bofC encodes a putative forespore regulator of the *Bacillus subtilis* σ^K checkpoint

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A mutation, *bofC1*, that restores σ^K activation in *Bacillus subtilis* strains unable to produce active σ^G has been identified. This mutation defines a new sporulation gene, *bofC*, that has been cloned and sequenced and encodes a 19 kDa protein. *bofC* is transcribed in the forespore by RNA polymerase associated with the transcription factors σ^F ($E\sigma^F$) and σ^G ($E\sigma^G$). BofC acts negatively on SpoIVB and the results described suggest that BofC regulates SpoIVB activity and its intercompartmental signalling role in the σ^K checkpoint.

Keywords: *Bacillus subtilis*, sporulation, checkpoint, gene regulation

INTRODUCTION

Spore formation is the predominant response of *Bacillus subtilis* to conditions of nutrient depletion. Development of the spore is dependent on coordinated programmes of gene expression in the two compartments (mother cell and forespore) of the differentiating cell. Controlling this are five sigma factors (σ^H , σ^F , σ^E , σ^G and σ^K) that confer different promoter specificities upon the RNA polymerase holoenzyme (Errington, 1993).

In the mother cell σ^K directs the expression of a large number of genes whose products are required for the final stages of spore maturation (e.g. synthesis of the proteinaceous coat which encases the dormant spore (Sandman *et al.*, 1988; Zheng & Losick, 1990). σ^K is synthesized initially as an inactive precursor, termed pro- σ^K , which must be cleaved at its N-terminus to be rendered functional (Lu *et al.*, 1990). Processing of pro- σ^K is controlled by an intercompartmental signal transduction pathway, termed the ' σ^K checkpoint', that couples mother cell σ^K -directed gene expression to σ^G -dependent gene expression in the forespore (Cutting *et al.*, 1990).

The major components of the σ^K checkpoint have been identified. In the mother cell, SpoIVFA, BofA and SpoIVFB are thought to form a hetero-oligomeric complex that resides in the outermost membrane layer of the forespore and controls processing of pro- σ^K

(Cutting *et al.*, 1991b; Ricca *et al.*, 1992; Resnekov *et al.*, 1996). SpoIVFB is the most likely candidate for the proteolytic enzyme although proteolytic activity has not been demonstrated (Lu *et al.*, 1995) and BofA and SpoIVFA are inhibitors of SpoIVFB activity. Pro- σ^K proteolysis is dependent on a signal originating from the forespore that relieves SpoIVFA and BofA-mediated inhibition of SpoIVFB. Accordingly, mutations that inactivate the BofA or SpoIVFA polypeptides (termed *bof* mutations, bypass of forespore gene expression) allow constitutive activity of SpoIVFB in the absence of forespore signalling (Cutting *et al.*, 1990). The signal itself is the product of σ^G -dependent gene expression within the forespore and one gene product, the 46 kDa SpoIVB protein, has been identified as the essential signalling molecule (Cutting *et al.*, 1991a). Genetic analysis has also shown that SpoIVB is most likely the only σ^G -dependent gene product necessary for signal transmission (Gomez *et al.*, 1995). SpoIVB contains a putative signal sequence that might allow it to transit the inner forespore membrane and directly, or through an intermediate enzymic reaction it catalyses, stimulate processing of pro- σ^K .

The σ^K checkpoint provides an elegant mechanism for fine-tuning gene expression and introduces a timing mechanism ensuring that activation of σ^K proceeds only when the appropriate stage of forespore development has been attained. In the absence of the checkpoint (e.g. in *bofA* or *spoIVFA* defective mutants), σ^K -directed gene expression proceeds prematurely, resulting in the early expression of the σ^K regulon which, in turn, results in a reduction in sporulation efficiency and the production of aberrantly formed spores (Cutting *et al.*, 1990).

We report here the identification of a new forespore-expressed gene, *bofC*, which encodes a putative regu-

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lator of SpoIVB function. The *bofC* gene product may therefore be involved in the fine-tuning of the σ^K checkpoint.

METHODS

Bacterial strains. Strains of *B. subtilis* are listed in Table 1. All strains used in this work were congenic derivatives of the prototrophic, Spo⁺, strain PY79. Strains lysogenic for *SPβ*:*gerE-lacZ* are referred to in the text by the name or allele of the strain lysogenized.

General methods. Standard *B. subtilis* methodology, including genetic analysis, was as described by Harwood & Cutting (1990). Sporulation was induced using the 'resuspension method' (Nicholson & Setlow, 1990). *SPβ* specialized transduction were performed using a lysate prepared from strain SC433 (*SPβ*:*gerE-lacZ*) as described by Cutting & Vander-Horn (1990). Primer extension reactions and preparation of *B. subtilis* mRNA were described previously (Cutting *et al.*, 1991b).

Isolation and mapping of the *bofC1* mutation. The *bofC1* mutation was identified inadvertently in a mutagenic screen for dominant alleles in the *spoIVF* operon. Cells of strain SC1008 (*spoIIIGA1 spoIVF⁺ chr::Tn917ΩHU144*) were mutagenized as described previously (Cutting *et al.*, 1991b) and DNA from these cells was used to transform strain SC1309 (*spoIIIGA1 spoIVF⁺ amyE::spoIVF⁺*) followed by selection for MLS^R (conferred by the transposon insertion *chr::Tn917ΩHU144*, which is approximately 30% linked, by transformation, to *spoIVF*). MLS^R transformants were screened for their Pig phenotype on DSM sporulation agar plates and one Pig⁺ Spo⁻ colony from about 100 000 examined was identified. The Pig⁺ phenotype (the brown coloration of sporulating colonies) is a marker of σ^K -directed gene expression and would, in this situation, result from a mutation bypassing the requirement of σ^G (encoded by *spoIIIG*) for activation of σ^K in the mother cell compartment. The pig⁺ mutation identified was 30% linked, by transformation, to the *chr::Tn917ΩHU144* insertion. However, complementation analysis by transformation with integrative plasmids (pSC212, pSC217, pSC220 and pSC223) that contain segments of the *spoIVF* operon (Cutting *et al.*, 1991b) indicated that the mutation was not allelic to *spoIVF*. To map the pig⁺ mutation, which was henceforth designated *bofC1*, we performed two- and three-factor transformation crosses using markers in the *spoIVF-spoVB* region and complementation analysis with integrative plasmids carrying segments of the *nic-spoVB* region of the chromosome (see Fig. 1 and Popham & Stragier, 1991).

Plasmid constructions. pDG850, pDG851, pDG855, pDG863 and pDG873 were provided by P. Stragier (Fig. 1 and Popham & Stragier, 1991). pDG855Δ*SacI* and pDG855Δ*EcoRV* were constructed by cleaving pDG855 with *NseI* and either *SacI* or *EcoRV*, blunting ends with T4 DNA polymerase and ligation. pSC60, pSC61, pSC63, pMG5, pMG7 and pMG28 were obtained by subcloning fragments from pDG855 (see Fig. 1) into the integrative vector pSGMU2 (Perego, 1993). pMG4 was constructed by 'chromosome walking' with *PstI*, using chromosomal DNA from strain PY79 transformed with pMG5. pMG17 was constructed by cloning a 954 bp PCR-amplified fragment of DNA containing the promoter and coding regions of *bofC* (nt 1493–2446 of Fig. 3) into the *Bam*HI–*Hind*III sites of pDG364. pDG364 allows insertion of cloned DNA at the *amyE* locus of the chromosome (Cutting & Vander-Horn, 1990).

