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Use of Green Fluorescent Protein for Visualization of Cell-Specific Gene Expression and Subcellular Protein Localization during Sporulation in *Bacillus subtilis*

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We report the use of the green fluorescent protein (GFP) of *Aequorea victoria* to visualize cell-specific gene expression and protein subcellular localization during sporulation in *Bacillus subtilis*. Sporangia bearing the gene (*gfp*) for the green fluorescent protein fused to genes under the control of the sporulation transcription factor σ^F exhibited a forespore-specific pattern of fluorescence. Forespore-specific fluorescence could be detected with fusions to promoters that are utilized with low (*csfB*) and high (*sspE-2G*) efficiency by σ^F-containing RNA polymerase. Conversely, a mother cell-specific pattern of fluorescence was observed in sporangia bearing a transcriptional fusion of *gfp* to a spore coat protein gene (*cotE*) under the control of σ^E and an in-frame fusion to a regulatory gene (*gerE*) under the control of σ^K. An in-frame fusion of *gfp* to *cotE* demonstrated that GFP can also be used to visualize protein subcellular localization. In sporangia producing the CotE-GFP fusion protein, fluorescence was found to localize around the developing spore, and this localization was dependent upon SpoIVA, a morphogenetic protein known to determine proper localization of CotE.

The green fluorescent protein (GFP) of the bioluminescent jellyfish *Aequorea victoria* has elicited much interest as a reporter protein for the visualization of gene expression and protein subcellular localization and has been used in a wide range of organisms, including *Escherichia coli*, *Caenorhabditis elegans*, and *Drosophila melanogaster* (3, 37). GFP is a 238-amino-acid (27-kDa) protein that emits green light when excited with blue light, the source of which in *A. victoria* is a calcium-activated photoprotein, aequorin (24, 25). The fluorophore is formed by an autocatalytic cyclization of three amino acid residues within GFP (4). Here we report the use of GFP to visualize cell-specific gene expression and protein localization during sporulation in *Bacillus subtilis.*

Sporulation in *B. subtilis* involves the formation of an asymmetrically positioned septum, which partitions the developing cell into forespore and mother cell compartments. Initially, the forespore and mother cell lie side by side, but later in development the forespore is engulfed by the mother cell, resulting in a cell within a cell. Differential gene expression between the mother cell and forespore is initially directed by RNA polymerase containing σ^E and σ^F, respectively (2, 10, 14, 21, 23, 35), and later σ^K and σ^G, respectively (16–19, 22, 36). Two examples of promoters that are under the control of σ^F are the promoters for *csfB*, a weakly expressed gene of unknown function (9), and *sspE-2G*, an artificial promoter that supports a high level of transcription (35). An example of a promoter under the control of σ^E is that of *cotE*, a gene that encodes a protein involved in the formation of the spore coat (11). An example of a promoter under the control of σ^K is that of *gerE*, a regulatory gene encoding a DNA-binding protein that activates late expression of spore coat genes (7, 39). These four promoters were used to drive transcription of *gfp* during sporulation. Forespore-specific fluorescence was observed in sporangia containing *gfp* fusions to *csfB* and *sspE-2G*, and mother cell-specific fluorescence was observed in sporangia with a transcriptional fusion of the reporter gene to *cotE* and a translational fusion to *gerE*. In addition to cell-specific gene expression, we were also able to visualize subcellular localization of CotE using an in-frame fusion of *gfp* to *cotE*. Subcellular localization of the CotE-GFP fusion protein depended on a protein (SpoIVA) known to guide coat assembly to the outside surface of the forespore (11, 29, 30, 32, 34).

MATERIALS AND METHODS

Growth conditions. For sporulation, cells were grown in Difco sporulation medium or by the resuspension sporulation method at either 25 or 37°C (26). The level of fluorescence signal seemed higher at 25°C, which may indicate that the protein has more optimal activity or folds more quickly at the lower temperatures, since GFP was isolated from an organism whose natural habitat is in the ocean.

