Interspecies interactions that result in *Bacillus subtilis* forming biofilms are mediated mainly by members of its own genus

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Many different systems of bacterial interactions have been described. However, relatively few studies have explored how interactions between different microorganisms might influence bacterial development. To explore such interspecies interactions, we focused on *Bacillus subtilis*, which characteristically develops into matrix-producing cannibals before entering sporulation. We investigated whether organisms from the natural environment of *B. subtilis*—the soil—were able to alter the development of *B. subtilis*. To test this possibility, we developed a coculture microcolony screen in which we used fluorescent reporters to identify soil bacteria able to induce matrix production in *B. subtilis*. Most of the bacteria that influence matrix production in *B. subtilis* are members of the genus *Bacillus*, suggesting that such interactions may be predominantly with close relatives. The interactions we observed were mediated via two different mechanisms. One resulted in increased expression of matrix genes via the activation of a sensor histidine kinase, KinD. The second was kinase independent and conceivably functions by altering the relative subpopulations of *B. subtilis* cell types by preferentially killing noncannibals. These two mechanisms were grouped according to the inducing strain's relatedness to *B. subtilis*. Our results suggest that bacteria preferentially alter their development in response to secreted molecules from closely related bacteria and do so using mechanisms that depend on the phylogenetic relatedness of the interacting bacteria.

Cell–cell interactions are a feature common to all living systems. Bacteria are no exception, and numerous mechanisms that use secreted products as signaling molecules are known (1, 2). Among these, the so-called "quorum sensing" systems are perhaps the best studied (3, 4). In quorum sensing, all bacterial cells within a population produce secreted molecules. Only when population densities are high is there a response to these compounds, thus allowing the bacteria to coordinate their behavior. However, it is clear that there is much more to bacterial cell–cell interactions than simply counting numbers and coordinating behavior. Secreted molecules also play key roles in microbial development so that different cell fates can arise and coexist within a single-species population (5, 6). In addition, in settings where multiple species coexist, their interactions often are mediated through extracellular compounds. Development in one microbe can be influenced by small molecules secreted by other species (7, 8).

We have been interested in understanding the role of interspecies interactions in the well-studied developmental processes that the soil bacterium *Bacillus subtilis* undergoes during biofilm formation. Biofilms are aggregations of cells held together by an extracellular matrix (9, 10). The matrix has two main components, an exopolysaccharide synthesized by the products of the *epsA-O* operon, and amyloid fibers encoded by the products of the *tapA* operon (formerly *yqxM*) (9, 11, 12). Within biofilms, several different cell types coexist, including subpopulations of motile cells, matrix-producing cells, and dormant spores (5, 6).

The regulation of the expression of the *epsA-O* and *tapA* operons has been studied extensively (10, 13–20). Both operons are controlled indirectly by the master regulator, phosphorylated Spo0A (Spo0A∼P) (11, 21, 22). This transcription factor modulates the expression of a large number of genes depending on its concentration (23, 24). Low levels of Spo0A∼P lead to the induction of transcription of the *epsA-O* and *tapA* operons, resulting in the production of extracellular matrix and thus biofilm formation (Fig. 1) (22). At high levels of Spo0A∼P, matrix genes are repressed, sporulation genes are induced, and these matrix-producing cells go on to become spores.

The matrix-producing cells have a second important physiological function: They are cannibals (Fig. 1) (25, 26). These cells produce two toxins, sporulation-delaying protein (SDP) and sporulation-killing factor (SKF) (25, 27). To survive, these cells also produce immunity proteins (28, 29). Both the toxin and immunity genes are under the control of Spo0A∼P. These toxins are able to kill *B. subtilis* cells not expressing immunity (those that have not yet phosphorylated Spo0A) as well as other microbes (30). This killing presumably provides the matrix-producing cannibal with nutrients and delays sporulation (25). The production of biofilm matrix thus is linked intrinsically to this mechanism of siblicide that prolongs the survival of this subpopulation of *B. subtilis* cells (25, 26).

The levels of Spo0A∼P are controlled by the action of membrane-bound histidine sensor kinases (KinA, KinB, KinC, KinD, and KinE) that directly and indirectly phosphorylate Spo0A in response to still poorly defined environmental cues (31). The activities of these kinases thus control differentiation in *B. subtilis* (31–33). KinC has a role in defining the matrix-producing cannibal subpopulation when it is activated by the self-produced molecule surfactin (34). Purified natural products functionally related to surfactin but produced by other bacteria also induce matrix synthesis in *B. subtilis* in a KinC-dependent manner (34). In addition, nisin, an antimicrobial produced by *Lactococcus*

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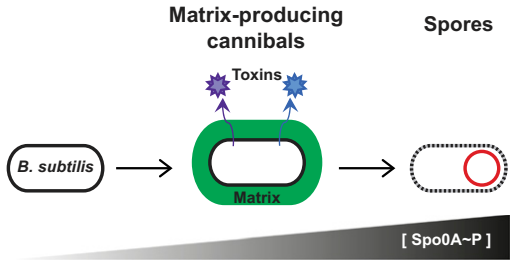

Fig. 1. Diagram illustrating the development of *B. subtilis* into matrix-producing cannibals and then spores. At low Spo0A~P levels *B. subtilis* activates genes required for the production of matrix and produces two cannibalism toxins. At high Spo0A~P levels sporulation begins.

lactis, induces a hypercannibalism phenotype in *B. subtilis* (26). Thus, small molecules from numerous bacteria are capable of influencing differentiation in *B. subtilis*. This observation suggests that *B. subtilis* development may be influenced by the presence of other bacteria in natural settings.

