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Use of Immunofluorescence To Visualize Cell-Specific Gene Expression during Sporulation in *Bacillus subtilis*

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We have adapted immunofluorescence microscopy for use in *Bacillus subtilis* and have employed this procedure for visualizing cell-specific gene expression at early to intermediate stages of sporulation. Sporangia were doubly stained with propidium iodide to visualize the forespore and mother cell nucleoids and with fluorescein-conjugated antibodies to visualize the location of β-galactosidase produced under the control of the sporulation RNA polymerase sigma factors σ^E and σ^F. In confirmation and extension of earlier reports, we found that expression of a *lacZ* fusion under the control of σ^E was confined to the mother cell compartment of sporangia at the septation (II) and engulfment (III) stages of morphogenesis. Conversely, σ^F-directed gene expression was confined to the forespore compartment of sporangia at postseptation stages of development. Little indication was found for σ^E- or σ^F-directed gene expression prior to septation or in both compartments of postseptation sporangia. Gene expression under the control of the forespore sigma factor σ^G also exhibited a high level of compartmentalization. A high proportion of sporangia exhibited fluorescence in our immunostaining protocol, which should be suitable for the subcellular localization of sporulation proteins for which specific antibodies are available.

Sporulation in *Bacillus subtilis* is a primitive developmental process that involves the generation of differentiated cell types (5, 20, 26). Soon after the initiation of sporulation, an asymmetrically positioned septum partitions the developing cell or sporangium into two compartments with unequal sizes: a small forespore cell and a large mother cell. The compartments each receive a chromosome from the last round of DNA replication but follow different programs of gene expression; certain genes are expressed in the forespore and others are expressed in the mother cell. This differential gene expression is chiefly governed by four transcription factors. Just after septation, gene expression is controlled by σ^F in the forespore and σ^E in the mother cell. Later in development, when the forespore has become engulfed by the mother cell, σ^F and σ^E are replaced by σ^G and σ^K, respectively (for reviews, see references 10 and 19).

The compartmentalization of σ^G and σ^K activities arises as a result of their cell-specific synthesis. However, both σ^F and σ^E are synthesized prior to septation (12; for a review, see reference 9) but are not active until after septation (7, 17, 22, 24). The regulatory mechanisms controlling the compartment-specific activities of these two transcription factors are complex (see, for example, references 1, 6, 14, and 18). The principal determinants of cell-specific gene expression are believed to be the regulatory proteins that control the activity of σ^F, because σ^F function sets in motion a chain of events that leads to the cell-specific appearance of σ^E, σ^G, and σ^K (19).

Several techniques have been used to investigate the compartmentalization of gene expression during sporulation (for a review, see reference 10). Cell-type-specific gene expression was originally inferred from the use of genetically mosaic sporangia (which contain one mutant and one wild-type chromosome) (5). This method makes it possible to classify genes on the basis of the requirement for their expression in a particular compartment but does not indicate whether gene expression is compartment specific. Physical separation of the contents of the mother cell and forespore (23, 40) made it possible to demonstrate the compartmentalization of σ^G- and σ^K-directed gene expression (4, 23). However, such fractionation methods can be used on sporangia only at a relatively late morphological stage (IV), and significant cross-contamination of fractions occurs with this technique.

The introduction of immunoelectron microscopy provided the first direct method for demonstrating cell-specific gene expression (11). By this method, thin sections of fixed cells are treated with antibodies against a protein whose synthesis might be compartment specific (7, 8, 11, 22, 39). A gold-conjugated secondary antibody is then used to visualize the protein. Use of this technique confirmed the compartmentalization of σ^G- and σ^K-directed gene expression (7, 11) and provided the first direct evidence that σ^F and σ^E act in a cell-specific fashion (7, 22, 38). Immunoelectron microscopy has also been used to determine the subcellular localizations of various sporulation proteins at very high resolution (8). However, the technique requires high technical proficiency (particularly in cell fixation and thin sectioning) and expensive equipment and is slow and laborious and not highly sensitive.

Recently, another method for studying compartmentalized gene expression during sporulation has been described (2, 17). This technique involves the use of a fluorogenic substrate (FDG and its derivatives, such as C_8FDG) for β-galactosidase to localize gene expression from *lacZ* fusions by fluorescence microscopy. This method is rapid, simple, and highly sensitive; however, it suffers from three significant limitations: it is limited to the detection of β-galactosidase, only a small proportion of sporangia are observed to give a detectable signal, and the hydrolyzed FDG is membrane permeable (13). The problem that only a low proportion of cells give a signal could be a consequence of the tendency of the fluorescent products of FDG and C_8FDG hydrolysis to leak rapidly out of cells (13).

This membrane permeability could be the basis for conflicting results concerning the extent to which σ^F-directed gene

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TABLE 1. *B. subtilis* strains used in this study

Strain	Genotype	Type of *lacZ* fusion	Reference or source
PY79	Prototrophic	None	43
BK338	*spoIIIG*Δ1	None	15
PM389	*spoIIIG-lacZ spoIIIG*Δ*1*	Translational	22
LDB41	*amyE*::*sspE*-2G-*lacZ spoIIIG*Δ::*neo*	Translational	L. Duncan
BZ184	*amyE*::*spoIID-lacZ*	Transcriptional	B. Zheng
PM8	*sspB-lacZ*	Translational	22

expression is compartmentalized that have been obtained by this method. In conformity with the earlier immunoelectron microscopy results described by Margolis et al. (22) and Driks and Losick (7), Lewis et al. (16, 17) found that the activities of σ^F and σ^E are highly compartmentalized. On the other hand, Bylund et al. (2) reported that σ^F activity is initially not compartmentalized and becomes compartmentalized only after an interval in which the transcription factor is apparently active throughout the sporangium. On this basis, Bylund et al. (2) proposed that σ^F activity becomes compartmentalized as sporulation progresses, perhaps as a consequence of the action of certain genes under the control of σ^F itself. However, these workers (2) could not exclude the possibility that the seemingly uncompartmentalized activity of σ^F was an artifact due to the diffusion of hydrolyzed C_8FDG from the forespore into the mother cell.

