Negative regulation of *Bacillus anthracis* sporulation by the Spo0E family of phosphatases

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ABSTRACT

The initiation of sporulation in *Bacillus* species is controlled by the phosphorelay signal transduction system. Multiple regulatory elements act on the phosphorelay to modulate the level of protein phosphorylation in response to cellular, environmental and metabolic signals. In *Bacillus anthracis* nine possible histidine sensor kinases can positively activate the system while two response regulator aspartyl phosphate phosphatases of the Rap family negatively impact the pathway by dephosphorylating the Spo0F intermediate response regulator. In this study, we have characterized the *B. anthracis* members of the Spo0E family of phosphatases that specifically dephosphorylate the Spo0A response regulator of the phosphorelay and master regulator of sporulation. The products of four genes were found to have potential to impact on the *B. anthracis* decision whether or not to sporulate by dephosphorylating Spo0A~P *in vivo* and *in vitro*. We propose that these proteins may contribute to maintain *B. anthracis* in the transition phase of growth during an active infection, therefore contributing to the virulence of this organism.

INTRODUCTION

The *Bacillus anthracis* exotoxins play a central role in the pathogenesis of this organism. The protective antigen (PA), in association with either lethal factor (LF) or edema factor (EF) can cause lethality and edema, respectively, to the infected hosts (3,5,23,24,43). Lately it has become evident that the production of these toxins is under control of the pathway regulating sporulation initiation (37). In fact, AtxA, an essential activator of toxin genes transcription, is negatively regulated by the transition phase regulator AbrB whose transcription is, in turn, regulated by the Spo0A response regulator. Spo0A is the master regulator of sporulation initiation and the final acceptor of activating phosphoryl groups in the phosphorelay signal transduction pathway that regulates the initiation of the developmental process, as it has been well characterized in *B. subtilis* (13,35). In this organism, five histidine sensor kinases may provide the activating phosphoryl group inputs into the system by autophosphorylating on a histidine residue, in response to signals, which is followed by transfer of the phosphoryl group to the intermediate response regulator Spo0F (15,16,18). From Spo0F the phosphoryl group is transferred to Spo0A through the Spo0B phosphotransferase (7). Once activated by phosphorylation, Spo0A promotes directly or indirectly the transcription of a myriad of genes required for sporulation while it represses genes whose products are not necessary during development (3,4,25,41). Negative inputs into the phosphorelay are provided by aspartyl phosphate phosphatases that specifically dephosphorylate the Spo0F response regulator (Rap proteins) or the Spo0A transcription factor (Spo0E proteins) (30,31). The Spo0F, Spo0B and Spo0A proteins of *B. subtilis* are essentially identical to their counterparts of

B. anthracis indicating that the phosphorelay pathway is functionally identical in controlling sporulation initiation in the two organisms (39).

Recently, we have identified nine genes on the *B. anthracis* genome, whose products may function as sporulation histidine sensor kinases (6). Furthermore, we have characterized two of the six Rap proteins of *B. anthracis* as aspartyl phosphate phosphatases of Spo0F with a possible role in the pathogenesis of this organism (5). Here we report the characterization of the Spo0E family of proteins and their role in negatively regulating the sporulation pathway of *B. anthracis*.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The *B. anthracis* Sterne strain 34F2 ($pXO1^+$, $pXO2^-$) was used in this study and transformation was carried out by the electroporation method described in Koehler et al (17). The *B. subtilis* JH642 (*trpC2*, *phe-1*) strain used in this study was transformed by the method of Anagnostopoulos and Spizizen (1).

Strains were grown in Schaeffer's sporulation medium (SM) (38) with antibiotics at the following concentrations: erythromycin 5μg/ml, lincomycin 25μg/ml, kanamycin 7.5μg/ml for *B. anthracis* and 2μg/ml for *B. subtilis.*

E. coli DH5α was used for plasmid construction and propagation. Cells were grown in LB medium supplemented with ampicillin (100μg/ml) or kanamycin (30μg/ml). *E. coli* SCS110 (Stratagene) was used as a source of unmethylated plasmid DNA.

Plasmid construction

The vectors used in this study were: pHT315 (2), a multicopy shuttle vector replicating in *E. coli*, *B. subtilis* and *B. anthracis*; pJM115 (28), a transcriptional *lacZ* fusion vector conferring kanamycin resistance and integrating by double crossover at the *amyE* locus of the *B. subtilis* chromosome; pTCVlac {Poyart, 1997 6478 /id}, a transcriptional *lacZ* fusion vector that replicates in *E. coli* and *B. anthracis* and confers resistance to kanamycin.

The BA1877, BA0046, BA2416 and BAyisI coding sequences and their promoter regions (approximately 200-300bp upstream the start site of translation) were amplified by PCR reaction from chromosomal DNA of strain 34F2 using the oligonucleotide pairs shown in Table 1 of the Supplemental Material. The 5' oligonucleotide introduced either an EcoRI site or a KpnI site while the 3' primer introduced a BamHI site. The fragments obtained were cloned in pHT315 digested with KpnI and BamHI or EcoRI and BamHI.

The *lacZ*-transcriptional fusion constructs were generated in pJM115 and pTCVlac as follows. The BA0046 promoter region was amplified with oligonucleotide primers BA00465'Kpn and BA00463'Bam2 (Table 1S), the fragment was digested with BamHI and cloned in the vectors digested with SmaI and BamHI. The BA1877 promoter was amplified with oligonucleotide primers BA18775'EcoRI and BA18773'Bam2 and cloned in EcoRI-BamHI digested vectors. The YisI fragment obtained with oligonucleotide primers BAYisI5'Eco and BAYisI3'Bam was digested with EcoRI and XmnI (this restriction site naturally occurring within the gene) and the fragment cloned in the vectors digested with EcoRI and SmaI. The BA2416 promoter was generated by EcoRI digestion of plasmid pHT315-2416 (the EcoRI site at the 5' end and originated from the vector multiple cloning site, while the EcoRI at the 3' end was naturally occurring within the BA2416 coding sequence) and it was cloned in the EcoRI digested vectors.

The BAYisI translational fusions to the *lacZ* gene of *E. coli* were first constructed in plasmid pJM115 by cloning the EcoRI-SalI digested fragments generated by PCR

amplification using oligonucleotide primers BAYisI5'Eco and BaYisI3'Sal1 or BAYisI3'Sal2 in the EcoRI-SalI digested vector. Because of the presence of two SalI sites in pTCVlac, the fragments were then recovered as EcoRI-SacI and recloned in EcoRI-SacI digested pTCVlac. Fidelity of PCR amplifications and fragment orientation were checked by DNA sequence analysis.

Protein expression and purification

The coding sequence of each *B. anthracis* Spo0E-like protein was amplified by PCR reaction using oligonucleotide primers that introduced an NdeI or NheI site at the 5'end and a BamHI site at the 3' end (Table 1S). After digestion with NdeI + BamHI or NheI + BamHI the fragments were cloned in similarly digested pET28 vector (Novagen) generating a fusion to 6 histidine codons at the 5' end of the genes. The plasmids obtained were transformed in *E. coli* BL21(DE3) and protein expression was obtained by growing the cells in LB broth in the presence of kanamycin. Expression was induced with IPTG 1mM at OD_{600} 0.7 and growth was continued for approximately 3 hours. The proteins were purified in denaturing conditions using a buffer containing 50mM Tris-HCl pH 8.0, 100mM KCL, 1mM β-mercaptoethanol, 5mM imidazole and 7M urea. After binding to a Ni-NTA agarose column (Qiagen), proteins were eluted using the same buffer with 50mM imidazole. After dialysis and concentration, the proteins were stored at -20ºC in a buffer containing 50mM Tris-HCl pH 8.0, 100mM KCL, 1mM DTT, 1.5M urea and 20% glycerol.