***gfp* fusion and bacterial strain constructions.** The *gfp* gene was released from plasmid TU#61, a gift from M. Chalfie (3), as a 741-bp *Kpn*I-*Eco*RI fragment. The ends of the fragment were rendered flush with T4 DNA polymerase, and the fragment was ligated to pBluescriptSKII(−) (Stratagene) that had been cut with *Eco*RV. A plasmid in which the 5′ end of *gfp* is on the *Sac*I side of pBluescriptSKII(−) is pCW8, which was used for all *gfp* fusions in this study. This plasmid places polylinker sequences in front of the AUG start site of *gfp*, in addition to 9 bp from the original *Kpn*I-*Eco*RI fragment.

sspE-2G was obtained by PCR amplification from pPS1280, a gift of P. Setlow (35), as a DNA segment from position −90 to position +78 relative to the *sspE* transcription start site, using as primers oligonucleotides that created an *Xba*I site at the 5′ end and a *Bam*HI site at the 3′ end. This PCR fragment was digested with *Xba*I and *Bam*HI and then ligated to pCW8 that had been digested with *Xba*I and *Bam*HI, yielding pCW31. This created an in-frame fusion of *sspE-2G* at its 21st codon, with an additional 12 codons (from the polylinker sequence) before the beginning AUG codon of *gfp* (confirmed by sequence analysis). This plasmid was digested with *Xho*I and *Xba*I, and its ends were rendered flush with DNA polymerase I Klenow fragment. The resulting 910-bp fragment was ligated to pER82 (a vector that enables integration of the plasmid into the *amyE* locus) (11) that had been digested with *Eco*RI and *Bam*HI with its ends rendered flush with DNA polymerase I Klenow fragment, yielding pCW33. This plasmid contained the *sspE-2G–gfp* fusion with the kanamycin resistance gene and segments of the *amyE* gene. It was linearized by digestion with *Nru*I to integrate the plasmid into the *amyE* locus by a double-reciprocal recombination. The linearized plasmid was integrated into the *amyE* locus of wild-type (PY79) and *spoIIIGΔ1* mutant (RL78) cells (Table 1) by selecting for kanamycin resistance (3 μg/ml) and screening for *amy* mutant cells (8), yielding strains CW335 and CW338, respectively.

The source of *csfB* was pAB53 (9), which contains *csfB* in a 1.6-kb *Hin*dIII-*Pst*I fragment cloned into pSGMU2 (13) that had been digested with *Sma*I and *Pst*I.

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TABLE 1. Bacterial strains

Strain	Genotype or description	Reference or source
PY79	Prototrophic	38
RL78	*spoIIIGΔ1*	Laboratory stock, mutation from reference 16 moved into PY78 by congression
RL327	*spoIVA67*	Laboratory stock, mutation from reference 12 moved into PY78 by congression
RL438	*spoIVCBΔ19 trpC2*	Laboratory stock, VO136 (27) moved into JH642 (1) by congression
CW271	*cotE*::pCW13 *cotE-gfp cat*	This paper
CW274	*amyE*::pCW15 *csfB-gfp* Kan^r *spoIIIGΔ1*	This paper
CW303	*cotE*::pCW13 *cotE-gfp cat spoIVA67*	This paper
CW335	*amyE*::pCW33 *sspE-2G–gfp* Kan^r	This paper
CW338	*amyE*::pCW33 *sspE-2G–gfp* Kan^r *spoIIIGΔ1*	This paper
CW347	*cotE*::pCW43 *cotEΔ-gfp cat spoIIIGΔ1*	This paper
AT2	*amyE*::pAT3 *gerE-gfp* Kan^r	This paper
AT3	*amyE*::pAT3 *gerE-gfp* Kan^r *spoIVCBΔ19*	This paper

The plasmid containing *gfp*, pCW8, was digested with *Pst*I, the ends were rendered flush with DNA polymerase I Klenow fragment, and then it was digested with *Hin*dIII, releasing a 760-bp fragment containing *gfp*. This fragment was ligated to pAB53 that had been digested with *Sna*BI and *Hin*dIII, yielding pCW14, in which *csfB* was joined in-frame at its 50th codon with six additional codons from a polylinker sequence before the first AUG of *gfp* (confirmed by sequence analysis). Next, pCW14 was digested with *Hin*dIII, and the resulting ends were rendered flush with DNA polymerase I Klenow fragment. The DNA was then partially digested with *Eco*RI, so as to release a 1.1-kb fragment containing an internal *Eco*RI site from the pCW8 portion of pCW14. This fragment, which contained the *csfB-gfp* fusion, was ligated to pER82 (11) that, in succession, had been digested with *Bam*HI, blunt ended with DNA polymerase I Klenow fragment, and then digested with *Eco*RI. This yielded pCW15, which contained the *csfB-gfp* in-frame fusion with the kanamycin resistance gene and segments of the *amyE* gene. This plasmid was linearized with *Nru*I and integrated into the *amyE* locus of a *spoIIIGΔ1* mutant (RL78) (Table 1) by a double-reciprocal recombination, by selecting for kanamycin resistance (3 μg/ml) and screening for *amy* mutant cells (8), yielding strain CW274.

