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# A Novel Mode of Regulation of the *Staphylococcus aureus* Catabolite Control Protein A (CcpA) Mediated by Stk1 Protein Phosphorylation<sup>\*[S]</sup>

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**Background:** The catabolite control protein A (CcpA) plays an important role in *Staphylococcus aureus* virulence, biofilm formation, and central metabolism.

**Results:** Phosphorylation of CcpA negatively affects its DNA binding activity and thus the expression of its target genes.

**Conclusion:** CcpA activity is modulated by Stk1 phosphorylation.

**Significance:** This study highlights that phosphorylation of CcpA represents a novel regulatory control mechanism for this major transcriptional factor.

The *Staphylococcus aureus* serine/threonine protein kinase Stk1 (also known as PknB) affects different key pathways such as cell wall metabolism, antibiotic susceptibility, and regulation of virulence. Here we report that the catabolite control protein A (CcpA), a highly conserved regulator of carbon catabolite repression and virulence in a number of Gram-positive pathogens, was efficiently phosphorylated *in vitro* and *in vivo* by Stk1 in *S. aureus*, whereas the CcpA homologues of *Bacillus subtilis* and *Bacillus anthracis* were not affected by the Stk1 orthologue PrkC. Mass spectrometry and mutational analyses identified Thr-18 and Thr-33 as the phosphoacceptors; both are located in the DNA binding domain of this protein. Electrophoretic mobility shift assays demonstrated that the CcpA DNA binding activity was completely abrogated for the phosphorylated CcpA. The physiological relevance of CcpA phosphorylation was assessed by generating CcpA phosphoablative (T18A/T33A) or phosphomimetic (T18D/T33D) mutants. In contrast to the wild-type and phosphoablative *ccpA* alleles, introduction of the phosphomimetic *ccpA* allele in a  $\Delta$ *ccpA* mutant failed to restore the parental biofilm formation profile and the transcription of *citZ* and *hla* to levels seen with the wild type. The strong up regulation of *ccpA* transcripts and CcpA level in the *ccpA* mutant trans-complemented with the phosphomimetic CcpA variant suggest furthermore that CcpA acts as a negative regulator of its own expression. Together, these findings demonstrate that Stk1-driven phosphorylation of CcpA inhibits its DNA binding activity toward its regulon in *S. aureus*, representing a novel

regulatory mechanism of CcpA activity in addition to the well known regulation via HprKP/Hpr in this clinically important pathogen.

Carbon catabolite repression is a widespread, global regulatory phenomenon in bacteria that allows modulation of the expression of genes and operons involved in carbon utilization and metabolism in the presence of the preferred carbon source(s). In carbon catabolite repression, the presence of a preferred carbon source usually induces the repression of genes and operons whose products are involved in the metabolism of alternative, less preferred carbon sources. One of the important and highly conserved regulators of carbon catabolite regulation in low-GC Gram-positive bacteria is the catabolite control protein A (CcpA),<sup>3</sup> a member of the LacI/GalR family of bacterial regulatory proteins, which has been intensively studied in *Bacillus subtilis* (for reviews, see Refs. 1 and 2). In the presence of glucose or other rapidly metabolized carbon sources, CcpA is activated by formation of a complex with its co-regulator Hpr that needs to be phosphorylated on residue Ser-46 by its cognate kinase HprKP. The CcpA·Hpr-Ser(P)-46 complex has an increased affinity for particular *cis*-acting sequences, termed catabolite-responsive element (*cre*) sequences, and thereby represses or enhances gene expression depending on the position of the *cre* in relation to the operator sequence (3, 4). In a number of Gram-positive pathogens such as *Bacillus anthracis* (5), *Clostridium difficile* (6), *Staphylococcus aureus* (7), *Staphylococcus epidermidis* (8), *Streptococcus pneumoniae* (9), and *Streptococcus pyogenes* (10), CcpA is also involved in the regulation of virulence determinants.

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<sup>3</sup> The abbreviations used are: CcpA, catabolite control protein A; STPK, Ser/Thr protein kinase; *cre*, catabolite-responsive element; TSB, tryptic soy broth; CcpA-P, phosphorylated CcpA; HprKP, Hpr Kinase Phosphatase; Hpr-P, phosphorylated Hpr; Bs, *B. subtilis*; Ba, *B. anthracis*.

# Regulation of CcpA by Ser/Thr Kinase Phosphorylation

**TABLE 1**

Strains and plasmids used in this study

Strains and plasmids	Genotype or description <sup>a</sup>	Source/Ref.
<b><i>E. coli</i> strains</b>		
10G	<i>E. coli</i> derivative ultracompetent cells used for general cloning; F <sup>−</sup> <i>mcrA</i> D( <i>mrr-hsdRMS-mcrBC</i> ) f80 <i>dlacZ</i> ΔM15 D <i>lacX74 endA1 recA1 araD139 D (ara, leu) 7697 galU galK rpsL nupG-tonA</i> ;	Lucigen
BL21(DE3)Star	F2 <i>ompT hsdSB</i> (rB2 mB2) <i>gal dcm</i> (DE3); used to express recombinant proteins in <i>E. coli</i>	Stratagene
<b><i>S. aureus</i> strains</b>		
RN4220	NCTC 8325-4 r <sup>−</sup> m <sup>+</sup> (restriction-negative, modification-positive)	26
SA113	ATCC35556, PIA-dependent biofilm producer, NCTC 8325 derivative, <i>rsbU</i> <sup>−</sup>	47
KS66	SA113 Δ <i>ccpA</i> , biofilm-negative SA113 derivative (Tet <sup>R</sup> )	7
ST1004	NCTC 8325-4 Δ <i>stk1</i> derivative	16
<b><i>E. coli</i> plasmids</b>		
pETPhos	pET15b (Novagen) derivative including the replacement of the thrombin site coding sequence with a tobacco etch virus protease site and Ser to Gly mutagenesis in the N-terminal His tag (Amp <sup>R</sup> )	22
pETPhos_ <i>ccpA</i>	pETPhos derivative used to express His-tagged fusion of CcpA (Amp <sup>R</sup> )	This work
pETPhos_ <i>ccpA</i> _T18A	pETPhos derivative used to express His-tagged fusion of CcpA_T18A (Amp <sup>R</sup> )	This work
pETPhos_ <i>ccpA</i> _T33A	pETPhos derivative used to express His-tagged fusion of CcpA_T33A (Amp <sup>R</sup> )	This work
pETPhos_ <i>ccpA</i> _T18A/T33A	pETPhos derivative used to express His-tagged fusion of CcpA_T18A/T33A (Amp <sup>R</sup> )	This work
pETPhos_ <i>ccpA</i> <sup>Bs</sup>	pETPhos derivative used to express His-tagged fusion of <i>B. subtilis</i> CcpA in <i>E. coli</i> (Amp <sup>R</sup> )	This work
pETPhos_ <i>ccpA</i> <sup>Bsa</sup>	pETPhos derivative used to express His-tagged fusion of <i>B. anthracis</i> CcpA in <i>E. coli</i> (Amp <sup>R</sup> )	5
pGEX_ <i>prkC</i>	pGEX-4T derivative used to express GST-tagged fusion of <i>B. subtilis</i> PrkC kinase domain in <i>E. coli</i> (Amp <sup>R</sup> )	This work
pCDFDuet-1	pET vector derivative designed for the co-expression of two proteins under <i>T7lac</i> promoter induction (Spec <sup>R</sup> )	Novagen
pDuet_ <i>ccpA</i>	pET vector derivative used for the co-expression of Stk1 and CcpA proteins under <i>T7lac</i> promoter induction (Spec <sup>R</sup> )	This work
<b><i>S. aureus</i> plasmids</b>		
pMK4_Pprot	pMK4-derived <i>E. coli</i> - <i>S. aureus</i> shuttle vector with a constitutive promoter (Amp <sup>R</sup> in <i>E. coli</i> /Cm <sup>R</sup> in <i>S. aureus</i> )	25
pMK4_Pprot_ <i>ccpA</i>	pMK4_Pprot derivative used to express His-tagged fusion of CcpA in <i>S. aureus</i> (Amp <sup>R</sup> in <i>E. coli</i> /Cm <sup>R</sup> in <i>S. aureus</i> )	This work
pMK4_Pprot_ <i>ccpA</i> _T18A/T33A	pMK4_Pprot derivative used to express His-tagged fusion of CcpA_T18A/T33A in <i>S. aureus</i> (Amp <sup>R</sup> in <i>E. coli</i> /Cm <sup>R</sup> in <i>S. aureus</i> )	This work
pCN34	<i>E. coli</i> - <i>S. aureus</i> shuttle vector (Amp <sup>R</sup> in <i>E. coli</i> /Km <sup>R</sup> in <i>S. aureus</i> )	27
pCN34_ <i>ccpA</i>	pCN34 derivative harboring <i>ccpA</i> and its native promoter (Amp <sup>R</sup> in <i>E. coli</i> /Km <sup>R</sup> in <i>S. aureus</i> )	This work
pCN34_ <i>ccpA</i> _ <i>Ala</i>	pCN34 with a <i>ccpA</i> derivative carrying the CcpA_T18A/T33A mutations under the control of the <i>ccpA</i> promoter (Amp <sup>R</sup> in <i>E. coli</i> /Km <sup>R</sup> in <i>S. aureus</i> )	This work
pCN34_ <i>ccpA</i> _ <i>Asp</i>	pCN34 with a <i>ccpA</i> derivative carrying the CcpA_T18D/T33D mutations under the control of the <i>ccpA</i> promoter (Amp <sup>R</sup> in <i>E. coli</i> /Km <sup>R</sup> in <i>S. aureus</i> )	This work

<sup>a</sup> Amp<sup>R</sup>, ampicillin-resistant; Cm<sup>R</sup>, chloramphenicol-resistant; Km<sup>R</sup>, kanamycin-resistant; Spec<sup>R</sup>, spectinomycin-resistant; Tet<sup>R</sup>, tetracycline-resistant; PIA, polysaccharide intercellular adhesin.

