Candicidin-producing *Streptomyces* support leaf-cutting ants to protect their fungus garden against the pathogenic fungus *Escovopsis*

Susanne Haeder[a], Rainer Wirth[b], Hubert Herz[c,d], and Dieter Spiteller[a,1]

[a]Department of Bio-Organic Chemistry, Max Planck Institute for Chemical Ecology, Hans-Knöll-Strasse 8, D-07745 Jena, Germany; [b]Department of Plant Ecology and Systematics, Technische Universität Kaiserslautern, Erwin-Schrödinger-Strasse 13, D-67663 Kaiserslautern, Germany; and [c]Smithsonian Tropical Research Institute, Apartado 0843-03092, Balboa, Ancón, Republic of Panamá; and [d]Biology Department, San Francisco State University, 1600 Holloway Avenue, San Francisco, CA 94132

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Leaf-cutting ants such as *Acromyrmex octospinosus* live in obligate symbiosis with fungi of the genus *Leucoagaricus*, which they grow with harvested leaf material. The symbiotic fungi, in turn, serve as a major food source for the ants. This mutualistic relation is disturbed by the specialized pathogenic fungus *Escovopsis* sp., which can overcome *Leucoagaricus* sp. and thus destroy the ant colony. Microbial symbionts of leaf-cutting ants have been suggested to protect the fungus garden against *Escovopsis* by producing antifungal compounds [Currie CR, Scott JA, Summerbell RC, Malloch D (1999) Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* 398:701–704.]. To date, however, the chemical nature of these compounds has remained elusive. We characterized 19 leaf-cutting ant–associated microorganisms (5 *Pseudonocardia*, 1 *Dermacoccus*, and 13 *Streptomyces*) from 3 *Acromyrmex* species, *A. octospinosus*, *A. echinatior*, and *A. volcanus*, using 16*S*-rDNA analysis. Because the strain *Streptomyces* sp. Ao10 proved highly active against the pathogen *Escovopsis*, we identified the molecular basis of its antifungal activity. Using bioassay-guided fractionation, high-resolution electrospray mass spectrometry (HR-ESI-MS), and UV spectroscopy, and comparing the results with an authentic standard, we were able identify candicidin macrolides. Candicidin macrolides are highly active against *Escovopsis* but do not significantly affect the growth of the symbiotic fungus. At least one of the microbial isolates from each of the 3 leaf-cutting ant species analyzed produced candicidin macrolides. This suggests that candicidins play an important role in protecting the fungus gardens of leaf-cutting ants against pathogenic fungi.

Acromyrmex | antifungal agent | symbionts | Attini | polyketides

Ants have fascinated people for a long time, in part because many aspects of their social organization appear so similar to those of humans (1). The highly evolved leaf-cutting ants of *Acromyrmex* and *Atta* spp. (Hymenoptera: Formicidae: Attini) have attracted particular attention because these ants cultivate a symbiotic fungus (Agaricales: mostly Lepiotaceae: Leucocoprineae) in specialized chambers of their nests. The ants cut leaves, preprocess them into small pieces, and feed them to the symbiotic fungus, which, in turn, serves as their major food source (2). However, their highly sophisticated behavior aside, leaf-cutting ants are both dominant herbivores of neotropical forests (2) and a serious problem to crop plants in South America (3).

Leaf-cutting ants, like other fungus-growing ants (tribe Attini), are threatened by specialized fungal pathogens; *Escovopsis* (Ascomycota: anamorphic Hypocreales) was shown to have detrimental effects on the health of the fungal gardens and, consequently, on the survival of the ant colony (4, 5). To avoid infection, leaf-cutting ants have evolved an elaborate cleaning behavior, carefully maintaining their fungus garden and placing old or suspicious material into waste chambers (6). In addition to mechanically removing waste, leaf-cutting ants secrete antimicrobial and antifungal agents from their metapleural glands [e.g., myrmicacin (3-hydroxydecanoic acid)], which help to protect against pathogens (7–10).

