Eukaryote-like Ser/Thr protein kinase PrkA modulates sporulation via regulating the transcriptional factor $\sigma^K$ in Bacillus subtilis

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Protein kinase A (PrkA), also known as AMP-activated protein kinase, functions as a serine/threonine protein kinase (STPK), has been shown to be involved in a variety of important biologic processes, including pathogenesis of many important diseases in mammals. However, the biological functions of PrkA are less known in prokaryote cells. Here, we explored the function of PrkA as well as its underlying molecular mechanisms using the model bacterium Bacillus subtilis 168. When PrkA is inhibited by 9-β-D-arabinofuranosyladenine (ara-A) in the wild type strain or deleted in the ΔprkA mutant strain, we observed sporulation defects in B. subtilis, suggesting that PrkA functions as a sporulation-related protein. Transcriptional analysis using the lacZ reporter gene demonstrated that deletion of prkA significantly reduced the expression of the transcriptional factor $\sigma^K$ and its downstream genes. Complementation of sigK gene in prkA knockout mutant partially rescued the phenotype of ΔprkA, further supporting the hypothesis that the decreased $\sigma^K$ expression should be one of the reasons for the sporulation defect resulting from prkA disruption. Finally, our data confirmed that Hpr (ScoC) negatively controlled the expression of transcriptional factor $\sigma^K$, and thus PrkA accelerated sporulation and the expression of $\sigma^K$ by suppression of Hpr (ScoC). Taken together, our study discovered a novel function of the eukaryotic-like STPK PrkA in spore development as well as its underlying molecular mechanism in B. subtilis.

Keywords: PrkA, serine/threonine protein kinase, sporulation, B. subtilis, the transcription factor $\sigma^K$, transcriptional regulation

Introduction

Protein phosphorylation is the principal mechanism by which extracellular signals are translated into cellular responses. A variety of protein kinases are responsible for the reversible phosphorylation at specific amino acid residues, including serine/threonine protein kinases (STPKs) that phosphorylate serine or threonine of the target proteins. Contrary to the signaling pathways predominantly carried out by STPKs in eukaryotes, two-component systems, consisting of His-kinase sensors and their associated response regulators, are the most common signal transduction system in prokaryotes. Therefore, serine, threonine, and tyrosine kinases had been previously thought to
be unique to eukaryotes until phosphorylation at Ser residues was identified in bacteria (Deutscher and Saier, 1988; Reizer et al., 1993). Recent data from genomic sequencing has further illustrated that the eukaryote-like STPKs exist widely in bacteria, suggesting that this well-characterized protein phosphorylation is also distributed in prokaryotes along with two-component systems (Bakal and Davies, 2000). For example, the genome of *Mycobacterium tuberculosis* contains 11 STPKs (Cole et al., 1998; Av-Gay and Everett, 2000). These STPKs are involved in a variety of processes such as development, cell growth, stress responses, primary and secondary metabolism, biofilm formation, antibiotic resistance, and virulence (Cozzone, 2005; Kristich et al., 2007; Wehenkel et al., 2008; Molle and Kremer, 2010; Ohlsen and Donat, 2010).

The AMP-activated protein kinase (AMPK), also called protein kinase A (PrkA), is an important type of STPK. In eukaryotic cells, AMPK is a highly conserved heterotrimeric protein consisting of a catalytic α subunit and regulatory β and γ subunits, and it is regulated by the intracellular ratio of AMP to ATP (Osler and Zierath, 2008). AMPK is activated under conditions of low cellular ATP and high cellular AMP. Therefore, AMPK functions as an energy sensor in cells, and a likely metabolic master switch to coordinate global metabolic response involving cellular uptake of glucose, glycogen synthesis and decomposition, β-oxidation of fatty acids, and mitochondrial biogenesis. At the meantime, AMPK also participates in pathogenesis of important diseases such as ischemic heart, diabetes, cancer, and even viral infection. As a result, it has become a research focus in recent years (Spasic et al., 2009; Kuznetsov et al., 2011).