We therefore hypothesized that other soil microbes, when grown next to *B. subtilis*, might trigger its differentiation into matrix-producing cannibals. To investigate these possible interspecies interactions, we developed a screen in which *B. subtilis* and soil microorganisms were cocultured. While the colonies grew, we monitored *B. subtilis* differentiation into matrix producers using a fluorescent reporter. In this way we identified a large number of soil organisms that induced differentiation in *B. subtilis*. The majority of these organisms were other members of the genus *Bacillus*. This result led us to explore how broadly distributed the ability to induce matrix production is among a range of phylogenetically diverse *Bacillus* species. We found that many *Bacillus* species induce matrix production in *B. subtilis*, and the mechanisms they use are grouped according to their phylogenetic relatedness to *B. subtilis*. We therefore suggest that the interspecies interactions that induce biofilm production in the environment correlate with phylogenetic relatedness.

Results

Screen to Identify Matrix-Inducing Soil Organisms. We designed an interspecies-interaction microcolony screen to identify microbes able to induce matrix production in *B. subtilis*. To monitor *B. subtilis* matrix production, we used a reporter strain in which the promoter of the *tapA* operon (P_{tapA}) drives the transcription of the YFP gene (*yfp*), P_{tapA}-*yfp* (35). By coculturing this strain with random environmental microbes on agar plates, we could mimic a multispecies environment. Within this complex mixture of colonies, the fluorescent reporter allowed us to identify matrix-inducing interspecies interactions. Screening was performed in dilute, buffered LB medium (0.1× LB, pH 7) in which *B. subtilis* normally does not express the matrix genes (34).

We first wanted to ensure that our chosen conditions permitted a diverse range of organisms to grow within the time scale of the screen. Therefore we plated a subset of the soil samples that would be used during screening in the absence of the *B. subtilis* reporter strain. Random microcolonies were isolated, and we sequenced their 16S rRNA genes. The environmental microbes that grew under these conditions were primarily Proteobacteria and Firmicutes, with representatives from a number of different genera (Fig. 2). The Firmicute representatives all fell within the order *Bacillales*. Rarefaction curves indicate that our sequence analysis accurately reflects the diversity of the bacteria that we cultivated (Fig. S1). Thus, although spore-forming *Bacillus* species are well represented in the cultivable soil isolates, our screen probed interactions between *B. subtilis* and a variety of phylogenetically diverse soil microbes.

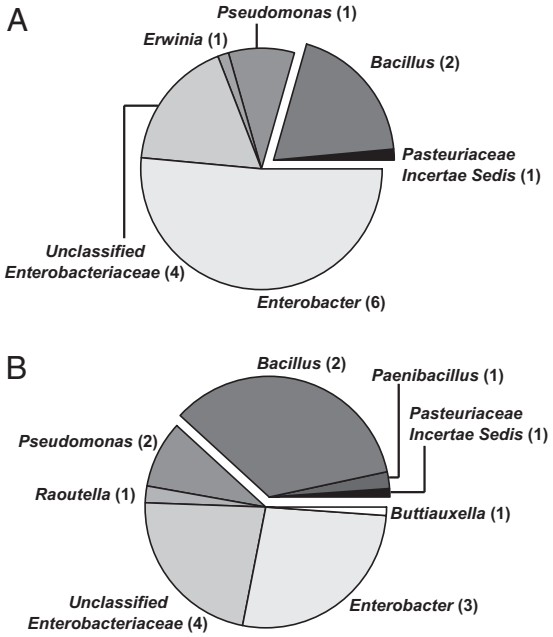

Fig. 2. The cultivable organisms growing under the microcolony coculture screen conditions are phylogenetically diverse. Soil organisms were selected systematically from random fields of view of soil A and soil B (grown as for coculture screen) and were isolated. (*A*) The 16S rRNA gene from 71 isolates from soil A and (*B*) 89 isolates from soil B were sequenced. The pie chart sectors are proportional to the number of representatives within each labeled group. The number of operational taxonomic units at the 99% level within that group is noted in parentheses. The darkly shaded exploded wedges represent the Firmicutes, whose representatives are all within the class Bacillales. All other sequences were from Proteobacteria.

We conducted interspecies-interaction microcolony screens by mixing the P_{tapA}-*yfp B. subtilis* reporter strain with soil samples from multiple sampling sites. These coculture plates were inspected visually using fluorescence microscopy to identify interspecies interactions resulting in matrix production in *B. subtilis* (i.e., activation of the fluorescent reporter, P_{tapA}-*yfp*). Matrix-inducing soil microbes (nonfluorescent colonies adjacent to fluorescing *B. subtilis* reporter colonies) were identified frequently (Fig. 3 *A*–*D*). The hit rate in the screen was high (see Fig. 4 for quantification), and a subset of positive hits (fewer than 100 isolates) was selected for further analysis in a secondary screen. The secondary screen confirmed that the isolated soil microbes induced fluorescence in the reporter strain. We used both the *B. subtilis* matrix reporter strain (P_{tapA}-*yfp*) and a cannibalism reporter strain (P_{skf}-*yfp*) in the secondary screen. As expected, in all cases in which we observed activation of P_{tapA}-*yfp*, we also observed fluorescence from P_{skf}-*yfp* (26).

