Fruiting body formation by *Bacillus subtilis*

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Spore formation by the bacterium *Bacillus subtilis* has long been studied as a model for cellular differentiation, but predominantly as a single cell. When analyzed within the context of highly structured, surface-associated communities (biofilms), spore formation was discovered to have heretofore unsuspected spatial organization. Initially, motile cells differentiated into aligned chains of attached cells that eventually produced aerial structures, or fruiting bodies, that served as preferential sites for sporulation. Fruiting body formation depended on regulatory genes required early in sporulation and on genes evidently needed for exopolysaccharide and surfactin production. The formation of aerial structures was robust in natural isolates but not in laboratory strains, an indication that multicellularity has been lost during domestication of *B. subtilis*. Other microbial differentiation processes long thought to involve only single cells could display the spatial organization characteristic of multicellular organisms when studied with recent natural isolates.

Microbes of many kinds enter complex pathways of development in response to cues from the environment (1–3). One of the best-studied pathways of microbial development is the process of endospore formation by the Gram-positive soil bacterium *Bacillus subtilis*. Decades of molecular genetic studies have led to deep insights into how a single cell gives rise to two differentiated cell types: the mother cell, which nurtures the developing spore, and the forespore, which matures into a dormant cell capable of surviving for long periods of time and of resisting environmental extremes (4, 5). In the past, these studies have focused on the development of a unicellular organism, a single cell undergoing differentiation in homogeneous suspension in liquid culture. In natural settings, however, microorganisms most commonly exist as multicellular communities exhibiting a high degree of structure (6, 7).

The present work began with an investigation of the ability of *B. subtilis* to form biofilms, i.e., surface-associated communities. Initial tests with several laboratory strains grown in standing cultures revealed the formation of thin and structureless pellicles on the surface of the liquid. Given that repeated passage of bacterial isolates in liquid culture can select for the loss of social behaviors (8), we decided to investigate pellicle formation by natural isolates of *B. subtilis*. Indeed, all of the natural isolates we tested formed robust pellicles with intricate web-like structures whose formation depended on genes required for entry into sporulation. Moreover, when sporulation was analyzed in the context of these structured, surface-associated communities, the process was found to display a previously unrecognized level of spatial organization.

Materials and Methods

Bacterial Strains and Media. Laboratory isolates of *B. subtilis*: 168 (*trpC2*) (9); PY79 (prototroph, derived from 168; from P. Youngman, University of Georgia, Athens, GA); JH642 (*trpC2 pheA1*, derived from 168; from J. A. Hoch, Scripps Research Institute, La Jolla, CA). Natural isolates of *B. subtilis*: NCIB3610 [prototroph, referred to as "WT" throughout; from A. L. Sonenshein and the *Bacillus* Genetic Stock Center (BGSC), Ohio State Univ., Columbus, OH]; X5 (prototroph, from BGSC); 1431 (prototroph, from BGSC); 1440 (prototroph, from BGSC). Minimal medium (MSgg): 5 mM potassium phosphate (pH 7)/100 mM Mops (pH 7)/2 mM $MgCl_2$/700 μM $CaCl_2$/50 μM $MnCl_2$/50 μM $FeCl_3$/1 μM $ZnCl_2$/2 μM thiamine/0.5% glycerol/0.5% glutamate/50 μg/ml tryptophan/50 μg/ml phenylalanine (adapted from ref. 10). LB medium: 1% Bacto tryptone/0.5% Bacto yeast extract/1% NaCl/1 mM NaOH (11). Media were solidified through addition of Bacto agar (Difco) to 1.5%, and the plates were allowed to dry at 25°C for 16 h before use.

