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

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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. volcancis*, using 16S-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 candididin macrolides. Candididin 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 candididin macrolides. This suggests that candididins play an important role in protecting the fungus gardens of leaf-cutting ants against pathogenic fungi.

*Acromyrmex* | antifungal agent | symbionts | Attini | polyketides

A nts 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: Leucocorpi-neae) 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 antifungal and microbial agents from their metapleurial glands [e.g., myrmecin (3-hydroxydecanic 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 weberti* (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 candididin 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 volcancis*) 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). 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).

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cardia, 1 Dermacoccus, and 1 Streptomyces. Two of the Pseudonocardia strains exhibited 16S-rDNA sequences that were highly similar (98.69%, 97.45%) to the 16S-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. albido flavus 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. albido flavus. From A. volcanus, we isolated 11 bacterial strains: 1 Pseudonocardia strain and 10 Streptomyces strains, some of which were highly similar to Streptomyces albido flavus 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 Streptomyces; 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 SiO2 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 C90H86O19N3 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−H2O]+ or m/z 928.48528 [M+H−mycosamine−H2O]+). 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 candA (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. 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).
showed that some complex microbial communities of leaf-cutting ants are likely extremely valuable bioactive secondary metabolites and have previously been isolated from leaf-cutting ants (5, 14, 16). However, because conventional isolation techniques yield only a single coevolved 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 candididin macrocles, which were highly active against E. weberi and E. aspergilloides. Candididin macrocles 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 candididins are highly active antifungals, their medical use is restricted to severe cases (28). Like other polyketide polyenes, candididins 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 candididin-producing microorganisms and do not face the risk that Escovopsis will adapt quickly. Recently, the candididin 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 candididin-producing microorganisms were on the ants’ bodies without having to isolate the microorganisms. Ninety nanomoles of candididin macrocles 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 candididin macrocles clearly inhibited the growth of E. weberi (Fig. S4). This result illustrates that candididin macrocles effectively help leaf-cutting ants to defend their fungus gardens against Escovopsis. In contrast, candididins 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. gongylrophorus was not significantly altered by candididins (Fig. S8).

Candididin Macrolides as Selective Antifungal Compounds Against Escovopsis. After the candididin macrocles were identified, their antifungal potential was addressed. In the agar diffusion assay against E. weberi, 90-nmol candididin macrocles created a large inhibition zone of 1.6 cm after 4 days of incubation (Fig. 2). Nine nanomoles of candididin macrocles 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. Candididins seemed to inhibit Escovopsis specifically, because the growth of other pathogenic fungi was either not or only weakly inhibited (Metharhizium anisopliae, Beauveria bassiana, and Lecanicillium lecanii) (Figs. S5–S7). Importantly, the ants’ symbiotic fungus L. gongylrophorus is not significantly affected by candididin macrocles (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).

Fig. 2. Antifungal activity assay of candididin macrocles (90 nmol) against E. weberi, B. bassiana, M. anisopliae, L. lecanii, and the symbiotic fungus L. gongylrophorus in comparison to the solvent control.
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 Muehl et al. (16) yielded an S. griseus strain from the fungus garden of Cyphomyrmex muelleri. This strain is closely related to our candidicidin-producing Streptomyces strains, and thus is highly likely to produce candidicidin macrolides. Consequently, candidicidin-producing Streptomyces 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 metabolite gland influences the growth of microbial symbionts on the ants’ bodies. However, the chemical basis of both observations remains unknown.

Even though candidicidin-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 candidicidin, we characterized an antifungal agent produced by the symbiotic bacteria of Acromyrmex leaf-cutting ants. Candidicidin proved to be efficient agents against the fungal pathogen Escovopsis. The identification of candidicidin 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. minax from DSMZ 1490, B. bassiana DSMZ 875, and L. lecanii DSMZ 3411 originating 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 obtained from A. volansus, 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 16S rDNA analysis, the primers BF (AGAGTTTGATCATGCTCAG) and 1949r (GGTACCTTGGTACGACCT) (33), F22 (GGAGTTGATCATGCTCAG) (34), and 16s (TTCGCGGACTTAACCAACAT) (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 fractionation 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 × 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 candidicidin macrolides dissolved in DMSO (2 mg/mL) were tested (4.5–90 nmol) (50); inhibition zones E. weberi: 90 nmol 0.1 cm, 95 nmol 0.2 cm, 13 nmol 0.13 cm, and 4.5 nmol 0.6 cm; inhibition zones E. aspergilloides: 90 nmol 1.4 cm, 45 nmol 0.13 cm, 18 nmol 0.1 cm, and 9 nmol 0.6 cm.

