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Effects of Phosphorelay Perturbations on Architecture, Sporulation, and Spore Resistance in Biofilms of *Bacillus subtilis*‡

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The spore-forming bacterium *Bacillus subtilis* is able to form highly organized multicellular communities called biofilms. This coordinated bacterial behavior is often lost in domesticated or laboratory strains as a result of planktonic growth in rich media for many generations. However, we show here that the laboratory strain *B. subtilis* 168 is still capable of forming spatially organized multicellular communities on minimal medium agar plates, exemplified by colonies with vein-like structures formed by elevated bundles of cells. In line with the current model for biofilm formation, we demonstrate that overproduction of the phosphorelay components KinA and Spo0A stimulates bundle formation, while overproduction of the transition state regulators AbrB and SinR leads to repression of formation of elevated bundles. Time-lapse fluorescence microscopy studies of *B. subtilis* green fluorescent protein reporter strains show that bundles are preferential sites for spore formation and that flat structures surrounding the bundles contain vegetative cells. The elevated bundle structures are formed prior to sporulation, in agreement with a genetic developmental program in which these processes are sequentially activated. Perturbations of the phosphorelay by disruption and overexpression of genes that lead to an increased tendency to sporulate result in the segregation of sporulation mutations and decreased heat resistance of spores in biofilms. These results stress the importance of a balanced control of the phosphorelay for biofilm and spore development.

In nature, bacteria occur predominantly in the form of multicellular communities known as biofilms (9). The gram-positive model organism *Bacillus subtilis* is capable of forming surface-associated communities of cells in a matrix of extracellular polymers organized in complex structures (2, 19). The formation of these structures in *B. subtilis* depends strongly on the growth conditions and is highly variable among strains. Unlike most laboratory *B. subtilis* strains, certain undomesticated strains are able to form colonies with elevated structures and fruiting bodies that preferentially produce spores at their tips (2, 46). In this work, we define *B. subtilis* biofilms as communities of cells embedded in a polymeric matrix, which can be either pellicles at an air-liquid interface or colonies with vein-like structures grown on semisolid agar surfaces. The master regulator in *B. subtilis*, which governs the transition from free-living (planktonic) cells in liquid medium to sessile cells in a surface-associated biofilm, was found to be SinR (28). A number of genes essential for biofilm formation are under direct control of SinR, such as the putative *epsA-epsO* operon, which encodes exopolysaccharide (EPS) synthesis, and the *yqxM-tasA-sipW* operon, which encodes components of the extracellular matrix. SinR represses the transcription of both operons (28). This activity is counteracted by the product of *sinI*, a small gene upstream of *sinR*, which binds to SinR, thereby releasing SinR from its target DNA (1). The expression of *sinI* is activated by the transcription factor Spo0A~P (45) and repressed by the pleiotropic AbrB regulator, which acts as a global repressor for genes active in the stationary phase during exponential growth (49). The master sporulation transcription factor, Spo0A, can be phosphorylated by the so-called phosphorelay, stimulating its capacity to bind DNA and activate or repress gene transcription (43). The genes repressed by Spo0A~P include *abrB* (18). Therefore, biofilm formation is influenced by the levels of AbrB, Spo0A, and SinI/R, as demonstrated in a number of previous studies (2, 19, 20, 28, 48). This renders the process of biofilm formation highly intertwined with the initiation of sporulation. Fujita and coworkers have shown that within the Spo0A regulon, categories of genes are present that respond to different threshold levels of Spo0A~P (12). In relation to biofilm formation, they showed that *abrB* and *sinI* respond at relatively low levels of Spo0A~P and that most sporulation genes respond at high levels. Altogether, these data indicate a versatile role for the sporulation phosphorelay directing multiple stationary-phase phenomena, including biofilm formation and sporulation.

In this study, we monitor the developmental program of bundle and spore formation in biofilms by the use of green fluorescent protein (GFP) reporter strains in combination with time-lapse fluorescence microscopy. We show that the standard laboratory *B. subtilis* 1A700 strain is able to form colonies

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‡ Supplemental material for this article may be found at http://jb.asm.org/.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Source/reference
Strains		
E. coli		
XL1-Blue	General cloning strain, Tc^r	Stratagene
MC1061	F$^-$ *araD139*Δ(*ara-leu*)*7696* Δ(*lac*)*X74 galU galK hsdR2 mcrA mcrB1 rspL*	6
M15/pREP4	*lac ara gal mtl recA*$^+$ *uvr*$^+$ (pREP4, *lacI* Km^r)	55
B. subtilis		
168 1A700	*trpC2*/parental strain in this study	32; Bacillus Genetic Stock Center
168 1A1	*trpC2*	Bacillus Genetic Stock Center
NCIB3610	Wild type	Bacillus Genetic Stock Center
abrB-gfp	P$_{abrB}$-*gfp* Cm^r	This study
IIA-gfp	P$_{spoIIA}$-*gfp* Cm^r	52
spoIIE-gfp	P$_{spoIIE}$-*gfp* Cm^r	This study
cotC-gfp	P$_{cotC}$-*cotC-gfp* Cm^r	This study
ΔabrB	*abrB*::Cm Cm^r	18
Δspo0A	*spo0A*::Km Km^r	54
ΔspoIIAC	*spoIIAC*::Km Km^r	2
ΔsinR	*sinR*::Sp Sp^r	47
ΔrapA	*rapA*::Cm Cm^r	51
Δspo0E	*spo0E*::Sp Sp^r	22
ΔsinI	*sinI*::Km	Gift from D. Dubnau
sin+	*sinR* multicopy; Cm^r	14
IIA/ΔrapA	P$_{spoIIA}$-*gfp* Cm^r *rapA*::Sp Sp^r	52
IIA/Δspo0E	P$_{spoIIA}$-*gfp* Cm^r *spo0E*::Sp Sp^r	52
IIA/ΔsinR	P$_{spoIIA}$-*gfp* Cm^r *sinR*::Sp Sp^r	This study
IIA/abrB	P$_{spoIIA}$-*gfp* Cm^r *amyE*::P$_{spac}$-*abrB* Sp^r	This study
IIA/kinA	P$_{spoIIA}$-*gfp* Cm^r P$_{spac}$-*kinA* Km^r	This study
IIA/sad67	P$_{spoIIA}$-*gfp* Cm^r *amyE*::P$_{spac}$-*sad67* Tc^r	52
Plasmids		
pQE30	Ap^r Cm^r; T5 promoter, six-His tag	8
pQE30-kinA	*kinA* inserted into pQE30	This study
pDG148-Stu	Ap^r Km^r P$_{spac}$	26
pKinA	P$_{spac}$-*kinA* Ap^r Km^r	This study
pGFP-abrB	Ap^r Cm^r P$_{abrB}$-*gfp*	This study
pGFP-IIE	Ap^r Cm^r P$_{spoIIE}$-*gfp*	This study
pCotC-GFP	Ap^r Cm^r P$_{cotC}$-*cotC-gfp*	This study

with complex architecture in the form of elevated bundles when grown on a chemically defined solid medium. Using a genetic approach, we demonstrate that bundle formation in this strain is regulated similarly to bundle and biofilm formation in an undomesticated *B. subtilis* strain (2). Furthermore, we illustrate that elevated bundles are formed prior to the initiation of sporulation and are the preferred sites for sporulation. Perturbations of the phosphorelay result in the segregation of sporulation mutations and decreased heat resistance of spores in biofilms, demonstrating the importance of a balanced control of the phosphorelay for biofilm and spore development.

