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Coupling of σ^G Activation to Completion of Engulfment during Sporulation of *Bacillus subtilis* Survives Large Perturbations to DNA Translocation and Replication

Genevieve Regan,[a] Mitsuhiro Itaya,[b] and Patrick J. Piggot[a]

Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, Pennsylvania, USA,[a] and Institute for Advanced Biosciences, Keio University, Tsuruoka, Yamagata, Japan[b]

Spore formation in *Bacillus subtilis* is characterized by activation of RNA polymerase sigma factors, including the late-expressed σ^G. During spore formation an asymmetric division occurs, yielding the smaller prespore and the larger mother cell. At division, only 30% of the chromosome is in the prespore, and the rest is then translocated into the prespore. Following completion of engulfment of the prespore by the mother cell, σ^G is activated in the prespore. Here we tested the link between engulfment and σ^G activation by perturbing DNA translocation and replication, which are completed before engulfment. One approach was to have large DNA insertions in the chromosome; the second was to have an impaired DNA translocase; the third was to use a strain in which the site of termination of chromosome replication was relocated. Insertion of 2.3 Mb of *Synechocystis* DNA into the *B. subtilis* genome had the largest effect, delaying engulfment by at least 90 min. Chromosome translocation was also delayed and was completed shortly before the completion of engulfment. Despite the delay, σ^G became active only after the completion of engulfment. All results are consistent with a strong link between completion of engulfment and σ^G activation. They support a link between completion of chromosome translocation and completion of engulfment.

Vegetative cells of *Bacillus subtilis* differentiate into highly resistant, dormant spores through a series of morphological changes. In response to starvation, the vegetative bacteria divide asymmetrically, yielding the smaller prespore (also called forespore) and the larger mother cell. The mother cell then engulfs the prespore and nurtures it as it develops into the mature spore. Ultimately, the mother cell lyses, releasing the mature spore. Changes in gene expression are coupled to the morphological changes (14, 22, 35). Of cardinal importance is the sequential, compartment-specific activation of four RNA polymerase sigma factors. The first of these, σ^F, is activated in the prespore after the asymmetric sporulation division. Then σ^E is activated in the mother cell. Following completion of engulfment, σ^G is activated in the prespore, and then σ^K is activated in the mother cell. Intercellular signals ensure that activation of succeeding sigma factors is dependent on the activity of the previous sigma factor in this sequence. The mechanisms for the activation of σ^F, σ^E, and σ^K are moderately well understood (14, 22, 29, 35). However, it is less clear how σ^G becomes activated. Here we focus on the coupling of σ^G activation to the completion of engulfment.

σ^G is encoded by *sigG*, also called *spoIIIG* (14, 22, 35), and becomes active upon the completion of engulfment (44). Prespore-specific transcription of *sigG* is initially directed by σ^F. Once active, σ^G directs transcription of its own structural gene in a positive feedback loop (25, 45). This mechanism provides a switch to rapid σ^G activity when needed during spore formation. However, activation of σ^G during vegetative growth is toxic, and premature activation during sporulation can block the process (28), so that this feedback loop must be tightly controlled. A number of approaches have been used to study σ^G activation, and they have identified a variety of controls, including those exercised by SpoIIAB, LonA, and CsfB. In general, these controls appear to prevent inappropriate activation of σ^G and thus to control the feedback loop. They do not signal activation at the correct time. Of the three proteins, CsfB has the most prominent role in that it prevents premature activation of σ^G in the prespore (5, 11, 26, 42). Transcription of *csfB* is initially directed by the prespore-specific factor σ^F (13, 42) and subsequently by σ^G, indicating complex regulatory circuits (42). Deletion of *csfB* results in a low level of σ^G activity in the prespore prior to the completion of engulfment but does not affect the large increase in σ^G activity that follows completion of engulfment (11, 26, 42). Thus, CsfB is inadequate to explain the postengulfment activation of σ^G. SpoIIAB is an anti-sigma factor with a key role in σ^F activation; its main role with respect to σ^G is thought to be to prevent inappropriate activation in the mother cell (8, 41). The protease LonA prevents activation of σ^G under nonsporulation conditions (40). A fourth protein, Fin, was recently shown to be necessary for an efficient transition from σ^F to σ^G activity in the prespore; in the absence of Fin, σ^G activity is substantially reduced (6). However, Fin does not affect the timing of σ^G activation upon completion of engulfment; rather, σ^G-directed *fin* transcription appears to enhance *fin* expression so as to ensure efficient σ^G activation (6). It appears to be an adjunct to the positive feedback loop of σ^G-directed *sigG* transcription in ensuring fast appearance of ample σ^G activity when it is needed.