In *S. aureus*, which is one of the leading causes for nosocomial infections and an important human pathogen responsible for a wide variety of diseases, including soft tissue infections, toxic shock syndrome, and necrotizing pneumonia (11), CcpA was shown to affect antibiotic resistance, biofilm formation, expression of toxins such as *hla* (encoding α-hemolysin) and *tst* (encoding toxic shock syndrome toxin), and infectivity of this bacterium (7, 12–15). Recent transcriptome and proteome analyses confirmed the broad effects of CcpA on gene expression in *S. aureus* and suggested this LacI/GalR type of regulator to be active in the presence and absence of glucose (13). However, the regulatory mechanism(s) that modulates the DNA binding properties of the *S. aureus* CcpA in the absence of glucose has not yet been identified.

Signal transduction through reversible protein phosphorylation is a key regulatory mechanism of both prokaryotes and eukaryotes. To overcome the stressful conditions imposed by a host, pathogens have evolved various protective and offensive responses generally achieved through cascades of phosphorylation reactions. Many of the stimuli encountered are transduced via sensor kinases in the membrane, allowing the pathogen to adapt for survival in hostile environments. In addition to the classical two-component systems, *S. aureus* contains one eukaryotic-like serine/threonine protein kinase (STPK), Stk1 (16, 17). Recent studies provided the first insights into the reg-

ulatory function of Stk1 and the molecular mechanisms of the Ser/Thr phosphorylation system in *S. aureus* (17). Interestingly, the Ser/Thr kinase Stk1 was identified to influence central metabolic processes in *S. aureus* (18) and the activity of important regulatory factors such as SarA (19), MgrA (20), and LuxS (21).

Therefore, we investigated the possible regulation of CcpA by Stk1. To this end, we have identified CcpA as a new substrate of Stk1 and identified its phosphorylation sites. This allowed us to address the role and contribution of phosphorylation in regulating CcpA activity. Importantly, with both *in vitro* and *in vivo* approaches, we provide for the first time evidence that phosphorylation negatively regulates the activity of CcpA, thus inhibiting biofilm formation and modifying CcpA target gene expression.

## EXPERIMENTAL PROCEDURES

**Bacterial Strains and Growth Conditions**—Strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37 °C in LB medium supplemented with 100 μg/ml ampicillin or 100 μg/ml spectinomycin when required. *S. aureus* isolates were plated on tryptic soy agar supplemented with 10 μg/ml chloramphenicol or 50 μg/ml kanamycin when required or grown in filter-sterilized tryptic soy broth (TSB) medium at 37 °C and 150 rpm with a culture to flask volume of 1:10.

TABLE 2

## Primers used in this study

Nterm, N-terminal; Cterm, C-terminal; forw, forward; rev, reverse.

	5' to 3' sequence <sup>a</sup>
<b>Primers</b>	
NtermCcpA	TAATAGCTCATATGACAGTACATATATGATGTAGCAAGAGAAGCGCG (NdeI)
CtermCcpA	TATGGATCCTTATTTTGTAGTTCCTCGGTATTCAATTCTGTGAGG (BamHI)
NtermCcpA <sup>BS</sup>	TAATAGCTGCTAGCATGAGCAATATTACGATCTACGAT (NheI)
CtermCcpA <sup>BS</sup>	TATGGATCCCTATGACTTGGTTGA CTTCTAAGCTC (BamHI)
NtermPrkC	TATGGATCCGTGCTAATCGGCAAGCGGATCAGCGGG (BamHI)
CtermPrkC	TATAAGCTTCTACACGGCCACTTTTTTCTTTTGCC (HindIII)
pMK4_NtermCcpA	TAATAGCTGGATCCATGACAGTTACTATATATGATGTAGCAAGAGAAGCG (BamHI)
pMK4_CtermCcpA	TATCTGCAGTTAGTGATGATGATGATGATGATGTTTTGTAGTTCCTCGGTATTC (PstI)
pCN34_NtermCcpA	TATGGATCCTGTTGAAGTAGAATATTATC (BamHI)
pCN34_CtermCcpA	GAAGAATTCTTATTTGTAGTTCCTCGGTATTCAATTCTGTG (EcoRI)
Nterm <i>hla</i> probe	TAATTAATACCCTTTTCTCTATTTTC
Cterm <i>hla</i> probe	GTTACTGAGCTGACTATACGTGTTTTTCAT
Nterm <i>citZ</i> probe	ATTGTAAAATTCATGGATTATTCAC
Cterm <i>citZ</i> probe	CTAAACCTCTTTGTAATTCGCCAT
Nterm <i>ald</i> probe	TGCCCATTCGCTTCATG TGAACC
Cterm <i>ald</i> probe	CTCCCTGGTATACCAATTTTCAT
Nterm <i>tst</i> probe	CACTTTGTTTTTGTCTATTTGTAAAC
Cterm <i>tst</i> probe	CATTAGTAATTTTTTATTCAT
Nterm <i>malR</i> probe	TGACTAAACAATCGATTTTAA
Cterm <i>malR</i> probe	GCAACATCTTTAATCGTAACCAT
Nterm <i>pckA</i> probe	GCTGGGTTCATTGGGTCCATGTC
Cterm <i>pckA</i> probe	CAGTGTATGTGTCTACTGACAT
<b>RT-PCR primers</b>	
<i>ccpA</i> forw	AAAGGCAATTTGCCAGATGC
<i>ccpA</i> rev	AATTGCTTCTTCGTCGCTGATAC
<i>citZ</i> forw	CCGTAGGTTCTCTGAAAGGGC
<i>citZ</i> rev	AACATCGTCATAACTTGTTCGTTTG
<i>gyrB</i> forw	GACTGATGCCGATGTGGA
<i>gyrB</i> rev	AACGGTGGCTGTGCAATA
<i>hla</i> forw	AACCCGGTATATGGCAATCAACT
<i>hla</i> rev	CTGCTGCTTCATAGAGCCATT
16 S rRNA forw	CCATAAAGTTGTTCTCAGTT
16 S rRNA rev	CATGTCGATCTACGATTACT

<sup>a</sup> Restriction sites are underlined and specified in parentheses.

**Cloning, Expression, and Purification of CcpA and Mutant Proteins from *S. aureus***—The *ccpA* gene was amplified by PCR using *S. aureus* N315 chromosomal DNA as a template with the primers listed in Table 2 containing an NdeI and a BamHI restriction site, respectively. The corresponding amplified product was digested with NdeI and BamHI and ligated into the pETPhos plasmid (22), a variant of pET15b (Novagen) that includes a tobacco etch virus protease site instead of the thrombin site and an N-terminal His tag free of Ser/Thr/Tyr residues, thus generating pETPhos\_*ccpA*. pETPhos\_*ccpA* derivatives harboring threonine to alanine substitutions at Thr-18 and/or Thr-33 of the *ccpA* open reading frame were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and resulted in the construction of plasmids pETPhos\_*ccpA*\_T18A, pETPhos\_*ccpA*\_T33A, and pETPhos\_*ccpA*\_T18A/T33A, respectively. The duet strategy was used to generate a hyperphosphorylated CcpA protein as described previously (23). The *ccpA* gene was cloned into the pCDF-Duet-1 vector containing the Stk1 kinase domain (21), generating plasmid pDuet\_*ccpA*, which was used to transform *E. coli* BL21(DE3)Star cells. pDuet\_*ccpA* *E. coli* cells were used to overexpress and purify His-tagged CcpA as described below. All constructs were verified by DNA sequencing. Recombinant strains harboring the different constructs were used to inoculate 200 ml of LB medium supplemented with ampicillin or spectinomycin, and resulting cultures were incubated at 37 °C with shaking until the optical density of the culture reached an  $A_{600}$  of 0.5. Isopropyl 1-thio- $\beta$ -D-galactopyranoside (1 mM

final) was added to induce the overexpression, and growth was continued for 3 h at 37 °C. Purifications of the His-tagged recombinants were performed as described by the manufacturer (Qiagen).

**Mass Spectrometry Analysis**—Purified His-tagged hyperphosphorylated CcpA from the *E. coli* strain carrying pDuet\_*ccpA* and co-expressing the Stk1 kinase was subjected to mass spectrometry without further treatment. Subsequent mass spectrometric analyses were performed as reported previously (24).

