kinA mRNA is missing a stop codon in the undomesticated *Bacillus subtilis* strain ATCC 6051

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Several features distinguish laboratory and undomesticated strains of *Bacillus subtilis*. For example, unlike the laboratory strain 168, the undomesticated strain ATCC 6051 is deficient in sporulation in a rich sporulation medium, 2× SG. ATCC 6051 cannot induce transcription of the *spoII*G operon, suggesting that this strain has a defect in initiation of sporulation. To determine the genetic difference between 168 and ATCC 6051, the DNA region responsible for the Spo[−] phenotype was transferred to strain 168. Genetic mapping and DNA sequencing analysis revealed that a stop codon (TAA) for *kinA* in 168 is replaced with Lys (TAT) in ATCC 6051, making the *kinA* open reading frame 201 bp longer in the undomesticated strain ATCC 6051. Introduction of *kinA* from strain 168 restored sporulation in ATCC 6051, indicating that the difference in *kinA* is responsible for the Spo[−] phenotype of ATCC 6051. A potential *p*-independent terminator is located upstream of a stop codon for the extended *kinA* open reading frame in ATCC 6051. Northern blot analysis showed that transcription of *kinA* terminated at this terminator, and *kinA* mRNA is missing a stop codon in ATCC 6051. Moreover, deletion of tmRNA suppresses the sporulation defect in ATCC 6051. These observations indicate that in ATCC 6051 the absence of a stop codon in *kinA* mRNA affects sporulation, probably by leading to rapid degradation of KinA via the *trans*-translation process. In ATCC 6051, the *kinA* mutation affects sporulation but not other Spo0A-dependent phenomena such as biofilm formation, which can be activated by a low level of Spo0A~P. This is due to the fact that KinA activity is kept low during the exponential phase via transcriptional and post-translational regulation. Thus, the stop-codon-less *kinA* probably affects only sporulation. DNA sequencing of 30 *B. subtilis* strains revealed that another strain also produces stop-codon-less *kinA* mRNA. This observation suggests that the lack of a stop codon for *kinA* mRNA may give rise to a selective advantage under certain conditions.

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INTRODUCTION

The Gram-positive soil bacterium *Bacillus subtilis* makes spores in response to nutrient depletion. Initiation of sporulation is controlled by the multi-component phosphotransfer system, the phosphorelay (Hoch, 1991, 1993). In response to nutrient depletion, a histidine kinase phosphorylates itself, and then this phosphate residue is sequentially transferred to Spo0F, Spo0B and finally Spo0A. Spo0A is a member of a response-regulator family of transcriptional regulators, and phosphorylated Spo0A

(Spo0A~P) activates transcription of early sporulation genes.

Spo0A~P directly regulates about 120 genes required for various stationary-phase phenomena including sporulation, competence development, production of degradative enzymes, cannibalism and biofilm formation (Molle *et al.*, 2003). Spo0A-regulated genes can be classified into two groups based on Spo0A dose dependency: low-threshold genes require a low level of Spo0A~P for activation or repression of transcription, whereas high-threshold genes require a high level of Spo0A~P for transcription (Fujita *et al.*, 2005). A representative low-threshold gene is *abrB*, whose expression is repressed by a low level of Spo0A~P. *AbrB* is a pleiotropic global repressor of many genes active in stationary phase (Hamon *et al.*, 2004; Strauch, 1991).

Abbreviations: Km^r, kanamycin resistant; Spo0A~P, phosphorylated Spo0A.

Two supplementary tables are available with the online version of this paper.

One target of AbrB is the *sipW* operon required for biofilm formation (Hamon *et al.*, 2004). By contrast, a high level of Spo0A~P is required for induction of sporulation genes such as the *spoIIA*, *spoIIE* and *spoIIG* operon genes (Fujita *et al.*, 2005). Thus, the level of Spo0A~P can determine cell fate.

In vivo and *in vitro* evidence has shown that five kinases, KinA, KinB, KinC, KinD and KinE, can phosphorylate Spo0F (Antoniewski *et al.*, 1990; Jiang *et al.*, 1999, 2000; Kobayashi *et al.*, 1995; LeDeaux & Grossman, 1995; Perego *et al.*, 1989; Trach & Hoch, 1993). At least under laboratory conditions, these kinases appear to have different roles in laboratory strain 168. KinA and KinB are major kinases for sporulation, since a *kinA kinB* double-mutant strain cannot make spores (Trach & Hoch, 1993). On the other hand, mutations in *kinC*, *kinD* or *kinE* reduce biofilm formation but not sporulation, whereas mutations in *kinA* or *kinB* do not affect biofilm formation (Hamon & Lazazzera, 2001; Jiang *et al.*, 1999; Kobayashi *et al.*, 1995; LeDeaux & Grossman, 1995; LeDeaux *et al.*, 1995). The function of Spo0A in biofilm formation is to repress *abrB* transcription, since *abrB* inactivation can restore biofilm formation in a *spo0A* mutant strain (Hamon & Lazazzera, 2001). In addition, although the *kinA kinB* double mutation does not affect *abrB* transcription, in a *kinA kinB kinC kinD* quadruple-mutant strain, the level of *abrB* transcription is elevated (Jiang *et al.*, 2000). Thus, KinC, KinD and perhaps KinE seem to produce a low level of Spo0A~P for repression of *abrB* transcription.