Because of the importance of this issue to our understanding of events involved in the establishment of cell type and the general need for an efficient, general, and reliable procedure for visualizing cell-specific gene expression and subcellular protein localization in *B. subtilis*, we have adapted indirect immunofluorescence methodology for use in *B. subtilis*. In our procedure, which was modified from those described by Maddock and Shapiro (21) and Pringle et al. (29), cells are chemically fixed and then rendered permeable to antibodies by treatment with lysozyme. This method has the following advantages: it is highly sensitive, it is simple and reliable, a very high proportion of cells give a strong signal, it is broadly applicable to any sufficiently abundant protein for which a high-titer antibody is available, and it does not involve the use of membrane-permeable fluorophores. Here, in confirmation and extension of the findings described by Losick and colleagues, Setlow and colleagues, and Errington and colleagues (7, 16, 17, 22, 38), we demonstrate that σ^F and σ^E are indeed compartment-specific transcription factors; a high proportion of sporulating cells exhibited fluorescence and, in almost all sporangia, the fluorescence was restricted to a single compartment.

MATERIALS AND METHODS

Bacterial strains used in this study. The strains of *B. subtilis* used in this study are isogenic with *B. subtilis* PY79 and are listed in Table 1. PY79 and BK338 were used as negative controls in immunofluorescence experiments to confirm antibody specificity. PM8 and PM389 both contain their respective *lacZ* fusions as Campbell integrants (23, 37).

Sporulation conditions and sample collection. Strains of *B. subtilis* were induced to sporulate either by the resuspension method (23, 35, 37) or by nutrient exhaustion in DS medium (32) as described by Sandman et al. (31). Similar results were obtained regardless of the growth temperature or sporulation medium. When fresh samples of sporulating cultures were required for immunofluorescence, it was often more convenient to induce sporulation overnight at 25°C rather than at 37°C. To do so, a single colony grown on Luria-Bertani (LB) agar was used to inoculate 2 ml of DS medium. With a fresh, young colony, this method gave reproducible sporulation: after 21 h of incubation at 25°C, the majority of sporulating cells had reached stage II, as judged by staining with 0.1 μg of 4′,6-diamidino-2-phenylindole (DAPI) per ml, while after 24 h, the majority of sporulating cells had reached stage III, and a few phase-grey endospores were visible by phase-contrast microscopy.

Immunofluorescence procedure. Immunofluorescence staining was performed by a modification of methods described for *Escherichia coli* by Maddock and Shapiro (21) and for yeast by Pringle et al. (29).

(i) Fixation and permeabilization of cells. One major difference between our protocol and that of Maddock and Shapiro (21) was that we fixed the bacteria directly in the sporulation or growth medium. This approach was taken because of the observation that the localization of some proteins in both *B. subtilis* and *E. coli* could be altered when the cultures were centrifuged and resuspended in phosphate-buffered saline (PBS) prior to fixation (27). One limitation of this method is that the degree of fixation is affected by medium components and the proper level of fixation must be determined empirically for each condition. We found that fixation of bacteria in complex media such as LB or DS medium required more time and glutaraldehyde than did fixation in resuspension medium.

Prior to fixation, the cultures were vortexed to disrupt any clumps of bacteria. A 0.25-ml volume of bacterial culture was mixed with concentrated fixative solution. Cells growing in DS or LB medium were fixed with a final concentration of 2.4% (vol/vol) paraformaldehyde, 0.04% (vol/vol) glutaraldehyde, and 30 mM Na-PO_4 buffer (pH 7.5) for 10 min at room temperature and 50 min on ice. Cells incubated in resuspension medium were fixed with a final concentration of 2.4% (vol/vol) paraformaldehyde, 0.01% (vol/vol) glutaraldehyde, and 30 mM Na-PO_4 buffer (pH 7.5) for 15 min at room temperature and 35 min on ice. The fixed bacteria were washed three times in PBS (pH 7.4) at room temperature and then resuspended in 90 to 125 μl of GTE (50 mM glucose, 20 mM Tris-HCl [pH 7.5], 10 mM EDTA). A freshly prepared lysozyme solution in GTE was added to a final concentration of 2 mg/ml. Samples (10 μl) were immediately distributed onto wells of a multiwell microscope slide (ICN Biochemicals) which had been treated with 0.1% (wt/vol) poly-L-lysine (Sigma). After 30 s, the liquid was aspirated from the wells, which were then allowed to dry completely. The slides were dipped in −20°C methanol for 5 min and then in −20°C acetone for 30 s and allowed to dry. A 10-μl volume of blocking solution (2% bovine serum albumin [BSA] in PBS [BSA-PBS]) was then added to each well, and the slides were then incubated for 15 min at room temperature. In some cases, 0.05% Tween 20 was added to the blocking agent; however, this had no discernible effect in these experiments.

(ii) Immunofluorescence staining of cells. Cells were incubated with a 1:1,500 dilution in BSA-PBS of mouse monoclonal anti-β-galactosidase antibodies (Promega) or rabbit polyclonal anti-β-galactosidase antibodies (5′-3′Inc.) for 1 h at room temperature and were washed 10 times with PBS. (Equivalent results were obtained with both antibodies. However, the mouse monoclonal antibodies were found to be inactivated when they were subjected to a cycle of freezing at −20°C and thawing.) Samples were then incubated with a 7.5-μg/ml solution of the fluorescein-conjugated secondary antibody in BSA-PBS (either fluorescein-conjugated goat anti-mouse immunoglobulin G or fluorescein-conjugated goat anti-rabbit immunoglobulin G; Jackson Immunoresearch) for 1 h at room temperature in the dark. Dilutions of the primary and secondary antibodies were prepared with BSA-PBS. Propidium iodide (PI; Molecular Probes) was added with the secondary antibody at a final concentration of 0.01 mg/ml. The samples were washed 10 times in PBS and then once with mounting medium. Slides were mounted in either Slow Fade in a PBS-glycerol solution (Molecular Probes) or a solution of *p*-ethylenediamine (Sigma), which was prepared as described by Pringle et al. (29). The final slides could be stored at −20°C for several days.

