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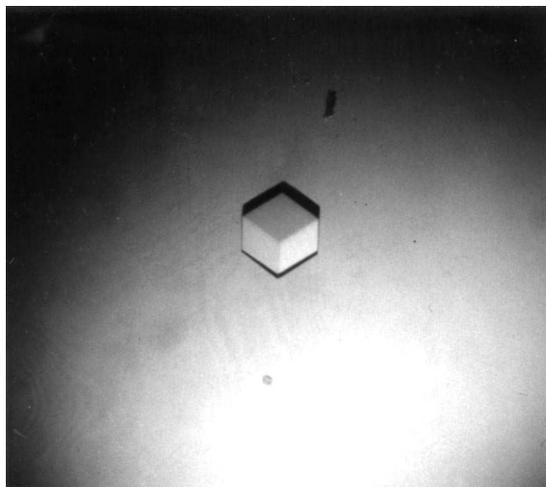
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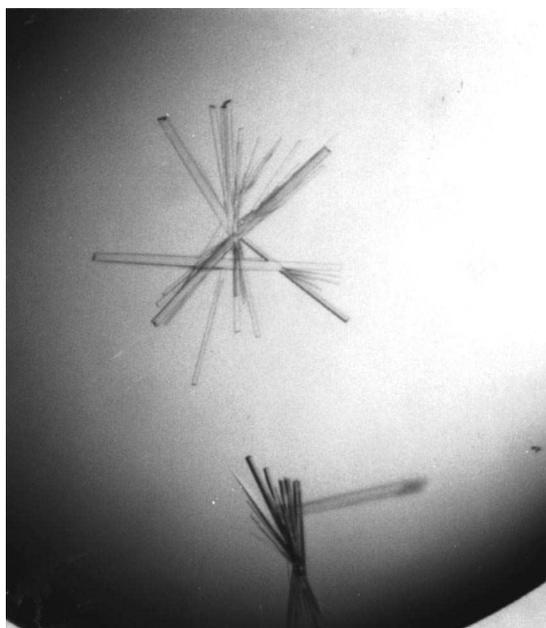




**Figure 2**  
Photomicrograph of the cubic crystals of the C-terminal fragment of Spo0A from *B. stearothermophilus*. The crystal shown is approximately  $0.1 \times 0.1 \times 0.1$  mm.



**Figure 3**  
Photomicrograph of the triclinic crystals of the C-terminal fragment of Spo0A from *B. stearothermophilus*.



**Figure 4**  
Photomicrograph of the crystals of the N-terminal fragment of Spo0A from *B. stearothermophilus*. The crystals shown are approximately  $0.3 \times 0.01 \times 0.01$  mm.

residue Asp56, which leads to expression of a specific set of genes essential for the entry into sporulation. These genes include *sinI* (Gaur *et al.*, 1988), *spoIIA* (Wu *et al.*, 1989), *spoIIE* (York *et al.*, 1992), *spoIIG* (Satola *et al.*, 1991) and *spo0A* itself (Strauch *et al.*, 1992). In addition, Spo0A-P is a repressor of gene expression, down-regulating the expression of AbrB, a regulatory protein that controls gene expression during the transition from exponential growth to stationary phase (Strauch & Hoch, 1993). The build-up of Spo0A-P to threshold levels triggers the developmental switch and commits the cell to sporulation.

Spo0A has a two-domain structure: the N-terminal or phospho-acceptor domain has a regulatory function, while the C-terminal domain is associated with DNA binding and transcriptional activation (Grimsley *et al.*, 1994). The N-terminal domain (N-Spo0A) shows significant sequence similarity to the CheY superfamily of phospho-acceptors, which include CheY, Spo0F, NarL and CheB, the three-dimensional structures of which are known (Volz & Matsumura, 1991; Madhusudan *et al.*, 1996; Baikalov *et al.*, 1996; Djordjevic *et al.*, 1998). The C-terminal domain of Spo0A (C-Spo0A) from *B. subtilis* shares no significant similarity to other sequences in the database, other than Spo0A homologues from other endospore-forming bacteria (Brown *et al.*, 1994), and it is not known which residues are responsible for making specific interactions with target promoter regions. The structure of this domain is obviously of great interest, as it could be associated with a novel mode of sequence-specific DNA recognition and will be vital in analysing the molecular mechanisms regulating entry into sporulation. In a previous comparative study of *spo0A* genes of endospore-forming bacteria, part of the gene encoding Spo0A from *B.*

*stearothermophilus* was cloned and sequenced (Brown *et al.*, 1994). Here, we describe the completed sequence and the purification of the intact protein and its two functional domains. We also describe the crystallization and preliminary crystallographic analysis of crystals of both the regulatory phospho-acceptor and the effector DNA-binding domains of Spo0A.