In vitro assays

Phosphorylation and dephosphorylation reactions were carried out essentially as previously described (5,32). The reactions were initiated by the addition of [γ^{32}P] ATP (specific activity 6,000Ci/mmol) and aliquots were taken at the time points indicated in the figures.

Sporulation assays

Strains were grown in SM liquid medium at 37ºC for the time indicated in the tables in the presence of erythromycin and lincomycin. Serial dilutions were plated in duplicate before and after treatment with chloroform (10% v/v). Percentage of sporulation was calculated as the ratio between viable counts (before $CHCl_3$ treatment) and spore counts (after $CHCl_3$ treatment).

B-galactosidase assay

Cultures for transcriptional and translational assays were grown in SM liquid medium. Kanamycin was added for the *B. anthracis* cultures carrying pTCVlac derivatives. Duplicate samples were collected at hourly intervals. β-galactosidase activity was carried out essentially as described (22).

RESULTS

Identification of the B. anthracis Spo0E-like proteins.

The Spo0E protein of *B. subtilis* (34) was used as query in a BLAST search (tblastn) against the first available *B. anthracis* genome (A2012). Four genes were identified as encoding putative Spo0E-like proteins based on two main criteria: i) protein size ranging between 40 and 90 amino acids; ii) the presence of the Spo0E signature sequence "SQELD" (30). Two genes were annotated as Bant0046 and Bant1877. A third gene was not identified in strain A2012 but was annotated as BA2416 when the genome of the Ames strain became available. The fourth gene is still not annotated in any of the available *B. anthracis* genomes because of a frame shift in the sequence. If the frame were to be corrected, the gene product would be most similar to the *B. subtilis* YisI (33% identity); we called this gene and its product BAYisI. A summary of the genes names used in this study and in the most commonly used *B. anthracis* strains is reported in Table 1 and their amino acid alignment is shown in Figure 1.

A fifth gene annotated as BA1655 in the genome of the Ames strain (NC_003997) encoding a Spo0E-like protein with a divergent signature motif (SRDLD) has been functionally and structurally characterized in Grenha at al (11).

In vivo analysis of B. anthracis Spo0E-like proteins.

The four *spo0E*-like genes were PCR amplified from the *B. anthracis* Sterne strain 34F2 using the oligonucleotide primers listed in Table 1S (Supplemental Materials) and cloned

in the multicopy shuttle vector pHT315. The resulting plasmids were transformed into *B. subtilis* wild type strain JH642 and, after passage through the *dam*$^-$ *E. coli* strain SCS110, electroporated into *B. anthracis* 34F2. The phenotype of the transformants obtained was analyzed on Schaeffer's sporulation agar medium and compared to the parental strain carrying the vector alone. As shown in Figure 2A, expression of BA1877 strongly affected sporulation of *B. anthracis* and inhibition was also observed with the expression of BA2416 while no effect was seen in the strains expressing BA0046 and BAYisI. When the same plasmids were transformed in the *B. subtilis* wild type strain JH642, sporulation inhibition was observed only by the overexpression of BA1877 (Figure 2B). A quantitative analysis of the sporulation efficiency of the strains constructed was also carried out in Schaeffer's liquid medium (Table 2). The results indicated that BA1877 expression is the most drastic in inhibiting sporulation even in liquid cultures in both organisms, while none of the other Spo0E-like proteins significantly affected the spore efficiency. The BA2416, which visibly affected sporulation in solid growth conditions, did not significantly affect the efficiency of spore formation in liquid culture, suggesting that perhaps different transcriptional regulatory mechanisms exhist between the two modes of cell growth.

In vitro analysis of B. anthracis Spo0E-like proteins.

In order to determine whether the product of the four genes were all capable of dephosphorylating the Spo0A response regulator, *in vitro* assays were carried out using purified components. The four coding sequences of the *B. anthracis* Spo0E-like genes were PCR amplified from strain 34F2 and cloned in the *E. coli* expression vector pET28

(Novagen) in order to create a fusion to six histidine codons at the N-terminal end. The proteins were purified on nickel-NTA agarose columns (Qiagen) and assayed in a phosphorelay dephosphorylation reaction using the KinA, Spo0F, Spo0B and Spo0A proteins of *B. subtilis*. Note that, despite the presence of a frame shift, the strain carrying the pET28-BaYisI plasmid expressed a protein whose molecular weight was approximately 9,200 Daltons as determined by denaturing gel analysis and MALDI-TOF Mass spectrometry (Figure 1S in the Supplemental Material) in good agreement with the theoretical molecular weight deduced from the amino acid sequence. This indicated that the frame shift had been repaired in *E. coli* and a full length protein of the expected size was produced (43). The results of the *in vitro* assay, shown in Figure 3, indicated that all four proteins were capable of dephosphorylating the phosphorelay. Upon addition of the Spo0E proteins, in fact, the phosphorylation level of each component of the pathway decreased. Since in an assay containing only KinA and Spo0F no dephosphorylation was observed upon addition of the Spo0E-like proteins (Figure 2S of the Supplemental Material), by extrapolation from the genetic and biochemical evidence so far accumulated on the Spo0E family of proteins, we concluded that the four *B. anthracis* members of the Spo0E family all acted by dephosphorylating the Spo0A protein. The activity of BA1877 was approximately 2 fold stronger than the activity of the other three proteins (Figure 3S of the Supplemental Material). This is in agreement with the *in vivo* data which indicated that BA1877 was extremely more active than BA0046, BA2416 and BAYisI.

Transcriptional and translational analyses.

In order to determine whether the different effects observed *in vivo* were due to differences in the transcriptional level of each gene, the promoter regions of the four *B. anthracis spo0E*-like genes were cloned in front of the promoterless *E. coli lacZ* gene in the transcriptional fusion vectors pJM115 and pTCVlac. The pJM115 derivatives were transformed in *B. subtilis* JH642 selecting for kanamycin resistance and integrated by double crossover at the *amyE* region. The pTCV *lac* derivatives were electroporated into strains 34F2 selecting for kanamycin resistance. Transcriptional analysis was carried out by means of β-galactosidase assays in Schaeffer's sporulation medium. The results shown in Figure 4 indicated that in *B. anthracis* (Figure 4A) the BA2416 gene was transcribed at the highest levels, compared to the other genes, and in a constitutive way, while the BA1877 and BAYisI were induced one hour before or around the transition from vegetative growth to stationary phase (T_0 in Figure 4) respectively. The promoter of BA0046 was inactive, at least in the growth conditions tested, as it never exceeded the level of activity detected in the control strain carrying a promoterless vector pTCVlac.

