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# Mutational analysis of the early forespore/mother-cell signalling pathway in *Bacillus subtilis*

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**Intercellular communication is a crucial phenomenon during spore development in *Bacillus subtilis*. It couples the establishment of a compartment-specific genetic program to the transcriptional activity of a $\sigma$ factor in the other compartment. It also keeps $\sigma$ factor activation in register with the morphological process. This study used directed mutagenesis to analyse the pathway that couples $\sigma^E$ activation in the mother-cell to activation of $\sigma^F$ in the forespore following asymmetric septation. Targets for mutagenesis in SpoIIGA (the receptor) were chosen based on the predicted topology of the protein when associated with the cell membrane. The results showed that a residue near the N terminus (D6), predicted to be exposed outside the cell, is required for receptor activity, whereas the major extracellular loop (between membrane domains IV and V) is dispensable for function. In contrast, mutations in SpoIIR (the signal) that partially blocked protein release (but not membrane translocation) had no effect on signal transduction. These results do not rule out the possibility that uncharacterized molecules intervene in the signalling pathway that establishes the mother-cell-specific developmental program during the early stage of sporulation.**

**Keywords:** *Bacillus subtilis*, sporulation, signalling pathway, membrane receptor

## INTRODUCTION

Sporulation in *Bacillus subtilis* is a developmental process initiated in response to nutrient exhaustion and resulting in the production of a dormant, heat-resistant, form: the mature spore. During this process, an ordered sequence of morphological events takes place, starting with the formation of an asymmetrically positioned septum that divides the sporangium into two unequal compartments: the forespore and the mother-cell (Errington, 1993). Each compartment contains a chromosome and engages in a specific genetic program governed by four $\sigma$ factors: $\sigma^F$ and $\sigma^G$ in the forespore, $\sigma^E$ and $\sigma^K$ in the mother-cell. $\sigma$ factor activation, which appears to be co-ordinated with the progression of the morphological stages, is organized in a cascade triggered by activation of $\sigma^F$ and followed by activation of $\sigma^E$, then $\sigma^G$ and finally $\sigma^K$ (Haldenwang, 1995). The molecular events leading to activation of $\sigma$ factors are schematically represented in a model called 'criss-cross' (Losick & Stragier, 1992) in which signals go back and forth through membrane structures between both compartments.

The first event in this scheme links $\sigma^E$ activation in the mother-cell to $\sigma^F$ activity in the forespore. It has been shown recently that transcriptional activity of $\sigma^F$ in the forespore results in the production of SpoIIR (Karow *et al.*, 1995; Londoño-Vallejo & Stragier, 1995; Zhang *et al.*, 1996) which triggers the conversion of inactive pro-$\sigma^E$ into active $\sigma^E$ in the mother-cell. The activation of $\sigma^E$, through cleavage of an N-terminal extension of 28 residues, also requires the presence of SpoIIGA, a membrane-associated protein with a large cytoplasmic domain bearing significant similarity to a family of aspartic proteases (Jonas *et al.*, 1988; Masuda *et al.*, 1990; Stragier *et al.*, 1988). Genetic evidence indicates that SpoIIGA itself is the protease responsible for pro-$\sigma^E$ processing during sporulation (Peters & Haldenwang, 1994). In addition, we have shown, using vegetatively growing cells, that SpoIIR is the only sporulation-specific protein required for activation of SpoIIGA-

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**Abbreviation:** PhoA, alkaline phosphatase.

dependent processing of pro-$\sigma^E$ (Londoño-Vallejo & Stragier, 1995). Moreover, culture supernatants from cells synthesizing SpoIIR are able to trigger production of mature $\sigma^E$ when added extracellularly to non-sporulating whole cells bearing SpoIIGA and pro-$\sigma^E$ (Hofmeister *et al*., 1995).

Based on these results, current models for the forespore/mother-cell signalling pathway between $\sigma^F$ and $\sigma^E$ propose two intervening molecules: SpoIIR (the signal) and SpoIIGA (the receptor). In an effort to understand the molecular basis of signal–receptor interactions, an analysis of both structural genes using site-directed mutagenesis was undertaken.

## METHODS

**In-frame deletions in *spoIIGA*.** The in-frame deletion in *spoIIGA* affecting codons 110–132 was created as follows: an *Eco*RI–*Pst*I fragment spanning the *spoIIG* promoter and the beginning of the gene (up to codon 109) (Guérout-Fleury *et al*., 1996) was subcloned between the *Eco*RI site of pUC9 and the *Pst*I site of pUC8, using a third enzyme (*Alw*NI) to reconstruct the vector. This introduced four codons downstream from the *Pst*I site, a *Sma*I site compatible and in-frame with the *Pvu*II site in codon 132 of *spoIIGA*. The reconstituted version of *spoIIGA* was integrated at *amyE* in the *B. subtilis* chromosome using pDG1662 (Guérout-Fleury *et al*., 1996). The large deletion affecting *spoIIGA* at its own locus between codons 27 and 272 has been described by Guérout-Fleury *et al*. (1996).

