Cyclic Di-AMP Impairs Potassium Uptake Mediated by a Cyclic Di-AMP Binding Protein in *Streptococcus pneumoniae*

Yinlan Bai,* Jun Yang, Tiffany M. Zarrella, Yang Zhang, Dennis W. Metzger, Guangchun Bai

Center for Immunology and Microbial Disease, Albany Medical College, Albany, New York, USA

Cyclic di-AMP (c-di-AMP) has been shown to play important roles as a second messenger in bacterial physiology and infections. However, understanding of how the signal is transduced is still limited. Previously, we have characterized a diadenylate cyclase and two c-di-AMP phosphodiesterases in *Streptococcus pneumoniae*, a Gram-positive pathogen. In this study, we identified a c-di-AMP binding protein (CabP) in *S. pneumoniae* using c-di-AMP affinity chromatography. We demonstrated that CabP specifically bound c-di-AMP and that this interaction could not be interrupted by competition with other nucleotides, including ATP, cAMP, AMP, phosphoadenylyl adenosine (pApA), and cyclic di-GMP (c-di-GMP). By using a bacterial two-hybrid system and genetic mutagenesis, we showed that CabP directly interacted with a potassium transporter (SPD_0076) and that both proteins were required for pneumococcal growth in media with low concentrations of potassium. Interestingly, the interaction between CabP and SPD_0076 and the efficiency of potassium uptake were impaired by elevated c-di-AMP in pneumococci. These results establish a direct c-di-AMP-mediated signaling pathway that regulates pneumococcal potassium uptake.

Over the past few decades, cyclic AMP (cAMP), cyclic GMP (cGMP), and cyclic di-GMP (c-di-GMP) have been shown to control important biological cascades in both prokaryotic and eukaryotic cells (1–4). In contrast, cyclic di-AMP (c-di-AMP) was recognized only recently as a potential new second messenger (5). This nucleotide is synthesized enzymatically from two molecules of ATP or ADP by diadenylate cyclase and cleaved to phosphoadenylyl adenosine (pApA) or AMP by distinct c-di-AMP phosphodiesterases (5–9). The presence of diadenylate cyclase has been experimentally demonstrated in *Bacillus subtilis* (5, 10, 11), *Thermotoga maritima* (5), *Listeria monocytogenes* (12), *Staphylococcus aureus* (7), *Mycobacterium tuberculosis* (6), *Streptococcus pyogenes* (13), *Chlamydia trachomatis* (14), and *Streptococcus pneumoniae* (9). Most bacteria possess only one diadenylate cyclase-encoding gene in their genomes, whereas three diadenylate cyclases have been reported for *B. subtilis* (10, 11, 15).

c-di-AMP has been shown to play important roles in bacterial physiology and infections. In *B. subtilis*, a triple diadenylate cyclase mutant was unable to be generated unless the bacterium was compensated with one of these cyclases, suggesting that the lack of c-di-AMP is detrimental for *B. subtilis* (10, 11). Moreover, c-di-AMP is associated with bacterial peptidoglycan homeostasis (7, 10), cellular resistance to antibiotics (7, 10, 16–18), bacterial size and biofilm formation (7, 19), chain length of *S. pneumoniae* (9), and bacterial infections (18–22). Additionally, c-di-AMP produced by *L. monocytogenes* has been shown to activate a host type I interferon response (12) mediated by host effector proteins STING and DDX41 (23–27).

As a second messenger, c-di-AMP's signaling mechanism remains largely unknown. Recently, a c-di-AMP binding transcription factor, DarR, has been identified in *Mycobacterium smegmatis* (28). The interaction between DarR and its target DNA ligands is enhanced in the presence of c-di-AMP *in vitro*. It has also been shown that DarR represses the expression of genes associated with fatty acid metabolism and transport (28). However, a direct regulatory role of c-di-AMP in DarR-controlled gene regulation has not been experimentally addressed. It was also reported that an *S. aureus* protein, KtrA, binds c-di-AMP and is required in potassium trans-

port (29). Additionally, three more c-di-AMP binding proteins were demonstrated in the same report (29). However, the regulatory roles of c-di-AMP through these proteins still remain largely unclear.

S. pneumoniae is a Gram-positive pathogen. This bacterium can cause pneumonia, septicemia, otitis media (OM), sinusitis, and meningitis. The pathogenesis of this bacterium is still not fully understood. Pneumococci possess a diadenylate cyclase (*dacA*) and two c-di-AMP phosphodiesterase encoding genes, *pde1* and *pde2* (9). Our previous study revealed that c-di-AMP influences bacterial growth and the chain length of *S. pneumoniae* (9). Most importantly, both of the c-di-AMP phosphodiesterase proteins contribute to pneumococcal virulence (9, 20). However, how the c-di-AMP signal is transduced in pneumococcus has not yet been explored. Here we report the identification of a c-di-AMP binding protein in *S. pneumoniae* which controls potassium uptake through interaction with a transmembrane protein, SPD_0076. We also demonstrate that the interaction between the two proteins is inhibited by c-di-AMP, which results in impaired potassium uptake by pneumococci.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All of the bacterial strains used in the present study are listed in Table 1. *S. pneumoniae* D39 (serotype 2; ATCC) and its derivatives were initially grown in Todd-Hewitt broth containing 0.5% (wt/vol) yeast extract (THY; BD Biosciences). A commercial chemically defined medium (CDM; purchased from JRH

Received 4 September 2013 Accepted 13 November 2013

Published ahead of print 22 November 2013

Address correspondence to Guangchun Bai, baig@mail.amc.edu.

* Present address: Yinlan Bai, Department of Microbiology, Fourth Military Medical University, Xi'an, China.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JB.01041-13>.

Copyright © 2014, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JB.01041-13