(iii) Microscopy and photography. Samples were observed and photographed with an Olympus BX60 fluorescence microscope equipped with a PM-30 automatic camera system. Two types of Olympus objectives were used. A UPlan Fluorite phase-contrast objective (magnification, ×100; numerical aperture, 1.3) was used for phase-contrast and some fluorescence observations, and a UPlan Fluorite Apochromatic objective (magnification, ×100; numerical aperture, 1.3) was used for most fluorescence observations and photography. PI staining was visualized by using an excitation cube unit (U-MWG; Olympus) with a wide-band-pass (510- to 550-nm) excitation filter and a long-pass (590-nm) barrier filter. Fluorescein-stained cells were visualized by using an excitation cube unit (U-MWIBA; Olympus) with a wide-band-pass (460- to 490-nm) excitation filter and a narrow-band-pass barrier filter (512- to 550 nm). No spectral crossover fluorescence was observed with these filter sets. Both stains could be visualized simultaneously by using a wide-band-pass (460- to 490-nm) excitation filter and a long-pass (515-nm) barrier filter (U-MWIB; Olympus).

Fluorescent photographs were obtained with Kodak Ektachrome (ASA 400 or ASA 100, and pushing the development to ASA 400) and Kodak T-MAX film (ASA 400). Phase-contrast photography was performed with Kodak T-MAX film (ASA 100). Cell counting and scoring were performed from color slides in which each field of cells was represented by separate PI (for nucleoid staining) and fluorescein (for protein localization) staining and a multiple exposure showing both fluorescent stains simultaneously (for determination of the relative locations of the two stained regions). At least 300 cells were scored for each strain.

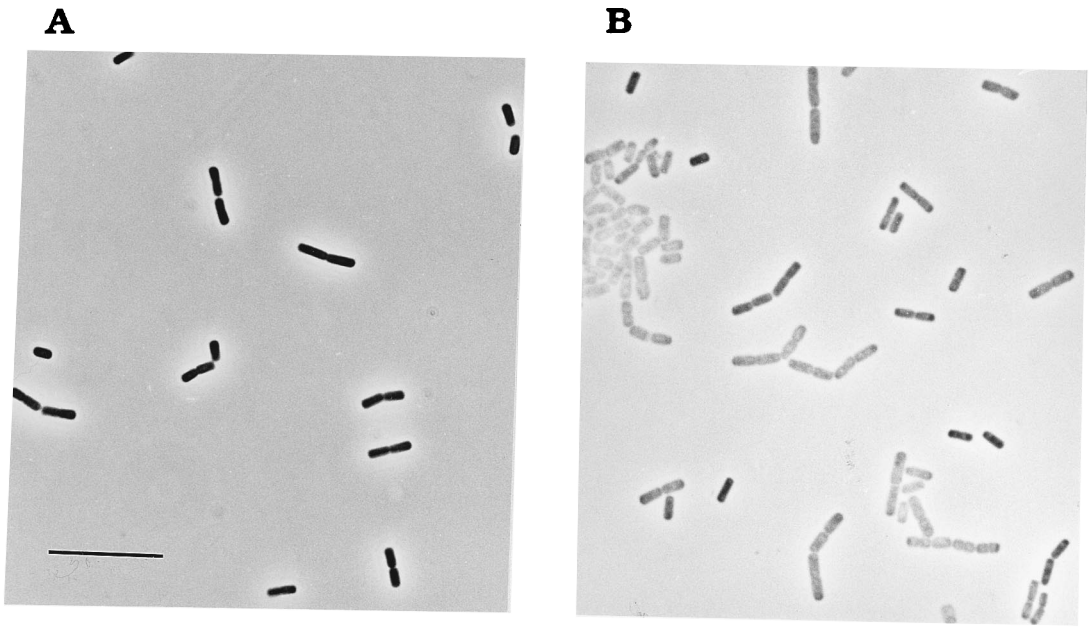

FIG. 1. Phase-contrast photomicrographs of *B. subtilis*. Cells of strain PM8 were collected 2 h after sporulation was induced at 37°C by the resuspension method and subjected to the immunostaining procedure described in Materials and Methods. (A) Cells after fixation; (B) cells after fixation, lysozyme treatment, and the subsequent steps of the immunostaining procedure, except that antibodies were omitted. Bar, 11.7 μm.

RESULTS

Phase-contrast observations and nucleoid staining of sporulating cells. Prior to immunolocalization of proteins with fluorescence, cells were fixed and then permeabilized with lysozyme to allow antibodies to penetrate the cell. Phase-contrast microscopy showed that fixed cells lost much of their contrast after lysozyme treatment and the subsequent steps of our immunostaining protocol (Fig. 1). Unless the fluorescence is extremely bright, this makes it difficult to observe fluorescent cells with both transmitted and fluorescent light. In addition, fluorescence is obscured under phase-contrast microscopy. Therefore, we used fluorescent nucleoid staining when performing immunofluorescence experiments to determine the orientation of cells and to locate the two compartments within sporulating cells. Staining of DNA with DAPI during sporulation in both *Bacillus megaterium* and *B. subtilis* has been well studied (34). We used PI in these experiments, since there is less spectral crossover between PI and fluorescein than between DAPI and fluorescein at the level of sensitivity required for immunofluorescence. Although PI stains both DNA and RNA, DAPI and PI give very similar staining patterns under these conditions (see also reference 16).

