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Dimer-induced signal propagation in Spo0A

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Summary

Spo0A, the response regulator protein controlling the initiation of sporulation in *Bacillus*, has two distinct domains, an N-terminal phosphoacceptor (or receiver) domain and a C-terminal DNA-binding (or effector) domain. The phosphoacceptor domain mediates dimerization of Spo0A on phosphorylation. A comparison of the crystal structures of phosphorylated and unphosphorylated response regulators suggests a mechanism of activation in which structural changes originating at the phosphorylatable aspartate extend to the $\alpha_4\beta_5\alpha_5$ surface of the protein. In particular, the data show an important role in downstream signalling for a conserved aromatic residue (Phe-105 in Spo0A), the conformation of which alters upon phosphorylation. In this study, we have prepared a Phe-105 to Ala mutant to probe the contribution of this residue to Spo0A function. We have also made an alanine substitution of the neighbouring residue Tyr-104 that is absolutely conserved in the Spo0As of spore-forming Bacilli. The *spo0A(Y104A)* and *spo0A(F105A)* alleles severely impair sporulation *in vivo*. *In vitro* phosphorylation of the purified proteins by phosphoramidate is unaffected, but dimerization and DNA binding are abolished by the mutations. We have identified intragenic suppressor mutations of *spo0A(F105A)* and shown that these second-site mutations in the purified proteins restore phosphorylation-dependent dimer formation. Our data support a model in which dimerization and signal transduction between the two domains of Spo0A are mediated principally by the $\alpha_4\beta_5\alpha_5$ signalling surface in the receiver domain.

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Introduction

Sporulation in *Bacillus subtilis* is controlled by an expanded two-component signal transduction system called a phosphorelay (Trach *et al*., 1991). Environmental signals trigger the autophosphorylation on histidine of up to five sensor kinases (Ireton *et al*., 1993; Jiang *et al*., 2000). The phosphoryl group is then transferred sequentially from the kinase to Spo0F, Spo0B and, finally, to the response regulator Spo0A. Spo0A consists of a receiver domain, which is phosphorylated on Asp-56 by the phosphorelay components, and an effector domain, which is capable of binding to specific DNA sequences, called 0A boxes, and regulating transcription. For the switch from stationary phase to sporulation to take place, a threshold concentration of phosphorylated Spo0A (Spo0A~P) has to be attained (Chung *et al*., 1994). The importance of Spo0A as an activator and a repressor of transcription of many stationary phase as well as sporulation-specific genes is emphasized by the finding that more than 500 transcripts in *B. subtilis* are dependent on Spo0A (Fawcett *et al*., 2000).

Spo0A dimerizes upon phosphorylation, with dimer formation mediated principally by the N-terminal receiver domain (Asayama *et al*., 1995; Lewis *et al*., 2002). The crystal structures of the receiver (N-Spo0A) and effector (C-Spo0A) domains of Spo0A from *Bacillus stearothermophilus* have been solved (Lewis *et al*., 2000a,b), the former in the phosphorylated form (Lewis *et al*., 1999). Recently, insight into the mode of DNA binding by Spo0A has emerged with the determination of the crystal structure of the effector domain of Spo0A in complex with a duplex oligonucleotide representing part of the *abrB* promoter (Zhao *et al*., 2002). Although the isolated effector domain is a monomer, in the structure of the DNA complex, C-Spo0A forms a dimer. It is also known that the isolated effector domains, created by genetic deletion or proteolysis, are capable of binding to DNA and altering the levels of transcription of Spo0A-controlled genes (Spiegelman *et al*., 1995). Unphosphorylated Spo0A does not bind to 0A boxes *in vitro* (Ladds *et al*., 2003), and thus cannot regulate transcription. Other experiments showed that the Spo0A effector domain alone is able to function as the transcription regulator in *B. subtilis* cells (Florek *et al*., 2002). It would appear that, in common with

other response regulators (Ellison and McCleary, 2000), the function of the Spo0A receiver domain is to inhibit the effector domain, and that phosphorylation serves to overcome this inhibition. What are unknown at present are (i) the nature of the intersubunit interactions in Spo0A dimers; (ii) the nature of the interdomain interactions responsible for inhibition in unphosphorylated Spo0A; and (iii) whether a common surface on the receiver domain is responsible for both sets of interactions.

In this study, we have prepared alanine substitutions of Tyr-104 and Phe-105 in order to investigate signalling between the N- and C-domains in Spo0A. Comparison of the crystal structure of N-Spo0A~P with structures of unphosphorylated response regulators suggested that the structural changes associated with phosphorylation of Spo0A are not extensive (Lewis *et al*., 1999). The most obvious change is a reorientation of the side-chain of Thr-84, which moves in the order of 5 Å so that its hydroxyl group makes a charge–dipole contact to an oxygen of the phosphoryl group. The repositioning of Thr-84 is accompanied by a structural rearrangement of the side-chain of Phe-105, which is redirected from an outward to an inward orientation with respect to the core of the receiver domain (Lewis *et al*., 1999). The available evidence from studies of other systems suggests that this 'aromatic switch' is a conserved aspect of signalling in response regulators (Birck *et al*., 1999; Cho *et al*., 2001; Lee *et al*., 2001; Park *et al*., 2002; Gardino *et al*., 2003; Hastings *et al*., 2003). To evaluate the contribution of Phe-105 to signalling in Spo0A directly, this residue was mutated to alanine. We also noted that the preceding residue, Tyr-104, which is on the surface of N-Spo0A~P, is invariant in Spo0A orthologues, although it is not conserved in the wider response regulator family. This suggests an important function for Tyr-104 that is confined to Spo0A and, accordingly, this amino acid residue was also substituted with alanine. We have analysed the effects of these mutations on Spo0A function *in vivo* and *in vitro*. These and other data are discussed in terms of a structural model for dimer formation and signal propagation.

Results

Alanine substitution of Tyr-104 and Phe-105

To evaluate the functional importance of Tyr-104 and Phe-105, we have introduced alanine substitutions at these positions by site-directed mutagenesis. The mutated *spo0A* alleles were introduced into the chromosome of wild-type *B. subtilis* PY79 by single cross-over recombination, creating the strains IB658 and IB659 respectively (Table 1). The two mutations have severe effects on sporulation, similar to that in strain IB220 in which the *spo0A* gene is disrupted (Table 2). Relative to the wild-type strain, the sporulation efficiency of the mutants is reduced 10^4- to 10^5-fold. To investigate the mutations further, recombinant Spo0A(Y104A) and Spo0A(F105A) were overproduced in *Escherichia coli* and purified to homogeneity for biophysical and biochemical assays.

Spo0A(Y104A) and Spo0A(F105A) fail to form dimers

Size exclusion chromatography on a Superdex 75 HR 10/30 gel filtration column was used to determine the oligomeric state of Spo0A(Y104A) and Spo0A(F105A). Both mutant proteins eluted from this column in the same volume as wild-type Spo0A, indicating that they are monomers (Fig. 1A–C and Table 3). To examine the phosphorylated forms, the three proteins were treated with 50 mM phosphoramidate. The elution volume of phosphoramidate-treated wild-type Spo0A decreased from 10.7 ml to 9.3 ml, indicating an increase in mass, which we have shown previously to result from dimer formation (Lewis *et al*., 2002). The elution volumes of the two mutant proteins showed a much smaller reduction, suggesting that these proteins are principally monomers. After treatment of all three samples with Spo0E, the Spo0A phosphate-specific phosphatase, the elution volumes were restored to those before treatment with phosphoramidate (data not shown).