In their pioneering work, Currie et al. (11) discovered symbiotic microorganisms on the integument of leaf-cutting ants as additional players in these interactions. Those microorganisms, identified as *Pseudonocardia* spp., were suggested to be part of a 50 million-year-old tripartite coevolutionary process. Microorganisms producing antifungal compounds coevolved to protect the ants' fungus against the pathogens *Escovopsis aspergilloides* and *Escovopsis weberi* (12, 13). However, recent evidence indicates that diverse microbial communities rather than a single symbiont exist in the leaf-cutting ants' gardens. This contradicts the prevailing view, according to which attine ants, their garden fungus, and the pathogens specifically coevolved with *Pseudonocardia*. To give an example, *Streptomyces* and *Burkholderia* are now thought to be involved in this multitrophic interaction (14–16). The studies of Kost et al. (14) and Mueller et al. (16) provide strong evidence that the microbial communities of leaf-cutting ants are flexible, highly diverse, and likely to result when microorganisms are recruited from the environment.

Although our knowledge about the ants' microbial symbionts has evolved dramatically in recent years (11), the chemical basis of their antifungal activities has not yet been elucidated. Using a bioassay-guided approach, we isolated an antifungal compound produced by a *Streptomyces* strain associated with leaf-cutting ants. Structure elucidation revealed candicidin macrolides that are highly active against the fungal pathogen *Escovopsis* but not against the ants' garden fungus *Leucoagaricus gongylophorus.*

Results

Isolation and Characterization of Microorganisms Associated with Leaf-Cutting Ants. Microorganisms from 3 leaf-cutting ant species (*Acromyrmex octospinosus*, *Acromyrmex echinatior*, and *Acromyrmex volcanus*) were isolated from individual worker ants or small fungus garden samples. *Streptomyces*-type microorganisms were isolated using selective agar plates (17), and their typical morphology was considered as the selection criterion (18). Microorganisms were found in all 3 ant species. 16*S*-rDNA analysis of the isolates allowed us to classify the isolates [supporting information (SI) Table S1 and Fig. S1].

Six strains were obtained from *A. octospinosus*: 4 *Pseudono-*

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[1]To whom correspondence should be addressed. E-mail: dspiteller@ice.mpg.de.

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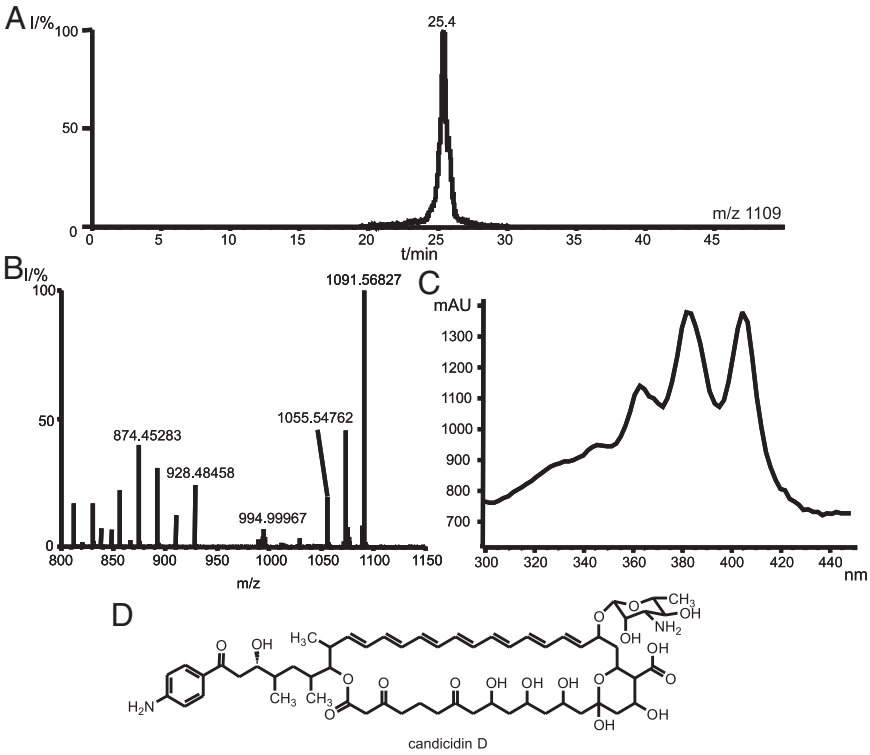