Identification of Soil Isolates That Induce Matrix Gene Expression. Next, we identified the soil isolates that induced fluorescence in the *B. subtilis* reporter strains. Sequencing of the 16S rRNA genes revealed that the bulk of the isolates were *Bacillus* species (Fig. 3*E* and Fig. S2). The majority of the sequenced strains were in the *Bacillus cereus/thuringiensis/mycoides* group, with a few representatives from other species, such as *Bacillus megaterium* and *Bacillus luciferensis*. *B. subtilis* was not identified among the 64 isolates sequenced. The only non-*Bacillus* isolate was identified as *Pseudomonas monteilli.* These results indicate that, by and large, in this assay *B. subtilis* responds to other members of the *Bacillus* genus.

A characteristic feature of the *Bacillus* genus is the ability of its constituents to form heat- and desiccation-resistant spores (36).

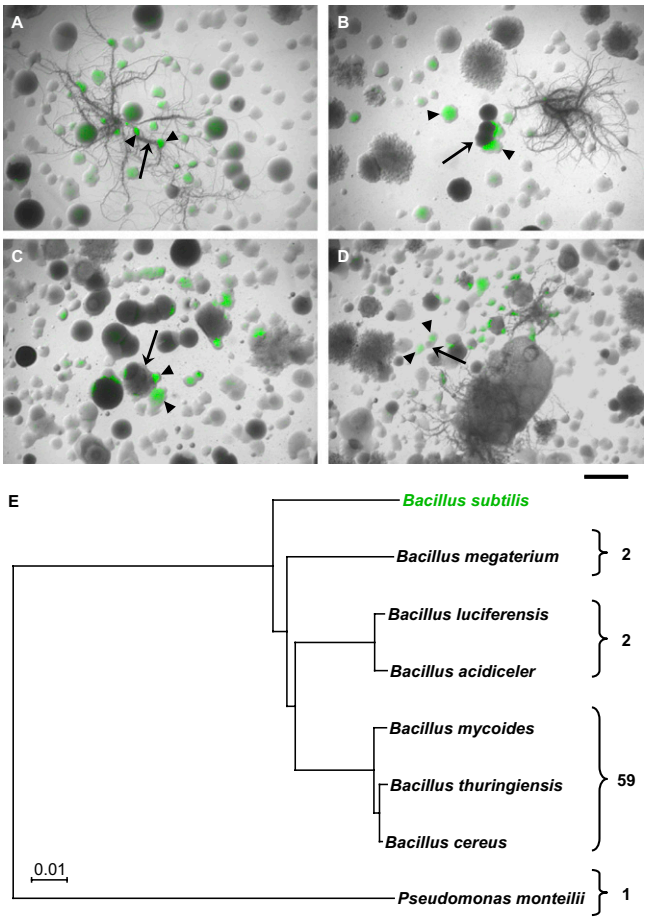

Fig. 3. The microcolony coculture screens reveal interspecies interactions between *B. subtilis* and soil organisms. (*A–D*) Representative images of microcolony coculture screens of the *B. subtilis* P_{tapA}-*yfp* reporter strain with four different soils. Bright-field images were overlaid with fluorescent images false-colored green. (Scale bar, 1 mm.) Fluorescent *B. subtilis* reporter colonies (arrowheads) are present in each field of view. Putative inducer colonies (arrows) are the nonfluorescent colonies in close proximity to the fluorescent reporter colonies. (*E*) Maximum-likelihood tree showing the identity of the organisms identified as P_{tapA}-*yfp* inducers. The number of sequenced representatives within each group of organisms is given on the right.

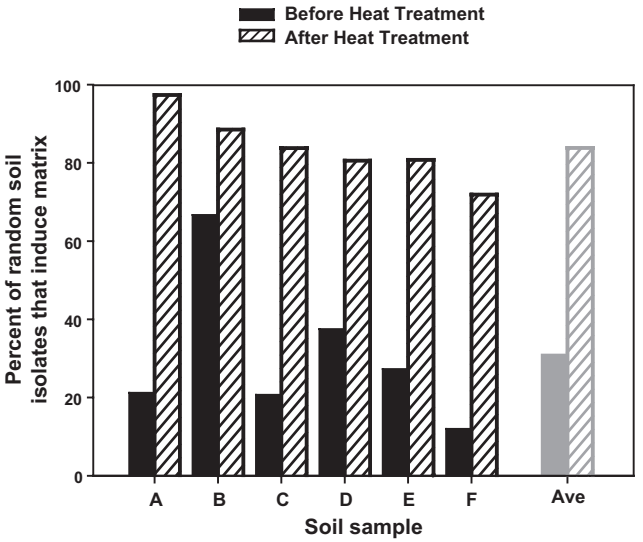

Fig. 4. The ability of random soil isolates and endospores to induce the P_{tapA}-*yfp* matrix reporter in *B. subtilis* is common. Random soil colonies from six soils used during coculture screening were tested for their ability to induce fluorescence from the *B. subtilis* P_{tapA}-*yfp* reporter both before (dark bars) and after (hatched bars) treatment at 80 °C for 30 min. A total of 507 soil colonies were tested, with an average of 40 colonies examined for each bar.

