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MICROBIOLOGY OF AQUATIC SYSTEMS

Antibiotic Activity Altered by Competitive Interactions Between Two Coral Reef–Associated Bacteria

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Abstract
Microbes produce natural products that mediate interactions with each other and with their environments, representing a potential source of antibiotics for human use. The biosynthesis of some antibiotics whose constitutive production otherwise remains low has been shown to be induced by competing microbes. Competition among macroorganism hosts may further influence the metabolic outputs of members of their microbiomes, especially near host surfaces where hosts and microbial symbionts come into close contact. At multiple field sites in Fiji, we collected matched samples of corals and algae that were freestanding or in physical contact with each other, cultivated bacteria from their surfaces, and explored growth-inhibitory activities of these bacteria against marine and human pathogens. In the course of the investigation, an interaction was discovered between two coral-associated actinomycetes in which an *Agrococcus* sp. interfered with the antibiotic output of a *Streptomyces* sp. Several diketopiperazines identified from the antibiotic-producing bacterium could not, on their own, account for the antibiotic activity indicating that other, as yet unidentified molecule(s) or molecular blends, possibly including diketopiperazines, are likely involved. This observation highlights the complex molecular dynamics at play among microbiome constituents. The mechanisms through which microbial interactions impact the biological activities of specialized metabolites deserve further attention considering the ecological and commercial importance of bacterial natural products.

Keywords Marine natural product · Antibiotic · Microbial ecology

Introduction

Interference competition among microbes may be observed through contact-dependent killing, the secretion of antimicrobial peptides, and the production of antibiotic small molecules by competing strains [1]. Ecological and environmental cues that mediate these interactions are context-specific and, in many cases, poorly understood. In natural settings, "antibiotics" may be deployed at subinhibitory concentrations that promote non-lethal transcriptional changes in neighboring microbes, altering their cellular functions [2]. The recognition that such small molecules are as likely to be involved in signaling and metabolic modulation as they are in microbial warfare, and that a molecule's effect may vary with its concentration or its combination with other molecules, has led some to advocate for calling these chemicals 'specialized metabolites' [3].

Genome mining efforts have revealed that microbial biosynthetic potential has been underestimated and that many gene clusters remain "cryptic" having yet to be linked to their small molecule products [4]. Functional annotation of bacterial genomes has allowed researchers to predict the chemical structures encoded by silent clusters and, in some cases, to determine conditions under which their biosynthesis is activated [5, 6]. A microbe harboring silent gene clusters is expected to minimize metabolic and other costs by upregulating biosynthesis when advantageous to the producer [7, 8]. Interspecific competition is one scenario in which

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specialized metabolites have been shown to be induced in response to co-culture [9].

It is known then that competitive interactions can lead microbes to induce or ramp up the production of bioactive chemicals. How these chemical interactions shape ecological outcomes among — and are themselves shaped by — the macroorganisms with which they associate is less obvious. Unraveling these relationships can prove complex as macroorganisms are ecologically multifaceted in their own right, engaged in cooperation and competition, acquiring resources, and being consumed and parasitized. The result is a set of reciprocal relationships in which metabolic outcomes and the fitness of hosts and symbionts are influenced by their associations with each other and with the environment in which they coexist and evolve.

One environment in which these complex dynamics among macro and microorganisms may be observed is on tropical marine reefs. Historically, hard corals which harbor diverse, metabolically active microbial communities have dominated many such ecosystems. Increased fishing pressure has greatly reduced the abundance of herbivorous fishes and, along with pollution and other aspects of anthropogenic global change, has led to macroalgal dominance on degraded reefs [10, 11]. Increased macroalgal cover has negatively affected hard corals, which experience bleaching due to exposure to allelopathic chemicals where they come into contact with algae [12–15]. In addition, decreased herbivore presence and increased algal dominance, often combined with eutrophication, can lead to increased levels of dissolved organic carbon and a trophic shift towards increased microbial biomass and energy use [16]. This process, known as "microbialization," can further harm corals by exacerbating eutrophication and causing disease outbreaks [17].

Coral microbiomes, including their composition, stability, influence on and regulation by hosts, and ability to protect the holobiont from pathogens, have been extensively investigated [18–24]. Epiphytic algal microbiomes also contribute to algal fitness through the fixation of nitrogen or production of vitamins [25]. Conversely, some algae produce chemical defenses that alter or inhibit bacterial colonization [26, 27]. Thus, at coral-algal interfaces, it is conceivable that the presence of algal allelochemicals and the potential for a resultant coral immune response, combined with the interactions among members of these hosts' microbiomes, may create a unique environment that gives rise to production of novel chemicals [28–33].

