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Compartmentalization of Gene Expression during Sporulation of *Bacillus subtilis* Is Compromised in Mutants Blocked at Stage III of Sporulation

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Mutations in the *spoIIIA* and *spoIIIJ* loci disrupt the compartmentalization of gene expression during sporulation of *Bacillus subtilis*. The breakdown in compartmentalization is not the cause of their being blocked in spore formation. Rather, it appears to be a consequence of the engulfed prespore's being unstable.

Cell differentiation is one of the fundamental processes in biology. Central to it is the compartmentalization of gene expression, so that different genes are expressed in different cell types. Spore formation by *Bacillus subtilis* has become a paradigm for the study of cell differentiation in prokaryotes. During sporulation, *B. subtilis* divides asymmetrically, resulting in two unequal-size cells, the smaller prespore and the larger mother cell. Following sporulation division, conventionally designated stage II, gene expression becomes compartmentalized. Distinct RNA polymerase sigma factors become active, σ^F in the prespore and σ^E in the mother cell (12). The mechanisms for compartmentalizing the activity of these sigma factors are not fully understood. Following division, the prespore is engulfed by the mother cell. The engulfed prespore (also called the forespore) develops into the mature spore. The mother cell is necessary for this process but ultimately lyses. Completion of engulfment (stage III) is associated with activation of other sigma factors, σ^G in the prespore and σ^K in the mother cell. Mutants that are blocked at stage III do not activate σ^G or σ^K (12).

A genetic test to analyze the compartmentalization of gene expression during sporulation was recently developed (7). The test is based on the *sacB*/SacY transcriptional antitermination system (2). The SacY protein regulates transcription of *sacB* (encoding levansucrase) by antitermination. In the absence of SacY, transcription of *sacB* terminates in the region 5′ to the *sacB* structural gene. When SacY is present, it binds to the RAT site in the nascent 5′ region, prevents termination, and so allows transcription of the *sacB* structural gene (2). Thus, SacY needs to be present during transcription of *sacB* in order to obtain levansucrase or, in our case, β-galactosidase from a *sacB′-′lacZ* translational fusion. The SacY protein is ordinarily subject to complex regulation, and we used a truncated derivative, SacY(1-55), that is free from this regulation (8). By placing *sacB′-′lacZ* and *sacY*(1-55) under the control of promoters directed by the same or different sigma factors, we were able to confirm that there was essentially complete compartmentalization of σ^F and σ^E activity during sporulation (7).

We have used mutagenesis with a Tn*10* derivative (9) to generate mutants with the compartmentalization of the activities of σ^F and σ^E disrupted and have obtained mutations in the *spoIIIA* and *spoIIIJ* loci. Here we explore the reason why such mutants might have compartmentalization disrupted. We distinguished two alternatives that would point to radically different roles for these *spoIII* loci. The first is that the mutations disrupt compartmentalization, and as a consequence sporulation cannot proceed beyond stage III. The second is that the mutations cause a block at stage III, and as a consequence the engulfed prespores are prone to lyse, causing loss of compartmentalization. In the first mechanism, disruption of compartmentalization should be apparent as soon as σ^F and σ^E become active. In the second mechanism, the activities of σ^F and σ^E should be compartmentalized when they first appear, and compartmentalization should be lost only following engulfment. We found the latter to be the case, as the loss of compartmentalization follows the completion of engulfment.

Isolation of mutants disrupted in compartmentalization of gene expression. A pool of *B. subtilis* mutants with mini-Tn*10* insertions (9) was screened for compartmentalization of σ^F and σ^E activities. The mutants were obtained in strain SL7643, which is a derivative of SA501 cured of SPβ (7); SA501 (8) has deletions of *sacY*, *sacB*, *licT*, and *sacT* to avoid possible interference with the antitermination system being used. Chromosomal DNA of the pooled mutants was purified and used to transform the tester strain SL9106, also a derivative of SA501, selecting for Tn*10*-associated chloramphenicol resistance. Strain SL9106 contains P_{cotEp1}-*sacB′-′lacZ*/P_{spoIIQ}-*sacY*(1-55) (the σ^E/σ^F configuration) (7). Among approximately 30,000 Cm^r transformants, 32 displayed β-galactosidase activity. Of these, one activated the cryptic endogenous *lacA* gene, and one mapped in *spoIIIE*, where insertions have previously been shown to disrupt compartmentalization (7, 17). The remaining Tn*10* mutations were tested by backcrossing into strain SL9106 and by transfer to strain SL9105 (which has the opposite configuration, σ^F/σ^E). Nine mutations that caused substantial β-galactosidase expression in both strains were then transferred into strains with σ^E- and σ^F-directed *gfp* fusions (SL9109 *spoIID-gfp*

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and SL10280 *spoIIQ-gfp*, respectively [kindly provided by David Hilbert and Vasant Chary]). Eight of the mutations caused a substantial loss of compartmentalization, as visualized with bacteria grown on Schaeffer's sporulation agar (11). This result indicated that the *sacB*/SacY screen was an effective predictor of loss of compartmentalization.

