Cell–cell signaling pathway activating a developmental transcription factor in *Bacillus subtilis*

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Transcription in the mother cell at early stages of sporulation in *Bacillus subtilis* is controlled by σ^E_, a σ factor that is synthesized in the predvisional cell as an inactive larger precursor, pro-σ^E_. Activation of σ^E depends on σ^F_, the factor that governs transcription in the forespore. Genetic experiments have indicated that transduction of the activation signal from the forespore to the mother cell requires the products of some genes belonging to the σ^F-controlled regulon. We have identified and characterized a σ^F-dependent gene, csfX, encoding a protein necessary and sufficient for triggering processing of pro-σ^E_. The CsfX protein contains a typical amino-terminal signal sequence suggesting that, although synthesized in the forespore, it may act across the septum to activate the membrane-bound enzyme that is responsible for pro-σ^E_ processing in the mother cell.

[Key Words: Sporulation, Bacillus subtilis, σ factor, secreted protein]

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Early during sporulation in *Bacillus subtilis* an asymmetric division event generates two cells with different but coordinated developmental fates [Losick and Stragier 1992; Errington 1993]. Differential gene expression in these two cells is largely governed by new transcription factors. It is initiated by activation of the σ^F_ factor in the smaller cell, the forespore [Margolis et al. 1991; Partridge et al. 1991], which is required for subsequent activation of the σ^E_ factor in the larger cell, the mother cell [Driks and Losick 1991; Losick and Stragier 1992]. σ^E_ is synthesized in the predvisional cell [Gholamhoseinian and Pigott 1989] as an inactive larger precursor, pro-σ^E_ [LaBell et al. 1987] the product of the spoIIGB gene [Stragier et al. 1984; Trempe et al. 1985a], which is cotranscribed with spoIIGA, the gene encoding the putative pro-σ^E_ processing enzyme [Stragier et al. 1988]. SpoIIGA is predicted to be a membrane-bound protein [Stragier et al. 1988; Peters and Haldenwang 1991] and is also synthesized before septation, suggesting that it is present on both sides of the septum. It has been hypothesized that σ^E_ is involved in generating the signal that triggers pro-σ^E_ processing, by inducing transcription of a gene, X, whose product acts vectorially from the forespore to the mother cell [Losick and Stragier 1992]. Further support of the idea that pro-σ^E_ processing is regulated by the products of some genes belonging to the σ^F-controlled regulon has been obtained recently [Shazand et al. 1995]. To start elucidating the molecular basis of this transduction pathway between the forespore and the mother cell, we have searched for genes controlled by σ^F_ (collectively designated by the csf acronym). This strategy has led to the identification and characterization of csfX, a σ^F-controlled gene encoding a protein necessary and sufficient for triggering processing of pro-σ^E_.

**Results**

The screening rationale

We have searched for σ^F-dependent genes by screening a plasmid library creating lacZ transcriptional fusions after integration into the *B. subtilis* chromosome through a single homologous recombination event [O’Reilly et al. 1994]. To allow easy identification of the csf–lacZ fusions, the plasmid library was introduced into a strain in which σ^E activity was dependent on the presence of xylose (by placing the regulatory gene spoIIE under the control of a xylose-inducible promoter). In such a strain the presence of xylose in sporulation agar plates leads to σ^E activity appearing at the normal time and being correctly compartmentalized [F. Arigoni and P. Stragier, unpubl.]. Because σ^E activation induces the whole sporulation cascade [Losick and Stragier 1992], a null mutation was introduced in spoIIGB, the gene encoding σ^E, to avoid expression of spo–lacZ fusions controlled by σ factors other than σ^F_. Furthermore, because pro-σ^E processing occurs in a spoIIE36 mutant, in which only 30% of the chromosome around the replication origin is segregated into the forespore [Wu and Errington 1994], we inferred that the csf genes required for activating σ^E were present in the chromosome segment that enters the forespore. There-
fore, we introduced this additional mutation in our recipient strain to focus our search on that region of the chromosome.

Of 47 lacZ fusions identified as being dependent on xylose on sporulation agar plates, 33 were found to be inactive in a strain carrying a null mutation in spolIA C, the gene encoding σE, and thus to be actually dependent on σE and not on xylose itself. DNA adjacent to the lacZ fusions was cloned for each candidate, and restriction and nucleotide sequence analyses revealed the presence of nine distinct families. Various deletions and disruptions were created in the immediate vicinity of the original fusion points. We checked the effect of these mutations on sporulation and σE activity on solid media, by introducing them into a wild-type strain containing a fusion of lacZ to the σE-controlled spoIID promoter [Rong et al. 1986].

