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ENVIRONMENTAL MICROBIOLOGY

The Plant Host Induces Antibiotic Production To Select the Most-Beneficial Colonizers

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ABSTRACT Microbial ecosystems tightly associated with a eukaryotic host are widespread in nature. The genetic and metabolic networks of the eukaryotic hosts and the associated microbes have coevolved to form a symbiotic relationship. Both the Gram-positive *Bacillus subtilis* and the Gram-negative *Serratia plymuthica* can form biofilms on plant roots and thus can serve as a model system for the study of interspecies interactions in a host-associated ecosystem. We found that *B. subtilis* biofilms expand collectively and asymmetrically toward *S. plymuthica*, while expressing a nonribosomal antibiotic bacillaene and an extracellular protease. As a result, *B. subtilis* biofilms outcompeted *S. plymuthica* for successful colonization of the host. Strikingly, the plant host was able to enhance the efficiency of this killing by inducing bacillaene synthesis. In turn, *B. subtilis* biofilms increased the resistance of the plant host to pathogens. These results provide an example of how plant-bacterium symbiosis promotes the immune response of the plant host and the fitness of the associated bacteria.

IMPORTANCE Our study sheds mechanistic light on how multicellular biofilm units compete to successfully colonize a eukaryote host, using *B. subtilis* microbial communities as our lens. The microbiota and its interactions with its host play various roles in the development and prevention of diseases. Using competing beneficial biofilms that are essential microbiota members on the plant host, we found that *B. subtilis* biofilms activate collective migration to capture their prey, followed by nonribosomal antibiotic synthesis. Plant hosts increase the efficiency of antibiotic production by *B. subtilis* biofilms, as they activate the synthesis of polyketides; therefore, our study provides evidence of a mechanism by which the host can indirectly select for beneficial microbiota members.

KEYWORDS *Bacillus subtilis*, biofilms, nonribosomal peptides, symbiosis

In the densely populated plant root rhizosphere, soilborne microorganisms compete for resources such as nitrogen and organic material. One of the strategies evolved by rhizosphere bacteria to gain advantage over the competitors is to form complex structures referred to as biofilms. A biofilm is a differentiated community in which the residing cells are held together by an organic extracellular matrix and biogenic minerals (1–4). In a biofilm, cells use a variety of mechanisms to communicate and coordinate activity within, as well as across, species (5, 6). In many instances, biofilms formed on the surfaces of plant roots are beneficial to their hosts, for example, by preventing the growth of bacterial and fungal pathogens (7–9), and thus can be used as biocontrol agents. In addition, soil microbiota community members, such as *Bacillus subtilis*, belong to the group of plant growth-promoting rhizobacteria (PGPR) which, when applied to soils, can stimulate plant growth and increase yields (10–13). In addition to beneficial interactions with plants, bacterial biofilms also have an active role in the bioremediation of contaminated soils (14) and in carbon dioxide sequestration (15) and thus are of enormous ecological importance.

Citation Ogran A, Yardeni EH, Keren-Paz A, Bucher T, Jain R, Gilhar O, Kolodkin-Gal I. 2019. The plant host induces antibiotic production to select the most-beneficial colonizers. Appl Environ Microbiol 85:e00512-19. https://doi.org/10.1128/AEM.00512-19.

Editor Rebecca E. Parales, University of California, Davis

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Received 3 March 2019
Accepted 11 April 2019
Accepted manuscript posted online 19 April 2019
Published 17 June 2019

B. subtilis is a Gram-positive soil bacterium used as a highly efficient biocontrol agent, protecting plants from both fungal and bacterial pathogens (16–20). This protection is proposed to be mediated by the formation of robust biofilms (7, 21) and by the production of a wide range of antibiotics (22) that shape interspecies interactions (23). *B. subtilis* strains can also defend plants indirectly by activating plant defenses through the classical induced systemic defense response (ISR), subsequently preventing the growth of fungal pathogens (24, 25). Therefore, understanding the mechanisms of physical and chemical interactions between *B. subtilis* and competing rhizosphere biofilms is of particular interest in rhizosphere ecology.

In this study, we explored the interactions between the biofilms of the Gram-positive *B. subtilis* and the Gram-negative beneficial soil bacterium *Serratia plymuthica* (26, 27). The two species simultaneously colonize plant roots, and thus, especially when studied in association with a host plant, can serve as a model for interspecies interactions in the rhizosphere. Furthermore, as both species can be beneficial to the plant, the study of three-party interactions between these colonizers and their host can provide insights into the assembly of complex beneficial communities in the soil, as well as in other host-associated microbiota communities.

RESULTS

***B. subtilis* biofilm actively expands toward a competing *S. plymuthica* colony.** When grown on a solid biofilm-inducing medium, *B. sutbilis* biofilms form symmetrical, circular colonies. To determine the effect of a competing *S. plymuthica* colony on the development of a *B. subtilis* biofilm, the two species were inoculated next to each other on a solid biofilm medium. After 2 days, the *B. subtilis* colony reached *S. plymuthica*, forming a thick wrinkle around its edge and penetrating toward its center. By the third day, *B. subtilis* biofilm completely engulfed the *S. plymuthica* colony, covering it with a thin, unstructured film and enclosing it within the circular wrinkle (Fig. 1A). The location and shape of the *B. subtilis* center of the biofilm did not change during the interaction with *S. plymuthica*. However, the biofilm colony advanced asymmetrically toward the *S. plymuthica* colony, breaking from its usual circular shape (Fig. 1A).

We next examined the mechanisms that could mediate this asymmetric expansion toward a competitor. Many bacterial cells are capable of directional swimming using their flagella (28). The genetic basis of flagellar swimming in *B. subtilis* is well known. *B. subtilis* has peritrichous flagella (29) that propel the cell forward (30, 31). Each flagellum is composed of a basal body, a hook, and a filament composed of many flagellin monomers (32). The flagellin is encoded by the *hag* gene, which is transcribed by a σ^D RNA polymerase encoded by *sigD* (33). In addition, flagellar rotation can also be controlled by chemotaxis (28). To assess whether motility and chemotaxis are responsible for the asymmetrical spreading of the *B. subtilis* biofilm, two motility mutants, the Δ*sigD* and Δ*hag* strains, and two chemotaxis mutants, the Δ*cheA* and Δ*cheY* strains (28, 31, 34), were tested. Again, the parental *B. subtilis* and the mutant strains were inoculated next to *S. plymuthica* at various distances and allowed to grow for 3 days. The asymmetry of each colony was quantified as the ratio between the vertical radius extending toward the *S. plymuthica* colony and the opposite vertical radius. Wild-type *B. subtilis* and all four mutants exhibited similar asymmetrical growth toward the *S. plymuthica* colony, at inoculation distances of $<$1.5 cm (Fig. 1B). Those results suggest that neither flagellar motility nor chemotaxis is required for the ability of *B. subtilis* to expand specifically in the direction of a competitor.

We next decided to examine which component of the *S. plymuthica* colony could promote this behavior. We repeated the experiment, but this time, the asymmetrical expansions toward live *S. plymuthica* cells, heat-killed *S. plymuthica,* or a supernatant of *S. plymuthica* spotted next to the *B. subtilis* colony were compared. The supernatant of *S. plymuthica* culture had no effect on the expansion of *B. subtilis*, further supporting the conclusion that the movement of *B. subtilis* biofilm was not initiated by signaling molecules secreted by the competitor and did not involve chemotaxis (Fig. 1C). On the other hand, *S. plymuthica* cells killed by heat promoted asymmetrical expansion,

