

Microbiota of Healthy Corals Are Active against Fungi in a Light-Dependent Manner

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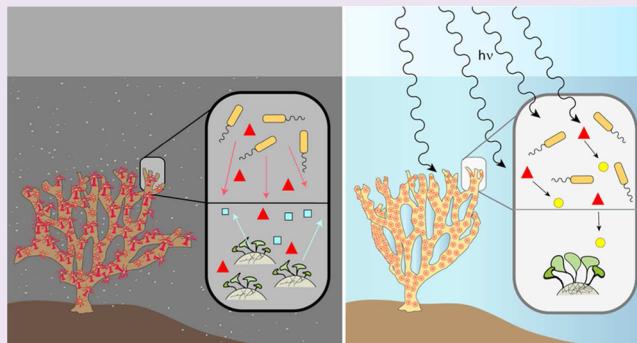
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S Supporting Information

ABSTRACT: Coral reefs are intricate ecosystems that harbor diverse organisms, including 25% of all marine fish. Healthy corals exhibit a complex symbiosis between coral polyps, endosymbiotic alga, and an array of microorganisms, called the coral holobiont. Secretion of specialized metabolites by coral microbiota is thought to contribute to the defense of this sessile organism against harmful biotic and abiotic factors. While few causative agents of coral diseases have been unequivocally identified, fungi have been implicated in the massive destruction of some soft corals worldwide. Because corals are nocturnal feeders, they may be more vulnerable to fungal infection at night, and we hypothesized that the coral microbiota would have the capability to enhance their defenses against fungi in the dark. A *Pseudoalteromonas* sp. isolated from a healthy octocoral displayed light-dependent antifungal properties when grown adjacent to *Penicillium citrinum* (*P. citrinum*) isolated from a diseased Gorgonian octocoral. Microbial MALDI-imaging mass spectrometry (IMS) coupled with molecular network analyses revealed that *Pseudoalteromonas* produced higher levels of antifungal polyketide alteramides in the dark than in the light. The alteramides were inactivated by light through a photoinduced intramolecular cyclization. Further NMR studies led to a revision of the stereochemical structure of the alteramides. Alteramide A exhibited antifungal properties and elicited changes in fungal metabolite distributions of mycotoxin citrinin and citrinadins. These data support the hypothesis that coral microbiota use abiotic factors such as light to regulate the production of metabolites with specialized functions to combat opportunistic pathogens at night.



Pathogenic fungal infections are an emerging threat to the earth's ecosystems and human health and food supply.^{1,2} Fungal diseases are also linked to the recent extinction of numerous animal species. *Batrachochytrium dendrobatidis*, a fungal amphibian pathogen, has been implicated in outbreaks of chytridiomycosis causing extinction of more than 200 amphibian species.^{3,4} *Pseudogymnoascus destructans*, a psychrophilic fungus responsible for the white-nose syndrome in bats, has decimated numerous bat populations in North American caves.^{5,6} Additionally, fungal infections are becoming more prevalent in aquatic environments as illustrated by the disease outbreak of sea fan corals in the Florida Keys (U.S.) attributed to *Aspergillus sydowii*.^{7,8} Contributing to these infections are

increased global travel, new agricultural techniques, climate change, and the unique ability of fungi to reproduce through both sexual and asexual mechanisms and to infect a broad range of hosts.^{1,2}

Combating emerging fungal infections requires improved understanding of the molecular interactions between fungi and the immune system of the infected species as well as the microbiota of the infected species as a function of abiotic