Sequencing of the *bofC* chromosomal region. To sequence the *bofC* chromosomal region denatured double-stranded DNA templates of the plasmids pSC60, pSC61, pSC63, pMG4, pMG5 and pMG7 were used as sequencing templates. The cloned inserts in these plasmids are flanked by sites recognized by the M13 'Universal' Forward (–40) and Reverse sequencing primers. The sequence obtained using these primers was extended by synthesizing additional sequencing primers using the new sequence information generated. All restriction sites were crossed, the DNA sequenced on both strands and several independent sequence determinations made.

Sequencing the *bofC1* allele. To sequence *bofC1* two primers, F9FOR and F9REV, were used to amplify chromosomal DNA of strain SC1643. F9FOR (GGGAATTCACGGCGTATCTTTTGCTGGG; nt 1713–1732 of Fig. 3) and F9REV (GGAAGCTTGGATTTCGGCGTGAAAATCTGATAG; nt 2446–2423 of Fig. 3) annealed upstream and downstream of the region known to contain *bofC1* (determined from genetic mapping experiments). The 734 bp PCR-amplified product was digested to completion with *Hind*III and *Eco*RI and cloned into the integrative plasmid pSGMU2 to create pSC67. Competent cells of SC500 (*spoIIIGA1*) were transformed with pSC67 DNA and approximately 60% of Cm^R transformants were Pig⁺ due to introduction of the *bofC1* mutation. The entire cloned fragment in pSC67 was sequenced on both DNA strands using primers that annealed to the polylinker cloning sites in pSGMU2 and with other oligonucleotide primers that annealed to the cloned DNA. Several independent plasmid clones obtained using this procedure were sequenced to authenticate our sequence determination.

Construction of the *bofC::neo* insertional mutant. To create an insertional mutation in *bofC* a neomycin-resistance cassette (*neo*) was inserted into the unique *Bsp*EI site within the *bofC* ORF. The *neo* cartridge was isolated as a 1.3 kb *Sma*I fragment from plasmid pBEST501 (Itaya *et al.*, 1989) and ligated to pSC60 (see above) which had been linearized (*Bsp*EI) and blunt-ended with S1 nuclease. Following transformation into *Escherichia coli* and selection for Nm^R (25 µg ml⁻¹) a plasmid, pMG3, was isolated which contained the neomycin-resistance cassette inserted at the *Bsp*EI site (see Fig. 1). pMG3 was linearized (*Hind*III) and used to transform *B. subtilis* strain PY79 with selection for Nm^R (2.5 µg ml⁻¹). Using this procedure Nm^R transformants should only arise by a double crossover (marker replacement) where the *bofC::neo* construct would replace homologous DNA at the *bofC* locus. To confirm that Nm^R transformants had arisen by marker replacement, and not due to integration of circular plasmid DNA by a Campbell-type mechanism, colonies were checked for sensitivity to chloramphenicol (pMG3 carries the *cat* gene). One such Nm^R Cm^S transformant was isolated (strain MG54) which contained a stable *bofC::neo* insertional mutation.

Construction of strains containing *bofC* at the *amyE* locus. To generate strains partially diploid for *bofC* we introduced the *bofC* gene at the *amyE* locus using pMG17, whose construction has been described above. In pMG17 the *bofC* and *cat* genes are flanked by the front and back portions of the *amyE* gene (Cutting & Vander-Horn, 1990). pMG17 was linearized (*Xho*I) and used to transform appropriate strains with selection for Cm^R. Transformants arising from a marker replacement (double crossover) recombination between the *amyE*(F)–*bofC*–*cat*–*amyE*(B) construct in pMG17 and the chromosomal copy of *amyE* were identified by their Amy⁻ phenotype (Cutting & Vander-Horn, 1990).

Table 1. *Bacillus subtilis* strains

Strain	Genotype	Source/reference
PY79	<i>spo</i> ⁺	Youngman <i>et al.</i> (1984)
SC433	<i>SPβ::gerE-lacZ</i>	Cutting <i>et al.</i> (1989)
BSA80*	<i>sigB314 rbsU::kan</i>	B. Haldenwang (University of Texas Health Science Center, San Antonio)
1A616*	<i>nic-82::Tn917 trpC2 SPβc2</i>	BGSC†
BK556	<i>spoIVCB23</i>	Kunkel <i>et al.</i> (1989)
VO1088*	<i>spoIVBΔ::spc trpC2</i>	Oke (1994)
SC500	<i>spoIIIGΔ1</i>	Cutting <i>et al.</i> (1990)
SC692	<i>spoIIIGΔ1 metB5</i>	Lab. stock
SC745	<i>bofB8 spoIIIGΔ1</i>	Cutting <i>et al.</i> (1990)
SC776	<i>bofB8 cat</i>	Cutting <i>et al.</i> (1990)
SC1008	<i>spoIIIGΔ1 chr::Tn917ΩHU144 spoIVF</i> ⁺	Lab. stock
SC1158	<i>spoIID66</i>	Cutting <i>et al.</i> (1991a)
SC1159	<i>spoIIAC1</i>	Cutting <i>et al.</i> (1991a)
SC1163	<i>spoIIG55</i>	Cutting <i>et al.</i> (1991a)
SC1297	<i>spoIVF</i> ⁺ <i>amyE::spoIVF</i> ⁺	Cutting <i>et al.</i> (1991b)
SC1309	<i>spoIIIGΔ1 amyE::spoIVF</i> ⁺ <i>spoIVF</i> ⁺	From SC1297 into SC500
SC1367	<i>spoIIIGΔ1 amyE::spoIVF bofC1</i>	This work
SC1643	<i>bofC1 spoIIIGΔ1</i>	This work
SC1703	<i>bofC1 spoIIIA53 chr::Tn917ΩHU144</i>	This work‡
SC1704	<i>spoIIIA53 chr::Tn917ΩHU144</i>	This work‡
MG24	<i>amyE::bofC-lacZ</i>	This work
MG51	<i>csbX::neo</i>	Gomez & Cutting (1997)
MG54	<i>bofC::neo</i>	This work
MG57	<i>csbX::neo spoIIIGΔ1</i>	From MG51 into SC500
MG60	<i>bofC::neo spoIIIGΔ1</i>	From MG54 into SC500
MG101	<i>spoIVBΔ::spc</i>	From VO1088 into PY79
MG103	<i>amyE::bofC-lacZ spoIIIGΔ1</i>	From MG24 into SC500
MG117	<i>bofC::neo spoIVBΔ::spc</i>	From MG54 into MG101
MG126	<i>bofB8 cat spoIVBΔ::spc</i>	From MG101 into SC776
MG196	<i>bofC::bofC-lacZ</i>	This work
MG237	<i>sigB314 rbsU::kan</i>	From BSA80 into PY79
MG242	<i>sigB314 amyE::bofC-lacZ rbsU::kan</i>	From MG24 into MG237
MG243	<i>sigB314 bofC::bofC-lacZ rbsU::kan</i>	From MG196 into MG237
MG253	<i>csbX::neo bofC1 spoIIIGΔ1</i>	This work§
MG257	<i>csbX::neo bofC1</i>	This work§
MG291	<i>spoIVBΔ::spc csbX::neo bofC1</i>	From MG257 into MG101

* These strains are not isogenic with PY79.