In *B. subtilis* (Figure 4B) the BA1877 promoter was the most active and, as in *B. anthracis*, the transcription was strongly induced approximately one hour before the transition phase. Induced at approximately the same time but at a lower level was also the promoter of BAYisI, while the BA2416 promoter was expressed throughout the growth with a highest level at T_0. The BA0046 was transcribed at a low level in vegetative phase but by transition phase its transcription was essentially at background level. The results help to explain why, despite the fact that all four Spo0E-like proteins are active *in vitro*, only BA1877 is visibly active *in vivo*. BA0046 does not appear to be

transcribed, at least in the assay conditions used in this study, consistent with a lack of phenotype, BA2416 was weakly active *in vitro* and it may also be weakly active *in vivo* since its level of expression is higher than BA1877 in *B. anthracis* but its inhibition of sporulation *in vivo* is less dramatic. In *B. subtilis*, BA2416 did not cause a sporulation deficient phenotype, consistent with a lower level of expression than BA1877.

BAYisI was transcriptionally regulated in a manner similar to BA1877, however, it was inactive *in vivo* because of a frameshift in the sequence that results in a truncated protein. Since significant frameshift repair was found to occur when BAYisI was expressed in *E. coli*, we analyzed the possibility of frameshift repair occurring in *B. anthracis* by means of translational *lacZ* fusion analyses. The two frames generated by the frameshift were each translationally fused to the promoterless *lacZ* gene of plasmid pTCVlac at the SalI site. The resulting plasmids, pTCV-YisISal1 and pTCV-YisISal2 were transformed into *B. anthracis* 34F2 and β-galactosidase assays were carried out on cultures grown in SM medium. As shown in Figure 5, the first frame, preceding the frameshift, is translated at relatively high levels when compared to the second frame, and followed a pattern of induction identical to the one observed with the transcriptional fusion. The second frame, fused to the *lacZ* gene downstream of the frameshift, produced a level of β-galactosidase that was 2-3 fold lower than the one produced by the first frame, but yet it was higher than the background level of activity generated by the vector alone. This suggested that some frameshift repair occurred but it did not result in a sufficient level of protein to generate a visible sporulation phenotype. Accordingly, we have not been able to detect the full length BAYisI protein when overexpressed from the pHT315 vector with a His-

tag at the carboxy terminal end that provided the epitope for Western Blot analysis (data not shown).

DISCUSSION

In this study we have characterized the Spo0E family of phosphatases in the anthrax causing organism *B. anthracis*. We have shown that, of the four genes encoding Spo0E-like proteins, two are expressed to produce proteins active in dephosphorylating the Spo0A transcription factor, one is not transcribed in the assay conditions used in this study but it can potentially produce a Spo0A-like targeting protein, while a fourth gene (BAYisI) is frameshifted and does not produce an active protein. A fifth gene encoding a Spo0E-like protein active on Spo0A~P (BA1655) is also present on the chromosome of *B. anthracis* and it characterization is reported in Grenha et al (11). All four proteins characterized in this study, when purified from an overexpressing *E. coli* strain, were shown to be able to dephosphorylate the Spo0A protein of the phosphorelay *in vitro*. Extensive frameshift repair was observed in *E. coli* for the BAYisI gene which allowed us to purify and test a full length protein *in vitro*. Although the differential level of activity observed among the four proteins could be a result of the purification procedures in denaturing conditions, we cannot exclude that *in vivo* the proteins may actually have different affinity for Spo0A~P as the *in vivo* data suggest.

The *B. anthracis* members of the Spo0E family of phosphatases were identified based on the size of the proteins and the presence of the Spo0E signature motif "SQELD" which is generally preceded by a hydrophobic residue two amino acids upstream of the serine residue, and two hydrophobic residues localized one amino acid downstream of the aspartate residue (30).

Pair score analysis indicated that the *B. anthracis* Spo0E proteins share between 21 (BA1877) and 32 (BA0046) percent of identical residues with the *B. subtilis* Spo0E protein. Among each other, the *B. anthracis* proteins share between 25 and 51% of identical residues with the highest score being between BA0046 and BA2416 clearly indicative of a relatively recent gene duplication event. Noticeable in the alignment in Figure 1 is the length of BA1877 (93aa) which resembles the length of *B. subtilis* Spo0E (85aa) whose carboxy-terminal 25 amino acids are known to be inhibitory to its phosphatase activity (27). Whether a similar C-terminal inhibitory effect exists on BA1877 remains to be determined. The other three proteins (BA0046, 56aa; BA2416, 49aa; BAYisI, potentially 59aa) resemble in size the YisI and YnzD proteins of *B. subtilis* (56 and 57aa, respectively) with identities ranging between 28 to 37%.

When expressed from a multicopy vector the most active protein *in vitro*, BA1877, was also the most active *in vivo* in both *B. anthracis* and *B. subtilis*. Although expressed at the highest level in *B. anthracis*, BA2416 was only moderately active in inhibiting sporulation in *B. anthracis* and inactive in *B. subtilis*. The results are consistent with a weak activity *in vitro* of the purified protein. The BA0046 and BAYisI did not affect sporulation *in vivo*, consistent with the absence of gene transcription and the frameshifted gene, respectively.

The initiation of sporulation is a critical phase in the life cycle of sporulating bacteria. In Bacilli, a complex signal transduction pathway, called phosphorelay, works to integrate a

myriad of signals that the cell receives from the environment as well as its own metabolism to ensure that the decision to sporulate or to remain in vegetative growth is the more appropriate for the bacterium survival. The key factor regulated by the phosphorelay is the Spo0A transcription regulator whose activation by phosphorylation results in the repression or activation of more than 500 genes (25). To ensure that the phosphorylated form of Spo0A is at the appropriate concentration at any given time during the cell cycle, a series of controls are positioned at the kinases level (8,42) or at the response regulator level within the phosphorelay (29). Aspartyl phosphate phosphatases of the Rap or Spo0E families modulate the phosphorylation level of Spo0F and Spo0A, respectively, to ensure that initiation of sporulation does not occur at inappropriate times. Each member of the Rap and Spo0E family of phosphatases of *B. subtilis* has been found to be transcriptionally regulated by physiological conditions antagonistic to sporulation such as growth or competence to DNA transformation (26,30,33). Typical among the members of the Rap and Spo0E families of proteins is a variety of transcription patterns indicative of differential regulatory controls. In *B. anthracis* we found that the four genes encoding Spo0E-like proteins are differentially controlled at the level of transcription with one promoter (BA0046) not being transcribed, at least in the assay condition used in this study. A certain correlation was observed in transcriptional patterns between *B. anthracis* and *B. subtilis*, although the lower level of BA2416 observed in *B. subtilis* compared the *B. anthracis* and with respect to the transcription level of BA1877, suggests that different transcriptional controls exist in these two organisms. Further studies will be necessary in order to unravel these

transcriptional control systems and establish whether they have any relevance to the pathogenesis of *B. anthracis*.

