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Suppression of Engulfment Defects in *Bacillus subtilis* by Elevated Expression of the Motility Regulon†

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During *Bacillus subtilis* sporulation, the transient engulfment defect of *spoIIB* strains is enhanced by *spoVG* null mutations and suppressed by *spoVS* null mutations. These mutations have opposite effects on expression of the motility regulon, as the *spoVG* mutation reduces and the *spoVS* mutation increases σ^D-directed gene expression, cell separation, and autolysis. Elevating σ^D activity by eliminating the anti-σ factor FlgM also suppresses *spoIIB spoVG*, and both *flgM* and *spoVS* mutations cause continued expression of the σ^D regulon during sporulation. We propose that peptidoglycan hydrolases induced during motility can substitute for sporulation-specific hydrolases during engulfment. We find that sporulating cells are heterogeneous in their expression of the motility regulon, which could result in phenotypic variation between individual sporulating cells.

A key step in *Bacillus subtilis* sporulation is engulfment, which mediates a dramatic rearrangement of the two cells required for sporulation, from two adjacent daughter cells to a sporangium in which one daughter cell (the forespore) is completely enclosed within the cytoplasm of another (the mother cell). This transformation is mediated by the migration of the mother cell membrane around the forespore, which is ultimately released into the mother cell cytoplasm (Fig. 1A). Engulfment depends on three conserved proteins, SpoIID, SpoIIM, and SpoIIP (1, 5, 17, 30), and is facilitated by the less-conserved SpoIIB protein (19, 23). These proteins participate in septal thinning (1, 5, 17, 19, 23, 30), during which septal peptidoglycan is degraded by the SpoIID peptidoglycan hydrolase and perhaps other enzymes (1). While *spoIID*, *spoIIM*, and *spoIIP* mutants are completely defective in septal thinning and membrane migration (Fig. 1B), a *spoIIB* mutant

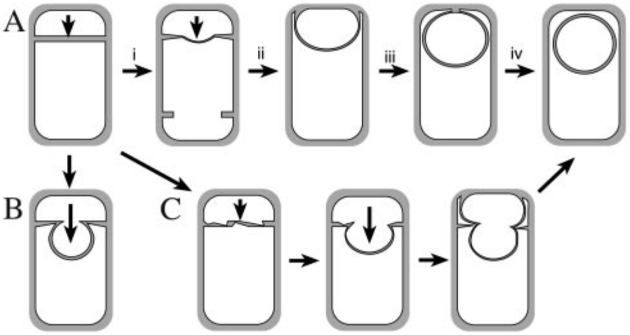

FIG. 1. Engulfment in *B. subtilis*. (A) Asymmetric division produces the small forespore and large mother cell. Septal thinning (step i) commences at the septal midpoint and proceeds towards the edges, followed by membrane migration up (step ii) and around (step iii) the forespore, until the membrane meets and fuses (step iv) to release the forespore into the mother cell cytoplasm. (B) In the absence of SpoIID, SpoIIM, or SpoIIP, septal thinning is blocked, and the forespore ultimately bulges into the mother cell. (C) In the absence of SpoIIB, septal thinning is slowly and uneven, forming a transient bulge, although engulfment is ultimately completed. In the absence of both SpoIIB and SpoVG, membrane migration is blocked.

TABLE 1. *Bacillus subtilis* strains used in this study[a]

