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Use of Integrational Plasmid Excision To Identify Cellular Localization of Gene Expression during Sporulation in *Bacillus subtilis*

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Sporulation in *Bacillus subtilis* is a simple developmental system involving the differentiation of two sister cells, the prespore and the mother cell. Many of the genes that regulate sporulation (*spo* genes) are thought to be expressed differentially. However, direct demonstration of differential gene expression, by fractionation of prespore and mother cell proteins, is possible only at a relatively late stage of development. H. De Lencastre and P. J. Piggot (J. Gen. Microbiol. 114:377–389, 1979) have described a genetic method for determining the cellular location of the requirement for *spo* gene expression. Here we describe a similar method based on the use of integrational plasmids that can insertionally inactivate any given *spo* gene. Loss of the integrated plasmid by homologous recombination leads to the restoration of *spo* gene function. If this occurs just before sporulation begins, the phenotypes of the progeny of heat-resistant spores should depend on whether the gene is required in the prespore or the mother cell. Thus, we show that for known prespore-specific genes, such as *spoIIIG* and *spoVA*, only phenotypically Spo$^+$ progeny that have lost the integrated plasmid are produced. In contrast, for mother-cell-specific genes, such as *spoIIIC* and *spoVJ*, a substantial proportion of the progeny are asporogenous, having retained the integrated plasmid. On the basis of our results, the *spoIID* and *spoIIIA* genes, which are expressed soon after division, appear to be required only in the mother cell compartment.

Spore formation in *Bacillus subtilis* is a simple developmental process involving two cell types, the prespore and the mother cell. About 50 genes that are essential for sporulation have been identified by *spo* mutations, and many of them have now been cloned and characterized. A complex pattern of interaction between the products of the genes is beginning to emerge. Mutations in genes that are expressed late in spore development often show epistatic interactions which indicate that there are at least two independent pathways of gene activation during spore development. It seems likely that one of these pathways represents mother-cell-specific gene expression and that the other represents prespore-specific gene expression (20, 21).

Various methods have been used to test whether expression of genes on these pathways is indeed localized. One method is based on mechanical disruption of the mother cell by forcing the sporulating bacteria through a small orifice at high pressure. Under appropriate conditions, the prespores remain intact and can be separated from the mother cell debris by centrifugation. The pellet (prespore fraction) and supernatant (mother cell fraction) may then be assayed for enzyme activity or for the presence of structural proteins such as the small acid-soluble proteins (26). Alternatively, the fractions can be assayed for β-galactosidase activity directed from fusions to *lacZ*. Thus, expression of genes such as *spoVA* (8), *0.3 kb* (23), and the *ssp* family (coding for the small acid-soluble proteins) (22) has been shown to be restricted to the prespore, while expression of *spoIIIC* (30), *spoVJ* (9), and *spoIVC* (18) has been shown to be restricted to the mother cell. The mother cell and prespore can only be separated effectively once the prespore has acquired sufficient resistance to mechanical shear, about 5 h after the initiation of sporulation. Hence, this method is not generally applicable; its use is restricted to studies of genes that are expressed relatively late during sporulation. It should certainly not be used to obtain information about the localization of expression of genes that are activated before or immediately after prespore engulfment is completed. Rupture of such immature sporangia would lead to prespore-specific material being found in the mother cell fraction. A further problem arises from the presence of nonsporulating cells (in practice, only 50 to 80% of the cells in a culture sporulate). Rupture of these cells results in underestimation of the relative concentration of any protein in the mother cell fraction.

Another method, one that is particularly suitable for studying genes, such as the *ssp* genes, that are strongly expressed, is immunoelectron microscopy (12). However, the majority of *spo* genes are expressed much less strongly than the *ssp* genes, and it remains to be established whether this technique can be used to localize β-galactosidase produced from *lacZ* fusions to more weakly expressed genes.

De Lencastre and Piggot (5) devised an ingenious genetic approach, which measures the cellular localization of the requirement for correct *spo* gene expression. *spo* mutant cells were transformed with wild-type DNA immediately before inducing sporulation. A proportion of the recombinant sporangioles (i.e., sporangia together with their prespores) were *spo*$^+$/*spo* mosaics with the wild-type allele residing on either the mother cell or the prespore chromosome. The phenotypes of the progeny obtained following selection for heat-resistant spores depends on whether expression of the particular *spo* gene was needed in the prespore or the mother cell (or both). For a gene whose correct expression was needed in the prespore, all heat-resistant progeny would be derived from prespores containing the wild-type allele, whether mosaic or not, i.e., irre-