To explore coral-algal competition as an ecological framework for the discovery of bioactive microbial metabolites, we evaluated the antibiotic capacity of bacteria cultured from macroorganism surfaces at sites of coral-algal physical contact as well as areas of no-contact. We hypothesized that, through complex ecological interactions among members of coral and algal microbiomes, the margins of coral-algal competition may serve as potential hotspots of antibiotic production, and that a growing understanding microbial ecology may be leveraged for natural products drug discovery. To assess microbial antibiotic potential and to investigate competitive, antagonistic, or other interactions and enable us to access antibiotics to attempt structural characterization, it was necessary to work with metabolically active bacteria. This led us to take a culture-based approach. Experiments integrating omics approaches are of future interest.

Methods

Collection and Preservation of Marine Bacteria

Bacteria were collected in the Yasawa Islands, Fiji, in the vicinity of Nacula Island, from the surfaces of algae and corals growing either alone or in contact with one another (Fig. S1). Sampling occurred in June of 2017. Reefs from which specimens were collected appeared healthy; no coral bleaching was observed, and some algal cover, typical of Fijian reefs, was noted. Small (1–5 g) specimens of algae and corals were collected via SCUBA or snorkel using sterile plastic whirlpacks in order to avoid directly touching the specimens, as well as to store them for later transport to an onshore laboratory for microbial isolation. Four categories of specimen were collected: (1) corals growing in the absence of algae or cyanobacteria, (2) corals that were in direct physical contact with the algae or cyanobacteria listed in "4," (3) algae or cyanobacteria not in contact with live coral, and (4) algae or cyanobacteria that were in direct physical contact with the live coral listed in "2." Microbes were then isolated from macroorganism specimens by agitating them in pure filtered seawater to dislodge the bacterial community. Serial dilutions of that wash were inoculated onto marine agar plates using a sterile cell spreader. Once growth was observed, 3 isolates were chosen from each plate for further subculture. The choice to prioritize the selection of 3 isolates per plate was a function of the limited supplies available in the field. See supplementary methods.

Preliminary Antibiotic Screening

Isolated bacterial strains were screened for antibiotic activity against methicillin-resistant *Staphylococcus aureus* (MRSA; ATCC 33,591), multi-drug resistant *Escherichia coli* (MDREC; ATCC BAA-1743), and the marine pathogen *Vibrio corallilyticus* CN2 using a novel growth inhibition assay. Briefly, the unique bacterial isolates from corals and algae were inoculated onto, and grown on a thin sheet of marine agar using a 96-pin replicator. Once isolate growth had occurred, the entire agar sheet was overlaid onto a layer

of soft agar impregnated with MRSA (in LB agar), MDREC (in LB agar), or *Vibrio coralliilyticus* CN2 (in marine agar). This setup allowed the marine bacteria and the pathogens each to be cultured using their preferred medium and also prioritized the detection of diffusible chemicals. Antibiotic activity was observed as zones of inhibition in the growth of the pathogen lawn. See supplementary methods.

Secondary Antibiotic Screening

Several isolates of interest observed in the primary screen to inhibit the growth of MRSA or *Vibrio corallilyticus*, including *Streptomyces* sp. CC-108–1 and *Agrococcus* sp. CA-87–1 which interacted with each other in addition to MRSA, were further tested. Each isolate active in the initial screen was cultured, by itself this time, on top of two, stacked sheets of marine agar. The sheets were then separated, and each (the top layer contained the colony and the bottom layer contained only metabolites that had diffused from the colony during its solo growth) was overlaid onto a layer of soft agar containing the indicator pathogen. Antibiotic activity was observed as a zone of inhibition in the growth of the pathogen lawn. See supplementary methods.

Colony PCR and Sanger Sequencing

Several isolates with antibiotic potential were subjected to 16S colony PCR, amplified using primers 27F (5′-AGA GTTTGATCCTGGCTCAG-3) and 1492R (5′-GGTTAC CTTGTTACGACTT-3′). Subsequent Sanger sequencing determined their identity to the genus level, and ribosomal RNA sequences were submitted to GenBank (OK184772—OK184808). See supplementary methods for more information regarding PCR reaction composition and cycling conditions.

MALDI-TOF Imaging Mass Spectrometry

Agar-based imaging mass spectrometry [34] was performed on an interaction discovered during preliminary screening between *Streptomyces* sp. CC-108–1, *Agrococcus* sp. CA-87–1, and MRSA, in an attempt to visualize chemical signals that may correlate with antibiotic distribution. See supplementary methods for further information regarding sample preparation and data collection.

Purification of Antibiotics from Streptomyces sp. CC-108–1

Natural products of *Streptomyces* sp. CC-108–1 were purified from 8 L of liquid culture by bioassay-guided fractionation. This was accomplished via chloroform liquid–liquid extraction and normal phase HPLC purification. HPLC fractions were assessed for bioactivity via disc diffusion assay; paper discs were soaked with HPLC fractions, allowed to dry, and overlaid onto a lawn of MRSA. Pathogen growth inhibition was then monitored. See supplementary methods.

