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Crystallization and preliminary X-ray analysis of the sporulation factor SpoIIAA in its native and phosphorylated forms

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Sporulation in *Bacillus* begins with an asymmetric cell division producing two progeny with identical chromosomes but different developmental fates. As such, it is a simple example of cellular differentiation. The establishment of cell type is controlled by a series of alternate RNA polymerase sigma subunits. The first compartment-specific sigma factor is σ^F, whose activity is controlled by SpoIIAB, an anti-sigma factor, and SpoIIAA, an anti-sigma factor antagonist which is phosphorylated by the kinase activity of SpoIIAB. Here, the preliminary crystallographic analysis of SpoIIAA and phosphorylated SpoIIAA from *B. sphaericus* in forms suitable for high-resolution structure determination are reported.

1. Introduction

The formation of a resistant spore is the ultimate response of *Bacillus subtilis* to a hostile environment representing perhaps the simplest example of cellular differentiation. Sporulation begins with an asymmetric cell division producing two cells of unequal size, a larger mother cell and smaller forespore. The mother cell engulfs the forespore and the two cells collaborate in constructing a complex proteinaceous coat around the developing spore. In the final step, the mother cell lyses, releasing the mature spore, which can lie dormant in the soil, germinating when favourable growth conditions are restored (Stragier & Losick, 1996).

Following asymmetric cell division, the mother cell and the forespore contain identical chromosomes, yet they follow different programmes of gene expression and have different fates. This is brought about by the sequential activation of alternate sigma factors within the respective compartments: σ^F and σ^G in the forespore and σ^E and σ^K in the mother cell (Losick & Stragier, 1992). These combine with core RNA polymerase and direct it to distinct sets of promoters. σ^F is the first forespore-specific RNA polymerase sigma factor to become active during sporulation. Although σ^F is present in the pre-divisional cell and partitions into both compartments upon formation of the asymmetric septum, it is activated only in the forespore.

σ^F activity and hence cell fate is governed by the interactions between SpoIIAB and SpoIIAA. The interplay between these components is complex and dependent on the phosphorylation status of SpoIIAA and the presence of adenine nucleotides. SpoIIAB is an anti-σ^F which acts by sequestering σ^F in a ternary complex with ATP in which the sigma factor is unable to combine with core RNA polymerase (Min *et al.*, 1993; Duncan & Losick, 1993). In the presence of ADP, SpoIIAB can also bind to SpoIIAA, forming a ternary complex in which the former is unavailable to inhibit σ^F (Min *et al.*, 1993; Duncan & Losick, 1993). Thus, SpoIIAA is termed an anti-σ^F antagonist or 'anti-anti-σ^F'. In the presence of ATP, however, SpoIIAB is a protein kinase (Min *et al.*, 1993) which specifically phosphorylates SpoIIAA on Ser58 (Najafi *et al.*, 1995). SpoIIAA$\sim$P does not interact with SpoIIAB, leaving the latter free to inhibit σ^F. Thus, the predominant forms in the pre-divisional cell and the mother cell are SpoIIAA$\sim$P and SpoIIAB–σ^F, because SpoIIAA is phosphorylated by the unopposed activity of SpoIIAB. In the forespore compartment, however, SpoIIE, a septum-bound protein phosphatase, becomes active (Arigoni *et al.*, 1996). SpoIIE dephosphorylates SpoIIAA$\sim$P so that the predominant forms in the forespore become SpoIIAA–SpoIIAB and σ^F (Alper *et al.*, 1994; Diederich *et al.*, 1994).

The genes for SpoIIAA, SpoIIAB and σ^F (SpoIIAC) are arranged sequentially in an operon. To gain insight into the fascinating but complex interactions among the proteins of this *spoIIA* operon, we have embarked on a programme of structural studies focusing initially on the crystallization of SpoIIAA. Whilst the structure of native SpoIIAA from *B. subtilis* has been previously determined by NMR (Kovacs *et al.*, 1998), little is known with regard to conformational changes accompanying phosphorylation. We have targeted the crystallization of phosphorylated SpoIIAA in addition to the native form in order to provide molecular details of the partner-

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Table 1
Crystallization conditions.

