Regulatory Interactions of a Virulence-Associated Serine/Threonine Phosphatase-Kinase Pair in *Bacillus anthracis*²

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In the current study, we examined the regulatory interactions of a serine/threonine phosphatase (BA-Stp1), serine/threonine kinase (BA-Stk1) pair in *Bacillus anthracis*. *B. anthracis* STPK101, a null mutant lacking BA-Stp1 and BA-Stk1, was impaired in its ability to survive within macrophages, and this correlated with an observed reduction in virulence in a mouse model of pulmonary anthrax. Biochemical analyses confirmed that BA-Stp1 is a PP2C phosphatase and dephosphorylates phosphoserine and phosphothreonine residues. Treatment of BA-Stk1 with BA-Stp1 altered BA-Stk1 kinase activity, indicating that the enzymatic function of BA-Stk1 can be influenced by BA-Stp1 dephosphorylation. Using a combination of mass spectrometry and mutagenesis approaches, three phosphorylated residues, T165, S173, and S214, in BA-Stk1 were identified as putative regulatory targets of BA-Stp1. Further analysis found that T165 and S173 were necessary for optimal substrate phosphorylation, while S214 was necessary for complete ATP hydrolysis, autophosphorylation, and substrate phosphorylation. These findings provide insight into a previously undescribed Stp/Stk pair in *B. anthracis*.

A profile of the intracellular signaling proteins that regulate transition of *Bacillus anthracis* from dormancy to expression of virulence factors is emerging. Like many prokaryotes, *B. anthracis* utilizes two-component histidine kinase systems to regulate physiological changes and the expression of virulence factors. These systems include the Spo0 histidine kinase-based phosphorelay pathway (32, 37) and the Bacillus respiratory response A and B system involved in regulating toxin expression (36). Unlike for histidine kinase systems, little is known about reversible serine/threonine phosphorylation events in *B. anthracis*. These systems are common to eukaryotic cells (3, 14, 25, 40) but were only recently found in prokaryotes to modulate a variety of metabolic and physiological processes (1, 2, 7, 11, 12, 15, 17, 24, 28, 35, 38). Whether reversible serine/threonine phosphorylation contributes to similar events in *B. anthracis* is not known.

The current paradigm for prokaryotic serine/threonine kinases (STK) is based in part on the structure of PknB, a serine/threonine kinase from *Mycobacterium tuberculosis* that is structurally related to eukaryotic Hanks-type kinases (39). PknB autophosphorylates and is dephosphorylated by an *M. tuberculosis* phosphatase, PstP, in order to alter kinase activity (4). Similar to the findings for PknB, Madec et al. identified critical autophosphorylated residues and autodephosphorylated domains of PrkC, an STK from *Bacillus subtilis* (22), which suggested that the phosphorylation state of these residues impacts the activation of PrkC (22). These studies suggested that prokaryotic STKs exhibited activities similar to those of their eukaryotic homologs and were regulated by cognate phosphatases. Hence, studies of serine/threonine phosphatase (STP)/STK pairs may help define a core regulatory module in bacterial physiology and virulence, wherein the kinase autophosphorylates following interaction with stimuli and is subsequently downregulated by a cognate phosphatase when stimulus levels decline.

Analysis of the *B. anthracis* genome indicates that this organism has a single phosphatase-kinase pair encoded within a putative operon. This operon, between nucleotides 3588319 and 3678099 in the genome of *B. anthracis* Sterne, contains eight candidate open reading frames (ORFs). Six of the potential ORFs encode proteins involved in translation and DNA metabolism, while the phosphatase-encoding ORF (stpk1) and the kinase-encoding ORF (stpk1) are paired at the 3’ end of this operon. Examination of the genome sequences of several other Gram-positive bacteria indicates that this putative operon and the general orientation of *stpk1* and *stp1* are conserved among members of the *Firmicutes* group of bacteria. *B. anthracis* Stp1 (BA-Stp1) and BA-Stk1 homologs influence a variety of bacterial processes. For example, homologs of BA-Stp1 and BA-Stk1 regulate growth in *Bacillus subtilis* (12), cell viability and segregation in *Streptococcus agalactiae* (28), competence in *Streptococcus pneumoniae* (26), and virulence in both *Streptococcus pyogenes* (17) and *Staphylococcus aureus* (9). Although kinases homologous to BA-Stk1 influence several bacterial processes in different species, the tandem association of this kinase with a phosphatase does not vary. This observation led us to hypothesize that the phosphatase (BA-Stp1) influences Ba-Stk1 activity by dephosphorylation.