**Overexpression and Purification of CcpA in *S. aureus***—The *ccpA* gene was amplified by PCR using *S. aureus* N315 chromosomal DNA as a template with the primers listed in Table 2 containing a BamHI and a PstI site, respectively. The PCR product harboring a C-terminal His-tagged fusion was digested with BamHI/PstI, enabling direct cloning into the pMK4-pProt expression vector cut with the same enzymes, thus generating pMK4\_*ccpA*, which allows constitutive expression in *S. aureus* as described previously (16, 25). The double mutant corresponding to T18A and T33A was generated using the QuikChange site-directed mutagenesis kit (Stratagene), generating the pMK4\_*ccpA*\_T18A/T33A construct. The resulting vectors were used to transform *S. aureus* strain RN4220 (26), which served as donor for transducing the plasmids into the *stk1* mutant ST1004 (*S. aureus* 8325-4  $\Delta$ *stk1*) (16). Purification of soluble CcpA proteins was performed on nickel-nitrilotriacetic acid-agarose beads as described by the manufacturer (Qiagen) and used for immunoblotting.



**In Vitro Kinase Assay**—*In vitro* phosphorylation was performed with 4  $\mu$ g of CcpA protein or mutant derivatives in 20  $\mu$ l of buffer P (25 mM Tris-HCl, pH 7.0, 1 mM dithiothreitol (DTT), 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 50  $\mu$ M ATP) with 200  $\mu$ Ci ml<sup>-1</sup> (65 nM) [ $\gamma$ -<sup>32</sup>P]ATP (PerkinElmer Life Sciences; 3000 Ci mmol<sup>-1</sup>) and 0.5  $\mu$ g of Stk1 kinase for 30 min at 37 °C. Control experiments were conducted under the same conditions but without kinase. Cloning, expression, and purification of the Stk1 kinase from *S. aureus* were described previously (21).

**Electrophoretic Mobility Shift Assays**—The DNA probes for electrophoretic mobility shift assays (EMSAs) were generated by PCR using *S. aureus* N315 chromosomal DNA as a template, which encompasses the promoter regions of *hla*, *citZ*, *ald*, *tst*, *malR*, and *pckA*, with respective primer pairs listed in Table 2. The 5'-ends of the double-stranded PCR products were labeled using [ $\gamma$ -<sup>32</sup>P]ATP and T4 polynucleotide kinase. A typical assay mixture contained (in 20  $\mu$ l) 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1  $\mu$ g of nonspecific competitor (poly(dI-dC)), 5% (v/v) glycerol, radioactive DNA probe (2000 cpm ml<sup>-1</sup>), and various amounts (0, 15, 65, 130, and 200 nM) of the purified CcpA proteins. After 30 min of incubation at room temperature, 20  $\mu$ l of this mixture was loaded onto a native 4% (w/v) polyacrylamide Tris borate-EDTA Ready Gel (Bio-Rad) and electrophoresed in 1% Tris borate-EDTA (v/v) buffer for 1 h at 100 V cm<sup>-1</sup>. Radioactive species were detected by autoradiography using direct exposure to films.

**Complementation Studies**—For the *trans*-complementation of the *S. aureus* SA113  $\Delta$ ccpA mutant KS66 (12), the following plasmids harboring the wild-type *ccpA* and 500 bp of the upstream region (pCN34-*ccpA*), the phosphoablative *ccpA* variant carrying the T18A/T33A modifications (pCN34-*ccpA*-*Ala*), and the phosphomimetic *ccpA* variant carrying the T18D/T33D modifications (pCN34-*ccpA*-*Asp*), respectively, were constructed as follows. The *ccpA* derivatives were synthesized by Genscript (Aachen, Germany) using the N315 sequence information as master template; amplified with specific primers (Table 2) introducing BamHI and EcoRI sites, respectively; and cloned into the pCN34 vector (27) digested with the same restriction enzymes. The resulting constructs were sequenced and electroporated into *S. aureus* strain RN4220, which served as donor for transducing the plasmids into KS66.

**Antibody Production and Immunoblotting**—Polyclonal anti-CcpA antibodies were raised by injecting 500  $\mu$ g of the His-tagged recombinant CcpA into rabbits (Eurogentec, Liege, Belgium). The resulting crude antisera were purified against the immobilized CcpA antigen. For the determination of CcpA, cytoplasmic protein extracts were isolated from *S. aureus* cell cultures grown for 5 h in TSB at 37 °C as described previously (28), and protein fractions (10  $\mu$ g/lane) were separated using SDS-PAGE, blotted onto a nitrocellulose membrane, and subjected to Western blot analysis using the antigen-purified polyclonal anti-CcpA antiserum. Phosphorylated CcpA derivatives were detected by immunoblotting as described (23) using anti-phosphothreonine, anti-phosphoserine, or anti-phosphotyrosine antibodies (Cell Signaling Technology), respectively.

**Measurement of Gene Expression by Quantitative Real Time PCR**—RNA isolation and real time RT-PCR were carried out as described by Chatterjee *et al.* (29) using the primer pairs listed

in Table 2. The level of mRNA was normalized against the internal controls *gyrB* and 16 S rRNA. Transcript amounts were expressed as the -fold difference relative to the control ( $2^{\Delta\Delta CT}$  where  $\Delta CT$  represents the difference in threshold cycle between the target and control genes).

**Biofilm Assay**—Bacterial cultures were grown in TSB supplemented with 0.1% (w/v) glucose in glass culture tubes with aeration (150 rpm) at 37 °C. Growth medium was inoculated with 0.5 McFarland unit. Biofilm formation on the glass surface and clumping of the cells were monitored after 18 h of growth.

**Cloning, Expression, and Purification of PrkC and CcpA from Bacillus Species**—The *ccpA* gene from *B. subtilis* was amplified by PCR using *B. subtilis* 168 chromosomal DNA as a template with the primers listed in Table 2 containing NheI and BamHI restriction sites, respectively. The corresponding amplified product was then digested with NheI and BamHI and ligated into the pETPhos plasmid, thus generating pETPhos-*ccpA*<sup>Ba</sup>, which was transformed and overexpressed, and the His-tagged CcpA was purified as described above. The *B. anthracis* *ccpA* recombinant vector was a kind gift from Marta Perego and purified as described by Chiang *et al.* (5) to generate CcpA<sup>Ba</sup>. The *prkC* kinase domain from *B. subtilis* was amplified by PCR using *B. subtilis* 168 chromosomal DNA as a template with the primers listed in Table 2 containing a BamHI and a HindIII restriction site, respectively. The corresponding amplified product was then digested with BamHI and HindIII and ligated into the pGEX(M) vector, thus generating pGEX-*prkC*, which was transformed and overexpressed, and the GST-tagged PrkC<sup>Bs</sup> was purified as described previously (30).

**Statistical Analysis**—The significance of changes was assessed using the Wilcoxon rank sum test. *p* values <0.05 were considered significant.

## RESULTS

**Stk1-mediated Phosphorylation of CcpA**—The *S. aureus* genome encodes one Ser/Thr protein kinase named Stk1 or PknB (16, 18). Although Stk1 appears to be involved in different key pathways such as cell wall metabolism, antibiotic susceptibility, and regulation of virulence (17–19, 31–33), only a little is known about the nature of the corresponding substrates. Because of our interests in Ser/Thr kinase regulation in *S. aureus* and in central metabolic pathways of this pathogen, we wished to investigate whether the CcpA might be regulated by Ser/Thr phosphorylation in this organism.

To establish whether the CcpA protein undergoes post-translational modification by phosphorylation, we first investigated *in vitro* whether CcpA could be phosphorylated in the presence of the purified Stk1 kinase. Stk1 was expressed as a His-tagged fusion protein and purified from *E. coli* as described previously (19, 21). The purified kinase was incubated with CcpA and [ $\gamma$ -<sup>32</sup>P]ATP, the proteins were resolved by SDS-PAGE, and the phosphorylation profile was analyzed by autoradiography (Fig. 1A). The *upper* bands illustrate the autokinase activity of Stk1, whereas the *lower* bands represent phosphorylated CcpA (CcpA-P) proteins. The presence of an intense radioactive signal indicated that CcpA was indeed phosphorylated by Stk1. As expected, no radioactive bands were observed in the absence of Stk1 in this assay. These data