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‡ Chromosomal DNA from strain SC1367 (*bofC1 spoIIIGΔ1 chr::Tn917ΩHU144*) was transformed into *spoIIIA53* cells (strain SC615; Cutting *et al.*, 1991a) using non-saturating DNA concentrations (less than 50 ng ml⁻¹). Selection was made for MLS^R conferred by *chr::Tn917ΩHU144*, which is 30 % linked to *bofC1* by transformation. Two isolates (SC1703 and SC1704) were identified from this cross and their genotypes confirmed by appropriate backcrosses.

§ A silent neomycin insertion in the *csbX* ORF (*csbX::neo*; Gomez & Cutting, 1997) was introduced into SC1643 (*spoIIIGΔ1 bofC1*) cells to create MG253. Next, chromosomal DNA prepared from MG253 was introduced into wild-type cells (PY79) with selection for Nm^R. One transformant was isolated (MG257) which was *spoIIIG*⁺ and the presence of the *bofC1* allele confirmed by backcrossing into a *spoIIIG* mutant strain.

|| Chromosomal DNA prepared from MG257 was introduced into MG101 with selection for Nm^R. More than 95 % of colonies contained *bofC1* by co-recombination which was verified by backcrossing.

Fig. 1. Physical map of the *bofC* region of the chromosome. (a) Physical map of the *bofC* chromosomal region. The map shows (to scale) the 27 kb region of DNA beginning at the *spoIVF* locus and extending to the *spoVB* gene. This region of the chromosome has been cloned and partially sequenced and the map constructed from other work describing the characterization of the *spoIVF*, *spoOB*, *pheA*, *nifS*, *nadB* and *spoVB* loci (Cutting *et al.*, 1991b; Popham & Stragier, 1991; Sun & Setlow, 1993; Trach & Hoch, 1989). The physical map linking the *nadB* locus with DNA contained in pDG851 has been surmised from published work but not confirmed by Southern hybridization analysis. Putative ORFs are indicated by arrows, the direction indicating the transcriptional orientation. Known genetic loci are shown below the arrows. Genetic markers used to map the *bofC1* mutation are shown above the physical map together with map linkages [expressed as (100 – % co-transformation)/100]. The precise position of *chr::Tn917ΩHU144* is not known but is less than 1 kb (to either side) from the *pheA* gene. The cloned segments of DNA contained in the integrative plasmids, pDG850, pDG851, pDG855, pDG858, pDG863 and pDG888, used to determine the chromosomal position of *bofC1* are shown. The +/– symbol indicates whether the corresponding plasmid was able to correct, by integrative recombination, the *bofC1* mutation that was located in the 3.3 kb region (indicated as a striped bar). (b) An expanded physical map of the 5 kb DNA insert contained in plasmid pDG855. The rectangles below the map show the four ORFs found in the 2664 bp region of DNA sequenced between the *PstI* and *HindIII* sites. The bottom of the figure shows the cloned inserts in the indicated integrational plasmids used to localize the *bofC1* mutation and for sequence analysis. The adjacent table indicates whether the cloned insert in these plasmids was able to correct, by integrative recombination, the *bofC1* mutation as described in the text. The table also shows whether plasmids pSC60, pSC61, pSC63, pMG7 and pMG28 disrupted the *bofC* transcriptional unit following integration into the *bofC* locus. Disruption of *bofC* caused a *BofC* phenotype (–) and was used to define the borders of the *bofC* locus. The cloned insert in pMG17 was generated by PCR amplification, and used for *in trans* complementation analysis. The construction of the *bofC::neo* insertional mutation is schematically illustrated as well as the cloned DNA used to construct the transcriptional fusion of *bofC* to *lacZ*. Restriction sites: *EcoRI* (E), *HindIII* (H), *BamHI* (B), *BglII* (G), *SmaI* (M), *StuI* (T), *DraI* (D), *SalI* (S), *NsiI* (N), *PstI* (P), *EcoRV* (V), *BspEI* (I), *SacI* (C). Symbols in parentheses indicate that the map is not complete for that particular enzyme. Symbols in (b) are numbered for multiple sites.

Construction of *bofC-lacZ* transcriptional fusions and measurement of β -galactosidase activity. The 918 bp *EcoRI*–*Bam*HI fragment from pSC63 was cloned into the *EcoRI*–*Bam*HI sites of plasmids pDG268 (Stragier *et al.*, 1988) and pTKlac (Kenney & Moran, 1987) (NB: the *Bam*HI site is derived from the polylinker cloning sites of pSC63). The resulting subclones, pMG1 and pMG23, respectively, contained the 5' region of *bofC* fused in front of the *lacZ* gene of *E. coli*. pDG268, like pDG364 (see above), allows insertion of DNA (here the *lacZ* fusion) at the *amyE* locus by marker replacement, while pTKlac allows insertion of the *lacZ* fusion in the site defined by the cloned insert (i.e. the normal chromosomal position of the gene) through a Campbell-type single crossover recombination. Strain MG24 (*amyE::bofC-lacZ*) was created by transforming cells (PY79) with linearized pMG1 and screening for *Amy*^S *Cm*^R colonies. Strain MG196 (*bofC::bofC-lacZ*) was created by transforming cells (PY79) with pMG23 followed by *Cm*^R selection. β -Galactosidase activity was determined as described by Nicholson & Setlow (1990).

RESULTS

Identification of a locus, *bofC*, involved in the intercompartmental regulation of σ^K -directed gene expression

As described in Methods we used a genetic screen to identify *bof* (bypass of forespore gene expression) alleles in the *spoIVF* (*bofB*) locus. Surprisingly, we identified one mutation (*bofC1*) that restored σ^K -directed gene expression (as indicated by the *Pig*⁺ phenotype) in a *spoIIIG* mutant (unable to produce σ^G), mapped near *spoIVF* (7% co-transformation linkage; Fig. 1) yet was not allelic. The *bofC1* mutation then, defines a fourth locus, *bofC*, which together with *spoIVB*, *bofA* and *spoIVF* (*bofB*), is involved in the intercompartmental signal transduction pathway that activates σ^K .

Since *bofC1* was able to restore σ^K -directed gene expression in a *spoIIIG* mutant we reasoned that it would be similar phenotypically to the *bofA* and *bofB* (*spoIVFA*) mutations. Both *bofA* and *bofB* alleles restore substantial amounts of σ^K -directed gene expression in *spoIIIG* cells but also allow premature expression of σ^K -dependent genes since the requirement of the forespore signal for processing of pro- σ^K has been bypassed.