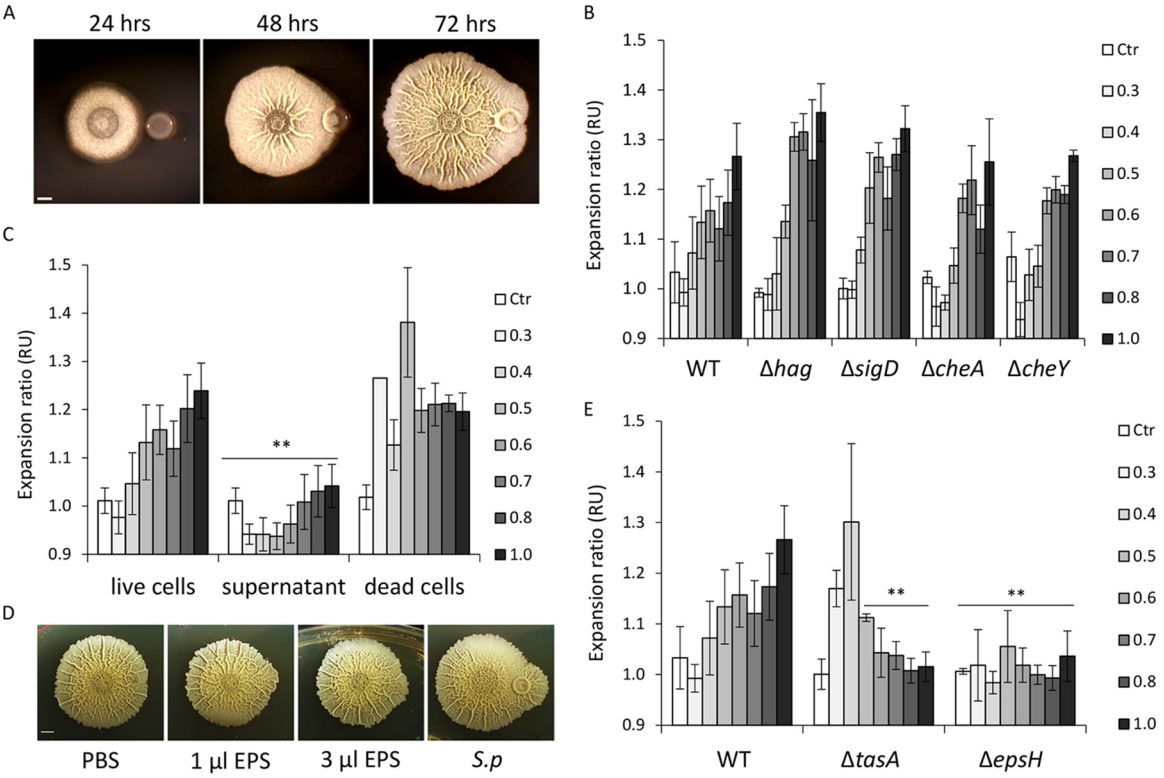

FIG 1 *B. subtilis* expands asymmetrically toward *S. plymuthica*. (A) Time course of interaction between *B. subtilis* and *S. plymuthica*, with no contact (24 h), initial contact (48 h), and engulfment (72 h). Top-down images of biofilm colonies grown on solid biofilm-inducing medium are shown; scale bar = 0.2 cm. (B) Quantification of asymmetric expansion of *B. subtilis* biofilm toward *S. plymuthica* biofilm. Wild-type (WT) *B. subtilis* and the indicated mutant strains were inoculated near *S. plymuthica* at different distances as indicated in the key (in cm), and incubated at 30°C for 3 days. The expansion ratio was defined as the ratio between the radius from the center of the *B. subtilis* biofilm to the *S. plymuthica* colony and the radius extending to the opposite side of the biofilm. *B. subtilis* biofilm grown alone served as a control (Ctr). RU, relative units. (C) Quantification of asymmetric expansion of *B. subtilis* biofilm toward live *S. plymuthica*, the supernatant of *S. plymuthica*, or dead *S. plymuthica* cells. (D) Top-down images of *B. subtilis* biofilm expanding toward PBS (negative control), indicated amounts of isolated *S. plymuthica* (*S.p*) EPS, or *S. plymuthica* colony (positive control). (E) Quantification of asymmetric expansion of *B. subtilis* biofilm toward *S. plymuthica* biofilm. WT *B. subtilis* and the indicated mutant strains were inoculated near *S. plymuthica* at different distances, as indicated by the key, and incubated at 30°C for 3 days. This experimental series was done in parallel to the experiment in panel B; therefore, the same WT control is used. **, $P < 0.005$ based on a two-tailed Student's *t* test of the entire indicated measurement series. Error bars represent standard deviations. All experiments were performed at least 3 times with at least two technical repeats.

similarly to live *S. plymuthica* cells (Fig. 1C). This raised the possibility that this collective movement could be aided by organic polymers associated with *S. plymuthica* cells. We therefore directly tested the possibility of *B. subtilis* movement being aided by the exopolysaccharide (EPS) of the competitor colony. EPS from an *S. plymuthica* colony was isolated and spotted next to *B. subtilis*. Indeed, *B. subtilis* was able to expand toward isolated EPS but not toward phosphate-buffered saline (PBS) (Fig. 1D). These results suggest that asymmetrical expansion might be achieved by a sliding motion and that EPS can be shared between different species to facilitate this motion.

It was previously shown that *B. subtilis* is capable of using its own matrix components, primarily EPS encoded by the *eps* operon, to slide toward new territories (35, 36). We therefore assessed the contribution of matrix components to asymmetric expansion. We tested the ability of the Δ*eps* and Δ*tasA* mutants (lacking the protein component of the extracellular matrix) to asymmetrically expand toward its competitor. The Δ*eps* mutant could not specifically expand toward *S. plymuthica* at any of the tested distances. The Δ*tasA* mutant was capable of asymmetric expansion, but only at shorter distances (Fig. 1E).

Taken together, these results demonstrate that *B. subtilis* biofilms are capable of directional movement toward their competitors. Interestingly, this collective migration