(Part of this work was presented in a duo presentation at the 3rd Conference on Functional Genomics of Gram-Positive Microorganisms, San Diego, Calif., 12 to 16 June 2005.)

MATERIALS AND METHODS

Strains, plasmids, and culture media. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown in LB at 37°C, and antibiotics were added when appropriate with the following end concentrations: ampicillin, 100 μg/ml; tetracycline, 10 μg/ml; and kanamycin, 25 μg/ml. *Bacillus subtilis* was cultured at 37°C in either LB or a defined 3-(*N*-morpholino)propanesulfonic acid (MOPS)-buffered growth medium (17), with modifications as described by Branda et al. (2). Final antibiotic concentrations were as follows: tetracycline, 6 μg/ml; erythromycin, 1 μg/ml; chloramphenicol, 5 μg/ml; kanamycin, 10 μg/ml; and spectinomycin, 100 μg/ml. Colonies were obtained by spotting 1 μl of an exponentially growing *Bacillus* culture on 1.5% MOPS agar plates with the appropriate antibiotics, using a Gilson pipette. Agar plates were sealed with parafilm to avoid evaporation, followed by incubation for 1 to 4 days at 30°C, unless specified otherwise. Numbers of CFU were determined on Trypticase soy broth (TSB) pour plates. The swarming abilities of wild-type and laboratory strains of *B. subtilis* were assessed by plating a 1-μl droplet of exponentially growing cells on a 0.3% agar plate, followed by overnight incubation at 30°C.

Recombinant DNA techniques and oligonucleotides. Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of *E. coli* were carried out as described by Sambrook et al. (44). Enzymes were obtained from Roche (Mannheim, Germany). *B. subtilis* was transformed as described before (53). The oligonucleotides used in this study are listed in Table 2. In all PCRs, *Pwo* DNA polymerase (Roche Molecular Biochemicals) and DNA of *B. subtilis* 168 1A700 were used.

Construction of mutant and GFP reporter strains. Construction of pKinA was carried out by amplification of *kinA* with primers kinA-F and kinA-R (Table 3). The resulting DNA fragment was digested with BamHI and HindIII and inserted into the linearized pQE30 vector, yielding pQE30-kinA. The DNA fragment encoding the histidine-tagged full-length *kinA* gene was amplified with primers PDG-F and PDG-R, followed by insertion into pDG148-Stu to yield pKinA by a ligation-independent cloning procedure (26). The DNA sequence of the entire open reading frame in pKinA was determined and showed one mismatch in the *kinA* gene (a silent mutation changing the codon for R351 from AGG to CGG). The plasmid pKinA was isolated from *E. coli* MC1061 and transformed to competent *B. subtilis* cells for overexpression of *kinA* under control of the IPTG

TABLE 2. Oligonucleotides used in this study[a]

Oligonucleotide	Sequence (5′ to 3′)	Description and position
abrB-F	CCATCGATCGGCATCTTGAAACCTCCTA	ClaI; 5′ end of P_{abrB}
abrB-R	GGAATTCCTTCATAAACATTCTCCTCCC	EcoRI; 3′ end of P_{abrB}
spoIIE-F	CCCAAGCTTGGCGGTGTAGCTCAGCTGGC	HindIII; 5′ end of P_{spoIIE}
spoIIE-R	TTCTGCAGTTCCATTCCTCTCATCTCCCACCTG	PstI; 3′ end of P_{spoIIE}
cotC-F	GGGGTACCCAGAATTTAAACAAGCAACAAGCGG	KpnI; 5′ end of P_{cotC}
cotC-R-transl	CCCAAGCTTGTAGTGTTTTTTATGCTTTTTATACTCTAC	HindIII; 3′ end of *cotC*
kinA-F	GCGGATCCGATGACGATGACAAAATGGAACAGGATACGCAGCATGTTAAC	BamHI; 5′ end of *kinA*
kinA-R	GCGCAAGCTTATTTTTTTGGAAATGAAATTTTAAACGC	HindIII; 3′ end of *kinA*
PDG-F	AAGGAGGAAGCAGGTATGAGAGGATCGCATCACCATCACCATCAC	LIC; 5′ end of pQE30-*kinA*
PDG-R	GACACGCACGAGGTTATTTTTTTGGAAATGAAATTTTAAACGCTG	LIC; 3′ end of pQE30-*kinA*

[a] Relevant restriction sites are underlined. Abbreviations: F, forward; R, reverse; LIC, ligation-independent cloning. Note that the use of the primers for *abrB* and *spoIIE* resulted in transcriptional GFP fusions; the primer for *cotC* resulted in a C-terminal translational GFP fusion under the control of its own promoter; and the primer for *kinA* resulted in a GFP fusion under the control of an IPTG-inducible promoter.

(isopropyl-β-D-thiogalactopyranoside)-inducible *spac* promoter. Expression of the *kinA* gene was verified by Ni affinity column chromatography, which allowed the isolation of full-length KinA protein from cell extracts of *E. coli*/pQE30-kinA and *B. subtilis*/pKinA (data not shown). To obtain strain IIA/kinA, strain IIA-gfp (52) was transformed with plasmid pKinA. Transformants were selected on LB agar plates containing kanamycin after overnight incubation at 37°C.

To construct plasmid pGFP-abrB, a PCR with the primers abrB-F and abrB-R was performed. The amplified fragment was subsequently cleaved with ClaI and EcoRI and ligated into the corresponding sites of pSG1151 (33) to generate plasmid pGFP-abrB. To construct plasmid pGFP-spoIIE, a PCR with the primers spoIIE-F and spoIIE-R was performed. The amplified fragment was subsequently cleaved with HindIII and PstI and ligated into the corresponding sites of pSG1151 to generate plasmid pGFP-spoIIE. To construct plasmid pCotC-GFP, a PCR with the primers cotC-F and cotC-R-transl was performed. The amplified fragment was subsequently cleaved with KpnI and HindIII and ligated into the corresponding sites of pSG1151 to generate plasmid pCotC-GFP. *B. subtilis* strains abrB-gfp, spoIIE-gfp, and cotC-gfp were obtained by Campbell-type integration (single crossover) of plasmids pGFP-abrB, pGFP-spoIIE, and pCotC-GFP, respectively, into the chromosome of *B. subtilis* 168 1A700. Transformants were selected on tryptone-yeast extract (TY) agar plates containing chloramphenicol after overnight incubation at 37°C. Correct integration was verified by PCR (data not shown). To obtain *B. subtilis* strains ΔabrB, Δspo0A, ΔspoIIAC, ΔsinR, and ΔsinI, parental strain *B. subtilis* 168 1A700 was transformed with chromosomal DNA of strains BD2238 (18), SWV215 (54), RL1265 (2), ΔsinR (47), and BD2641 (gift from D. Dubnau), respectively. To obtain strain sin+, parental strain *B. subtilis* 168 1A700 was transformed with plasmid pIS119 (14). Transformants were selected on TY agar plates containing the appropriate antibiotics after overnight incubation at 37°C. To obtain *B. subtilis* strains IIA/ΔsinR and IIA/ΔabrB, strain IIA-gfp (52) was transformed with chromosomal DNA from strains DS92 (28) and BD2238 (18), respectively. Transformants were selected on TY agar plates containing the appropriate antibiotics after overnight incubation at 37°C.