Comparing to the well-described functions of AMPK in eukaryotic cells, few investigations of AMPK have been reported in prokaryotes. The first gene encoding a prokaryotic PrkA was cloned in *Bacillus subtilis*. Though the sequence of this PrkA exhibited distant homology to eukaryotic proteins, it phosphorylated a 60-kDa target protein at a Ser residue. However, the biological functions of PrkA remained unclear because no phenotypic change was obtained when the *prkA* gene was deleted (Fischer et al., 1996). Later, it has been demonstrated that the transcription of *prkA* was regulated by the spore-specific sigma factor σE (Eichenberger et al., 2003; Steil et al., 2005). Its role was also involved in spore formation because of its localization of spore coat and the decreased sporulation efficiency in *prkA* knockout mutant (Eichenberger et al., 2003). However, how PrkA works requires further elucidation. Additionally, another prokaryotic STPK PrkA was identified in a Gram-positive rod-shaped bacterium *Listeria monocytogenes* (Lima et al., 2011). Analysis of its potential interaction partners through proteomic approaches suggested that the signal transduction pathways mediated by PrkA in *L. monocytogenes* may affect a variety of fundamental functions, such as protein synthesis, cell wall metabolism, and carbohydrates metabolism (Lima et al., 2011). Since the relatively poor understanding on prokaryotic PrkA, we investigate STPK PrkA in *B. subtilis* strain 168 and demonstrate its roles in sporulation as well as the underlying molecular mechanism.

### Materials and Methods

#### Bacterial Strains, Plasmids, and Media

The strains of *B. subtilis* and *Escherichia coli* as well as the plasmids used in this study are listed in Table 1. All *B. subtilis* strains were derivatives of *B. subtilis* 168, and those constructed in this work were prepared via transformation with plasmid DNA, confirmed by PCR analysis, and sequenced to ensure that the targeted changes were made. The oligonucleotides primers used for PCR amplification in this study are listed in Table 2.

The strains were grown in Luria–Bertani medium, and sporulation was induced in 2x SG medium, a modified Schaeffer’s medium containing beef extract 0.3 g/L, peptone 0.5 g/L, MgSO₄•7H₂O 0.5 g/L, KCl 2.0 g/L, 10⁻⁴ M MnCl₂, 10⁻³ M Ca(NO₃)₂, 10⁻⁶ M FeSO₄, 7H₂O and 0.1% glucose (Leighton and Doi, 1971). Antibiotics were used at the following concentrations: chloramphenicol 5 μg/ml, kanamycin 5 μg/ml, and spectinomycin 100 μg/ml.

#### Genetic Manipulation

The double crossover of homologous recombination method was used to construct ∆prkA mutant (PRKA5E), ∆hpr mutant (HPR5E), and ∆prkA∆hpr mutant (PRKA5E HPR5E) of *B. subtilis*. Two homologous fragments of the *prkA* gene, chloramphenicol resistant gene were amplified via PCR. The three fragments were linked by overlapping PCR and inserted into a pMD19-T vector to obtain plasmid pPRKA5E. *B. subtilis* 168 was transformed with plasmid pPRKA5E to generate the ∆prkA mutant strain PRKA5E. For the construction of ∆hpr mutant (HPR5E) and ∆prkA∆hpr mutant (PRKA5E HPR5E), two homologous fragments of the *hpr* gene and erythromycin resistant gene were amplified via PCR, connected by overlap PCR, and inserted into a pEASY-T5 vector to obtain plasmid pHPR5E. The plasmid pHPR5E was finally transformed into *B. subtilis* 168 and ∆prkA mutant (PRKA5E) to obtain the ∆hpr mutant (HPR5E) and ∆prkA∆hpr mutant (PRKA5E HPR5E), respectively.

The encoding gene of the transcriptional factor σK is formed due to a developmental DNA rearrangement of the two separate coding regions (*spoIVCB* and *spoIIC*). To complement the expression of *SigK*, *spoIVCB*, and *spoIIC* were amplified via PCR, linked together by overlapping PCR. The linked PCR product was digested with *Hind*III and *SphI* at primer–incorporated restriction sites, and inserted into a *Hind*III/*SphI* -digested pDG148 vector to obtain plasmid pSIGPRKA5E. The recombinant plasmids pSIGPRKA5E and the blank vector pDG148 were, respectively, transformed into the ∆prkA mutant strain to obtain the bacterial strain SIGPRKA5E that complement the expressions of the *sigK* genes and the corresponding control strain PDG148PRK5E.