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description ^a	Reference or source
Strains		
<i>S. pneumoniae</i>		
D39	<i>S. pneumoniae</i> serotype 2	53
ST581	D39 derivative; Strep ^r	9
ST2734	ST581 $\Delta pde1 \Delta pde2$; Strep ^r (c-di-AMP phosphodiesterase mutant)	9
ST2796	D39 $\Delta cabP$::Janus cassette; Kan ^r	This study
ST2797	D39 ΔSPD_0076 ::Janus cassette; Kan ^r	This study
ST2798	D39 $\Delta SPD_0076 \Delta cabP$::Janus cassette; Kan ^r	This study
ST2803	ST2797(pST2799); Kan ^r Erm ^r	This study
ST2804	ST2797(pVA838); Kan ^r Erm ^r	This study
ST2805	ST2796(pST2815); Kan ^r Erm ^r	This study
ST2806	ST2796(pVA838); Kan ^r Erm ^r	This study
ST2807	ST581 ΔSPD_0430 ::Janus cassette; Kan ^r	This study
ST2808	ST581 ΔSPD_0429 ::Janus cassette; Kan ^r	This study
ST2809	ST581 $\Delta SPD_0429 \Delta SPD_0430$::Janus cassette; Kan ^r	This study
ST2810	ST2809 but with the Janus cassette removed; Strep ^r	This study
ST2811	ST2810 $\Delta SPD_0076 \Delta cabP$::Janus cassette; Kan ^r	This study
<i>E. coli</i>		
DH5 α	<i>E. coli</i> strain used for cloning	Laboratory stock
BL21(DE3)	<i>E. coli</i> strain used for expression	Novagen
ST2789	BL21(DE3)(pST2788); Kan ^r	This study
BTH101	<i>E. coli</i> Δcya used for bacterial two-hybrid	35
ST2835	BTH101(pKT25)(pST2833); Kan ^r Ap ^r	This study
ST2836	BTH101(pST2834)(pUT18); Kan ^r Ap ^r	This study
ST2837	BTH101(pST2834)(pST2833); Kan ^r Ap ^r	This study
ST2838	BTH101(pKT25-zip)(pUT18-zip); Kan ^r Ap ^r	This study
ST2845	BTH101(pKT25)(pUT18); Kan ^r Ap ^r	This study
ST2860	BTH101(pST2853)(pUT18); Kan ^r Ap ^r	This study
ST2861	BTH101(pST2853)(pST2833); Kan ^r Ap ^r	This study
LB2003	F ⁻ <i>kup1</i> $\Delta kdpABC5 \Delta trkA rpsL metE thi rha gal$	37
ST2927	LB2003(pMBC664); Ap ^r	This study
ST2934	LB2003(pST2821); Ap ^r	This study
ST2941	LB2003(pST2822); Ap ^r	This study
ST2996	BTH101(pST2994)(pST2833); Kan ^r Ap ^r	This study
ST2997	BTH101(pST2994)(pST2982); Kan ^r Ap ^r	This study
ST2998	BTH101(pST2981)(pST2995); Kan ^r Ap ^r	This study
ST2999	BTH101(pST2834)(pST2995); Kan ^r Ap ^r	This study
Plasmids		
pVA838	<i>E. coli</i> - <i>S. pneumoniae</i> shuttle vector; Erm ^r	34
pST2799	pVA838 carrying SPD_0076 promoter and ORF; Erm ^r	This study
pST2815	pVA838 carrying SPD_0076 promoter and <i>cabP</i> ORF; Erm ^r	This study
pET28a(+)	Expression plasmid; Kan ^r	Novagen
pST2788	pET28a(+) carrying <i>S. pneumoniae cabP</i> ORF; Kan ^r	This study
pKT25	Two-hybrid vector; Kan ^r	35
pUT18	Two-hybrid vector; Ap ^r	35
pKT25-zip	pKT25 carrying zip control; Kan ^r	35
pUT18-zip	pUT18 carrying zip control; Ap ^r	35
pST2833	pUT18 carrying SPD_0076 ORF; Ap ^r	This study
pST2834	pKT25 carrying <i>cabP</i> ORF; Kan ^r	This study
pMBC664	pBR322 carrying <i>E. coli crp</i> promoter; Ap ^r	36
pST2821	pMBC664 carrying SPD_0076- <i>cabP</i> ; Ap ^r	This study
pST2822	pST2821 carrying <i>M. tuberculosis disA</i> subcloned from pST2852; Ap ^r	This study
pST2852	pMBC664 carrying <i>M. tuberculosis disA</i> for expression of <i>disA</i> ; Ap ^r	This study
pST2853	pST2834 carrying <i>M. tuberculosis disA</i> expression sequence subcloned from pST2852; Ap ^r	This study
pST2981	pKT25 carrying SPD_0076 ORF; Kan ^r	This study
pST2982	pUT18 carrying <i>cabP</i> ORF; Ap ^r	This study
pST2994	pKT25 carrying <i>M. tuberculosis disA</i> ORF; Kan ^r	This study
pST2995	pUT18 carrying <i>M. tuberculosis disA</i> ORF; Ap ^r	This study

^a Strep^r, streptomycin resistance; Kan^r, kanamycin resistance; Erm^r, erythromycin resistance; Ap^r, ampicillin resistance.

Bioscience) and a formulated CDM were prepared as described previously (30), except that all the potassium salts were dropped out in the formulated CDM. This potassium-free CDM was then supplemented with various concentrations of KCl, as specified below. Bacterial stocks were thoroughly washed with the potassium-free CDM prior to inoculation. *Escherichia coli* strains were grown either in Luria-Bertani (LB) broth or on LB agar plates.

Affinity chromatography. *S. pneumoniae* D39 derivative ST581 was grown in 50 ml of THY overnight at 37°C with 5% CO₂. Bacteria were harvested, and the pellet was resuspended into 10 ml of phosphate-buffered saline (PBS) containing 0.5% (vol/vol) Triton X-100 followed by sonication for 5 min with 5 s on and 5 s off. The bacterial debris was removed by centrifugation at 12,000 rpm and 4°C for 20 min. The supernatant was collected, filtered with a 0.45-μm filter, and loaded onto a 2'-AHC-c-di-AMP agarose column (BioLog). The column was then washed with 20 ml of PBS containing 1% (vol/vol) Triton X-100 at a flow rate of 1 ml/min. Subsequently, the proteins were eluted from the column with 5 ml of 9 M urea in PBS containing 1% (vol/vol) Triton X-100. The eluted samples were analyzed using SDS-PAGE.

MS analysis. The following experiment was performed by the University at Albany Proteomics Facility of the Center for Functional Genomics, Rensselaer, NY. Briefly, the designated band was excised, followed by reduction, alkylation, and in-gel tryptic digestion. The peptide mixture was extracted and analyzed with a QSTAR XL mass spectrometer coupled with a Waters/Micromass CapLC. The C₁₈ PicoFrit column (100-μm inside diameter by 16-cm resin length) was custom-packed with a Vydac Everest resin (5 μm, 300 Å). The high-performance liquid chromatography (HPLC) flow rate of 9.2 μl/min was split to a 200-nl/min microspray flow rate. The electrospray voltage was set at 2.2 kV. The QSTAR XL mass spectrometer was operated in an information-dependent acquisition mode whereby following the interrogation of mass spectrometry (MS) data, ions were selected for MS/MS analysis based on their intensity (20 cps) and charge state (+2, +3, +4). Rolling collision energies were chosen automatically based on the *m/z* and charge state of the selected precursor ions. Tandem MS spectra were acquired as peptides eluted from the liquid chromatograph (LC) column by scanning a mass range of *m/z* 350 to *m/z* 1,500. All MS/MS spectra obtained from the experiment were used to search the NCBI nonredundant database using the Mascot database search program with a species-limited search filter for *Streptococcus* spp. Variable modifications, including deamidation (N, Q), oxidation (M), and carbamidomethylation (C), were also considered for the search.

