KapB is a lipoprotein required for KinB signal transduction and activation of the phosphorelay to sporulation in *Bacillus subtilis*

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Summary

KinB is one of the two major histidine kinases that provide phosphate input in the phosphorelay to produce Spo0A–P, the key transcription factor controlling the initiation of sporulation. A search for insertion mutants affected in activation of KinB-dependent sporulation led to the identification of the *lgt* locus encoding the lipoprotein glyceryltransferase required for the lipid modification of prolipoproteins before their cleavage and translocation across the cytoplasmic membrane. In parallel, a putative lipoprotein signal peptide cleavage site was detected in KapB, known to be strictly required for KinB-mediated sporulation and located downstream of KinB in a single transcription unit. Using PhoA peptide fusions, we have shown that KapB signal-peptide can direct active alkaline phosphatase to the outer surface of the cytoplasmic membrane in an LGT-dependent manner, strongly suggesting that KapB is a lipoprotein tethered to the outer face of the cytoplasmic membrane via a lipid anchor. As KapB proved to be dispensable for expression of the *kinBkapB* operon, a chimeric kinase was built consisting of KinA sensor domain fused to KinB kinase domain (KinA*-B) to assess (i) the involvement of KapB in catalysis of the kinase reaction, and (ii) the ability of KinB to phosphorylate Spo0F *in vivo*. It was shown that KapB is dispensable for both *in vivo* and *in vitro* activation of the phosphorelay by the KinA*-B chimera and that KinA*-B phosphorylates Spo0F directly *in vitro*. Models for the role of KapB in regulating KinB activity are discussed.

Introduction

In *Bacillus subtilis*, the initiation of sporulation is primarily controlled by the Spo0A transcription factor, the active (phosphorylated) state of which is regulated by a sophisticated multicomponent signal transduction system termed the phosphorelay (Burbuly et al., 1991; Hoch, 1995). Two autophosphorylating sensor kinases, KinA and KinB, input phosphate into the system. In the case of KinA, the phosphorylated group was shown to be transferred from KinA–P to the response regulator Spo0F and subsequently to Spo0A–P, by the phosphotransferase Spo0B (Burbuly et al., 1991). Although genetic data support the notion that Spo0F is an obligate part of the pathway from KinB to Spo0A (Trach and Hoch, 1993), biochemical evidence indicating direct phosphorylation of Spo0F by KinB has been lacking so far because of the membrane location of KinB.

Several lines of evidence indicate that KinA and KinB play distinct roles in the initiation of sporulation (Dartois et al., 1996) and are likely to be regulated by different input signals. Although KinA is a cytoplasmic kinase (Perego et al., 1989; Antoniewski et al., 1990), the KinB amino domain consists exclusively of six membrane-spanning helices with virtually no outside loop. In addition, *kinB* displays a unique feature in that it is transcribed together with the downstream *kapB* ORF, the product of which is either required for the function of KinB or plays a regulatory role in the expression of the *kinB kapB* transcription unit (Trach and Hoch, 1993).

To gain some insight into the response regulator specificity of KinB, a chimeric KinA*-B signal transducer was built that allowed *in vitro* studies on the role of KinB and KapB in activation of the phosphorelay. In this report, we present biochemical evidence that the hybrid KinA*-B kinase can directly phosphorylate the Spo0F response regulator *in vitro* and that phosphorylation of Spo0F by KinB is KapB-independent *in vitro*. We further show that KinB-mediated sporulation in vivo requires functional KapB protein. We also demonstrate that KapB is anchored to the outer surface of the cytoplasmic membrane and carries a typical lipoprotein modification site required for its function. A model is proposed in which KapB interacts with the transmembrane sensor domain of KinB to transport or otherwise process an extracellular signalling compound.
Results

Mutation of the lgt gene encoding lipoprotein glyceryl transferase prevents KinB-dependent sporulation

As mentioned above, KinA and KinB are the two major kinases that act as phosphate sources for the phosphorelay. KinB displays unique properties in that it is expressed and activated before KinA, it responds to distinct input signals (Dartois et al., 1996; 1997) and requires the product of the co-transcribed kapB ORF for its function (Trach and Hoch, 1993). To specifically investigate the KinB signalling pathway, transposon mutagenesis was carried out using the spectinomycin mini-Tn10 delivery vector pIC333 (Steinmetz and Richter, 1994) in the ΔkinA96 strain MB340, where sporulation is entirely dependent on KinB. The mini-Tn10 insertion library was screened for Spo0 mutants (see Dartois et al., 1996 for details). To discriminate between mutants with defects in spo0 genes from those affected in kinB or in KinB-signalling genes, the transposon insertions were transferred into the wild-type strain (KinA⁺) and those that did not alter the recipient Spoo phenotype on plates were assumed to affect only the KinB pathway. This conclusion was verified by monitoring expression of spoIAlacZ and spoIG–lacZ fusions in both the wild-type and KinA⁻ strains carrying the transposon mutation as a means to assess SpooA–P accumulation resulting from the activation of KinA and/or KinB. One of the most drastically affected mutants, carrying transposon insertion Tn10-53, was further characterized.

The Tn10-53 sporulation phenotypes observed on plates in the wild-type and KinA⁻ backgrounds were confirmed by quantifying the corresponding sporulation efficiencies in Schaeffer sporulation medium. As expected, the Tn10-53 mutation caused a 1000-fold decrease of sporulation efficiency in the KinA⁻ background, whereas no significant effect could be detected when the insertion mutation was introduced in the wild-type strain JH642. Accordingly, the maximum expression levels of stage II genes remained similar when the Tn10-53 insertion was transferred in the wild-type strain JH642 (data not shown), whereas expression of spoIAlacZ and spoIG–lacZ in the KinA⁻ strain carrying the Tn10-53 was decreased to that of a double kinA kinB mutant (Fig. 1).