Because of the preponderance of *Bacillus* species identified as matrix inducers, we wondered if the ability to induce matrix production in *B. subtilis* was a common feature of spore formers present in our soil samples. To test this possibility, we selected six soils used for screening and examined them either directly or after heat treatment (30 min at 80 °C) to select for *Bacillus* spores. These soil samples were plated at concentrations equivalent to those used during screening, and random colonies were selected from each sample. Organisms were isolated and assayed for their ability to induce fluorescence in the *B. subtilis* matrix reporter strain. The ability to induce matrix was pervasive among the random cultivable soil isolates, ranging from 12% to 67% (Fig. 4, dark bars), and an even higher percentage of spore formers from these soils (72–97%) was capable of inducing fluorescence in the reporter (Fig. 4, hatched bars).

We noted that the overall percentages of soil organisms able to induce the P_{tapA}-*yfp* reporter (21% for soil A and 67% for soil B) (Fig. 4) were approximately proportional to the number of organisms within the *Bacillales* order from these soils (21% for soil A and 39% for soil B) (Fig. 2). Furthermore, more than 70% of *Bacillus* species present in the soil as spores can induce *B. subtilis* to produce matrix. Thus, the data are consistent with the conclusion that many of the environmental organisms capable of inducing matrix production in *B. subtilis* are likely other *Bacillus* species. In conjunction with our results from the specific isolates identified in the coculture screen, it is clear that close phylogenetic relatives of *B. subtilis* are particularly apt at stimulating biofilm formation in *B. subtilis* when grown in coculture.

To determine the phylogenetic breadth of *Bacillus* species that are capable of inducing matrix production in *B. subtilis*, we analyzed an assortment of 14 phylogenetically diverse, well-characterized *Bacillus* species, including many type strains. The majority of these other *Bacillus* species induced the *B. subtilis* matrix and cannibalism reporters under our conditions. We also examined the induction capability of the *B. subtilis* reporter itself, along with 13 other *B. subtilis* subsp. *subtilis* and *B. subtilis* subsp. *spizizenii* strains. Only two of these closely related strains induced the P_{skf}-*yfp* reporter (Fig. S3). Thus, in this assay, *B. subtilis* produces matrix in response to different *Bacillus* species more often than it does in response to *B. subtilis* strains. Because we selected our screening conditions to minimize self-induction of matrix production, this result was as anticipated. We next investigated the mechanism by which *B. subtilis* responds to the matrix-inducing molecule(s) from its phylogenetic relatives.

Role of Sensor Kinases in Matrix Induction. We wanted to determine which of the five sensor kinases known to phosphorylate Spo0A were involved in mediating the observed interspecies interactions. We thus compared the induction of P_{skf}-*yfp* in wild-type and kinase-mutant strains. If *B. subtilis* requires one of the sensor kinases to respond to the other *Bacillus* species and differentiate into matrix-producing cannibals, then no fluorescence would be expected in the corresponding sensor kinase-mutant reporter strain. The results obtained for four bacteria that induced P_{skf}-*yfp* are shown in Fig. 5. Interestingly, we observed no effects with mutants in *kinA*, *kinB*, *kinC*, or *kinE,* regardless of the inducing strain used. The matrix-induction produced by *Bacillus megaterium* and *Bacillus cereus* was abolished or reduced in the *kinD* mutant (Fig. 5*A*). Therefore, we concluded that, at least for these

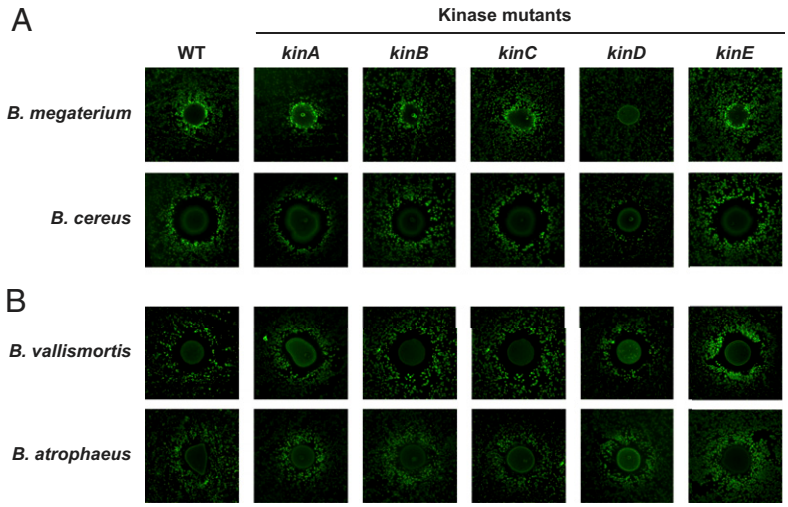

Fig. 5. *kinD* is necessary for some but not all interspecies interactions leading to induction of *B. subtilis* matrix production and cannibalism. Inducing organisms were tested for their ability to activate the P_{skf}-*yfp* reporter in wild-type and kinase deletion mutants of *B. subtilis* using the secondary screen format. (*A*) *B. megaterium* and *B. cereus* have reduced activation of the P_{skf}-*yfp* reporter in the absence of *kinD*. (*B*) *B. vallismortis* and *B. atrophaeus* do not require any of the sensor kinases for induction of the P_{skf}-*yfp* fluorescent reporter.

bacteria, the interspecies interactions proceeded along the anticipated Spo0A~P pathway. However, the matrix-induction caused by the other two strains (*Bacillus vallismortis* and *Bacillus atrophaeus*) was not affected in any of the kinase mutants (Fig. 5*B*). This result led us to consider a possible role of cannibalism in this latter type of interaction.