Sedimentation velocity analytical ultracentrifugation (AUC) experiments were performed on purified preparations of wild-type Spo0A, Spo0A(Y104A) and Spo0A(F105A). The molecular weights of the three proteins were observed to be 27.2, 26.7 and 25.3 kDa, respectively, confirming that all three proteins are monomers (Table 3). Subsequent AUC analysis of the phosphoramidate-treated proteins gave observed molecular weights of 28.4 and 27.3 kDa for Spo0A(Y104A)~P and Spo0A(F105A)~P respectively. Two species with molecular weights of 25.3 and 48.4 kDa were observed for wild-type Spo0A (Table 3). The data suggest that a significant proportion of the wild-type Spo0A sample forms dimers upon exposure to phosphoramidate. In contrast, the mutant proteins remain as monomers. The absence of dimers of the Ala-104 and Ala-105 mutants may be caused by their failure to (i) be significantly phosphorylated in the presence of phosphoramidate or (ii) assemble into dimers after phosphorylation.

Spo0A(Y104A) and Spo0A(F105A) are not impaired in phosphorylation

To establish whether Spo0A(Y104A) and Spo0A(F105A) are phosphorylated by phosphoramidate, we used a non-denaturing polyacrylamide gel electrophoresis (PAGE) assay. The phosphorylated and unphosphorylated forms of Spo0A have very low mobilities in conventional poly-

Table 1. Bacterial strains, plasmids and oligonucleotides used in this study.

Strain or name	Genotype, phenotype, sequence and relevant characteristics	Source or reference
E. coli		
MM294	*endA1 hsdR17 supE44 thi-1 recA*$^+$	Backman *et al.* (1976)
JM110	*rpsL thr leu thi-1 lacY galK galT ara tonA tsx dam dcm supE44* Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]	Yanish-Perron *et al.* (1985)
BL21(DE3)	*hsdS gal* (λ*c*lts857 *ind*1 *S*am7 *nin5 lacUV5*-T7 gene1)	Novagen
B. subtilis		
PY79	Prototroph	Youngman (1990)
IB220	*spo0A::kan*	Schmeisser *et al.* (2000)
IB658	PY79::pBG15YA, Spo$^-$	This work
IB659	PY79::pBG15FA, Spo$^-$	This work
IB685	Spo$^+$ mutant after treatment of IB659 with EMS Spo0AF105AT94M	This work
IB686	Spo$^+$ mutant after treatment of IB659 with EMS Spo0AF105AQ121R	This work
IB687	Spo$^+$ mutant after treatment of IB659 with EMS Spo0AF105AH162R	This work
IB690	Spo$^+$ mutant after treatment of IB659 with EMS Spo0AF105AQ90R	This work
Plasmids		
pBG15	Amp^r, Cm^r, pGEM-3zf(+)-cat-1, promoterless *spo0A* gene	Youngman (1990)
pBG15YA	Same as pBG15 with mutation Spo0AY104A	This work
pBG15FA	Same as pBG15 with mutation Spo0AF105A	This work
pET26b(+)	Km^r, *lacI*; T7 promoter	Novagen
pET0AY	Km^r, *lacI*; T7 promoter, *spo0AY104A*	This work
pET0AF	Km^r, *lacI*; T7 promoter, *spo0AF105A*	This work
pET0ASF1	Km^r, *lacI*; T7 promoter, *spo0AF105AT94M*	This work
pET0ASF2	Km^r, *lacI*; T7 promoter, *spo0AF105AQ121R*	This work
pET0ASF3	Km^r, *lacI*; T7 promoter, *spo0AF105AQ90R*	This work
pET0ASF4	Km^r, *lacI*; T7 promoter, *spo0AF105AH162R*	This work
Oligonucleotides		
R1	5′-TGAGAATAAAGGCGGACGCGCCT-3′ introduces Tyr-104Ala mutation	
R2	5′-TGAGAATAGCGTAGGACGCGCCT-3′ introduces Phe-105Ala mutation	
MUT7	5′-GCTCGGTACCCGGAGATCC-3′ eliminates *Bam*HI site of pBG15	
M4	5′-GTTTTCCCAGTCACGAC-3′	
RV	5′-CAGGAAACAGCTATGAC-3′	
N0A	5′-GGAGGAAGACATATGGAGAAAATTAAAGTTTGTGT-3′ introduces *Nde*I site	
C0A	5′-ATAAGCTCAAGCTTAAGAAGCCTTATGCTCTAACCT-3′ introduces *Hin*dIII site	
ABRBTOP	5′-GGATTTTGTCGAATAATGACGAAGAAAAAT-3′	
ABRBBOT	5′-ATTTTTCTTCGTCATTATTCGACAAAATCC-3′	
ATNESCC	5′-TTAATATTCCTTATATAT-3′	
ATNESGG	5′-ATATATAAGGAATATTAA-3′	

acrylamide gels, and the two forms are not resolved. We therefore treated the samples with limiting concentrations of trypsin, which cleaves Spo0A in the linker region that separates the N- and C-terminal domains (Grimsley *et al.*, 1994; Muchová *et al.*, 1999). The isolated effector domain fragment has a very low mobility in these native gels; moreover, the phosphorylated and unphosphorylated forms of the receiver domain can be clearly resolved. As

Table 2. Sporulation efficiency of strains with mutations in Spo0A compared with wild-type *B. subtilis.*

Strain	Mutation in Spo0A	Other name	Location	Sporulation efficiency (%)
PY 79	Wild type	–		100
IB658	Y104A	–	β5	0.01
IB659	F105A	–	β5	0.002
IB220	*spo0A::kan*	–		0.0004
IB685 (*sfa*-1)	F105A, T94M	–	β5, α4	56
IB686 (*sfa*-2)	F105A, Q121R	*sof*-108	β5, α5	62
IB687 (*sfa*-4)	F105A, H162R	*suv*-4	β5, αA	17
IB690 (*sfa*-3)	F105A, Q90R	*coi*-15	β5, β4–α4 loop	18