Construction of Mutants. PY79 mutants RL891 (*spo0A*::*erm*), RL2242 (*spo0A*::*spc*), RL1265 (*sigF*::*kan*, from P. Stragier, Institut de Biologie Physico-Chemique, Paris, France), and 168 mutant RL102 (*spo0H*::*cat*) were used as donor strains in transferring the mutant alleles into the WT strain by means of PBS1 phage transduction with standard methods (11). In the case of the *spo0A* mutants, transduction was associated with loss of motility; however, motile *spo0A* variants were isolated through a procedure based on a chemotaxis assay in which bacteria swim toward an attractant contained within a capillary tube (12). All of these mutants grew as well as the WT parent strain in agitated MSgg cultures, and none produced spores when grown in or on MSgg or Difco sporulation medium (11). In other experiments, a collection of 60 knock-out alleles of Spo0A-controlled genes was generated in PY79 by using a long-flanking-homology PCR strategy (ref. 13 and S.B.-Y. and R.L., unpublished results). Two of these mutations (*yveQ*::*tet* and *yveR*::*tet*) were associated with defective biofilm formation and were introduced into WT by transduction; the resulting mutants grew like WT in agitated MSgg cultures, were motile, and produced spores. Note that *yveQ* and *yveR* are the seventh and eighth genes of a predicted 16-gene operon, so their disruption could affect expression of other genes within the operon. Finally, a mutant *sfp* allele was introduced into the WT strain fortuitously, as follows. A construct in which *gfp* expression is placed under the control of a constitutive promoter (P*spac*^{c}-*gfp*::*spc*) was integrated into the *amyE* locus of PY79 by means of standard methods (11). This construct was then moved from PY79 to WT by transduction, and two classes of transductants were recovered: those that produced surfactin (called strain EG219) and those that did not (called EG220). Unlike WT and other surfactin-producing natural isolates, common laboratory strains such as PY79 contain a frameshift mutation that disrupts *sfp*, which encodes an enzyme required for surfactin synthesis (14). It seemed likely that the PBS1 phage, which can package up to 10% of a host's genome (11), had in some instances transferred from PY79 to WT both the desired construct integrated at *amyE* and the mutant *sfp* allele (*amyE* is about 60 kb from *sfp* on the chromosome). Sequence analysis confirmed that the *sfp* locus of EG219 contained no deleterious mutations, whereas that of EG220 contained the specific muta-

Abbreviations: WT, wild type; BGSC, *Bacillus* Genetic Stock Center; EPS, exopolysaccharide; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

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tion characteristic of laboratory strains. Phenotypic differences between EG219 and EG220 were attributed to the latter strain's inability to produce surfactin because a *srfAA*::*erm* mutant (constructed in the same way as the *yveQ* and *yveR* mutants), which carries a WT *sfp* allele but is unable to produce surfactin (because of the disruption of the first subunit of the nonribosomal peptide synthetase that produces surfactin; ref. 15), behaved like EG220 under all conditions tested. Furthermore, the morphological phenotypes of both mutants—reduced spreading of colonies and lack of aerial structures in both colonies and biofilms—could be partially complemented extracellularly through the close proximity of surfactin-producing strains or the addition of purified surfactin (Sigma). Both EG220 and the *srfAA* mutant grew like WT in agitated MSgg cultures, were motile, and produced spores. Moreover, aside from expressing green fluorescent protein, EG219 behaved like WT under all conditions tested, and, therefore, EG220 could be directly compared with WT. Finally, it should be noted that for all of these mutants, multiple transductants were analyzed in parallel, and, in general, they gave similar results; the exceptions (*spo0A* and EG219 vs. EG220) are discussed above.

Analysis of Sporulation-Specific Gene Expression. An in-frame translational fusion of the *Escherichia coli lacZ* gene with the *B. subtilis sspE* gene (*sspE-lacZ*::*cat*), which is expressed only during the later stages of sporulation (16), was integrated into the endogenous *sspE* locus of PY79 through transformation, and then moved into the WT genetic background through PBS1-phage transduction by using standard methods (11). Similarly, a *spoIID-lacZ*::*cat* fusion construct, which is expressed only during sporulation (17), was integrated into the *amyE* locus of PY79 and then moved into WT. As a control for a constitutively expressed fusion, we used *lacZ* fused to the P*spac*^{c} promoter (M. Fujita and R.L., unpublished results), which we moved from PY79 into WT. These strains were grown in or on MSgg supplemented with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal; 300 mg/ml final concentration) and photographed with a dissection microscope equipped with a charge-coupled device video camera. Aside from their expression of β-galactosidase, all of these strains were indistinguishable from WT in terms of planktonic, biofilm, and colony growth, as well as sporulation efficiency.

Microscopy and Measurement of Spore Formation. WT bacteria were grown in agitated LB cultures at 37°C to an OD_{600} of 1.0–2.0 and then diluted 1,000-fold with MSgg. Aliquots of 40 ml each from the diluted culture were transferred to 100-ml Pyrex beakers, each equipped with a side-arm stop-cock. These cultures were incubated at 25°C without agitation, and at 12 h intervals, three were harvested. A sample from below the air–medium interface was withdrawn by means of the stop-cock (to avoid disruption of the biofilm), and then samples from the pellicle were harvested. Each sample was examined at high magnification (600×) with an Optiphot-2 phase-contrast microscope (Nikon) equipped with a charge-coupled device video camera system (Optronics Engineering, Goleta, CA) and a computer interface. Note that the images in Figs. 3 and 4*D*) represent successive stages of biofilm development, the exact timing of which varied from culture to culture. The biofilm samples then were passed through a sterile 23-gauge needle several times, and all samples were vigorously and extensively vortexed before each was diluted 1,000-fold with MSgg. At this point, each sample was split into two fractions: one was incubated at 80°C (to kill vegetative cells but not spores; ref. 11), and the other was incubated at 25°C for 20 min. The effectiveness of heat-killing was confirmed by phase-contrast microscopy. Appropriate dilutions of each fraction were plated on LB agar and incubated at 37°C for 16 h. Total colony-forming units per ml of sample was calculated from the number of colonies derived from the 25°C fraction dilutions, and spores per sample was calculated from the number of colonies derived from the 80°C fraction dilutions.