We introduced a *gerE-lacZ* fusion (*gerE* is a member of the σ^K regulon; Cutting *et al.*, 1989) by specialized transduction into isogenic strains containing the following alleles: *spo*⁺; *spoIIIG* Δ 1; *spoIIIG* Δ 1 *bofC1*; and *spoIIIG* Δ 1 *bofB*8. When sporulation was induced *gerE*-directed β -galactosidase synthesis in wild-type (*spo*⁺) cells initiated 3.5–4 h following induction and was essentially abolished in *spoIIIG* Δ 1 cells (Fig. 2a). In *spoIIIG* Δ 1 *bofB*8 cells *gerE-lacZ* expression initiated about 30–45 min prematurely. In contrast, *gerE*-directed β -galactosidase synthesis was significantly delayed in *spoIIIG* Δ 1 *bofC1* cells, initiating approximately 3 h later than in *spo*⁺ cells and reaching a maximum level of activity almost 15 h after the initiation of sporulation. This delay was also observed using *lacZ* transcriptional fusions to two other members of the σ^K regulon, *cotA* and *cotD* (data not shown).

Introduction of the *bofC1* mutation into a *spoIIIA53* strain also restored σ^K activation, although σ^K -dependent gene expression started 2 h, not 3 h, later than in the wild-type strain (Fig. 2b). *spoIIIA* is an operon containing eight genes, transcribed by *E σ^E* , that is known to be involved in the activation of σ^G (Stragier, 1992). In contrast, the *bofC1* mutation did not restore σ^K -directed gene expression in strains carrying the mutations *spoIVFB152* or *spoIVCB23* (*spoIVCB* provides the 5' end of the composite gene, *sigK*, that encodes σ^K).

Fig. 2. *bofC1* bypasses the requirement of σ^G for σ^K activation and is a loss-of-function mutation. σ^K activity was monitored by measuring *gerE*-directed β -galactosidase synthesis at the indicated times after the induction of sporulation (t_0). Strains were lysogenic for *SP β ::gerE-lacZ* and only the relevant alleles are shown. (a) PY79 *spo*⁺ (■), SC500 *spoIIIG* Δ 1 (○), SC1643 *bofC1 spoIIIG* Δ 1 (△), SC745 *bofB8 spoIIIG* Δ 1 (▼). (b) PY79 *spo*⁺ (■), SC1704 *spoIIIA53* (◇), SC1703 *bofC1 spoIIIA53* (◆). (c) PY79 *spo*⁺ (■), SC500 *spoIIIG* Δ 1 (○), MG60 *spoIIIG* Δ 1 *bofC::neo* (◆), SC1643 *spoIIIG* Δ 1 *bofC1* (△), MG54 *bofC::neo* (◇), MG257 *csbX::neo bofC1* (●). Background levels of β -galactosidase detected in *spo*⁺ cells containing no *gerE-lacZ* fusion have been subtracted.

Orf1 ➔

PstI * * * * * 100
 CTGCAGCGGCTGACAATAGCAGGCCGACAACGGTTGAGGTGTCAACAGCTGATTTTGTGATGAAGGATAAACCGCATTCTTTTCTTGAACGTATAA
 A A A D N S R P T T V E V S T A D F V M K D K P H F F F L E R Y K
 * * * * * 200
 GGATTCATATGAGGAGGAGATTCTCCGTTTTCAGAGAAGCGATCGGCACAAACCAGGAGACTCCCTGCACCGGAATGACGGTTTACAGGCCGGGAGGATC
 D S Y E E E I L R F A E A I G T N Q E T P C T G N D G L Q A G R I
 * * * * * 300
 GCCAGAGCAGCACAGCAATCGCTTGGCATGCCTGTAGCATTGAGCACACTGAAAAATCGCTTTTAACTCTAACAGGATTACAATTACAGCAAG
 A R A A Q Q S L A F G M P V S I E H T E K I A F

Orf2 (CsbX) ➔

-10 * * * * * EcoRI 400
 CTTGGGTATATACTCCATTGATACTTTAAGTAGGCGGTGGAGAAAATGAATACAGTACATGCTAAAGGAAATGTTTGAACAAAATCGGAATTCCTTCTC
 M N T V H A K G N V L N K I G I P S
 * * * * * 500
 ACATGGTTTGGGGTTATATTGGCGTTGTCATCTTTATGGTTGGAGACGGCCTCGAACAAGCTGGCTGTCTCCTTTTCTCGTTGATCATGGTCTCAGTAT
 H M V W G Y I G V V I F M V G D G L E Q G W L S P F L V D H G L S M
 * * * * * 600
 GCAGCAATCCGCATCGTTATTTACCATGTACGGCATTGCTGTACCATCTCAGCTTGGCTTTCAGGAACGTTTGGGAACTTGGGGGCCGAGAAAAACG
 Q Q S A S L F T M Y G I A V T I S A W L S G T F V E T W G P R K T
 * * * * * 700
 ATGACTGTCGGATTGCTTGCATTATCCTCGGTTCCGCCGCTTTTATCGGCTGGGCGATTCTCATATGTATTATCCGGCTCTCTTGGGCAGCTATGCTC
 M T V G L L A F I L G S A A F I G W A I P H M Y Y P A L L G S Y A
 * * * * * 800
 TTAGAGGCTTGGGATATCCGCTGTTTGCATACCTTTTCTCGTATGGGTGTCATACAGCACCTCTCAAAATATCTTGGAAAAGCCGCTGGCTGTTTGG
 L R G L G Y P L F A Y S F L V W V S Y S T S Q N I L G K A V G W F W
 * * * * * 900
 GTTTATGTTTACGTGCGGCCCTTAACGTGCTCGGTCGGTCTTATCCAGCTATGAGTTCGGCCTTGGAGAAATCAATACGCTTGGAGCGCTTACTG
 F M F T C G L N V L G P F Y S S Y A V P A F G E I N T L W S A L L
 * * * * * 1000
 TTTGTGGCGGCAGGCGGAATCTTGCCTTATTTTAAACAAAGATAAATTACTCCGATACAAAAACAAGATCAGCCGAAATGGAAAGAAGTGTCAAGG
 F V A A G G I L A L F F N K D K F T P I Q K Q D Q P K W K E L S K
 * * * * * 1100
 CATTACGATTATGTTTGAACCCCTAAGGTAGGCATCGGCGGAGTGGTCAAGACGATTATGCGATAGGACAATTTGGATTGGCCATCTTCTCTCTAC
 A F T I M F E N P K V G I G G V V K T I N A I G Q F G F A I F L P T
 * * * * * 1200
 TTATTTAGCAGATACGGGTATTCGGTTTCGGAATGGCTGCAAAATATGGGGGACTCTGTTTTTGTGAATATTGTGTTAATATCATTTCGGTGCAGTC
 Y L A R Y G Y S V S E W L Q I W G T L F F V N I V F N I I F G A V
 * * * * * 1300
 GGAGATAAACTAGCGTGGCGCAATACGGTCATGTGGTTTGGCGGTGTCGGATCGGGTATTTTCTGGCGCTGTATTACAGCCCTCAGCTGATCGGGCATC
 G D K L A W R N T V M W F G G V G C G I F L A L Y Y T P Q L I G H
 * * * * * 1400
 AGTATTGGGTGCTCATGATTATCGCCTGCTACGGCGCGGCTTTAGCAGGGTACGTCCCATTAAGCGCGCTTTTGGCGACGCTTGACCTGATAATAA
 Q Y W V L M I I A C C Y G A A L A G Y V P L S A L L P T L A P D N K
 * * * * * 1500
 AGGTGCTGCCATGTGAGTGTGAATTTGGGATCTGGTTTATGTGCGTTTATCGCGCGGGCATCGTGAGTCTTTTCATTGGGCGGCTCGGTGTCAGGTGGC
 G A A M S V L N L G S G L C A F I A P G I V S L F I G P L G A G G
 * * * * * 1600
 GTGATTGGATCTTTCGGGATTGTATTTTTCAGTGCCTTTCTGACGCGATTCTTGACAATTTCTGAGCAAAGCACAGATGTGTATACGGAGGAACGGT
 V I W I F A A L Y F F S A F L T R F L T I S E Q S T D V Y T E E R
 * * * * * 1700
 TTTGTCAGGAAAAATGTGCAGACAAATTTTGATAAAACGGTTAAAGCAGTACCTCTTTGGTCAAACTAAATCATAAAGCTGTGCAAGAGGTTAGAGGGTG
 F V R E N V Q T N F D K T V K Q