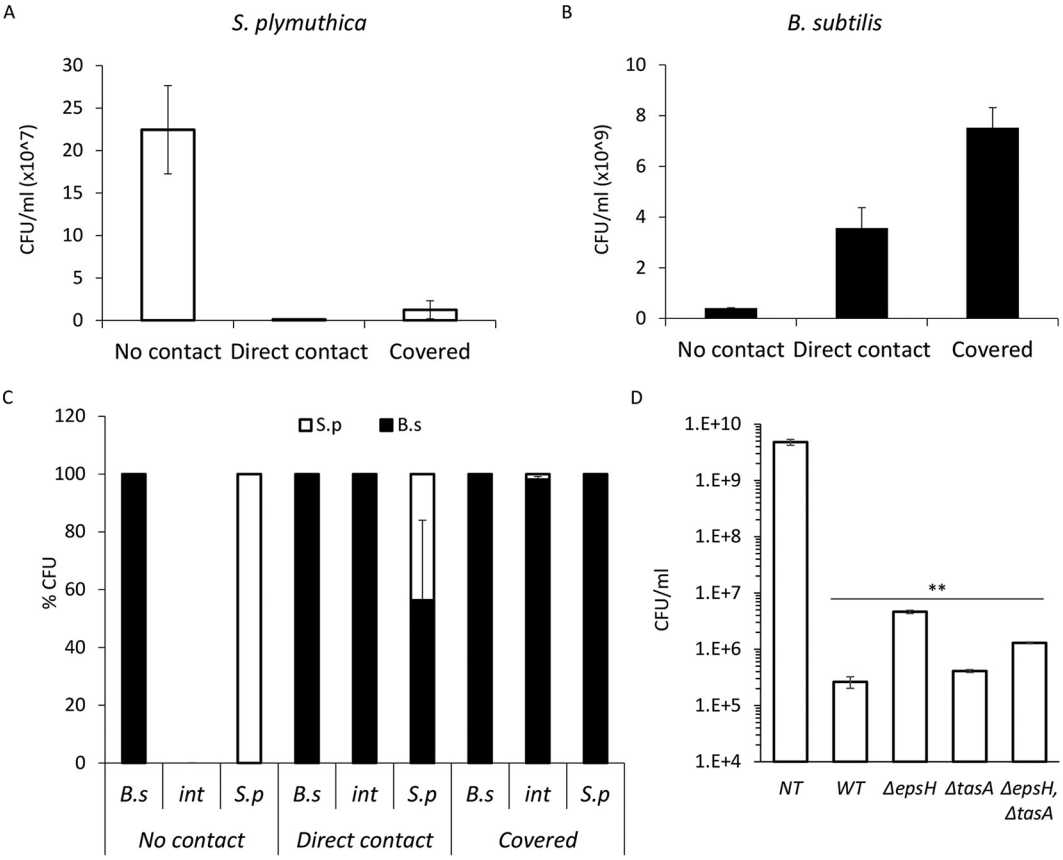

FIG 2 *B. subtilis* eliminates *S. plymuthica* cells. (A) The number of CFU of *S. plymuthica* isolated from the area of interaction at 24 h (no contact), 48 h (direct contact), and 72 h (covered) after inoculation. Error bars represent standard deviations of the results from 5 biological replicates. (B) CFU counts of *B. subtilis* isolated from the area of interaction at 24 h (no contact), 48 h (direct contact), and 72 h (covered) after inoculation. Error bars represent standard deviations of the results from 5 biological replicates. (C) Relative CFU counts at each stage of interaction. The interacting colonies were divided into three areas, as follows: *B.s*, the area of the *B. subtilis* colony most distant from the interaction area; *int*, the area of direct interaction; and *S.p*, the area of the *S. plymuthica* colony most distant from the interaction area. Each section was separately harvested, sonicated, and plated to determine the number of replicative cells of each species. The experiment was repeated at 24 h (no contact), 48 h (direct contact), and 72 h (covered) after inoculation. (D) Total CFU counts of *S. plymuthica* following the interaction with wild-type *B. subtilis* (WT) and the indicated strains. Colonies were inoculated 0.3 cm apart to compensate for the expansion defect. **, $P < 0.005$ based on a two-tailed Student's *t* test, compared to *S. plymuthica* colony grown alone (untreated control [NT]). Error bars represent standard deviations. All experiments were performed at least 3 times with at least three technical repeats.

occurs on top of 1.5% agar, which is a concentration 2-fold higher than the maximal concentration permitting sliding in *B. subtilis* monocultures (37), consistent with similar observations that were recently made for additional interspecies interactions between *B. subtilis* and *Streptomyces* spp. (38, 39). This movement does not require flagellar motility or chemotaxis. Instead, the cells in the expanding biofilm colony are likely sliding on the extracellular matrix produced by their own colony and, here, also by their competitor.

The ability of *B. subtilis* to eliminate *S. plymuthica* cells is mediated by bacillaene and by the extracellular protease AprE, but not by ECM. Next, we tested the outcome of the above-described interaction between the competing species regarding their viability. We determined the absolute (Fig. 2A and B) and relative (Fig. 2C) CFU of both species before contact with the competitor, at the time of the initial direct contact, and after engulfment. Contact with *B. subtilis* led to a dramatic reduction in the viability of *S. plymuthica* (Fig. 2A and C), while the number of viable *B. subtilis* cells actually increased during the interaction (Fig. 2B and C). Thus, upon reaching its competitor, *B. subtilis* was able to efficiently kill it. Unlike the ability to slide in the direction of *S. plymuthica*, the ability to eradicate it was not dependent on extracellular matrix (ECM)

production, as all matrix mutants reduced the amount of viable *S. plymuthica* cells when inoculated at a short distance to compensate for their inability to expand toward it (Fig. 2D; see also Fig. S1 in the supplemental material).

A nonribosomal peptide that contributes to the biocontrol capacity of *B. subtilis* is surfactin, a surface- and membrane-acting lipopeptide (40, 41). Surfactin mediates both movement by reducing surface tension to promote collective flagellar and sliding motility (42, 43) and toxicity of *B. subtilis* toward biofilm competitors from related species (44). While the Δ*srfAA* mutant had a defect in expansion toward *S. plymuthica* colonies consistent with its role in promoting sliding motility (data not shown), it was still capable of eradicating the colony cells after the formation of a direct contact (Fig. S2), further suggesting that the ability of *B. subtilis* to kill its competitor is independent from motility. Taken together, these results demonstrate that asymmetric expansion creates the opportunity for contact but is not essential for the eradication of *S. plymuthica* by *B. subtilis*.

B. subtilis has a repertoire of antibacterial molecules it can produce to kill competitors. To determine which of those molecules are involved in the elimination of *S. plymuthica* in our system, we tested which antibiotics are secreted by *B. subtilis* under the conditions used in this study. We allowed *B. subtilis* to form biofilm colonies on solid medium and analyzed the medium to identify molecules secreted by the bacterial cells using liquid chromatography-mass spectrometry analysis (Fig. S3). We detected the presence of bacillaene (45, 46), a polyketide antibiotic synthesized by enzymatic complex encoded in a *pks* gene cluster and previously implicated in bacterial predator-prey interactions (47) (Fig. 3A, left). We therefore set out to test whether this is the molecule responsible for killing the competing colony.

To determine the role of bacillaene in competitor eradication, we tested the ability of the Δ*pks* mutant, which is unable to produce bacillaene (Fig. 3A, right), to kill *S. plymuthica*. As expected, the engulfing wild-type *B. subtilis* colony promoted the death of *S. plymuthica* cells, as reflected by a drop in the number of viable cells. However, when engulfed by the Δ*pks* mutant, *S. plymuthica* cells retained stable cell counts (Fig. 3B). Moreover, when the *S. plymuthica* cells interacting with the wild-type *B. subtilis* were examined by light and electron microscopy, there was a clear impact on their morphology, and an increased number of lysed cells was observed. No such effect could be detected in cells interacting with the Δ*pks* mutant strain; the cells retained an intact morphology and a thick extracellular matrix (Fig. 3C and D). Next, we extracted the molecules secreted by a *B. subtilis* colony and examined their effect on planktonic growth of *S. plymuthica*. Molecules secreted into the solid medium by the wild-type strain, but not the Δ*pks* mutant, inhibited the growth of *S. plymuthica* (Fig. S4). Moreover, deletion of the *pks* operon completely eliminated the toxicity of *B. subtilis* conditioned medium against *S. plymuthica* (Fig. 3E). Interestingly, the *pks* operon is highly conserved in bacilli residing exclusively in the rhizosphere but not in *B. cereus* and *B. anthracis*, which reside in a broader range of hosts (Fig. S5). Consistent with the hypothesis that polyketide synthesis (PKS) is relevant to the competitiveness of *B. subtilis* in the soil, the Δ*pks* mutant could not eliminate an additional Gram-negative competitor from a different genus, *Pseudomonas chlororaphis* (Fig. S6).

In addition to antibiotics, *B. subtilis* biofilm cells are also capable of producing extracellular proteases that allow them to "mine" extracellular material, such as dead cells, breaking these macromolecules down into amino acids that can then be imported and used for further growth (48). As the CFU of *B. subtilis* increased following interaction with *S. plymuthica* cells (Fig. 2B), we decided to examine the contribution of the main extracellular proteases (AprE, Vpr, and Mpr) to the ability of *B. subtilis* to overcome its competitor during the interaction. The deletion of *aprE*, but not of the other proteases, inhibited the eradication of *S. plymuthica* (Fig. 3F and S7). However, its effect on viability of the competitor was less dramatic than that of *pks* (Fig. 3F), suggesting an indirect mechanism. One intriguing possibility is that consuming dead competitor cells provides *B. subtilis* with a growth advantage, and the increased rate of growth further enhances its ability to successfully compete and eradicate its neighbor.