Role of Subpopulation Dynamics in Matrix Induction. How did these *Bacillus* species stimulate matrix production if they did not lead to an increase in Spo0A phosphorylation via one of the known sensor kinases? One possibility is that the matrix-inducing strains might produce two or more compounds that activate two or more kinases, and the additive effect of these compounds might lead to the observed activation. Although this notion was possible, there was a more parsimonious explanation. We hypothesized that the observed increase in P_{skf}-*yfp* fluorescence could be attributed to an increase in the number of cells of the matrix-producing cannibal subpopulation rather than to changes in transcription mediated by the kinases. A cannibalistic toxin produced by the matrix-inducing strain to which the *B. subtilis* cells also were immune would have the effect of increasing the relative proportion of the matrix-producing cannibal subpopulation.

To investigate this possibility indirectly, we tested whether these matrix-inducing strains were able to kill a *B. subtilis* strain that could not produce cannibal toxins or resistance. Cells lacking the *spo0A* gene can never produce matrix-producing cannibals, because Spo0A~P levels cannot accumulate to activate this development. We determined whether the matrix-inducing strains produced a larger killing halo on a lawn of a *spo0A*-mutant strain than on a wild-type *B. subtilis* lawn. We spotted the matrix-inducing strains on LB agar and let them grow until a mature colony was formed, allowing them time to produce any potential toxins. We then superimposed an agar slab embedded with either wild-type *B. subtilis* or *spo0A* mutant cells and examined the overlays after growth to determine the extent of the killing, if any. Some *Bacillus* species produced no halo

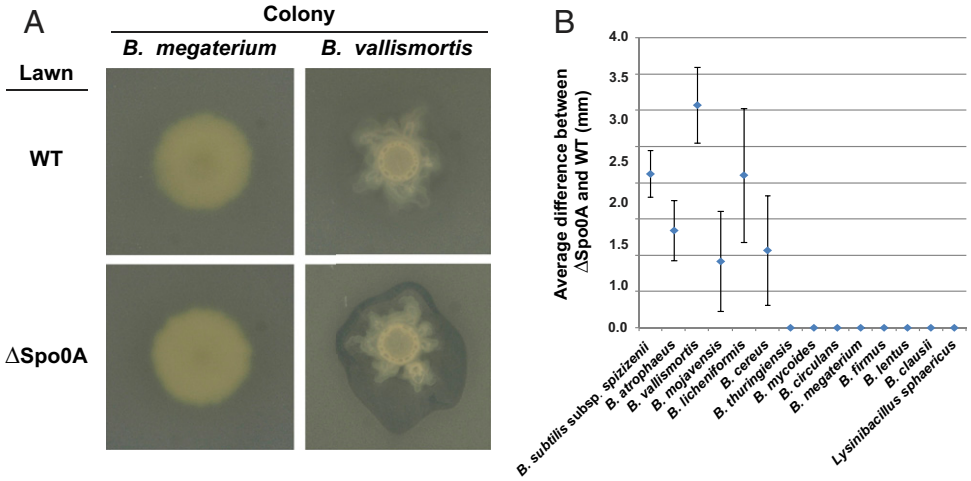

Fig. 6. *B. subtilis* possesses a Spo0A-dependent immunity to some *Bacillus* species. (*A*) *B. megaterium* and *B. vallismortis* colonies overlaid with embedded agar lawns of either wild-type (WT) or ΔSpo0A *B. subtilis*. *B. megaterium* does not kill either *B. subtilis* strain; *B. vallismortis* kills ΔSpo0A but not WT *B. subtilis*. (*B*) Graph showing quantification of the average difference in the halo size between the ΔSpo0A and WT overlays. At least three independent experiments were averaged, and each experiment was performed in duplicate. Error bars show SEM.

(*B. megaterium*) or a halo of the same size with either *B. subtilis* strain, but others (such as *B. vallismortis*) produced a significantly larger killing zone on the *spo0A*-mutant cells than on wild-type cells (Fig. 6). Thus, *B. subtilis* possesses an Spo0A-dependent immunity to these strains, perhaps resulting from resistance to a cannibalism-like toxin. This immunity could result in the appearance of enhanced matrix production in *B. subtilis* because of the death of all subpopulations of cells not producing matrix and not immune to cannibalism.

Verification That Induction Is via Secreted Molecules. Although the chemical identity of these matrix-inducing compounds is not yet known, we verified that these developmental effects were the result of secreted compounds. We used approaches that allowed us to separate the production of compounds by the matrix inducers temporally and spatially from the matrix-induction assay of *B. subtilis.* This separation allowed us to test whether any secreted compounds induced the P_{skf}-*yfp* reporter even in the presence of additional nutrients. To do so we used three different approaches. We grew the matrix-inducing organisms and then removed the colony by coring out and UV-sterilizing the agar; we used cellophanes to separate the matrix inducers from the agar; and we examined the effects of conditioned medium (CM) on colony development as well as on fluorescence induction. The results from these assays indicate that different molecules are produced by the matrix-inducing organisms (Table S1).

Two Matrix-Inducing Mechanisms Cluster with the Relatedness of the Inducing Strain. The microbes that induced *B. subtilis* matrix production via KinD and those that produced an Spo0A-specific zone of killing fell largely into two groups. We mapped the mechanism of matrix induction onto the phylogenetic tree of these strains (Fig. 7). The strains inducing *B. subtilis* matrix production via the Spo0A-dependent immunity mechanisms were the closest relatives of *B. subtilis* (including the two *B. subtilis* matrix-inducing strains). The strains inducing *B. subtilis* matrix production via the KinD-dependent mechanism were more distantly related to *B. subtilis*. Two strains, *B. cereus* and *Bacillus licheniformis*, induced matrix via both mechanisms.