SpoIIAA crystal form	Bst-I	Bst-II	Bst∼P	Bsp and Bsp∼P-I	Bsp∼P-II
Dimensions (mm)	0.1 × 0.05 × 0.02	0.1 × 0.01 × 0.01 and 0.2 × 0.05 × 0.02	0.2 × 0.05 × 0.05	Each 0.1 × 0.03 × 0.01	0.3 × 0.1 × 0.1
Precipitant	30% PEG 4K, 200 m*M* Li_2SO_4	1.5 *M* Li_2SO_4	18% PEG 2K MME, 200 m*M* Li_2SO_4	15% PEG 4K, 200 m*M* $MgCl_2$	30% PEG 5K MME, 200 m*M* $(NH_4)_2SO_4$
Additive	None	2–5% DMF or 25–50 m*M* NaSCN	15% PEG 400	None	None
Buffer (100 m*M*), pH	Tris–HCl, 8.5	Tris–HCl, 7.5	Tris–HCl, 8.5	Tris–HCl, 8.5	MES, 6.5
Cryoprotectant	Mother liquor	20% glycerol	Mother liquor	20% PEG 4K	Mother liquor

switching mechanism for controlling sigma-factor activity.

2. Methods: molecular cloning, purification and crystallization of SpoIIAA from three distinct species of Bacilli

2.1. Purification of *B. subtilis* SpoIIAA

For the overexpression of SpoIIAA from three species of Bacilli, *B. subtilis*, *B. stearothermophilus* and *B. sphaericus*, the coding sequences for SpoIIAA were cloned into T7-RNA polymerase-based pET vectors and expressed in the DE3-lysogenized *Escherichia coli* strain BL21. For *B. subtilis* SpoIIAA (Bsu SpoIIAA) we utilized a clone, pEAA, kindly provided by M. D. Yudkin, University of Oxford, to direct overexpression of SpoIIAA (Min *et al.*, 1993). *E. coli* BL21 (DE3) cells, freshly transformed with plasmid pEAA, were grown in LB media containing 100 µg ml^{-1} ampicillin at 310 K until the optical density at 600 nm reached 0.6. Expression of recombinant protein was induced by the addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 1 m*M*. After 4 h induction, the cells were harvested by centrifugation and frozen at 193 K for later use. For purification, the frozen cell pellet was resuspended in buffer *A*, which comprises 25 m*M* Tris–HCl pH 7.5, 10% glycerol, 5 m*M* EDTA, 2 m*M* DTT, 0.2 *M* NaCl and 0.1 m*M* of the protease inhibitor 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF). The cells were lysed by sonication and the lysate was clarified by centrifugation. The protein was purified to homogeneity by a combination of ammonium sulfate fractionation and hydrophobic interaction and gel-filtration chromatography (Diederich *et al.*, 1994).

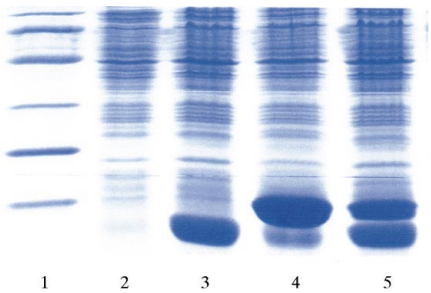

Figure 1
SDS–PAGE (15% gel) of the hyper-expression of SpoIIAA, SpoIIAB and SpoIIAA∼P/SpoIIAB. The example shown is for gene products from *B. stearothermophilus*. Lanes 3, 4 and 5: soluble cell extracts from induced *E. coli* cells expressing Bst SpoIIAA, SpoIIAB and SpoIIAA+AB, respectively. Lane 2, non-induced cell extract; lane 1, protein molecular-weight markers of 97.4, 66.2, 45.0, 31.0, 21.5 and 14.4 kDa.

2.2. Cloning and purification of SpoIIAA from *B. stearothermophilus* and *B. sphaericus*

Fragments encompassing the *spoIIAA* coding sequence were amplified by PCR methods. A pUC18 clone containing a 2.5 kbp *Sal*I DNA fragment from *B. stearothermophilus* DSM22 (Park & Yudkin, 1997) was used to generate an *Nde*I–*Bam*HI fragment for cloning into the expression plasmid pET26b. *B. sphaericus* ATCC 14577 chromosomal DNA was used to amplify an *Nco*I–*Bam*HI fragment for cloning into the plasmid pET28a. Incorporation of the *Nco*I site at the initiation codon was accompanied by substitution of the second codon for one specifying alanine. The sequence data have been deposited with the accession code AJ278291.

