



Regulation of Biofilm Aging and Dispersal in *Bacillus subtilis* by the Alternative Sigma Factor SigB

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ABSTRACT Bacterial biofilms are important in natural settings, biotechnology, and medicine. However, regulation of biofilm development and its persistence in different niches is complex and only partially understood. One key step during the biofilm life cycle is dispersal, when motile cells abandon the mature biofilm to spread out and colonize new niches. Here, we show that in the model bacterium *Bacillus subtilis* the general stress transcription factor SigB is essential for halting detrimental overgrowth of mature biofilm and for triggering dispersal when nutrients become limited. Specifically, SigB-deficient biofilms were larger than wild-type biofilms but exhibited accelerated cell death, significantly greater sensitivity to different stresses, and reduced dispersal. Interestingly, the signal detected by SigB to limit biofilm growth was transduced through the RsbP-dependent metabolic arm of the SigB regulatory cascade, which in turn positively controlled expression of SinR, the master regulator of biofilm formation and cell motility. This novel SigB-SinR regulatory circuit might be important in controlling the fitness of biofilms (either beneficial or harmful) in diverse environments.

IMPORTANCE Biofilms are crucial for bacterial survival, adaptation, and dissemination in natural, industrial, and medical systems. Sessile cells embedded in the self-produced extracellular matrix of the biofilm benefit from a division of labor and are protected from environmental insults. However, as the biofilm ages, cells become stressed because of overcrowding, starvation, and accumulation of waste products. How does the sessile biofilm community sense and respond to stressful conditions? Here, we show that in *Bacillus subtilis*, the transcription factors SigB and SinR control whether cells remain in or leave a biofilm when metabolic conditions become unfavorable. This novel SigB-SinR regulatory circuit might be important for controlling the fitness of biofilms (either beneficial or harmful) in diverse environments.

KEYWORDS *Bacillus subtilis*, biofilm aging, biofilm dispersal, sigma B, stress activation

Bacteria form multicellular and cooperative communities known as biofilms, which are critical for bacterial survival, adaptation, and dissemination in natural, industrial, and medical systems (1, 2). Sessile cells embedded in the self-produced extracellular matrix of a biofilm benefit from a division of labor and are protected from environmental insults (3). However, as the biofilm ages, the population of cells becomes stressed due to overcrowding, nutrient restriction, and waste product accumulation. However, thus far it has not been resolved how a sessile biofilm community senses and responds to stressful conditions (1, 2).

One strategy that is believed to be beneficial for rejuvenation of a stressed biofilm is triggering the escape of motile cells. This phenomenon, known as biofilm dispersal (4, 5), is a natural step of the biofilm life cycle and is important for many pathogenic

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bacteria with regard to the transmission, exacerbation, and spread of infections, especially in periodontitis, cystic fibrosis, pneumonia, and catheter-associated endocarditis (1, 5, 6). Proficiency in flagellum synthesis, which is essential for swimming and swarming activities, is required for the ability to disperse from liquid (pellicle) and solid (colony) biofilms (5–8). Regardless, the molecular mechanisms that coordinate the aging and rejuvenation of biofilm through cell dispersal in Gram-positive bacteria are poorly understood (4).

Bacillus subtilis is a Gram-positive, endospore-forming bacterium that serves as a model organism for the study of different prokaryote phenomena (e.g., biofilm development) at both physiological and molecular levels (4, 9–13). In *B. subtilis*, SinR is considered a master regulator that controls entry into two alternative physiological states: a motile state, activated by SinR, in which cells are able to swim or swarm (on liquid or wet surfaces, respectively), and a nonmotile state, repressed by SinR, in which cells form a sessile multicellular biofilm (14, 15). Under conditions that favor planktonic growth (i.e., bacterial growth with shaking), SinR activates the genes required for flagellum synthesis (*hag*) and cell chain separation (*lytA* and *lytF*) and represses those required for a sessile lifestyle (i.e., *eps* and *tasA*) (10). When *B. subtilis* develops in media that support a sessile lifestyle (i.e., bacterial growth without shaking), a subset of the population produces and secretes the complex polysaccharides (extracellular polysaccharides, or EPSs) and proteins, mainly the amyloid-like fiber TasA and the hydrophobin BslA, that will constitute the main components of the extracellular matrix of the biofilm (4). Notably, the operons responsible for the synthesis of EPS and TasA (the 15 genes of the *epsA-epsO* (*epsA-O*) operon and the three-cistron *tapA-sipW-tasA* operon, respectively) are repressed by SinR (14, 15).

In *B. subtilis*, other bacilli, *Listeria*, and *Staphylococcus*, the transcription factor SigB controls the general stress response that comprises more than 200 genes, the products of which confer the bacterium with resistance to multiple stresses (16, 17). Activation of SigB is controlled by the partner-switching RsbV-RsbW-SigB module (16, 18, 19). Under nonstress conditions, SigB is maintained in an inactive state in a complex with the anti-sigma factor/kinase RsbW; the third partner, RsbV, is inactive because of phosphorylation by RsbW (20–23). However, under stress, release of SigB from the inactive SigB::RsbW complex is achieved via the dephosphorylated form of the anti-sigma factor RsbV. In *B. subtilis*, activation (dephosphorylation) of RsbV, and therefore SigB activation, is achieved by alternative phosphatases that sense energy or environmental stress (RsbP or RsbU phosphatase, respectively) (20–23). SigB is also activated by cold shock (24, 25) independently of RsbP, RsbU, and RsbV activities (24).

Here, we describe a novel mechanism involving the transcription factors SigB and SinR by which *B. subtilis* regulates whether a cell remains in or leaves a biofilm when environmental and metabolic conditions become unfavorable.

RESULTS

SigB and its role during the biofilm life cycle. In planktonic cultures, expression of at least 200 genes (~5% of the genome) is estimated to be under direct or indirect control of the general stress sigma factor SigB (16, 17). Induction of this SigB-dependent regulon confers the cell with general, unspecific, and preventive stress protection (16, 17). We sought to determine if SigB is activated during biofilm formation and, if so, what its role is during biofilm development. To address this issue, we monitored the activity of SigB during growth of the *B. subtilis* reference strain JH642 (Table 1) under biofilm-supporting conditions. This domesticated laboratory strain is able to form a substantial biofilm (i.e., floating pellicles) under developmental control (26–28) when cultivated in nutritionally enriched media such as LBY broth (Luria-Bertani broth fortified with 4% yeast extract) (29). As shown in Fig. 1A, the low β -galactosidase activity driven from the SigB-dependent *ctc* promoter (16, 17) during the first 20 to 30 h of cultivation indicated that SigB was not very active when the biofilm was juvenile. However, the transcription factor SigB became active at later incubation times (after 40 h), with the highest activity occurring when the biofilm reached its plateau of

