JOURNAL OF BACTERIOLOGY, Dec. 2005, p. 8340–8349 Vol. 187, No. 24
0021-9193/05/$08.00+0 doi:10.1128/JB.187.24.8340–8349.2005

Catabolite Control Protein A (CcpA) Contributes to Virulence and Regulation of Sugar Metabolism in *Streptococcus pneumoniae*

Ramkumar Iyer,[2] Nitin S. Baliga,[3] and Andrew Camilli[1,2]*

Howard Hughes Medical Institute[1] and Department of Molecular Biology & Microbiology, Tufts University, 136 Harrison Avenue, Boston, Massachusetts 02111,[2] and Institute for Systems Biology, 1441 N. 34th Street, Seattle, Washington 98103[3]

Received 8 July 2005/Accepted 2 October 2005

We characterized the role of catabolite control protein A (*ccpA*) in the physiology and virulence of *Streptococcus pneumoniae*. *S. pneumoniae* has a large percentage of its genome devoted to sugar uptake and metabolism, and therefore, regulation of these processes is likely to be crucial for fitness in the nasopharynx and may play a role during invasive disease. In many bacteria, carbon catabolite repression (CCR) is central to such regulation, influencing hierarchical sugar utilization and growth rates. CcpA is the major transcriptional regulator in CCR in several gram-positive bacteria. We show that CcpA functions in CCR of lactose-inducible β-galactosidase activity in *S. pneumoniae*. CCR of maltose-inducible α-glucosidase, raffinose-inducible α-galactosidase, and cellobiose-inducible β-glucosidase is unaffected in the *ccpA* strain, suggesting that other regulators, possibly redundant with CcpA, control these systems. The *ccpA* strain is severely attenuated for nasopharyngeal colonization and lung infection in the mouse, establishing its role in fitness on these mucosal surfaces. Comparison of the cell wall fraction of the *ccpA* and wild-type strains shows that CcpA regulates many proteins in this compartment that are involved in central and intermediary metabolism, a subset of which are required for survival and multiplication in vivo. Both in vitro and in vivo defects were complemented by providing *ccpA* in *trans*. Our results demonstrate that CcpA, though not a global regulator of CCR in *S. pneumoniae*, is required for colonization of the nasopharynx and survival and multiplication in the lung.

Carbon metabolism and its regulation are central to prokaryotic life. Sugars serve as the most facile source of carbon and energy, both of which are needed to replenish essential nucleotide cofactors and other metabolites in the cell. When faced with a wide variety of carbon and energy sources, a bacterium has to make metabolic decisions, opting for preferential use of one source over another in order to maintain optimal growth (70, 74). Simultaneous utilization of all available sugars would be metabolically inefficient and would lead to slower growth. The ability to utilize preferred sugars depends on a regulatory process called carbon catabolite repression (CCR) (69, 74, 79). CCR causes silencing of genes specific for the utilization of nonpreferred sugars until the cell has consumed the preferred sugar(s).

CCR has been studied in considerable detail in the model free-living, gram-positive bacterium *Bacillus subtilis* (71, 74, 79). The main global regulator of CCR in this organism is catabolite control protein A (CcpA) (10, 31). CcpA belongs to the LacI/GalR family of activator-repressor transcription factors and influences the expression of a wide range of catabolic operons in *B. subtilis* (4, 24, 25, 30, 33, 36, 67, 71, 78, 79). CcpA has also been identified to function in the regulation of catabolic operons and catabolite repression in many *Streptococcus* spp. (1, 17, 59, 77). CcpA has also shown to be required for biofilm formation in *Streptococcus mutans* (80). Candidate genes or operons that are subject to CcpA-dependent CCR are often identifiable by the presence of an operator sequence, called the catabolite-repressible element (*cre*), to which CcpA binds (1, 37, 48, 56).

The affinity of CcpA for *cre* sequences is enhanced by binding to another protein, the histidine phosphoprotein (HPr). HPr is an integral component of the phosphoenolpyruvate-dependent phosphotransferase system (PTS), where it normally functions in the transfer of high-energy phosphate from phosphoenolpyruvate to the enzyme II complex during sugar uptake (55, 57). The presence of a preferred sugar, such as glucose, in the medium activates phosphorylation of HPr on a conserved serine residue at position 46 by the Hpr kinase, which itself is activated by metabolites such as the high-energy glycolytic intermediate fructose-1,6-bisphosphate (8, 21, 54, 58, 73). CcpA interacts with the phosphoserine form of HPr, P∼Ser-HPr, to form a dimeric complex. This interaction increases the affinity of CcpA for the *cre*. Binding of this dimeric complex typically causes repression of promoters, facilitating CCR (1, 13, 14). CcpA residues involved in binding of P∼Ser-HPr (38) and those involved in binding of *cre* (37) have been characterized, and the crystal structure of the CcpA–P∼Ser-HPr complex has been recently solved (63).

CCR has also been shown to play a role in the regulation of virulence factors in some gram-positive pathogens (23, 46). The coordinate regulation of virulence genes with carbon utilization genes may be critical for fitness when pathogens compete with other microbes for niche colonization. CCR may be needed during infection of host compartments where multiple sugars are available to the pathogen. *Streptococcus pneumoniae* is, under normal conditions, a resident of the human nasopharynx. Mucosal immunity prevents extensive colonization and, together with serum antibodies, prevents invasive disease. However, under certain conditions that are not well under-

* Corresponding author. Mailing address: Department of Molecular Biology & Microbiology, Tufts University, 136 Harrison Avenue, Boston, MA 02111. Phone: (617) 636-2144. Fax: (617) 636-2175. E-mail: andrew.camilli@tufts.edu.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Source or reference
S. pneumoniae		
AC353	Spontaneous Sm^r derivative of TIGR4	27
RI1932	AC353 Δ*ccpA*::*cat*	This work
RI1933	RI1932 Δ*ccpA*::*cat* ΔSP0474-SP0478::*aad9-ccpA*	This work
E. coli		
AC578	DH5α λpir(pR412); Ap^r Sp^r	This work
AC1000	DH5α λpir(pAC1000); Ap^r Cm^r	This work
Plasmids		
pAC1000	*S. pneumoniae* suicide vector; Cm^r	28
pR412	Contains *magellan5* minitransposon; Ap^r Sp^r	45

stood, most commonly in the very young or the elderly, the bacteria spread to the lung and cause pneumonia with further potentially serious complications such as bacteremia and meningitis (27, 52). Middle ear infections by this bacterium are also fairly common and severe in young children (27, 52, 76).

S. pneumoniae has a large fraction of its transporters in the genome devoted to the uptake and metabolism of sugars, and these include classical PTS, ATP-binding cassette, and ion gradient-driven transporters (72). The ability of *S. pneumoniae* to metabolize a wide range of sugars may confer a fitness boost in certain host niches. For example, *S. pneumoniae* may be able to utilize a wide variety of host sugars from surface glycoproteins, thereby contributing to its effective growth and colonization of the nasopharynx, as well as during invasive disease (72). Indeed, CcpA from *S. pneumoniae* (also called RegM) shares ~54% identity with *B. subtilis* CcpA and has been recently shown to contribute to the virulence of the D39 serotype 2 strain of *S. pneumoniae* in a murine bacteremia model (22). However, the role of CcpA during colonization or infection at mucosal surfaces in the host is not known. Given these observations, it is clear that a thorough understanding of the pathogenesis of *S. pneumoniae* will require study of its sugar metabolism and basic physiology.

In this work, we characterized the role of CcpA in more detail by studying its physiological role in vitro, as well as in the virulence of a serotype 4 strain of *S. pneumoniae* in murine pneumonia and nasopharyngeal carriage models. In vitro, the *ccpA* strain showed increased total α-glucosidase activity, confirming an earlier report that CcpA acts as a repressor of the synthesis of this enzyme (22). CcpA is required for complete catabolite repression of the total cellular β-galactosidase. Deletion of *ccpA* severely compromised the virulence of *S. pneumoniae* in a pneumonia model and, in addition, severely compromised colonization of the nasopharynx. To begin to address the role of CcpA during colonization and infection, we compared the proteins that are differentially expressed on the surface of the cell in the wild-type and *ccpA* mutant strains. This analysis led to the identification of many surface-localized metabolic enzymes that were down-regulated in the *ccpA* strain; some of these enzymes have previously been shown to play a role in in vivo fitness and/or virulence. For two of these candidates (enolase and Hpr), loss of *ccpA* did not alter the synthesis of the respective mRNAs. This suggests a defect at the posttranscriptional level causing decreased translocation to the cell surface. This role for a carbon regulator in the control of genes associated with metabolism and in vivo fitness connects the physiological process of carbon and sugar metabolism with colonization and disease and unexpectedly reveals CcpA-dependent, surface-localized metabolic enzymes with a potential role in invasion and virulence in the lung.

MATERIALS AND METHODS

Bacterial strains, plasmids, and primers for DNA manipulations. Refer to Table 1 for a list of relevant strains and plasmids and to Table 2 for the primers used in this study.

Construction of the *ccpA* mutant. The *ccpA* mutant was constructed by replacing the entire 1,011-bp coding sequence with a chloramphenicol (CM) resistance cassette, *cat*. The *cat* cassette, which has its own promoter and conferred CM resistance on *Escherichia coli* and *S. pneumoniae*, was PCR amplified from pAC1000 by using primers catF1 and NcatR1. One-kilobase-pair DNA fragments containing the regions 5′ and 3′ of the *ccpA* gene were PCR amplified from AC353 genomic DNA by using two primer pairs, ccpupF-ccpupR and ccpdnF-ccpdnR. One of the primers in each of the pairs was designed to have a 20-bp overlap with the 5′ and 3′ sequences of the *cat* cassette. The *cat* cassette was fused to these two PCR products by splicing by overlap extension (SOE) (32). The final PCR product was introduced into *S. pneumoniae* TIGR4 by natural transformation as previously described (7). The double-recombination event was selected for by plating on medium containing 4 μg/ml CM and was confirmed by PCR and DNA sequencing.