Results and Discussion

Fig. 1*A* shows that a standing culture of the laboratory strain PY79 (referred to as "LAB") formed an extremely thin, fragile, and smooth pellicle that lacked a distinctive macroscopic architecture. In contrast, the undomesticated WT strain NCIB3610 formed a thick pellicle with an intricate vein-like appearance on its surface. When spotted on minimal agar plates, the *B. subtilis* strains produced colonies with morphological features characteristic of their corresponding pellicles. Lab strain colonies were thin and smooth (and small, because of lack of surfactin production; see below), whereas WT colonies were thick and structurally complicated (Fig. 1*B*).

Closer examination of the WT colonies revealed a highly structured community (Fig. 1*C*), the architecture of which was best viewed by scanning electron microscopy. As shown in Fig. 1*D*, the WT colonies consisted of many tongue-like columns that projected up from the agar surface. Higher magnification revealed that these aerial structures were composed mainly of long chains of cells bundled together in parallel alignment (Fig. 1*D Right*). Such structures were also a prominent feature of pellicles formed on the surfaces of standing liquid cultures (data not shown).

Aerial structures are the predominant sites for sporulation in filamentous bacteria and fungi (1–3). Similarly, the spore-filled fruiting bodies of the myxobacteria can be considered aerial projections. Thus, we investigated the possibility that sporulation preferentially occurs within the aerial structures projecting from the surfaces of *B. subtilis* colonies and pellicles. To test this possibility, a construct in which *lacZ* is fused to a gene (*sspE*) that is expressed late in sporulation (16) was integrated into the chromosome of WT, and the resulting strain was grown on solid medium that contained the chromogenic galactoside X-Gal (18). We observed a striking spatial pattern of coloration in which blue dye preferentially accumulated at the tips of the columns of cells that arose from the colony surface (Fig. 2). This pattern indicated that sporulation-specific gene expression was most intense near the top of the aerial structures. A similar spatial pattern of sporulation-specific gene expression was observed in the pellicles of standing cultures, and equivalent observations were made by using *lacZ* fused to a different sporulation gene (*spoIID*) (data not shown). In contrast, cells harboring *lacZ* that was fused to a constitutively active promoter (P*spac*^{c}) generated uniformly blue aerial structures (data not shown). Thus, our results indicate that discrete physical structures projecting from the surfaces of WT colonies and pellicles serve as sites of sporulation within these communities. The spatial organization of cell types within these highly structured communities is reminiscent of fruiting bodies, and, therefore, we refer to them as such henceforth.

Formation of the *B. subtilis* fruiting bodies followed a distinctive developmental pathway with marked spatial organization. Standing cultures initially contained only planktonic cells that were highly motile and of unit length (Fig. 3, 12 h). After 12–24 h of incubation, the population density in the medium had reached about 3×10^7 colony forming units per ml (cfu/ml), and a pellicle had begun to form. By 36–48 h, the cell density in the liquid phase of the culture had dropped sharply to about 3×10^5 cfu/ml, and this drop correlated with substantial growth of the pellicle. Unlike the remaining planktonic cells, which retained their motility and unit length, the cells at the air–medium interface became nonmotile and formed long chains that were aligned and bound together (Fig. 3, 36 h) presumably by an exopolysaccharide matrix (see below). By 60 h, some cells within the chains had begun to sporulate, and by 96 h more than 50%