Natural Product Characterization by Nuclear Magnetic resonance spectroscopy and high-resolution mass spectrometry

NMR spectra (^{1}H, ^{13}C, DEPT, COSY, HSQC, HMBC) were acquired for pure compounds using an 800 MHz Bruker Avance IIIHD spectrometer with a 3-mm cryoprobe. Spectra were recorded in deuterated chloroform ($CDCl_3$) and processed using MestReNova v. 14.0.0. High-resolution mass spectra were acquired using a Thermo Scientific LTQ Orbitrap XL ETD hybrid linear ion trap/orbitrap tandem mass spectrometer. Sample introduction was performed via direct injection in methanol.

Results

From 143 Fijian coral and algal specimens, a total of 313 bacterial isolates were preserved (Table S1). Antibiotic screening of these environmental bacterial isolates led to the discovery of 54 bacteria exhibiting antibiotic activity towards MRSA, the coral pathogen *Vibrio corallilyticus*, or both (Fig. 1; Table S2). Only three isolates, CA-173–1 (isolated from *Acropora* sp.), CC-48–3 (isolated from an *Acropora* sp. in contact with *Amphiroa* sp.), and AC-192–2 (*Chlorodesmis* sp. in contact with an *Acropora* sp.), all later identified as *Bacillus subtilis*, inhibited growth of MDREC under the chosen laboratory growth conditions, and this indicator was therefore removed from further consideration. Antibiotic activity did not appear to correlate with the source of the strains; bioactive isolates were cultivated from both algae and corals. 16S rDNA sequencing revealed that the inhibitory bacteria included members of the genera *Bacillus*, *Pseudoalteromonas*, *Microbulbifer*, *Streptomyces*, and *Shewanella*, all of which have been noted previously for antibiotic production [23, 35, 36]. Forty-one of 54 bacterial isolates retained their ability to inhibit the growth of MRSA and/or *V. corallilyticus* in a secondary screen (Fig. 2; Table S2). A greater number of the antibiotic-producing bacteria inhibited the growth of Gram-positive MRSA than Gram-negative *V. corallilyticus*, although 15 strains were active against both. These included bacteria belonging to the genera *Bacillus* (11), *Microbulbifer* (1), *Streptomyces* (2), and *Pseudoalteromonas* (1). The secondary screen also sought to identify instances in which antibiotic upregulation may occur, by comparing the antibiotic response when the environmental bacterium and pathogen were co-cultured

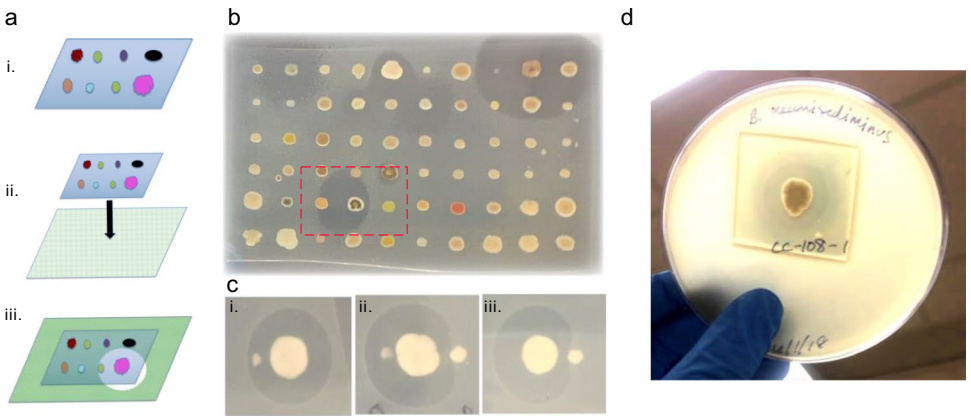

Fig. 1 Antibiotic production by a library of environmental bacterial isolates against the human pathogen methicillin-resistant *Staphylococcus aureus* (MRSA) was assessed via an agar overlay assay (**a**). The environmental isolate array (**a**, i) was overlaid onto a lawn of soft agar containing MRSA (**a**, ii) and antibiotic production by environmental bacteria was detected as a zone of growth inhibition in the MRSA lawn (**a**, iii). An interaction in which one environmental isolate affected the ability of another to inhibit the growth of MRSA was detected in this way (**b**, dashed box). Targeted interaction assays with the environmental isolates nearest the antibiotic-producing isolate established that the isolate (*Paracoccus* sp., strain CA-113–2) to the left of the antibiotic producer (*Streptomyces* sp., strain CC-108–1) was not stimulating antibiotic production by the antibiotic-producing isolate (**c**, i), but rather that the yellow isolate to the right of the antibiotic-producer (*Agrococcus* sp., strain CA-87–1) was responsible for impairing its ability to inhibit MRSA growth (**c**, iii). The antibiotic-producing isolate also affected the growth of a lawn of the marine bacterium *Bacillus oceanisediminus* (**d**)

and when the pathogen was exposed to the diffused metabolites of the environmental bacterium in the absence of the colony itself. This assay was not without important caveats which are discussed in the subsequent section. Generally, molecules diffused from *Streptomyces* spp. and *Bacillus* spp. colonies were sufficient to inhibit pathogen growth, while for other taxa, live bacteria had to be present for pathogen growth to be inhibited.