**PCR-directed mutagenesis.** Point mutations in *spoIIGA* were introduced by PCR as described by Picard *et al*. (1994). The following oligonucleotides (complementary strand) carrying the desired point mutations (underlined) were synthesized: YA4, CCAAATGACATCTAAGGCCATTTTCACATC; DA6, GCCAAATGACGGCCAAATAGATTTTCAC; and VA7, TAACAGCCAAATCGCGTCTAAATAGATTTTC. The PCR reactions used a Δ*Pst*I derivative of pDG181 (promoterless *spoIIGA* cloned into pUC18; Stragier *et al*., 1988) which carried the 5′ end of the gene (beyond the start codon). After PCR, the amplified products were used to reconstitute the structural gene through ligation to the *spoIIGA* 3′ end derived from a *spoIIGA–HA*1 construction (kindly provided by N. Frandsen, IBPC, Paris) and carrying the HA1 epitope tag (Field *et al*., 1988). The tag, inserted at position M120 of SpoIIGA, has the sequence HAMVPT-YPYDVPDYASRPGLGTMACM (epitope underlined). The mutated genes, as well as the wild-type, were placed under the control of the *spoIIA* promoter (Stragier *et al*., 1988) and integrated at the *amyE* locus of a *B. subtilis* strain carrying a large deletion in *spoIIGA* (Guérout-Fleury *et al*., 1996). Alternatively, they were placed under the control of the inducible promoter *spac* within the *B. subtilis* replicative vector pDG178 (Stragier *et al*., 1988) which carries the whole *spoIIG* operon.

The point mutation DE6 was introduced by PCR using the synthetic oligonucleotide GCCAAATGACCTCGAGATAG-ATTTTCACATC (complementary strand) and a vector carrying the *spoIIG* promoter and the beginning of the *spoIIGA* gene (described above) as DNA template. The whole gene was reconstituted and integrated at the *amyE* locus as indicated above.

Point mutations in *spoIIR* (GV21 and AM23) were introduced in the same way using the synthetic oligonucleotide (coding strand) CCGGAGCGCTCGTAGTGCTCATGAAAGAAG-AGACGGCAC and a subcloned fragment of *spoIIR* (with or without promoter) as DNA template. The amplified product was used for reconstitution of *spoIIR* either under the control of its own promoter or under the control of the inducible promoter *spac* (Yansura & Henner, 1984). Both constructions were integrated at *thrC* in the *B. subtilis* chromosome using pDG1664 (Guérout-Fleury *et al*., 1996). In addition, the *Bsp*HI site overlapping the AM23 mutation allowed us to construct a version of *spoIIR* lacking the first 22 codons after several subcloning steps aimed at replacing the Shine–Dalgarno sequence in front of the methionine. This version was also integrated at *thrC* in the chromosome under the control of the *spoIIR* promoter. Alternatively, it was placed under the control of the *spac* promoter.

All point mutations introduced by PCR were confirmed by DNA sequencing. In the case of *spoIIGA*(*DA6*), when the mutated allele was introduced in single copy into a wild-type strain, the presence of the point mutation in the chromosome after transformation was confirmed by PCR and sequencing.

**Constructions of *phoA* (alkaline phosphatase) and *lacZ* (β-galactosidase) fusions to *spoIIGA*.** *phoA* fusions to *spoIIGA* were constructed by cloning a DNA fragment encoding the soluble domain of PhoA (derived from plasmid pPHO7; Gutierrez & Devedjian, 1989) in-frame behind several promoterless *spoIIGA* fragments. These *spoIIGA* fragments were generated using the natural restriction sites *Nhe*I (codon 65), *Tsp509*I (codon 87), *Bsp*HI (codon 120) and *Stu*I (codon 180). A *Bam*HI restriction site was introduced by PCR at codon 36. The *spoIIGA–phoA* fusions were placed under the control of the inducible promoter *spac* in the plasmid pDG148 (Stragier *et al*., 1988). *lacZ* fusions to *spoIIGA* codons 36, 87 and 180 were also constructed and placed under the control of *spac*. *phoA* and *lacZ* fusions to *spoIIGA*(*DA6*) were derived from wild-type fusions using the unique *Nhe*I site. *phoA* fusions were introduced in *Escherichia coli* DH4B (*phoA*$^-$) and *lacZ* fusions in *E. coli* IBPC5311 (*lacZ*$^-$). PhoA and β-galactosidase activities were determined in whole cells as described by Brickman & Beckwith (1975) and Miller (1972). Accumulation of PhoA protein fusions was determined by Western blotting using specific anti-PhoA rabbit antibodies (20 μg ml$^{-1}$) (Rockland). Protein extracts from induced *E. coli* cultures were subjected to SDS-PAGE (8%) followed by electrotransfer onto nitrocellulose. After overnight incubation (4 °C), antibodies were revealed by anti-rabbit IgG antibodies conjugated to PhoA (1:5000 dilution) (Promega).

