Lipopeptide-mediated bacterial interaction enables cooperative predator defense

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Edited by Joan E. Strassmann, Washington University in St. Louis, St. Louis, MO, and approved December 7, 2020 (received for review July 6, 2020)

Bacteria are inherently social organisms whose actions should ideally be studied within an interactive ecological context. We show that the exchange and modification of natural products enables two unrelated bacteria to defend themselves against a common predator. Amoebal predation is a major cause of death in soil bacteria and thus it exerts a strong selective pressure to evolve defensive strategies. A systematic analysis of binary combinations of coisolated bacteria revealed strains that were individually susceptible to predation but together killed their predator. This cooperative defense relies on a *Pseudomonas* species producing syringafactin, a lipopeptide, which induces the production of peptidases in a *Paenibacillus* strain. These peptidases then degrade the innocuous syringafactin into compounds, which kill the predator. A combination of bioprospecting, coculture experiments, genome modification, and transcriptomics unravel this novel natural product-based defense strategy.

natural products | cooperative defense | lipopeptides | amoebae | *Pseudomonas*

Within their habitats, microorganisms rarely occur as isolated monocultures, rather they gregariously interact with a variety of other species (1, 2). Within this ecological framework, many complex microbial traits can only be established by the cooperation of various organisms, a common phenomenon in natural microbial communities (1). Secreted small molecules or natural products often play crucial roles in these microbial interactions in which they are required to ensure survival and maintenance of multipartite associations (3). For instance, chemical entities known as quorum-sensing signals function as a means of communication when coordinating community efforts (4, 5). Furthermore, defensive molecules of bacterial origin can participate in protecting the producers' hosts from parasites, pathogens, or microbial competitors (6–8). The multifarious biological activities of microbial natural products have rendered them indispensable in the development of antibiotics, anticancer agents, and immunomodulators, as well as other drugs (9) and the need for novel structures is urgent.

Most efforts toward identifying new natural products or in investigating their biological/ecological role in a laboratory setting make use of microbial monocultures. Yet many compounds are only produced when required within a polymicrobial environment (10, 11). Furthermore, some molecules may be secreted by one species and chemically modified by another one to yield a novel bioactive compound (12, 13). These are just two of the reasons why cultivation of individual microorganisms in a laboratory setting may only reveal a minute fraction of the metabolic space encountered in nature. An analysis of microbial cocultures within their ecological context thus carries the potential to identify new molecular entities with unprecedented biological functions. In particular, events that subject polymicrobial associations to a strong evolutionary selection pressure, such as predation, may lead to the emergence of these cooperative traits. The bacterivorous protist *Dictyostelium discoideum* (14, 15), a well-established model organism, is ideally suited to study microbial predation in the laboratory (16, 17). As single cells, these amoebae are chemotactically attracted to bacteria and feed on them by phagocytosis, a mechanism they share with macrophages that engulf pathogens (18, 19). Upon feeding, the unicellular amoebae divide by binary fission until starvation, when ~10^5 cells aggregate to form a fruiting body—hence they are known as social amoebae (17). The resulting fruiting bodies allow for the dissemination of amoebal spores into new habitats. A number of predator-resistance mechanisms have emerged in bacteria, which coevolved with amoebae (19, 20). Previous studies have shown that secreted natural products of bacterial origin can play an important role in preventing predation since they can act as particularly potent amoebicides (20–22).

Here, we extend the concept of antipredator defense based on secreted amoebicides to cooperatively interacting microorganisms. An analysis of pairwise combinations of bacteria obtained from the habitat of amoebae led to the identification of bacterial isolates, which can only antagonize their predator when cultured together. Eventually, by using a combination of microscopy, genome modification, chemical characterization, and transcriptomic analysis, we reveal the underlying mechanism of this interaction and identify the resulting molecular entities that mediate cooperative defense against bacterial predators.

Results and Discussion

Combinatorial and Metabolic Screening of Bacteria Reveals Cooperative Defense Traits. A collection of 58 distinct bacterial strains (Fig. 1) was isolated from litter-rich forest soil, a habitat shared by both social amoebae and bacteria (23). The ability of these bacterial strains to resist amoebal predation was assessed using a plaque assay, in which vegetative *D. discoideum* AX2 cells were deposited onto a solid medium that was previously inoculated with the bacterial strain. Successful predation resulted in

Significance

Natural products are important mediators in interacting microbial communities. Here, we show that bacteria can defend themselves against a common predator by teaming up. This form of cooperative defense relies on the production of a linear lipopeptide by a *Pseudomonas* species, which induces the production of peptidases and proteases in a *Paenibacillus* species. These enzymes degrade the lipopeptide into fragments which are highly toxic to the amoebal predator. Investigating microbial interactions enables identification of novel chemical entities with potent biological functions.

Author contributions: S.Z., R.M., and P.S. designed research; S.Z., R.M., and L.R. performed research; S.Z., R.M., S.C., and P.S. analyzed data; and S.Z., R.M., S.C., and P.S. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

This paper is a winner of the 2021 Cozzarelli Prize.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2013759118/-/DCSupplemental.

Published February 1, 2021.