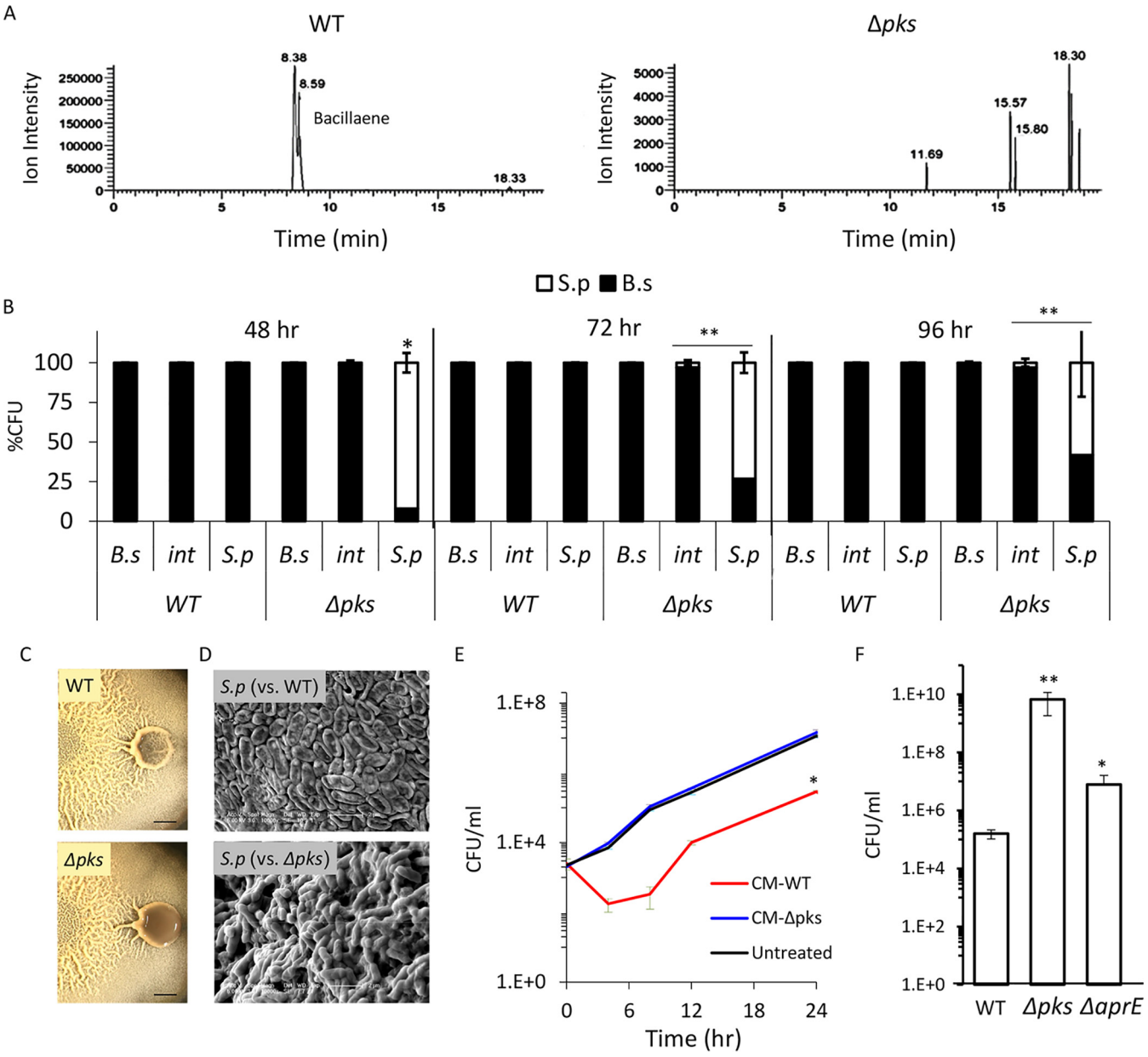

FIG 3 *B. subtilis* eliminates *S. plymuthica* cells in a PKS-dependent manner. (A) Liquid chromatography-mass spectrometry of a wild type (right) and Δ*pks* mutant (left) biofilm colonies extracted with isopropanol. The chromatogram is focused on *m/z* 581.3585 and related masses. The peaks at retention times of 8.38 min and 8.59 min in the WT extract represent two isomers of bacillaene and are fully absent in the Δ*pks* mutant extract. (B) Relative CFU counts of *B. subtilis* WT or Δ*pks* mutant and *S. plymuthica* at each stage of the interaction. The interacting colonies were divided into three areas as follows: *B.s*, the area of the *B. subtilis* colony most distant from the interaction area; *int*, the area of direct interaction; *S.p*, the area of the *S. plymuthica* colony most distant from the interaction area. Each section was separately harvested, sonicated, and plated to determine the number of replicative cells of each species. The experiment was repeated at 48 h, 72 h, and 96 h after inoculation. (C) Top-down images of interaction between *S. plymuthica* and either WT *B. subtilis* or Δ*pks* mutant after 4 days of incubation. Scale bar = 0.2 cm. (D) SEM images of *S. plymuthica* from the interaction zone between and either WT *B. subtilis* or Δ*pks* mutant. Cells were harvested and visualized after 48 h of incubation at 30°C. (E) Growth curves of *S. plymuthica* in liquid biofilm medium, supplemented with 50% (vol/vol) of *B. subtilis* WT or Δ*pks* mutant conditioned medium (CM). Experiments were performed at 30°C. (F) The number of CFU represents *S. plymuthica* replicative cells, at 72 h after coinoculation with WT *B. subtilis*, Δ*pks* mutant, and Δ*aprE* mutant strains. *, $P < 0.01$; **, $P < 0.005$ based on a two-tailed Student's *t* test of the entire indicated measurement series. Error bars represent standard deviations. All experiments were performed at least 3 times with at least three technical repeats.

Overall, our results separate two biological properties that contribute to the success of *B. subtilis* during the interaction with *S. plymuthica*, EPS-dependent sliding movement that leads to asymmetric expansion toward its competitor, and bacillaene-dependent killing once the two colonies make contact (summarized in Table S1).

Plant host promotes *pks*-dependent killing via Spo0A. Both *B. subtilis* and *S. plymuthica* reside in the soil rhizosphere of temperate habitats, where the temperature