## 2. Materials and methods

### 2.1. Cloning and overexpression of *B. stearothermophilus* spo0A

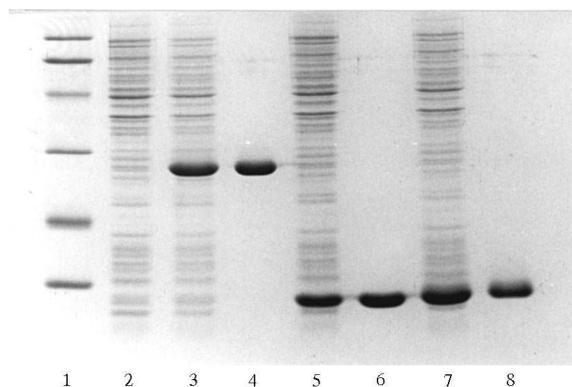
Specific primers known to anneal only within the most highly conserved regions of the effector domain of *spo0A* were used for PCR amplification from chromosomal *B. stearothermophilus* DNA (Brown *et al.*, 1994). The PCR product served as a high-stringency hybridization probe for Southern blots prepared with *Hind*III-digested genomic *B. stearothermophilus* DNA. The construction of a *B. stearothermophilus* subgenomic library of *Hind*III fragments 3.7–7.0 kbp in length into the plasmid pBR322, transformation into *E. coli* MM294 and subsequent screening of this library were all performed using standard protocols (Ausubel *et al.*, 1987). Candidate recombinant plasmids were sequenced to verify the presence of the *spo0A* homologue and the sequence deposited at the EMBL sequence database under the accession number AJ002297 (Fig. 1).

For high-level protein production, *spo0A* and fragments specifying the protein domains were subcloned by PCR methods into the T7-RNA polymerase-based expression vector (Studier & Moffatt, 1986) pET26b (Novagen) and expressed in *E. coli* strains BL21 (DE3) and B834 (DE3). Single-stranded plasmid DNA was prepared from all derivative clones and sequenced to ensure that no PCR-derived mutations had occurred. Cell cultures were grown in LB media containing  $100 \mu\text{g ml}^{-1}$  ampicillin or  $30 \mu\text{g ml}^{-1}$  kanamycin as appropriate. At an optical density of 0.6 at 600 nm, protein expression was induced by the addition of IPTG to a final concentration of 1 mM. After 4 h further growth, cells were harvested by centrifugation and the cell pellet frozen at 193 K until use.

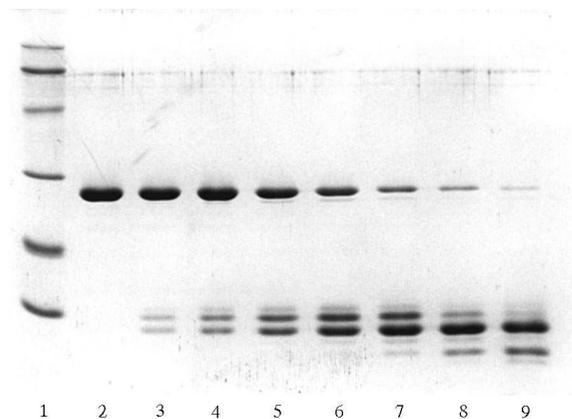
### 2.2. Purification and proteolysis of intact *B. stearothermophilus* Spo0A

Cells were resuspended in 50 ml of 25 mM MES buffer pH 5.6 containing 5 mM EDTA, 2 mM DTT, 1 mM AEBSF (aminoethyl benzenesulfonyl fluoride) before disruption

by sonication. The cell extract was clarified by centrifugation at  $19000 \text{ rev min}^{-1}$  for 60 min before loading onto an S-Sepharose column pre-equilibrated in buffer *A* (25 mM MES pH 5.6, 2 mM EDTA, 1 mM DTT). Spo0A was eluted with a linear gradient of 0–1 M NaCl. Fractions containing Spo0A were pooled, diluted 1 in 4 with buffer *A* and loaded onto a pre-equilibrated heparin-Sepharose column. Spo0A was eluted with a linear gradient of 0–1 M NaCl. Fractions containing Spo0A were pooled, concentrated and dialysed into a buffer of 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM DTT before further purification using a Superdex S75 16/60 gel-filtration column.