Two different *cotE* fusions to *gfp* were created for this study. The first was an in-frame translational fusion of *gfp* to the 3′ end of *cotE* (*cotE-gfp*) to demonstrate subcellular localization of the fusion protein. The second was a fusion in which the entire coding sequence of *cotE* was deleted (*cotEΔ-gfp*), which put *gfp* under the control of the *cotE* promoter, with translational signals from the *cotE* Shine-Dalgarno sequence.

The in-frame translational fusion to *cotE* was made by digesting pCW8 with *Pst*I and *Hin*dIII and rendering the ends flush with DNA polymerase I Klenow fragment, releasing a 760-bp fragment containing *gfp*. This fragment was ligated to pOR100 (31) that had been digested with *Bbs*I and its ends were rendered flush with DNA polymerase I Klenow fragment, yielding pCW13. This created an in-frame fusion to *cotE* 10 codons from the 3′ end, with six codons from polylinker sequence before the first AUG of *gfp* (confirmed by sequence analysis). This was integrated into the chromosome of wild-type (PY79) and *spoIVA* mutant (RL327) (Table 1) cells by a single-reciprocal Campbell-like recombination at or directly upstream of the *cotE* locus by selecting for chloramphenicol resistance (5 μg/ml), yielding CW271 and CW303, respectively.

The *cotE* transcriptional fusion (*cotEΔ-gfp*) was made by digesting pCW13 with *Sph*I, releasing an approximately 700-bp fragment containing the *cotE* promoter and Shine-Dalgarno sequence and additional upstream sequence. This fragment was rendered flush with DNA polymerase I Klenow fragment and then ligated to pCW8 that had been cut with *Eco*RI with its ends rendered flush with mung bean nuclease. This resulted in pCW37, which contained a transcriptional fusion that placed the expression of *gfp* under the control of the *cotE* promoter, along with the translation under the control of the *cotE* Shine-Dalgarno sequence. The resulting junction between the *cotE* sequence (boldface) and the *gfp* sequence (lightface) is as follows (the Shine-Dalgarno sequence and the ATG start codon are in uppercase): **aagaagaacAAGGAGG**cgattagaaaaaATGagt. This was confirmed by sequence analysis. To place a *B. subtilis* marker into this plasmid, the 1.1-kb *Sal*I-*Bam*HI fragment (containing the kanamycin resistance gene) from pER82 (11) that had been rendered flush with DNA polymerase I Klenow fragment was ligated to pCW37 that had been digested with *Sma*I. This resulted in pCW43, which contained the transcriptional fusion of the *cotE* promoter to *gfp* and the kanamycin resistance gene. This plasmid was integrated into the *cotE* locus (or just upstream of it) by a single-reciprocal Campbell-like recombination by selecting for kanamycin resistance (5 μg/ml), resulting in strain CW347.

The source of *gerE* was pSGMU101 (5), which was digested with *Xcm*I and blunt ended with DNA polymerase I Klenow fragment. The 510-bp fragment was ligated to pCW8 that had been digested with *Eco*RI and *Bam*HI with its ends rendered flush with DNA polymerase I Klenow fragment, yielding pAT2. This created an in-frame fusion between *gerE* at its 51st codon, with seven codons of polylinker sequence before the first AUG of *gfp* (confirmed by sequence analysis). This plasmid was digested with *Hin*dIII and *Xba*I, and then its ends were rendered flush with DNA polymerase I Klenow fragment. The 1.3-kb fragment was ligated to pER82 (11) that had been digested with *Eco*RI and blunt ended with DNA polymerase I Klenow fragment. This yielded pAT3, which contained the *gerE-gfp* in-frame fusion with the kanamycin resistance gene and segments of the *amyE* gene. This plasmid was linearized by digestion with *Nru*I and integrated into the *amyE* locus of a wild-type strain (PY79) and a *spoIVCBΔ19* strain (RL438) (Table 1) through a double-reciprocal recombination, by selecting for kanamycin resistance (5 μg/ml) and screening for *amy* mutant cells (8), yielding strains AT2 and AT3, respectively.