The transition phase, defined as the growth phase between vegetative growth and stationary phase, is a critical time in a bacterium life cycle. For a number of spore forming bacteria, the transition phase is characterized by the production of secondary metabolites, virulence factors and toxins. The key regulator of this phase is the Spo0A response regulator. One of the genes repressed by Spo0A at the beginning of the transition phase is *abrB* whose gene product generally acts as a preventer of stationary gene product expression (40,41). Repression of *abrB* by Spo0A~P is required for production of peptide antibiotics in *B. subtilis* and *B. brevis* and proteases in *B. subtilis* (10,20,21). Directly or indirectly Spo0A is required for crystal toxin production in *B. thuringiensis* and *B. sphaericus*, the enterotoxin of *Clostridium perfringens* or the production of solvent in *C. acetobutylicum* (9,12,14,19). Recently the role of Spo0A in repressing *abrB* transcription has been found to be a requirement for full expression of toxin genes in *B. anthracis* (37). Thus it has become even more clear now how important it is for cell survival and pathogenicity to precisely regulate the level of Spo0A~P so that secondary metabolites and toxins are produced only at the appropriate time. In *B. anthracis*, as in *B. subtilis*, the Spo0E-like proteins are probably the last checkpoint on the phosphorelay to ensure the appropriate timing of toxin production. Furthermore, during an infection, the Spo0E-like proteins may contribute to maintain the level of Spo0A~P such that *abrB* is repressed, thus toxins are produced, but sporulation does not progress further. We have recently proposed that such a scenario occurs in *B. anthracis*

cells within an infected body (6). Since macrophages have the ability to phagocytose and destroy spores while encapsulated bacteria escape this defense mechanism (24), a pathogenesis-induced prevention of sporulation has been hypothesized. Weak or inhibited sporulation histidine sensor kinases together with a plasmid encoded Rap phosphatase have been shown to be able to prevent spore formation and affect the level of AbrB in the cell (5,6). The Spo0E-like proteins identified in this study are likely to provide an additional level of control to ensure that spores are not formed while an infection is in its full development. The differential level of expression observed in our transcriptional studies in batch cultures are indicative of differential regulatory mechanisms that can modulate time and level of gene expression during an active infection.

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Table 1. Nomenclature of the Spo0E family of proteins in *B. anthracis* strains.

This Work[a]	**A2012**[b]	**Sterne**[b]	**Ames**[b]
	NZ_AAACO2000001	NC_005945	NC_003997
BA0046	Bant0047	BAS4809	BA5174
BA2416	NA[c]	NA	BA2416
BA1877	Bant1968	BAS1251	NA
BAYisI	NA[d]	NA[d]	NA[d]
	1617906-1617731	1121865-1121690	1121973-1121798

a) As of 05/02/2003, when the first BLAST search was carried out
b) As of 03/29/06
c) NA = Not annotated
d) The frame shifted ORF is not annotated but it is located within the nucleotides indicated.

Table 2. Sporulation efficiency of *B. subtilis* JH642 and *B. anthracis* 34F2 strains carrying the *spo0E*-like genes on the multicopy plasmid pHT315.

Plasmid	Viable Count	Spore Count	% of Spores[a]
B. subtilis			
pHT315	3.4×10^8	1.3×10^8	38.2 (± 2.0)
pHT315-1877	2.4×10^8	1.7×10^3	0.0007 (± 0.001)
pHT315-0046	4.3×10^8	1.4×10^8	32.5 (± 2.8)
pHT315-2416	3.3×10^8	1.3×10^8	39.4 (± 0.9)
pHT315-YisI	3.7×10^8	1.4×10^8	37.8 (± 5.8)
B. anthracis			
pHT315	1.8×10^8	5.0×10^7	27.7 (± 4.6)
pHT315-1877	8.5×10^7	3.6×10^4	0.04 (± 1.5)
pHT315-0046	2.5×10^8	7.2×10^7	28.8 (± 2.1)
pHT315-2416	2.9×10^8	6.8×10^7	23.4 (± 3.6)
pHT315-YisI	2.3×10^8	6.6×10^7	28.6 (± 6.2)

a) Strains were grown for 48 hours at 37ºC in Schaeffer's sporulation medium. The results shown are the average of two independent experiments. Deviation from the mean is shown in parenthesis.

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FIGURE LEGENDS

Figure 1: Amino acid sequence alignment of the *B. anthracis* Spo0E-like proteins. Proteins were aligned with the ClustalW program. Asterisks indicate identical residues in all five sequences; columns denote conserved substitutions. The four *B. anthracis* (BA) proteins analyzed in this study are aligned against the *B. subtilis* Spo0E protein (34). The truncated proteins resulting from the frameshift in the BAYisI gene are indicated with 1 and 2.

Figure 2: Sporulation phenotypes of *B. anthracis* (**A**) and *B. subtilis* (**B**) strains expressing the Spo0E-like proteins from the multicopy plasmid pHT315. Strains in **A** are as follows: 1, pHT315; 2, pHT315-YisI; 3, pHT315-0046; 4, pHT315-1877; 5, pHT315-2416; 6, pHT315. Strains in **B** are as follows: 1, pHT315; 2, pHT315-1877; 3, pHT315-2416; 4, pHT315-YisI; 5, pHT315-0046.

Strains were streaked on SM plates and incubated at 37ºC for 48 hours.

Figure 3: Phosphorelay dephosphorylation assay. Purified Spo0E-like proteins of *B. anthracis* were tested *in vitro* for their ability to dephosphorylate the *B. subtilis* phosphorelay KinA (0.2μM) , Spo0F (2.5μM), Spo0B (0.2μM), Spo0A (2.5μM) were incubated in the presence or absence of the *B. anthracis* BA1877 (**A**), BA0046 (**B**), BAYisI (**C**) and BA2416 (**D**) proteins purified from *E. coli* at 5μM final concentration.

The reactions were initiated by the addition of ATP and aliquots were withdrawn at the indicated times. The position of the phosphorylated proteins is indicated by the arrows.

Figure 4: Transcriptional analysis of the *B. anthracis spo0E*-like genes in *B. anthracis* (**A**) and *B. subtilis* (**B**). Cultures were grown in SM liquid medium. Symbols: -●- BA0046; -■- BAYisI; -▲- B1877; -◆- BA2416; -▼-pTCVlac vector. The growth curve of strains carrying the BA0046 construct is shown as representative of all growth curves (-○-).

Figure 5: Translational analysis of BAYisI in *B. anthracis*. *B. anthracis* carrying pTCV-YisISalI (-●-) or pTCV-YisISal2 (-■-) were grown in SM liquid medium together with a strain carrying the vector clone (-▼-). Samples were taken at hourly intervals before and after the transition (T_0) from vegetative to sporulation phase. The growth curve of the strain carrying the vector is shown as representative of all growth curves (-▽-).

Figure 1S: MALDI TOF analysis of the BAYisI protein isolated from *E. coli*. The sequence of one possible protein resulting from the frameshift repair is shown with the His-tag sequence at the N-terminal end. The predicted molecular weight of this protein is also shown.

Figure 2S: Phosphorelay dephosphorylation assay. Purified Spo0E-like proteins of *B. anthracis* were tested for their ability to dephosphorylate KinA (0.2μM) or Spo0F

(2.5μM). BA1877, BA0046, BA2416 and BAYisI were added at 5μM final concentration. The reactions were initiated by the addition of ATP. Aliquots were withdrawn at the indicated times. The position of the phosphorylated KinA and Spo0F proteins is indicated by the arrows. The asterisk denotes a dimer form of Spo0F.