Recently, it has been recognized that the laboratory strain 168 is not representative of *B. subtilis* (Earl *et al.*, 2007), as it has several features that distinguish it from undomesticated strains. For example, the 168 strain is deficient in swarming motility (Kearns & Losick, 2003), poly- γ -glutamate synthesis (Stanley & Lazazzera, 2005), antibiotic production (Nakano *et al.*, 1988; Tsuge *et al.*, 1999) and biofilm formation (Branda *et al.*, 2001). These differences are caused by genetic differences among strains in at least the *sfp*, *swrA* and *degQ* genes (Kearns *et al.*, 2004; Nakano *et al.*, 1992; Stanley & Lazazzera, 2005; Tsuge *et al.*, 1999). One undomesticated strain, ATCC 6051 (NCIB 3610), can produce these antibiotics and form biofilms, and has been used by many laboratories to study multi-cellular behaviour. Unlike strain 168, strain ATCC 6051 forms aerial structures that serve as preferential sites for sporulation (Branda *et al.*, 2001). However, sporulation is conditional in ATCC 6051. It sporulates well in MSgg minimal medium (Branda *et al.*, 2001) but we have found that it does not make spores efficiently in rich sporulation media such as DSM and 2 $\times$ SG. This makes ATCC 6051 different, as other undomesticated strains can form aerial structures with spores in DSM and 2 $\times$ SG. Here, we demonstrate that a functionally relevant genetic difference exists between the *kinA* gene in strain 168 versus that in strain ATCC 6051.

METHODS

Bacterial strains. The *B. subtilis* strains used in this study are listed in Table 1. The *B. subtilis* ATCC 6051 and NBRC strains were

obtained from the American Type Culture Collection (ATCC) and NITE Biological Resource Center (NBRC), respectively. All strains with a W prefix are derivatives of ATCC 6051. The *B. subtilis* (*natto*) strain BEST 195 was isolated from Miyagono natto (Itaya & Matsui, 1999). In all cases, *B. subtilis* strains were maintained on TBABM, 2 $\times$ SG or LB (Difco) (Kobayashi, 2007). *E. coli* JM105 was used for construction and maintenance of plasmids. Antibiotics were used at the following concentrations: ampicillin, 30 μ g ml⁻¹; chloramphenicol, 5 μ g ml⁻¹; erythromycin/lincomycin, 0.5 μ g ml⁻¹ and 25 μ g ml⁻¹, respectively; kanamycin, 10 μ g ml⁻¹; spectinomycin, 100 μ g ml⁻¹.

Identification of *kinA* variation in ATCC 6051. The DNA region responsible for the Spo⁻ phenotype of ATCC 6051 was transferred to strain 168 by conjugation. For this, 1 ml of 168 competent cells was mixed with chromosomal DNA (0.5 μ g) isolated from an ATCC 6051 derivative strain, W1151, that harbours *spoIID-lacZ* (kanamycin-resistant, Km^r) at the *amyE* locus, and then Spo⁻ and white colonies were screened among Km^r transformants on TBABM plates containing 100 μ g X-Gal ml⁻¹. One such transformant was designated K477. To identify the DNA region responsible for the Spo⁻ phenotype, K477 was transformed with chromosomal DNA from 285 regulator mutants (Kobayashi, 2007) and β -galactosidase activity of each transformant was examined on TBABM plates supplemented with 100 μ g X-Gal ml⁻¹. The *spo* mutation was mapped to the chromosomal region between *ykuM* and *splA*.

Construction of *B. subtilis* mutant strains. Mutant strains were constructed using an overlap-extension PCR technique (Kobayashi, 2007). Antibiotic-resistance cassettes were amplified by PCR using the primers pUC-F and pUC-R from pCBB31(*cat*), pAE41(*erm*), pDG780(*kan*) and pDG1726(*spc*) (Guerout-Fleury *et al.*, 1995; Kobayashi *et al.*, 1995). As ATCC 6051 has low competence, the mutant alleles were first introduced into strain 168 and then transferred into ATCC 6051 by transformation with chromosomal DNA from the newly generated 168 mutant strains. Transformation of *B. subtilis* was carried out using a standard protocol (Dubnau & Davidoff-Abelson, 1971). Primers used for construction of mutant alleles are listed in Supplementary Table S1 (available with the online version of this paper).

The 3' region of *kinA* was amplified from 168 or ATCC 6051 chromosomal DNA by PCR using the primers *kinA*-F9 and *kinA*-R9. After digestion with *Eco*RI and *Hind*III, the resultant fragments were inserted into pCA191, a chloramphenicol-resistant (Cm^r) derivative of pUC19, to generate pCAkinA¹⁶⁸ and pCAkinA⁶⁰⁵¹, respectively. These plasmids were then used to transform strain 168 and Cm^r transformants were selected. The DNA sequence of *kinA* in the transformants was determined; *kinA*¹⁶⁸ and *kinA*⁶⁰⁵¹ strains were selected among these transformants.

The promoter region of *spoIID* was amplified from ATCC 6051 chromosomal DNA by PCR using the primers *spoIID*-P-F and *spoIID*-P-R. After digestion with *Eco*RI and *Bam*HI, the DNA fragment was inserted into pDLK2, which contains a *lacZ* reporter between the *amyE*-up and -down sequences. The resultant plasmid, pDLKspoIID, was used to transform strain 168 and a transformant harbouring *amyE::spoIIDp-lacZ* was used in further analyses.

Northern blot analysis. *B. subtilis* strains were grown in 2 $\times$ SG at 37 °C and cells were harvested at the indicated time points. Total RNA was isolated via the method described by Igo & Losick (1986). Northern blotting was performed as described previously (Kobayashi, 2007). To prepare digoxigenin (DIG)-labelled RNA probes, DNA fragments were amplified by PCR using the primers described in Table S1.