In the current study, we analyzed the importance of BA-Stp1 and BA-Stk1 in the virulence of *B. anthracis* and assessed the
biochemical interactions between these two proteins. Results from these studies indicate that this phosphatase-kinase pair contributes to the virulence of *B. anthracis*, as mutants lacking BA-Stp1 and BA-Stk1 exhibit decreased lethality in a mouse model of pulmonary anthrax. Furthermore, a series of biochemical analyses reveal an interaction between BA-Stk1 and BA-Stp1 where BA-Stk1 autophosphorylates in order to enter a putative step in downregulating kinase activity as the levels of stimuli subside. Moreover, we have identified candidate serine and threonine residues that appear to modulate kinase activity. These findings provide insight into a previously undescribed serine/threonine phosphatase-kinase system in *B. anthracis*.

**MATERIALS AND METHODS**

**Bacterial strains, cell lines, and growth conditions.** *B. anthracis* Sterne strain 7702, as described by Cataldi et al. (6), and *Escherichia coli* were grown in brain heart infusion broth (BHI) and Luria-Bertani (LB) medium, respectively, at 37°C.

Abelson murine leukemia virus-transfected murine macrophages derived from ascites of BALB/c mice (termed RAW 264.7) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The RAW 264.7 cell line was maintained in tissue culture-grade T-75 flasks grown in the presence of Dulbecco’s modified essential medium (DMEM) (ATCC) supplemented with 10% fetal bovine serum (FBS) (ATCC) at 37°C with 5% CO2 in a humidified incubator.

**Spore preparation and infection experiments.** *B. anthracis* Speck Sterne 7702 parent or the *B. anthracis* Sterne 7702 parent strain and the Sterne 7702 parent or the *B. anthracis* Sterne 7702 in BHI. To construct His-BA-Stp1, a 753-bp fragment was PCR amplified with forward primer 5′-CCGGAATTCGCCATTAGTGCTAGAAAGT6C-3′ and reverse primer 5′-CGGAATTCGCTTATAACGGTCATTTAAGAGAAC3′-3′ incorporating EcoRI restriction sites. The PCR product was subsequently ligated to a pGEM-T-easy vector (Promega) using TA overhangs to generate the plasmid construct pGEM-PK101, which was transformed into E. coli JM109. Transformed clones were screened for ampicillin resistance, and DNA sequencing of the isolated plasmid confirmed clones positive for the insert. Inverse PCR was then performed using pGEM-PK101 as a template with right primer 5′-CCGGAATTCGCCATTAGTGCTAGAAAGT6C-3′ and left primer 5′-CCGGAATTCGCTTATAACGGTCATTTAAGAGAAC3′-3′ incorporating BamHI restriction sites. The Inverse PCR product was then sequenced in pGEM-T-easy and the sequence was confirmed by submission to the National Biotechnology Center (NBIC) and the sequence was confirmed by sequence analysis.

**Expression and purification of His-tagged BA-Stp1 and His-tagged BA-Stk1.** Genomic DNA was isolated from an overnight culture of *B. anthracis* Sterne 7702 in BHI. To construct His-BA-Stp1, a 753-bp fragment was PCR amplified with forward primer 5′-CCGGAATTCGCCATTAGTGCTAGAAAGT6C-3′ and reverse primer 5′-CGGAATTCGCTTATAACGGTCATTTAAGAGAAC3′-3′ incorporating EcoRI restriction sites. The PCR product was subsequently ligated to a pGEM-T-easy vector (Promega) using TA overhangs to generate the plasmid construct pGEM-PK101, which was transformed into E. coli JM109. Transformed clones were screened for ampicillin resistance, and DNA sequencing of the isolated plasmid confirmed clones positive for the insert. Inverse PCR was then performed using pGEM-PK101 as a template with right primer 5′-CCGGAATTCGCCATTAGTGCTAGAAAGT6C-3′ and left primer 5′-CCGGAATTCGCTTATAACGGTCATTTAAGAGAAC3′-3′ incorporating BamHI restriction sites. The Inverse PCR product was then sequenced in pGEM-T-easy and the sequence was confirmed by submission to the National Biotechnology Center (NBIC) and the sequence was confirmed by sequence analysis.