Recombinant SpoIIAA proteins from *B. stearothermophilus* (Bst SpoIIAA) and *B. sphaericus* (Bsp SpoIIAA) were expressed in a fashion similar to that described above for Bsu SpoIIAA, except that kanamycin (at a concentration of 30 µg ml^{-1}) was the antibiotic used to maintain plasmid selection. The proteins were purified to homogeneity by ion-exchange and gel-filtration chromatography using Source Q and Superdex 75, respectively.

2.3. Purification of SpoIIAA∼P from *B. stearothermophilus* and *B. sphaericus*

For the production of SpoIIAA∼P from both *B. stearothermophilus* and *B. sphaericus*, DNA fragments encompassing the co-cistronic *spoIIAA* and *spoIIAB* genes were amplified and cloned into pET26b and pET28a, respectively. Induction of cultures harbouring pET-AA+AB leads to overexpression of both SpoIIAB and SpoIIAA, so that the SpoIIAA becomes phosphorylated by the kinase activity of SpoIIAB. Bst and Bsp SpoIIAA∼P proteins were purified following the same procedures as for the native proteins and were separated from residual unphosphorylated material by high-resolution MonoQ ion-exchange chromatography.

Using these procedures, all three SpoIIAA and both SpoIIAA∼P homologues could be purified to apparent homogeneity, as judged by three different electrophoretic methods in conjunction with MALDI-TOF mass spectrometry (Fig. 1). For crystallization of Bsu SpoIIAA, the purified protein was concentrated to 10 mg ml^{-1} in a buffer of 10 m*M* Tris–HCl pH 7.5, 1 m*M* DTT. For crystallization of the Bst and Bsp SpoIIAA homologues, the purified proteins were each concentrated to 5 mg ml^{-1} in a buffer of 10 m*M* Tris–HCl pH 8.5, 50 m*M* NaCl, 1 m*M* DTT. For the phosphorylated forms, the buffer pH was 7.5, in an attempt to stabilize the covalent phosphoryl linkage.

2.4. Crystallization of SpoIIAA and X-ray analysis

Crystallization conditions for SpoIIAA from the three species of Bacilli were established using a sparse-matrix screening approach and hanging-drop vapour diffusion. Briefly, 1 µl of concentrated protein was mixed with an equal volume of well solution and suspended on a siliconized glass cover slip above 1 ml of well solution at 291 K. For X-ray data collection, a single crystal was transferred directly into mother liquor supplemented where specified with a cryoprotectant (Table 1). The crystal was mounted in a small loop of fine rayon fibre and flash-frozen in a stream of nitrogen gas at 120 K. Diffraction data were integrated and scaled using the *HKL* suite of programs (Otwinowski & Minor, 1997) and all further data analysis was performed using programs

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Table 2
Data-collection statistics.

Crystal form	BstAA∼P	BspAA	BspAA∼P-I	BspAA∼P-II
Wavelength (λ)	0.87	0.93	0.98	1.54
X-ray source	PX9.6, SRS	ID14-EH2, ESRF	PX14.2, SRS	Rotating Cu anode
Detector	ADSC CCD	MAR CCD	ADSC CCD	MAR image plate
Space group	$P3_{(1/2)}2_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters: a, b, c (Å)	48.8, 48.8, 115.5	36.8, 53.5, 101.3	37.2, 52.1, 100.4	51.1, 61.5, 65.7
Resolution (highest shell)	3.0 (3.11–3.0)	1.61 (1.67–1.61)	2.25 (2.29–2.25)	2.44 (2.53–2.44)
No. of measurements	10128	118510	32567	29900
No. of unique reflections	3114	25927	9363	8075
Completeness (%)	90.2 (96.2)	96.4 (87.1)	97.9 (95.8)	99.5 (98.7)
Redundancy	3.25	4.57	3.48	3.70
R_{sym}	0.110 (0.147)	0.076 (0.24)	0.042 (0.135)	0.041 (0.074)
$I/\sigma(I)$	11.7 (6.5)	19.8 (5.4)	26.7 (8.2)	18.6 (9.8)
V_M for 1/2 molecules in the asymmetric unit (Å^3 Da^{-1})	3.5/1.8	3.9/1.9	3.7/1.9	4.0/2.0
Solvent content for 1/2 molecules in the asymmetric unit (%)	64.7/29.5	67.8/35.6	66.8/33.6	68.8/37.7

from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion: crystallographic analysis of SpoIIAA crystals