Genetic crosses between the eight strains with impaired compartmentalization indicated that the mutations mapped in two distinct loci. Flanking DNA from the inserts was recovered and sequenced, and the loci were identified as *spoIIIJ* (seven mutations) and *spoIIIAB* (one mutation; Tn*10* insertion 428 nucleotides downstream from the presumed start of translation). The ninth mutation, which resulted in β-galactosidase activity in SL9105 and SL9106 but had little effect on the compartmentalization of σ^E- and σ^F-directed *gfp* expression, mapped in *ybdB* (Tn*10* insertion 457 nucleotides downstream from the presumed start of translation); it was not studied further. The *ybdB* locus, now designated *skf*, is associated with production of an antibiotic that kills nonsporulating bacteria in the same population (4). The *spoIIIAB* mutation and a *spoIIIJ* mutation with Tn*10* inserted 472 nucleotides downstream from the presumed start of translation were used for further study. Mutations in the *spoIIIJ* and *spoIIIA* loci block sporulation at stage III and prevent activation of σ^G and hence σ^K (3, 10, 12, 14, 15); the engulfed prespores of *spoIIIA* mutants have been reported to be unstable (10, 16).

Compartmentalization of gene expression is gradually lost during sporulation of *spoIIIA* and *spoIIIJ* mutants. The following questions arise for *spoIIIA* and *spoIIIJ*: do the mutations directly affect the compartmentalization of σ^F and σ^E activity, or do they indirectly influence the process, because they block sporulation at the stage of engulfment, and the mutant prespore is unstable and breaks down, resulting in loss of compartmentalization? To distinguish between these possibilities, compartmentalization of gene expression during sporulation was monitored by observing expression of *spoIIQ-gfp* (directed by σ^F) and *spoIID-gfp* (directed by σ^E). We reasoned that if the primary effect of *spoIIIA* and *spoIIIJ* mutations is on compartmentalization, then we should be able to observe impaired compartmentalization at the earliest times of green fluorescent protein (GFP) expression and that the extent of impairment should not increase with time during sporulation. In contrast, if loss of compartmentalization was a consequence of an engulfment or other defect, then GFP expression should initially be compartmentalized; bacteria displaying uncompartmentalized expression should appear only after they reach the impaired stage. It is thought that σ^F and σ^E continue to be active in the absence of σ^G and σ^K activity (7), so that either mechanism for the breakdown in compartmentalization would result in β-galactosidase activity with the *sacB*/SacY system. The *sacB*/SacY system was used to analyze populations and not single cells and so did not satisfactorily distinguish between the two possibilities; expression of *gfp* was found to be more suitable for analyzing single cells.

Strains with *spoIIQ-gfp* and *spoIID-gfp* transcriptional fusions were induced to sporulate in modified Schaeffer's sporulation medium (11). They were examined by fluorescence microscopy at various times after the start of spore formation, defined as the end of exponential growth. Conditions for microscopy were essentially as previously described (5, 13, 18).

TABLE 1. Effect of mutations in *spoIIIAB* and *spoIIIJ* on the compartmentalization of σ^F- and σ^E-directed *gfp* expression

Strain	Relevant genotype	σ factor[a]	Proportion (%)[b] of GFP-expressing bacteria displaying uncompartmentalized expression at time (h) after the end of exponential growth					
			5	6	7	8	9	10
SL10280	+	σ^F	0	0	0	1	0	1
SL10271	*spoIIIAB*	σ^F	0	0	0	3	21	31
SL10253	*spoIIIJ*	σ^F	0	0	0	0	5	23
SL11371	+	σ^E	0	0	0	0	0	0
SL10307	*spoIIIAB*	σ^E	0	0	0	0	24	62
SL10319	*spoIIIJ*	σ^E	1	0	2	5	14	50

[a] σ factor directing *gfp* transcription; σ^F activity was detected with a *spoIIQ-gfp* fusion, and σ^E activity was detected with a *spoIID-gfp* fusion.

[b] At least 40% of bacteria were expressing GFP, except for SL10253 (5 h, 14%) and SL10280 (5 h, 38%). At least 200 GFP-expressing organisms were counted for each sample. Samples were stained with FM4-64 (Molecular Probes) to enhance visualization of sporulation septa (13).