Discussion

We were interested in determining which soil microbes might influence *B. subtilis* development. To address this question, we devised a screen that allowed us to identify microbes that, when grown in the vicinity of *B. subtilis*, would induce *B. subtilis* to make an extracellular matrix. Although the growth conditions used permitted the growth of a phylogenetically diverse collection of microbes, secreted molecules leading to matrix production came primarily from members of the *Bacillus* genus. Thus, as *B. subtilis* evaluates its environment to control its development, the microbes that prove most influential are its close relatives, although primarily not those of the same species. Interestingly, matrix inducers more closely related to *B. subtilis* elicited this effect through a mechanism reminiscent of cannibalism, whereas more distantly related bacteria induced extracellular matrix formation via the activation of the sensor histidine kinase KinD (Fig. 7).

KinD was described recently as a "checkpoint" protein controlling the progression from matrix producers to spore formers (37). KinD appears to have a dual function, acting as a phosphatase until sufficient matrix is produced and then becoming a kinase that helps attain the high Spo0A~P levels required for sporulation (37). It is possible that the *Bacillus* species identified in our screen produce matrices that somehow influence KinD activity.

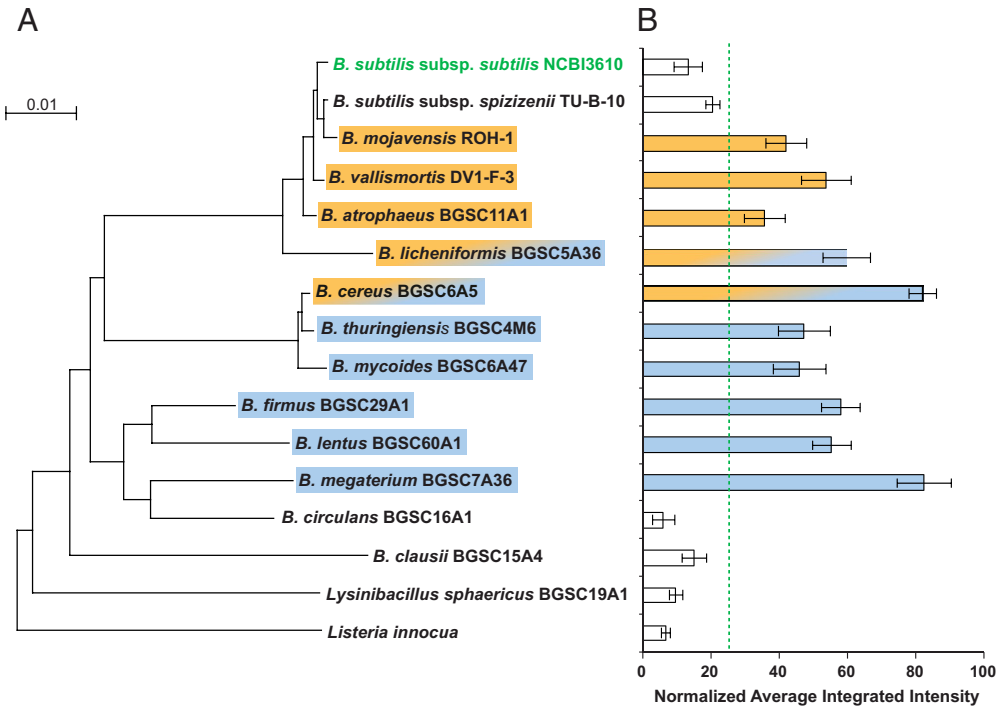

Fig. 7. The mechanisms used to induce matrix-producing cannibals in *B. subtilis* are segregated based on phylogeny. (*A*) Maximum-likelihood tree of the strains tested. The strains able to induce the P_{tapA}-*yfp* and P_{skf}-*yfp* reporters in *B. subtilis* are highlighted by boxes. *B. subtilis* possesses an Spo0A~P-dependent immunity to the organisms in orange; organisms in blue induce *B. subtilis* in a KinD-dependent manner. *B. cereus* and *B. licheniformis* demonstrated both activities. (*B*) Fluorescence induction for each strain was quantified from at least three independent experiments (error bars show SEM). The normalized average intensities of the inducers were significantly different from those of the noninducers using the Wilcoxon rank sum test ($P = 0.0006$). The boundary between these groups' normalized average intensities is indicated by the green dashed line. An analysis of means test ($\alpha = 0.05$) also indicates that the average intensities of the noninducing organisms are significantly below the mean threshold of the measured values (58).

Here we show that *B. subtilis* possesses an Spo0A-dependent immunity against the killing activities produced by *Bacillus* species that are closely related to it. As a result, when *B. subtilis* is grown near these close relatives, cells unable to induce the Spo0A regulon are killed. We propose that this killing could lead to an increase in the fraction of the population that is composed of matrix-producing cannibals. These close relatives probably do not produce the same toxins as *B. subtilis*, because no *skf* or *sdp* operons are found in their genomes. However, they may produce other toxins to which *B. subtilis*' cannibalism immunity genes provide cross-immunity. The Spo0A-dependent immunity of *B. subtilis* to these close relatives may be related instead to more general, intrinsic mechanisms of cellular immunity, such as those under the control of the transcription factors sigma W or sigma X, which have a role in providing immunity to SDP and other antimicrobials in *B. subtilis* (38–40).

