Cell, Vol. 104, 269–279, January 26, 2001, Copyright ©2001 by Cell Press

Replication Initiation Proteins Regulate a Developmental Checkpoint in *Bacillus subtilis*

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Summary

We identified a signaling pathway that prevents initiation of sporulation in *Bacillus subtilis* when replication initiation is impaired. We isolated mutations that allow a replication initiation mutant (*dnaA*) to sporulate. These mutations affect a small open reading frame, *sda*, that was overexpressed in replication initiation mutants and appears to be directly regulated by DnaA. Mutations in replication initiation genes inhibit the onset of sporulation by preventing activation of a transcription factor required for sporulation, Spo0A. Deletion of *sda* restored activation of Spo0A in replication initiation mutants. Overexpression of *sda* in otherwise wild-type cells inhibited activation of Spo0A and sporulation. Purified Sda inhibited a histidine kinase needed for activation of Spo0A. Our results indicate that control of *sda* by DnaA establishes a checkpoint that inhibits activation of Spo0A and prevents futile attempts to initiate sporulation.

Introduction

Many organisms have checkpoint mechanisms that block cell cycle progression or development in response to DNA damage or defects in replication. DNA-dependent checkpoints have been identified that regulate development in *Bacillus subtilis* (Ireton and Grossman, 1992; Ireton and Grossman, 1994; Ireton et al., 1994; Lemon et al., 2000). When starved, cells of *B. subtilis* can develop into endospores that are resistant to environmental stresses (reviewed in Hoch, 1995; Stragier and Losick, 1996; Burkholder and Grossman, 2000; Levin and Losick, 2000). *B. subtilis* initiates sporulation by altering its cell cycle. During growth, in the presence of adequate nutrients, cells elongate and divide symmetrically. During sporulation, in response to nutrient deprivation, cells divide asymmetrically into a large cell and a small cell joined at the division septum. The small cell subsequently develops into an endospore engulfed by the larger cell. Gene expression is required in both cell types for successful sporulation, and each cell type must receive an intact genome.

Mutations in factors involved in replication initiation or chromosome segregation inhibit sporulation, as does DNA damage (Mandelstam et al., 1971; Dunn et al., 1978; Ireton and Grossman, 1992; Ireton and Grossman, 1994; Ireton et al., 1994; Lemon et al., 2000). These conditions inhibit sporulation prior to asymmetric cell division, apparently by preventing activation of the transcriptional regulator Spo0A, or by directly inhibiting expression of Spo0A-dependent genes. Spo0A is required for sporulation-specific gene expression and is activated by phosphorylation via a multicomponent phosphorelay (Burbulys et al., 1991). Three histidine kinases, KinA, KinB, and KinC, autophosphorylate, then donate phosphate to Spo0F. Phosphate is then transferred from Spo0F to Spo0B and finally from Spo0B to Spo0A (reviewed in Hoch, 1995; Burkholder and Grossman, 2000). *kinA* and *kinB* are required to activate Spo0A during sporulation and are largely redundant, so that deletion of either alone causes a modest decrease in sporulation frequency (Perego et al., 1989; Antoniewski et al., 1990; Trach and Hoch, 1993; Kobayashi et al., 1995; LeDeaux and Grossman, 1995; LeDeaux et al., 1995).

We describe a signaling pathway that regulates activation of Spo0A in response to defects in the replication initiation machinery. Three genes are known to be required for replication initiation, but not elongation, in *B. subtilis*, *dnaA*, *dnaB*, and *dnaD* (Gross et al., 1968; Karamata and Gross, 1970; Ogasawara et al., 1986; Moriya et al., 1990; Bruand et al., 1995b). Temperature-sensitive mutations in these genes inhibit sporulation, apparently by preventing activation of Spo0A (Mandelstam et al., 1971; Ireton and Grossman, 1994; Lemon et al., 2000). *B. subtilis* DnaA, like *E. coli* DnaA, is an essential site-specific DNA binding protein required for replication initiation (reviewed in Yoshikawa and Wake, 1993; Messer and Weigel, 1996; Moriya et al., 1999). DnaA binds to sites in the origin and promotes assembly of the replisome. In *E. coli*, DnaA also positively and negatively regulates expression of some genes by binding to sites in their promoter regions (reviewed in Messer and Weigel, 1996; Messer and Weigel, 1997). The most extensively characterized example is the negative autoregulation of *dnaA*. *B. subtilis* DnaA also appears to negatively regulate its own expression, indicating that this role of DnaA is highly conserved (Moriya et al., 1999). The activity of DnaA is regulated by ATP binding and hydrolysis, and DnaA-ATP is the active form for replication initiation and the well-characterized examples of transcriptional regulation (Sekimizu et al., 1987; Speck et al., 1999).

The functions of *B. subtilis* DnaB and DnaD in replication initiation are less well characterized. (*B. subtilis* DnaB is not related to the DnaB helicase of *E. coli*; the *B. subtilis* replicative helicase is named DnaC.) Unlike the universally conserved DnaA protein, homologs of DnaB and DnaD have been found only in some gram-positive bacteria. DnaB plays a role in anchoring origin DNA to the membrane (Winston and Sueoka, 1980), and DnaB and DnaD are required for primosome function (Bruand et al., 1995a).

To begin to understand how DnaA regulates the initiation of sporulation and activation of Spo0A, we isolated and characterized suppressor mutations that allow a *dnaA* mutant to sporulate. The mutant allele, *dnaA1*, renders cells temperature sensitive for replication initia-

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Table 1. *sda* Mutations Suppress the Sporulation Defect of *dnaA1* Mutants

Strain	Relevant Genotype	Sporulation Frequency[a]
BB641	*dnaA*$^+$ *sda*$^+$	0.60
BB640	*dnaA1 sda*$^+$	1.2×10^{-4}
BB643	*dnaA1 sda1*	0.64
BB649	*dnaA1 sda3*	0.55
BB652	*dnaA1 sda4*	0.54
BB655	*dnaA1 sda5*	8.6×10^{-2}
BB751	*dnaA1* Δ*sda*	1.0
BB644	*dnaA*$^+$ *sda1*	0.46
BB650	*dnaA*$^+$ *sda3*	0.55
BB653	*dnaA*$^+$ *sda4*	0.85
BB656	*dnaA*$^+$ *sda5*	0.63
BB750	*dnaA*$^+$ Δ*sda*	0.84

[a] Cells were grown in DS medium at 30°C. Viable cells/ml ranged from 1.9×10^8 (BB751) to 9.7×10^8 (BB640).

tion and growth (Moriya et al., 1990). At permissive temperature, *dnaA1* mutants grow at wild-type rates but are still defective for sporulation; the mutants sporulate at frequencies as much as several thousand-fold lower than wild-type strains (S. Moriya, personal communication; Lemon et al., 2000).

Suppressor mutations that allow the *dnaA1* mutant to sporulate affected a small open reading frame that we have named *sda* (suppressor of *dnaA*). We found that the *sda* gene product is an inhibitor of sporulation and that purified Sda inhibits activity of the sporulation kinase KinA in vitro. Expression of *sda* was increased in *dnaA1* and other replication initiation mutants, and loss of function mutations in *sda* uncoupled initiation of sporulation from control by the replication initiation genes. The Sda signaling pathway provides a checkpoint that couples phosphorylation of Spo0A and initiation of sporulation to the function of replication initiation proteins.

Results

Isolation of Extragenic Suppressors that Allow *dnaA1* Mutants to Sporulate

At permissive temperatures, *dnaA1* mutants grow at wild-type rates (Moriya et al., 1990) but are unable to sporulate efficiently (Table 1) (Lemon et al., 2000). We isolated extragenic suppressor mutations that restored the ability of a *dnaA1* mutant to sporulate (Experimental Procedures). The *dnaA1* mutant was mutagenized with EMS and from ~10^5 colonies screened, we identified five independent mutants that were sporulation proficient (Spo$^+$) and still temperature sensitive for growth. DNA transformation and mapping experiments indicated that each mutant contained a single mutation, unlinked to *dnaA*, that suppressed or partially suppressed the *dnaA1* sporulation defect (Table 1). We named these mutations *sda* for suppressors of *dnaA1*.