The novel format of the preliminary antibiotic assay in which 96 bacteria were simultaneously evaluated for antibiotic production led to the discovery of an unexpected interaction between two bacteria isolated from acroporan corals (Fig. 1b). The presence of a reproducible, skewed zone of MRSA inhibition caused by one of the isolates suggested that an adjacent bacterium was altering the effectiveness of an antibiotic produced by the first. It was unclear whether antibiotic production was induced by the isolate to its left, towards which the zone of inhibition was skewed, or if the antibiotic was interfered with by the isolate to its right. To resolve these possibilities, pairwise interactions between the antibiotic producer and each adjacent isolate were performed (Fig. 1c). The skewed zone of MRSA inhibition was reproduced when the antibiotic producer was paired with the isolate to its right. Furthermore, the zone of inhibition was oriented away from the adjacent isolate indicating that either the production or effectiveness of the antibiotic was impaired by its neighbor. 16S rDNA sequencing revealed the antibiotic producer to be a member of Actinobacterial genus *Streptomyces* (strain CC-108–1) (Table S2). The isolate responsible for skewing the zone of inhibition was also an Actinobacteria of the genus *Agrococcus* (strain CA-87–1), and this effect was not observed with any other strains grown adjacent to *Streptomyces* CC-108–1. In addition, growth impairment of the marine Gram-positive bacterium *Bacillus oceanisediminus* CNY-977 by CC-108–1 suggested that the antibiotic in question is broad-spectrum or at least effective against multiple Gram-positive bacteria (Fig. 1d).

MALDI-TOF imaging mass spectrometry (MALDI-IMS) permitted partial visualization of the chemical basis for the tripartite interaction in which an *Agrococcus* sp. CA-87–1 interfered with the antibiotic activity of *Streptomyces* sp. CC-108–1 against MRSA (Fig. 3). Several mass-to-charge ratio signals representing probable molecular ions of diffusible chemicals secreted by the streptomycete were observed, but none exhibited the skewed pattern observed in the MRSA lawn complicating assignment of any of the mass spectral signals to the antibiotic, suggesting that the antibiotic affected by its neighbor is not one of the mass spectral signals shown.

High-performance liquid chromatography (HPLC) fractionation of a crude extract of a liquid culture of *Streptomyces* sp. CC-108–1 yielded multiple fractions with antibiotic activity against MRSA, which was chosen as the indicator bacterium for purification efforts as it was easier to manipulate than *V. corallilyticus*. Structural elucidation of compounds for which there was sufficient material for purification was achieved by nuclear magnetic resonance (NMR) spectroscopy and high-resolution mass spectrometry (MS), revealing that major metabolites were of the diketopiperazine (DKP) family of natural products (Fig. 4). Comparison