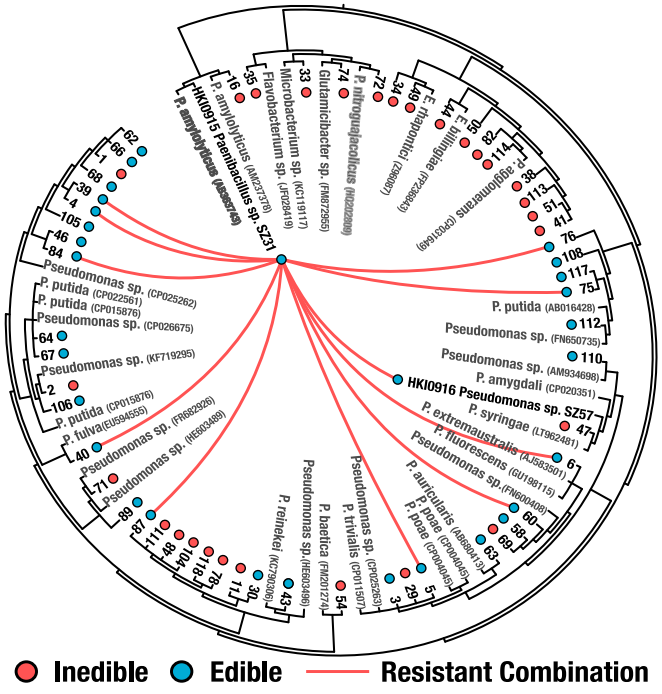

Fig. 1. The phylogeny of 58 unique bacterial isolates bioprospected from forest soil is depicted as a maximum likelihood tree. The phylogeny is anchored with 34 reference strains that are the nearest relatives of strains used in the study to indicate their phylogenetic affiliation. The isolates have been marked as inedible (i.e., resistant; red dot) or edible (i.e., vulnerable; blue dot) with respect to amoebal predation. The combination of edible strains that eventually became inedible to *D. discoideum* AX2 have been indicated by the red lines joining the two bacterial strains.

the emergence of grazing plaques, i.e., zones devoid of bacteria, which were eventually covered with amoebal fruiting bodies (24). Conversely, the absence of both grazing plaques and fruiting bodies showed that the bacteria had resisted amoebal predation (*SI Appendix*). We thus divided the collection of bacteria into 30 inedible (resistant) and 28 edible (vulnerable) strains. Phylogenetic typing based on 16S ribosomal RNA (rRNA) gene sequencing revealed that amoeba-resistant traits were not exclusive to any particular bacterial group (Fig. 1). As antipredator defenses often emerge in polymicrobial communities with individual members being vulnerable, we tested combinations of vulnerable strains for resistance against predators. In the absence of cooperative behavior, we expected such a combination to be edible. Screening the 378 binary combinations of 28 vulnerable strains resulted in 11 combinations that could resist the amoebal predator. Phylogenetically, these 11 pairings were always between a gram-negative *Pseudomonas* strain and the single gram-positive *Paenibacillus* sp. SZ31 (HKI0915) (Fig. 1). Both bacterial genera are well-known producers of natural products that also have broad biocontrol applications (25, 26). Interestingly, combination between a related gram-negative bacterium (e.g., *Burkholderia* sp.) and *Paenibacillus* sp. was also shown to cooperatively produce many potent antimicrobial compounds (27). As a result, it seemed plausible that amoebicidal natural products may be involved in the observed antipredator defense mechanism.

Genomic and Metabolic Characterization of Bacteria that Cooperatively Kill Their Predator. In order to probe their secondary metabolomes, liquid chromatography-mass spectrometry (LC-MS) analysis of the *Paenibacillus* sp. SZ31 (HKI0915) and the 11 *Pseudomonas* strains alone, as well as in coculture, was performed. In particular, we were interested in the emergence of novel, potentially amoebicidal, natural products in any one of the cocultures. Only the coculture between one of the *Pseudomonas* strains (SZ57) and the *Paenibacillus* strain showed this effect, which can be seen by the presence of additional peaks in the high-performance liquid chromatography (HPLC) profiles in Fig. 2. From monocultures of *Pseudomonas* sp. SZ57 (HKI0916), we were able to isolate and elucidate the structure of syringafactins A and C (Fig. 2 and *SI Appendix*, Figs. S18 and S19 and Tables S5 and S6). These related lipo-octapeptides were previously reported as microbial biosurfactants (28). On the other hand, the monoculture of *Paenibacillus* sp. SZ31 did not show any prominent secondary metabolites (Fig. 2 and *SI Appendix*, Figs. S15 and S16).

The metabolites that appeared in the coculture of SZ57 and SZ31 seemed to be peptidolytic degradation products of the syringafactins. Tandem mass spectrometry (MS–MS) analysis was used to determine the position of cleavage. Interestingly, the degradation profiles differed when the coculture was performed in liquid or on solid medium (Fig. 2 and *SI Appendix*, Figs. S15 and S16). In liquid medium, only their C-terminal amino acids were hydrolyzed, resulting in the appearance of syringafactins A_{-1} and C_{-1}, whereas on solid medium, shorter degradation products, namely syringafactins X_{-3}, X_{-4}, and X_{-7}, were obtained (syringafactins A and C differ at amino acid 6; when removed, the cleaved products between the two congeners are identical, as indicated by the letter X).

Next, we aimed at understanding if these novel metabolites indeed result from degradation of syringafactins. To this end, we assembled the genomes of both SZ31 and SZ51 constructed from short read sequencing (Illumina) and searched for secondary metabolite gene clusters using antiSMASH (29). In the genome of *Pseudomonas* sp. SZ57, we identified the *syf* biosynthetic gene cluster (BGC), required for the generation of the