Expression and purification of CabP. The open reading frame (ORF) of *S. pneumoniae cabP* was amplified by PCR with primers Pr2974 and Pr2975 using *S. pneumoniae* D39 genomic DNA as the template. The PCR product was digested with NdeI and HindIII and cloned into pET28a(+) plasmid (Novagen) between the NdeI and HindIII sites, and the sequence was verified. This recombinant plasmid was designated pST2788 and maintained in *E. coli* BL21(DE3) as the expression strain to produce N-His-tagged CabP. A single colony of the expression strain was picked and grown in LB broth containing 25 μg/ml of kanamycin overnight as seeds. Five hundred milliliters of LB broth containing 25 μg/ml of kanamycin was inoculated with 5 ml of an overnight culture and was shaken at 37°C with aeration to an optical density (OD) at 600 nm (OD₆₀₀) of 0.6. The production of CabP was induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 h at room temperature. Bacteria were harvested and resuspended into 40 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 500 mM NaCl, 10 mM imidazole, and 10% glycerol). The suspension was sonicated for 10 min on ice with 5 s on and 10 s off. The bacterial debris was removed by centrifugation at 13,000 rpm for 20 min at 4°C. The supernatant was then loaded on a column with nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen) and washed with 50 ml of buffer I (50 mM Tris-HCl [pH 7.5], 500 mM NaCl, and 30 mM imidazole), followed with 50 ml of buffer II (50 mM Tris-HCl [pH 7.5], 500 mM NaCl, and 50 mM imidazole). The protein was finally eluted with 5 ml of elution buffer (50 mM Tris-HCl [pH 7.5], 500 mM NaCl, and 250 mM imidazole) and

dialyzed twice with PBS and once with PBS containing 10% (vol/vol) glycerol. The purity of the purified protein was determined by SDS-PAGE. The concentration of the purified protein was determined with a Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). The protein was then aliquoted and stored at -80°C until use.

Gel filtration. Size exclusion chromatography was performed with a Superdex-200 column connected to a GradiPhrac automatic sampler (Amersham Biosciences). The column was equilibrated and eluted with the running buffer (10% glycerol in PBS at pH 7.4) at a constant flow rate of 0.5 ml/min. The molecular mass of the proteins was determined by using a gel filtration standard (Bio-Rad) per the instructions in *Gel Filtration Principles and Methods* (31).

Synthesis of ³²P-labeled c-di-AMP. The reaction mixture for labeling contained 40 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 100 mM NaCl, 1 mM ATP, 2.5 μM purified *M. tuberculosis* DisA (also referred to as DacA) (6), and 20 μCi of [α-³²P]ATP (MP Biomedicals). After incubation at 37°C for 1 h, the c-di-AMP was separated by thin-layer chromatography (TLC) as previously reported (6) and was extracted into 500 μl of water.

Electrophoretic mobility shift assay (EMSA). The reaction mixture (10 μl) contained 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 5 mM CaCl₂, 5 mM KCl, 10% (vol/vol) glycerol, and the concentrations of purified protein indicated below. The reaction was initiated by the addition of ~0.01 pmol of ³²P-labeled c-di-AMP. For competition experiments, 1,000-fold unlabeled nucleotide was provided. The samples were incubated for 30 min at room temperature and then separated on 8% native PAGE gels. Gels were dried and analyzed as previously reported (32).

Construction and complementation of mutants. *S. pneumoniae* mutant strains were generated in an ST581 background, except where specified, by homologous recombination as reported earlier (30, 33) using primers listed in Table S1 in the supplemental material. Therefore, ST581 is defined as the wild type (WT) in this text. To generate the quadruple mutant, the SPD_0430-SPD_0429 locus was first replaced with the Janus cassette in ST581. The kanamycin-resistant and streptomycin-sensitive mutant was then unmarked to select a kanamycin-sensitive and streptomycin-resistant mutant, which we designated ST2810. The SPD_0076-*cabP* locus was then replaced with the Janus cassette in ST2810 to generate ST2811, which is kanamycin resistant and streptomycin sensitive.

To complement the ΔSPD_0076 mutant (ST2797), the SPD_0076 upstream region and its ORF were cloned into pVA838 (34). To complement the Δ*cabP* mutant, the *cabP* ORF was linked with the SPD_0076 promoter sequence by overlapping PCR, and this DNA fragment was then cloned into pVA838. The complementary plasmids were transformed into respective mutants. Mutants transformed with pVA838 were used as controls.

Bacterial two-hybrid analysis. Interaction between CabP and SPD_0076 was determined using a bacterial two-hybrid system (35). This system is based on the interaction-mediated reconstruction of adenylate cyclase activity in an *E. coli* Δ*cya* strain, BTH101. An interaction between bait and prey proteins results in the production of cAMP, which is reported by expression of β-galactosidase and monitored by a catalytic reaction using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as a substrate. In this study, ORFs of *cabP* and SPD_0076 were cloned into pKT25 and pUT18, respectively. The constructs were cotransformed into BTH101. For the expression of *M. tuberculosis* *disA* in the two-hybrid system, *disA* was first cloned into pMBC664 (36), controlled by an *E. coli* *crp* promoter to generate pST2852. The expression cassette was then subcloned into pST2834 to express both *disA* and *cabP-cyaA*. Bacterial culture was diluted 1:10⁴ and spotted in 2 μl onto LB plates containing 80 μg/ml of X-Gal, 0.5 mM IPTG, 100 μg/ml of ampicillin, and 25 μg/ml of kanamycin. Plates were incubated for 16 h at 37°C.

Detection of c-di-AMP in *E. coli*. *E. coli* strains for two-hybrid analysis were grown 16 h in 5 ml of LB broth containing 100 μg/ml of ampicillin and 25 μg/ml of kanamycin. The growth was determined by measuring the OD₆₂₀. The bacteria were harvested by centrifugation at 13,000 rpm for 1 min. The pellet was resuspended in 0.5 ml of 50 mM Tris-HCl (pH 8.0), sonicated for 20 s, and heated for 10 min at 95°C. Bacterial debris was

removed and the supernatant was taken to determine c-di-AMP levels using an enzyme-linked immunosorbent assay (ELISA) (patent pending) as we described earlier (9). The amount of c-di-AMP was normalized by the OD₆₂₀ of the culture.

Functional complementation of *E. coli* mutant LB2003. The SPD_0076-*cabP* locus was amplified by PCR using the D39 genomic DNA as the template. This fragment was digested with MfeI and BamHI and then cloned into pMBC664 between the EcoRI and BamHI sites to generate pST2821. The *disA* expression cassette was cut from pST2852, blunted using Klenow, and then cloned into pST2821 at the SmaI site to generate pST2822. Plasmids pMBC664, pST2821, and pST2822 were individually transformed into *E. coli* LB2003, a potassium transport-deficient mutant (37). These recombinant strains were grown in KLM medium and a minimal medium containing 2 mM KCl. KLM medium is a potassium-rich medium prepared with the same recipe as LB broth except that NaCl was replaced with the same amount of KCl. The minimal medium contains 46 mM Na₂HPO₄, 23 mM NaH₂PO₄, 8 mM (NH₄)₂SO₄, 1 mM sodium citrate, 0.4 mM MgSO₄, 1 mg/liter of thiamine, 10 mM glucose, and desired concentrations of KCl (37). Ampicillin was supplemented at a concentration of 100 µg/ml. Bacterial growth was monitored every 2 h for OD₆₂₀.

RESULTS

Identification of a c-di-AMP binding protein in *S. pneumoniae*.

