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Analysis by Fluorescence Microscopy of the Development of Compartment-Specific Gene Expression during Sporulation of *Bacillus subtilis*

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The use of a fluorogenic substrate, 5-octanoylaminofluorescein-di-β-D-galactopyranoside, for β-galactosidase has made it possible to visualize enzyme activity in individual cells of sporulating populations of *Bacillus subtilis* by fluorescence microscopy. *lacZ* fusions to different sporulation-associated genes have been used to investigate the cell compartmentalization of gene expression during sporulation. A strain with a *lacZ* fusion to *sspA*, a gene which is transcribed by E-σ^G at a late stage of sporulation, displayed predominantly compartment-specific fluorescence. Expression of the early-expressed *spoIIA* locus, which includes the structural gene for σ^F, was seen not to be compartmentalized. Populations of strains with *lacZ* fusions to *gpr* and *dacF*, genes which are transcribed by E-σ^F at intermediate stages of sporulation, included some organisms showing uncompartmentalized fluorescence and others showing compartment-specific fluorescence; the proportion showing compartment-specific fluorescence increased in samples taken later in sporulation. Several possible explanations of the results obtained with *gpr* and *dacF* are considered. A plausible interpretation is that σ^F activity is initially not compartmentalized and becomes compartmentalized as sporulation progresses. The progression to compartmentalization does not require the activities of the sporulation-specific factor σ^E or σ^G but may require some product of σ^F activity.

Differentiation of one cell type into a different cell type is a fundamental process in biology. A primitive example of cell differentiation is provided by spore formation of *Bacillus subtilis*. Spore formation is triggered in vegetatively growing cells by nutrient depletion (24). This leads to an asymmetric division (by convention, stage II of sporulation [29]) giving rise to two distinct cells, the smaller of which is called the prespore and the larger of which is called the mother cell. These two cells have radically different developmental fates. The prespore is engulfed by the mother cell (stage III of sporulation). It then undergoes a series of changes as it develops into the mature, resistant, dormant spore. The mother cell, which is required for this development, ultimately lyses. The engulfed prespore is sometimes referred to as the forespore, and we use this term here to distinguish it from the prespore formed at stage II.

The first evidence that gene expression during sporulation might be cell compartment specific came from biochemical studies that separated mother cell extracts from forespore extracts. These studies showed clearly that a number of biochemicals were differentially distributed between the mother cell and forespore (1, 5, 9, 34). Similar analysis was later performed on strains containing fusions to *lacZ* of a variety of sporulation-associated genes; the distribution of β-galactosidase activity gave clear evidence of the compartmentalization of transcription (recently reviewed in reference 6). The limitations of this biochemical approach are that (i) the fractions are never pure, so that it is possible to deduce predominant, but not exclusive, expression in a particular cell type, and (ii) the fractionation works well only when the developing forespore is substantially tougher than the mother cell, i.e., 1 h or more after engulfment. Thus, the approach is inadequate for the analysis of compartmentalization of gene expression during the crucial period before engulfment when it is thought likely that expression becomes compartmentalized.

A distinctive genetic approach to compartmentalization used genetic mosaics in which one copy of the chromosome was mutant and the other was wild type. With certain assumptions about the time of expression, this approach led to the conclusion that some genes had to be expressed in the prespore or forespore, whereas other genes need be expressed only in the mother cell (3). Again, it was not possible to deduce when transcription of a particular gene became compartmentalized, nor was it possible to determine whether transcription was completely compartmentalized.

Electron microscopy of immunogold-tagged proteins has given the clearest evidence of compartmentalization. Small acid-soluble proteins were localized almost exclusively in the forespore (8). This result agrees with prior biochemical fractionation studies (32). It is consistent with the transcription of the corresponding *ssp* genes by RNA polymerase containing σ^G (E-σ^G), as previous biochemical studies had indicated that σ^G was localized in the forespore (14). Immunogold labeling of β-galactosidase has been used successfully to identify the site of expression of several other sporulation-specific loci fused to *lacZ* (4, 16, 38). It confirmed that E-σ^G activity was confined to the forespore and provided a clear demonstration that E-σ^K activity on *gerE* and *cotD* was confined to the mother cell (4).

The compartmentalized activities of σ^G and σ^K result, at least in part, from the action of σ^F and σ^E, which are required for transcription of the structural genes for σ^G and σ^K (6, 15). The genes encoding σ^F and σ^E are transcribed and translated before septation (10, 21), and σ^F and σ^E appear to be activated, in some way, by formation of the spore septum