Colony microscopy. Colonies of *Bacillus subtilis* were observed and photographed using the stereoscopic 0.8 to 8× zoom microscope SMZ-1000 (Nikon Corporation, Tokyo, Japan), equipped with two 10× oculars and a 0.5× objective (working distance, 123.6 mm). The microscope contained an epifluorescence attachment and a DXM-1200 digital camera system (Nikon). The EclipseNet software package version 1.16.2 was used for control of the camera and image processing. Fluorescent *B. subtilis* reporter strains were monitored by the use of an HBO 103W/2 mercury short arc lamp (Osram Inc., Augsburg, Germany) and a long pass GFP filter (ex 460-500, DM505, BA510). All fluorescence pictures were taken with an exposure time of 1 s and default color balance settings (red gain, 30; green gain, 10; blue gain, 50) and processed identically.

Time-lapse movies were shot over a period of 4 days, with the experimental setup in a climate room at 30°C. To enhance the contrast between green fluorescent and nonfluorescent cells in the colony, red background illumination was applied by the insertion of a 580-nm long pass filter into the fiber optic 150 W KL 1500 LCD AC halogen light source (Schott Inc., Mainz, Germany). Heating of the specimen by the red background light was prevented by guiding the light via the optical fibers through 25 mm water in a transparent container. The agar plate with the growing colony was put in a closed glass petri dish containing water to prevent dehydration of the agar plate. A layer of Repel-Silane (Pharmacia Inc., Uppsala, Sweden) was applied on the lid of the petri dish to prevent condensation. Shots of 1-s exposure (3 s for strain cotC-gfp) were taken every 10 min with the DXM-1200 digital camera system in the presence of background red light and blue excitation light. A home-built mechanical shutter controlled the blue-light pulses of 10 s, which were delivered to the specimen every 10 min. Synchronization was done by a home-built timing device that delivered output pulses to start the camera via the ACT-1 version 2.20 software package (Nikon) and pulses to open the mechanical shutter for 10 s. A schematic representation of the experimental setup used for time-lapse fluorescence is presented in Fig. S1 in the supplemental material. All pictures were taken with identical camera settings and imported into Windows Movie Maker version 5.1 (Microsoft Corporation). Movies were created with a minimal picture duration and transition duration of 125 ms and 250 ms, respectively.

Single-cell microscopy and flow cytometry. To perform single-cell analysis on specific areas within a colony, a selected region was dissected using a scalpel and placed into a 2-ml screw-cap tube containing 1 ml of MOPS medium. Dissection was performed by the use of a magnifying glass and a surgical scalpel blade (Lance Blades Limited, Sheffield, United Kingdom). To homogenize the cells, tubes were placed in a mini-bead beater (BioSpec Products, Bartlesville, Okla.) for 1 min. Next, cells were prepared for microscopy and applied to agarose slides to fix the cells as described before (53), and images were acquired using an Axiophot microscope equipped with an AxioVision camera (Zeiss, Oberkochen, Germany). Fluorescent signals of GFP were visualized using set 09 (excitation, 450 to 490 nm; emission, >520 nm) (Zeiss). AxioVs20 software (Zeiss) was used for image capturing, and figures were prepared for publication using Corel Graphics Suite 11 (Corel Corporation). For flow cytometric analysis, spores were isolated as mentioned below. Spores were diluted 100-fold in 0.2 μM filtered minimal medium and directly measured on a Coulter Epics XL-MCL flow cytometer (Beckman Coulter, Mijdrecht, The Netherlands) operating an argon laser (488 nm). For each sample, at least 20,000 spores were analyzed. Data containing the green fluorescent signals were collected by a fluorescein isothiocyanate filter, and the photomultiplier voltage was set between 700 and 800 V. Data were captured using XL2 software (Beckman Coulter) and further analyzed using WinMDI 2.8 software (http://facs.scripps.edu/software.html). Figures were prepared for publication using WinMDI 2.8 and Corel Graphics Suite 11.

Spore inactivation. Cell suspensions (1 μl) were spotted on MOPS plates at 30°C. Colonies were resuspended after 5 days of incubation in 1 ml of sterile 0.9% NaCl and homogenized by mild sonication (Branson Sonifier 250) including 10 treatments of six consecutive pulses of 500-ms duration (duty cycle at 50%), which was interrupted by cooling in ice water for ~2 minutes. Output control was set at 3 (on a scale from 0 to 10). It should be noted that this lengthy

TABLE 3. Multicellular behavior in wild-type and domesticated strains of *B. subtilis*[a]

Strain	Bundle formation	Fruiting body formation	Swarming
NCIB3610	+	+	+
168 BGSC 1A1	−	−	−
168 BGSC 1A700	+	−	−

[a] Forms of multicellular behavior were observed after 2 days of growth on agar plates at 30°C. +, presence; −, absence.

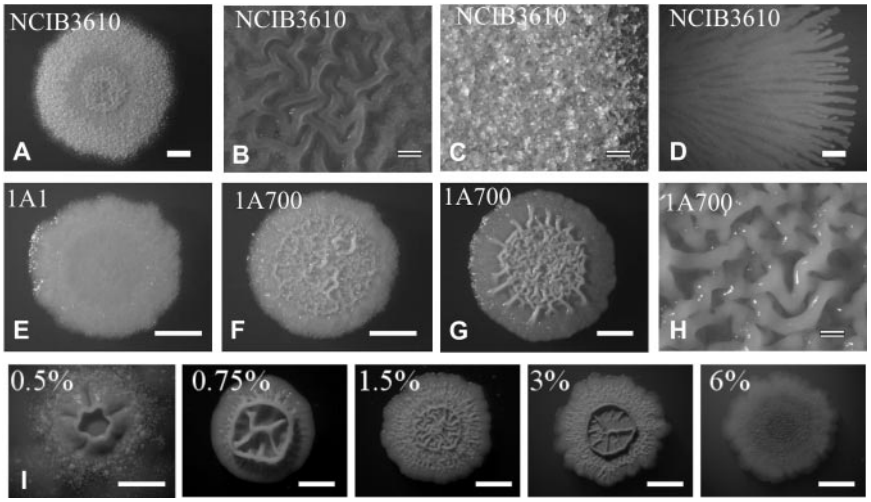

FIG. 1. Forms of multicellular development in domesticated and undomesticated strains of *B. subtilis*. Cells were spotted onto MOPS-based agar plates as described in Materials and Methods (single white bars are 2 mm; double white bars are 0.2 mm). (A) Colony of the wild-type strain NCIB3610. (B) Higher magnification of the center of the colony shown in panel A showing the presence of bundles. (C) Higher magnification of the edges of the colony shown in panel A demonstrating the presence of fruiting bodies. (D) Swarming patterns of strain NCIB3610 spotted on a 1% agar plate. (E) Laboratory 168 strain 1A1 shows no complex architectural structures. (F and G) Laboratory 168 strain 1A700 after 2 and 4 days, respectively. (H) Higher magnification of the center of the colony shown in panel G showing the presence of bundles. (I) Formation of the bundles of strain 1A700 depends on the agar concentration (after 6 days of growth).

sonication treatment was required only to homogenize spore suspensions from colonies of the *sinR* mutant, but it was applied to colonies from all strains. Cells and spores were counted in the hemocytometer. Spores were purified by the water washing method (36). Before heat inactivation, all spore suspensions were treated for 10 min at 70°C for heat activation of spores and killing of residual cells. Subsequent microscopy inspections showed that the wild type and all mutants yielded phase bright, isolated spores. Heat inactivation of spores was carried out for 20 min at 100°C. Viability counts of heat-inactivated and untreated spores were carried out in duplicate in TSB pour plates after overnight incubation at 37°C. To analyze dipicolinic acid (DPA) release, spore suspensions were diluted to an optical density at 600 nm of 0.5 by use of a NanoDrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). Total DPA content was determined by measuring the release of DPA from autoclaved spore suspensions (22 min at 121°C). Heat inactivation for the DPA release measurements was carried out by the addition of 20 μl of spore suspensions to 380 μl of preheated 0.9% NaCl solutions in a heating block, followed by incubation for a period of 20 min at 98°C. Fluorescence monitoring of DPA release was carried out as described before (31).