**Figure 5**  
17% SDS-PAGE analysis of the expression and purification of intact *B. stearothermophilus* Spo0A and its two domains. Lane 1, low molecular-weight markers (97.4, 66.2, 45, 31, 21.5 and 14.4 kDa); lane 2, total cell protein from uninduced *E. coli* BL21 (DE3) harbouring pET0ABst; lane 3, total cell protein from induced *E. coli* BL21 (DE3) harbouring pET0ABst; lane 4, purified intact *B. stearothermophilus* Spo0A; lane 5, total cell protein from induced *E. coli* BL21 (DE3) harbouring pETC0ABst; lane 6, purified C-terminal *B. stearothermophilus* Spo0A; lane 7, total cell protein from induced *E. coli* BL21 (DE3) harbouring pETN0ABst; lane 8, purified N-terminal *B. stearothermophilus* Spo0A.



**Figure 6**  
15% SDS-PAGE of the Spo0A fragments resulting from the series of trypsin-digest reactions. Lane 1, low molecular-weight markers (97.4, 66.2, 45, 31, 21.5 and 14.4 kDa); lanes 2 to 9 show intact Spo0A (10  $\mu\text{g}$ ) and protein digested with 0.25, 0.5, 1.25, 2.5, 5, 12.5 and 25 ng of trypsin, respectively.

For the preparation of the two individual domains, purified Spo0A protein was incubated with trypsin at a ratio of 2000:1 at room temperature in a buffer of 20 mM Tris-HCl pH 7.5, 10 mM  $\text{MgCl}_2$ , 100 mM NaCl, 1 mM DTT. Proteolysis was quenched after 30 min by the addition of the protease inhibitor AEBSF to a final concentration of 1 mM. The digested protein was immediately applied to a 1 ml HiTrap heparin-Sepharose column linked in series to a 1 ml HiTrap Q-Sepharose column pre-equilibrated in a buffer of 20 mM Tris-HCl pH 7.5, 1 mM DTT. Under these conditions, the C-terminal DNA-binding domain was adsorbed onto the heparin matrix, whereas the N-terminal phospho-acceptor domain passes directly through this column and is bound by the Q-Sepharose resin. The columns were separated and the proteins were eluted individually by the application of a 0–1 M NaCl gradient. Peptide sequencing and mass-spectrum analyses were performed by Jeff Keen of the University of Leeds using the PE Applied Biosystems 477A liquid-pulse peptide sequencer and MicroMass TOFSpec laser-desorption apparatus, respectively.

### 2.3. Purification of Spo0A domains

The protocols for the over-expression and purification of the individual phospho-acceptor and DNA-binding domains of Spo0A were adapted from those used for the intact protein. C-Spo0A protein was purified by a combination of S-Sepharose, heparin-Sepharose chromatography and, as the final step, Mono-S cation-exchange chromatography. N-Spo0A expressing cells were disrupted by sonication in 20 ml of 50 mM Tris-HCl pH 7.5, 2 mM DTT, 1 mM EDTA, 1 mM AEBSF (buffer *B*), the lysate was clarified by centrifugation and proteins were precipitated by the addition of ammonium sulfate to 40% saturation. After resuspending the pellet in buffer *B*,

ammonium sulfate was added to a final concentration of 1.5 M and the sample was loaded onto a phenyl-Sepharose column pre-equilibrated in the same buffer. N-Spo0A was eluted with a linear gradient of 1.5–0 M ammonium sulfate. Fractions containing N-Spo0A were pooled and dialysed against buffer *B* before further purification with Mono-Q anion-exchange chromatography.

### 2.4. Crystallization of the domains of Spo0A

For crystallization experiments, each of the two domains was desalted and concentrated to  $10 \text{ mg ml}^{-1}$  in a buffer of 10 mM Tris-HCl pH 7.5, 1 mM DTT. Initial crystallization conditions were screened by the 'sparse-matrix' approach at 289 K by hanging-drop vapour diffusion (Jancarik & Kim, 1991). Briefly, 1  $\mu\text{l}$  of concentrated protein was mixed with an equal volume of well solution and suspended on a siliconized glass cover slip over 1 ml of well solution.