Orf3 (BofC) ➔

-35 -10 +1
 TTTGTCAGGAAAAATGTGCAGACAAATTTTGATAAAACGGTTAAAGCAGTACCTCTTTGGTCAAACTAAATCATAAAGCTGTGCAAGAGGTTAGAGGGTG
 F V R E N V Q T N F D K T V K Q M

Fig. 3. For legend see opposite.

Following *bofC* is the 5' end of an ORF, designated ORF4, which extends to the *HindIII*₍₂₎ site. This ORF is preceded by a putative ribosome-binding site and sequences (aaGACt and TAcAAT) that could correspond to the -35 and -10 consensus sequence of promoters recognized by the vegetative RNA polymerase, $E\sigma^A$ (Moran, 1993). The 105 codons of ORF4 we have identified would encode a polypeptide that has significant homology to the 5' region of the *ruvA* gene of *E. coli*. The *ruvA* gene encodes a 22 kDa polypeptide involved in homologous recombination that is induced by DNA damage as part of the SOS response and is regulated by the LexA protein (Shinagawa *et al.*, 1988). Upstream of the putative -35 promoter sequence there is a sequence (GAAC-N₄-GTTa) of high similarity to the 'Cheo Box' sequence (GAAC-N₄-GTTC) that functions as an operator in controlling the expression of *din* genes in *B. subtilis*. *din* (DNA-damage-inducible) genes are regulated as a response to DNA damage by the DinR protein in a manner similar to LexA-controlled genes in *E. coli* (Cheo *et al.*, 1991). Earlier work has shown that the UV-sensitive mutation, *recB2*, maps in the *pheA-nic* region of the chromosome (Hoch & Anagnostopoulos, 1970).

Upstream of *bofC* are two further ORFs, ORF1 and ORF2. Only the 3' end of ORF1, encoding 90 amino acid residues, has been sequenced but this would terminate at nucleotide 272 of Fig. 3. ORF2 contains 434 codons that could encode a polypeptide of 47714 Da. In other work (Gomez & Cutting, 1997) we have found that this ORF is under the control of σ^B during stationary-phase growth and we have named it *csbX* (controlled by sigma B).

To identify the *bofC1* mutation in this region we used two oligonucleotide primers to amplify the region of DNA containing virtually the entire coding region of *bofC* (ORF3) from strain SC1643 containing the *bofC1* allele. The cloned DNA was sequenced on both strands and one base change corresponding to a C to T transition (nucleotide 1984 of Fig. 3) was identified converting the serine codon at position 96 to a phenylalanine codon.

***bofC1* is a loss-of-function mutation**

We constructed a *bofC1 spoIIIG*⁺ strain (MG257) and found that during sporulation the *bofC1* mutation caused no impairment of sporulation efficiency or the production of heat resistant spores (results not shown). The *bofC1* allele, being a missense suppressor, may not necessarily affect the function of BofC in the presence of σ^G . Therefore, to determine the requirements of BofC for spore formation we created an insertional mutation in the *bofC* ORF (see Fig. 1 and Methods). Although this was not a complete knockout mutation we expected, at most, no more than half of the BofC polypeptide to be synthesized in this construct. We then compared the phenotypes associated with the *bofC::neo* (strain MG54) and *bofC1* (strain MG257) mutations. The *bofC::neo* strain was Spo⁺ and indistinguishable from

the congeneric strains PY79 (*spo*⁺) and MG257 (*bofC1*), and it produced normal levels of heat-resistant spores (results not shown). We measured σ^K -directed gene expression (using *SP β gerE-lacZ*) in these strains and found that there was a noticeable difference in the kinetics of gene expression, compared with the wild-type strain, PY79. Specifically, there was a slight delay and a reduction in maximum levels of *gerE*-directed β -galactosidase synthesis in both mutants (Fig. 2c).

Since the *bofC::neo* and *bofC1* mutants were similar phenotypically we wondered if the BofC phenotype was caused by the absence of the BofC protein. To address this we introduced the *bofC::neo* construct into *spoIIIG41* cells (strain MG60) and measured *gerE-lacZ* expression (Fig. 2c). In these cells significant levels of σ^K -directed gene expression were restored although, as was the case with *bofC1*, there was a substantial delay in the initiation of gene expression (~3 h). Examination of the double mutant revealed that spore formation had advanced to stage IV-V with the production of phase-grey sporelets as was found with the *bofC1 spoIIIG41* mutant.

***bofC* is monocistronic**

To determine the transcriptional borders of *bofC* we employed a method routinely used in *B. subtilis*. Since the BofC phenotype is due to loss of function of the *bofC* gene product we reasoned that a plasmid containing neither the transcription start nor termination signals would, upon a Campbell-type integration into the chromosome in *spoIIIG41* cells, disrupt the *bofC* gene resulting in a Pig⁺ phenotype. We used the plasmids pSC60, pSC61, pSG63, pMG7 and pMG28, each carrying a segment of the DNA covering part of *bofC* (see Fig. 1 and Methods). These plasmids contain the *cat* gene, which allows insertion of the plasmid DNA into the chromosomal region (defined by the cloned insert) by selection for Cm^R. When pSC60, pSC61, pSC63 and pMG7 were introduced into *spoIIIG41* cells a Pig⁻ (*SpoIIIG*⁻) phenotype resulted, showing that the transcriptional start (pSC63, pMG7) and termination signals (pSC60, pSC61) must be contained within the cloned DNA in these plasmids. pMG28, however, containing DNA internal to the *bofC* ORF produced a Pig⁺ phenotype, which must have resulted from disruption and inactivation of *bofC*. In each cross, the Pig phenotype was confirmed both by phase-contrast microscopy and by measuring *gerE-lacZ* expression in cells lysogenized with *SP β ::gerE-lacZ* (data not shown). These results imply that the *bofC* locus lies between the *EcoRI*₍₂₎ and *HindIII*₍₂₎ sites (the upstream and downstream boundaries of the *B. subtilis* DNA insert in pMG7 and pSC60/pSC61, respectively, as shown in Fig. 1) located at positions 917 and 2659, respectively, of Fig. 3.