Bioassay-Guided Fractionation and Structure Elucidation. Streptomyces sp. Ao10 was grown in 500-mL Erlenmeyer flasks fitted with stoppers and containing 200 mL of liquid SFM medium. The flasks were incubated at 28 °C on a rotating shaker (Infor Multitron 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 SiO2-column chromatography (60 M SiO2 50 cm × 4 cm; Macherey-Nagel). 1 L of ethylacetate, 1 L of MeOH, and 1 L of water. Two hundred-well fraction collector. HPLC conditions were 3 min 100% A, in 27 min to 100% B, 10 min equilibration. The fractions were monitored on plates that 2 days earlier had been inoculated with one of the test colonies. Fifty microliters of the supernatant was analyzed by LC-UV-MS. The sample was then vortexed and centrifuged to pellet the cells. Fifty microliter aliquots of the supernatant were analyzed by LC-MS-MS.

Screening Microbial Isolates for Candicidin Production. All microorganisms isolated from the different leaf-cutting ants (Table 5) were screened for their ability to produce candidicidin. 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 microliter aliquots of the supernatant were analyzed by LC-UV-MS.

Screening for Candidicidin Biosynthetic Genes. Primers specific for the candidicidin 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 E. coli culture, and the resulting PCR products were sequenced using the primers ECOLOGY.

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regions from fscA, fscM, and fscP were amplified by PCR using fscAfor (ATGACGACCGCCAGGATCGTGGTGDGTCAGTGCCG), fscMfor (GGTTCTGGCCCAGGCACTGGTGGACGCCGTAGCC), and fscPfor (GGTTCTGGCCCAGGACGAGGTGACGCCGTAGCC) and fscPrev (ATGACGACCGCCAGGATCGTGGTGTCAGTGCCG) and fscMrev (CGGCTTGTCCAGGGTCAGGCTGATGCCG), fscArev (GGCGGCGAGGTGACGCCGTAGCC) and fscPrev (ATGACGACCGCCAGGATCGTGGTGDGTCAGTGCCG). 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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Fig. S1. Phylogenetic tree based on the 16S-rDNA sequences from the 20 microorganisms isolated from *A. octospinosus*, *A. echinatior*, and *A. volcanus* and comparison with related microorganisms. Candicidin-producing microorganisms are highlighted in bold. Bootstrap values for n = 1,000 are given at the branching points in percentages for values higher than 65.
Fig. S2. *E. aspergilloides*, 8 days: first row, 45 nmol candididin; second row, 90 nmol candididin; third row, control
Fig. S3. *E. weberi*, 4 days: first row, 90 nmol candicidin; second row, control
Fig. S4. *E. weberi*, 5 days: first row, 9 nmol candicidin; second row, control (paper-disk assay)
Fig. S5. *M. anisopliae*, 5 days: first row, control; second row, 90 nmol candididin.
Fig. S6.  *B. bassiana*, 5 days: first row, control; second row, 90 nmol candididin
Fig. S7. *L. lecanii*, 4 days: first row, 90 nmol candicidin; second row, control
Fig. S8. L. gongylophorus, 12 days: first row, 90 nmol candicidin; second row, control
Table S1. 16S-rDNA analysis of 19 microorganisms isolated from leaf-cutting ants (A. octospinosus, A. echinatior, and A. volcanus) and their potential to produce candicidins

<table>
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<th>Isolated strain/accession number</th>
<th>Phylogenetic analysis, related strain/accession number</th>
<th>Similarity, %</th>
<th>Isolated from</th>
<th>Candidcidin production</th>
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<td>Dermacoccus sp. VM7/DQ238836.1</td>
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<td>Streptomyces sp. Av25_4/FJ490534</td>
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<td>95.94</td>
<td>A. volcanus</td>
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<td>Streptomyces albidoflavus strain /NRRL B-24475 DQ978978.1</td>
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<td>A. echinatior colony 2</td>
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