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**Genetic construction of a Δba-stp1 Δba-stk1 double mutant of *B. anthracis* Sterne.** Allelic replacement was used to construct a double mutant lacking expression of BA-Stp1 and BA-Stk1. Briefly, a 3.75-kb fragment comprising 1,500-bp flanking regions sharing sequence similarity to the upstream and downstream regions of the ba-stp1 gene was PCR amplified from *B. anthracis* Sterne 7702 genomic DNA using forward primer 5′-CCGGAATTCGCTTATAACGGTCATTTAAGAGAAC3′-3′ and reverse primer 5′-CGGAATTCGCTTATAACGGTCATTTAAGAGAAC3′-3′ incorporating EcoRI restriction sites. The PCR product was subsequently ligated to a pGEM-T-easy vector (Promega) using TA overhangs to generate the plasmid construct pGEM-PK101, which was transformed into E. coli JM109. Transformed clones were screened for ampicillin resistance, and DNA sequencing of the isolated plasmid confirmed clones positive for the insert. Inverse PCR was then performed using pGEM-PK101 as a template with right primer 5′-CCGGAATTCGCCATTAGTGCTAGAAAGT6C-3′ and left primer 5′-CCGGAATTCGCTTATAACGGTCATTTAAGAGAAC3′-3′ incorporating BamHI restriction sites. The Inverse PCR product was then sequenced in pGEM-T-easy and the sequence was confirmed by submission to the National Biotechnology Center (NBIC) and the sequence was confirmed by sequence analysis.

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Measurement of BA-Stk1cat kinase activity in vitro. Kinase activities of 1.25 μM BA-Stk1cat wild-type, alanine mutant (T165A, S173A, S214A, T290A), or aspartic acid mutant (T165D, S173D, S214D, T290D) were incubated with 4 mM MOPS, pH 7.2, 5 mM MnCl2, 0.05 mM DTT with 10 μM ATP (Cell Signaling Technology). After incubation for 90 min at 37°C, kinase activity was assessed using the Kinase-Glo luminescent kinase assay platform (Promega) and the luminescent signal was measured in a Victor 3 (Perkin Elmer) plate reader. The luminescent signal inversely correlates to the amount of kinase activity. The percent relative activity of BA-Stk1cat site-directed mutants was calculated as an inverse of units of luminescence, compared to that of the wild-type BA-Stk1cat. All reactions were performed in triplicate.

RESULTS

Identification and characterization of a serine/threonine phosphatase-kinase pair in B. anthracis. A search of the B. anthracis Sterne genome revealed putative orthologs of eu- karyotic STKs and phosphatases. In all, four putative STKs and six putative serine/threonine phosphatases (STPs) were identified. Of particular interest to our studies was a pair of ORFs juxtaposed at the 3’ end of a putative operon, as this was the only closely related STP/STK pair identified in B. anthracis. The STP (termed BA-Stp1) and STK (termed BA-Stk1) are encoded by BAS3714 and BAS3713 (GenBank accession numbers AAT56015 and AAT56016), respectively. No other closely associated STP/STK pair was identified within the genome of B. anthracis.
To characterize BA-Stp1/BA-Stk1 in *B. anthracis* Sterne, a double mutant (Δstp1 Δstkl) was constructed by allelic replacement using a Ω Kan’ cassette consisting of the aphA-3 gene conferring kanamycin resistance, and the mutant was termed *B. anthracis* STPK101. In vitro analysis of *B. anthracis* STPK101 found that the mutant grew in liquid medium, sporulated, and germinated with efficiency similar to that of the parent strain (data not shown). Thus, we next determined whether the virulence of *B. anthracis* STPK101 was similar to that of the parent strain.

Macrophages are considered the primary site of *B. anthracis* spore germination following infection (13); hence, *B. anthracis* STPK101 was assessed for growth and survival in these cells. When RAW 264.7 macrophage-like cells were infected with spores of the parent *B. anthracis* Sterne strain or *B. anthracis* STPK101, the total number of CFU of *B. anthracis* STPK101 recovered at 10 h postinfection were significantly reduced compared to results for the parent strain (Fig. 1A). A comparison between STPK101 and the parent strain found no differences in the rate of growth in tissue culture medium alone (data not shown), suggesting that the observed defect was due to attenuated germination, growth, or survival of the mutant within the macrophage.

Next, to assess the importance of the phosphatase-kinase pair in virulence of *B. anthracis*, the 50% lethal doses (LD<sub>50</sub>) of the parent *B. anthracis* Sterne strain and *B. anthracis* STPK101 were compared using a mouse model of pulmonary anthrax. As shown in Fig. 1B and C, the LD<sub>50</sub> of *B. anthracis* STPK101 (4.5 × 10<sup>5</sup> spores) was 10-fold higher than the LD<sub>50</sub> of the *B. anthracis* parent strain (4.5 × 10<sup>4</sup> spores). Collectively, these data indicate that the phosphatase-kinase pair contributes to the survival of *B. anthracis* during infection of cultured macrophages and following infection in a mouse model of pulmonary anthrax.

