful exoglycosidase for the structural elucidation of glycolipids. Anal. Biochem. **165:**465–469.
26. **Lanie, J. A., W. L. Ng, K. M. Kazmierczak, T. M. Andrzejewski, T. M. Davidsen, K. J. Wayne, H. Tettelin, J. I. Glass, and M. E. Winkler.** 2007. Genome sequence of Avery's virulent serotype 2 strain D39 of *Streptococcus pneumoniae* and comparison with that of unencapsulated laboratory strain R6. J. Bacteriol. **189:**38–51.
27. **Martin, B., O. Humbert, M. Camara, E. Guenzi, J. Walker, T. Mitchell, P. Andrew, M. Prudhomme, G. Alloing, R. Hakenbeck, et al.** 1992. A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*. Nucleic Acids Res. **20:**3479–3483.
28. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
29. **Miwa, Y., A. Nakata, A. Ogiwara, M. Yamamoto, and Y. Fujita.** 2000. Evaluation and characterization of catabolite-responsive elements (cre) of *Bacillus subtilis*. Nucleic Acids Res. **28:**1206–1210.
30. **Philips, B. J., J. X. Meguer, J. Redman, and E. H. Baker.** 2003. Factors determining the appearance of glucose in upper and lower respiratory tract secretions. Intensive Care Med. **29:**2204–2210.
31. **Schumacher, M. A., G. S. Allen, M. Diel, G. Seidel, W. Hillen, and R. G. Brennan.** 2004. Structural basis for allosteric control of the transcription regulator CcpA by the phosphoprotein HPr-Ser46-P. Cell **118:**731–741.
32. **Shivers, R. P., S. S. Dineen, and A. L. Sonenshein.** 2006. Positive regulation of *Bacillus subtilis ackA* by CodY and CcpA: establishing a potential hierarchy in carbon flow. Mol. Microbiol. **62:**811–822.
33. **Simpson, C. L., and R. R. Russell.** 1998. Identification of a homolog of CcpA catabolite repressor protein in *Streptococcus mutans*. Infect. Immun. **66:**2085–2092.
34. **Sonenshein, A. L.** 2005. CodY, a global regulator of stationary phase and virulence in Gram-positive bacteria. Curr. Opin. Microbiol. **8:**203–207.
35. **Spik, G., G. Strecker, B. Fournet, S. Bouquelet, J. Montreuil, L. Dorland, H. van Halbeek, and J. F. Vliegenthart.** 1982. Primary structure of the glycans from human lactotransferrin. Eur. J. Biochem. **121:**413–419.
36. **Tettelin, H., K. E. Nelson, I. T. Paulsen, J. A. Eisen, T. D. Read, S. Peterson, J. Heidelberg, R. T. DeBoy, D. H. Haft, R. J. Dodson, A. S. Durkin, M. Gwinn, J. F. Kolonay, W. C. Nelson, J. D. Peterson, L. A. Umayam, O. White, S. L. Salzberg, M. R. Lewis, D. Radune, E. Holtzapple, H. Khouri, A. M. Wolf, T. R. Utterback, C. L. Hansen, L. A. McDonald, T. V. Feldblyum, S. Angiuoli, T. Dickinson, E. K. Hickey, I. E. Holt, B. J. Loftus, F. Yang, H. O. Smith, J. C. Venter, B. A. Dougherty, D. A. Morrison, S. K. Hollingshead, and C. M. Fraser.** 2001. Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. Science **293:**498–506.
37. **Titgemeyer, F., and W. Hillen.** 2002. Global control of sugar metabolism: a gram-positive solution. Antonie Leeuwenhoek **82:**59–71.
38. **Turinsky, A. J., F. J. Grundy, J. H. Kim, G. H. Chambliss, and T. M. Henkin.** 1998. Transcriptional activation of the *Bacillus subtilis ackA* gene requires sequences upstream of the promoter. J. Bacteriol. **180:**5961–5967.
39. **Warner, J. B., and J. S. Lolkema.** 2003. CcpA-dependent carbon catabolite repression in bacteria. Microbiol. Mol. Biol. Rev. **67:**475–490.
40. **Weickert, M. J., and S. Adhya.** 1992. A family of bacterial regulators homologous to Gal and Lac repressors. J. Biol. Chem. **267:**15869–15874.
41. **Weickert, M. J., and G. H. Chambliss.** 1990. Site-directed mutagenesis of a catabolite repression operator sequence in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **87:**6238–6242.
42. **Weiser, J. N., D. Bae, H. Epino, S. B. Gordon, M. Kapoor, L. A. Zenewicz, and M. Shchepetov.** 2001. Changes in availability of oxygen accentuate differences in capsular polysaccharide expression by phenotypic variants and clinical isolates of *Streptococcus pneumoniae*. Infect. Immun. **69:**5430–5439.
43. **West, A. H., and A. M. Stock.** 2001. Histidine kinases and response regulator proteins in two-component signaling systems. Trends Biochem. Sci. **26:**369–376.
44. **Xayarath, B., and J. Yother.** 2007. Mutations blocking side chain assembly, polymerization, or transport of a Wzy-dependent *Streptococcus pneumoniae* capsule are lethal in the absence of suppressor mutations and can affect polymer transfer to the cell wall. J. Bacteriol. **189:**3369–3381.
45. **Yother, J., G. L. Handsome, and D. E. Briles.** 1992. Truncated forms of PspA that are secreted from *Streptococcus pneumoniae* and their use in functional studies and cloning of the *pspA* gene. J. Bacteriol. **174:**610–618.
46. **Zahner, D., and R. Hakenbeck.** 2000. The *Streptococcus pneumoniae* beta-galactosidase is a surface protein. J. Bacteriol. **182:**5919–5921.
47. **Zeng, L., Y. Dong, and R. A. Burne.** 2006. Characterization of *cis*-acting sites controlling arginine deiminase gene expression in *Streptococcus gordonii*. J. Bacteriol. **188:**941–949.
48. **Zysk, G., R. J. Bongaerts, E. ten Thoren, G. Bethe, R. Hakenbeck, and H. P. Heinz.** 2000. Detection of 23 immunogenic pneumococcal proteins using convalescent-phase serum. Infect. Immun. **68:**3740–3743.