Use of green fluorescent protein for detection of cell-specific gene expression and subcellular protein localization during sporulation in *Bacillus subtilis*

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Wild-type and mutant forms of the gene encoding green fluorescent protein (GFP) from *Aequorea victoria* have been introduced into *Bacillus subtilis* as translational fusions to the prespore-specific and mother-cell-specific genes *dacF* and *spoIVA*. In both cases, the protein was readily detected by fluorescence microscopy, and its synthesis was correctly localized. The S65T substitution, which improves the quantum yield and rate of development of fluorescence, also produced a spectral shift that allowed the protein to be co-localized with DNA, after staining with 4',6-diamidino-2-phenylindole. Three different translational fusions to the N-terminal region of GFP all produced active protein. Moreover, a full-length SpoIVA-GFP fusion showed proper targeting to the surface of the spore, albeit at low temperature and in the presence of wild-type SpoIVA protein. A mutation in the *gfp* gene which changes the light emitted by the protein from green to blue was found not to be useful because of the intrinsic autofluorescence of *B. subtilis* in the blue part of the spectrum.

Keywords: sporulation, *Bacillus subtilis*, green fluorescent protein, bacterial differentiation, protein localization

INTRODUCTION

During sporulation in *Bacillus subtilis*, an asymmetric cell division produces a small prespore and much larger mother cell, which go on to differentiate. Differentiation is driven by the expression of distinct programmes of gene expression in the two cells. A number of methods have been used to demonstrate the cell specificity or compartmentalization of gene expression during sporulation, including physical separation of the two cells and assay of the separated fractions, analysis of the dependence of spore formation on specific genes in genetic mosaics, differential permeability of the prespore and mother cell to enzymic substrates, and immunoelectron microscopy (reviewed by Errington, 1993). Over the past few years, cytological methods based on fluorescence microscopy and either fluorogenic enzyme substrates (Bylund *et al.*, 1994; Lewis *et al.*, 1994a, b) or immunofluorescence (Harry *et al.*, 1995; Pogliano *et al.*, 1995) have been introduced.

More recently, the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has been utilized as a reporter for cell-specific gene expression in a number of systems (Chalfie *et al.*, 1994). GFP is a small (27 kDa), intrinsically fluorescent protein. Fluorescence results from an autocatalytic cyclization between amino acids 65 and 67 to give the heterocyclic ring Ser-dehydroTyr-Gly (Cody *et al.*, 1993). The wild-type form is excited maximally at 395 nm (with a minor peak at 470 nm) and emits green light at 509 nm (Chalfie *et al.*, 1994). GFP is known to be active in both eukaryotes and prokaryotes (Chalfie *et al.*, 1994) and has been used to study the subcellular localization of both N- and C-terminal protein fusions in *Drosophila* (Wang & Hazelrigg, 1994). Useful mutations that alter the spectral properties of wild-type GFP have also been reported. One such mutation changed Tyr 66 to His (Y66H), resulting in a protein that emitted blue fluorescence (Heim *et al.*, 1994). Two groups (Heim *et al.*, 1995; Delagrave *et al.*, 1995) reported 'red shift' mutations that showed increased quantum yields and a new maximal excitation peak at 490 nm. The S65T mutant reported by Heim *et al.* (1995) also forms the heterocyclic fluorescent tripeptide more rapidly than the wild-type

Abbreviations: BFP, blue fluorescent protein; DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein.

protein, with half maximal fluorescence developing in 20 min versus 2 h, due to the elimination of a dehydration step. Compared with other methods of cytological detection of gene expression, GFP has the advantages that fixation and exogenous addition of substrates are not required (although it requires an aerobic environment). Thus, real-time analysis of cell-specific gene expression is possible in live samples, as has been reported for *Drosophila* (S. Kain, personal communication).