At stage II, after the sporulation septum has been formed, the forespore nucleoid condenses and appears as a small bright dot when it is stained with PI, whereas the mother cell nucleoid appears larger and more diffuse (34). Examples of PI-stained cells at this stage are shown in Fig. 2A. Between stages II and III, the mother cell nucleoid begins to condense and DNA in the forespore decondenses as the volume of the maturing forespore increases (Fig. 2C). Later, at approximately stage III to stage IV, the nucleoids of both compartments are similar in density and in size, as shown in Fig. 2E (see also later figures). By stage IV and later, the forespore nucleoid retains the same size but often appears less bright and sometimes has the shape of a ring (whose significance is the subject of an unpublished report [28]). Because it was difficult to distinguish between late-stage-II and early-stage-III sporangia on the basis of nucleoid staining alone, we defined two broad morphological classes that do not precisely correspond to stages defined by electron microscopy. Class A includes sporangia in which the forespore nucleoid was significantly more dense and smaller than the mother cell nucleoid. This class would include sporangia from stage II to early stage III. Class B includes sporangia in which the forespore and mother cell nucleoids are essentially the same size and would include sporangia at stage III and later. Using this classification system for counting sporulating cells, we saw a significant increase in the proportion of class B sporangia with time, indicating that class A and class B correspond to earlier and later stages of sporulation, respectively (data not shown).

Immunolocalization of β-galactosidase in sporulating cells. To demonstrate cell-specific gene expression by immunofluorescence, we used strains containing *spo-lacZ* fusions under the control of σ^F, σ^E, or σ^G. The immunofluorescence experiments were carried out as described in Materials and Methods using anti-β-galactosidase antibodies and secondary antibodies that had been conjugated with fluorescein. In contrast to the compartment-specific fluorescence results presented below, immunostaining of vegetative cells bearing a *lacZ* fusion to the ribosomal operon *rrnO* revealed a pattern of strong, uniform fluorescence across the entire bacterium (data not shown).

(i) Immunolocalization of β-galactosidase in cells containing a σ^E-controlled *lacZ* fusion. We used a *spoIID-lacZ* transcriptional fusion, which was contained in strain BZ184, as a reporter for σ^E-directed gene expression (3, 30, 36). Examples of sporangia of strain BZ184 stained with PI and immunostained with fluorescein are shown in the black-and-white photomicrographs of Fig. 2, in which it can be seen that the immunostaining was substantially restricted to the mother cell (Fig. 2B, D, and F). That σ^E-directed β-galactosidase synthesis was restricted to the mother cell is shown clearly by the color photomicrographs of Fig. 3, in which the relative locations of

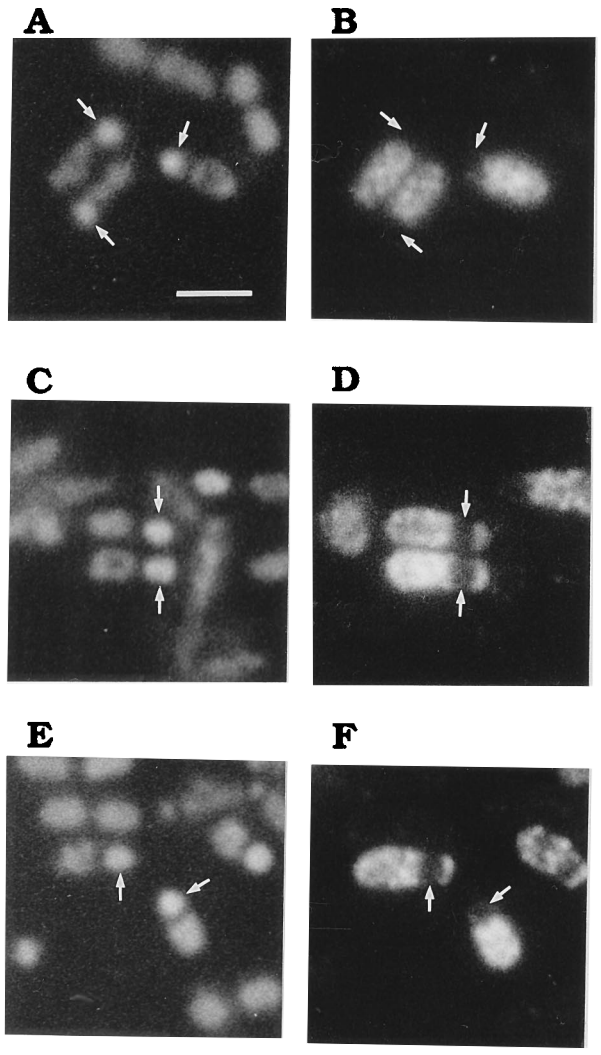

FIG. 2. Immunolocalization of β-galactosidase in sporulating cells containing a *spoIID-lacZ* fusion by fluorescence microscopy. BZ184 cells were collected during sporulation and stained with PI and immunostained with fluorescein as described in Materials and Methods. (A, C, and E) Fluorescence from PI staining; and (B, D, and F) fluorescence from fluorescein immunostaining. The same cells are shown in panels A and B, in panels C and D, and in panels E and F. The arrows indicate the positions of the forespore nucleoids and are at right angles to the longitudinal axis of the cell. Bar, 2.4 μm.

fluorescence from PI (red) and fluorescein (green) can be compared directly. The top two panels of Fig. 3 show a microscopic field of *spoIID-lacZ*-bearing sporangia displaying either both PI staining and fluorescein immunostaining (Fig. 3A) or the same sporangia showing just fluorescein immunostaining (Fig. 3B). Figure 3A and Table 2 illustrate the high efficiency of the antibody staining; almost all of the cells that were deemed to be class A or class B sporangia on the basis of the nucleoid (PI) staining pattern also exhibited immunofluorescence. Moreover, in almost all of these cells, the fluorescence was limited to the mother cell (99% [Table 2]).