RESULTS

Colony architecture in *Bacillus subtilis*. *Bacillus subtilis* is capable of growing as a multicellular community with a high degree of spatial organization, exemplified by colonies with complex architectural features (2). Here, we study the spatial and temporal differentiation within these colonies by using specific GFP reporter strains. As colony architecture in *B. subtilis* can be considered a broad term, we make a clear distinction in this work between two levels of multicellular organization. First, bundles of cells of 150- to 170-μm thickness are formed which erect from the agar surface in the center of the colonies (Fig. 1A and B). Second, relatively small structures of 10- to 20-μm thickness that rise from the surface of the colony are formed at the edges of the colony (Fig. 1A and C) and have been defined as fruiting bodies and characterized as preferential sites for spore formation (2). We characterized the parental *B. subtilis* 168 strain in this study, *Bacillus* Genetic Stock Center (BGSC) code 1A700, and compared it to the lab strain 1A1 and the wild-type isolate NCIB3610 (http://www.bgsc.org/). We found that on chemically defined MOPS-based medium, but not on rich LB medium, 1A700 formed bundles in the center of the colony, and the cells coordinately formed a ring-shaped structure that erects from the surface (Fig. 1F to H). However, the other 168 strain available from the BGSC (1A1) did not form this type of complex architecture (Fig. 1E). The latter colony appeared as a flat structure without any spatial organization. A similar phenotype was observed for the PY79 laboratory strain (2). None of the 168 lab strains was capable of fruiting body formation or swarming under the conditions used, as shown by the wild-type isolate NCIB3610 (Fig. 1A to D and Table 3) (27). The formation of a ring-shaped structure appears to depend on how the strain is inoculated on the agar, since this shape is established only when cells are dispensed in droplets in which the highest cell densities can be found at the edges. Due to capillary flow, cells within a liquid drop spotted on a dry solid surface always disperse with the highest densities at the edges, comparable to a coffee stain (10). These initial regions with high cell density subsequently form the ring-shaped structure. This suggests that a high population density is an important trigger for bundle formation. Furthermore, the degree of bundle elevation depends strongly on the percentage of agar within the semisolid growth medium (Fig. 1I).

Bundle formation in *B. subtilis* 1A700 is phosphorelay and SinR dependent. An important common characteristic of biofilm formation is the critical dependence on the presence of extracellular substances that constitute a polymeric matrix in which cells are embedded (4, 9). Recent studies have shown that SinR is the master regulator of biofilm formation (28). SinR represses multiple genes essential for biofilm formation, such as the *epsA-epsO* operon and the *yqxM-tasA-sipW* operon, for which products are included in the extracellular matrix

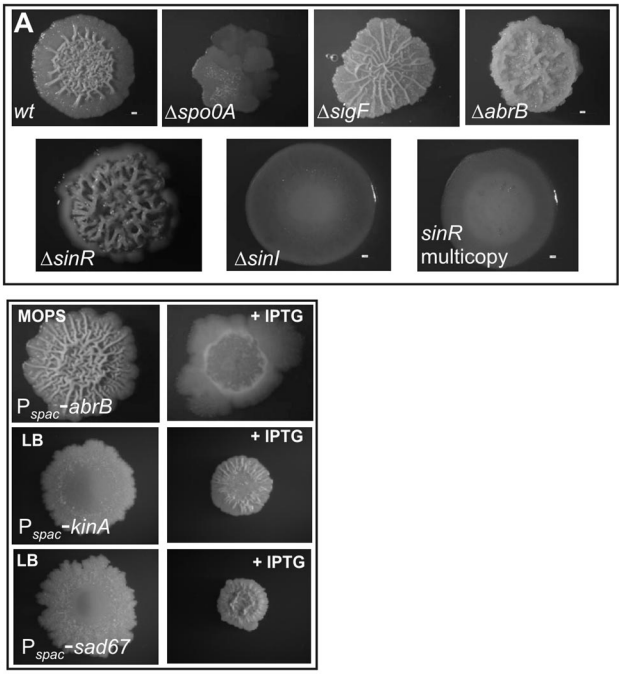

FIG. 2. Regulation of biofilm formation in *B. subtilis* 168 1A700. (A) Microscopic images of *B. subtilis* 1A700 (wt) and mutants derived from this strain. Cells were spotted onto MOPS-based agar plates and incubated for 4 days at 30°C as described in Materials and Methods. (B) Strains were spotted on agar in the absence (left panel) or presence (right panel) of moderate IPTG concentrations: P*spac-abrB*, 50 μM IPTG; P*spac-kinA*, 200 μM IPTG; and P*spac-sad67*, 200 μM IPTG. Strain IIA/abrB was spotted on MOPS-based agar (upper panel), while strains IIA/kinA (middle panel) and IIA/sad67 (lower panel) were spotted on LB-based agar.

(28). Furthermore, the AbrB and Spo0A regulators were shown to control the expression of genes required for efficient biofilm formation (2, 19, 20). It has been described that Spo0A activity, but not sporulation, is required for biofilm development (19). Mutants at later stages of sporulation did not affect biofilm formation, and biofilm formation could be restored in a *spo0A abrB* double mutant (2, 19). Colonies of undomesticated strains grown on solid agar plates were shown to form vein-like structures similar to the bundles observed in this study (2, 28). Furthermore, it was shown that mutations in *spo0A* or *sinI* abolished the formation of these structures (2, 28). To test whether the formation of elevated bundles in the laboratory *B. subtilis* 1A700 strain, as shown in Fig. 1, is regulated in a fashion similar to that of bundle formation in wild-type *B. subtilis* strains, we adopted a genetic approach in which we mutated the genes encoding the major players in biofilm formation. Mutations in *spo0A*, *sigF*, *abrB*, *sinR*, and *sinI* were introduced in *B. subtilis* 1A700 as described in Materials and Methods, and colonies were examined using microscopy. As shown in Fig. 2A, inactivation of *spo0A* resulted in the loss of any spatial structure within the colony, indicating that Spo0A activity is required to form the elevated structures. A mutation in the operon encoding the sporulation-specific sigma factor SigF did not alter bundle formation, indicating that actual spore formation is not a requirement. The Δ*abrB* strain showed a slight increase in the extent of bundle formation. However, a more prominent effect on bundle formation was found in the *sinR* mutant, in which a dramatic increase in the amount of elevated structure and increased production of mucous substances on the surface of the colonies were observed. Furthermore, bundle formation was completely abolished in a *sinI* mutant and in a strain containing *sinR* on a multicopy plasmid (Fig. 2A).