Sporulation assay. *B. subtilis* strains were grown in 2 $\times$ SG medium at 37 °C and sporulation was assayed at 24 h after the end of the

Table 1. *B. subtilis* strains used in this study

Strains*	Genotype	Source or reference†
168 derivatives		
168	<i>trpC2</i>	C. Anagnostopoulos, CNRS, France
K477	<i>trpC2 ΔamyE::spoIID-lacZ (kan) kinA⁶⁰⁵¹</i>	This study
K440	<i>trpC2 ΔkinA::cat</i>	This study
K441	<i>trpC2 ΔkinB::kan</i>	This study
K442	<i>trpC2 ΔkinA::cat ΔkinB::kan</i>	This study
K439	<i>trpC2 kinA⁶⁰⁵¹::pCAkinA⁶⁰⁵¹(cat)</i>	This study
K443	<i>trpC2 kinA⁶⁰⁵¹::pCAkinA⁶⁰⁵¹(cat) ΔkinB::kan</i>	This study
ATCC 6051 derivatives		
ATCC 6051	Wild-type	American Type Culture Collection
W1151	<i>ΔamyE::spoIIDp-lacZ (kan)</i>	This study
W918	<i>ΔkinA::cat</i>	This study
W1051	<i>ΔkinB::kan</i>	This study
W924	<i>ΔkinC::erm</i>	This study
W925	<i>ΔkinD::spc</i>	This study
W885	<i>ΔkinE::pMutin (erm)</i>	YKRQd (Kobayashi <i>et al.</i> , 2003)→ATCC 6051
W1056	<i>ΔkinA::cat ΔkinB::kan</i>	This study
W1046	<i>kinA¹⁶⁸::pCAkinA¹⁶⁸(cat)</i>	This study
W1077	<i>kinA¹⁶⁸::pCAkinA¹⁶⁸(cat) ΔkinC::erm</i>	This study
W1078	<i>kinA¹⁶⁸::pCAkinA¹⁶⁸(cat) ΔkinD::spc</i>	This study
W1125	<i>ΔssrA::erm</i>	This study
W1148	<i>ΔkinA::cat ΔssrA::erm</i>	This study
W1128	<i>ΔkinB::kan ΔssrA::erm</i>	This study
W1149	<i>ΔkinA::cat ΔkinB::kan ΔssrA::erm</i>	This study
W1037	<i>ΔkinC::erm ΔkinD::spc</i>	This study
WTF2	<i>ΔabrB::cat</i>	Kobayashi (2007)
W1045	<i>ΔabrB::cat ΔkinC::erm ΔkinD::spc</i>	WTF2 (Kobayashi, 2007)→W1037
W1129	<i>ΔkipI::erm Δsda::kan</i>	This study
W1163	<i>ΔkinD::spc ΔkipI::erm Δsda::kan</i>	This study
W1130	<i>kinA¹⁶⁸::pCAkinA¹⁶⁸(cat) ΔkipI::erm Δsda::kan</i>	This study
W1136	<i>kinA¹⁶⁸::pCAkinA¹⁶⁸(cat) ΔkinD::spc ΔkipI::erm Δsda::kan</i>	This study
NBRC 16449 derivatives		
NBRC 16449	Wild-type	NITE Biological Resource Center
K493	<i>ΔkinA::cat</i>	This study
K494	<i>ΔkinB::kan</i>	This study
K496	<i>ΔkinA::cat ΔkinB::kan</i>	This study
K495	<i>ΔssrA::erm</i>	This study
K497	<i>ΔkinA::cat ΔssrA::erm</i>	This study
K498	<i>ΔkinB::kan ΔssrA::erm</i>	This study
K499	<i>ΔkinA::cat ΔkinB::kan ΔssrA::erm</i>	This study

*All strains with W prefix are derivatives of ATCC 6051.

†Arrows indicate the direction of donor-to-recipient transformation for strain construction.

exponential phase (T_{24}). The number of spores per ml culture was determined by identifying the number of heat-resistant c.f.u. (80 °C for 10 min) on LB plates. The number of viable cells was determined by counting the total number of c.f.u. at T_{24} with one exception: viable cells from strain ATCC 6051 and its derivatives were measured at T_3 because the number of viable cells from this strain had decreased by T_{24} .

Pellicle formation. Pellicle formation was examined in $2 \times$ SGG medium [$2 \times$ SG plus 1% (w/v) glycerol] as described previously (Kobayashi, 2007). Cells were observed via phase-contrast microscopy

using a DMRE-HC microscope (Leica) combined with a digital CCD camera (1300Y, Roper Science).

Isolation of *B. subtilis* and related strains from soil. To isolate wild *Bacillus* strains, soil samples were gathered from several places in the garden at Setsunan University. Approximately 2 g soil samples were dissolved in 2 ml of 10 mM Tris/HCl (pH 7.2) and then boiled at 95 °C for 5 min. From this, 0.1 ml of each sample was then spread onto LB plates and incubated at 37 °C. Forty-eight *Bacillus* strains were isolated based on sporulation ability and colony morphology. Because it is not known if these strains are *B. subtilis*, only strains that

contained a *kinA* region that could be amplified by PCR using the primers *kinA*-F7 and *kinA*-R8 were used in the analysis. Notably, no specific DNA was amplified from *Bacillus amyloliquefaciens* or *Bacillus licheniformis* genomic DNA when tested by PCR with these primers (data not shown).

RESULTS AND DISCUSSION

Failure of *B. subtilis* ATCC 6051 to sporulate in 2× SG is attributable to a variation in *kinA*

The undomesticated *B. subtilis* strain ATCC 6051 (hereafter, 6051) produced spores at lower frequency than the laboratory strain 168 in 2× SG sporulation medium (Table 2). Moreover, transcription of the *spoIIG* operon was not induced in 6051 grown in 2× SG medium, suggesting that this strain has a defect in initiation of sporulation (Fig. 1a). To identify a genetic determinant responsible for the 6051 Spo[−] phenotype, we first sought to transfer the DNA region responsible for the Spo[−] phenotype from 6051 to 168, as 6051 is not suitable for genetic manipulation. To do this, 168 was transformed with chromosomal DNA isolated from 6051 *amyE::spoIIG-lacZ* (Km^r), and white and Spo[−] colonies were screened among Km^r transformants on TBABM plates containing X-Gal. One white, Spo[−] transformant was isolated and used for further analysis.

