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Zinc regulates the activity of kinase-phosphatase pair (BasPrkC/BasPrpC) in *Bacillus anthracis*

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Abstract *Bacillus anthracis* Ser/Thr protein kinase PrkC (BasPrkC) is important for virulence of the bacterium within the host. Homologs of PrkC and its cognate phosphatase PrpC (BasPrpC) are the most conserved mediators of signaling events in diverse bacteria. BasPrkC homolog in *Bacillus subtilis* regulates critical processes like spore germination and BasPrpC modulates the activity of BasPrkC by dephosphorylation. So far, biochemical and genetic studies have provided important insights into the roles of BasPrkC and BasPrpC; however, regulation of their activities is not known. We studied the regulation of BasPrkC/BasPrpC pair and observed that Zn^{2+} metal ions can alter their activities. Zn^{2+} promotes BasPrkC kinase activity while inhibits the BasPrpC phosphatase activity. Concentration of Zn^{2+} in growing *B. anthracis* cells was found to vary with growth phase. Zn^{2+} was found to be lowest in log phase cells while it was highest in spores. This variation in Zn^{2+} concentration is significant for understanding the antagonistic activities of BasPrkC/BasPrpC pair. Our results also show that BasPrkC activity is modulated by temperature changes and kinase inhibitors. Additionally, we identified Elongation Factor Tu (BasEf-Tu) as a substrate of BasPrkC/BasPrpC pair and assessed the impact of their regulation on BasEf-Tu phosphorylation. Based on these results, we propose Zn^{2+} as an important regulator of BasPrkC/BasPrpC mediated phosphorylation cascades. Thus, this study reveals additional means by which BasPrkC can be activated leading to autophosphorylation and substrate phosphorylation.

Keywords *Bacillus anthracis* · PrkC · PrpC · Zinc · Phosphorylation · Ef-Tu · Kinase inhibitors · Thermostability · ICP-OES

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Introduction

Ser/Thr and Tyr mediated phosphorylation is one of the most important post-translational events in signaling of bacteria. We had earlier reported that in *Bacillus anthracis*, the etiological agent of anthrax, loss of key Tyrosine kinase(s) may lead to gain of novel dual specificity protein kinases (phosphorylates Ser/Thr and Tyr) (Arora et al. 2012; Mattoo et al. 2008). In wake of

these observations, it can be speculated that Ser/Thr protein kinases (STPKs) of *B. anthracis* are unique and may be regulated by novel mechanisms, uncommon to other *Bacillus* or bacterial species. Of the three characterized kinases, two (PrkD and PrkG) were shown to be dual specificity protein kinases (Arora et al. 2012). The third kinase BasPrkC (*B. anthracis* PrkC) was a bonafide STPK, speculated to be an "infection specific kinase" (Bryant-Hudson et al. 2011).

Bacillus species exhibit a particular bimodal lifestyle alternating between the vegetative and the spore phases (Pilo and Frey 2011). *B. anthracis* spores infect humans through the respiratory tract and germinate in phagosomes of alveolar macrophages, leading to inhalational anthrax, the most fatal manifestation of the disease (Alvarez et al. 2010). Germinated vegetative cells produce toxins and cause death of the host. Thus, spore germination is a crucial step for the initiation of pathogenesis (Alvarez et al. 2010; Baweja et al. 2008; Khanna and Singh 2001; Singh et al. 1989, 1999; Zaman et al. 2005). In *Bacillus subtilis*, PrkC senses 'muropeptides' as a signal for spore germination (Shah et al. 2008). *B. anthracis* mutant strains-BasΔ*prkC* (single gene mutant) and BasΔ*prkC*Δ*prpC* (double gene mutant with Ser/Thr phosphatase PrpC) were found to be severely defective for growth and virulence in the host as compared to the parental strain (Bryant-Hudson et al. 2011; Shakir et al. 2010), indicating their role in spore germination during infection. BasPrpC inactivates BasPrkC by dephosphorylation, thus ceasing the signals emanating from BasPrkC (Shakir et al. 2010).Therefore, BasPrkC helps in establishing infection and BasPrpC regulates kinase activity and so the balance between the two is crucial to the process.

STPKs have evolved two properties essential for their function: the ability to recognize and phosphorylate their cognate substrates, and sensitive means of regulation (Biondi and Nebreda 2003). PrpC-homologs are usually co-transcribed with PrkC homologs in most bacterial species, and PrpC reverses the effects of PrkC-mediated phosphorylation (Beltramini et al. 2009; Faucher et al. 2008; Jin and Pancholi 2006; Novakova et al. 2005). If they both are co-transcribed and expressed together, then the question remains that how their opposing activities are regulated at the same time. In this study, we have identified Zn^{2+} metal ion as a common regulator of BasPrkC/BasPrpC, which can affect auto- and trans-activities of these proteins.

Materials and methods

In silico analysis

To generate a structural model, BLAST was performed against pdb structure database using $BasPrkC_c$ (1–282 aa) and PrpC (full length) sequences as queries. For BasPrkC, *Staphylococcus aureus* PknB (PDB id: 4EQM) and for BasPrpC, *Streptococcus agalactiae* Ser/Thr phosphatase (PDB id: 2PK0)—were found to possess maximum sequence homology and selected as templates (Rakette et al. 2012; Rantanen et al. 2007). Modeller version 9.10 was used to generate 50 models for each protein-$BasPrkC_c$ and BasPrpC. The models were corroborated by Verify_3D program (http://nihserver.mbi.ucla.edu/SAVES/).

Bacterial strains and growth conditions

Escherichia coli strain DH5α (Novagen) was used for cloning and BL21 (DE3) (Stratagene) was used for the expression of recombinant proteins. *E. coli* cells were grown and maintained with constant shaking (220 rpm) at 37 °C in LB broth (Difco) supplemented with 100 µg/ml ampicillin (Sigma) when needed. *B. anthracis* Sterne strain was grown in LB broth at 37 °C with shaking at 220 rpm. For solid media, LB-Agar (Difco) was used for both *E. coli* and *B. anthracis*.

Growth curve of *B. anthracis* Sterne strain in the presence of $ZnCl_2$

B. anthracis primary culture was inoculated from a single colony and grown till OD_{600} of 1.0. Then secondary cultures were inoculated from primary culture (1:1,000 dilutions). To study the effect of Zn^{2+}, $ZnCl_2$ (Sigma) was added to the cultures at the concentrations of 1 mM, 5 mM and 10 mM, in duplicates. Absorbance was taken at 600 nm and plotted against time.