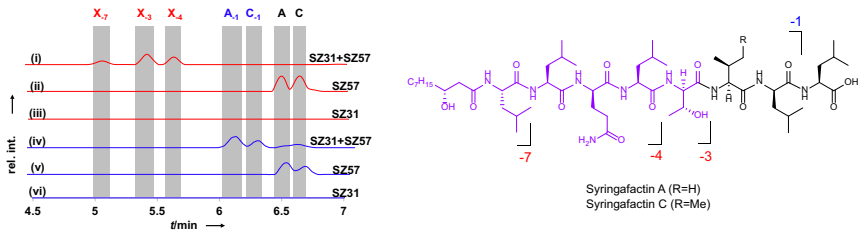

Fig. 2. (*Left*) HPLC profile of culture extracts of *Pseudomonas* sp. SZ57 and *Paenibacillus* sp. SZ31 strains, as well as coculture of both strains (ultraviolet detection at λ = 210 nm). Red profiles represent cleavage products identified on solid medium and blue ones in liquid medium. (*Right*) Syringafactin and peptidolytic degradation products thereof. Syringafactins A and C are detected in cultures of SZ57. Syrigafactins A_{-1} and C_{-1} are produced in liquid medium, whereas syrigafactins X_{-3}, X_{-4}, and X_{-7} (X = A or C) are observed on solid medium. Syringafactin X_{-n} indicates a truncated syringafactin metabolite, with n amino acid residue(s) removed from the C-terminal end. Please note that since amino acid 6 differs in syringafactins A and C, degradation products of the same length with shorter than 6 amino acids are identical for syringafactin A and C (purple part of the structure).

syringafactins. The genome of *Paenibacillus* did not code for any BGCs that could be related to syringafactins or any of the shorter congeners. We generated the mutant *Pseudomonas* sp. SZ57Δ*syf*, which was unable to produce syringafactins, and cocultured it with *Paenibacillus* sp. SZ31. As expected, the coculture did not contain any syringafactin or its shorter congeners and was not toxic to amoeba, unlike the combination between wild-type (WT) SZ57 and SZ31 (Fig. 3). This indicates that the *Pseudomonas* strain SZ57 provides syringafactins to the microbial mixture for subsequent degradation.

Growth Dynamics of Bacteria in Mono- and Coculture. Mixed microbial cultivations require a knowledge of the growth dynamics of each individual strain in order to adjust their ratios in coculture. A comparison of growth dynamics of *Pseudomonas* sp. SZ57 and *Paenibacillus* sp. SZ31 revealed that SZ31 attained stationary phase much earlier than SZ57 (*SI Appendix*, Fig. S2). This result correlates with the fact that their 3:1 coculture (based on the inoculum ratio both on solid and in liquid media) displayed the most pronounced amoebicidal phenotype. Furthermore, this accounts for the observation that cocultures with insufficient amounts of SZ31 (i.e., ratios of 2:1 or 1:1) would become more vulnerable to predation. Interestingly, the use of a 3:1 coculture of SZ31 and SZ57 also mimics naturally observed biomass ratios of gram-positive and gram-negative bacteria in predator-rich rhizosphere soil (30).

A comparison of growth curves of WT SZ57 and the syringafactin deficient mutant was done in both rich and minimal medium containing glucose as carbon source. Under these conditions, both the strains had similar growth dynamics indicating that the gene deletion did not offer any obvious growth advantage to the gene-deletion mutant SZ57Δ*syf* in these media (*SI Appendix*, Fig. S1).

Eventually, exposing *Paenibacillus* sp. SZ31 (HKI0915) to varying doses of syringafactin A for a prolonged period of time (5 d) did not alter its growth dynamics relative to an untreated sample (*SI Appendix*, Fig. S3). Similarly, a comparison of growth dynamics in which SZ31 was exposed to syringafactin A or a combination of syringafactins A and C, showed only a slight difference in growth rates (*SI Appendix*, Fig. S4) indicating that syringafactins do not detrimentally affect the growth of *Paenibacillus* sp. SZ31.

Identification of Effector Molecules Mediating Antipredator Defense. Since the coculture of *Pseudomonas* sp. SZ57Δ*syf* and *Paenibacillus* sp. SZ31 was edible to amoebae (Fig. 2 and *SI Appendix*, Fig. S5), production of syringafactins appears necessary for establishing the cooperative defense trait. Syringafactins A and C were not toxic to amoebae even at concentrations up to and well above 100 $\mu g \cdot mL^{-1}$. We then determined the toxicity of the respective degradation products.

Indeed, syringafactins A_{-1} and C_{-1} (1:1, wt/wt) produced in a liquid coculture were highly toxic for the amoebae with IC_{50} (*D. discoideum*) = 6.7 ± 1.2 $\mu g \cdot mL^{-1}$ (mean ± SEM, $n = 3$). The peptidolytic-degradation products produced on a solid medium, syringafactins X_{-3}, X_{-4}, and X_{-7} (3:2:1, wt/wt/w), displayed lower toxicity with IC_{50} (*D. discoideum*) = 23.0 ± 5.6 $\mu g \cdot mL^{-1}$ (mean ± SEM, $n = 3$). The ratios of the syringafactin cleavage products mirrored those produced during predatory challenge (*SI Appendix*, Figs. S26 and S27). While the exact mode of action remains unclear, the peptidolytic-degradation products display altered physicochemical properties (lipophilicity, solubility, complex formation, etc.) compared to intact syringafactins. These properties may in turn affect the interaction with or permeation of the amoebal cell membrane.

As amoebal predators in soil prefer and thrive in regions of high moisture (31), the greater toxicity associated with degradation products from liquid medium could extend the spatial range of the predatory defense in soils with high moisture content.

Amoebicide Production Is Independent of the Presence of Predators. Often, chemical defenses are induced by or deployed in the presence of predators whose populations not only depend on the presence of their prey (32) but also on conducive environmental conditions that foster bacterial growth (33). For instance, a *Pseudomonas fluorescens* strain that resists predation by the amoebae *Naegleria Americana* via the production of cyclic lipopeptides (massetolide and viscosin) increases their production titers in the presence of the predator (34). In order to test if such a change is also induced in our system, we compared metabolite production of the *Paenibacillus–Pseudomonas* coculture in the absence and the presence of the predator *D. discoideum*. We did not observe any substantial change in quantities of defensive molecules either in liquid or on solid medium (*SI Appendix*, Figs. S7 and S8). This indicates that in our system the amoebicidal metabolites are constitutively produced as an "always on" defense mechanism. It may thus be possible that this cooperative defense system is not triggered by the direct presence of predators, but rather dependent on environmental factors that enable predators to thrive, such as moisture levels.