Two types of immunostaining patterns were observed in sporulating cells carrying the *spoIID-lacZ* fusion. Figure 2B shows examples of sporangia that displayed one rectangular staining region. These likely represent sporangia that have not yet undergone or completed engulfment. If so, then our immunostaining results support the view that σ^E-directed gene expression is compartmentalized prior to the stage of engulfment. Figure 2D shows examples of sporangia in which an additional small region of fluorescence could be seen at the end of the sporangium. These likely represent sporangia in which the forespore has been completely engulfed within the mother cell (stage III). A similar pattern has been observed previously in immunoelectron microscopy experiments and in fluorescence microscopy experiments with a fluorogenic substrate for β-galactosidase in strains expressing *lacZ* under the control of σ^E (7, 16, 17). The small region of fluorescence presumably represents cytoplasm at the pole of the mother cell which surrounds the engulfed forespore. In keeping with this interpretation, we observed an increase in the proportion of cells showing these two staining regions in cells judged to be more advanced by nucleoid staining (15 and 50% for classes A and B, respectively).

(ii) Immunolocalization of β-galactosidase in cells containing σ^F- or σ^G-controlled *lacZ* fusions. To examine σ^F-directed gene expression, we used two strains bearing different σ^F-controlled *lacZ* fusions: PM389, which contains a *spoIIIG-lacZ* fusion; and LDB41, which contains an *sspE*-2G-*lacZ* fusion. The promoter for *spoIIIG* is used by σ^F-RNA polymerase both in vitro and in vivo (22, 24, 25, 33, 39). This promoter is also recognized by the product of its own gene, σ^G, but with lower relative affinity (37). *sspE* is normally transcribed by σ^G-RNA polymerase (39). However, the *sspE*-2G-*lacZ* construct contains a mutant derivative of *sspE* that is used more efficiently by σ^F-RNA polymerase than by σ^G-RNA polymerase both in vitro and in vivo (37). To prevent any transcription of the *spoIIIG-lacZ* and *sspE*-2G-*lacZ* fusions by σ^G-RNA polymerase, strains containing these fusions also carried a null mutation in the *spoIIIG* gene (Table 1).

Representative cells of PM389 (*spoIIIG-lacZ*) stained with PI and fluorescein are shown in Fig. 4. Immunostaining with fluorescein (Fig. 4B and D) showed staining regions close to the cell poles that were similar to the shape of the forespore nucleoid. The positions of these immunostained regions corresponded to the forespore nucleoid, at least in cases in which it could be distinguished from the mother cell nucleoid (Fig. 4A and C). In cases in which the two nucleoids could not be distinguished, the circular shape of the fluorescein-stained region was interpreted as corresponding to the forespore compartment. An example of a class A sporangium is shown at the top of Fig. 4A and B. Some representative class B sporangia are shown in Fig. 4C and D. The color images in the lower portion of Fig. 3C and D show a microscopic field of *spoIIIG-lacZ*-containing sporangia visualized either by both PI staining and fluorescein immunostaining (Fig. 3C) or by fluorescein immunostaining alone (Fig. 3D). Most of the sporulating cells appear to be at stage II or III. Once again, the efficiency of the immunostaining was very high; 92% of the class B sporangia were fluorescent (Table 2).

A similar pattern of forespore-specific immunostaining was obtained with strain LDB41, which contains the *sspE*-2G-*lacZ* fusion (Table 2 and data not shown) (although the proportion [71%] of sporangia exhibiting fluorescence was lower). In other work (27) in which cells of strain LDB41 were examined at a relatively early time in sporulation when sporangia were readily found that had not yet reached the stage of a condensed forespore nucleoid, among cells that exhibited immunostaining, only a forespore-specific pattern of expression of the *sspE*-2G-*lacZ* fusion was observed. In toto, these results reinforce the view that σ^F is active after septation and predominantly or exclusively in the forespore.

The *sspB* gene is under the control of σ^G (23, 39). Figure 5 shows representative sporangia of strain PM8, which contains