Strain	Genotype	Reference or source
PY79	Wild type	35
KP52	Δ*spoIIB*::*erm spoVG*::Tn*917*	19
KP84	*spoIIAC-lacZ*	28
KP174	Δ(*spoIIAA-AC*)::*spec*	2
KP343	Δ*spoIIB*::*erm*	19
KP548	*spoVG*::Tn*917*::*spec*	23
KP535	Δ*spoVS*::*spec*	27
KP787	Δ*spoIIB*::*erm spoVG*::Tn*917* Δ*spoVS*::*spec*	This study
KP798	Δ*sinR*::*neo*	6
KP799	Δ*sinR*::*neo* Δ*spoVS*::*spec*	This study
KP812	*flgM*Δ*80*	21
KP813	*sigD*::pLM5	10
KP814	Δ*spoIIB*::*erm spoVG*::Tn*917*::*spec*	This study
KP815	Δ*spoIIB*::*erm spoVG*::Tn*917*::*spec flgM*Δ*80*	This study
KP818	P_{hag}(−UP)-*lacZ*	6
KP819	*flgM*Δ*80* P_{hag}(−UP)-*lacZ*	This study
KP820	*sigD*::pLM5 (*cm*^{r}) P_{hag}(−UP)-*lacZ*	This study
KP821	Δ*spoVS*::*spec*, P_{hag}(−UP)-*lacZ*	This study
KP822	Δ*sinR*::*neo* P_{hag}(−UP)-*lacZ*	This study
KP823	*spoVG*::Tn*917*, P_{hag}(−UP)-*lacZ*	This study
KP826	Δ*spoIIB*::*erm spoVG*::Tn*917* Δ*spoVS*::*spec lytABC*::*neo*	This study
KP827	Δ*spoIIB*::*erm spoVG*::Tn*917* Δ*spoVS*::*spec lytD*::*tet*	This study
KP828	Δ*spoIIB*::*erm spoVG*::Tn*917* Δ*spoVS*::*spec lytABC*::*neo lytD*::*tet*	This study
KP829	*flgM*Δ*80 sigD*::pLM5	This study
KP830	Δ*spoVS*::*spec sigD*::pLM5	This study
KP930	*spoIIAC-lacZ*::*tet rvtA11*::*spec*	This study
KP931	*spoIIAC-lacZ*::*tet rvtA11*::*spec* Δ*cotE*::*cm* Δ*spoVS*::*spec*	This study
KP932	*spoIIAC-lacZ*::*cm* Δ*abrB*::*tet*	This study
KP933	*spoIIAC-lacZ*::*cat* Δ*abrB*::*tet*, Δ*spoVS*::*spec*	This study
KP934	*spoIIAC-lacZ*::*cat flgM*Δ*80*	This study
KP936	*spoIIAC-lacZ*::*cat* Δ*spoVS*::*spec*	This study
KP937	*spoIIAC-lacZ*::*cat* Δ*sinR*::*neo*	This study
KP938	*spoIIAC-lacZ*::*cat spoVG*::Tn*917*	This study

[a] All strains are PY79 derivatives except those with *flgM*Δ*80,* which are JH642 derivatives.

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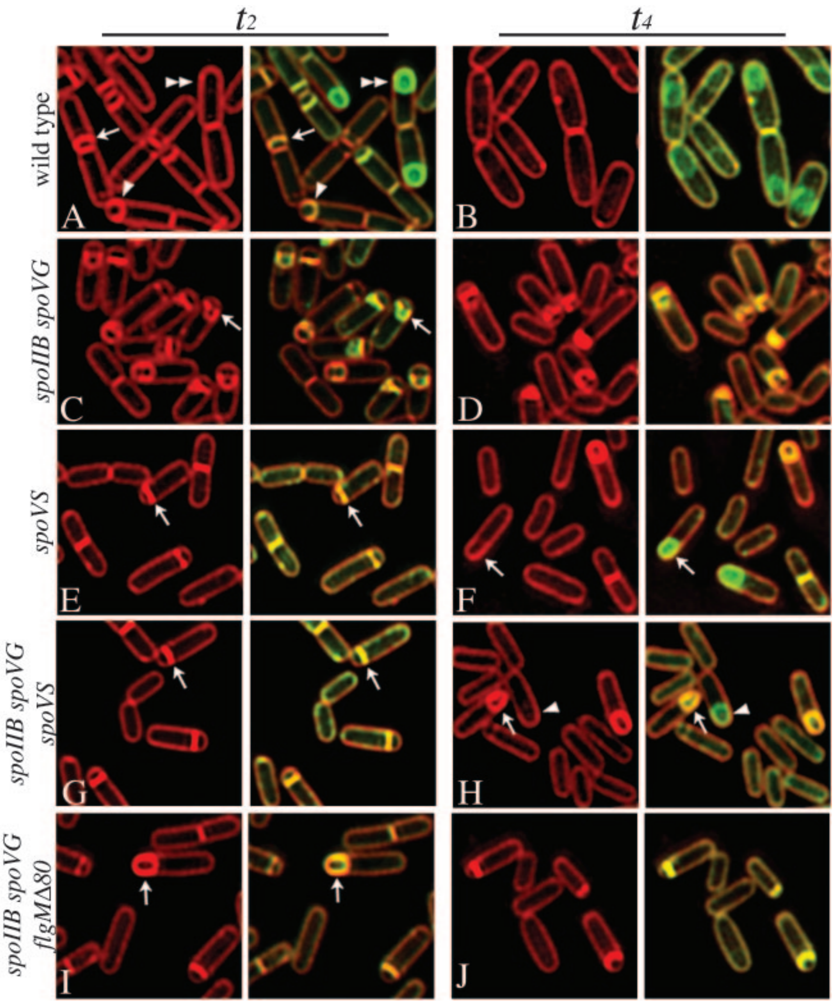