Construction of the *ccpA* complementation strain. For complementation of the *ccpA* mutation, *ccpA*, along with its native ribosome-binding site, was amplified by PCR with primers Spccp1 and ccp2. Spccp1 contained a 36-bp sequence that overlaps the 3′ end of the *aad9* (spectinomycin [SP resistance) gene (29). *aad9* and its promoter were amplified by PCR with primers spcF1 and spcR1 from plasmid pR412 (45). The product of the Spccp1-and-ccp2 PCR was joined to *aad9* by using SOE to generate the *aad9-ccpA* cassette. This PCR construct was flanked by 800-bp DNA identical to the regions 5′ and 3′ of the *lacE* PTS operon (SP0474 to SP0478) to target the cassette to this region of the chromosome. The *lacE* 5′ region, LacE(up), was amplified from AC353 genomic DNA by using the primer pair lacEF1-lacER1. In a separate PCR, the *aad9-ccpA* cassette was amplified by using primers lacESp and ccp2, creating a fragment that has a sequence overlap with the 3′ end of the lacEF1-lacER1 product. Both of these two fragments were fused by using SOE to create LacE(up)-*aad9-ccpA*. In a similar manner, the *lacE* downstream region, LacE(down), was PCR amplified by using the primer pair CplacE-lacER2, in which the former has a sequence overlap with the 3′ end of *ccpA*. This piece was fused to the LacE(up)-*aad9-ccpA* cassette by using SOE to create the final complementation cassette LacE(up)-*aad9-ccpA*-LacE(down). The complementation construct completely replaced the coding region of the *lacE* operon and expressed *aad9* and *ccpA* constitutively from the *aad9* promoter. The *ccpA* mutant was transformed with the complementation cassette, and the double-recombination event was selected for by plating on medium containing 100 μg/ml SP and was confirmed by PCR and DNA sequencing. We have previously determined that a deletion-insertion in this locus affected neither in vitro growth nor virulence (data not shown).

TABLE 2. Sequences of primers used in this study

Primer	Contig no.; coordinates or source	Sequence[a] (5′ to 3′)
ccpupF	AE007488; 11641–11660	GCTGCCAAGTATGTCACCAA
ccpupR	AE007489; 358–339	GAAGAAGGTTTTTATATTACAGCTCCAAAAAAATCAGGGAATCGAGA
ccpdnF	AE007489; 1370–1390	CATCAAGCTTATCGATACCGTCCTTTTCCTGTCCTTTCTAT
ccpdnR	AE007489; 2390–2368	AAAGTATTAGTCGCAGAAGATCA
ccpF	AE007489; 487–504	GAGGTTGGGCAATCGTTG
ccpR	AE007489; 1069–1049	AATAGCGATGAAGATAACGAG
ccpF0	AE007488; 11593–11610	AGGGCTGCTGACAAAGGA
ccpR0	AE007489; 2430–2411	AGTGCAGGTTCGACTACTTT
catF1	pAC1000	CGGTATCGATAAGCTTGATG
NcatR1	pAC1000	TGGAGCTGTAATATAAAAACCTTC
lacEF1	AE007358; 10181–10200	TTTAGAGGCTCCTATTTTTT
lacER1	AE007359; 96–114	CACCGGAACTCCTTTTTTT
lacER2	AE007359; 7860–7841	CCTATCTGGTCAGTATCGGA
lacESp	AE007359; 114–96	AAAAAAAGGAGTTCCGGTGCCCAGATCTACCGCTCTAGAACTAGTGGATCCC
SpcR1	pR412	CCCAGATCTCAATTTTTTTATAATTTTTTTAATCTG
SpcF1	pR412	CCCAGATCTACCGCTCTAGAACTAGTGGATCCC
Ccp1	AE007489; 1390–1370	ATAGAAAGGACAGGAAAAGGA
Ccp2	AE007489; 339–358	TCTCGATTCCCTGATTTTTT
Spccp1	AE007489; 1390–1370	CAGATTAAAAAAATTATAAAAAAATTGAGATCTGGGATAGAAAGGACAGGAAAAGGA
CplacE	AE007489; 358–339	AAAAAATCAGGGAATCGAGAGCTGTGTAGTAAGTTTTTCCA
NlacEF	AE007358; 10261–10283	GGGTATTGTGTGGATTAAAAAGG
NlacER	AE007359; 7736–7713	ACTGGTTTCTACAGGCTTGATTAG
76rpa	AE007418; 8260–8277	TAATACGACTCACTATAGGGAGATCACCACACATACCAGCC
Sal76	AE007418; 8051–8071	AGCGTCGACTCAATCAAAATTAACGTATTCTT
28rpa	AE007414; 1192–1173	TAATACGACTCACTATAGGGAGAGCCATTTCGATAGCTTCAA
Sal128	AE007414; 1417–1396	AGCGTCGACTTATTTTTTAAGGTTGTAGAAT
1414F	AE007438; 6294–6276	ATGTCTAAAACAGTAGTAC
1414R	AE007438; 6118–6140	TAATACGACTCACTATAGGGAGTTAGAATTTTTTACGTTTACGAG

[a] Underlined sequences are complementary to the *S. pneumoniae* genomic DNA. Coordinates are provided in the middle column.

Bacterial growth and media. *S. pneumoniae* was routinely grown in Todd-Hewitt broth (THB) or on blood agar plates containing 100 μg/ml streptomycin (SM) at 37°C in a 5% CO_2 atmosphere. For broth cultures, 1.5 μl/ml Oxyrase (Oxyrase, Inc.) was added in addition and cultures were typically grown to a final optical density at 600 nm (OD_{600}) of 0.5 to 0.6. For the sugar hydrolysis assays, *S. pneumoniae* was grown in a semidefined minimal medium (SDMM) without BSA (39) containing 100 μg/ml SM for the wild type, 100 μg/ml SM and 4 μg/ml CM for the *ccpA* mutant, and 100 μg/ml SM and 100 μg/ml SP for the Δ*ccpA*/*ccpA*$^+$ complemented strain. Fresh catalase (3 U/ml; Sigma-Aldrich) was added to the SDMM before inoculation with *S. pneumoniae* in order to facilitate optimal growth. Wild-type or *ccpA* mutant cells were grown in SDMM containing the appropriate sugar(s) as the primary carbon source for growth curve and generation time determinations. Bacteria were grown in SDMM in the presence of an inducing sugar (lactose, maltose, cellobiose, or raffinose) either alone or in combination with a repressing sugar (glucose or sucrose) to create catabolite-repressing conditions. The concentration of sugar was 10 mM in all cases. There was negligible growth in SDMM in the absence of added sugar.

Animal infections. All animal infections were done with 8- to 10-week-old female Swiss-Webster mice (Taconic Laboratories). Mice provided with continuous food and water were housed in accordance with guidelines provided by the Tufts University Department of Laboratory Animal Medicine. Bacteria were recovered from plates of tryptic soy agar (TSA) with 5% sheep's blood, subcultured in prewarmed THB containing Oxyrase, and grown at 37°C for 3 to 4 h to an OD_{600} of 0.4. One milliliter of cultured cells was centrifuged at 10,000 rpm for 2 min in a Microfuge, and the cells were resuspended in 500 μl of prewarmed THB. For competition assays, mutant and wild-type bacteria were mixed 1:1 and inoculated intranasally at the following doses: 10^7 CFU in a 40-μl volume for lung infections and 10^5 CFU in a 10-μl volume for nasopharyngeal colonization. Prior to inoculation, mice were anesthetized by isoflurane inhalation. Similar volumes and concentrations of bacteria were used for single-strain infections. Immediately after inoculation of mice, 10^6 CFU of the input mixture was also used to inoculate 10 ml of prewarmed THB containing 100 μg/ml SM and 1.5 μl/ml Oxyrase. Growth of this in vitro competition culture was carried out at 37°C in 5% CO_2 for 5 h, after which the cells were diluted and plated on blood agar plates supplemented with 100 μg/ml SM. Lung infection was carried out for 42 h and nasopharyngeal colonization for 7 days, after which the mice were sacrificed. Lungs were removed and placed in 3 ml of THB containing 20% glycerol and mechanically homogenized. The homogenates were appropriately diluted and spread on blood agar plates supplemented with 100 μg/ml SM. Bacteria were recovered from the mouse nasopharynx by allowing 1 ml of phosphate-buffered saline to flow into the trachea and out through the nasal passages with collection of the flowthrough on Parafilm. Appropriate dilutions of the flowthrough were plated on blood agar supplemented with 100 μg/ml SM. For competition assays, the ratio of the *ccpA* mutant or the *ccpA*-complemented strain to the wild type in the output from each mouse was determined by replica plating on blood agar plates supplemented with 100 μg/ml SM and 4 μg/ml CM or 100 μg/ml SM and 100 μg/ml SP, respectively.

Colony size measurements. Individual colony sizes of wild-type and *ccpA* cells were magnified with the 10× Plan-NEOFLUAR lens on an Axioplan 2 microscope (Zeiss) and an Orca cooled, charge-coupled device camera (Hamamatsu Photonics). Images were recorded by Openlab 3.1.7 software (Improvision).