**SpoIIR–levansucrase hybrid proteins.** SpoIIR–levansucrase hybrid proteins were constructed by fusing a *Rsa*I–*Bcl*I DNA fragment from *spoIIR* (spanning the promoter and the first 40 codons) in-frame with a modified *sacB* gene (encoding the soluble form of levansucrase; a gift from M. F. Petit-Glatron, Institut Jacques Monod, Paris). The *spoIIR* fragment encoded either the wild-type or the mutated SpoIIR N-terminal signal sequence. The hybrids were integrated at an ectopic site (*thrC*) in the *B. subtilis* chromosome. All experiments involving levansucrase were performed in strains carrying a disruption in the structural gene *sacB*. The disruption was introduced in the *B. subtilis* chromosome by marker replacement using a tetracycline resistance cassette (Guérout-Fleury *et al*., 1995) cloned between sites Asp718 (codon 354) and *Sac*II (codon 360).

**Sporulation assays.** All *B. subtilis* strains used are derivatives of JH642, except for the protein secretion experiments

described below. The deletion-disruption in *spoIIR* has been described previously (Londoño-Vallejo & Stragier, 1995). Cells were allowed to sporulate by nutrient exhaustion in liquid DS medium at 37 °C (Schaeffer *et al*., 1965). Samples were withdrawn at different times during sporulation and analysed for $\beta$-galactosidase activity or pro-$\sigma^E$ processing as described by Frandsen & Stragier (1995) and Londoño-Vallejo & Stragier (1995). Heat-resistant (80 °C, 10 min) spores were counted after 30 h of culture in sporulating medium.

**Induction of gene expression under non-sporulating conditions.** Conditions of induction of the *spac* promoter have been described by Frandsen & Stragier (1995). In experiments including *spac–spoIIG* and *spac–spoIIR*, *B. subtilis* cell cultures were induced early during exponential growth and samples were withdrawn at different times for $\beta$-galactosidase determination or $\sigma^E$ immunodetection, as described previously (Londoño-Vallejo & Stragier, 1995). Immunodetection of SpoIIGA–HA1 was performed in samples withdrawn 3 h after induction. Cell lysates were prepared by osmotic shock (Breitling & Dubnau, 1990) and membrane and soluble fractions, obtained through differential centrifugation, were subjected to SDS-PAGE (12%) followed by electrotransfer onto nitrocellulose. Blots were incubated with the anti-HA1 monoclonal antibody (5 μg ml$^{-1}$, 4 °C, overnight) (Boehringer) and the anti-mouse secondary antibody conjugated to PhoA (1:5000 dilution) (Hyclone).

**Detection of SpoIIR–levansucrase hybrids during sporulation and exponential growth.** SpoIIR–levansucrase protein hybrids were detected using purified anti-levansucrase antibodies. The antibodies were affinity-purified using purified levansucrase and a rabbit anti-levansucrase serum (both kindly provided by M. F. Petit-Glatron) as follows. Nitrocellulose-bound levansucrase (~100 μg) was incubated with the anti-levansucrase antibodies (1:20 dilution) at 4 °C overnight with continuous shaking. Following extensive washing with TBS (10 mM Tris, pH 8, 150 mM NaCl) the antibodies were eluted by treatment with glycine (0·2 M, pH 2·8) for 10 min, after which the solution was neutralized with Tris base (40 mM) and equilibrated with TBS.

Protein extracts to be analysed by Western blotting were prepared either from sporulating cells or after induction during vegetative growth. For studies during sporulation, *spoIIR–sacB* constructions were introduced into a *spoIIAC561* strain carrying a deletion-disruption in *sacB*. Cells were allowed to sporulate in DS medium and samples were withdrawn at different times. For studies during vegetative growth, *spoIIR–sacB* constructions were introduced into the protease-deficient strain GP283 (Hofmeister *et al*., 1995), which carried a plasmid containing the gene for $\sigma^F$ under the control of *spac* (Londoño-Vallejo & Stragier, 1995). Induction by addition of IPTG was performed as above and samples were processed after 2 h. Membrane fractions were prepared by differential centrifugation of cell lysates (Breitling & Dubnau, 1990) and were analysed by SDS-PAGE (10%) followed by electrotransfer onto nitrocellulose. Supernatants from IPTG-induced cell cultures were filtered (0·2 μm) and concentrated using Centriplus10 (Amicon), as described by (Hofmeister *et al*. (1995). Affinity-purified anti-levansucrase antibodies were used for immunodetection at 1:10 dilution (4 °C, overnight) followed by incubation with an anti-rabbit secondary antibody (1:5000 dilution) coupled to PhoA (Promega).

## RESULTS

### Membrane topology of SpoIIGA and prediction of potential sensor domains

Computer analysis of the SpoIIGA amino acid sequence has predicted the presence of five membrane-spanning domains in the N-terminal half of the protein (Stragier *et al*., 1988). The distribution of positive charges found in the connecting loops also predicted that the large soluble C-terminal domain is exposed to the cytosol and that the N-terminus of the protein and two loops (between domains II and III, and IV and V) are found outside the cell (Stragier *et al*., 1988) (Fig. 1). Indirect evidence for the cytoplasmic localization of the C-terminal domain has been provided by Peters & Haldenwang (1991) through the construction of a translational *lacZ* fusion to the 3′ end of the gene, yielding an active $\beta$-galactosidase fusion in *E. coli*. More evidence in support of the proposed membrane topology for SpoIIGA has been obtained through the construction of five *phoA* translational fusions at the positions indicated in Table 1. Two of these fusions (positions L65 and M120) yielded enzymically active PhoA fusions in *E. coli*, indicating their extracellular localization. *phoA* translational fusions to three other positions (L36, N87 and G180) yielded inactive products, whereas the corresponding *lacZ* fusions resulted in high $\beta$-galactosidase activity (Table 1).