Inductively coupled plasma optical emission spectrometry (ICP-OES)

ICP-OES was performed with *B. anthracis* Sterne strain, starting with spore phase. Spores were prepared from 4 day old cultures, as discussed previously

Table 1 Primers used in the study

Genes	Vector	Restriction sites	Primers (5′ → 3′)[a]	Reference
BasPrkC	pProEx-HTc	FP-*Bam*HI	TAGGTGAAGT*GGATCC*TCCTGCTGATTGGAAAACGC	This study
(*bas3713*) full length		RP-*Xho*I	TCTCTAGAAAGAAAC*TCGAG*TGTATTATTATTGTGTG	This study
BasEf-Tu	pGEX-5X-3	FP-*Bam*HI	CCTATATAAACTAAGGAGG*GGATCC*GAATGGCTAAAGC	This study
(*bas0108*)		RP-*Xho*I	GGGTTTTTTATATC*ACTCGAG*ATTACTCAACGATAGTAGC	This study
BasPrkC$_c$	pProEx-HTc	FP-*Bam*H1	TAGGTGAAGT*GGATCC*TCCTGCTGATTGGAAAACGC	This study
(*bas3713*)		RP-*Xho*1	TGTAATTAAAATC*TCGAG*TCATTTATTACTTCGTTTG	This study
Kinase domain				
BasPrpC	pProEx-HTc and pGEX-5X-3	FP-*Bam*HI	GCGCAAAGAAGAGACGAGG*GGATCC*AGATGAAAGCCGTGTTTCT	This study
(*bas3714*)		RP-*Xho*I	CGTTTTCCAATCAGCACG*CTCGAG*TTCACCTACTTTCGTTTGTCG	This study
BasPrkC$_c^{K40M}$	pProEx-HTc	FP	CGGGATGTAGCGGTG*ATG*ATATTAAGACTCGAC	This study
		RP	GTCGAGTCTTAATATC*ATC*ACCGCTACATCCCG	This study
pknB	pProEx-HTc	(Gupta et al. 2009)		
pknJ-kd	pProEx-HTc	(Arora et al. 2010)		

[a] Restriction/mutation sites have been italicized

(Setlow and Setlow 1987). Purified spores were heated at 70 °C for 40 min to kill any residual vegetative cells and germination efficiency was estimated. Approximately 1.5×10^9 spores were used for analysis of Zn^{2+}. For analysis of Zn^{2+} in cells, $\sim 1 \times 10^9$ spores were inoculated in 500 ml LB medium, with an initial OD_{600} of 0.08. Cells were harvested at different phases of growth—early log (2 h old culture, $OD_{600} = 0.2$), log (4 h old culture, $OD_{600} = 1.0$), stationary (9 h old culture, $OD_{600} = 2.9$) and 24 h old culture ($OD_{600} > 3$). Harvested cells were washed with deionized, autoclaved MilliQ water and dried completely, followed by estimation of dry-weight. Dry cells were resuspended in 1 % HNO_3 (Merck) and lysed by sonication for 5 min. Clarified samples were processed for ICP-OES in 6 replicates, as described previously (Francois et al. 2012; Liu et al. 2004). ICP-OES was performed at Shriram Institute for Industrial Research, Delhi, India. The ICP-OES system was calibrated with 1 % HNO_3 and standard curve was prepared by different dilutions of Zn^{2+} standards within the limits of detection (ranging from 0.01 to 0.5 ppm). The emission line used for the Zn^{2+} analysis was 213.857 nm. Water sample containing 1 % HNO_3 was taken as blank, which had very low intensity (approximately 20.813 counts/s) below the detection limit of ICP-OES of Zn^{2+} in water sample indicating very low amount of the metal.

Cloning of mutagenesis of *B. anthracis* genes and protein purification

For cloning the full length kinase *basprkC* (*bas3713*, 1–657aa), its catalytic domain *basprkC*$_c$ (1–337aa) and phosphatase *basprpC* (*bas3714*), the genes were PCR amplified from *B. anthracis* genomic DNA, using gene specific forward and reverse primers. The resulting PCR products were cloned into the *Bam*HI and *Xho*I sites of pProEx-HTc vector and/or pGEX-5X-3. The details of primers and plasmids are provided in Table 1. Gene encoding BasEf-Tu (*bas0108*) was cloned similarly into the vectors pGEX-5X-3 and pProEx-HTc. The clones were confirmed with restriction digestion and DNA sequencing (Biolinkk).

To generate Lysine (Lys40) mutant of BasPrkC, site-directed mutagenesis was carried out using the QuikChange®XL site-directed mutagenesis kit (Stratagene) as per the manufacturer's instructions, using

HTc-BasPrkC$_c$ as template. The clones were confirmed with DNA sequencing.

The recombinant plasmids were transformed, overexpressed in *E. coli* BL-21 (DE3) and the proteins were purified by Ni^{2+}-NTA or Glutathione Sepharose affinity columns (Qiagen) as described (Gupta et al. 2009). The purified proteins were visualized by coomassie stained SDS polyacrylamide gel and the concentrations were estimated by Bradford assay (Bio-Rad).

In vitro kinase assays

In vitro kinase assays of the full length kinase and its catalytic domain (1 μg) were carried out in kinase buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES] [pH 7.2], 5 mM $MgCl_2$ and 5 mM Dithiothreitol [DTT]) containing 2 μCi [γ-^{32}P]ATP (BRIT, India) followed by incubation at 25 °C for 30 min or as indicated. Phosphorylation of BasEf-Tu (5 μg) was also carried out similarly. Reactions were terminated by 5X SDS sample buffer followed by boiling at 100 °C for 5 min. Proteins were separated by SDS-PAGE and analyzed by PhosphorImager (Personal Molecular Imager-PMI, Bio-Rad). For determining ionic requirements of BasPrkC$_c$, in vitro kinase assays were performed as described (using 2 μM BasPrkC), except that kinase buffer contained only 20 mM HEPES [pH 7.2] and 5 mM DTT and various concentrations of divalent cations ($MgCl_2$/$MnCl_2$/$ZnCl_2$/$CaCl_2$/$NiCl_2$) were included additionally. In the in vitro kinase inhibition assays, the inhibitors were added to the indicated concentrations and the reactions were carried out as described previously. The control reactions contained DMSO (solvent in which inhibitors were dissolved), which had no effect on kinase activity. The images were quantitated by PMI software (Bio-Rad).

Protein thermostability assays and calculation of melting temperature

BasPrkC$_c$, BasPrkC$_{fl}$ (full length, 1–657aa) and PknB were desalted using PD10-desalting columns (GE healthcare) and re-dissolved in 20 mM Tris–Cl [pH 7.4]. Thermostability assays were performed in UV spectrophotometer (Cary 100 UV–Vis Spectrophotometer, Agilent Technologies) by measuring the absorbance of proteins (500 nM each) at 280 nm at increasing temperatures (40 °C–95 °C, 0.5 °C/min). Melting temperature was also calculated using CD spectroscopy (Jasco J-815 CD spectrometer) with increasing temperatures (40 °C–90 °C). The melting curve was plotted (absorbance versus temperature) and melting temperature (T_m) was calculated at the temperature where 50 % denaturation was observed. Subsequently, fraction of unfolded protein ($\alpha_{unfolded}$) at any temperature was calculated as described earlier and plotted against temperature (Kumar et al. 2011; Owczarzy 2005). At any point, the fraction of melted peptide, θ is calculated from the standard formula, $\theta = (A - A_L)/(A_U - A_L)$, where A, A_L, and A_U are sample absorbance, absorbance of the lower baseline, and absorbance of the upper baseline, respectively.

To assess the activity of kinases at higher temperatures, in vitro kinase reactions of BasPrkC$_c$ were performed at indicated temperatures between 25 °C and 60 °C for 30 min. In another assay, BasPrkC$_{fl}$ and PknB were first incubated at different temperatures—35 °C, 40 °C, 45 °C or 50 °C for 30 min and then the kinase assays were performed at same temperature.

Immunoblotting to identify phosphorylated residues

To detect the phosphorylated residues, immunoblotting with α-pSer and α-pThr antibodies was performed as described previously (Gupta et al. 2009; Arora et al. 2012; Sajid et al. 2011a). The blotting was carried out with primary antibodies α-pSer and α-pThr (Invitrogen) at 1:10,000 dilution and goat anti-rabbit IgG secondary antibodies (Bangalore Genei) (1:10,000) for 1 h each at room temperature. The blots were developed using SuperSignal® West Pico Chemiluminescent Substrate kit (Pierce Protein Research Products) according to manufacturer's instructions.