#### Sporulation Assays

The bacterial strains were cultured in Luria–Bertani medium and shaken overnight at 37°C. The overnight cultures were transferred to 2x SG medium to induce sporulation. Spores were assayed at 12, 24, and 36 h. The spore numbers per milliliter were measured by plating onto LB agar medium after applying a heat treatment (80°C for 15 min). The number of viable cells per
### TABLE 1 | Bacterial strains and plasmids used in this study.

| Strain or plasmid | Genotype/description | Source or reference |
|-------------------|-----------------------|---------------------|
| **Strains**       |                       |                     |
| Bacillus subtilis | Wild type (WT)        | From Bacillus Genetic Stock Center |
| PRKASE            | prkA::cat             | This work(PRKASE, 168) |
| SPOIVB            | spoIVB (pS::pDG1728, reporter, control) | This work(SPOIVB, 168) |
| GERDBS            | gerD (pD::pDG1728, reporter, control) | This work(GERD, 168) |
| GEREBS            | gerE (pE::pDG1728, reporter, control) | This work(GERE, 168) |
| SPOIVBPRKASE      | spoIVB (p::pDG1728, reporter) | This work(SPOIVB, PRKASE) |
| GERDPRKASE        | gerD (p::pDG1728, reporter) | This work(GERD, PRKASE) |
| GEREPRKASE        | gerE (p::pDG1728, reporter) | This work(GERE, PRKASE) |
| SIGKPRKASE        | sigK (p::pDG1728, reporter, control) | This work(SIGKPRKASE, PRKASE) |
| PDG148PRKASE      | pDG148, control       | This work(pDG148, PRKASE) |
| RSIGKBS           | sigK (p::pDG1728, reporter, control) | This work(RSIGK, 168) |
| RSIGEBS           | sigE (p::pDG1728, reporter, control) | This work(RSIGE, 168) |
| RHRPBS            | hpr (p::pDG1728, reporter, control) | This work(RHRPR, 168) |
| RGLNRBS           | glnR (p::pDG1728, reporter, control) | This work(RGLNR, 168) |
| RSIGDBS           | sigD (p::pDG1728, reporter, control) | This work(RSIGD, 168) |
| RSIGKPRKASE       | sigK (p::pDG1728, reporter) | This work(RSIGK, PRKASE) |
| RSIGEPRKASE       | sigE (p::pDG1728, reporter) | This work(RSIGE, PRKASE) |
| RHRPRPKASE        | hpr (p::pDG1728, reporter) | This work(RHRPR, PRKASE) |
| RGLNRPKASE        | glnR (p::pDG1728, reporter) | This work(RGLNR, PRKASE) |
| RSIGDPRKASE       | sigD (p::pDG1728, reporter) | This work(RSIGD, PRKASE) |
| Escherichia coli  | lacZYA-argF, endA1, recA1, hsdR17, supE44, thi-1, gyrA96, relA1, proA | TaKaRa |
| DH5x              |                       |                     |

| Plasmids          | Genotype/description | Source or reference |
|-------------------|----------------------|---------------------|
| pMD19-T           | Amp                  | TaKaRa |
| pDG1728           | Bla, emr, spc, spoIVG-lacZ, amyE, Pspac | Bacillus Genetic Stock Center |
| pDG148            | kanR, ampR, lacI, phr1, Ppen, Ppsec | Bacillus Genetic Stock Center |
| pPRKASE           | Amp, cat, prkA       | This work |
| pSPOIVB           | Amp, spc, spoIVB-lacZ | This work |
| pGERD             | Amp, spc, gerD-lacZ | This work |
| pSIGKPRKASE       | kanR, ampR, sigK     | This work |

(Continued)

### TABLE 1 | Continued

| Strain or plasmid | Genotype/description | Source or reference |
|-------------------|----------------------|---------------------|
| pRSIGK            | Amp, spc, SigK-lacZ  | Synthesized by Shanghai General Co. |
| pRSIGE            | Amp, spc, SigE-lacZ  | Synthesized by Shanghai General Co. |
| pRHRPR            | Amp, spc, Hpr-lacZ   | Synthesized by Shanghai General Co. |
| pRGLNR            | Amp, spc, GlnR-lacZ  | Synthesized by Shanghai General Co. |
| pRSIGD            | Amp, spc, SigD-lacZ  | Synthesized by Shanghai General Co. |

milliliter was counted, both before and after the heat treatment, as total CFU on LB plates. Sporulation frequency is determined as the ratio of the number of spores per milliliter to the number of viable cells per milliliter (LeDeaux and Grossman, 1995). Data for each strain is from at least three independent experiments.