Since SpoIIGA activity is controlled through an extracellular signal (and assuming that the topology of the protein in *E. coli* can be extrapolated to *B. subtilis* cells) segments exposed on the outer face of the membrane constitute potential sensor domains. In the predicted

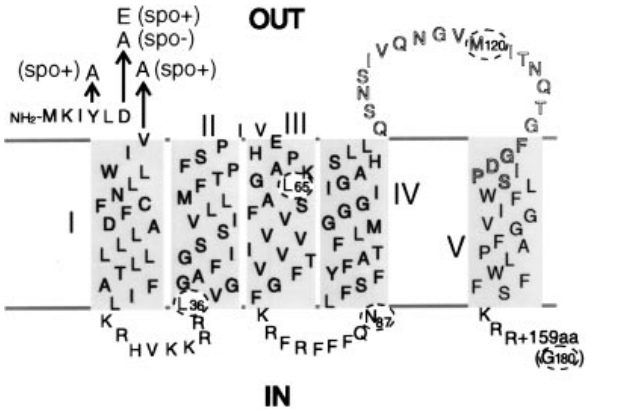


***Fig. 1.*** Schematic representation of the proposed membrane-associated structure of SpoIIGA, based on sequence and fusion analysis using *phoA*/*lacZ*. Transmembrane domains are numbered I–V and their residues are represented in a helical net. The C-terminal cytoplasmic domain is not shown. The amino acid positions fused to *phoA* or *lacZ* are numbered and indicated by dashed circles. Outlined characters between domains IV and V designate residues dispensable for function. Amino acid replacements affecting positions that are conserved in *C. acetobutylicum* and their effects on sporulation (spo+, spo-) are indicated by arrows.

***Table 1.*** PhoA and *β*-galactosidase activities obtained from PhoA and *β*-galactosidase fusions in SpoIIGA

Several PhoA and *β*-galactosidase fusions were constructed at the indicated amino acid positions in SpoIIGA (ND, not done). The enzymic activities were determined 4–5 h after the addition of IPTG to exponentially growing *E. coli* cells carrying the respective constructions. The results of a typical experiment are shown.

| Amino acid | PhoA activity (Miller units) | *β*-Galactosidase activity (Miller units) |
|---|---|---|
| L36 | 13 | 1375 |
| L65 | 210 | ND |
| N87 | 5 | 1158 |
| M120 | 287 | ND |
| G180 | 10 | 9722 |

***Table 2.*** PhoA and *β*-galactosidase activities obtained from PhoA and *β*-galactosidase fusions in the SpoIIGA(DA6) mutated protein

PhoA and *β*-Galactosidase fusions were constructed at the indicated positions in the SpoIIGA(DA6) mutated protein (DA6) and compared to the activities of the respective wild-type fusions (WT) under the same experimental conditions. ND, Not done.

| Amino acid | Fusion protein | PhoA activity (Miller units) | *β*-Galactosidase activity (Miller units) |
|---|---|---|---|
| M120 | WT | 113 | ND |
| | DA6 | 143 | ND |
| G180 | WT | 8 | 23456 |
| | DA6 | 16 | 25185 |

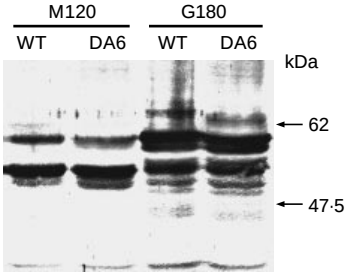


***Fig. 2.*** PhoA fusions to wild-type (WT) and SpoIIGA(DA6) (DA6). A Western blot analysis using specific antibodies against PhoA shows the relative accumulation of PhoA fusion proteins in cell extracts from the *E. coli* strains assayed in Table 2; the top bands in each lane correspond to the expected full-length fusion proteins.

topology, the extracellular loop between membrane domain 2 and 3 appears to be extremely short; therefore, only the larger extracellular loop (between domains IV and V) and the N-terminal end of the protein were defined as targets for directed mutagenesis (Fig. 1).

A copy of *spoIIGA* carrying the in-frame deletion ΔQ110–S132, which affects the whole extracellular loop between domains IV and V (Fig. 1) and replaces it by the sequence MSRG, was introduced at an ectopic site (*amyE*) in the chromosome. This deleted copy fully complemented ($\sim 3 \times 10^8$ spores ml$^{-1}$) a large in-frame deletion affecting *spoIIGA* at its own locus (no spores) (Guérout-Fleury *et al*., 1996). This result clearly indicates that the big extracellular loop in SpoIIGA is not required to sense the activating signal or, presumably, to transduce it to the catalytic domain inside the cell.