Two different crystal forms of C-Spo0A were obtained. The first form (C-I) grew slowly over a period of 6–8 weeks from 1.6 M sodium/potassium phosphate in HEPES buffer at pH 7.5 to a maximum size of  $0.1 \times 0.1 \times 0.1 \text{ mm}$  and exhibited a cubic morphology (Fig. 2). The second C-terminal domain crystal form (C-II) grew typically from 10% (w/v) PEG 4000 and 50 mM  $\text{Li}_2\text{SO}_4$  buffered with 50 mM Tris-HCl pH 7.5. These crystals appeared after 2–3 d and continued to grow for a week, reaching a typical size of  $0.2 \times 0.1 \times 0.1 \text{ mm}$  (Fig. 3). Crystals of the N-terminal domain of Spo0A grow readily and quickly from 225 mM  $\text{CaCl}_2$  in MOPS buffer at pH 6.5. They are long but extremely thin with maximum dimensions  $0.3 \times 0.01 \times 0.01 \text{ mm}$  (Fig. 4). It might be significant that calcium chloride is the precipitant for these particular crystals and that divalent cations are essential for phosphoryl transfer and, presumably, electrostatic stabilization in this multi-component phosphorelay.

### 2.5. X-ray analysis

For X-ray analysis, a single crystal of C-I was harvested directly into mother liquor containing 25% ethylene glycol as a cryoprotective agent. After 2 min, the crystal was mounted in a small loop of fine rayon fibre and flash-frozen in a stream of  $\text{N}_2$  at 120 K. Similarly, a single crystal of C-II was transferred into mother liquor and the cryoprotectant, PEG 400, was gradually and slowly introduced to this mother liquor in steps of 5% up to a concentration of 20%, after which the crystal was mounted and frozen as

above. Complete native data were collected from both these crystals on an R-AXIS IIC imaging-plate detector using Cu  $K\alpha$  radia-

tion provided by a Rigaku rotating-anode generator equipped with MSC double-focusing mirrors.

Finally, a single crystal of N-Spo0A was harvested into mother liquor supplemented with 25% ethylene glycol as a cryoprotectant before being mounted and frozen in the manner described above. Complete native data were collected on a MAR Research imaging-plate detector using synchrotron radiation on the EMBL BW7B beamline at the DORIS storage ring, DESY, Hamburg at a wavelength of 0.84 Å. Data in all cases were processed with the program *DENZO* (Otwinowski & Minor, 1997) and scaled with the program *SCALEPACK* (Otwinowski & Minor, 1997). All other data analyses were performed with the *CCP4* suite of programs (Collaborative Computational Project, Number 4, 1994).

### 3.2. Analysis of the sequence of *B. stearothermophilus* Spo0A

Analysis of the amino-acid sequence of *B. stearothermophilus* Spo0A reveals a high degree of similarity to the sequence of *B. subtilis* Spo0A, indicating that the structures of domains of *B. stearothermophilus* Spo0A will be virtually indistinguishable from those of its extensively characterized *B. subtilis* homologue. Approximately 110 highly conserved amino acids comprise the C-terminal domain and ~120 slightly less well conserved residues describe the N-terminal domain. These are linked by a stretch of ~30 poorly conserved amino acids which are believed to constitute a flexible hinge region (highlighted in Fig. 1; Brown *et al.*, 1994). Residues in the 'aspartic acid pocket', D10, D11 and D56 (the site of phosphorylation), are all conserved in the *B. stearothermophilus* Spo0A protein, as is K106, a residue which is believed to be involved in the activation of the effector domain. A variety of point mutations in the N-terminal domain of *B. subtilis* Spo0A which can suppress substitutions/deletions in other components of the phosphorelay have been described. These include *coi*, *sob*, *sof* and *sos* (Spiegelman *et al.*, 1995, and references cited therein). Similarly, mutations that cause a sporulation-deficient phenotype in the C-terminal domain of *B. subtilis* Spo0A have also been isolated *e.g.* *suvs*, *spo0A9V* and *spo0A153* (Spiegelman *et al.*, 1995, and references cited therein). The amino-acid residues affected are conserved in Spo0A from *B. stearothermophilus*. Therefore, all the residues which have so far have been implicated in the function of *B. subtilis* Spo0A are conserved in Spo0A from *B. stearothermophilus*.

## 3. Results and discussion

### 3.1. Molecular cloning and expression of *B. stearothermophilus* spo0A

PCR amplification from *B. stearothermophilus* chromosomal DNA generated a 270 bp product that was used as a hybridization probe to Southern blots prepared with *Hind*III-digested genomic *B. stearothermophilus* DNA. Screening of a subgenomic library led to the isolation of 21 putative positive clones, six of which were analysed further and shown to harbour a 4 kbp insert. DNA sequencing verified the presence of the complete *spo0A* gene. The gene encoding Spo0A was sub-cloned into plasmid pET26b using PCR both to introduce convenient restriction sites for cloning (*Nde*I and *Bam*HI) and to modify the initiation codon from TTG to ATG. This construct, pET0ABst, directed high levels of soluble protein production in suitable *E. coli* expression strains (Fig. 5).