TABLE 1 Strains used in this work

Strain	Relevant phenotype and/or genotype	Comment and/or source ^a
JH642	<i>B. subtilis</i> wild-type strain	Laboratory collection (12)
NCIB3610	<i>B. subtilis</i> Marburg strain	Laboratory collection (47)
RG4365	<i>B. subtilis</i> natto strain	Laboratory collection (37)
MR644	JH642 $\Delta sigB::cat$	Laboratory collection (25)
RG5567	RG4365 $\Delta sigB::cat$	MR644→RG4365 (this work)
RG5568	NCIB3610 $\Delta sigB::cat$	MR644→NCIB3610 (this work)
MR655	JH642 <i>amyE::PsigB-gfp::cat</i>	Laboratory collection (24)
MR101	JH642 <i>amyE::Pctc-lacZ::cat</i>	Laboratory collection (25)
NRS2289	JH642 <i>amyE::PbslA-lacZ::kan</i>	Laboratory collection (12)
RG5569	NRS2289 $\Delta sigB::cat$	NRS2289→JH642
RG5570	JH642 <i>amyE::PepsG-lacZ::kan</i>	Laboratory collection (12)
RG5571	RG5570 $\Delta sigB::cat$	RG5570→JH642
RG5572	JH642 $\Delta rsbU::kan$	Laboratory collection (24)
RG5573	JH642 $\Delta rsbP::spc$	Laboratory collection (24)
RG5574	JH642 $\Delta rsbUP::kan-spc$	Laboratory collection (24)
RG5575	RG5570 <i>amyE::Pctc-lacZ::cat</i>	MR101→RG5570 (this work)
RG5576	RG5571 <i>amyE::Pctc-lacZ::cat</i>	MR101→RG5571 (this work)
RG5577	RG5572 <i>amyE::Pctc-lacZ::cat</i>	MR101→RG5572 (this work)
RG438	JH642 <i>amyE::PsinR-lacZ::spc</i>	Laboratory collection (40)
RG432	JH642 $\Delta sinR::ery$	Laboratory collection (40)
RG4576	NCIB3610 $\Delta sinR::ery$	RG432→NCIB3610 (this work)
RG5578	MR644 $\Delta sinR::ery$	RG432→MR644 (this work)
RG5580	RG438 $\Delta sigB::cat$	RG438→MR644 (this work)
RG4500	JH642 <i>amyE::Pspo0A-lacZ::ery</i>	Laboratory collection (37)
RG4501	RG4500 $\Delta sigB::cat$	MR644→RG4500 (this work)
RG4503	JH642 <i>amyE::PabrB-lacZ::ery</i>	Laboratory collection (40)
RG4504	RG4503 $\Delta sigB::cat$	MR644→RG4503 (this work)
RG4505	JH642 <i>amyE::PsinI-lacZ::ery</i>	Laboratory collection (40)
RG4506	RG4505 $\Delta sigB::cat$	MR644→RG4505 (this work)
RG4507	JH642 <i>amyE::PcodY-lacZ::ery</i>	Laboratory collection (13)
RG4508	RG4507 $\Delta sigB::cat$	MR644→RG4507 (this work)
RG4509	JH642 <i>amyE::PcomA-lacZ::ery</i>	Laboratory collection (13)
RG4510	RG4509 $\Delta sigB::cat$	MR644→RG4509 (this work)
RG4511	JH642 <i>amyE::PdegSU-lacZ::ery</i>	Laboratory collection (13)
RG4512	RG4511 $\Delta sigB::cat$	MR644→RG4511 (this work)
RG4513	JH642 <i>amyE::Prok-lacZ::ery</i>	Laboratory collection (13)
RG4514	RG4513 $\Delta sigB::cat$	MR644→RG4513 (this work)
RG4515	JH642 <i>amyE::PluxS-lacZ::ery</i>	Laboratory collection (47)
RG4516	RG4515 $\Delta sigB::cat$	MR644→RG4515 (this work)
RG4517	JH642 <i>amyE::PsigH-lacZ::ery</i>	Laboratory collection (25)
RG4518	RG4517 $\Delta sigB::cat$	MR644→RG4517 (this work)
RG4519	JH642 <i>amyE::PtrnA-lacZ::ery</i>	Laboratory collection (13)
RG4520	RG4519 $\Delta sigB::cat$	MR644→RG4518 (this work)
TB24	NCIB3610 $\Delta hag::spc$	Laboratory collection (12)
RG4521	RG5568 $\Delta hag::spc$	TB24→RG5568 (this work)
TB25	TB24 $\Delta bsIA::kan$	Laboratory collection (12)
RG4522	TB24 $\Delta sinR::ery$	RG432→TB24 (this work)
RG4523	MR101 $\Delta sinR::ery$	RG432→MR101 (this work)
RG5581	NCIB3610 $\Delta rsbU::kan$	RG5572→NCIB3610 (this work)
RG5582	NCIB3610 $\Delta rsbP::spc$	RG5573→NCIB3610 (this work)
RG5583	NCIB3610 $\Delta rsbUP::kan-spc$	RG5581→RG5582 (this work)

^aComments are formatted as DNA from donor strain→receptor strain.

growth (50 h). Although SigB activity declined after reaching maximal expression (300 Miller units [MU] at 50 h of development), the level was still significantly higher (220 MU at 70 h of development) than that when the biofilm was younger (120 MU at 20 h of development) (Fig. 1A). The pattern of fluorescence protein production by a JH642-isogenic strain harboring the *sigB-gfp* reporter fusion (strain MR655) (Table 1) confirmed the presence of SigB activity under biofilm-supporting conditions (Fig. 1B to D). The activation pattern of SigB during the biofilm life cycle (Fig. 1A) was similar to the previously reported SigB activity pattern in planktonic cultures of *B. subtilis* (21, 30, 31), as shown in Fig. S1 in the supplemental material. Overall, these results suggest that for genetically equivalent (kin) cells living as a sessile biofilm (Fig. 1) and in a free