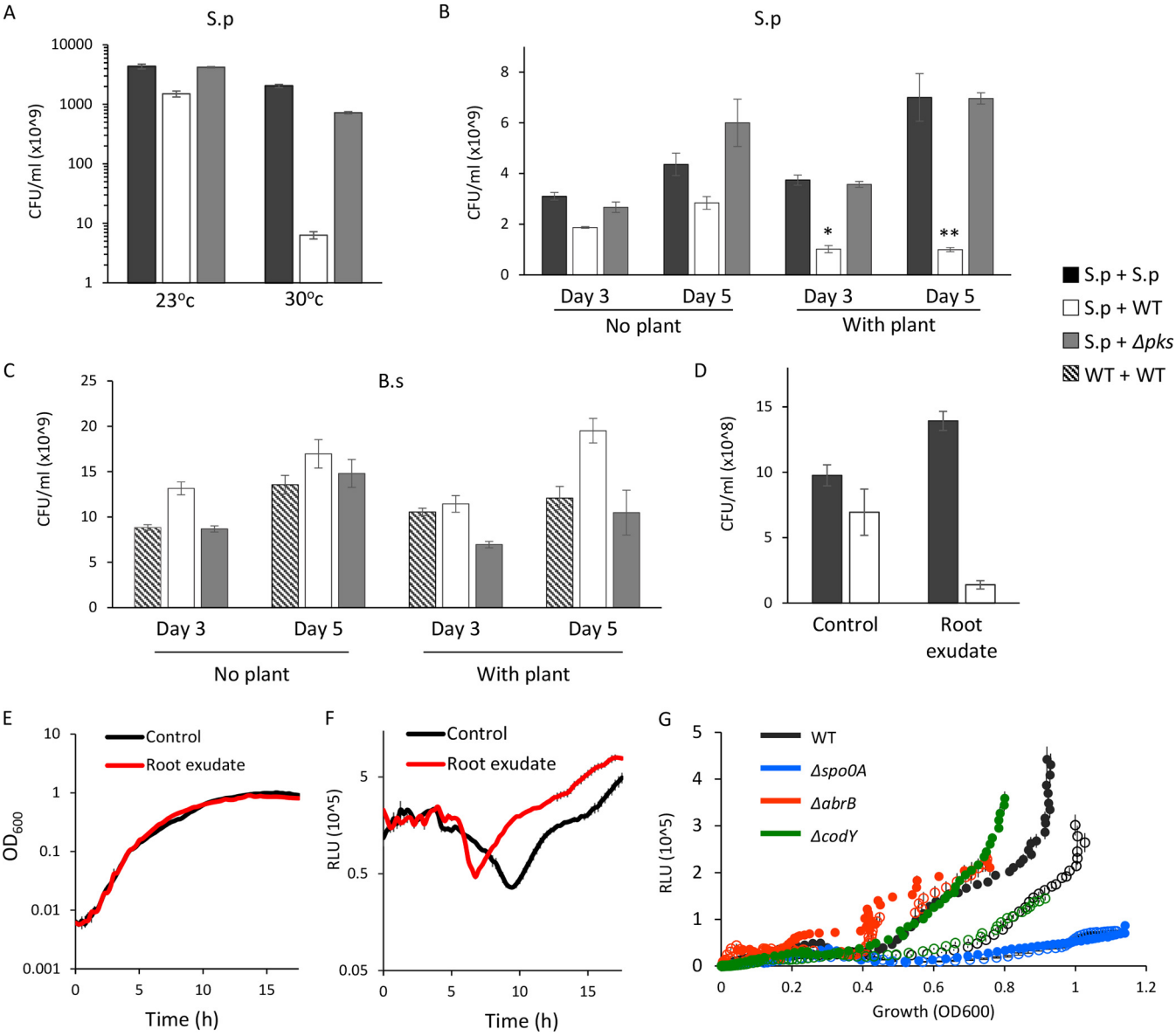

FIG 4 *pks*-dependent killing of *S. plymuthica* by *B. subtilis* is induced by the plant host at an environmental temperature. (A) Relative CFU counts of *S. plymuthica* inoculated alone (S.p + S.p, black bars), next to WT *B. subtilis* (S.p + WT, white bars) or Δ*pks* mutant (S.p + Δ*pks*, gray bars). The interacting colonies were incubated at 30°C and 23°C for 3 and 5 days, respectively, harvested, and plated to determine the number of replicative cells. (B and C) Relative CFU counts of *S. plymuthica* (B) and *B. subtilis* (C) after interaction in proximity of 0.8 cm to *E. sativa* seedling (with plant) and without seedling (no plant) for 3 and 5 days at 23°C. (B) *S. plymuthica* CFU were obtained after interaction with either another *S. plymuthica* biofilm (S.p + S.p, black bars), WT *B. subtilis* biofilm (S.p + WT, white bars) or with Δ*pks* mutant *B. subtilis* biofilm (S.p + Δ*pks*, gray bars). (C) *B. subtilis* CFU were obtained after interaction with either another *B. subtilis* biofilm (WT + WT, striped bars) as a control, *S. plymuthica* biofilm (WT + S.p, white bars), or Δ*pks* mutant with *S. plymuthica* (Δ*pks* + S.p, gray bars). (D) Relative CFU counts of *S. plymuthica* after interaction with either another *S. plymuthica* biofilm (S.p + S.p, black bars) as control, or WT *B. subtilis* (S.p + WT, white bars) on a biofilm-inducing solid medium pretreated with root exudate or plant growth medium (control) for 5 days at 23°C. (E) Growth (OD_{600}) P_{pksC}-*lux* strain treated with root exudate (red) or plant growth medium (black). (F) Growth-normalized luminescence (relative light units [RLU]) of P_{pksC}-*lux* strain treated with root exudate (red) or plant growth medium (black). (G) Luminescence (in RLU) of *B. subtilis* carrying the P_{pksC}-*lux* construct in WT or the indicated deletion mutants. Open circles represent the untreated control, while the full circles represent the addition of root exudate to the growth medium. *, $P < 0.01$; **, $P < 0.005$ based on a two-tailed Student's *t* test. Error bars represent the standard deviations. The results are averages of the results from a representative experiment performed with at least three technical repeats out of three independent experiments.

fluctuates between 15°C and 23°C (49). We therefore decided to test whether *pks*-dependent killing also occurs at the environmental temperature. Surprisingly, while being highly effective in killing *S. plymuthica* at 30°C, we found that *B. subtilis* could not efficiently kill it at 23°C (Fig. 4A). However, in the natural niche, additional species are present, most importantly, the host plant. Therefore, we decided to set up a plant-host model system. *Eruca sativa* (salad rocket) is a winter annual undomesticated wild

flowering plant that grows in nature mostly around the Mediterranean basin (50). It belongs to the Brassicaceae family, which includes various natural plant hosts for both *Bacillus* and *Serratia* species (51).

In the presence of an *E. sativa* plant host at 23°C, the killing of *S. plymuthica* was increased from 2-fold to 26-fold in a *pks*-dependent manner (Fig. 4B). Again, the killing was linked to predation and resulted in an increased number of *B. subtilis* cells, and this result could not be attributed to growth induction of *B. subtilis* by the host (Fig. 4C). In contrast, while the growth of *S. plymuthica* was indeed improved when grown together with *E. sativa* (Fig. 4B), it still could not overcome the *pks*-dependent killing in three-party interaction experiments.

Even in the absence of the plant, the addition of root exudate was sufficient to induce the killing of *S. plymuthica* by *B. subtilis*, suggesting that this induction is mediated by a factor secreted by the plant (Fig. 4D). In order to better understand the molecular mechanism of induction of *pks*-dependent killing, we tested the effect of the root exudate on the expression of the *pks* operon. As shown, purified root exudate of *E. sativa* had no impact on cell growth (Fig. 4E) but had significantly ($P < 10^{-9}$) induced *pks* expression (Fig. 4F). In contrast, we could detect no induction of *pks*, *eps,* or *tasA* expression by direct contact with an *S. plymuthica* colony during expansion and engulfment (Fig. S8) or by its conditioned medium (data not shown).

The transcription of the *pksA-R* operon (the operon containing *pksA* and *pskR* and the genetic material in between) initiates from a promoter located upstream of *pksC* and is regulated by two master regulators (52), CodY, which senses the levels of branched amino acids (53, 54), and the transition phase regulator AbrB (55). In turn, AbrB is inhibited by the master regulator of sporulation and biofilm development, Spo0A (56). Previous publications indicated that plant exudates of tomato and *Arabidopsis* spp. can induce Spo0A by several independent mechanisms (57, 58) and therefore may repress AbrB. We used a luciferase reporter driven by the *pksC* promoter to measure the induction of *pks* expression by root exudate in the wild type and in the Δ*spo0A*, Δ*abrB,* and Δ*codY* mutant strains. The activation of the *pks* promoter by the root exudate required Spo0A and AbrB but not CodY (Fig. 4G). Two target genes of Spo0A (*sinI* and *sdpA*) activated by low Spo0A-P were also induced by exudates of *E. sativa* similarly to the *pks* operon (Fig. S9). Altogether, these results support the hypothesis that the plant increases the *pks*-dependent killing of *S. plymuthica* by *B. subtilis* by inducing the transcription of the *pks* operon in an Spo0A-dependent manner.

***pks*-induced killing of *S. plymuthica* allows *B. subtilis* to enhance the systemic resistance of the plant.** Finally, we wondered whether the plant host has an underlying preference toward *B. subtilis*. Both *B. subtilis* and *S. plymuthica* are considered biocontrol species (40, 59). Therefore, we explored their relative contributions to the activation of the plant's immune system. Both bacterial species were inoculated on the root, either alone or in competition, and the bacteria were allowed to form root-associated communities. Next, the pathogen *Pseudomonas syringae* was injected directly into the leaves of precolonized *E. sativa* plants. After a week, the area of leaf necrosis was measured (Fig. 5A). As no direct contact occurred between the root-associated biofilms and *P. syringae*, enhanced resistance is the readout of the immune response of the plant hosts. Biofilms containing *B. subtilis* alone or together with *S. plymuthica*, but not *S. plymuthica* alone, provided significantly enhanced systemic resistance (Fig. 5B). When inoculated alone, the Δ*pks* mutant could provide protection similar to that of the wild-type *B. subtilis*, further demonstrating that *pks* is not required for this aspect of interaction. However, when inoculated together with *S. plymuthica*, the Δ*pks* mutant offered no protection to the plant (Fig. 5C). This was due to its inability to compete with *S. plymuthica*, as demonstrated by the CFU count (Fig. 5D). This result is a strong indication that the *pks* operon is fundamental to *B. subtilis*-plant interactions in competitive environments. Furthermore, these results could indicate a mechanism by which plants shape the rhizosphere communities by specifically regulating antibiotic production.