In our previous study, a diadenylate cyclase and two c-di-AMP phosphodiesterase proteins were identified in *S. pneumoniae* (9). However, it is unknown how c-di-AMP signaling is transduced in this pathogen. In the present study, we identified c-di-AMP binding proteins by c-di-AMP affinity chromatography using 2'-AHC-c-diAMP-agarose resin (BioLog). Since this product had not been demonstrated for purification of a c-di-AMP binding protein, we first mixed equal concentrations of *M. tuberculosis* DisA protein and bovine serum albumin (BSA) and ran the mixture through the column. Our result showed that only DisA was present in the final elution (Fig. 1A), which is consistent with the observation that the DisA ortholog in *B. subtilis* binds c-di-AMP (5). Bacterial lysates of *S. pneumoniae* ST581, a streptomycin-resistant D39 derivative, were then applied to purify any c-di-AMP binding proteins. A protein band with an apparent molecular mass of ~21 kDa was enriched (Fig. 1B). This protein was identified as SPD_0077 by mass spectrometry, with a calculated molecular mass of 24 kDa (see Fig. S1 in the supplemental material), and was annotated as a Trk family potassium transport protein (38). We designated this protein CabP for c-di-AMP binding protein. CabP shares 33% identity in amino acid sequence with *S. aureus* KtrA (see Fig. S2 in the supplemental material), which is a typical RCK (regulator of conductance of K⁺) protein (29).

CabP binds c-di-AMP specifically. To further characterize CabP, we overexpressed this protein in *E. coli* and purified it to homogeneity. The apparent molecular mass of the His-tagged protein is ~28 kDa (Fig. 2A). Gel filtration analysis with purified CabP revealed that it formed a highly stable octamer in solution with an estimated molecular mass of ~210 kDa (Fig. 2B), which is equivalent to an octamerized His-CabP with a calculated molecular mass of 208 kDa. This finding is also consistent with the oligomerization of *B. subtilis* KtrA (39, 40).

Multiple approaches were then performed to characterize the interaction between CabP and c-di-AMP. First, equal amounts of purified CabP and His-tagged *S. pneumoniae* PdxR (30) were run on SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane, and incubated with ³²P-labeled c-di-AMP. The results showed that CabP, but not the PdxR control, bound c-di-AMP

FIG 1 Identification of CabP as a c-di-AMP binding protein in *S. pneumoniae*. (A) Test of the column. A mixture containing the same concentrations of purified *M. tuberculosis* DisA and BSA was loaded onto a c-di-AMP affinity column (Biolog) and separated as described in Materials and Methods. The eluted samples were analyzed using SDS-PAGE and stained with Coomassie bright blue. Lane M, molecular mass standards with sizes (in kDa) noted on the left side of the gel. (B) Purification of c-di-AMP binding proteins in *S. pneumoniae*. The samples eluted from the affinity chromatography were analyzed using SDS-PAGE and staining with Coomassie bright blue. Lys, bacterial lysates; lane M, molecular mass standards with sizes (in kDa) noted on the left side of the gel; lanes 1 to 6, eluted samples.

(Fig. 3A), indicating that c-di-AMP binds CabP specifically. In a native gel, the mobility of ³²P-labeled c-di-AMP was retarded by CabP and *M. tuberculosis* DisA but not by the PdxR control (Fig. 3B), further supporting the binding specificity of CabP. CabP bound with c-di-AMP at a *K_d* (dissociation constant) of ~150 nM (Fig. 3C), which is similar to the interaction between *S. aureus* KtrA and c-di-AMP (29). Additionally, free ³²P-labeled c-di-AMP was recovered from the reaction with CabP in the presence of an excessive amount of unlabeled c-di-AMP, but not with unlabeled NAD⁺, NADH, NADP⁺, NADPH, c-di-GMP, 3',5'-cAMP (Fig. 3D), ATP, AMP, pApA, or 2',3'-cAMP (see Fig. S3 in the supplemental material), indicating that the c-di-AMP binding site in CabP is highly specific to c-di-AMP.

CabP is required for pneumococcal potassium uptake. CabP was annotated as a Trk family protein (38), implying that this protein might be involved in potassium uptake. Interestingly, *cabP* is located in an operon with the gene for SPD_0076, which contains 10 transmembrane helices and is predicted to be a TrkH protein (38). SPD_0076 is the ortholog of *B. subtilis* KtrB. Both Trk and Ktr transporters are functional for potassium uptake, although they differ in the cation cotransported with potassium (41). In the *S. pneumoniae* genome, there is another locus that also contains the genes for TrkA- and TrkH-like proteins, which are SPD_0430 and SPD_0429, respectively (Fig. 4A). SPD_0430 has

FIG 2 Purification of recombinant CabP. (A) SDS-PAGE of purified CabP. Lane M, molecular mass markers; lanes 1 to 3, eluted protein. (B) Gel filtration of CabP monitored at 280 nm. Retention volumes of molecular mass standards (in kDa) are indicated. mAu, milliabsorbance units.

two TrkA domain repeats, while SPD_0429 possesses 10 transmembrane helices, similar to SPD_0076. To evaluate the roles of these proteins in potassium uptake, we generated mutants as listed in Fig. 4A and grew them in a potassium dropout CDM that was then supplemented with various concentrations of KCl. The complete CDM contains 170 mM sodium salts and approximately 10 mM potassium salts (42). We first titrated the potassium concentrations in CDM for growth of the WT and the quadruple mutant (ST2811). After 8 h of incubation, the quadruple mutant exhibited a significant defect in growth at low concentrations of potassium, whereas it grew better than the WT in the CDM containing 10 mM

potassium (Fig. 4B). Surprisingly, the WT grew significantly in the potassium dropout CDM while inoculated with stocks prepared from the potassium-rich CDM, even though the stocks were thoroughly washed with the potassium dropout CDM prior to inoculation. However, a second passage of the WT in the potassium-dropout CDM was unable to grow (data not shown), indicating that potassium is an essential nutrient for pneumococci and that the growth of the first generation relies on the intracellular pool of potassium. In contrast, after inoculation of the two double mutants into CDM, only the Δ SPD_0076 Δ cabP mutant (ST2798), and not the Δ SPD_0430 Δ SPD_0429 mutant (ST2809), failed to

FIG 3 Interaction between CabP and c-di-AMP. (A) CabP protein and *S. pneumoniae* PdxR control were loaded for SDS-PAGE, followed by staining with Coomassie bright blue (Staining), or transferred onto a PVDF membrane and then incubated with labeled c-di-AMP (Blotting). The membrane was finally exposed on a phosphor screen and scanned with a Storm 860 phosphorimager. (B) Mobility of 32 P-labeled c-di-AMP in the presence of 4 μ M each purified protein as indicated. *M. tuberculosis* DisA served as a positive control, and *S. pneumoniae* PdxR served as a negative control. (C) Mobility of 32 P-labeled c-di-AMP in the absence or presence of various concentrations of purified CabP. (D) Mobility of 32 P-labeled c-di-AMP in the presence of 1 μ M purified CabP and an excessive amount of each unlabeled nucleotide as indicated.