To identify the DNA region responsible for the Spo[−] phenotype, the Spo[−] hybrid strain was transformed with chromosomal DNA isolated from mutants of strain 168 with Cm^r cassettes at genes encoding regulators on the chromosome (Kobayashi, 2007). In a transformation assay, *ykuM::cat* and *splA::cat* alleles had >50 % linkage with

the Spo[−] phenotype. Moreover, the *cheV* allele, which is located in the region between *ykuM* and *splA*, had >90 % linkage with the Spo[−] phenotype. As *kinA*, which encodes a histidine kinase for sporulation (Antoniewski *et al.*, 1990; Perego *et al.*, 1989), is located upstream of *cheV*, it seemed reasonable to propose that the *kinA* is responsible for the Spo[−] phenotype. To address this, the DNA region containing *kinA* was amplified by PCR and used for transformation. Spo⁺ transformants did appear after transformation of the Spo[−] hybrid strain with DNA containing *kinA* but did not appear after transformation with DNA lacking *kinA* (data not shown). Moreover, DNA sequencing of the entire *kinA* gene in strain ATCC 6051 revealed that a stop codon, TAA, present in strain 168 *kinA* is TAT (Lys) in strain 6051. The nucleotide difference results in a 201 bp extension of the *kinA* coding region in 6051, which extends the coding region into *pata* (Fig. 1b).

To ask if this difference is responsible for the Spo[−] phenotype, *kinA* gene in 168 (referred to as *kinA*¹⁶⁸) was replaced by *kinA* of 6051 (referred to as *kinA*⁶⁰⁵¹). It has been shown that initiation of sporulation is controlled by two kinases, KinA and KinB, in the laboratory strain 168. Mutations in *kinA* or *kinB* have little or no effect on sporulation but a combination of *kinA* and *kinB* mutations severely reduces sporulation (Trach & Hoch, 1993). As shown in Table 2, *kinA*⁶⁰⁵¹ had a similar effect on sporulation with the *kinA* mutation in the 168 background; that is, *kinA*⁶⁰⁵¹ reduced sporulation slightly on its own and dramatically when in combination with the *kinB* mutation. In the 6051 background, the *kinB* mutation had a striking negative effect on sporulation, decreasing to the level observed for the *kinA kinB* double-mutant strain (Table 2). Introduction of *kinA*¹⁶⁸ restored the ability of

Table 2. Sporulation frequency of kinase mutants

The number of viable cells and spores was determined at *T*₂₄ as the mean of three independent cultures. The number of viable cells of ATCC 6051 strains was determined at *T*₃.

Strain	Genotype	Cells ml ^{−1}	Spores ml ^{−1}	Frequency (%)
168 background				
168	Wild-type	1.7 × 10 ⁹	1.4 × 10 ⁹	82.4
K440	Δ <i>kinA</i>	1.9 × 10 ⁹	4.1 × 10 ⁸	21.2
K441	Δ <i>kinB</i>	2.0 × 10 ⁹	9.0 × 10 ⁸	45
K442	Δ <i>kinA</i> Δ <i>kinB</i>	1.1 × 10 ⁹	1.1 × 10 ²	1.0 × 10 ^{−5}
K439	<i>kinA</i> ⁶⁰⁵¹	9.0 × 10 ⁸	2.1 × 10 ⁸	23.3
K443	<i>kinA</i> ⁶⁰⁵¹ Δ <i>kinB</i>	1.0 × 10 ⁹	5.5 × 10 ⁴ *	5.5 × 10 ^{−3}
ATCC 6051 background				
ATCC 6051	Wild-type	5.6 × 10 ⁸	7.9 × 10 ⁶	1.4
W918	Δ <i>kinA</i>	7.5 × 10 ⁸	1.3 × 10 ⁷	1.7
W1051	Δ <i>kinB</i>	3.3 × 10 ⁸	1.2 × 10 ⁴	3.6 × 10 ^{−3}
W1056	Δ <i>kinA</i> Δ <i>kinB</i>	1.2 × 10 ⁹	7.3 × 10 ³	6.1 × 10 ^{−4}
W1046	<i>kinA</i> ¹⁶⁸	7.6 × 10 ⁸	1.6 × 10 ⁹	100

*Most of colonies became Spo⁺. Since *kinA*⁶⁰⁵¹ was introduced by Campbell-type integration of plasmid pCA*kinA*⁶⁰⁵¹, it was probably caused by replacement of *kinA*⁶⁰⁵¹ with *kinA*¹⁶⁸ through excision and reintegration of the plasmid.

Fig. 1. The *kinA* mRNA is missing a stop codon in ATCC 6051. (a) Northern blot analysis of the *spoIIGA* operon. *B. subtilis* strains ATCC 6051 and 168 were grown in 2× SG and RNA samples were prepared from cells taken at the time points shown in Fig. 3(b). As a control, a membrane stained with methylene blue to visualize rRNA is shown. (b) Top: schematic representation of gene organization in the *kinA* region. The promoter and terminator for *kinA* transcription are shown. Bottom: nucleotide sequence of the *kinA* 3' region in strain 168, along with the corresponding amino acid sequence. The nucleotide difference in strain ATCC 6051 is indicated above the sequence. The change results in elongation of the *kinA*-coding region by 201 bp (67 codons). (c) Northern blot analysis of *kinA*. RNA samples were isolated from cells taken at time point 7 as indicated in Fig. 4(b). RNA size makers are shown on the left.