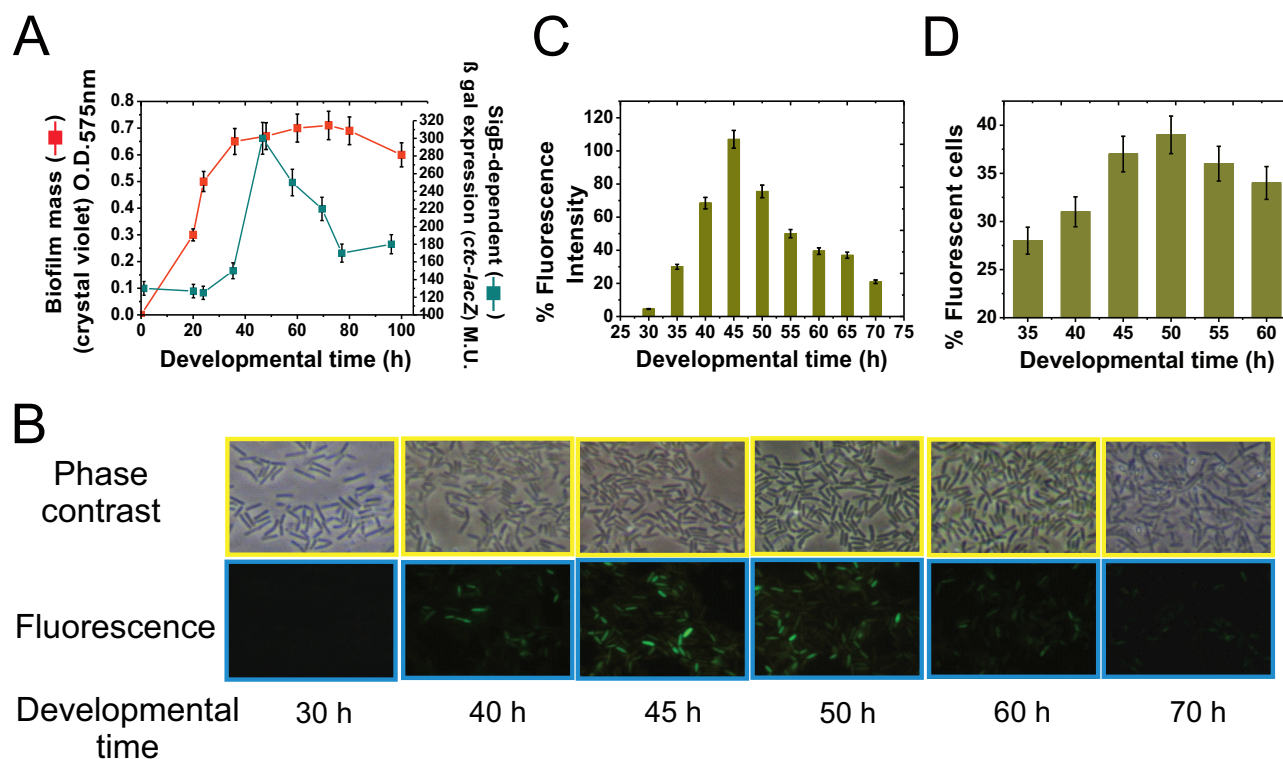


FIG 1 SigB expression during the biofilm cell cycle. (A) Kinetics of biofilm formation and expression of the SigB-dependent *ctc* gene in biofilm-supporting LBY static culture. O.D._{575nm}, optical density at 575 nm; β -Gal, β -galactosidase. (B) Representative photographs from inner biofilm regions of GFP-expressing isogenic JH642 cells (strain MR655) at the indicated development times. Cells from exterior regions of the biofilm did not exhibit significant SigB-directed fluorescence (data not shown). Representative results from three independent experiments performed in parallel are shown. (C) Relative fluorescence intensity, indicated as a percentage of maximal (100%) SigB-directed *gfp* expression by the JH642 isogenic strain MR655 (*amyE::P_{SigB}-gfp::cat*). Maximal fluorescence (100%) was considered to occur at 45 h of biofilm incubation. All of the other relative fluorescence levels, at the different times, were calculated as a percentage of the maximum fluorescence observed at 45 h of biofilm development. (D) Percentage of cells taken from inner regions of the biofilm that express GFP (MR655 strain). Each data point shown in panels A, C, and D is the mean \pm SEM from a representative experiment performed in triplicate.

planktonic state (Fig. S1), entry into the stationary phase of growth represents a stressful and threatening condition that *B. subtilis* might at least partially manage via activation of SigB and enhanced expression of its regulon. The other adaptive pathway that *B. subtilis* biofilm cells might employ for stress management at the onset of the stationary phase is sporulation (9, 11, 32). However, sporulation is largely prevented in nutrient-enriched media because it is subject to nitrogen and carbon catabolite control (9, 32). Under our experimental growth conditions in rich medium (i.e., LBY), the sporulation frequency of the wild-type strain after 50 h of biofilm development was less than 0.05% (1.8×10^8 viable cells and 1.1×10^5 spores ml^{-1} , respectively). Therefore, the sporulation pathway does not significantly influence biofilm stress adaptation under our experimental conditions.

To evaluate the effect of SigB activity on biofilm development, we monitored biofilm formation in JH642 cells with and without SigB activity (JH642 and MR644 strains, respectively) (Table 1). At early times of incubation (from 0 to 30 h of development), there was no significant difference in biofilm generation between SigB-proficient and SigB-deficient cultures. However, at later times of biofilm development, the ΔsigB cells produced more biofilm than did wild-type cells, and when the wild-type biofilm had reached the stationary phase and halted growth (50 h of incubation), the ΔsigB biofilm continued to grow (Fig. 2A). Synthesis of the main biofilm matrix components (e.g., the hydrophobin BslA and exopolysaccharide) (4, 33) continued during the stationary phase of the ΔsigB biofilm but ceased in the case of the wild-type biofilm, finally resulting in a 2.5-fold higher mass for the former than the latter (Fig. 2B and C). To assess whether the biofilm phenotype observed in ΔsigB cells derived from the JH642

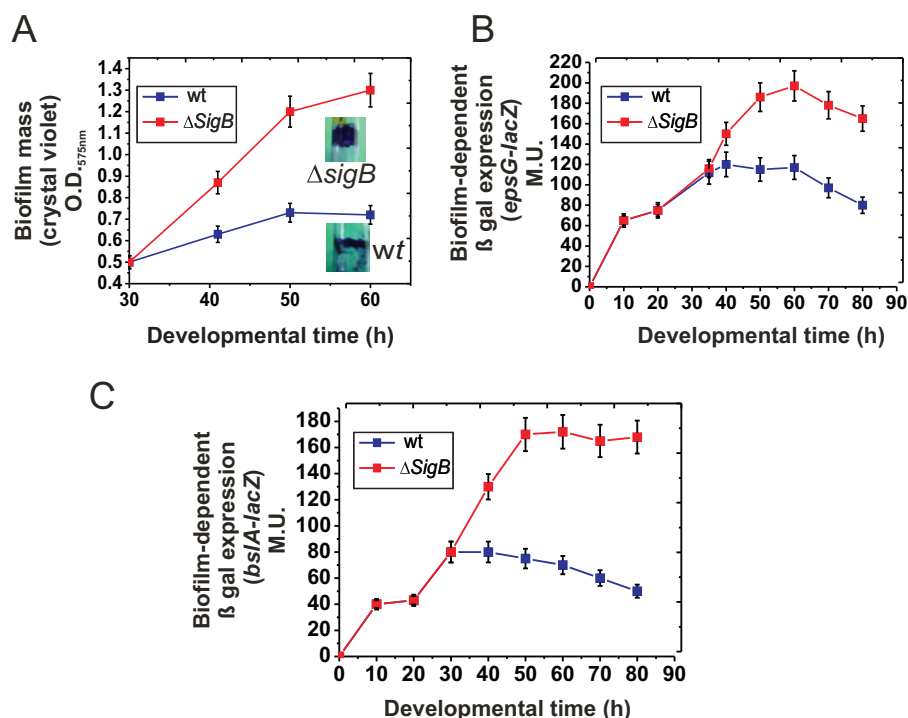


FIG 2 SigB regulates biofilm growth. (A) Rate of biofilm (floating pellicle) formation by wild-type (wt) JH642 and isogenic Δ SigB cells grown in static LB-Y broth. Inset photographs are representative of typical biofilms developed for 50 h and stained with crystal violet. (B) β -Galactosidase activity of the biofilm-reporter fusion *epsG-lacZ::amyE* in wild-type and isogenic Δ SigB cells grown in static LB-Y broth. A typical output of three independent experiments performed in parallel is shown. (C) β -Galactosidase activity of the biofilm-reporter fusion *bsIA-lacZ::amyE* in wild-type and isogenic Δ SigB cells grown in static LB-Y broth. Each data point is the mean \pm SEM. A typical output of three independent experiments performed in parallel is shown.

strain was due to its domesticated nature (29, 34–36), we analyzed the effect of SigB on biofilm formation in wild (undomesticated) strains of *B. subtilis*. The increased production of biofilm by SigB-deficient cells relative to the amount produced by wild-type cells was independent of the genetic pedigree (domesticated versus undomesticated) of different wild *B. subtilis* isolates (i.e., the Marburg- and natto-related strains NCIB3610 and RG4365, respectively) (34, 37) (Table 1 and Fig. S2A). Additionally, the observed increase in the amount of Δ SigB biofilms compared to that of wild-type biofilm was independent of the growth medium used (MSgg or LB-glycerol-manganese broth) (data not shown) as well as the supportive surface (i.e., glass or plastic; tubes, bottles, or microtiter wells) (Fig. S2B). Therefore, SigB acts as a negative regulator of biofilm formation in domesticated and undomesticated (wild) *B. subtilis* strains.