It is clear that the development of *B. subtilis*, and in particular the expression of matrix and cannibalism genes, can be influenced by extracellular bacterial molecules. The compound surfactin is produced by *B. subtilis* itself and acts as a quorum-sensing molecule to increase the subpopulation of matrix-producing cannibals via the action of the sensor kinase KinC (34). In addition, KinC was activated similarly by functionally related compounds produced by Actinobacteria such as valinomycin and amphotericin (34). Because of our screening conditions, it is not surprising that we did not find any Actinobacteria that induced matrix-producing cannibals in *B. subtilis*. However, our finding that *B. subtilis* possesses an Spo0A-dependent immunity to the killing action of other *Bacillus* species suggests that many antibiotics produced by soil bacteria could influence the development of *B. subtilis.*

Our understanding of the natural ecology of *B. subtilis* in the soil is incomplete (41, 42), and there are still serious challenges in knowing whether results obtained from bacteria in the laboratory are relevant to their counterparts in the soil (43). Studies examining how bacteria are distributed spatially in soil indicate that cells generally are clustered locally in small colonies (44, 45). Investigators looking specifically at *Bacillus* found them in groups of 1–40 cells depending on the substrate, averaging five cells per colony (43, 46). Although it seems feasible that cells at such proximities could interact, we also do not know the concentrations at which natural products are produced natively in soil (47). These gaps in our knowledge make it impossible to determine whether these molecules evolved for the purpose of altering bacterial development or whether that function is a nonadaptive by-product of another ecological role.

It is highly likely that the molecules mediating the observed interactions evolved for reasons unrelated the interactions themselves. Nevertheless, such molecules may be present in the microhabitat of *B. subtilis* and have the ability to influence its development. Thus, it is tempting to speculate about the consequences of such interspecies interactions in natural environments. These interactions could benefit the inducing organism by reducing their competition for resources: in *B. subtilis*, matrix-producing cannibals and swimming subpopulations are mutually exclusive (22, 48). Alternatively, increases in this subpopulation of *B. subtilis* cells could be advantageous to both interacting partners, allowing them to be enclosed jointly by a common matrix within a multispecies biofilm. Finally, matrix-producing cannibals could represent a bet-hedging strategy for *B. subtilis* with regards to survival in complex microbial communities (49). By maintaining a subpopulation of matrix-producing cannibal cells, *B. subtilis* safeguards those cells against antibiotics produced by other bacteria while also producing its own toxic arsenal. Our results show that *B. subtilis* increases its relative subpopulation of matrix-producing cannibal cells in response to antibiotics produced by closely related organisms. Thus, by allowing the death of a subpopulation of its own cells, *B. subtilis* may enhance its ability to survive when confronted with other organisms in coculture.

Methods

Strains and Culture Conditions. Strains used and generated in this work are listed in Table S2.

The *B. subtilis* strain was NCIB3610 from our laboratory collection. New strains were generated by using SPP1 phage transduction (50). Lysate from ZK3757 (3610 *amyE*::P_{skf}-*yfp*, from our strain collection) was transduced into various kinase mutant strains, and spectinomycin-resistant colonies were selected. Recipient strains (from our strain collection) were HV1204 for ZK4814, CA051 for ZK4815, DL147 for ZK4816, DL153 for ZK4817, and HV1205 for ZK4818. All plates contained 20 mL, poured using a Wheaton Unispense liquid dispenser. Coculture and secondary screen plates were 0.1× LB broth (BD Difco), 100 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS, pH 7) (Sigma), and 1.5% (wt/vol) agar. The halo overlay plates were 1× LB, 1.5% (wt/vol) agar. Liquid LB broth contained no agar.

Reporter Preparation. *B. subtilis* reporter strains were cultured in LB broth to mid-log stage, diluted to an OD_{600} of 0.02, and regrown to mid-log stage at least twice to reduce background fluorescence levels. At mid-log stage after these dilutions, glycerol was added [15–20% (vol/vol) final solution], and aliquots were frozen at −80 °C. The number of colony-forming units per milliliter of aliquot was determined by plating serial dilutions on LB plates before use.

Soil Preparation. Soil was collected from the Boston, MA, area. The top ∼0.5 cm of soil was discarded, and samples below that level were collected. Soil A and Soil B were collected at the same location 3 mo apart (in August and November, respectively). Sterile saline [0.85% (wt/vol) NaCl] was added at a ratio of 10 mL/g of soil, and this suspension was either vortexed for 1 min or blended in a Waring blender for three 1-min cycles, with 1-min rests on ice. Glycerol was added [15–20% (vol/vol) final solution], aliquots were frozen at −80 °C, and the number of colony-forming units per milliliter was determined as with the reporters.

Coculture and Secondary Screens. Screen plates were grown at ambient room temperature (24–26 °C). For the coculture screen, reporter and soil aliquots were thawed and diluted independently in saline or 0.1× LB broth, and 25,000 cfu of each were spread simultaneously on plates using 3-mm glass beads. After 24–26 h of growth, plates were examined visually using a Zeiss Stemi SV6 stereoscope attached to a fluorescence illumination system (X-cite 120, Lumen Dynamics). Putative matrix-inducing soil colonies were selected and isolated. For the secondary screen, the test organisms were resuspended in 0.1× LB broth to an OD_{600} of 0.5, and 3 μL was spotted onto a dried plate freshly inoculated with a *B. subtilis* reporter microcolony lawn. After 24–26 h of growth, the plates were examined for fluorescence using a Typhoon 9600 fluorescence imager [GE Healthcare; 488-nm excitation, 526-nm emission, 500-V (photomultiplier tube), 100 μm resolution, 3 mm scan height].