Dephosphorylation assays of BasPrkC$_c$ and BasEf-Tu using BasPrpC

Phosphorylated BasPrkC$_c$ (1 μg) and BasEf-Tu (5 μg) were subjected to dephosphorylation by BasPrpC (1 μg). Dephosphorylation reaction was carried out at 25 °C for increasing time-points up to 30 min. Reactions were terminated by adding 5X SDS sample buffer followed by boiling for 5 min at 100 °C. The samples were resolved on SDS-PAGE and the signals were analyzed by PhosphorImager. The phosphatase

activity of BasPrpC was determined by performing *p*NPP hydrolysis assay. BasPrpC (1 μM) was added to a reaction mixture containing phosphatase assay buffer (50 mM Tris pH 8.0, 5 mM DTT, 40 μM or 4 mM $MnCl_2$, depending on the range of Zn^{2+}) and 10 mM *p*NPP in a 96-well plate and incubated at 37 °C for indicated time points and absorbance was read at 405 nm (Microplate reader, Bio-Rad). $ZnCl_2$ was added to the reactions, in lower range (1–100 μM) and in higher range (0.1–1 mM), as indicated.

The effect of Zn^{2+} was studied by first performing the kinase assay for 30 min. To this reaction, different concentrations of $ZnCl_2$ and BasPrpC (1 μg) were added and incubated for additional 30 min (25 °C). Reactions were terminated by 5X SDS sample buffer followed by boiling at 100 °C for 5 min. Proteins were separated by SDS-PAGE and analyzed by PhosphorImager.

Results

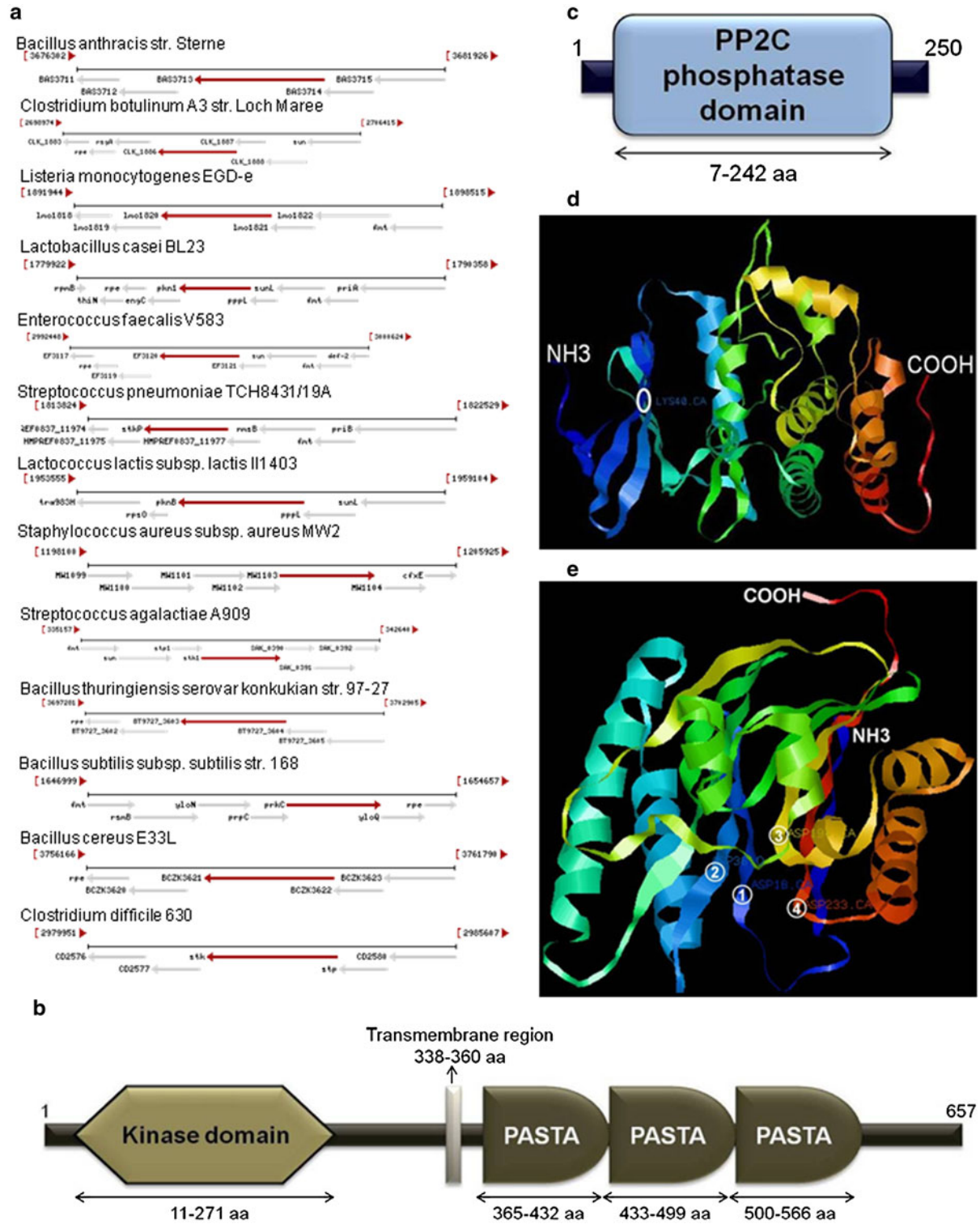
Genomic organization and domain architecture of PrkC–PrpC pair in bacteria

BasPrkC and its homologs are known to regulate key processes in different bacteria: spore germination in *Bacillus* species, antimicrobial resistance and intestinal persistence in *Enterococcus faecalis*, physiology and cell growth in *Mycobacterium* and *Corynebacterium* species (Kristich et al. 2007; Shah et al. 2008; Molle and Kremer 2010; Fiuza et al. 2008). Genomic organization of orthologs of BasPrkC-BasPrpC pair is investigated in diverse gram positive bacteria such as *B. subtilis*, *Clostridium botulinum, Listeria monocytogenes*, *Lactobacillus lactis, E. faecalis, Streptococcus pneumoniae, S. aureus* etc. (Fig. 1a). The orthologs of BasPrkC–BasPrpC show strong conservation in various gram positive bacteria. Our results indicate that BasPrkC–BasPrpC pair is co-transcribed in *B. anthracis* (data not shown), which is also supported by similar observations of Bryant-Hudson et al. (2011). The homologs of this pair are also known to be co-transcribed in other bacterial species (Debarbouille et al. 2009; Jin and Pancholi 2006; Madec et al. 2002).

BasPrkC and its homologs possess exclusive domain architecture: extracellular C-terminal Penicillin-binding protein And Serine/Threonine kinase Associated (PASTA) domain and intracellular N-terminal catalytic domain (Bryant-Hudson et al. 2011; Ruggiero et al. 2011). BasPrkC has three PASTA domains, oriented back-to-back in the same direction (Fig. 1b). These domains are separated from the intracellular kinase (catalytic) domain via a hydrophobic transmembrane region and a juxtamembrane region. BasPrpC is a cytosolic protein, unlike its *Mycobacterium tuberculosis* homolog-PstP, which is a membrane protein. BasPrpC belongs to the PP2C-class of metal dependent phosphatases (PPM family) and targets Ser/Thr phosphorylated proteins including PrkC (Chopra et al. 2003; Shakir et al. 2010; Sajid et al. 2011b) (Fig. 1c). To find structural details, we performed homology modeling of $BasPrkC_c$ and BasPrpC using primary amino acid sequences, as described in materials and methods. The critical residues of verified $BasPrkC_c$ and BasPrpC structures are labeled (Fig. 1d, e).