### 3.3. Proteolysis of intact *B. stearothermophilus* Spo0A

Limited proteolytic digestion of intact Spo0A yields two N-terminal fragments of  $M_r = 14600$  and  $15400$  and two C-terminal fragments of  $M_r = 13400$  and  $14200$ , as determined by mass spectrometry and peptide sequencing. Five cycles of amino-acid sequencing of the C-terminal domains of Spo0A gave the sequences AAPAP and DNKPK. These sequences indicate that trypsin has cleaved immediately after residues Lys130 and Arg138, generating two fragments with predicted  $M_r$  of 14250 and 13460, respectively, in agreement with the mass-spectrum data. The two corresponding N-terminal fragments, residues 2–130 and 2–138, have expected  $M_r$  values of 14590 and 15360, respectively, which is again consistent

Section  $l = 0$

BS

Section  $k = 0$

AS

Section  $h = 0$

CS

**Figure 7**

Pseudo-precession photographs of the three zero layers from C-Spo0A crystal form C-II.

**Table 1**  
Data-collection statistics.

Values in parentheses refer to the highest resolution shell.

Crystal form	C-Spo0A C-I	C-Spo0A C-II	N-Spo0A
Space group	$P2_13$	$P1$	$P2_12_12_1$
Resolution (Å)	15–4.0 (4.14–4.0)	25–2.5 (2.59–2.5)	30–2.0 (2.03–2.0)
Number of measurements	40417	27379	173534
Number of unique reflections	8675	13688	39774
Redundancy	4.7	2.0	4.4
Completeness (%)	99.9 (100)	95.7 (83.2)	99.9 (99.9)
$R_{\text{sym}}^\dagger$	0.122 (0.278)	0.042 (0.104)	0.085 (0.293)
$I/\sigma(I)$	10.8 (5.2)	16.1 (7.0)	17.5 (5.2)

$$^\dagger R_{\text{sym}} = \frac{\sum_{hkl} \sum_i |I_{hkl} - \langle I_{hkl} \rangle|}{\sum_{hkl} \sum_i \langle I_{hkl} \rangle}$$

with the mass-spectrum data. Trypsin cleavage therefore takes place within the non-conserved hinge region of Spo0A connecting the two putative domains. Cleavage after the residue equivalent to Arg138 of *B. stearothermophilus* Spo0A has been observed in tryptic proteolysis experiments of *B. subtilis* Spo0A (Grimsley *et al.*, 1994; Fig. 1). Based on these results, fragments of *spo0A* which encode the N-terminal (residues 1–130) or C-terminal (139–260) domains of Spo0A were amplified by PCR and subcloned into pET26b for high-level protein expression in suitable strains of *E. coli* (Fig. 5). Proteins were purified to homogeneity as judged by electrophoretic methods.

### 3.4. Crystallographic analysis of crystals of the C-terminal domain

Analysis of the diffraction data from C-Spo0A crystal form C-I reveals that the crystal belongs to the primitive cubic space group  $P2_13$ , with unit-cell dimensions  $a = b = c = 145.1 \text{ \AA}$  (Table 1). The  $I/\sigma(I)$  in the outermost shell (4.14–4.0 Å) of these data is quite high (5.2), suggesting that with a more intense source of X-rays higher order reflections may be successfully recorded. Assuming that there are eight molecules in the asymmetric unit, the  $V_m$  of these crystals is  $2.77 \text{ \AA}^3 \text{ Da}^{-1}$  and the solvent content is 47.0%, which is close to the most commonly observed values in protein crystals (Matthews, 1968). With between four and 12 molecules ( $M_r = 13400$ ) in the asymmetric unit, the  $V_m$  of these crystals would range from 4.75 to  $1.58 \text{ \AA}^3 \text{ Da}^{-1}$  and the solvent content from 73.9 to 21.0%, well within the observed range for protein crystals (Matthews, 1968). Self-rotation functions calculated with a variety of input parameters do not unambiguously reveal the presence of more than one non-crystallographic twofold axis. The native Patterson is featureless. Furthermore, the C-terminal domain exists as a monomer in solution, as

determined by size-exclusion chromatography, and thus it is difficult to determine with any certainty the exact number of molecules in the asymmetric unit.