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TABLE 1. Strains used

Strain	Genotype and/or derivation	Source and/or reference
BR151	*trpC2 metB10 lys-3*	F. E. Young
IA680	*trpC2 xglA1 xglR1*	BGSC[a]; 7
MB24	*trpC2 metC3 rif-2*	22
SL617	*spoIIGB55 leuA8*; NG12.12 (22) into MB21 (12)	3
SL4168	*trpC2 metB10 lys-3 amyE::rrnO-lacZ*	R. Rudner
SL4741	*trpC2 metB10* SPβ::*spoIIA-lacZ*; fusion moved to BR151 by PBS1-mediated transduction	43
SL4834	*trpC2 metC3 rif-2 amyE::dacF-lacZ*; an *Eco*RV-*Dra*I fragment encompassing the *dacF* promoter (36) was cloned into pDH32 (33) and inserted at the *amyE* locus in MB24	R. Schuch
SL5072	*trpC2 metB10 lys-3 amyE::dacF-lacZ*; fusion transformed into BR151 with DNA from SL4834	This study
SL5137	*spoIIGB55 leuA8 amyE::dacF-lacZ*; fusion transformed into SL617 with DNA from SL4834	This study
SL5336	*trpC2 metC3 rif-2 amyE::gpr-lacZ*; a *Kpn*I-*Hin*dIII fragment containing the *gpr* promoter (40) subcloned in pDH32 (33) and inserted at the *amyE* locus of MB24	R. Schuch
SL5367	*spoIIGB55 leuA8 amyE::gpr-lacZ*; fusion transformed into SL617 with DNA from SL5336	This study
SL5429	*trpC2 metB10 lys-3 amyE::gpr-lacZ*; fusion transformed into BR151 with DNA from SL5336	This study
SL5430	*trpC2 metB10 lys-3 amyE::spoIIIG-lacZ*; fusion (30) transformed into BR151 with DNA from strain RS78	R. Schmidt
SL5453	*spoIIAC1 trpC2 dacF*::pPP293 (3 copies)	R. Schuch
SL5465	*spoIIIGΔPB trpC2 metB10 lys-3 amyE::gpr-lacZ*; fusion from SL5336 transformed into a strain with a *Pst*I-*Bam*HI fragment of *spoIIIG* deleted	M. Karow
SL5466	*spoIIIGΔPB trpC2 metB10 lys-3 amyE::dacF-lacZ*; fusion from SL4834 transformed into a strain with a *Pst*I-*Bam*HI fragment of *spoIIIG* deleted	M. Karow
SL5530	*trpC2 metB10 lys-3 sspA'-'lacZ*; translation fusion (17) transformed into BR151	This study
SL5531	*trpC2 metB10 lys-3* SPβ::*dacF-lacZ*; an *Eco*RI-*Hae*III fragment encompassing the *dacF* promoter (44) was inserted into pGV34 (45) and transferred to BR151 via ZB307 (47)	J.-J. Wu
SL5911	*trpC2 xglA1 xglR1 amyE::gpr-lacZ*; fusion from SL5336 transformed into IA680 at a low DNA concentration (0.01 μg/ml)	This study
SL5915	*trpC2 xglA1 xglR1 dacF*::pPP293 (3 copies); fusion from SL5453 transformed into IA680 at low DNA concentration	This study
SL5917	*spoIIAC1 trpC2 xglA1 xglR1 dacF*::pPP293 (3 copies); fusion from SL5453 transformed into SL5746 at low DNA concentration	This study

[a] BGSC, *Bacillus* Genetic Stock Center.

(reviewed in references 6, 15, and 37). Expression of the *spoIID* locus, which is transcribed by E-σ^E, was detected, by immunogold electron microscopy, in the mother cell after septation and before engulfment, indicating that compartmentalization of gene expression was already operative at this stage (4). Transcription by RNA polymerase containing a mutated form of σ^F (16) and transcription of a mutated promoter by unmutated σ^F (38) have been localized to the forespore, and expression of a mutated form of σ^F was also detected in the prespore (16).

There is thus an extensive body of evidence that gene expression becomes compartmentalized during sporulation, and this is first seen in the activities of σ^F and σ^E (reviewed in references 6, 15, and 37). As σ^F is required for σ^E activation, it seems plausible that the activity of σ^F may be pivotal to compartmentalization (15). Although the immunogold technique has been very useful for identifying compartmentalized gene expression, it has not been sensitive enough to detect the expression of genes such as *gpr* and *spoIIIG*, which are normally transcribed by RNA polymerase containing unmutated σ^F (16, 39).

We report here the development of a method employing a fluorescent probe for β-galactosidase in order to study the activity of σ^F-controlled genes fused to *lacZ* by fluorescence microscopy. The probe provides a more sensitive method for localizing the expression of *lacZ* fusions than does immunoelectron microscopy. The use of a fluorescent probe for the detection of β-galactosidase produced by single cells of *Escherichia coli* was described by Rotman and coworkers some 30 years ago (26, 27). The procedure has recently been adapted to fluorescence-activated cell sorting analysis of microorganisms (19,28,41). Here we used the fluorescent probe 5-octanoylaminofluorescein-di-β-D-galactopyranoside (FDG) to display enzyme activity within individual sporulating organisms of *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are described in Table 1. Strains used for analysis with FDG were isogenic derivatives of BR151, with the exceptions of SL5137, SL5367, SL5912, SL5915, and SL5917. SL5137 and SL5367 were derived via SL617 from MB19 (22) and are isogenic with MB24; SL5911, SL5915, and SL5917 are isogenic with IA680. Fusions to *lacZ* were transcriptional except for the fusion in strain SL5530, which was translational. Plasmid pPP293 containing a *dacF-lacZ* fusion has been described previously (43).