For the experiment of PrkA inhibitor, 250 µM 9-β-D-arabinofuranosyladenine (ara-A) from Sigma Co. was added into the 2x SG medium before adding overnight culture.

### β-Galactosidase Assays

Plasmid pDG1728 containing the lacZ gene, obtained from the Bacillus Genetic Stock Center (BGSC), was used for constructing reporter vectors for transformation. Segments of the reporter of spoIVB, gerD, and gerE genes were amplified via PCR, digested with EcoRI and BamHI at the corresponding primer-incorporated restriction sites, and inserted into the EcoRI/BamHI-digested pDG1728 vector to obtain plasmids pSPOIVB, pGERD, and pGERE.

To analyze the regulation of gene expression for the transcriptional factor σK, five nested fragments that contained the truncated promoter region of σK were designed to fuse to pDG1728. Those five fragments above were sent to Shanghai General Co. Ltd for synthesis. All the reporter plasmids, including pSIGK, pSIGE, pHPR, pGLNR, and pSIGD, were successfully constructed. Those constructed reporter plasmids were then transformed into wild type (WT) strain B. subtilis 168 and the mutant ΔprkA, respectively.

After the strains harboring lacZ fusions were cultured at 37°C in 2x SG medium to induce sporulation, they were assayed for β-galactosidase activity as previously described (Ferrari et al., 1988). β-galactosidase was assayed using o-nitrophenyl-β-D-galactopyranoside as the substrate and is reported in Miller units. Three repeats were performed at each time point.

### Real-Time PCR Assays

The cells of strains B. subtilis 168, PRKASE, HPR5E, and PRKAK5E HPR5E were grown for 12 h at 37°C in 2x SG medium, respectively, and 5 ml cultures were harvested by centrifugation. The total RNA was isolated using RNA extracting kit (Tiangen, China) following the treatment of DNaseI to avoid DNA contaminant. RNAclean Kit (BioTech, China) was then employed to further purify the total RNA. RNA concentration was determined.
TABLE 2 | The oligonucleotide primers used in this study.

| Name     | Sequence (5'→3') | Function and Source |
|----------|------------------|---------------------|
| PRKA1F   | GACAGCGGGAAGGACTGAG | pPRKASE             |
| PRKA1R   | CCAACCGTCATGTGGCCTCAAT | pPRKASE             |
| CATF     | CAGTTGACGGGAGTATGGAGCACT | pPRKASE           |
| CATRI    | ATATGCTCATATTCTTCGCTCCAAT | pPRKASE           |
| PRKA2F   | ATTAGCGTGGAGCCTGCAAGGC | pPRKASE             |
| PRKA2R   | CCGCATTTCAGCAGCCTTTCGT | pPRKASE             |
| SPOVB     | GATTTGATTTTCTGCGCAGATCCCA | pSPOVB             |
| SPOVBR   | GATCTCTCTATTGCGTTGAATCA | pSPOVB             |
| GDRF     | GATCTATTGCCCTCAACAATAATC | pGERD             |
| GDRD     | GATCTCTACAAAAAAAAGGGCCACTCA | pGERD             |
| GREF     | GATCTCAATATATTCATTGGAACGTC | pGERE             |
| GERER    | GATCCACGGTTTTTGACTGATAAA | pGERE             |
| RSCPVOBVF | CTGTGTAATCTTTAGCTTACTG | Realtime-PCR       |
| RTPVOBVR | GTTGTGTATTTGCTTCCCTTCTT | Realtime-PCR       |
| RTPVOCBF | GATGAAAATGCCAAGAAACAT | Realtime-PCR       |
| RTPVOCBR | AAGTCTCTGCGATCCTACCT | Realtime-PCR       |
| RSCPVOICF | GTAGATAGCTGACGGGCTCAAT | Realtime-PCR       |
| RSCPVOICR | AAGCCTCTGCGATCCTACCT | Realtime-PCR       |
| RHCEREF | GATAAGACAACAAAAAGGGAGATTG | Realtime-PCR     |
| RHCERED | GATACGTGACGGGCGAGAAATC | Realtime-PCR       |
| RTMGBF   | GCAGGCTTATGCGAGGGCG | Realtime-PCR       |
| RTMGBR   | ATGATGATGAGGGCGAGGAAATC | Realtime-PCR     |
| RSPVOIDF | AGCTGACAAAACAGGCGCAT | Realtime-PCR       |
| RSPVOIDR | CCATGGGCTTCTGCGCTGCTGC | Realtime-PCR       |
| RSPVOBVF | TATCGATGCTGCGGGAAGGAAA | Realtime-PCR       |
| RSPVOBVR | CGTAGGAATCTGCCGACACAA | Realtime-PCR       |
| SIK1F    | AAAGCCATTGTCAGGCTGATAAT | pSIgKPRKASE       |
| SIK1R    | CATTACAAGGAAAGGGGGTTCAGA | pSIgKPRKASE       |
| SI1F     | TTTATCCTTCAAGAGTTACGCC | pSIgKPRKASE       |
| SI1R     | CTTTTTGTAATG | pSIgKPRKASE       |