Four of the *sda* mutations, *sda1*, *sda3*, *sda4*, and *sda5*, mapped to a single locus (Experimental Procedures). We cloned and sequenced the mutations and found that, according to the annotations for the genome sequence of *B. subtilis* (Kunst et al., 1997), the mutations were in the region between two genes of unknown function, *yqeF* and *yqeG* (Figure 1A). This first led us to suspect that the *sda* mutations affected the expression of the downstream *yqeG* operon. However, placing a wild-type copy of the *yqeF-yqeG* intergenic region (contained in plasmid pBB130) at a second site in the chromosome of a *dnaA1 sda1* strain was sufficient to complement the *sda1* phenotype (Figure 1B). That is, the complemented strain was Spo$^-$, like a *dnaA1* strain, instead of being Spo$^+$ like the *dnaA1 sda1* parent. The *sda4* and *sda5* mutant alleles were similarly complemented by the *yqeF-yqeG* intergenic region. This indicated that the intergenic region alone contained the functional locus targeted by the *sda* mutations and that the *sda* mutations were recessive, loss-of-function mutations. Additional complementation tests narrowed the locus targeted by the *sda* mutations to a 559 bp region immediately upstream of *yqeG* (Figure 1B).

To further define the locus targeted by the *sda* mutations, we tested the ability of fragments spanning the left and right halves of the 559 bp region to complement the *sda* mutations and found that they did not (Figure 1B). However, when the right half of the region was placed under control of the LacI-repressible-IPTG-inducible promoter, P_{spac}, the *sda* mutations were complemented in the presence of IPTG. These data indicate that the *sda* mutations impaired the expression or function of a gene lying in the right half of the region driven by a promoter lying in the left half.

sda Is a Small Open Reading Frame Encoding an Inhibitor of Sporulation

The right half of the 559 bp region contains many potential small open reading frames. *sda5* was predicted to introduce a missense mutation in two of three small open reading frames reading from left to right. One of these (*sda*) was preceded by a potential ribosome binding site optimally spaced from a nonstandard AUU start codon (Figure 1C). A second possible start codon, AUG, occurs later in the same reading frame. This frame is predicted to encode a 52 or 46 residue polypeptide, depending on the translation start site. This open reading frame had not been identified previously in the annotations to the genome sequence (Kunst et al., 1997).

We found that this open reading frame (*sda*) encodes a protein product that is a potent inhibitor of sporulation. We placed *sda* under the control of the promoter $P_{spac\text{-}hy}$ (J. Quisel and A. D. G., submitted), a stronger version of the LacI-repressible-IPTG-inducible promoter P_{spac}. The $P_{spac\text{-}hy}$-*sda* fusion was integrated into the *B. subtilis* chromosome at a heterologous site in an otherwise wild-type (*dnaA*$^+$) strain. Sporulation was potently inhibited when cells carrying $P_{spac\text{-}hy}$-*sda* were grown in the presence of IPTG, whereas cells carrying $P_{spac\text{-}hy}$ without an insert sporulated at wild-type frequencies (Table 2). In the absence of IPTG, basal expression of $P_{spac\text{-}hy}$-*sda* moderately inhibited sporulation (Table 2).

The ability of *sda* to inhibit sporulation was completely eliminated by a mutation in the predicted open reading frame that replaced the third codon from the AUG start with an ochre stop codon (Table 2). The ability of *sda*-(K3ochre) to inhibit sporulation was restored in a strain carrying the *sup-3* lysine-tRNA suppressor allele (Table 2). These results demonstrate that the *sda* open

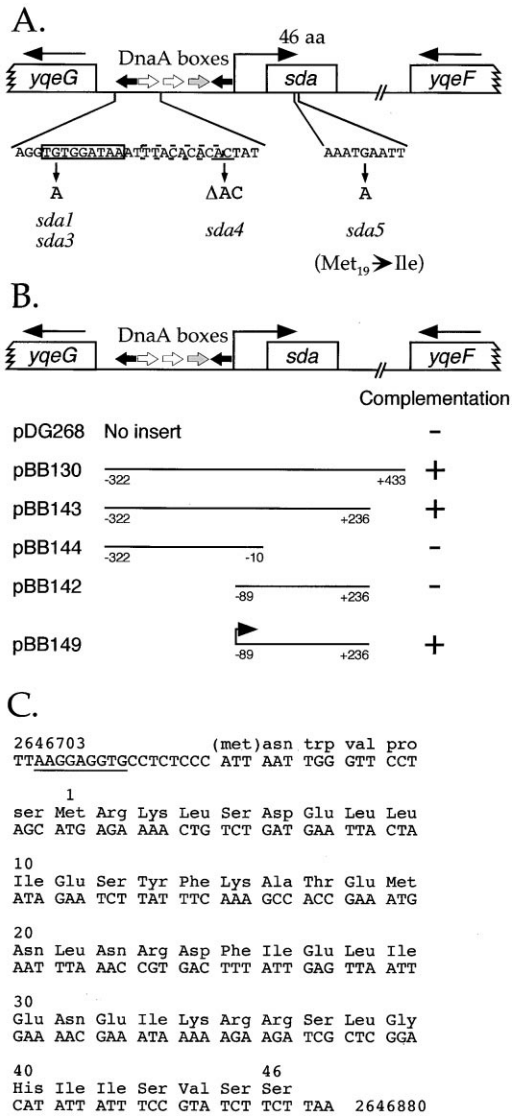

Figure 1. The *sda* Region of the Chromosome

(A) Map of the region between *yqeF* and *yqeG* (not drawn to scale). The transcription start site of *sda* is indicated by a vertical line with an arrow at the top and the direction of transcription of *yqeF* and *yqeG* is indicated by arrows above the genes. The thick black arrows represent sequences perfectly matching the DnaA binding site consensus sequence (5′-TTATCCACA-3′) (Fuller et al., 1984; Fukuoka et al., 1990), and the light gray and white arrows indicate sites differing from the consensus sequence by one or two mismatches, respectively. The DnaA binding sites overlapping the *sda1*, *sda3*, and *sda4* mutations are shown as solid and dashed boxes.

(B) Identifying the *sda* locus by complementation analysis. The indicated portions of the *yqeF-yqeG* intergenic region were integrated at the *amyE* locus by double crossover of the listed plasmids. The endpoints of the insertions are numbered relative to the A of the *sda* AUG start codon. pBB149 contains a LacI-repressible-IPTG-inducible promoter, P_{spac}, indicated by a bent arrow. *dnaA1 sda1* strains with the integrated inserts were tested for sporulation proficiency on DS medium plates at 37°C. Identical results were obtained with *dnaA1 sda3, dnaA1 sda4,* and *dnaA1 sda5* strains. IPTG (1 mM) was added to plates when screening strains transformed with plasmid pBB149, and complementation was dependent on the presence of IPTG.

(C) The *sda* open reading frame. Two translation start codons in the same reading frame are indicated, and a potential ribosome binding site is underlined. Residues of the Sda polypeptide are numbered relative to the second start codon. Both translation start codons are used when Sda is overexpressed with its own translation initiation signals in *E. coli*, though translation of the 46 residue form is favored. Both the 46 and 52 residue forms of Sda are active in vitro (see Experimental Procedures).

reading frame encodes an inhibitor of sporulation and that the protein product (and not just RNA) is necessary for function.

Deleting the entire *sda* open reading frame (Experimental Procedures) suppressed the sporulation defect of *dnaA1* mutants, like the previously isolated *sda* mutant alleles (Table 1), confirming that *sda* is responsible for inhibiting sporulation in *dnaA1* mutants. Δ*sda dnaA*$^+$ cells sporulated at higher frequencies than *sda*$^+$ *dnaA*$^+$ cells, indicating that expression of *sda* in wild-type cells has a small inhibitory effect on sporulation. The Δ*sda* mutation had no effect on the growth rates of otherwise wild-type cells in rich or minimal medium and did not render cells temperature sensitive for growth (data not shown). Thus *sda* is not required for growth of wild-type cells under any conditions tested.

sda Is Overexpressed in *dnaA1* Mutants

Since overexpressing *sda* from a heterologous promoter inhibited sporulation, we suspected that *sda* was overexpressed in *dnaA1* mutants. We compared expression of an *sda-lacZ* transcriptional fusion in *dnaA*$^+$ and *dnaA1* strains and found that *sda-lacZ* was overexpressed in *dnaA1* mutants, especially as cells entered stationary phase (Figure 2A). Primer extension analysis detected a single *sda* transcript with a 5′ end 102 bp upstream from the AUG start codon (Figure 1A). The 5′ end is appropriately spaced downstream from a likely sigma A-dependent promoter. Levels of the primer extension product in samples taken from *dnaA1* and *dnaA*$^+$ strains grown as in Figure 2A correlated with *sda-lacZ* expression (data not shown).