FIG. 2. Effect of the *spoVS* mutation on engulfment. Sporulation was induced by resuspension (33), and samples taken 2 h (t_2) (A, C, E, G, and I) and 4 h (t_4) (B, D, F, H, and J) later were stained with FM 4-64 (red) and MitoTracker Green (green) as previously described (24, 29). (A and B) Wild-type (PY79) sporangia after septation (arrow) and during engulfment (arrowhead). The septum stains approximately two times more brightly than the cytoplasmic membrane, because it contains two parallel membranes, while the engulfing membrane stains approximately three times more brightly, because it contains three membrane layers (Fig. 1A) (24). After membrane fusion (double arrowhead), FM 4-64 is excluded from the forespore (29), which is stained only with MitoTracker Green. (C and D) The *spoIIB spoVG* double mutant (KP52) shows sporangia with flat polar septa or bulges (arrow). (E and F) The *spoVS* mutant (KP535) is delayed in polar septation but engulfs normally (E, arrow) and completes membrane fusion (F, arrow). (G and H) The *spoIIB spoVG spoVS* triple mutant (KP787) exhibits few bulges (G, arrow) and completes membrane migration (H, arrow) and fusion (H, arrowhead). (I to J) The *spoIIB spoVG flgMΔ80* triple mutant (KP815) shows few bulges and completes engulfment (I, arrow).

shows slow and uneven septal thinning but ultimately completes engulfment and produces spores (Fig. 1C). Two additional proteins have been proposed to play a role in septal thinning: SpoVG, whose absence exacerbates the engulfment defect of *spoIIB* (19, 23), and SpoVS, whose absence suppresses a *spoIIB spoVG* double mutant, allowing engulfment to proceed (27). Here, we provide evidence that the *spoVS* mutation causes decreased expression of early-sporulation genes and increased expression of motility genes and that motility-specific genes, likely peptidoglycan hydrolases, can suppress septal thinning defects.

To investigate the mechanism of *spoVS*-mediated suppression of *spoIIB spoVG*, we used an in vivo membrane fusion assay that employs two membrane stains, the membrane-impermeable FM 4-64 and the membrane-permeable stain MitoTracker Green (strains of *B. subtilis* used in this study are shown in Table 1) (29). During engulfment in the wild type, the engulfing membrane smoothly curves around the forespore (Fig. 2A, arrow and arrowhead), with the septal membrane staining twice as brightly as other regions, due to the presence of two membranes (24). Ultimately, the migrating membrane meets and fuses to release the forespore into the mother cell cytoplasm. After membrane fusion, FM 4-64 is excluded from the forespore, resulting in a green forespore enclosed within the red mother cell (Fig. 2A, double arrowhead). In *spoIIB spoVG*, the growing forespore pushes through the unthinned septum, forming a bulge that is typical of septal thinning defective mutants (Fig. 2C to D). In the *spoIIB spoVG spoVS* triple mutant, sixfold-fewer bulges were observed than in the *spoIIB spoVG* mutant (6% versus 35% at $t_{3.0}$) (Fig. 2G and H;

TABLE 2. Effect of *spoVS* and *flgM* mutations on polar septation, engulfment, and spore formation