The products of SigB-controlled genes allow planktonic cells to adapt to and survive different environmental and/or metabolic stresses (21, 30, 31, 38). To examine the role of SigB in stress adaptation and the survival of embedded biofilm cells, we monitored the survival of SigB-proficient and SigB-deficient biofilms after exposure to particular stresses at different times after the start of biofilm formation. A drastic drop in pH (Fig. 3A) and heat shock, alcohol stress, and biocide treatments (Fig. S3) all resulted in higher resistance in wild-type biofilms during the stationary phase than in SigB-deficient biofilms that had reached the stationary phase. Thus, despite the fact that SigB-deficient cells produced thicker biofilms, they were more susceptible to stressful conditions. Interestingly, the cell destiny of the biofilms differed significantly. As shown in Fig. 3B, a higher proportion of SigB-deficient cells than wild-type cells displayed cell death at advanced times of biofilm development (15% and 55% of cellular survival in Δ SigB and wild-type biofilms after 60 h of biofilm development, respectively) in the

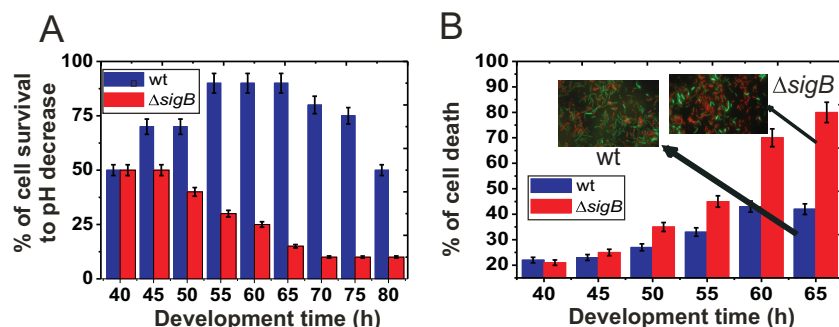


FIG 3 SigB regulates biofilm fitness. (A) Cell survival in wild-type JH642 and $\Delta sigB$ (strain MR644) biofilms after a drastic decrease in pH. At the indicated developmental times, wild-type and $\Delta sigB$ biofilms were exposed to a pH of 3.0 for 1 h before viable cells were counted, as indicated in Materials and Methods. A typical output of three independent experiments performed in parallel is shown. (B) Cell survival in wild-type JH642 and $\Delta sigB$ (strain MR644) biofilms of different ages were measured as the percentage of the total number of fluorescent cells observed in 10 different fields after staining with the LIVE/DEAD BacLight kit (green and red cells are alive and dead cells, respectively). Inset photographs are representative of several pictures taken at the indicated time. Each data point is the mean \pm SEM from a representative experiment performed in triplicate.

absence of external stress. Therefore, SigB-deficient cells produce more biofilm than do wild-type cells but die faster.

The RsbP-dependent pathway controls SigB activation during biofilm development. We next explored the regulatory route (Fig. 4A) that controls activation of the alternative sigma factor SigB during biofilm development. To this end, we monitored biofilm formation in isogenic JH642 strains with altered pathways of SigB activation (i.e., RG5572- $\Delta rsbU$, RG5573- $\Delta rsbP$, and RG5574- $\Delta rsbUP$ strains) (Table 1). For the $\Delta rsbU$ strain, the amount and kinetics of biofilm formation were very similar to those displayed by wild-type cells (Fig. 4B and C). Interestingly, the biofilm-forming capacity of $\Delta rsbP$ and $\Delta rsbUP$ strains resembled that of $\Delta sigB$ cells (Fig. 4B and C). These results strongly suggest that during biofilm development in LBY broth, the RsbP route is responsible for activation of SigB inside the biofilm. Monitoring of β -galactosidase activity from the SigB-dependent *ctc-lacZ*-reporter fusion in *B. subtilis* cells without RsbP and/or RsbU activities confirmed the proposed conclusion (Fig. 4D). The RsbP route senses energy depletion (nutrient deprivation) in planktonic cultures of *B. subtilis* (16, 17, 21, 23), and therefore it can be assumed that this route also senses energy (nutrient) depletion inside the biofilm. However, the possibility that RsbP senses an unknown signal that might be present exclusively in the biofilm and absent from (or weaker in) planktonic cultures cannot be excluded.

The effect of SigB on biofilm formation was independent of incubation temperature and not related to particular temperatures and the edge of the growth range (24, 25) (Fig. S4). Exposure of aged biofilms lacking either the RsbP or RsbU route of environmental or energy stress (strong pH variation or acute ATP depletion, respectively) sensing confirmed that the RsbP-dependent pathway controls activation of SigB and confers general, unspecific, and preventive stress protection (16, 17) to aging biofilms (Fig. 4E and F).

To further support the current findings, we monitored biofilm development in the well-studied biofilm-forming strain NCIB3610 (34) and isogenic derivatives affected in different routes of SigB activation (Fig. S5). The data obtained confirmed that the RsbP-dependent route controls SigB activation under biofilm-supporting conditions in domesticated and undomesticated *B. subtilis* isolates (strains JH642 and NCIB3610, respectively) (29, 34).

SigB maintains SinR levels in sessile *B. subtilis* populations. To assess how SigB prevents biofilm overgrowth, we tested the best-characterized *B. subtilis* biofilm regulators in SigB-proficient and SigB-deficient isogenic strains under biofilm-supporting conditions. SigB did not significantly affect expression of the genes encoding the