A "feeding tube" channel, AA-AH · Q, has been shown to be necessary for σ^G to become active after the completion of engulfment. The components of the channel are encoded by eight genes expressed in the mother cell under σ^E control, *spoIIIAA* through *spoIIIAH*, and one gene expressed in the prespore under σ^F con-

Received 14 August 2012 **Accepted** 11 September 2012
Published ahead of print 14 September 2012
Address correspondence to Patrick J. Piggot, piggotp@temple.edu.

doi:10.1128/JB.01470-12

trol, *spoIIQ*. The AA-AH · Q feeding tube provides a link between the mother cell and prespore, which is necessary for σ^G activation (4, 30) and for prespore integrity (16). No specific activator of σ^G has been shown to move through this channel. Rather, the channel is thought to be relatively nonspecific (4), needed after completion of engulfment for transport into the prespore of something (possibly ATP) required for σ^G activity. Notably, following completion of engulfment, it is also needed to obtain activity of σ^F and artificially expressed T7 RNA polymerase in the prespore. Both σ^F and T7 RNA polymerase are active before the completion of engulfment without the need for the feeding tube, whereas σ^G is not. Thus, although the feeding tube is necessary to ensure σ^G activity after engulfment, it is insufficient to explain why σ^G becomes active only after the completion of engulfment.

Here we tested the robustness of the link of σ^G activation to engulfment by perturbing engulfment. We took three approaches, based on the assumption that chromosome replication and chromosome translocation into the prespore are completed before engulfment is completed. The first approach, delaying replication and translocation, was to use derivatives of *B. subtilis* with 0.9 and 2.3 Mb of *Synechocystis* PCC6803 DNA inserted into the 4.2-Mb *B. subtilis* chromosome (24); the *Synechocystis* inserts lack *rrn* genes and appear to be largely silent (24). The second approach, affecting termination of replication and hence potentially the sequence of translocation events, was to use a strain in which a region containing the termination-associated *TerI*, *TerII*, and *rtp* loci had been relocated away from the normal replication termination site at 172°, resulting in termination of replication at 145° relative to the origin of replication at 0° (17, 19). The third approach, in which translocation was delayed, was to use a strain with a reduced SpoIIIE DNA translocase activity (3).

During the process of engulfment, the septal membrane undergoes a series of changes. When it is first formed, the septum is straight (designated stage IIi) (Fig. 1). The septum then becomes curved, bowing into the mother cell (stage IIii), as a result of the progressive loss of wall material starting from the center of the septal disk. The annulus of attachment of the septal membrane to the peripheral cell membrane then moves toward the cell pole (stage IIiii). Finally, the engulfing membranes fuse at the cell pole, releasing the completely engulfed prespore within the mother cell (stage III) (22, 23). The time from septation to completion of engulfment is not known precisely; the best estimate is about 70 min at 37°C (12, 32). Different studies indicate that chromosome replication is completed by stage IIi (2, 18, 21). However, in striking contrast to vegetative division, chromosome partitioning is not complete at the time of the sporulation division; approximately 70% of the chromosome destined for the prespore is still in the mother cell (47, 48). The DNA translocase SpoIIIE pumps the remaining chromosome into the prespore (1, 48). Completion of translocation of the chromosome into the prespore is estimated to take about 15 to 20 min (3, 27, 36) and occurs by stage IIiii (2). We found that insertion of 2.3 Mb of *Synechocystis* DNA into the *B. subtilis* chromosome delayed completion of translocation by at least 90 min, which nevertheless still preceded completion of engulfment and the activation of σ^G. Activation of σ^G also occurred after completion of engulfment in strains with impaired DNA translocase activity and with the site of termination of replication relocated. We infer that the completion of chromosome translocation into the prespore is a requirement for completion of engulfment, which is in turn required for activation of σ^G.

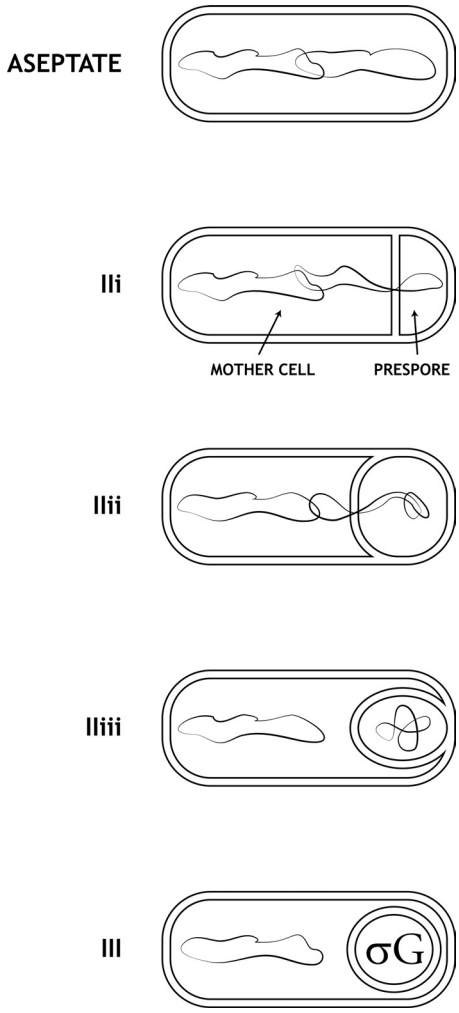

FIG 1 Illustration of the stages of engulfment of the prespore by the mother cell.