The suppressor mutations upstream of *sda*, *sda1* and *sda4*, reduced *sda-lacZ* expression to negligible levels in both wild-type and *dnaA1* mutant cells (Figure 2A and data not shown). The mutations are 123–142 bp upstream of the transcription start site, and might identify an upstream activating region. Analysis of the DNA sequence in this region identified two consensus DnaA binding sites and three sites that differ from the consensus sequence by one or two mismatches (Figure 1A). The two DnaA binding sites furthest from *sda* overlap the sites of the *sda1* and *sda4* mutations, and the gene-proximal DnaA binding site is 44 bp upstream of the transcription start site (Figure 1A).

Since a mutation in *dnaA* activates *sda* expression, and since the promoter region of *sda* contains consensus DnaA binding sites, it is likely that DnaA directly regulates transcription of *sda*. If DnaA activates *sda* expression, then *dnaA1* apparently causes increased activation, and the *sda1* and *sda4* mutations may reduce binding of DnaA to the *sda* regulatory region. If DnaA represses *sda* expression, then *dnaA1* probably reduces DnaA binding, and the *sda1* and *sda4* mutations affect an overlapping activating sequence.

Table 2. Translation of *sda* Is Required to Inhibit Sporulation

Strain[a]	$P_{spac\text{-}hy}$ Insert[b]	Presence of *sup-3*	IPTG	Sporulation Frequency[c]
BB535	None	−	−	0.38
BB537	*sda*$^+$	−	−	4.5×10^{-2}
BB537	*sda*$^+$	−	+	1.3×10^{-7}
BB612	None	+	+	0.19
BB613	None	−	+	5.2×10^{-2}
BB614	*sda*$^+$	+	+	7.5×10^{-7}
BB617	*sda*$^+$	−	+	6.5×10^{-8}
BB615	*sda*(K3ochre)	+	+	1.4×10^{-6}
BB616	*sda*(K3ochre)	−	+	0.19

[a] Strains BB612-617 carry the *metB5* allele, which is suppressed by *sup-3*. The *sup*$^+$ *metB5* strains BB613 and BB617 sporulate somewhat less efficiently than their isogenic *sup-3 metB5* (Met$^+$) counterparts BB612 and BB614 despite the presence of L-methionine in the medium.
[b] The $P_{spac\text{-}hy}$ promoter provides 10- to 20-fold higher basal and IPTG-induced expression levels than the P_{spac} promoter, from which it was derived, and is somewhat leaky in the absence of IPTG (J. Quisel and A. D. G., submitted).
[c] Cells were grown in DS medium at 30°C in the presence or absence of 1 mM IPTG. Viable cells/ml ranged from 3.3×10^8 (BB613) to 1.8×10^9 (BB537 + IPTG).

sda Is Overexpressed in *dnaB19* Mutants

The temperature-sensitive mutation *dnaB19* inhibits replication initiation and sporulation when cells are shifted to nonpermissive temperature (Karamata and Gross, 1970; Mandelstam et al., 1971; Ogasawara et al., 1986; Ireton and Grossman, 1994). We monitored expression of an *sda-lacZ* transcriptional fusion in *dnaB19* cells following a shift to nonpermissive temperature and induction of sporulation. *sda-lacZ* was overexpressed in the *dnaB19* strain compared to the wild-type control (Figure 2B). This pattern of *sda-lacZ* expression is similar to that seen in the *dnaA1* mutant (Figure 2A) as well as a *dnaD23* mutant (data not shown). *sda-lacZ* was also overexpressed in the *dnaB19* strain at permissive temperature, but the expression levels were lower than at nonpermissive temperature (Figure 2B).

The Replication Initiation Gene *dnaB* Controls Sporulation through *sda*

Inhibition of Spo0A activation in the *dnaB* mutant was due to overexpression of *sda*. We monitored Spo0A activation using a *lacZ* transcriptional fusion to a gene (*spoIIE*) whose expression requires activated Spo0A (Guzman et al., 1988; York et al., 1992). In wild-type cells, *spoIIE-lacZ* expression is induced shortly after the initiation of sporulation (Figure 2C). *spoIIE-lacZ* expression is strongly inhibited in *dnaB* mutants, consistent with a block in Spo0A activation (Ireton and Grossman, 1994). Deletion of *sda* restored *spoIIE-lacZ* expression, indicating that overexpressed Sda inhibits Spo0A activation in the *dnaB* mutant. Expression of *spoIIE-lacZ* in the *dnaB19* Δ*sda* strain is actually higher than in the wild-type strain, perhaps due to a failure to progress to the next stage of spore development, in which expression of *spoIIE* decreases.

Similar results were obtained when *spoIIE-lacZ* expression was monitored in *dnaD23 sda*$^+$ and *dnaD23* Δ*sda* mutants (data not shown), indicating that overexpressed Sda inhibits Spo0A activation in *dnaD* mutants, as in *dnaB* mutants.

Loss of the *sda* Checkpoint Reduces the Viability of Sporulating *dnaB19* Cells

Although deleting *sda* restored the ability of *dnaB19* mutants to activate Spo0A-dependent gene expression at nonpermissive temperature, it did not restore the ability of the mutants to sporulate. The *dnaB19* and the *dnaB19* Δ*sda* strains produced approximately 60–200-fold fewer spores than wild-type (Table 3, last column), indicating that *dnaB19* mutants are not only defective in activating Spo0A due to induction of Sda, but are also defective in a subsequent step of sporulation in the absence of *sda*.

The defect of *dnaB19* mutants at the subsequent step of spore development is apparently often lethal. The *dnaB19* Δ*sda* strain had 5–15-fold fewer viable cells compared to the *dnaB19* and *dnaB*$^+$ strains 20 hr after the initiation of sporulation (Table 3), despite having roughly equal numbers of viable cells (58%–73%) at the time spore development would usually begin (Table 3). Thus, production of Sda in *dnaB19* mutants appears to prevent cells from beginning spore morphogenesis under conditions in which they often cannot form mature spores successfully and may perish trying.

The inability of the *dnaB* mutants to sporulate in the absence of *sda* stands in marked contrast to the *dnaA* mutants, which can sporulate once the *sda*-dependent block to sporulation is removed. The sporulation defect of the *dnaB* mutants occurs at nonpermissive temperature, when replication initiation is inhibited. The inhibition of replication initiation may be directly responsible for the subsequent failure of *dnaB sda* double mutants to sporulate. Activation of the *sda* pathway in the *dnaB* mutants probably occurs as a result of the replication initiation defect and serves to protect cells from it. On the other hand, the sporulation defect of the *dnaA* mutants occurs at permissive growth temperature, when replication initiation is functioning at least adequately enough to sustain wild-type growth rates. Activation of the *sda* pathway in the *dnaA* mutants at permissive growth temperature probably reflects a defect in the DnaA-dependent regulation of *sda* expression, and perhaps a modest alteration in replication initiation.