To further define the *bofC* operon we constructed a merodiploid for *trans* complementation analysis. Two synthetic oligonucleotide primers were used to amplify

Fig. 4. *bofC-lacZ* expression. (a, b) *bofC*-directed β -galactosidase synthesis was determined at the indicated times following the induction of sporulation (t_0). All strains contained a *bofC-lacZ* transcriptional fusion introduced at the *amyE* locus by DNA-mediated transformation and with the exception of the indicated allele were isogenic. (a) PY79 *spo*⁺ (■), SC1159 *spoIIAC1* (□), BK556 *spoIVCB23* (●), SC500 *spoIIIGΔ1* (○). (b) PY79 *spo*⁺ (■), SC1158 *spoIID66* (△), SC1163 *spoIIGB55* (▲), SC1159 *spoIIAC1* (□). Background levels of β -galactosidase observed in *spo*⁺ cells containing no *bofC-lacZ* fusion have been subtracted. (c) *bofC*-directed β -galactosidase synthesis was determined in *spoIIIGΔ1* cells containing the *bofC-lacZ* fusion at the *amyE* locus (strain MG103) and either pSDA4 (*Pspac-IIAC*) or pDG298 (*Pspac-IIIG*). Cultures were grown in 2×YT medium with phleomycin (0.1 μ g ml⁻¹); at an OD₆₀₀ of 0.3 the culture was split and IPTG added to one portion (final concentration 1 mM) and growth of both cultures continued. Samples were removed and assayed for β -galactosidase activity at the indicated times after IPTG induction. ◇, pSDA4 without IPTG; ◆, pSDA4 plus IPTG; ▽, pDG298 without IPTG; ▼, pDG298 plus IPTG.

DNA covering the *bofC* ORF and an additional 205 bp upstream of the *bofC* start codon and 236 bp downstream of the *bofC* translational stop signal. We predicted that this segment (nt 1493–2446 of Fig. 3) would contain the putative *bofC* regulatory elements but separate it from other possible coding regions (ORF2 and ORF4). The amplified DNA product was cloned and introduced at the *amyE* locus of cells containing *bofC1 spoIIIGΔ1* (SC1643) or *bofC::neo spoIIIGΔ1* (MG60). The phenotype of the resulting transformants was *Pig*⁻, demonstrating *in trans* complementation (confirmed by analysis of *gerE*-directed β -galactosidase synthesis; results not shown) and the recessive nature of the *bofC1* and *bofC::neo* alleles.

bofC expression

To study the expression of *bofC* during sporulation we constructed a transcriptional fusion of *bofC* to the *lacZ* gene of *E. coli* at the *amyE* locus. To monitor *bofC-lacZ* expression cells were induced to sporulate and sampled for β -galactosidase activity at appropriate times following the induction of spore formation. Fig. 4 shows that *bofC*-directed β -galactosidase synthesis commenced at about 1 h (t_1) after the start of sporulation and produced two clearly identifiable temporal phases of expression. The first phase, starting at t_1 peaked at t_2 and following a brief decline resumed with the second phase at $t_{2.5}$, which continued to elevate the level of *bofC*-directed β -galactosidase synthesis to a maximum at t_5 .

To determine the dependence of *bofC* expression on the products of other *spo* genes we introduced, by DNA-mediated transformation, the *bofC-lacZ* fusion into a collection of *spo* mutants (all congenic with the *spo*⁺ strain PY79). *bofC*-directed β -galactosidase synthesis was determined at various times during sporulation in parallel with an isogenic *spo*⁺ strain containing the *bofC-lacZ* fusion (strain MG24). The pattern of *bofC* expression from some typical experiments is shown in Fig. 4(a, b). *bofC* expression was essentially abolished in a *spoIIAC* mutant (unable to produce σ^F). In contrast, the *spoIID*, *spoIIG* and *spoIIIG* mutations (which impair the production of active σ^G) affected the 'biphasic' pattern of *bofC*-directed β -galactosidase synthesis by reducing the second phase of *bofC-lacZ* expression. In addition, the *spoIID66* mutation caused a noticeable reduction in the first phase of expression while the *spoIIG55* mutation caused an elevation in the first phase. The *spoIVCB* mutant, though, did not have impaired *bofC* expression but showed an elevation in the second phase of expression.

As further proof that *bofC* is controlled by both σ^F and σ^G we induced the synthesis of an active form of either σ^F or σ^G during vegetative growth and measured *bofC*-directed β -galactosidase synthesis at appropriate times thereafter. For these experiments we used two autonomously replicating plasmids, pSDA4 (Shazand *et al.*, 1995) and pDG298 (Sun *et al.*, 1989) to facilitate induction of σ^F and σ^G respectively. These plasmids contain the structural genes of σ^F (*spoIIAC*; pSDA4) and σ^G (*spoIIIG*; pDG298) fused downstream, and under the

Fig. 5. Mapping the 5' terminus of *bofC* mRNA. (a) The transcriptional start point of *bofC* was determined by primer extension using the primer OMG13. For σ^F/σ^G -induced cultures (lanes 1–4), total RNA was prepared from SC500 (*spoIIIGΔ1*) cells growing exponentially and containing either pSDA4 or pDG298 (which allow expression of σ^F or σ^G , respectively, after IPTG induction). Following addition of IPTG (1 mM) total RNA was collected and used for cDNA synthesis using labelled OMG13. Lanes: 1, pSDA4 without IPTG; 2, pSDA4 plus IPTG; 3, pDG298 without IPTG; 4, pDG298 plus IPTG. For sporulating cultures (lanes 5–7), RNA was prepared from *spo*⁺ cells (PY79) at 0 h (lane 5), 2.5 h (lane 6) and 5 h (lane 7) after the initiation of spore formation. The nucleotide sequencing ladder shown on the right was generated using [γ -³²P]ATP-labelled OMG13 and a plasmid template containing *bofC*. An extension product with the same 5' termini was obtained using the primer OMG14 for *bofC* (results not shown).

control of, the IPTG-inducible *spac* promoter. Fig. 4(c) shows that addition of IPTG to vegetatively growing *spoIIIGΔ1* cells containing pSDA4 or pDG298 resulted in significant induction of *bofC*-directed β -galactosidase synthesis.

As a definitive characterization of *bofC* regulation we defined the 5' terminus of *bofC* mRNA by primer extension analysis, using the primers OMG13 and OMG14 (shown in Fig. 3) and total RNA prepared from (1) exponentially growing *spoIIIGΔ1* cells in which either σ^F or σ^G was induced or (2) sporulating cells (*spo*⁺). As shown in Fig. 5, in vegetatively growing cells producing either σ^F or σ^G , a transcript was identified that started 25 bp upstream of the *bofC* ORF. The same transcript was observed in sporulating cell extracts at 2.5 and 5 h but not at 0 h.