Careful examination of the diffraction data from C-Spo0A form C-II revealed that these crystals are in the triclinic space group  $P1$  with unit-cell parameters  $a = 43.5$ ,  $b = 53.6$ ,  $c = 53.8 \text{ \AA}$ ,  $\alpha = 89.8$ ,  $\beta = 111.9$ ,  $\gamma = 111.6^\circ$  (Table 1). These curious cell dimensions resemble those expected from the triclinic indexing of a C-centred monoclinic crystal. However, further processing of the data collected from one orientation of a single crystal proved impossible to reduce successfully in any space group other than  $P1$ . Furthermore, the  $(hk0)$ ,  $(h0l)$  and  $(0kl)$  layers reveal a lack of symmetry in the diffraction pattern (Fig. 7) and we are therefore certain that the space group is  $P1$ . Assuming that there are three molecules in the asymmetric unit, the  $V_m$  of these crystals is  $2.66 \text{ \AA}^3 \text{ Da}^{-1}$  and the solvent content is 53.4%, which is similar to the most commonly observed values in protein crystals (Matthews, 1968). A large peak, some 80% of the origin peak height on the  $\kappa = 120^\circ$  section, was observed in Patterson self-rotation studies, which is compatible with the premise that there are three molecules per asymmetric unit.

### 3.5. Crystallographic analysis of crystals of the N-terminal domain

A native data set was initially collected from a frozen crystal of N-Spo0A using a rotating-anode source of X-rays, which indicated that the crystals exhibit primitive orthorhombic symmetry with unit-cell dimensions  $a = 69.3$ ,  $b = 73.0$ ,  $c = 114.5 \text{ \AA}$ . Analysis of the reflections along the principal axes revealed the presence of three orthogonal twofold screw axes, defining the space group as  $P2_12_12_1$ . However, the crystal diffracted X-rays weakly: even with hour-long exposures, it was possible to measure reflections to only  $3.5 \text{ \AA}$  spacing [overall

$R_{\text{sym}} = 0.139$ ,  $I/\sigma(I) = 7.6$ ; in the highest resolution shell these values are 0.287 and 3.7, respectively]. The same frozen crystal was subsequently exposed to synchrotron radiation, allowing data to be extended to  $2.0 \text{ \AA}$  resolution (Table 1). These data confirm the space group to be  $P2_12_12_1$  and underline the advantages of using synchrotron radiation in macromolecular crystallography.

Assuming that there are four molecules ( $M_r$  of 14700) in the asymmetric unit, the  $V_m$  of these crystals is  $2.45 \text{ \AA}^3 \text{ Da}^{-1}$  and the solvent content is 49.3%. Patterson self-rotation studies reveal the presence of a large peak,  $\sim 90\%$  of the origin peak height, on the  $\kappa = 90^\circ$  section, which is consistent with the premise that there are four molecules per asymmetric unit. Furthermore, analysis of the  $(00l)$  layer reveals that reflections of the order  $004n$  are mostly strong and reflections of the order  $002n$  are mostly weak, consistent with a pseudo-fourfold screw axis of symmetry. We have been unable to observe a tetrameric form of this domain in solution, and thus believe that the four molecules in the asymmetric unit are merely arranged in an interesting way.

A search for heavy-metal derivatives of crystals of both the functional domains of Spo0A is now under way. In the absence of suitable crystals of the intact protein, we hope that the structures of the two domains of Spo0A will, in combination, provide detailed information on the way that Spo0A regulates late growth responses in *Bacillus*, on how specific DNA-promoter sequences are recognized, on how its function as a DNA-binding protein is regulated by phosphorylation and on the role of divalent cations in phosphoryl transfer. Furthermore, insights into the conformational switch brought about by phosphorylation of Spo0A will have broader implications for our understanding of signal transduction mediated through the large family of two-domain response-regulator proteins.

We would like to thank Alfred Antson and Paul Gollnick for collecting X-ray data at DESY on N-Spo0A, Zbyszek Dauter and Eleanor Dodson for help in analysing the C-Spo0A X-ray data, referee 2 for some insightful comments regarding the X-ray data, Ian Evans for technical assistance and Jeff Keen of the University of Leeds for peptide sequencing and mass-spectrum analysis. This work has been supported by the Wellcome Trust, grant 047031/Z/96/Z/PMG/MJD, by grant 2027 from the Slovak Academy of Sciences, the EU Programme

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