6051 to sporulate. Taken together, the results strongly suggest that *kinA*⁶⁰⁵¹ is non-functional and is responsible for the Spo[−] phenotype of strain 6051.

The *kinA*⁶⁰⁵¹ transcript lacks a stop codon

In strain 168, the *kinA* stop codon is followed by a ρ -independent terminator sequence, which is conserved at the corresponding position of *kinA*⁶⁰⁵¹ (Fig. 1b). Northern blot analysis revealed that the size of the *kinA* transcript is the same in 168 and 6051 and matches the predicted size of 1902 nt, which is based on the distance from the transcriptional start site to the terminator (Fig. 1c). These observations suggest that *kinA* transcription terminates at this terminator sequence upstream of a stop codon of the extended *kinA* in 6051. Thus, the *kinA*⁶⁰⁵¹ transcript lacks a stop codon.

Stop-codon-less mRNA prevents release of ribosomes from the mRNA, which in turn causes ribosomes to stall on the mRNA. Bacteria have a specific mechanism, termed *trans*-translation, for rescue of stalled ribosomes on incomplete or damaged mRNAs (Karzai *et al.*, 2000; Withey & Friedman, 2003). The *ssrA* gene codes for tmRNA, which possesses both a tRNA-like domain and an mRNA-like coding sequence that together enable tmRNA to function as both a tRNA and an mRNA. This system rescues ribosomes stalled on the mRNAs and directs addition of a proteolysis tag to the C-termini of aberrant protein products, thus targeting them for proteolysis by the Clp protease complex (Gottesman *et al.*, 1998; Wiegert & Schumann, 2001). Thus, the stop-codon-less mRNA of

*kinA*⁶⁰⁵¹ may be a substrate for tmRNA, leading to rapid degradation of KinA⁶⁰⁵¹ protein. To address this possibility, the effect of a mutation in *ssrA* on sporulation in strain 6051 was examined. As shown in Table 3, the *ssrA* mutation restored sporulation in wild-type and *kinB* mutant strains, whereas the *ssrA* mutation did not restore sporulation in the *kinA* and *kinA kinB* mutant strains. Thus, the *ssrA* mutation suppresses the sporulation defect of strain 6051 in a *kinA*⁶⁰⁵¹-dependent manner. These results indicate that the absence of a stop codon in *kinA*⁶⁰⁵¹ mRNA affects sporulation, probably leading to rapid degradation of KinA⁶⁰⁵¹ via the *trans*-translation process.

Table 3. Effect of the *ssrA* mutation on sporulation

The number of viable cells (T_3) and spores (T_{24}) was determined as the mean of three independent cultures.

Strain	Genotype	Cells ml ^{−1}	Spores ml ^{−1}	Frequency (%)
ATCC 6051	Wild-type	1.4×10^9	1.2×10^7	0.9
W1125	$\Delta ssrA$	5.4×10^8	4.4×10^8	81.5
W1148	$\Delta kinA \Delta ssrA$	7.7×10^8	4.0×10^7	5.2
W1056	$\Delta kinB$	2.1×10^9	4.0×10^3	1.9×10^{-4}
W1128	$\Delta kinB \Delta ssrA$	4.8×10^8	2.9×10^8	60.4
W1149	$\Delta kinA \Delta kinB \Delta ssrA$	6.6×10^8	2.5×10^2	3.8×10^{-5}

KinA is specifically required for sporulation

We next asked if introduction of *kinA*¹⁶⁸ into strain 6051 affects other Spo0A-dependent phenomena. In the laboratory strain 168, pellicle formation is negatively controlled by AbrB, expression of which is repressed by a low level of Spo0A~P (Fujita *et al.*, 2005; Hamon & Lazazzera, 2001). As has been shown for the laboratory strain (Hamon & Lazazzera, 2001), in strain 6051 pellicle formation appears to be controlled by other kinases, KinC and KinD, as *kinC* and *kinD* mutant strains form extremely thin pellicles (Fig. 2a, W924 and W925). However, unlike the situation in the laboratory strain, a mutation in *kinE* did not affect pellicle formation in strain 6051 (Fig. 2a, W885). Introduction of *kinA*¹⁶⁸ did not affect pellicle formation in the wild-type strain and could not restore pellicle formation of *kinC* and *kinD* mutant strains (Fig. 2a, W1077 and W1078). However, when these strains harboured *kinA*¹⁶⁸ they formed spores on pellicles efficiently, even in a *kinC* or *kinD* mutant background (Fig. 2b)

As expected, based on a previous report (Hamon & Lazazzera, 2001), the *abrB* mutation bypassed the requirement for KinC and KinD in pellicle formation (Fig. 2a, W1045). Transcriptional analysis of *abrB* revealed that KinC and KinD are responsible for production of a low level of Spo0A~P, which can repress *abrB* transcription (Fig. 3a). By contrast, mutation of *kinA* and *kinB* did not affect transcription of *abrB* (Fig. 3a), and introduction of

*kinA*¹⁶⁸ also did not affect *abrB* transcription (Fig. 3c, W1046). These observations suggest that KinA is distinct from KinC and KinD. KinA appears to be specifically required for sporulation, whereas KinC and KinD are involved in repression of *abrB* transcription.

Spo0A~P controls repression of *abrB* transcription and direct induction of sporulation genes, but the latter requires a higher level of Spo0A~P than the former (Fujita *et al.*, 2005). We wondered why KinA¹⁶⁸ cannot substitute for KinC or KinD despite the fact that KinA¹⁶⁸ can accumulate enough Spo0A~P to induce sporulation, even in the *kinC* or *kinD* mutant background (Fig. 2b). To address this, transcription of these kinases was compared by Northern blot analysis. When *B. subtilis* cells were grown in 2 × SG, *kinC* and *kinD* were expressed when cells were in the exponential phase, whereas transcription of *kinA*, *kinB* and *kinE* was induced after the exponential phase (Fig. 3b).