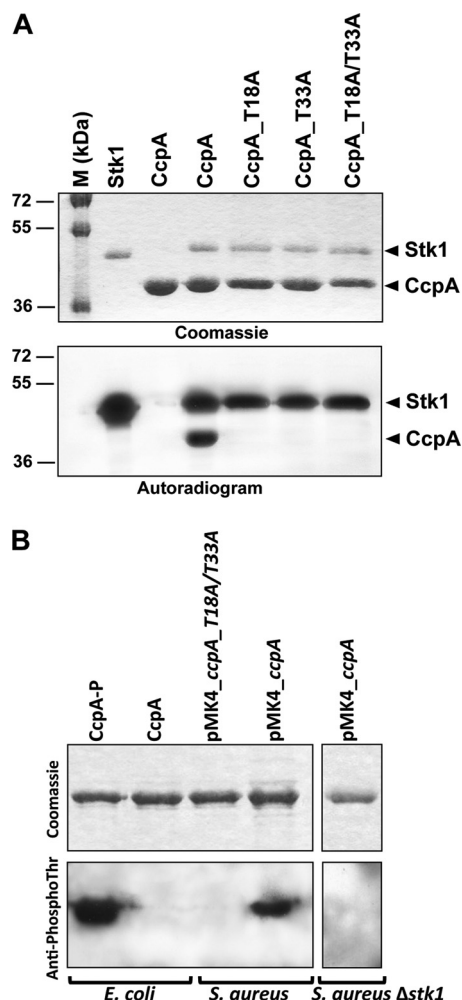


FIGURE 1. *A*, *in vitro* phosphorylation of CcpA and mutant derivatives. The recombinant Stk1 kinase encoded by the *S. aureus* genome was expressed and purified as a His-tagged fusion in *E. coli* and incubated in the presence of [ $\gamma$ - $^{33}$ P]ATP with either the purified His-tagged *S. aureus* CcpA (CcpA\_WT) or the mutated variant CcpA\_T18A (harboring a Thr to Ala substitution at Thr-18), CcpA\_T33A (harboring a Thr to Ala substitution at Thr-33), or CcpA\_T18A/T33A (harboring Thr to Ala substitutions at Thr-18 and Thr-33). Samples were separated by SDS-PAGE, stained with Coomassie Blue (*upper panel*), and visualized by autoradiography (*lower panel*). The upper bands illustrate the autokinase activity of Stk1, and the lower bands represent phosphorylated CcpA proteins. Standard proteins of known molecular masses were run in parallel (*M* (kDa) lane). *B*, *in vivo* phosphorylation of CcpA. Five micrograms of recombinant CcpA proteins purified from either *E. coli* (pDuet\_ccpA and pET-Phos\_ccpA, generating a phosphorylated CcpA-P and an unphosphorylated CcpA, respectively), *S. aureus* (pMK4\_ccpA and pMK4\_ccpA\_T18A/T33A), or *S. aureus*  $\Delta$ stk1 (pMK4\_ccpA) were analyzed by SDS-PAGE after staining with Coomassie Blue (*upper panel*) and detected on an independent SDS-polyacrylamide gel by immunoblotting using anti-phosphothreonine antibodies (Cell Signaling Technology) (*lower panel*).

indicated that CcpA is phosphorylated by Stk1 and suggested that this protein is regulated via this Ser/Thr kinase.

**CcpA Is Phosphorylated on Two Threonine Residues, Thr-18 and Thr-33**—Mass spectrometry (MS) was subsequently used to identify the number and nature of phosphorylation sites on CcpA as reported previously for other STPK substrates (21, 24, 34–38). Phosphorylated CcpA-P was purified from *E. coli* co-expressing Stk1 and CcpA (pDuet\_ccpA) and subjected to mass spectrometry analysis after tryptic digestion. We obtained a sequence coverage of 97%, bearing all possible Ser and Thr residues. The ProteinPilot® database searching software (using the

Paragon method with phosphorylation emphasis) was then used to detect and identify the phosphorylated peptides. The MS/MS spectra unambiguously identified the presence of two phosphate groups on peptide(14–30) (Fig. 2*A*) and peptide(31–42) (Fig. 2*B*), thus indicating that CcpA is phosphorylated on two threonines, corresponding to Thr-18 and Thr-33.

To confirm the phosphorylation sites identified by MS/MS, we substituted Thr-18 and Thr-33 by alanines either individually or together by site-directed mutagenesis to introduce either single or double mutations (Thr to Ala) in CcpA. The corresponding CcpA\_T18A, CcpA\_T33A, and CcpA\_T18A/T33A proteins were expressed and purified as His-tagged proteins in *E. coli* BL21(DE3)Star cells harboring either pETPhos\_ccpA\_T18A, pETPhos\_ccpA\_T33A, or pETPhos\_ccpA\_T18A/T33A constructs. Following incubation with Stk1 and [ $\gamma$ - $^{33}$ P]ATP, SDS-PAGE/autoradiogram revealed that all three mutant proteins were no longer phosphorylated by this kinase (Fig. 1*A*). Surprisingly, single Thr to Ala substitutions were already sufficient to abrogate phosphorylation.

**In Vivo Phosphorylation of CcpA**—To assess the relevance of our *in vitro* phosphorylation findings, we also investigated the *in vivo* phosphorylation of CcpA in a recombinant *S. aureus* RN4220 strain by Western blot analyses using anti-phosphothreonine, anti-phosphoserine, or anti-phosphotyrosine antibodies, respectively. First, the specificities of the antibodies for the phosphorylated isoforms were determined using the CcpA isoforms purified from either *E. coli* or *E. coli* co-expressing Stk1 based on the strategy described by Molle *et al.* (23). Only the phosphorylated CcpA isoform derived from pDuet\_ccpA (CcpA-P) reacted with the anti-phosphothreonine antibodies as expected, whereas the unphosphorylated CcpA protein derived from the pETPhos\_ccpA construct (CcpA) failed to give a signal with these antibodies (Fig. 1*B*). To assess the phosphorylation state of CcpA in *S. aureus*, we cloned the open reading frames of *ccpA* and *ccpA\_T18A/T33A* (harboring the Thr to Ala substitutions at Thr-18 and Thr-33) as C-terminal His-tagged fusions into the shuttle vector pMK4-pProt and transformed the resulting plasmids, pMK4\_ccpA and pMK4\_ccpA\_T18A/T33A, respectively, into the *S. aureus* strain RN4220. Testing the His tag-purified proteins derived from these RN4220 derivatives in a Western blot analysis using anti-phosphothreonine antibodies revealed a clear signal for the wild-type CcpA, whereas no such signal was detectable with the mutated CcpA (Fig. 1*B*). This demonstrated that CcpA is phosphorylated in *S. aureus in vivo* as well and that phosphorylation occurs at Thr-18 and Thr-33 both *in vitro* and in *S. aureus*. Moreover, no signal could be detected with either the anti-phosphoserine or the anti-phosphotyrosine antibodies, confirming that CcpA is only phosphorylated at threonine residues (data not shown). To confirm the direct phosphorylation of CcpA by Stk1 *in vivo*, we transduced the pMK4\_ccpA vector into the *stk1*-null mutant strain ST1004 (16) and overexpressed and purified the CcpA protein from this strain devoid of the Stk1 kinase. As shown in Fig. 1*B*, we could not detect any phosphorylated CcpA isoform when overexpressed in the *S. aureus*  $\Delta$ stk1 strain, strongly supporting that CcpA is a substrate of Stk1 *in vivo*.

**Structural Analysis of CcpA**—To get more insight into the possible role of the double phosphorylation on CcpA by Stk1,

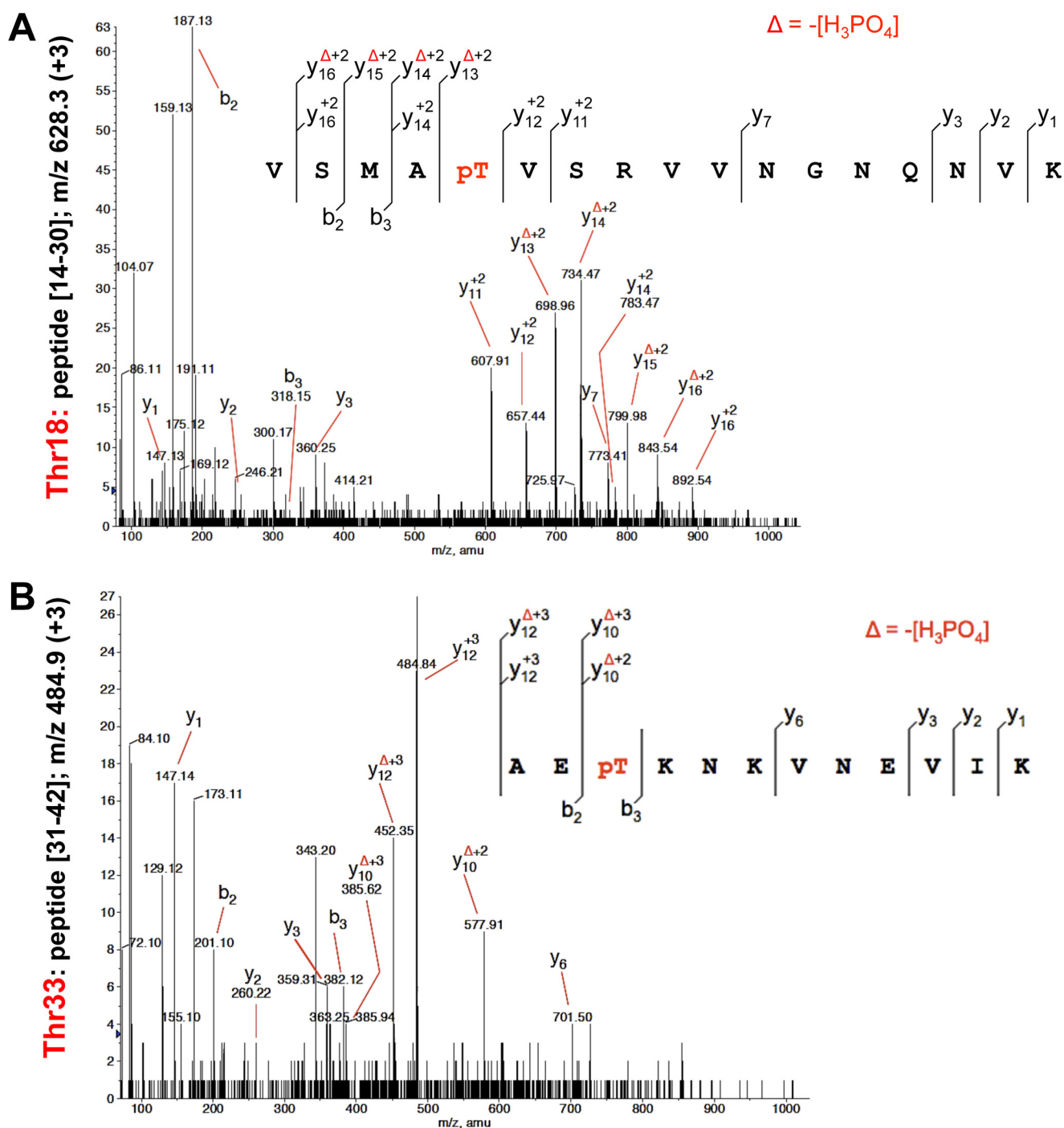


FIGURE 2. **Identification of the CcpA phosphorylation sites.** A, MS/MS spectra at m/z 628.3 (+3) of peptide(14–30) of CcpA. Localization of the phosphate group on Thr-18 was shown by observation of the “y” C-terminal daughter ion series. Starting from the C-terminal residue, all y ions lose phosphoric acid (–98 Da) after the phosphorylated residues. B, MS/MS spectra at m/z 484.9 (+3) of peptide(31–42) of CcpA. Localization of the phosphate group on Thr-33 was shown by observation of the y C-terminal daughter ion series. Starting from the C-terminal residue, all y ions lose phosphoric acid (–98 Da) after the phosphorylated residues.