Figure 3S: Quantitation of phosphorylated proteins in the phosphorelay reactions in the presence of the *B. anthracis* Spo0E-like proteins. The reactions shown in Figure 3 were quantitated using the ImageQuant software (Molecular Dynamics/Amersham). For each gel, the total phosphorylation in each lane (KinA, Spo0F, Spo0B and Spo0A) was measured in pixel and expressed as a percentage of the total phosphorylation level of the last time point of the control reaction carried out without the phosphatase. The 100% of phosphorylation is the 15' time point for the BA1877 gel, the 20' time point for the BA0046 and BAYisI gels, and the 30' time point for the BAYisI gel.

```
Ba0046          -------MNVTKLNDRIEAKKKELIYLVEKYG-THHKVISFSQELDRLLNLLIELKTKKKRYSL---------------------------- 56
Ba2416          -------MELVKLEKVIEIKKEELLYLVSDYGIQHEKVLALSQEIDKLINYFMFLK----------------------------------- 49
BaYisi1         -----------MFEQAIEKNVKK-------------------------------------------------------------------- 12
BAYisI2         -----------------KKREKMIYFAERYGMTSQKTVDCSQELDRLLNVIWHVHTDVHPNQTLDTHTQ---------------------- 52
Ba1877          MFTVKRKYTLEKLSRDIHMKREEMIQLGLTSGLNSMETIQVSQELDKLILQYQCYKEKQTPKWLSIIKAPIFQIGYEGKSSNFWRMLVAGFMK 93
BsSpo0E         ---MGGSSEQERLLVSIDEKRKLMIDAARKQGFTGHDTIRHSQELDCLINEYHQLMQENEHSQGIQGLVKKLGLWPRRDVMPAYDANK----- 85
                            :   *  *:   ::     *      :  ***:* *:
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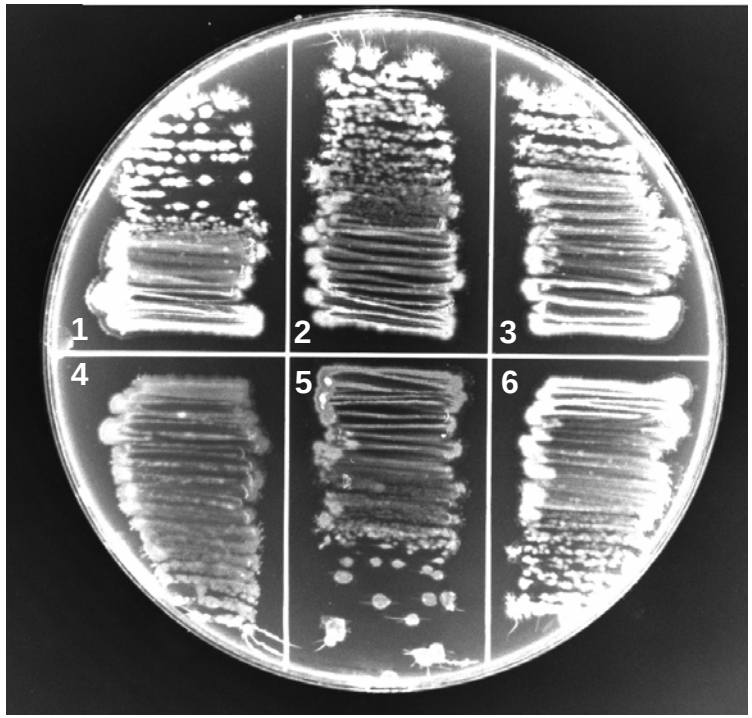
Bongiorni et al.
Fig.1

A



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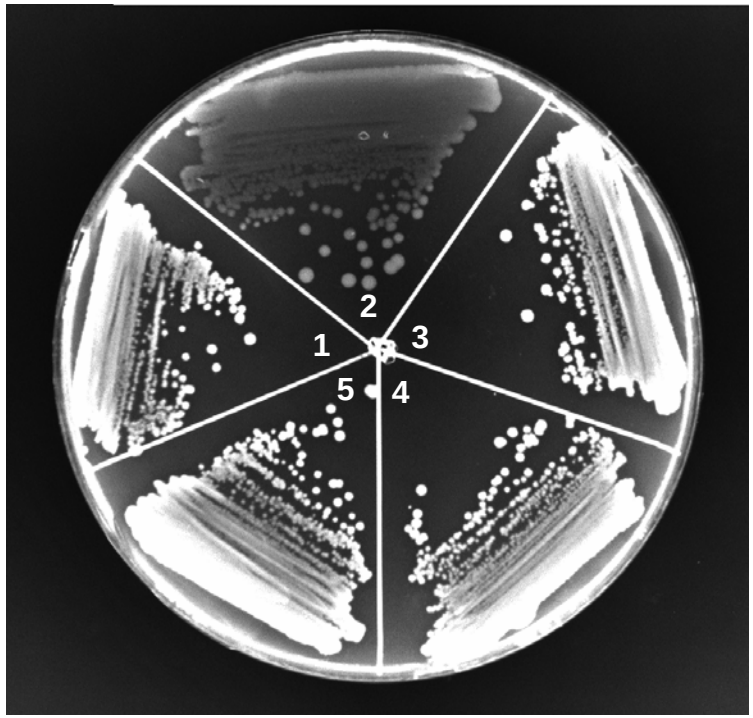
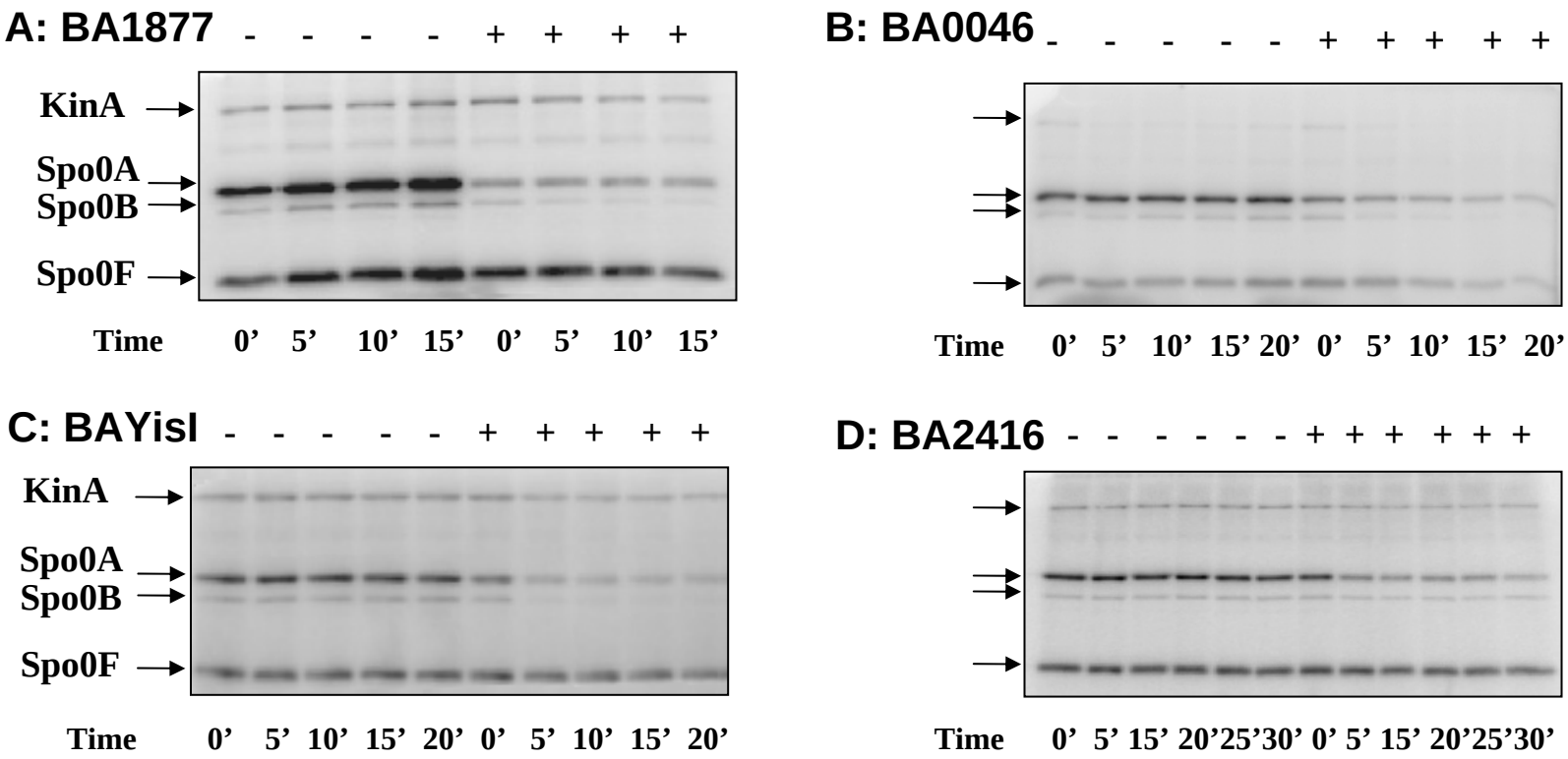