As the phosphatases KipI and Sda inhibit activity of KinA (Burkholder *et al.*, 2001; Wang *et al.*, 1997), we hypothesized that KinA¹⁶⁸ activity is inhibited by these phosphatases during the early growth phase, and therefore KinA¹⁶⁸ cannot substitute for KinC or KinD. To address this, *kinA*¹⁶⁸ was introduced into a *kipI sda kinD* triple-mutant strain. In the absence of *kipI* and *sda*, KinA¹⁶⁸ could restore pellicle formation of the *kinD* mutant strain (Fig. 2a, W1136). Thus, as expected, KinA activity is

Fig. 2. Pellicle formation and sporulation in kinase mutant strains. (a) To analyse pellicle formation, a fresh colony was used to inoculate 2 × SGG medium in the 3 cm diameter well of a culture dish. The cultures were then incubated at 30 °C for 24 h without agitation. (b) Sporulation on pellicles. After 48 h incubation, cells were taken from pellicles and analysed by phase-contrast microscopy. Bars, 5 μm.

Fig. 3. KinA activity is controlled by transcriptional and post-translational regulation. (a) Northern blot analysis of *abrB*. RNA samples were prepared from cells taken at the time points shown in (b). As a control, a membrane stained with methylene blue to visualize rRNA is shown. (b) Expression of kinase genes in strain ATCC 6051. Left panel, growth of ATCC 6051 in 2x SG. Arrows indicate the time points at which samples were taken for RNA isolation. Transcription of kinase genes was detected by Northern blotting. (c) *KipI*, *Sda* and *KinD* inhibit *KinA*¹⁶⁸ activity. Transcription of *abrB* was analysed at the time points shown in (b).

inhibited by these phosphatases. Moreover, mutations of *kipI* and *sda* also partly restored pellicle formation of the *kinD* mutant strain alone (Fig. 2a, W1163), suggesting that these phosphatases also inhibit activity of other kinases.

To confirm that these effects resulted from regulation of *abrB*, transcription of this gene was examined. As was the case for pellicle formation, introduction of *kinA*¹⁶⁸ did not affect *abrB* transcription in the wild-type and *kinD* mutant strains (Fig. 3c, W1046 and W1078). However, it should be noted that, unlike the case for pellicle formation, the *kinD* mutation did not affect *abrB* transcription in agitated culture (Fig. 3c, W925). By contrast, *KinA*¹⁶⁸ could strongly repress transcription of *abrB* in the absence of *kinD*, *kipI* and *sda* (Fig. 3c, W1136). However, *KinA*¹⁶⁸ did not affect transcription of *abrB* in the presence of *kinD*, even when *kipI* and *sda* were also absent (Fig. 3c, W1130). Since *KinA* and *KinD* phosphorylate *Spo0F*, these kinases may compete for *Spo0F*. Thus, these observations suggest that *KinA* is inhibited not only by the *KipI* and *Sda* phosphatases but also by competition with *KinD* in interaction with *Spo0F*.

Although *kinA* transcription is low during the exponential phase (Fig. 3b), *KinA*¹⁶⁸ strongly represses transcription of *abrB* in the absence of *kipI*, *sda* and *kinD*. This observation suggests the possibility that the kinase activity of *KinA* is stronger than that of *KinD*, which may enable *KinA* to induce sporulation. This possibility is supported by a previous report that, *in vitro*, *KinC* and *KinD* are less active

on *Spo0F* than *KinA*; however, it should be noted that truncated forms of *KinC* and *KinD* were used in the study (Jiang *et al.*, 1999).

Each kinase involved in the phosphorelay has a distinct function under the conditions we tested. These differences are caused by regulation at at least three levels: transcriptional regulation, control of kinase activity by phosphatases, and competition among kinases. By these regulations, *KinA* seems to be specific for sporulation that requires a high level of *Spo0A*~P. Thus, the stop-codon-less *kinA* affects only sporulation but not other *Spo0A*~P-related functions in strain 6051.

The *kinA* gene in other *B. subtilis* strains

We were interested to learn if the stop-codon-less form of the *kinA* gene exists in other *B. subtilis* strains. To address this, we determined the DNA sequence of the *kinA* 3' region in 30 *B. subtilis* strains: two *B. subtilis* subsp. *subtilis* strains, one *B. subtilis* subsp. *spizizenii* strain, three *B. subtilis* subsp. *subtilis* strains isolated from natto, and 24 strains newly isolated from soil. The DNA sequence of the stop codon region of *kinA* in each strain is shown in Fig. 4(a). Interestingly, the *kinA* sequence around a stop codon varied among strains, which can be classified into three groups. In the first group, the *kinA* stop codon region is the same as that of strain 168. In the second group, there is a 1 bp deletion downstream of the stop codon. And in

the third group, there are two 1 bp deletions, and conceptual translation shows that the KinA protein produced by strains in this group is four amino acids longer than KinA¹⁶⁸. These deletions are in a poly-A stretch around the position that corresponds to a stop codon for *kinA*¹⁶⁸. The A-to-T substitution in *kinA*⁶⁰⁵¹ also occurred in the poly-A stretch and, furthermore, *kinA* of NBRC 101239 has an A-to-G substitution in the poly-A stretch that does not affect the amino acid sequence. These observations imply that the sequence of the poly-A stretch may be susceptible to spontaneous mutations.