An extensive screen led to crystals of Bsu SpoIIAA with plate-like morphology which appeared within 48 h from 2 *M* $(NH_4)_2SO_4$ buffered with 100 m*M* Tris–HCl pH 8.5 in the presence of 200 m*M* NaCl, growing to maximum dimensions of 0.1 × 0.1 × 0.01 mm. Unfortunately, these crystals deteriorated rapidly, becoming unusable after a further 24 h. Diffraction of X-rays produced from a rotating copper-anode source was at best weak. It was possible in rare cases to determine that the crystals belonged to a primitive orthorhombic space group, with unit-cell parameters a = 51.9, b = 84.6, c = 153.9 Å. The weakness of the diffraction combined with the instability of the crystals prompted us to examine SpoIIAA from other species of Bacillus for their suitability for structural studies.

We first turned to SpoIIAA from the moderate thermophile *B. stearothermophilus*. Two crystal forms of Bst SpoIIAA were obtained. Both appeared overnight and had a rod-like morphology (Table 1). Crystal form I belongs to a primitive orthorhombic space group, with approximate unit-cell parameters a = 49, b = 58, c = 106 Å, while the form II crystals (Fig. 2*a*) are in space group $C2$, with approximate unit-cell parameters a = 111, b = 48, c = 107 Å, β = 101°. A trigonal bipyramidal crystal form of Bst SpoIIAA∼P was also obtained (Table 1; Fig. 2*b*). Despite the large size of these crystals, they do not diffract beyond 3 Å spacing even when exposed to synchrotron radiation (Table 2). Moreover, the diffraction from all the Bst crystals examined was very diffuse, with most crystals showing distinct signs of splitting and/or disorder. This problem could not be overcome despite different cryoprotection and crystal 'annealing' regimes being explored.

Since suitable crystals of Bsu and Bst SpoIIAA proved elusive, we targeted the protein from *B. sphaericus*, which has the greatest degree of divergence based on a phylogeny of known SpoIIAA sequences (Park & Yudkin, 1997). The initial crystals of Bsp SpoIIAA had plate-like morphology. Although they proved impossible to reproduce, they did provide effective microseeds for the successful growth of further batches of crystals (Fig. 2*c*). These crystals have yielded complete native data to 1.6 Å resolution (Table 2). Analysis of the diffraction data reveals that the crystals belong to the primitive orthorhombic space group $P2_12_12_1$.

Two crystal forms of Bsp SpoIIAA∼P (Bsp∼P) were obtained. The first was grown by streak seeding, with the initial microseeds taken from crystals of Bsp SpoIIAA. Crystals of the second form were grown more recently without the requirement for seeding (Table 1; Fig. 2*d*). Complete native data from both crystal forms have been acquired (Table 2). The space group and unit-cell parameters of the seeded Bsp∼P-I crystal form are similar to those of Bsp SpoIIAA from which they were seeded. However, we were unable to scale data sets from these two together, implying either a difference in molecular packing or differences in structure between the native and phosphorylated forms of SpoIIAA. To verify the presence and persistence of a covalently attached phosphoryl group, Bsp∼P crystals were washed, crushed and the dissolved protein analysed by non-denaturing gel electrophoresis. As shown in Fig. 3, the native and phosphorylated forms of *B. sphaericus* SpoIIAA are readily distinguished by this technique; moreover, the protein in the crystal is fully phosphorylated.