Here we demonstrate that GFP can be fused to sporulation genes and used to detect compartmentalized gene expression in *B. subtilis*. The S65T variant of GFP can be visualized in cells that have been counter-stained with the DNA specific dye 4',6-diamidino-2-phenylindole (DAPI). Synthesis of the reporter protein can then be related to the stage of morphological development in each cell. A fusion to full-length SpoIVA protein was initially found not to exhibit the expected assembly into the spore coat (Driks *et al.*, 1994). However, by reducing the incubation temperature and complementing with a wild-type copy of the *spoIV_A* gene, we were able to detect normal-looking assembly, suggesting that fusions to GFP may be useful for studying the subcellular targeting of proteins in this organism. While this work was in progress, we learned that similar results were being obtained in another laboratory (Arigoni *et al.*, 1995; Webb *et al.*, 1995).

METHODS

Bacterial strains. The *Escherichia coli* strain used for all cloning was DH5 α , F⁻ *endA1 hsdR17* ($r_K^- m_K^-$) *supE44 thi-1 λ^- recA1 gyrA96 relA1 $\Delta(lacZYA-argF)$ U169 ϕ 80dlacZ Δ M15* (Gibco-BRL). *Bacillus subtilis* strains used are listed in Table 1. Strains containing integrated plasmids were constructed by transforming strain SG38 with plasmid DNA and plating on nutrient agar containing chloramphenicol (5 μ g ml⁻¹). Strain 1026 was constructed by transforming strain 1025 with chromosomal DNA from strain 901 (Wu *et al.*, 1995), and selecting on nutrient agar plates containing chloramphenicol and kanamycin (5 μ g ml⁻¹ each). The *spoIV_A* defect in strain 1022 was complemented by lysogeny with phage ϕ 105J108 (*spoIV_A*⁺) (East & Errington, 1989) as described by Errington (1990).

Construction of plasmids. The plasmids used are also listed in Table 1. All recombinant plasmids were checked by PCR cycle sequencing using the protocol described by ABI. Oligonucleotides were synthesized (40 nmol scale) using an ABI 394 DNA synthesizer and diluted as required for PCR and sequencing.

pSG1125, a vector for generation of translational fusions to GFP, was constructed as follows. Wild-type *gfp* was obtained from plasmid TU#58 (M. Chalfie, Columbia University, NY, USA) by PCR using the oligonucleotides: 5'-TTTCTGCAG-ATGAGTAAAGGAGAAGAAGACTT-3' and 5'-CGGACACT-TTAGTGTAGATCTGAAGTCTGGAC-3'. The purified product was digested with *Pst*I and *Bgl*II and inserted into *Pst*I/*Bam*HI-digested pSG1301 (Stevens *et al.*, 1992). pSG1137

Table 1. *B. subtilis* strains and plasmids used in this work

Strain/ plasmid	Genotype	Construction, source or reference
<i>B. subtilis</i>		
SG38	<i>trpC2 amyE</i>	Errington & Mandelstam (1986)
901	<i>trpC2 Ω(spoIIIGA::aph-A3)901</i>	Wu <i>et al.</i> (1995)
1022	<i>spoIV_A::pSG1141 (spoIV_{A'}-gfpS65Tcat) trpC2 amyE</i>	This work
1024	<i>spoIV_A::pSG1145 (spoIV_A^{291'}-gfpS65T cat) trpC2 amyE</i>	This work
1025	<i>dacF::pSG1147 (dacF'-gfpS65T cat) trpC2 amyE</i>	This work
1026	<i>dacF::pSG1147 (dacF'-gfpS65T cat) Ω(spoIIIGA::aph-A3)901 trpC2 amyE</i>	This work
Plasmids		
TU#58	<i>bla gfp</i>	Chalfie <i>et al.</i> (1994)
pMS100	<i>bla cat dacF'-lacZ</i>	M. E. Sharpe (unpublished)
pSG1125	<i>bla cat gfp</i>	Cloning vector for C-terminal translational fusions to GFP
pSG1135	<i>bla cat bfp</i>	Cloning vector for C-terminal translational fusions to BFP
pSG1137	<i>bla cat gfpS65T</i>	Cloning vector for C-terminal translational fusions to GFP65T
pSG1141	<i>bla cat gfpS65T spoIV_A (723-1782)*</i>	1059 bp <i>Hind</i> III- <i>Pst</i> I fragment of PCR-amplified <i>spoIV_A</i> DNA cloned into <i>Hind</i> III/ <i>Pst</i> I-digested pSG1137
pSG1145	<i>bla cat gfpS65T spoIV_A (723-1191)</i>	468 bp <i>Hind</i> III- <i>Eco</i> RI fragment of PCR-amplified <i>spoIV_A</i> DNA cloned in-frame into <i>Hind</i> III/ <i>Eco</i> RI-digested pSG1137
pSG1147	<i>bla cat gfpS65T dacF (312-601)†</i>	289 bp <i>Hind</i> III- <i>Xba</i> I (filled) fragment of pMS100 cloned into <i>Hind</i> III/ <i>Pst</i> I-digested pSG1137 (blunted)
pSG1301	<i>bla cat</i>	Stevens <i>et al.</i> (1992)