Fluorescence Quantification. Typhoon data files (.gel) were loaded into Metamorph 7.1 (Molecular Devices), and brightness and contrast were adjusted linearly. Thresholding eliminated the pixel intensity of the agar. The average maximum intensity plus the signal-to-noise ratio (from at least 12 independent regions containing no microcolonies) was used as the lower bound in an inclusive threshold. Concentric regions of interest were defined around each colony spot. From the innermost, the regions of interest enclosed (1) the colony spot itself, (2) the microcolonies immediately surrounding the colony spot (potential induction area), (3) a spacer region, and (4) distant microcolonies (background intensity values). The average integrated intensity per area was determined by the intensity of region 2 minus the intensity of region 4. The resulting values were normalized by the background values to account for interplate variability. Values from each replicate assay were scaled relative to the maximum measured intensity for that assay to reduce noise and allow comparisons between biological replicates. Values for at least three independent experiments were averaged.

Halo Overlay Assay. Inducer strains were resuspended to OD_{600} = 0.5 in LB broth, spotted onto 20-mL LB plates, and grown for 5 d at 30 °C. Wild-type and ΔSpo0A *B. subtilis* strains were grown in LB medium at 37 °C to OD_{600} ∼1.0 and then were diluted 1:100 in LB agar [1.5% (wt/vol)] that had been melted and cooled to 50 °C. Ten-milliliter agar slabs embedded with *B. subtilis* were allowed to set and then were flipped onto the inducing strain colonies. After growth for 24 h at 30 °C, killing halos were measured as the distance between the outer edge of the colony and the *B. subtilis* halo, if any, and the difference between ΔSpo0A and wild-type halos was determined. Values for at least three independent experiments were averaged, with each experiment performed in duplicate.

Tests for Secreted Molecules. Agar was 0.1× LB, 100 MOPS unless noted. For cored plates, 3 μL of the inducers at OD_{600} = 0.5 were spotted onto 15-mL plates and grown for 2 d at 30 °C. Agar plugs containing the colonies were removed using sterilized corers, and the plates were UV-irradiated for 1 h. For cellophane separations, sterile cellophane (500PUT; Innovia Films, Inc.) was placed on 15-mL agar plates. Five milliliters of agar and then 3 μL of the inducer strain were added, and colonies were grown for 2 d at 30 °C before cellophanes and the top agar was removed. Then 5 mL of agar was added to the plates, and *B. subtilis* microcolony lawns were spread as for secondary screens. For CM, matrix inducers were grown in 0.1× LB medium or 1× LB medium for 4 d at 30 °C. Cultures were centrifuged, and the supernatant was filtered to sterilize it. Concentrated CM was tested for induction activity in a secondary screen. Matrix preparations were performed on cell pellets from the liquid cultures as described (11). For morphological tests, 20 μL of CM from LB cultures was spotted three successive times onto Minimal Salts glycerol glutamate (MSgg) plates (22) before 2 μL of *B. subtilis* was added. Colonies were grown for 3 d at 30 °C and were examined for a wrinkly phenotype indicative of hypercannibalism (26).

Sequence Analysis and Phylogenetic Trees. 16S rRNA genes were amplified in 50-μL PCR reactions using the 27F and 1492R primers (51), Qiagen Taq polymerase, and a DYAD DNA Engine Peltier Thermal Cycler (MJ Research). Amplicons were purified using a Qiagen PCR purification kit and sequenced using the 27F primer at the Dana-Farber/Harvard Cancer Center DNA Resource Core. Gene sequences were inspected manually for base-caller errors and were trimmed by removing any ambiguous trailing or leading bases using Sequencher (GeneCodes, Inc.). For soil clone library analysis, all sequences were longer than 450 bp. Sequences were checked for chimeras using Bellerophon, version 3 (52), and putative chimeras were removed from downstream analyses. The sequences were grouped into operational taxonomic units (OTUs) based on 99% sequence identity, and one representative of each OTU was compared with sequences in the National Center for Biotechnology Information database using BLAST. Rarefaction analysis was carried out using a Rarefaction Calculator (http://www2.biology.ualberta.ca/jbrzusto/rarefact.php). The Chao-1 nonparametric species richness estimator was used to estimate the total number of similar clusters of sequenced isolates (53, 54). 16S rRNA sequences from the soil analysis were deposited in GenBank (accession nos. JF501232–JF501388). For inducing isolates, all sequences were longer than 700 bp except for one, which was 576 bp. For phylogenetic trees, sequences were inserted into SILVA database dendrogram version 98 in ARB using parsimony (55, 56). Maximum-likelihood trees were constructed in ARB with novel and reference sequences selected from the ARB database using randomized axelerated maximum likelihood for high performance computing (RAxML-VI-HPC) v. 2.2 under the GTRCAT model of evolution (57). For constructing the tree, a *Listeria* sp. was used as an outgroup and subsequently was pruned. Bootstrap values greater than 50% (of 1,000 replicates) are shown. 16S rRNA gene sequences of inducing isolates were deposited in GenBank (accession nos. JF496856–JF496919).

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