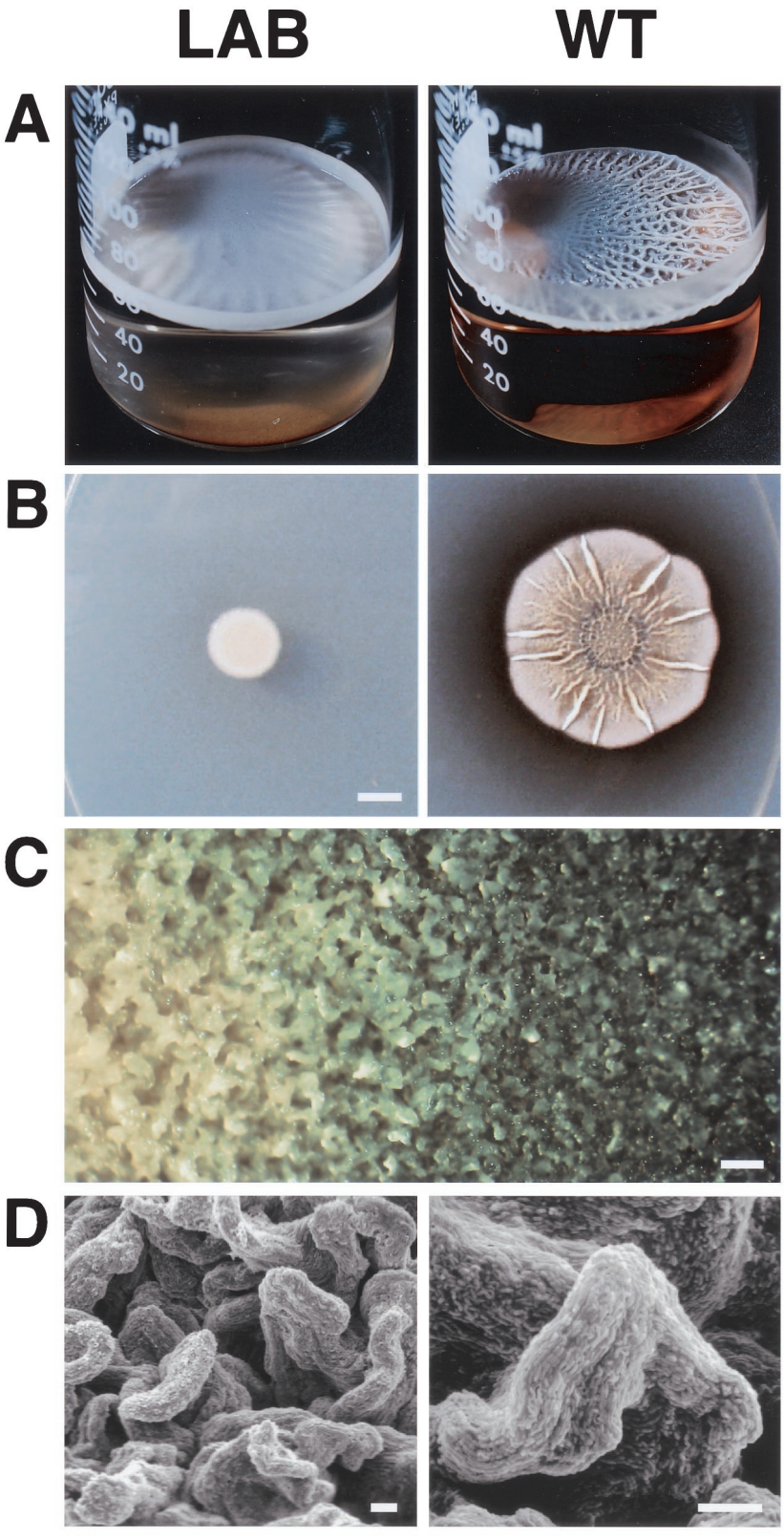

Fig. 1. Architecture of *B. subtilis* pellicles and colonies. (*A*) LAB and WT pellicles. Overnight cultures were diluted 1,000-fold into MSgg medium, and 60 ml was transferred to 150-ml Pyrex beakers. These cultures were incubated at 25°C without agitation for 5 days and then photographed. (*B*) LAB and WT colonies. Five microliters from overnight cultures were spotted onto a dry minimal agar plate. The plate was incubated at 25°C for 5 days and then photographed. (Bar = 5 mm.) (*C*) Close-up of the edge of a WT colony. A colony was grown at 25°C for 2 days and then photographed at 16× magnification with a dissection microscope equipped with a charge-coupled device video camera. (Bar = 100 μm.) (*D*) Scanning electron micrographs of a WT colony. A colony similar to that shown in *C* was photographed at 600× magnification (*Left*) and 1,000× magnification (*Right*). (Bars = 10 μm.)

of the viable cells within the pellicle had become spores. In contrast, virtually no spores (<5% spores per cfu) were detected in the planktonic phase of the culture, even after 120 h of incubation. These observations provide further evidence for the spatial and temporal organization of cellular differentiation during the formation of *B. subtilis* biofilms.