Genotype	Spores/ml	% Sporangia (t_2)[a]	% Sporangia with bulges (t_3)[b]	% Sporangia fused (t_4)
Wild type (PY79)	4×10^8	55 (775)[c]	0 (347)	84 (248)
spoVS	1×10^6	6 (1,342)	0 (547)	46 (623)
spoIIB	2×10^8	45 (1,670)[d]	37 (699)	7 (345)
spoVG	9×10^8	41 (391)[d]	0 (301)	40 (251)
spoIIB spoVG	9×10^4	55 (1,211)	35 (1,073)	0 (401)
spoIIB spoVG spoVS	2×10^6	1 (312)	6 (1,270)	21 (1,459)
*spoIIB spoVG flgM*Δ*80*	2×10^6	30 (573)	5 (761)	9 (474)
spoIIB spoVG spoVS lytABC[e]	5×10^5	ND[f]	13 (615)	35 (764)
*spoIIB spoVG flgM*Δ*80 lytABC*[e]	3×10^5	ND[f]	14 (336)	34 (126)

[a] Percent sporangia is determined by dividing the number of sporangia by the total number of total cells (in parentheses) in the fields scored.

[b] Percent sporangia with bulges or fused is calculated by dividing the number of sporangia with the indicated phenotype by the total number of sporangia (in parentheses).

[c] The number in parentheses indicates the total number of cells scored (vegetative cells and sporangia).

[d] Data for percent sporangia at t_2 are from Perez et al. (23) and are included for comparison only.

[e] The additional deletion of *lytD* had no effect on suppression (data not shown).

[f] ND, not determined.

Table 2), and engulfment was complete in 21% of *spoVS spoIIB spoVG* sporangia versus 0% of *spoIIB spoVG* sporangia at t_4 (Fig. 2; Table 2). The *spoVS* mutation also weakly suppressed *spoIID* and *spoIIM* strains, reducing the frequency of bulges twofold (from 64% to 31% in *spoIIM* and from 45% to 21% in *spoIID*) but not supporting the completion of engulfment or increased spore production (see Fig. S1 and Table S1 in the supplemental material). These results suggest that the *spoVS* mutation is a general suppressor of septal thinning defects.

We noted that *spoVS* strains showed fewer polar septa than the wild type (Fig. 2E and F; Table 2). This was due to decreased expression of early sporulation genes such as *spoIIAC* (Fig. 3F) and *spoIIE* (see Fig. S3 in the supplemental material), which require the Spo0A~P transcription factor that governs entry into sporulation (7). Expression of Spo0A~P-dependent genes is regulated by kinases and phosphatases that modulate the level of phosphorylated Spo0A (22, 32) and by various transcription factors that inhibit Spo0A~P-dependent gene expression (7–9). We found that the defect in polar septation and Spo0A~P-dependent gene expression could be substantially rescued by a *sinR* mutation, which eliminates one of these transcription factors, but not by *spo0Asad*, which encodes a Spo0A protein active without phosphorylation (11), *rvtA11*, which encodes a Spo0A protein that can be phosphorylated by an alternative kinase (16), or *abrB* or *spo0J-soj* mutations, which eliminate repressors of Spo0A~P-dependent gene expression (25, 34; see Fig. S3 in the supplemental material). These results suggest that the *spoVS* mutant has increased SinR activity.

SinR is a transcription factor that directly regulates biofilm formation (13), inhibits Spo0A~P-dependent gene expression, and activates σ^D-dependent motility genes (4, 6, 13, 18, 26), perhaps indirectly (13). Thus, if the *spoVS* strain has elevated SinR activity, it should also show increased expression of motility genes. Indeed, the *spoVS* strain showed approximately twofold elevated expression of the flagellin gene (*hag-lacZ*) relative to the wild type, while the *spoVG* mutation (enhancer of *spoIIB*) showed approximately fivefold reduced σ^D activity (Fig. 3G). This suggests that SpoVS directly or indirectly governs SinR activity, similar to two other recently described proteins, YlbF and YmcA (13). Strains lacking these proteins share some phenotypes with *spoVS* strains, with continued SinR activity, a failure to form biofilms, and growth that is slightly slower than that of the wild type (*spoVS* data are not shown) (13).