**Characterization of BA-Stp1.** Because BA-Stp1 and BA-Stk1 appeared to be important in *B. anthracis* virulence, we began to analyze these proteins in more detail. We predicted that BA-Stp1 could dephosphorylate BA-Stk1, which made it critical to first establish that BA-Stp1 is indeed a phosphatase. Experiments using p-NPP as a synthetic substrate found that BA-Stp1 exhibits phosphatase activity and requires divalent metal ions, Mn<sup>2+</sup> or Mg<sup>2+</sup>, to hydrolyze p-NPP (data not shown). Such a dependence on divalent cations suggested that BA-Stp1 could be a PP2C family phosphatase (1, 3, 5). This was confirmed by further biochemical analysis which revealed that BA-Stp1 is inactivated by nonspecific inhibitors such as sodium pyrophosphate but is unaffected by specific PPP and PP2B phosphatase inhibitors such as okadaic acid and calyculin A (data not shown).

Next, we performed experiments designed to identify the types of residues dephosphorylated by BA-Stp1. Synthetic phosphopeptides with phosphate groups at threonine [RRA(pT)VA], serine [RRA(pS)VA], or tyrosine [RRLIEDAE(pY)AARG] residues were used as substrates in a malachite green/phosphate detection assay. Phosphate release increased when phosphothreonine or phosphoserine peptides were used as substrates but not with the phosphotyrosine peptide (Fig. 2A). Using these synthetic substrates, we also determined the kinetic parameters of the dephospho-
phosphorylation reaction. The $V_{\text{max}}$ and $K_m$ values for the phosphothreonine peptide were calculated to be 115.3 pmol min$^{-1}$ $\mu$g$^{-1}$ and 121.5 $\mu$M, respectively. Similarly, the $V_{\text{max}}$ and $K_m$ values for the phosphoserine peptide were found to be 172.7 pmol min$^{-1}$ $\mu$g$^{-1}$ and 248.6 $\mu$M, respectively. The $K_m$ value of BA-Stp1 for the phosphotyrosine peptide was above the range for accurate determination (data not shown), which is consistent with low levels of Pi detected in the phosphate release assay. Error bars represent the standard errors of the means. Statistical analysis was performed with analysis of variance ($P < 0.0001$).

Examination of BA-Stp1 identified residues (D18, D36, D194, and D233) which corresponded to metal-complexed sites in human PP2Cα, leading us to predict that BA-Stp1 might share an enzymatic mechanism with human PP2Cα (8, 16). To address this possibility, single aspartic acid mutants D18A, D36A, D194A, and D233A were generated in BA-Stp1 and each mutant was assayed for phosphatase activity using RRA(pS)VA or RRA(pT)VA as substrates. As shown in Fig. 2B, substituting alanine for these critical aspartic acid residues eliminated phosphatase activity of BA-Stp1, supporting our prediction that BA-Stp1 is similar mechanistically to the human PP2Cα.

**BA-Stk1 is a substrate of BA-Stp1.** Having confirmed that BA-Stp1 is a phosphatase, we next sought to determine the interaction of this phosphatase with BA-Stk1. To test whether BA-Stp1 dephosphorylates phosphorylated on serine, threonine, or tyrosine residues. These activities were then compared with BA-Stp1 mutants containing alanine substitutions at critical aspartic acid residues. (A) Analysis of phosphoresidue specificity of BA-Stp1. Dephosphorylation of synthetic phosphopeptides by BA-Stp1 was measured using a malachite green phosphate detection system. BA-Stp1 (concentrations ranging from 0 to 600 nM) was incubated with 50 $\mu$M phosphothreonine peptide-RRA(pT)VA (black bars), 50 $\mu$M phosphoserine peptide-RRA(pS)VA (gray bars), and 50 $\mu$M phosphotyrosine peptide-RRLIEDAE(pY)AARG (white bars). Phosphatase activity was detected at 600 nm and measured in terms of picomoles of phosphate (Pi) released. Error bars represent standard deviations. (B) Analysis of phosphatase activity of site-directed aspartic acid mutants of BA-Stp1. Phosphatase activity of site-directed aspartic acid mutants of BA-Stp1 was measured in comparison to that of wild-type BA-Stp1. Using 50 $\mu$M RRA(pS)VA (black bars) and 50 $\mu$M RRA-(pT)VA (gray bars) as substrates, the phosphatase activity of 2 $\mu$M site-directed mutants D18A, D36A, D194A, and D233A and 2 $\mu$M wild type BA-Stp1 was measured as picomoles of phosphate released. Error bars represent the standard errors of the means. Statistical analysis was performed with analysis of variance ($P < 0.0001$).