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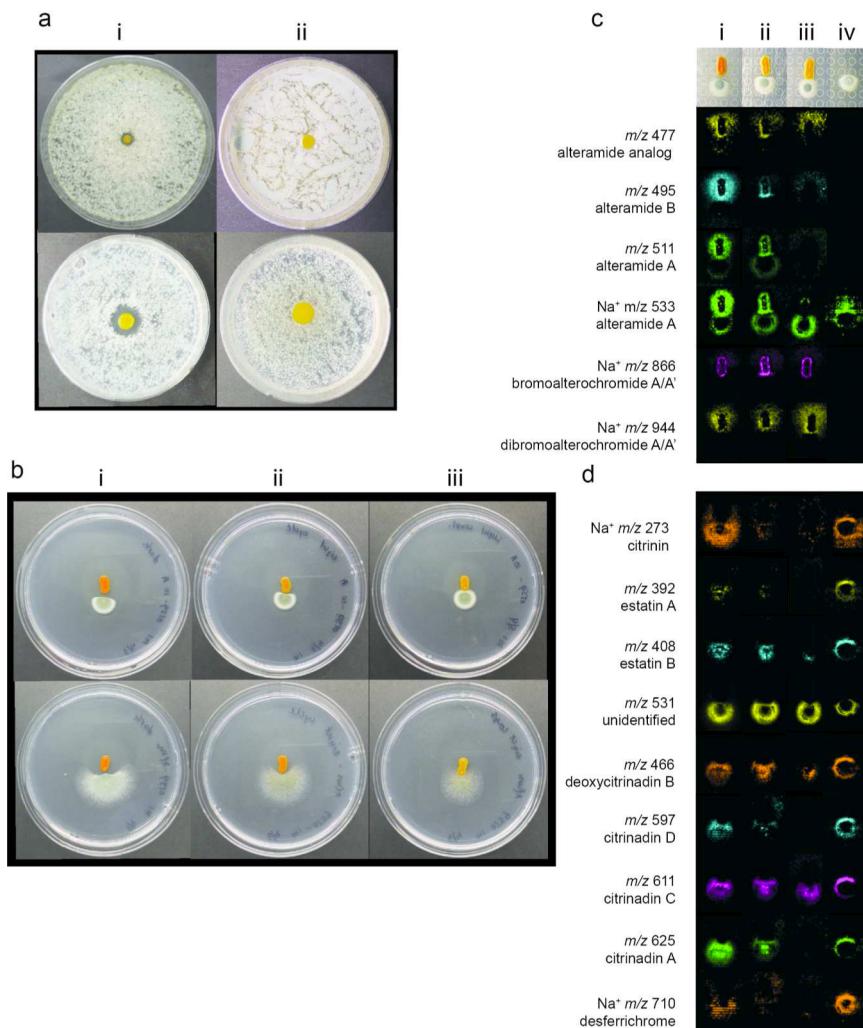


Figure 1. Fungal inhibition by OT59 and MALDI-IMS. (a) Fungal lawn inhibition by OT59 incubated in (i) continuous dark compared to incubation under (ii) continuous light exposure. Top row *P. citrinum* lawn (10 cm o.d. Petri dish), bottom row *A. fumigatus* lawn (5 cm o.d. Petri dish). (b) Side-by-side interaction of OT59 and *P. citrinum* (top row) compared to OT59 and *A. fumigatus* (bottom row) in (i) continuous dark, (ii) 12 h dark-light cycle, and (iii) continuous light exposure. (c) Comparison of selected bacterial and (d) fungal metabolites observed by MALDI-IMS in the interaction between OT59 (top) and *P. citrinum* (bottom) in (i) continuous dark, (ii) 12 h dark-light cycle, and (iii) continuous light. In column (iv) alteramide A (top) is applied 0.5 cm above a *P. citrinum* (bottom) inoculum prior to incubation.

factors such as light. Commensal microbial communities protect the host against invading pathogens,⁹ and disrupting beneficial microbiota may leave the host vulnerable to pathogenic fungal infections. Therefore, probiotic treatment of the host with endogenous bacterial strains that produce antifungal compounds has proven to be a promising strategy.¹⁰