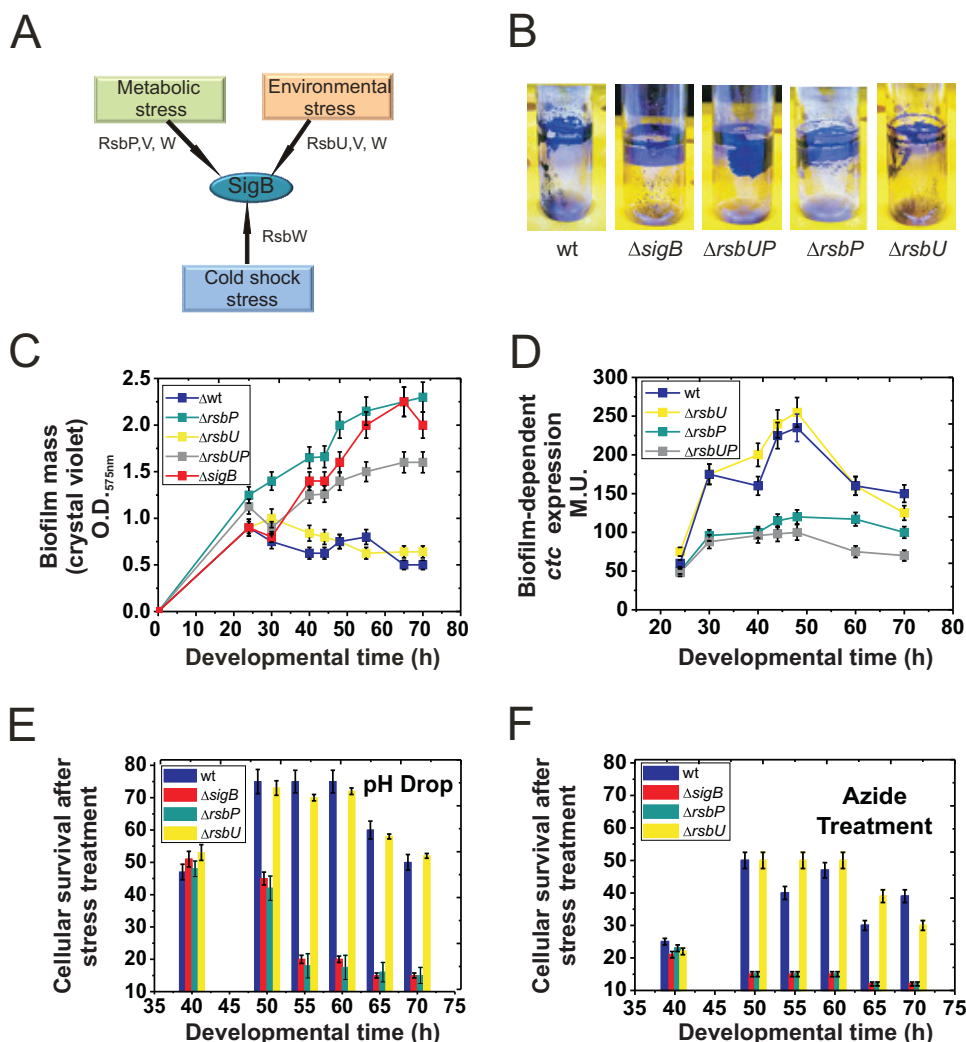


FIG 4 SigB input during the biofilm cell cycle. (A) Cartoon summarizing the three possible routes of SigB activation (see the text for details). (B) Fifty-hour-old biofilms, as observed after crystal violet staining, of wild-type JH642 and isogenic mutants with altered routes of SigB activation: strain MR644 ($\Delta sigB$), strain RG5572 ($\Delta rsbU$), strain RG5573 ($\Delta rsbP$), and strain RG5574 ($\Delta rsbUP$) (Table 1). Representative pictures of five independent experiments are shown. (C) Rate of biofilm (pellicle) formation by the wild type and different isogenic mutants with altered SigB activity shown in panel B. A typical output of three independent experiments performed in parallel is shown. (D) β -Galactosidase activity of the SigB-dependent reporter fusion *ctc-lacZ::amyE* in wild-type and isogenic mutants with altered SigB activity grown in static LB broth. (E and F) Cell survival in biofilms of wild-type JH642 and isogenic mutants with altered SigB activity after a drastic decrease in pH (E) or treatment with azide (F). The percentage of cellular survival is referenced to the number of CFU of biofilms of the same age that had not been exposed to stress. For panels B to F, typical output \pm SEM from five independent experiments is shown.

following direct and indirect key regulators of biofilm formation: ComA (39), Spo0A (29), AbrB (26), SigH (34), SinI (40), CodY (41), DegSU (42), LuxS (37), TnrA (32), and Rok (39) (Fig. S6). However, SigB was required to maintain expression of *sinR* (40) during the stationary phase of sessile cultures of *B. subtilis* (Fig. 5A). Interestingly, SinR is a master (negative) regulator of *B. subtilis* biofilm formation because it represses expression of the key operons, namely, *epsA-O* and *tapA-sigW-tasA*, involved in the synthesis of exopolysaccharide and the TasA amyloid fibers of the biofilm matrix, respectively (14, 15). Therefore, in $\Delta sigB$ cells, lower levels of *sinR* expression would result in increased biofilm formation. To obtain further experimental evidence that supports this interpretation, we measured biofilm growth in *B. subtilis* cultures with and without SigB and/or SinR activities. Interestingly, the increased biofilm formation observed for the $\Delta sinR$ strain (relative to the final size of the wild-type biofilm) (Fig. 5B and C) was quite similar

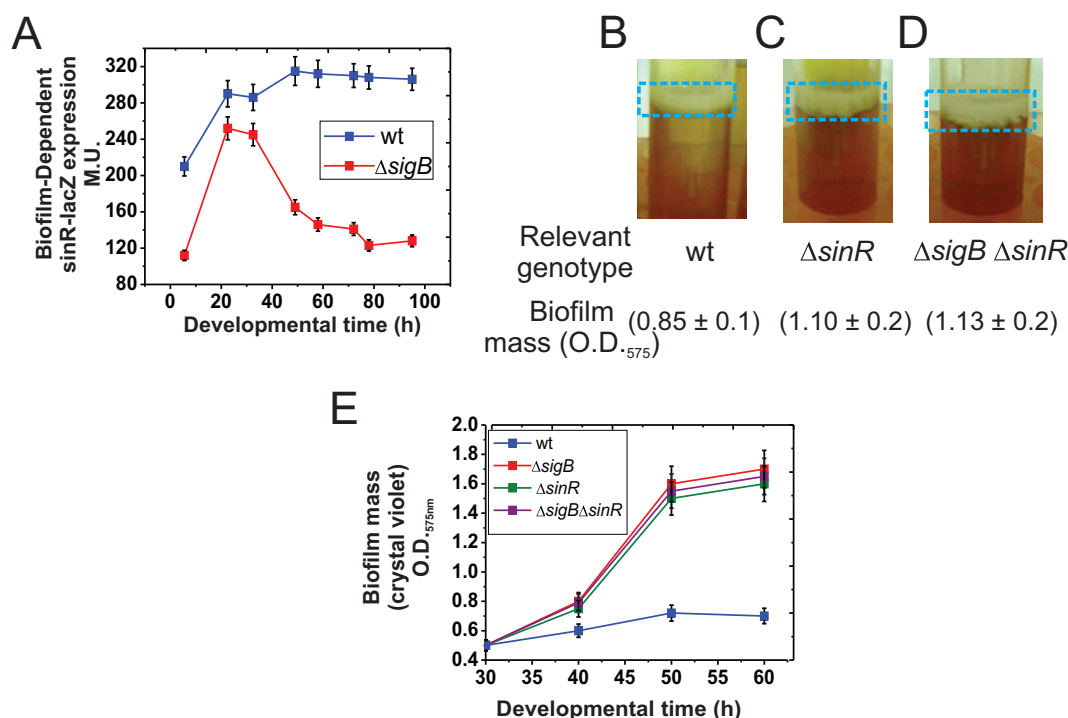


FIG 5 SigB regulates *sinR* expression. (A) β -Galactosidase activity from the *sinR-lacZ::amyE* reporter fusion in wild-type and isogenic $\Delta sigB$ cells grown in static LB. Typical output from one of four independent experiments is shown. (B to D) SinR and SigB do not generate additive effects on biofilm growth. Fifty-hour-old representative biofilms developed by standing cultures of the wild type (strain JH642) and isogenic mutants with altered SinR ($\Delta sinR$, strain RG432) and/or SigB ($\Delta sigB \Delta sinR$, strain RG5578) activity. Biofilm mass was measured using the crystal violet technique, as indicated. Similar results were observed in several independent experiments. (E) Rate of biofilm formation by the wild type and different isogenic mutants with altered SigB, SinR, and SigB-SinR activities. Each data point in panels A and E is an average \pm SEM from five independent representative experiments.

to that observed for $\Delta sinR \Delta sigB$ cells (Fig. 5D). Thus, biofilm growth (Fig. 5E) did not increase above the level observed in SigB-deficient cells by inactivating the natural negative regulator of biofilm formation, SinR. Additionally, SinR did not affect SigB expression (Fig. S7). Overall, these results indicate that SigB operates upstream of SinR to control the growth and fate of *B. subtilis* biofilms. The finding that the biofilm phenotype and its size were almost identical in $\Delta sigB$, $\Delta sinR$, and $\Delta sigB \Delta sinR$ strains strongly suggests that SinR is the main target of SigB to regulate biofilm fate.