Fig. 1. Isolation of candicidin D from *Streptomyces* sp. Ao10. LC-MS chromatogram of the active fraction (A), ESI-HR-MS/MS spectrum of $[M+H]^+$ 1109.57938 (*B*); UV spectrum of candicidin D (*C*); and structure of candicidin D (*D*).

cardia, 1 *Dermacoccus*, and 1 *Streptomyces*. Two of the *Pseudonocardia* strains exhibited 16*S*-rDNA sequences that were highly similar (98.69%, 97.45%) to the 16*S*-rDNA sequences published by Zhang et al. (19). The streptomycete *Streptomyces* sp. Ao10 originated from the *A. octospinosus* fungus garden and is similar to *S. albidoflavus* NRRL B-24475 (98.70%). Two strains were obtained from *A. echinatior*; one was identified as a *Pseudonocardia* and the other as closely related to *S. albidoflavus.* From *A. volcanus*, we isolated 11 bacterial strains: 1 *Pseudonocardia* strain and 10 *Streptomyces* strains, some of which were highly similar to *Streptomyces albidoflavus* and *Streptomyces griseus.*

Antifungal Screening and Structure Elucidation of an Antifungal Compound from Ao10. In an initial screening for antifungal activity against the pathogens *E. aspergilloides* and *E. weberi* (4, 5), the isolated bacterial strains were cocultivated on soy flour medium (SFM) agar plates, together with the fungal pathogen. *Streptomyces* sp. Ao10 was selected from this screening because it strongly inhibited the growth of *Escovopsis*. In addition, the closely related strains *Streptomyces* sp. Av25_1, Av26_5, Av28_2, Av28_3, and Ae32_2 inhibited the growth of the pathogenic fungus *Escovopsis*. Some of our *Pseudonocardia* that were closely related to Zhang's isolates (19) appeared to be less effective against *Escovopsis* than were the selected *Strepomyces*; some *Pseudonocardia* were even overgrown by the fungus.

To isolate the compounds that are responsible for inhibiting the growth of *E. weberi* and *E. aspergilloides*, we focused on *Streptomyces* sp. Ao10. The supernatant of a *Streptomyces* sp. Ao10 culture was extracted with *n*-butanol, and the residue of the concentrated extract was subjected to SiO_2 column chromatography followed by RP18 medium pressure liquid chromatography (MPLC) separation. The progress of all purification steps was evaluated using the agar diffusion assay against *Escovopsis* as a bioassay. Finally, the bioactive fractions were both analyzed by LC-UV-MS and purified by HPLC for further bioactivity testing (Fig. 1).

The active fraction exhibited a characteristic UV spectrum with 4 distinct maxima at 408 nm, 384 nm, 364 nm, and 344 nm and a $[M+H]^+$ ion at 1109.57938. High-resolution electrospray mass spectrometry (HR-ESI-MS) of the quasimolecular ion provided the molecular formula $C_{59}H_{84}O_{18}N_2$ for the antifungal compound. As a result of the information from the UV spectrum that is characteristic for polyenes (18) and the results of a literature search based on the elementary composition of the antifungal compound, candicidin was proposed as the antifungal compound (20, 21).