Bacteria were grown in a minimal-glucose-exhaustion sporulation medium (3) based on the medium of Ramaley and Burden (25). Amino acids (50 μg/ml) were added as required. Cultures were grown aerobically in Erlenmeyer flasks (1:10 medium-to-flask volume ratio) with agitation (120 rpm) at 37°C. Growth was monitored by turbidity at 600 nm (LKB Ultrospec IIE). Media were inoculated with 1/100 volume of cultures of exponentially growing bacteria (optical density at 600 nm, between 0.4 and 0.6). The initiation of sporulation was defined as the end of exponential growth. Time is expressed as the time in hours after the end of exponential growth (t_1, 1 h; t_2, 2 h; etc.). The frequency of sporulation was determined by phase-contrast microscopy of samples at t_{16}.

Fixation. Cells were fixed in 0.0001% glutaraldehyde. Control experiments indicated that such fixation did not affect β-galactosidase activity. Variation of the time of treatment of

fixed cells with FDG (Molecular Probes, Eugene, Ore.) did not affect the proportions of cells showing distinct fluorescence patterns. However, unfixed cells often became lytic and gave variable results as the time of FDG treatment was extended from 1 to 5 h.

FDG staining procedure. The FDG staining was accomplished as follows. Culture samples (1 ml) were harvested by centrifugation, fixed for 1 min, and washed once in chloroquine (300 μM) and once in A buffer (18). The pellet was then either adjusted to 10% dimethyl sulfoxide and frozen in liquid nitrogen or resuspended in 50 μl of Z buffer (18) and used directly or placed onto coverslips and cryopreserved by the freeze-plunge method of Parks et al. (20).

Fifteen microliters of the cell suspension was applied to a coverslip treated with 0.1% polylysine. The coverslip was placed onto 15 μl of 10 mM FDG (in 20% dimethyl sulfoxide), sealed with paraffin wax, and kept in the dark at room temperature for 1 to 5 h. (Variation in the time of treatment did not affect the proportions of the different fluorescing cell types.) The coverslip was then unsealed, washed by rapid submersion in water, placed onto a drop of Slow Fade (Molecular Probes) on a microscope slide, resealed with paraffin wax, and observed.

Microscopy. All microscopy was conducted with a Zeiss Photomicroscope II (Carl Zeiss, Oberkochen, Germany). β-Galactosidase activity of FDG-treated cells was observed by 460- to 500-nm epifluorescence, using a band pass excitation filter and a long-pass 520-nm barrier filter which allowed wavelengths greater than 520 nm emitted by the specimen to be visualized. Visualization of cells was enhanced by observing cells illuminated simultaneously with epifluorescent and transmitted phase-contrast light. Images of FDG-stained material were recorded on Kodak Tri-X 35-mm film (Eastman Kodak Co., Rochester, N.Y.). The percentage of cells fluorescing and the localization of fluorescence were determined visually. For most samples, the percentage of cells fluorescing was determined by counting at least 300 cells; the localization of fluorescence to the different cell types was determined separately.

RESULTS

FDG as a probe for β-galactosidase activity in *B. subtilis* strains containing different genes fused to *lacZ*. FDG is a substrate for β-galactosidase that, when cleaved, becomes a fluorescent compound easily detected by fluorescent light microscopy. We established conditions for FDG penetration, and subsequent cleavage by β-galactosidase, using *B. subtilis* containing an *rrnO-lacZ* fusion. We anticipated that *rrnO* would be expressed in most, if not all, cells throughout growth and much of sporulation. Using the procedure described in Materials and Methods, we consistently detected fluorescence in 85 to 96% of populations sampled during vegetative growth and during the first 5 h (i.e., up to t_5) after the end of exponential growth. The fluorescence was confined to the bacteria with little or no detectable leakage into the surrounding medium. The parent strain containing no *lacZ* fusion, BR151, displayed no fluorescence during vegetative growth or during the early stages of sporulation. At t_5, however, a variable proportion (from 0 to 10% in different experiments) of BR151 cells had detectable fluorescence, presumably resulting from endogenous *B. subtilis* β-galactosidase activity.