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spective of the genotype of the mother cell chromosome. However, in the case of a gene whose product was needed only in the mother cell, mosaic sporangioles of the appropriate class (i.e., *spo*$^+$ on the mother cell chromosome) could give rise to genotypically asporogenous (*spo*) spores. Following selection for heat resistance, these survivors would give rise to Spo$^-$ progeny. Since some cells would be transformed before the ultimate round of DNA replication was completed, there would always be some Spo$^+$ survivors following heat selection. These would arise from cells in which both the prespore and the mother cell chromosome carried the *spo*$^+$ allele. The localization of the requirement for the functional *spo* gene product therefore could be deduced from the ratios of the sporulation phenotypes of the progeny of the heat-resistant survivors.

Approximately 99% of the progeny of a *spoVA* strain were Spo$^+$, indicating that correct expression of the wild-type gene in the prespore was required for heat-resistant spore development. Subsequent work (8) has confirmed this finding and shown that *spoVA* is indeed expressed only in the prespore. In the case of strains carrying mutations in genes such as *spoIIID*, *spoIVA*, *spoVB*, and *spoVE*, up to 50% of the colonies obtained after heat treatment were Spo$^-$, implying that the genotype of the prespore chromosome with respect to these genes is unimportant; it is sufficient for the wild-type gene to be expressed only in the mother cell.

There are, however, certain limitations to the method of De Lencastre and Piggot (5). First, the competence of all of the *spo* strains was unreliable under their experimental conditions. Second, the method could not be used for strains harboring *spo* mutations that confer an oligosporogenous phenotype. Such mutants, e.g., those with defects in *spoIIIA*, characteristically produce a significant background of heat-resistant spores that retain the original *spo* mutation and are phenotypically Spo$^-$. Such individuals cannot formally be distinguished from those arising from mosaic sporangioles.

In this report, we describe a more generally applicable method based on mosaic formation for analyzing the localization of the requirement for *spo* gene expression. This method is based on the use of integrational plasmids, which recombine into the chromosome by a Campbell-like mechanism, causing gene disruption if they contain a DNA fragment internal to an operon (24). Reversal of the integration event, which occurs at a frequency of 10^{-4} to 10^{-5} per bacterial generation (31, 32), leads to precise excision of the plasmid and restoration of gene function (Fig. 1). Spontaneous excision of an integrated plasmid at the onset of sporulation can give rise to mosaic sporangioles (Fig. 2).

We have used this approach to confirm that the products of genes known to be expressed only in the prespore or only in the mother cell are also required only in these cells for sporulation and extended the analysis to determine in which compartments the products of genes that are expressed immediately after septation are required. We show that the product of *spoIIIG* is indeed only required in the prespore while, surprisingly, the products of *spoIID* and *spoIIIA* are only required in the mother cell for sporulation.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and integrational plasmids used in this study are listed in Tables 1 and 2. *B. subtilis* SG38 was transformed with plasmids as described previously (1, 15). Selection was for chloramphenicol resistance on Oxoid nutrient agar containing chloramphenicol (5 μg/ml). Each of the integrational plasmids contains an internal segment of a sporulation operon; their integration in the *B. subtilis* chromosome generates a Spo$^-$ phenotype (24).

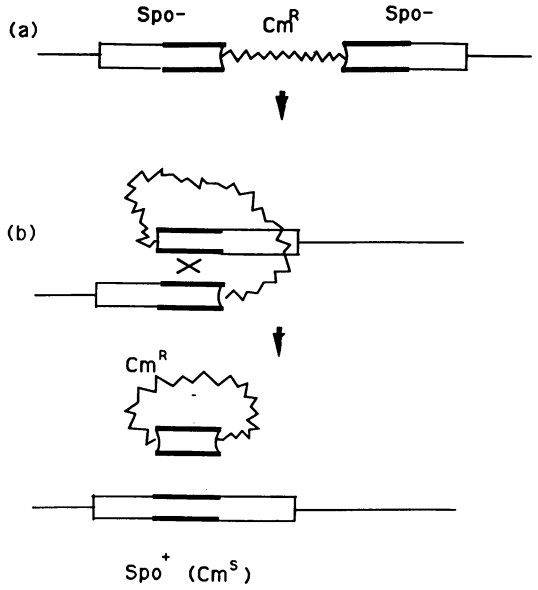

FIG. 1. Recombination between directly repeated internal segments within a *spo* locus leads to precise excision of an integrated plasmid and restoration of *spo* gene function. (a) Insertion of an integrational plasmid by homologous recombination has led to the structure shown, in which the plasmid sequences (∿∿∿) are flanked by duplicated copies of an internal segment of a *spo* gene (open box). Neither gene copy remains intact. The thick lines represent the directly repeated sequences generated by plasmid insertion. (b) Homologous recombination between the direct repeats leads to precise plasmid excision and restoration of gene function.

