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A Rebeccamycin Analog Provides Plasmid-Encoded Niche Defense

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Supporting Information

ABSTRACT: Bacterial symbionts of fungus-growing ants occupy a highly specialized ecological niche and face the constant existential threat of displacement by another strain of ant-adapted bacteria. As part of a systematic study of the small molecules underlying this fraternal competition, we discovered an analog of the antitumor agent rebeccamycin, a member of the increasingly important indolocarbazole family. While several gene clusters consistent with this molecule's newly reported modification had previously been identified in metagenomic studies, the metabolite itself has been cryptic. The biosynthetic gene cluster for 9-methoxyrebeccamycin is encoded on a plasmid in a manner reminiscent of plasmid-derived peptide antimicrobials that commonly mediate antagonism among closely related Gram-negative bacteria.

Many, arguably most, of our clinically used antibacterial agents are produced by other bacteria, and this observation has led to the widely held if poorly sourced view that these molecules evolved to mediate bacterial competition in natural environments.[1,2] The bacterial symbionts of fungus-growing ants, Actinobacteria in the genus *Pseudonocardia*, provide a model system in which to define the ecological roles of these bacterially produced small molecules. Fungus-growing ants collect plant material, which they feed to the fungal crops in their gardens, and their bacterial symbionts provide chemical defenses against pathogenic fungi and other microbial competitors.[3] The bacterial symbionts are fed by the ants, and their greatest existential threat is displacement by another *Pseudonocardia* strain. An earlier phenotypic analysis confirmed that *Pseudonocardia* produce diffusible small molecules, antibiotics, that suppress other *Pseudonocardia* strains, and in this report we begin to establish the molecular and genetic basis for this competition between these closely related bacteria.[4]

We began with a small set of ant-associated *Pseudonocardia* and used an intruder assay to assess competition between them. In this assay, one strain, the 'resident,' is placed in the center of a Petri dish and allowed to establish a colony before a second strain, the 'intruder,' is introduced around the dish's periphery. The zone of inhibition, if present, represents the ability of the resident to suppress the intruder.[4] A few of these assays are shown in Figure 1.

We focused on two strains, BCI1 and BCI2, which are both *Pseudonocardia* isolates collected from *Apterostigma dentigerum* ant colonies on Barro Colorado Island, Panama. The two ant colonies, which were located near each other on a small island, represent the same ant species and grow identical fungal crops in their gardens. By usual taxonomic standards the BCI1 and BCI2 bacterial isolates are also identical: they share 100% 16S rDNA sequence identity, and their chromosomal DNA shares ~98.3% sequence identity across the entire nucleotide sequence (see below). However, as illustrated in Figure 1, their performance in the intruder assay differs dramatically. BCI1 had no measurable defensive activity against any intruder strains, while BCI2 produced sizable inhibitory zones (≥1.4 cm) against all intruder strains except itself (Figure 1).

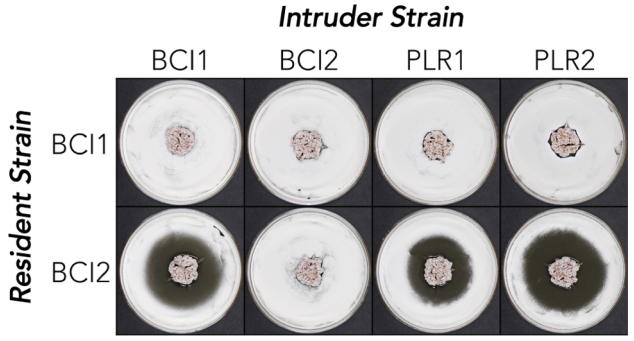

Figure 1. Intruder assay for ant-associated *Pseudonocardia* strains. The ability of the resident strain (center) to suppress growth of the intruder (periphery) is judged by the size of the zone of inhibition.

To investigate the molecular basis for the puzzling dichotomy between the taxonomic and genetic identity and the starkly different assay performance, we tested the organic soluble extracts from bacterial cultures. The BCI1 extract had no activity, while the BCI2 extract potently inhibited a panel of six ant-associated *Pseudonocardia* (Figure S2). We used activity against *Pseudonocardia* strain PLR1 for activity-guided fractionation, and C_{18} flash chromatography followed by reverse-phase HPLC led to a single molecule with potent inhibitory activity. High-resolution ESI-MS indicated a highly unsaturated, dichlorinated structure with a molecular formula of $C_{28}H_{23}Cl_2N_3O_8$ ($[M + H]^+$ calcd 600.0935, expt 600.0920), which is absent from natural product databases. We noted a distinctive UV−vis spectrum (λ_{max} 207, 244, 296, 317, 401 nm) similar to the dichlorinated indolocarbazole natural product rebeccamycin (**1**). 1D and 2D NMR revealed the anti-*Pseudonocardia* compound to be an unreported analog of rebeccamycin bearing a methoxy group, 9-methoxyrebeccamycin (**2**). All ^1H and ^{13}C resonances for the indolocarbazole core and the pendant 4-*O*-methylglucose of rebeccamycin were evident in this compound's 1D ^1H and ^{13}C spectra and could be assigned by COSY and HMBC couplings (Figure S6). HMBC couplings