Cooperative Defense Acts against Different Amoebae and Is Effective in Soil-Like Environments. Additionally, to better understand how these bacterial strains interact in the soil environment and to rule out that predation is an in vitro artifact, we buried a slide in an artificial soil-like porous environment (35) inoculated with the two strains and subsequently introduced the predator into the mixture. We observed successful predation when *D. discoideum* was introduced in artificial soil for individual *Pseudomonas* and *Paenibacillus* strains (*SI Appendix*, Fig. S12). When combined, the strains remained together on the slide indicating the absence of predation (*SI Appendix*, Fig. S12). Hence, the antipredator

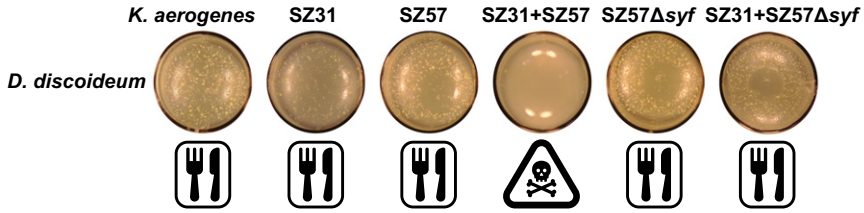

Fig. 3. An amoebal plaque assays was performed by adding vegetative *D. discoideum* AX2 amoebae onto lawns of respective bacteria on solid agar. The presence of fruiting bodies indicates that the amoebae can feed on the bacteria. *K. aerogenes* was used as a food source for the amoeba and served as edible control. The absence of grazing plaques indicates that the bacteria, or a combination of bacteria, is inedible to the amoebae. AX2 cells could graze on all bacterial strains individually (SZ31, SZ57, and on the syringafactin-deficient *Pseudomonas* sp. SZ57Δ*syf*). Only the combination of *Paenibacillus* sp. SZ31 and *Pseudomonas* sp. SZ57 is inedible to *D. discoideum* AX2, as indicated by the absence of macroscopic fruiting bodies. A combination of *Paenibacillus* sp. SZ31 and syringafactin-deficient *Pseudomonas* sp. SZ57Δ*syf* was also edible.

defense of the coculture can also occur in structurally complex soil(-like) environments. Gram staining and differential plating of the cocultures exposed to amoeba in a standard plaque assay also provided visual and growth-based evidence of survival of both strains in coculture. This further confirms the ability of the *Pseudomonas–Paenibacillus* coculture to survive and multiply in the presence of the predator (*SI Appendix*, Fig. S13). Live/dead staining with fluorescein diacetate of *D. discoideum* cells exposed to syringafactin A_{-1} and C_{-1} (10 μg · mL^{-1}) showed extensive cell death as compared to untreated controls (*SI Appendix*, Figs. S10 and S11).

Subsequently, we examined if the coculture could withstand predation by two *D. discoideum* wild isolates, which are better acclimated to soil (36), when compared to the highly passaged laboratory strain AX2. Wild isolates *D. discoideum* QS161 and QS160 showed the same behavior as AX2 in our system (*SI Appendix*, Fig. S6). This indicates that co-occurrence of both the *Pseudomonas* and *Paenibacillus* strains may be a broad-spectrum defense against predators, underscoring the importance of investigating bacterial interactions in this context.

Predators such as *D. discoideum* feed upon bacteria in soil ecosystems, allowing them to accumulate large amounts of nutrients from the bacterial biomass they feed on (37, 38). Thus, killing of the predator could putatively enhance nutrient availability for the soil microbial community (39).

Syringafactins Induce the Production of Peptidases in *Paenibacillus*. With an understanding of the molecular entities that prevent amoebal predation, we next determined how syringafactins are degraded in the coculture. Although lipopeptides are known to be toxic to gram-positive bacteria (26, 40, 41), *Paenibacillus* sp. SZ31 tolerated very high concentrations of syringafactins, up to 50 μg · mL^{-1} (*SI Appendix*, Fig. S3). Furthermore, addition of syringafactin to cultures of SZ31 rendered the otherwise vulnerable strain resistant to amoebal predation. Similar supplementation of other edible bacteria (e.g., *Klebsiella aerogenes*) did not result in a defensive phenotype (*SI Appendix*, Fig. S5). Combined with results from the structural analysis of the degradation products, we suspected that the *Paenibacillus* strain produces DL-carboxypeptidase and/or other peptidases to degrade the syringafactins. However, neither the conditioned medium nor the lysate of the *Paenibacillus* strain were able to degrade syringafactin A or C. Together with our previous findings, this suggests that peptidases in SZ31 may be induced by syringafactins. Thus, we compared the global transcriptome of an SZ31 liquid culture with that exposed to syringafactin A. Comparative transcriptomic analysis revealed an up-regulation of transcripts associated with both proteases and peptidases encoded within the *Paenibacillus sp.* SZ31 genome (Fig. 4 and *SI Appendix*, Fig. S9 and Table S2). Since syringafactins are composed of both D- and L-amino acids, induction of both D- and L-specific peptidases seem necessary for the emergence of the cooperative amoebicidal trait. Furthermore, it is conceivable that this defense strategy may have evolved from a detoxification mechanism for defense against other lipopeptides (40, 41).