GFP visualization procedures. An Olympus BX60 microscope was used with a PM-30 exposure control unit and a UPlan Fluorite phase-contrast objective (magnification, ×100; numerical aperture, 1.3). For GFP viewing, either of two mirror cube units for fluorescein isothiocyanate visualization was used: (i) a wide-band-pass (460- to 490-nm) excitation filter with a long-pass (515-nm) barrier filter (U-MWIB; Olympus) and (ii) a wide-band-pass (460- to 490-nm) excitation filter and a narrow-band-pass (515- to 550-nm) barrier filter (U-MWIBA; Olympus). The camera was set at the SFL-Auto mode, with a spot size of 30. Kodak T-max (ASA 100 and ASA 400) film was used; when ASA 100 was used, the exposure control unit was set for ASA 400. For the weak *csfB-gfp* fusion, relatively long exposure times were required (~3 min), while the other fusions required 10- to 40-s exposure times. To reduce background caused by the medium, cells were spun down and resuspended in water. For pictures that were taken with simultaneous phase contrast and fluorescence light, the phase-contrast light level was adjusted so that the light was bright enough to show the cells but dim enough that the fluorescent light could be seen. This technique seemed to enhance the fluorescence, because the exposure times were shorter than those required by fluorescent light alone (about half as long), yet the fluorescence still had a good signal in the photographs. An additional technique that seemed to enhance the fluorescence was a brief (2- to 3-s) pre-exposure to shorter wavelengths of light by using a mirror cube unit with a narrow-band-pass (360- to 370-nm) excitation filter (U-MNU or U-MNUA; Olympus), which was noted previously (3).

RESULTS AND DISCUSSION

Cell-specific expression. Two promoters (*sspE-2G* and *csfB*) were used to place the transcription of *gfp* under the control of σ^F. The *sspE-2G* promoter is an artificial promoter that is strongly recognized by σ^F RNA polymerase. It was created by a modification of *sspE*, a gene with a strong promoter that is normally under the control of σ^G, a forespore-specific transcription factor that appears after σ^F. The presence of two guanine residues at the −15 and −16 positions (relative to the transcription start site) of *sspE* is responsible for enabling the mutated promoter to be efficiently recognized by the σ^F RNA polymerase (35). To ensure that transcription from the *sspE-2G–gfp* fusion was due to σ^F alone, the fusion was examined in cells that were mutant in the gene (*spoIIIG*) for σ^G. In the *spoIIIG* mutant cells carrying the *sspE-2G–gfp* fusion, green fluorescence was observed selectively in a limited region at one end of the sporangium (Fig. 1A). This region corresponded to the forespore, as determined by DNA staining with propidium iodide in separate experiments. The forespore can be recognized as an area of intense DNA staining, because of the more