The N-terminal end of SpoIIGA, predicted to contain only 6 or 7 aa, stands out by the conservation of some of these residues (Y4, D6, V7) in the SpoIIGA homologue found in the sporulating bacterium *Clostridium acetobutylicum* (Wong *et al*., 1995). The predicted overall structure and membrane topology of this protein are very similar to the ones proposed for *B. subtilis* SpoIIGA: five transmembrane domains at their N-terminal half followed by a cytoplasmic domain containing highly conserved amino acid stretches forming the presumed catalytic site (not shown). However, the extracellular connecting loops are less well conserved, underscoring the potential importance of the N-terminal residues. Alanine replacements at positions Y4, D6 and V7 were introduced through PCR mutagenesis and the respective alleles were tested for their ability to complement the Δ*spoIIGA* strain. YA4 and VA7 mutated genes showed full capacity to complement the sporulation defect whereas the DA6 allele showed a degree of complementation two to three orders of magnitude below the normal level ($3 \times 10^5$–$1 \times 10^6$ spores ml$^{-1}$). The N-terminal DA6 point mutation apparently did not affect the overall SpoIIGA protein structure in the membrane since PhoA or *β*-galactosidase fusions to SpoIIGA(DA6) behaved identically to the wild-type fusions in *E. coli* (Table 2 and Fig. 2). No dominant negative effect on sporulation was observed when the *spoIIGA*(*DA6*) allele was introduced in an otherwise wild-type strain, the presence in the chromosome of the mutated allele being confirmed by PCR and sequencing. Interestingly, a DE6-mutated *spoIIGA* allele was able to fully complement the sporulation defect of the Δ*spoIIGA* strain when introduced at an ectopic site (*amyE*).

### The N-terminal domain of SpoIIGA is essential for SpoIIGA-dependent $\sigma^E$ activation

Experiments were performed to follow the kinetics of pro-$\sigma^E$ processing and of transcription of $\sigma^E$-dependent

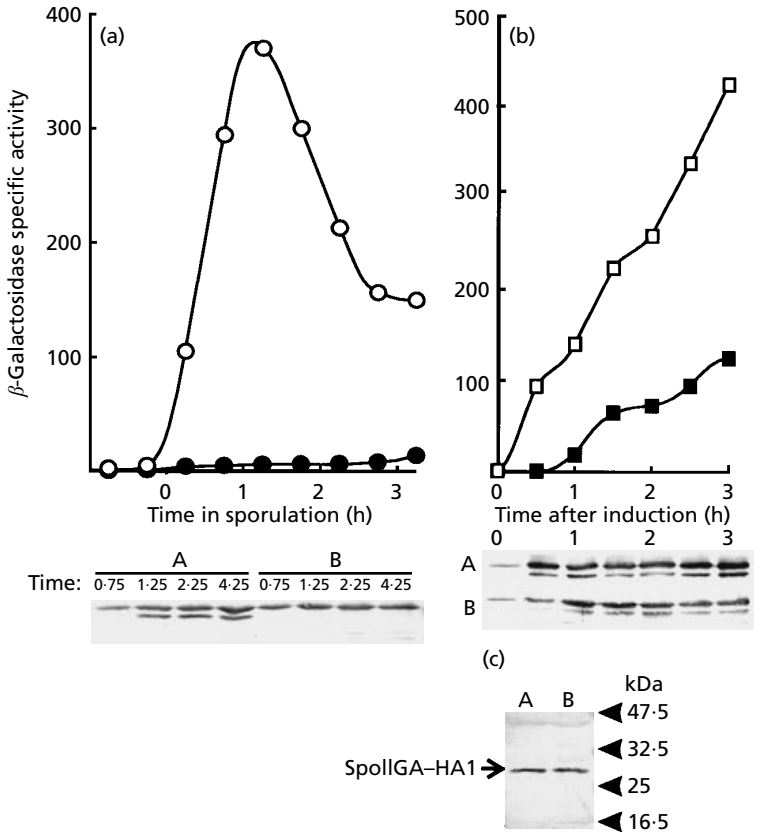


***Fig. 3.*** Effects of the DA6 mutation in SpoIIGA function. (a) Time course of expression during sporulation of a *spoIID–lacZ* transcriptional fusion (dependent on $\sigma^E$) in a Δ*spoIIGA* strain carrying either *spoIIGA–HA1* (○, top; A, lower) or *spoIIGA*(*DA6*)*–HA1* at the *amyE* locus (●, top; B, lower). The lower part shows an immunoblot analysis of pro-$\sigma^E$ processing in the same set of strains, samples being harvested at the times indicated (h). (b) Time course of expression of the *spoIID–lacZ* transcriptional fusion in strains engineered to produce pro-$\sigma^E$, SpoIIR and either SpoIIGA–HA1 (□, top; A, lower) or SpoIIGA(DA6)–HA1 (■, top; B, lower) in response to IPTG. The lower part shows an immunoblot analysis of pro-$\sigma^E$ processing in the same set of strains and samples. (c) SpoIIGA immunoblot analysis, using anti-HA1 monoclonal antibody and membrane protein extracts from cells harvested at the last time point in (b). Lanes: A, SpoIIGA–HA1; B, SpoIIGA(DA6)–HA1. Approximate mobility: 30 kDa (expected: 37·5 kDa). β-Galactosidase activity is expressed as nmol 2-nitrophenyl-β-D-galactopyranoside hydrolysed $min^{-1}$ (mg protein)$^{-1}$.

genes during sporulation in the Δ*spoIIGA* strain carrying a *spoIIGA*(*DA6*) allele. In the typical experiment shown in Fig. 3(a), pro-$\sigma^E$ processing was undetectable and expression of *spoIID–lacZ* (a member of the $\sigma^E$ regulon; Rong *et al.*, 1986) was highly depressed when compared to the control strain.