Our results highlight the importance of natural product modification as a powerful mechanism to control outcomes of ecological interactions (13). Here, lipopeptide modifications result in bacterial resistance to amoebal predators (Fig. 5). A recent study also demonstrated that a lipopeptide of a pathogen (*Pseudomonas tolaasii*) is modified by a "helper" bacterium to protect the mushroom *Agaricus bisporus* from the pathogen's virulence factors (42). Furthermore, lipopeptide modifications are commonly used for niche protection—a prominent trait in soil-dwelling *Streptomyces* strains against a competing *Bacillus* sp. (43). Together, these findings highlight the importance of cooperative modification of natural products as a robust strategy

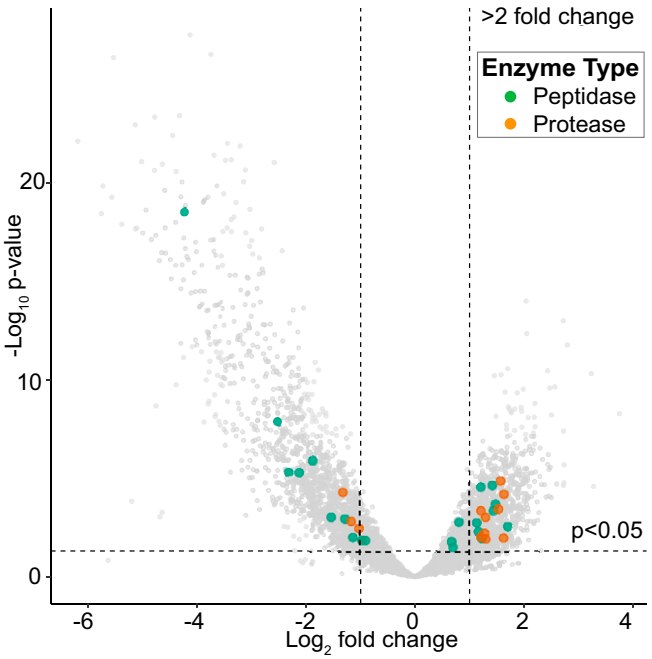

Fig. 4. Transcriptomic analysis of *Paenibacillus* sp. SZ31 treated with syringafactin 10 μg · mL^{-1} compared to no syringafactin treatment as control in KB broth. A volcano plot of log-transformed fold change relative to control (*x*-axis) against log-transformed FDR-corrected *P* values (*y*-axis) is depicted. Two vertical dotted lines depict fold-change boundaries, above and below which genes had a greater than twofold increase or decrease in expression relative to controls, respectively. The single horizontal dotted line designates the threshold above which the fold change of the transcripts is significant. Predicted peptidases (green) and proteases (orange) that are significantly up- or down-regulated are shown. All other annotated genes are shown in gray.

deployed within soil bacterial communities to serve a number of key functions in the soil ecosystem.

Conclusion

In summary, we describe a cooperative interaction of phylogenetically distant bacteria, which enables them to evade amoebal predation (Fig. 5). The mechanism underlying this effect relies on the secretion of the syringafactins, lipopeptides produced by a *Pseudomonas* strain. This compound family induces the production of peptidases in a *Paenibacillus* strain, which leads to the partial cleavage of the syringafactins. Although syringafactins themselves are not toxic to amoebae, the mixture of various syringafactin degradation products become toxic to amoeba resulting in cooperative bacterial defense. Such a polymicrobial bacteria–amoeba platform is very well suited to study microbial interactions that are based on the production, exchange, and modification of natural products, and we believe this is just one of many fascinating microbial dialogs that will enrich our understanding of the ecological roles of natural products. Furthermore, we outline an efficient strategy that allows us to discover bioactive natural products, which are only produced in polymicrobial communities. The conversion of a vulnerable bacterial strain into a resistant strain in its natural habitat, therefore, is a result of complementary traits of the members of the community that can result in robust community-level defense against predators. Our findings show that evolutionary selection pressures not only act on individual organisms that produce distinct compounds but also on combined gene pools of bacterial communities that allow for the cooperative production of secondary metabolites.

Methods

For a full description of methods used, refer to *SI Appendix*.

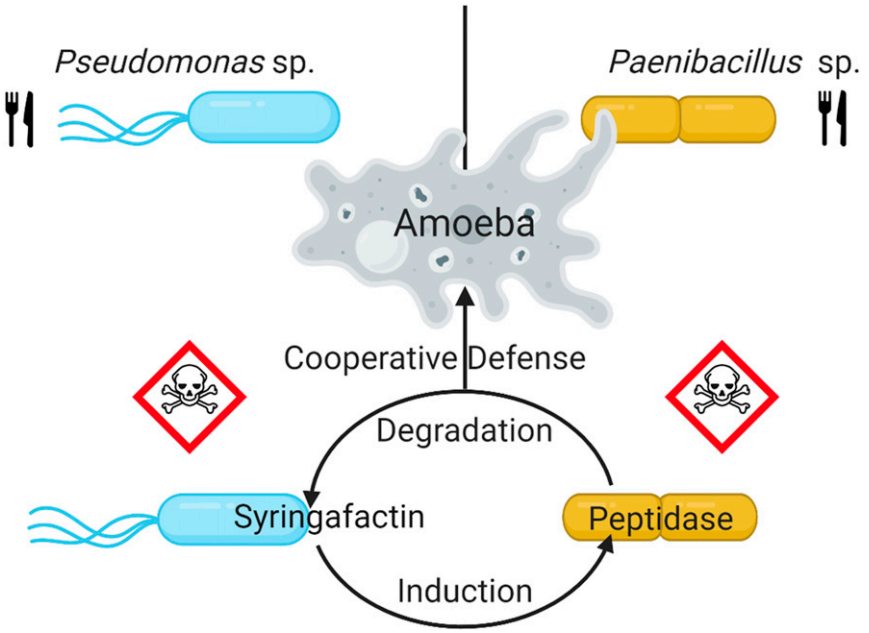

Fig. 5. Schematic overview of the underlying mechanism that leads to cooperative defense between a *Pseudomonas* strain (blue) and a *Paenibacillus* strain (yellow). Individually, each of the strains are food sources to the amoeba (*Top*). When in coculture (*Bottom*), the secreted syringafactins produced by the *Pseudomonas* strain induce the production of peptidases in the *Paenibacillus* strain, which degrade the lipopeptide. These cleavage products are strongly amoebicidal.