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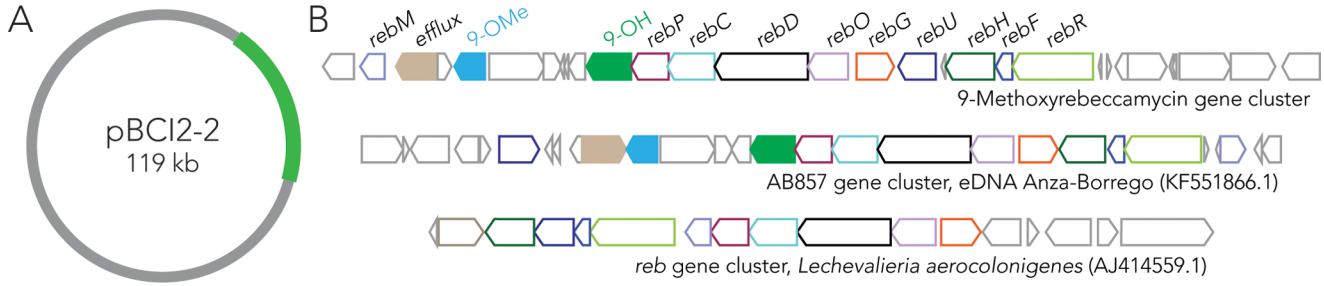
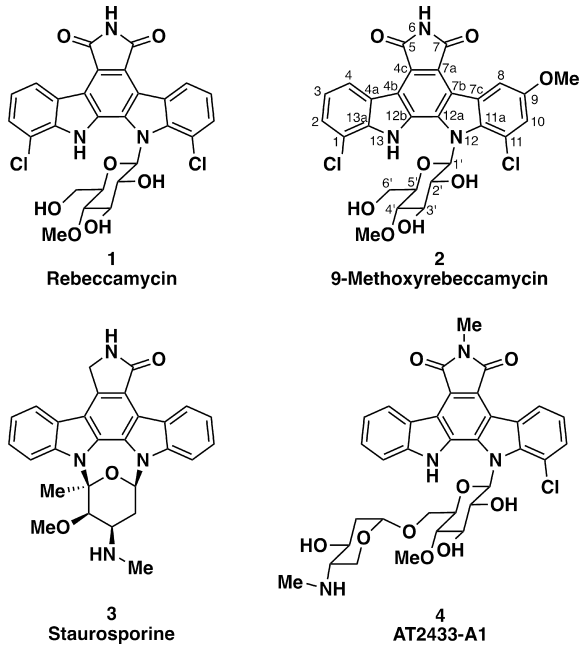


Figure 2. Genetic basis of BCI2 niche defense. (A) BCI2-specific plasmid encoding the BGC for 9-methoxyrebeccamycin. (B) The 9-methoxyrebeccamycin BGC and two related clusters. The conserved biosynthetic genes are named and color-coded.

indicated that the additional methoxy group in **2** is at position C9 on the glycosylated indole moiety. This placement is corroborated by a characteristic J_{meta} of 1.9 Hz between aromatic protons at C8 and C10. The absolute stereochemistry of the sugar was not assigned in this study and is shown as that of rebeccamycin.

Rebeccamycin (**1**) was first isolated from the Panamanian soil Actinobacterium *Lechevalieria aerocolonigenes* (formerly *Saccharothrix aerocolonigenes*) in a screen for antiproliferative agents.[5,6] It belongs to the indolocarbazole structural class of tryptophan dimer natural products that are well-known in drug discovery efforts.[7] The founding member of the indolocarbazoles, first described in 1977, is the kinase inhibitor staurosporine (**3**).[8] Many indolocarbazole natural products are now known, though the maleimide moiety is limited to rebeccamycin, its close analog AT2433-A1 (**4**),[9] and a handful of other structures. Recent bioinformatic analysis of soil metagenomes from the desert southwest of the United States indicated the existence of other rebeccamycin analogs, though the gene products of these biosynthetic gene clusters (BGCs) have not been reported.[10] To our knowledge, 9-methoxyrebeccamycin is the only naturally occurring molecule sharing rebeccamycin's dichlorinated indolocarbazole core. Rebeccamycin's antiproliferative activity sparked both a medicinal chemistry effort to advance analogs as chemotherapeutics and biosynthetic engineering efforts to generate novel indolocarbazoles.[11,12] Several rebeccamycin analogs have now been in clinical trials for a variety of cancers.[11,13] Rebeccamycin's antitumor activity has been ascribed to mammalian topoisomerase I inhibition.[14]