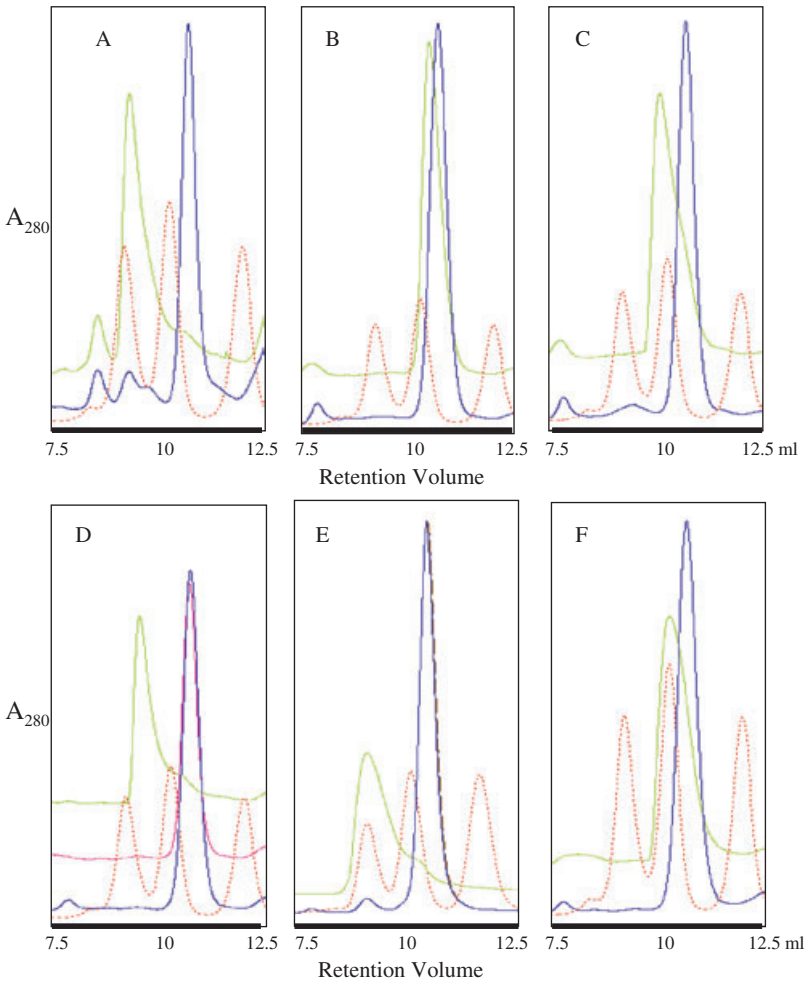

Fig. 1. Gel filtration chromatograms of Spo0A proteins. Blue, untreated Spo0A proteins; green, Spo0A proteins after incubation for 30 min in the presence of 50 mM phosphoramidate.
A–F. Chromatograms for the following unphosphorylated and phosphorylated Spo0A proteins: (A) wt Spo0A; (B) Spo0A(Y104A); (C) Spo0A(F105A); (D) Sfa-1; (E) Sfa-3; and (F) Sfa-4. The column was run in 20 mM Tris-HCl buffer (pH 7.5), 10 mM $MgCl_2$ and 250 mM NaCl. The absorbance at 280 nm was recorded in a flow cell with a path length of 2 mm. The solid red trace in (D) corresponds to phosphorylated Sfa-1 protein after treatment with the phosphatase Spo0E. Sfa-2 behaved in the same way as Sfa-3 (E, data not shown). The dotted red lines are the chromatograms for a mixture of molecular weight standards, these being, from left to right, bovine serum albumin (67 000 Da), ovalbumin (43 000 Da) and chymotrypsinogen (25 000 Da). Blue dextran (≈ 2000 kDa) was used in order to determine the void volume of the column.

may be seen in Fig. 2 (lanes 1 and 2), treatment of wild-type Spo0A with phosphoramidate causes the mobility of the receiver domain fragment to be noticeably lowered, its R_f value (relative to bromophenol blue) changing from 0.56 to 0.48. Treatment of the Spo0A(F105A) (Fig. 2, lanes 4 and 5) and Spo0A(Y104A) (Fig. 2, lanes 7 and 8) mutant proteins with phosphoramidate also changed the mobility of the receiver domain fragments generated by proteolysis. In these cases, the change is more modest from $R_f = 0.56$ for the unphosphorylated species to $R_f = 0.52$ for the phosphorylated forms.

The lowering of the gel mobility caused by phosphoramidate is more striking for wild-type Spo0A, consistent with the observation that it forms a dimer upon phosphorylation whereas the two mutant proteins do not. It has been shown that dimer formation is mediated by the

Table 3. Molecular weights of Spo0A proteins obtained by gel filtration and AUC.

	Unphosphorylated		Phosphorylated	
Spo0A protein	GF retention volume (ml)	AUC molecular weight (kDa)	GF retention volume (ml)	AUC molecular weight (Da)
Wild type	10.7	27.2	9.3	25.3, 48.4
Y104A	10.7	26.7	10.5	28.4
F105A	10.7	25.3	10.1	27.3
F105A, Q90R (Sfa-3)	10.7	27.3, 25.2[a]	9.8	25.2, 48.2
F105A, T94M (Sfa-1)	10.7	27.2	9.5	45.6
F105A, Q121R (Sfa-2)	10.7	28.0	9.4	32.1, 57.6
F105A, H162R (Sfa-4)	10.7	24.8	10.3	29.0, 48.0

a. Treated with phosphoramidate and then Spo0E94.

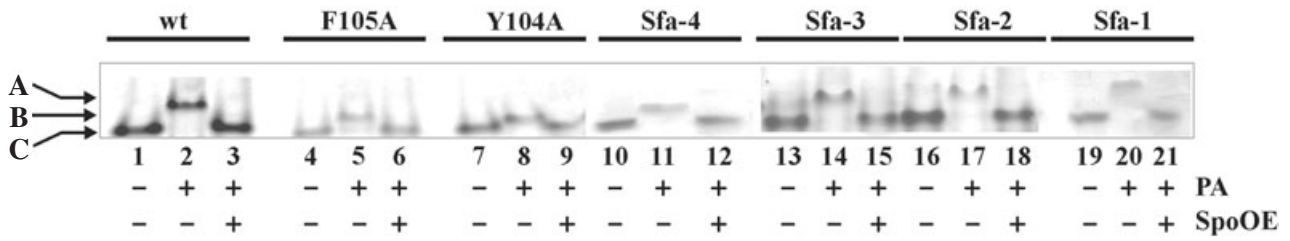

Fig. 2. Phosphorylation of wt Spo0A and mutant proteins. Non-denaturing 12.5% PAGE of the Spo0A fragments resulting from digest reactions with trypsin in a ratio of 2500:1. Lanes 1, 4, 7, 10, 13, 16 and 19 represent non-phosphorylated N-Spo0A protein domains. Lanes 2, 5, 8, 11, 14, 17 and 20 are phosphorylated N-Spo0A protein domains after treatment with phosphoramidate. Lanes 3, 6, 9, 12, 15, 18 and 21 are N-Spo0A protein domains after treatment with phosphoramidate and subsequent dephosphorylation by Spo0E94. The positions of different forms of N-Spo0A are marked at the left-hand side (A, dimer form of phosphorylated N-Spo0A; B, monomer form of phosphorylated N-Spo0A; C, monomer form of non-phosphorylated N-Spo0A. The presence of 50 mM phosphoramidate (PA) and/or 5 μM Spo0E94 is indicated + and –, respectively, at the bottom of the figure. Each lane contains 4 μg of protein.

receiver domain (Lewis *et al*., 2002). The decrease in the gel mobility of the receiver domains of the Spo0A mutants upon phosphorylation results from an increase (+1) in the net charge of the migrating species; the additional negative charge arising from phosphorylation of the carboxylate is offset by the +2 charge of a tightly bound magnesium ion. A decrease in gel mobility was also observed upon phosphorylation of Spo0F (Zapf *et al*., 1996), which has a very similar tertiary structure to N-Spo0A (Madhusudan *et al*., 1997), even though Spo0F is a monomer in both unphosphorylated and phosphorylated forms. Thus, the native gel electrophoresis method allows us to distinguish three different forms of N-Spo0A: phosphorylated dimers, phosphorylated monomers and unphosphorylated monomers (Fig. 3, arrows A–C).

To prove that the diminished gel mobilities result from phosphorylation of the active site aspartates, the phosphoramidate-treated protein samples were incubated with Spo0E as described in *Experimental procedures*. It can be seen in Fig. 2 (lanes 3, 6 and 9) that the gel mobilities of the tryptic fragments of all three proteins are restored to those of the unphosphorylated receiver domain fragments. We conclude that each of the three proteins is phosphorylated by phosphoramidate and that the phosphorylated forms are substrates for Spo0E regardless of whether or not they form dimers.