When GFP fluorescence was first detected, about 3 h after the end of exponential growth, it was almost completely compartmentalized in the *spoIIIA* and *spoIIIJ* mutants as well as the *spo*$^+$ strain. This observation applies to both σ^F-directed (*spoIIQ-gfp*) and σ^E-directed (*spoIID-gfp*) fusions. Expression remained largely compartmentalized for several hours (Table 1). However, by 8 to 10 h after the start of sporulation, fluorescence became uncompartmentalized in an increasing proportion of the mutant bacteria (Table 1). The transition from compartmentalized to uncompartmentalized expression was observed consistently for the *spoIIIAB* and *spoIIIJ* mutants, although the extent of the breakdown in compartmentalized expression varied from experiment to experiment (data not shown).

In the sporulation system used, there is a substantial period, about 3 h, between the end of exponential growth and the formation of the sporulation septum, and by 5 h after the end of exponential growth, 86 to 100% of the GFP-expressing population were at stage II of sporulation. Cultures were examined in detail 7 h after the end of exponential growth, before there was significant breakdown in compartmentalization. Engulfment was complete in about 50% of the GFP-expressing bacteria of the *spoIIIJ* and *spo*$^+$ strains and in a substantial portion of the *spoIIIAB* strain (Table 2) (in other experiments, that portion also reached 50%). Similar results were obtained with *spoIIQ-gfp* (σ^F-directed) and *spoIID-gfp* (σ^E-directed) fusions. Thus, completion of engulfment occurred well before substantial numbers of mutant bacteria displayed uncompartmentalized expression. It is inferred that the effect of the *spoIIIAB* and *spoIIIJ* mutations on compartmentalization was most likely a consequence of the instability of the engulfed prespore that led to mixing of mother cell and prespore cytoplasms. This effect contrasts with the loss of compartmentalization caused by insertional inactivation of *spoIIIE*, where the defect occurs at stage II and is most likely the consequence of a small pore in the septum permitting small proteins such as sigma factors and GFP to cross the septum (17; D. Hilbert, V. Chary, and P. Piggot, unpublished obser-

TABLE 2. Stage of sporulation reached by bacteria displaying compartmentalized expression of σ^F- and σ^E-directed *gfp* fusions 7 h after the end of exponential growth

Strain	Relevant genotype	σ factor	Proportion (%)[a] of bacteria displaying compartmentalized GFP expression at the indicated stage[b]		
			II_i	II_{ii}–II_{iii}	III
SL10280	+	σ^F	18	17	65
SL10271	*spoIIIAB*	σ^F	35	40	25
SL10253	*spoIIIJ*	σ^F	15	18	67
SL11371	+	σ^E	11	38	51
SL10307	*spoIIIAB*	σ^E	19	33	48
SL10319	*spoIIIJ*	σ^E	21	23	56

[a] At least 100 GFP-expressing organisms were counted for each sample; σ^F activity was detected with a *spoIIQ-gfp* fusion, and σ^E activity was detected with a *spoIID-gfp* fusion.

[b] The stages of sporulation are as follows: II_i, spore septum formed; II_{ii}–II_{iii}, partial engulfment of the prespore by the mother cell; III, complete engulfment (6, 10). In bacteria scored as having reached stage III, the prespore had clearly detached from the mother cell (10), and the prespore was not detected by FM4-64 staining (13); the prespore was visualized by prespore- or mother cell-specific GFP expression.

vations). Inactivation of the *spoIIAB* locus results in uncompartmentalized σ^F activity and blocks spore septum formation (1); the mutants display little σ^E activity and so would not be detected with the *sacB*/SacY system. The only mutations causing severe defects in compartmentalization that we detected in 30,000 Tn*10* mutants screened, with a system requiring both σ^F and σ^E activity, were located in *spoIIIE* (7), *spoIIIA*, and *spoIIIJ*. The transposon insertion could have a polar effect on genes downstream in the *spoIIIA* and *spoIIIJ* operons. We have not tested for this possibility. Disruption of the gene downstream of *spoIIIJ* does not affect spore formation (3, 12), so that *spoIIIJ* inactivation is the most likely cause of the effect described here. Nonpolar mutations in each of the eight genes in the *spoIIIA* operon result in the same stage III sporulation phenotype (12, 15), suggesting that disruption of other genes in the operon would have the effect described here. Our results suggest that insertional inactivation of a very limited number of loci results in uncompartmentalized activities of both σ^F and σ^E.

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