It is unclear if SpoVS directly or indirectly modulates SinR or σ^D activity, and it remains possible that *spoVS* mutation has additional direct or indirect effects on gene expression. We therefore tested if elevated σ^D activity was sufficient to suppress *spoIIB spoVG* by inactivating the anti-sigma factor for σ^D, FlgM (21). As expected, *flgM* strains have increased σ^D-directed gene expression (Fig. 3G). Further, like the *spoVS* mutation, the *flgM* mutation increased spore production by *spoIIB spoVG* ~20-fold (from 9×10^4 to 2×10^6) (Table 2), reduced the frequency of bulges ~7-fold (from 35% to 5%), and increased the proportion of sporangia that completed the final step of engulfment, membrane fusion, from 0% to 9% (Fig. 2I and J; Table 2). Thus, increased σ^D activity is sufficient to suppress the engulfment defect of *spoIIB spoVG* to a similar extent as the *spoVS* mutation.

B. subtilis cells often grow in short chains of cells that remain connected by septal peptidoglycan (Fig. 3A and C), which ultimately is split by peptidoglycan hydrolases to allow daughter cell separation (31). We noted that *spoVS* strains rarely grew in chains (Fig. 3B), suggesting an increased activity of peptidoglycan hydrolases involved in cell separation. Indeed, the σ^D regulon includes genes that encode peptidoglycan hydrolases that mediate cell separation (14, 15, 20), which likely facilitates motility by the generation of single cells (3). The overexpression of peptidoglycan hydrolases in *spoVS* and *flgM* strains was confirmed by an autolysis assay, which measures the lysis of bacteria whose growth has been arrested (3, 12). Both strains lysed faster than the wild type; after 4 h, optical density at 600 nm was reduced by 60% in the wild type versus 84% in *spoVS* and 94% in *flgM* (Fig. 3D). The elevated autolysis of *flgM* and *spoVS* strains was eliminated by a *sigD* mutation, which inactivates σ^D (Fig. 3E), and by a *lytABC* mutation, which eliminates the major σ^D-directed autolysin (see Fig. S2 in the supplemental material). The *lytABC* mutation also reduced but did not eliminate *spoVS*- and *flgM*-mediated suppression of *spoIIB spoVG*, increasing bulges and reducing spore formation to ~25% of suppressed levels (Table 2). We propose that *spoVS* and *flgM* suppress septal thinning defects by increasing expression of peptidoglycan hydrolases involved in cell separation and speculate that several such hydrolases are required for full suppression.

The increased σ^D activity in *spoVS* and *flgM* strains could be due to either an increased σ^D activity in all sporangia or an increased fraction of sporangia with σ^D activity. To test these possibilities, we used immunofluorescence microscopy to visualize Spo0A and σ^D activity in sporulating cultures. To detect Spo0A activity, we used antibodies to σ^F, the production of