Results

Effect of PrkA Inhibitor ara-A on Sporulation

To determine a possible role of PrkA in B. subtilis, the PrkA inhibitor ara-A was added to decrease the activity of PrkA, and then a series of phenotypes, such as vegetative growth, cell morphology, and sporulation were assayed. Our results showed that the addition of ara-A significantly affected the spore numbers. Specifically, statistical analysis revealed that the sporulation frequencies in the ara-A-treatment groups reduced to 0.65 ± 0.14%, 4.61 ± 1.13%, and 14.1 ± 2.42% at 12, 24, or 36 h, respectively, compared to 20.6 ± 2.45%, 23.3 ± 1.60%, and 47.2 ± 4.09% in the negative controls at the same time points (P < 0.01; Figure 1). Therefore, the significant differences between ara-A-treatment groups and the negative controls suggested that STPK PrkA in B. subtilis might be involved in sporulation. However, no obvious differences were observed in vegetative growth and cell morphology between the cultures treated and not treated with PrkA inhibitor ara-A (data not shown).

Disruption of Gene prkA Results in Decreased Sporulation

To confirm the roles of PrkA in sporulation, the prkA deletion strain (PRKA5E) was successfully constructed and its phenotypes were determined. The vegetative growth of ΔprkA mutant in either LB medium or 2x SG medium was similar to that of the WT strain (Figure 2A), illustrating that the PrkA protein has no effect on the vegetative growth on B. subtilis. However, the sporulation frequencies in ΔprkA mutant strain decreased significantly with 0%, 24.08 ± 1.16%, and 36.09 ± 3.44%, respectively, at 12, 24 or 36 h; while the WT strain had about 19.63 ± 3.83%, 48.31 ± 3.07%, and 49.26 ± 1.20% of the sporulation frequencies at these time points, respectively, (P < 0.01; Figure 2B). Compared the two sets of data between the ΔprkA mutant and

by measuring absorbance at 260 nm using a UV spectrophotometer. After random-primed cDNAs were generated, qPCR analysis was performed with SYBR Green JumpStart Taq Ready Mix for qPCR kit (Sigma–Aldrich Co.) following manufacturer’s instructions. The partial sequence of 16S rRNA was used as an internal control. The PCR amplification used 40 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 40 s on ABI PRISM 7000 Real-Time PCR.

The real-time PCR experiments were repeated three times for each reaction using independent RNA sample.

Statistical Analysis

All the data were expressed as the mean ± SD. Statistical comparisons were performed by a one-way analysis of variance (ANOVA) followed by Dunnett’s t-test.
FIGURE 2 | Disruption of the gene prkA decreases sporulation in B. subtilis 168. (A) The vegetative growth rates of ΔprkA mutant and B. subtilis 168 strain in either LB medium or 2x SG medium. ΔprkA mutant (■) and B. subtilis 168 in LB medium (♦); ΔprkA mutant (●) and B. subtilis 168 in 2x SG medium (▲).

(B) Sporulation frequencies in WT strain B. subtilis 168, strain with ara-A, ΔprkA mutant strain, and ΔprkA mutant with ara-A. *P < 0.01; n.s represented no statistical significance.

the WT strain, it was also suggested the sporulation was postponed by the deletion of gene prkA since no spores were available in ΔprkA at the time point of 12 h. Moreover, to determined whether the inhibitor ara-A could affect the sporulation through some other pathways besides inhibition of PrkA, we added the ara-A in the ΔprkA mutant strain again. It was found that ara-A had little inhibitory activity in sporulation when the gene prkA was knockout, and the sporulation frequencies in ΔprkA mutant with the treatment of ara-A were 0%, 20.43 ± 2.61%, 35.28 ± 4.04%, respectively, at 12, 24, 36 h (P > 0.05; Figure 2B). Our data from the ΔprkA mutant confirmed the previous report that PrkA is a sporulation-related protein (Eichenberger et al., 2003).