MATERIALS AND METHODS

Media. *B. subtilis* was grown at 37°C in modified Schaeffer's sporulation medium (MSSM) or on Schaeffer's sporulation agar (15, 33, 39). When required, the medium contained erythromycin at 1.5 μg/ml, neomycin at 3.5 μg/ml, chloramphenicol at 5 μg/ml, or spectinomycin at 100 μg/ml. *Escherichia coli* was grown on Luria Bertani lysogeny broth (LB) or LB agar, containing ampicillin at 100 μg/ml when required.

Strains. The *B. subtilis* strains used are shown in Table 1. Strain SL14525 was constructed by introducing the P_{spoIIQ}-*gfp* fusion into BEST7003 by double crossover from pGR6. pGR6 was constructed from pVK315 by replacing a P_{sspA}-*yfp* fusion with P_{spoIIQ}-*gfp* derived from pVK57 (kindly provided by Vasant Chary). To insert a σ^F-directed *gfp* reporter at various locations, the plasmid pGR14 was constructed by removing the erythromycin resistance gene and the region of homology to the 3′ end of *thrC* from pVK208 (kindly provided by Vasant Chary) and then inserting a spectinomycin resistance gene. The region of homology to the 5′ end of *thrC* was replaced with different regions of homology to generate a series of plasmids that could integrate by single crossover (Campbell mechanism) at various locations. A PCR-amplified fragment of *ylnF* replaced the *thrC* region in pGR14 to yield the plasmid pGR17. SL15634 was generated by Campbell integration of pGR17 into SU227. The *thrC* region in pGR14 was replaced with a PCR-amplified fragment of *ynaE* to yield pGR19, which was used to make SL15648. In pGR22 the *thrC*

TABLE 1 *B. subtilis* strains used

Strain	Relevant genotype or characteristics	Reference or source
BEST7003	No *Synechocystis* insert	24
BEST8817	890-kb *Synechocystis* insert	24
BEST7527	2.3-Mb *Synechocystis* inserts	24
BR151	Laboratory parental strain	F. E. Young
SL10969	BR151 *sspA*::P_{sspA}-*gfp*	Laboratory stock
SL14360	BEST7003 *sspA*::P_{sspA}-*gfp*	This study
SL14361	BEST8817 *sspA*::P_{sspA}-*gfp*	This study
SL14378	BEST7527 *sspA*::P_{sspA}-*gfp*	This study
SL14469	SpoIIIE(D584A) *sspA*::P_{sspA}-*gfp*	This study
SL14525	BEST 7003 *ppsB*::P_{spoIIQ}-*gfp*	This study
SL14539	BEST 7527 *ppsB*::P_{spoIIQ}-*gfp*	This study
SL14854	BEST 7003 *spoIIQ*::P_{spoIIQ}-*gfp*	This study
SL14856	BEST 7527 *spoIIQ*::P_{spoIIQ}-*gfp*	This study
SL15103	SU227 *amyE*::P_{sspA}-*gfp*	This study
SL15634	SU227 P_{spoIIQ}-*gfp@ylnF*	This study
SL15648	SU227 P_{spoIIQ}-*gfp@ynaE*	This study
SL15660	SU227 P_{spoIIQ}-*gfp@amyE*	This study
SL15671	BEST 7003 *amyE*::$P_{spoIIIG}$-*gfp*	This study
SL15673	BEST 7527 *amyE*::$P_{spoIIIG}$-*gfp*	This study
SpoIIIE(D584A)	Slow SpoIIIE DNA translocase	3
SU227	Replication termination occurs at 145°	7, 17,19

region was replaced by a region of *amyE* from the plasmid pVK2 (kindly provided by Vasant Chary); pGR22 was used to construct SL15660. Further details of strain construction are available on request. Plasmids were constructed and maintained in *Escherichia coli* DH5α.

Spore formation. Cultures were grown in MSSM in conical flasks where the medium occupied no more than 10% of the flask volume. The flasks were incubated at 37°C and shaken at 150 rpm on an orbital shaker. Growth was monitored by measuring the optical density at 600 nm. The end of exponential growth was defined as the start of spore formation. Cultures were analyzed by phase-contrast microscopy and by a heat killing assay (20 min at 80°C) to determine the extent of spore formation (9).

Fluorescence microscopy. The conditions used for the growth and imaging of samples were similar to those described previously (2, 10). Samples (300 μl) were taken from cultures grown at 37°C in MSSM and mixed with FM4-64 to a final concentration of 1 ng/μl. The bacteria were allowed to settle for 10 min before being visualized with a Leica TCS SP5 confocal microscope. The assessment of engulfment was as described previously (2). The names of the intermediates in the process are essentially those suggested by Illing and Errington (23). At stage IIi, the sporulation septum is straight; at stage IIii, it bulges into the mother cell; at stage IIiii, the points of attachment of the septum to the peripheral membrane have moved toward the pole of the cell; at stage III, engulfment is complete, resulting in a detached prespore entirely within the mother cell. The engulfed prespore often moved away from the pole of the mother cell, suggestive of later stages of sporulation; however, subsequent stages could not be distinguished confidently, and all completely engulfed prespores are designated as having reached stage III. With the strains and conditions used here, FM4-64 efficiently stained completely engulfed prespores, although it does not do so under other conditions (43). Images were analyzed with a Leica TCS SP5 confocal system. Green fluorescent protein (GFP) emission was captured between 500 and 540 nm and FM4-64 emission between 600 and 794 nm; both fluorophores were excited at 488 nm.