Regulation of BasPrkC activity

To elucidate the regulatory mechanisms of BasPrkC kinase activity, effects of few known and some unique cofactors were investigated. STPKs require various divalent cations as cofactors for their activity (Arora et al. 2010; Av-Gay et al. 1999; Udo et al. 1997). In line with other bacterial STPKs, BasPrkC was also shown to be active in the presence of Mg^{2+} and Mn^{2+} ions, although it was not clear if it requires both ions or either one is sufficient (Shakir et al. 2010). Activity of $BasPrkC_c$ (1–337aa, cytosolic domain) was assessed by in vitro kinase assays in presence of several metal ions, which indicated that it can also utilize Zn^{2+} ion as a cofactor in addition to Mg^{2+} or Mn^{2+} for its activity (Fig. 2a). To understand the specific effect of Zn^{2+} on BasPrkC activity, in vitro kinase assays were performed with increasing Zn^{2+} concentrations. An increase in phosphorylation was observed with increasing Zn^{2+} concentrations—from 0.01 to 0.5 mM, with as low as 0.01 mM Zn^{2+} (approx. 653 ppb) being able to activate BasPrkC in absence of other metal ions (Fig. 2b, Fig. 1 online supplemental resource). We could not observe any changes in BasPrkC activity below the given Zn^{2+} ion concentrations. This may be due to the lack of activation of BasPrkC at concentration of Zn^{2+} below 0.01 mM or technical limitation of in vitro kinase assays. $BasPrkC_c$ was not active in the presence of Ca^{2+} and Ni^{2+}, thus confirming the specificity of BasPrkC for selected metal ions. To find the effect of Zn^{2+} on other *B.*


a
Bacillus anthracis str. Sterne
Clostridium botulinum A3 str. Loch Maree
Listeria monocytogenes EGD-e
Lactobacillus casei BL23
Enterococcus faecalis V583
Streptococcus pneumoniae TCH8431/19A
Lactococcus lactis subsp. lactis Il1403
Staphylococcus aureus subsp. aureus MW2
Streptococcus agalactiae A909
Bacillus thuringiensis serovar konkukian str. 97-27
Bacillus subtilis subsp. subtilis str. 168
Bacillus cereus E33L
Clostridium difficile 630
c
1
PP2C phosphatase domain
250
7-242 aa
d
NH3
COOH
e
COOH
NH3
b
Transmembrane region
338-360 aa
1
Kinase domain
PASTA
PASTA
PASTA
657
11-271 aa
365-432 aa
433-499 aa
500-566 aa

◀ **Fig. 1** Genomic and domain organization of BasPrkC and BasPrpC. **a** Conservation of BasPrkC/BasPrpC pair in different bacterial species is shown. The kinase gene is indicated by *red arrow*. The genomic organization was adapted from NCBI. **b** Diagrammatic representation of domain organization of BasPrkC, showing N-terminal kinase domain and C-terminal PASTA domains, separated by transmembrane region. **c** Diagrammatic representation of BasPrpC domain organization, a PP2C-family phosphatase. The domain organization was adapted from SMART domain analysis. **d** PDB view of Ribbon model of BasPrkC_c. The model indicates N- and C-terminals and the critical Lys40 residue (encircled). **e** PDB view of Ribbon model of BasPrpC. The model indicates N- and C-terminals and the critical residues (encircled) as—(1) Asp18 (2) Asp36 (3) Asp194 (4) Asp233. Both the tertiary structure models were derived from the primary sequences of the proteins, using Modeller version 9.10 and corroborated by Verify_3D program. (Color figure online)

anthracis kinases, PrkD and PrkG were also probed. There was no effect of Zn^{2+} on PrkD activity, while the activity of PrkG was reduced (data not shown).

Regulation of BasPrpC activity

Ser/Thr phosphatase BasPrpC dephosphorylates and thus inactivates BasPrkC (Shakir et al. 2010). In an earlier study on a homolog of BasPrpC in *M. tuberculosis*—PstP, we reported the regulation and inhibition of phosphatase activity by Zn^{2+} (Sajid et al. 2011b). Thus, the effect of Zn^{2+} on BasPrpC was also analyzed by a biochemical assay using *para*-nitrophenol phosphate (*p*NPP). Since BasPrpC requires Mn^{2+} for its activity, all the inhibition assays were performed in the presence of excessive Mn^{2+} ions. We found that Zn^{2+} inhibits BasPrpC activity in the concentration range of 1–100 μM (approx. 65.38–6,538 ppb), in presence of 40 μM Mn^{2+} (Fig. 3a). On performing assays at a higher concentration of Mn^{2+} (4 mM), Zn^{2+} was able to inhibit BasPrpC in range of 0.1 mM to 1.0 mM (Fig. 2 online supplemental resource). Thus, the effective inhibition of BasPrpC by Zn^{2+} also depends on relative concentration of Mn^{2+} ions, which is usually higher than Zn^{2+} in the *B. anthracis* cells (Tu et al. 2012). To further investigate the overall role of Zn^{2+} in regulating activity of both proteins—BasPrkC and BasPrpC, we studied the combined effect of Zn^{2+} and Mn^{2+} on their activities. Dephosphorylation of Bas PrkC$_c$ by BasPrpC was proportionately inhibited in the presence of increasing Zn^{2+} concentrations (Fig. 3b). Thus, the equilibrium of kinase-phosphatase reaction shifts towards the kinase activity in the presence of Zn^{2+}, leading to enhanced phosphorylation. Interestingly, Zn^{2+} also inhibited *B. anthracis* growth significantly (Fig. 3c), indicating that it plays an important role in *B. anthracis* physiology.

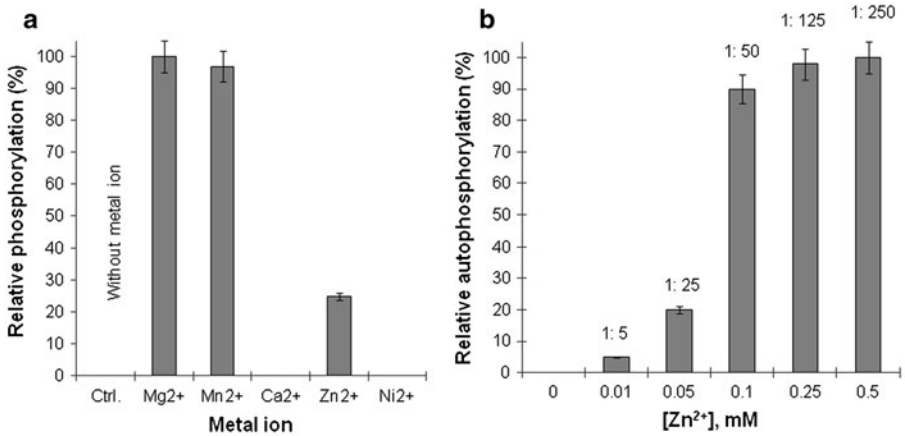


Fig. 2 Ionic regulation of BasPrkC. **a** Activity of Bas PrkC$_c$ (1 μg each) in the presence of various metal ions (10 mM each). Maximum phosphorylation of Bas PrkC$_c$ was observed in the presence of Mg^{2+}, which was taken as 100 % and relative phosphorylation was calculated in the presence of other ions (using PMI image analysis software). It is evident from the figure that Bas PrkC$_c$ can utilize Zn^{2+} in addition to Mg^{2+} and Mn^{2+} for its activation. **b** Activity of Bas PrkC$_c$ (2 μM) was assessed with increasing concentrations of Zn^{2+} in the in vitro kinase assays. The representative coomassie stained SDS polyacrylamide gel images have been shown in Fig. 1 online supplemental resource. Maximum activity (at 0.5 mM Zn^{2+}) was taken as 100 % and relative phosphorylation intensity was calculated. The molar ratio of BasPrkC: Zn^{2+} is indicated above the corresponding column. The experiments were repeated three times and the *error bars* represent the standard deviation (SD) of three individual values

