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Different Roles for KinA, KinB, and KinC in the Initiation of Sporulation in *Bacillus subtilis*

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Activation (phosphorylation) of the transcription factor encoded by *spo0A* is essential for the initiation of sporulation in *Bacillus subtilis*. At least three histidine protein kinases are involved in the phosphorylation of Spo0A. Under some growth conditions, KinA was the primary kinase, but under other conditions, KinB had the more critical role. KinC was required for the initial activation of Spo0A, even in the presence of KinA and KinB.

Under appropriate conditions, cells of the gram-positive soil bacterium *Bacillus subtilis* differentiate to form dormant heat-resistant endospores. One of the key factors determining whether cells initiate sporulation is the accumulation of the active phosphorylated form of the transcription factor encoded by *spo0A* (reviewed in reference 7). Spo0A belongs to the response regulator family of transcription factors (1, 20) which usually receive phosphate on an aspartate residue from histidine protein kinases known as sensor kinases (1, 20). The histidine protein kinases autophosphorylate on a histidine residue and then serve as a phosphate donor to a cognate response regulator. Unlike most response regulators, Spo0A does not receive phosphate directly from a histidine protein kinase. Rather, phosphorylation of Spo0A requires the transfer of phosphate through the phosphorelay (5). The sporulation kinases first serve as phosphate donors to the response regulator encoded by *spo0F*. Phosphate is then transferred from Spo0F to Spo0B and finally to Spo0A (5, 7). The physiological function of the phosphorelay seems to be to integrate the many signals that regulate the initiation of sporulation (8, 10–13) and to generate a threshold concentration of Spo0A~P necessary for the initiation of sporulation (6).

Spo0A~P is a transcriptional activator and repressor, depending on the location of the target binding site. Spo0A~P represses transcription of *abrB* (22, 27) and activates transcription of several sporulation genes, including *spoIIA* (28), *spoIIE* (32), and *spoIIG* (3, 4). The *abrB* gene product is a transcriptional repressor, and repression of *abrB* by Spo0A~P causes activation of genes that are normally repressed by AbrB (31, 34). Less Spo0A~P is needed for repression of *abrB* than for activation of the *spoII* genes, since several mutations that decrease activation of Spo0A have little or no effect on *abrB* expression while causing a decrease in expression of *spoII* genes (6, 19, 21, 26, 29).

Three histidine protein kinases are involved in the initiation of sporulation: KinA (2, 21), KinB (29), and KinC (15, 16). Under most sporulation conditions tested, *kinA* null mutations cause a small reduction in the sporulation frequency while null mutations in either *kinB* or *kinC* cause little or no decrease in sporulation frequency (15, 16, 29). The experiments described

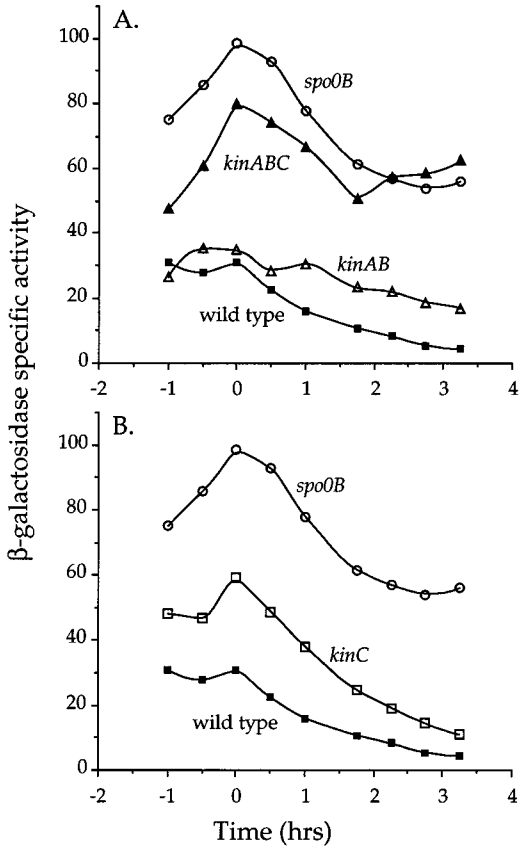

FIG. 1. *abrB-lacZ* expression in different kinase mutants in DS medium. The time of exit from exponential growth is defined as time zero (T_0). The *abrB-lacZ* fusion used is a transcriptional fusion located in the chromosome at the *amyE* locus and was a gift from Mark Strauch (22, 27). The *cat* marker associated with the fusion was converted to *cat*::*neo* (Cm^s Neo^r) by using pIK105 as described (9). (A) Wild type, JRL1018; *kinAB*, JRL1036; *kinABC*, JRL1039; *spo0B*, JRL1058. (B) Wild type, JRL1018; *kinC*, JRL1035; *spo0B*, JRL1058. The data in panels A and B are from the same experiment, and the datum points for the wild type and *spo0B* are the same. Similar effects of the *kinC* mutation on expression of *abrB* were observed in 2× SG medium (data not shown). Strains containing the *abrB-lacZ* fusion were made by transforming the kinase mutants described in Table 1 with chromosomal DNA containing the fusion *amyE*::(*abrB-lacZ cat*::*neo*). The *spo0B* allele was *spo0BΔPst* (31) and was introduced into JH642 by cotransformation with *phe*$^+$ (16).