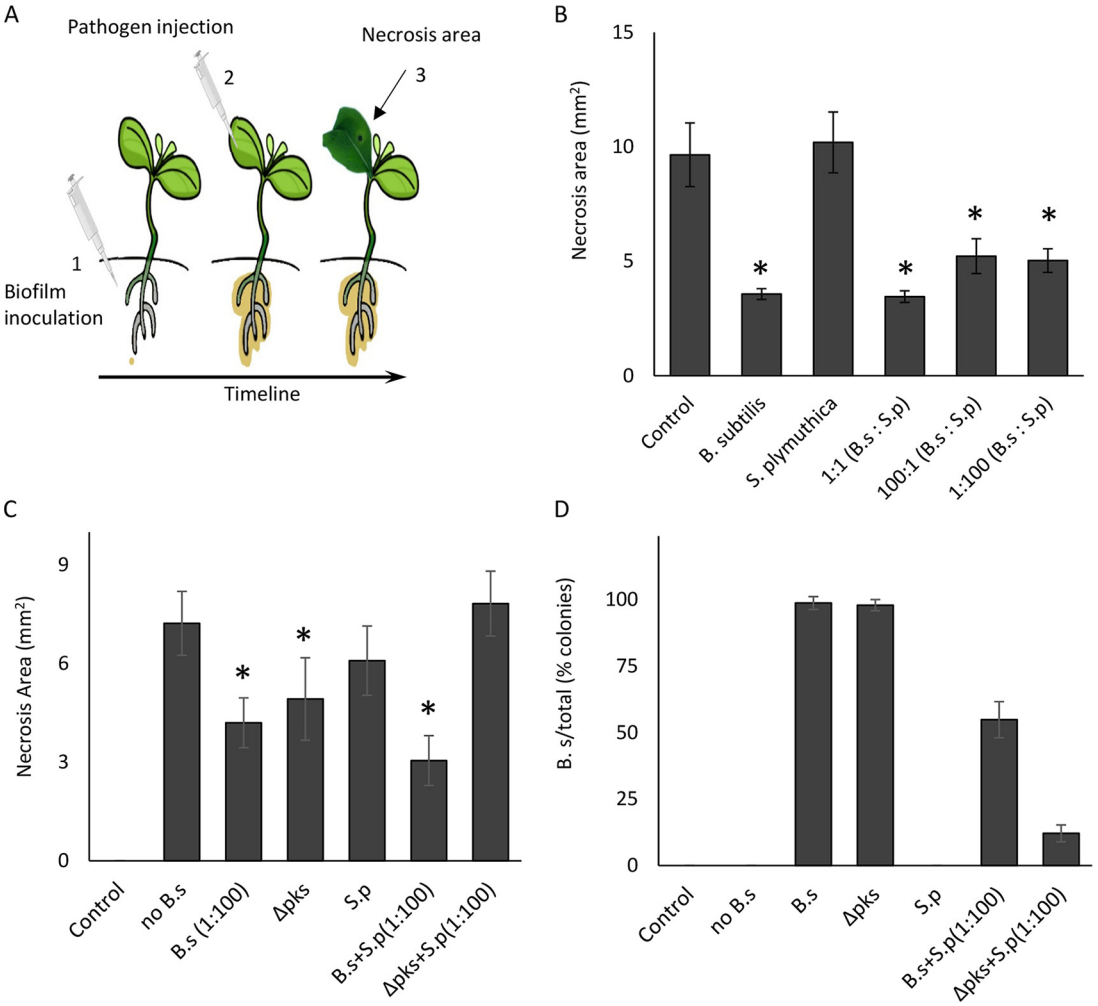

FIG 5 The effects of *B. subtilis* and *S. plymuthica* on systemic resistance of their plant host. (A) Diagram of the experimental setting. Biofilms were established on *E. sativa* plant roots prior to infection of their leaves with *P. syringae*. (B) Quantification of necrosis area (in square millimeters) on *E. sativa* leaves infected by the pathogen *P. syringae*. The necrosis area was measured in *E. sativa* seedlings whose roots were inoculated with *B. subtilis*, *S. plymuthica,* and their combination in ratios of 1:1, 100:1, and 1:100. (C) Quantification of necrosis area (in square millimeters) on *E. sativa* leaves infected by the pathogen *P. syringae*. The necrosis area was measured in *E. sativa* seedlings whose roots were inoculated with wild type (WT) *B. subtilis* (diluted 1:100 in LB to an OD equivalent to that for the 1:100 ratio in panel B), Δ*pks* mutant, *S. plymuthica*, or combinations of the strains as indicated in the figure. (D) CFU counts of *B. subtilis* isolated from the samples from panel C. After their leaves were harvested and analyzed, bacteria were harvested from the roots of the plants, and the relative CFU counts of *B. subtilis* were determined. *, $P < 0.005$. Results represent averages of the results from a representative experiment performed with at least five technical repeats out of three independent experiments. Error bars represent standard deviations.

DISCUSSION

In a biofilm, cells use a variety of mechanisms to coordinate activity within the community, as well as across species (5, 6). In many instances, biofilms are beneficial to their hosts; e.g., biocontrol agents form biofilms on the surfaces of plant roots, thereby preventing the growth of various pathogens (7), similarly to probiotic bacteria in the gut (60).

In natural environments, such as the rhizosphere, beneficial bacterial communities are surrounded by a multitude of other organisms, including other bacteria. *B. subtilis*, a biocontrol agent whose genome contains numerous gene clusters that control the expression of antibiotics, generally resides within this complex environment (16, 61). It is capable of a variety of chemical interactions with its competitors, as follows: pirated siderophores promote sporulation (62), *B. cereus* produces thiopeptide antibiotic that promotes *B. subtilis* biofilm formation (63), surfactin is involved in competition between

B. subtilis and *B. simplex* (44), and bacillaene promotes the competitiveness of *B. subtilis* versus the Gram-positive bacterium *Streptomyces coelicolor* and the Gram-negative bacterium *Myxococcus xanthus* (47, 64). Collectively, these findings indicate that the presence of a diverse arsenal of antibiotics assists *B. subtilis* in competition against various species in its natural habitats.

The mechanisms underlying activation and deployment of this arsenal of antibiotics remain largely elusive. One such possible mechanism would be sensing and responding to other species competing for the same ecological niche (61, 65). Thus, studying social interactions between *B. subtilis* biofilms and bacterial species invading their territory and the outcomes of such interactions on synthesis of relevant antibiotics is of particular interest.

In this work, we characterized the interactions between *B. subtilis* and *S. plymuthica,* an additional biocontrol agent that suppresses bacterial and fungal pathogens (66–71). When encountering an *S. plymuthica* colony, *B. subtilis* encircled and engulfed the colony in a flagellum- and chemotaxis-independent manner (Fig. 1). However, matrix components, such as exopolysaccharides and TasA, were necessary for *B. subtilis* to advance asymmetrically toward the *S. plymuthica* colony. Flagellum-independent surface motility has been shown to promote symmetrical expansion of *B. subtilis* during the growth of bacterial biofilms (43, 72–75). This behavior was suggested to improve access to nutrients while bacterial consumption equals uptake and starvation occurs (72). Our results suggest an additional layer of ecological significance to EPS-dependent collective motility, as it leads to the success of *B. subtilis* in competitive interspecies interactions.

When *B. subtilis* colonies encircled *S. plymuthica*, the secreted antibiotic bacillaene, produced by the *pks* cluster (45), eliminated the competitor colony. The secretion of the antibiotic and ability to move toward and engulf the competing colony were independent, suggesting that *pks* products may be diffusion limited and reach the minimal effective threshold only over short distances, as occurs when two competing colonies merge (Fig. 2 and 3). Alternatively, the *pks* products may remain bound to the bacterial surface and extracellular polymers, requiring direct contact. The presence of bacillaene determined the outcome of the interaction, resulting in the predation of *S. plymuthica* by *B. subtilis* (Fig. 3).

Finally, we found that the plant host played an important role in the regulation of the bacterial interactions (Fig. 4 and 5). The plant host induced *pks*-dependent killing of *S. plymuthica* by *B. subtilis* at an environmental temperature. In turn, *B. subtilis*, but not *S. plymuthica*, enhanced the systemic immunity of the plant host. As tomato (but not Brassicaceae) was recently found to inhibit Spo0A during overgrowth of *B. subtilis* biofilms (76), Spo0A induction could be highly host and temperature specific.