* Numbers in parentheses relate to the sequence numbers given in Stevens *et al.* (1992).

† Numbers in parentheses relate to the sequence numbers given in Wu *et al.* (1992).

Fig. 1. Plasmid map of pSG1137, a vector for construction of C-terminal fusions to GFP565T. The multiple cloning site upstream of GFP565T is shown with restriction sites, nucleotide sequence, and amino acid translation. Unique restriction sites are also indicated downstream of the GFP565T coding sequence: *cat*, chloramphenicol resistance; *bla*, ampicillin resistance. Plasmids pSG1125 and pSG1135 are exactly the same, except that they contain coding sequences for wild-type and GFPY66H, respectively.

Table 2. Cellular location of GFP during sporulation

Strain	Time (no. cells)	Location of fluorescence (no. cells)			Cells at morphological stage (%)		
		Prespore	Mother cell	Whole cell	II	III	IV
1022	t_4 (198)	0	77	0	12	88*	
	t_5 (176)	0	84	0	7	93*	
1025	t_3 (451)	293	0	1	58	34	8
	t_4 (124)	110	0	0	17	44	39
					One 'prespore'		Two 'prespores'
1026†	t_3 (441)	238	0	0	48	52	
	t_4 (118)	65	0	0	37	63	

* Since phase-bright spores are not produced in *spoIV-A* mutants, these cells are scored as being stage $\geq$ III.

† Since strain 1026 is an abortive disporic mutant, cells were scored as having either one or two fluorescent prespore-like cells.

(Fig. 1) was obtained by *in vitro* mutagenesis (Sambrook *et al.*, 1989) using the oligonucleotide 5'-ACTACTTTCACCTATG-GTGTT-3', which changes amino acid 65 of *gfp* from Ser to Thr to give GFP565T (Heim *et al.*, 1995). pSG1135 was also made by *in vitro* mutagenesis using the oligonucleotide 5'-ACTACTTTCACCTATG-GTGTT-3', which changes amino acids 65 and 66 of GFP to Thr and His, respectively. Mutants were obtained with changes solely at position 66 (His) and at positions 65 and 66. Heim *et al.* (1994) reported that changing amino acid 66 from Tyr to His produced a blue fluorescent protein (BFP) with a major absorbance peak at 382 nm and emission at 448 nm. The rationale in also incorporating the S65T mutation was that a brighter more rapidly forming BFP might be produced. It has subsequently been reported that such an effect is not observed (S. Kain, personal communication), so only the Y66H single mutant was used in experiments reported below.

A *dacF'*-*S65Tgfp* translational fusion was constructed as follows. Plasmid pMS100 (M. E. Sharpe, personal communication), containing a 289 bp fragment of *dacF* including the promoter, was digested with *Xba*I, end-filled with the Klenow fragment of DNA polymerase, then digested with *Hind*III. The promoter fragment was purified and ligated to pSG1137 that had been digested with *Pst*I, blunted with Klenow, and digested with *Hind*III. This gave plasmid pSG1147, in which the first 33 codons of *dacF* were fused in-frame with *gfpS65T*. A *dacF'*-*bfp*

fusion, in plasmid pSG1146 was produced in the same way, except pSG1135 was the vector plasmid.