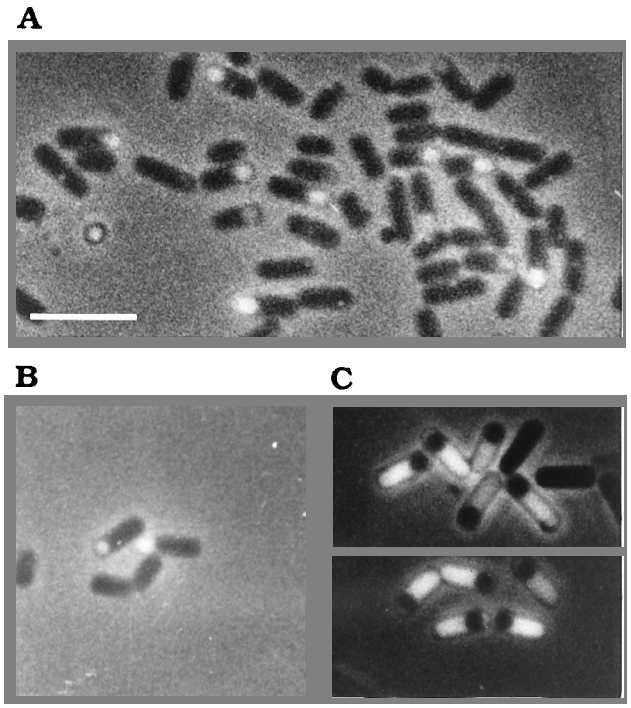

FIG. 1. Cell-specific fluorescence of *gfp* fusions. Sporangia were visualized by using phase light and fluorescent light simultaneously. Sporulation was induced by the Difco sporulation medium nutrient exhaustion method and sporangia were photographed as described in Materials and Methods. All of the strains used in this experiment are blocked at the engulfment stage of sporulation because of the presence of the *spoIIIGΔ1* mutation (Table 1). (A) Sporangia of strain CW338 bearing *sspE-2G–gfp* at 2.5 h of sporulation. Bar, 5 μm. (B) Sporangia of strain CW274 bearing *csfB-gfp* at approximately 2 h of sporulation. (C) Sporangia of strain CW347 bearing the transcriptional fusion *cotE*Δ*-gfp* at approximately 3 h of sporulation.

condensed state of the chromosome in the forespore than in the mother cell (14, 20, 33). It should be noted that, because of spectral crossover, propidium iodide is not optimal for use with GFP. The red fluorescence from propidium iodide can be seen even with the green narrow-band-pass barrier filter (U-MWIBA), which interferes with viewing the relatively weaker fluorescence of GFP. However, in multiple-exposure color photographs, the overlap between the red fluorescence of the condensed forespore nucleoid and the green fluorescence of the *sspE-2G–gfp* fusion could be clearly seen.

The green fluorescence was seen as early as 2 h after the start of sporulation at 37°C (that is, after the resuspension in sporulation medium), and the intensity of the fluorescence increased as sporulation continued, persisting for over 24 h (in cells that survived that long without lysing). Among hundreds of *spoIIIG* mutant sporangia examined, very few in which the entire sporangium exhibited fluorescence were observed, in agreement with previous studies demonstrating that σ^F is active only in the forespore (Table 2) (see the article by Harry et al. [14] for a discussion of previous electron microscopy and immunofluorescence microscopy studies of cell-specific gene expression in *B. subtilis*).

The second promoter that was used to drive *gfp* transcription was that of *csfB*, a recently discovered gene that was identified on the basis of its being under the control of σ^F. The *csfB* promoter was chosen because the peak level of expression of a *csfB-lacZ* fusion is only 10 Miller units in *spoIIIG* mutant cells (9). As a comparison, a *sspE-2G–lacZ* fusion has a peak expression level of about 200 Miller units in *spoIIIG* mutant cells (35). Strikingly, we were able to detect forespore-specific fluorescence with the *csfB-gfp* fusion, albeit at a low level, despite the fact that *csfB* is a relatively weak promoter (Fig. 1B). Also, fluorescence was limited to the forespore; little whole-cell fluorescence was observed among hundreds of cells examined (Table 2). Our result with the *csfB-gfp* fusion demonstrates that GFP is a highly sensitive reporter that can be used to detect even low levels of gene expression.

TABLE 2. Fluorescence patterns of cells containing σ^F- or σ^E-controlled *gfp* fusions

Strain	Fusion	No. of fluorescent cells[a]	% of fluorescent cells		
			Whole cells[b]	Mother cells	Forespores
CW338	*sspE-2G–gfp*	329	0.3	0	99.7
CW274	*csfB-gfp*	418	1.9	0	98.1
CW347	*cotE*Δ*-gfp*	522	2.5	97.5	0

[a] Combined results from both the resuspension and nutrient exhaustion method as described in Materials and Methods.
[b] Most whole cells that fluoresced appeared to be lysed.