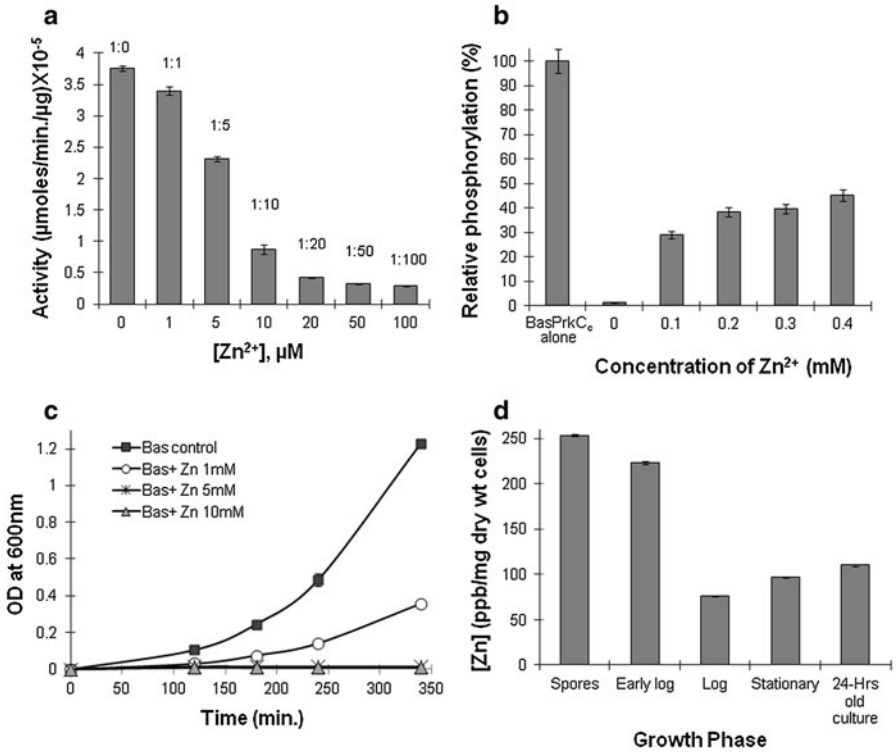


Fig. 3 Effect of Zn^{2+} ions and estimation of Zn^{2+} concentration in *B. anthracis* cells. **a** Effect of Zn^{2+} on the activity of BasPrpC (1 µM) was evaluated by *p*NPP assay. Zn^{2+} reduced the activity of phosphatase in a concentration dependent manner. The molar ratio of BasPrpC:Zn^{2+} is indicated above the corresponding column, with constant Mn^{2+} (40 µM). As clearly evident, as low as 1 µM Zn^{2+} inhibited the activity of BasPrpC by 10 %, even in the presence of excess of Mn^{2+}. **b** Phosphorylation of $BasPrkC_c$ was measured in the presence of BasPrpC and increasing concentrations of Zn^{2+}. The phosphorylation on $BasPrkC_c$ without BasPrpC and Zn^{2+} was taken as 100 % and relative phosphorylation was calculated. In all the histograms, each value is average ± SE of three observations. **c** Growth curve of *B. anthracis* Sterne strain cells was performed in the absence and presence of Zn^{2+} ions (1–10 mM). Each value is average ± SE of two observations. **d** The cellular concentration of total Zn^{2+} content was estimated in *B. anthracis* Sterne strain cells, growing at different stages, starting with spores. The estimates are provided in parts per billion of Zn^{2+} (ppb) per mg of dry weight of the cells. The experiment was performed once in six replicates and the error bars represent the mean SD. value of six replicates. The concentrations were calculated using the Zn^{2+} standard curve (Fig. 3 online supplemental resource)

Concentration of Zn^{2+} in *B. anthracis* cells

In prokaryotic cells, Zn^{2+} is present and required in minute quantities (McDevitt et al. 2011). As observed in Fig. 3c, even 1 mM additional Zn^{2+} in media is toxic to *Bacillus* cells. To find the role of Zinc in cellular development of *B. anthracis*, we tried to estimate the concentration of Zn^{2+} ions inside *B. anthracis* spores and germinating cells, during different growth phases. We expected Zn^{2+} to be present in very less quantity in the cells, since it was found to be toxic at higher concentrations. To determine Zn^{2+} content, we utilized the technique "Inductively Coupled Plasma Optical Emission Spectrometry" (ICP-OES), which is routinely used for determination of trace elements in biological samples (Graham et al. 2009; Tu et al. 2012). Samples were prepared in 1 % HNO_3, diluted in de-ionized MilliQ water (although traces of Zn^{2+} may be present in MilliQ water, which are below the detection limit of ICP-OES, data not shown). In log phase cells ($OD_{600} = 1.0$), the concentration of Zn^{2+} was estimated to be 76.5 ppb/mg dry weight of cells. On comparing the various developmental phases of the cells, viz. spores, germinating spores, stationary phase and late stationary phase cells, the concentration of Zn^{2+} varied in all the samples (Fig. 3d, Fig. 3 online supplemental resource). Interestingly, spores contained highest amount of Zn^{2+} (>250 ppb/mg dry wt.). This concentration decreases as the spores started germinating and proceed to subsequent growth phases of culture,

ultimately being lowest in log phase cells. Stationary phase cells also had higher Zn^{2+} content as compared to log phase cells, albeit less than that of spores. Thus, this analysis directly depicts that Zn^{2+} content of the *B. anthracis* cells vary according to the growth phase and physiological environments. This variation may proportionately alter the functioning of enzymes that are stimulated or inhibited by cellular Zn^{2+} concentration (like BasPrkC, BasPrpC, ribosomal proteins, superoxide dismutase or lethal factor).