The Csfx protein is required for pro-σE processing

One locus was found to be essential for sporulation and for expression of spoIID–lacZ. Sequence comparison indicated that the gene initially fused to lacZ, and now called csfx [Fig. 1], lies in a region sequenced in the frame of the Bacillus Genome Sequencing Project [accession no. Z38002] and that it is identical to the ipc-27d open reading frame located at 327° on the chromosome. The Csfx protein is predicted to contain 224 residues with a typical signal sequence, followed by a potential cleavage site after residue 23, and a carboxy-terminal region extremely rich in charged residues, especially glutamic acid.

The availability of a large sequence from that region allowed us to refine our inactivation procedure and to disrupt precisely either csfx or the genes located immediately upstream or downstream of csfx [Fig. 1]. The latter mutations had no obvious phenotype, whereas inactivation of csfx blocked sporulation and prevented σE activity, on plate and in liquid media [Fig. 2]. Immunoblot analysis using a monoclonal antibody directed against σE showed that the defect in σE activity was attributable to the absence of the processing of its precursor [Fig. 2]. When grown in sporulation conditions and examined by phase-contrast microscopy, the csfx mutant was found to accumulate disporic forms, a feature characteristic of the absence of σE activity [Lewis et al. 1994; not shown]. Mutations in the same locus have been obtained independently by screening for clones displaying σE but no σF activity [Karow et al. 1995].

Figure 2. Blockage of pro-σE processing in a csfx mutant. (Top) The time course expression of a transcriptional fusion [integrated at the amyE locus] of the lacZ gene to the σE-dependent spoIID promoter in a wild-type (●) and in a csfx strain (○). Time indicates hours in sporulation. (Bottom) An immunoblot analysis of pro-σE processing in the same set of strains, samples being harvested at the hours indicated.

csfx is controlled exclusively by σE

The csfx open reading frame is preceded immediately by a sequence similar to that found in other σE-dependent promoters [Sun et al. 1991]. A DNA fragment containing the csfx gene and only 82 bp of sequence upstream of its putative transcription start was able to fully complement in trans the sporulation defect of the csfx mutant, indicating that all the signals required for correct expression of csfx are located in that short interval.

To follow the expression of csfx we used the original csfx–lacZ fusion, which was moved to the ectopic amyE locus. In a wild-type strain containing this fusion, β-galactosidase synthesis starts 1 hr after the onset of sporulation and continues for the next hour (Fig. 3A). It is reduced to the background level in a spoIID mutant (no σE), it is not affected by a spoIIG mutation (no σG), and it doubles in a spoIIGB mutant (no σI), as already de-
How σF activates σE

Figure 3. Dependence of csfX transcription on σF. (A) Time course expression of a transcriptional csfX-lacZ fusion integrated at the amyE locus in a wild-type strain (○), as compared to strains disrupted for the σ factors σF (spolIAC, ●), σE (spolIGB, ▲), and σG (spolIIIG, □). The background level of β-galactosidase activity observed in the absence of lacZ fusion has been subtracted. Time indicates hours in sporulation. (B) Time course expression of the same csfX-lacZ fusion in a wild-type strain (○), as compared with strains carrying the spolIAC561 (altered σF, ●) or the spolIIIE36 (chromosome segregation defect, ▲) mutation. Note the difference in scale. Time indicates hours in sporulation. (C) Time course expression of the same csfX-lacZ fusion during exponential growth in cells engineered to produce either σF (○), or σG (▲ or □), in response to isopropyl β-D-thiogalactoside (IPTG). Time indicates hours after addition of IPTG. No β-galactosidase was accumulated in the absence of IPTG.

The CsF protein is sufficient for pro-σE processing

We wondered whether genes other than csfX were required for activation of pro-σE processing. It has been shown previously that expression of the whole σE-dependent regulon during exponential growth activates SpoIIGA-mediated processing of pro-σE (Shazand et al. 1995). A similar experiment in which only the csfX gene was artificially expressed during growth, together with the spolIG operon encoding SpoIIGA and pro-σE, indicated that the CsF protein is sufficient for activation of pro-σE processing and expression of a σE-dependent lacZ fusion (Fig. 4). Moreover, expression of csfX independent of σE early during sporulation (by placing the csfX gene under the control of the spoIIGA promoter) allows pro-σE processing to occur in the complete absence of σE (Fig. 5). Altogether, these results demonstrate that csfX is the only gene controlled by σF required for activation of σE.