RNase protection assays (RPAs). Total RNA was isolated from 20 ml of *S. pneumoniae* wild-type and *ccpA* mutant cells grown in THB supplemented with streptomycin (100 μg/ml) and Oxyrase (1.5 μl/ml). Cells were pelleted at 4,000 rpm for 10 min. RNA was isolated from the cell pellet with a QIAGEN RNeasy kit in accordance with the recommendations of the manufacturer (QIAGEN). Template DNA for the generation of riboprobes was PCR amplified with primer sets 76rpa-Sal76, 28rpa-Sal128, and 1414F-1414R. One of the primers in each pair had the T7 promoter sequence (5′-TAATACGACTCACTATAG GGAG-3′) so that the resultant PCR piece could be directly used as a template for the generation of riboprobes with a MaxiScript III in vitro transcription kit (Ambion). Synthesized probes were purified on a 4% denaturing polyacrylamide gel containing 8 M urea. RPAs were carried out as described by the manufacturer with an RPAIII kit (Ambion). The protected fragments were visualized by exposing each gel to a phosphorimaging screen (Kodak) and analyzed with a Storm 860 scanner and IQMac V1.2 imaging software. The relative amount of each protected fragment in each assay was normalized to the amount of SP1414 (*rpsU*) protected RNA in each lane.

Enzyme assays. (i) α-Glucosidase. α-Glucosidase activity was assayed as previously described (9). Enzyme activity was monitored by following the increase in absorbance at 420 nm due to enzymatic release of *p*-nitrophenol from *p*-nitrophenyl-α-D-glucoside. The reaction mixture contained 0.2 ml of *p*-nitrophenyl-

glucoside (2 mg/ml) in 250 mM potassium phosphate buffer (pH 7.0). To this, 0.8 ml of 50 mM potassium phosphate buffer (pH 7.0) was added to bring the final volume to 1 ml. One milliliter of growing bacteria at an OD_{600} of 0.4 was pelleted in a Microfuge at 10,000 rpm for 1 min and resuspended in 100 μl of 50 mM potassium phosphate buffer (pH 7.0). One microliter of a 10% Triton X-100 stock was added to 100 μl of mid-log-phase cells of *S. pneumoniae*, and the cells were incubated at 37°C for 10 min to lyse the cells. The reaction was initiated by addition of 50 μl of cell extract. Enzyme activity was expressed as nanomoles of *p*-nitrophenol released per unit of time per unit of volume of cell extract.

(ii) β-Glucosidase. β-Glucosidase activity was assayed as previously described (49). Fifty-microliter aliquots of mid-log-phase *S. pneumoniae* cells were washed and resuspended in 50 μl of 50 mM Tris-HCl (pH 7.4). Five microliters of 1 M $MgCl_2$ and 50 μl of 100 mM *p*-nitrophenyl-β-D-glucopyranoside were added to 0.895 ml of 50 mM Tris-HCl (pH 7.4) to get a final volume of 0.95 ml. The reaction was then initiated by addition of 50 μl of the cell suspension. Enzymatic release of *p*-nitrophenol was monitored at 405 nm, and activity was expressed as nanomoles of *p*-nitrophenol released per unit of time per unit of volume of cell suspension.

(iii) α-Galactosidase. α-Galactosidase activity was assayed as previously described (60). One milliliter of 1 M $MgCl_2$, 3.1 μl of 1.43 M β-mercaptoethanol, and 90 μl of a 1 mg/ml solution of *p*-nitrophenyl-α-D-galactopyranoside were added to 0.896 ml of 100 mM sodium phosphate (pH 7.5). Ten microliters of Triton X-100 cell extract of *S. pneumoniae* was added to the reaction mixture. Enzymatic release of *p*-nitrophenol was monitored at 405 nm, and activity was expressed as nanomoles of *p*-nitrophenol released per unit of time per unit of volume of cell extract.

(iv) β-Galactosidase. β-Galactosidase activity was assayed as previously described (47). One microliter of 1 M $MgCl_2$ and 3.5 μl of 14.3 M β-mercaptoethanol were added to 0.945 ml of 100 mM sodium phosphate buffer (pH 7.5) containing 0.8 mg/ml *o*-nitrophenyl-β-D-galactopyranoside. Fifty microliters of a Triton X-100 cell extract of *S. pneumoniae* was added to the reaction mixture. The enzymatic release of *o*-nitrophenol was monitored at 420 nm, and activity was expressed as nanomoles of *o*-nitrophenol released per unit of time per unit of volume of cell extract.

Cell wall fractionation and two-dimensional (2-D) gels. Cell wall fraction was prepared by overnight digestion with mutanolysin enzyme as previously described (5). The protein concentration in the sample was estimated with the bicinchoninic acid protein assay kit (Pierce) before loading of isoelectric focusing strips. One hundred micrograms of total protein was resuspended in 180 μl of buffer containing pH 4 to 7 range ampholytes from Invitrogen and loaded directly onto the focusing strips. 2-D gels were run with the Zoom IPG runner system and subsequently 4 to 12% Bis-Tris Zoom polyacrylamide gels to separate proteins in the second dimension. Proteins in the gel were stained with Coomassie brilliant blue.

Protein identification. Protein spots were excised from Coomassie-stained gels and dehydrated in 100% acetonitrile for 10 min. The acetonitrile was removed, and the tube with the gel piece was vacuum centrifuged until dry. The gel was rehydrated at 4°C for 45 min in 5 μl of buffer containing trypsin (12 ng/μl of buffer) and 50 mM NH_4HCO_3. The buffer was replaced with 10 μl of 20 mM NH_4HCO_3, and the digestion was carried out for 18 h at 37°C. Peptides were then extracted from the gel by three exchanges of 50 μl of 5% formic acid in 50% acetonitrile (20 min of incubation each time) at room temperature. The supernatants were pooled and vacuum centrifuged to a dry pellet. The proteins were resuspended in 100 μl of 0.4% acetic solution prior to mass spectrometer analysis.

Tandem mass spectrometry. In brief, tryptic peptides from in-gel-digested spots were analyzed by μLC-ESI-MS/MS with an LCQ-DECA mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with a C_{18} trap and an analytical nano-LC column-emitter. To prevent carryover between runs, a fresh trap and a fresh column-emitter were used for every run. Mass spectra were searched against both of the published *S. pneumoniae* TIGR4 and R6 genomes to match fragment ion peaks to theoretical peaks. Probability of peptide identification was determined with PeptideProphet, and assignment of peptides to proteins was assessed by ProteinProphet (38).

RESULTS

Growth characteristics of the *ccpA* strain. We generated a *ccpA* deletion-insertion mutation (Δ*ccpA*::*cat*) in the encapsulated serotype 4 *S. pneumoniae* TIGR4 strain. The resultant *ccpA* strain produced smaller colonies on blood agar plates than did the parent strain. The average colony size of the wild type (0.58 ± 0.03 mm) was ~22% larger than that of the *ccpA* mutant (0.46 ± 0.05 mm). This is similar to the observation made for a *ccpA* strain in the D39 (serotype 2) *S. pneumoniae* background (22). In that report, the small-colony phenotype was attributed to the lowered transcription from the capsule locus, causing closer packing of bacterial cells within colonies. To test if a similar phenomenon was occurring in the TIGR4 *ccpA* strain, the Quellung reaction was used to qualitatively measure capsule expression. There was no detectable difference in the Quellung reaction between *ccpA* and wild-type cells (data not shown).

TABLE 3. Generation times of wild type and *ccpA* mutant in SDMM with different sugars

Sugar (class)[a]	Mean generation time (min) ± SD	
	Wild type (n = 3)	*ccpA* (n = 3)
Glucose (monosaccharide)	46 ± 4	53 ± 8
Sucrose (disaccharide)	40 ± 3	47 ± 9
Lactose (disaccharide)	61 ± 2	62 ± 5
Raffinose (trisaccharide)	59 ± 8	76 ± 11[b]
N-Acetylglucosamine (amino sugar)	54 ± 1	53 ± 4
Mannitol (sugar alcohol)	104 ± 17[c]	103 ± 11[c]

[a] Each sugar was used at 10 mM.
[b] Significant at $P < 0.05$.
[c] Average of two independent determinations.

An alternative explanation for the small-colony phenotype is a reduced growth rate. It is known that loss of *ccpA* in *B. subtilis* causes a general growth defect that arises from amino acid auxotrophy, specifically, Glu, Met, and the branched-chain amino acids Ile, Leu, and Val (41). We therefore tested for growth defects of the *S. pneumoniae ccpA* strain in SDMM with a variety of sugars as the sole carbon source. However, there was no statistically significant difference in generation time on most of the carbon sources tested, except raffinose (Table 3). The *ccpA* strain was mildly outcompeted (twofold) in in vitro competitions with wild-type cells in rich broth (~10 generations of growth), suggesting a mild decrease in fitness in complex media. Consistent with this, when grown separately, the generation time of the *ccpA* strain (55 ± 0.7 min) was increased in rich broth compared to that of the wild type (47 ± 2 min). Finally, we measured the numbers of CFU per colony of the wild-type and *ccpA* strains. A single *ccpA* colony had approximately 30% fewer CFU compared to the wild type ($4.8 \times 10^5 \pm 0.7 \times 10^5$ and $7.0 \times 10^5 \pm 0.6 \times 10^5$ for *ccpA* and the wild type, respectively). This is consistent with the mild competitive defect observed in complex broth that we propose accounts for the small-colony phenotype.