The HR-ESI-MS/MS fragmentation pattern of the antifungal compound from *Streptomyces* sp. Ao10 perfectly matched the fragmentation of candicidin D (e.g., *m/z* 1091.56827 $[M+H-H_2O]^+$ or *m/z* 928.48458 $[M+H-\text{mycosamine}-H_2O]^+$). The isolated compound exhibited the same chromatographic behavior and ESI-MS/MS fragmentation as an authentic candicidin D standard. In addition to the dominant peak at *m/z* 1109, a coeluting signal at *m/z* 1111 was observed. Structural variants of candicidin macrolides are known to be produced by the candicidin biosynthesis gene clusters from *S. griseus* (22) and *Streptomyces* FR-008 (23, 24).

Subsequently, primers specific for candicidin biosynthesis genes were used to amplify characteristic regions of the genes fscA, fscM, and fscP by PCR from the genomic DNA of *Streptomyces* sp. Ao10 (22, 23). Sequencing the amplified products revealed 99% identity to canA (FJ490545), fscM (FJ490546), and fscP (FJ490547). Consequently, we conclude that *Streptomyces* sp. Ao10 produces candicidin macrolides that are highly active against *Escovopsis*. The total DNA of the microorganisms associated with the leaf-cutting ants was prepared from a single *A. echinatior* worker and used as a template to amplify fscP (FJ490548), a gene that is specific for candicidin biosynthesis. Sequence comparison with the gene from *Streptomyces* FR-008 (23) provided direct evidence of the presence

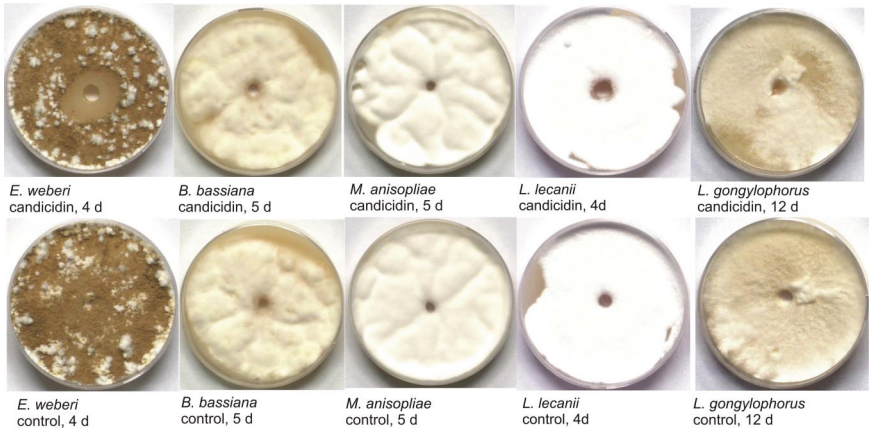

Fig. 2. Antifungal activity assay of candicidin macrolides (90 nmol) against *E. weberi*, *B. bassiana*, *M. anisopliae*, *L. lecanii*, and the symbiotic fungus *L. gongylophorus* in comparison to the solvent control.

of candicidin-producing microorganisms on the leaf-cutting ants' bodies.

Having identified candicidin macrolides as an antifungal principle of *Streptomyces* sp. Ao10, we screened for candicidins and related polyketide polyenes in the other microbial isolates from leaf-cutting ants. Six of 19 microorganisms were able to produce candicidin macrolides. At least 1 associated microorganism in all 3 leaf-cutting ant species (*A. octospinosus*, *A. echinatior*, and *A. volcanus*) was found to produce candicidins. All candicidin-producing microorganisms belonged to *Streptomyces* (Table S1).