Discussion

The early forespore–mother cell transduction pathway

The whole sporulation cascade of gene expression has been proposed to be controlled by a crisscross mechanism in which signals flow from one cell to the other where they induce the next developmental step by activating a new σ factor. Three such transduction pathways have been postulated, between σF and σE, between σE and σG, and between σG and σK (Losick and Stragier 1992). Until now, the latter case was the best understood at the genetic level, but it has not yet been possible to find conditions allowing activation of σK under artificial conditions, a prerequisite for analyzing the role of the various partners in the pathway.

Because such conditions have already been defined for σF, by inducing simultaneously σF, SpoIIGA, and pro-σE during exponential growth (Shazand et al. 1995), the identification of the csfX gene has allowed us to unravel the complete transduction pathway leading from σF to σE. The csfX gene is controlled exclusively by σF and is therefore presumably transcribed only in the forespore (Margolis et al. 1991). As expected from the existence of
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fully complements the sporulation defect of a csfX mutant, suggesting that the αE molecules processed before asymmetric septation (if any) do not interfere with the sporulation process.

The role of CsFX

The sequence of the CsFX protein does not give any clue about its function. However, it is very likely that CsFX plays a catalytical role because experiments in which csfX was under the control of the inducible spac promoter have shown that the background level of csfX expression in the absence of inducer is sufficient for production of 5% of the wild-type level of heat-resistant spores (not shown). The presence of a signal sequence indicates that CsFX is presumably translocated outside of the forespore cytoplasm where it could act, directly or indirectly, on the SpoIIGA molecules embedded in the septum membrane on the mother cell side (Fig. 6). Previous experiments have shown that the septum is not absolutely required for transducing the CsFX signal to SpoIIGA and that conditions can be found where αE is

Figure 4. Activation of SpoIIGA-mediated pro-αE processing by CsFX during exponential growth. [Top] The time course expression of a transcriptional fusion of the lacZ gene to the αE-dependent spoIIGA promoter (integrated at the amyE locus) in cells engineered to produce pro-αE, as well as SpoIIGA (●), CsFX (○), or both (▲), in response to IPTG. Time indicates hours after addition of IPTG. No β-galactosidase was accumulated in the absence of IPTG. [Bottom] An immunoblot analysis of pro-αE processing after addition of IPTG, in the same set of strains and the same samples.

pro-αE processing in a spoIIE36 mutant, the csfX gene is located in the region of the chromosome that belongs to the 30% closest to the replication origin. It should be noted that the very high level of csfX transcription observed in this background was completely abolished by inactivating spoIAC, or by moving the csfX-lacZ fusion from the amyE locus (which segregates into the forespore in this mutant) to thrC (which does not), indicating that the overexpression observed is still dependent on αE and is forespore compartmentalized (not shown).

Inducing csfX together with spoIIGA and spoIIGB during exponential growth is sufficient to get efficient pro-αE processing and expression of a αE-dependent lacZ fusion. In addition to confirming that SpoIIGA is the pro-αE processing enzyme as suggested originally [Stragier et al. 1988], these experiments indicate that no other csf gene is involved in the pathway, as demonstrated further by the bypass of αE for pro-αE processing observed when csfX is expressed through an early sporulation promoter independent of αE. The PsplolIG-csfX construct has no negative effect on sporulation of a wild-type strain and

Figure 5. Bypass of αE for activation of αE by CsFX during early sporulation. [Top] The time course expression of a spoIIG-lacZ fusion (integrated at the thrC locus) in cells containing a second copy of the csfX gene under the control of the spoIIG promoter (integrated at the amyE locus), and in which spoIAC (the gene encoding αE) is either intact (●) or disrupted (○). Time indicates hours in sporulation. [Bottom] An immunoblot analysis of pro-αE processing in the same set of strains, with samples being harvested at the hours indicated.
processed in the same cell where CsfX is synthesized [Shazand et al. 1995]. Therefore, the action of CsfX on the SpolIGA molecules present in the forespore membrane remains a matter of speculation. CsfX appears to generate a short-range signal, as we have not been able to observe any extracellular complementation when mixing csfX and spoIG clones. Interestingly, these experiments allowed us to detect a pigment difference between these two classes of mutants, which are both devoid of active $\sigma^R$. This suggests that CsfX might modify some feature of the bacterial envelope, including the septal space, which, in turn, would activate SpolIGA processing activity. Such a signal transduction pathway is reminiscent of the coupling of $\sigma^R$ activation in the mother cell to spoIVB expression directed by $\sigma^G$ in forespore at late stages of sporulation [Cutting et al. 1991]. The homologous roles played by CsfX and SpoIVB at different stages of development might reflect related enzymatic functions. Presumably both proteins synthesized in the forespore are involved in remodeling of the peptidoglycan present in the space between the forespore and the mother cell. In addition, or as a mere consequence of their enzymatic function, they activate a membrane-bound protease that triggers a new transcription program in the mother cell.