Using PBS1 transduction, the Tn10-53 insertion was localized at about 306° on the B. subtilis genetic map. The insertion target sequence was rescued together with the mini-Tn10 transposon as described previously (Dartois et al., 1996), and sequenced. The transposon was found to be inserted in a lgt-like gene encoding the lipoprotein glyceryl transferase that participates in post-translational lipid modification of prolipoproteins before their processing by the signal peptidase II (Sankaran and Wu, 1994a). The deduced protein sequence of the B. subtilis lgt-like gene was aligned with the Lgt protein sequences from Staphylococcus aureus and Salmonella typhimurium that were both proven biochemically to have LGT activity (Gan et al., 1993; Qi et al., 1995). The alignment shown in Fig. 2A revealed 56% identity with the S. aureus enzyme, whereas the three Lgt proteins appeared to share 20% identity and 18% similarity. Among the conserved amino acids are the residues corresponding to H103-GGLIG-108, Y235 and H196 that were found to play an important role in the function of Escherichia coli LGT (Qi et al., 1995; Sankaran et al., 1997).

Sequencing upstream of the lgt gene region revealed the 3’ end of a putative ORF transcribed in the same orientation and ending 13 bp from the start codon of lgt. A computer search of the NCBI databases revealed extensive similarities between the putative encoded peptide and the hydroxymethylglutaryl-CoA reductase from Mycoplasma species (Fig. 2B). After the lgt gene and in the same direction of transcription, two more ORFs were found and designated OrfX and OrfY (Fig. 2B). The product of OrfX appears to be an integral membrane protein showing no similarity with any other sequence available in the databases. OrfY was only partially sequenced and appears...
to be homologous to a series of bacterial gene products of unknown function. The nucleotide and deduced amino acid sequence of the lgt region are available from GenBank under accession no. U63310.

The KapB protein carries motifs typical of prolipoproteins

Bacterial prolipoproteins have a signal peptidase II consensus sequence (L,V,I)(A,S,T,G)(G,A)C in which the invariant cystine is the first residue of the mature peptide and undergoes specific lipid modification, starting with the transfer of a glyceryl moiety by the LGT enzyme and followed by signal peptide cleavage (von Heijne, 1989). After translocation, the lipid-modified mature protein remains bound to the outer face of the cytoplasmic membrane through its N-terminal lipidic extension. The observation that the lgt::Tn10 mutation causes a KinB phenotype raised the possibility that a lipoprotein could be involved in activation of the KinB pathway to sporulation initiation.

The kapB gene belongs to the kinB kapB operon and encodes a 128 residue polypeptide required for KinB-mediated sporulation. Upon close examination of the KapB amino acid sequence, a motif was detected that fits the consensus of prolipoprotein cleavage sites: I–T–A–C (Fig. 3).
When crude extracts were prepared from a strain carrying \textit{kapB} on a replicative plasmid under a strong constitutive promoter (pJV198), a 15 kDa band was detected in the cytoplasmic fraction that was absent in the strain harbouring the vector only and corresponded to the size expected for the putative KapB propeptide ($M_r = 14,669$) (data not shown). In addition, a 12–13 kDa band was present in the membrane fraction and absent in the control strain, which could be in agreement with the approximate molecular size of the putative lipid-modified mature KapB (Fig. 3). Taken together, these observations prompted us to ask whether KapB was a lipoprotein and experiments were undertaken to alter the invariant cysteine residue using site-directed mutagenesis to prevent lipoprotein formation.

The Cys-26 was substituted with Ala, Pro, Ser and Thr. As \textit{kapB} belongs to the \textit{kinBkapB} operon, the mutated \textit{kapB} alleles were integrated by Campbell-type single cross-over at the \textit{kapB} chromosomal locus to maintain the stoichiometry between \textit{KinB} and \textit{KapB}. Transformation of the \textit{KinA}– strain JH12638 with plasmids pJV204 (‘\textit{kinBkapBCI26P}’), pJV205 (‘\textit{kinBkapBCI26T}’), pJV208 (‘\textit{kinBkapBCI26S}’), and pJV209 (‘\textit{kinBkapBCI26A}’), or pJV216 gave two types of colonies that either exhibited the parental phenotype (\textit{KinA}–) or were \textit{Spo0} on Schaeffer sporulation medium. This is the expected result from homologous recombination if the mutated \textit{kapB} alleles are non-functional. Sporulation efficiencies of the different mutants were decreased 200- to 20,000-fold compared with the parental strain, whereas deletion of the \textit{kapB} gene in JH12638 results in a 20,000-fold decrease (Table 1).

These results clearly indicate that the Cys-26 residue of KapB is essential for KinB-dependent signal transduction pathway.

**KapB signal peptide can direct alkaline phosphatase to the outer surface of the cytoplasmic membrane**

\textit{E. coli} alkaline phosphatase (AP) has been used extensively as a reporter for protein secretion in \textit{B. subtilis} and for analysing topology of membrane proteins (Manoil and Beckwith, 1986; Payne and Jackson, 1991). To verify that KapB is a lipoprotein and to determine whether the Cys-26 residue is involved in lipid modification and signal peptide cleavage of the KapB propeptide, a translational fusion was made between \textit{kapB} putative signal sequence followed by the first 20 codons of the mature sequence, and the mature coding sequence of \textit{E. coli} \textit{phoA} gene (see Experimental procedures). The chimeric capB–\textit{phoA} gene was subcloned in the pMA5 expression vector (Zyprian and Matzura, 1986; Dartois 	extit{et al.}, 1994) and the recombinant plasmid pJV215 was introduced by transformation into the alkaline phosphatase-deficient strains MH3402 (\textit{PhoA–PhoB–}) and JH16724 (\textit{PhoA–PhoB–Lgt–}). Similar translational fusions containing the C26–P and C26–T mutated signal sequences were constructed (pJV217 and pJV218 respectively), as well as a control plasmid carrying the \textit{phoA} mature sequence devoid of signal sequence (pJV216). The production of secreted active AP was monitored on XP indicator plates (see Experimental procedures). As can be seen in Fig. 4, the \textit{kapB}–\textit{phoA} hybrid gene directed secretion of active AP in a Lgt-dependent fashion, whereas the mutated KapB signal peptides where the invariant Cys residue was substituted with Pro or Thr proved unable to direct AP translocation. To further localize the mature \textit{KapB–PhoA} chimera, strain MH3402 [pJV215] was grown in liquid medium and AP activity was quantified using, as the enzyme source, culture supernatants, intact cells, protoplasts or the soluble fraction of cell lysates. Significant AP activity (7.0 units) could be detected only when protoplasts were used as the enzyme source, whereas the levels of AP activity found in the supernatant or cytoplasmic fractions or with intact cell cultures were close to the background level (around 1.0 unit).