To substantiate the mutant analyses, we introduced IPTG-inducible variants of *abrB*, *kinA*, and *spo0A-sad67* in our parental strain. Upon artificial induction of AbrB, elevated structures were completely abolished when grown under biofilm-forming conditions (Fig. 2B, upper panel). When the primary phosphorelay kinase KinA was overproduced, bundle formation was stimulated on rich medium, which normally does not induce biofilm formation (Fig. 2B, middle panel). Overproduction of KinA causes elevated intracellular levels of phosphorylated Spo0A (39). Therefore, we also examined the effect of overproduction of a constitutively active form of Spo0A, Spo0A-Sad67 (24), on bundle formation in rich medium. As shown in Fig. 2B (lower panel), the formation of elevated bundles is strongly stimulated upon induction of Spo0A-Sad67. Moderate IPTG induction levels were applied due to the toxicity of Sad67 (24). Overall, these results show that the formation of elevated bundles in the laboratory strain *B. subtilis* 1A700 requires the sporulation phosphorelay and demonstrate that SinR is the major regulator involved in bundle formation.

Elevated structures are the preferred sites of sporulation. To correlate colony growth, bundle formation, and sporulation, we monitored the sites for vegetative growth and sporulation within colonies by construction and analysis of a number of GFP reporter strains derived from *Bacillus subtilis* 168 BGSC code 1A700. To visualize vegetatively growing cells within the colony, we constructed a strain in which the *gfp* gene is under control of the *abrB* promoter. It has previously been demonstrated that this promoter is specifically active in exponentially growing, nonsporulating cells and repressed by the key sporulation regulator Spo0A (18, 53). To identify the sites of activation of sporulation-specific gene expression within colonies, we fused *gfp* to the promoters of the *spoIIA* and *spoIIE* operons. These promoters are under direct positive control of Spo0A~P (34). The *spoIIA* operon contains three genes, *spoIIAA*, *spoIIAB*, and *sigF*, the forespore-specific sigma factor.SpoIIEdephosphorylatesSpoIIAA~P,andtheunphosphorylated SpoIIAA protein associates with the SigF-SpoIIAB complex, thereby releasing SigF (43). To visualize actual spore formation within the colonies, we constructed a full translational fusion of the gene encoding the abundant spore coat protein CotC (21) to the *gfp* gene under control of its own promoter. A C-terminal GFP fusion was constructed instead of an N-terminal fusion to avoid possible loss of the GFP marker by removal of the N-terminal part of the protein due to post-translational processing (25). Correct timing and localization of the P_{cotC}-CotC-GFP fusion to the forespore were confirmed by single-cell fluorescence microscopy (Fig. 3A, lower right corner).

The above-mentioned GFP reporter strains were inoculated onto solid agar plates containing MOPS medium and incubated at 30°C. The gene expression patterns within the colonies were followed from 1 to 4 days using time-lapse fluorescence microscopy as described in Materials and Methods.

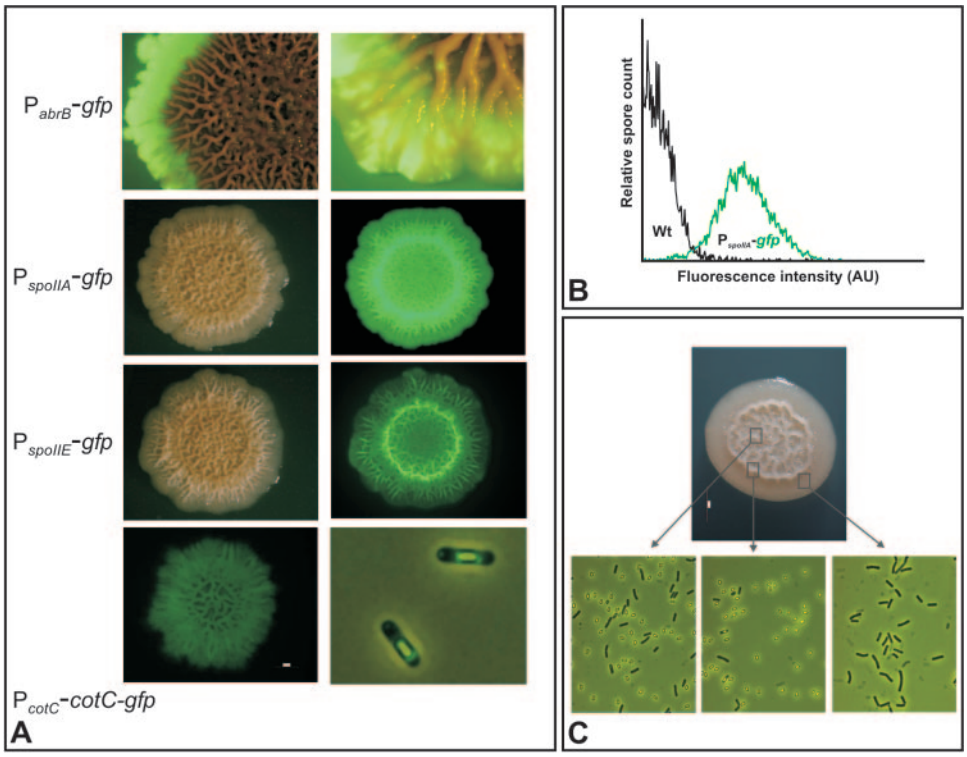

FIG. 3. Bundles are preferential sites for sporulation. (A) Cells were spotted onto MOPS-based agar plates and incubated for 4 days at 30°C, unless specified otherwise, and fluorescence microscopic images were taken as described in Materials and Methods. The edge of a colony from strain abrB-gfp (P_{abrB}-*gfp*), incubated for 14 days, was excited with blue light in a background of white light (left panel) and at a higher magnification (right panel). Strains IIA-gfp (P_{spoIIA}-*gfp*) and spoIIE-gfp (P_{spoIIE}-*gfp*) were captured with white light (left panel) and excited with blue light (right panel). A colony from strain cotC-gfp (P_{cotC}-*cotC-gfp*) was captured by excitation with blue light (left panel). A single-cell fluorescent microscopic image of cells from strain cotC-gfp is depicted in the right panel (overlay between white light and fluorescence). Interestingly, CotC-GFP accumulates mostly at the poles of the forespore and is significantly less abundant at the flanks of the forespore. (B) Spores from strains *B. subtilis* 1A700 (Wt) and IIA-gfp (P_{spoIIA}-*gfp*) were isolated from 5-day-old colonies grown on MOPS-based agar at 30°C. Spores were purified, and fluorescence was determined by flow cytometry as described in Materials and Methods. Green fluorescence intensity is indicated by arbitrary units (AU). (C) Single-cell microscopy analysis of a typical wild-type *B. subtilis* 1A700 colony grown on MOPS-based agar. Rectangles indicate the dissection sites taken for microscopy.