The waters of Panama are home to a large number of coral reefs with a diverse octocoral fauna on both sides of the isthmus¹¹ that harbor unique microbes. Most coral species feed actively at night by expanding their polyps and extending their tentacles to sting their prey with their nematocysts but rely on photosynthetic activity of their endosymbiotic algae during the day when they contract.¹² Because of this behavior, corals are expected to have additional mechanisms to protect themselves from potential pathogens. The holobiont is proposed to play a critical role in the defense against pathogens by secretion of specialized metabolites including antifungal, antibiotic, and signaling molecules.^{13–15} Our collection of coral-associated bacteria obtained from these waters was mined for the production of specialized metabolites to combat infectious

diseases including fungal infections in a light-dependent manner. We hypothesized that a colored *Pseudoalteromonas* sp. isolated from a healthy specimen of *Leptogorgia alba* (Gulf of Panama) might have the ability to absorb light and possibly respond to light-dark cycles by producing specialized metabolites in the dark that confer protection against pathogens. Indeed, we observed that *Pseudoalteromonas* sp. OT59 was able to inhibit the growth of a marine fungus *P. citrinum* isolated from the necrotic tissue of a diseased gorgonian octocoral (*Eunicea* sp.) found in Caribbean Panama, but only when grown in the dark. *Pseudoalteromonas* sp. OT59 also exhibited an antifungal effect on other fungal species from both terrestrial and marine environments. Exposure of *Pseudoalteromonas* sp. OT59 to light completely abolished the antifungal phenotype. Here, we applied two mass spectrometry tools, MALDI-IMS of microbes grown on agar^{16,17} and molecular network analysis of corresponding extracts,¹⁸ to decipher the chemotype responsible for this remarkable light-dependent fungicidal phenotype. The antifungal compounds produced by the coral-associated *Pseudoalteromonas* sp. OT59

are alteramides, which are inactivated upon light exposure and responsible for the dark-light-dependent antifungal phenotype. Detailed NMR analysis revealed a revised stereochemistry for this class of molecules. The mass spectrometry techniques further provided insight on the effects of the antifungal agent alteramide A on the secondary metabolite output of *P. citrinum* and facilitated annotation of novel congeners of molecular families produced by the marine bacterium and fungus. These findings demonstrate how abiotic factors such as light exposure can impact the metabolic output of the commensal coral microbiota and its ability to fight off fungal pathogens.

RESULTS AND DISCUSSION

Light-Dependent Fungal Inhibition by OT59. An associated, pigmented *Pseudoalteromonas* sp. (OT59) was isolated from a specimen of Pacific octocoral *Leptogorgia alba* collected at ca. 12 m deep (Supplementary Figure S1a). The light levels at that depth decrease exponentially to <30% of the incoming irradiation due to absorption and scattering.¹⁹ In the dark, OT59 displayed robust antifungal activity against *P. citrinum* isolated from the necrotic tissue of a diseased Caribbean octocoral (*Eunicea* sp.) found at 6 m deep (Figure 1a, Table 1, and Supplementary Figure S1b) and inhibited

which roughly corresponds to 15% exposure at 8–12 m of ocean depth where the corals were collected based on an yearly average irradiance level of $1050 \mu\text{E m}^{-2} \text{s}^{-1}$ at the equator during a 12 h light period.¹⁹ The dark versus light dependent phenotype was confirmed using side-by-side inoculations of OT59 and *P. citrinum* or *Aspergillus fumigatus* as representative fungal strains (Figure 1b). Constant light exposure abrogated the antifungal phenotype for all fungal species except *Trichoderma virens* (Figure 1a, photographs in Supplementary Figure S3). Incubation at the $149 \mu\text{E m}^{-2} \text{s}^{-1}$ exposure level in a 12 h on-off cycle, representative of a day-night cycle, also showed fungal inhibition although less pronounced than under complete exclusion of light (Figure 1b).