To place *gfp* under the control of a promoter recognized by σ^E, a transcriptional fusion to the *cotE* gene was constructed. The *cotE*Δ*-gfp* fusion was then introduced into a *spoIIIG* mutant, so that the pattern of fluorescence could be directly compared with the σ^F-controlled pattern of fluorescence observed in the *spoIIIG* mutant cells described above. Sporangia bearing the *cotE*Δ*-gfp* fusion exhibited a large region of fluorescence whose pattern clearly corresponded to the mother cell (Fig. 1C). This was confirmed in separate experiments with propidium iodide staining, which showed that the condensed chromosome was limited to the circular zone lacking green fluorescence. Among the sporangia examined, fluorescence was almost exclusively limited to the mother cell with little or no fluorescence being observed in the forespore (Table 2). This confirms previous studies indicating that σ^E-directed gene expression is strictly confined to the mother cell (once again, see the discussion by Harry et al. [14]).

To examine *gfp*-directed fluorescence at a later stage in sporulation, an in-frame fusion to the σ^K-controlled *gerE* gene was constructed. Sporangia bearing this fusion exhibited a pattern of fluorescence similar to that of the *cotE*Δ*-gfp* fusion (Fig. 2A and B), which corresponded to the mother cell. However, the fluorescence was not as bright as expected from the level of expression of a *gerE-lacZ* fusion (180 Miller units [7]). For comparison, *gerE-gfp* was less bright than *csfB-gfp* even though a *csfB-lacZ* fusion is expressed at much lower levels. The reason for the relatively low intensity of fluorescence for *gerE-gfp* is not known; it may be that the GerE moiety of the GerE-GFP fusion protein interferes with the formation of the fluorophore, or it may indicate that the environment within sporangia at later stages is not conducive to the formation of the fluorophore. As a test of the latter possibility, the *gerE-gfp* fusion was introduced into a *spoIVCBΔ19* mutant, in which the first 19 codons of the coding sequence for σ^K are deleted. This deletion results in the production of a σ^K protein that does not need to undergo processing to be active, which normally delays the onset of σ^K-directed expression by an hour (6). In this strain, fluorescence was indeed brighter, but still not at expected levels (Fig. 2C and D). These results indicate that there may not be a strict correlation between levels of β-galactosidase activity and levels of fluorescence observed in gene fusions, or that some feature in the mother cell of late stages prevents normal formation of the fluorophore.

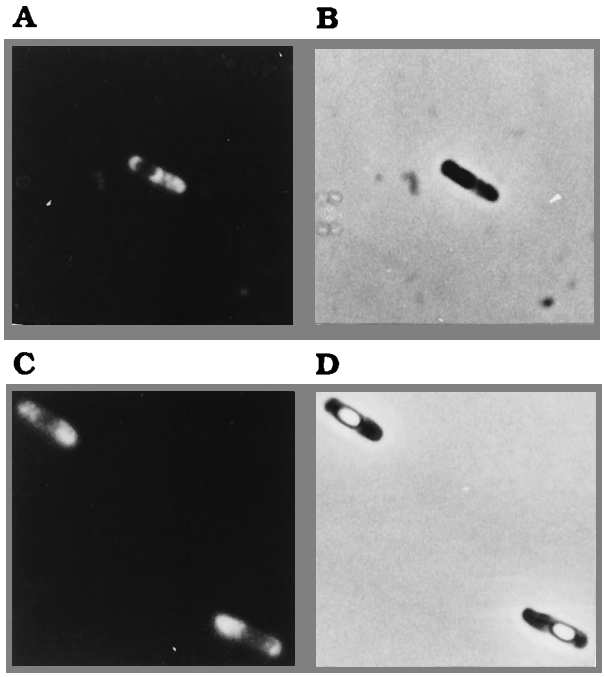

FIG. 2. Fluorescence of sporangia bearing a *gerE-gfp* fusion. Sporulation was induced by the resuspension method and sporangia were photographed as described in Materials and Methods. (A) Fluorescence photograph of a sporangium of strain AT2, bearing *gerE-gfp* at approximately 4 h of sporulation. (B) Phase-contrast photograph of the sporangium in panel A. (C) Fluorescence photograph of a *sigK*Δ*19* mutant sporangia of strain AT3, bearing the *gerE-gfp* fusion, at 5 h of sporulation. (D) Phase-contrast photograph of the sporangia in panel C.