Expression of *gfp* from the *abrB* promoter initially takes place throughout the colony, but with increased time, expression decreases in the center and is observed mainly at the borders of the colony (data not shown). After 14 days of growth of this colony, brown-pigmented, elevated bundles can be identified (Fig. 3A, top right). This brown pigmentation likely results from enzymatic activity of the CotA spore coat protein (23). The pigmented structures are flanked by patches containing GFP, produced under the control of the *abrB* promoter, at the same distance from the center of the colony. This indicates that spatial differentiation and not simply growth is responsible for this phenomenon. In contrast, GFP produced under the control of the *spoIIA* and *spoIIE* promoters is observed mostly in the center of the colony (Fig. 3A). As we took these pictures after 4 days of incubation, we checked whether the observed fluorescence originated from fluorescent spores. Extraction of spores from colonies, followed by purification and fluorescence-assisted flow cytometry, indicated that spores from the IIA-gfp strain (P_{spoIIA}-*gfp*) are much more fluorescent than spores of the parental *B. subtilis* strain (Fig. 3B). The observed green fluorescence of spores is most likely caused by entrapment of GFP within the forespore compartment, as expression of the *spoIIA* promoter takes place very early in the sporulation process, prior to formation of the asymmetric septum. Indeed, previous studies indicate that expression of the *spoIIA* operon is not compartmentalized (5). We further established that spores are formed predominantly in the elevated bundles by colonies that express CotC-GFP. This translational fusion results in green fluorescent spores with GFP present predominantly at the poles, as confirmed on a single-cell level (Fig. 3A). To further validate the GFP reporter observations, we performed single-cell analyses on different areas within a single colony. Colonies from the wild-type *B. subtilis* 1A700 strain were dissected, and cells were examined using single-cell microscopy. As depicted in Fig. 3C, dissected material from the flat edges of a colony contain mainly vegetative growing cells. The elevated bundles in the ring-like structure, however, contain predominantly mature spores, while the middle of the colony shows a mix of vegetative and sporulating cells. Overall, these results show that spore formation occurs preferentially in the elevated structures of a colony.

Sequential activation of elevated bundles and spore formation in biofilms. Recently, Fujita et al. (12) demonstrated that categories of genes respond to different threshold levels of

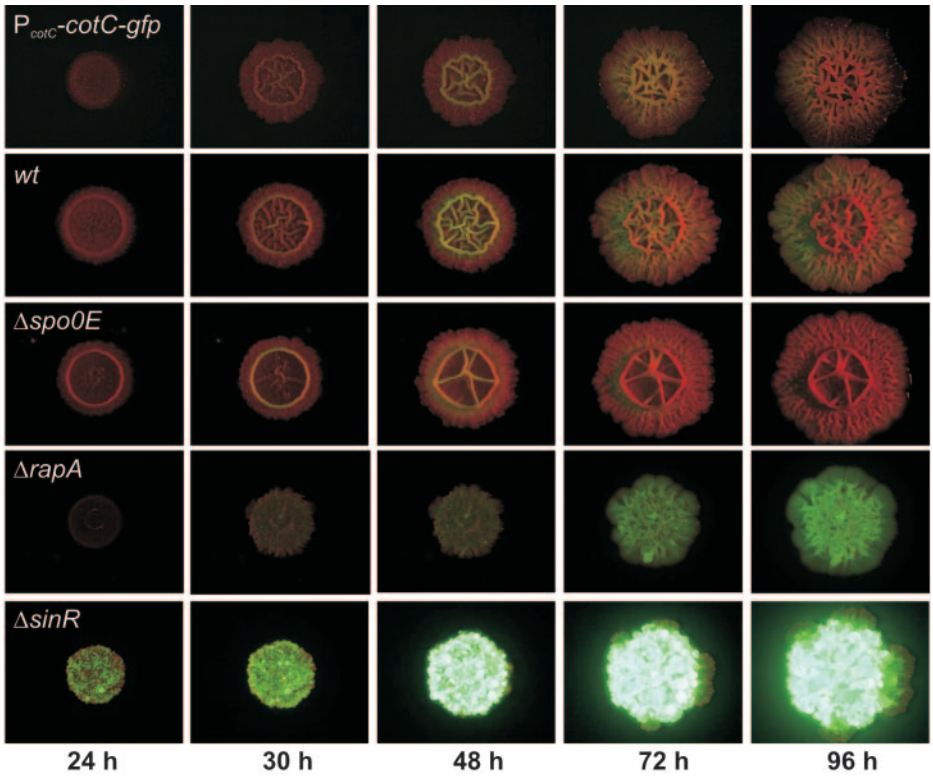

FIG. 4. Effect of perturbations in the phosphorelay on biofilm formation monitored by time-lapse fluorescence microscopy. Cells of strains *B. subtilis* cotC-gfp (P_{cotC}-*cotC-gfp*), IIA-gfp (P_{spoIIA}-*gfp* [wt]), IIA/Δspo0E (P_{spoIIA}-*gfp* [Δ*spo0E*]), IIA/ΔrapA (P_{spoIIA}-*gfp* [Δ*rapA*]), and IIA/ΔsinR (P_{spoIIA}-*gfp* [Δ*sinR*]) were spotted onto MOPS-based agar plates and incubated at 30°C, and time-lapse fluorescent microscopic images were taken as described in Materials and Methods. Images of colonies after 24, 30, 48, 72, and 96 h of incubation are shown. Time-lapse movies can be found in the supplemental material.

Spo0A~P within the Spo0A regulon, which was previously characterized by chromatin immunoprecipitation in combination with microarray analysis (34). Importantly, they showed that *abrB* and *sinI* are repressed and activated, respectively, at threshold levels lower than those required to activate sporulation genes. Since *abrB* and *sinI* regulate biofilm formation (20, 28), Fujita and coworkers suggested that biofilm formation can be seen as a prelude to efficient spore formation (12). As bundle formation in our model strain appears to be regulated similarly to biofilm formation in wild-type strains (Fig. 2), we wondered whether the bundles are established before sporulation is initiated. Using time-lapse fluorescence microscopy of strains IIA-gfp (P_{spoIIA}-*gfp*) and cotC-gfp (P_{cotC}-*cotC-gfp*), we demonstrated that the elevated bundles were present before the sporulation gene program was activated (Fig. 4) (see Fig. S3 in the supplemental material for movies). For wild-type colonies, approximately 24 h after inoculation, elevated structures in the form of bundles became visible. Bundle formation was subsequently followed by expression of the *gfp* gene from the *spoIIA* promoter (3 to 5 h after bundle formation) and expression of *cotC-gfp* from the *cotC* promoter (7 to 8 h after bundle formation).

From an environmental and evolutionary perspective, it can be envisioned that for optimal spore dispersal to different niches, it is important for the spores to be present in an elevated structure within the multicellular community.