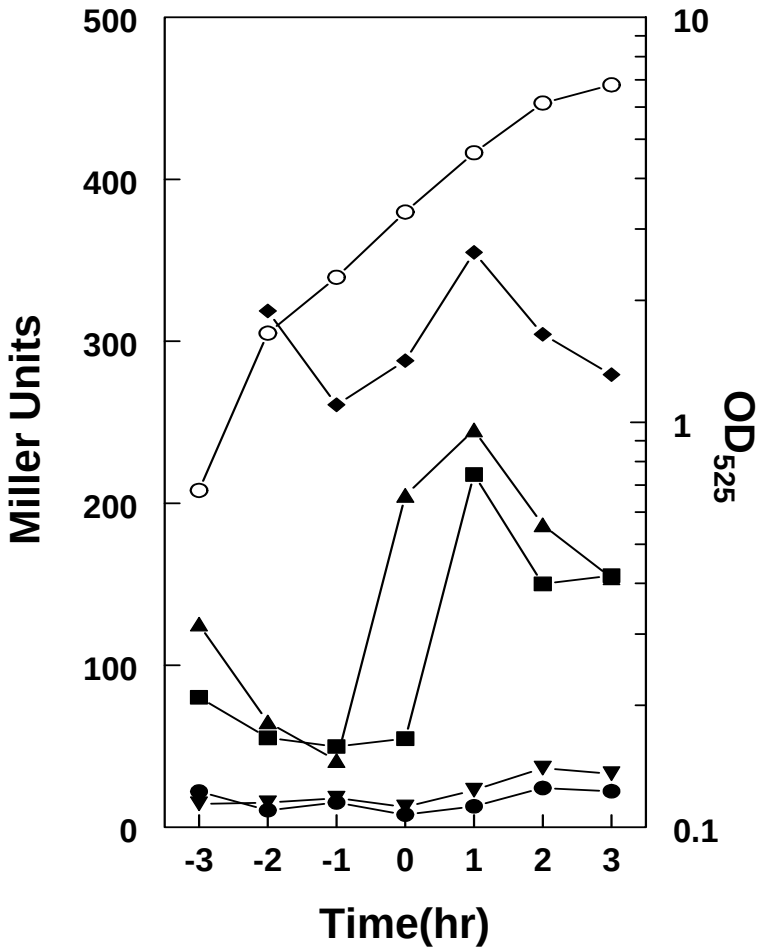


Bongiorni et al.
Fig.2



Bongiorni et al.
Fig.3

A



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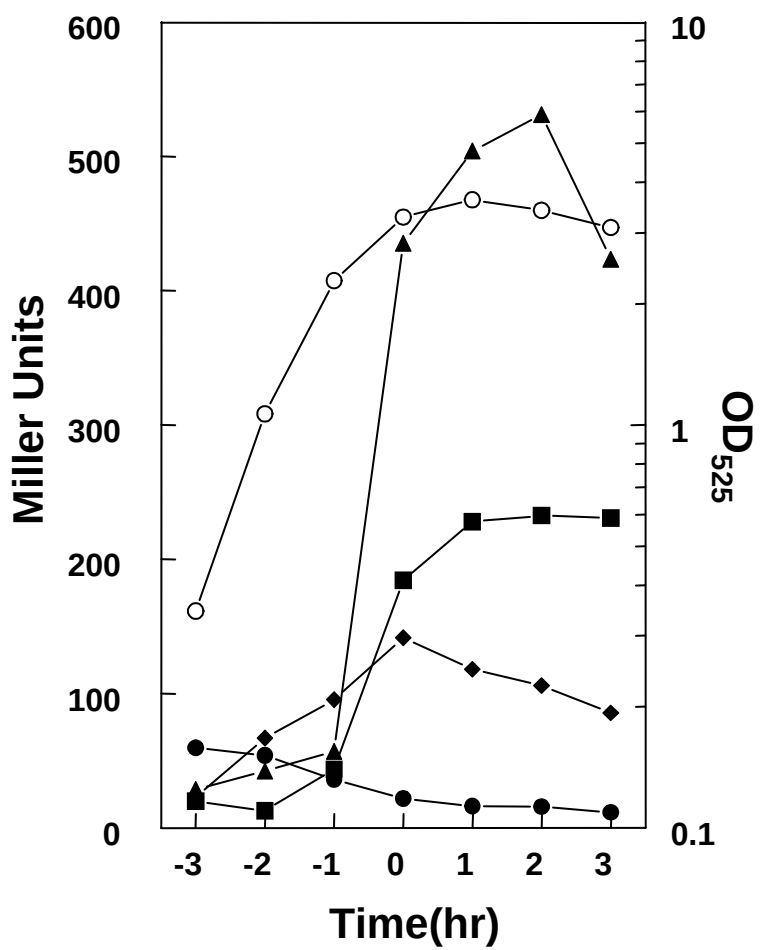
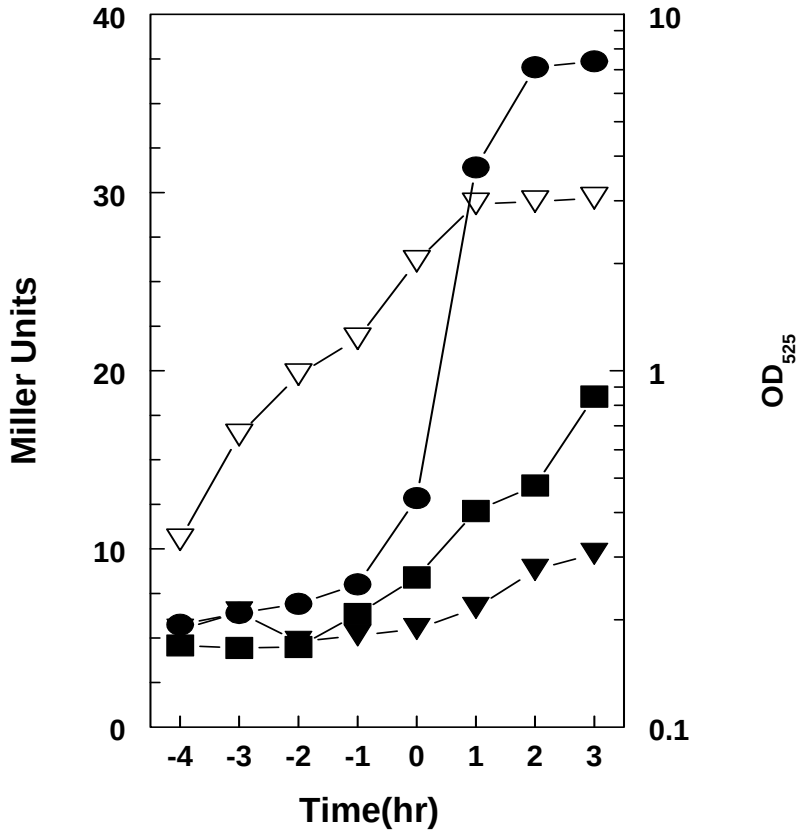


Bongiorni et al.
Fig.4



Bongiorni et al.
Fig.5

Table 1S. Oligonucleotide primers used in this study.

Name	Sequence[a]
BA00465'Kpn	TATTAGGTACCGGTATCCTCTGGATG
BA00463'Bam	TAGATGGATCCTTTCTGAATCTAG
BA00463'Bam2	ATCCGTAGGATCCAACGAGATAGATC
BA00465'Nde	TTCATTTCACATATGAACGTAACGAAAC
BA18775'EcoRI	TAAACGAATTCCTTTCTATATTATC
BA18773'Bam	CAGTTGGATCCGAAATTCCATTACAC
BA18773'Bam2	GAAAGGGATCCTAATGTGTACTTCCT
BA18775'Nhe	TGAATGGCTAGCGTAAAAAGGAAGTAC
BA24165'Kpn	AGAGAGGTACCGATTTGTTATGAATATG
BA24163'Bam	TCCCCGGATCCTGAAATGTACGTATG
BA24165'Nde	GAAGTAAAACCATATGGAATTAGTGAAATTGG
BAYisi5'Eco	TAATTGAATTCGCATCCGTAAGAAAAAAAC