Time lapse microscopy. Bacteria were inoculated into 5 ml MSSM and grown overnight at 30°C, without shaking. Microscope slides were prepared essentially as described by Veening et al. (46). Agarose pads were prepared on the slides within 1.7- by 2.8-cm Gene Frames (Thermo Scientific) with 1.5% low-melting-point agarose in MSSM (50). Pads contained FM4-64 at a final concentration of 0.4 ng/ml. Pads were inoculated with 0.3 μl of early-exponential-phase culture, sealed with a 22- by 40-mm no. 1.5 cover glass (Fisher Scientific) and incubated for approximately 4 h at 30°C. Slides were then monitored with a TCS SP5 confocal microscope (Leica) contained within a temperature-controlled microscope environmental chamber which had been prewarmed to 30°C. For each slide, a field was chosen and focused upon, and the equipment was left to equilibrate for 2 h. After 2 h, the focus was adjusted, and bacteria were imaged at regular intervals using Leica time lapse software with autofocusing. GFP levels were quantified with the Leica TCS SP5 confocal system software. Derivatives of strain BEST7003 formed spores very poorly under these conditions and were not analyzed by time lapse microscopy.

Other methods. The methods used to transform *B. subtilis* and to prepare plasmid and chromosomal DNA were essentially as described previously (33, 34).

RESULTS

Insertion of 2.3 Mb *Synechocystis* DNA into the *B. subtilis* chromosome delayed engulfment of the prespore by the mother cell. *B. subtilis* strains harboring different *Synechocystis* DNA inserts (24) were tested for their ability to form spores in liquid media. Of those strains, BEST7527 was chosen for detailed study because it had the largest inserts of strains that formed substantial numbers of spores, although the extent varied from experiment to experiment. The BEST7527 derivative SL14378 formed 46% and 37% of the heat-resistant spores formed by the parental strain SL14360 with no inserts in the experiments described below. The inserts in BEST7527 are located origin distal to the ~1.1 Mb of the *B. subtilis* chromosome that is ordinarily present in the prespore when it is first formed (49). There are a 908-kb insert in the right arm of the *B. subtilis* chromosome and two fragments totaling 1,387 kb in the left arm (Fig. 2) (24). The progression through the stages of engulfment for strains with (SL14378) and without (SL14360) the inserts is shown in Fig. 3.

For the parental strain with no insert, the sporulation division was first observed about 3 h after the end of exponential growth, and half the sporulating population had formed the septum by 4 h (stage IIi, strain SL14360) (Fig. 3A). Engulfment was completed (stage III) within 60 to 90 min of septation, similar to times reported previously for *B. subtilis* (12, 32). The strain containing the 2.3-Mb insert, SL14378, appeared to be slightly delayed in formation of the sporulation division septum compared to the parent strain. It was considerably delayed in the progression through engulfment stages IIii and IIiii, with half the sporulating population

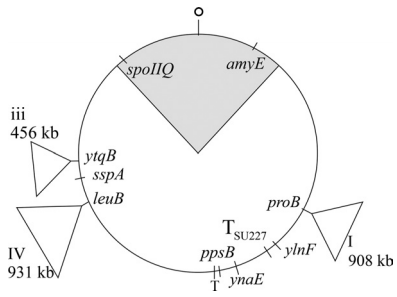

FIG 2 Schematic diagram of the *B. subtilis* chromosome showing the approximate location of *Synechocystis* DNA insertions in BEST7527 and of different genetic loci. BEST8817 had an 890-kb insert (not shown) in the position of insert iii of BEST7527. O, origin of replication; T, terminus; T_{SU227}, terminus of replication in strain SU227. The shaded area indicates the portion of the chromosome that is in the prespore at the time of the sporulation division.

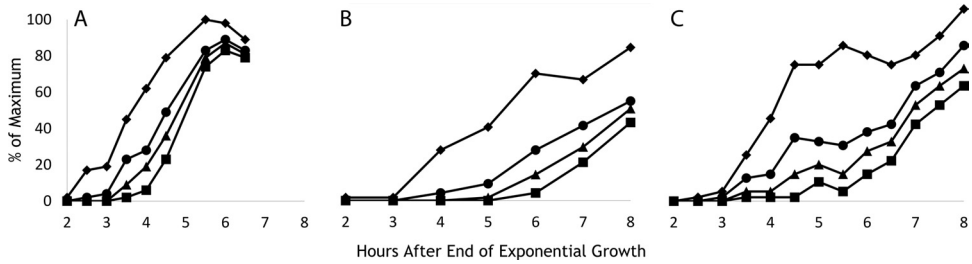

FIG 3 Effect of insertion of *Synechocystis* DNA in *B. subtilis* on the progression of engulfment. The symbols indicate bacteria that had reached a given stage of engulfment (and may have progressed beyond it): diamonds, stage IIi; circles, stage IIii; triangles, stage IIiii; squares, stage III. Values are percentages of the maximum number that reached stage IIi. (A) Strain SL14360, which contains no *Synechocystis* DNA; for each point, at least 200 organisms were scored. (B and C) Independent experiments with strain SL14378, which contains 2.3 Mb *Synechocystis* DNA. (B) At least 100 organisms were scored for each point (except for 5 h, for which 89 were scored). (C) At least 200 organisms were scored for each point (except for 5 and 5.5 h, for which at least 140 organisms were scored).