Candicidin Macrolides as Selective Antifungal Compounds Against *Escovopsis*. After the candicidin macrolides were identified, their antifungal potential was addressed. In the agar diffusion assay against *E. weberi*, 90-nmol candicidin macrolides created a large inhibition zone of ∅ 1.6 cm after 4 days of incubation (Fig. 2). Nine nanomoles of candicidin macrolides was clearly sufficient to inhibit the growth of *E. weberi* (∅ 1.3-cm inhibition zone) and *E. aspergilloides* (∅ 0.6-cm inhibition zone) in the agar diffusion assay (Figs. S2–S4). At 4.5 nmol, however, even though growth is still visibly inhibited, the minimum concentration necessary for the inhibition of *E. weberi* to occur is reached. Candicidins seemed to inhibit *Escovopsis* specifically, because the growth of other pathogenic fungi was either not or only weakly inhibited (*Metarhizium anisopliae*, *Beauveria bassiana*, and *Lecanicillium lecanii*) (Figs. S5–S7). Importantly, the ants' symbiotic fungus *L. gongylophorus* is not significantly affected by candicidin macrolides (Fig. 2 and Fig. S8).

Discussion

To isolate an antifungal compound from microorganisms associated with leaf-cutting ants, we focused on *Streptomyces*-type bacteria. These microorganisms are both well known for their valuable bioactive secondary metabolites (25) and have previously been isolated from leaf-cutting ants (5, 14, 16). However, despite our selective isolation procedure, we obtained 19 strains, with 13 of them belonging to *Streptomyces* (Table S1 and Fig. S1). This supports the recent finding that diverse microbial communities are associated with leaf-cutting ants (14, 16) rather than a single coevolved *Pseudonocardia* strain, as previously believed (12, 13). Because conventional isolation techniques yield only a small proportion of the naturally occurring microorganisms, the microbial communities of leaf-cutting ants are likely extremely complex (26).

Because our initial screening for their antifungal potential against *Escovopsis* showed that some *Streptomyces* exhibited pronounced activity, we isolated the antifungal compound from the *Streptomyces* sp. Ao10. We did not opt for a *Pseudonocardia* because most of our *Pseudonocardia* isolates did not appear to be very active against *Escovopsis*, an observation supported by previous studies (16). *Streptomyces* sp. Ao10 has been isolated directly from the *A. octospinosus* fungus garden. The presence of microorganisms producing antifungal compounds in the fungus garden that is the target of *Escovopsis* may provide the most efficient protection to the symbiotic fungus, whereas the bacterial biofilms on the body of the leaf-cutting ants may mainly protect individuals and help to distribute beneficial microorganisms. This conclusion is also supported by the observation that leaf-cutting workers preparing leaf material as a substrate for the symbiotic fungus inoculate the leaf material with microorganisms (27).

The bioassay-guided fractionation of the highly active *Streptomyces* sp. Ao10 yielded candicidin macrolides, which were highly active against *E. weberi* and *E. aspergilloides*. Candicidin macrolides were isolated in the 1950s (20), but elucidating their structure took until 1979 (21). Their name reflects the antifungal properties of the compounds against the human pathogenic fungus *Candida albicans*. Although candicidins are highly active antifungals, their medical use is restricted to severe cases (28). Like other polyketide polyenes, candicidins interact with sterols in the fungal cell membranes, causing K^+-leakage and inducing cell death (29). The relatively rare development of resistance to polyketide polyenes among pathogenic fungi (28) might explain why leaf-cutting ants benefit from the presence of candicidin-producing microorganisms and do not face the risk that *Escovopsis* will adapt quickly. Recently, the candicidin biosynthetic gene cluster was characterized from both *Streptomyces* sp. FR-008 (23) and *S. griseus* (22), which allowed us to confirm its presence by PCR with gene-specific primers not only in *Streptomyces* sp. Ao10 but in total microbial DNA from individual *A. echinatior* worker ants. Thus, we could directly prove that candicidin-producing microorganisms were on the ants' bodies without having to isolate the microorganisms. Ninety nanomoles of candicidin macrolides caused a large inhibition zone against *E. weberi* and *E. aspergilloides* in the agar diffusion assay (Fig. 2 and Figs. S2–S4); this zone was not overgrown even after 14 days of incubation. In the same assay, only 9 nmol of candicidin macrolides clearly inhibited the growth of *E. weberi* (Fig. S4). This result illustrates that candicidin macrolides effectively help leaf-cutting ants to defend their fungus gardens against *Escovopsis*. In contrast, candicidins did not inhibit the growth of the insect pathogenic fungi *M. anisopliae*, *B. bassiana*, and *L. lecanii* (Figs. S5–S7). In particular, the growth performance of the symbiotic fungus *L. gongylophorus* was not significantly altered by candicidins (Fig. S8).