PrkA Regulates the Expression of the Transcriptional Factor σK and its Target Genes

Endospore formation is a very complex multi-stages process, in which many cell type-specific, compartmentalized programs of gene expression are controlled by the cell type-specific activity of RNA polymerase sigma factors (Hilbert and Piggot, 2004). Meanwhile, the promoter region of prkA contains a cis-acting element of the spore specific sigma factor σE and it has also been reported to be transcribed by σE (Eichenberger et al., 2003; Steil et al., 2005). To define the specific stages that PrkA protein was involved during sporulation, three sporulation-related genes that are controlled by three sigma factors of later stages were selected to construct the reporter plasmids to examine transcription by assaying β-galactosidase activities. Those three sporulation-related genes included spoIVB (controlled by σF), gerD (controlled by σG), and gerE (controlled by σK).

The promoter regions of those three genes were cloned and fused to the reporter plasmid pDG1728 to drive the expression of β-galactosidase. Altogether, six strains, including SPOIVBPRKA5E, GERDPRKA5E, GERERPRKA5E as well as the corresponding control strains SPOIVBBS, GERDBS, GEREBBS, were successfully constructed by transforming those three recombinant plasmids into the parent strains.
Yan et al. prkA modulates sporulation via σ^K of ΔprkA and B. subtilis 168, respectively. After analyzing their β-galactosidase activities, we found that the expression level of gene gerE, one of the target genes of the transcriptional factor σ^K, decreased significantly in the ΔprkA mutant strain compared to that in the corresponding control strains (Figure 3A).

To further verify our experimental results from the assay of β-galactosidase activity, we compared the expression levels of spoIVB, sigK, and gerE using real-time PCR between B. subtilis 168 and ΔprkA mutant strain. At the same time, we also assayed the transcription of those genes mmmB, spoVB, and spoIID that are known to be under the control of σ^K. The encoding gene for the sigma factor of σ^K includes two separate coding regions of spoIVCB and spoIIIC as mentioned above. It was found that the disruption of gene prkA led to decreased expressions of the encoding genes of σ^K (spoIVCB and spoIIIC). The expression level of gerE, which is controlled by σ^K, showed a reduction similar to the result from β-galactosidase assay. In contrast, compared to the WT strain, the transcription of mmmB, spoVB, and spoIID were differentially increased in the ΔprkA mutant. The gene spoIVB controlled by σ^K had the similar mRNA level to WT strain (Figure 3B). Thus our results suggested that PrkA was likely involved in regulating the synthesis of transcriptional factor σ^K, affected the expression of its downstream target genes, and finally caused the deficient sporulation.

The Complementary Expression of sigK Genes Rescued the Defect of Sporulation in ΔprkA Mutant Strain

Based on the above hypothesis that the decreased sporulation level of ΔprkA mutant strain was caused by the reduced expressions of the transcriptional factor σ^K, we designed an experiment to elevate the expression of sigK in ΔprkA mutant strain. After the recombinant plasmid pSIGKPRKA5E and the blank vector pDG148 were transformed into ΔprkA mutant, the spore numbers and sporulation frequencies were determined. Compared to the ΔprkA mutant strain, the sporulation of SIGKPRKA5E strain significantly improved to about 60% of sporulation capability in WT B. subtilis 168 (Figure 4). Thus, the data is consistent with the hypothesis that elevated expression of σ^K-regulated genes could partially improve the sporulation defect in ΔprkA mutant.

PrkA Positively Controls the Transcriptional Factor σ^K Mediated by the Motif of Hpr (ScoC)

To understand how PrkA positively control the expression of the transcriptional factor σ^K that is required at the later stage
DBTBS (Database of Transcriptional Regulation in B. subtilis) was firstly employed to identify the potential cis-activating elements within the promoter region of sigK, and four potential transcription factor binding motifs of SigE, Hpr (ScoC), GlnR, and SigD were found at the 5% significance level. Then, a series of reporter fusions containing the truncated sigK promoter regions to lacZ were successfully constructed. Specifically, five nested fragments with a common downstream end and variable upstream ends were fused to promoter-less lacZ gene in pDG1728, respectively (Figures 5A, B). Those constructed plasmids were transformed into the ΔprkA mutant strain and the WT B. subtilis 168. The β-galactosidase activity in each strain was measured.