we looked at various crystal structures of CcpA from different homologues. For our structural analysis depicted in Fig. 3, we used the tertiary complexed form of CcpA, Hpr-Ser(P)-46, and its target DNA from *Bacillus megaterium* (39). The high degree of conservation between the *S. aureus* and *B. megaterium* CcpA amino acid sequences (sequence identity, 57% overall and 82% for the first 60 residues representing the sole DNA binding domain) and their target DNAs allowed us to use this structure

for our analysis. This structural complex described the two-component allosteric DNA binding activation mechanism. The two phosphorylated threonine residues are located on helices 2 and 3 of the helix-turn-helix domain responsible for the DNA binding (Fig. 3). Both residues established multiple contacts with the DNA phosphate through the hydroxyl groups of the lateral chain of both threonines. The phosphorylation of one of these threonines will not only generate a steric clash but also



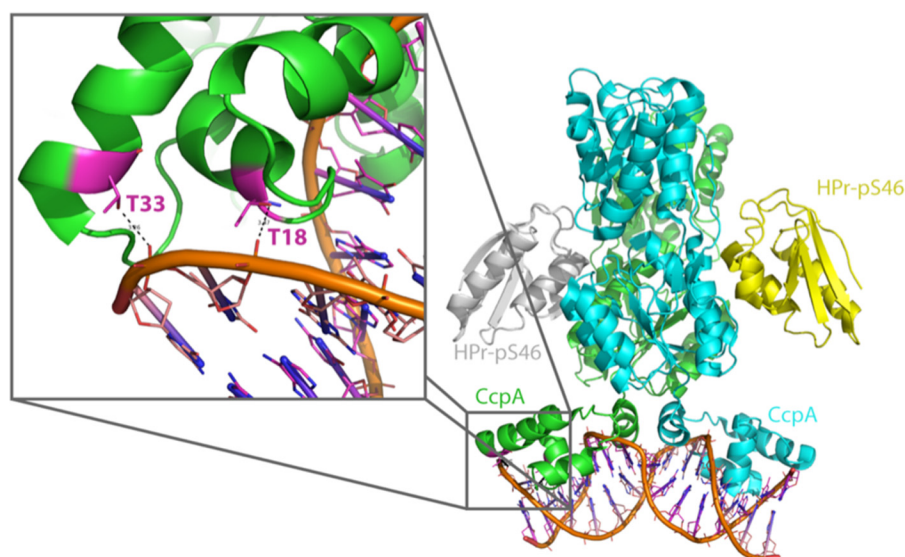


FIGURE 3. **Mapping of the Thr-18 and Thr-33 phosphorylation sites on CcpA.** A schematic representation of the crystal structure of the tertiary complex of CcpA (in blue and green), Hpr-Ser(P)-46 (HPr-pS46; in yellow), and its target DNA (in gray) from *B. megaterium* (Protein Data Bank code 1RZR) is shown. The close view highlights the interaction of threonines 18 and 31 with the phosphate moieties of the DNA.

cause an electrostatic repulsion between the DNA binding domain and its target DNA.

**Phosphorylation Negatively Regulates CcpA-DNA Binding Properties**—The structural mapping of the phosphorylation sites suggested that the phosphorylation of CcpA might negatively influence the DNA binding activity of this protein. To test this hypothesis, we analyzed the binding capacities of the non-phosphorylated CcpA form (CcpA) and that of the hyperphosphorylated CcpA form (CcpA-P) to a couple of gene promoter elements that were recently shown to be affected by this regulatory protein (Fig. 4C) (7, 12–14). Although the unphosphorylated CcpA interacted with all the double-stranded DNA probes tested in our electrophoretic mobility shift assays (Fig. 4, A and B), the phosphorylated CcpA-P failed to shift any of these probes (Fig. 4A; only the *hla* probe profile is shown), indicating that CcpA-P had lost its ability to bind to its *cre* sequences. On the contrary, our EMSAs carried out with the CcpA\_T18A/T33A phosphoablative mutant protein showed that this CcpA derivative retained its DNA binding activity (Fig. 4A; only the *hla* probe profile is shown), confirming the critical role of phosphorylation on the DNA binding capacity of this regulatory protein. It is noteworthy to emphasize that the *S. aureus* CcpA does not essentially require the association with Hpr being phosphorylated by its cognate HprKP kinase (HPr-P) for efficient binding to its DNA targets (15). This observation was confirmed in our EMSAs for which the DNA binding activity of CcpA was assayed in the absence of Hpr-P, which did not essentially modify the DNA binding properties in this *in vitro* assay (Fig. 4B).

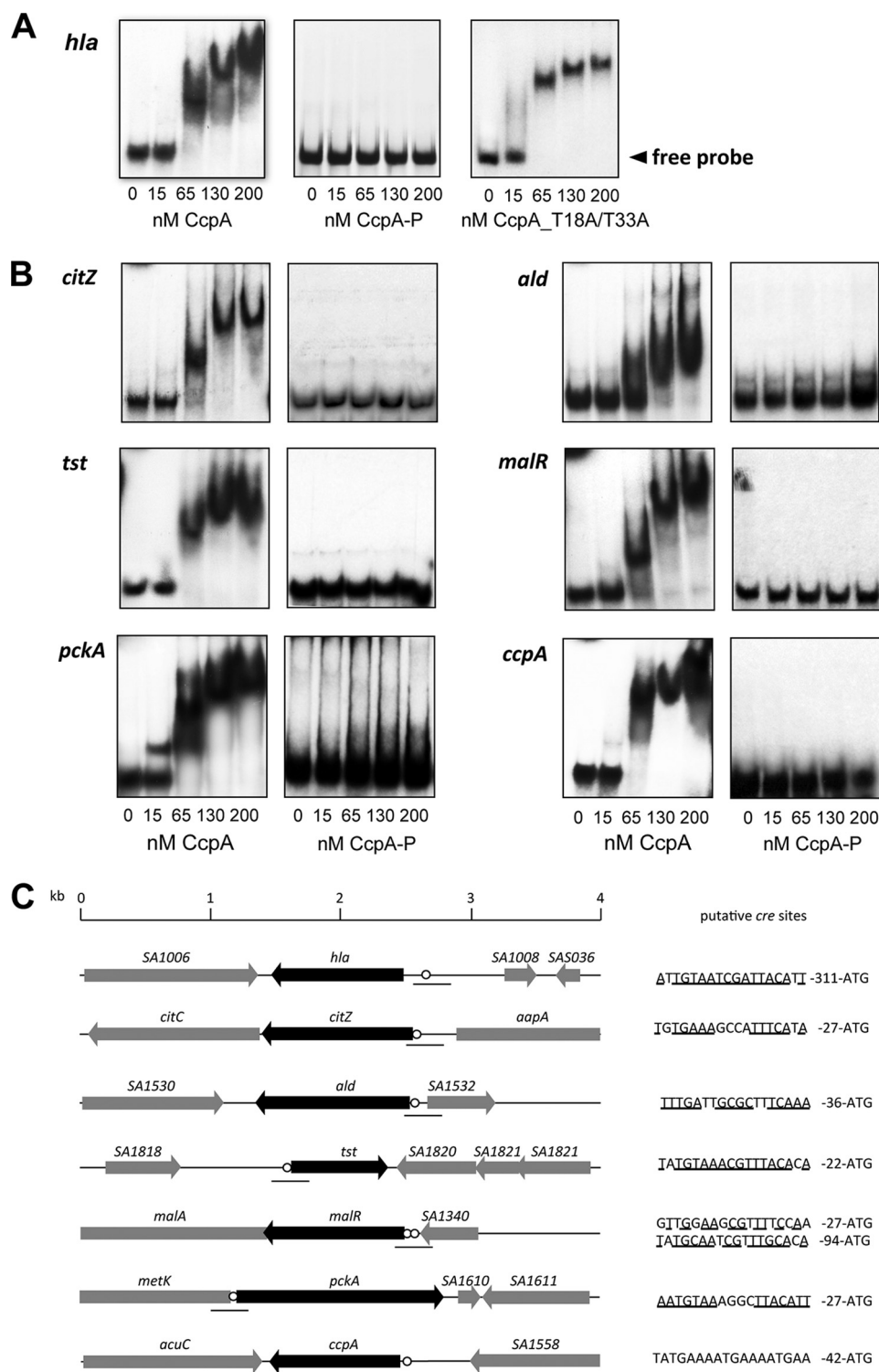
**Lack of Complementation with the CcpA Phosphomimetic Mutant in  $\Delta$ ccpA Mutant Strain**—Acidic residues such as aspartic acid (Asp) qualitatively mimic the phosphorylation effect with regard to functional activity (24, 34, 36, 38, 40, 41). Therefore, phosphoablative (Thr to Ala replacements) and phosphomimetic (Thr to Asp replacements) *ccpA* alleles were generated and cloned into the *E. coli*-*S. aureus* shuttle plasmid