Notably, of 19 bacterial isolates from leaf-cutting ants, 6 produced candicidin macrolides. In fact, we found candicidin-producing microorganisms associated with all leaf-cutting ant species analyzed (*A. octospinosus*, *A. echinatior*, and *A. volcanus*). Moreover, the microbial screening by Mueller et al. (16) yielded an *S. griseus* strain from the fungus garden of *Cyphomyrmex muelleri*. This strain is closely related to our candicidin-producing *Streptomyces* strains, and thus is highly likely to produce candicidin macrolides. Consequently, candicidin-producing *Strepomyces* appear to be widespread in fungus gardens of leaf-cutting ants. In this context, it is interesting that Zhang et al. (19) concluded in their behavioral study that *Acromyrmex* ants are able to select for beneficial microorganisms. Furthermore, Poulsen et al. (30) suggest that the metapleural gland influences the growth of microbial symbionts on the ants' bodies. However, the chemical basis of both observations remains unknown.

Even though candicidin-producing *Streptomyces* sp. seem to be widespread among leaf-cutting ants, other antifungal compounds likely help to protect the fungus garden. *Pseudonocardia autotrophica*, which is closely related to the *Pseudonocardia* strains of leaf-cutting ants, has been found to contain a cryptic gene cluster specific for polyketide polyenes (31). However, we did not observe an antifungal polyene produced by our *Pseudonocardia* isolates. In addition to polyene macrolides, an as yet uncharacterized depsipeptide (32) and an unknown antifungal from *Burkholderia* (15) may contribute to the protection of the symbiotic fungus of different leaf-cutting ant species. These studies suggest that according to the microbial diversity among leaf-cutting ant–associated bacteria (14, 16), there is considerable variety of antifungal compounds.

In summary, with the identification of candicidins, we characterized an antifungal agent produced by the symbiotic bacteria of *Acromyrmex* leaf-cutting ants. Candicidins proved to be efficient agents against the fungal pathogen *Escovopsis.* The identification of candicidin macrolides as active antifungal compounds in the leaf-cutting ants' ecosystem opens a door for future studies of the chemical ecology of the microbial communities associated with leaf-cutting ants.

Materials and Methods

Fungal Cultures. *E. aspergilloides* CBS 423.93 and *E. weberi* CBS 110660 were obtained from the Centralbureau voor Schimmelcultures. *L. gongylophorus* was an isolate from the fungus garden of *Atta colombica*. *M. anisopliae* DSMZ 1490, *B. bassiana* DSMZ 875, and *L. lecanii* DSMZ 3411 originated from the German Collection of Microorganisms and Cell Cultures. The strains were maintained on SFM agar plates.

Collection of the Ants and Fungus Garden Samples. Leaf-cutting ants and fungus garden samples were taken from *A. volcanus*, *A. octospinosus*, and *A. echinatior* colonies (colonies A, B, C, and 1, respectively) collected in Gamboa, Panama. In addition, microorganisms were isolated from the fungus garden of an established laboratory colony of *A. echinatior* (colony 2) collected in 2002 in Panama.

Isolation of Microorganisms. Microorganisms from the body of leaf-cutting ants were isolated by touching each ant with a sterile toothpick and drawing this over *Streptomyces* isolation agar (SIA) (17) medium agar plates. To collect microorganisms from the fungus garden, 2 mL of sterile water was added to a few milligrams of fungus garden. After samples were vortexed for 2 min, the aqueous supernatant was either plated directly onto SIA agar plates or diluted further before plating. After growing for 3 to 14 days, *Streptomyces*-type colonies were identified by their morphology, picked, and isolated by transfer onto new plates. Such pure colonies were maintained on SFM agar plates (20 g of soy flour, 20 g of mannitol, 20 g of agar, and 1 L of water) (25).