In the five strains containing the reporter plasmids transformed into the WT B. subtilis 168, we found that strain WT-P2 had an obviously lower β-galactosidase activity than WT-P1 (P < 0.01). Strain WT-P1 contained an additional transcriptional motif of SigE than strain WT-P2. However, WT-P2 had much lower β-galactosidase activity than WT-P3 that missed the cis-activating element of Hpr (ScoC; P < 0.01; Figure 5C). The β-galactosidase activities in WT-P4 and WT-P5 had no statistical differences (P > 0.05). Based on the experimental data, we confirmed that, in WT strain B. subtilis 168, the expression of sigK was under the transcription factor of SigE that had been described previously (Kroos et al., 1989). However, it was firstly shown in our study that the transcription factor Hpr (ScoC) negatively regulated the expression of sigK.

In the analysis of strains with plasmids transformed into ΔprkA mutant, the β-galactosidase activity in prkA-P2 was much lower than in prkA-P1 (P < 0.01), while prkA-P1 was similar to that in the corresponding strain transformed from WT B. subtilis 168. However, no significant difference was observed between prkA-P2 and prkA-P3 (P > 0.05; Figure 5C). Since the reporter plasmids prkA-P2 had an additional binding motif of Hpr (ScoC) that potentially controlled sigK expression negatively in the WT strain, the absence of PrkA inhibited Hpr (ScoC) to regulate the expression of sigK anymore, suggesting that PrkA increased the expression of sigK via the transcription factor binding motif of Hpr (ScoC).

**Hpr (ScoC) Functions Epistatic to PrkA in the Spore Development**

It was shown in our β-galactosidase assay that the motif Hpr (ScoC) negatively regulated the expression of sigK in the WT B. subtilis 168. To further confirm such a conclusion, we assessed if the mRNA levels of spoIIC and spoIVCB were influenced by the gene hpr. In Δhpr mutant, the mRNA levels of both spoIIC and spoIVCB increased about 4.5-fold compared to the WT B. subtilis 168 (P < 0.01; Figure 6A), suggesting that Hpr indeed negatively controls the expression of sigK. Moreover, we also constructed the double mutant ΔprkAΔhpr and detected the expression of sigK again. It was shown that ΔprkAΔhpr had the similarly higher mRNA levels than the WT (P < 0.01; Figure 6A).

Then, we examined whether Hpr functioned in the spore development. Our result showed that the sporulation frequencies in Δhpr strain were 20.22 ± 2.02%, 41.04 ± 2.11%, 43.97 ± 0.90% at 12, 24, 36 h, respectively, which were obviously higher than that of the WT strain (12.25 ± 1.01%, 26.24 ± 3.00%, and 33.43 ± 3.72% at these time points; P < 0.01; Figure 6B). Likewise, the double mutant ΔprkAΔhpr had the comparable sporulation frequencies as Δhpr mutant (P > 0.05; Figure 6B). Together, these experiments confirmed that Hpr (ScoC) negatively regulated the expression of sigK in B. subtilis 168, and functioned epistatic to PrkA in the spore development.

**Discussion**

Under starvation or other environmental stress, Gram-positive bacterium initiates a series of sporulation-related
factors (Driks, 1999; Errington, 2003). The cascade of those four sigma sporulation gene expressions occur at the right time and place demonstrated as one of the most important events to ensure that application in biocontrol agents (Nicholson, 2002; Spencer, the pathogenesis in pathogenic bacterium or agricultural bacteria have become an important factor in influencing Thus, the abilities to form tough, resistant endospores in detergents, and hydrolytic enzymes (Nicholson et al., 2000). high temperatures, ionizing radiation, chemical solvents, efficiently kill other bacterial forms. Such conditions include treatments that would otherwise rapidly and efficiently kill other bacterial forms. Such conditions include high temperatures, ionizing radiation, chemical solvents, detergents, and hydrolytic enzymes (Nicholson et al., 2000). Thus, the abilities to form tough, resistant endospores in bacteria have become an important factor in influencing the pathogenesis in pathogenic bacterium or agricultural application in biocontrol agents (Nicholson, 2002; Spencer, 2003). Furthermore, the process of sporulation involves in an unusually mechanism of asymmetric cell division, and attracts much attentions in biological research (Harry, 2001; Ben-Yehuda and Losick, 2002). As a kind of typical pattern of microorganisms, the best-studied paradigm of spore-forming is B. subtilis.