Growth and sporulation conditions. Each strain was grown in casein hydrolysate medium (27) containing chloramphenicol (5 μg/ml) to maintain selection pressure for the integrated plasmid and was then resuspended in sporulation medium (27) without antibiotic selection. After 10 h at 37°C (i.e., at t_{10}), samples were plated either directly or after heat treatment (85°C, 10 min) to measure the number of viable or heat-resistant CFU, respectively. In each case, samples were plated both on nutrient agar and on nutrient agar containing chloramphenicol (5 μg/ml) to measure the proportion of survivors that still contained integrated plasmids.

RESULTS AND DISCUSSION

The integrational plasmids capable of disrupting the various *spo* genes used in this study are indicated in Table 2. The plasmids all had small inserts of relatively similar size (between 320 and 800 bp) to reduce any potential effect of size of the region of homology on the excision rate. Each of the plasmids generated a Spo$^-$ phenotype when transformed into *B. subtilis* SG38.

The resultant strains (Table 1) were grown in casein hydrolysate medium containing chloramphenicol and then resuspended in antibiotic-free sporulation medium. Ten hours after resuspension, samples were plated to measure the viable CFU and, after heat treatment, to measure the number of spores (Table 3). All of the strains containing integrated plasmids had a similar number of viable CFU at t_{10}, and there was no substantial difference between the counts on selective and nonselective media. Selection for

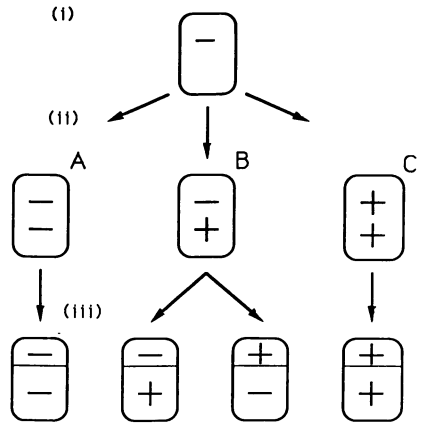

FIG. 2. Schematic diagram of possible mosaic sporangioles that would arise from excision of integrational plasmids at the onset of sporulation. (i) Spo⁻ vegetative cell containing a single chromosome disrupted by an integrational plasmid. (ii) During replication and entry into sporulation, the integrational plasmid may excise from one (B), both (C), or neither (A) of the chromosomes to give the genotypes shown (−, *spo*; +, *spo*⁺). (iii) The chromosomes segregate into the prespore and mother cell. The formation of a spore depends on the segregation of *spo*⁺ and *spo* alleles and the compartment in which the wild-type allele is expressed. The phenotypes of the progeny of these spores (selected by heat treatment) are shown as follows: Spo⁻, Spo⁻ Cm^r progeny; Spo⁺, Spo⁺ Cm^s progeny; 0, no spores.

heat-resistant spores was used to measure the frequency of plasmid excision. Frequency ranged from 2.4×10^{-5} to 3.3×10^{-4} heat-resistant cells per viable cell, which is consistent with recent reports on the stability of reiterated sequences in the *B. subtilis* chromosome (31, 32), and appeared to be related to the size of the insert in the integrational plasmid.

The progeny of the survivors of heat treatment were scored for Cm Spo phenotype. As was expected (9), a large number (29%) of the progeny from the heat-resistant spores

TABLE 1. Bacterial strains

Strains	Relevant genotype	Reference or origin
SG38	*trpC2 spo*⁺	7
46.1	*trpC2 spoIID-lacZ cat*	3
623.1	*trpC2 spoVJ-lacZ cat*	9
630	*trpC2 spoIIIA-lacZ cat*	Illing and Errington, unpublished results
NI1	*trpC2 spoIID*::pLD8	This study
NI2	*trpC2 spoVJ*::pSGMU137	This study
NI3	*trpC2 spoVA*::pSGMU196	This study
NI4	*trpC2 spoIIIA*::pSGMU410	This study
NI5	*trpC2 spoIIIG*::pSGMU422	This study