To explore the mechanisms underlying the development of spatial organization of sporulation within these communities, we disrupted genes required for sporulation in the WT strain and analyzed the mutants with regard to pellicle and colony morphology. We found that mutants lacking Spo0A, the key transcriptional regulator that governs entry into sporulation (19, 20),

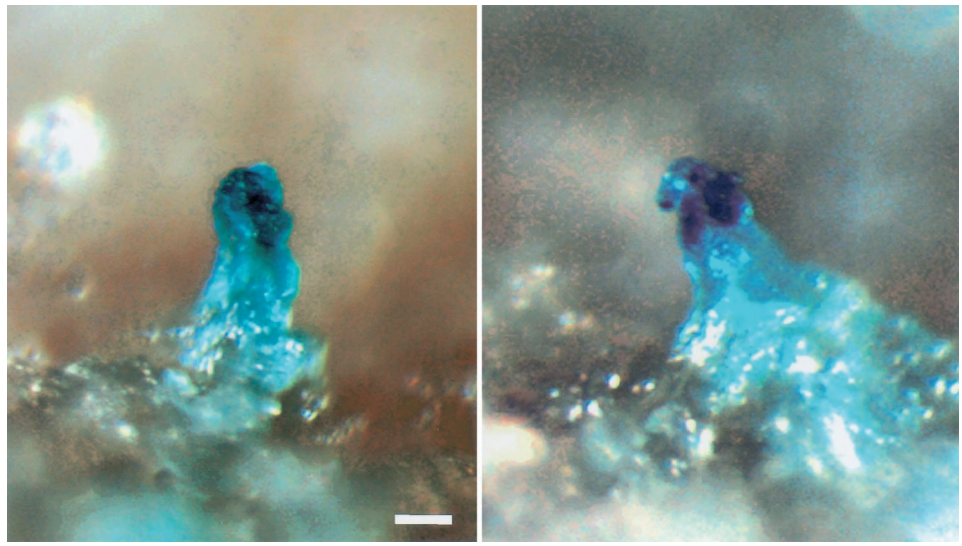

Fig. 2. Sporulation sites on the surfaces of WT colonies. WT(*sspE-lacZ*) bacteria were streaked on a minimal agar plate containing X-Gal (300 μg/ml), incubated at 25°C for 3 days, and then photographed at 130× magnification as described for Fig 1*C*. (Bar = 50 μm.)

failed to form pellicles in standing cultures (Fig. 4*A*) and produced mucoid, unstructured colonies (Fig. 4 *B* and *C*). A mutant (*spo0H*) lacking σ^H, a sigma factor that controls expression of proteins involved in the early stages of sporulation (4, 21), showed similar but less severe phenotypes: extremely thin pellicles and flat colonies whose surfaces lacked aerial structures (Fig. 4 *A–C*). In contrast, a mutant (*sigF*) lacking σ^F, the first compartment-specific sigma factor produced during sporulation (4, 21), formed pellicles and colonies that, save for the absence of spores, closely resembled those formed by WT (Fig. 4 *A–C*). These results indicate that Spo0A and σ^H, long recognized as key regulators of the initial steps of sporulation (4, 19–21), also play a critical role in the formation of the aerial structures that serve as sites of sporulation within surface-associated *B. subtilis* communities.

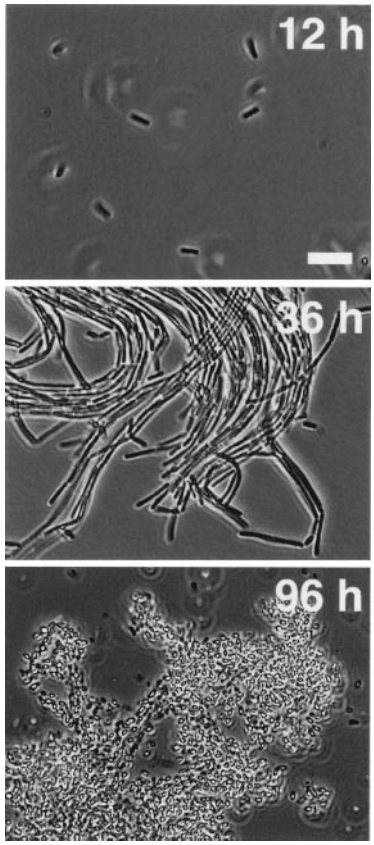

Fig. 3. *B. subtilis* pellicle development at the cellular level. WT cultures similar to those shown in Fig. 1*A* were incubated at 25°C without agitation. At the times indicated, samples were withdrawn from the air–medium interface and examined at 600× magnification with a phase-contrast microscope equipped with a charge-coupled device video camera. (Bar = 5 μm.)