To confirm our prediction that BA-Stk1 was isolated from *E. coli* in a recombinant form, it was necessary to show that the residues dephosphorylated by BA-Stp1 arose from autophosphorylation and not through cross-phosphorylation by *E. coli* kinases during isolation of the protein. To accomplish this, we incubated BA-Stk1cat with [$\gamma$-32P]ATP, thereby allowing the predicted autophosphorylation to occur. The 32P-labeled BA-Stk1cat was then incubated with BA-Stp1, or buffer control, and the level of incorporated 32P was determined by autoradiography. As shown in Fig. 3C, when labeled BA-Stk1cat was incubated with BA-Stp1 there was a precipitous decrease in the level of detectable phosphorylated kinase. Within 30 min following the incubation start, there was a greater than 90% reduction in the level of detectable phosphorylated kinase, indicating that BA-Stp1 dephosphorylates phosphorylated BA-Stk1.

To confirm that BA-Stk1 was a substrate of BA-Stp1, we examined the dephosphorylated kinase using anti-phosphoserine antibodies (Fig. 4A). As shown in the immunoblot, upon incubation with shrimp alkaline phosphatase, there was no detectable decrease in the phosphoserine signal. However, when BA-Stp1 was added to BA-Stk1cat, a precipitous reduction in the levels of phosphoserine was observed. Prolonged incubation (30 min) resulted in hydrolysis of the phosphate group from serine residues on BA-Stk1cat to levels that were no longer detectable by the anti-phosphoserine antibodies. Finally, when a similar analysis was performed using an inactive phosphatase mutant, BA-Stp1D18A, there was no detectable change in the level of phosphorylated BA-Stk1cat, further suggesting that this reaction requires catalytically active phosphatase.

Previous work by Madec et al. demonstrated that autophosphorylation is critical to the kinase activity of *B. subtilis* PrkC (22), suggesting that BA-Stp1-mediated dephosphorylation could alter the kinase activity of BA-Stk1. Hence, we next analyzed the impact of BA-Stp1 dephosphorylation on the kinase activity of BA-Stk1. To accomplish this, His-tagged BA-
Stk1_cat was incubated with recombinant BA-Stp1 or BA-Stp1D36A, both of which had the His tags removed. This approach was taken in order to separate BA-Stk1_cat and BA-Stp1 prior to performing the kinase assay. After separation of the two proteins using nickel affinity chromatography, BA-Stk1_cat was incubated with $[^{32}P]ATP$, and levels of autophosphorylated kinase were determined across a time course. As shown in Fig. 4B, pretreatment of the kinase with BA-Stp1 resulted in a reduction in the amount of $[^{32}P]P_i$ incorporated during autophosphorylation. In contrast, pretreatment of BA-Stk1_cat with the inactive phosphatase mutant, BA-Stp1 D36A, did not alter levels of kinase activity.

Because it was difficult to absolutely exclude the effects of trace amounts of phosphatase in these experiments, a second experimental approach was utilized to confirm that dephosphorylation reduced the kinase activity of BA-Stk1. After separation of the two proteins using nickel affinity chromatography, BA-Stk1_cat was incubated with $[^{32}P]ATP$, and levels of autophosphorylated kinase were determined across a time course. As shown in Fig. 4B, pretreatment of the kinase with BA-Stp1 resulted in a reduction in the amount of $[^{32}P]P_i$ incorporated during autophosphorylation. In contrast, pretreatment of BA-Stk1_cat with the inactive phosphatase mutant, BA-Stp1 D36A, did not alter levels of kinase activity.

Identification of autophosphorylated residues on BA-Stk1. Phosphorylation and dephosphorylation appeared to be important for regulating the kinase activity of BA-Stk1_cat, and this heightened our interest in identifying residues phosphorylated in BA-Stk1_cat. Indeed, we predicted that such residues are involved in important aspects of BA-Stk1’s enzymatic activity and might be subjected to regulation via BA-Stp1 dephosphorylation. Thus, in the next series of experiments we mapped phosphorylated regions in BA-Stk1. Autophosphorylated BA-Stk1_cat was excised from an SDS-PAGE gel and trypsin digested, and the phosphopeptides were isolated using immobilized gallium. The enriched phosphopeptide pool was then subjected to LC-MS-MS analysis. The MS-MS spectra identified four phosphorylated peptides of BA-Stk1_cat based on sequences from the NCBI nonredundant protein database bank (Table 1). Of these peptides, Stk1:150-183, with the sequence VTDFGIATATSATTITHTNSVLGSVHYLSPEQAR, was predicted to have a cluster of seven phosphoresidues. When BA-Stk1_cat was subjected to dephosphorylation by BA-Stp1, we observed a loss in the level of phosphorylated amino acids within this peptide fragment, indicating that BA-Stp1 hydrolyzes phosphate groups of this phosphopeptide (data not shown). A similar observation was made for the peptide Stk1:150-183, with the sequence VTDFGIATATSATTITHTNSVLGSVHYLSPEQAR, indicating that serine residues on this peptide are dephosphorylated by BA-Stp1. Similar peptides were found by Madec et al. to be phosphorylated in PrkC (22), suggesting that PrkC and BA-Stk1 share autophosphorylation domains.