BAYisi3'Bam	TACAAGGATCCTTTCGTTTTTAAAATAAAC
BAYisi5'Nde	CGATTCATATGTTTGAGCAAGCG
BAYisI3'SalI	ATCGCGTCGACAAACATTGAAATCGGCTC
BAYisI3'Sal2	CCATAGTCGACAGCAAAATAAATCATTTTTTCAC

a) Regions underlined denote the restriction site.

BAYisI (predicted MW 9,255.35)
MGSSHHHHHHSSGLVPRGSHMFEQAIEKKREKMIYFAERYGMTSQKTVDCSQELDRLLNVIWHVHTDVHPNQTLDTHTQ

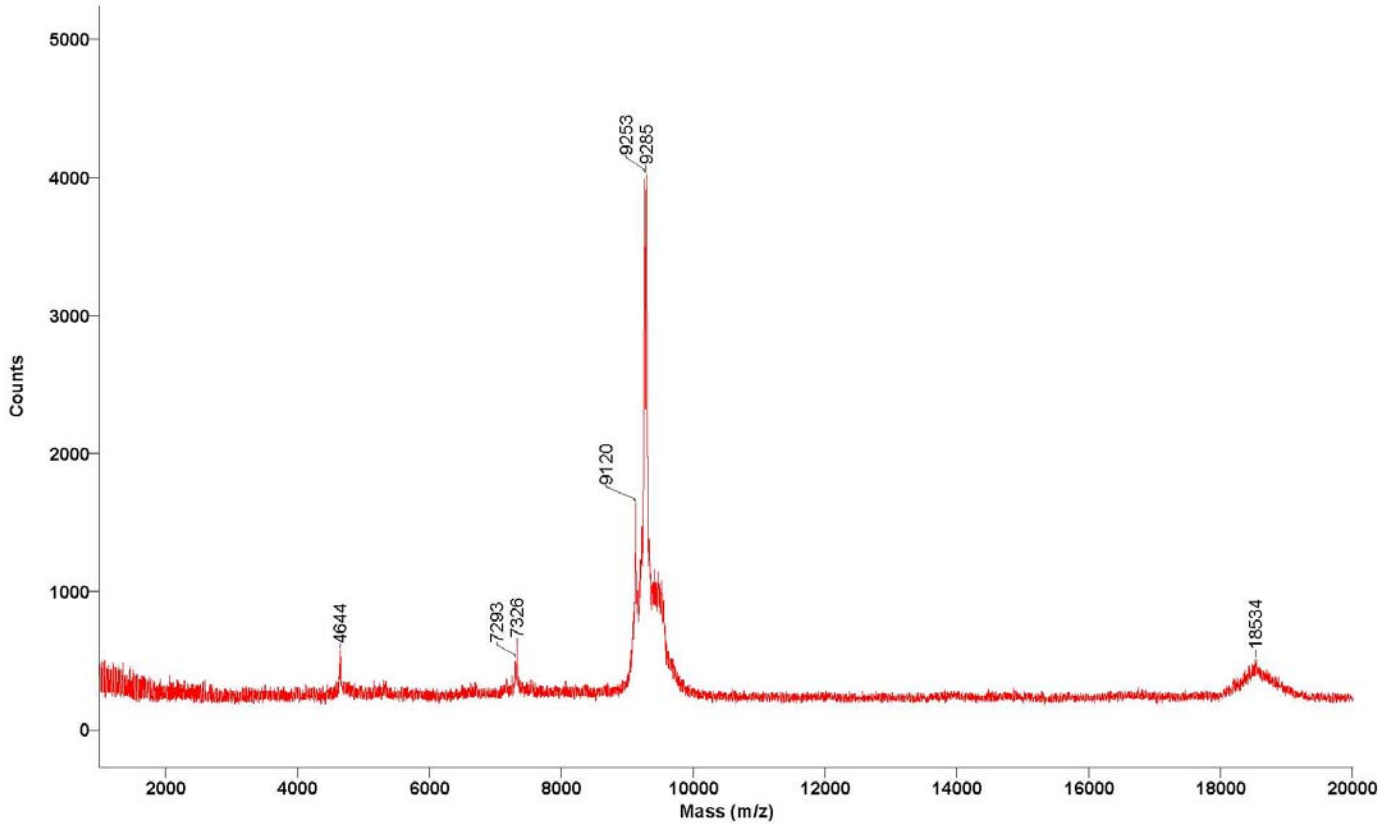


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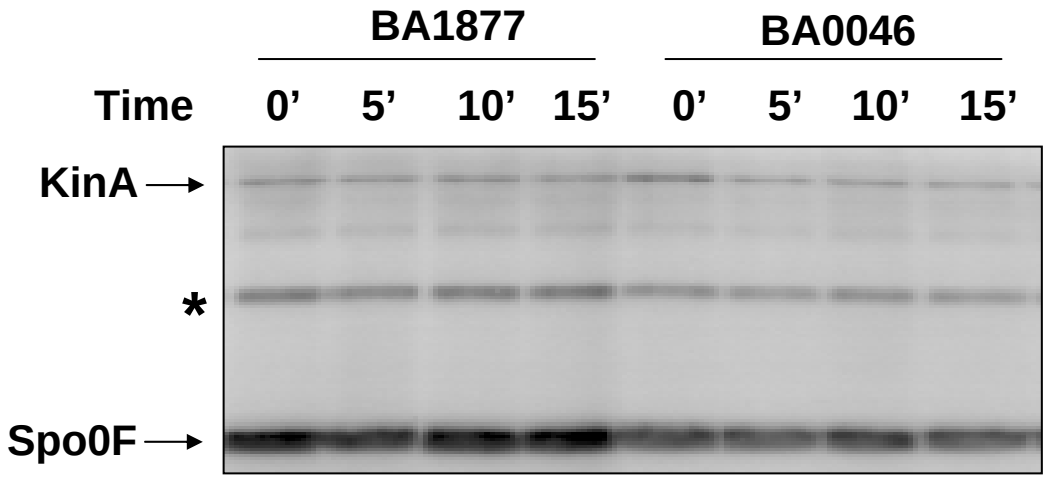
Fig.1S



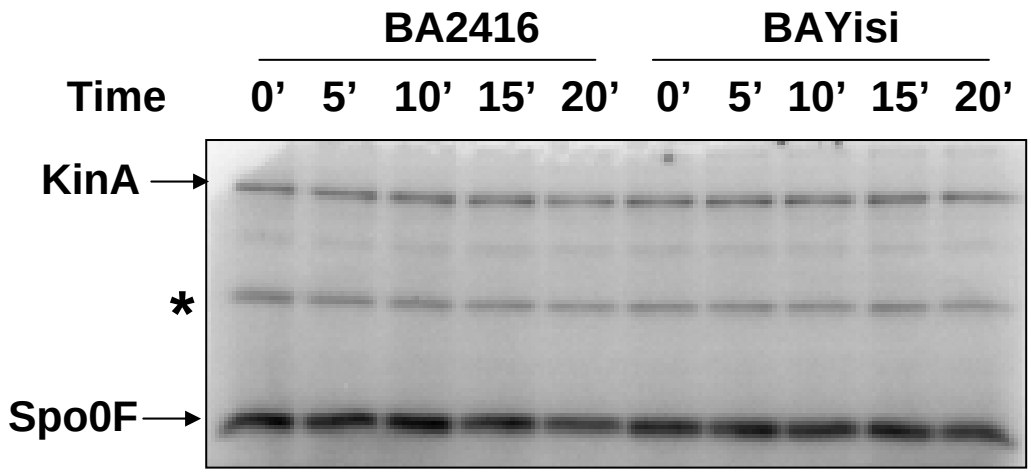


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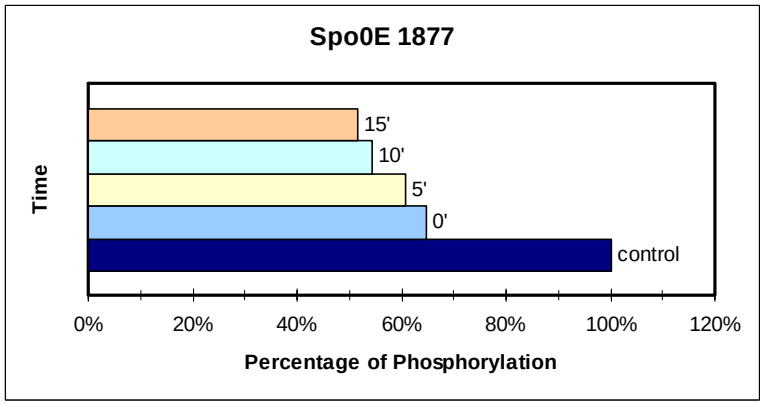
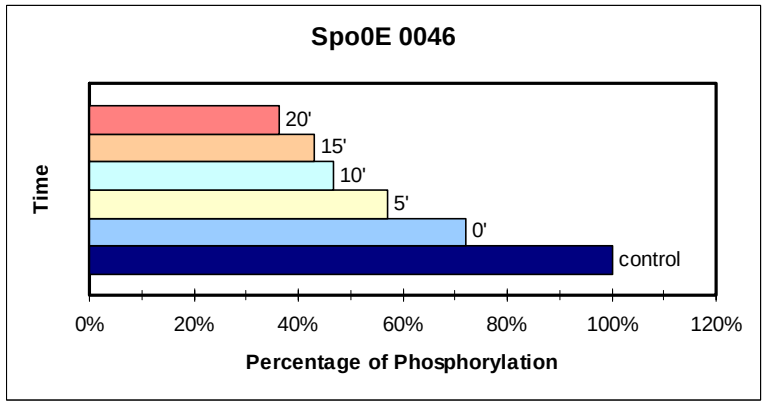
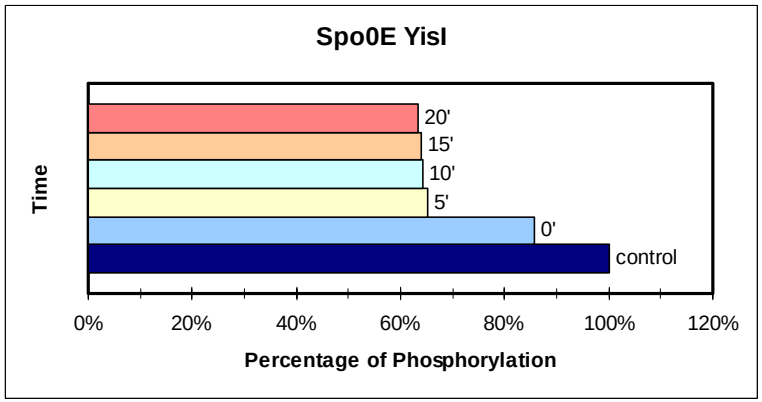
Fig.2S







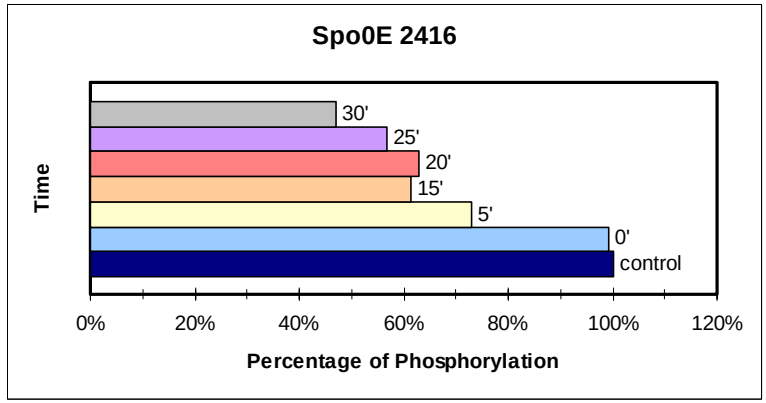


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Fig. 3S