completing engulfment only about 3 h after they had formed the septum (Fig. 3B and C).

σ^G activity was detected only in bacteria that had completed engulfment, even when engulfment was delayed. To test the effect on σ^G activation of a greatly enlarged chromosome and the resulting delay in engulfment, a transcriptional σ^G-directed P_{sspA}-*gfp* fusion was used. The fusion was inserted at the *sspA* locus (Fig. 2). σ^G activity was detected in almost all prespores when engulfment had been completed. In contrast no expression was detected in any bacteria that had not completed engulfment (strain SL14378) (Table 2; examples of organisms at the different stages of engulfment are shown in Fig. 4A). The same result was obtained with the parental strain with no *Synechocystis* inserts (strain SL14360) (Table 2; Fig. 4B); namely, σ^G activity was detected only after the completion of engulfment. In contrast, σ^F-directed gene expression was detected soon after septation (strain SL14856, which has the *Synechocystis* inserts) (Table 2; Fig. 4C). Transcription from the *sigG* promoter was assessed with a transcriptional *gfp* fusion. It was detected well before the completion of engulfment (strain SL15673) (Table 2).

We also tested the effect of a large *Synechocystis* insert in just one arm of the *B. subtilis* chromosome. Strain SL14361 is derived from BEST8817 (24) and has 890 kb inserted in the left arm of the chromosome (in the position of insert iii) (Fig. 2). Such a large, asymmetrically located insertion might potentially disrupt the processes leading to σ^G activation. However, σ^G activity was again detected in almost all prespores that were completely engulfed but in no prespores that had not been completely engulfed (strain SL14631) (Table 2).

Completion of chromosome translocation into the prespore was delayed by the 2.3-Mb DNA insertion and occurred shortly before completion of engulfment. Genes transcribed by RNA polymerase containing σ^F are expressed only in the prespore. Those located within about 1.1 Mb spanning the origin of replication of the circular 4.2-Mb chromosome are present in the prespore at the time of septation (49) and are expressed soon after septation (20). However, expression of genes located near the terminus requires their translocation into the prespore before they are expressed (3, 27, 49, 51). Expression of terminus-proximal σ^F-directed genes serves as an indicator of translocation of the terminus region into the prespore.

We used the σ^F-directed reporter P_{spoIIQ}-*gfp* at origin-proximal and origin-distal locations to assess chromosome translocation in BEST7527 derivatives. In strain SL14856, the reporter was inserted at the origin-proximal *spoIIQ* locus (Fig. 2). As discussed above, expression was detected soon after septation (strain SL14856) (Table 2), with GFP fluorescence being observed in some bacteria at stage IIi and in most bacteria from stage IIii onwards. In contrast, when the reporter was inserted at *ppsB*, located close to the chromosome terminus (Fig. 2), no expression was detected at stages IIi or IIii (strain SL14539) (Table 2). GFP expression began to be detected only at stage IIiii, and by stage III most prespores displayed fluorescence. σ^F-directed genes located near the terminus are weakly expressed compared to the same gene at an origin-proximal location (27) (P. Xenopoulos and P. J. Piggot, unpublished results). Consequently, the number expressing at IIiii may be an underestimate. Nevertheless, it seems reasonable to infer that in the BEST7527 background, chromosome

TABLE 2 Effect on prespore-specific transcription of different perturbations of chromosome translocation and replication

Strain	Perturbation[a]	σ tested	Promoter[b]	No. with GFP fluorescence/total at stage			
				IIi	IIii	IIiii	III
SL14360	None	σ^G	P_{sspA}	0/263	0/87	0/59	432/439
SL14361	0.9-Mb insert	σ^G	P_{sspA}	0/169	0/46	0/60	365/372
SL14378	2.3-Mb insert	σ^G	P_{sspA}	0/564	0/212	0/153	505/523
SL14539	2.3-Mb insert	σ^F	P_{spoIIQ}(ter)	0/445	0/227	14/218	261/261
SL14856	2.3-Mb insert	σ^F	P_{spoIIQ}(ori)	15/196	124/147	261/262	806/806
SL15673	2.3-Mb insert	σ^F/σ^G	P_{sigG}[c]	49/308	196/273	306/325	1,026/1,027
SL15103	Ter at 145°	σ^G	P_{sspA}	0/291	0/207	0/190	270/353
SL14469	Slow translocase	σ^G	P_{sspA}	0/94	0/37	0/36	177/178

[a] Insertion of *Synechocystis* DNA, termination of replication (Ter) at 145°, or slow SpoIIIE (D584A) DNA translocase.
[b] Promoter driving expression of *gfp*. ter and ori, promoter located near the terminus and origin.
[c] Promoter of the structural gene for σ^G.