**Materials and methods**

**Cloning of csfX**

The integrative plasmid library (O’Reilly et al. 1994) was introduced in the recipient strain MO1809 [trpC2 phe-1 xylA861 spoIIE2 kan spoIIIB arm spoIIIIE36 amyE::[PsyIA-spoIIE spoIIEC spec] and the transformants selected on sporulation agar plates, in the presence of chloramphenicol (5 $\mu$g/ml), xylose (2.5 mm), and 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactopyranoside (100 $\mu$g/ml). Blue colonies were purified and checked for their color in the absence of xylose. The selected strains were grown in the presence of 50 $\mu$g/ml of chloramphenicol to amplify the integrated lacZ plasmids, which were rescued by restricting chromosomal DNA with EcoRI, followed by ligation and transformation of Escherichia coli [Perego 1993]. In the case of the csfX locus, a 2.5-kb fragment was recovered that corresponds to the initial Sau3A fragment cloned upstream of lacZ in plM783 [O’Reilly et al. 1994]. Partial nucleotide sequence was obtained using a primer hybridizing just downstream of the fusion point. DNA extending 2.7 kb farther downstream was cloned by a similar procedure after restricting nonamplified chromosomal DNA with PvuII.

**lacZ fusions**

The original fusion between csfX and lacZ was recovered as a PstI–Clal fragment that contained 354 bp upstream of the putative transcription start of csfX as well as the junction with lacZ at a Sau3A site located at codon 40 in csfX. This fusion was cloned in the pDG268 vector [Antoniewski et al. 1990] and introduced into the chromosome at the amyE locus [Shimotsu and Henner 1986]. The spoIID–lacZ transcriptional fusion integrated at the amyE locus has been described previously as well as the conditions of culture and $\beta$-galactosidase assay (Stragier et al. 1988). All of the strains used are derivatives of strain JH642. $\beta$-Galactosidase activity is expressed as nanomoles of 2-nitrophenyl-$\beta$-D-galactopyranoside hydrolyzed per minute per milligram of protein.

**Induction of $\sigma$ factors during growth**

Conditions of induction of the spoIIE promoter [Yansura and Henner 1984] by IPTG during exponential growth have been described previously (Frandsen and Stragier 1995). The genes encoding the various sporulation $\sigma$ factors were cloned downstream of the spoIIE promoter on a multicopy plasmid providing resistance to erythromycin. To avoid interferences attributable to autoregulation, competition, or inhibition, the strains carried chromosomal disruptions of spoIIG (the gene encoding $\sigma^R$), when inducing $\sigma^R$ or $\sigma^G$, or of both sigB and rsbW (the genes encoding $\sigma^B$ and its inhibitor), when inducing $\sigma^B$.

**Heterochronic expression of csfX**

The csfX-coding sequence was cloned as a BfaI–XhoI fragment downstream of the spoIIE promoter, in a plasmid allowing integration by marker exchange at the chromosomal thrC locus [a gift of A.-M. Guérout-Fleury, Institut de Biologie Physico-Chimique, Paris, France]. The BfaI site overlaps the putative csfX transcription start, and the XhoI site is located 120 bp downstream of the csfX reading frame. The Pspac–csfX construct fully complements the sporulation defect of a csfX mutant when grown in the presence of 1 mM IPTG. The multicopy plasmids carrying various parts of the spoIIG operon (encoding SpolIGA and pro-$\sigma^R$) under the control of the spoIIE promoter have been described previously [Stragier et al. 1988]. The BfaI–XhoI fragment carrying the csfX-coding sequence was also cloned downstream of the spoIIGA promoter [which is activated shortly after the onset of sporulation [Kenney and Moran 1987]].
in a plasmid allowing integration by marker exchange at the chromosomal amyE locus.

Immunoblot analysis

Clarified crude extracts prepared by ultrasonication were analyzed by polyacrylamide gel electrophoresis (PAGE; 12.5% acrylamide) in the presence of SDS. Proteins were detected by immoblotting (Sambrook et al. 1989), using a monoclonal antibody directed against αs (Trempy et al. 1985b) and a secondary antibody conjugated to alkaline phosphatase (Hyclone).

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