SinR is also essential for flagellum-mediated *B. subtilis* motility (swimming and swarming), and *sinR* mutant strains are completely nonmotile (12, 43, 44). Therefore, if SigB is required to maintain *sinR* expression, it would be expected that flagellum-dependent motility would also be affected by SigB. Accordingly, swimming (Fig. 6A and C) and swarming (Fig. 6B and D) were reduced in $\Delta sigB$ cells compared to their levels in wild-type cells. However, in contrast to biofilm thickness, motility displayed SigB-dependent, SigB-independent, and SinR-dependent components. Conversely, sliding proficiency, which is another type of social motility behavior that is flagellum independent (and therefore independent of SinR activity) (12), was not affected by inactivating SigB (Fig. S8). In summary, the results confirmed that SigB-dependent downregulation of biofilm overgrowth is mediated by controlling the activity of SinR.

SigB regulates biofilm dispersal. Although dispersal is likely to be crucial for bacterial survival, the gene network and signal transduction pathways controlling this phenomenon in bacilli are completely unexplored (4). In old (mature) biofilms, surface-attached cells detach and disperse into the environment to search for new food supplies and/or to colonize new sites. Studies of biofilm dispersal in Gram-negative bacteria and Gram-positive cocci have focused on identifying signals that trigger dispersal, one of which is nutrient availability (5, 7, 8, 45). Accordingly, we found that

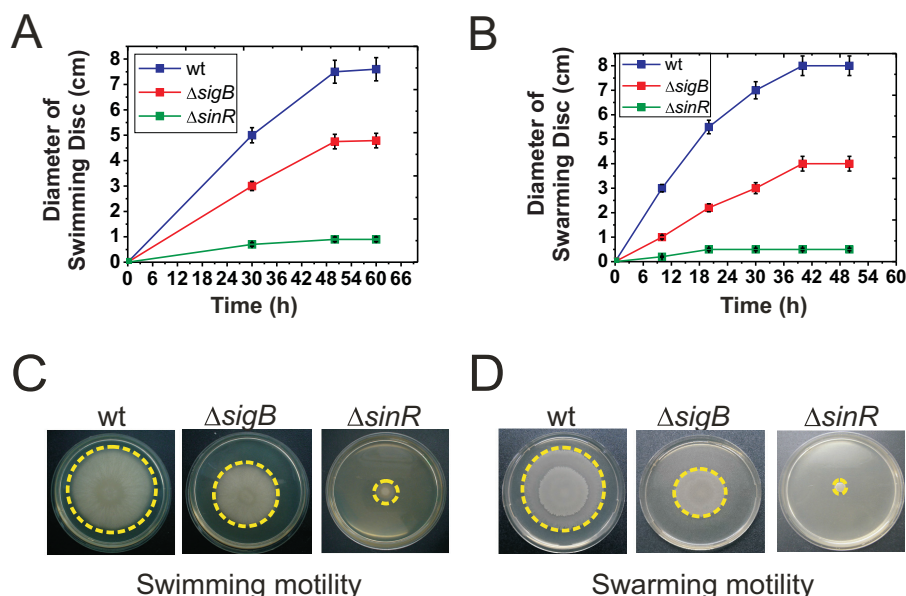


FIG 6 SigB regulates flagellum-dependent motilities in *B. subtilis*. (A) Swimming proficiencies of wild-type (strain JH642) and isogenic mutants with altered SigB or SinR activity (strains MR644 and RG432, respectively). (B) Swarming proficiencies of the wild type (strain NCIB3610) and isogenic mutants with altered SigB or SinR activity (strains RG5568 and RG4576, respectively). For the swarming experiments, the NCIB3610 strain was utilized instead of the JH642 strain, because the latter strain does not swarm. (C and D) Representative pictures from several independent experiments of top-viewed swimming (C) or swarming (D) cells after 48 h of incubation are shown. Each data point in panels A and B is the mean \pm SEM from a representative experiment performed in triplicate.

RsbP (which, in planktonic cultures, senses the nutritional status of the population) activated SigB inside the biofilm (Fig. 4), which in turn maintained the expression levels of the cell motility regulator SinR (Fig. 5). Therefore, it can be predicted that dispersal, a biofilm property that relies on motility proficiency, is also positively affected by the alternative sigma factor SigB. To test this hypothesis, we monitored the cell dispersal of wild-type and $\Delta sigB$ *B. subtilis* biofilms over time. Because biofilm dispersal has not been previously studied in Gram-positive bacilli, we adapted methodologies used to monitor the dispersal of other bacteria (8, 46). In one case (Fig. 7A), we quantified dispersal from a solid biofilm (colony) by counting the dispersed (swimming) cells (in CFU) present in the medium surrounding the biofilm (8); in the second case, we measured formation of a cellular ring (biofilm pellicle) located on top of a preformed biofilm from where dispersed cells produced the aforementioned ring (8) (Fig. 7B). As expected, in both cases, significantly less dispersal (2.5- to 3.5-fold) occurred for $\Delta sigB$ biofilms than for wild-type biofilms (Fig. 7A and B). The reduction in the number of cells dispersing from the biofilm might be a direct consequence of the lack of SigB activity or an indirect consequence of the reduced number of living cells observed in aging $\Delta sigB$ biofilms than in wild-type biofilms (Fig. 3B). However, dispersal also requires proficiency in cellular motility (5–8, 45, 46), and in *B. subtilis* this property is regulated by SinR (43, 44), whose levels are reduced in $\Delta sigB$ biofilms (Fig. 5A). Furthermore, as shown in Fig. 7A, the reduced dispersal of $\Delta sigB$ biofilm cells (compared with the dispersal of wild-type biofilm cells) is evident at early times of incubation (before 50 h) well before significant differences in cell death between both types of biofilms occur (Fig. 3B). Again, and similar to motility, dispersal displayed a SigB-dependent but also a SigB-independent but still SinR-dependent component, because dispersal was further reduced in a $\Delta sinR$ mutant compared to that of a $\Delta sigB$ mutant. In summary, the results presented allow us to hypothesize that the physiological role of SigB during the biofilm life cycle is to halt overgrowth of a mature biofilm and to prevent massive cell death through SinR-mediated dispersal activation when environmental and/or