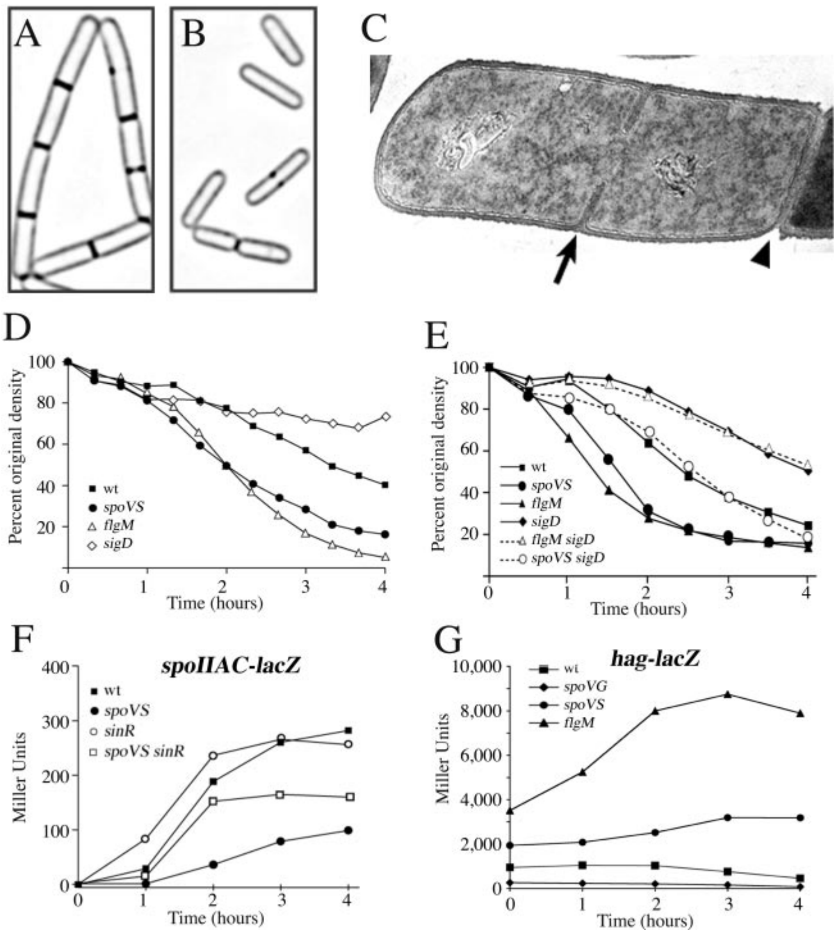

FIG. 3. Cell separation, autolysis, and Spo0A~P- and σ^D-directed gene expression. (A and B) FM 4-64 membrane staining showing the wild type (PY79) growing in chains (A) and *spoVS* (KP535) growing as single cells (B). (C) Electron micrograph showing a vegetative PY79 cell dividing in the middle (arrow) and still connected to its sister cell by septal peptidoglycan in the progress of being split by autolytic enzymes (arrowhead); samples were prepared as previously described (23). (D and E) Sodium azide-induced autolysis during vegetative growth, measured by taking samples from a growing culture, adding sodium azide to a concentration of 0.05 M, and following the optical density at 600 nm over time at 37°C with continued aeration (3). The wild type (squares), *sigD* (open diamonds; KP813), *spoVS* (circles; KP535), and *flgM*Δ*80* (open triangle; KP812) trains sare shown. (E) The following strains are shown: wild type (squares); *sigD* (filled diamonds; KP813); *spoVS* (circles; KP535); *flgM*Δ*80* (triangle; KP812), *flgM sigD* (open triangle; KP829), and *spoVS sigD* (open circle; KP830). (F) Spo0A~P-dependent expression of *spoIIAC-lacZ* in the wild type (squares; KP84), *spoVS* (circles; KP936), *sinR* (open circles; KP937), and *spoVS sinR* (open squares). Samples were harvested at the indicated time after the initiation of sporulation by resuspension at 37°C. (G) σ^D-directed expression of *hag-lacZ* during a resuspension sporulation in the wild-type (squares; KP818), *spoVG* (diamonds; KP823), *spoVS* (circle; KP821), and *flgM*Δ*80* (triangle; KP819) strains.

which requires Spo0A~P; to detect σ^D activity, we used antibodies to β-galactosidase produced from the *hag-lacZ* fusion. After 2.5 h of sporulation, σ^D activity was observed in 16% of wild-type sporangia (cells containing a sporulation septum visualized by FM 4-64 membrane staining) (Fig. 4A; Table 3), compared to 36% of *spoVS* sporangia and 72% of *flgM*Δ*80* sporangia (Fig. 4B to C; Table 3). Thus, *spoVS* and *flgM* strains have two to four times more sporangia expressing motility genes than the wild type, which would likely result in an increased number of sporangia with elevated levels of peptidoglycan hydrolases involved in cell separation. Clearly, even a wild-type population of sporulating cells displays heterogeneity in the activity of the motility regulon.

These results demonstrate an unanticipated link between expression of the σ^D-directed motility regulon and the ability of sporulating cells to complete the phagocytosis-like process

TABLE 3. Expression of σ^D and Spo0A~P-directed genes at $t_{2.5}$ of sporulation

Genotype	Cell morphology[a]	Percentage in class	Percentage of cells in morphological class expressing:			
			σ^F	*hag-lacZ*	Both	Neither
Wild type	Vegetative[b]	41	46	10	4	39
(244)[c]	Sporulating	59	81	0	16	3
spoVS	Vegetative[b]	85	9	46	27	18
(185)[c]	Sporulating	15	57	0	36	7
*flgM*Δ*80*	Vegetative[b]	71	2	69	26	2
(171)[c]	Sporulating	29	26	0	72	2

[a] Vegetative cells do not contain a polar septum, while sporulating cells are defined as sporangia that contain a polar septum in any stage of engulfment.