While its antitumor activity has attracted the most attention, rebeccamycin is also a potent antibacterial agent, as is AT2433-A1 (**4**).[6,9] We quantified the antibacterial activity of 9-methoxyrebeccamycin in an agar-based assay. It has potent, nanomolar activity against a small panel of *Pseudonocardia* (590 nM against BCI1 and 150 nM against PLR1); roughly 2-fold more potent than rebeccamycin (Table S3). Not surprisingly, BCI2 is strongly resistant to both 9-methoxyrebeccamycin and rebeccamycin (>300 μM).

PacBio sequencing of BCI1 and BCI2 revealed that each had a circular chromosome and a megaplasmid that were essentially identical in gene content (Figure S9). A comparison using *in silico* DNA/DNA hybridization calculations yielded a value of ~98.3% across the entire chromosomes and ~96.3% between conserved megaplasmids (pBCI1−1 and pBCI2−1; Table S6). In addition to these two conserved replicons, each organism carried a third strain-specific replicon: BCI1 has an unstable linear plasmid (pBCI1−2, 207 kb), while BCI2 harbors a stable circular plasmid (pBCI2−2, 119 kb) with a rebeccamycin-like BGC (Figure 2). Niche defense is thus a plasmid-specific trait.

The plasmid-borne 9-methoxyrebeccamycin BGC contains the full suite of genes required for rebeccamycin biosynthesis previously annotated for the rebeccamycin producing strain *Lechevalieria aerocolonigenes*. The 9-methoxyrebeccamycin cluster additionally encodes enzymes that could install the 9-methoxy group specific to this molecule. These enzymes include a flavin-dependent monooxygenase and a SAM-dependent *O*-methyltransferase that likely perform the tailoring chemistry (Figure 2B, green and blue shaded arrows). We also compared the 9-methoxyrebeccamycin BGC to other reported rebeccamyin-like gene clusters from eDNA.[10] AB857, derived from a California desert soil sample, was the most similar (Figure 2B). This BGC has been predicted to encode a rebeccamycin analog, though the product of this cluster has not been identified.[10] All rebeccamycin biosynthesis genes are conserved, but interestingly the AB857 BGC also contains the tailoring enzymes that presumably install the 9-methoxy group, which are located on a shared subcluster. Notably, 4/7 rebeccamycin-like gene clusters—the two presented above, AB1533, and NM747[10]—encode for the tailoring enzymes for the 9-methoxy group, suggesting that it is a relatively common natural modification to the rebeccamycin scaffold, discovered 30 years ago. Identification of these putative tailoring enzymes could be useful for biosynthetic engineering of rebeccamycin analogs. Knockout of the methyltransferase, for example, would yield a phenolic handle for further elaboration into analogs.

BCI2's high tolerance for 9-methoxyrebeccamycin implies a resistance mechanism on the pBCI2-2 plasmid. The integral membrane transporter *RebT*, a putative efflux pump in the major facilitator family, has been shown to confer rebeccamycin resistance when heterologously expressed in an otherwise

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sensitive Actinomycete, *Streptomyces albus*.[15] A distinct member of this transporter family is encoded in both the BCI2 9-methoxyrebeccamycin and eDNA AB857 clusters (Figure 2B, brown shaded arrows) and is the most likely candidate for self-resistance. An uncharacterized putative transporter gene, *rebU*, is also present in all three clusters and could also contribute to resistance.

A few plasmid-encoded antibiotic BGCs have been reported, although the rapid pace of complete bacterial genome sequencing is likely to reveal many more.[16,17] Indeed, we have recently discovered other examples of plasmid-derived BGCs from ant-associated *Pseudonocardia* isolates.[18] Plasmid-encoded niche defense was first reported long ago in *E. coli* and other Gram-negative bacteria for the colicins and microcins—ribosomally synthesized peptide antimicrobials—which are thought to provide narrow spectrum defense against closely related bacteria.[19−21]

This analysis of niche defense in an increasingly well-studied multilateral symbiosis demonstrates a clear population-level diversity between the two seemingly identical strains we examined. This diversity argues that the fungus-growing ant system will be an even more fruitful source of molecular diversity than was originally thought.[22] And the production of a new version of a therapeutically promising molecule that had been identified genetically is an additional argument for further exploration of this system.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b09794.

Experimental details, supplemental figures, data tables, and spectra (PDF)

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Notes

The authors declare no competing financial interest.

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