Sda Inhibits the Signaling Pathway Activating Spo0A

Genetic data indicated that overexpressed Sda, like *dnaA1* and *dnaB* mutants (Ireton and Grossman, 1994; Lemon et al., 2000), inhibited sporulation by preventing activation (phosphorylation) of the transcription factor

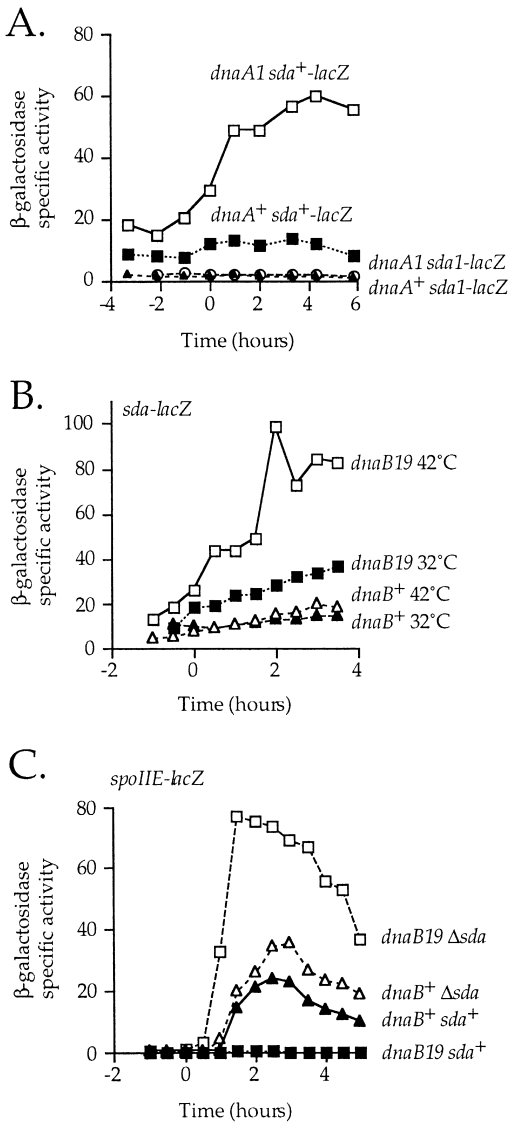

Figure 2. *sda* and Gene Expression

(A) *sda* is overexpressed in a *dnaA1* mutant, and the *sda1* mutation inhibits *sda* expression. Cells were grown in DS medium at 30°C. At the indicated times, samples were removed to assay β-galactosidase specific activity. Time zero is the end of exponential growth. Symbols: closed squares, *dnaA*$^+$ *amyE*::(*sda*$^+$-*lacZ*) (BB505); open squares, *dnaA1 amyE*::(*sda*$^+$-*lacZ*) (BB507); closed triangles, *dnaA*$^+$ *amyE*::(*sda1-lacZ*) (BB509); open circles, *dnaA1 amyE*::(*sda1-lacZ*) (BB511).

(B) *sda* is overexpressed in *dnaB19* mutants. Duplicate cultures were grown in defined minimal medium with required amino acids at 32°C. At an OD_{600} of 0.3–0.4, one culture from each pair was shifted to 42°C and the other was maintained at 32°C. After 1 hr, cells were induced to sporulate by the addition of mycophenolic acid (30 μg/ml). At the indicated times, samples were removed to assay β-galactosidase specific activity. Symbols: closed triangles, *dnaB*$^+$ *amyE*::(*sda-lacZ*) (BB625) at 32°C; open triangles, *dnaB*$^+$ *amyE*::(*sda-lacZ*) (BB625) at 42°C; closed squares, *dnaB19 amyE*::(*sda-lacZ*) (BB623) at 32°C; open squares, *dnaB19 amyE*::(*sda-lacZ*) (BB623) at 42°C.

(C) Deletion of *sda* restores Spo0A-dependent gene expression in *dnaB* mutants. Cells were grown as described in (B). Symbols: closed triangles, *dnaB*$^+$ *sda*$^+$ *amyE*::(*spoIIE-lacZ*) (BB854); open triangles, *dnaB*$^+$ Δ*sda amyE*::(*spoIIE-lacZ*) (BB860); closed squares, *dnaB19 sda*$^+$ *amyE*::(*spoIIE-lacZ*) (BB855); open squares, *dnaB19* Δ*sda amyE*::(*spoIIE-lacZ*) (BB862).

Spo0A. Spo0A is activated by a phosphotransfer pathway consisting of histidine kinases (KinA, B, and C) and the phosphotransfer proteins Spo0F and Spo0B (Burbulys et al., 1991). Normally, KinA or KinB is required to activate Spo0A during sporulation (Trach and Hoch, 1993; LeDeaux et al., 1995). A mutant allele of *spo0A*, *spo0A*^{rvtA11}, permits activation of Spo0A by the kinase KinC in the absence of KinA, KinB, Spo0F, or Spo0B (Kobayashi et al., 1995; LeDeaux and Grossman, 1995). When the KinA/KinB/Spo0F/Spo0B-dependent pathway is missing or inhibited, KinC is required for sporulation of *spo0A*^{rvtA11} mutants (Kobayashi et al., 1995; LeDeaux and Grossman, 1995). The sporulation defect caused by *dnaA1* is suppressed by *spo0A*^{rvtA11} in a KinC-dependent manner (Lemon et al., 2000). Similarly, we found that the inhibition of sporulation caused by overexpressing Sda was suppressed by *spo0A*^{rvtA11} and that this suppression was largely dependent on the presence of *kinC* (Table 4). These results indicate that Sda directly or indirectly inhibits the pathway required for phosphorylation of Spo0A.

Sda Inhibits the Histidine Kinase KinA

We found that Sda acts directly on one of the components of the phosphorylation pathway activating Spo0A. We purified functional tagged versions of Sda, KinA, and Spo0F (Experimental procedures). As shown previously (Perego et al., 1989; Burbulys et al., 1991), incubation of KinA and Spo0F with ATP resulted in the accumulation of Spo0F-phosphate (Spo0F∼P) (Figure 3A). Sda inhibited the accumulation of Spo0F∼P (Figure 3A).

Sda inhibited the accumulation of Spo0F∼P by inhibiting the activity of KinA. The autophosphorylation activity of KinA results in the accumulation of KinA∼P when KinA is incubated with ATP (Figure 3B, left panel) (Perego et al., 1989). Sda inhibited accumulation of KinA∼P (Figure 3B). In contrast, Sda was much less effective at inhibiting autophosphorylation of the cytoplasmic kinase domain of the closely related histidine kinase KinC (Figure 3B, right panel), indicating that the inhibition of KinA autophosphorylation was relatively specific.

Two lines of evidence indicate that Sda has at least one target in vivo in addition to KinA. First, overexpressing Sda in mutants lacking *kinA* potently inhibited sporulation (approximately six orders of magnitude) compared to a *kinA* strain lacking the Sda expression construct. Thus, *sda* does not require *kinA* to inhibit sporulation. Second, KinA is partially redundant with KinB under all growth conditions tested (Trach and Hoch, 1993; LeDeaux et al., 1995), so that inactivation of either kinase alone has only a small effect on sporulation frequencies. Since overexpressed Sda greatly inhibits sporulation (Table 2), it is likely that KinB is a second target inhibited by Sda. KinB is an integral membrane protein (Trach and Hoch, 1993), and it has been difficult to obtain functional preparations of the cytoplasmic kinase domain to test this possibility in vitro.

Though Sda inhibits KinA and likely inhibits KinB, it appears to have a much smaller effect on KinC in vivo. This conclusion is based on the finding that *spo0A*^{rvtA11} permits cells overexpressing Sda to sporulate at relatively high frequencies only if KinC is present (Table 4). Thus, KinC is not inhibited significantly by Sda. The small effect of overexpressed Sda on sporulation in *kinC*$^+$

Table 3. Deleting *sda* Does Not Restore the Ability of *dnaB* Mutants to Form Viable Spores

Strain[a]	Relevant Genotype	Viable cells/ml at t_0	Viable cells/ml at t_{20}	Spores/ml at t_{20}
BB854	*dnaB*$^+$ *sda*$^+$	3.8×10^8	4.1×10^8	4.2×10^7
BB855	*dnaB19 sda*$^+$	3.0×10^8	1.6×10^8	6.6×10^5
BB860	*dnaB*$^+$ Δ*sda*	4.0×10^8	2.7×10^8	2.7×10^7
BB862	*dnaB19* Δ*sda*	2.2×10^8	2.8×10^7	2.2×10^5

[a] Cells were grown in defined minimal medium as described in the legend to Figure 2B.
Sporulation was induced at 42°C by the addition of mycophenolic acid (30 μg/ml) at time zero (t_0). Viable cells and spores were assayed at the indicated times.

spo0A^{rvtA11} cells could be due to weak inhibition of KinC, consistent with the in vitro experiments in which Sda inhibited KinC autophosphorylation less efficiently than KinA autophosphorylation (Figure 3B).