The wild-type and DA6 *spoIIGA* versions used in this experiment carried the epitope tag HA1 (Field *et al.*, 1988) at position M120, and both versions were placed under the control of a relatively strong stage 0 promoter (*spoIIA*) (Errington & Mandelstam, 1986; Wu *et al.*, 1991). However, both proteins were undetectable in Western blots using the anti-HA1 monoclonal antibody (not shown); this suggested a very low level of expression of this particular gene during sporulation.

To circumvent this problem, a system was used in which *spoIIG* (encoding both SpoIIGA and pro-$\sigma^E$) as well as *spoIIR* are controlled by an inducible promoter (Londoño-Vallejo & Stragier, 1995; Stragier *et al.*, 1988). Induction of these genes during vegetative growth (non-sporulation conditions) results in pro-$\sigma^E$ processing and transcription of $\sigma^E$-dependent genes (Londoño-Vallejo & Stragier, 1995). After induction, the strain bearing the mutated version of *spoIIGA–HA1* showed impaired $\sigma^E$ processing and consequently low $\sigma^E$ transcriptional activity when compared to the control strain (Fig. 3b). The residual activity observed for SpoIIGA(DA6) was still dependent on the presence of *spoIIR* (not shown). Western blot analysis showed that both wild-type and SpoIIGA(DA6) proteins accumulated in cell-membrane fractions and were undetectable in the cytosol (not shown). Although, as shown in Fig. 3(c), SpoIIGA(DA6) seemed to accumulate at a slightly lower level than the wild-type, protein instability would not fully account for the fourfold difference between the $\sigma^E$ transcriptional activities detected in the same strains (Fig. 3b).

## Release of SpoIIR is not necessary for SpoIIGA activation

Analysis of the SpoIIR amino acid sequence predicted that the protein is exported through the membrane and secreted due to a typical N-terminal signal peptide (Karow *et al.*, 1995). Those predictions were confirmed by the finding that a protein factor possessing the characteristics of SpoIIR is released in the culture supernatants of cells induced for $\sigma^F$ activity (Hofmeister *et al.*, 1995). The SpoIIR secretion signal peptide contains the highly conserved amino acids glycine and alanine at positions −3 and −1, respectively, from the presumed cleavage point (Nagarajan, 1993).

To test whether release from the membrane is necessary for SpoIIR activity, point mutations were introduced by PCR at these conserved positions (GV21 and AM23). These mutations were expected to block cleavage but

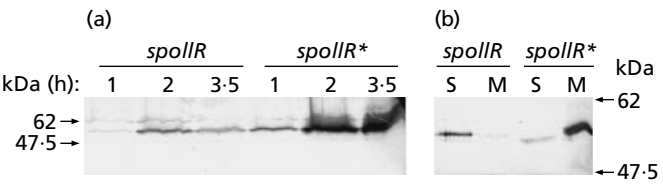


***Fig. 4.*** Effect of point mutations in the SpoIIR putative cleavage site on protein release. (a) Immunoblot analysis, during sporulation, of strains expressing levansucrase fused either to the wild-type (*spoIIR*) or to the mutated (*spoIIR**) SpoIIR N-terminal signal sequence. Membrane protein samples from cells harvested at the times indicated (h) are shown. Antibodies were directed against the levansucrase moiety. Expected mobility (of the unprocessed protein): 54·8 kDa. (b) Immunoblot analysis of *B. subtilis* cultures engineered to produce active $\sigma^F$ during exponential growth. Cells expressing either SpoIIR–levansucrase (*spoIIR*) or SpoIIR(GV21–AM23)–levansucrase (*spoIIR**) in response to $\sigma^F$ were harvested 2 h after induction. An immunoblot performed on cell membrane (M) and culture supernatant (S) proteins from both strains is shown. Antibodies were directed against the levansucrase moiety.

should allow normal protein translocation through the membrane (Nagarajan, 1993). Sporulation tests showed that the *spoIIR* mutated version (GV21–AM23) fully complemented ($3 \times 10^8$ spores $ml^{-1}$) a *spoIIR* disruption-deletion (0 spores $ml^{-1}$) when introduced at the *thrC* locus in the *Bacillus* chromosome. This result indicates that the GV21–AM23 mutations in SpoIIR do not interfere with protein function nor, presumably, with protein membrane translocation during sporulation.

A different version of *spoIIR*, which completely lacks the signal sequence, was also tested. When expressed under its own promoter, this version of *spoIIR* failed to complement ($4 \times 10^2$ spores $ml^{-1}$) the Δ*spoIIR* strain. On the other hand, the same version under the control of the inducible promoter *spac* (and whose expression is presumably not compartmentalized) was able to partially complement ($10^7$ spores $ml^{-1}$) the *spoIIR* null mutation. Taken together, these results are in agreement with the predicted requirement of membrane translocation during normal sporulation conditions for SpoIIR to activate the mother-cell genetic program.