pCN34 under the control of the *ccpA* promoter to *trans*-complement the *S. aureus* SA113  $\Delta$ ccpA mutant KS66 (12). In these constructs, wild-type, phosphoablative (T18A/T33A), and phosphomimetic (T18D/T33D) CcpA isoform expression was monitored by Western blotting using anti-CcpA antibodies. As expected, *S. aureus* SA113  $\Delta$ ccpA failed to express CcpA, whereas increased amounts of CcpA were synthesized in the KS66 derivatives complemented with the phosphoablative *ccpA* variant (pCN34\_ccaAla) and the *ccpA* wild-type allele (pCN34\_ccpA), respectively, probably due to a multicopy effect of the introduced plasmids (Fig. 5A). In the KS66 derivative complemented with the phosphomimetic *ccpA* variant (pCN34\_ccpA\_Asp), however, a marked increase in CcpA was detected in line with the transcriptional data showing a 63-fold increase in *ccpA* transcripts in the pCN34\_ccpA\_Asp-harboring KS66 derivative compared with the wild type (Fig. 5B), suggesting that the expression of this transcriptional regulator is negatively autoregulated in *S. aureus*.

The impact of constitutive CcpA phosphorylation on gene expression of specific CcpA-regulated target genes was next examined by determining the transcription of *citZ* encoding the tricarboxylic acid cycle enzyme citrate synthase and *hla* (encoding  $\alpha$ -hemolysin) in SA113 and its derivatives (Fig. 5B). In line with our previous findings (7, 14), we found a significant increase in *citZ* transcription in the *ccpA* mutant KS66 in the early exponential growth phase (*i.e.* after 3 h of growth in TSB supplemented with 0.1% (w/v) of glucose), whereas transcription of *hla*, which is expressed mainly during the later stages of growth, was markedly down-regulated in KS66 after 8 h of growth compared with the wild type. *Trans*-complementation of the *ccpA* mutant with the *ccpA* wild-type allele (pCN34\_ccpA) and the phosphoablative *ccpA* variant (pCN34\_ccpA\_Ala) led to the production of *citZ* and *hla* transcripts to levels seen in the wild type, whereas the introduction of the T18D/T33D-carrying CcpA version (pCN34\_ccpA\_Asp) into KS66 did not alter the transcription of both genes (Fig. 5B). This



## Regulation of CcpA by Ser/Thr Kinase Phosphorylation

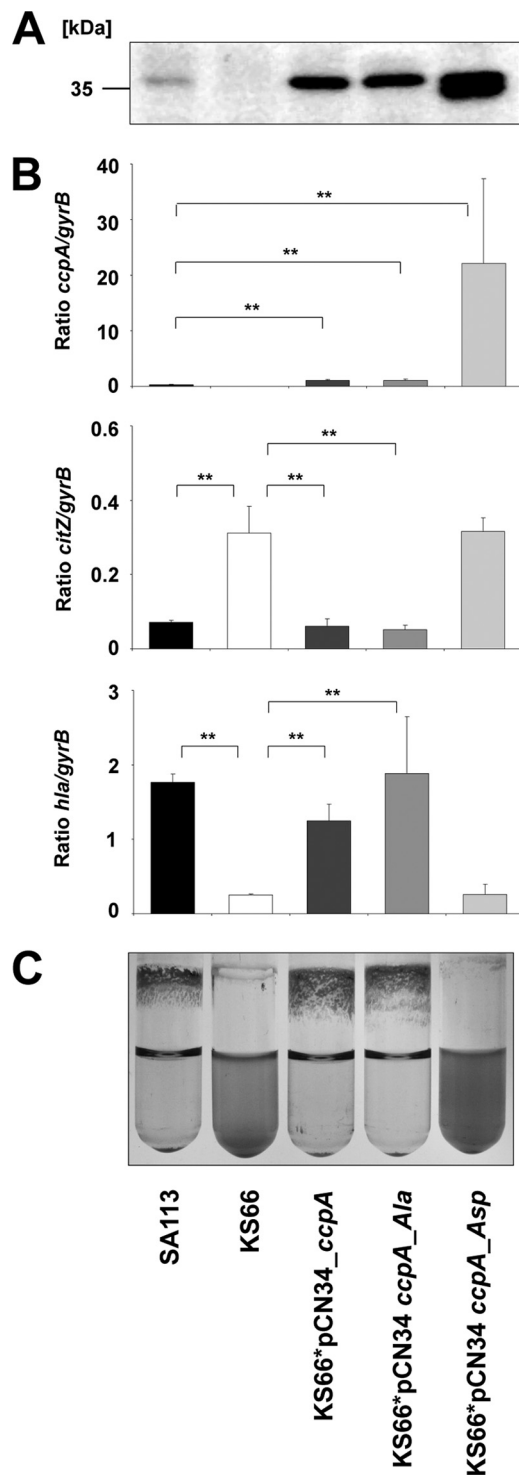


**FIGURE 4. DNA binding activity of CcpA derivatives.** A gel EMSA of CcpA binding to the *cre* sequences of the *hla*, *citZ*, *ald*, *tst*, *malR*, *ccpA*, and *pckA* promoters. The *cre* regions of target genes were amplified by PCR; radioactively labeled; and incubated with 15, 65, 130, or 200 nM purified CcpA. A, binding of the unphosphorylated CcpA, CcpA-P, or the CcpA\_T18A/T33A double mutant to the *hla* promoter region. B, binding of the unphosphorylated CcpA and CcpA-P to the *cre* sequences of the *citZ*, *ald*, *tst*, *malR*, *ccpA*, and *pckA* promoters. The CcpA\_T18A/T33A double mutant is not represented as all the probes tested produced profiles identical to those of CcpA. C, schematic representation of the *cre* sequences (circles) and open reading frames (arrowed boxes) of the promoter elements studied. The putative *cre* sequences and the probes (lines) used for the EMSA experiments are indicated. The annotation/numbering of genes are based on the genome sequence of *S. aureus* strain N315.

result suggests that the phosphomimetic CcpA variant is unable to modulate the expression of CcpA-regulated target genes.

Whether phosphorylation of CcpA affects biofilm formation as described previously for a *ccpA* knock-out mutant (12) was

investigated by growing the SA113 derivatives in glucose-supplemented TSB under aeration in glass tubes. Under these conditions, SA113 forms a strong biofilm on the glass surface and shows a high degree of cell clumping, whereas cells of the *S.*



**FIGURE 5. Phenotypic complementation of the *ccpA* phenotype in *S. aureus*.** **A**, Western blot analysis of cytosolic protein extracts obtained from 6-h cultures of *S. aureus* strain SA113, its respective  $\Delta$ *ccpA* mutant KS66, and the KS66 derivatives complemented with the wild-type allele (KS66\**p*CN34\_ *ccpA*), the CcpA\_T18A/T33A variant (KS66\**p*CN34\_ *ccpA*\_Ala), and the CcpA\_T18D/T33D version (KS66\**p*CN34\_ *ccpA*\_Asp) with the polyclonal anti-CcpA antibody SY490. **B**, quantitative transcript analysis of *ccpA*, *citZ*, and *hla* of *S. aureus* strain SA113 and its derivatives grown in TSB supplemented with 0.1% (w/v) glucose for 3 (*ccpA* and *citZ*) and 8 h (*hla*) at 37 °C. **C**, biofilm formation capacities of *S. aureus* strains SA113 and its derivatives grown in TSB supplemented with 0.1% (w/v) glucose for 18 h at 37 °C. The results are representative of at least two independent experiments (A and C) or are presented as the mean  $\pm$  S.D. (error bars) of five independent experiments each determined in duplicate (B). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  for wild type