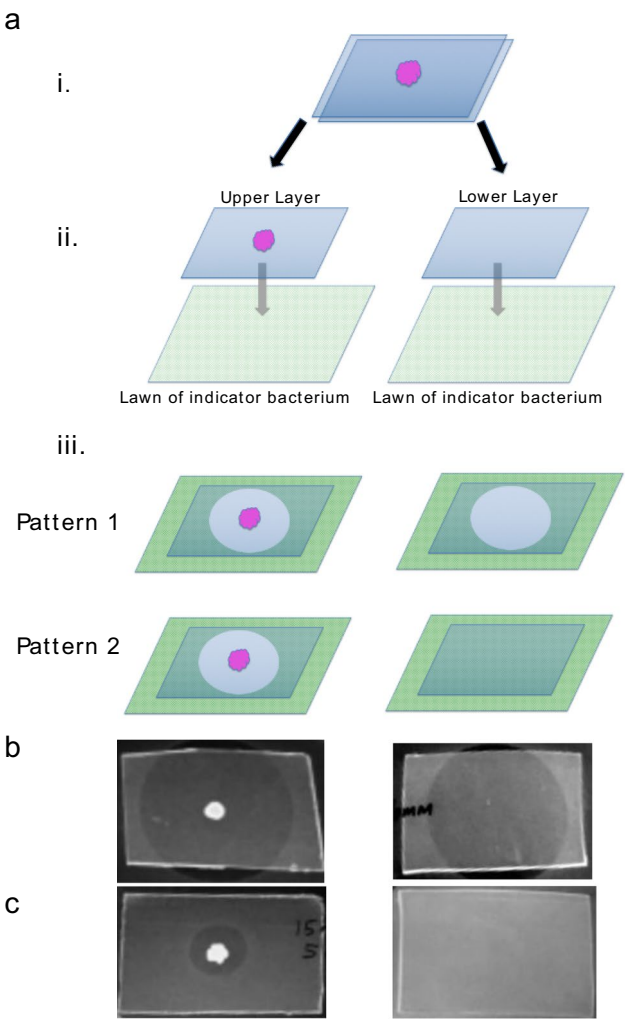

Fig. 2 To look for signs that antibiotic activity from environmental bacteria may be induced or upregulated through interaction with the indicator pathogen, a double-layer agar assay was performed. The environmental bacterium (pink) was grown on a double layer of agar (**a**, i). Layers were separated and placed onto a lawn of the indicator organism (**a**, ii). Two distinct patterns of antibiotic distribution were observed (**a**, iii): presence of a zone of pathogen inhibition on both the colony-free bottom agar layer and the colony-containing top agar layer (**b**, *Streptomyces* sp. CA-187–2) versus the presence of a zone of inhibition only on the top colony-containing agar layer (**c**, *Streptomyces* sp. CC-108–1)

of spectroscopic data to data previously reported for diketopiperazines in the literature, including HRMS and comparison of NMR ^{1}H and ^{13}C chemical shifts, supported structural assignments of *cyclo*(Leu-Pro), *cyclo*(Phe-Pro), and *cyclo*(Gly-Pro) [37–40]. Additional NMR data, including H–H couplings via COSY and C-H couplings via HSQC and HMBC, were used to further confirm the structural assignment. Evidence for additional DKPs was present, but structures were not fully elucidated due to low natural abundances and low isolated yields. Absolute configurations of the identified diketopiperazines were not confirmed, but literature reports indicate that *cyclo*(Leu-Pro) most commonly incorporates amino acids of either the *D* or *L* configuration, whereas for *cyclo*(Phe-Pro), a mixture of *D*- and *L*-amino acids is more typical; *cyclo*(Gly-Pro) most often occurs with *L*-Pro [41].

DKPs, including those identified here, have been previously described to display antibiotic activity (de Carvalho and Abraham, 2012). However, commercially obtained *cyclo*(Gly-*L*-Pro) [cyclo(Gly-*S*-Pro); Santa Cruz Biotechnology, Dallas, TX USA] and *cyclo*(*L*-Leu-*L*-Pro) [cyclo(*S*-Leu-*S*-Pro); Chem Impex, Wood Dale, IL, USA] (250 µg) each failed to inhibit MRSA growth in our hands (data not shown).

Discussion

Around 17% of 313 bacterial strains isolated from Fijian corals and seaweeds were observed to inhibit the growth of the pathogens MRSA, MDREC, and/or *Vibrio coralliilyticus* indicating that this bacterial library is a resource for natural product discovery. Our finding that 17% of 313 cultivated strains exhibited antibiotic activity is somewhat lower than previous studies: one investigation of cultured bacteria from a soft coral reported 48% of isolates with antimicrobial activity [23]. A separate investigation of scleractinian corals found that 25–70% of culturable, mucus-associated bacterial isolates displayed antimicrobial bioactivity [24]. Other studies sampled coral extracts, including coral proteins and enzymes, rather than strains cultivated from corals [18]. Our reported rate of antimicrobial activity reflects the corals and algae from which the bacteria were derived, as well as the culture conditions and the indicator pathogens we selected.

Despite increased opportunity for bacterial competition in the unique environment at sites where corals and macroalgae come into direct contact, no obvious pattern in prevalence of antibiotic-rich bacteria or in their antibiotic potency was observed to be associated with bacterial strains cultivated from the margins of coral-algal competition. This observation does not support our original hypothesis that ecological interactions between the microbiomes of seaweeds and corals in direct physical contact are potential hotspots of antibiotic production. Rather, many of the same bacterial taxa that inhibited pathogen growth were cultured from both coral and algal surfaces, regardless of whether they were alone or in contact. As only a small fraction of marine bacteria are readily culturable, it may be that our methodology failed to capture antibiotic mediated interactions between coral and algal microbiomes. It is also possible that lab cultivation of these strains failed to elicit responses that arise in the field, where simultaneous competition among many organisms and other environmental triggers, including contributions