Sda Is Conserved in Other *Bacillus* Species

Sda is highly conserved in the four closest relatives of *B. subtilis* to be sequenced to date, *Bacillus firmus*, *Bacillus halodurans*, *Bacillus stearothermophilus*, and *Bacillus anthracis* (Figure 4A), but is not found in other organisms. In contrast, homologs of *yqeG*, the gene upstream of *sda*, are found in most other gram-positive bacteria. The DnaA binding sites are found only in the four *Bacillus* species with *sda* (DNA sequence upstream of *sda* is not available in the fifth). The orientation and spacing of the DnaA boxes is highly conserved among the *Bacillus* species (Figure 4B), whereas the other noncoding sequences are not well conserved. These observations indicate that the DnaA boxes probably play an important conserved role in regulating *sda* expression.

Discussion

We have identified a small protein, Sda, that mediates a developmental checkpoint inhibiting sporulation in response to defects in the replication initiation machinery of *B. subtilis*. Sda is overexpressed in mutants of the replication initiation factors DnaA, DnaB, and DnaD. It is likely that DnaA directly regulates transcription of *sda*, given that the promoter region of *sda* contains several consensus and nearly consensus DnaA binding sites. The regulation of *sda* by DnaA establishes a checkpoint that prevents cells from inappropriately attempting to sporulate when replication initiation is impaired. We propose that defects in DnaB and DnaD, and possibly other conditions affecting replication initiation, alter the balance of active DnaA in the cell and thus regulate *sda* expression through DnaA. Sporulation in wild-type cells is regulated by cell cycle or replication cycle progression. That is, cells are proficient to initiate sporulation only during a limited window of the cell or replication cycle (Dawes and Mandelstam, 1970; Mandelstam and Higgs, 1974; Keynan et al., 1976; Hauser and Errington, 1995). We speculate that the DnaA-Sda pathway might be responsible for this regulation.

DnaA and Transcriptional Regulation

DnaA is ideally suited to regulate the Sda signaling pathway in response to the replication cycle and defects in replication initiation. The activity of DnaA, both in replication initiation and transcriptional regulation, is controlled by conditions related to replication cycle progression and chromosome copy number. DnaA binds and hydrolyzes ATP, and DnaA-ATP is the active form for replication initiation and the well-characterized examples of transcriptional regulation (Sekimizu et al., 1987; Speck et al., 1999). The ratio of DnaA-ATP to DnaA-ADP in the cell, and hence the fraction of active DnaA, is regulated in a cell cycle–dependent manner by ongoing replication (Kurokawa et al., 1999). This regulation is mediated in part by the β subunit sliding clamp of DNA polymerase III, which stimulates ATP hydrolysis by DnaA (Katayama et al., 1998). Chromosome copy number may also regulate DnaA activity by titrating levels of free DnaA in the cell as the concentration of DnaA binding sites varies (Hansen et al., 1991; Kitagawa et al., 1998; Roth and Messer, 1998; Christensen et al., 1999).

The simplest model for the function of DnaA in regulating *sda* expression is that DnaA-ATP is a repressor of *sda* transcription and that the *dnaA1*, *dnaB*, and *dnaD* mutations relieve, or partly relieve, repression. In this view, the *sda1* and *sda4* mutations could reduce expression of *sda* either by disrupting the promoter or a site for an activator. However, these mutations are 123 to 142 bp upstream from the 5′ end of the *sda* mRNA. If the 5′ end of the mRNA is a processed end and not the transcription start site, then these two *sda* mutations

Table 4. *spo0A*^{rvtA11} Bypasses the Sporulation Defect of Strains Overexpressing *sda*, and the Bypass Is Partially Dependent on *kinC*

Strain	$P_{spac\text{-}hy}$ Insert	Relevant Genotype	Sporulation Frequency[a]
BB573	None	*spo0A*$^+$	0.24
BB575	None	*spo0A*^{rvtA11}	0.14
BB811	None	*spo0A*^{rvtA11} *kinC*	0.43
BB576	*sda*$^+$	*spo0A*$^+$	5.2×10^{-7}
BB578	*sda*$^+$	*spo0A*^{rvtA11}	1.1×10^{-2}
BB813	*sda*$^+$	*spo0A*^{rvtA11} *kinC*	5.1×10^{-5}

[a] Cells were grown in 2 × SG medium at 30°C in the presence of 1 mM IPTG. Viable cells/ml ranged from 1.5×10^8 (BB575) to 2.1×10^9 (BB576).

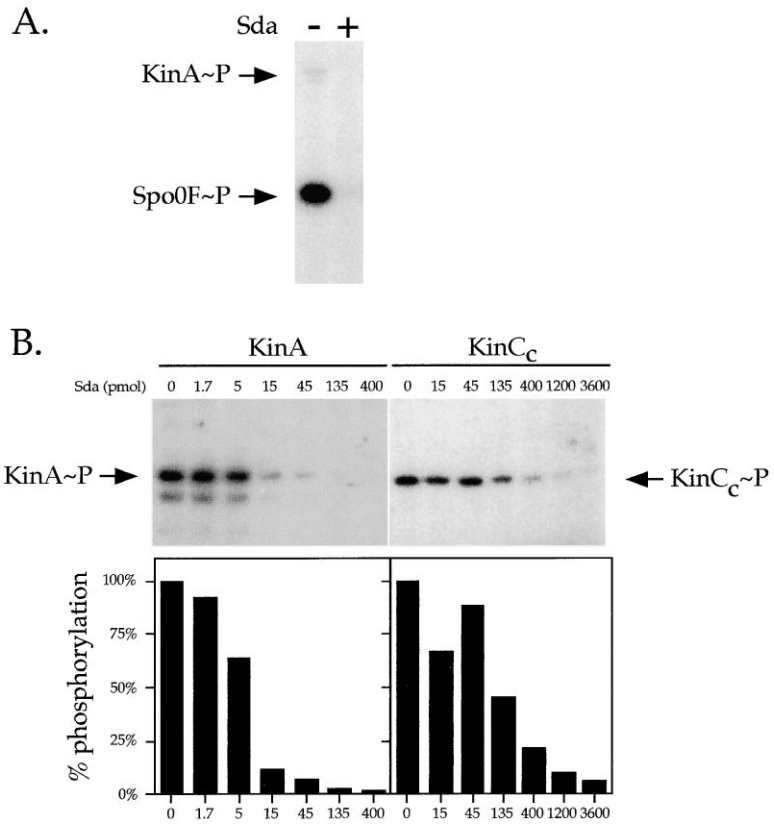

Figure 3. Sda Inhibits the Accumulation of Spo0F∼P and KinA∼P In Vitro

Kinase assays were performed as described in Experimental Procedures, and the accumulation of phosphorylated proteins was monitored by SDS-PAGE and autoradiography.

(A) Reactions (30 μl) contained 0.37 μM KinA-C-his$_6$ (11 pmol), 7.8 μM Spo0F-C-his$_6$, and 0.5 mM ^{32}P-gamma-ATP. Sda-C-his$_6$ (1.75 μM) was added as indicated. Reactions were incubated at 25°C for 30 min and then stopped by adding EDTA and placing on ice. Samples were electrophoresed on a 13.9% Tris-Tricine SDS polyacrylamide gel.

(B) Reactions (25 μl) contained 4 pmol KinA-C-his$_6$ (left panel) or 4 pmol GST-N-KinC$_c$ (the C-terminal cytoplasmic kinase domain of KinC fused to GST; right panel), the indicated amounts of Sda$_{46}$, and 0.5 mM ^{32}P-gamma-ATP. Reactions were incubated at 25°C for 7 min and then stopped by adding EDTA and placing on ice. Samples were electrophoresed on a 10% Tris-glycine SDS polyacrylamide gel. The relative levels of phosphorylated KinA and KinC$_c$ are shown in the bar graphs below the autoradiograms. The KinA-C-his$_6$ preparation contains degradation products that also autophosphorylate and are seen as lower molecular weight bands in the autoradiograms.

could be in the promoter. If the 5′ end of the mRNA actually represents the transcription start site and not a processed end, then it is unlikely that the mutations are in the promoter per se. Rather, they probably affect an as yet unidentified activator.