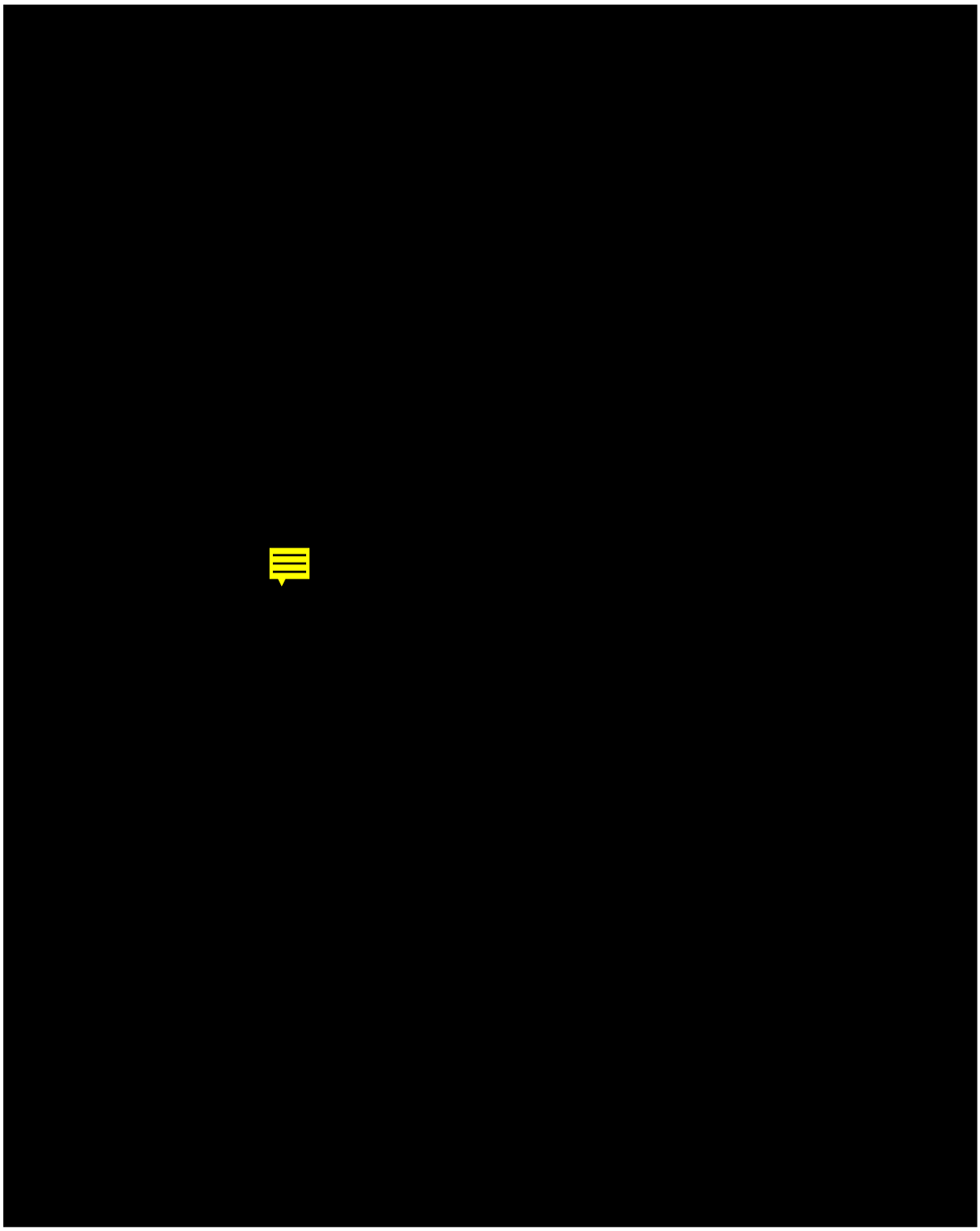

FIG. 3. Color images obtained by fluorescence microscopy of sporangia that had been stained with PI and immunostained with fluorescein. Sporulating cells were collected and stained with PI and fluorescein as described in the legend to Fig. 2. (A and B) The same field of cells from a sporulating culture of strain BZ184, containing the σ^E-controlled *spoIID-lacZ* fusion; (C and D) the same field of cells from a sporulating culture of strain PM389, containing the σ^F-controlled *spoIIIG-lacZ* fusion. Fluorescence from both PI (red) and fluorescein (green) (A and C) and from fluorescein alone (B and D) is shown. Bar, 12.3 μm.

an *sspB-lacZ* fusion, stained with PI and immunostained with fluorescein. The sporangia shown in Fig. 5A and B are representative of class B sporangia. The two sporangia in Fig. 5D that show the strongest immunostaining represent class B sporangia that are at approximately stage IV, since the forespore nucleoids are less bright and have begun to assume a ring shape (Fig. 5C). The sporangium located in the top left region of Fig. 5C is a class A sporangium and is not stained with

TABLE 2. Immunofluorescence staining patterns of cells containing σ^F-, σ^E-, or σ^G-controlled *lacZ* fusions

Strain	Fusion	Class[a]	No. of sporangia counted[b]	% of sporangia fluorescing	Fluorescence pattern of cells as % of fluorescent cells counted		
					Whole cell	Mother cell	Forespore
BZ184	*spoIID-lacZ* (σ^E)	A	160	97	1	99	0
		B	153	97	1	99	0
PM389	*spoIIIG-lacZ* (σ^F)	A	271	75	0	0	100
		B	173	92	0	2	98
LDB41	*sspE*-2G-*lacZ* (σ^F)	A	229	71	0	1	99
		B	144	70	0	0	100
PM8	*sspB-lacZ* (σ^G)	A	125	35	9	9	82
		B	441	74	1	0	99

[a] All fluorescent bacteria exhibited either the A or B nucleoid morphology, both of which are described in the text.
[b] All scoring was performed by using cells from at least two independent experiments.

fluorescein. As with the *spoIIIG-lacZ* and *sspE*-2G-*lacZ* fusion-bearing strains PM389 and LDB41, immunostaining with strain PM8 was substantially confined to the forespore, with a high proportion of the fluorescent sporangia of the *sspB-lacZ*-bearing strain showing exclusively forespore-specific fluorescence (Table 2). Interestingly, some (18%) of the class A sporangia that were fluorescent seemed not to show forespore-specific staining (Table 2). We have no explanation for this observation; however, we note that only a small proportion of class A sporangia were fluorescent; the large majority of strain PM8 sporangia that were fluorescent were of class B. Therefore, among all fluorescent sporangia observed (that is, those of classes A and B), 97% exhibited a forespore-specific pattern.

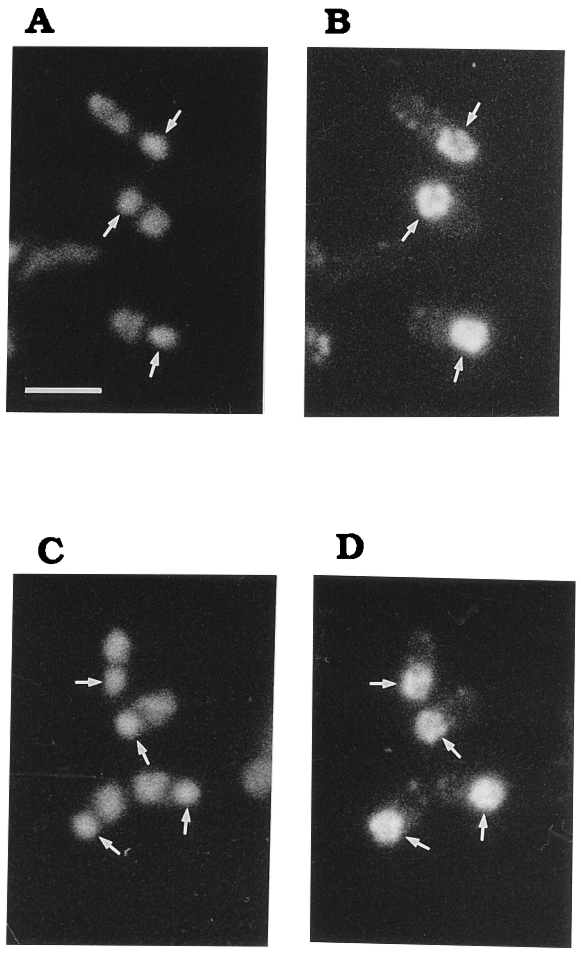

FIG. 4. Immunolocalization of β-galactosidase in sporulating cells containing a *spoIIIG-lacZ* fusion by fluorescence microscopy. Cells of strain PM389 were collected during sporulation and subjected to immunofluorescence and PI staining as described in the legend to Fig. 2. (A and C) Fluorescence from PI staining; (B and D) fluorescence from fluorescein immunostaining. The same cells are shown in panels A and B and in panels C and D. The arrows indicate the positions of the forespore nucleoids and are at right angles to the longitudinal axis of the cell. Bar, 2.4 μm.