Mass Spectrometric Analysis of Interaction between OT59 and *P. citrinum*. MALDI-IMS was employed to study the molecular differences between the side-by-side interaction of OT59 and *P. citrinum* in the dark, light, and during a 12 h dark-light cycle. This minimally invasive method preserves and visualizes the spatial distributions of multiple metabolites including fungal inhibitors at the site of interaction directly on agar and can aid the dereplication process of the bioactive molecules.^{16,17} A large number of metabolites were produced by both the bacterial (selected examples in Figure 1c, columns i–iii) and fungal species (Selected examples in Figure 1d, columns i–iii). Molecular network analysis of extracts from OT59, *P. citrinum*, and a side-by-side interaction of OT59 and *P. citrinum* was used as a complementary technology to facilitate the identification of the metabolites observed in this image (Figure 2, Supplementary Figure S4). Metabolites with comparable MS/MS fragmentation patterns that form clusters in a molecular network, are often members of the same molecular family, and can expedite annotation of the cluster neighbors including related congeners not described before.^{18,20}

Comparing the MALDI-IMS of the fungal-bacterial interaction under light and dark conditions identified similar metabolites that exhibited significant differences in distribution and intensity, for both the fungal and the bacterial strain. In particular, OT59 produced ions $[\text{M} + \text{H}]^+$ 477, 495, and 511 and corresponding adducts $[\text{M} + \text{Na}]^+$ 499, 517 and 533, which were abundantly secreted in the media when incubated in the dark, but nearly absent under constant light exposure (Figure 1c). This observation was confirmed by HPLC coupled to off-line mass spectrometry analysis (Supplementary Figure S5a). In contrast, a family of brominated metabolites with mass ranges from *m/z* 844 to 958 showed similar intensities in HPLC and a similar distribution in the MALDI-IMS for the different conditions (dark, day-night cycle, light). These *Pseudoalteromonas*-produced metabolites were identified through molecular network analysis as bromoalterochromides exemplified by $[\text{M} + \text{Na}]^+$ 866 (bromoalterochromide A/A') and $[\text{M} + \text{Na}]^+$ 944 (dibromoalterochromide A/A') (Figure 2, Supplementary Figures S4, S5a, and S11–S14).^{20–22}

Isolation and Identification of Antifungal Compounds. We hypothesized that the bacterial metabolites *m/z* 477, 495, and 511, extensively secreted only when OT59 was grown in the dark, might be responsible for the observed inhibitory phenotype. A mass-spectrometry-guided isolation scheme was employed to confirm whether *m/z* 477, 495, and 511 were indeed responsible for the antifungal activity and to elucidate their structures. The growth of OT59 on M1 agar and isolation of metabolites were all performed under exclusion of light to preserve antifungal activity. Purified metabolites were subjected to disk diffusion antifungal assays with *P. citrinum* as

Table 1. Inhibition of a Variety of Fungal Species by OT59 in a Lawn Assay^a in the Dark Compared to Constant Light Exposure at $149 \mu\text{E m}^{-2} \text{s}^{-1}$

	fungus strain ^b	diameter of inhibition zone (mm) dark	diameter of inhibition zone (mm) at $149 \mu\text{E m}^{-2} \text{s}^{-1}$
1	<i>Penicillium citrinum</i>	8	0
2	<i>Aspergillus fumigatus</i> F0075	9	0
3	<i>Aspergillus fumigatus</i> Af293	11	0
4	<i>Aspergillus flavus</i> NRRL 3357	11	0
5	<i>Aspergillus oryzae</i> RIB40	7	0
6	<i>Aspergillus terreus</i> FGSC A1156	9	0
7	<i>Aspergillus nidulans</i> FGSC A4	14	0
8	<i>Aspergillus niger</i> NRRL 3	8	0
9	<i>Aspergillus versicolor</i> F0073	11	0
10	<i>Penicillium chrysogenum</i> ATCC 28089	10	0
11	<i>Trichoderma virens</i> CNL 910	13	11
12	<i>Acremonium</i> species CNC 890	11	0
13	<i>Fusarium</i> species CNL 292	7	0
14	<i>Fusarium</i> species CNT 021F	13	0

^aLawns of test fungi on M1 agar with OT59 inoculated in the center. Data reported are averages of triplicate experiments. ^bOrigin of the fungi: marine (entries 1, 11–14), terrestrial (entries 2–10).

various other fungal species derived from both terrestrial (Table 1, entries 2–10) and marine environments (Table 1, entries 11–14). This inhibitory phenotype was not observed in the light at irradiance levels of 66, 149, and $323 \mu\text{E m}^{-2} \text{s}^{-1}$ (Supplementary Figure S2). For comparative mass spectrometry experiments, a light level of $149 \mu\text{E m}^{-2} \text{s}^{-1}$ was used,