In agreement with the AP phenotypes observed on XP plates, strain MH3402 carrying plasmid pJV216, pJV217 or pJV218 did not produce a significant amount of active AP, nor did strain JH16724 (\textit{lgt::Tn10}) harbouring the

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**Table 1. Effect of site-directed mutagenesis of the \textit{KapB} Cys26 residue on sporulation efficiency through the \textit{KinB} pathway.**

<table>
<thead>
<tr>
<th>\textit{kapB} allele</th>
<th>Wild-type</th>
<th>C26A</th>
<th>C26P</th>
<th>C26S</th>
<th>C26T</th>
<th>$\Delta$kapB::\textit{spc}</th>
</tr>
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<tbody>
<tr>
<td>Per cent spores$^b$</td>
<td>4.2</td>
<td>7 $\times$ 10$^{-3}$</td>
<td>1.1 $\times$ 10$^{-3}$</td>
<td>2.5 $\times$ 10$^{-2}$</td>
<td>1.7 $\times$ 10$^{-4}$</td>
<td>1.5 $\times$ 10$^{-4}$</td>
</tr>
</tbody>
</table>

\textit{a.} \textit{kapB} mutant alleles were introduced in the parent strain JH12638 (\textit{KinA}–).

\textit{b.} Sporulation frequencies are expressed in percentage of spores relative to viable cells.

wild-type hybrid gene (Fig. 4). Thus, the signal peptide and first 20 residues of the KapB propeptide can direct active alkaline phosphatase to the outer surface of the cytoplasmic membrane and this translocation process requires functional LGT protein. These observations strongly suggest that KapB is a lipoprotein tethered to the outer face of the cytoplasmic membrane via a lipid anchor.

The *KinA<sup>8</sup>-B* hybrid transducer phosphorylates Spo0F in vitro in a *KapB*-independent manner

As a first step to further understand the role of KapB in KinB expression or activity, expression of a kinB-lacZ transcriptional fusion integrated at the chromosomal amyE locus was monitored in the wild-type (JH16169) and *ΔkapB::spc* (JH16549) strains. Both timing and level of expression of *kinB-lacZ* were identical in the two backgrounds (data not shown). Similar results were obtained when the transcriptional fusion was integrated at the *kinB* locus, indicating that KapB does not affect expression of the *kinB* operon whether in cis or in trans.

Previous attempts to overexpress and purify KinB from *E. coli* were unsuccessful, most probably the result of the hydrophobic nature of the six putative transmembrane helices that exclusively compose the amino domain of KinB. Consequently, biochemical evidence of direct phosphorylation of Spo0F by the KinB kinase could not be obtained. To overcome this problem and specifically investigate the role of KapB in KinB-catalysed kinase reaction, a chimera was built that contained the N-terminal sensor domain of KinA fused to the C-terminal kinase domain of KinB. Given that KapB is strictly required for KinB activity in vivo while being completely dispensable for KinA expression or activity, the underlying rationale was to design a soluble hybrid kinase to assess (i) the ability of KinB to autophosphorylate and catalyse phosphotransfer to Spo0F in vivo and in vitro, and (ii) the involvement of KapB in catalysis of the kinase reaction. On the basis of limited staphylococcal V8 protease digestion data (L. Wang and J. A. Hoch, in preparation), the fusion site was positioned at the S207 E208 K209 motif (KinB co-ordinates) common to both kinases, located at the beginning of the highly conserved catalytic domain and 15 amino acids upstream of the putative phosphorylated histidine residue (Fig. 5). The corresponding hybrid gene, named *kinA<sup>8</sup>-B*, was engineered and cloned into *E. coli* expression vector pET16b using a strategy that neither requires nor creates any restriction site at the junction (see Experimental procedures). To compare the enzymatic properties of the isolated C-terminal domain of KinB (KinB<sub>C</sub>) with those of the *kinA<sup>8</sup>-B* hybrid kinase, a truncated form of *kinB*, encoding the kinase domain starting at the Ser207 residue was cloned into the pET16b expression vector. The recombinant *kinA<sup>8</sup>-B*, KinB<sub>C</sub> and KapB proteins were purified from *E. coli*, as described in Experimental procedures.