***16S*-rDNA Analysis of Isolated Symbionts.** Genomic DNA was prepared from 3–7-day-old 25-mL cultures grown in liquid SFM medium following ''procedure B'' (25). For 16*S* rDNA analysis, the primers 8F (AGAGTTTGATCATGGCTCAG) and 1492r (GGTTACCTTGTTACGACTT) (33), fD2 (GAGTTTGATCATGGCTCAG) (34), and 16Sr (TTGCGGGACTTAACCCAACAT) (35) were used. The PCR products were gel-purified and sequenced using the same primers. A minimum size of *ca.* 1,400 bp was used for database comparison (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi, simrank algorithm). Phylogenetic analyses using the neighbor joining method (bootstrap value, $n = 1{,}000$) were conducted with MEGA version 4.1 (36).

Bioassay of the Antifungal Potential. *E. aspergilloides*, *E. weberi*, *M. anisopliae*, *B. bassiana*, *L. lecanii*, and *L. gongylophorus* were used as test organisms in the agar diffusion assay against isolated microorganisms, culture extracts, or pure samples. In the initial screening, the growth performance of *E. aspergilloides* was monitored on plates that 2 days earlier had been inoculated with one of the isolated microorganisms on one side of the plate. For the bioassay-guided fractionation, 100 μL of mycelium or spore suspensions (*ca.* 5 mg wet weight/mL Luria–Bertani medium) of the test organisms was spread onto SFM plates (5.5-cm diameter). A 6-mm hole was cut in the middle of the plate, or a piece of filter paper was placed onto the agar plate, to apply 5–200 μL of the test solution or an appropriate solvent control (e.g., MeOH, DMSO). To assay *L. gongylophorus*, a SFM agar piece (0.5 cm $\times$ 0.5 cm) with the fully grown fungus was used to inoculate the test plate. The inhibition zones were monitored after 3 to 14 days at 28 °C. All assays were performed at least in triplicate and were compared with equally prepared solvent controls. Various amounts of purified candicidin macrolides dissolved in DMSO (2 mg/mL) were tested (4.5–90 nmol) (*SI*): inhibition zones *E. weberi*: 90 nmol $\varnothing$ 1.6 cm, 9 nmol $\varnothing$ 1.3 cm, and 4.5 nmol $\varnothing$ 0.6 cm; inhibition zones *E. aspergilloides*: 90 nmol $\varnothing$ 1.4 cm, 45 nmol $\varnothing$ 1.3 cm, 18 nmol $\varnothing$ 1 cm, and 9 nmol $\varnothing$ 0.6 cm.