CcpA in sugar metabolism. *S. pneumoniae* can grow on many sugars, including maltose, cellobiose, raffinose, and lactose, when provided as the sole carbon source (Table 3; data not shown). The presence of these sugars induces the enzymes α-glucosidase (9, 26, 62), β-glucosidase (34, 43, 49), α-galactosidase (2, 3, 60), and β-galactosidase (15, 66), respectively. While these activities were chosen as representative candidates to investigate the role of *ccpA* in CCR of sugar metabolism, we do not know the identities of the genes that are specifically

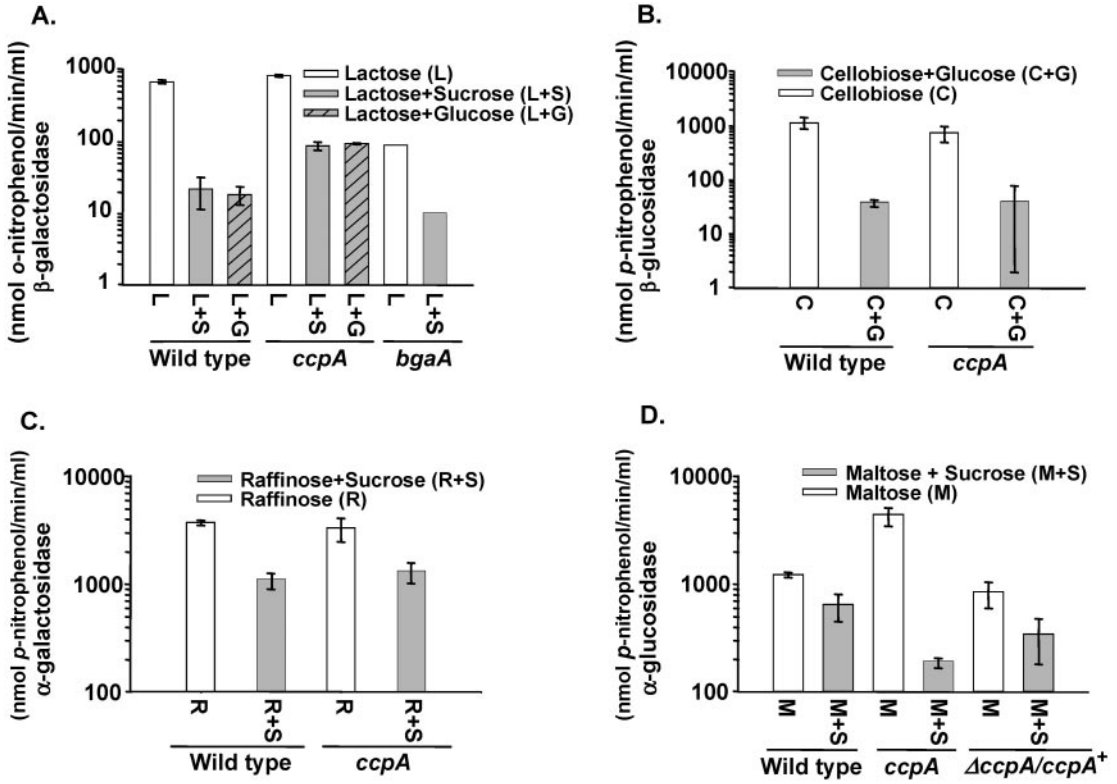

FIG. 1. CCR of sugar-degrading enzymes in *S. pneumoniae*. (A) β-Galactosidase activity in wild-type and *ccpA* and *bgaA* mutant cells in the presence of the inducer lactose (L) alone compared to activity in the presence of the repressing sugar glucose (L+G) or sucrose (L+S). (B) β-Glucosidase activity in wild-type and *ccpA* mutant cells in the presence of the inducer cellobiose (C) compared to activity in the presence of the repressing sugar glucose (C+G). (C) α-Galactosidase activity in wild-type and *ccpA* mutant cells in the presence of the inducer raffinose (R) alone compared to activity in the presence of the repressing sugar sucrose (R+S). (D) α-Glucosidase activity in wild-type and *ccpA* mutant cells in the presence of the inducer maltose (M) alone compared to activity in the presence of the repressing sugar sucrose (M+S).

responsible for these enzymes, with the exception of *bgaA*, which encodes the cell wall-localized β-galactosidase. The wild-type and *ccpA* strains were grown in the presence of the inducing sugar either alone or in combination with a repressing sugar, glucose or sucrose, and CCR was measured. The presence of either glucose or sucrose in the growth medium repressed lactose-inducible β-galactosidase in wild-type cells 30-fold (Fig. 1A, compare bar 1 with bars 2 and 3) and was taken as an indication of CCR. CCR was relieved about threefold in the *ccpA* strain (Fig. 1A, bars 5 and 6), indicating that CcpA mediates part of the CCR of β-galactosidase activity in the presence of glucose or sucrose.

In contrast, loss of *ccpA* in D39 led to an increase in lactose-induced β-galactosidase in the absence of a repressing sugar (22). This implies inherent differences in CcpA-mediated regulation of β-galactoside metabolism between serotype 2 and 4 strains. A major portion (~85%) of the total cellular β-galactosidase activity measured in TIGR4 above arises from the *bgaA* gene (82) (Fig. 1A, compare bars 1 and 7). Deleting *bgaA* allowed us to monitor the remaining β-galactosidase activity. The BgaA-independent β-galactosidase activity is about sevenfold lower than that of the wild type (Fig. 1A, compare bars 1 and 7). It is repressed about 10-fold in the presence of the repressing sugar (Fig. 1A, compare bars 7 and 8), which is ~3-fold less than the repression seen in the wild type.

In contrast to β-galactosidase activity, β-glucosidase and α-galactosidase activities were still subject to CCR in the absence of CcpA (Fig. 1B and C). This suggests that another regulator(s), which may or may not be redundant with CcpA, mediates CCR of these genes. The possibility that CcpA could still regulate the expression of these genes in the absence of the CcpA-independent mechanism cannot be excluded. Indeed, redundancy in CCR and regulation of carbon metabolism is not unprecedented since this has been observed in the regulation of the *B. subtilis* glycerol operon (12, 14).

Loss of CcpA leads to significantly increased CCR of α-glucosidase activity in the presence of the repressing sugar compared to the wild type (Fig. 1D, bars 2 and 4). This indicates the existence of an alternative regulator that mediates CCR of α-glucosidase in TIGR4 in the absence of CcpA. We also observed that loss of *ccpA* produced a significant increase in total cellular α-glucosidase activity under inducing conditions (Fig. 1D, compare bars 1 and 3). This indicates that CcpA represses this system in the wild type when an inducer is present as the sole carbon source. This defect was restored to the wild type by complementation of *ccpA* in *trans* (Fig. 1D,

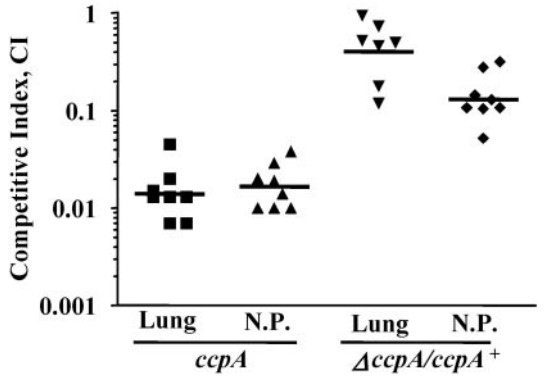

FIG. 2. Role of *ccpA* in pneumonia and nasopharyngeal colonization in mice. Loss of *ccpA* causes attenuation of the *ccpA* mutant (*ccpA*) in an in vivo competition with the wild type in the pneumonia (Lung) and nasopharyngeal carriage (N.P.) models of infection. Provision of a single copy of *ccpA* complements both defects (Δ*ccpA*/*ccpA*$^+$). Horizontal bars represent geometric means.

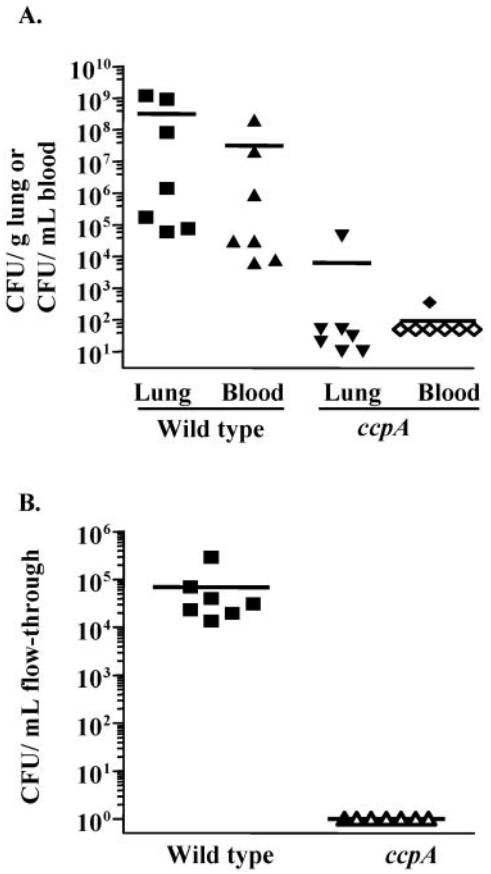

FIG. 3. Single-strain pneumonia and nasopharyngeal colonization of mice with the wild type and the *ccpA* mutant. The *ccpA* strain (*ccpA*) is attenuated in both pneumonia (A) and nasopharyngeal colonization (B) compared to the wild type. Open symbols represent data at or below the limit of detection (~10^0 per mouse). Horizontal bars represent arithmetic means.

bars 5 and 6). Thus, CcpA can also function as a negative regulator in *S. pneumoniae*.