Fusions of *spoIV-A* to *gfpS65T* were constructed as follows. *spoIV-A* was amplified from chromosomal DNA of strain SG38 using the following oligonucleotides: 5'-ATCGGCACACGA-AAGCTTATTC AAGAACAC-3', corresponding to nucleotides (nt) 709–738 of the sequence of Stevens *et al.* (1992), and 5'-AGAGGTCTACCGGTACTGCAGGATGATGGC-3', corresponding to nt 1800–1771 of the sequence of Stevens *et al.* (1992). Amplification with these oligonucleotides inserted a unique *Hind*III site at nt 723 and a unique *Pst*I site at nt 1782. To construct plasmid pSG1141, the *Hind*III-*Pst*I fragment was inserted into *Hind*III/*Pst*I-digested pSG1137. This produced an in-frame fusion of the promoter-distal part of *spoIV-A* to the complete coding sequence of *gfpS65T* (Table 1). The fusion junction between *spoIV-A* and *gfpS65T* was such that no additional amino acids would be introduced between the final amino acid of SpoIVA and the first of GFP565T.

To fuse *gfpS65T* to a truncated *spoIV-A* gene, the 1059 bp PCR product described above was digested with *Eco*RI and *Hind*III, and ligated to *Eco*RI/*Hind*III-digested pSG1137. The resulting plasmid, pSG1145, contained a 468 bp insertion encoding amino acids 135–291 of SpoIVA fused in-frame with the *gfpS65T* gene via a 12 bp bridge. After transformation of *B. subtilis* this construction would lead to the synthesis of a fusion protein with

Fig. 2. Compartment-specific expression of a *dacF'*-*gfpS65T* fusion in strain 1025 at t_4 . (a) GFP fluorescence; (b) DAPI fluorescence; (c) phase-contrast image; (d) images of (a) and (b) merged, showing co-localization of GFP fluorescence and the DAPI-stained prespore nucleoid. Cells at stages II, III and IV of sporulation are indicated. Bar, 4 μ m.

the following sequence: SpoIVA¹⁻²⁹¹-Glu-Phe-Leu-Gln-GFPS65T.

spoIV A fusions with the wild-type *gfp* gene were constructed in the same way, except that the vector plasmid was pSG1125.

Transformation of *B. subtilis*. Transformations were done by standard laboratory techniques (Anagnostopoulos & Spizizen, 1961; Jenkinson, 1983).

Induction of sporulation. Cells were grown and induced to sporulate by the resuspension method of Sterlini & Mandelstam (1969) as specified by Partridge & Errington (1993). Sporulation medium was supplemented with 2 μ g chloramphenicol ml⁻¹.

Visualization of fluorescence. At appropriate times, 200 μ l samples of cells were taken, pelleted by centrifugation, and resuspended in 200 μ l sterile water. A 3 μ l aliquot of cell suspension was then mixed with 1 μ l DAPI (1 μ g ml⁻¹; Sigma) on a slide pre-coated with 0.01% polylysine (Sigma). A coverslip, also pre-coated with polylysine, was then placed on top. Cells were viewed by epifluorescence microscopy using a Zeiss Axiovert 135TV epifluorescence microscope with a 100 W mercury lamp source, a $\times 100$ Plan-Neofluar oil-immersion objective lens (numerical aperture 1.3), and a $\times 2.5$ optovar in place. A SYS2000 800 $\times$ 1200 pixel cooled CCD camera (Digital Pixel Advanced Imaging Systems) was attached to the microscope camera port and images were captured with Lucida V 2.x software (Kinetic Imaging).