Solvent-content calculations suggest the presence of either one or two molecules in the asymmetric unit (Matthews, 1968; Table 2). Self-rotation functions calculated for both Bsp SpoIIAA and Bsp∼P-I with a variety of input parameters reveal no obvious non-crystallographic symmetry elements. Furthermore, it is unlikely that there are two molecules oriented similarly in the asymmetric unit because native Pattersons are featureless. In contrast, the native

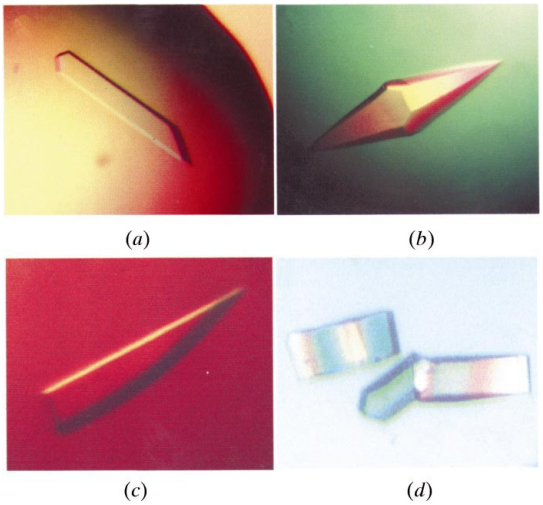

Figure 2
Crystal photomicrographs. (*a*) Bst SpoIIAA-II, (*b*) Bst SpoIIAA∼P, (*c*) Bsp SpoIIAA and (*d*) Bsp SpoIIAA∼P-II.

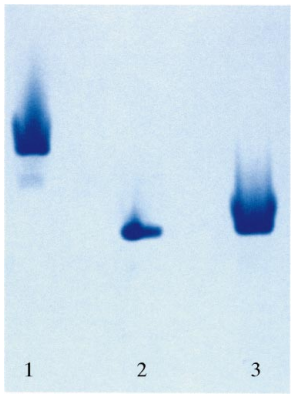

Figure 3
Coomassie-stained non-denaturing 8.75% polyacrylamide-gel electrophoresis (pH 8.8) of *B. sphaericus* SpoIIAA samples. Lanes 1 and 3 were loaded with samples of purified SpoIIAA and SpoIIAA∼P, respectively; lane 2 contains a sample of washed and dissolved BspAA∼P-II crystals.

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Patterson for Bsp∼P-II reveals the presence of a 15σ peak (11% of the origin height) at u = 0.12, v = 0, w = 0.5, which is apparent even at 2.5 Å spacing (Fig. 4). This indicates that there are two molecules in the asymmetric unit in approximately the same orientation.

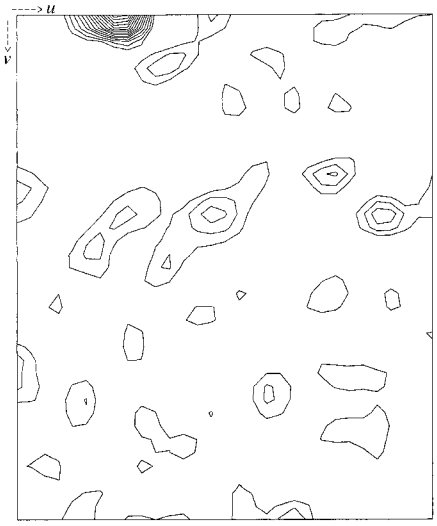

Figure 4
Native Patterson calculated using all X-ray data from crystal form Bsp∼P-II. The section shown is of w = 0.5 and is plotted across the asymmetric unit for space group $P2_12_12_1$. The peak at u = 0.12, v = 0, w = 0.5 is some 11% of the origin height and strongly suggests that there are two molecules in the asymmetric unit that are in approximately the same orientation.

We have attempted to solve the structure of Bsp SpoIIAA and SpoIIAA∼P by molecular replacement using the coordinates of Bsu SpoIIAA determined by multidimensional heteronuclear NMR as a search model (Kovacs *et al.*, 1998). However, this approach has not yielded satisfactory solutions. We have, therefore, prepared selenomethionine-substituted protein in preparation for a multiple wavelength anomalous dispersion experiment to determine the crystallographic phases for SpoIIAA. In combination, the structures of phosphorylated and non-phosphorylated SpoIIAA are expected to provide details on a molecular level as to how the activity of a crucial alternative sigma factor of Bacillus is regulated.

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