Spo0A(Y104A) and Spo0A(F105A) do not bind to the abrB *promoter*

To examine whether the mutant proteins can bind to DNA, we constructed a 30 bp duplex DNA fragment derived from the *abrB* promoter and containing a 0A box sequence (5′-TGACGAA-3′) using the synthetic oligonucleotides ABRBTOP and ABRBBOT (Table 1). This DNA fragment was used in gel mobility shift assays. Expression of *abrB* is repressed by Spo0A~P early in sporulation, and the interactions of Spo0A~P with 0A boxes at this promoter have been studied in detail (Strauch *et al*., 1990). As shown in Fig. 3A (lane 3), the *abrB* promoter DNA fragment is shifted efficiently by 2.5 ng μl^{-1} wild-type Spo0A~P. The same concentrations of Spo0A(Y104A)~P and Spo0A(F105A)~P fail to bring about a gel mobility shift. Weak binding of the mutant proteins is observed only at 10-fold higher concentrations (Fig. 3B and C). No binding to DNA was detected upon incubation with unphosphorylated wild-type Spo0A even at elevated protein concentrations (data not shown). The specificity of the interaction between Spo0A~P and the *abrB* promoter was demonstrated in competition assays in which unlabelled specific and non-specific DNAs were included in the incubation mixes. The non-specific competitor DNA, formed from oligonucleotides ATNESCC and ATNESGG (Table 1), was not able to compete with ^{32}P-labelled *abrB* DNA (0.19 pmol) in DNA–Spo0A~P complex formation even when present in 100-fold molar excess (19 pmol) (Fig. 3D). The presence of Spo0A~P in the retarded ^{32}P-labelled *abrB* DNA band was confirmed by Western blotting, probing with a polyclonal anti-Spo0A antibody (data not shown).

The search for suppressor mutations

If the sporulation defect resulting from the *spo0A(F105A)* and *spo0A(Y104A)* mutations is caused by the inability of the encoded proteins to dimerize upon phosphorylation, second-site mutations in the dimer-forming surface might be able to compensate for the F105A and Y104A substitutions and restore sporulation. We therefore performed an *in vivo* search for suppressor mutations as described in *Experimental procedures*. In our search for suppressors of *spo0A(F105A)*, six well-growing colonies were isolated that exhibited a clear Spo$^+$ phenotype. Back-transformation experiments showed that the sites of all six compensatory suppressor mutations were linked to *spo0A*. Amplification of the *spo0A* region of each of these Spo$^+$ colonies by polymerase chain reaction (PCR) methods

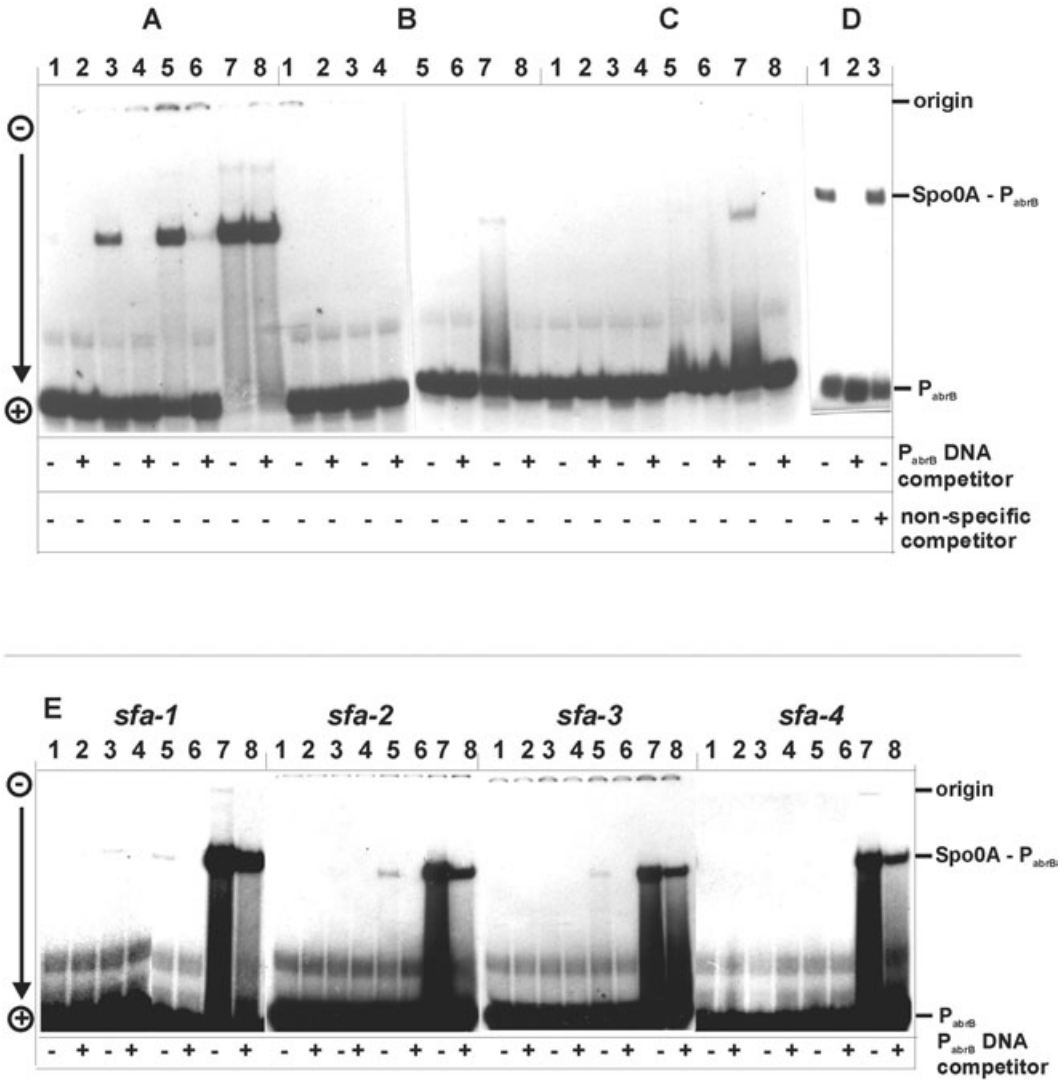

Fig. 3. Gel mobility shift assay experiments.
A, B, C and E. Binding of phosphorylated Spo0A, Spo0AF105A, Spo0AY104A and suppressor proteins to the *abrB* promoter (P_{abrB}).
D. The specificity of Spo0A binding to P_{abrB}. Phosphorylated Spo0A proteins were incubated with 0.19 pmol of ^{32}P-labelled P_{abrB} DNA as described in *Experimental procedures*. Reaction mixtures were separated on a native 8% polyacrylamide gel and then analysed by autoradiography. The positions of P_{abrB} DNA, Spo0A–P_{abrB} complex as well as the origins of the gel lanes are indicated on the right. The direction of migration was towards the anode as indicated on the left. The presence of 19 pmol of specific inhibitor (non-labelled P_{abrB}) or 19 pmol of non-specific inhibitor is indicated + and –, respectively, at the bottom of the autoradiogram. The amount of protein used for the experiments in (D) was 50 ng in lanes 1–3. Increasing amounts of protein was used for (A), (B), (C) and (E) as follows: lanes 1 and 2, 20 ng; lanes 3 and 4, 50 ng; lanes 5 and 6, 200 ng; and lanes 7 and 8, 500 ng.

and subsequent DNA sequencing of the entire coding regions revealed that the suppressor mutations were at four different sites (Table 2). Three of the six isolates shared a mutation at codon 94: an ACG transitional mutation to ATG, corresponding to a threonine to methionine substitution. We have named this mutation *sfa-1* (suppressor of F105A mutation). Two other suppressor mutations, Gln-121Arg (*sfa-2*) and Gln-90Arg (*sfa-3*), mapped to the N-terminal domain of Spo0A. The fourth suppressor mutation, His-162Arg (*sfa-4*), mapped to the C-terminal domain. The characteristics of the suppressor mutants are listed in Table 2, and the location of the second-site mutations in the structure of Spo0A is indicated in Fig. 4. These suppressor mutations restore the sporulation efficiency of *spo0A(F105A)* to between 17% and 62% of that of wild-type *spo0A* strains (Table 2).