The *kinA* gene in NBRC 16449 has an insertion upstream of a stop codon that results in a longer *kinA* coding sequence (Fig. 4b). Although we did not determine the entire sequence of the insertion element, the sequence is significantly similar to that of IS660 in *Oceanobacillus iheyensis* HTE 83 (data not shown). To our surprise, the terminus of the IS insertion was the ρ -independent terminator sequence and thereby, the IS insertion

introduced a new potential ρ -independent terminator, preceding the potential stop codon (Fig. 4c). NBRC 16449 sporulated well in 2 × SG medium (see Supplementary Table S2, available with the online version of this paper). As is the case for ATCC 6051, the *kinB* mutation reduced sporulation in the NBRC 16449 background. Moreover, the *ssrA* mutation restored sporulation ability of the *kinB* mutant strain in a *kinA*-dependent manner. These observations suggest that NBRC 16449 also produces a stop-codon-less *kinA* mRNA.

It is easy to imagine that sporulation, which blocks an increase in cell number, can be disadvantageous under some conditions, particularly as in natural environments, bacteria live in competition with other organisms. The *kinA* mutation reduces the frequency of initiation of sporulation but does not affect phenomena such as biofilm formation, which is important for survival in natural environments. Moreover, the *kinA* mutation does not abolish sporulation: the *kinA* mutant strain produces

Fig. 4. Comparison of the *kinA* gene region among *B. subtilis* strains. (a) Comparison of the *kinA* 3' region in the following strains: *B. subtilis* subsp. *subtilis*, 168, ATCC 6051, NBRC 13722 and NBRC 3134; *B. subtilis* subsp. *spizizenii*, NBRC 101239; *B. subtilis* subsp. *subtilis* var. *natto*, BEST 195, NBRC 3335 and NBRC 16449. Of the SUBC strains, 24 were newly isolated from soil. Stop codons are underlined. The ρ -independent terminator sequence is indicated by an inverted arrow. The position of an insertion element in strain NBRC 16449 is indicated by an upward-pointing arrow. (b) Insertion in the strain NBRC 16449 *kinA* gene. The 3' region of *kinA* was amplified from chromosomal DNA from strain 168 (lane 2) or NBRC 16449 (lane 3) by PCR. Left, a DNA size marker (λ HindIII digest; lane 1). (c) The nucleotide sequence of the 3' region of *kinA* in strain NBRC 16449. The insertion is shaded and the ρ -independent terminator sequence is indicated by an inverted arrow. The conceptual translation is shown below the nucleotide sequence.

spores at a low frequency in rich sporulation medium and makes spores normally in minimal medium (LeDeaux *et al.*, 1995). These observations suggest that the *kinA* mutation may give rise to a selective advantage under certain conditions. However, ATCC 6051 and NBRC 16449 have unique variations that produce stop-codon-less *kinA* mRNAs. In addition, the stop-codon-less *kinA* is functional in an *ssrA* mutant background. These observations imply that the stop-codon-less *kinA* may be functional under certain conditions. However, we could not find conditions that induce sporulation in a stop-codon-less *kinA*-dependent manner. Investigating a possible function for the stop-codon-less *kinA*⁶⁰⁵¹ will be a subject of future study.