Cells expressing *lacZ* fused to the sporulation-associated genes used in this study displayed three predominant patterns of fluorescence when treated with FDG (Fig. 1). The fluorescence in Fig. 1A corresponds to a complete cell, and this

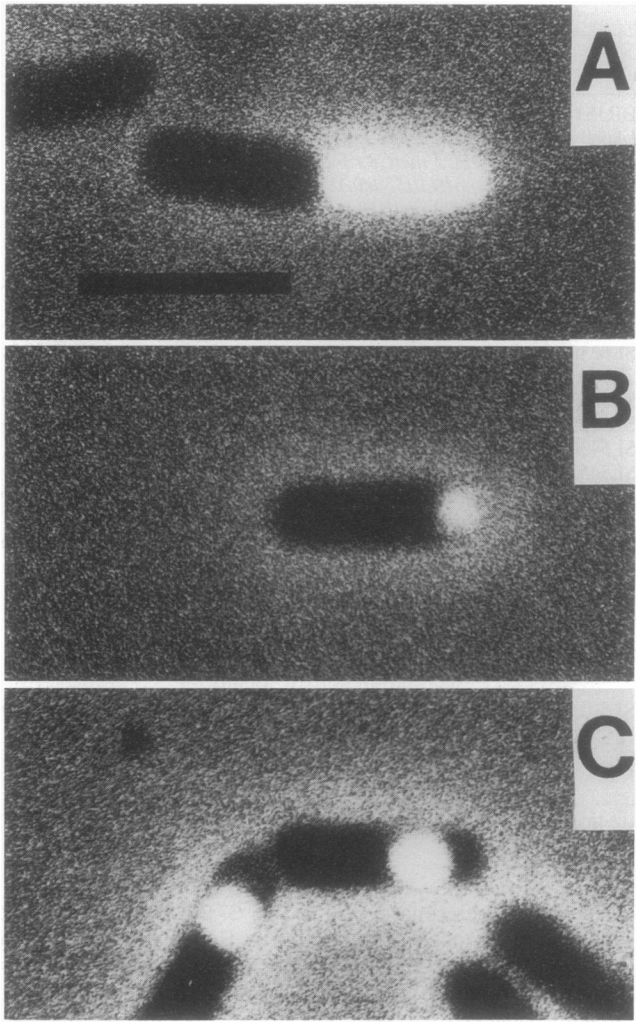

FIG. 1. Photomicrograph of *B. subtilis* cells exposed to FDG and illuminated simultaneously with epifluorescent and transmitted phase-contrast light sources. The presence of β-galactosidase activity results in hydrolysis of FDG to a fluorescent product which is seen in the micrographs as the bright area within cells. (A) Whole-cell fluorescence, *gpr-lacZ* fusion, strain SL5429; (B) prespore fluorescence, *spoIIIG-lacZ* fusion, strain SL5430; (C) forespore fluorescence, *spoIIIG-lacZ* fusion, strain SL5430. The bar in panel A applies also to panels B and C and represents 2.5 μm.

fluorescence pattern is designated whole-cell fluorescence. The fluorescent regions in Fig. 1B and C have the appearance of compartmentalized fluorescence in the prespore and the forespore, respectively, and we provisionally designate them as showing prespore-specific and forespore-specific fluorescence, respectively. As sporulation proceeds, forespores become distinguishable by phase-contrast microscopy. Prespores are not generally distinguishable by phase-contrast microscopy, and the FDG staining interfered in some way with our attempts at nucleoid staining with 4′,6-diamidino-2-phenylindole. Thus, we were unable to confirm directly our prespore designations and, in some cases, our forespore designations; these designations should therefore be considered tentative. Some cells, particularly of strains containing *gpr-lacZ* and *dacF-lacZ* fusions, appeared to show whole-cell fluorescence with somewhat stronger fluorescence in the prespore. Such cells were difficult

TABLE 2. Patterns of fluorescence observed upon FDG treatment of strains containing *spoIIA-lacZ*, *sspA-lacZ*, or no fusion

Strain	Fusion	% Sporulation	Time of sample (h after t_0)	Cells fluorescing as % of cells examined	No. of fluorescent cells counted[a]					Whole-cell fluorescence as % of fluorescing cells
					Whole cell	Prespore	Forespore	Early mother cell	Late mother cell	
SL4741	*spoIIA-lacZ*	66	2	2	94	7	1	1	0	91
			3	57	156	2	0	3	0	97
			4	40	140	2	1	7	0	93
SL5530	*sspA-lacZ*	70	2	1	8	0	97	0	0	8
			3	4	13	0	155	0	0	8
			4	4	3	0	120	0	0	2
			5	30	38[b]	0	205	0	0	16[b]
BR151	None	68	2	0						
			3	2[c]	27[c]	0	0	0	0	100
			4	2.6[c]	36[c]	0	0	0	0	100
			5	8.8	88	0	0	0	0	100

[a] The designations of cell types are as described in the text; the three common types are illustrated in Fig. 1.
[b] Most or all of the whole-cell fluorescence at t_5 could be a consequence of endogenous β-galactosidase.
[c] Very weak fluorescence.

to distinguish from cells showing uniform whole-cell fluorescence and were recorded as displaying whole-cell fluorescence. In addition to the predominant patterns, rare cells were observed displaying early (i.e., stages II to III) mother cell-specific fluorescence or late (i.e., stage III onward) mother cell-specific fluorescence.