Alternatively, instead of functioning as a repressor, DnaA-ATP might be an activator of *sda* transcription. In this model, the *dnaA1*, *dnaB*, and *dnaD* mutations might cause an increase in the amount of DnaA bound at the sites in the *sda* promoter region, perhaps due to increased levels or reduced sequestration of DnaA at the origin. The *sda1* and *sda4* mutations would work by reducing or preventing binding of DnaA and thus decreasing expression of *sda*. Distinguishing between the two models, DnaA as a repressor versus DnaA as an activator of *sda* transcription, will provide new insights into the regulation of DnaA activity and its function in replication and transcription.

Inhibitors of Histidine Protein Kinases

Sda inhibits accumulation of the autophosphorylated form of the histidine protein kinase KinA, and probably also inhibits the activity of KinB. A third, closely related histidine kinase, KinC (48% identity between KinA and KinC; 34% identity between KinB and KinC), also activates Spo0A via the Spo0F-Spo0B phosphorelay, but Sda has relatively little effect on KinC. Determining the structural basis for this specificity may help to elucidate how specificity is achieved in histidine kinase signaling pathways.

Another inhibitor of KinA, KipI, has been described previously (Wang et al., 1997). Unlike Sda, KipI appears to act specifically on KinA without any effect on KinB. Overexpressing KipI in a wild-type strain inhibits sporulation to roughly the same extent as does deleting *kinA*, while overexpressing KipI in a *kinA* null mutant has no additional effect on sporulation frequency (Wang et al., 1997). In contrast, overexpressing Sda in a *kinA* null mutant still potently inhibits sporulation. The role of KipI in regulating sporulation is unclear. Other protein inhibitors of specific histidine kinases or related histidine phosphotransfer proteins have been identified, including PII, SixA, and FixT (Garnerone et al., 1999; and references therein). No sequence similarities are apparent between Sda, KipI, or any of the other inhibitors identified so far. Given the central role played by histidine kinases in regulating adaptive responses, there will probably be more examples of kinase inhibitors involved in checkpoint mechanisms.

Perspective

The DnaA-Sda signaling pathway is remarkable for the economy with which coordination is established between replication initiation and the onset of spore development. Such coordination seems to ensure that cells only try to initiate sporulation under conditions in which they are likely to be able to complete spore morphogenesis successfully. The continuing identification of other signaling pathways in bacteria that coordinate cell cycle–dependent events with growth and development will be important for understanding how organisms maximize their survival in changing environments.

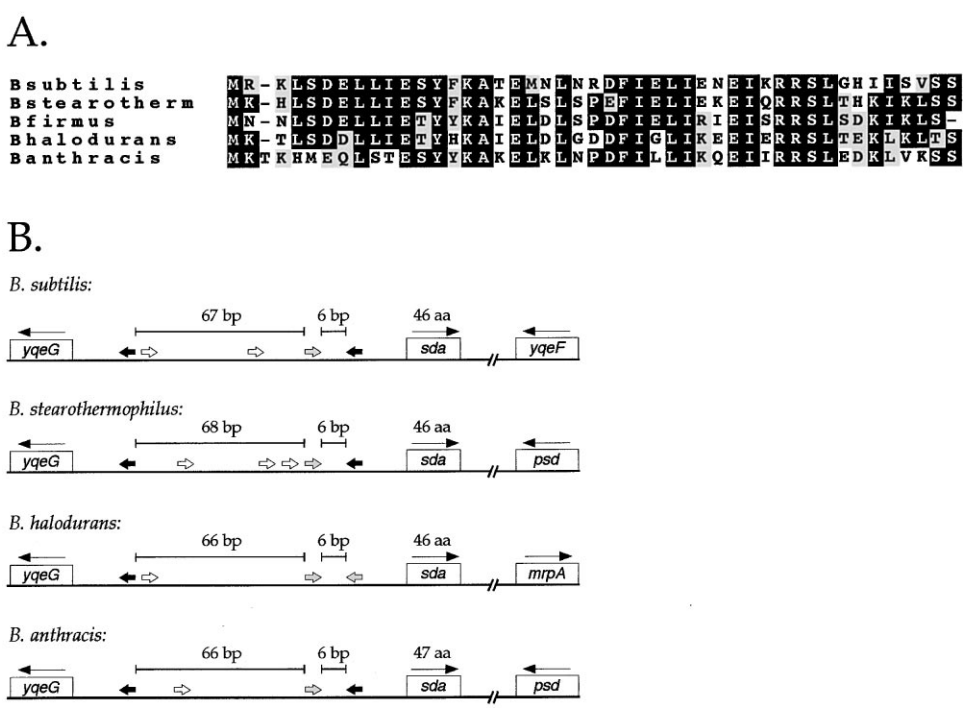

Figure 4. Conservation of Sda and the Upstream DnaA Binding Sites in Other *Bacillus* Species

(A) Alignment of Sda sequences from *B. subtilis* (the 46 residue form), *B. stearothermophilus* (70% identical to *B. subtilis* Sda), *B. firmus* (62% identical), *B. halodurans* (54% identical), and *B. anthracis* (51% identical). Two somewhat less well-conserved paralogs of *sda* are also found in *B. halodurans* (not shown), and they do not have DnaA binding sites in their upstream regions.

(B) Schematic representations of the *sda* loci. The arrows indicate the approximate location and orientation of predicted DnaA binding sites. The black arrows represent sequences perfectly matching the DnaA binding site consensus sequence (5′-TTATCCACA-3′), and the light gray and white arrows indicate sites differing from the consensus sequence by one or two mismatches, respectively. The regions are not drawn to scale. The functions of the *yqeF* and *yqeG* genes are unknown. The *psd* and *mrpA* genes are predicted to encode phosphatidyl serine decarboxylase and a multiple resistance Na^+/H^+ antiporter, respectively. Sequence data for the region upstream of *B. firmus sda* was not available.

Experimental Procedures

Media

Cells were grown in Difco nutrient broth sporulation (DS) medium, 2× SG medium (twice the nutrient broth of DS medium plus 0.1% glucose), S7-defined minimal medium (using 50 mM MOPS buffer instead of 100 mM), or Luria-Bertani (LB) medium (Miller, 1972; Harwood and Cutting, 1990), as indicated, supplemented with appropriate antibiotics, as needed. Defined minimal medium was supplemented with 1% glucose, 0.1% glutamate, and required amino acids (40 μg/ml). Sporulation was induced in defined minimal medium by the addition of mycophenolic acid (30 μg/ml).

Strains and Plasmids

The *B. subtilis* strains used are listed in Table 5. All the *sda* strains were made by crossing the originally isolated mutations into unmutagenized strain backgrounds. Standard techniques were used for strain constructions (Harwood and Cutting, 1990). Most of the mutant alleles and linked markers have been described previously (Karamata and Gross, 1970; Ogasawara et al., 1986; Antoniewski et al., 1990; Moriya et al., 1990; Garrity and Zahler, 1993; Ireton and Grossman, 1994; Bruand et al., 1995b; LeDeaux and Grossman, 1995; Lemon et al., 2000; and references therein).

DNA from the *sda* region of the chromosome used for complementation tests (Figure 1B) and *lacZ* fusions was amplified by PCR from genomic DNA, cloned into the *amyE* integration vector pDG268 (Antoniewski et al., 1990), and recombined into the chromosome at *amyE* by double crossover. Fragments in the *sda-lacZ* fusion plasmids pBB159, pBB160, and pBB161 extend from −322 to +56 relative to the A of the AUG start codon of *sda* and were amplified by PCR from genomic DNA from wild-type, *sda1*, and *sda4* strains, respectively.

For overexpression in *B. subtilis*, DNA fragments containing *sda* were cloned downstream from the promoters P_{spac} (in pDR67) or $P_{spac\text{-}hy}$ (in pPL82), and integrated by double crossover into *amyE*. pBB149 (P_{spac}-*sda*) contains the region from 89 bp upstream to 236 bp downstream of the A of the ATG start codon cloned into pDR67 (Ireton et al., 1993). pBB166 ($P_{spac\text{-}hy}$-*sda*$^+$) and pBB167 {$P_{spac\text{-}hy}$-*sda*(*K3ochre*)} contain the region −82 to +150, relative to the A of the ATG start codon, cloned into pPL82 (J. Quisel and A. D. G., submitted). The *sda*(*K3ochre*) mutation (AAA to TAA) was made using the Quikchange site-directed mutagenesis kit (Stratagene) and verified by DNA sequencing.