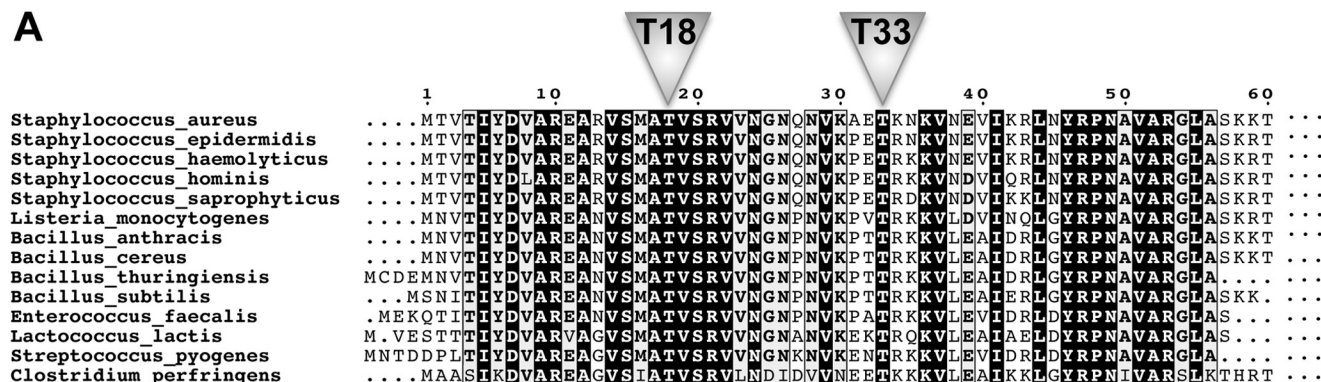
*aureus* SA113  $\Delta$ *ccpA* mutant KS66 remain mainly planktonic (12). Here we found that both phenotypes could be restored by complementation with either the wild-type allele or the phosphoablative *ccpA* variant (Fig. 5C). In contrast, introduction of CcpA\_T18D/T33D (*ccpA*\_Asp) failed to restore the parental biofilm profile and clumping, indicating that the phosphomimetic isoform is unable to complement the lack of CcpA activity in biofilm formation presumably as a consequence of its impaired DNA binding activity toward specific regulated genes involved in these processes. It is noteworthy that these effects were not due to a tertiary structure change as confirmed by analysis of the trypsinolysis kinetics of wild-type and mutated CcpA proteins (supplemental Fig. S1) and were consistent with the structural analysis indicating that introduction of Asp at positions 18 and 33 does not seem to induce steric or electrostatic conflicts that could alter the protein fold (Fig. 3). Thus, it can be inferred that CcpA phosphorylation in *S. aureus* reduces/abolishes its activity, supporting the view that this post-translational modification plays a role in regulating CcpA activity.

**CcpA Orthologue Phosphorylation and Cross-phosphorylation between Species**—Given the high degree of similarity between the CcpA proteins of *S. aureus* and bacilli and based on the finding that the Thr-18 and Thr-33 sites are well conserved among CcpA orthologues (Fig. 6A), we wondered whether the CcpAs from *Bacillus* species might be phosphorylated as well. Interestingly, CcpA from *B. subtilis* (CcpA<sup>Bs</sup>) could not be phosphorylated by its cognate kinase PrkC (Fig. 6B). This result raised the question whether the phosphoregulation of CcpA might be restricted to pathogens, which led us to test the corresponding CcpA orthologue of *B. anthracis* (CcpA<sup>Ba</sup>). As presented in Fig. 6B, no phosphorylation signal could be detected with CcpA<sup>Ba</sup>, indicating that the STPK-driven control of CcpA activity does not seem to be present in bacilli.

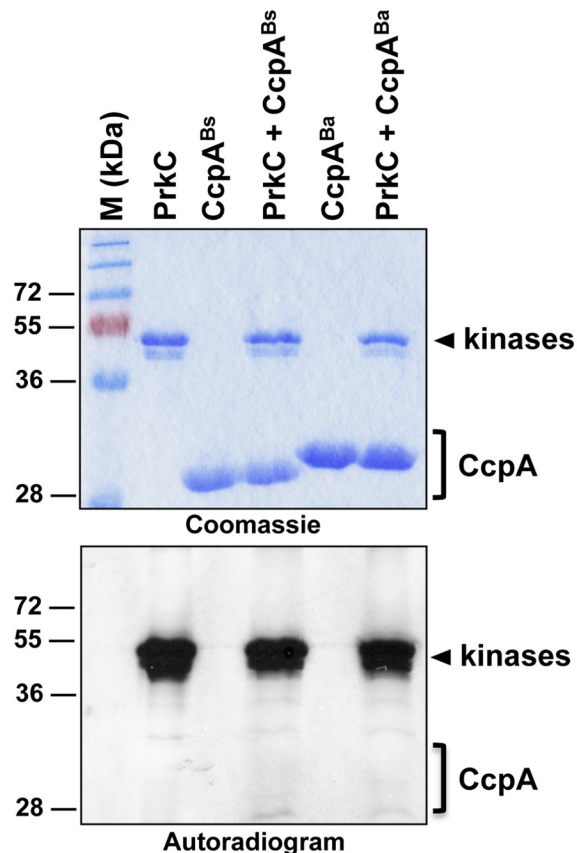
To determine whether the lack of CcpA phosphorylation observed with the *Bacillus* orthologues might be due to STPK specificity, we investigated the Ser/Thr cross-phosphorylation between the *S. aureus* kinase and the *B. subtilis* CcpA and vice versa. As presented in Fig. 6C, we found that the *B. subtilis* kinase PrkC was able to cross-phosphorylate CcpA from *S. aureus*, whereas the CcpA orthologue from *B. subtilis* remained unphosphorylated in the presence of Stk1. These findings suggest a critical role for the substrate phosphorylation site environment rather than for the kinase specificity at least under *in vitro* conditions. Interestingly, the *in silico* comparison of the DNA binding domains of the CcpA orthologues identified a strict conservation around Thr-18 (residues 14–26), whereas the conservation around Thr-33 was much lower (Fig. 6A). Of note, as mentioned above, the conservation of the N-terminal DNA binding domain of the CcpA protein is very high within orthologues (>80%), and the only divergent area is centered around Thr-33. All *Bacillus* CcpAs contained a proline at position 31, which might explain the absence of phosphorylation in this genus. This proline is likely to N-cap one of the  $\alpha$ -helices of the CcpA DNA binding domain, thus stabilizing this struc-

versus *ccpA* mutant and for *trans*-complemented derivatives versus *ccpA* mutant, respectively (Wilcoxon rank sum test).

A



B



C

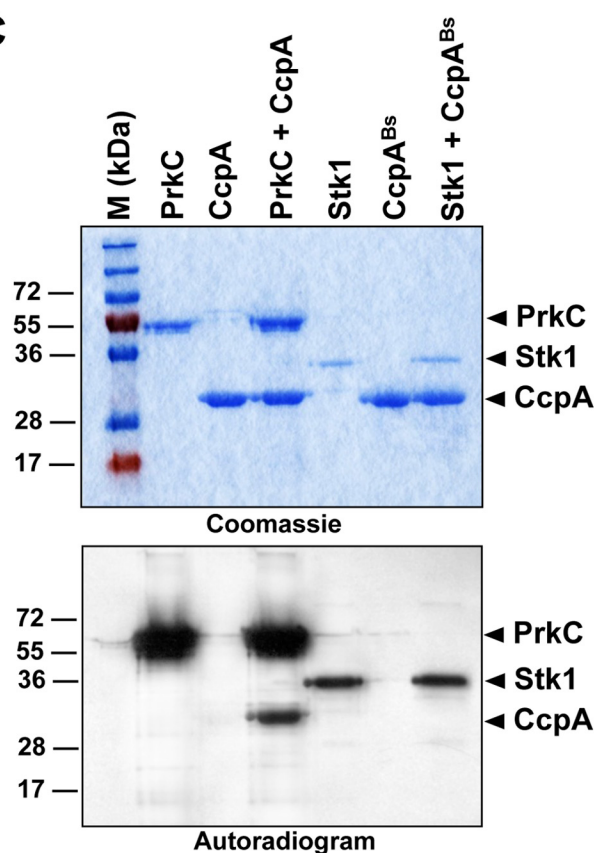


FIGURE 6. A, conservation of the phosphoacceptors in CcpA orthologues within the DNA binding domain. The multiple sequence alignment of CcpA orthologues was performed using ClustalW and ESPript. Numbering of the amino acids corresponds to the CcpA from *S. aureus* strain COL. Residues conserved in all species are presented in black boxes. The positions of phosphorylated sites of CcpA from *S. aureus* are indicated by triangles. B, *in vitro* phosphorylation of CcpA from *B. subtilis* and *B. anthracis* orthologues. The recombinant PrkC kinase encoded by the *B. subtilis* genome was expressed and purified as a GST-tagged fusion in *E. coli* and incubated in the presence of [ $\gamma$ - $^{32}$ P]ATP with either the purified His-tagged *B. subtilis* CcpA (CcpA<sup>Bs</sup>) or *B. anthracis* CcpA (CcpA<sup>Ba</sup>). The vector allowing CcpA<sup>Ba</sup> overexpression and purification was kindly provided by Perego and co-workers (5). Samples were separated by SDS-PAGE, stained with Coomassie Blue (upper panel), and visualized by autoradiography (lower panel). Standard proteins of known molecular masses were run in parallel (M (kDa) lane). C, *in vitro* cross-phosphorylation. The recombinant PrkC kinase encoded as a GST-tagged fusion was incubated in the presence of [ $\gamma$ - $^{32}$ P]ATP with the purified His-tagged *S. aureus* CcpA. In parallel, the recombinant His-tagged fusion Stk1 kinase encoded by the *S. aureus* genome was incubated in the presence of [ $\gamma$ - $^{32}$ P]ATP with the purified His-tagged *B. subtilis* CcpA (CcpA<sup>Bs</sup>). Samples were separated by SDS-PAGE, stained with Coomassie Blue (upper panel), and visualized by autoradiography (lower panel). The upper bands illustrate the autokinase activity of the kinases, and the lower bands represent phosphorylated CcpAs. Standard proteins of known molecular masses were run in parallel (M (kDa) lane).

ture. Therefore, unfolding of the region critical for Stk1 kinase recognition may be in part altered by the presence of this proline residue in *Bacillus* species. Moreover, it remains to be investigated whether the CcpA orthologues of other G<sup>+</sup> organisms such as *Clostridium perfringens*, *Lactococcus lactis*, and *S. pneumoniae*, which do not possess a proline residue at position 31 (Fig. 6A), might be regulated by Ser/Thr phosphorylation.