Analysis of expression of the *spoIIA* locus. The *spoIIA* locus includes the structural gene for σ^F (39). It is transcribed by E-σ^H (42, 43). A strain containing a transcriptional fusion of *lacZ* to *spoIIA* displayed predominantly whole-cell fluorescence (Table 2). At t_3 and t_4 of sporulation, fluorescence was detected in 57 and 40% of the cells, respectively. The culture sporulated efficiently (66%), and it is deduced that transcription of the *spoIIA* locus in the sporulating population was not compartmentalized. This conclusion is consistent with previous studies which indicated that the locus is transcribed before the spore septum is formed (10).

Analysis of expression of a gene transcribed by E-σ^G. There is strong evidence that the *ssp* genes are transcribed primarily or exclusively in the forespore (17, 32) by E-σ^G (39). Analysis of strain SL5530, containing an *sspA-lacZ* fusion, indicated that β-galactosidase activity was primarily confined to the forespore (Table 2). In samples taken at t_2 through t_4, more than 90% of fluorescing organisms showed forespore specificity, although relatively few cells were fluorescing. Substantially more fluorescing organisms (30%) were detected at t_5, as expected from previous studies about the timing of *sspA* expression (6, 15). Again the preponderance showed forespore specificity. Precise interpretation of the t_5 result was difficult because endogenous β-galactosidase activity became a significant factor from t_5 onward. In the t_5 sample from a parallel culture of the parent strain BR151, which lacked the *sspA-lacZ* fusion, 9% of cells fluoresced and all displayed whole-cell fluorescence (Table 2). This endogenous activity could account for most or all of the whole-cell fluorescence observed with strain SL5530 at t_5. However, the proportion of BR151 cells displaying fluorescence at t_5 varied from 0 to 10% in other experiments, making an accurate correction for endogenous fluorescence difficult.

Analysis of expression of genes transcribed by E-σ^F. FDG analysis of strain SL5429, which contained the E-σ^F-transcribed gene fusion *gpr-lacZ*, revealed a pattern of fluorescing cells different from the pattern obtained with the strain containing the *sspA-lacZ* fusion (Table 2). Consistent with *gpr* being expressed earlier than *sspA* (40), there was prespore-specific in addition to forespore-specific fluorescence (Table 3). The prespore-specific/forespore-specific ratio declined with time during sporulation, suggesting a progression from prespore- to forespore-specific expression.

There was a substantial proportion of fluorescing cells which displayed whole-cell fluorescence, and this proportion also declined with time. A parallel culture of the parental strain that contained no fusion, BR151, showed no fluorescing cells in samples taken at t_2, t_3, and t_4 (Table 3). Thus, we think that the whole-cell fluorescence detected with strain SL5429 resulted from *gpr-lacZ* expression. To investigate this further, we constructed a strain, SL5911, in which a *gpr-lacZ* fusion was introduced into the genetic background of a strain isolated by Errington and Vogt (7) and reported to have very low endogenous β-galactosidase activity. The pattern of fluorescing cell types for SL5911 (Table 4) was very similar to that obtained with SL5429, and again the frequencies of whole-cell and of prespore/forespore-specific fluorescence in t_2, t_3, and t_4 samples were substantially above the frequencies obtained with the parental strain that lacked the fusion. By t_5, most or all of the whole-cell fluorescence with strains SL5429 and SL5911 may have resulted from the endogenous activity rather than the *gpr-lacZ* fusion. The proportion of fluorescing cells displaying whole-cell fluorescence for SL5911, as for SL5429, declined with time, suggesting a temporal change from whole-cell toward compartment-specific expression. A similar pattern of fluorescent cell types was also observed (Table 5) with strain SL5072, which contained *lacZ* fused to another E-σ^F-transcribed gene, *dacF* (31). The experiments with *gpr-lacZ* and *dacF-lacZ* were repeated several times, and the same overall pattern was always observed, although there was day-to-day variation in the absolute numbers of fluorescing cell types.

The *spoIIIG* gene is also transcribed by E-σ^F (38). Analysis of a strain (SL5430) that contained a *spoIIIG-lacZ* fusion indicated that, as for strains carrying the other σ^F-controlled genes, cells displayed prespore/forespore-specific fluorescence as well as whole-cell fluorescence (Table 3). The number of cells displaying whole-cell fluorescence was above the level of fluorescence obtained with a parallel culture of strain BR151 which contained no fusion (Table 3). The significance of the low proportion of fluorescing cells displaying whole-cell fluorescence compared with results obtained with *gpr-lacZ* and *dacF-lacZ* fusions is unclear. It may reflect a difference in the regulation of *spoIIIG* (21), or it may be a consequence of the

TABLE 3. Patterns of fluorescence observed upon FDG treatment of *spo*$^+$ strains containing transcriptional *lacZ* fusions to genes transcribed by E-σ^F

Strain	Fusion	% Sporulation	Time of sample (h after t_0)	Cells fluorescing as % of cells examined	No. of fluorescent cells counted[a]					Whole-cell fluorescence as % of fluorescing cells
					Whole cell	Prespore	Forespore	Early mother cell	Late mother cell	
SL5429	*gpr-lacZ*	81	2	3	89	36	73	2	0	45
			3	10	60	22	98	0	0	33
			4	20	51	25	121	2	0	26
			5	17	11	17	172	0	0	6[b]
SL5430	*spoIIIG-lacZ*	78	3	15	45	82	73	0	0	22
			4	15	39	27	110	0	0	24
			5	20	58[b]	44	157	0	0	22[b]
BR151	None	69	2	0	0	0	0	0	0	
			3	0	0	0	0	0	0	
			4	0	0	0	0	0	0	
			5	10	90	0	8	0	2	90

[a] The designations of cell types are as described in the text; the three common types are illustrated in Fig. 1.
[b] Most or all of the whole-cell fluorescence at t_5 could be a consequence of endogenous β-galactosidase.

relatively weak expression of *spoIIIG-lacZ* being closer to the threshold of sensitivity of the method.