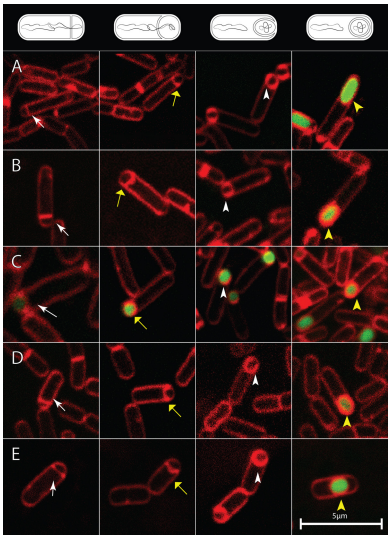

FIG 4 Examples of bacteria showing the activation of *gfp* transcription directed by σ^G or by σ^F at different stages of engulfment. White arrows, stage IIi; yellow arrows, stage IIii; white arrowheads, stage IIiii; yellow arrowheads, stage III. Diagrams of the different stages are shown at the top. Membranes were stained with FM4-64 (red). (A) Strain SL14378 with a 2.3-Mb *Synechocystis* insert and σ^G-directed P_{sspA}-*gfp*; B, strain SL14360, σ^G-directed P_{sspA}-*gfp* in BEST7003 background with no insert; C, strain SL14856 with a 2.3-Mb insert and origin-proximal σ^F-directed P_{spoIIQ}-*gfp*; (D) strain SL15103 with relocated termination of replication at 145° and σ^G-directed P_{sspA}-*gfp*; (E) strain SL14469 with slow DNA translocase and σ^G-directed P_{sspA}-*gfp*. Bar, 5 μm (all images).

translocation was considerably delayed and was not completed until shortly before completion of engulfment.

σ^G activation remained coupled to completion of engulfment after relocation of the site for termination of replication and after impairment of DNA translocase function. As described above, the robustness of the link(s) between translocation, engulfment, and σ^G activation was tested by examining the effects of large inserts of foreign DNA in the *B. subtilis* chromosome. We wished to test the effect of disrupting termination of replication, and hence potentially the sequence of translocation events, without large inserts of foreign DNA. For this purpose we used derivatives of a strain (SU227) in which termination of replication occurs at about 145° and not 172° as the result of relocation of key termination loci (17, 19). Sporulation efficiency was reduced to about 10%. Nevertheless, substantial numbers of sporulating organisms were present in batch cultures, and σ^G became active only after completion of engulfment (strain SL15103) (Table 2; examples of organisms at the different stages of engulfment are shown in Fig. 4D). To explore this relationship further, time lapse microscopy was performed to visualize σ^G activation in individual organisms forming spores on agarose pads. To assess σ^G activity, green fluorescence was measured in individual cells as they transitioned from septation to completion of engulfment. σ^G activity was detected in organisms that had completed engulfment but not in organisms that had not completed engulfment (strain SL15103) (Fig. 5B). The pattern was very similar to that of a derivative of our standard *B. subtilis* 168 strain, in which there had been no manipulation of the terminus region (strain SL10969) (Fig. 5A).

The relocation of the terminus of replication did not uncouple σ^G activation from completion of engulfment. However, it remained possible that the relocation disturbed chromosome translocation and uncoupled that process from engulfment. To test for this possibility, time lapse studies were preformed using a σ^F-directed reporter, P_{spoIIQ}-*gfp*, at different chromosomal locations. When located near the origin, at 28° (*amyE*), the reporter was expected to already be in the prespore at the time of septation. Indeed, GFP expression began soon after septum formation (strain SL15660) (Fig. 5C). Two locations were tested, 140° (*ylnF*) and 161° (*ynaE*), that flanked the replication termination site in SU227 (Fig. 2). In both cases (strains SL15634 and SL15648) (Fig. 5D and E, respectively), expression also began soon after septation and well before the completion of engulfment (although it was perhaps slightly delayed compared to expression at the 28° location). Thus, translocation into the prespore of these terminus-proximal loci preceded completion of engulfment.

We also tested σ^G activation in a *B. subtilis* strain in which translocation was delayed by about 30 min because of a mutation that reduced the efficiency of the DNA translocase SpoIIIE (3). Again, in batch cultures, σ^G activity was detected only after the completion of engulfment (strain SL14469) (Table 2; examples of organisms at the different stages of engulfment are shown in Fig. 4E). This conclusion was reinforced by time lapse microscopy of individual organisms forming spores on agarose pads (strain SL14469) (Fig. 5F). Interestingly, σ^G activity was first detected some time after completion of engulfment with SL14469, in contrast to strain SL10969 with wild-type *spoIIIE*, where σ^G activity was detected soon after completion of engulfment (Fig. 5A). The reason for the slow activation after engulfment in the strain with the inefficient translocase is not known. It was not apparent in batch culture (Table 2) and might result from the different temperatures used for batch cultures (37°C) and time lapse studies (30°C); this possibility was not tested.