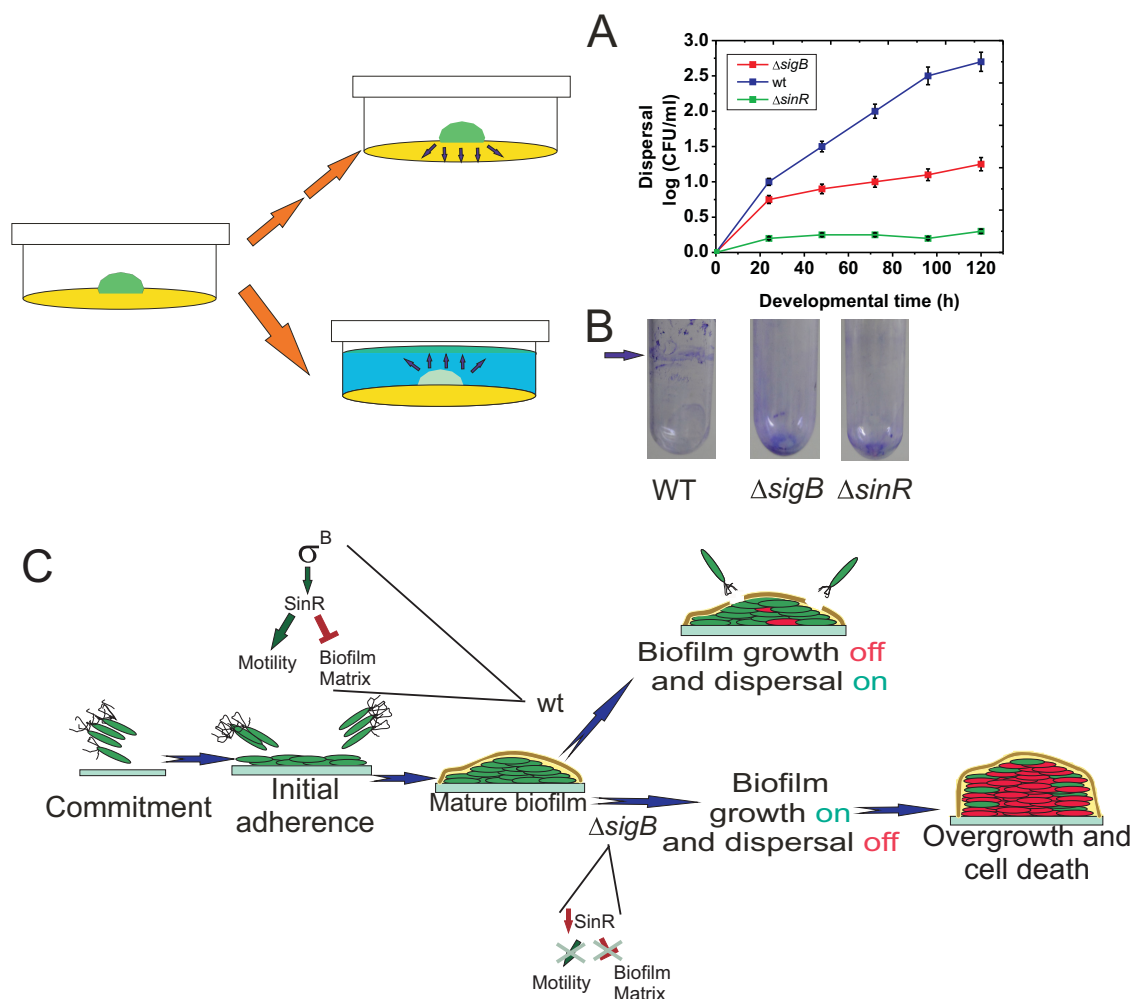


FIG 7 SigB regulates biofilm dispersal. (A and B) Quantification of cell dispersal from wild-type and $\Delta sigB$ biofilms. (A) Colonies of JH642 and isogenic $\Delta sigB$ and $\Delta sinR$ derivatives (strains MR644 and RG432, respectively) developed on 2-cm-diameter wells poured with LB medium (yellow in the cartoon) prepared with 0.8% agar and incubated for 30 h at 37°C, as indicated in Materials and Methods. At the indicated times, the agar (yellow in the cartoon) surrounding each solid biofilm (green in the cartoon) was carefully removed, and trapped cells were eluted and washed before appropriate serial dilutions were plated on LB agar plates as described in Materials and Methods. After 24 h of incubation at 37°C, the number of CFU ml⁻¹ was calculated and plotted. The means \pm SEM from 10 independent experiments are shown. (B) Colonies of wild-type (JH642), $\Delta sigB$ (MR644), and $\Delta sinR$ (RG432) strains were developed for 30 h at 37°C on 0.2 ml of LB medium solidified with 2% agar at the bottom of a glass tube. The colonies were then carefully covered with 2.5 ml of LB broth (blue in the cartoon) and incubated for another 15 h at 25°C to visualize the cellular ring (new green pellicle biofilm in the cartoon) produced at the liquid-air interface by the dispersed cells (see Materials and Methods for details). Ring cells adhered to the well walls were visualized by crystal violet staining (arrow). In the case of $\Delta sigB$ and $\Delta sinR$ biofilms, the staining at the bottom of the tubes represents the dispersal-defective SigB and SinR cells adhered to the glass bottom. A typical result from 10 independent experiments is shown. (C) Proposed model for the role of SigB during the biofilm life cycle and its regulation on *sinR* (see the text for details).

metabolic conditions become suboptimal or detrimental to maintaining the fitness of the population (Fig. 7C).

DISCUSSION

The idea of what we now recognize as a bacterial biofilm likely emerged in the 17th century, when Anton Van Leeuwenhoek observed and described microbial aggregates adhered to his teeth. However, it was not until forty years ago that the first reports pointing to the clinical relevance of biofilms appeared (47). Since then, accumulating evidence indicates that biofilms are important for the persistence, evolution, and dissemination of bacteria in multiple environments (2). Not all bacterial biofilms are harmful; indeed, some biofilms play critical and beneficial roles in nature. For example, plant-beneficial bacteria form biofilms in the plant rhizosphere and phyllosphere to

colonize plant tissues (roots and leaves, respectively) and promote plant growth (48), and animal and human probiotics produce beneficial biofilms at the gut mucosa to improve host health and immunity (49, 50). Interestingly, it was recently demonstrated that probiotic *B. subtilis* can prevent *S. aureus* biofilm formation and colonization in both humans and mice when the bacilli were coadministered with the diet (51). In addition, other useful biofilms are used in industry, for example, to reduce steel corrosion and to explore novel compounds of biotechnological interest (52).

However, the formation of biofilms provokes stressful conditions for the cells involved (53, 54). When conditions are favorable, altruism exists between cells in the biofilm (kin cooperation), but when conditions become unfavorable, competition arises (kin rivalry). How kin cooperation and kin competition are resolved at the community level is beginning to be understood (5, 33, 55–57). For example, as a biofilm grows in size, the resident bacteria become crowded, and cells in the inner parts will experience reduced access to nutrients and electron acceptors and will accumulate waste products and toxin-like by-products (54). How do trapped biofilm cells cope with this self-generated life-threatening situation? Larger colonies produce greater detachments of individual cells than do smaller colonies, an observation that inspired the proposal that a certain maturation stage of the colony, as defined by its size, is linked to the nutritional status of the inner parts of the community to trigger dispersal events (58, 59). Supporting this interpretation, a positive relationship between dispersal proficiency and biofilm size has been reported for the pathogens *Streptococcus intermedius* and *Pseudomonas aeruginosa* (60, 61). In *P. aeruginosa*, swimming biofilm cells appear to initiate detachment when the size of the colony reaches a threshold diameter (60). In the early steps of infection by *S. intermedius*, the high-molecular-mass polysaccharide hyaluronan (HA), a major component of the extracellular matrix of connective tissue, allows bacterial adherence and biofilm formation. A common mechanism used by *S. intermedius* and other bacteria to allow dispersal involves the production of extracellular enzymes that degrade adhesive components of the biofilm extracellular matrix (7, 8, 45). Accordingly, hyaluronidase-defective mutant strains of *S. intermedius* are deficient in dispersal, forming 31% more biofilm than a wild-type strain in medium supplemented with HA (61). Importantly, and in accordance with our results, Singh et al. recently reported that the general stress-responsive transcription factor RpoS (SigS), the SigB functional homolog present in Gram-negative bacteria, is also activated by starvation to trigger biofilm dispersal in the human pathogen *Vibrio cholerae* (62).