Kinase activity of serine/threonine mutants of BA-Stk1_cat. Madec et al. performed an extensive mutational analysis of candidate phosphorylated residues in the activation loop of PrkC by substituting alanine for threonines and serines in this domain of the kinase. PrkC mutants that could no longer phosphorylate substrates were then identified (22). Yet,
whether these mutants were defective in ATP hydrolysis and autophosphorylation or whether sustained phosphorylation of these residues influenced activity was not determined. Moreover, whether BA-Stk1 utilized similar residues for autophosphorylation was not known. Thus, we examined representative serine/threonine residues for their contribution to regulation of BA-Stk1cat activity. The first two residues, T165 and S173, were selected as representatives of targets phosphorylated within the activation loop (T165) or outside the activation loop (S173) of the enzymatic domain of BA-Stk1cat. S214 was selected from the identified phosphopeptide Stk1: 208-221. Finally, T290 was selected as a representative of targets phosphorylated in the juxtamembrane region of BA-Stk1 (spanning 55 residues) that is implicated in the regulation of eukaryotic kinases (34). Alanine substitutions of these residues in BA-Stk1cat were made, and the impact of these mutations on kinase activity and autophosphorylation was determined. Kinase activity of the mutants was measured in terms of ATP hydrolysis by analyzing the consumption of ATP in a luminescence-based assay. As shown in Fig. 5A, the kinase activity of mutants S173A and S214A was reduced compared to that of wild-type BA-Stk1cat, suggesting that phosphorylation at residues S173 and S214 is important for BA-Stk1cat kinase activity. Mutants T165A and T290A, however, did not show reduced kinase activity, as levels of ATP hydrolysis were comparable to those of wild-type BA-Stk1cat.

To further test the importance of phosphorylation on these residues, aspartic acid substitutions were used to mimic a phosphorylated state of BA-Stk1 and the mutated proteins were tested for kinase activity by measuring levels of ATP hydrolysis. As shown in Fig. 5B, the kinase activity of S173D and S214D was similar to that of wild-type BA-Stk1cat, suggesting that the phosphorylated state of residues S173 and S214 is critical for kinase activity.

**FIG. 4.** BA-Stp1 dephosphorylation of BA-Stk1 and corresponding decrease in autophosphorylation. Purified BA-Stk1 was subjected to a dephosphorylation reaction by BA-Stp1 and examined for changes in autophosphorylation activity. (A) Western blot analysis of BA-Stk1 serine phosphorylation following incubation with BA-Stp1. BA-Stk1 was incubated under the indicated conditions and examined for dephosphorylation using polyclonal anti-phosphoserine antibodies at the indicated time points. Lanes: 1 to 3, BA-Stk1 incubated in reaction buffer alone; 4 to 6, BA-Stk1 incubated with shrimp alkaline phosphatase (SAP); 7 to 9, BA-Stk1 incubated with BA-Stp1; 10 to 12, BA-Stk1 incubated with enzymatically inactive BA-Stp1 D36A. (B) Autoradiograph of BA-Stk1 autophosphorylation following treatment with BA-Stp1. Prior to the autophosphorylation reaction, BA-Stk1 was incubated with BA-Stp1 or the enzymatically inactive BA-Stp1 D36A for 30 min. Untagged BA-Stp1 and BA-Stp1 D36A were removed from the mixture by affinity chromatography, and the kinase activity of His-tagged BA-Stk1 was examined by analyzing autophosphorylation using [γ-32P]ATP. Reactions were stopped at 0, 15, 30, and 60 min, and the products were analyzed by SDS-PAGE followed by autoradiography. Lanes: 1 to 4, control BA-Stk1; 5 to 8, BA-Stp1-treated BA-Stk1; 9 to 12, BA-Stp1 D36A-treated BA-Stk1. (C) Autoradiograph of BA-Stk1 autophosphorylation and MBP phosphorylation following treatment with BA-Stp1. BA-Stk1 was preincubated with BA-Stp1 or buffer control for 30 min to allow for the dephosphorylation reaction. Subsequently, autophosphorylation and kinase activity were analyzed using MBP and [35S]ATP-S. Reactions were stopped at 15, 30, and 60 min, and the products were analyzed by SDS-PAGE followed by autoradiography.