Perturbations in the phosphorelay affect bundle formation and reduce spore resistance. Phosphatases of the phosphorelay are important in ensuring proper timing of sporulation gene expression and correct integration of environmental signals (reviewed in references 38 and 42). To test the importance of a balanced phosphorelay, which directs bundle formation in our strain (Fig. 2), we performed time-lapse fluorescence on phosphorelay mutants. Previously, it was demonstrated that mutations in the *rapA* and *spo0E* phosphatases caused more cells to initiate sporulation and, in the case of *spo0E*, prematurely sporulate (41, 52). Colonies of the *spo0E* or *rapA* mutant grown on solid agar were shown to rapidly accumulate Spo segregants (see Fig. S2 in the supplemental material) and form so-called papillae because of the increased pressure to sporulate (37, 40, 41). In this study, similar observations were made when either KinA or Spo0A-Sad67 was overproduced, stressing the fact that an unbalanced phosphorelay induces the segregation of sporulation mutants. Thus, to perturb correct signaling of the phosphorelay and examine how this affects bundle formation, spore formation, and spore resistance within colonies, we introduced a *rapA* and a *spo0E* mutation in a strain containing a sporulation-specific GFP reporter (P_{spoIIA}-*gfp*). Since a *sinR* mutation leads to a highly disturbed bundle phenotype, we also introduced a *sinR* mutation in our reporter strain. As shown in Fig. 4, the formation of elevated bundles was less coordinated in mutants for the phosphatases, although the phenotype of

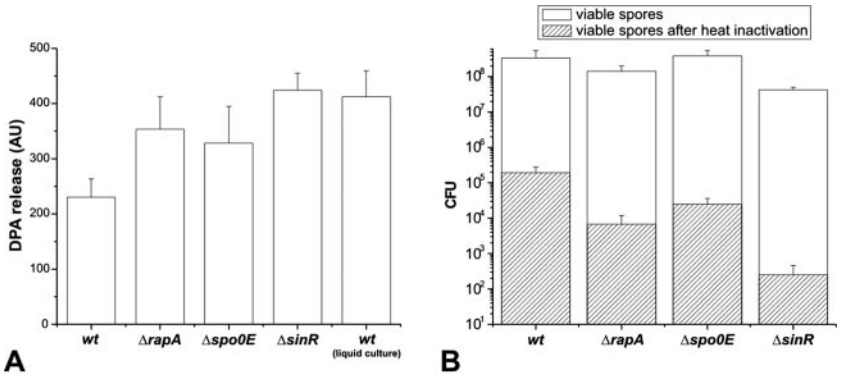

FIG. 5. Spores from biofilms of Δ*rapA*, Δ*spo0E*, and Δ*sinR* strains show decreased heat resistance. Spores were harvested and treated as described in Materials and Methods. Results are from two independent experiments, both performed in duplicate. The standard errors in these experiments are indicated by bars. (A) DPA release upon heat inactivation is indicated by arbitrary units (AU) of fluorescence intensity. (B) The numbers of CFU after overnight incubation at 37°C on TSB pour plates are depicted on the *y* axis.

the *spo0E* mutant was subtler than that of the *rapA* mutant. It should be noted that although the fluorescence intensities of GFP in the *spo0E* mutant were not significantly higher, fluorescence could be observed slightly earlier after bundle formation (~2 h) compared to that of the wild-type strain, indicating disturbance of the normal regulatory control (Fig. 4). Furthermore, sporulation was less homogeneous in the *rapA* mutant, as indicated by the presence of dark (non-GFP) patches. In addition, Δ*rapA* colonies appeared flatter than those of either the wild-type or Δ*spo0E* strain. Expression of *gfp* from the *spoIIA* promoter was extremely high in the *sinR* mutant, and bundles were either more abundantly present or deformed, possibly resulting from a very high EPS production, as *sinR* colonies were covered with droplets of a mucous substance (Fig. 4). After prolonged incubations on agar plates, colonies of the *spo0E*, *rapA*, and *sinR* mutants contained increased numbers of Spo segregants and cells with extremely high levels of GFP production from the *spoIIA* promoter (see Fig. S2 in the supplemental material). Besides increasing the tendency to initiate sporulation in the *spo0E* and *rapA* mutants, the presence of secondary mutants within the colonies could contribute to the altered bundle phenotypes.

The above-described results show that when the phosphorelay is perturbed, bundle formation and sporulation are disturbed in colonies. This raises the question of whether spores within these colonies show altered resistance properties. To answer this question, we isolated spores from colonies of the wild-type, Δ*rapA*, Δ*spo0E*, and Δ*sinR* strains. Spores were purified and tested by a recently developed assay for their heat resistance based on heat-induced release of DPA as indicated in Fig. 5A (31). All mutants showed a higher release of DPA upon heat treatment, indicative of reduced resistance. It should be noted that the total DPA amounts per spore were virtually the same in all strains (data not shown). These results were substantiated by counts of CFU in an independent experiment. As shown in Fig. 5B, the most prominent effects were observed in spores from *sinR* mutants, showing a 100-fold decrease in heat resistance compared to that of wild type, while *rapA* and *spo0E* mutants showed only a 10-fold decrease in heat resistance. In contrast, comparisons between spores isolated from mutants and the wild-type strain grown in liquid medium did not show decreased resistance properties (15; data not shown). The mechanism responsible for the decrease in heat resistance for spores isolated from colonies of *rapA* and *sinR* mutants and its possible correlation to biofilm development have not yet been resolved. In general, spores isolated from liquid media are less heat resistant compared to spores isolated from agar plates (S. Brul and S. J. Oomes, unpublished observations) (Fig. 5A). This suggests that there is an additional level of resistance gained in spores isolated from agar plates. The underlying mechanism responsible for this phenomenon is currently under investigation.

DISCUSSION

It is becoming more and more obvious that, in natural habitats, bacteria are present predominantly in dense biofilms. The ability to form complex multicellular structures, however, is often lost in laboratory strains (2). The genetic program that regulates biofilm formation in *Bacillus subtilis* has been elucidated in some detail (2, 3, 19, 20, 28, 48). It was shown that there is shared use of regulators that govern biofilm and spore formation. In these reports, *B. subtilis* strains that are genetically accessible and that show complex colony morphology were chosen. In the present study, we show that the sequenced and commonly used laboratory strain *B. subtilis* 168 1A700 (32) is able to form elevated structures (bundles) with complex architecture but lacks the ability to swarm or form fruiting bodies (Fig. 1 and Table 3). Bundles were previously described as long nonseparated chains of cells bound together as cables (28). Here, we use the term "bundles" to describe the macroscopic "veins" that form within a colony. Bundle formation is most prominent in the center of colonies, in the form of a ring-shaped structure (Fig. 1). This suggests that a high cell density is an important trigger for bundle formation, as is the case for sporulation (16). We show that the master biofilm regulator, SinR, regulates the formation of these bundles in the *B. subtilis* 168 laboratory strain (Fig. 2). By artificially inducing AbrB, bundle formation is completely abolished. The same holds true for a *sinI* mutant or SinR overproduction. Activation of the sporulation phosphorelay, by overproduction of KinA or Spo0A-Sad67, resulted in stimulation of bundle

formation, most likely by activation of *sinI*. The actual formation of spores is not required, since bundles are still formed in a *sigF* mutant. Overall, this demonstrates that the gene regulatory pathways governing sporulation and bundle formation are highly intertwined, as was previously established for the wild-type isolate, the *B. subtilis* NCIB3610 strain (2, 28).