FIG 4 Growth of mutants in CDM with various concentrations of potassium. Bacterial growth was determined at 8 h postinoculation for all panels. (A) Domain and genetic organizations of the annotated Trk proteins in the *S. pneumoniae* genome. The deleted regions in the mutant strains as listed are indicated with black lines. (B) Growth of WT and the quadruple mutant (ST2811) in a formulated CDM contains 5-fold serial dilutions of KCl. The commercial CDM (CDM), which contains ~10 mM potassium salts, was used as a control. (C) Growth of the Δ SPD_0076 Δ cabP (ST2798) and Δ SPD_0430 Δ SPD_0429 (ST2809) double mutants in the formulated CDM with indicated concentrations of KCl. ST2811 was used as a control. (D) Growth of Δ cabP (ST2796) and Δ SPD_0076 (ST2797) mutants in the formulated CDM containing the indicated concentrations of KCl. ST2798 was used as a control. All the growth data shown in panels B through D are the means of three independent experiments. The error bars denote the SEMs. (E) Growth of the complemented Δ SPD_0076 and Δ cabP mutants in the formulated CDM. Complemented strains were generated by transformation of the plasmid expressing either SPD_0076 or cabP into the respective "host." Strains transformed with pVA838 plasmid (Control) served as controls.

grow at low concentrations of potassium (Fig. 4C), indicating that only the SPD_0076-cabP locus plays a central role in pneumococcal potassium uptake. Furthermore, neither the Δ cabP (ST2796) nor the Δ SPD_0076 (ST2797) strain was able to grow in CDM with low concentrations of potassium (Fig. 4D). The growth defect detected with both single mutants could be restored by expressing the respective genes but not by transformation with the vector control (Fig. 4E; see also Fig. S4 in the supplemental material). Notably, Δ cabP, Δ SPD_0076, and Δ SPD_0076 Δ cabP strains displayed growth rates similar to that of the WT in THY broth (data not shown). These results indicate that both CabP and SPD_0076 are required for potassium uptake by pneumococci.

c-di-AMP reduces the interaction between CabP and SPD_0076. The gene arrangement and the role of CabP and SPD_0076 imply that the two proteins might interact directly for potassium uptake. This hypothesis is consistent with the potassium transport models in other bacteria (29, 43). To test this hypothesis, an *E. coli* two-hybrid experiment was performed. Our results showed that introduction of both CabP and SPD_0076 in this system exhibited a functional adenylate cyclase that is reflected by the catalytic activity of β -galactosidase with X-Gal as the

substrate and the production of blue colonies (Fig. 5A), similar to results with a positive control provided with the system (35). In contrast, introduction of either CabP or SPD_0076 alone did not result in any color response (Fig. 5A). This result indicates that CabP and SPD_0076 interact physically, which is consistent with the growth phenotype of Δ cabP and Δ SPD_0076 (Fig. 4D). Interestingly, coexpression of *M. tuberculosis* *disA* significantly reduced the color of the strain harboring both CabP and SPD_0076, and the production of c-di-AMP in *disA*-expressing strains was verified by measurement of the bacterial c-di-AMP levels (Fig. 5B). Meanwhile, direct interaction of DisA with either CabP or SPD_0076 was not detected using the two-hybrid system (see Fig. S5 in the supplemental material). This result suggests that c-di-AMP, rather than DisA protein itself, reduces the interaction between CabP and SPD_0076.

c-di-AMP impairs potassium uptake by CabP-SPD_0076 complex. We have reported that *S. pneumoniae* possesses two c-di-AMP phosphodiesterases, Pde1 and Pde2. A Δ pde1 Δ pde2 double mutant (ST2734) exhibited significantly elevated c-di-AMP levels compared to those of the parental strain (9). Since c-di-AMP reduced the interaction between CabP and SPD_0076, we

FIG 5 Role of c-di-AMP in potassium uptake. (A) Interaction between CabP and SPD_0076 determined in a two-hybrid system. The indicated recombinant *E. coli* strains were spotted onto LB plates containing 80 μ g/ml of X-Gal. ST2838 is a positive control, whereas ST2845 is a negative control. The data shown are representative of three repeat experiments. (B) Detection of bacterial c-di-AMP in the indicated *E. coli* strains. Note that only ST2860 and ST2861 exhibited detectable c-di-AMP levels. The data shown are the means of three independent experiments. The error bars denote the SEMs. (C and D) Growth of the WT (C) and ST2734 (D) in CDM with indicated concentrations of KCl. The data shown are the means of three independent experiments. The error bars denote the SEMs.

hypothesized that an increase in c-di-AMP levels in pneumococci might reduce bacterial potassium uptake. To test this hypothesis, the growth of the $\Delta pde1\Delta pde2$ mutant in CDM with various concentrations of potassium was compared to that of the parental strain. Our result showed that the growth of the WT was not significantly changed in CDM containing 0.1 to 10 mM KCl for the first 10 h. A slightly reduced growth rate was detected with the WT grown in potassium-free CDM during this growth period (Fig. 5C). The $\Delta pde1\Delta pde2$ mutant grew very slowly in CDM with 10 mM KCl; therefore, the growth curve was determined up to 22 h. The result showed that the growth rate of the $\Delta pde1\Delta pde2$ mutant was reduced in response to low levels of potassium in a dose-dependent manner (Fig. 5D). This result suggests that the potassium uptake ability of pneumococci is impaired by c-di-AMP.

To further confirm the role of c-di-AMP in bacterial potassium uptake, we coexpressed *S. pneumoniae cabP* and SPD_0076 in the

potassium transport-deficient *E. coli* mutant LB2003 (37). Our results showed that LB2003 was unable to grow in a minimal medium containing 2 mM KCl (not shown), similar to the observation in a previous study (44). However, the defective growth of LB2003 in this medium could be rescued by coexpression of *cabP* and SPD_0076 but not by the vector control (Fig. 6). Interestingly, LB2003 expressing *M. tuberculosis disA* along with the *cabP*-SPD_0076 strain failed to grow in this low-potassium medium (Fig. 6), whereas all three strains were able to grow in the potassium-rich medium (data not shown). It is known that *E. coli* does not possess diadenylate cyclase (7, 11); thus, our results indicate that c-di-AMP reduces potassium uptake by CabP-SPD_0076 complex when they are coexpressed in *E. coli*.

Taking these findings together, we conclude that c-di-AMP impairs potassium uptake by reducing the interaction between CabP and SPD_0076 (Fig. 7).

FIG 6 Growth of the LB2003 recombinant strains in minimal medium containing 2 mM KCl. (A) Expression of SPD_0076, *cabP*, and *M. tuberculosis disA* (*Mtb_disA*) in LB2003. These genes were cloned in pMBC664, in which the expression is controlled by an *E. coli crp* promoter as illustrated, and were transformed into LB2003 to generate the indicated strains. (B) Growth curve of the LB2003 recombinant strains as indicated in panel A in minimal medium containing 2 mM KCl. The data shown are the means of three independent experiments. The error bars denote the SEMs.

FIG 7 Model of c-di-AMP-controlled potassium uptake by *S. pneumoniae*. In the absence of c-di-AMP, CabP interacts with SPD_0076 and results in efficient potassium uptake. In the presence of c-di-AMP, the interaction between CabP and SPD_0076 is impaired, and the potassium uptake is significantly reduced. Therefore, the requirement of the environmental potassium threshold for bacterial growth is elevated.