| TABLE 1. Phosphopeptides of BA-Stk1cat identified by mass spectrometry |
|-----------------------------|-----------------------------|
| Peptide name                | Peptide sequence           |
| Stk1:45-55................... | D[pS]NNEEFIKR              |
| Stk1:150-183................ | VTP[ST]p[TP][TP][PS][pT][pT][pT] |
| Stk1:208-221................ | QPF[ST]p[PS]AVALALK        |
| Stk1:264-273................ | D[pT]ALYPER               |

a Phosphopeptides identified in autophosphorylated Ba-Stk1 by LC-MS-MS analysis of tryptic digests. Residues in parentheses were identified as predicted phosphorylation sites based on a search of the NCBI nonredundant protein database search of all eubacteria (Firmicutes group).
To confirm the role of these residues in the BA-Stk1 kinase activity, both alanine and aspartic acid mutants of T165, S173, S214, and T290 were analyzed in a myelin basic protein (MBP) phosphorylation assay using radiolabeled ATP. Analysis of autophosphorylation activity of the BA-Stk1 mutants demonstrated that the mutants undergo autophosphorylation to levels slightly reduced in comparison to, or similar to, that of wild-type BA-Stk1cat (Fig. 5C). However, comparison of MBP phosphorylation by the mutants and wild-type BA-Stk1cat shows that kinase activities of both alanine and aspartic acid mutants of T165 and S173 are significantly reduced (Fig. 5C). Calculation of the percent phosphorylation of MBP shows that mutants of T165 and S173 exhibit only 20% and 2% phosphorylation, respectively, compared to wild-type BA-Stk1cat (Fig. 5D). These results suggest that the presence of the phosphate moiety on these residues is required for binding and/or phosphorylation of substrates. Though T165A and T165D had no detectable defect in ATP hydrolysis as demonstrated in Fig. 5A and Fig. 5B, both mutants were attenuated in substrate phosphorylation (Fig. 5C and Fig. 5D). More importantly, the native phosphoserine at position 173 is essential both for ATP hydrolysis (Fig. 5A) and for substrate phosphorylation (Fig. 5C and Fig. 5D).

Analysis of MBP phosphorylation by mutant S214A also shows reduced activity; however, this defect is rescued by the aspartic acid substitution on the same site (Fig. 5D), suggesting that the phosphorylation state of residue S214 is important not only for hydrolysis of ATP (Fig. 5A) but for phosphorylation of substrates as well (Fig. 5D). Though residue T290 was predicted as being phosphorylated in kinases homologous to BA-Stk1, alanine or aspartic acid substitutions at this site had no apparent effect on autophosphorylation or substrate phosphorylation (Fig. 5D).

DISCUSSION

The current findings provide insight into a paired serine/threonine phosphatase and kinase in B. anthracis. Several recent studies have shown that Stp/Stk pairs contribute to bacterial virulence and physiology, but the underlying molecular mechanisms and the regulation of these proteins remain poorly understood. To this end, the data provided herein address important gaps in our understanding of how the activity of a bacterial Stk is regulated by a partner phosphatase through dephosphorylation. The results of this study indicate that BA-Stp1 regulation of BA-Stk1 is a complex system of multiple...
residues targeted for dephosphorylation, with some phosphoresidues important only for autophosphorylation, while other residues are necessary for both ATP hydrolysis and substrate phosphorylation.

Analysis of the enzyme kinetic parameters ($K_m$ and $V_{max}$) indicated that BA-Stp1 dephosphorylates phosphoethreonine and phosphoserine residues with $K_m$ values similar to those reported for phosphatases, such as PphA from *Synechocystis* sp. strain PCC 6803 (30) and PrpZ from *Salmonella enterica* (18). Thus, these biochemical and enzymatic characterizations of BA-Stp1 all correspond to the expected activities of a PP2C phosphatase. The data shown in Fig. 3 and 4 indicate that BA-Stp1 utilizes this PP2C phosphatase activity to modulate BA-Stk1, wherein dephosphorylation leads to a decline in kinase activity. Importantly, this reaction appears to be more specific, as BA-Stp1 exhibited a higher affinity toward BA-Stk1 than generic substrates ($K_m$, 9.65 μM versus 248.6 μM).