The *dacF-*, *gpr-*, and *spoIIIG-lacZ* fusions discussed above were inserted into the *amyE* locus. To test whether the fluorescence patterns obtained were influenced by the location of the *lacZ* fusion, strains that had *dacF-lacZ* fusions at SPβ (SL5531) and at *dacF* (SL5915) were tested. Substantial numbers of whole-cell, prespore-specific, and forespore-specific fluorescent cells were detected whatever the location of the fusion (Table 5). The strain with the fusion inserted at *dacF* gave a higher proportion of fluorescing cells that displayed whole-cell fluorescence. The significance of this difference is unclear; we were unable to obtain a strain with a single copy of *dacF-lacZ* at *dacF* to test if copy number might be a factor.

The *dacF* gene is located immediately upstream of the *spoIIA* operon, which encodes σ^F and its regulators (44). This facilitated the study of the expression of *dacF* in an isogenic pair of strains differing only in a nonsense mutation, *spoIIAC1* (46), in the structural gene for σ^F. The *spoIIAC1* mutation blocks expression of *dacF* (31) and largely prevented the whole-cell and prespore/forespore-specific fluorescence associated with *dacF-lacZ* expression (strain SL5917; Table 5).

Effects of *spoIIIG* and *spoIIGB* mutations on the expression of *gpr* and *dacF*. σ^F has overlapping promoter specificity with σ^G, and the *gpr* and *dacF* promoters are recognized by RNA polymerase holoenzyme containing either sigma factor (31, 40). To determine if the progression from whole-cell to compartment-specific fluorescence observed with the Spo$^+$ strains was caused in part by the activity of σ^G, the effect of a deletion in *spoIIIG*, the structural gene for σ^G (14, 39), was tested. The same general pattern of fluorescence was obtained with strain SL5465 (*spoIIIGΔPB gpr-lacZ*) (Table 6) as was obtained with the *spo*$^+$ strain SL5429. Similar results were obtained with the *spoIIIGΔPB* strain SL5466, which contained a *dacF-lacZ* fusion (Table 6). As with the corresponding *spo*$^+$ strains, SL5429 and SL5072, the percentage of fluorescing cells displaying whole-cell fluorescence and the prespore/forespore expression ratio declined with time into sporulation. The results indicate that σ^G is not required for the compartmentalization of expression of these genes.

A possible role of σ^E in compartmentalization was also tested. The expression of *dacF-lacZ* and *gpr-lacZ* in the absence of functional σ^E was analyzed in strains containing a *spoIIGB55* mutation (36). As with *spo*$^+$ strains, samples taken relatively early in sporulation displayed predominantly whole-cell fluorescence (Table 6), and samples taken later showed a substantial switch to prespore-specific expression. For both fusions, the cells scored as prespore specific at t_5 included organisms with prespores at both ends, corresponding to the abortively disporic phenotype which is characteristic of *spoIIGB* and certain other *spoII* mutants (13, 24, 29). As mentioned previously for other strains, the proportion showing

TABLE 4. Patterns of fluorescence observed upon FDG treatment of strains with reduced endogenous background (7)

Strain	Fusion	% Sporulation	Time of sample (h after t_0)	Cells fluorescing as % of cells examined	No. of fluorescent cells counted[a]					Whole-cell fluorescence as % of fluorescing cells
					Whole cell	Prespore	Forespore	Early mother cell	Late mother cell	
SL5911	*gpr-lacZ*	92	2	5	78	25	11	0	0	68
			3	9	45	35	22	0	0	44
			4	3	30	14	82	0	0	24
			5	4	22[b]	1	112	0	0	16[b]
IA680	None	86	2	0	0	0	0	0	0	
			3	0.6	2	1	0	0	0	
			4	0.6	1	0	3	0	0	
			5	0.9	5	0	0	0	0	

[a] The designations of cell types are as described in the text; the three common types are illustrated in Fig. 1.
[b] Most or all of the whole-cell fluorescence at t_5 could be a consequence of endogenous β-galactosidase.