Effect of temperature on stability and activity of BasPrkC

Bacillus spores are known to withstand high temperatures (Spotts Whitney et al. 2003). Since *B. subtilis* PrkC is shown to be involved in spore germination process (Shah et al. 2008), we sought to determine the thermostability of BasPrkC (in the temperature range of 40 °C–95 °C). With increase in temperature of the BasPrkC, hyperchromic effect was observed by CD spectroscopy and UV melting assays. $BasPrkC_c$ was observed to be very stable protein with a melting temperature (T_m) of ~75 °C. The fraction of unfolded protein ($\alpha_{unfolded}$) at a given temperature was also calculated (Fig. 4a and Fig. 4 online supplemental resource). In addition to thermostability, the enzymatic activity of $BasPrkC_c$ at different temperatures was also measured to study its effect on kinase activity. As shown in Fig. 4b, $BasPrkC_c$ was active in the temperature range of 25 °C to 50 °C (Fig. 4b and Fig. 5 online supplemental resource). Similar results were observed when $BasPrkC_c$ was pre-incubated at 35 °C–50 °C for 30 min and then the kinase assays were performed (data not shown). Validation of these observations was carried out by performing similar assays with $BasPrkC_{fl}$ and PknB,

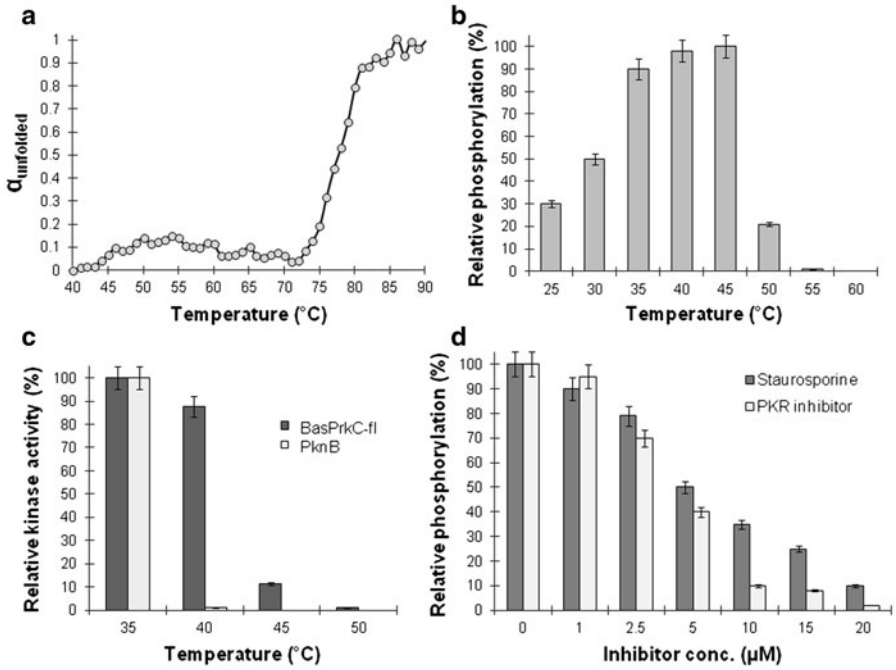


Fig. 4 Effect of temperature and kinase inhibitors on autophosphorylation of BasPrkC **a** Thermal denaturation of $BasPrkC_c$ (500 nM) was measured by CD spectroscopy and the unfolded fraction ($\alpha_{unfolded}$) at a given temperature was calculated which was then plotted against temperature (40 °C–90 °C). **b** Autophosphorylation activity of $BasPrkC_c$ (1 μg) was determined at different temperatures (25 °C–60 °C) in the in vitro kinase assays. Maximum phosphorylation was observed at 45 °C, which was taken as 100 % and relative phosphorylation was calculated. The corresponding image representations have been shown in Fig. 5 online supplemental resource. **c** The activity of $BasPrkC_{fl}$ was compared with its close homolog PknB of *M. tuberculosis*, at various temperatures. The proteins were pre-incubated at indicated temperatures and then used for kinase assays at the same temperatures. Both kinases were active at 35 °C (taken as 100 %). While PknB lost its activity beyond 35 °C, BasPrkC-fl was active till 45 °C. **d** Activity of $BasPrkC_c$ was measured in the presence of kinase inhibitors (0–20 μM). The activity without any added inhibitor was taken as 100 % and relative phosphorylation was calculated. The corresponding representative gel images have been shown in Fig. 6 online supplemental resource. Each value is average ± SE of three observations

the mycobacterial homolog of BasPrkC. While PknB does not exhibit any activity beyond 40 °C, $BasPrkC_{fl}$ was very active till 45 °C (Fig. 4c).

Inhibition of BasPrkC activity by protein kinase inhibitors

Activation of BasPrkC is dependent on its autokinase activity, as also known for several other STPKs (Johnson et al. 1996; Bryant-Hudson et al. 2011). In order to understand the mode of kinase regulation, molecules that can suppress BasPrkC autophosphorylation may be very useful. In this regard, the ability of four known kinase inhibitors was studied to assess $BasPrkC_c$ inhibition. The inhibitors used in this study were: staurosporine, which acts against Protein kinase C (Tamaoki et al. 1986); KN-93, an antagonist of Ca^{2+}/CaM kinase II (Rokolya and Singer 2000); PKR inhibitor, an antagonist of RNA dependent protein kinases (Ruvolo et al. 2008); and Genistein, which suppresses the activities of Tyrosine kinases (Arora et al. 2012; Akiyama et al. 1987). Both staurosporine and PKR inhibitor decreased the autophosphorylation of $BasPrkC_c$ (Fig. 4d and Fig. 6 online supplemental resource), while no inhibition was noticed with KN-93 and Genistein (data not shown).

Reversible phosphorylation of BasEf-Tu, a common substrate of BasPrkC/BasPrpC

The effect of BasPrkC regulators was also investigated on substrate phosphorylation efficiency. In *M. tuberculosis*, PknB regulates protein synthesis by phosphorylating an essential protein, elongation factor Tu (Ef-Tu) (Sajid et al. 2011a). Ef-Tu homologs are also known to be phosphorylated by PrkC homologs in other bacterial pathogens (Sajid et al. 2011a). BasEf-Tu was indeed phosphorylated with $BasPrkC_c$ in a time-dependent manner for 30 min and relative phosphorylation was calculated (Fig. 5a, b and Fig. 7a online supplemental resource). For dephosphorylation assay, BasEf-Tu—previously phosphorylated by $BasPrkC_c$, was incubated with BasPrpC. Time-dependent dephosphorylation of BasEf-Tu was observed, confirming the reversibility of phenomena (Fig. 5c and Fig. 7b online supplemental material). Immunoblot analysis of BasEf-Tu using α-pSer and α-pThr antibodies indicated that both Ser and Thr residues were phosphorylated (Fig. 8 online supplemental resource).