Here, we show that SigB-deficient cells are unable to sense stress and maintain SinR levels as the biofilm ages (Fig. 4 and 5). As a consequence of such metabolic imbalance, the biofilm continued to grow (Fig. 2) and became less resistant to diverse stresses (Fig. 3; see also Fig. S3 in the supplemental material); dispersal was also downregulated (Fig. 7). How SigB regulates *sinR* expression is an unsolved question. The only known signal that regulates (activates) the expression of *sinR* is alcohol stress (40), which is also known as one of the best inducers of the SigB-dependent general stress response (16, 17). The simplest scenario for the influence of SigB on *sinR* expression would be direct binding of SigB to the *sinR* promoter. However, even the most comprehensive characterization of the transcriptional landscape of *B. subtilis* performed to date (63) did not reveal a SigB-dependent promoter directly upstream of *sinR* or in front of *sinI*. Unfortunately, all studies on regulation of SigB activity have so far been uniquely confined to the planktonic growth style of *B. subtilis* but not related to its sessile life style. Therefore, it is likely that SigB activates *sinR* expression indirectly via an unidentified pathway that operates under biofilm-supporting conditions. The likely target of this regulation is the promoter directly upstream of *sinR* (Fig. 5A), because no effect was observed on expression of *sinI* (Fig. S6C).

Because SigB and its functional homologs (i.e., SigS) are also present in pathogenic bacteria (17, 62, 64–66) and likely perform similar functions, blocking expression or activity of SigB or its homologs in pathogens might constitute a novel strategy for fighting harmful biofilms. Nonetheless, *B. subtilis* and other bacilli (i.e., *Bacillus coagulans*, *B. amyloliquefaciens*, and *B. thuringiensis*) have beneficial effects on agriculture and

human health (13, 48, 51, 52, 67). In both situations, formation of a beneficial biofilm at the level of the plant rhizosphere or the human gut mucosa would be desired, and industrial/medical interventions with molecules or treatments that induce SigB might contribute to this goal.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. The *B. subtilis* strains used in this study are summarized in Table 1. Bacterial cultures were maintained in Luria-Bertani (LB) broth; for biofilm formation, LB was fortified with 4% yeast extract (LBY medium) as described previously (29). When appropriate, antibiotics were included at the following final concentrations: 1 μ g/ml erythromycin (Ery), 5 μ g/ml kanamycin (Kan), 5 μ g/ml chloramphenicol (Cat), and 50 μ g/ml spectinomycin (Spc). Transformation of *B. subtilis* to obtain isogenic derivatives of the parental strains was carried out as previously described (12). The cultures used to assess β -galactosidase activity were grown at 37°C in LBY broth without shaking (static cultures) (29). For each time point, 5 ml of LBY inoculated with 20 μ l of an overnight culture of the corresponding strain was incubated in glass test tubes until the indicated developmental time. After the corresponding incubations, each pellicle biofilm was disrupted (dissolved) by vortexing during at least 3 min at room temperature and centrifuged at 4,500 rpm for 10 min, and the cellular (biofilm) pellet was resuspended in 1 ml of Z buffer for measuring of specific β -galactosidase activity (expressed in Miller units, or MU) as described previously (29). An inverted fluorescence microscope (Axiovert 25; Zeiss) was used to visualize green fluorescent protein (GFP) expression, and images were photographed, recorded, and analyzed with an image capture system (Olympus software and Fiji software). The LIVE/DEAD BacLight kit from Thermo-Fisher was used to evaluate the viability of biofilm cells according to the manufacturer's recommendations. For the case of SigB-directed GFP expression during biofilm formation, 10 different microscope fields for each developmental time point were analyzed in triplicate, and the percentage of fluorescent cells refers to the total number of GFP-expressing and non-GFP-expressing cells counted at that time. For sporulation efficiency, *B. subtilis* biofilms were developed in LBY broth for 48 h, disrupted, and resuspended in sterile water. The biofilm cells were serially diluted, and appropriate dilutions were plated on LB agar plates and incubated for 24 h at 37°C to determine the total number of viable cells (spores plus vegetative cells). The same serial dilutions were treated with 10% CHCl₃ for 5 min before plating to determine the number of spores (32).

Biofilm formation and dispersal. The different *B. subtilis* strains used in this study were grown overnight at 37°C in LB medium with antibiotic supplementation (if appropriate); 20 μ l of each culture was diluted in 5 ml of fresh LBY broth for pellicle formation in test tubes or as indicated in the figure legends when the biofilm was developed in microplates or bottles. The tubes (or microplates or bottles, as indicated) were statically incubated at 37°C for the indicated times. The amount of biofilm was measured by the crystal violet technique (29).

For dispersal experiments, a solid biofilm (colony) was developed on the center of 2-cm-diameter wells (12-well microtiter plates) containing LBY medium with 0.8% agar; this agar concentration allows swimming cells to enter the agar surrounding the solid biofilm. At the indicated times, the soft agar surrounding the solid biofilm was carefully removed and soaked (at 50 rpm) in 5.0 ml of sterile water supplemented with a sub-MIC amount of nalidixic acid (0.1 μ g/ml) to avoid cellular duplication for 2 h at room temperature. After this incubation time, the undissolved soft agar was carefully removed and the solution containing the eluted cells was centrifuged at 4,500 rpm for 15 min at room temperature. The supernatant was discarded and the cellular pellet was dissolved in 1 ml of sterile water, and appropriate serial dilutions were plated on LB agar plates. After 24 h of incubation at 37°C, the number of CFU per milliliter was calculated and plotted. In another set of experiments, biofilm dispersal was detected by the formation of a second biofilm (a ring or pellicle) on top of the first biofilm (8). To this end, a solid biofilm (colony) was developed on 0.2 ml of LBY medium solidified with 2.0% agar for 30 h at 37°C, as indicated above, on the bottom of a glass tube. After this incubation time, 2.5 ml of LBY broth was carefully added through the wall of the glass tube to completely cover the preformed solid biofilm (without detaching or disturbing it), and the sample was incubated for 15 h at 25°C. Formation of a cellular ring (located at the liquid-air interface) indicated the formation of a second biofilm by cells dispersed from the first biofilm.

Stress treatments. Isogenic JH642 and Δ sigB cells were subjected to different types of environmental and metabolic stresses, as described previously (31). For pH stress, biofilms were developed for the indicated times in 48-well microtiter plates at 37°C, and 500 μ l of a pH 3.0 HCl solution was carefully added to the top of the biofilm. After 1 h of incubation at 25°C, the biofilms were disrupted and washed, and appropriate serial dilutions were plated on LB agar plates. CFU were counted after 24 h of incubation at 37°C, and the percentage of cell survival was referenced to the number of CFU of biofilms of the same age that had not been exposed to HCl. For azide treatment, 300 μ l of 50 mM azide solution was carefully added to the top of biofilms developed in 48-well microtiter plates at 37°C. After 1 h of incubation at 25°C, the biofilms were disrupted and washed; appropriate serial dilutions were then plated on LB agar plates and incubated for 24 h at 37°C before CFU counting. Each data point in the figures represents the mean \pm standard errors of the mean (SEM) of a representative experiment performed in triplicate.

Spreading (swimming, swarming, and sliding) experiments. To assess surface (swarming and sliding) and swimming motilities, LB plates fortified with 0.7% or 0.3% agar, respectively, and dried for 1 h were inoculated at the center with 1 μ l of an 8×10^7 -cells ml⁻¹ culture grown to mid-log phase at 37°C in LB broth. The inoculated petri dishes were then incubated at 37°C for the indicated times. The developed swimming, swarming, and sliding cellular discs were visualized using a Stemi 2000 (Zeiss)

stereomicroscope with the KL1500LCD (Zeiss) illumination system. A PowerShot A80 (Canon) system was used to capture images.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00473-18>.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

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We have no conflicts of interest to declare.

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