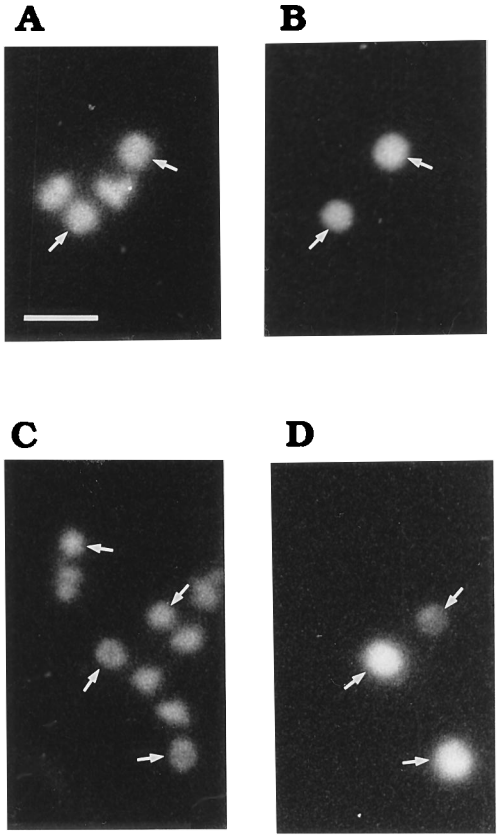

FIG. 5. Immunolocalization of β-galactosidase in sporulating cells containing an *sspB-lacZ* fusion by fluorescence microscopy. Sporulating cells of strain PM8 were stained with both PI and fluorescein as described in the legend to Fig. 2. (A and C) Fluorescence from PI staining; (B and D) fluorescence from fluorescein immunostaining. The same cells are shown in panels A and B and in panels C and D. The arrows indicate the positions of the forespore nucleoids and are aligned at right angles to the longitudinal axis of the cell. Bar, 2.4 μm.

In strains PM389 (*spoIIIG-lacZ*) and PM8 (*sspB-lacZ*), a greater number of class B than class A sporangia stained with fluorescein (Table 2). Furthermore, the average class B sporangium fluoresced more brightly than did the average class A sporangium. This is consistent with the view that expression of *spoIIIG* and *sspB* continued after engulfment. In contrast, equal numbers of class A and class B sporangia were immunostained in strain BZ184 (*spoIID-lacZ*; Table 2). This could indicate that *spoIID* expression had shut off or was diminishing after engulfment.

DISCUSSION

We have developed immunofluorescence techniques for use with *B. subtilis* and have shown that this method can be used to localize β-galactosidase produced under the control of RNA polymerase sigma factors that act in a cell-specific fashion. This method is reliable and easy to perform. Importantly, a high proportion of cells treated by our procedure exhibit a strong signal. In addition, our data indicate that this method is more sensitive than immunoelectron microscopy, which could not detect β-galactosidase produced from fusions of *lacZ* to *spoIID* (7) or *spoIIIG* (22) present in single copy. It appears that the level of sensitivity obtained with immunofluorescence is similar to that obtained with the fluorogenic substrate for β-galactosidase FDG, although we have not compared the two methods directly. Unlike the fluorescent hydrolysis product of FDG, however, the fluorophore in immunofluorescence is immobilized and cannot diffuse within or leak out of the sporangium. Finally, immunofluorescence is broadly applicable to any protein that is present in sufficient quantity and for which a high-titer antibody is available. Elsewhere, we (28) will report the use of the methods described herein to demonstrate the subcellular localization at high resolution of the sporulation proteins CotE and SpoIVA, which surround the forespore, and the α/β-type SASP, which, our results indicate, cause the forespore nucleoid to condense into a ring-like structure.

The results reported herein confirm and extend previous immunoelectron microscopy and fluorescence microscopy findings based on the use of the fluorogenic substrate FDG that σ^E, σ^F, and σ^G are cell-specific transcription factors (7, 8, 16, 17, 22). Recently, however, data obtained by Bylund et al. (2) using the FDG-derivative C_8FDG indicated that σ^F is initially active throughout the sporangium and becomes compartmentalized only as sporulation progresses. In light of the fact that only a small proportion of sporangia yielded a signal by the FDG method and the known membrane permeability of the hydrolysis products of FDG and C_8FDG, it seems likely that the whole-cell fluorescence observed by Bylund et al. (2) was due to leakage from the forespore into the mother cell (13). In any event, in our study, in which the fluorophore was immobilized, few if any sporangia were detected in which fluorescence was not confined, within the limits of detection, to the forespore. Of course, we cannot exclude the possibility that there is a low level or brief period of σ^F activity in the predivisional sporangium or in the mother cell; however, if so, this activity must be substantially lower than that in the forespore or must persist for only a very short time, and the β-galactosidase thus produced must be unstable. Further independent evidence in support of the view that σ^F activity is substantially restricted to the forespore and does not exhibit a period of substantial activity throughout the sporangium comes from recent experiments based on the use of a gene fusion encoding green fluorescence protein, which can be detected in living sporangia without the need for any fixation procedure (41).