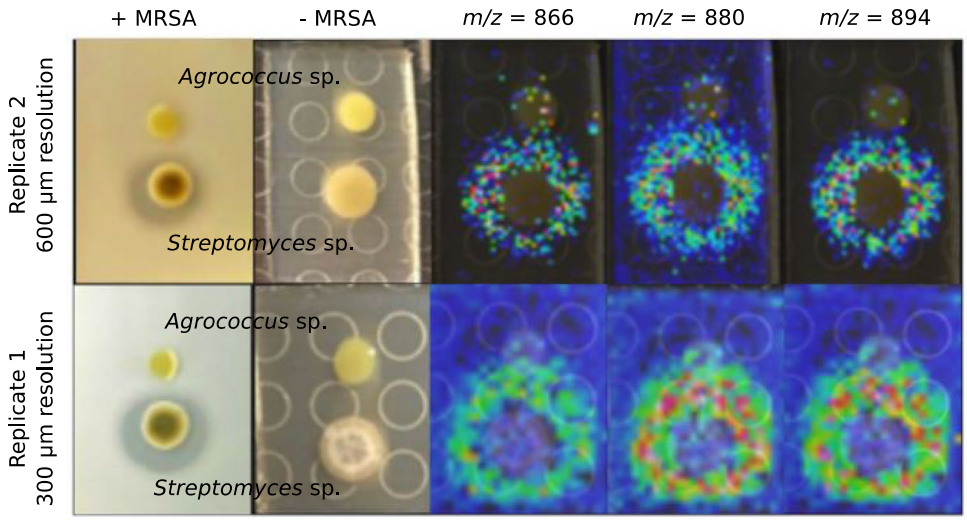

Fig. 3 Imaging mass spectrometry detection of diffusible molecules produced by *Streptomyces* sp. CC-108–1. Diffusible compounds were detected, but did not exhibit the skewed morphology that was observed in the MRSA lawn at the interface between *Streptomyces* sp. CC-108–1 and *Agrococcus* sp. CA-87–1

from their microorganism hosts, could stimulate antibiotic production. The pathogens against which we evaluated the antibiotic activity of marine bacteria (MRSA, MDREC, and *V. corallilyticus*) may also have biased outcomes; marine bacteria may produce more species-specific (narrow spectrum) antibiotics when confronted with other ecologically relevant competitors. While we did not observe increased antibiotic activity by microbes isolated from coral-algae interactions, our results do agree with other reports that indicate that coral and algal microbiomes produce antimicrobial compounds that may serve a defensive function for the holobiont [18, 21, 24].

It is possible that among the sampled bacteria, there is at least some regulation of antibiotic production in response to competition, based on the results of the secondary screen. For many bacterial taxa including *Microbulbifer* sp. *Pseudoalteromona*s sp., and *Shewanella* sp., pathogen inhibition (MRSA or *V. corallilyticus*) was only observed when the marine bacterium was physically present; the diffused metabolites of the marine bacterium in the absence of the actual colony were insufficient to inhibit growth of the pathogen. This presents several possibilities: (1) antibiotics/enzymes diffused too poorly or slowly for their effects to be captured by the assay setup, (2) inhibitory antibiotics/enzymes are produced only during later phases of growth of the marine bacteria, (3) inhibition of the pathogen in the presence of the marine bacterium colony is due to nutrient depletion or factors other than antibiotic production [42], or (4) the marine bacterium detected the pathogen and up-regulated its antibiotic/enzyme production, but did not produce antibiotics when grown alone. These possibilities must all be addressed before concluding that induction of antibiotic production in environmental bacteria occurred in response to the presence of the pathogen. For several *Streptomyces* and *Bacillus* species, the antibiotic producer did not need to be physically present; unknown metabolites and/or enzymes secreted into the agar were sufficient to inhibit pathogen growth.

We observed an unexpected instance in which a coral-associated actinobacterium (*Agroccocus* sp. CA-87–1) impaired the ability of a second coral-associated actinobacterium (*Streptomyces* sp. CC-108–1) to produce antibiotic activity. As both of these bacteria were isolated from the same coral genus, *Acropora*, our observation of one coral-associated bacterium diminishing the ability of another coral-associated bacterium to impair pathogen growth is an interesting case that may have implications for our understanding of the protective ability of coral microbiomes. While many studies have investigated the ability of coral-associated microbes to protect corals against diseases caused by marine pathogens [43], microbes are typically tested individually against pathogens of interest [23, 43], or the antibiotic potential of coral or its mucus extract is measured [18, 24]. Both of these methods overlook possible inhibitory interactions and chemical transformations of antibiotics that could occur among members of a microbial community, as well as the interplay that occurs between a microbiome and the rest of the holobiont. Our 96 bacterial isolate overlay assay (designed to evaluate the contribution of diffusible compounds produced by marine bacteria while excluding the contributions of contact-mediated competitive mechanisms such as Type VI secretion systems, outer membrane exchange, and contact-dependent inhibition), fortuitously captured a chemically mediated interaction between two bacteria [44]. Many more such interactions may occur, a situation made even more complex by the ecological and spatial dynamics of the community as a whole, beyond pairwise interactions. This suggests that even for microbiomes composed of similar species, these communities could differ in their chemical profiles, as the molecules that are present can interact with and modify each other, beyond the