DISCUSSION

Diadenylate cyclase and c-di-AMP phosphodiesterase have been characterized in several bacteria. However, the knowledge about the bacterial signaling cascade of c-di-AMP as a newly recognized messenger is still in an early stage. So far, only a c-di-AMP binding transcription factor and a potassium transport-associated protein have been characterized in *M. smegmatis* and *S. aureus*, respectively (28, 29). In this study, we identified CabP as a c-di-AMP binding protein and demonstrated that c-di-AMP reduced potassium uptake via interference of the CabP-SPD_0076 interaction in *S. pneumoniae* (Fig. 7). Interestingly, in a most recent report, three out of the four c-di-AMP binding proteins identified in *S. aureus* have been implicated in potassium transport (29). Therefore, the role of c-di-AMP in controlling bacterial potassium uptake is likely a common scheme. In our experiments using c-di-AMP chromatography, we also observed that multiple proteins were eluted, with CabP as the most abundant protein (Fig. 1B). In this study, *S. pneumoniae* DacA was not significantly enriched by affinity chromatography. It is likely either that DacA has lower affinity to c-di-AMP than CabP or that DacA differs from DisA in binding c-di-AMP. Our future study will explore other potential c-di-AMP binding proteins in pneumococci.

CabP belongs to the Trk/Ktr/HKT family of proteins. Bacterial Trk and Ktr systems are multimeric, each complex consisting of a hydrophobic ion-conducting transmembrane subunit and at least one peripheral regulatory subunit (41). The major difference between the Trk and Ktr subfamilies is the cation they cotransport with K^+ . Trk transporters are thought to utilize H^+ , while Ktr transporters utilize Na^+ (41). Both *S. aureus* KtrA and *S. pneumoniae* CabP are peripheral regulatory subunits for potassium uptake. Potential cations they might cotransport with K^+ have not been experimentally identified. Furthermore, it is unknown whether KtrA or CabP has any other biological roles aside from potassium uptake that are mediated by c-di-AMP, which warrants further investigation.

We showed that the c-di-AMP binding site in CabP was highly specific to c-di-AMP and that the binding of c-di-AMP by CabP was not affected by competition with the tested nucleotides, including ATP, cAMP, AMP, pApA, and c-di-GMP. Such results have also been noted with *M. smegmatis* DarR and *S. aureus* KtrA (28, 29). These observations suggest that c-di-AMP signaling is likely distinct from that of other well-known signaling nucleotides, including cAMP and c-di-GMP. In contrast, a cAMP recep-

tor protein (CRP) is capable of binding c-di-GMP in some bacteria (45–48). *S. aureus* KtrA and its homologs in other bacteria have been shown to bind ATP at the N terminus (29, 39, 49). Additionally, ADP, AMP, and NAD(H) have been reported to interact with Trk or Ktr proteins (49, 50), indicating that these nucleotides may also play a substantial role in coordinating potassium transport, despite this not having been shown *in vivo* (41). Diadenylate cyclase is found mostly in Gram-positive bacteria (15, 51), and it is very likely that another nucleotide(s) might play a role similar to that of c-di-AMP for potassium uptake, particularly in bacteria that lack c-di-AMP.

The structural studies of Trk/Ktr/HKT family transporters have been focused on regulatory KtrA and the RCK/NTN domain of these proteins (41). However, cocrystallization of c-di-AMP with these proteins has not been explored. It is still unknown how c-di-AMP interacts with CabP. We have shown that CabP forms a stable octamer in solution, which is consistent with the oligomerization of *B. subtilis* KtrA (39, 40) but differs from earlier observations of RCK domains as a tetrameric model (52). Nonetheless, oligomerization of CabP seems unnecessary for binding c-di-AMP, since the interaction between c-di-AMP and CabP was detected with a monomeric CabP when analyzed by denaturing SDS-PAGE (Fig. 3A). It is possible that c-di-AMP alters the conformation of CabP, which affects the interface between CabP and the potassium transporter SPD_0076.

In conclusion, this study revealed a direct role for c-di-AMP in controlling potassium uptake through interference of a protein-protein interaction between CabP and the potassium transporter. Since potassium is an essential nutrient for all living organisms, the regulatory role of c-di-AMP provides intriguing insights into bacterial physiology.

ACKNOWLEDGMENTS

We thank Joseph Wade, Zhaoqing Luo, and Inga Hänelt for providing *E. coli* strains, Hongmin Li for assistance with gel filtration, and Qishan Lin for assistance with proteomics.

This study was supported by National Institutes of Health grant DC006917 to D.W.M.

REFERENCES

- Gomelsky M. 2011. cAMP, c-di-GMP, c-di-AMP and now cGMP: bacteria use them all! *Mol. Microbiol.* 79:562–565. <http://dx.doi.org/10.1111/j.1365-2958.2010.07514.x>.
- Kalia D, Merrey G, Nakayama S, Zheng Y, Zhou J, Luo Y, Guo M, Roembke BT, Sintim HO. 2013. Nucleotide, c-di-GMP, c-di-AMP, cGMP, cAMP, (p)ppGpp signaling in bacteria and implications in pathogenesis. *Chem. Soc. Rev.* 42:305–341. <http://dx.doi.org/10.1039/c2cs35206k>.
- McDonough KA, Rodriguez A. 2012. The myriad roles of cyclic AMP in microbial pathogens: from signal to sword. *Nat. Rev. Microbiol.* 10:27–38. <http://dx.doi.org/10.1038/nrmicro2688>.
- Römling U, Galperin MY, Gomelsky M. 2013. Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. *Microbiol. Mol. Biol. Rev.* 77:1–52. <http://dx.doi.org/10.1128/MMBR.00043-12>.
- Witte G, Hartung S, Buttner K, Hopfner KP. 2008. Structural biochemistry of a bacterial checkpoint protein reveals diadenylate cyclase activity regulated by DNA recombination intermediates. *Mol. Cell* 30:167–178. <http://dx.doi.org/10.1016/j.molcel.2008.02.020>.
- Bai Y, Yang J, Zhou X, Ding X, Eisele LE, Bai G. 2012. *Mycobacterium tuberculosis* Rv3586 (DacA) is a diadenylate cyclase that converts ATP or ADP into c-di-AMP. *PLoS One* 7:e35206. <http://dx.doi.org/10.1371/journal.pone.0035206>.
- Corrigan RM, Abbott JC, Burhenne H, Kaever V, Grundling A. 2011. c-di-AMP is a new second messenger in *Staphylococcus aureus* with a role in controlling cell size and envelope stress. *PLoS Pathog.* 7:e1002217. <http://dx.doi.org/10.1371/journal.ppat.1002217>.