Fig. 6. *bofC* mutations do not bypass the requirement of SpoIVB for σ^K activation. The indicated strains, lysogenic for *SPβ::gerE-lacZ*, were induced to sporulate and *gerE*-directed β -galactosidase synthesis was measured at the indicated times following the initiation of sporulation (t_0). Other than the indicated allele all strains were isogenic: PY79 *spo*⁺ (■), SC500 *spoIIIGΔ1* (○), SC745 *spoIIIGΔ1 bofB8* (▼), MG101 *spoIVBΔ::spc* (△), MG291 *spoIVBΔ::spc bofC1* (▲), MG117 *spoIVBΔ::spc bofC::neo* (▽), MG126 *spoIVBΔ::spc bofB8* (●). Background levels of β -galactosidase observed in *spo*⁺ cells containing no fusion have been subtracted.

bofC mutations do not bypass the requirement of SpoIVB for σ^K activation

Since *bofC1* bypasses the requirement of *spoIIIG* for σ^K activation it should likewise bypass *spoIVB* since this gene is σ^G -controlled. In recent work, however, we have found that *spoIVB* is transcribed at very low levels by $E\sigma^F$ (Gomez & Cutting, 1996). We introduced the *bofC1* or *bofC::neo* alleles into a *spoIVBΔ::spc* insertional mutant and lysogenized cells with *SPβ::gerE-lacZ* and measured β -galactosidase activity during sporulation. As a control, we also examined a *spoIVBΔ::spc bofB8* double mutant (MG126) in which the requirement of SpoIVB in the σ^K checkpoint is bypassed. Our results (Fig. 6) show that the *bofC* alleles did not restore σ^K -directed gene expression in the *spoIVB* mutant whereas *bofB8* restored significant levels of *gerE-lacZ* expression. Interestingly, *gerE-lacZ* expression in the *bofB8 spoIVBΔ::spc* cells was approximately 1 h later than the premature expression seen in *spoIIIGΔ1 bofB8* cells.

csbX and *bofC* could be cotranscribed during stationary phase

We wondered whether *bofC* could be cotranscribed from the upstream σ^B -dependent *csbX* promoter, since as mentioned earlier, no transcriptional terminator follows *csbX*. To address this we first constructed a *bofC-lacZ* fusion contained at the *bofC* locus (*bofC::bofC-lacZ*) as opposed to the *amyE* locus (see Methods). In this construct *bofC* expression could be

Fig. 7. *csbX* and *bofC* are transcribed during the stationary phase. *bofC*-directed β -galactosidase synthesis was measured before and after the end of exponential growth (t_0) in LB supplemented with glucose (5%) and glutamine (0.2%). $\square$, *amyE::bofC-lacZ* (MG24); $\circ$, *bofC::bofC-lacZ* (MG196); $\blacksquare$, *sigB314 amyE::bofC-lacZ* (MG242); $\bullet$, *sigB314 bofC::bofC-lacZ* (MG243). Background levels of β -galactosidase observed in *spo*⁺ cells containing no fusion have been subtracted.

controlled not only by the σ^F/σ^G -dependent promoter but also by any other regulatory elements that might exist upstream of *bofC* (including the *csbX* promoter). During sporulation we found that *bofC* expression was identical whether *bofC*-directed β -galactosidase synthesis was driven by *bofC-lacZ* at the *bofC* locus or at *amyE* (results not shown). However, when *bofC* expression was measured during growth in LB supplemented with glucose and glutamine (i.e. under conditions where sporulation is catabolite repressed (Schaeffer *et al.*, 1965) we found that *bofC* was expressed at the end of exponential phase when *bofC-lacZ* was placed at the *bofC* locus but not when *bofC* was placed at the *amyE* locus (Fig. 7). Moreover, this expression was abolished in a *sigB314* mutant, showing that this stationary-phase expression was under σ^B control.

DISCUSSION

The σ^K checkpoint provides an elegant device for regulating σ^K -directed gene expression during sporulation. Four developmental proteins facilitate this: SpoIVFA, SpoIVFB, BofA and SpoIVB. The first three are the most likely candidates for the processing complex that cleaves pro- σ^K to its mature form in the mother cell compartment. SpoIVB's function is to signal processing by transiting the forespore membranes and interacting directly, or indirectly, with the BofA-SpoIVFA-SpoIVFB complex.

Our results clearly show that the *bofC* gene product is also involved in the σ^K checkpoint. The *bofC1* or *bofC::neo* alleles show similarities to the *bofA* and *bofB* suppressor mutations. First, *bofC1* restores σ^K -

directed gene expression in a mutant unable to synthesize σ^G and so uncouples the requirement of forespore gene expression for pro- σ^K activation. Second, *bofC1* bypasses a *spoIIIA* mutant whose gene products are required for σ^G activation. Interestingly, σ^K -directed gene expression was turned on earlier in a *bofC1 spoIIIA53* mutant than in a *bofC1 spoIIIG41* mutant. Possibly, in a *spoIIIA53* mutant a low level of active σ^G could be produced, explaining why σ^K -directed gene expression was turned on earlier than in a *spoIIIG41* cells. Third, the *bofC1* (or *bofC::neo*) mutation confers an advanced sporulation phenotype upon *spoIIIG41* (or *spoIIIA53*) cells which is attributed to the aberrant synthesis and assembly of coat proteins onto the immature forespore in the absence of σ^G -dependent gene expression (Cutting *et al.*, 1990). Finally, insertional inactivation of *bofC* produces a Bof phenotype showing that loss of function of the *bofC* gene product is required for suppression [this is true also for null mutations in the *bofA* and *bofB* (*spoIVFA*) genes]. Thus, the *bofC* gene product would most likely act negatively in the σ^K checkpoint, which is also true for the *bofA* and *bofB* mutations.

The *bofC* mutations are, however, distinct from *bofA* and *bofB* in three crucial respects: (1) the compartment in which the gene defined by the mutation is expressed, (2) timing of σ^K activation in *bof spoIIIG41* strains and (3) their ability to bypass the requirement of SpoIVB for intercompartmental signalling. Thus, the similarity in phenotype produced by the *bofC* alleles with that produced by the *bofA* and *bofB* mutations must be considered superficial. These differences are discussed further below.

bofC is forespore expressed

The requirements for *bofC* transcription are most similar to the prespore-expressed genes *gpr* and *dacF*, which are controlled first by σ^F and then by σ^G (Partridge & Errington, 1993). Most indicative is the 'biphasic' pattern of gene expression seen as two peaks of activity representing the periods where first σ^F and then σ^G is functional in the prespore. For *gpr/dacF* transcription, as with *bofC*, the *spoIIAC* gene is absolutely required since this gene encodes σ^F , essential not only for the first phase of prespore gene expression but also for transcription of *spoIIIG*, which encodes σ^G , the sigma factor directing the second phase of compartmentalized gene expression. Mutations blocking σ^F - or σ^G -directed gene expression have been well documented and can be used to define whether a gene is forespore or mother cell expressed (Partridge & Errington, 1993; Stragier, 1992). Thus, the *spoIIIG* and *spoIIID* mutants block only the second, σ^G -controlled, phase of *bofC* expression since these genes encode proteins required for activating σ^G in the forespore chamber. Likewise, a *spoIVCB* mutant did not impair *bofC* expression since this gene encodes pro- σ^K , which is required only for the mother cell programme of gene expression. Together, the temporal 'biphasic' pattern of gene expression and genetic

dependence are diagnostic of a $E\sigma^F$ -controlled gene expressed in the prespore chamber (beginning at t_1), which is later controlled, following engulfment, by $E\sigma^G$ (from $t_{2.5}$ onwards). Supporting dual σ^F/σ^G control we have shown also that *bofC* expression can be artificially induced in vegetatively growing cells by induction of σ^F or σ^G and we have mapped the 5' terminus of *bofC* mRNA, placing it downstream of a putative σ^F/σ^G -recognized promoter.