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REFERENCES

- Antoniewski, C., Savelli, B. & Stragier, P. (1990). The *spoIIJ* gene, which regulates early developmental steps in *Bacillus subtilis*, belongs to a class of environmentally responsive genes. *J Bacteriol* **172**, 86–93.
- Branda, S. S., Gonzalez-Pastor, J. E., Ben-Yehuda, S., Losick, R. & Kolter, R. (2001). Fruiting body formation by *Bacillus subtilis*. *Proc Natl Acad Sci U S A* **98**, 11621–11626.
- Burkholder, W. F., Kurtser, I. & Grossman, A. D. (2001). Replication initiation proteins regulate a developmental checkpoint in *Bacillus subtilis*. *Cell* **104**, 269–279.
- Dubnau, D. & Davidoff-Abelson, R. (1971). Fate of transforming DNA following uptake by competent *Bacillus subtilis*. I. Formation and properties of the donor-recipient complex. *J Mol Biol* **56**, 209–221.
- Earl, A. M., Losick, R. & Kolter, R. (2007). *Bacillus subtilis* genome diversity. *J Bacteriol* **189**, 1163–1170.
- Fujita, M., Gonzalez-Pastor, J. E. & Losick, R. (2005). High- and low-threshold genes in the Spo0A regulon of *Bacillus subtilis*. *J Bacteriol* **187**, 1357–1368.
- Gottesman, S., Roche, E., Zhou, Y. & Sauer, R. (1998). The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system. *Genes Dev* **12**, 1338–1347.
- Guerout-Fleury, A. M., Shazand, K., Frandsen, N. & Stragier, P. (1995). Antibiotic-resistance cassettes for *Bacillus subtilis*. *Gene* **167**, 335–336.
- Hamon, M. A. & Lazazzera, B. A. (2001). The sporulation transcription factor Spo0A is required for biofilm development in *Bacillus subtilis*. *Mol Microbiol* **42**, 1199–1209.
- Hamon, M. A., Stanley, N. R., Britton, R. A., Grossman, A. D. & Lazazzera, B. A. (2004). Identification of AbrB-regulated genes involved in biofilm formation by *Bacillus subtilis*. *Mol Microbiol* **52**, 847–860.
- Hoch, J. A. (1991). *spo0* genes, the phosphorelay, and the initiation of sporulation. In *Bacillus subtilis and Other Gram-positive Bacteria: Biochemistry, Physiology, and Molecular Genetics*, pp. 747–755. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington, DC: American Society for Microbiology.
- Hoch, J. A. (1993). Regulation of the onset of the stationary phase and sporulation in *Bacillus subtilis*. *Adv Microb Physiol* **35**, 111–133.
- Igo, M. M. & Losick, R. (1986). Regulation of a promoter that is utilized by minor forms of RNA polymerase holoenzyme in *Bacillus subtilis*. *J Mol Biol* **191**, 615–624.
- Itaya, M. & Matsui, K. (1999). Conversion of *Bacillus subtilis* 168: natto producing *Bacillus subtilis* with mosaic genomes. *Biosci Biotechnol Biochem* **63**, 2034–2037.
- Jiang, M., Tzeng, Y. L., Feher, V. A., Perego, M. & Hoch, J. A. (1999). Alanine mutants of the Spo0F response regulator modifying specificity for sensor kinases in sporulation initiation. *Mol Microbiol* **33**, 389–395.
- Jiang, M., Shao, W., Perego, M. & Hoch, J. A. (2000). Multiple histidine kinases regulate entry into stationary phase and sporulation in *Bacillus subtilis*. *Mol Microbiol* **38**, 535–542.
- Karzai, A. W., Roche, E. D. & Sauer, R. T. (2000). The SsrA-SmpB system for protein tagging, directed degradation and ribosome rescue. *Nat Struct Biol* **7**, 449–455.
- Kearns, D. B. & Losick, R. (2003). Swarming motility in undomesticated *Bacillus subtilis*. *Mol Microbiol* **49**, 581–590.
- Kearns, D. B., Chu, F., Rudner, R. & Losick, R. (2004). Genes governing swarming in *Bacillus subtilis* and evidence for a phase variation mechanism controlling surface motility. *Mol Microbiol* **52**, 357–369.
- Kobayashi, K. (2007). *Bacillus subtilis* pellicle formation proceeds through genetically defined morphological changes. *J Bacteriol* **189**, 4920–4931.
- Kobayashi, K., Shoji, K., Shimizu, T., Nakano, K., Sato, T. & Kobayashi, Y. (1995). Analysis of a suppressor mutation *ssb* (*kinC*) of *surOB20* (*spo0A*) mutation in *Bacillus subtilis* reveals that *kinC* encodes a histidine protein kinase. *J Bacteriol* **177**, 176–182.
- Kobayashi, K., Ehrlich, S. D., Albertini, A., Amati, G., Andersen, K. K., Arnaud, M., Asai, K., Ashikaga, S., Aymerich, S. & other authors (2003). Essential *Bacillus subtilis* genes. *Proc Natl Acad Sci U S A* **100**, 4678–4683.
- LeDeaux, J. R. & Grossman, A. D. (1995). Isolation and characterization of *kinC*, a gene that encodes a sensor kinase homologous to the sporulation sensor kinases KinA and KinB in *Bacillus subtilis*. *J Bacteriol* **177**, 166–175.
- LeDeaux, J. R., Yu, N. & Grossman, A. D. (1995). Different roles for KinA, KinB, and KinC in the initiation of sporulation in *Bacillus subtilis*. *J Bacteriol* **177**, 861–863.
- Molle, V., Fujita, M., Jensen, S. T., Eichenberger, P., Gonzalez-Pastor, J. E., Liu, J. S. & Losick, R. (2003). The Spo0A regulon of *Bacillus subtilis*. *Mol Microbiol* **50**, 1683–1701.
- Nakano, M. M., Marahiel, M. A. & Zuber, P. (1988). Identification of a genetic locus required for biosynthesis of the lipopeptide antibiotic surfactin in *Bacillus subtilis*. *J Bacteriol* **170**, 5662–5668.
- Nakano, M. M., Corbell, N., Besson, J. & Zuber, P. (1992). Isolation and characterization of *sfp*: a gene that functions in the production of the lipopeptide biosurfactant, surfactin, in *Bacillus subtilis*. *Mol Genet* **232**, 313–321.
- Perego, M., Cole, S. P., Burbulys, D., Trach, K. & Hoch, J. A. (1989). Characterization of the gene for a protein kinase which phosphorylates the sporulation-regulatory proteins Spo0A and Spo0F of *Bacillus subtilis*. *J Bacteriol* **171**, 6187–6196.
- Stanley, N. R. & Lazazzera, B. A. (2005). Defining the genetic differences between wild and domestic strains of *Bacillus subtilis* that affect poly-gamma-DL-glutamic acid production and biofilm formation. *Mol Microbiol* **57**, 1143–1158.
- Strauch, M. A. (1991). AbrB, a transition state regulator. In *Bacillus subtilis and Other Gram-positive Bacteria: Biochemistry, Physiology,*

and *Molecular Genetics*, pp. 757–764. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington, DC: American Society for Microbiology.

Trach, K. A. & Hoch, J. A. (1993). Multisensory activation of the phosphorelay initiating sporulation in *Bacillus subtilis*: identification and sequence of the protein kinase of the alternate pathway. *Mol Microbiol* **8**, 69–79.

Tsuge, K., Ano, T., Hirai, M., Nakamura, Y. & Shoda, M. (1999). The genes *degQ*, *pps*, and *lpa-8* (*sfp*) are responsible for conversion of *Bacillus subtilis* 168 to plipastatin production. *Antimicrob Agents Chemother* **43**, 2183–2192.

Wang, L., Grau, R., Perego, M. & Hoch, J. A. (1997). A novel histidine kinase inhibitor regulating development in *Bacillus subtilis*. *Genes Dev* **11**, 2569–2579.

Wiegert, T. & Schumann, W. (2001). SsrA-mediated tagging in *Bacillus subtilis*. *J Bacteriol* **183**, 3885–3889.

Withey, J. H. & Friedman, D. I. (2003). A salvage pathway for protein structures: tmRNA and *trans*-translation. *Annu Rev Microbiol* **57**, 101–123.

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