DISCUSSION

During spore formation, σ^G becomes active after the completion of engulfment (44). Once activated, σ^G directs transcription of its own structural gene, *sigG*, in a positive feedback loop (25), so that there is the potential for strong, inappropriate activation. Premature activation of σ^G can be highly detrimental (28), and a series of controls have been identified that prevent its occurrence: CsfB prevents premature activation in the prespore (5, 11, 26, 42), SpoIIAB prevents activation in the mother cell (8, 41), and the protease LonA prevents activation under nonsporulating conditions (40). Mutations causing premature σ^G activation map to the genes encoding these regulators. Artificial overexpression of *sigG* can also uncouple σ^G activation from the completion of engulfment (16). Despite these findings, the nature of the link between activation and engulfment has remained enigmatic. Here we explored its robustness by testing the effect of perturbations of chromosome replication and translocation, without affecting the known σ^G regulators by mutation or overexpression. Our principal finding is that there is indeed a strong link between the completion of engulfment and activation of σ^G. In addition, the results are consistent with a link between the completion of chromosome translocation and the completion of engulfment.

The greatest effect on engulfment resulted from insertion of 2.3 Mb of *Synechocystis* DNA in the *B. subtilis* chromosome. The presence of the insert delayed completion of engulfment by at least 90 min. The results were consistent with chromosome translocation also being substantially delayed and with the delay in engulfment