Since *B. subtilis* was first described by Ferdinand Cohn in the late 1800s, it was shown to specialize in the production of metabolites (77). Many of the biosynthetic pathways for these metabolites are conserved either across the entire *Bacillus* genus or within specific phylogenetic clades. Fengycin, bacillaene, iturin, and difficidin were essentially observed only within the *B. subtilis* group. This suggests that the different environmental niches inhabited by members of the *B. subtilis* clade may select for conservation of metabolites with distinct (or potentially redundant) beneficial functions (78). We here describe one such function, that of outcompeting *S. plymuthica* in nonfavorable growth temperature.

The complexity of these three-player interactions suggests that *B. subtilis* biofilms can be considered a part of the plant host microbiome, with the host actively promoting the establishment of the most beneficial bacterial community. Our findings provide a simple example of high-order interactions that shape microbiomes; the host modulates antibiotic production in the desired bacterial colonizers, providing the colonizers a clear advantage over less beneficial potential residents.

TABLE 1 Bacterial strains used in this work[a]

Strain	Genotype	Reference or source
B. subtilis		
NCIB 3610	Wild type	79
IKbs0222	Δ*hag*::*tet*	80
IKbs0210	Δ*sigD*::*tet amyE*::P_{hag}-*gfp* (Cam)	Lab collection
IKbs0225	Δ*cheA*::*tet*	81
IKbs0226	Δ*cheY*::*tet*	81
IKbs0008	Δ*epsH*::*tet*	82
IKbs0005	Δ*tasA*::*kan*	83
IKbs0227	Δ*srfAA*::*mls*	80
IKbs0042	Δ*vpr*::*kan*	Lab collection
IKbs0041	Δ*mpr*::*tet*	Lab collection
IKbs0036	Δ*aprE*::*mls*	Lab collection
IKbs0224	Δ*pksA-R*::*spec*	46
IKbs0035	Δ*pksA-E*::*tet*	45
IKbs0085	*sacA*::P_{sdpA}-*lux* (Cam)	57
IKbs236	*lacA*::P_{sinI}-*lux* (Cam)	84
IKbs0887	*sacA*::P_{pksC}-*lux* (Cam)	This study
IKbs0888	Δ*spo0A*::*kan sacA*::P_{pksC}-*lux* (Cam)	*spo0A*::*kan* (85) × IKbs0887
IKbs0889	Δ*abrB*::*kan sacA*::P_{pksC}-*lux* (Cam)	*abrB*::*kan* (85) × IKbs0887
IKbs0891	Δ*codY*::*kan sacA*::P_{pksC}-*lux* (Cam)	Δ*codY*::*kan** × IKbs0887
Serratia plymuthica IC1270	Wild type	L. Chernin
Pseudomonas chlororaphis PC-449	Wild type	R. Kolter

[a]All *B. subtilis* strains are derived from NCIB 3610 and were constructed as described in Materials and Methods. Cam, chloramphenicol resistance.

MATERIALS AND METHODS

Strains and media. All experiments were performed with the natural *B. subtilis* isolate NCIB 3610 and its derivatives. The strains used in this study are listed in Table 1 and were referenced previously (45, 46, 57, 79–85). A natural isolate of *S. plymuthica* was received from the lab of Leonid Chernin, and a natural isolate of *P. chlororaphis* was received from the lab of Roberto Kolter. 16S rRNA gene analysis was used to confirm the species of the *S. plymuthica* and *P. chlororaphis* strains. Assays were carried out with the biofilm-inducing medium MSgg, which contains 5 mM potassium phosphate, 100 mM morpholinepropanesulfonic acid (MOPS) (pH 7), 2 mM $MgCl_2$, 50 μM $MnCl_2$, 1 μM $ZnCl_2$, 2 μM thiamine, 0.5% glycerol, 0.5% glutamate, 50 μg/ml tryptophan, 50 μg/ml phenylalanine, 50 μg/ml threonine, 700 μM $CaCl_2$, and 50 μM (for growth assays) or 125 μM (for biofilm assays) $FeCl_3$ (79, 86). Solid MSgg medium was obtained by adding Bacto agar (Difco) to a final concentration of 1.5%. When necessary, selective medium was prepared with LB agar or LB broth, supplemented with 100 μg/ml ampicillin (Amp; AG Scientific), 10 μg/ml kanamycin (Kan; AG Scientific), 10 μg/ml chloramphenicol (Cam; Amresco), 10 μg/ml tetracycline (Tet; Amresco), 100 μg/ml spectinomycin (Spec; Tivan Biotech), and 1 μg/ml erythromycin (Erm; Amresco) plus 25 μg/ml lincomycin (Linc; Sigma-Aldrich). To create the transcriptional *luc* reporter, the promoter of *pksC* was amplified from genomic DNA (gDNA) of *B. subtilis* NCIB 3610 with the following primers: primer 5′ P_{pksC}-*lux* P1, GTCCTAGTAAGGTCGACAGGAGGACTCTCTGCAAATCGCCCGGCCATTCGATAAAGG, and primer 3′ P_{pksC}-*lux* P2, GTATGTAAGCAAAAAGTTTCCAAATTTCATTCTCTCAAAGCCACCCTTCCGATTAGT, and further integrated into pBS3Clux by restriction-free cloning. After confirmation by sequencing, pBS3Clux was cut and integrated into the bacterial genome of *B. subtilis* NCIB 3610 at the neutral *sacA* locus.

The deletion of *codY* was generated using the long-flanking homology (LFH) PCR mutagenesis protocol of Wach (87), replacing an endogenous locus with a kanamycin resistance gene from pIKec14. The primers used were as follows: P1, TCGATATGGATGAAGTCGGCCAGGAA; P2, CAATTCGCCCTATAGTGAGTCGTCCGCAGCTTGCAGCATGGAGTTAATA; P3, CCAGCTTTTGTTCCCTTTAGTGAGTCAGGCTTATATCAAGGCGAGAAATGTAGTT; and P4, TTCTGTAAGGCACCCACTCTCCATTC.

The product was first introduced by transformation into strain *B. subtilis* PY79 and the deletion further integrated into the NCIB 3610 wild type or mutant by transformation. Transformation of *B. subtilis* PY79 and NCIB 3610 by natural competence with linearized plasmid or genomic DNA was done as described previously (88) and as performed previously to assess natural competence in NCIB 3610 (89).

Bacillaene extraction. Bacillaene was extracted from the biofilms of strain NCIB 3610. A single *B. subtilis* wild-type (WT) colony, isolated on a solid LB plate, was inoculated into 3 ml of LB broth and grown overnight at room temperature, and 2 μl of the culture was plated onto solid MSgg medium. The plate was incubated for 3 days at 30°C to allow proper biofilm development. A ~44-mm^2 MSgg agar segment was cut out of the plate (together with a piece of the biofilm) and crushed manually, and 700 μl of isopropanol was added to it. The isopropanol-containing crushed MSgg mixture was then incubated at 50°C for 1 h, with occasional vortexing, followed by centrifugation (14,000 rpm for 5 min) to precipitate MSgg agar flakes. Supernatant was extracted and evaporated using a SpeedVac. As a control, the same procedure was performed with noninoculated solid-MSgg medium. To verify that the activity of the extract was *pks* dependent and not due to the extraction of surfactin, the procedure was also carried out

with the Δ*pks* mutant. To analyze the activity of the extracted molecules, 6.6 μl dimethyl sulfoxide (DMSO) and 193.4 μl distilled water (dW) were added to the evaporated residues and vortexed to dissolve them (resulting in a molecule concentration of 3.5×). For *S. plymuthica* growth curves, liquid MSgg was supplemented with 30% of the 3.5× solution, achieving a final concentration of ~1×. Growth assays were performed as follows: cells were grown at room temperature with agitation for 18 h in a microplate reader (Synergy 2; BioTek), and the optical density at 600 nm (OD_{600}) was measured every 15 min.