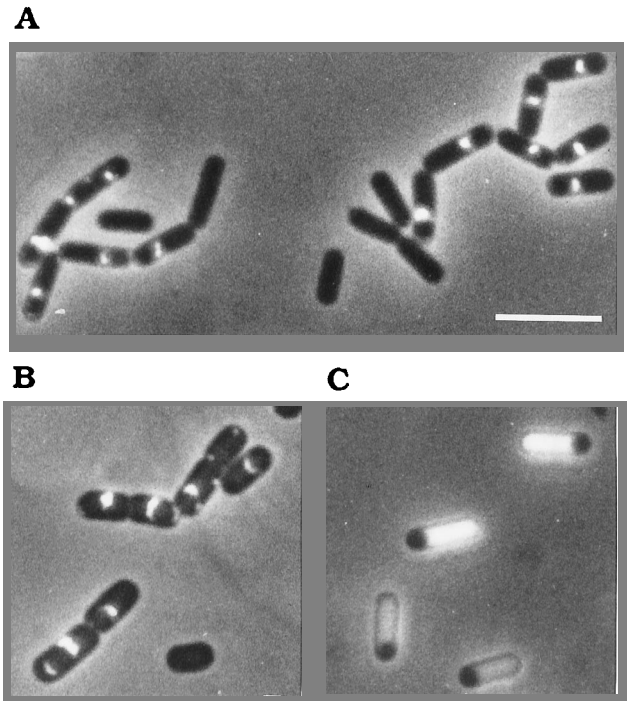

FIG. 3. Subcellular localization of CotE-GFP fusion protein. Sporulation was induced by the resuspension method of sporulation and sporangia were photographed as described in Materials and Methods. Sporangia of the *spo*$^+$ strain CW271, which contains an in-frame *cotE-gfp* fusion, are shown at 2.5 (A) and 3.5 (B) h of sporulation. (C) Sporangia in the *spoIVA* mutant strain CW303, which also contains an in-frame *cotE-gfp* fusion, is shown at 3.5 h of sporulation. Bar, 5 μm.

Subcellular localization. To use GFP to visualize protein subcellular localization in sporulating cells, we constructed an in-frame fusion of *gfp* to *cotE*, whose product, a coat protein, has been shown to localize to a region around the forespore (11, 30). In sporangia producing the CotE-GFP fusion protein, fluorescence was concentrated in a zone located in close proximity to the forespore, with the level of fluorescence increasing as sporulation progressed (Fig. 3A and B). Fluorescence was initially observed at the mother cell side of the forespore (Fig. 3A), subsequently appearing at the opposite end of the forespore (Fig. 3B) and eventually surrounding the forespore, albeit unevenly, at late stages. The accumulation of the CotE-GFP fusion protein at the mother cell side of the forespore in wild-type sporangia is similar to the pattern previously observed by immunofluorescence microscopy and to a lesser extent by immunoelectron microscopy (11, 30). As genetic evidence that the observed subcellular localization of the fusion protein was, at least in part, a meaningful representation of coat morphogenesis, we examined the pattern of fluorescence in a mutant of *spoIVA*, a gene that is known to guide coat assembly to the region surrounding the forespore (11, 29, 30, 32, 34). In contrast to the pattern observed in wild-type sporangia, fluorescence was uniformly distributed in the mother cell of the mutant sporangia (Fig. 3C).

Nonetheless, the appearance of free spores released from sporangia of strain CW271 (*spo*$^+$) expressing the CotE-GFP fusion was somewhat abnormal. Under simultaneous phase-contrast and fluorescence microscopy, mature spores exhibited an asymmetrical pattern of fluorescence (Fig. 4A). When spores were viewed by phase-contrast microscopy alone, the regions of fluorescence were seen to correspond to patches of phase-dark material alongside the phase-bright spore cores (Fig. 4B). Evidently, coat morphogenesis was somewhat aberrant in the presence of the fusion protein. As a comparison, free spores released from wild-type sporangia expressing the *sspE-2G-gfp* fusion appeared normal, with fluorescence (Fig. 4C) corresponding exactly with the spore as viewed by phase-contrast microscopy (Fig. 4D and E). Also, note that the phase-bright spore cores are encircled evenly by darker layers of spore coat.