GFP fluorescence was viewed with HQ:fluorescein isothiocyanate (FITC) filter set 41001 (Chroma Technology) (excitation wavelength 450–505 nm; long pass 505 nm; emission 510–560 nm). The exposure time was 3 s for all GFP images, except for those of Fig. 5, which were for 10 s. Phase contrast images (300 ms exposure) were then obtained with the HQ:FITC filter set still in place. Finally, 100 ms DAPI images

Fig. 3. Compartment-specific expression of a *dacF'*-*gfpS65T* fusion in a *spoIIg* (abortive disporic) mutant strain 1026 at t_3 . Parts (a–d) and scale bar as in Fig. 2. Due to the low density of cells in the field, the image was cropped to exclude some blank spaces.

were obtained with the narrow band set for DAPI (filter set 31013, Chroma; excitation wavelength 355–375 nm; LP 400 nm; emission 435–490 nm). Separate background images were obtained for GFP, phase contrast and DAPI images, and these were subtracted from raw images during processing. Image overlays and micrograph figures were prepared with Adobe Photoshop version 3.0.4.

RESULTS

Vectors for construction of fusions to GFP

The coding sequence of *gfp* was amplified by PCR as described in Methods and cloned into plasmid pSG1301 to generate a convenient vector with which to make fusions that could be readily introduced into *B. subtilis*. The resultant plasmid, pSG1125, contains the following useful functions: an origin of replication that functions in *E. coli* but not in *B. subtilis*; antibiotic resistance determinants, *bla* for selection in *E. coli* and *cat* for *B. subtilis*; and multiple cloning sites for generating C-terminal fusions to the *gfp* gene. The plasmid [which was based originally on pBluescript KS(–); Stratagene] also contains the phage f1 origin for generation of single-stranded DNA, which facilitates the construction of new *gfp* derivatives by site-directed mutagenesis. Two mutant forms of *gfp* were constructed in the course of this work. The S65T substitution was introduced into pSG1125 to generate plasmid pSG1137 (Fig. 1), which was used as the

Fig. 4. Compartment-specific expression of a *spoIVA'*-*gfpS65T* fusion in strain 1022 at t_4 . Parts (a-d) and scale bar as in Fig. 2.

Fig. 5. Subcellular assembly of SpoIVA-GFPs65T around the developing prespore in strain 1022 lysogenic for phage $\phi 105J108$ (*spoIVA*⁺). The culture was induced to sporulate by resuspension at 25 °C and samples were taken at t_{13} (a-c) and t_{18} (d, e). The upper panels show merged GFP (green) and DAPI (blue) images. The lower panels show corresponding phase-contrast images. Bar, 2 μ m.

vector for the construction of the various fusions described below. This substitution was reported to have several beneficial effects as discussed above (Heim *et al.*, 1995). The shift in the peak emission wavelength was particularly useful because it allowed co-localization of the mutant protein and DNA (stained with DAPI).

Several of the plasmids described below were also modified to carry the Y66H substitution. This change was reported to alter the spectral properties of GFP so that it emits blue light rather than green (Heim *et al.*, 1994). However, use of this protein in *B. subtilis* turned out to be impractical, because of cell autofluorescence at the emission peak of this modified protein (results not shown).

GFP as a marker for prespore-specific gene expression

A 289 bp fragment of DNA containing the promoter and first 33 codons of the prespore-specific (σ^F -dependent) *dacF* gene (Schuch & Piggot, 1994) was cloned into

plasmid pSG1137 to generate an in-frame fusion to *gfpS65T* (see Methods). The resultant plasmid, pSG1147, was transformed into Spo⁺ *B. subtilis* strain SG38, with selection for chloramphenicol resistance. Cells transformed with this type of plasmid arise by single crossover homologous recombination (Duncan *et al.*, 1978). A transformant, designated strain 1025, was induced to sporulate by the resuspension method (Sterlini & Mandelstam, 1969) and at intervals the cells were viewed by fluorescence microscopy (Fig. 2). Fluorescence in the emission range expected for GFPs65T began to be detected at about t_2 (2 h after the initiation of sporulation), which is similar to the time of expression of a *dacF-lacZ* fusion reported by Schuch & Piggot (1994). The proportion of fluorescent cells increased to 61 % at t_3 , and by t_4 virtually all of the prespores (89 %) fluoresced (Table 2).