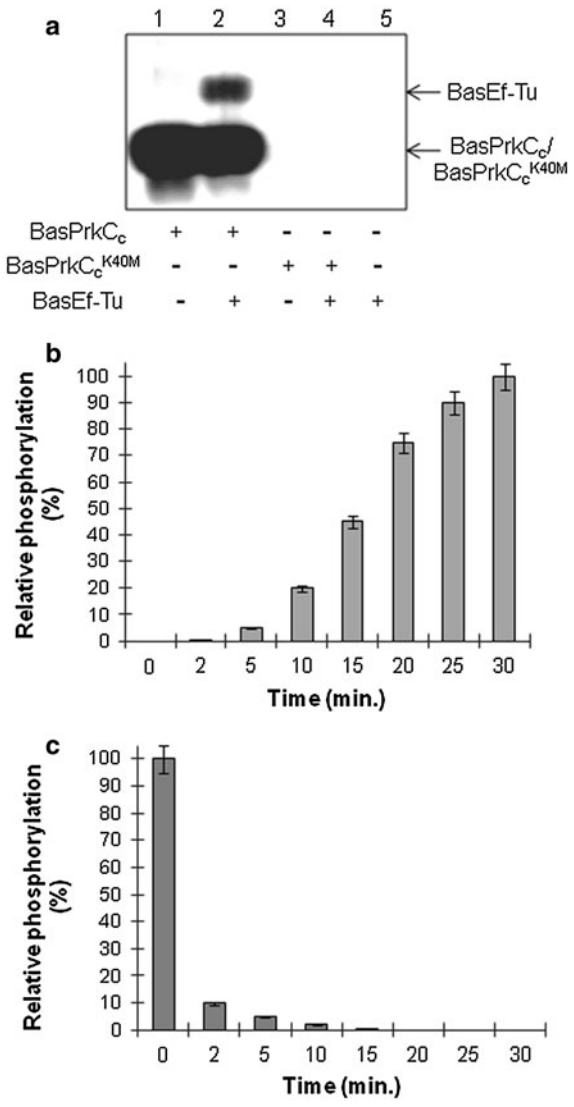


Fig. 5 Reversible phosphorylation of BasEf-Tu. **a** In vitro kinase assays illustrating $BasPrkC_c$-mediated phosphorylation of BasEf-Tu. As shown in the autoradiogram, no phosphorylation was observed when kinase dead mutant ($BasPrkC_c^{K40M}$) or substrate alone was used. **b** BasEf-Tu (5 μg) was phosphorylated by $BasPrkC_c$ (1 μg) in a time-dependent manner, up to 30 min. The phosphorylation signal after 30 min was taken as 100 % and relative phosphorylation was estimated using PMI image analysis software. **c** BasEf-Tu (5 μg) was phosphorylated by $BasPrkC_c$ (1 μg) and then dephosphorylated by BasPrpC (1 μg) in a time-dependent manner. Dephosphorylation was evaluated by measuring signal intensity (zero minute taken as 100 % phosphorylation). Relative phosphorylation was measured after incubating with BasPrpC for various time-points. The representative gel images have been shown in Fig. 7 online supplemental resource. Each value is average ± SE of three observations

M. tuberculosis STPK PknJ (Arora et al. 2010) and BasPrpC served as positive and negative controls, respectively. Thus, BasEf-Tu is phosphorylated by $BasPrkC_c$ on both Ser and Thr residues and is dephosphorylated by BasPrpC.

Regulation of BasEf-Tu phosphorylation by BasPrkC

We assessed the roles of several factors that can affect kinase activity such as Zn^{2+}, temperature and kinase inhibitors on BasPrkC mediated reversible phosphorylation of BasEf-Tu. BasEf-Tu was first phosphorylated by $BasPrkC_c$ using standard in vitro kinase assay and then dephosphorylated by BasPrpC in the presence of Zn^{2+}. As observed in Fig. 6a, Zn^{2+} partially protected the dephosphorylation of BasEf-Tu from BasPrpC, ultimately enhancing the extent of phosphorylation (Fig. 6a).

Furthermore, BasEf-Tu was phosphorylated by $BasPrkC_c$ at different temperatures. Phosphorylation of BasEf-Tu was observed till 45 °C, but a comprehensible increase in phosphorylation levels could not be detected due to precipitation of BasEf-Tu at higher temperature. Subsequently, the effect of BasPrkC antagonists was also assessed on its phosphotransfer potential and relative phosphorylation of BasEf-Tu was calculated. The phosphorylation of BasEf-Tu was reduced by almost 70 % in the presence of 20 μM staurosporine and PKR inhibitor (Fig. 6b).

Discussion

Phosphorylation is a proficient mechanism for regulation of many proteins, catalyzed by their cognate kinases. Phosphoproteomic studies of diverse bacterial species have revealed that in each of the proteome, more than 70 proteins are phosphorylated on Ser/Thr residues (Macek et al. 2007; Macek et al. 2008; Sun et al. 2010; Prisic et al. 2010). In *B. subtilis*, muropeptide-activated PrkC was shown to phosphorylate translation elongation factor Ef-G that acts as a germination signal for spores (Shah et al. 2008; Shah and Dworkin 2010), thus highlighting the importance of phosphorylation in physiology of *Bacillus*. While *B. subtilis* PrkC has broad role in other cellular processes, its *B. anthracis* homolog is important for virulence (Bryant-Hudson et al. 2011; Shakir et al. 2010); however, regulation of its activity is not well understood. The present study reports various ways by which BasPrkC can be activated and can phosphorylate its substrates.

STPKs are widely known to utilize metal ions as cofactors for their activity and metal-ion binding can induce conformational changes in STPKs, resulting in altered activity profiles (Newton 2001). Structural data on BasPrkC homologs—PknB of *M. tuberculosis* and Protein Kinase A of eukaryotes, indicates the presence of two binding sites of divalent ions coordinated to ATP (Ortiz-Lombardia et al. 2003). Amongst the three divalent cations tested in this study (Mg^{2+}, Mn^{2+} and

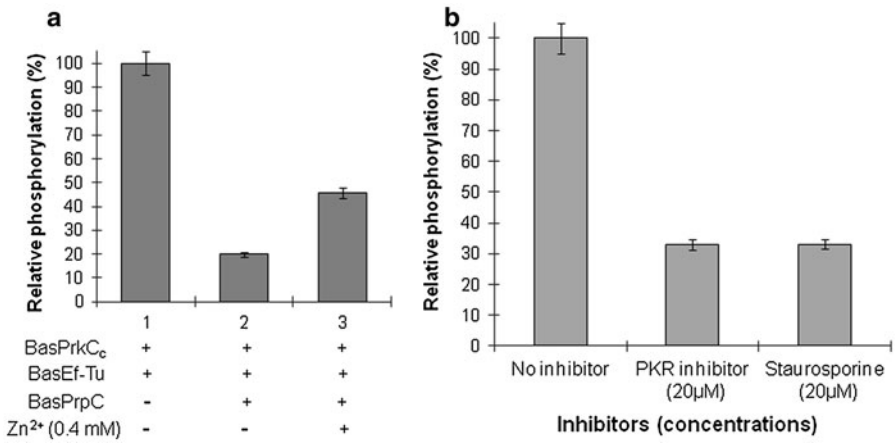


Fig. 6 Effect of BasPrkC regulators on its phosphotransfer potential. **a** Effect of Zn^{2+} on the phosphorylation of BasEf-Tu was studied. BasEf-Tu phosphorylated by $BasPrkC_c$ without BasPrpC and Zn^{2+} was taken as 100 % and relative phosphorylation was calculated when BasPrpC was added, with or without Zn^{2+}. **b** Relative phosphorylation of BasEf-Tu by $BasPrkC_c$ was studied in the presence of staurosporine and PKR inhibitor. The values are average ± SE of three individual observations

Zn^{2+}), Mg^{2+} is thought to be the preferred cofactor of STPKs while Mn^{2+} is a coveted cofactor of Tyrosine kinases (Herberg et al. 1999; Swarup et al. 1984; Waas and Dalby 2003).