Use of GFP in *B. subtilis*. Our results show that GFP is a useful tool for visualization of gene expression and subcellular localization of proteins in *B. subtilis*. Indeed, *sspE-2G–gfp* and *cotE*Δ*-gfp* should be the reporter fusions of choice for visualizing cell-specific gene expression during sporulation. Even higher levels of sensitivity could be possible in the future by the use of mutant forms of GFP that exhibit enhanced fluorescence (15). Among the advantages of GFP are that it does not require exogenous substrates (unlike luciferase) and that the cells do not need to be treated with lysozyme and chemically fixed (unlike immunostaining with fluorophore-conjugated antibodies) (14, 30). All pictures shown in the present article are of live cells, whose structure could be visualized with phase-contrast microscopy (unlike cells studied by immunofluorescence microscopy, in which the lysozyme treatment destroys cell integrity) (14, 30). Also, in all *gfp* fusions tested, a high percentage of sporulating cells (80 to 90%) exhibited fluorescence. A possible limitation of GFP is that the protein is reported to require several hours to fold properly and to undergo autocatalytic cyclization to create the fluorophore (15). However, in our experiments, fluorescence from *sspE-2G*- and *cotE*-directed synthesis of GFP was observed as early as 2 h

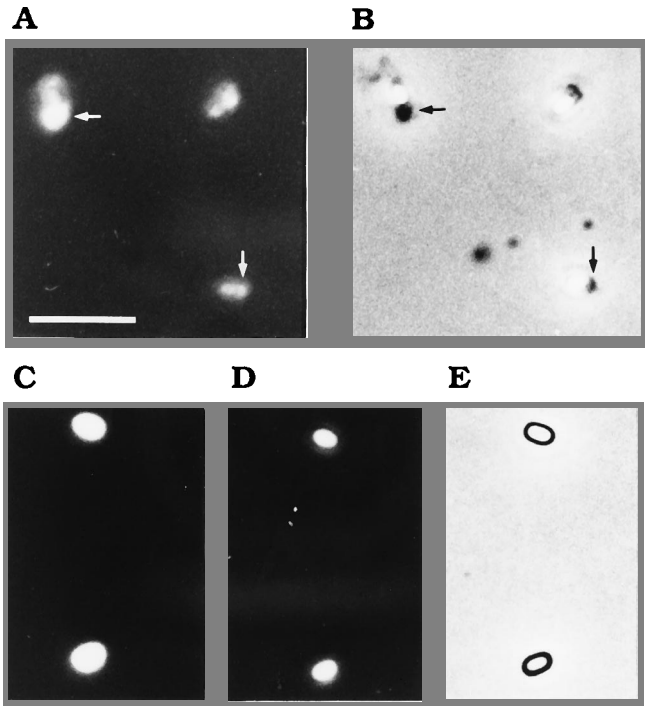

FIG. 4. Fluorescence of free spores. Sporulation was induced by the Difco sporulation medium nutrient exhaustion method and sporangia were photographed as described in Materials and Methods. (A) Simultaneous phase-contrast and fluorescence photograph of free spores at approximately 24 h after the start of sporulation, released from sporangia of strain CW271 (*spo*$^+$), producing the CotE-GFP fusion protein. Bar, 5 μm. (B) Phase-contrast photograph of spores in panel A. Note that spores are seen as phase-bright objects in both panels A and B while the CotE-GFP containing material is seen as fluorescent material in panel A and as phase-dark material alongside phase-bright spores in panel B, as indicated by arrows. (C through E) Free spores released from sporangia of strain CW335 (*spo*$^+$) bearing the *sspE-2G–gfp* fusion, at approximately 24 h after the start of sporulation. (C) Fluorescence alone; (D) simultaneous phase contrast and fluorescence; (E) phase contrast alone.

after the start of sporulation at 37°C. Given that σ^F and σ^E become active approximately 80 min after the start of sporulation (28), we estimate that in *B. subtilis* GFP begins to fluoresce shortly after its synthesis, with a lag time of no more than 1 h and possibly much less. Once synthesized, GFP seems to be stable over long periods. Mature spores displayed fluorescent green halos of CotE-GFP even 12 or more hours after their release from sporangia. Also, sporangia containing the σ^F- and the σ^E-controlled fusions continued to exhibit fluorescence for as long as 24 h.

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