Bacterial Cocultures. *Pseudomonas* sp. SZ57 and *Paenibacillus* sp. SZ31 were inoculated individually in 3 mL King's B (KB) broth from their respective glycerol stocks and incubated overnight at 22 °C under shaking conditions. After incubation, cells were washed with sterile 1 × phosphate-buffered saline (PBS) and resuspended in the same buffer, such that the OD_{600} (optical density at 600 nm) of both the cultures was 0.1. The individual cultures were then added to KB broth in the ratio of 1:3 (SZ57:SZ31, vol/vol). For cocultures on solid medium, the same procedure was followed as described above, except the initial OD_{600} was adjusted to 0.05, and 100 μL of the mixture of SZ57 and SZ31 was added to each KB plate. The coculture of *Pseudomonas* sp. SZ57 and *Paenibacillus* sp. SZ31 in KB broth was incubated for 4 d at 22 °C while shaking at 160 rpm. The combined culture of these bacteria on KB agar plates was continued for 7 d at 22 °C (27).

Generation of the Gene Deletion Mutant Δ*syf* in *Pseudomonas* sp. SZ57. For the generation of marker-less genomic-deletion mutants, a gentamicin resistance (*gentR*) selection and sucrose counter-selection (*sacB*) method was used. The corresponding pEXG2-based suicide vector (44) was constructed using the Gibson Assembly method. The parent plasmid pEXG2 was linearized using HindIII and EcoRI restriction enzymes. Left and right homology arms (LA and RA) were PCR amplified from genomic DNA of *Pseudomonas* sp. SZ57 using primer pairs LA *syf* forward/reverse and RA *syf* forward/reverse, respectively (*SI Appendix*, Table S1). Both these primers included an 18 bp sequence adjacent to the PCR fragment, complementary to the linearized vector (the primers were designed using http://nebuilder.neb.com/). The LA and RA were ligated into double-digested pEXG2 vector using the standard Gibson Assembly protocol (New England Biolabs) to yield the respective plasmid pEXG2Δ*syf*.

The vectors were transformed into chemically competent *Escherichia coli* Top10 cells via heat shock at 42 °C, and after 1 h outgrow in super optimal broth with catabolite repression medium, cells were plated on Luria–Bertani (LB) agar plates containing 15 $\mu g \cdot mL^{-1}$ gentamicin. Single colonies obtained the next day were checked via colony PCR for the presence of plasmid containing the right-sized insert, and positive clones were inoculated in antibiotic-containing liquid medium for plasmid extraction the following day. Plasmids were purified from positive *E. coli* Top10 clones using QIAprep Spin Miniprep Kit (Qiagen) and then validated by Sanger sequencing. For subsequent intergeneric conjugation, chemically competent *E. coli* S17-λ-pir were initially transformed with pEXG2Δ*syf*. Biparental mating was then performed using a standard protocol. Briefly, an overnight culture of donor (*E. coli* S17-λ-pir and pEXG2Δ*syf*) and acceptor (SZ57) strains were diluted to a starting $OD_{600} = 0.1$ and were then grown to an $OD_{600} = 0.6$ at 37 °C in LB medium supplemented with 15 $\mu g \cdot mL^{-1}$ gentamicin, and at 22 °C in LB medium without any antibiotics, respectively. After the appropriate OD_{600} was reached, recipient and donor strains were mixed in ratios of 1:1, 1:2, and 1:3 (vol/vol). The mixed cultures were then washed twice with sterile, deionized water. Mating spots (30 μL in deionized water) were placed on LB agar plates and incubated at 28 °C overnight. Following the incubation period, the mating spots were resuspended in 200 μL LB broth without antibiotics. After mixing, 100 μL of this cell suspension was plated on LB agar plates supplemented with 5 $\mu g \cdot mL^{-1}$ gentamicin and 100 $\mu g \cdot mL^{-1}$ ampicillin. After 48 h at 28 °C, single colonies of transconjugants were picked up from the plates and allowed to grow in 200 μL LB medium at 28 °C for 4 h. The cultures were then streaked onto LB (without NaCl) plates containing 10% (wt/wt) sucrose for selection of double-crossover knock-out mutants. The identity of deletion mutants was confirmed by colony PCR using DreamTaq Green PCR 2× Master Mix (Thermo Scientific) and primer pairs including both up- and downstream regions of the homology arms (*SI Appendix*, Table S1). Finally, a *syf*-knock-out mutant, created by a markerless deletion of the C-starter domain in the syringafactin biosynthetic gene cluster, was obtained.

Plaque Assay. Edibility of SZ57, SZ31, SZ57Δ*syf*, and combinations of these bacterial strains was tested using a *D. discoideum* AX2-based plaque assay in a 24-well plate format. Briefly, bacterial strains to be tested were first cultured overnight in SM/5 broth. The next day, 30 μL of each of these cultures or their combinations was added to individual SM/5 agar wells of the 24-well plate (in triplicates) and left to dry for 2 h. A total of 10,000 amoebae cells were added onto individual bacterial lawns. Overnight culture of *K. aerogenes*, a food bacterium for AX2, was used as a positive control for the assay. Plates were subsequently incubated at 22 °C for a period of 5 to 6 d. At the end of the incubation periods, wells were checked for the presence of amoebal fruiting bodies (indicating that amoeba could graze successfully on these bacteria) and photographs were taken. Inability of AX2 to form clear plaques or fruiting bodies on any individual bacterium lawn or combinations thereof was taken as an indication of their unpalatability or toxicity. The assay was performed in duplicates and photographs were acquired using a Canon EOS 800D camera.