In contrast, the *bofA* and *spoIVF* (*bofB*) genes are expressed beginning at t_1 in the mother cell chamber under the control of $E\sigma^E$ (Cutting *et al.*, 1991b; Ricca *et al.*, 1992). The spatial localization of *bofC*, *spoIVF* and *bofA* suggests that their gene products may act at different steps and use different mechanisms in the intercompartmental signal transduction pathway leading to σ^K activation.

Timing of σ^K activation in *spoIIIGΔ1* cells

Both the *bofC1* and *bofC::neo* mutations restored, at least partially, σ^K -directed gene expression in strains unable to produce active σ^G (and presumably processing of pro- σ^K) and this phenotype is well documented for the *bofA* and *bofB* mutations (Cutting *et al.*, 1990). However, in one important respect the *bofC* alleles differ and this is in the timing of σ^K -directed gene expression. *bofA/bofB* mutations allow premature processing ($t_{3.5}$, approximately 30 min early) of pro- σ^K since SpoIVFB is constitutively active, presumably because the inhibitory action of SpoIVFA/BofA on SpoIVFB is relieved. In such strains then, as soon as pro- σ^K is synthesized it is promptly cleaved. Interestingly, pro- σ^K is actually synthesized approximately 60 min prior to processing (t_3), yet the earliest possible time at which cleavage can occur is 30 min after synthesis of pro- σ^K ($t_{3.5}$). This delay in processing, even when SpoIVFB is constitutively active, has been attributed to the time required for the putative processing complex to assemble into the outer forespore membranes (Gomez *et al.*, 1995). In contrast, the *bofC* mutations introduced a substantial delay of about 3 h before σ^K -directed gene expression commenced (t_{7-8}). This delayed σ^K -dependent gene expression implies that the *bofC* mutations (or the absence of BofC) do not facilitate constitutive activity of SpoIVFB.

BofC does not act ahead of SpoIVB

Since SpoIVFB is constitutively active in *bofA* and *bofB* mutants, pro- σ^K processing commences in the absence of any signal (SpoIVB) from the forespore. Accordingly, a *bofA* or *bofB* mutation will restore σ^K -dependent gene expression in *spoIIIG* or *spoIVB* mutant strains. In contrast, *bofC* mutations bypass the requirement of σ^G for σ^K activation but do not bypass mutations in the *spoIVB* gene. In other work we have found that *spoIVB* expression is controlled not only by $E\sigma^G$ but also by $E\sigma^F$ (Gomez & Cutting, 1996) and that in a *spoIIIG* mutant SpoIVB is still synthesized at very low levels, which are

not enough to trigger σ^K activation in the mother cell. That *spoIVB* can be transcribed by both $E\sigma^F$ and $E\sigma^G$ provides a hint about how the BofC phenotype is produced. In the absence of active σ^G (due to a null mutation in *spoIIIG* or *spoIIIA*) σ^F -directed expression of SpoIVB would be sufficient to trigger σ^K activation, albeit delayed, but only if BofC is absent. Thus we conclude that BofC is a negative regulator, and that its absence compensates for the reduction in SpoIVB production in strains unable to produce active σ^G . *bofC* mutations then, can not be considered true *bof* alleles since they do not uncouple the forespore and mother cell programmes of gene expression.

A simple explanation for BofC's function could be repression of *spoIVB* transcription. We have explored this possibility and found that a *bofC* mutation does not enhance *spoIVB-lacZ* expression in a *spoIIIG* mutant (data not shown). We are left, then, with BofC as a negative regulator of SpoIVB activity or function.

Four mechanisms for how BofC and SpoIVB interact can be proposed. First, BofC could be directly involved in the assembly and translocation of SpoIVB through the forespore membrane(s). Second, BofC could inhibit a 'hypothetical' enzymic activity of SpoIVB, probably by direct protein-protein interaction. Third, BofC could be involved in the proteolytic turnover of SpoIVB. Finally, BofC could prevent or block the proposed interaction of SpoIVB with the BofA-SpoIVFA-SpoIVFB processing complex in the outer forespore membranes.

Although our work clearly shows that BofC could negatively regulate SpoIVB function and that *bofC* must be considered a component of the σ^K checkpoint, no obvious function (which is attributed to other checkpoint genes) can be associated with *bofC*.

Another surprising finding was the difference in timing of σ^K -directed gene expression in *spoIIIGΔ1 bofB8* or *spoIVBΔ::spc bofB8* cells. σ^K first appeared active at t_3 in *spoIIIGΔ1 bofB8* cells while in *spoIVBΔ::spc bofB8* cells σ^K -directed gene expression commenced some 60 min later, and about 30 min later than in *spo+* cells. Previous work has shown that the *bofB* (*spoIVFB*) alleles allow constitutive processing of pro- σ^K and premature σ^K -directed gene expression (Cutting *et al.*, 1990; Lu *et al.*, 1990). Here we find that in a *spoIVB* mutant this does not occur. We reason that the membrane-bound processing complex consisting of BofA, SpoIVFB and the mutant form of SpoIVFA (encoded by *bofB8*) could still be responsive to the low levels of σ^F -produced SpoIVB in *spoIIIGΔ1* cells, allowing premature signalling and processing of pro- σ^K .

The *bofC* transcriptional unit

Although our work clearly suggests that *bofC* is monocistronic during development we present evidence suggesting that *bofC* could be transcribed by read-through from the upstream gene *csbX* in stationary-phase conditions, when sporulation is repressed and in a

σ^B -dependent manner. The physiological significance of this probable read-through transcription is, for now, unclear, but it is unlikely that the *bofC* cistron of the *csbX-bofC* transcript is actually translated. Examination of the nucleotide sequence shows that there exists a region centred approximately -43 nt from the *bofC* start codon, that could base-pair with the *bofC* ribosome-binding site (centred at -11) and block its availability to ribosomes. The sporulation transcript starts at -25 nt from the *bofC* start codon and therefore could not form any secondary structure. A similar situation has been described for the *spoIIIG* gene, which is initially cotranscribed with the *spoIIG* operon but translation is prevented by a region of dyad symmetry separating the *spoIIG* and *spoIIIG* cistrons (Stragier, 1992).

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