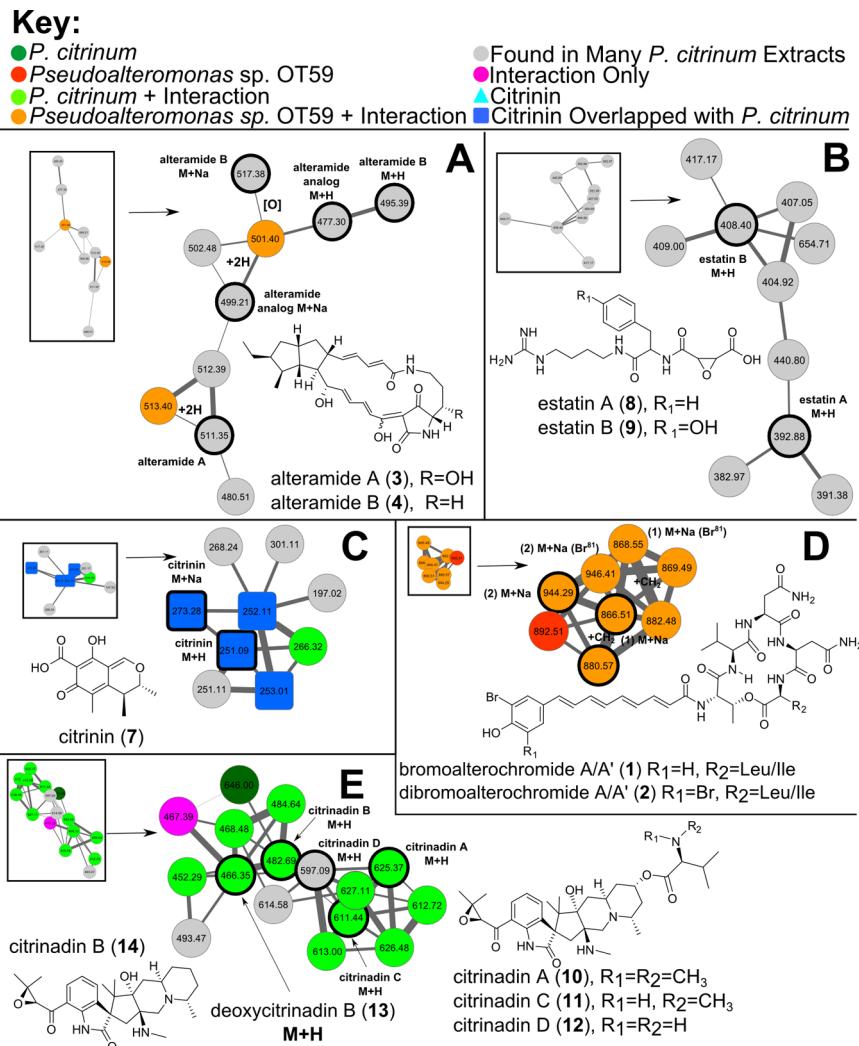


Figure 2. Zoom in of MS/MS network analysis (Supplementary Figure S4) of *n*-BuOH extracts from OT59, *P. citrinum*, and the interaction of OT59 and *P. citrinum*. Colors indicated in the key correspond to the compound source, and shapes are used to highlight overlap with known standards. Nodes bolded by black lines are the compounds of interest observed from the IMS analysis and are labeled with their compound name. Each node represents a parent mass with corresponding MS/MS fragmentation pattern; lines depict the relatedness between the compounds as measured by their MS/MS fragmentation.¹⁸

the fungal test species at escalating doses of the isolated metabolites (0, 5, 10, 20 μ g). OT59 metabolites *m/z* 511 and 495 were found to have fungal inhibition with fungal clearances zones of 13 and 10 mm (diameter) at 20 μ g, respectively. The third metabolite (*m/z* 477) isolated from the same HPLC purification scheme as *m/z* 511 and 495 showed weaker activity with an antifungal inhibition zone of 7 mm at 20 μ g. (Supplementary Figure S7).