LC-MS Analysis. For the detection of secondary metabolites from mono- and cocultures, LC-MS measurements were performed on a Shimadzu UHPLC-MS System (LC-30AD, SPD-M30A, and LCMS-2020). The system is equipped with an electrospray ion source and a Kinetex C18 column (50 × 2.1 mm, particle size 1.7 μm, pore diameter 100 Å, Phenomenex). Column oven was set to 40 °C; scan range of MS was set to *m/z* 150 to 2,000 with a scan speed of 10,000 atomic mass units/s and event time of 0.25 s under positive and

negative mode. Desolvation line temperature was set to 250 °C with an interface temperature of 350 °C and a heat block temperature of 400 °C. The nebulizing-gas flow was set to 1.5 L · min^{-1} and dry-gas flow to 15 L · min^{-1}. If not otherwise stated, the following standard LC method was used: flow rate = 0.7 mL · min^{-1}; 0 to 0.5 min: 10% (vol/vol) MeCN in water containing 0.1% formic acid; 0.5 to 8.5 min: linear gradient 10 to 100% MeCN in water containing 0.1% formic acid; 8.5 to 11.5 min: 100% MeCN in water containing 0.1% formic acid; and injection volume: 10 μL. LC-MS results were analyzed using LabSolutions Postrun and Browser (version 5.60).

Data Availability. Genome sequences of both *Pseudomonas* sp. SZ57 (HKI0916) and *Paenibacillus* sp. SZ31 (HKI0915) are available in the National Center for Biotechnology Information (NCBI) (*Pseudomonas* sp. SZ57: NZ_WIBD00000000; *Paenibacillus* sp. SZ31: JABBEA000000000). Raw transcriptomic data are available in Figshare (https://doi.org/10.6084/m9.figshare.13585583.v1). All other study data are included in the article and/or *SI Appendix*.

ACKNOWLEDGMENTS. We are grateful for financial support from the Leibniz Association and the Werner Siemens-Stiftung. This work was supported by the Deutsche Forschungsgemeinschaft (German Research Foundation) under Germany's Excellence Strategy—EXC 2051—Project ID 390713860 and the collaborative research cluster SFB1127/2 ChemBioSys—Project ID 239748522—as well as an Exploration Grant of the Boehringer Ingelheim Foundation. We are grateful to the Europäischer Fonds für regionale Entwicklung for an instrument grant and to the DECHEMA for a Max-Buchner Fellowship. We also thank A. Perner and M. Flak for LC-HRMS and LC-MS/MS analysis and H. Heineke for NMR measurements.