TABLE 2. Plasmids

Plasmid[a]	Relevant genotype	Size of insert (bp)	Origin or reference
pSGMU91	*cat spoIIG*⁺ *spoIIIG646*	4,400	Insert from ϕ105J95 (6) subcloned into pSGMU2 (10)
pLD8	*cat 'spoIID'*	601	19
pSGMU137	*cat 'spoVJ'*	750	9
pSGMU196	*cat 'spoVA'*	800	4
pSGMU410	*cat 'spoIIIA'*	550	Illing and Errington, unpublished results
pSGMU422	*cat 'spoIIIG'*	320	*Hin*dIII-*Pst*I fragment subcloned from pSGMU91

[a] All have an origin of replication that allows autonomous replication in *Escherichia coli* but do not replicate in *B. subtilis*. Each is a derivative of pSGMU2 (10), and all except pSGMU91 contain an internal segment of a sporulation operon such that integration in *B. subtilis* generates a Spo⁻ phenotype.

of strain NI2 (*spoVJ*) were Spo⁻ Cm^r. These Spo⁻ Cm^r cells were presumably derived from mosaic sporangioles in which the *spoVJ* integrational plasmid was lost from the mother cell chromosome but retained in the prespore. This finding indicates that it is sufficient to express the *spoVJ* gene only in the mother cell during sporulation. The Spo⁻ Cm^r survivors are directly analogous to the Spo⁻ survivors described by De Lencastre and Piggot (5). The remainder of the survivors, which were Spo⁺, were presumably derived by loss of the integrational plasmid from both chromosomes.

In contrast, almost all (>99%) of the progeny of the heat-resistant spores from strain NI3 (*spoVA*) were Spo⁺ Cm^s. This behavior was expected for a plasmid which disrupted a gene required in the prespore; excision from the prespore chromosome would be necessary for a heat-resistant spore to develop. The progeny of heat-resistant survivors should be all Spo⁺ and Cm^s. Rare Spo⁻ Cm^r progeny may conceivable arise from reintegration of excised plasmids. Alternatively, it is also formally possible, though not likely in this instance, that plasmid insertion gives rise to an oligosporogenous phenotype (see below).

There was a 100-fold difference between the number of Spo⁻ Cm^r survivors obtained from a strain in which a mother-cell-specific gene was disrupted and the number obtained from a strain in which a prespore-specific gene was disrupted (compare the results for *spoVA* and *spoVJ*). These results show that the Spo Cm phenotype of heat-resistant survivors can be a valid indicator of the compartment in which a *spo* gene is required during spore development.

The product of *spoIIIG* is a transcription activator (σ^G) that is required for expression of prespore-specific genes (29), and it has recently been suggested that *spoIIIG* is also expressed only in the prespore (29). We found (Table 3) that >99% of the heat-resistant progeny from strain NI5 were Spo⁺ Cm^s, confirming that the *spoIIIG* product is needed only in the prespore. This result is in good agreement with the findings of Gholamhoseinian and Piggot (13), who reported a similar result by using the method of De Lencastre and Piggot (5).

The results obtained with strains NI1 and NI4 were unexpected; they suggested that the products of *spoIID* and *spoIIIA* are required only in the mother cell compartment for spore development.

The *spoIID* gene is generally considered to be controlled directly by σ^E, since the σ^E form of RNA polymerase is both necessary and sufficient for *spoIID* expression in vivo (28), and the purified enzyme directs transcription from the cor-

TABLE 3. Frequencies and phenotypes of heat-resistant CFU arising from loss of integrational plasmids

Strain	*spo* locus inactivated	Viable CFU/ml at t_{10}[a]		Heat-resistant CFU/ml at t_{10}[a]		% Cm^r among heat-resistant progeny
		Total	Cm^r	Total	Cm^r	
SG38		2.2×10^8	<10	1.4×10^8	<10	
NI1	*spoIID*	4.1×10^8	3.8×10^8	1.0×10^4	1.1×10^3	11
NI2	*spoVJ*	2.1×10^8	1.3×10^8	2.1×10^4	6.1×10^3	29
NI3	*spoVA*	1.4×10^8	1.0×10^8	3.3×10^4	9.4×10^1	0.3
NI4	*spoIIIA*	3.2×10^8	3.0×10^8	5.0×10^4	1.5×10^4	30
NI5	*spoIIIG*	3.3×10^8	2.8×10^8	3.3×10^3	0.3×10^1	0.1

[a] Average of three independent experiments.