Isopropanol extracts were analyzed by liquid chromatography-mass spectrometry. High-performance liquid chromatography (HPLC) separations were carried out using a Kinetex hexyl-phenyl column (2.1 by 150 mm; particle size, 2.6 μm; Phenomenex). The mass spectrometer was operated in positive ionization mode, and the ion source parameters were as follows: spray voltage, 3.5 kV; capillary temperature, 300°C; ion-transfer optics parameters optimized using automatic tune option; sheath gas rate (arb), 35; and auxiliary gas rate (arb), 15. Mass spectra were acquired in the range of *m/z* 150 to 2,000 Da. The liquid chromatography-mass spectrometry (LC-MS) system was controlled, and data were analyzed using the Chromeleon and Xcalibur software (Thermo Fisher Scientific, Inc.). Bacillaene was detected at *m/z* 581.3585. Dihydrobacillaene was not detected.

Effect of *S. plymuthica* supernatant, cells debris, and ECM on *B. subtilis*. To acquire *S. plymuthica* supernatant and dead cells, an overnight LB culture of *S. plymuthica* grown at room temperature was inoculated into liquid MSgg medium (dilution of 1:100) and incubated at 30°C for 24 h. Cultures were then centrifuged (14,000 rpm, 10 min), and the supernatant was filtered, evaporated by SpeedVac, and rehydrated with distilled water (dW), achieving a 20× concentration. The remaining cells were washed with dW and resuspended in 2 ml dW. Cells were heat killed at 85°C for 25 min. For the interaction, 3 μl of either the 20× supernatant or the dead cells was inoculated next to a 2-μl *B. subtilis* LB culture (grown at room temperature overnight) and then incubated at 30°C for 3 days. For EPS extraction, *S. plymuthica* cells were grown at room temperature, inoculated into liquid MSgg medium (dilution of 1:100), and grown for 3 days without shaking to generate floating biofilms. Growing medium was collected and filtered, and ECM was precipitated with isopropanol as previously described (90). Solvent was evaporated overnight in a chemical hood.

Imaging of the interaction between biofilms. Single *B. subtilis* or *S. plymuthica* colonies, isolated on solid LB plates, were inoculated into 3 ml of LB broth (Difco) and grown overnight at room temperature. For interaction analysis, 2 μl of each culture was inoculated onto solid MSgg medium at the desired distances. Plates were incubated at 23°C and 30°C for 5 and 3 days, respectively. Cells were visualized using a SteREO Discovery.V20 microscope equipped with a PlanApo S 0.5× FWD 134-mm objective or a PlanApo S 1.0× FWD 60-mm objective (Zeiss) attached to an Axiocam camera. Data were captured and analyzed using the Zen Pro AxioVision suite software (Zeiss, Oberkochen, Germany) and ImageJ2 (91).

For quantification of a symmetric spreading of *B. subtilis* biofilm colony, the radius ratio was determined by dividing a vertical radius extending from the center of the biofilm toward the *S. plymuthica* colony by the vertical radius extending to the opposite side.

Scanning electron microscopy. Two-day-old interactions of *B. subtilis* WT or the Δ*pks* mutant with *S. plymuthica* were fixed overnight at 4°C with 2% glutaraldehyde, 3% paraformaldehyde, 0.1 M sodium cacodylate (pH 7.4), and 5 mM $CaCl_2$. Samples were washed twice for 15 min with double-distilled water (ddW) and dehydrated with a series of ethanol washes at 30%, 50%, 70%, 96% and 100%. Samples were then dried overnight on filter paper (Whatman) at room temperature, mounted, and stored in a vacuum. The mounted samples were sputtered with gold-palladium before examination by scanning electron microscopy (SEM) XL30 with field emission gun (FEI).

Analysis of bacterial populations during interaction. To analyze the cell numbers for each strain during the interaction between *B. subtilis* and *S. plymuthica*, colonies were seeded as described above and incubated for the required time period. As shown in Fig. 1 and 2A to D, the interacting colonies were divided into three sections, as follows: (i) *B. subtilis*, consisting of the entire *B. subtilis* biofilm, excluding the thick wrinkle that surrounded *S. plymuthica*; (ii) the manually separated wrinkle, containing cells from the contact area (also designated interaction area); and (iii) *S. plymuthica*, consisting of the entire *S. plymuthica* colony, excluding the cells that were attached to the wrinkle. For other experiments, both interacting colonies were harvested, with no further separation. Colonies of *B. subtilis* and *S. plymuthica* grown alone served as controls.

Each sample was harvested, resuspended in 500 μl phosphate-buffered saline (PBS), and mildly sonicated (3× for 5 s at 15%). The samples were then diluted, plated on solid agar, and incubated at 30°C overnight to allow formation of colonies. Colonies formed by the two species are easily distinguishable on LB medium due to differences in size and colony shape. For *B. subtilis* biofilm mutants, confirmation of the identity of the colonies was performed by dual plating on nonselective LB and LB with the appropriate antibiotics.

Plant colonization assay. To test bacterial interaction over roots, seeds of the annual *E. sativa* (Brassicaceae), originated from natural population (92), were germinated on Nitsch (93) agar plates at 25°C with an 8-h/16-h day/night photoperiod. One-week-old seedlings were transferred to solid MSgg medium plates, and bacteria were inoculated (as described above) on the next day at a distance of 0.8 cm from the root and between the inoculants. Samples were incubated in a growth chamber (MRC) with 8-h/16-h day/night photoperiods at 23°C and 30°C for 5 and 3 days, respectively. For interspecies interactions population assessment, the overall community was separated from the root, mildly sonicated, and plated to detect single colonies in triplicate.

Root exudate extraction and bioactivity assessment. Root exudate of *E. sativa* seedlings was extracted from Nitsch agar growth medium 2 weeks from germination. After seedlings were removed, exudate was obtained by extracting Nitsch agar with isopropanol (3:2), followed by vigorous vortexing and incubating at room temperature (RT) for 10 min. Samples were centrifuged in 10,000 rpm for 10 min to separate the aqueous phase. Exudate samples were evaporated in a SpeedVac concentrator (Eppendorf) and rehydrated in dW. Prior to biofilm inoculation, plates were treated with either 50-μl drops of root exudate or Nitsch agar (plant growth medium) extraction used as a control.

Expression analysis. *pks* expression in the presence of plant metabolites was analyzed as follows: the luminescence intensity of cells harboring a P_{pksC}-*lux* transcriptional promoter fusion was analyzed. Cells were grown to a mid-logarithmic phase, diluted 1:100 in 150 μl liquid MSgg medium, either with or without root exudate. Cells were grown in a 96-well microplate (Thermo Scientific, Roskilde, Denmark) with agitation at 30°C for 16 h in a microplate reader (Synergy 2; BioTek, Winooski, VT, USA), and the luminescence intensity and the optical density at 600 nm (OD_{600}) were measured every 15 min.

Statistical methods. All studies were performed in duplicate or triplicate at least three separate and independent times. Data are expressed as average values ± standard deviations of the means. For triple-party interactions, data are expressed as average values ± standard errors. Parametric testing was performed when appropriate after confirming that raw data were normally distributed. A paired Student's *t* test was used to determine if the set of treated versus the untreated control are different from each other, and *P* values of less than 0.1 were considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00512-19.

SUPPLEMENTAL FILE 1, PDF file, 1.5 MB.

ACKNOWLEDGMENTS

We thank Leonid Chernin for kindly providing us *Serratia* and *Pseudomonas* strains. We thank Oz Barazani for kindly providing us *Eruca sativa* seeds. We thank Yael Helman and Assaf Vardi for critical reading of our manuscript.

The Kolodkin-Gal lab is supported by ISF-icore grant 152/1 and Israel Science Foundation research grant 119/2016 and by the Skoll Center for Environment and Climate.

I.K.-G., E.H.Y., and A.O. designed the experiments; E.H.Y., A.O., R.J., T.B., and A.K.-P. performed the experiments; E.H.Y., A.O., A.K.-P., and I.K.-G. analyzed the data; O.G. provided the reagents; T.B. provided the methodologies; and I.K.-G., A.O., E.H.Y., and A.K.-P. wrote the manuscript.

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