Autophosphorylation of *kinA<sup>8</sup>-B* hybrid kinase and phosphotransfer from *kinA<sup>8</sup>-B* to Spo0F were assayed in the presence of [γ-<sup>32</sup>P]-ATP. Each reaction was run in parallel with KinA as a control. As can be seen in Fig. 6A, similar efficiencies of autophosphorylation and phosphotransfer to Spo0F were obtained with *kinA<sup>8</sup>-B* and KinA. A His-tagged version of the *kinA<sup>8</sup>-B* chimera was also expressed and purified from *B. subtilis* strain TS101B[pJV190] and proved to display the same enzymatic activity as the renatured *kinA<sup>8</sup>-B* prepared from *E. coli* (data not shown). To determine whether the catalytic
domain of KinB devoid of any KinA sequence was able to direct both autophosphorylation and phosphotransfer activities, the same set of reactions was run with KinB C as the kinase. Figure 6B (lanes 1 and 2) clearly shows that KinB C autophosphorylates and catalyses phosphotransfer to Spo0F. Finally, KinB C was incubated together with purified phosphorylated Spo0F and appeared to be able to catalyse the reverse phosphotransfer reaction from Spo0F—P to the kinase (Fig. 6B, lanes 3 and 4). These results demonstrate that the kinase domain of KinB is able to catalyse phosphotransfer to Spo0F in vitro. We then asked whether KapB could modulate the catalytic activity of KinA’-B. KapB was purified to homogeneity and mixed at increasing concentrations with KinA’-B alone or KinA’-B and Spo0F in the presence of ATP (see Experimental procedures). Even when the KinA’-B/KapB molar ratio was 1:20, no significant effect could be detected on either autophosphorylation or phosphotransfer activity (data not shown). These observations together with the fact that KapB is a lipoprotein located at the outer surface of the cytoplasmic membrane suggest that KapB must be involved in signalling to the N-terminal transmembrane domain of KinB or in signal transfer from the sensor domain to the kinase domain.

KapB is dispensable for in vivo activation of the phosphorelay by the KinA’-B chimeric kinase

We checked the ability of the hybrid kinase to initiate phosphotransfer through the phosphorelay in vivo. Plasmid pJV181 harbouring the promoterless kinA’-B kapB operon on the pH7181 B. subtilis integrative vector was introduced into the Spo0 strain JH16567 (KinA’-B KinB’) to give strain JH16577. Homologous recombination at the 5’ end of the kinA locus brought the kinA’-B kapB donor fragment under the kinA promoter and gave rise to Spo0+ colonies, strongly suggesting that KinA’-B

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Fig. 5. Domain swap chimera of KinA and KinB. The large box in each kinase molecule represents the highly conserved catalytic domain containing the phosphorylated histidine residue (His—P) and the conserved ATP-binding motifs (vertical bars). The six hatched boxes in KinB symbolize the transmembrane helices that constitute its sensor domain. The amino acid sequence surrounding the chimera fusion site is shown at the bottom. The sequences and co-ordinates preceding and following the S–E–K pattern common to both kinases originate from KinA and KinB respectively.

Fig. 6. Phosphorylation of the Spo0F response regulator by the chimeric KinA’-B kinase and by the catalytic domain of KinB (KinB C). A. Autophosphorylation (lanes 1 and 2) and transphosphorylation (lanes 3 and 4) reactions were carried out at room temperature for 1 h as described under Experimental procedures. After SDS–PAGE, the film was exposed for 15 min at −70°C using an intensifying screen. B. Autophosphorylation of KinB C (lane 1) and phosphotransfer to Spo0F (lane 2) were carried out under standard conditions (see Experimental procedures). Reaction mixtures containing [γ-32P]Spo0F—P (lane 3) in the presence of KinB C (lane 4) were incubated at room temperature for 3 min. The film was exposed for 40 min at −70°C using an intensifying screen.

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can complement the absence of the two major kinases of the phosphorelay in vivo and is able to stimulate production of the key transcription factor Spo0A–P. To determine whether complementation could be due to direct phosphorylation of Spo0A in vivo, plasmid pJV181 was introduced into strain JH649 (spoOF221) to give strain JH16730. The resulting transformants remained Spo0A–. To rule out the possibility that Spo0F was required for expression of the hybrid kinase gene from the kinA promoter, expression of a kinA–lacZ fusion was monitored in the spoOF221 mutant and proved to be slightly elevated compared with the wild-type level (V. Dartois and J. A. Hoch, unpublished). Therefore, the above observation indicates that Spo0F is an obligate component in the phosphotransfer from KinA–B to Spo0A and that KinA–B is unable to transfer the phosphate group directly to either Spo0B or Spo0A in vivo.

To determine whether KapB was required for KinA–B activity, plasmid pJV188 harbouring the kinA–B gene alone was integrated in the chromosome of a KinA–B–kapB mutant (JH16579). Homologous recombination at the 5′ end of the kinA locus gave rise to Spo0A− colonies, indicating that KinA–B remained able to restore sporulation to the wild-type level in the absence of functional KapB and that KapB is therefore dispensable for KinA–B phosphotransfer activity in vivo as well as in vitro.

Discussion

Among the genes encoding bacterial two-component sensor kinases, kinB is unique in that it is co-transcribed with kapB, a gene known to be strictly required for KinB-mediated activation of the phosphorelay (Trach and Hoch, 1993). However, the function of the KapB protein has remained a mystery. Furthermore, the response regulator target of KapB was not found in the same operon but was speculated to be Spo0F. Using the KinA–B–Chimeric signal transducer and a truncated version of KapB consisting of the kinase domain alone, we have obtained experimental evidence indicating that KinB phosphorylates the Spo0F response regulator in vitro, confirming that phosphotransfer from KinB to Spo0F is an obligate step in KinB-mediated activation of the phosphorelay in vivo. The efficient phosphorylation of Spo0F by KinB in vitro also suggests that the N-terminal sensor domain is dispensable for KinB autophosphorylation and phosphotransfer activity. In the native kinase, the N-terminal domain could rather interact with the kinase domain to prevent constitutive autophosphorylation and/or phosphotransfer and this negative modulation could be relieved upon signalling to the sensor domain. Interestingly, KapB proved to be totally dispensable for activation of the phosphorelay by KinA–B in vivo, as well as for efficient phosphotransfer from KinA–B or KapB to Spo0F in vitro. Furthermore, expression of the kinBkapB operon remained unchanged in the presence or absence of functional KapB. Therefore, it seems most likely that KapB is involved in signal transduction to KinB, perhaps as the ligand recognition component of a KapB–KinB complex.