The Δ*sda* mutation replaces the entire *sda* coding region (−40 to +142 relative to the A of the AUG start codon) with an NcoI restriction site. The mutation was created by PCR amplifying a 2963 bp fragment upstream of *sda*, including the entire *aroD* gene, and a 1286 bp fragment downstream of *sda*, using oligonucleotides that introduced NcoI sites at the *sda*-proximal ends of both fragments and heterologous restriction sites at the *sda*-distal ends. The fragments were cloned into a plasmid by three-way ligation and the mutation was verified by PCR and restriction digest. The mutation was introduced by double crossover into the chromosome of an *aroD120* mutant (BB503), selecting for *aroD*$^+$ transformants and screening for the *sda* mutation by PCR. *sda* is ~95% linked to *aroD* by transformation.

The *spoIIE-lacZ* fusion (in pBB174) extends from −181 to +5 relative to the *spoIIE* transcription start site (York et al., 1992).

EMS Mutagenesis

Strain KPL2 (*dnaA1*) was grown in DS medium at 37°C. Cells in mid-exponential growth were resuspended in defined minimal medium and 0.6% methanesulfonic acid ethyl ester (EMS) was added. Cells were aliquoted into eight tubes, incubated with aeration for 30 min at 37°C, washed 3× with DS medium (cell survival was approximately 14%), and diluted into DS medium. After growth overnight, cells were plated on DS medium plates (~2600 colonies/plate, 5 plates/

Table 5. *B. subtilis* Strains Used

Strain	Genotype
JH642	*trpC2 pheA1* (Perego et al., 1988)
KPL2	JH642 *dnaA1*-Tn*917*ΩHU163 (*erm*)
BB505	JH642 *dnaA*$^+$-Tn*917*ΩHU163 (*erm*) *amyE*::(*sda-lacZ cat*)
BB507	JH642 *dnaA1*-Tn*917*ΩHU163 (*erm*) *amyE*::(*sda-lacZ cat*)
BB509	JH642 *dnaA*$^+$-Tn*917*ΩHU163 (*erm*) *amyE*::(*sda1-lacZ cat*)
BB511	JH642 *dnaA1*-Tn*917*ΩHU163 (*erm*) *amyE*::(*sda1-lacZ cat*)
BB513	JH642 *dnaA*$^+$-Tn*917*ΩHU163 (*erm*) *amyE*::(*sda4-lacZ cat*)
BB515	JH642 *dnaA1*-Tn*917*ΩHU163 (*erm*) *amyE*::(*sda4-lacZ cat*)
BB535	JH642 *dnaA*$^+$-Tn*917*ΩHU163 (*erm*) *amyE*::($P_{spac\text{-}hy}$ *cat*)
BB537	JH642 *dnaA*$^+$-Tn*917*ΩHU163 (*erm*) *amyE*::($P_{spac\text{-}hy}$-*sda*$^+$ *cat*)
BB573	JH642 *dnaA*$^+$-Tn*917*ΩHU163 (*erm*) *amyE*::($P_{spac\text{-}hy}$ *cat*) *spo0A*$^+$-*spc*
BB575	JH642 *dnaA*$^+$-Tn*917*ΩHU163 (*erm*) *amyE*::($P_{spac\text{-}ht}$ *cat*) *spo0A*^{rvtA11}-*spc*
BB576	JH642 *dnaA*$^+$-Tn*917*ΩHU163 (*erm*) *amyE*::($P_{spac\text{-}hy}$-*sda*$^+$ *cat*) *spo0A*$^+$-*spc*
BB578	JH642 *dnaA*$^+$-Tn*917*ΩHU163 (*erm*) *amyE*::($P_{spac\text{-}hy}$-*sda*$^+$ *cat*) *spo0A*^{rvtA11}-*spc*
BB612	*sup-3 metB5 amyE*::($P_{spac\text{-}hy}$ *cat*) (Met$^+$)
BB613	*sup*$^+$ *metB5 amyE*::($P_{spac\text{-}hy}$ *cat*)
BB614	*sup-3 metB5 amyE*::($P_{spac\text{-}hy}$-*sda*$^+$ *cat*) (Met$^+$)
BB615	*sup-3 metB5 amyE*::($P_{spac\text{-}hy}$-*sda*(K3ochre) *cat*) (Met$^+$)
BB616	*sup*$^+$ *metB5 amyE*::($P_{spac\text{-}hy}$-*sda*(K3ochre) *cat*)
BB617	*sup*$^+$ *metB5 amyE*::($P_{spac\text{-}hy}$-*sda*$^+$ *cat*)
BB623	JH642 *dnaB19-zhb-83*::Tn*917* (*erm*) *amyE*::(*sda-lacZ cat*)
BB625	JH642 *dnaB*$^+$-*zhb-83*::Tn*917* (*erm*) *amyE*::(*sda-lacZ cat*)
BB640	JH642 *dnaA1*-Tn*917*ΩHU163 (*erm*) *sda*$^+$
BB641	JH642 *dnaA*$^+$-Tn*917*ΩHU163 (*erm*) *sda*$^+$
BB643	JH642 *dnaA1*-Tn*917*ΩHU163 (*erm*) *sda1*
BB644	JH642 *dnaA*$^+$-Tn*917*ΩHU163 (*erm*) *sda1*
BB649	JH642 *dnaA1*-Tn*917*ΩHU163 (*erm*) *sda3*
BB650	JH642 *dnaA*$^+$-Tn*917*ΩHU163 (*erm*) *sda3*
BB652	JH642 *dnaA1*-Tn*917*ΩHU163 (*erm*) *sda4*
BB653	JH642 *dnaA*$^+$-Tn*917*ΩHU163 (*erm*) *sda4*
BB655	JH642 *dnaA1*-Tn*917*ΩHU163 (*erm*) *sda5*
BB656	JH642 *dnaA*$^+$-Tn*917*ΩHU163 (*erm*) *sda5*
BB750	JH642 *dnaA*$^+$-Tn*917*ΩHU163 (*erm*) Δ*sda*
BB751	JH642 *dnaA1*-Tn*917*ΩHU163 (*erm*) Δ*sda*
BB811	JH642 *dnaA*$^+$-Tn*917*ΩHU163 (*erm*) *amyE*::($P_{spac\text{-}hy}$ *cat*) *spo0A*^{rvtA11}-*spc* Δ*kinC*::*kan*
BB813	JH642 *dnaA*$^+$-Tn*917*ΩHU163 (*erm*) *amyE*::($P_{spac\text{-}hy}$-*sda*$^+$ *cat*) *spo0A*^{rvtA11}-*spc* Δ*kinC*::*kan*
BB845	JH642 *dnaD*$^+$-Tn*917*ΩHU151 (*erm*) *sda*$^+$ *amyE*::(*spoIIE-lacZ cat*)
BB846	JH642 *dnaD23*-Tn*917*ΩHU151 (*erm*) *sda*$^+$ *amyE*::(*spoIIE-lacZ cat*)
BB850	JH642 *dnaD*$^+$-Tn*917*ΩHU151 (*erm*) Δ*sda amyE*::(*spoIIE-lacZ cat*)
BB852	JH642 *dnaD23*$^+$-Tn*917*ΩHU151 (*erm*) Δ*sda amyE*::(*spoIIE-lacZ cat*)
BB854	JH642 *dnaB*$^+$-*zhb-83*::Tn*917* (*erm*) *sda*$^+$ *amyE*::(*spoIIE-lacZ cat*)
BB855	JH642 *dnaB19-zhb-83*::Tn*917* (*erm*) *sda*$^+$ *amyE*::(*spoIIE-lacZ cat*)
BB860	JH642 *dnaB*$^+$-*zhb-83*::Tn*917* (*erm*) Δ*sda amyE*::(*spoIIE-lacZ cat*)
BB862	JH642 *dnaB19-zhb-83*::Tn*917* (*erm*) Δ*sda amyE*::(*spoIIE-lacZ cat*)
BB874	JH642 *dnaD*$^+$-Tn*917*ΩHU151 (*erm*) *amyE*::(*sda-lacZ cat*)
BB876	JH642 *dnaD23*-Tn*917*ΩHU151 (*erm*) *amyE*::(*sda-lacZ cat*)
BB881	JH642 *dnaA*$^+$-Tn*917*ΩHU163 (*erm*) *amyE*::($P_{spac\text{-}hy}$-*sda*$^+$ *cat*) *kinA*::*spc*
BB886	JH642 *dnaA*$^+$-Tn*917*ΩHU163 (*erm*) *amyE*::($P_{spac\text{-}hy}$ *cat*) *kinA*::*spc*

pool; ~100,000 colonies screened altogether) and screened for Spo$^+$ colonies after two days of incubation at 37°C.