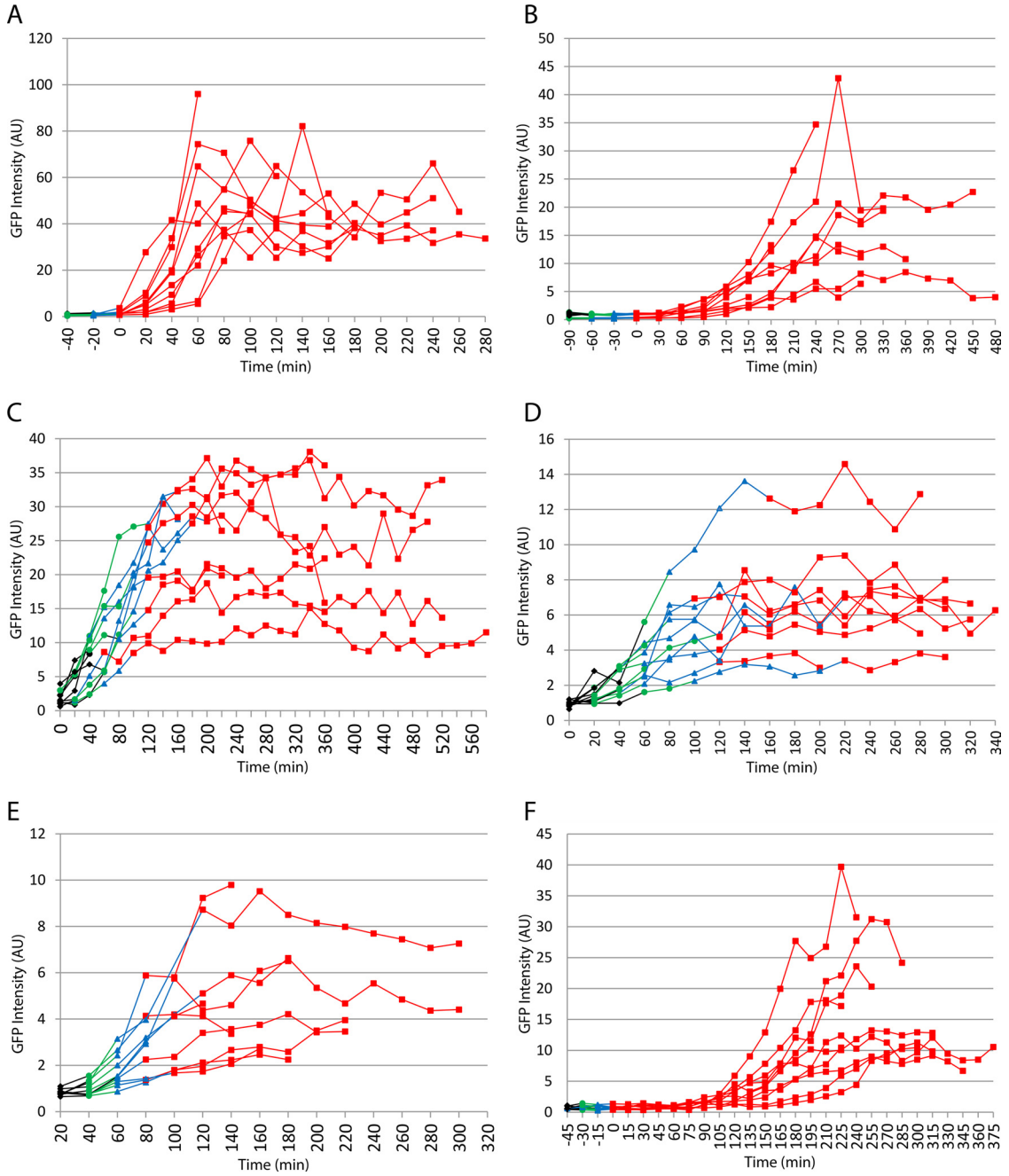

FIG 5 Activation of σ^G- and σ^F-directed gene expression in individual cells analyzed by time lapse microscopy. Time traces of individual cells. (A) SL10969, standard strain, σ^G reporter; (B) Strain SL15103, termination of replication at 145° and σ^G reporter; (C) SL15660, termination of replication at 145° and σ^F reporter at 28°; (D) SL15634, termination of replication at 145° and σ^F reporter at 140°; (E) SL15648, termination of replication at 145° and σ^F reporter at 161°; (F) SL14469; slow DNA translocase and σ^G reporter. Bacteria were maintained at 30°C, and successive images were captured at the indicated times. Expression from *gfp* transcriptional fusions was observed as green fluorescence, which was measured in arbitrary units that are not comparable between strains. For strains expressing a σ^F-directed reporter, times are normalized (time zero) to the first image to show the sporulation septum (stage IIi). For strains expressing a σ^G-directed reporter, time traces of individual bacteria are normalized (time zero) to the first image showing completion of engulfment (stage III). The different stages of engulfment are indicated by different colors: black is stage IIi, green is stage IIii, blue is stage IIiii, and red is stage III.

being a consequence of the delay in the completion of translocation. Even with such a long delay, activation of σ^G was detected only after engulfment had been completed, suggesting a robust linkage between the two processes.

In wild-type *B. subtilis*, it takes about 15 to 20 min to complete translocation of the origin-distal ~3.1 Mb of the chromosome from the mother cell into the prespore (3, 27, 36). We do not know how much DNA is in the prespore when it is first formed in strains containing 2.3 Mb of *Synechocystis* DNA. However, the long time of translocation of the rest of the chromosome into the prespore seems disproportionate to the size of the additional DNA. A possible explanation is that the prolonged delay in translocation

caused by the *Synechocystis* DNA results from the lack of directional skew in copies of the octamer GAGAAGGG, named SRS6, in the *Synechocystis* DNA (31, 38). In the *B. subtilis* chromosome, the orientation of SRS6 is highly skewed and directs the SpoIIIE translocase to the terminus (37).

Two other sets of experiments also tested the effects of perturbations of chromosome replication and translocation. In one, a SpoIIIE translocase was used in which translocation is delayed by about 30 min (3). Again, despite the delayed translocation, σ^G became active only after completion of engulfment (Table 2; Fig. 5F). In the other, a strain was used in which termination of replication occurred at 145° relative to the origin at 0° on the circular chromosome map, rather than at the normal position, 172° (17, 19). Relocating the terminus of replication had no effect on σ^G activation, which occurred only after the completion of engulfment (Table 2; Fig. 5B). It also appeared not to delay completion of translocation, as terminus-proximal σ^F-directed reporters were expressed before the completion of engulfment. Given the role of SRS6 octamers in directing the DNA translocase toward the terminus region (37), this result was somewhat surprising. However, there are just four SRS6 sequences between 145° and 172°, one pointing toward 172° and three away from it, so changing the terminus of replication within this region may not greatly affect translocation.

Together, the various experiments indicate that there is a robust link ensuring that σ^G becomes active only upon the completion of engulfment. They do not indicate what the link is. Nevertheless, the link is central to activation of σ^G. Probably the most dramatic changes upon the completion of engulfment are, first, that the prespore is no longer in direct contact with the external medium, being completely surrounded by the mother cell, and, second, that it is separated from the mother cell by two opposed membranes. The requirement for the AA-AH · Q channel (4, 30) emphasizes the effect of completing engulfment but does not provide a sufficient explanation for σ^G activation. The channel is not needed for σ^F or artificially expressed T7 RNA polymerase to be active in the prespore before engulfment completion, but it is needed for them to be active after the completion of engulfment. Hence, the idea that the channel is a feeding tube, necessary to provide some key metabolite (possibly ATP) (4). But before completion of engulfment, enough key metabolite was present in the prespore to ensure σ^F activity and T7 polymerase activity, so why is σ^G not active? Completion of engulfment presumably causes a general shock to the prespore. It could cause a conformational change in a regulatory protein that is associated with the engulfment machinery. It could reduce the uptake of micronutrients into the prespore. It could, at least transiently, disrupt energy generation in the prespore. Any of these effects could be sufficient to trip the autocatalytic loop to activate σ^G expression, overcoming the inhibitory effects of regulators such as CsfB.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants GM43577 (to P.J.P.) and T32AI07101 (to G.R.) from the National Institutes of Health.

We are very grateful to Gerry Wake, Liz Harry, David Rudner, and Vasant Chary for kindly providing strains. We thank Tina Buttaro, Vasant Chary, and Panos Xenopoulos for many helpful discussions.

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