A mini-Tn10 insertion in the lgt gene, encoding the lipoprotein glyceroltransferase, was found to inactivate KinB-dependent sporulation, strongly suggesting that a lipoprotein could be involved in activation of the phosphorelay through the KinB sensor kinase. In E. coli and Salmonella typhimurium, Lgt was reported to be essential and only temperature-sensitive lgt mutant strains have been isolated (Williams et al., 1989; Gan et al., 1993). The B. subtilis mutant isolated in this study carries a Tn10 insertion located in the carboxyl-encoding region of the lgt gene (Fig. 2) and exhibits partial growth deficiency (small colonies on LB plates and increased doubling time in liquid medium, data not shown). These observations raise the possibility that a truncated Lgt enzyme is produced in the Tn10 mutant that has retained residual Lgt activity and modifies prolipoproteins to a lower extent than the wild-type enzyme, or exhibits altered specificity and modifies only a subset of the lipoprotein population. Alternatively, the Tn10-truncated Lgt may be completely inactive, although lgt-like gene(s) could exist in B. subtilis whose product could modify specific subsets of prolipoproteins. Amino acid alignment of B. subtilis Lgt with its E. coli and S. typhimurium homologues indicated that the putative Tn10-truncated Lgt had retained the conserved residues (H103-GGLIG108, Y235 and H196) found to play an important role in the function of E. coli Lgt (Sankaran et al., 1997). Given the essential character of bacterial Lgt proteins, no attempts were undertaken to delete the complete lgt gene from the chromosome of B. subtilis.

The KapB protein appeared to be a possible candidate for the required lipoprotein, based on inspection of its amino acid sequence (Fig. 3). Accordingly, several lines of evidence identified KapB as a lipoprotein. First, site-directed mutagenesis of the invariant Cys residue, which undergoes lipid modification before signal peptide cleavage by the signal peptidase II (Tokunaga et al., 1982), inhibited KinB-mediated sporulation. To minimize alteration of the secondary and tertiary structure of the protein, amino acid substitutions were chosen according to a matrix of preferred amino acid exchanges within conserved topological structures (Bordo and Argos, 1991). As all four changes (C26→P; C26→A; C26→S and C26→T) drastically affected KapB function, it can be reasonably assumed that the observed KapB-deficient phenotype is due to the loss of an essential residue rather than to some general conformational change. Second, translational fusion between the signal sequence of kapB and the mature sequence of phoA allowed translocation of the recombinant KapB–PhoA peptide in an lgt-dependent manner, whereas the modified
C26—P signal peptide proved unable to direct the alkaline phosphatase across the cytoplasmic membrane. Finally, crude extracts obtained from a strain overexpressing KapB allowed the detection of an additional 15 kDa band in the cytoplasmic fraction while a 12 kDa band was present in the insoluble membrane fraction. The presence of residual unprocessed KapB in the cytoplasm could be due to saturation of the processing machinery after overexpression of the KapB protein.

These observations have led us to propose the provisional model presented in Fig. 7. Histidine kinases are known to function as homodimers and KinB C behaves as a dimer in solution (data not shown). The KapB protein is also known to dimerize in solution (data not shown) and appears to be a lipoprotein tethered to the outer surface of the cytoplasmic membrane via a lipid anchor. Furthermore, kinB and kapB are transcribed together as an operon. Therefore, one could speculate that KapB functions as a receptor or ligand-binding protein that interacts with the sensor domain of KinB to stimulate activity of the kinase domain by affecting its conformation or accessibility. A similar example of such a mechanism is the Uhp sugar-phosphate transport system of E. coli in which UhpC, an integral membrane protein, stimulates the accessibility and activity of the UhpB sensor kinase upon binding of glucose-6-phosphate (Kadner, 1995). Alternatively, KapB dimers together with the sensor domain of KinB dimers, composed of 12 transmembrane segments, may form a gated pore or channel through which the signal molecule could enter the cell and transduce a signal, thereby relieving the intramolecular inhibition exerted by the N-terminal domain on the kinase activity. Such a transporting system could be roughly compared to ABC-type transporters in which a solute-binding lipoprotein interacts with integral membrane protein components that translocate the substrate or ligand across the membrane (Fath and Kolter, 1993).

These models predict that the activating signal is a small molecule serving as a means to communicate an external signal to the cytoplasm. This may represent a mechanism for communication between the membrane outer-surface cell wall and the cytoplasm. As KinB is predominantly an exponential phase protein (Dartois et al., 1996), it seems less likely to function in response to cell density signals. On the other hand, there is no real evidence of direct interaction between KapB and KinB, and KapB could as well serve as a signal transduction mediator for another protein located at the cell surface that ultimately interacts with KinB. It will be interesting to design experiments to choose among these possibilities.

**Experimental procedures**

**Bacterial strains and genetic techniques**

All the strains used in this study are listed in Table 2. E. coli transformations were performed by electroporation of the K-12 strain TG1 [Δlac-proAB supE thi hsdS20 F’ [traD36 proA + proB + lacI Q lacZ M15] (Gibson, 1984) using the BioRad Gene Pulser according to the procedure provided by the supplier. Selection was done on LB broth supplemented when appropriate with ampicillin or spectinomycin 100 μg ml⁻¹. B. subtilis strains were maintained on Schaeffer sporulation medium and transformed with chromosomal or plasmid DNA according to the procedure of Anagnostopoulos and Spizizen (1961). Selection was carried out using erythromycin 0.5 μg ml⁻¹; chloramphenicol 5 μg ml⁻¹; kanamycin 2 μg ml⁻¹; spectinomycin 100 μg ml⁻¹; tetracycline 8 μg ml⁻¹.