Despite multiple efforts, we were not able to isolate any suppressor mutations of *spo0A(Y104A)*, arguing that it is impossible to compensate for the substitution of this tyrosine by alanine, at least by the subset of amino acid codons accessible by a single base change within the spectrum of the mutagenesis protocol.

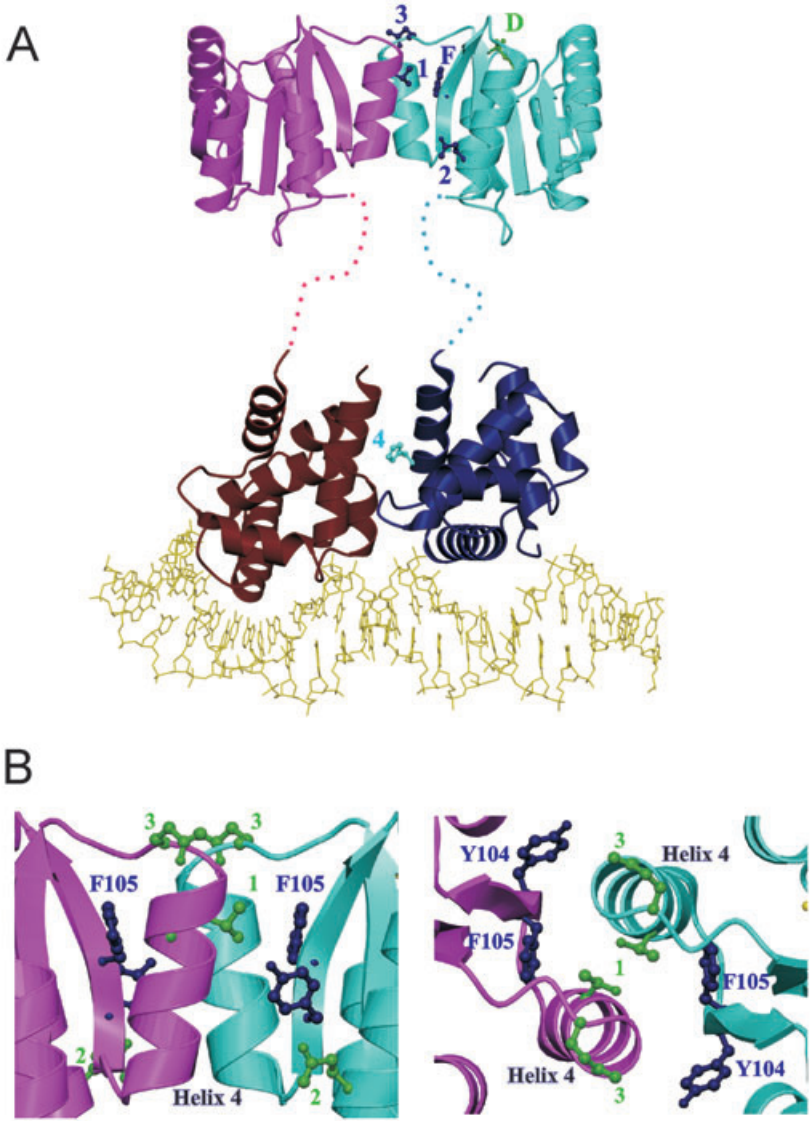

Fig. 4. A. Model of activated Spo0A derived from X-ray structures of the individual domains. For this model, we have used PDB accession codes 1QMP for N-Spo0A (Lewis *et al*., 1999) and 1LQ1 for C-Spo0A in complex with DNA (Zhao *et al*., 2002). The dimeric form of Spo0A was constructed by least-squares superimposition of equivalent residues in the structures of phosphorylated N-Spo0A (1QMP; Lewis *et al*., 1999), a monomer in the crystal and dimeric N-FixJ (1D5W; Birck *et al*., 1999). The linkers between the functional domains of Spo0A in this dimer are drawn as dotted lines: no structural information is currently available to determine the path of this part of the structure. In this model, each protomer in a Spo0A dimer is coloured red and blue; however, the effector domains are each shaded darker than their corresponding receiver domains. Note that, in our model, the receiver domains dimerize upon phosphorylation in a head-to-head fashion, whereas the effector domains make intermolecular contacts in a head-to-tail manner. DNA is drawn in yellow and the phosphorylatable aspartic acid (Asp-56) is drawn in green and labelled 'D'. The site of the F105A mutation is coloured dark blue and labelled 'F'. The positions of the suppressors of this Spo$^-$ variant within the receiver domain are also shaded dark blue and are labelled '1' for *sfa-1* (T94M); '2' for *sfa-2* (Q121R) and '3' for *sfa-3* (Q90R). In the effector domain, '4' denotes the position of *sfa-4* (H162R) and is situated in the interface between the two C-Spo0A domains bound to DNA and coloured light blue. For clarity, the position of Asp-56 and the mutants of Spo0A described in this article are only drawn in one molecule (the right-hand) of the dimer.

B. Close-up views of the proposed dimer interface of Spo0A. The panel on the left is in the same orientation as Fig. 4A, and the panel on the right is orthogonal to this, looking down from above (compare this panel with fig. 2 of Birck *et al*., 1999). Ball and stick models indicate the positions of Y104 and F105 (dark blue) and the three Sfa suppressors (green) in the receiver domain and the proximity of Sfa-1 (T94M) and Sfa-3 (Q90R) to the dimer interface.

Characterization of the suppressor mutant proteins

To elucidate how the suppressor mutations restore sporulation, we overexpressed and purified Sfa-1, Sfa-2, Sfa-3 and Sfa-4 proteins and characterized them *in vitro* as described above for the single mutants. Gel filtration experiments showed that all four of the purified suppressor mutant proteins have retention volumes similar to those of wild-type Spo0A and Spo0A(F105A), suggesting that these proteins are monomers. After treatment with phosphoramidate, the retention volumes of Sfa-1(F105A, T94M), Sfa-2(F105A, Q121R) and Sfa-3(F105A, Q90R) were markedly lowered indicating a preponderance of dimers. Their retention volumes of 9.5, 9.4 and 9.8 ml, respectively, are lower than that of Spo0A(F105A)~P (10.1 ml) and closer to that of wild-type Spo0A~P (9.3 ml; Fig. 1, Table 3). In contrast, the retention volume for Sfa-4(F105A, H162R) is higher at 10.3 ml (Fig. 1, Table 3).

Sedimentation velocity AUC experiments with all four suppressor mutant proteins confirmed that they are monomers in the unphosphorylated form (Table 3). The phosphorylated forms of Sfa-1(F105A, T94M), Sfa-2(F105A, Q121R) and Sfa-3(F105A, Q90R) have higher molecular masses, 45–57 kDa, similar to the wild-type Spo0A dimer (Table 3). The smallest change in molecular mass after phosphorylation was observed in the case of Sfa-4(F015A, H162R), which stays predominantly in the form of a monomer upon phosphorylation (Table 3). All these results are consistent with those observed by gel filtration (Fig. 1).

Non-denaturing PAGE of the trypsin-generated receiver domains also indicated that dimer formation is restored in Sfa-1(F105A, T94M), Sfa-2(F105A, Q121R) and Sfa-3(F105A, Q90R) after phosphorylation by phosphoramidate (Fig. 2, lanes 20, 17 and 14 respectively). The phosphoramidate-treated proteins are substrates for Spo0E, which stimulated the dissociation of the dimer forms of the N-Spo0A mutants to monomers, as observed with wild-type N-Spo0A (Fig. 2, lanes 3, 15, 18 and 21). In contrast, the mobility of Sfa-4(F105A, H162R) in these gels is indistinguishable from that of Spo0A(F105A), suggesting that the receiver domain of this protein is impaired in dimer formation after phosphorylation (Fig. 2, lane 11).