The *m/z* 511, 495, and 477 ions clustered together in the molecular network (Figure 2), and inspection of the AntiMarin database²³ for their exact masses (*m/z* 511.2794, 495.2830, and 477.2732) suggested the polycyclic tetrameric macrolactams alteramide A²⁴ or maltophilin²⁵ as possible matches for the *m/z* 511.2794 compound. On the basis of comparable MS/MS fragmentation, the products with *m/z* 495.2830 and *m/z* 477.2732 were hypothesized to be dehydroxylated analogues of either alteramide A or maltophilin (Supplementary Figures S15–S16). While maltophilin is a known antifungal agent,²⁵ to our knowledge neither alteramide A nor the dehydroxylated congeners have reported antifungal activities.^{24,26} Characterization by 1D and 2D NMR of the *m/z* 511.2793 ion revealed a

match with alteramide A (Supplementary Figure S23–28 and S36 and Tables S1–3). Although the connectivity matched with the carbon skeleton reported for alteramide A, key NOEs were observed between H6 and protons H4, H7a, and H14 (Figure 3a) that suggest H6 is beta and on the opposite plane as H13. This contrasted with the alteramide A structure proposed by Shigemori et al.,²⁴ which places both H6 and H13 *syn* on the alpha site. Conformational energy minimization of our proposed structure of alteramide A indicated that pairs of conformations for both *E* and *Z* tautomers could accommodate the observed NOEs in the NMR (Supplementary Figure S6, Supplementary Methods).

The NMR of compound with *m/z* 495.2840 was very similar to alteramide A, with significant changes in chemical shifts for protons attached to C-23, C-25, and C-26, and was identified as the C-25 dehydroxy congener alteramide B (Supplementary Figures S29–36, Tables S4–6), previously suggested as the biosynthetic precursor for alteramide A.²⁴ Our NMR analysis indicated that the skeleton was identical to that of alteramide A isolated from OT59 with H6 beta and on the opposite plane as H13 (Figure 3a). The third antifungal metabolite *m/z* 477 was

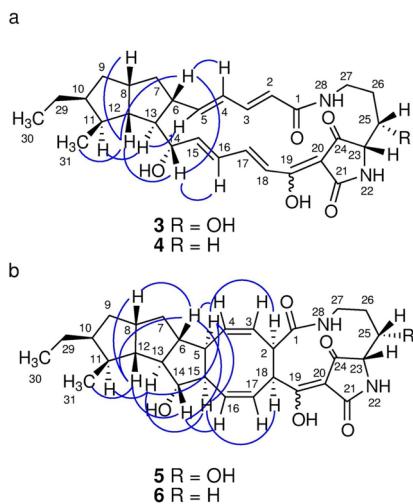


Figure 3. Key NOE correlations indicating the revised stereochemistry of H6. (a) Alteramides A (3) and B (4) and (b) intramolecular cyclized alteramides A (5) and B (6).

not isolated in sufficient quantity and purity for a full NMR analysis, but MS/MS fragmentations and UV spectrum (Supplementary Figures S16 and S5b) suggest it to be another alteramide congener. Recently, 6-*epi*-alteramide A and B isolated from a *Streptomyces* sp. demonstrated identical stereochemistry at C-6 as the alteramides in our studies.²⁷ In light of our data, we suggest a revision of the original stereochemistry rather than denomination of new epimers.