An independent line of evidence that σ^F activity is highly compartmentalized comes from the discovery that the sporulation mutation *spoIIIE36* causes a chromosome position effect. Sun et al. (38) found that *lacZ* fused to a σ^F-controlled promoter is active when it is present near the origin of the chromosome replication in an *spoIIIE36* mutant and completely inactive when it is distal to the origin. This phenomenon has been explained by Wu and Errington (42), who showed that the *spoIIIE36* mutation impairs chromosome translocation into the forespore, allowing only what is presumed to be the origin-proximal region of the chromosome to enter the smaller sporangial compartment. The observed inactivity of σ^F-controlled *lacZ* fusions situated distally to the origin in an *spoIIIE36* mutant is therefore difficult to reconcile with the view that σ^F goes through a period in which it is active outside the forespore.

The issue of the extent to which σ^F and σ^E activities are compartmentalized is significant because it has an important bearing on models for how cell fate is determined. If σ^F initially becomes active only in the forespore, then its compartmentalization must be determined by early-appearing sporulation proteins, such as SpoIIAA, SpoIIAB, and SpoIIE (22). Alternatively, as pointed out by Bylund et al. (2), if σ^F is initially active throughout the sporangium, then genes under its control could be involved in its compartmentalization. Likewise, the activation of pro-σ^E by proteolytic processing is known to depend on a recently discovered gene (*spoIIR* or *csfX*) under the control of σ^F (14, 18). If σ^F activity is exclusively restricted to the forespore (a stronger inference than can be drawn from our present experiments), then, as proposed by Margolis et al. (22), the *spoIIR* gene product must act in an intercellular fashion to trigger activation of pro-σ^E in the mother cell. Alternatively, if σ^F is transiently active throughout the sporangium, then the *spoIIR* product could exert its effect on the pro-σ^E processing enzyme from within the mother cell. It will be interesting to see which of these models is correct as further insights into the mechanisms governing the establishment of cell type emerge.

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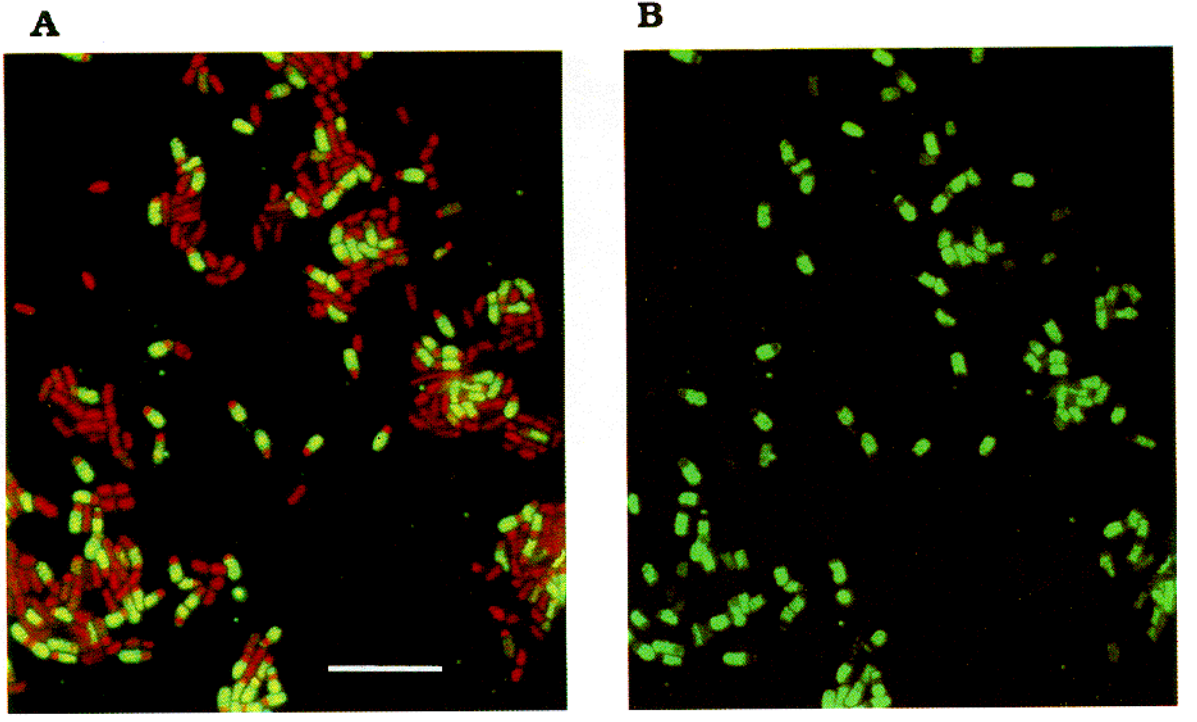
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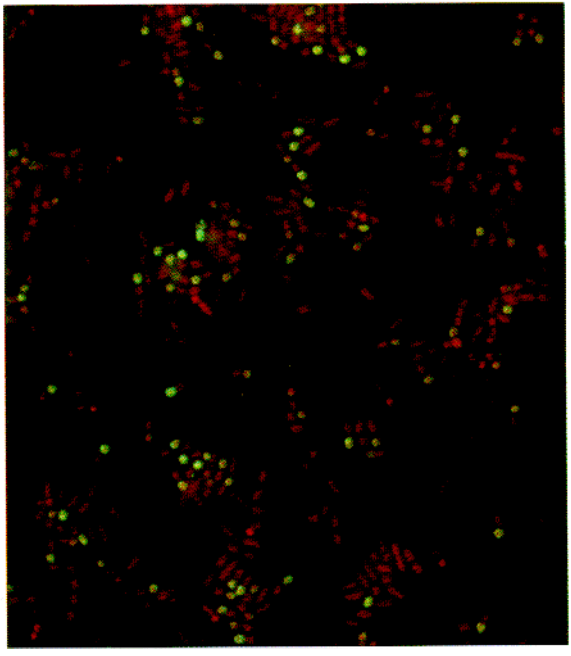

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