For transposon mutagenesis, the mini-Tn10 delivery vector pIC333 (Steinmetz and Richter, 1994) was used as previously described (Dartois et al., 1996).

The sporulation efficiency of B. subtilis strains was determined by the nutrient exhaustion method of Schaeffer. Cells were grown in Schaeffer medium at 37°C for 16 h after the transition between exponential and stationary growth phases. Serial dilutions of sporulating cells were plated before and after treatment with 0.1 vol. chloroform to obtain a viable cell count and a spore count. To determine the location of the Tn10-53 transposon insertion, PBS1 transducing lysates were prepared from strain JH16053 and used to transduce (Takahashi, 1963) the kit of auxotrophic strains of Dedonder et al. (Dedonder et al., 1977). The mini-Tn10 was found to be linked to hisA (30% recombination) and more distantly to thrC (94% recombination) and cttA (96% recombination). The position of the Tn10-53 was calculated according to these data combined with three-point-crosses analyses.

**β-Galactosidase assay**

B. subtilis strains harbouring lacZ fusions were assayed for β-galactosidase activity as previously described (Ferrari et
### Table 2. B. subtilis strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCL1050</td>
<td>trpC2 leuA metB ΔlpA estB::cat</td>
<td>Dartois et al. (1994)</td>
</tr>
<tr>
<td>JH642</td>
<td>trpC2 phoT</td>
<td>laboratory stock</td>
</tr>
<tr>
<td>MB340</td>
<td>trpC2 spolIF96</td>
<td>Mueller and Sonenshein (1992)</td>
</tr>
<tr>
<td>MH3402</td>
<td>ΔphoA::cat phoB::Tn917 mls^R</td>
<td>M. Hulett</td>
</tr>
<tr>
<td>JH12638</td>
<td>kina::Tn9171HJ19 (Em^R)</td>
<td>Perego et al. (1989)</td>
</tr>
<tr>
<td>JH16125</td>
<td>kina::Tn917 amyE::(spolIAA--)lacZ cat</td>
<td>pJV101→JH12638</td>
</tr>
<tr>
<td>JH16141</td>
<td>kina::Tn917 amyE::(spolIAA--lacZ cat) tgt::Tn10</td>
<td>Tn10-S3→JH16125</td>
</tr>
<tr>
<td>JH16169</td>
<td>amyE::(kinB--lacZ cat)</td>
<td>Dartois et al. (1996)</td>
</tr>
<tr>
<td>JH16180</td>
<td>kina::Tn917 amyE::(spolIAA--lacZ cat) ΔkinB::tet</td>
<td>JH12849→JH16125</td>
</tr>
<tr>
<td>JH16305</td>
<td>kina::Tn917 amyE::(spolIAA--lacZ cat) aphA3</td>
<td>pJT103→JH1638</td>
</tr>
<tr>
<td>JH16309</td>
<td>kina::Tn917 amyE::(spolIAA--lacZ cat) aphA3</td>
<td>JH12849→JH1638</td>
</tr>
<tr>
<td>JH16332</td>
<td>kina::Tn917 amyE::(spolIAA--lacZ cat) aphA3</td>
<td>Tn10-S3→JH16305</td>
</tr>
<tr>
<td>JH16511</td>
<td>kinB::(kinB--lacZ)</td>
<td>pJV142→JH1642</td>
</tr>
<tr>
<td>JH16549</td>
<td>amyE::(kinB--lacZ cat) ΔkapB::spc</td>
<td>pJV154→JH16169</td>
</tr>
<tr>
<td>JH16563</td>
<td>ΔkinA::cat</td>
<td>Perego et al. (1989)</td>
</tr>
<tr>
<td>JH16567</td>
<td>ΔkinA::cat ΔkinB::tet</td>
<td>JH12849→JH16553</td>
</tr>
<tr>
<td>JH16579</td>
<td>ΔkinA::cat ΔkinB::tet ΔkapB::spc</td>
<td>pJV154→JH16567</td>
</tr>
<tr>
<td>JH16724</td>
<td>ΔphoA::cat phoB::Tn917 mls^R</td>
<td>Tn10-S3→MH3402</td>
</tr>
</tbody>
</table>

**a.** All JH and MH strains are derived from JH642.  
**b.** cat is the chloramphenicol acetyltransferase gene from pC194 (Horinouchi and Weisblum, 1982); aphA3 is the Streptococcus faecalis kanamycin resistance gene (Trieu-Cuot and Courvalin, 1983) and spc is the spectinomycin adenyltransferase gene from Staphylococcus aureus Tn554 (Murphy, 1985).  
**c.** Indicates construction by transformation.

**Nucleic acid manipulations**

The mini-Tn10 insertion and flanking regions were rescued in a single step, taking advantage of the pUC origin of replication present on the transposon. The chromosome of strain JH16053 was totally digested with HindIII and EcoRI, and fragments were self-ligated at low DNA concentration (2 μg ml^-1). The ligation mixtures were precipitated and transformed into E. coli TG1 for spectinomycin resistance. One clone from each was retained for further analysis and designated pJV53H and pJV53E. Sequencing reactions were carried out on both strands of double-stranded DNA purified with Qiagen-tips 20 (Qiagen) using the dyeoxy chain termination sequencing kit from United States Biochemicals and synthetic primers.