Because of the lack of specific probes against SpoIIR, the effects of the GV21–AM23 mutation on protein release were tested by fusing the first 30 aa of either the wild-type or the mutated SpoIIR to the soluble domain of levansucrase (lacking the N-terminal signal peptide) (Borchert & Nagarajan, 1991; Petit-Glatron *et al.*, 1990). An antiserum specific for levansucrase (kindly supplied by M. F. Petit-Glatron) was used for the detection of the SpoIIR–levansucrase hybrid proteins in culture supernatants or membrane protein extracts.

In a first approach, the fate of the two SpoIIR–levansucrase hybrid proteins during sporulation was determined. Since the fusions were placed under the control of the *spoIIR* promoter, which is expressed at a very low level (Karow *et al.*, 1995; Londoño-Vallejo & Stragier, 1995), the constructions were introduced into a *spoIIAC561* genetic background (Illing & Errington, 1991). These strains, which produced a modified form of $\sigma^F$ resulting in a higher expression of *spoIIR*, were allowed to engage in sporulation and cell samples were analysed by Western blotting at different times. As shown in Fig. 4(a), signals corresponding to the SpoIIR–levansucrase hybrids were visible in membrane fractions from both strains. However, only the mutated version accumulated with time, suggesting that its release from the membrane was somewhat impaired by the changes introduced at the putative cleavage site of SpoIIR, whereas most of the wild-type SpoIIR–levansucrase hybrid was presumably released into the medium.

Next, the fate of the hybrid SpoIIR–levansucrase proteins after $\sigma^F$ induction was determined by probing the supernatant and membrane extracts from exponentially growing cell cultures. As shown in Fig. 4(b), the wild-type SpoIIR signal sequence mediated secretion of SpoIIR–levansucrase, which was found almost exclusively in the supernatant, whereas mutations in the SpoIIR putative cleavage site led to accumulation of the hybrid protein in the membrane with a small proportion being detected in solution. Because the observed relative mobilities of the secreted form in the wild-type and the membrane-associated form in the mutant were not different in the gel system used here, it is not known whether protein accumulation in the membrane actually resulted from blockage of cleavage in the signal sequence of SpoIIR.

The mutations introduced in the signal sequence of SpoIIR, although they partially inhibited protein release, do not appear to affect its function, namely the activation of SpoIIGA. This conclusion is reinforced by induction experiments during vegetative growth in which the kinetics and efficiency of this activation is indistinguishable from that observed with the wild-type SpoIIR (Fig. 5a). To further test the possibility that protein release might not be required for SpoIIR signalling, a conventional N-terminal membrane domain, found in the forespore-specific membrane protein SpoIIQ (domain 1-MREEEKKTSQVKKLQQF-FRKRWVFPAIYLVSAAVILTAVLWYQSVSNDEV-KD-52, with the same orientation and with no putative cleavage site; Londoño-Vallejo *et al.*, 1997), was substituted for the signal peptide in SpoIIR. The *spoIIQ–spoIIR* version was able to fully complement ($3 \times 10^8$ spores $ml^{-1}$) the sporulation defect when integrated at the *amyE* locus in the *B. subtilis* chromosome of a Δ*spoIIR* strain. This observation suggests that the SpoIIQ membrane domain mediated translocation of SpoIIR through the forespore membrane and further supports the idea that release of SpoIIR within the interseptal space might not be required to activate SpoIIGA during sporulation.

Finally, the ability of the SpoIIR mutated proteins to activate SpoIIGA in the same cell compartment was explored. To do this, the *spoIIR*(*GV21–AM23*) allele (or the 'cytoplasmic' *spoIIR* version) was substituted for