Mapping the *sda* Mutations

A chloramphenicol resistance marker linked to the *sda3* mutation was obtained as follows. Genomic DNA from a *dnaA1 sda3* strain (BB294) was partially digested with Sau3AI and cloned into the integration vector pGEMcat (Harwood and Cutting, 1990). The resulting genomic DNA library was integrated by single crossover into a *dnaA1* strain, selecting for chloramphenicol resistance (Cm^R) and screening for sporulation-proficient (Spo$^+$) transformants. Cm^R Spo$^+$ transformants were backcrossed to determine the linkage between the Cm^R and Spo$^+$ phenotypes. One strain (BB351) was isolated in which a *dnaA1* suppressor mutation was ~80% linked by transformation to the Cm^R marker. To confirm linkage of the Cm^R marker to the *sda* mutations, genomic DNA from BB351 was backcrossed into a *dnaA1* strain to obtain a Cm^R Spo$^-$ (Sda$^+$) transformant (BB374). BB374 genomic DNA was then used to transform the appropriate *dnaA1 sda* strains, selecting for Cm^R and determining the frequency of Sda$^+$ (Spo$^-$) transformants. *sda1*, *sda3*, *sda4*, and *sda5* were ~60%–80% linked to the Cm^R marker.

To determine the integration site of the Cm^R marker, genomic DNA from an integrated strain was digested with HinDIII, and clones of the recircularized plasmid were recovered in *E. coli* for sequencing. One arm of the original plasmid insert began in the *yqeK* gene and continued upstream. To further map the *sda* mutations, regions of the chromosome upstream of *yqeK* were amplified by PCR from an *sda*$^+$ strain and cloned into pGEMcat. The cloned fragments were integrated by single crossover into *dnaA1 sda* strains to test for reversion of the Sda$^-$ (Spo$^+$) phenotype. From this, *sda1, sda2, sda3,* and *sda5* were localized to the *yqeF-yqeG* intergenic region and plasmids carrying the mutations were sequenced, together with a clone of the wild-type region.

Sporulation and β-Galactosidase Assays

Sporulation frequencies were determined as the ratio of heat resistant (80°C for 20 min) colony forming units to total colony forming units. Assays were done ~20 hr after the end of exponential growth unless otherwise noted. β-galactosidase specific activity ({ΔA_{420} per min per ml of culture per OD_{600}} × 1000) was determined as described (Miller, 1972) after pelleting cell debris.

Primer Extension

Strains BB640 (*dnaA1*) and BB641 (*dnaA*$^+$) were grown as described in Figure 2A. RNA was isolated using RNeasy Maxi-columns (Qiagen). Primer extensions were performed essentially as described (Ausubel et al., 1990) using 50 μg RNA/reaction and primers with 3′ ends at positions +46, −44, and, −140 relative to the A of the *sda* AUG start codon.

Protein Purifications

Full-length KinA, Spo0F, and Sda were expressed as C-terminal his_6 fusion proteins in *E. coli* from plasmids pBB173, pGK10, and pBB179, respectively, and affinity purified on Ni^{2+}-NTA resin according to the manufacturer's protocol (Qiagen).

The C-terminal cytoplasmic kinase domain of KinC ("$KinC_c$"; codons 60 to 428 out of 428 total) was expressed as an N-terminal GST fusion protein in *E. coli* from plasmid pJQ32 and purified from inclusion bodies. Material was solubilized in 8 M urea and refolded by stepwise dialysis against 20 mM Tris-Cl, pH 8.0, 1 mM EDTA, 10 mM KCl, 1 mM DTT. Following dialysis, NaCl (150 mM) and DTT (5 mM) were added and GST-$KinC_c$ was purified using glutathione Sepharose 4B (Pharmacia) according to the manufacturer's protocol, eluting at room temperature and supplementing the PBS-based wash and elution buffers with 0.1% Triton.

Sda used in Figure 3A was expressed as a C-terminal his_6 fusion protein in *E. coli* from plasmid pBB179 (the sequence fused to the C terminus of Sda was GGRRASVLEHHHHHH, which includes a heart muscle kinase phosphorylation site; the fusion protein is active in vivo). The purification yielded predominantly the 46 residue form, but also low levels of the 52 residue form of Sda (both fused to his_6) as determined by N-terminal sequencing and mass spectrometry. Sda used in Figure 3B was a gift from S. Rowland and G. King (University of Connecticut Health Science Center, Farmington, CT). It was expressed as an N-terminal GST fusion protein, affinity purified on glutathione sepharose, cleaved with thrombin at the fusion junction, and purified to homogeneity, yielding the 46 residue form of Sda with the dipeptide Gly–Ser fused to the N terminus (Sda_{46}). There appears to be no functional difference between the 46 and 52 residue forms of Sda. Homogeneous preparations of each (prepared from N-terminal GST fusions) inhibited KinA in vitro with equal apparent affinities, as well as exhibiting equal lower affinities for KinC (W. F. B., S. Rowland, G. King, and A. D. G. unpublished). Based on the expression results in *E. coli* and the sequence alignments in Figure 4, the 46 residue form of Sda is likely the predominant form expressed in *B. subtilis*.

Protein concentrations were determined by A_{280} or by Bradford assay (Bio-Rad) using BSA as a standard.

Kinase Assays

Kinase assays were performed essentially as described (Zapf et al., 1998) in 50 mM N-(2-hydroxyethyl)piperazine-N′-(3-propanesulfonic acid) (EPPS), pH 8.5, 50 mM KCl, 20 mM $MgCl_2$, 5% glycerol with 0.5 mM ATP (25 μCi gamma-^{32}P-ATP/reaction) in 25 or 30 μl at 25°C. Reactions were stopped by adding EDTA (100 mM, pH 8) and placing on ice. Samples were heated for 10 min at 37°C in SDS-loading buffer and run on SDS-polyacrylamide gels. Following electrophoresis, gels were washed 3× for a total of 30 min in 20 mM Tris-Cl, pH 8, 1 mM EDTA, 20% methanol, exposed for ~12 hr on a Phosphorimager plate (Molecular Dynamics), and quantified using ImageQuant software (Molecular Dynamics). Gels were then stained with Coomassie Blue to verify loadings.

Sequence Analysis

Preliminary genome sequence data was searched at the NCBI website (http://www.ncbi.nlm.nih.gov/). The *Bacillus stearothermophilus yqeG* and *sda* genes were found in two separate contigs in the latest release of the genome sequence searched (searched Aug 11, 2000 at NCBI). The contig sequence data and the proposed assembled sequence used in our analysis is available upon request.

Acknowledgments

We thank J. Mendoza for preliminary primer extension results, S. Rowland and G. King for kindly providing purified Sda, J. Quisel and P. Levin for plasmids pJQ32 and pPL82, and S. Zahler and the Bacillus Genetic Stock Center for the *sup-3 metB5* strain CU1962. We also thank K. Schneider, R. Britton, and J. Lindow for comments on the manuscript and members of the Grossman lab for many helpful discussions.

The unpublished genome sequence data referred to in this work was made available to the public by sequencing projects funded by the Office of Naval Research, the National Institute of Allergy and Infectious Diseases, the U. S. Department of Energy, the National Science Foundation, and the Beowulf Genomics Initiative of the Wellcome Trust.

This work was supported in part by Public Health Service grant GM41934 and W. F. B. was supported in part by a postdoctoral fellowship from the American Cancer Society.

Received October 5, 2000; revised December 8, 2000.

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