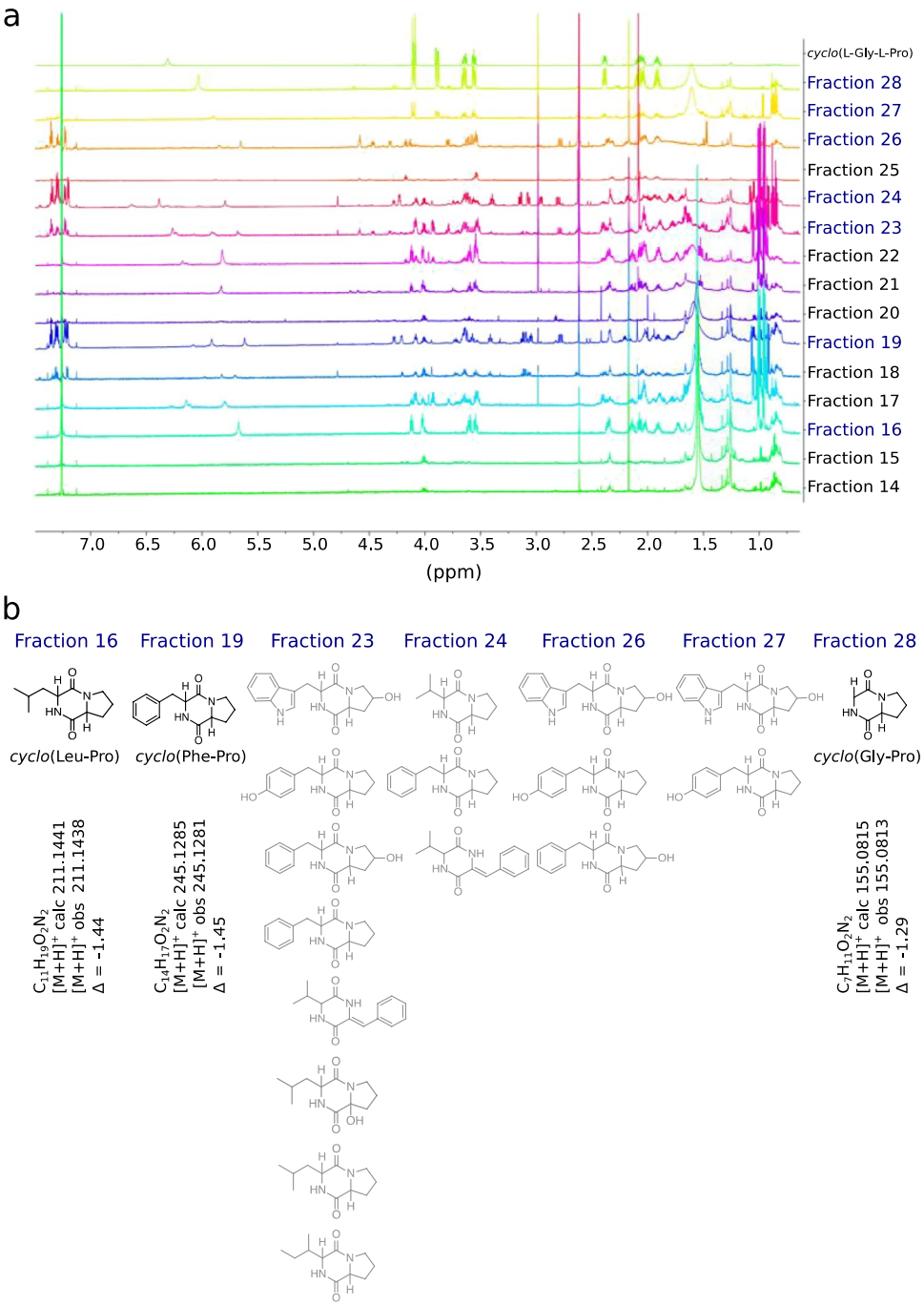

Fig. 4 ^{1}H NMR spectra of HPLC fractions generated from the bioactive extract of the antibiotic-producing strain *Streptomyces* sp. CC-108–1 (**a**). Fractions denoted by blue text were also analyzed by high-resolution mass spectrometry (HRMS). Molecular structures confirmed by both NMR spectroscopy and HRMS are shown in black (**b**). Molecular structures shown in gray are proposed structures based on HRMS and ^{1}H NMR spectroscopy but were not fully confirmed due to low yields. *Cyclo*(Leu-Pro) and *cyclo*(Phe-Pro) have been reported to have antibiotic activity. *Cyclo*(Gly-Pro) has been reported to have neuroactive properties

more commonly considered circumstance in which bacteria induce antibiotic production in each other.

Several competing explanations for antibiotic inhibition observed between the two coral-associated Actinobacteria were considered. Antibiotic biosynthesis by the streptomycete CC-108–1 may have been directly impeded by *Agrococcus* sp. CA-87–1. Alternatively, *Agrococcus* sp. CA-87–1 may have degraded the antibiotic(s) produced by its competitor. Chemically mediated antibiotic degradation resulting from bacterial competition has been previously documented as has the enzymatic degradation of DKPs, specifically [45, 46]. Yet another possibility is that CA-87–1 dominates exploitation of limiting resources, depleting nutrients required by *Streptomyces* sp. CC-108–1 to efficiently produce antibiotic(s). Finally, CA-87–1 may somehow shield the indicator pathogen MRSA from negative effects of the CC-108–1 antibiotic, potentially through impact on pH or nutrition to the benefit of MRSA.