The appearance of the areas of fluorescence, small, roughly spherical and situated close to the cell pole, was consistent with the GFP being specifically synthesized in the prespore compartment (Lewis *et al.*, 1994a, b). Because there was no detectable spectral crossover between the

fluorescence emitted by GFPS65T and the DNA-specific fluorescent dye DAPI (results not shown), it was possible to counterstain cells to view their chromosomes. Superimposition of the two fluorescent images (Fig. 2d) confirmed that the GFP fluorescence always co-localized with the prespore nucleoid. The DAPI staining also allowed three different morphological classes of cells exhibiting GFP fluorescence to be distinguished (Fig. 2d). At stage II, the prespore DNA is tightly compacted into the small prespore compartment and its maximum fluorescence is considerably greater than that of the mother cell. At stage III, when prespore engulfment is complete, the prespore DNA occupies a larger space and consequently has a reduced maximum fluorescence. Later in development, at about stage IV, the prespore DNA becomes refractory to staining by DAPI (Setlow *et al.*, 1991). As expected, at t_4 , the fluorescent prespores tended to have reached a later stage of development than at t_3 (Table 2). In general, the level of GFP fluorescence also increased in accordance with the morphological progression (Fig. 2).

To confirm the prespore localization of *dacF-gfpS65T* and demonstrate its effectiveness as a cytological marker, a *spoIIG* mutation was introduced into strain 1025 to give strain 1026. *spoIIG* mutations eliminate the mother-cell-specific sigma factor σ^E and lead to a 'disporic' phenotype, in which prespore-like cells are formed at both poles of the sporulating cell, leaving a central anucleate compartment. As shown in Fig. 3, GFP fluorescence was readily detected at either one or both poles of the sporulating cells of strain 1026. A slightly lower proportion of the cells exhibited fluorescence than with strain 1025, probably because *dacF* is transcribed by RNA polymerase containing either σ^F or the later prespore-specific sigma factor σ^G (Schuch & Piggot, 1994), and the *spoIIG* mutation eliminates the contribution made by σ^G (Partridge & Errington, 1993). The detection of cells in which only one prespore was fluorescing, especially in the earlier t_3 sample, was consistent with previous experiments showing that the prespore-like cells of these mutants form sequentially (Lewis *et al.*, 1994b).

GFP as a marker for mother-cell-specific gene expression

To test whether GFP could be used to study mother-cell-specific gene expression, a fusion to the *spoIVA* gene was constructed, again using pSG1137 and the GFPS65T mutant gene. Plasmid pSG1141 was transformed into SG38, again generating the fusion by single crossover homologous recombination (strain 1022). In this case, the whole of the *spoIVA* coding sequence was fused in-frame with *gfp* (see Table 1). Unexpectedly, the resultant transformants were Spo^- , suggesting that the fusion protein is non-functional (see below). The pattern of fluorescence exhibited by the *spoIVA-gfp* fusion was quite different from that of *dacF-gfp*. Fluorescence was restricted to the mother cell compartment, as revealed by the DAPI staining (Fig. 4; Table 2). Cells at different stages of sporulation showed a pattern of GFP

fluorescence consistent with the protein occupying the whole of the mother cell cytoplasm; at stage II, a single cylindrical region of staining to one side of the prespore DNA was visible, whereas at stage III (visible in Fig. 4), fluorescence was additionally detectable in the mother cell cytoplasm at the distal side of the engulfed prespore. In this experiment, no attempt was made to distinguish between cells at stages III and IV, because the loss of DAPI staining which occurred in wild-type sporulating cells at stage IV was less obvious in the *spoIVA* mutant cells.

Similar results were obtained with strain 1024, in which *gfp* was fused to the N-terminal coding half of the *spoIVA* gene rather than to the whole gene (results not shown).