## DISCUSSION

Recent studies in various *S. aureus* strains suggest that Ser/Thr phosphorylation by Stk1 plays an important role in central metabolic processes such as cell wall metabolism, glycolysis, the citrate cycle, purine and pyrimidine synthesis, translation, quorum sensing, and virulence (17, 19, 21, 31, 32) presumably due to the regulation of selected global regulators. In line with this



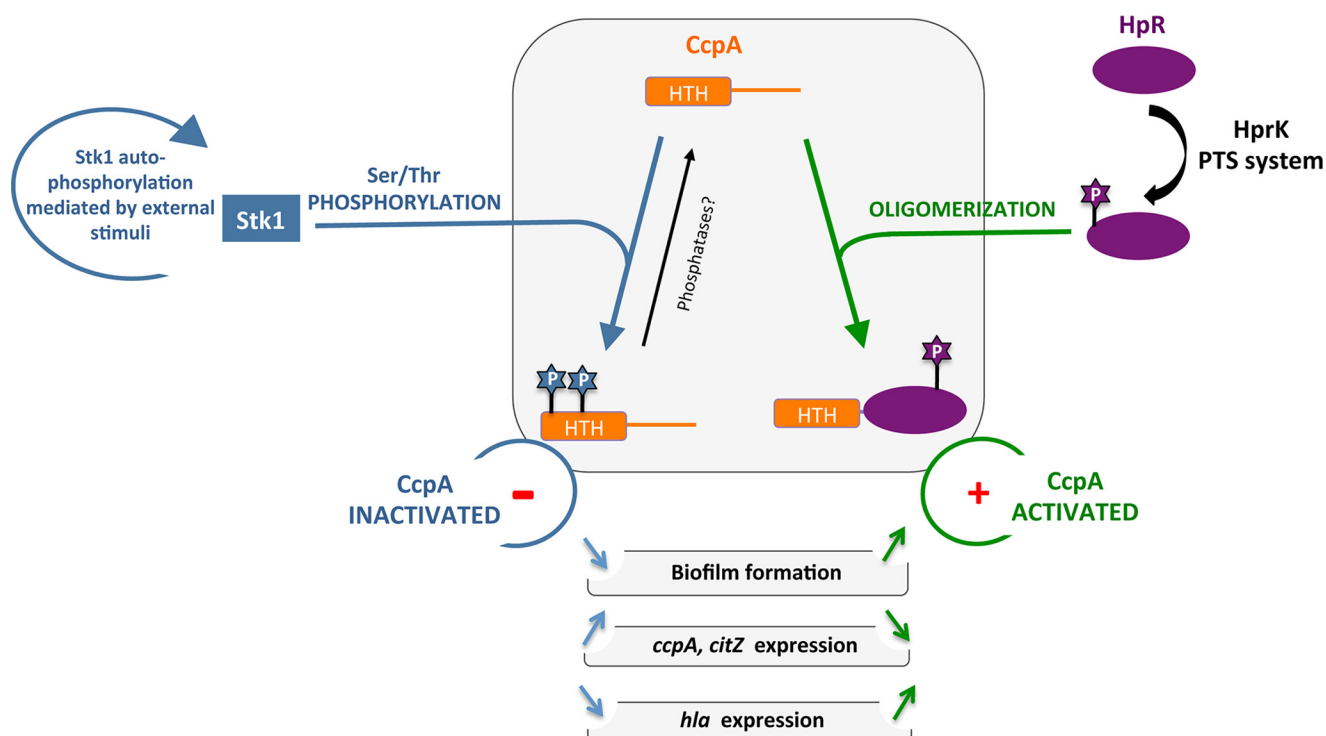


FIGURE 7. **Proposed regulatory role of the different isoforms of CcpA as a molecular switch in carbon catabolite repression and virulence gene expression in *S. aureus*.** The unphosphorylated form of CcpA binds to its target DNA sequences, and this binding is modulated via the HprKP/Hpr system, which is thought to promote the binding of CcpA to its DNA targets if CcpA is complexed with Hpr-Ser(P)-46. The Stk1-mediated phosphorylation of CcpA inactivates the DNA binding activity of this regulatory molecule, thereby preventing the transcriptional control of its target genes. *PTS*, PhosphoTransferase System; *HTH*, helix-turn-helix domain.

assumption, Stk1 was recently shown to affect the activities of regulatory factors such as MgrA, SarA, and LuxS (19–21). With the present study, we extend previous work demonstrating the regulatory role of Stk1-mediated phosphorylation in central metabolism and virulence pathways by providing first evidence of a Ser/Thr kinase phosphorylation-dependent regulation of the major transcriptional regulator CcpA. This mechanism is of special relevance as CcpA contributes to the pathogenicity of *S. aureus* (7, 8, 12, 14, 15). We demonstrated that phosphorylation of Thr-18 and Thr-33 residues abrogated CcpA DNA binding activity *in vitro*. Moreover, in the case of growing bacteria, a phosphomimetic mutant displayed a major alteration of the biofilm formation, probably due to the lack of CcpA activity, similar to what was observed for a  $\Delta$ ccpA mutant (12). Likewise, the CcpA phosphomimetic strain exhibited reduced transcription of *citZ* and increased transcription of *hla* similar to the  $\Delta$ ccpA mutant profile, confirming that phosphorylation prevents CcpA regulation toward its target genes *in vivo*. Previous studies identified Stk1 to affect the expression of a variety of proteins, including virulence determinants like hemolysins (32). Of notable interest is the *S. aureus* *stk1* mutant for which transcription of the *hla* gene was increased (32), thus supporting the view that the increased  $\alpha$  toxin expression in the  $\Delta$ stk1 mutant could be linked, at least in part, to the phosphorylation state of CcpA.

Our data presented here now strongly suggest that the activity of this *S. aureus* global regulator is not only modulated by metabolic signals that lead to the formation of the coregulator Hpr-Ser(P)-46 but also by Stk1 (Fig. 7). However, the environ-

mental stimuli that activate Stk1 kinase activity have not yet been identified, making it difficult to predict under which conditions CcpA activity might be affected by Stk1 phosphorylation. However, our findings presented here and elsewhere that this regulatory circuit does not seem to be present in the closely related genus *Bacillus* (Fig. 7) and that *ccpA* transcription appears to be autoregulated in staphylococci (Fig. 5 and Ref. 42) but not in bacilli (43, 44) highlight that some fundamental differences in the regulation of CcpA activity between staphylococci and bacilli exist. Moreover, in combination with our recent observations that CcpA also seems to regulate the expression of some of its target genes in the absence of glucose (13) and that CcpA can readily bind to its *cre* sequences in absence of its coregulator Hpr-Ser(P)-46 (Fig. 4 and Ref. 15), it is tempting to speculate that the staphylococcal CcpAs might be fairly active even in the absence of Hpr-Ser(P)-46 and therefore need to be controlled by further transcriptional and post-translational mechanisms, whereas the *Bacillus* CcpA homologues might not bind efficiently to their *cre* sequences in the absence of Hpr-Ser(P)-46 and thus do not need to be controlled by these regulatory circuits.

In summary, this study provides conceptual advances in our understanding of how pathogenic bacteria adapt to their physiology. This work also strengthens the biological importance of CcpA in the physiology and virulence of *S. aureus* as such Ser/Thr kinase-dependent fine-tuning mechanism is not ubiquitous.

Our results furthermore suggest that displacement of the CcpA/CcpA-P balance in favor of the phosphorylated isoform leads to biofilm inhibition and modification of gene expression.

Although very challenging, future studies should now focus on the identification of the extracellular cues that are sensed by the Stk1 kinase and lead to CcpA phosphorylation. However, it remains unclear under which conditions and to what extent Stk1 phosphorylates CcpA in this pathogen, how this phosphorylation affects the expression of the regulon controlled by this LacI/GalR type of regulator, and whether this phosphorylation might be reversible by a serine/threonine phosphatase, such as Stp, that is co-transcribed with Stk1 (45). Moreover, the lack of this regulatory circuit in the closely related genus *Bacillus* together with the observed low degree of conservation in the amino acid sequence surrounding Thr-33 of the CcpA orthologues analyzed here suggests that this regulatory control mechanism of CcpA might only be present in some of the G<sup>+</sup> organisms harboring CcpA and Stk1 orthologues. Investigations are currently ongoing to address these questions.

From an application standpoint, the selective inhibition of CcpA activity through constitutive phosphorylation may strongly impair *S. aureus* pathogenicity at least in terms of biofilm formation, thus opening new and original perspectives for future drug development. It is indeed noteworthy that small molecules such as bryostatin (46) can activate STPK, which may be of great therapeutic value in inhibiting *S. aureus* biofilm formation *in vivo*.

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