8. Rao F, See RY, Zhang D, Toh DC, Ji Q, Liang ZX. 2010. YybT is a signaling protein that contains a cyclic dinucleotide phosphodiesterase domain and a GGDEF domain with ATPase activity. *J. Biol. Chem.* 285: 473–482. <http://dx.doi.org/10.1074/jbc.M109.040238>.
9. Bai Y, Yang J, Eisele LE, Underwood AJ, Koestler BJ, Waters CM, Metzger DW, Bai G. 2013. Two DHH subfamily 1 proteins in *Streptococcus pneumoniae* possess cyclic di-AMP phosphodiesterase activity and affect bacterial growth and virulence. *J. Bacteriol.* 195:5123–5132. <http://dx.doi.org/10.1128/JB.00769-13>.
10. Luo Y, Helmann JD. 2012. Analysis of the role of *Bacillus subtilis* sigma(M) in beta-lactam resistance reveals an essential role for c-di-AMP in peptidoglycan homeostasis. *Mol. Microbiol.* 83:623–639. <http://dx.doi.org/10.1111/j.1365-2958.2011.07953.x>.
11. Mehne FM, Gunka K, Eilers H, Herzberg C, Kaever V, Stulke J. 2013. Cyclic di-AMP homeostasis in *Bacillus subtilis*: both lack and high-level accumulation of the nucleotide are detrimental for cell growth. *J. Biol. Chem.* 288:2004–2017. <http://dx.doi.org/10.1074/jbc.M112.395491>.
12. Woodward JJ, Iavarone AT, Portnoy DA. 2010. c-di-AMP secreted by intracellular *Listeria monocytogenes* activates a host type I interferon response. *Science* 328:1703–1705. <http://dx.doi.org/10.1126/science.1189801>.
13. Kamegaya T, Kuroda K, Hayakawa Y. 2011. Identification of a *Streptococcus pyogenes* SF370 gene involved in production of c-di-AMP. *Nagoya J. Med. Sci.* 73:49–57.
14. Barker JR, Koestler BJ, Carpenter VK, Burdette DL, Waters CM, Vance RE, Valdivia RH. 2013. STING-dependent recognition of cyclic di-AMP mediates type I interferon responses during *Chlamydia trachomatis* infection. *MBio* 4:e00018–13. <http://dx.doi.org/10.1128/mBio.00018-13>.
15. Römling U. 2008. Great times for small molecules: c-di-AMP, a second messenger candidate in Bacteria and Archaea. *Sci. Signal.* 1:pe39. <http://dx.doi.org/10.1126/scisignal.133pe39>.
16. Banerjee R, Gretes M, Harlem C, Basuino L, Chambers HF. 2010. A *mecA*-negative strain of methicillin-resistant *Staphylococcus aureus* with high-level beta-lactam resistance contains mutations in three genes. *Antimicrob. Agents Chemother.* 54:4900–4902. <http://dx.doi.org/10.1128/AAC.00594-10>.
17. Griffiths JM, O'Neill AJ. 2012. Loss of function of the GdpP protein leads to joint beta-lactam/glycopeptide tolerance in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 56:579–581. <http://dx.doi.org/10.1128/AAC.05148-11>.
18. Witte CE, Whiteley AT, Burke TP, Sauer JD, Portnoy DA, Woodward JJ. 2013. Cyclic di-AMP is critical for *Listeria monocytogenes* growth, cell wall homeostasis, and establishment of infection. *MBio* 4:e00282–13. <http://dx.doi.org/10.1128/mBio.00282-13>.
19. Pozzi C, Waters EM, Rudkin JK, Schaeffer CR, Lohan AJ, Tong P, Loftus BJ, Pier GB, Massey RC, O'Gara JP. 2012. Methicillin resistance alters the biofilm phenotype and attenuates virulence in *Staphylococcus aureus* device-associated infections. *PLoS Pathog.* 8:e1002626. <http://dx.doi.org/10.1371/journal.ppat.1002626>.
20. Cron LE, Stol K, Burghout P, van Selm S, Simonetti ER, Bootsma HJ, Hermans PW. 2011. Two DHH subfamily 1 proteins contribute to pneumococcal virulence and confer protection against pneumococcal disease. *Infect. Immun.* 79:3697–3710. <http://dx.doi.org/10.1128/IAI.01383-10>.
21. Schwartz KT, Carleton JD, Quillin SJ, Rollins SD, Portnoy DA, Leber JH. 2012. Hyperinduction of host beta interferon by a *Listeria monocytogenes* strain naturally overexpressing the multidrug efflux pump MdrT. *Infect. Immun.* 80:1537–1545. <http://dx.doi.org/10.1128/IAI.06286-11>.
22. Yamamoto T, Hara H, Tsuchiya K, Sakai S, Fang R, Matsuura M, Nomura T, Sato F, Mitsuyama M, Kawamura I. 2012. *Listeria monocytogenes* strain-specific impairment of the TetR regulator underlies the drastic increase in cyclic di-AMP secretion and beta interferon-inducing ability. *Infect. Immun.* 80:2323–2332. <http://dx.doi.org/10.1128/IAI.06162-11>.
23. Burdette DL, Monroe KM, Sotelo-Troha K, Iwig JS, Eckert B, Hyodo M, Hayakawa Y, Vance RE. 2011. STING is a direct innate immune sensor of cyclic di-GMP. *Nature* 478:515–518. <http://dx.doi.org/10.1038/nature10429>.
24. Jin L, Hill KK, Filak H, Mogan J, Knowles H, Zhang B, Perraud AL, Cambier JC, Lenz LL. 2011. MPYS is required for IFN response factor 3 activation and type I IFN production in the response of cultured phagocytes to bacterial second messengers cyclic-di-AMP and cyclic-di-GMP. *J. Immunol.* 187:2595–2601. <http://dx.doi.org/10.4049/jimmunol.1100088>.
25. Sauer JD, Sotelo-Troha K, von Moltke J, Monroe KM, Rae CS, Brubaker SW, Hyodo M, Hayakawa Y, Woodward JJ, Portnoy DA, Vance RE. 2011. The N-ethyl-N-nitrosourea-induced Goldenticket mouse mutant reveals an essential function of Sting in the in vivo interferon response to *Listeria monocytogenes* and cyclic dinucleotides. *Infect. Immun.* 79: 688–694. <http://dx.doi.org/10.1128/IAI.00999-10>.
26. Bowie AG. 2012. Innate sensing of bacterial cyclic dinucleotides: more than just STING. *Nat. Immunol.* 13:1137–1139. <http://dx.doi.org/10.1038/ni.2469>.
27. Parvatiyar K, Zhang Z, Teles RM, Ouyang S, Jiang Y, Iyer SS, Zaver SA, Schenk M, Zeng S, Zhong W, Liu ZJ, Modlin RL, Liu YJ, Cheng G. 2012. The helicase DDX41 recognizes the bacterial secondary messengers cyclic di-GMP and cyclic di-AMP to activate a type I interferon immune response. *Nat. Immunol.* 13:1155–1161. <http://dx.doi.org/10.1038/ni.2460>.
28. Zhang L, Li W, He ZG. 2013. DarR, a TetR-like transcriptional factor, is a cyclic di-AMP responsive repressor in *Mycobacterium smegmatis*. *J. Biol. Chem.* 288:3085–3096. <http://dx.doi.org/10.1074/jbc.M112.428110>.
29. Corrigan RM, Campeotto I, Jegannathan T, Roelofs KG, Lee VT, Grunlding A. 2013. Systematic identification of conserved bacterial c-di-AMP receptor proteins. *Proc. Natl. Acad. Sci. U. S. A.* 110:9084–9089. <http://dx.doi.org/10.1073/pnas.1300595110>.
30. El Qaidi S, Yang J, Zhang JR, Metzger DW, Bai G. 2013. The vitamin B6 biosynthesis pathway in *Streptococcus pneumoniae* is controlled by pyridoxal 5'-phosphate and the transcription factor PdxR and has an impact on ear infection. *J. Bacteriol.* 195:2187–2196. <http://dx.doi.org/10.1128/JB.00041-13>.
31. GE Healthcare. Gel filtration principles and methods.
32. Bai G, McCue LA, McDonough KA. 2005. Characterization of *Mycobacterium tuberculosis* Rv3676 (CRP_{Mt}), a cyclic AMP receptor protein-like DNA binding protein. *J. Bacteriol.* 187:7795–7804. <http://dx.doi.org/10.1128/JB.187.22.7795-7804.2005>.
33. Lu L, Ma Y, Zhang JR. 2006. *Streptococcus pneumoniae* recruits complement factor H through the amino terminus of CbpA. *J. Biol. Chem.* 281: 15464–15474. <http://dx.doi.org/10.1074/jbc.M602404200>.
34. Macrina FL, Evans RP, Tobian JA, Hartley DL, Clewell DB, Jones KR. 1983. Novel shuttle plasmid vehicles for *Escherichia-Streptococcus* transgeneric cloning. *Gene* 25:145–150. [http://dx.doi.org/10.1016/0378-1119\(83\)90176-2](http://dx.doi.org/10.1016/0378-1119(83)90176-2).
35. Karimova G, Pidoux J, Ullmann A, Ladant D. 1998. A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proc. Natl. Acad. Sci. U. S. A.* 95:5752–5756. <http://dx.doi.org/10.1073/pnas.95.10.5752>.
36. Bai G, Schaak DD, Smith EA, McDonough KA. 2011. Dysregulation of serine biosynthesis contributes to the growth defect of a *Mycobacterium tuberculosis* *crp* mutant. *Mol. Microbiol.* 82:180–198. <http://dx.doi.org/10.1111/j.1365-2958.2011.07806.x>.
37. Stumpe S, Bakker EP. 1997. Requirement of a large K⁺-uptake capacity and of extracytoplasmic protease activity for protamine resistance of *Escherichia coli*. *Arch. Microbiol.* 167:126–136. <http://dx.doi.org/10.1007/s002030050425>.
38. Lanie JA, Ng WL, Kazmierczak KM, Andrzejewski TM, Davidsen TM, Wayne KJ, Tettelin H, Glass JL, Winkler ME. 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. <http://dx.doi.org/10.1128/JB.01148-06>.
39. Vieira-Pires RS, Szollosi A, Morais-Cabral JH. 2013. The structure of the KtrAB potassium transporter. *Nature* 496:323–328.
40. Albright RA, Ibar JL, Kim CU, Gruner SM, Morais-Cabral JH. 2006. The RCK domain of the KtrAB K⁺ transporter: multiple conformations of an octameric ring. *Cell* 126:1147–1159. <http://dx.doi.org/10.1016/j.cell.2006.08.028>.
41. Corratgé-Faillie C, Jabnoute M, Zimmermann S, Very AA, Fizames C, Sentenac H. 2010. Potassium and sodium transport in non-animal cells: the Trk/Ktr/HKT transporter family. *Cell. Mol. Life Sci.* 67:2511–2532. <http://dx.doi.org/10.1007/s00018-010-0317-7>.
42. van de Rijn I, Kessler RE. 1980. Growth characteristics of group A streptococci in a new chemically defined medium. *Infect. Immun.* 27: 444–448.
43. Holtmann G, Bakker EP, Uozumi N, Bremer E. 2003. KtrAB and KtrCD: two K⁺ uptake systems in *Bacillus subtilis* and their role in adaptation to hypertonicity. *J. Bacteriol.* 185:1289–1298. <http://dx.doi.org/10.1128/JB.185.4.1289-1298.2003>.
44. Mosimann M, Goshima S, Wenzler T, Luscher A, Uozumi N, Maser P.