To test the DNA-binding characteristics of the phosphorylated suppressor proteins, we have performed gel mobility shift assays using an *abrB* promoter DNA. The experiments clearly show that all four phosphorylated suppressor proteins bind the *abrB* promoter fragment, albeit less efficiently than the wild-type protein (Fig. 3A and E). The band associated with the complex after incubation with 50 ng of protein is noticeably fainter for the mutants than it is for wild-type Spo0A (Fig. 3A and E). Phosphorylated Sfa-3(F105A, Q90R) and Sfa-4(F105A, H162R) bind the DNA fragment less tightly than Sfa-1(F105A, T94M) and Sfa-2(F105A, Q121R), and higher protein concentrations are required to shift the same fraction of the DNA.

Discussion

Defects in dimerization impair interdomain signal transduction

In this study, we have investigated the contributions of Tyr-104 and Phe-105 to Spo0A function. These residues are found on strand β5 at the heart of the $\alpha_4\beta_5\alpha_5$ surface (the '455' face) of the protein. Across the response regulator family, there is abundant evidence suggesting that this surface is important in the molecular events that follow phosphorylation (Zapf *et al*., 2000; Park *et al*., 2002; Gardino *et al*., 2003; Hastings *et al*., 2003). Residues from α4 and β5 of CheY interact with the P2 domain of CheA (McEvoy *et al*., 1998), and a similar set of residues found in helix α4 and strand β5 of beryllofluoride-activated CheY mediates binding of the flagellar motor protein, FliM (Lee *et al*., 2001). In the chemotaxis response regulator, CheB, helix α4 and strand β5 form part of the surface of the receiver domain that inhibits its methylesterase effector domain (Djordjevic *et al*., 1998). The dimerization of FixJ is mediated by residues situated on helix α4 and strand β5 of the receiver domain (Birck *et al*., 1999; Da Re *et al*., 1999). A key residue in the FixJ dimer interface is Asp-100, which corresponds to Tyr-104 of Spo0A (Birck *et al*., 1999). Finally, in Spo0A itself, phosphorylation of the receiver domain leads to marked structural changes in the vicinity of Phe-105.

Cells harbouring the *spo0A(F105A)* and *spo0A(Y104A)* alleles are severely impaired in sporulation (Table 2). The similarity in the behaviour of Spo0A(F105A) and Spo0A(Y104A) to wild-type Spo0A in the course of purification and their capacity to participate in phosphotransfer reactions suggest that loss of biological function in the mutant proteins is not caused by folding defects. Gel filtration, AUC and electrophoresis experiments reveal that these mutant proteins are impaired in phosphorylation-mediated dimer formation relative to the wild-type Spo0A (Table 3; Figs 1 and 2). This may account for their failure to bind to the *abrB* promoter in gel mobility shift experiments (Fig. 3). Our results clearly show that cells with Spo0A~P monomers are deficient in sporulation and cells with Spo0A~P dimers sporulate. These observations indicate that dimerization is a key link between phosphorylation, sequence-specific DNA binding and transcription regulation by Spo0A.

Suppressors of spo0A(F105A) *reveal important residues for interdomain signalling*

Our results suggest that both Spo0A(Y104A) and Spo0A(F105A) are defective in intramolecular domain–

domain signalling; phosphorylation in the receiver domain does not lead to DNA binding by the effector domain. To explore this further, we searched for suppressor mutations that restore the function of the mutant Spo0A proteins *in vivo*. Surprisingly, we isolated *spo*$^+$ colonies only for strain IB659, which carries the *spo0A(F105A)* allele. We found four different intragenic suppressor mutations, with the second-site mutation in three of them (*sfa-1*, *sfa-2* and *sfa-3*) mapping to the receiver domain and that in the fourth (*sfa-4*) mapping to the effector domain of Spo0A (Table 2).

What is the mechanism of suppression? If the effect of the Phe-105Ala mutation is simply to destabilize the dimer form of Spo0A~P, we would expect that mutations elsewhere on the dimer interface might restore function by providing compensating intermolecular contacts that restore dimer stability. Equally, if the effect of the Phe-105Ala mutation is to strengthen intramolecular domain–domain interactions in Spo0A that sequester the effector domain in an inactive state, mutations elsewhere on this surface that weaken domain–domain interactions would help to restore function. As it is possible that the '455' surface, on to which our mutations map, is used in both intra- and intermolecular contacts, distinguishing between these possibilities is not easy.

The locations of the mutations in the crystal structure are indicated in Fig. 4, which shows a model for the Spo0A dimer built with reference to the crystal structures of (i) N-FixJ~P in which the 455 face of the molecule mediates contacts between a pair of receiver domains related by a dyad axis of symmetry (Birck *et al*., 1999); and (ii) C-Spo0A bound to DNA in which a pair of effector domains sits side-by-side on the DNA (Zhao *et al*., 2002). An interesting feature of the model is the contrasting head-to-head organization of the receiver domains and the head-to-tail organization of the effector domains (Lewis *et al*., 2001). This is expected to be facilitated by the extended linker connecting the two domains that confers conformational independence in the phosphorylated form. The variability in the length and sequence of the linker (Brown *et al*., 1994) is consistent with the absence of defined structure in the interdomain peptide. The data presented here are interpreted in terms of the model presented in Fig. 4 and structural knowledge of how phosphorylation of the active site aspartic acid residue influences the 455 interface in Spo0A.

A comparison of the crystal structures of phosphorylated and unphosphorylated N-Spo0A reveals that the outward to inward movement of the side-chain of Phe-105 (marked 'F' in Fig. 4) upon phosphorylation can be accommodated only if helix α4 moves away from the protein core. There would otherwise be severe steric hindrance to the inward movement of Phe-105 by Thr-94, which lies on the inside face of helix α4. The movement of α4 is hinged towards the C-terminus of the helix, with Ala-97 acting as the fulcrum. The aromatic ring of Phe-105 fills the void created by the movement of Thr-84 towards the aspartyl-phosphate, and we can envisage how this could be mimicked in the Ala-105 mutant if Thr-94 is replaced by the bulkier methionine residue in Sfa-1 (marked '1' in Fig. 4).