[b] Cells containing σ^F in the vegetative cell category likely have initiated sporulation, but have not yet synthesized the polar septum. Indeed, in many of these cells the chromosome was rearranged into the axial filament structure that precedes polar spetation.

[c] Total cells scored.

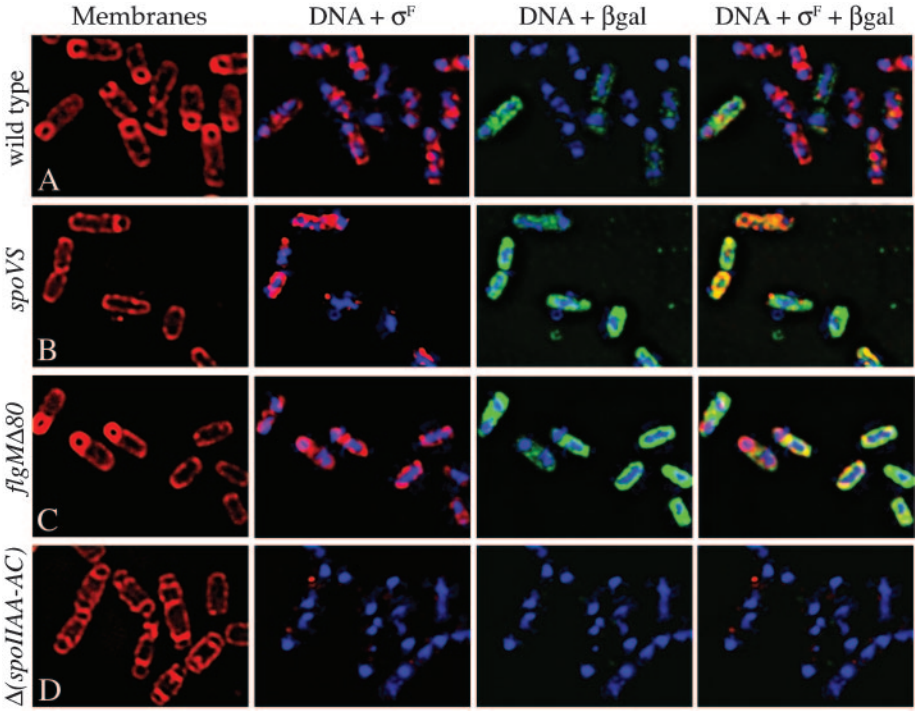

FIG. 4. Immunofluorescence microscopy to examine Spo0A~P- and σ ^{D}-directed gene expression. Sporulation was induced by resuspension, and samples were harvested at $t_{2.5}$ and processed for immunofluorescence microcopy (23), staining with membrane stain FM 4-64 (red, left) and the DNA stain 4′,6′-diamidino-2-phenylindole(DAPI; blue) and using antibodies specific for σ^F (magenta) and for β-galactosidase produced by *hag-lacZ* (green). The wild-type strain (KP818) (A), the *spoVS* strain (KP821) (B), the *flgM*Δ*80* strain (KP819) (C), and the negative control strain KP174 Δ(*spoIIAA-AC*) which has a deletion in the gene encoding σ^F and no *lacZ* fusion (D) are shown.

of engulfment during sporulation. Apparently, the continued expression of σ^D-directed genes during sporulation allows engulfment in the septal thinning defective *spoIIB spoVG* strain, perhaps by providing additional hydrolases that contribute to septal thinning. Because there is cell-to-cell variation in the level of motility gene expression in individual sporulating cells, these observations might explain why there is also cell-to-cell variation in the phenotypes of engulfment mutants, with variations in whether they divided at the second site in the mother cell and whether they have a flat or bulged septum (24). We anticipate the discovery of additional examples in which a cell's proteomic "memory" of past gene expression alters its behavior during subsequent transcriptional responses or developmental pathways.

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