Subcellular localization of SpoIVA-GFP

Although the above results confirmed the cell specificity of synthesis of the SpoIVA-GFP fusion proteins, it appeared that the full-length fusion protein in strain 1022 was not, like native SpoIVA, targeted to the assembling spore coat (Driks *et al.*, 1994). The fact that the cells with this fusion became Spo^- , despite having the whole of the SpoIVA-coding sequence, indicated that addition of GFP to the C-terminal tail of SpoIVA interferes with the function of the latter. However, discrete localization of the hybrid fluorescent protein was obtained by use of two adjustments to the method. First, the experiments were done at lower temperatures (20–22 °C). This resulted in detection of discrete regions of higher fluorescence generally located between the prespore and the mother cell nucleoids (data not shown). Second, a wild-type copy of the *spoIVA* gene was introduced into strain 1022. In this case, a culture resuspended at low temperature gave rise to cells in which the expected pattern of assembly of the hybrid SpoIVA-GFPS65T protein around the prespore was readily detected (Fig. 5). The cells shown in (a–c) were obtained at t_{13} and those in (d) and (e) at t_{18} . They represent cells apparently having reached stages IIii, IIiii, III, IV and V, respectively (Errington, 1993). At stage IIii (Fig. 5a), the prespore septum is largely intact, and SpoIVA-GFPS65T has assembled in the middle of the septum at the site where septal hydrolysis is first observed (Driks *et al.*, 1994). During stage IIiii (Fig. 5b), septal hydrolysis is completed and the initial stages of prespore engulfment have been initiated. The SpoIVA-GFPS65T fusion can be seen assembled on the surface of the outer prespore membrane. By stage III (Fig. 5c), the prespore has been fully engulfed and the SpoIVA-GFPS65T fusion appears to completely surround the prespore. At stage IV (Fig. 5d), the prespore becomes refractory to DAPI staining, which occurs at about the time that cortex formation is initiated. At stage V (Fig. 5e), the developing spore has become phase bright. The region of fluorescence now completely surrounds the developing spore. At later stages of development, GFP fluorescence disappeared, which may indicate that the fusion protein becomes buried by the outer layers of the coat.

DISCUSSION

We have demonstrated that GFP has considerable potential for use as a cytological marker for gene expression during sporulation in *B. subtilis*. Presumably, it may find many other applications in situations in which cell-to-cell heterogeneity of gene expression is liable to occur.

Fusions to prespore-specific and mother-cell-specific genes both gave the expected patterns of expression. In combination with DAPI staining, we were able to distinguish several distinct morphological stages of sporulation in unfixed cells. Such fusions should be of use in a number of future studies, e.g. in identifying and characterizing mutants with altered morphology, or in studying the fate of sporulating cells after reversal of starvation (G. Parker & J. Errington, unpublished). Although our preliminary experiments with BFP proved to be unfruitful, it is likely that other colour variants of GFP, or different fluorogenic proteins, will become available, and these will open up the possibility of double labelling, co-localization experiments.

Protein fusions to GFP are beginning to be used extensively to study subcellular protein localization in eukaryotic systems. In the preliminary experiments reported here, we have been able to detect targeting of a SpoIVA-GFP fusion protein to the surface of the prespore, where SpoIVA is known to participate in the assembly of the spore coat (Driks *et al.*, 1994). The Spo⁻ phenotype of the strain with the fusion of the full-length SpoIVA and GFP proteins may be due to impaired folding of the SpoIVA moiety. Perhaps at lower temperature folding is more efficient and a degree of targeting to the surface of the spore is revealed. Irrespective of whether this explanation is correct, introduction of wild-type SpoIVA protein by complementation with phage ϕ 105J108 appeared to allow correct assembly of the fusion protein into the spore coat. It is possible that modifications such as production of an N-terminal fusion to GFP, or introduction of a short 'linker region' between the two protein domains, might facilitate folding and allow proper targeting to occur at higher temperatures and/or in the absence of wild-type protein. It seems likely that further application of methods based on fusions to GFP will bring about major advances in our understanding of the subcellular organization of bacterial cells.

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