Recently, Kearns and Losick showed that the laboratory *B. subtilis* PY79 strain, which is unable to form bundles on agar plates (2), is present predominantly as long chains of sessile cells during exponential growth in liquid cultures (30). The wild-type *B. subtilis* 3610 strain, however, occurs mainly as motile single cells or pairs under these conditions. Interestingly, our *B. subtilis* 168 1A700 strain shows a phenotype similar to that of the wild-type 3610 strain in liquid cultures (data not shown). It was found that the gene product of *swrA*, which is needed for efficient swarming (29), is responsible for the activation of a large operon required for cell motility (30). In the PY79 lab strain, *swrA* contains a single-base-pair insertion, thereby causing a frameshift. It was demonstrated that deletion of the insertion occurs at higher frequencies than typical point mutations, and cells readily revert to the wild-type *swrA* allele (29). These data suggest that the 1A700 strain has a wild-type copy of the *swrA* gene and offer an explanation as to why cells of our laboratory strain are able to form complex multicellular structures and occur as motile cells in liquid media, in contrast to most other *B. subtilis* laboratory strains. Restoring the function of *swrA* in the PY79 lab strain was not sufficient to regenerate the rough colony phenotype, indicating that the *B. subtilis* 168 1A700 strain used in this study may differ at other genetic loci as well from PY79 (D. Kearns, personal communication).

Using specific GFP reporter strains in combination with time-lapse fluorescence microscopy, we were able to pinpoint actual sites of sporulation within growing colonies (Fig. 3 and 4). Since the GFP variant used in this study (GFPmut1) has a short maturation time and can already be detected in vivo 8 min after transcriptional induction, the occurrence of fluorescence from our reporter strains gives a good indication of the actual start of transcription (7). These experiments show that bundles are established prior to actual spore formation. The preferred sites for spore formation are within the elevated bundles. Similarly, in wild-type *B. subtilis* strains, sporulation takes place preferably within fruiting bodies at the tip of the colony (2). The production of spores at elevated structures within a colony may increase a spore's chances of being relocated in order to find a new, more-nutrient-rich environment. Thus, the formation of spore-containing bundles could be part of a spore dispersal strategy.

Fujita and coworkers recently resolved the functioning of the Spo0A regulon using a combination of genetic and biochemical techniques (12, 13). Within this regulon, they defined four categories of genes responding to different levels of Spo0A~P (genes that require low and high levels of Spo0A~P to be activated or repressed, respectively). Most of the sporulation-specific genes (e.g., the *spoIIA* and *spoIIE* operons) belong to the category that requires high levels of Spo0A~P for activation, while genes involved in biofilm formation respond at lower levels of Spo0A~P (e.g., *abrB* and *sinI*). The latter gene has been categorized as a high-threshold gene, but is, within this category, stimulated at lower Spo0A~P levels than the sporulation genes (12). The observation of these Spo0A~P level-dependent transcription responses led to the hypothesis that biofilm formation occurs prior to sporulation. Indeed, we could show, using time-lapse fluorescence microscopy, that bundles are created before spores are formed (Fig. 4) (see the supplemental material for movies).

It can be envisioned that via different threshold levels of Spo0A~P, fine-tuned timing of bundle and spore formation is important in generating spores that are maximally resistant to various environmental insults. To test this hypothesis, we perturbed signals from the Spo0A phosphorelay and disturbed temporal regulation by mutating genes encoding the regulatory phosphatases RapA and Spo0E. These phosphatases hydrolyze Spo0F~P and Spo0A~P, respectively, which results in lower intracellular levels of Spo0A~P (42). Previously, we have shown that the initiation of sporulation is regulated by a hypersensitive "bistable" switch which requires cells to reach a threshold level of Spo0A~P in order to induce sufficient activation of the *spoIIA* operon (52). Furthermore, the fraction of cells initiating sporulation is modulated by the phosphorelay phosphatases. Accordingly, a greater fraction of cells initiates sporulation in both *rapA* and *spo0E* mutant backgrounds. Counterintuitively to the findings for liquid cultures, bundle formation and expression of GFP from the *spoIIA* promoter in colonies of the *rapA* mutant seem less coordinate, or more heterogeneous (Fig. 4). These heterogeneous colonies are most likely formed by the increased pressure to sporulate and by the presence of secondary mutants within the colony that accumulate as a result of this pressure (40). Besides the accumulation of sporulation-deficient cells in the Δ*spo0E*, Δ*rapA*, and Δ*sinR* colonies that show no detectable expression of the *gfp* gene from the *spoIIA* promoter, cells that demonstrate extremely high *PspoIIA* activity could be identified (see Fig. S2 in the supplemental material). We have no clear explanation for the nature of cells expressing high levels of *gfp* under the control of *PspoIIA*, which in contrast to the sporulation-deficient cells, revert back to cells with wild-type levels of *PspoIIA* activity at relatively high frequencies (data not shown).

The *spo0E* gene is repressed by AbrB and thus requires Spo0A~P for its expression (50). The *rapA* gene is activated by the competence quorum-sensing system ComA/P and, at later stages, is activated and repressed by Spo0A~P (12, 35, 52). Bundles of the *rapA* mutant seem more uncoordinated, and colonies appear flatter than those of both the wild-type strain and the *spo0E* mutant (Fig. 4). These results suggest that the phosphoryl drain by Spo0E from Spo0A~P, which is required for coordinate sporulation, can be complemented partly by another phosphorelay phosphatase, such as RapA (hence, the mild bundle phenotype). A *rapA* mutation, however, seems to be insurmountable. This could be due to the fact that *spo0E* can be expressed only to a low level, once all the AbrB is titrated away from its promoter, while *rapA* expression is activated by the ComA/P system as well as by Spo0A~P and is capable of reaching higher levels. Overall, these results indicate that when the signals to sporulate are provoked by these gene deletions, spores are formed prematurely, secondary mutations accumulate, and as a result, bundles and spores are formed in a less coordinate manner.

Next, we investigated whether spores that were not formed in the regularly timed manner (in which cells first contribute to bundle formation before differentiating into a spore) show

altered resistance properties. Therefore, we extracted spores from colonies of Δ*sinR*, Δ*rapA*, and Δ*spo0E* strains and compared their heat resistance profiles to spores isolated from wild-type colonies. Spores from colonies that displayed deregulated bundle formation demonstrated reduced heat resistance (Fig. 5). We propose two possible explanations for this result which are not mutually exclusive. First, spores from these mutant colonies could have accumulated secondary mutations that have a negative effect on heat resistance. Second, the time span of sporulation is shortened in these mutants, by premature activation of the unidirectional gene regulatory cascade of feed-forward loops governing spore formation (11). If the latter interpretation is correct, then the effect of reduction of this time span on heat resistance appears more drastic for spores isolated from agar plates than those isolated from liquid cultures. Many differences in growth conditions within liquid cultures and agar plates could contribute to this effect, such as nutrient availability and levels of hydration. However, increased EPS levels, as produced by colonies of a *sinR* mutant, seem to have no positive effect on the spore's heat resistance.

This work may contribute to the reevaluation of the common microbiologist's view of microorganisms as unicellular life forms, since we demonstrate complex coordinated bacterial behavior using GFP reporter strains in combination with time-lapse fluorescence microscopy. Sensing and signal transfer mechanisms for adaptation processes have evolved to systems that are accurately tuned towards their ambient environment. Therefore, it is relevant to study these processes under the conditions in which they predominantly take place. However, as shown in this work, studies of biofilms suffer from the complication of temporal and spatial differentiation, resulting from either genetic or physiological variation.

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ADDENDUM IN PROOF

Sequencing of the *swrA* gene of strain 1A700 revealed that it contains a frameshift identical to the one found in the PY79 strain, indicating that 1A700 must differ from PY79 at at least one other genetic locus.

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