TABLE 5. Effects of different locations of a *dacF-lacZ* fusion on the fluorescence pattern observed upon FDG treatment of *spo*$^+$ strains

Strain[a]	Fusion location	% Sporulation	Time of sample (h after t_0)	Cells fluorescing as % of cells examined	No. of fluorescent cells counted[b]					Whole-cell fluorescence as % of fluorescing cells
					Whole cell	Prespore	Forespore	Early mother cell	Late mother cell	
SL5072	*amyE*	80	3	3	80	68	56	0	0	39
			4	8	55	81	64	0	1	28
SL5531	SPβ	62	4	15	86	39	75	0	0	43
SL5915	*dacF*	73	3	11	150	37	28	0	0	72
			4	7	153	13	59	2	0	68
SL5917	*dacF*	0[c]	3	0.9	6	1	0	7[d]	0	
			4	1.1	5	2	0	28[d]	0	

[a] Strains SL5915 and SL5917 were analyzed in parallel experiments; strains SL5072 and SL5531 were analyzed in a separate set of experiments.
[b] The designations of cell types are as described in the text; the three common types are illustrated in Fig. 1.
[c] Strain contains *spoIIAC1* mutation.
[d] Abortively disporic appearance with fluorescence in the mother cell.

whole-cell fluorescence at t_5 may have been inflated by endogenous β-galactosidase activity. For the *spoIIGB* and the *spoIIIG* mutants, both the number of fluorescing cells at t_2 and the proportion of fluorescing cells displaying whole-cell fluorescence tended to be higher than for the corresponding *spo*$^+$ strains. The reason for this is not known.

DISCUSSION

The use of a fluorogenic substrate, FDG, for β-galactosidase has enabled us to visualize enzyme activity in individual cells of *B. subtilis* by fluorescence microscopy. Further, it has enabled us to localize activity to what we suggest are different cell types involved in spore formation (Fig. 1). It had previously been deduced that *spoIIA* is transcribed before the spore septum is formed, and thus transcription should not be compartmentalized (10, 21). The results obtained with the *spoIIA-lacZ* fusion (Table 2) provide the most direct demonstration to date that, indeed, transcription of *spoIIA* is not compartmentalized during the early stages of sporulation. The demonstration that *sspA-lacZ* expression appears to be confined to the forespore during sporulation (Table 2) agrees with the previous demonstration by immunogold labeling that the small acid-soluble protein A, encoded by *sspA*, is located in the forespore (8) and with biochemical fractionation studies indicating that *sspA* transcription is largely confined to the forespore (17). The agreement with results of studies that used other methods gives us confidence that the FDG approach is valid.

The results obtained with the σ^F-controlled genes *gpr*, *dacF*, and *spoIIIG* show expression compartmentalized to the forespore. This finding is consistent with two immunogold studies, one using a modified σ^F (16) and one using a modified σ^F-controlled promoter (38). The greater sensitivity of FDG than of immunogold has enabled us to detect expression of single copies of unmutated promoters by unmutated σ^F. The studies here also show compartmentalization of expression of the three loci in what we infer is the prespore (Fig. 1B; Tables 3 and 4). This finding indicates compartmentalization of σ^F activity before engulfment, which had previously been demonstrated only for a modified form of σ^F active on the *ctc* promoter (16). As might be expected, there was an increase in the ratio of forespore-specific compared with prespore-specific fluorescence with time during sporulation (Tables 3 and 4). This is consistent with a progression from prespore- to forespore-specific expression. Compartmentalization was observed

TABLE 6. Patterns of fluorescence observed upon FDG treatment of *spoIIIG* and *spoIIGB* mutant strains containing transcriptional fusions to genes transcribed by E-σ^F

Strain	Fusion	*spo* mutation	Time of sample (h after t_0)	Cells fluorescing as % of cells examined	No. of fluorescent cells counted					Whole-cell fluorescence as % of fluorescing cells
					Whole cell	Prespore	Forespore	Early mother cell	Late mother cell	
SL5465	*gpr-lacZ*	*spoIIIGΔPB*	2	20	158	18	41	0	0	73
			3	30	138	15	169	0	0	43
			4	45	72	2	175	0	0	29
SL5466	*dacF-lacZ*	*spoIIIGΔPB*	2	22	117	43	133	0	0	40
			3	15	88	25	91	0	0	43
			4	20	62	7	105	0	0	36
SL5137	*dacF-lacZ*	*spoIIGB55*	3	20	97	3	0	0	0	97
			4	5[a]	31	69	0	0	0	31
			5	20	18[b]	82[c]	0	0	0	18[b]
SL5367	*gpr-lacZ*	*spoIIGB55*	3	10	84	16	0	0	0	84
			4	15	82	18	0	0	0	82
			5	ND[d]	39[b]	61[c]	0	0	0	39[b]

[a] The reason for the low value in this sample is not known; in separate experiments with a *spoIIGB55 dacF-lacZ* strain, the t_4 sample gave 20% fluorescence.
[b] Most or all of the whole-cell fluorescence at t_5 could be a consequence of endogenous β-galactosidase.
[c] Includes 15 cells displaying fluorescence at both ends (abortively disporic cells).
[d] ND, not done.

in strains lacking σ^E and σ^G (Table 4), confirming that those σ factors are not required for the process.