In structured microbial communities such as biofilms, the formation and maintenance of multicellular aggregates are mediated by an extracellular matrix (6, 7) that is predominantly composed of exopolysaccharides (EPS; ref. 22). Of the *B. subtilis* genes whose transcription is under the control of both Spo0A (23) and σ^H (R. Britton, P. Eichenberger, J.E.G.-P., E. Ruiz, N. Comella, A. D. Grossman and R.L., unpublished results), two—*yveQ* and *yveR*—seem to encode EPS biosynthetic enzymes.[¶] We found that in standing cultures, *yveQ* and *yveR* mutants generated thick but very fragile pellicles that usually split and sank to the bottom of the culture vessel (Fig. 4*A*). The surfaces of these pellicles and of colonies formed by the mutants were smooth and lacked aerial structures (Fig. 4 *A–C*). Moreover, phase-contrast microscopy revealed that at the cellular level, the development of *yveQ* and *yveR* mutant biofilms (Fig. 4*D*) differed from that of WT biofilms (Fig. 3). Although the mutants did proliferate as long chains of cells at the air–medium interface of standing cultures, these chains were neither aligned nor bound together; rather, the mutant chains formed only very loose aggregates. Growth of the aggregates coincided with a dramatic decrease in chain length, leading to formation of pellicles consisting of short chains of cells densely packed together in disarray. Interestingly, the *spo0H* mutant, which shows reduced expression of *yveQ* and

[¶]The predicted YveQ polypeptide (367 residues) is similar in sequence to EpsJ (17% identity over 263 residues), a protein thought to be involved in polymerization of EPS repeating units in *Streptococcus thermophilus* (24). Likewise, the predicted YveR polypeptide (344 residues) is similar in sequence to a number of known or predicted glycosyltransferases, including EpsI of *S. thermophilus* (30% identity over 275 residues; ref. 24). In fact, *yveQ* and *yveR* are located within a predicted operon encompassing 16 genes, most of which resemble genes involved in polysaccharide biosynthesis, modification, and export.

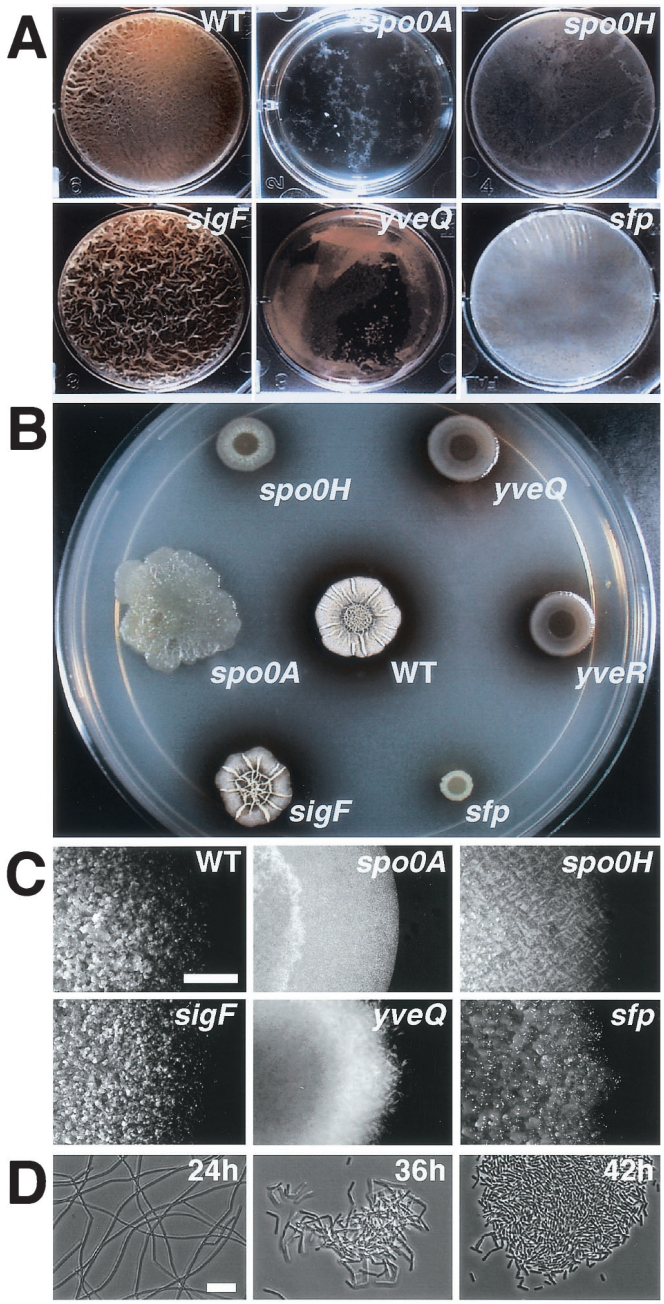

Fig. 4. Developmental properties of various *B. subtilis* mutants. Starting cultures were grown as described in the legend of Fig. 1; see text for description of each mutant. (*A*) Mutant pellicles. All starting cultures were diluted 1,000-fold, and 12 ml was transferred to the wells of 6-well microtiter plates. These cultures were incubated and photographed as described for Fig. 1*A*. (*B*) Mutant colonies at low magnification. Five microliters from each starting culture was spotted on an MSgg agar plate, and the plate was incubated and photographed as described for Fig. 1*B*. (*C*) Mutant colonies at high magnification. Colonies were incubated and photographed as described in Fig. 1*C*. (Bar = 0.5 μm.) (*D*) Development of *yveQ* mutant pellicles at the cellular level. Cultures similar to those of *A* were incubated at 25°C without agitation; at the times indicated, samples were withdrawn from the air–medium interface and examined by phase-contrast microscopy as described in the legend for Fig. 3.