rect start point in vitro (25). Stragier et al. (28) have argued that *spoIID* is actively transcribed in both the mother cell and the prespore after septation. This would appear to agree with the conclusion of Carlson and Haldenwang (2) that σ^E is present in both the mother cell and the prespore compartments. However, the latter conclusion was based on measurements of σ^E in prespore and mother cell extracts prepared from cells harvested only 3.5 h after the onset of sporulation. This is well before the prespore has acquired any resistance to mechanical shear, and cross-contamination of prespore and mother cell fractions might therefore be expected in these experiments (see introduction). The results shown indicate that at least some mother cell material (alkaline phosphatase) was present in the prespore fraction. There also appeared to be rather less σ^E protein in the prespore fraction than in the mother cell fraction. Taken together, these observations raise the possibility that σ^E is more abundant in the mother cell than in the prespore. In any case, our results do not necessarily contradict the supposition that *spoIID* is transcribed in both compartments; however, they do imply that the *spoIID* product is required only in the mother cell for successful spore development.

spoIIIA, on the other hand, has been reported to play a key role in the control of prespore-specific gene expression (8, 16, 22, 23), and the result presented here indicating that the *spoIIIA* products are required only in the mother cell appears to be contradictory. However, in the accompanying paper (14), we report that *spoIIIA* mutations have a relatively minor effect on the expression of at least some prespore-specific genes. Some of the previous reports indicating a more marked effect on prespore-specific expression were based on a mutant strain, IS37, that was originally thought to carry the *spoIIIA53* mutation; in fact, it carries a *spoOA* mutation. In addition, after an initial burst of expression, *spoIIIA* is repressed by the product of the well-characterized *spoIIID* gene (N. Illing and J. Errington, unpublished results), which is specifically concerned with the regulation of mother cell events and is probably expressed only in the mother cell (17). De Lencastre and Piggot (5) concluded on the basis of their transformation experiments that the *spoIIID* product is required only in the mother cell. Taken together, these results suggest that transcription of *spoIIIA* is controlled by mother-cell-specific genes and that the product of *spoIIIA* is required only in the mother cell. Localization of *spoIIIA* expression in the mother cell does not, of course, preclude it from affecting gene expression in the prespore.

As stated above, it is formally possible that oligosporogeny could account for the appearance of the Spo^- Cm^r survivors in these experiments. Insertion-deletion mutations in all of the *spo* loci considered here generate asporogenous phenotypes (data not shown) except for those in *spoIIIA*, in which such mutations results in a significant level of oligosporogeny (Illing and Errington, unpublished results). We measured the number of heat-resistant spores made by strain 630, which carries a *lacZ cat* insertion in the *spoIIIA* locus, to quantify the contribution that oligosporogeny might make to the number of Spo^- Cm^r survivors from strain NI4. The level of oligosporogeny (3.4×10^3 heat-resistant survivors per ml of culture; average of three independent experiments) represents less than 1/10 the number of heat-resistant survivors obtained with strain NI4 (5×10^4 heat-resistant survivors per ml of culture). If the frequency of heat-resistant Spo^- survivors from strain NI4 is corrected for the incidence of oligosporogeny, the proportion of Cm^r Spo^- survivors drops to 25%, which is still much higher than would be expected for a gene that is required in the prespore. Similar proportions of Spo^- Cm^r survivors were observed with various other integrational plasmids that disrupt the *spoIIIA* operon, indicating that this is not simply a leaky insertional phenotype (results not shown).

An alternative explanation for the appearance of Spo^- Cm^r survivors would be that the disrupted sporulation operon is expressed before septation. The protein products could then be distributed into both the prespore and the mother cell after septation, irrespective of the genotypes of the chromosomes segregating into each compartment. However, this argument cannot be used to explain the results described here, as both *spoIID* and *spoIIIA* are known to be expressed after septation (3; N. Illing, unpublished results).

The principles underlying the method that we have described are similar to those exploited by De Lencastre and Piggot (5). However, the new method has two important advantages. First, the frequency of plasmid excision is about 10-fold greater than that of transformation to Spo^+, which permits analysis of mutations causing an appreciable degree of oligosporogeny (see above). Second, the progeny of genetically asporogenous spores can be selected directly and scored easily on the basis of the plasmid-borne antibiotic resistance gene. Selection was not possible with the earlier method, and accurate scoring required relatively laborious microscopic examination of bacteria from individual colonies. The main disadvantage of the new method lies in the requirement for an integrational plasmid that disrupts the gene of interest. However, most of the *spo* genes have now been cloned, and problems are likely to arise only with very small genes such as *spoIIID*. Integrational plasmids with small inserts (200 bp) disrupting such genes may not be easy to construct, since they may recombine with the bacterial chromosome relatively inefficiently.

In conclusion, the method described here offers an additional simple test for the localization of gene action during sporulation. This form of analysis, which provides information about the cellular compartment in which gene expression is required during sporulation, should prove to be a

useful adjunct to the various methods already available for determining the localization of gene expression during endospore development.

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