Elucidation of the mechanism underlying the observed interaction between *Streptomyces* sp. CC-108–1 and

Agrococcus sp. CA-87–1 would be facilitated by identification of the antibiotic(s) involved. Efforts to isolate the antibiotic(s) led to purification and characterization of three diketopiperazines (DKPs) from antibiotic fractions of CC-108–1 extracts which reproducibly inhibited pathogen growth. Several additional DKPs were observed via NMR and high-resolution MS although full structural characterization was not successful due to low yields of each DKP, even from scaled up cultivation. DKPs are common bacterial natural products that possess a variety of bioactivities including but not limited to, antibiotic and antifungal activity, neuroprotection, quorum sensing inhibition, chemotherapeutic, and immunosuppressant effects [41, 47]. There are also indications that DKPs may play a role in mediating inter-kingdom ecological interactions; cyclo(D-His-L-Pro) up-regulates *Vibrio fischeri* bioluminescence in vitro and has been detected in the bobtail squid light organ [48]. A multitude of compounds have already been discovered within this small molecule class and investigation of the biosynthetic machinery behind their production in bacteria suggests that much additional structural diversity is likely [49].

The DKPs identified from *Streptomyces* sp. strain CC-108–1, *cyclo*(Gly-Pro), *cyclo*(Leu-Pro), and *cyclo*(Phe-Pro) were previously reported to exhibit broad spectrum antibiotic activity [47]. However, no single DKP identified in the current study accounted for the antibiotic activity of the bioactive chromatographic fraction consisting of a complex mixture of DKPs; commercially obtained DKPs *cyclo*(Gly-*L*(*S*)-Pro), *cyclo*(*L*(*S*)-Leu-*L*(*S*)-Pro) did not inhibit MRSA at concentrations similar to those in CC-108–1 cultures (data not shown). It may be that purchased compounds did not have the same absolute stereochemical configurations as those DKPs isolated from CC-108–1 or that specific mixtures of DKPs at particular concentrations are necessary to achieve antibiotic disruption. Synergy between multiple DKPs in magnifying antibiotic activity has been previously demonstrated [50]. It may also be that a minor non-DKP compound that was not identified was responsible for the antibiotic activity. MS imaging data revealed the secretion of higher mass compounds by CC-108–1, although it is unknown whether these exhibit antibiotic properties, nor did they show the skewed pattern observed in the MRSA inhibition assay making it less likely that these compounds are the antibiotics of interest. Low molecular weight metabolites like DKPs would not have been visible in this imaging experiment due to the selected *m/z* window and matrix interference. Thus, attribution of the mechanism underlying the complex interaction between the two actinomycetes was hindered by lack of knowledge regarding the nature of the antibiotic(s) whose activity was affected by bacterial competition, preventing us from ruling out competing hypotheses of antibiotic degradation, inhibition of biosynthesis, exploitation competition, and pathogen rescue.

While the original hypothesis that coral-algal competition is reflected in antibiotic profiles of coral- and algal-associated bacteria was not supported by the findings of this study, our ecologically focused sampling of four different pairings of coral and macroalgae/cyanobacteria taxa, either "alone" or in physical contact with each other, led to a library of 313 bacteria from coral reef environments to serve as a resource for future discovery and experimentation. Furthermore, while this study was limited to bacteria that were successfully cultured, the hypothesis that competition at organismal interfaces leads to bioactive microbiomes that produce protective chemicals by microbes warrants further investigation. The observation of one coral-associated bacterium interfering with the antibiotic activity of another coral-associated bacterium presents additional ecological dynamics for consideration. We propose that the "sum" of all chemically mediated interactions within a coral microbiome has greater implications for the protective capacity of the holobiont than do the individual biosynthetic potentials of community members.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00248-022-02016-6.

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Author Contribution SJM, PJ, and JK conceived of and designed experiments. SJM, AD, SV, JG, KS, PJ, and JK contributed to fieldwork, microbial isolation efforts, and subsequent experiments. The manuscript was written by SJM and JK with input from all authors.

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Data Availability 16S sequences for the microbes used in this study have been deposited in GenBank (OK184772-OK184808). All other data generated or analyzed during this study are included in this published article (and its supplementary information files) unless otherwise noted. Microbial isolates are stored at the Georgia Institute of Technology and are available upon request.

Code Availability Not applicable.

Declarations

Ethics Approval The research conducted here complied with the Convention on the Trade in Endangered Species of Wild Fauna and Flora (CITES permit #17FJ/NV2000284) and abided by the legal agreement between the Georgia Institute of Technology, Scripps Institution of Oceanography, The University of the South Pacific, and the Fijian Government.

Consent to Participate Not applicable.

Consent for Publication All authors have reviewed the manuscript and consent to its publication.

Conflict of Interest The authors declare no competing interests.

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