Role of CcpA in virulence. CcpA has been shown to contribute to the growth of strain D39 in a murine model of bacteremia (22). The D39 *ccpA* strain also showed a significant decrease in transcription of the capsular locus, and the authors attributed some of the attenuation of virulence to this defect. In order to investigate the role of CcpA in the colonization and infection of mucosal surfaces, we tested the role of *ccpA* in the TIGR4 strain in murine models of pneumonia and nasopharyngeal colonization. The *ccpA* strain was outcompeted by the wild type in both the pneumonia (competitive index [CI] of 0.015) and nasopharyngeal carriage (CI of 0.02) models (Fig. 2). Competition assays in vitro in THB revealed a mild growth defect in the *ccpA* strain (CI = 0.5). Attenuation of virulence in the lung was almost completely complemented by constitutive expression of *ccpA* in *trans*; however, the defect in the nasopharynx was only partially complemented (Fig. 2). It is possible that constitutive expression of *ccpA* in *trans* is not able to fully complement due to slight over- or underexpression or, alternatively, due to incorrect temporal expression. The sum of these results demonstrates that significant attenuation of virulence and colonization results from the loss of CcpA.

To test the possibility that the attenuation seen in the *ccpA* strain was a result of competition by the wild type for limited resources, we also performed single-strain infections. Forty-two hours after inoculation of the lungs, the wild type was recovered in large numbers from the lung (3×10^8 CFU/g) and the bloodstream (3×10^7 CFU/ml), whereas the *ccpA* mutant was recovered at levels approximately 5 orders of magnitude lower from either site (Fig. 3A). Similar results were obtained from experiments to test colonization of the nasopharynx: The wild type was recovered in large numbers (7×10^4 CFU/ml), whereas the *ccpA* mutant was recovered at or below the limit of detection (Fig. 3B). It is formally possible that the wild-type cells might be able to complement some of the *ccpA* deficits in vivo, and this could explain the more severe attenuation in the single-strain infections compared to the competition experiments. These results show that CcpA plays an important role in lung infection and colonization of the mouse nasopharynx.

Identification of CcpA-regulated surface proteins. CcpA is a global regulator of several catabolic operons in *B. subtilis*. However, the nature of the genes regulated by CcpA in serotype 4 *S. pneumoniae* is unclear. Given the minimal growth defects of the *ccpA* strain observed in vitro but the substantial defects exhibited in the mouse lung and nasopharynx, we wanted to identify *ccpA*-regulated factors that could underlie the observed lack of fitness in vivo. We looked for the subset of *ccpA*-dependent proteins that are cell wall associated, since such factors may mediate important interactions with the host to facilitate colonization and multiplication. Figure 4 shows representative 2-D gel profiles of the cell wall fractions of the wild-type and *ccpA* strains. Some prominent differences in protein spots between the wild-type and *ccpA* strains are highlighted with arrows. While some spots were up-regulated in the *ccpA* strain (arrows in panel B), others were absent or reduced in the *ccpA* strain (open arrows in panel A). Provision of a wild-type copy of *ccpA* in *trans* restored the cell wall protein profile to that of the wild type (Fig. 4C), showing that the

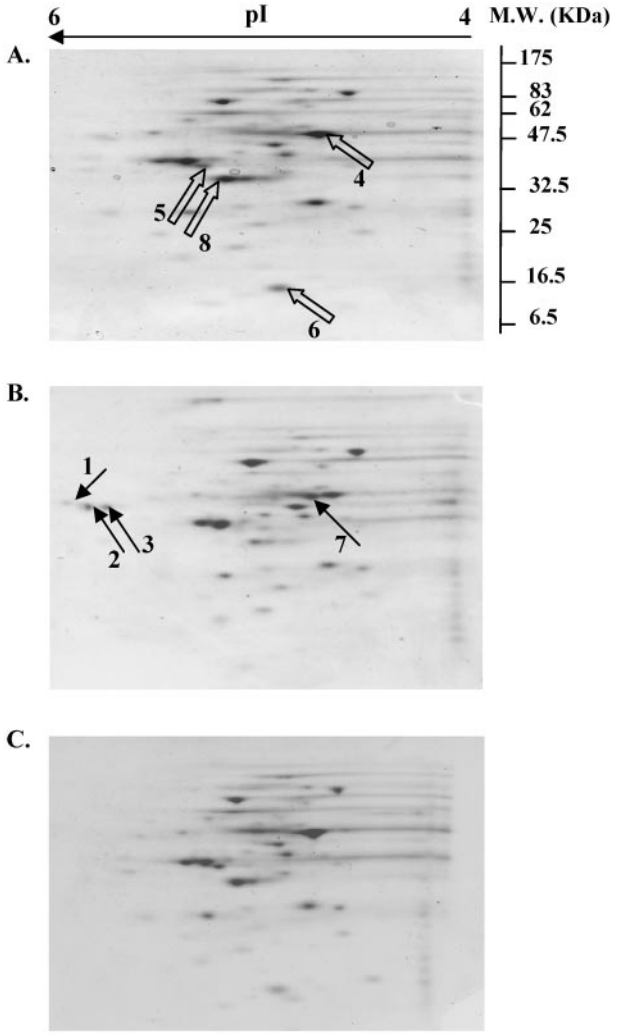

FIG. 4. 2-D gels of cell wall fraction of *S. pneumoniae*. Panels: A, wild type; B, *ccpA* mutant (*ccpA*)$^-$; C, *ccpA*-complemented strain (Δ*ccpA*/*ccpA*$^+$). Arrows indicate differences in the protein profiles. M.W., molecular mass.

altered expression of proteins in the cell wall arose from the lack of CcpA. These results are consistent with a dual function of CcpA as both an activator and a repressor. Table 4 lists a subset of proteins identified in this analysis by peptide fingerprinting by mass spectrometry.

Among the proteins that were down-regulated in the *ccpA* strain are ones associated with PTSs (SP1177, HPr) (57), glycolysis (SP0605, aldolase; SP1128, enolase), pyrimidine nucleotide biosynthesis (SP0764) (61), and lactate-to-pyruvate interconversion (SP1220) (1). SP1128 has also been shown to bind plasminogen, thereby facilitating host invasion, and contributes to virulence potential (6, 20). Some of the proteins that are derepressed in the *ccpA* strain are associated with sugar uptake (SP1580, SP2108) (50, 60) and organic acid metabolism (SP0715, SP1588) (64, 68).

To see if the down-regulation of proteins in the cell wall in the *ccpA* strain was due to a general decreased transcription of these genes versus defects in translocation of the proteins, we carried out RPAs. We used [^{32}P]UTP-labeled riboprobes to quantify the amounts of the SP1128 (enolase) and SP1176 (*ptsI*) transcripts. SP1176 is located immediately downstream of SP1177 (*ptsH*/*hpr*) and is predicted to be in an operon with this gene. Both SP1176 (*ptsI*) (Fig. 5A) and SP1128 (enolase) (Fig. 5B) were transcribed to the same extent in the wild-type and *ccpA* strains. The absence of a difference at the level of transcription suggests that the underlying cause for the decreased levels of HPr and the enolase in the cell wall is post-transcriptional in nature.

DISCUSSION

Given its lack of a functional electron transport chain and tricarboxylic acid cycle, *S. pneumoniae* is predicted to be highly dependent on external sugars for its energy requirements (72). In this study, we tested the role of CcpA as a central player in mediating the CCR and virulence of this bacterium.

Loss of CcpA affects the catabolite repression of only one of the four sugar-metabolizing enzymes tested. Even this is only a partial effect. The result suggests the presence of alternative regulatory systems that are operative independently of *ccpA*, implying that CcpA may not be a universal regulator of CCR in *S. pneumoniae*. Unexpectedly, loss of CcpA led to greatly increased catabolite repression of α-glucosidase activity in the presence of sucrose, pointing to the existence of perhaps another transcription factor that is capable of relaying carbon catabolite regulatory signals.

B. subtilis has been shown to possess, in addition to CcpA, two other functionally related regulators, CcpB (11), which is involved in catabolite repression of the gluconate and xylose operons, and CcpC (35), which regulates catabolite repression of the aconitase and citrate synthase genes. Although searching the *S. pneumoniae* genome for CcpA paralogs and for orthologs of *B. subtilis* CcpB and CcpC identified potential relatives, their role in CCR, if any, remains untested. Deleting these genes individually or in combination with *ccpA* may reveal parallel CcpA-like catabolite repression pathways in *S. pneumoniae*. Searching the TIGR4 genome for orthologs of the *B. subtilis* glycolytic regulator CggR (16) and the gluconeo-

TABLE 4. CcpA-dependent cell wall-associated proteins

Protein class and name	Function	Spot(s)[a]
Up-regulated in *ccpA* strain		
SP1580	MsmK ATP-binding protein	1
SP0715	Lactate oxidase	2, 3
SP1588	Pyruvate–2-oxoglatarate and dihydrolipoamide dehydrogenase (E3) complex	7
SP2108	Maltose-binding protein	7
Down-regulated in *ccpA* strain		
SP1128	Enolase	4
SP1220	Malate/lactate dehydrogenase	5
SP1177	HPr component of PTS	6
SP0605	Fructose-1,6-bisphosphate aldolase	8
SP0764	Dihydroorotate dehydrogenase	8

[a] Spots in Fig. 4.