yveR (R. Britton, P. Eichenberger, J.E.G.-P., E. Ruiz, N. Comella, A. D. Grossman and R.L., unpublished results), followed an intermediate course of pellicle development: its chains were partially aligned and loosely bound together but tended to break down into shorter chains, resulting in pellicles that were much less organized than those formed by WT but more so than those formed by the *yveQ* and *yveR* mutants (data not shown). In contrast, the *spo0A* mutant, which overexpresses *yveQ* and *yveR* (23), initially formed long chains that were tightly bound and aligned, although many of the cells within these chains lysed (data not shown). These results suggest that an EPS matrix produced through a process that involves *yveQ* and *yveR* mediates the cell–cell interactions required for production of the complex architecture characteristic of *B. subtilis* biofilms.

Surfactants are known to be important for the erection of aerial hyphae in fungi and the streptomycetes, caused at least in part by their ability to lower the surface tension of water (25–27). The phenotype of mutants unable to make surfactin is consistent with a similar role for this surfactant in *B. subtilis* fruiting body formation. A mutant (*sfp*) lacking the phosphopantetheinyltransferase that activates the nonribosomal peptide synthetase that produces surfactin (28) formed thick but completely flat pellicles (Fig. 4*A*). Similarly, *sfp* mutant colonies were flat and small, spreading very little on the agar surface (Fig. 4 *B* and *C*). Low-magnification microscopy revealed that these mutant colonies did form projecting columns, but these grew laterally and eventually fused with each other (Fig. 4*C*), leading to small colonies lacking aerial structures.

We have found that WT isolates of *B. subtilis* exhibit a high degree of spatial organization during the development of surface-associated communities. Most strikingly, the architecture of these communities includes fruiting bodies, the tips of which preferentially support spore-specific gene expression. The observation that extracellular signals are involved in sporulation had already indicated that high population density is an important signal in this developmental pathway (29). Without a clear community structure for context, however, the idea that sporulation in *B. subtilis* is essentially a single-cell response has persisted. The results presented here challenge that view. We have demonstrated that, when observed within the context of a surface-associated multicellular community, sporulation is a social phenomenon with a high degree of temporal and spatial organization. Indeed, under these circumstances, *B. subtilis* forms spore-filled fruiting bodies that could facilitate spore dispersal, as is the case for other microbes (3).

The temporal and spatial steps leading to fruiting body formation have been defined by observation and mutant analysis. Late during exponential growth or at entry into stationary phase, motile planktonic *B. subtilis* cells appear to migrate to the air–water interface, probably as an aerotactic response (30, 31). Upon reaching the interface, these cells differentiate into long chains of nonmotile cells that are aligned and bound together, presumably by an exopolysaccharide matrix. These chains represent the fabric from which higher-order structures are woven. Mutants that fail to form ordered and bound chains also fail to form spore-filled fruiting bodies (*yveQ*, *yveR*, and *spo0H*). Mutants that do not synthesize surfactin still form ordered chains of cells, yet they fail to form mature aerial structures (*sfp* and *srfAA*). This finding represents another case of a common solution among distantly related organisms, because fungi and the streptomycetes also secrete biosurfactants that play a critical role in the development of aerial mycelia (25–27). Once aerial structures are formed, sporulation first occurs at the tips of these structures.

The traditional view of bacteria as strictly unicellular organisms is increasingly being replaced by the that microbial communities often behave as multicellular organisms (32, 33). In analyzing the process of biofilm formation by *B. subtilis*, we were led to the surprising finding that spore formation, long seen as a process involving only a single cell, is tightly intertwined with the development of multicellular communities. It is likely that

many other microbial developmental processes that are currently considered unicellular will exhibit multicellular features when observed in the context of structured communities such as biofilms. It is of particular interest that WT isolates of *B. subtilis* produced more elaborate and robust structured communities as compared with laboratory strains. Microbial domestication for the purposes of laboratory investigation has almost invariably meant repeated selections for fast growth in liquid culture. It is likely that, in many instances, this process has led to the loss of multicellular attributes (e.g., ref. 8). In the future, it will be important to work with recently isolated microbes and to observe them in the context of structured communities to fully appreciate their developmental potential.