During sporulation in B. subtilis, a strict program of sigma factors activation, including $\sigma^H$, $\sigma^E$, $\sigma^F$, and $\sigma^K$, has been demonstrated as one of the most important events to ensure that sporulation gene expressions occur at the right time and place (Driks, 1999; Errington, 2003). The cascade of those four sigma factors ($\sigma^E$, $\sigma^F$, $\sigma^G$, and $\sigma^K$) is triggered in a sequential order and their biological functions are involved in the engulfment of the smaller cell by the larger sibling, the formation of forespore and the mother cell, and final the assembly of spore coat (Piggot and Hilbert, 2004). Their roles are mainly compartment specific, but also interactional. The sigma factors $\sigma^E$ and $\sigma^K$ function in the forespores, where the transcription of spoIIIG gene (encoding $\sigma^G$) depends on the RNA polymerase containing $\sigma^F$-subunit. Meanwhile, it has also been suggested that the $\sigma^E$-directed signals from the mother cell are necessary for the formation and activation of $\sigma^G$ (Piggot and Hilbert, 2004). The activities of $\sigma^E$ and $\sigma^K$ are confined within the mother cells, where the synthesis of $\sigma^K$ is under the control of $\sigma^E$. But after translation, $\sigma^K$ will remain as an inactive precursor in the mother cell until its activation by a proteolysis event, in which the $\sigma^G$-controlled gene spoIVB plays an important role (Wakeley et al., 2000; Campo and Rudner, 2007). Because sporulation itself is a complex multi-gene regulation process, a gene regulatory network should exist to accurately control the expression of sigma factor.

Our current investigation suggested that though the expression of $\sigma^K$ was controlled by the well-known sigma factor of $\sigma^E$, PrkA can also negatively regulated Hpr (ScoC) to induce the expression of sigK. In mammalian cells, STPK PrkA belongs to AMPK superfamily, and acts as a master regulator of the main energy metabolism pathway. However, few investigations of PrkA have been reported in prokaryotic cells and thus its function requires further elucidated. Our results here demonstrated that the absence of PrkA significantly reduced the sporulation in B. subtilis 168 that is also consistent with the results
FIGURE 6 | Hpr (ScoC) negatively regulates the expression of σK in B. subtilis 168, and functions epistatic to PrkA in sporulation. (A) Hpr (ScoC) increases the expression of the transcriptional factor σK. Real-time PCR was employed to detect the relative expression levels of the transcriptional factor σK, which included spoIIIC and spoIVCB. (B) Effect of the gene hpr on sporulation. Sporulation frequencies in WT strain B. subtilis 168, ΔprkA mutant, Δhpr mutant, and ΔprkAΔhpr mutant were determined at 12, 24, 36 h, respectively. ∗P < 0.01.

FIGURE 7 | A proposed model of PrkA regulating the expression of sigK in B. subtilis 168.

PrkA as a sporulation-related protein. To analyze the specific stages of sporulation that PrkA influences, the expression levels of the important sigma factors as well as their downstream genes were assayed using reporter gene of lacZ and qPCR. Our data further suggested that the decreased expression of the transcriptional factor σK and its downstream genes caused by the absence of PrkA were the main reason of reduced sporulation. This hypothesis was confirmed by the complementary expression of sigK in ΔprkA mutant. Combining the results from bioinformatics analysis and reporter gene activity assay, our study revealed that PrkA controls the expression of σK via the transcription factor binding motifs of Hpr (ScoC). Meanwhile, we have also noticed an indirect pathway described in previous literature: Hpr (ScoC) influencing σE expression by negatively regulating SinI, SinR, and Spo0A (Koide et al., 1999; Kodgire and Rao, 2009), and ultimately decreasing σK (Figure 7). In our transcriptional analysis, it consistently illustrated that prkA-P1 strain (ΔprkA mutant with the reporter fusion containing SigE binding motif) had lower β-galactosidase activity than that WT-P1 (WT strain with the reporter fusion containing SigE binding motif; Figure 5).

Conclusion

Our current investigation confirms the biological role of PrkA in sporulation. In addition, we revealed the underlying mechanism of how PrkA influenced the expression of sigma factor σK by the negative regulation of Hpr (ScoC).
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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