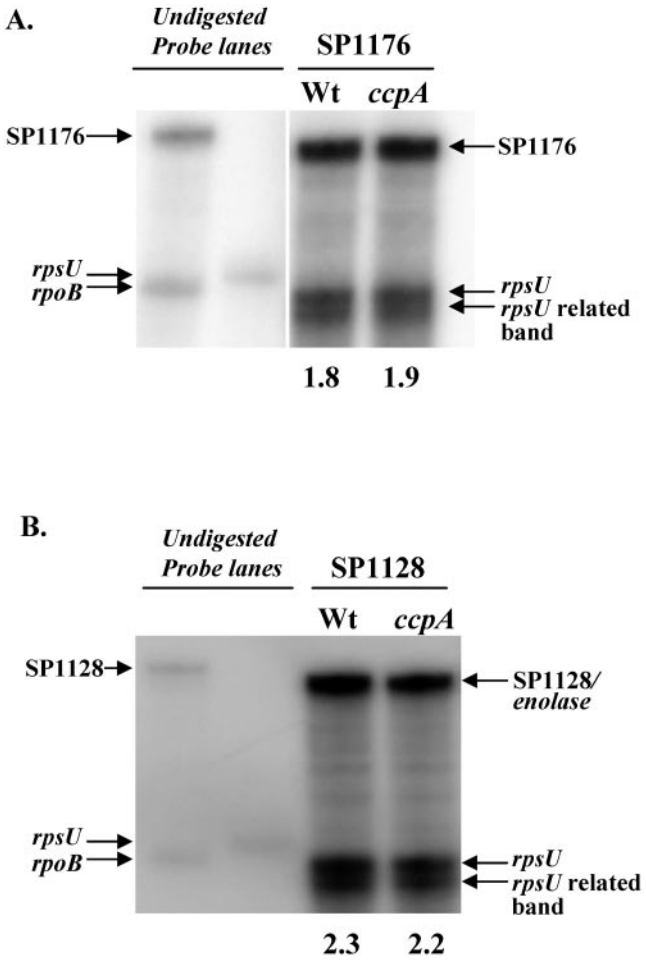

FIG. 5. RPA to analyze mRNA levels of SP1176 (*ptsI*) (A) and SP1128 (enolase) (B) in wild-type (Wt) strain AC353 and *ccpA* strain RI1932. Riboprobes for SP1176 (*ptsI*), SP1128 (enolase), and SP1414 (*rpsU* loading control) were generated and hybridized to 3 μg of RNA from the three *S. pneumoniae* strains. RNA was harvested from cells grown in THB to an OD_{600} of 0.3. Undigested riboprobes of SP1176, SP1414, and SP1128 in the absence of *S. pneumoniae* RNA are indicated. An *rpoB* riboprobe is also indicated but was not used in the experiments. The value below a lane is the relative abundance of the SP1176 or SP1128 transcript in the wild-type or *ccpA* strain normalized to SP1414 (*rpsU*), as calculated by densitometry with ImageQuant TL software (Amersham Biosciences).

genic gene CCR regulator CcpN (65) revealed a putative ortholog for CggR (SP0247), while CcpN had no matches. While the existence of such parallel pathways remains a viable explanation, inducer exclusion (74) via the phospho-Ser HPr protein could also account for the CcpA-independent CCR observed for the β-glucosidase and α-galactosidase activities.

Because *S. pneumoniae* is an obligate commensal and clinically important pathogen of humans, we also wanted to examine if CcpA influences the colonization and virulence characteristics of this organism in a murine model. In contrast with the dispensability of CcpA for growth in vitro, the *ccpA* strain was drastically attenuated for infection of the lung and colonization of the nasopharynx. While providing *ccpA* in *trans* to the mutant restores virulence in the lung, the rescue of nasopharyngeal colonization is only partial, albeit significant. This could be due to aberrant expression of *ccpA* from the complementation construct. CcpA naturally autoregulates its own expression in some bacteria due to the presence of a *cre* sequence upstream of the gene (19, 42). Inspection of the region upstream of *ccpA* in *S. pneumoniae* reveals the presence of a candidate *cre* about 140 bp upstream of the start codon. CcpA autoregulation might be required for the optimal regulation of target genes in vivo.

The large attenuation of colonization and virulence of the *ccpA* strain suggested that CcpA is directly or indirectly regulating genes that are required for in vivo fitness. Our characterization of the cell wall proteome revealed that CcpA serves to activate the expression of some proteins while negatively regulating others. Since CcpA has been shown to be important in central metabolism in other bacteria, the presence of metabolic enzymes in the list of up- and down-regulated cell wall proteins is not entirely surprising. Among the former class is a metabolic protein, enolase, which is known to be required for survival and multiplication of *S. pneumoniae* in vivo (6). Enolase (SP1128) is a glycolytic enzyme that normally converts 2-phosphoglycerate to generate phosphoenolpyruvate. However, some enolase has been shown to be cell wall associated and to bind host plasminogen, which, when converted to plasmin, facilitates invasion of host tissues by degrading the extracellular matrix (6, 20, 81). Another metabolic enzyme, dihydroorotate dehydrogenase (SP0764), is down-regulated in the *ccpA* strain. Dihydroorotate dehydrogenase is normally involved in pyrimidine biosynthesis (61) and is induced in vivo (44). In addition, microarray analysis revealed that a dihydroorotate dehydrogenase gene is up-regulated in bacteria attached to a pharyngeal epithelial cell line in vitro (51). Fructose-1,6-bisphosphate aldolase (SP0605), a cytoplasmic glycolytic enzyme, splits fructose-1,6-bisphosphate into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. This enzyme has also been shown to be cell wall associated and antigenic in mice (40), although a possible role in infection has not been investigated. CcpA has been shown to positively regulate glycolysis in *B. subtilis* (75). The involvement of CcpA as a positive regulator of these potentially dual-role metabolic enzymes in *S. pneumoniae* is a novel observation that warrants further investigation.

HPr, which is one of the chief players in PTS-mediated sugar uptake, is normally a cytoplasmic protein. It has been shown to also localize to the surface in *Streptococcus suis* cells (18). Cell surface-associated HPr, although retaining function, was shown to be devoid of the N-terminal methionine residue, a modification that may lead to its altered location. The down-regulation of HPr (SP1177) and enolase (SP1128) in the *ccpA* strain cell wall could reflect an overall decrease in the expression of these proteins in the cell due to decreased transcription of these genes or a defect in the efficiency of presentation on the cell surface. Our RPA results reveal that SP1176 (*ptsI*), SP1177 (*ptsH/hpr*), and SP1128 (enolase) are transcribed to the same extent in the *ccpA* strain as in the wild type, suggesting that the differences are likely to arise from changes at the posttranscriptional level.

We additionally found that CcpA functions as a repressor of some cell wall proteins in *S. pneumoniae*. Among the derepressed proteins observed is lactate oxidase (SP0715) (64), which is involved in metabolic processes resulting in the for-

mation of acetyl phosphate (53), a high-energy precursor in the cell. The two protein spots corresponding to lactate oxidase on 2-D gels differed in pI but not molecular weight, indicating a possible posttranslational modification. It is unknown how this enzyme is exported and retained in the cell wall compartment in *S. pneumoniae*. Other cell wall proteins subject to derepression in the *ccpA* strain include components of sugar binding and uptake systems like MsmK (SP1580), a maltose-binding periplasmic protein (SP2108), and a member of the dihydrolipamide dehydrogenase family (SP1588). Among these candidates, dihydrolipamide dehydrogenase has been shown to be required for survival and multiplication of *S. pneumoniae* in vivo (68). However, it is not clear if derepression of these factors has a role in the observed attenuation in colonization and virulence by the *ccpA* strain.

Our results highlight an unappreciated role for CcpA as a transcriptional regulator that controls the expression of genes with varied functions in metabolism in *S. pneumoniae*. Prominent on the list of altered CcpA-regulated candidates are proteins associated with functions in central and intermediary cellular metabolism. These changes in basic cellular metabolism could be responsible for the observed defects in vivo. Indeed, the loss of CcpA also causes down-regulation of glycolytic enzymes like enolase and other metabolic enzymes. Some of these are known to contribute to virulence and might meet specific metabolic needs during infection. It is conceivable that misregulation of these factors adversely affects colonization of the nasopharynx and survival in the lungs during infection, leading to rapid clearance. The requirement for CcpA as a regulator in determining colonization and infection of mucosal surfaces by *S. pneumoniae*, and the potential for a similar role for CcpA-independent CCR mechanisms, opens up new regulatory roles for carbon regulators in the control of CCR and sugar metabolism in the context of pathogenesis.

ACKNOWLEDGMENTS

We are grateful to A. L. Sonenshein for critically reading the manuscript and for helpful discussions to C. Squires and the Camilli lab for suggestions, to S. Donahue for help with proteomic analysis, to A. Tischler for assistance with RPAs, and to Y. Yamaichi, H. Kimsey, and the Waldor lab for help with experiments.

A.C. is an investigator of the Howard Hughes Medical Institute (HHMI). This work was supported by the HHMI. N.S.B. was supported by NSF grant EF-0313754.