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TABLE 1. Relative sporulation frequencies of different kinase mutants in different media

Strain	Relevant genotype[a]	Relative sporulation frequency[b] in[c]:		
		2×SG medium	DS medium	Minimal medium
JH642	Wild type	1 (3.9×10^8)	1 (1.9×10^8)	1 (1.3×10^8)
AG522	*kinA*	0.1 (6.3×10^7)	8.0×10^{-2} (1.9×10^7)	1.2 (1.1×10^8)
NY120	*kinB*	0.67 (2.3×10^8)	0.13 (2.7×10^7)	7.1×10^{-2} (6.0×10^6)
JRL920	*kinC*	0.77 (4.1×10^8)	0.45 (1.0×10^8)	0.91 (1.3×10^8)
NY121	*kinA kinB*	1.9×10^{-6} (1.7×10^3)	$<5 \times 10^{-8}$ (<10)	2.1×10^{-7} (20)
JRL1046	*kinA kinC*	3.3×10^{-2} (2.3×10^7)	6.8×10^{-2} (1.5×10^7)	0.41 (7.3×10^7)
JRL1004	*kinB kinC*	0.43 (3.8×10^8)	0.15 (4.5×10^7)	1.2×10^{-2} (2.0×10^6)
JRL1007	*kinA kinB kinC*	$<3 \times 10^{-8}$ (<10)	$<2 \times 10^{-7}$ (<10)	3×10^{-7} (60)

[a] The JH642 strain is *trpC2 pheA1* (22), and all strains used were derived from JH642. The *kinA* allele is *kinA*::Tn*917* (2, 24). The *kinB* allele is a deletion-insertion mutation that contains the spectinomycin resistance (*spc*) cassette from pJL73 (16) inserted between the *Sac*II site at codon 232 (of 428) of *kinB* and the *Bss*HII site downstream of *kapB*, the gene downstream of *kinB* (29). Our clone of the *kinB* operon was isolated in two steps, on the basis of the published sequence (29). First, a small fragment from the 5′ end of the operon was amplified by PCR and cloned into the integrative vector pGEM::*cat* (33) to generate pNY13. pNY13 was recombined into the chromosome of wild-type cells, and chromosomal DNA was used to clone the entire operon to generate pNY113. This plasmid contains the entire *kinB* operon from 77 bp upstream of the transcriptional start site (29) to the *Bam*HI site downstream of *kapB*. The *spc* cassette was then cloned between the *Sac*II site in *kinB* and the *Bss*HII site downstream of *kapB*, deleting part of *kinB* and all of *kapB*, to yield pNY118. The Δ*kinBkapB*::*spc* mutation was recombined into the chromosome by selecting for Spc^r and checking for Cm^s (loss of the pGEM*cat* vector) to be sure that there was a double crossover. The *kinC* allele is *kinC*::pLK124, a disruption caused by integrating a plasmid containing an internal fragment of *kinC* (16).

[b] Relative sporulation frequency is the number of spores per milliliter as a fraction of the number of viable cells per milliliter, normalized to the control (JH642) in a given experiment. Samples to be tested were serially diluted in minimal salts, and the number of spores was measured as heat-resistant (80°C for 15 min) CFU on Luria-Bertani plates. Viable cells were measured, before heat treatment, as total CFU on Luria-Bertani plates. The numbers in parentheses are heat-resistant spores per milliliter. Data for each medium are from a representative experiment, and similar results were obtained for at least three independent experiments.

[c] 2×SG is a rich sporulation medium and contains nutrient broth and 0.1% glucose (17). DS is the nutrient broth medium of Schaeffer et al. (25). The minimal medium was S7 medium (30) as used previously (14), except that glucose was used at 0.1%.

below indicate that under some sporulation conditions *kinB* mutations cause a more severe sporulation defect than do *kinA* mutations, indicating that although they are partly redundant, the requirements for KinA or KinB depend on the sporulation medium. A *kinC* null mutation was found to affect regulation of *abrB*, both in otherwise wild-type cells and in the *kinA kinB* double mutant. In addition, *kinC* was found to be required for the low frequency of sporulation seen under some conditions for the *kinA kinB* double mutant.