A graphical summary of our data is presented in Fig. 6. Unlike histidine kinases, whose phosphorylation status can either turn on or turn off a single phosphotransferase mechanism, BA-Stk1 exhibits a complex assembly of critical autophosphorylated residues, some of which contribute to ATP hydrolysis and autophosphorylation and others of which contribute only to substrate phosphorylation. For example, in the absence of phosphorylation of S173 and S214, substrate phosphorylation is attenuated but autophosphorylation increases. The phosphorylation of S173 and S214 may, therefore, shift the kinase from an autophosphorylating state to substrate phosphorylation. While it is difficult to absolutely exclude the influence of subtle conformational changes occurring from the alanine substitution, the aspartic acid substitutions further support the idea that the balance between these two biochemical activities is influenced by the phosphorylation of these residues. Indeed, when phosphorylation is simulated at S214 by aspartic acid substitution, autophosphorylation is reduced and substrate phosphorylation is enhanced. In a corresponding manner, when the residue cannot be phosphorylated due to an alanine substitution, Stk1 increases autophosphorylation and decreases substrate phosphorylation. Conversely, mimicking phosphorylation of S173 by aspartic acid substitution reduced autophosphorylation and substrate phosphorylation, indicating that the addition of a phosphate group to this residue has an overall negative effect on kinase activity. Finally, aspartic acid substitution at T165 slightly increased autophosphorylation, suggesting that phosphorylation of this residue enhances kinase activity. We predict that T165 is one of the key residues dephosphorylated by BA-Stp1 leading to downregulation of kinase activity and that the presence of the threonine at position 165 is necessary to allow for binding and/or phosphorylation of target substrates. Collectively, we observed that phosphorylation of some residues in BA-Stk1 promotes kinase activity, while phosphorylation of other residues represses kinase activity. These results reveal a sophisticated mechanism of control for BA-Stk1 kinase activity, wherein a network of phosphorylated residues are altered to either activate or inactivate the enzymatic activity of this signaling protein in *B. anthracis*.

A comparison of our findings for BA-Stp1 relative to those for *B. subtilis* PrpC is instructive. Like BA-Stp1, PrpC is believed to be functionally coupled with its partner kinase, PrkC. Moreover, PrpC was found to dephosphorylate PrkC in earlier work by Obuchowski and colleagues (27) and this is similar to our current results on Ba-Stp1 dephosphorylation of Ba-Stk1. However, the impact of dephosphorylation on kinase activity has not been determined for the PrpC/PrkC pair. PrpC/PrkC also differs from BA-Stp1/BA-Stk1 in that the phosphatase-kinase pair appears to regulate late-stationary-growth-phase events (e.g., sporulation and biofilm formation) in *B. subtilis*, an effect we did not observe in *B. anthracis*. Despite several attempts, we could not detect or demonstrate any difference in sporulation efficiency between the parent *B. anthracis* Sterne strain and *B. anthracis* STPK101 (data not shown). In fact, extensive analysis of *B. anthracis* STPK101 did not find any notable growth defect under *in vitro* conditions, and a prominent phenotype became apparent only when infection models were used. These data suggest that BA-Stp1/BA-Stk1 may have been refined by *B. anthracis* for growth and virulence *in vivo*, while PrpC/PrkC have evolved to regulate events more important to survival of *B. subtilis* in the environment. Very recently, Shah et al. (33) reported a role for *B. subtilis* PrkC in sensing muropeptides released during germination, and *B. anthracis* was among the list of other bacteria for which this group defined this system. The study by Shah et al. found that null mutants lacking the gene for PrkC and BA-Stk1 were unable to sense muropeptide and peptidoglycan as a germinant. Thus, although we did not observe a defect in germination of the mutant *in vitro*, it is reasonable to

FIG. 6. Graphic summary of BA-Stk1 phosphoresidues targeted by BA-Stp1 and their contribution to specific kinase activities. Four residues are represented in the figure and include the two serines and two threonines predicted as sites of phosphorylation dephosphorylated by BA-Stp1. This model is derived from a series of experiments using a combination of mutagenesis approaches and kinase assays to determine the role of each residue in the activity of BA-Stk1. The symbols † and ‡ denote increases or decreases in activity. Overall kinase activity (KA) is measured in terms of levels of autophosphorylation plus substrate phosphorylation.

Overall, BA-Stp1 utilizes this PP2C phosphatase activity to modulate BA-Stk1, wherein dephosphorylation leads to a decline in kinase activity. Importantly, this reaction appears to be more specific, as BA-Stp1 exhibited a higher affinity toward BA-Stk1 than generic substrates ($K_m$, 9.65 μM versus 248.6 μM).
suspect that the attenuation of virulence could be due to defects in the ability of \textit{B. anthracis} to regulate peptidoglycan-related germination during the establishment of disease.

Our findings report the importance of a critical serine/threonine phosphatase-kinase pair in the virulence of \textit{B. anthracis} and highlight a mechanism by which a bacterial phosphatase modulates the activity of its partner kinase at the molecular level. We hypothesize that BA-Stk1 acts as a sensor for regulating virulence in response to stimuli and does so by autophosphorylation, and as the stimuli subsides BA-Stp1 serves to inactive the kinase by dephosphorylation. Further studies of the phosphatase-kinase association between BA-Stp1 and BA-Stk1 will provide new insights into identification of novel target substrates and the importance of reversible phosphorylation as a regulatory mechanism in \textit{B. anthracis}.

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