REFERENCES

1. **Asanuma, N., T. Yoshii, and T. Hino.** 2004. Molecular characterization of CcpA and involvement of this protein in transcriptional regulation of lactate dehydrogenase and pyruvate formate-lyase in the ruminal bacterium *Streptococcus bovis*. Appl. Environ. Microbiol. **70:**5244–5251.
2. **Aslanidis, C., K. Schmid, and R. Schmitt.** 1989. Nucleotide sequences and operon structure of plasmid-borne genes mediating uptake and utilization of raffinose in *Escherichia coli*. J. Bacteriol. **171:**6753–6763.
3. **Barletta, R. G., and R. Curtiss, 3rd.** 1989. Impairment of melibiose utilization in *Streptococcus mutans* serotype c *gtfA* mutants. Infect. Immun. **57:**992–995.
4. **Belitsky, B. R., H. J. Kim, and A. L. Sonenshein.** 2004. CcpA-dependent regulation of *Bacillus subtilis* glutamate dehydrogenase gene expression. J. Bacteriol. **186:**3392–3398.
5. **Bender, M. H., R. T. Cartee, and J. Yother.** 2003. Positive correlation between tyrosine phosphorylation of CpsD and capsular polysaccharide production in *Streptococcus pneumoniae*. J. Bacteriol. **185:**6057–6066.
6. **Bergmann, S., M. Rohde, G. S. Chhatwal, and S. Hammerschmidt.** 2001. α-Enolase of *Streptococcus pneumoniae* is a plasmin(ogen)-binding protein displayed on the bacterial cell surface. Mol. Microbiol. **40:**1273–1287.
7. **Bricker, A. L., and A. Camilli.** 1999. Transformation of a type 4 encapsulated strain of *Streptococcus pneumoniae*. FEMS Microbiol. Lett. **172:**131–135.
8. **Brochu, D., and C. Vadeboncoeur.** 1999. The HPr(Ser) kinase of *Streptococcus salivarius*: purification, properties, and cloning of the *hprK* gene. J. Bacteriol. **181:**709–717.
9. **Chasin, L. A., and B. Magasanik.** 1968. Induction and repression of the histidine-degrading enzymes of *Bacillus subtilis*. J. Biol. Chem. **243:**5165–5178.
10. **Chauvaux, S.** 1996. CcpA and HPr(ser-P): mediators of catabolite repression in *Bacillus subtilis*. Res. Microbiol. **147:**518–522.
11. **Chauvaux, S., I. T. Paulsen, and M. H. Saier, Jr.** 1998. CcpB, a novel transcription factor implicated in catabolite repression in *Bacillus subtilis*. J. Bacteriol. **180:**491–497.
12. **Darbon, E., P. Servant, S. Poncet, and J. Deutscher.** 2002. Antitermination by GlpP, catabolite repression via CcpA and inducer exclusion triggered by P-GlpK dephosphorylation control *Bacillus subtilis* glpFK expression. Mol. Microbiol. **43:**1039–1052.
13. **Deutscher, J., E. Kuster, U. Bergstedt, V. Charrier, and W. Hillen.** 1995. Protein kinase-dependent HPr/CcpA interaction links glycolytic activity to carbon catabolite repression in gram-positive bacteria. Mol. Microbiol. **15:**1049–1053.
14. **Deutscher, J., J. Reizer, C. Fischer, A. Galinier, M. H. Saier, Jr., and M. Steinmetz.** 1994. Loss of protein kinase-catalyzed phosphorylation of HPr, a phosphocarrier protein of the phosphotransferase system, by mutation of the *ptsH* gene confers catabolite repression resistance to several catabolic genes of Bacillus subtilis. J. Bacteriol. **176:**3336–3344.
15. **de Vos, W. M., I. Boerrigter, R. J. van Rooyen, B. Reiche, and W. Hengstenberg.** 1990. Characterization of the lactose-specific enzymes of the phosphotransferase system in *Lactococcus lactis*. J. Biol. Chem. **265:**22554–22560.
16. **Doan, T., and S. Aymerich.** 2003. Regulation of the central glycolytic genes in *Bacillus subtilis*: binding of the repressor CggR to its single DNA target sequence is modulated by fructose-1,6-bisphosphate. Mol. Microbiol. **47:**1709–1721.
17. **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.
18. **Dubreuil, J. D., M. Jacques, D. Brochu, M. Frenette, and C. Vadeboncoeur.** 1996. Surface location of HPr, a phosphocarrier of the phosphoenolpyruvate:sugar phosphotransferase system in *Streptococcus suis*. Microbiology **142**(Pt. 4)**:**837–843.
19. **Egeter, O., and R. Bruckner.** 1996. Catabolite repression mediated by the catabolite control protein CcpA in *Staphylococcus xylosus*. Mol. Microbiol. **21:**739–749.
20. **Ehinger, S., W. D. Schubert, S. Bergmann, S. Hammerschmidt, and D. W. Heinz.** 2004. Plasmin(ogen)-binding α-enolase from *Streptococcus pneumoniae*: crystal structure and evaluation of plasmin(ogen)-binding sites. J. Mol. Biol. **343:**997–1005.
21. **Frey, N., S. Nessler, S. Fieulaine, K. Vaillancourt, M. Frenette, and C. Vadeboncoeur.** 2003. The HPr(Ser) kinase of *Streptococcus salivarius*: a hexameric bifunctional enzyme controlled by glycolytic intermediates and inorganic phosphate. FEMS Microbiol. Lett. **224:**67–72.
22. **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.
23. **Gilbreth, S. E., A. K. Benson, and R. W. Hutkins.** 2004. Catabolite repression and virulence gene expression in *Listeria monocytogenes*. Curr. Microbiol. **49:**95–98.
24. **Grundy, F. J., A. J. Turinsky, and T. M. Henkin.** 1994. Catabolite regulation of *Bacillus subtilis* acetate and acetoin utilization genes by CcpA. J. Bacteriol. **176:**4527–4533.
25. **Grundy, F. J., D. A. Waters, T. Y. Takova, and T. M. Henkin.** 1993. Identification of genes involved in utilization of acetate and acetoin in *Bacillus subtilis*. Mol. Microbiol. **10:**259–271.
26. **Guffanti, A. A., and W. A. Corpe.** 1975. Maltose metabolism of *Pseudomonas fluorescens*. J. Bacteriol. **124:**262–268.
27. **Hava, D. L., and A. Camilli.** 2002. Large-scale identification of serotype 4 *Streptococcus pneumoniae* virulence factors. Mol. Microbiol. **45:**1389–1406.
28. **Hava, D. L., C. J. Hemsley, and A. Camilli.** 2003. Transcriptional regulation in the *Streptococcus pneumoniae rlrA* pathogenicity islet by RlrA. J. Bacteriol. **185:**413–421.
29. **Hemsley, C., E. Joyce, D. L. Hava, A. Kawale, and A. Camilli.** 2003. MgrA, an orthologue of Mga, acts as a transcriptional repressor of the genes within the *rlrA* pathogenicity islet in *Streptococcus pneumoniae*. J. Bacteriol. **185:**6640–6647.
30. **Henkin, T. M.** 1996. The role of CcpA transcriptional regulator in carbon metabolism in *Bacillus subtilis*. FEMS Microbiol. Lett. **135:**9–15.
31. **Henkin, T. M., F. J. Grundy, W. L. Nicholson, and G. H. Chambliss.** 1991. Catabolite repression of α-amylase gene expression in *Bacillus subtilis* involves a trans-acting gene product homologous to the *Escherichia coli lacI* and *galR* repressors. Mol. Microbiol. **5:**575–584.
32. **Horton, R. M., Z. L. Cai, S. N. Ho, and L. R. Pease.** 1990. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. BioTechniques **8:**528–535.
33. **Hueck, C. J., and W. Hillen.** 1995. Catabolite repression in *Bacillus subtilis*:

a global regulatory mechanism for the gram-positive bacteria? Mol. Microbiol. **15:**395–401.
34. **Jiresova, M., Z. Dobrova, J. Naprstek, P. Rysavy, and J. Janecek.** 1983. Induction of β-D-glucosidase in *Streptomyces granaticolor*. Folia Microbiol. **28:**379–385.
35. **Jourlin-Castelli, C., N. Mani, M. M. Nakano, and A. L. Sonenshein.** 2000. CcpC, a novel regulator of the LysR family required for glucose repression of the *citB* gene in *Bacillus subtilis*. J. Mol. Biol. **295:**865–878.
36. **Kim, H. J., A. Roux, and A. L. Sonenshein.** 2002. Direct and indirect roles of CcpA in regulation of *Bacillus subtilis* Krebs cycle genes. Mol. Microbiol. **45:**179–190.
37. **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.
38. **Kraus, A., E. Kuster, A. Wagner, K. Hoffmann, and W. Hillen.** 1998. Identification of a co-repressor binding site in catabolite control protein CcpA. Mol. Microbiol. **30:**955–963.
39. **Lacks, S.** 1966. Integration efficiency and genetic recombination in pneumococcal transformation. Genetics **53:**207–235.
40. **Ling, E., G. Feldman, M. Portnoi, R. Dagan, K. Overweg, F. Mulholland, V. Chalifa-Caspi, J. Wells, and Y. Mizrachi-Nebenzahl.** 2004. Glycolytic enzymes associated with the cell surface of *Streptococcus pneumoniae* are antigenic in humans and elicit protective immune responses in the mouse. Clin. Exp. Immunol. **138:**290–298.
41. **Ludwig, H., C. Meinken, A. Matin, and J. Stulke.** 2002. Insufficient expression of the *ilv-leu* operon encoding enzymes of branched-chain amino acid biosynthesis limits growth of a *Bacillus subtilis ccpA* mutant. J. Bacteriol. **184:**5174–5178.
42. **Mahr, K., W. Hillen, and F. Titgemeyer.** 2000. Carbon catabolite repression in *Lactobacillus pentosus*: analysis of the *ccpA* region. Appl. Environ. Microbiol. **66:**277–283.
43. **Marasco, R., C. T. Lago, and M. De Felice.** 1995. Utilization of cellobiose and other β-D-glucosides in *Agrobacterium tumefaciens*. Res. Microbiol. **146:**485–492.
44. **Marra, A., J. Asundi, M. Bartilson, S. Lawson, F. Fang, J. Christine, C. Wiesner, D. Brigham, W. P. Schneider, and A. E. Hromockyj.** 2002. Differential fluorescence induction analysis of *Streptococcus pneumoniae* identifies genes involved in pathogenesis. Infect. Immun. **70:**1422–1433.
45. **Martin, B., M. Prudhomme, G. Alloing, C. Granadel, and J. P. Claverys.** 2000. Cross-regulation of competence pheromone production and export in the early control of transformation in *Streptococcus pneumoniae*. Mol. Microbiol. **38:**867–878.
46. **Milenbachs, A. A., D. P. Brown, M. Moors, and P. Youngman.** 1997. Carbon-source regulation of virulence gene expression in *Listeria monocytogenes*. Mol. Microbiol. **23:**1075–1085.
47. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
48. **Miwa, Y., K. Nagura, S. Eguchi, H. Fukuda, J. Deutscher, and Y. Fujita.** 1997. Catabolite repression of the *Bacillus subtilis gnt* operon exerted by two catabolite-responsive elements. Mol. Microbiol. **23:**1203–1213.
49. **Monedero, V., O. P. Kuipers, E. Jamet, and J. Deutscher.** 2001. Regulatory functions of serine-46-phosphorylated HPr in *Lactococcus lactis*. J. Bacteriol. **183:**3391–3398.
50. **Nieto, C., M. Espinosa, and A. Puyet.** 1997. The maltose/maltodextrin regulon of *Streptococcus pneumoniae*. Differential promoter regulation by the transcriptional repressor MalR. J. Biol. Chem. **272:**30860–30865.
51. **Orihuela, C. J., J. N. Radin, J. E. Sublett, G. Gao, D. Kaushal, and E. I. Tuomanen.** 2004. Microarray analysis of pneumococcal gene expression during invasive disease. Infect. Immun. **72:**5582–5596.
52. **Paton, J. C., and P. Giammarinaro.** 2001. Genome-based analysis of pneumococcal virulence factors: the quest for novel vaccine antigens and drug targets. Trends Microbiol. **9:**515–518.
53. **Pericone, C. D., S. Park, J. A. Imlay, and J. N. Weiser.** 2003. Factors contributing to hydrogen peroxide resistance in *Streptococcus pneumoniae* include pyruvate oxidase (SpxB) and avoidance of the toxic effects of the Fenton reaction. J. Bacteriol. **185:**6815–6825.
54. **Poncet, S., I. Mijakovic, S. Nessler, V. Gueguen-Chaignon, V. Chaptal, A. Galinier, G. Boel, A. Maze, and J. Deutscher.** 2004. HPr kinase/phosphorylase, a Walker motif A-containing bifunctional sensor enzyme controlling catabolite repression in gram-positive bacteria. Biochim. Biophys. Acta **1697:**123–135.
55. **Postma, P. W., and J. W. Lengeler.** 1985. Phosphoenolpyruvate:carbohydrate phosphotransferase system of bacteria. Microbiol. Rev. **49:**232–269.
56. **Ramseier, T. M., J. Reizer, E. Kuster, W. Hillen, and M. H. Saier.** 1995. *In vitro* binding of the CcpA protein of *Bacillus megaterium* to cis-acting catabolite responsive elements (CREs) of gram-positive bacteria. FEMS Microbiol. Lett. **129:**207–213.
57. **Reizer, J., S. Bachem, A. Reizer, M. Arnaud, M. H. Saier, Jr., and J. Stulke.** 1999. Novel phosphotransferase system genes revealed by genome analysis—the complete complement of PTS proteins encoded within the genome of *Bacillus subtilis*. Microbiology **145**(Pt. 12)**:**3419–3429.
58. **Reizer, J., C. Hoischen, F. Titgemeyer, C. Rivolta, R. Rabus, J. Stulke, D. Karamata, M. H. Saier, Jr., and W. Hillen.** 1998. A novel protein kinase that controls carbon catabolite repression in bacteria. Mol. Microbiol. **27:**1157–1169.
59. **Rogers, J. D., and F. A. Scannapieco.** 2001. RegG, a CcpA homolog, participates in regulation of amylase-binding protein A gene (*abpA*) expression in *Streptococcus gordonii*. J. Bacteriol. **183:**3521–3525.
60. **Rosenow, C., M. Maniar, and J. Trias.** 1999. Regulation of the α-galactosidase activity in *Streptococcus pneumoniae*: characterization of the raffinose utilization system. Genome Res. **9:**1189–1197.
61. **Rowland, P., O. Bjornberg, F. S. Nielsen, K. F. Jensen, and S. Larsen.** 1998. The crystal structure of *Lactococcus lactis* dihydroorotate dehydrogenase A complexed with the enzyme reaction product throws light on its enzymatic function. Protein Sci. **7:**1269–1279.
62. **Schonert, S., T. Buder, and M. K. Dahl.** 1998. Identification and enzymatic characterization of the maltose-inducible α-glucosidase MalL (sucrase-isomaltase-maltase) of *Bacillus subtilis*. J. Bacteriol. **180:**2574–2578.
63. **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.
64. **Seki, M., K. Iida, M. Saito, H. Nakayama, and S. Yoshida.** 2004. Hydrogen peroxide production in *Streptococcus pyogenes*: involvement of lactate oxidase and coupling with aerobic utilization of lactate. J. Bacteriol. **186:**2046–2051.
65. **Servant, P., D. Le Coq, and S. Aymerich.** 2005. CcpN (YqzB), a novel regulator for CcpA-independent catabolite repression of *Bacillus subtilis* gluconeogenic genes. Mol. Microbiol. **55:**1435–1451.
66. **Shaw, G. C., H. S. Kao, and C. Y. Chiou.** 1998. Cloning, expression, and catabolite repression of a gene encoding β-galactosidase of *Bacillus megaterium* ATCC 14581. J. Bacteriol. **180:**4734–4738.
67. **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.
68. **Smith, A. W., H. Roche, M. C. Trombe, D. E. Briles, and A. Hakansson.** 2002. Characterization of the dihydrolipoamide dehydrogenase from *Streptococcus pneumoniae* and its role in pneumococcal infection. Mol. Microbiol. **44:**431–448.
69. **Stulke, J., and W. Hillen.** 1999. Carbon catabolite repression in bacteria. Curr. Opin. Microbiol. **2:**195–201.
70. **Stulke, J., and W. Hillen.** 1998. Coupling physiology and gene regulation in bacteria: the phosphotransferase sugar uptake system delivers the signals. Naturwissenschaften **85:**583–592.
71. **Stulke, J., and W. Hillen.** 2000. Regulation of carbon catabolism in *Bacillus* species. Annu. Rev. Microbiol. **54:**849–880.
72. **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.
73. **Thevenot, T., D. Brochu, C. Vadeboncoeur, and I. R. Hamilton.** 1995. Regulation of ATP-dependent P-(Ser)-HPr formation in *Streptococcus mutans* and *Streptococcus salivarius*. J. Bacteriol. **177:**2751–2759.
74. **Titgemeyer, F., and W. Hillen.** 2002. Global control of sugar metabolism: a gram-positive solution. Antonie Van Leeuwenhoek **82:**59–71.
75. **Tobisch, S., D. Zuhlke, J. Bernhardt, J. Stulke, and M. Hecker.** 1999. Role of CcpA in regulation of the central pathways of carbon catabolism in *Bacillus subtilis*. J. Bacteriol. **181:**6996–7004.
76. **Tuomanen, E.** 1999. Molecular and cellular biology of pneumococcal infection. Curr. Opin. Microbiol. **2:**35–39.
77. **van den Bogaard, P. T., M. Kleerebezem, O. P. Kuipers, and W. M. de Vos.** 2000. Control of lactose transport, β-galactosidase activity, and glycolysis by CcpA in *Streptococcus thermophilus*: evidence for carbon catabolite repression by a non-phosphoenolpyruvate-dependent phosphotransferase system sugar. J. Bacteriol. **182:**5982–5989.
78. **Warner, J. B., B. P. Krom, C. Magni, W. N. Konings, and J. S. Lolkema.** 2000. Catabolite repression and induction of the Mg^{2+}-citrate transporter CitM of *Bacillus subtilis*. J. Bacteriol. **182:**6099–6105.
79. **Warner, J. B., and J. S. Lolkema.** 2003. CcpA-dependent carbon catabolite repression in bacteria. Microbiol. Mol. Biol. Rev. **67:**475–490.
80. **Wen, Z. T., and R. A. Burne.** 2002. Functional genomics approach to identifying genes required for biofilm development by *Streptococcus mutans*. Appl. Environ. Microbiol. **68:**1196–1203.
81. **Whiting, G. C., J. T. Evans, S. Patel, and S. H. Gillespie.** 2002. Purification of native α-enolase from *Streptococcus pneumoniae* that binds plasminogen and is immunogenic. J. Med. Microbiol. **51:**837–843.
82. **Zahner, D., and R. Hakenbeck.** 2000. The *Streptococcus pneumoniae* β-galactosidase is a surface protein. J. Bacteriol. **182:**5919–5921.