Phenotypes caused by *kinA* or *kinB* null mutations depend on the sporulation medium. KinA appears to be the major kinase under most sporulation conditions. *kinA* null mutants sporulated at a frequency of approximately 4 to 10% that of the wild type when grown in DS medium (nutrient broth) (25) or the richer 2×SG medium (twice the nutrient broth as DS medium plus 0.1% glucose) (Table 1), consistent with published data (2, 18, 21, 23, 24). However, when sporulation was induced by the exhaustion of glucose from cultures grown in defined minimal medium with glucose (0.1%) as the carbon source, the *kinA* mutant was able to sporulate at or near the wild-type frequencies (Table 1). In contrast, the sporulation frequency of the *kinB* mutant was similar to that of the wild type in 2×SG medium and approximately 5 to 10% that of the wild type in DS medium or minimal medium (Table 1). The phenotype of *kinB* in DS medium was somewhat variable and seemed to depend on the specific preparation of DS medium; this is consistent with effects reported by others (29). On the other hand, the sporulation defect of the *kinB* mutant in minimal glucose medium was highly reproducible but was observed only if the culture had undergone at least four to five doublings after inoculation and before entry into stationary phase. The requirement for a certain number of cell divisions before observing the Spo$^-$ phenotype of the *kinB* mutant suggests that an activator of sporulation had accumulated in the preculture that was used as the inoculum and that this factor must be diluted below a certain level in order for the *kinB* phenotype to be observed. This factor could be a metabolite or a gene product that could substitute for KinB, perhaps another kinase. Taken together, our results indicate that under some conditions KinB is the major sporulation kinase and KinA plays a minor role and that the different sporulation sensor kinases can respond to different nutritional conditions. These effects could reflect differences in the expression of *kinA* and *kinB* or in the regulation of kinase activity, or both.

KinC is required for the residual sporulation seen in a *kinA kinB* double mutant in the rich sporulation medium (2×SG). *kinA kinB* double mutants had a much more severe sporulation defect than did either single mutant under all conditions tested (Table 1), consistent with previous findings (29). In 2×SG medium the *kinA kinB* double mutant consistently produced approximately 10^3 spores per ml, at least 100- to 1,000-fold more than in DS medium or minimal medium (Table 1). This small but reproducible level of sporulation in 2×SG medium was entirely dependent on *kinC* as the *kinA kinB kinC* triple mutant produced <10 spores per ml in 2×SG medium (Table 1).

KinC is required for normal regulation of *abrB* expression. Transcription of the stationary-phase regulatory gene *abrB* is repressed by Spo0A~P (22, 27), but a *kinA kinB* double mutant has little or no effect on the expression of an *abrB-lacZ* fusion (29). To see if KinC is involved in the expression of *abrB*, we measured the expression of an *abrB-lacZ* fusion in strains containing different combinations of *kinA*, *kinB*, and *kinC* mutations.

KinC was responsible for most of the regulation of *abrB-lacZ* via the phosphorelay in the *kinA kinB* double mutant. Expression of *abrB* in the *kinA kinB* double mutant in DS medium was similar to that in the wild type, as previously reported (29), although the amount of β-galactosidase at times after entry into stationary phase (T_0) was reproducibly ~2-fold higher in the double mutant (Fig. 1A). The amount of β-galactosidase accumulated before entry into stationary phase in the double mutant was less than that in a *spo0B* mutant (Fig. 1), indicating that there must be at least one other source of phosphate and that phosphate must be transferred to Spo0A via Spo0B and the phosphorelay (29). KinC was a major source of that phosphate in the absence of KinA and KinB, as expression of *abrB* was higher in the *kinA kinB kinC* triple mutant

than in the *kinA kinB* double mutant (Fig. 1A). The initial accumulation of β-galactosidase in the triple mutant was somewhat lower than that in the *spo0B* mutant, suggesting that there might be yet another minor source of phosphate for Spo0A.

A *kinC* mutation also caused increased expression of *abrB* in otherwise wild-type (*kinA*$^+$ *kinB*$^+$) cells. Expression of *abrB* was reproducibly higher in the *kinC* mutant before and at the time of entry into stationary phase (Fig. 1B). Shortly after entry into stationary phase, β-galactosidase specific activity began to decrease; this was similar to the decrease seen for wild-type cells. This substantive decrease was due to KinA and KinB, as there was much less of a decrease in the kinase triple mutant (Fig. 1A).

Our results on the effects of kinase mutations on *abrB* expression indicate that different kinases that contribute to the phosphorelay and the production of Spo0A~P may be active at different times during the growth and stationary phases. The contribution of KinC seems to be greater before entry into stationary phase, while KinA and KinB appear to contribute more after entry into stationary phase. In addition, it appears that the type of medium also affects which kinase is more important for the developmental process. Both the growth stage and medium effects could be at the level of expression or activity of the kinases, or both, but there is not yet enough information available concerning the regulation of expression or activity of the kinases to distinguish these possibilities.

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