Lipopeptide-mediated bacterial interaction enables cooperative predator defense

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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 syringafacin, a lipopeptide, which induces the production of peptidases in a Paenibacillus strain. These peptidases then degrade the innocuous syringafacin into compounds, which kill the predator. A combination of bioprospecting, coculture experiments, genome modification, and transcriptomics unravel this novel natural product-based defense strategy.

Results and Discussion

Cooperative Defense Traits. 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 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.

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.
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 indecisive (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 cooperate in producing 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 sy biosynthetic gene cluster (BGC), required for the generation of the

![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 chored with 34 reference strains that are the nearest relatives of strains used in the study to indicate their phylogenetic affiliation. The isolates have been chored with 34 reference strains that are the nearest relatives of strains used in the study to indicate their phylogenetic affiliation. The isolates have been chored with 34 reference strains that are the nearest relatives of strains used in the study to indicate their phylogenetic affiliation.](https://doi.org/10.1073/pnas.2013759118 Lipopeptide-mediated bacterial interaction enables cooperative predator defense)

![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 $\lambda_e = 210$ nm). Red profiles represent cleavage products identified on solid medium and blue ones in liquid medium. (Right) Syringafactin A and peptidolytic degradation products thereof. Syringafactins A and C are detected in cultures of SZ57. Syringafactins A$_1$ and C$_1$ are produced in liquid medium, whereas syringafactins 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).](https://doi.org/10.1073/pnas.2013759118 Lipopeptide-mediated bacterial interaction enables cooperative predator defense)
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. SZ57Δsyf, which was unable to produce syringafactins, and co-cultured 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 SZ57Δsyf 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. SZ57Δsyf 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 μg · mL⁻¹. We then determined the toxicity of the respective degradation products.

Indeed, syringafactins A₁ and C₁ (1:1, wt/wt) produced in a liquid coculture were highly toxic for the amoebae with IC₅₀ (*D. discoideum*) = 6.7 ± 1.2 μg · mL⁻¹ (mean ± SEM, n = 3). The peptidolytic-degradation products produced on a solid medium, syringafactins X₃, X₄, and X₇ (3:2:1, wt/wt), displayed lower toxicity with IC₅₀ (*D. discoideum*) = 23.0 ± 5.6 μg · 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
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, and C, (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 supplementations 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 D-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, Figs. 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 taiwanensis) 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 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.
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 \(_{600}\) was adjusted to 0.05, and 100 \(\mu\)L of this cell suspension was plated on KB agar plates and incubated at 28 °C overnight. Following the incubation period, the mating spots were resuspended in 200 \(\mu\)L LB broth without antibiotics. After mixing, 100 \(\mu\)L 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 \(\mu\)L 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- knockout 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, SZ57Δsyf, 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 \(\mu\)L 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 ionization source and a Kinetex C18 column (50 × 2.1 mm, particle size 1.7 \(\mu\)m, 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 ion modes.
negative mode. Desorption 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 (HK01916) and Paenibacillus sp. SZ31 (HK01915) are available in the National Center for Biotechnology Information (NCBI) (Pseudomonas sp. SZ57: NZ_WIBDD00000000; Paenibacillus sp. SZ31: JABBEA0000000000). Raw transcriptomic data are available in Figshare (https://doi.org/10.6084/m9.figshare.13858583.v1). All other data studies are included in the article and/or SI Appendix.

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