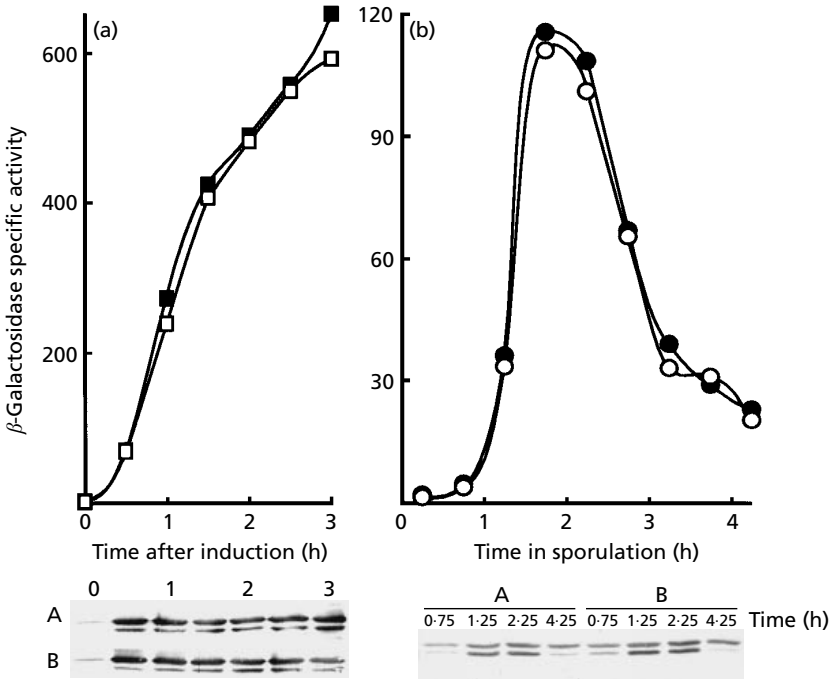


***Fig. 5.*** Efficiency of SpoIIGA activation by SpoIIR(GV21–AM23). (a) Time course of expression of the *spoIID–lacZ* transcriptional fusion in strains engineered to produce pro-$\sigma^E$, SpoIIGA and either SpoIIR (□, top; A, lower) or SpoIIR(GV21–AM23) (■, top; B, lower) in response to IPTG. The latter strain carried a *spoIIR* null mutation in the chromosome. The lower part shows an immunoblot analysis of pro-$\sigma^E$ processing in the same set of strains and samples. (b) Time course of expression during sporulation of a *spoIID–lacZ* transcriptional fusion in MO 1300 strains carrying either a wild-type *spoIIR* allele at its own locus (○, top; A, lower) or a null mutation in *spoIIR* and the allele SpoIIR(GV21–AM23) at an ectopic site (●, top; B, lower). The lower part shows an immunoblot analysis of pro-$\sigma^E$ processing in the same set of strains, samples being harvested at the times indicated (h). $\beta$-Galactosidase activity is expressed as nmol 2-nitrophenyl-$\beta$-D-galactopyranoside hydrolysed $min^{-1}$ (mg protein)$^{-1}$.

the wild-type allele in the *B. subtilis* strain MO1300 (P**spoIIA*–Δ*spoIIAB*). In this strain, SpoIIAB, the anti-$\sigma$ factor that prevents $\sigma^F$ activation before septation (Duncan & Losick, 1993), is absent; this allows $\sigma^F$ activity to appear before septation, a second mutation in the *spoIIA* promoter keeping active $\sigma^F$ levels low enough to prevent immediate cell lysis (Shazand *et al.*, 1995). Nevertheless, active $\sigma^F$ triggers activation of $\sigma^E$, which leads to definitive blockage of asymmetric septation (Shazand *et al.*, 1995). Since activation of $\sigma^E$ in MO1300 is not due to a long-range intercellular signal (Londoño-Vallejo & Stragier, 1995), it is better explained by the ability of SpoIIR to activate SpoIIGA molecules inserted on the same membrane surface from which the signal is released. As shown in Fig. 5(b), SpoIIR(GV21–AM23) is as efficient as the wild-type protein in triggering pro-$\sigma^E$ processing by SpoIIGA in non-compartmentalized cells. No pro-$\sigma^E$ processing was detected in the MO1300 genetic context when the 'cytoplasmic' version of SpoIIR was used to replace the wild-type *spoIIR* gene (not shown), suggesting that membrane translocation does favour the interaction between signal and receptor.

## DISCUSSION

A genetic approach to investigate the presumed interactions between the forespore signal (SpoIIR) and the mother-cell receptor (SpoIIGA) was followed. Based on protein sequence analysis and experimental evidence, mutations in two extracellularly exposed domains of SpoIIGA were designed. The results showed that the large extracellular loop between membrane domains IV and V is dispensable for function. On the other hand, an aspartate at position 6 appears to be important for triggering pro-$\sigma^E$ proteolytic activation. However, the reasons for the loss of function in the SpoIIGA(DA6) protein remain obscure.

The induction experiments during vegetative growth suggested that impairment in $\sigma^E$ activation by SpoIIGA(DA6) is not caused by protein instability or by interference with the correct targeting of the protein to a specific location in the cell (for example, the sporulation septum). Interestingly, a more conservative amino acid change (to glutamate) preserves the function of the protein. Perhaps this negatively charged residue is part of a surface which contacts either SpoIIR itself or another protein involved in signal transduction.

This report also presents evidence suggesting that membrane translocation of SpoIIR is required for full SpoIIGA activation. On the other hand, mutations in the putative cleavage site of SpoIIR that partially block release of the fusion protein SpoIIR–levansucrase do not affect protein function. In this case, the release of small amounts of SpoIIR could theoretically be enough to activate SpoIIGA (Hofmeister *et al.*; 1995). However, the replacement of the whole signal sequence in SpoIIR by a conventional membrane domain did not affect its function either, suggesting that membrane-exported SpoIIR may not need to be released to activate SpoIIGA.

Clearly, more powerful experiments are required to demonstrate a direct interaction between SpoIIR and SpoIIGA or else the participation of intermediate molecules. An *in vitro* reconstitution of the system would provide the best tool to test the relevance and topology of partner interactions as well as to understand the molecular mechanisms leading to receptor activation. Alternatively, directed mutagenesis aimed at

modifying the properties of the SpoIIGA transmembrane domains could shed light on the way signal transduction takes place during the early stages of spore development.

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