**Plasmid construction and site-directed mutagenesis**

Plasmid pJV101 was obtained by cloning the 1.5 kb HindIII-PvuII fragment containing the spolIIA promoter region (Wu et al., 1992) into pJM116, which is a pDH32 derivative carrying an expanded multiple-cloning site (Perego, 1993). The promoter region of spolIIA was recovered from a HindIII–EcoRI digestion of plasmid pUC118pA (Satola et al., 1992) and subcloned in pJM115 (Perego, 1993) to give pJT103. Plasmid pJV198 contains the kapB ORF from the upstream Clai site until the stop codon subcloned at the Sphi site of the B. subtilis expression vector pMA5 (Dartois et al., 1994) after Klenow polymerase fill-in of both insert and vector. The E. coli replicon was removed by SstI digestion and self-ligation before transformation into B. subtilis strain BCL1050. Site-directed mutagenesis of the KapB Cys-26 residue was carried out by PCR. A 1.1 kb upstream fragment starting within the kinB gene and ending at the mutation site was amplified using oligonucleotides 5'–GCA TGAATCTTGA TGGTGAATAGTTTGGAACTGT GCT ATCCCATCTGACCTCCGC–3' and 5'–GACGAGA AGACAGCAGCGCTGCGACGGCAGCAGCTATT TTAATGAAAG–3' (C26→A); 5'–AGGAGA AAGCAGCAGCGCTGCGACGGCAGCAGCTATT TTAATGAAAG–3' (C26→G); 5'–AGGAGA AAGCAGCAGCGCTGCGACGGCAGCAGCTATT TTAATGAAAG–3' (C26→C); or 5'–AGGAGA AAGCAGCAGCGCTGCGACGGCAGCAGCTATT TTAATGAAAG–3' (C26→T), and digested with EcoRI and Bsal to liberate 5'–GGT–3' asymmetric overhang directly preceding the Cys codon. Four 380 bp fragments harbouring each of the Cys-26 modifications were amplified using the following oligonucleotides: 5'–AGGAGAAGAAGCAGCGCTGCGACGGCAGCAGCTATT TTAATGAAAG–3' (C26→A); 5'–AGGAGAAGAAGCAGCGCTGCGACGGCAGCAGCTATT TTAATGAAAG–3' (C26→G); 5'–AGGAGAAGAAGCAGCGCTGCGACGGCAGCAGCTATT TTAATGAAAG–3' (C26→C); or 5'–AGGAGAAGAAGCAGCGCTGCGACGGCAGCAGCTATT TTAATGAAAG–3' (C26→T), and digested with EcoRI and BamHI and Bsal liberated 5'–AGGC–3' cohesive ends preceding the mutated Cys-26 codon. Three-way ligations were performed between (i) the pJM103 integrational vector (Perego, 1993) cleaved with EcoRI and BamHI; (ii) the EcoRI-Bsal 1.1 kb fragment; and (iii) each of the four 380 bp fragments carrying the Cys-26 mutations and digested with BamHI and Bsal. The recombinant clones were designated pJV204 (C26→A), pJV205 (C26→T), pJV208 (C26→C), and pJV209 (C26→G) and the inserts were entirely sequenced to verify that no additional mutation had been introduced in the course of PCR amplification and cloning processes. To construct the kapB–phoA fusions, the phoA mature coding sequence was recovered from a BglII-HindIII digestion of pBE30 secretion vector (Payne and Jackson, 1991; Nagarajan et al., 1992) and subcloned between HindIII and HindIII restriction sites of the pJM103 integrational vector to give plasmid pJV211. A region encompassing the 3' end of kinB, the kapB signal sequence and the first 20 codons of the mature sequence, was then amplified using B. subtilis...
chromosomal DNA as the template and oligonucleotides 5'-GATGAAATTCTATGTTGATGCTCGCT-3' and 5'-GCTTCGATTCCTCGTCGCCGGATGGTATCAGC-3', digested with EcoRV, and subcloned at the unique EcoRV site of plasmid pJV211. Insert orientation and in-frame fusion were verified by restriction pattern analysis and sequencing. Digestion of the resulting plasmid pJV212 with HindIII released a 1.6 kb fragment containing the kapB'-phoA hybrid gene that was subsequently subcloned at the HindIII site of the B. subtilis pMA5 expression vector to give plasmid pJV215 after SstI deletion of the E. coli replication. Similar translational fusions containing the C265-P and C265-T mutant signal sequences were constructed in parallel using the same pair of oligonucleotides and plasmids pJV204 or pJV205 as templates respectively. The mutated kapB'-phoA fusions were recovered by HindIII digestion and subcloned into pMA5 to give plasmids pJV217 and pJV218 respectively. The control plasmid pJV216 was obtained by subcloning the phoA mature sequence recovered from pBE30 (EcoRV–HindIII) between the EcoRV and HindIII sites of pMA5.

Plasmid pJV186 harbouring the kinA'-B hybrid gene was constructed according to the following strategy. The 5' region of the kinA gene, encoding the putative sensor domain of KinA, was amplified by PCR using the oligonucleotides 5'-GATGATCAGGCTCTATCG-3' (NcoI) and 5'-GATAGTCTTCGGATCCGATCGATTCTT-3' (BamHI) and ligated into the B. subtilis expression vector pMA5 (Dartois et al., 1994) after Klenow polymerase fill-in of both insert and vector. The resulting fragments were ligated, the asymmetric cohesive ends allowing unidirectional ligation of fragments A and B. The multimers were then resolved by NcoI/BamHI digestion and subcloned at the SphI site of the B. subtilis expression vector pMA5 (Dartois et al., 1994). After Klenow polymerase fill-in of both insert and vector. Plasmids pJV181 and pJV198 were obtained by subcloning the XbaI-BamHI inserts of plasmids pJV170 and pJV186, respectively, into the integrative vector pH181 (Lereclus and Arantes, 1992) previously treated with the same restriction enzymes. Plasmid pJV172 carries the 3' region of kinB, encoding the catalytic domain of the kinase, amplified by PCR using the oligonucleotides 5'-CACCTCATGAAAATCTGAACTGAGATGGTGAG-3' (BspHI) and 5'-CGAGGATCCGTCAGATCGATTCTT-3' (BamHI) and ligated with Ncol/BamHI-treated pET16b vector. Finally, plasmid pJV164 contains the kapB ORF amplified with the oligonucleotides 5'-TGACCCATGGCAGCTTTGAGCAGGCTCTATCG-3' (NcoI) and 5'-CCAAGATCCGGAGAATCGATTTAACCGAACTG-3' (BamHI) and ligated into the pET16b vector.