BasPrkC and BasPrpC homologs are found to be conserved in several other pathogenic and non-pathogenic bacteria. Autophosphorylation of kinase and extent of phosphotransfer depends on the relative activities of both BasPrkC and BasPrpC. Both BasPrkC and BasPrpC require Mg^{2+}/Mn^{2+} for their activities, whereas Zn^{2+} has alternate effects on both these proteins and overall favors the phosphorylation events. Thus, variation in Zn^{2+} concentration can regulate the phosphorylation levels of substrates. Zn^{2+} mediated regulation of phosphorylation in *B. anthracis* could be very important as the BasPrkC/BasPrpC pair is co-expressed in the cell (Bryant-Hudson et al. 2011) and no regulatory mechanism is known, which could control their antagonistic activities simultaneously. In absence of such control, BasPrpC would inactivate BasPrkC and dephosphorylate its substrates. Our results indicate that presence of Zn^{2+} can inhibit dephosphorylation by BasPrpC while promoting the BasPrkC kinase activity. Interestingly in *B. subtilis*, PrkC is thought to regulate protein translation by phosphorylating Ef-G and CpgA during spore germination while Zn^{2+} is required by multiple ribosomal proteins (Gabriel and Helmann 2009; Nanamiya et al. 2004; Nanamiya et al. 2006; Pompeo et al. 2012). The inverse regulation of kinase/phosphatase pair, coupled by Zn^{2+} and the BasPrkC dependent phosphorylation of BasEf-Tu may correlate the regulation of translational efficiency to Zn^{2+} availability. Interestingly, the role of Zn^{2+} in eukaryotes is well known in extracellular signal transduction, secondary messenger metabolism and protein phosphorylation (Beyersmann and Haase 2001). Increase in extracellular Zn^{2+} enhances Tyrosine phosphorylation and Mitogen activated protein kinase activity in murine fibroblasts (Beyersmann and Haase 2001). In a recent study, activity of receptor Tyrosine phosphatase-β is shown to be inhibited by picomolar concentrations of free Zn^{2+} ions using a more sensitive fluorescence based Tyrosine phosphatase assay (Wilson et al. 2012). The role of Zn^{2+} is most crucial for eukaryotic Protein kinase C where it not only modulates the kinase activity but also its localization (Beyersmann and Haase 2001; Lehel et al. 1995; Zalewski et al. 1991).

A number of studies have highlighted the importance of Zn^{2+} in *Bacillus* physiology (Hantke 2005; Natori et al. 2007). The anthrax lethal toxin contains Zinc-dependent metalloprotease lethal factor and Zinc is crucial for its biological role and toxicity (Klimpel et al. 1994; Singh et al. 1999). For *B. subtilis*, even 5 mM (0.004 %) colloidal suspension of ZnO nanoparticles could inhibit more than 95 % of growth (Jones et al. 2008). In a comparative study of various bacilli, vegetative cells of *B. anthracis* Sterne strain were inactivated by AgION (a silver- and Zinc-containing zeolite) after 2 h of contact with antimicrobial-coated stainless steel (Galeano et al. 2003). In our analysis, we observed that *B. anthracis* growth is inhibited in the presence of $ZnCl_2$. The concentration of Zn^{2+} in the *Bacillus* cells varies with developmental phases. Spores have high Zn^{2+} content, which start to decrease as spores start germinating and continue till cells reach log phase. This indicates that actively growing cells in log phase do not require high Zn^{2+}, rather presence of additional 1 mM Zn^{2+} in culture inhibited growth (Fig. 3c). The cellular levels of Zn^{2+} again start rising as cells reach stationary phase. Taken together, these studies further substantiate the importance of BasPrkC and BasPrpC co-regulation, which respond to Zn^{2+} reciprocally and may help in maintaining cellular development. In our previous study, we showed that the expression of *prkC* is highest in late log phase, indicating its presence in stationary and sporulation phases (Arora et al. 2012). Since the role of BasPrkC has been previously indicated in sporulation and spore germination (Madec et al. 2002; Shah et al. 2008), the high content of Zn^{2+} in spores may have a correlation with BasPrkC activation.

However, certain precaution is needed while extrapolating these results as the Zinc concentration tested for in vitro assays was quite high as compared to the concentration observed in vivo. Additionally, our results depict the total concentration of Zinc in the cell, rather than free Zn^{2+} ions, which might actually be lesser. The term "free" here refers to specify the labile Zinc that is freely available for binding by newly synthesized Zinc metalloproteins. Attempts to measure free Zinc in bacteria have indicated a wide range of concentrations (10^{-9}–10^{-15} M) (Eide 2006; Outten and O'Halloran 2001; Maret 2013; Haase et al. 2013; Wang nd Fierke 2013). Since living cells are exposed to variations from its surrounding environment, the levels of free Zn^{2+} may also vary in different

environmental conditions—in culture or inside the host cells. This level is usually maintained by Zinc transporters and Zinc uptake regulators, which are responsible for balancing the Zinc content from being limiting or being too high to be toxic (Eide 2006). Thus, further studies are needed to validate how the oscillating levels of free Zn^{2+} in the cells may help in precise regulation of BasPrkC and BasPrpC activities.

Bacteria are known to survive in harsh conditions like extreme temperatures. In such conditions, the ability of macromolecules such as proteins to adapt and remain functional is critical for bacterial survival (Miller et al. 2010). In case of *B. anthracis*, spores are known to withstand high temperatures (Spotts Whitney et al. 2003). Thus, it is interesting to know that BasPrkC is the first bacterial STPK to exhibit thermostability at 75 °C, and is able to phosphorylate itself and its substrates even at 45 °C. The factors enhancing the thermostable nature of BasPrkC, as suggested for other thermostable proteins, could be greater hydrophobicity, better packing, increased hydrogen bonding and salt bridges (Kumar et al. 2000). Hence, further structural analyses are required to substantiate the thermostable nature of BasPrkC.

In the case of eukaryotic cellular signaling, it has been observed that pharmacological approaches could be helpful in identifying the substrates and their role in cellular responses (Lizcano and Alessi 2002). In view of the role of BasPrkC in spore germination, compounds that can retard BasPrkC activation may be projected as the basic class of compounds against anthrax. We evaluated several classes of inhibitors known to be active against different protein kinases. Staurosporine was previously shown to reduce spore germination in *B. subtilis* at a very low concentration (10 pM) by interfering with BasPrkC (Shah et al. 2008). Staurosporine and PKR inhibitors are diverse compounds that target ATP binding site in STPKs (Jammi et al. 2003; Ruegg and Burgess 1989; Li et al. 2009). We observed that 20 μM of staurosporine and PKR inhibitor substantially reduced the autophosphorylation ability of $BasPrkC_c$ in vitro, although phosphorylation of BasEf-Tu by $BasPrkC_c$ was not inhibited completely.

Ef-Tu was found to be phosphorylated by PrkC-homologs in *B. subtilis* (Levine et al. 2006), *E. coli* (Lippmann et al. 1993), *Thermus thermophilus* (Lippmann et al. 1993), *L. monocytogenes* (Archambaud et al. 2005) and *M. tuberculosis* (Sajid et al. 2011a), and thus could be proposed as a conserved substrate. Therefore, we performed phosphorylation and dephosphorylation assays to validate that it is also a common substrate of BasPrkC/BasPrpC in *B. anthracis*. Further, immunoblotting experiments were also carried out to confirm the phosphorylation of BasEf-Tu on Ser and Thr residues.

After identification of BasEf-Tu as BasPrkC substrate, we tried to find the factors that can affect the kinase activity and substrate phosphorylation. Zn^{2+} modulates the catalytic efficiencies of BasPrkC and BasPrpC, which in turn affects phosphorylation of BasEf-Tu, ultimately leading to enhanced phosphorylation with increasing Zn^{2+} concentrations. Additionally, the modulators of BasPrkC—temperature and kinase inhibitors—also affect the BasEf-Tu phosphorylation.

Thus, although the role of these two co-expressing and yet antagonistic enzymes has been discussed in different bacterial species, it was still not clear that how both enzymes work together efficiently. It appears that phosphorylation level of BasPrkC may be controlled through Zn^{2+} ions as it inhibits the activity of BasPrpC and activates BasPrkC.

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