Bioassay-Guided Fractionation and Structure Elucidation. *Streptomyces* sp. Ao10 was grown in 500-mL Erlenmeyer flasks fitted with springs for aeration and containing 200 mL of liquid SFM medium. The flasks were incubated at 28 °C on a rotating shaker (Infors Mutitron II MT25) shaker (200 rpm) for 7 days. From 2 to 10 L of culture was used for the bioassay-guided fractionation and purification of the antifungal compound. After harvesting, the cultures were centrifuged at 5,000 *g* for 30 min. The supernatant was extracted 3 times with an equal volume of 1-butanol. The extract was concentrated using a roti-vap. The residue was then resuspended in 20–50 mL of MeOH and subjected to SiO_2-column chromatography (60 M SiO_2, 50 cm $\times$ 4 cm; Macherey-Nagel): 1 L of ethylacetate, 1 L of MeOH, and 1 L of water. Two hundred-milliliter fractions were collected, concentrated, redissolved in 4 mL of MeOH, and tested in the agar diffusion assay (50 μL) against *E. aspergilloides.* Bioactive fractions were combined and subjected to RP 18 MPLC (40–63 μm, LiChroprep RP18 resin, 45 cm $\times$ 4 cm; Merck) using gradient elution at a flow rate of 10 mL: 100% A 0 min, in 30 min 100% B, 100% B 15 min (A: water, B: MeCN). Twenty-milliliter fractions were collected, concentrated, redissolved in 4 mL of MeOH, and used for the bioassay (50 μL). The bioactive fraction was subjected to LC-MS analysis and HPLC separation using a Phenomenex Synergi Polar RP column (250 mm $\times$ 2 mm, 5 μm). A HP1100 system with a diode array detector (DAD) (Agilent) hooked to a Thermo Fisher LTQ or a Gilson 207 fraction collector was used. HPLC conditions were 3 min 100% A, in 27 min to 100% B, 10 min 100% B (A: water 0.1% AcOH, B: MeCN 0.1% AcOH), with a flow rate of 0.25 mL/min. Retention time of candicidin D was 25.4 min (UV: 408 nm, 384 nm, 364 nm, and 344 nm). High-resolution ESI-MS was performed with a Thermo Fisher Orbitrap injecting the purified samples directly via a syringe pump. Measured HR-ESI-MS was 1109.57938, and calculated HR-ESI-MS was 1109.57974 ($C_{59}H_{85}O_{18}N_2$). HR-ESI-MS/MS of 1109 was as follows: $[M+H-H_2O]^+$ 1091.56827 ($C_{59}H_{83}O_{17}N_2$), $[M+H-3H_2O]^+$ 1055.54762 ($C_{59}H_{79}O_{15}N_2$), $[M+H-\text{mycosamine}-H_2O]^+$ 928.48458 ($C_{53}H_{70}O_{13}N$), 874.45283 ($C_{53}H_{64}O_{10}N$). Semipreparative HPLC was performed with a Grom-Sil ODS 5 ST RP18 column (250 mm $\times$ 10 mm, 5 μm; Alltech) using the fraction collector. HPLC conditions were 3 min 100% A, in 27 min to 100% B, 10 min 100% B (A: water 0.1% AcOH, B: MeCN 0.1% AcOH), at a flow rate of 3 mL/min and with a retention time of 20 min.

Screening Microbial Isolates for Candicidin Production. All microorganisms isolated from the different leaf-cutting ants (Table S1) were screened for their ability to produce candicidins. The strains were cultivated for 7 days in 100 mL of SFM medium at 28 °C/200 rpm. One milliliter of MeOH was added to 1 mL of the culture broth; the sample was then vortexed and centrifuged to pellet the cells. Fifty microliters of the supernatant was analyzed by LC-UV-MS.

Screening for Candicidin Biosynthetic Genes. Primers specific for the candicidin biosynthetic genes were used to amplify characteristic fragments from isolated microorganisms as well as directly from individual ants (*A. echinatior*). DNA of microorganisms associated with individual ants was obtained by washing an ant in 1 mL of sterile water, transferring the resulting solution into a new Eppendorf tube, and centrifuging it. The pellet was utilized for DNA isolation using the Promega DNA Isolation Kit following its protocol for genomic DNA isolation from Gram-positive microorganisms. About 1,000 kb

regions from fscA, fscM, and fscP were amplified by PCR using fscAfor (ATGGTGCCCGTCCACGCACACGACTACGTGACCGATCCGC) and fscArev (GGCGGCCAGCACCTCGGCGACGGCGGTCACCACCAG), fscMfor (TCGCTGGGCGAGGTGGTCCCCGAACTGT) and fscMrev (CGGCTTGTCCAGGGTCAGGCTGATGCCG), and fscPfor (GGTTCTGGCCCAGGCACTGGTGGACGCCGTAGCC) and fscPrev (ATGACGACCAGCCCCGGCCCGACGGTGGTCGACTTCCCAC). PCR products were gel-purified and sequenced using the gene-specific primers. The sequences were compared by Blast search to the corresponding genes from *Streptomyces* sp. FR-008 (23) and *S. griseus* (22).

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