AP activity assays

AP activity was detected on LB medium supplemented with 50 μg ml⁻¹ XP (5-bromo-4-chloro-3-indolyl phosphate, Sigma). Plates were incubated at 37°C for 16 h. AP activity was quantified by measuring the conversion of p-nitrophenyl phosphate (pNPP, Sigma) to p-nitrophenol (pNP) in CHES buffer 0.8 M pH 9.5, pNPP 20 mM. Typically, MH3402 [pJV215] and MH3402 [pJV216] transformants were grown in LB medium supplemented with 20 μg ml⁻¹ kanamycin. Two hundred and fifty microlitres of overnight cultures was centrifuged. Both the supernatant and the cell fractions were recovered. Cell pellets were resuspended in 250 μl of 0.5 M sucrose, 20 mM sodium citrate, 20 mM MgCl₂ and 2 mg ml⁻¹ lysozyme, and incubated for 30 min at 37°C. Protoplasts were pelleted by centrifugation at 9000 r.p.m. for 1 min. To recover the cytoplasmatic fraction, protoplasts were resuspended in 100 μl of cold water, subjected to two freeze–thaw cycles, and the membrane fraction was removed by centrifugation at 15000 r.p.m. for 45 min. Supernatants and whole-cell cultures were diluted 10-fold in the reaction buffer; protoplasts were resuspended directly in the reaction buffer and cytoplasmatic fractions were diluted fivefold. The reaction was conducted in a total volume of 0.5 ml for 1 h at 37°C, then stopped by the addition of 100 μl of K₂HPO₄ 13% and the A₄₂₀ was read.

Expression and preparation of KinA'-B, KinBc and KapB

BL21 DE3 [pJV186] and BL21 DE3 [pJV172] cells were grown in LB broth (1 l) to an optical density of 0.6 (at 600 nm), at which point IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 1 mM. After continuing growth for 2–4 h, both proteins were found sequestered within inclusion bodies. Several attempts to keep the proteins in solution by varying IPTG concentrations, time of induction and growth temperature proved unsuccessful. Therefore, formation of these insoluble complexes was used as a purification step. The cells were pelleted, resuspended in 8 ml of lysis buffer (10 mM Tris-HCl pH 7.8, 10 mM KCl, 5 mM diithiothreitol, 1 mM phenylmethylsulphonyl fluoride) and disrupted by sonication. The samples were then centrifuged for 30 min at 10000 × g, and the supernatants were discarded. The insoluble pellets containing the bulk of KinA'-B and KinBc, were washed in 50 mM Tris-HCl pH 7.8, 0.1% sodium deoxycholate, 0.02% lysozyme, 1 mM EDTA for 1 h at 4°C to remove cell debris and contaminating macromolecules. After centrifugation for 30 min at 10000 × g, the pellets were resuspended in 8 ml of lysis buffer. A 1 ml aliquot of each suspension was solubilized

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to a final protein concentration of 100–200 mM in 50 mM Tris-HCl pH 7.8, 50 mM KCl, 5 mM dithiothreitol, 1 mM EDTA and stirred gently for 1 h at room temperature. The unsolubilized fraction was pelleted by centrifugation for 30 min at 10,000 × g and the supernatants were recovered. Renaturation of denatured KinAΔB and KinBC was carried out by step-dialysis at 4°C against 21 mM urea in buffer B (20 mM Tris-HCl pH 7.8, 10 mM KCl, 5 mM dithiothreitol) and then 210 mM urea in buffer B. The samples were concentrated using a CentriPrep-30 centrifugal concentrator (Amicon) for KinAΔB and a CentriPrep-10 for KinBC. Samples were dialysed against 40% glycerol in buffer B before pooling and stored at −20°C.

KapB was overexpressed from strain BL21 DE3 [pJV164] as described by Novagen (Madison, WI). The purified protein was purified on a Ni2+-NTA column, taking advantage of the presence of two neighbouring His pairs in the intrinsic aa sequence of KapB (Trach and Hoch, 1993). The purification procedure was a modification of the Qiagen protocol that included a single wash step with 25 mM Tris pH 8.0; 300 mM NaCl. KapB was precipitated with 40–60% (NH4)2SO4 and finally purified to homogeneity by gel filtration on a S16/60 Pharmacia column.

Autophosphorylation and phosphotransfer assay

The phosphorylation assays were performed in a 40 μl reaction volume containing 50 mM EPPS buffer pH 8.5; 50 mM KCl; 20 mM MgCl2; 0.1% gelatin and 5% glycerol. Standard protein concentrations were as follows: 1 μM KinA, 1 μM KinAΔB, 1 μM KinBC, 10 μM Spo0F and 1–20 μM KapB.

The reaction was initiated by the addition of 60 μCi of [γ32P] ATP and 0.1 mM unlabelled ATP. The reaction mixture was incubated at room temperature for 60 min, stopped by the addition of 0.2 vols of 5x protein-loading dye (250 mM Tris-HCl pH 6.8, 20% glycerol, 0.1% bromophenol blue, 1% SDS and 150 mM β-mercaptoethanol) and placed on dry ice until loading on a 15% acrylamide gel. Electrophoresis was carried out as previously described (Burbulys et al., 1991). Purified Spo0F was phosphorylated as described above by incubation for 5 min at room temperature in the presence of KinA and [γ32P]ATP. Spo0F−P was separated from KinA by gel filtration chromatography. Diphosphorylation of Spo0F−P was examined by incubating 1 μM of purified Spo0F−P in the presence of 1 μM KinBC for 3 min at room temperature.

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