2010. A Trk/HKT-type K⁺ transporter from *Trypanosoma brucei*. Eukaryot. Cell 9:539–546. <http://dx.doi.org/10.1128/EC.00314-09>.
45. Chin KH, Lee YC, Tu ZL, Chen CH, Tseng YH, Yang JM, Ryan RP, McCarthy Y, Dow JM, Wang AH, Chou SH. 2010. The cAMP receptor-like protein CLP is a novel c-di-GMP receptor linking cell-cell signaling to virulence gene expression in *Xanthomonas campestris*. J. Mol. Biol. 396: 646–662. <http://dx.doi.org/10.1016/j.jmb.2009.11.076>.
 46. Fazli M, O'Connell A, Nilsson M, Niehaus K, Dow JM, Givskov M, Ryan RP, Tolker-Nielsen T. 2011. The CRP/FNR family protein Bcam1349 is a c-di-GMP effector that regulates biofilm formation in the respiratory pathogen *Burkholderia cenocepacia*. Mol. Microbiol. 82:327–341. <http://dx.doi.org/10.1111/j.1365-2958.2011.07814.x>.
 47. Leduc JL, Roberts GP. 2009. Cyclic di-GMP allosterically inhibits the CRP-like protein (Clp) of *Xanthomonas axonopodis* pv. citri. J. Bacteriol. 191:7121–7122. <http://dx.doi.org/10.1128/JB.00845-09>.
 48. Tao F, He YW, Wu DH, Swarup S, Zhang LH. 2010. The cyclic nucleotide monophosphate domain of *Xanthomonas campestris* global regulator Clp defines a new class of cyclic di-GMP effectors. J. Bacteriol. 192: 1020–1029. <http://dx.doi.org/10.1128/JB.01253-09>.
 49. Johnson HA, Hampton E, Lesley SA. 2009. The *Thermotoga maritima* Trk potassium transporter—from frameshift to function. J. Bacteriol. 191: 2276–2284. <http://dx.doi.org/10.1128/JB.01367-08>.
 50. Schlösser A, Hamann A, Bossemeyer D, Schneider E, Bakker EP. 1993. NAD⁺ binding to the *Escherichia coli* K(+) uptake protein TrkA and sequence similarity between TrkA and domains of a family of dehydrogenases suggest a role for NAD⁺ in bacterial transport. Mol. Microbiol. 9:533–543. <http://dx.doi.org/10.1111/j.1365-2958.1993.tb01714.x>.
 51. Corrigan RM, Grundling A. 2013. Cyclic di-AMP: another second messenger enters the fray. Nat. Rev. Microbiol. 11:513–524. <http://dx.doi.org/10.1038/nrmicro3069>.
 52. Roosild TP, Miller S, Booth IR, Choe S. 2002. A mechanism of regulating transmembrane potassium flux through a ligand-mediated conformational switch. Cell 109:781–791. [http://dx.doi.org/10.1016/S0092-8674\(02\)00768-7](http://dx.doi.org/10.1016/S0092-8674(02)00768-7).
 53. Avery OT, Macleod CM, McCarty M. 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types: induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. J. Exp. Med. 79:137–158. <http://dx.doi.org/10.1084/jem.79.2.137>.