A substantial proportion of fluorescing cells of the strains with *gpr-lacZ* or *dacF-lacZ* fusions displayed activity that was not compartmentalized, with the proportion generally declining as sporulation progressed (Tables 3, 4, and 6). This result was surprising, and it is necessary to determine if the whole-cell fluorescence is an artifact or if it reflects β-galactosidase activity. The extent of whole-cell fluorescence was substantially greater than the fluorescence detected with parental strains, lacking any fusion, that were analyzed at the same time (Tables 3 and 4). Similarly, the whole-cell fluorescence was greatly curtailed by a *spoIIAC1* mutation (Table 5). It seems reasonable to suggest that the fluorescence reflects the activity of E-σ^F on the *lacZ* fusion. It should be noted that if there is an artifact, then it is not detected with the *sspA-lacZ* fusion and so is not a problem after engulfment. Nor is the switch from whole-cell to compartment-specific fluorescence confined to genes transcribed by E-σ^F, as preliminary studies of FDG hydrolysis by bacteria containing a *spoIID-lacZ* fusion, which is transcribed by E-σ^E, have suggested a progression from whole-cell-specific to mother cell-specific fluorescence (2). Two possible artifacts are (i) that FDG treatment damages the prespore septal structure and so causes artifactual whole-cell fluorescence and (ii) that the fluorescent product of FDG hydrolysis can leak through the intact spore septum from the prespore to the mother cell even though β-galactosidase activity is compartmentalized. The observed changes in septal structure during sporulation do not favor either of these explanations. The septal structure separating the prespore from the mother cell appears more robust when it is first completed than it does subsequently, and it also appears more robust than the forespore membrane upon engulfment (23), yet the proportion of whole-cell fluorescence generally declined rather than increased with time during sporulation (Tables 3, 4, and 6). In addition, varying the time of FDG treatment from 1 to 5 h did not cause any change in the proportion of cells showing uncompartmentalized expression (unpublished observations). Thus, we think it unlikely that the whole-cell fluorescence is an artifact, although we cannot exclude the possibility, as we have not been able to examine the septal structure of cells displaying uncompartmentalized expression. In the following paragraphs, we consider different interpretations of the alternative conclusion, namely, that the location of fluorescence does indicate the location of β-galactosidase activity.

The proportion of cells that fluoresced with strains containing *gpr-lacZ* and *dacF-lacZ* fusions was generally in the range of 10 to 30%, although all Spo$^+$ strains sporulated efficiently (62 to 81%). It is therefore formally possible that some or all of the fluorescing cells (whole cell or compartment specific) were from the nonsporulating population. This possibility is particularly intriguing for the cells displaying whole-cell fluorescence and cannot be discounted. For example, it may be that the sporulating cells display compartmentalized expression and that the nonsporulating cells display whole-cell fluorescence. If this were the case, it would imply that σ^F-controlled genes are expressed first in a relatively high proportion of the nonsporulating bacteria in a noncompartmentalized fashion and at a later time display compartmentalized expression in a lower proportion of the sporulating bacteria of the same population. Another interpretation of the results, which we think is more plausible, is that the bulk of the fluorescing cells are from the sporulating population, and that σ^F activity is initially not compartmentalized but becomes compartmentalized in the prespore, remaining compartmentalized when the prespore is engulfed by the mother cell to become the forespore. A shift from noncompartmentalized to compartmentalized expression might also help explain the apparently anomalous behavior of the E-σ^E-transcribed *spoIIM* locus; one set of experiments indicated that it had to be expressed in the prespore or forespore, whereas another set indicated that at least later in sporulation, expression was largely confined to the mother cell (35).

The low proportion of fluorescing cells detected with σ^F-transcribed genes compared with the proportion of cells sporulating indicates heterogeneity of expression within the population. It is presumably a consequence of the weak expression of the genes and of the limitations of the detection system. That cells were scored as showing either whole-cell or compartment-specific fluorescence also indicates the limitations of the detection system, rather than indicating a quantal transition from whole-cell to prespore-specific fluorescence. Indeed, some *gpr-lacZ* and *dacF-lacZ* cells appeared to display stronger prespore-specific fluorescence superimposed on weaker whole-cell fluorescence; such cells were difficult to distinguish from cells showing uniform whole-cell fluorescence and were recorded as displaying whole-cell fluorescence. We would speculate that cessation of β-galactosidase synthesis in the mother cell and turnover of existing enzyme, combined with continued synthesis in the prespore, resulted in the switch from whole-cell to prespore-specific fluorescence; the method of recording the data exaggerates the speed of the transition.

If we assume that the move from noncompartmentalized to compartmentalized expression by σ^F in sporulating organisms does indeed occur, then it seems plausible that it is an early product, or products, of σ^F action which brings about compartmentalization. The early product could, for example, cause changes to the spore septum that lead to compartmentalization (11, 23). Such a hypothesis is compatible with earlier models for compartmentalization (15). In order to analyze this problem further, it will clearly be important to establish the precise stage of sporulation in the cells displaying noncompartmentalized activity.

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