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1. Losick, R. & Shapiro, L., eds. (1984) *Microbial Development* (Cold Spring Harbor Lab. Press, Plainview, NY).
2. Dworkin, M. (1985) *Developmental Biology of the Bacteria* (Benjamin/Cummings Science, San Francisco).
3. Brun, Y. V. & Shimkets, L. J., eds. (2000) *Prokaryotic Development* (Am. Soc. Microbiol., Washington, DC).
4. Piggot, P. & Losick, R. (in press) in *Bacillus subtilis and Its Closest Relatives: From Genes to Cells*, eds. Sonenshein, A. L., Hoch, J. A. & Losick, R. (Am. Soc. Microbiol., Washington, DC), in press.
5. Setlow, P. (in press) in *Bacillus subtilis and Its Closest Relatives: From Genes to Cells*, eds. Sonenshein, A. L., Hoch, J. A. & Losick, R. (Am. Soc. Microbiol., Washington, DC), in press.
6. O'Toole, G. A., Kaplan, H. B. & Kolter, R. (2000) *Annu. Rev. Microbiol.* **54,** 49–79.
7. Davey, M. E. & O'Toole, G. A. (2000) *Microbiol. Mol. Biol. Rev.* **64,** 847–867.
8. Velicier, G. J., Kroos, L. & Lenski, R. E. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 12376–12380.
9. Burkholder, P. R. & Giles, N. H. (1947) *Am. J. Bot.* **34,** 345–348.
10. Freese, E., Heinze, J. E. & Galliers, E. M. (1979) *J. Gen. Microbiol.* **115,** 193–205.
11. Harwood, C. R. & Cutting, S. M., eds. (1990) *Molecular Biological Methods for Bacillus* (John Wiley & Sons, Chichester, U.K.).
12. Ordal, G. W. & Gibson, K. J. (1977) *J. Bacteriol.* **129,** 151–155.
13. Wach, A. (1996) *Yeast* **12,** 259–265.
14. Nakano, M. M., Corbell, N., Besson, J. & Zuber, P. (1992) *Mol. Gen. Genet.* **232,** 313–321.
15. Peypoux, F., Bonmatin, J. M. & Wallach, J. (1999) *Appl. Microbiol. Biotechnol.* **51,** 553–563.
16. Mason, J. M., Hackett, R. H. & Setlow, P. (1988) *J. Bacteriol.* **170,** 239–244.
17. Stragier, P., Bonamy, C. & Karmazyn-Campelli, C. (1988) *Cell* **52,** 697–704.
18. Sambrook, J. & Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
19. Ferrari, F. A., Trach, K., LeCoq, D., Spence, J., Ferrari, E. & Hoch, J. A. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 2647–2651.
20. Grossman, A. D. (1995) *Annu. Rev. Genet.* **29,** 477–508.
21. Stragier, P. & Losick, R. (1996) *Annu. Rev. Genet.* **30,** 297–341.
22. Sutherland, I. (2001) *Microbiology* **147,** 3–9.
23. Fawcett, P., Eichenberger, P., Losick, R. & Youngman, P. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 8063–8068. (First Published June 27, 2000; 10.1073/pnas.140209597)
24. Stingele, F., Neeser, J. R. & Mollet, B. (1996) *J. Bacteriol.* **178,** 1680–1690.
25. Wösten, H. A. B., Van Wetter, M. A., Lugones, L. G., Van der Mei, H. C., Busscher, H. J. & Wessels, J. G. H. (1998) *Curr. Biol.* **9,** 85–88.
26. Tillotson, R. D., Wösten, H. A. B., Richter, M. & Willey, J. M. (1998) *Mol. Microbiol.* **30,** 595–602.
27. Van Wetter, M. A., Wösten, H. A. B. & Wessels, J. G. H. (2000) *Mol. Microbiol.* **36,** 201–210.
28. Lambalot, R. H., Gehring, A. M., Flugel, R. S., Zuber, P., LaCelle, M., Marahiel, M. A., Reid, R., Khosla, C. & Walsh, C. T. (1996) *Chem. Biol.* **3,** 923–936.
29. Grossman, A. D. & Losick, R. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 4369–4373.
30. Wong, L. S., Johnson, M. S., Zhulin, I. B. & Taylor, B. L. (1995) *J. Bacteriol.* **177,** 3985–3991.
31. Nakano, M. M. & Zuber, P. (1998) *Annu. Rev. Microbiol.* **52,** 165–190.
32. Shapiro, J. A. & Dworkin, M., eds. (1997) *Bacteria as Multicellular Organisms* (Oxford Univ. Press, New York).
33. Shapiro, J. A. (1998) *Annu. Rev. Microbiol.* **52,** 81–104.