Sfa-3 also forms dimers upon phosphorylation (Figs 1 and 2, Table 3). Gln-90, which is substituted by Arg in Sfa-3 (marked '3' in Fig. 4) is situated on helix α4. As this is part of the proposed dimer interface (Fig. 4), it is conceivable that the mutation strengthens the dimer interface and compensates for the disruption caused by replacement of Phe-105 by Ala. It is interesting to note that the corresponding residue in Spo0F is Glu-86. Alanine substitution of this residue in Spo0F leads to a sporulation-defective phenotype (Tzeng and Hoch, 1997). In the Spo0F–Spo0B complex, Glu-86 of Spo0F is adjacent to the Spo0B binding surface (Zapf *et al*., 2000). Mutation to alanine may therefore alter the kinetics of phosphotransfer between Spo0F and Spo0B. The Gln-90 to Arg substitution associated with *sfa-3* is obviously similar to the Gln-90 to Lys change encoded in the *coi-15* allele of *spo0A*. A characteristic of *coi* mutants is 'inappropriate sporulation' in which normal nutritional signals are not required (Olmedo *et al*., 1990). Although the suppressor mutation in Sfa-2 (marked '2' in Fig. 4) also enables Spo0A harbouring Ala-105 to dimerize, Arg-121, which is on helix-5, is not integral to the dimer interface in our model. It may be that this suppressor acts by destabilizing intramolecular domain–domain interactions. Genetic evidence suggests that helix-5 is involved in interdomain interactions in PhoB (Allen *et al*., 2001), and mutation of a residue (Thr-109) on this same helix increases the affinity of FixJ for DNA (Saito *et al*., 2003). The Gln-121 to Arg substitution occurs elsewhere as a single substitution mutation in the Spo0A variant, Sof-108. *sof108* strains can sporulate even when *spo0F* has been deleted (*sof* – suppressor of *spo0F* deletion; Spiegelman *et al*., 1990; Cervin and Spiegelman, 1999). It is assumed, but not proved, that the Arg-121 mutation allows Spo0A to receive the phosphoryl group directly from one of the sporulation sensor kinases. Bypass of the phosphorelay may therefore also take place with Sfa-2. The fact that we could not isolate any suppressors of the Tyr-104 to Ala mutation reinforces the notion that this residue is important for Spo0A function. Tyr-104 is invariant among the 23 Spo0A orthologues sequenced to date, although it is not conserved across the response regulator family, implying that this residue has a discrete function in Spo0A. This residue appears to have co-evolved with the effector domains in response regulators. In >95% of CheB sequences, the equivalent residue is aspartic acid, as it is in all OmpR, PhoB and FixJ sequences, whereas glycine is found at the equivalent position in the NarL, GacA, UhpA, LuxR and DegU sub-

families. Glycine is also commonly found at this position within the CheY subfamily, allowing the close approach of Leu-15 of the FliM peptide (Lee *et al*., 2001). Hence, within response regulator subfamilies, this residue is conserved, while variance is permitted across the whole family. This pattern of conservation across families of response regulators argues that the equivalent residue in other response regulators has an important – but not necessarily the same – role.

The most surprising suppressor is *sfa-4* in which the suppressing mutation is His-162 (marked '4' in Fig. 4) to Arg. His-162 is located in the C-terminal domain of Spo0A. This same mutation was isolated previously as a suppressor of *spo0A9V* (Ala-257Val) and Ser-250His (Perego *et al*., 1991; Schmeisser *et al*., 2000). This mutation is only the second intragenic *spo0A* suppressor that lies in the opposing domain to the original mutation. The other, *sof114* (Asp-92Tyr), was isolated as a suppressor of *spo0A9V* (Perego *et al*., 1991). Asp-92 is also situated on helix α4, sandwiched between Gln-90 and Thr-94, which are mutated in *sfa-1* and *sfa-3* respectively. Although Phe-105 and Asp-92 are situated on the same protein '455' face, His-162 (site of *sfa*-4) and Ala-257 (site of *spo0A9V*) are on opposite sides of the effector domain, and it is not possible for both His-162 and Ala-257 simultaneously to contact the '455' signalling surface within a monomer. However, in a Spo0A dimer, intermolecular contacts between residues His-162 and Ala-257 could occur, as revealed by the crystal structure of C-Spo0A in complex with DNA (Zhao *et al*., 2002). Similar interactions may also explain the suppression by *suv-3* and *suv-4* of the Ser-250His and *spo0A9V* mutations (Schmeisser *et al*., 2000).

Model of Spo0A activation through phosphorylation and dimerization

We propose the following model of Spo0A action (Fig. 4). Spo0A becomes phosphorylated by the phosphorelay on Asp-56, and the concomitant conformational changes, mediated via Thr-84 and Phe-105 side-chain movements, result in dimerization. Dimerization disrupts transcription-inhibitory contacts between the receiver and effector domains of Spo0A. The most important dimer-forming contacts involve the N-terminal domains with additional dimer contacts mediated through the C-terminal domains upon binding to DNA (Lewis *et al*., 2002; Zhao *et al*., 2002). Dimerization thus activates the DNA-binding and transcription activation/repression functions of the effector domain. According to the structural and mutagenesis studies, helix α4 of N-Spo0A may be involved not only in dimerization, but also in contacts with the effector domain before activation. Such a model is supported by the data on suppressor mutants, such as suv-3, suv-4, sof-114 and sfa-4. In addition to helix α4, other regions of the receiver domain, such as the surface encompassing Asp-75, may be involved in interactions with the effector domain (Cervin and Spiegelman, 2000).

The biochemical characterization of a variety of Spo0A variants, in which the genetic lesions are located in the receiver domain (Cervin and Spiegelman, 1999; 2000), together with the structure of N-Spo0A~P (Lewis *et al*., 1999), reinforces the significance of phosphorylation-induced conformational changes in the receiver domain for the activation of the effector domain. Complex structural changes in Spo0A must occur on phosphorylation, involving changes in intramolecular contacts and dimerization. Helices αA, αB and αF of the effector domain and α4 of the receiver domain appear to be important constituents of the signal transduction pathway in Spo0A. The molecular mechanism of transduction of the phosphorylation signal from N-Spo0A to C-Spo0A is the crucial, unanswered question concerning the function of Spo0A.

Experimental procedures

Bacterial strains, culture media and genetic techniques

All bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise indicated, *E. coli* and *B. subtilis* cultures were grown in LB medium (Ausubel *et al*., 1987). Transformation of *B. subtilis* and assays of sporulation efficiency were performed as described previously (Harwood and Cutting, 1990). Sporulation was induced by nutrient exhaustion in Difco sporulation medium (DSM). After 24 h incubation at 37°C, aliquots of the culture were serially diluted and plated on to LB plates before and after a 15 min incubation at 80°C. The sporulation efficiency was defined in terms of colony-forming units (cfu) as follows: (cfu of spores/viable cfu)/(wt viable spores/wt total viable cfu).

Site-directed mutagenesis and DNA sequencing

Site-directed mutagenesis of *spo0A* was carried out with the LA PCR *in vitro* mutagenesis kit (TaKaRa) according to the manufacturer's instructions. The integrative plasmid pBG15, which contains a promoterless *spo0A* gene on a 1.9 kb *Bam*HI–*Hin*dIII fragment, was used as the template for the PCRs. Suitable mutagenic oligonucleotides were designed to introduce alanine substitutions at Tyr-104 and Phe-105, each introducing a double base change, in anticipation of a subsequent search for suppressor mutations. Each of the two PCR products was digested with *Bam*HI and *Hin*dIII and recloned into pBG15 digested with the same restriction enzymes, creating plasmids pBG15YA (Tyr-104Ala) and pBG15FA (Phe-105Ala). After exposure of the ligation products to competent *E. coli* strain MM294, several ampicillin-resistant transformants were isolated, analysed and subsequently sequenced by the dideoxy chain termination reaction. The DNA sequences of all the primers used are listed in Table 1. Plasmids pBG15YA and pBG15FA were each used

to transform the wild-type *B. subtilis* strain PY79 to chloramphenicol resistance. Transformants arising from a single cross-over recombination event created the strains IB658 and IB659 respectively. The sporulation efficiency of these strains was analysed as described above.

Plasmid constructions

The mutated *spo0A* genes were amplified by PCR using the primers N0A and C0A (Table 1) to introduce flanking *Nde*I and *Hin*dIII sites for subsequent insertion between these same sites in pET26b(+) (Novagen). This generated the plasmids pET0AY, pET0AF, pET0ASF1, pET0ASF2, pET0ASF3 and pET0ASF4. Several clones were obtained, analysed and sequenced to verify that no inadvertent, PCR-derived mutations had arisen.