1. S. Mitri, K. R. Foster, The genotypic view of social interactions in microbial communities. *Annu. Rev. Genet.* **47**, 247–273 (2013).
2. A. E. Little, C. J. Robinson, S. B. Peterson, K. F. Raffa, J. Handelsman, Rules of engagement: Interspecies interactions that regulate microbial communities. *Annu. Rev. Microbiol.* **62**, 375–401 (2008).
3. V. V. Phelan, W. T. Liu, K. Pogliano, P. C. Dorrestein, Microbial metabolic exchange–The chemotype-to-phenotype link. *Nat. Chem. Biol.* **8**, 26–35 (2011).
4. K. Papenfort, B. L. Bassler, Quorum sensing signal-response systems in Gram-negative bacteria. *Nat. Rev. Microbiol.* **14**, 576–588 (2016).
5. M. Whiteley, S. P. Diggle, E. P. Greenberg, Progress in and promise of bacterial quorum sensing research. *Nature* **551**, 313–320 (2017).
6. D. C. Oh, M. Poulsen, C. R. Currie, J. Clardy, Dentigerumycin: A bacterial mediator of an ant-fungus symbiosis. *Nat. Chem. Biol.* **5**, 391–393 (2009).
7. J. Kroiss *et al*., Symbiotic Streptomycetes provide antibiotic combination prophylaxis for wasp offspring. *Nat. Chem. Biol.* **6**, 261–263 (2010).
8. P. Stallforth *et al*., A bacterial symbiont is converted from an inedible producer of beneficial molecules into food by a single mutation in the *gacA* gene. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 14528–14533 (2013).
9. D. J. Newman, G. M. Cragg, Natural products as sources of new drugs from 1981 to 2014. *J. Nat. Prod.* **79**, 629–661 (2016).
10. G. P. van Wezel, K. J. McDowall, The regulation of the secondary metabolism of *Streptomyces*: New links and experimental advances. *Nat. Prod. Rep.* **28**, 1311–1333 (2011).
11. A. A. Brakhage, Regulation of fungal secondary metabolism. *Nat. Rev. Microbiol.* **11**, 21–32 (2013).
12. K. Scherlach, B. Busch, G. Lackner, U. Paszkowski, C. Hertweck, Symbiotic cooperation in the biosynthesis of a phytotoxin. *Angew. Chem. Int. Ed. Engl.* **51**, 9615–9618 (2012).
13. R. Wang, M. R. Seyedsayamdost, Opinion: Hijacking exogenous signals to generate new secondary metabolites during symbiotic interactions. *Nat. Rev. Chem.* **1**, 1–8 (2017).
14. P. Cosson, T. Soldati, Eat, kill or die: When amoeba meets bacteria. *Curr. Opin. Microbiol.* **11**, 271–276 (2008).
15. C. J. Weijer, *Dictyostelium* morphogenesis. *Curr. Opin. Genet. Dev.* **14**, 392–398 (2004).
16. R. Froquet, E. Lelong, A. Marchetti, P. Cosson, *Dictyostelium discoideum*: A model host to measure bacterial virulence. *Nat. Protoc.* **4**, 25–30 (2009).
17. R. Herbst, M. Günther, P. Stallforth, *Chemical Ecology of Dictyostelium Discoideum. Comprehensive Natural Products III* (Elsevier, 2019).
18. J. Boulais *et al*., Molecular characterization of the evolution of phagosomes. *Mol. Syst. Biol.* **6**, 423 (2010).
19. J. D. Dunn *et al*., Eat prey, live: *Dictyostelium discoideum* as a model for cell-autonomous defenses. *Front. Immunol.* **8**, 1906 (2018).
20. C. Matz, S. Kjelleberg, Off the hook–How bacteria survive protozoan grazing. *Trends Microbiol.* **13**, 302–307 (2005).
21. M. Klapper, S. Götze, R. Barnett, K. Willing, P. Stallforth, Bacterial alkaloids prevent amoebal predation. *Angew. Chem. Int. Ed. Engl.* **55**, 8944–8947 (2016).
22. M. Klapper, J. Arp, M. Günther, P. Stallforth, The role of bacterial natural products in predator defense. *Synlett* **29**, 537–541 (2018).
23. D. A. Brock *et al*., Diversity of free-living environmental bacteria and their interactions with a bacterivorous amoeba. *Front. Cell. Infect. Microbiol.* **23**, 411 (2013).
24. J. Arp *et al*., Synergistic activity of cosecreted natural products from amoebae-associated bacteria. *Proc. Natl. Acad. Sci. U.S.A.* **115**, 3758–3763 (2018).
25. H. Gross, J. E. Loper, Genomics of secondary metabolite production by *Pseudomonas* spp. *Nat. Prod. Rep.* **26**, 1408–1446 (2009).
26. E. N. Grady, J. MacDonald, L. Liu, A. Richman, Z. C. Yuan, Current knowledge and perspectives of *Paenibacillus*: A review. *Microb. Cell Fact.* **15**, 203 (2016).
27. O. Tyc *et al*., Exploring bacterial interspecific interactions for discovery of novel antimicrobial compounds. *Microb. Biotechnol.* **10**, 910–925 (2017).
28. A. D. Berti, N. J. Greve, Q. H. Christensen, M. G. Thomas, Identification of a biosynthetic gene cluster and the six associated lipopeptides involved in swarming motility of *Pseudomonas syringae* pv. tomato DC3000. *J. Bacteriol.* **189**, 6312–6323 (2007).
29. K. Blin *et al*., antiSMASH 5.0: Updates to the secondary metabolite genome mining pipeline. *Nucleic Acids Res.* **47**, W81–W87 (2019).
30. N. Fanin *et al*., The ratio of Gram-positive to Gram-negative bacterial PLFA markers as an indicator of carbon availability in organic soils. *Soil Biol. Biochem.* **128**, 111–114 (2019).
31. B. N. Singh, Studies on soil acrasieae; the active life of species of Dictyostelium in soil and the influence thereon of soil moisture and bacterial food. *J. Gen. Microbiol.* **1**, 361–367 (1947).
32. T. L. Loh, J. R. Pawlik, Chemical defenses and resource trade-offs structure sponge communities on Caribbean coral reefs. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 4151–4156 (2014).
33. H. Chen, R. Athar, G. Zheng, H. N. Williams, Prey bacteria shape the community structure of their predators. *ISME J.* **5**, 1314–1322 (2011).
34. M. Mazzola, I. de Bruijn, M. F. Cohen, J. M. Raaijmakers, Protozoan-induced regulation of cyclic lipopeptide biosynthesis is an effective predation defense mechanism for *Pseudomonas fluorescens*. *Appl. Environ. Microbiol.* **75**, 6804–6811 (2009).
35. L. Ma *et al*., Hydrogel-based transparent soils for root phenotyping in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **116**, 11063–11068 (2019).
36. D. A. Brock, T. E. Douglas, D. C. Queller, J. E. Strassmann, Primitive agriculture in a social amoeba. *Nature* **469**, 393–396 (2011).
37. J. Johnke *et al*., Multiple micro-predators controlling bacterial communities in the environment. *Curr. Opin. Biotechnol.* **27**, 185–190 (2014).
38. Z. Gao, I. Karlsson, S. Geisen, G. Kowalchuk, A. Jousset, Protists: Puppet masters of the rhizosphere microbiome. *Trends Plant Sci.* **24**, 165–176 (2019).
39. J. I. Rilling *et al*., Current opinion and perspectives on the methods for tracking and monitoring plant growth–promoting bacteria. *Soil Biol. Biochem.* **130**, 205–219 (2018).
40. S. Götze, P. Stallforth, Structure, properties, and biological functions of nonribosomal lipopeptides from pseudomonads. *Nat. Prod. Rep.* **37**, 29–54 (2020).
41. N. Geudens, J. C. Martins, Cyclic lipodepsipeptides from *Pseudomonas* spp.-biological Swiss-army knives. *Front. Microbiol.* **9**, 1867 (2018).
42. R. Hermenau, S. Kugel, A. J. Komor, C. Hertweck, Helper bacteria halt and disarm mushroom pathogens by linearizing structurally diverse cyclolipopeptides. *Proc. Natl. Acad. Sci. U.S.A.* **117**, 23802–23806 (2020).
43. B. C. Hoefler *et al*., Enzymatic resistance to the lipopeptide surfactin as identified through imaging mass spectrometry of bacterial competition. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 13082–13087 (2012).
44. L. R. Hmelo *et al*., Precision-engineering the *Pseudomonas aeruginosa* genome with two-step allelic exchange. *Nat. Protoc.* **10**, 1820–1841 (2015).