Protein isolation and purification

Cultures of *E. coli* strain BL21 (DE3) harbouring the pET0A derivatives were grown at 37°C in LB media containing 30 μg ml^{-1} kanamycin until an optical density of 0.6 at 600 nm (OD_{600}) was reached. Recombinant protein expression was induced by the addition of IPTG to a final concentration of 1 mM. After 4 h of further growth at 30°C, cells were harvested by centrifugation, and the cell pellets were frozen at −70°C until use. Cell pellets were resuspended in 10 ml of buffer A [20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM dithiothreitol (DTT), 250 mM NaCl, 1 mM AEBSF] before disruption by sonication. The cell lysate was clarified by centrifugation before loading on to a Heparin Sepharose column, pre-equilibrated in buffer A. Mutant Spo0A proteins were eluted in buffer A with a linear gradient of 250–1000 mM NaCl. Fractions containing Spo0A were pooled and concentrated before further purification using a Superdex S75 16/60 gel filtration column. Purified proteins were stored at −20°C in filtration buffer, supplemented with glycerol (10% v/v).

Spo0A phosphorylation and dephosphorylation

Spo0A proteins were treated with phosphoramidate, which can serve as a specific phosphoryl group donor for the active-site aspartate residues in a number of response regulators (Buckler and Stock, 2000). Protein samples were concentrated to 1 mg ml^{-1} in a buffer of 20 mM Tris-HCl, pH 7.5, 2 mM DTT, 250 mM NaCl, 10 mM $MgCl_2$. Phosphorylation was achieved by the addition of phosphoramidate to a final concentration of 50 mM for 30 min at 37°C (Lewis *et al*., 2002). After removal of phosphoramidate by Sephadex G-25, samples were placed on ice before further immediate analysis by analytical ultracentrifugation, gel filtration experiments, DNA binding or trypsin digestion, followed by electrophoresis. Dephosphorylation of wild-type Spo0A and mutant proteins was performed with a hyperactive form of Spo0E, Spo0E94, as described previously (Lewis *et al*., 2002). Spo0A (30 μM) samples were dephosphorylated by Spo0E94 at the final concentration of 5 μM for native PAGE experiments, and 550 μM Spo0A samples were dephosphorylated by Spo0E94 at the final concentration of 100 μM for gel filtration experiments.

Proteolysis of intact Spo0A

Spo0A proteins were incubated with trypsin at a molar ratio of 2500:1 at 16°C (Muchová *et al*., 1999) in 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT. The digestion products were immediately analysed by non-denaturing, 12.5% PAGE.

Analytical ultracentrifugation

Sedimentation velocity experiments were performed in a Beckman XL/I analytical ultracentrifuge. Protein samples were prepared with A_{280} readings of 0.2 for Spo0A(Y104A) and Spo0A(F105A), 0.5 for Sfa-2, 0.8 for Sfa-4, 1.0 for Sfa-3 and 1.2 for wild-type Spo0A, corresponding to concentrations of 5, 5, 12.5, 20, 25 and 30 μM. Unphosphorylated Spo0A samples were prepared in 20 mM Tris-HCl, pH 7.5, 250 mM NaCl, 2 mM EDTA, phosphorylated protein in the same buffer with the addition of 25 mM $MgCl_2$ and 50 mM phosphoramidate, and Spo0E-treated samples as for the phosphorylated protein with the addition of the protein phosphatase. Protein samples (≈ 400 μl) were centrifuged in charcoal-filled Epon double-sector centrepieces with identical buffers as reference, at 40 000 r.p.m. Sedimentation was observed by scanning absorbance at 280 nm until the plateau region was lost. Data were analysed with the program SEDFIT (Schuck, 2000) using a simple c(s) model (distribution of sedimentation coefficients without assumption of size or number of species) then transformed to c(M) (distribution of molecular weight). The calculated molecular weights depend on the partial specific volume, and buffer density and viscosity were calculated using the program SEDNTERP (Laue *et al*., 1992) using phosphate for phosphoramidate and neglecting the contribution from Spo0E where used.

In vivo *mutagenesis using ethyl methanesulphonate (EMS)*

Strains IB658 and IB659 were mutagenized by plating on DSM agar plates containing appropriate antibiotics and incubated at 30°C overnight. A sterile piece of filter paper with a diameter of ≈1 cm was placed on the surface of the cell lawn. A volume of 20–35 μl of ethyl methanesulphonate (EMS) was dropped onto the filter paper, and the cultures were incubated at 37°C. After incubation for 24 or 36 h, plates were exposed to chloroform vapour for 15 min to kill non-sporulating cells. The plates were incubated further overnight at 37°C to allow outgrowth of possible spores. Single colonies were picked from these DSM plates and streaked out on to LB plates containing the appropriate antibiotics. Chromosomal DNA was prepared from each of the single colonies and used as the template for PCRs to amplify the *spo0A* gene for DNA sequencing in order to determine the presence and sequence of the intragenic suppressors.

DNA preparation for the gel mobility shift assays

An aliquot of 100 ng (11 pmol) of ABRBTOP oligonucleotide (Table 1) was heated for 5 min at 95°C, quickly cooled in an ice bath and labelled with 20 μCi (5 pmol) of [γ-^{32}P]-ATP (ICN;

4000 Ci mmol^{-1}) in a total volume of 25 μl (Ausubel *et al*., 1987). The incorporation of radioactive label to DNA was determined (Kormanec and Farkasovsky, 1993) before mixing the labelled ABRBTOP oligonucleotide with an equimolar amount of ABRBBOT oligonucleotide (Table 1). This mixture was heated for 5 min at 95°C, annealed for 20 min at 65°C and slowly cooled to 20°C. Unincorporated radionucleotides were removed from duplex DNA by desalting using Sephadex G-50 microtip columns. Specific competitor DNA was prepared by the same annealing procedure using unlabelled *abrB*-specific oligonucleotides. Non-specific competitor DNA was prepared by annealing equimolar amounts of the ATNESCC and ATNESGG oligonucleotides (Table 1) at an annealing temperature 35°C.

Gel mobility shift assays

Gel mobility shift assays were performed essentially as described by Ausubel *et al*. (1987). Protein samples were incubated with 20 000 c.p.m. (0.19 ng) of ^{32}P-labelled *abrB*-specific DNA with or without competitor DNA for 20 min at 30°C in a solution containing 40 mM Tris-acetate, pH 8.0, 40 mM sodium acetate, 10 mM magnesium acetate, 0.1 mM DTT, 10 μg ml^{-1} BSA, 1 mM EDTA and 5% (v/v) glycerol in a total volume of 20 μl. Spo0A and the mutant Spo0A proteins were phosphorylated with phosphoramidate before the binding reaction as described above. Samples were loaded on to an 8% polyacrylamide gel in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 7.9) containing 2% (v/v) glycerol. The gel was electrophoresed at 30°C and 150 V in 1× TAE buffer until the bromophenol blue had migrated to within 2 cm from the bottom of the gel. The gel was then transferred to Whatman paper, dried and analysed by autoradiography.

Western blot analysis

Gel mobility shift assays were carried out as described above with 50 μg of Spo0A per reaction. The part of the gel destined for blotting was cut from the rest of the gel, and the products of the binding reaction were transferred to a PROTRAN BA 85 (NC) membrane by a semi-dry blotting procedure. Immunodetection of Spo0A was carried out with a polyclonal anti-Spo0A antibody (rabbit) diluted 1:500 in blocking buffer followed by anti-rabbit IgG–alkaline phosphatase reaction.

Gel filtration

Samples of 100 μl of each Spo0A protein, at concentrations of 550 μM, were applied to a Pharmacia Superdex 75 HR 10/30 gel filtration column, equilibrated in a buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 250 mM NaCl. According to the manufacturer, this column effectively separates globular proteins in the molecular weight range 3000–70 000 Da.

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