Exposure of alteramide A and B in methanol to indirect sunlight induced an internal [4 + 4] cycloaddition as evidenced by the disappearance of the downfield NMR signals corresponding to the conjugated diene protons (consistent with reports for alteramide A).²⁴ Photocyclized alteramides A and B were inactive when tested in the disk diffusion antifungal assay up to 40 μ g against *P. citrinum* (Supplementary Figure S7). Full NMR analysis of the photocyclized alteramide A (Supplementary Figures S37–43, Tables S7–9) confirmed the structure as characterized by Shigemori et al.²⁴ However, as was noted above, key NOEs between H6 and H4, H14, H12, and H8 (weak), NOEs between H13 and H5, H11, and H15 indicated that H6 was beta and *trans* from H13 (Figure 3b). Similar NOEs were observed for the photocyclized alteramide B (Supplementary Figures S44–51, Tables S10–12). For both photocyclized alteramides, two distinct conformations (*E* and *Z* forms) were observed that were not interchangeable on the NMR time scale at the temperatures investigated (298–318 K)²⁸ (Supplementary Figures S51–52), while slightly broadened proton resonances were observed for H-15 to H-18 in alteramides A and B (Supplementary Figure S36).

Therefore, the alteramides are responsible for the observed OT59 antifungal activity when grown in the dark. Light exposure diminishes production of alteramides and induces a photochemical intramolecular [4 + 4] cycloaddition, eliminating remaining antifungal activity. OT59 was able to maintain reduced antifungal activity during a day-night cycle, implying that nighttime alteramide production provided some daytime protection. Light exposure itself affects the growth of fungi and their metabolite production including mycotoxins.²⁹ Light has an inhibitory growth effect on the *Penicillium* species, and therefore the need for antifungal production by OT59 to protect the coral would be higher in the dark as observed in our

studies. Environmental changes in abiotic factors including light exposure can differentially affect the growth of various fungal species²⁹ and potentially affect the health of corals harboring *Pseudoalteromonas* species.

Effect of Alteramide A on *P. citrinum*. MALDI-IMS was used to compare the fungal metabolites secreted by *P. citrinum* when co-incubated with OT59 in the dark, dark-light cycle, and light (Figure 1c, columns i–iii) to when *P. citrinum* was exposed to one of the antifungals, alteramide A (Figure 1c, column iv). This technique yielded a bird's-eye view of the changes in fungal metabolite distribution due to adjacent OT59 under the various light exposure conditions compared to the direct effect of *Pseudoalteromonas*-produced alteramide A on *P. citrinum* in the dark. Observed metabolites were identified using the molecular network analysis (Figure 2, Supplementary Figure S4).^{18,20}

P. citrinum secretes multiple metabolites in the interaction with OT59 or alteramide A with different spatial distributions or intensities when incubated in the dark, light, or dark-light cycle, suggesting their function most likely has been affected, either positively or negatively (examples in Figure 1d). Citrinin (Na^+ form *m/z* 273) increased in the presence of OT59 specifically when grown in the dark. When *P. citrinum* was co-incubated with alteramide A, higher intensities of citrinin (Figure 1d, column iv) were also observed, suggesting that the alteramide played a role in the upregulation of this mycotoxin (Supplementary Figures S8, S17). Citrinin is a bacteriostatic antibiotic,³⁰ and the increased production by *P. citrinum* under the influence of OT59 underlines its defensive role against other organisms. In contrast, fungal metabolites estatin A and B were clearly suppressed (or consumed) in the interaction with OT59 as deduced by comparison with *P. citrinum* control grown in the dark (Supplementary Figure S9, S18). Suppression of estatins was not observed to the same extent in the interaction of *P. citrinum* with alteramide A, suggesting that other bacterial factors were involved. Although estatins A and B have been isolated from *Myceliophthora thermophila* and characterized as thiol protease inhibitors,³¹ to our knowledge no reports exist that they are also produced by *P. citrinum*.

Reduced levels of unidentified fungal metabolites (*m/z* 515, 531, and 547) correlated with lack of hyphae and fungal growth inhibition (*m/z* 531 shown in Figure 1c, compare columns i and iv). In contrast, a set of citrinadin metabolites was concentrated at the interface of OT59 and *P. citrinum* when incubated in the dark but were more centrally distributed when grown in the light. Alteramide A (Figure 1c and d, column iv) induced a similar distribution of the citrinadins. Multiple citrinadin congeners with similar distributions as citrinadin A were observed and putatively assigned as citrinadin C, D and deoxycitrinadin B based on MS/MS fragmentation (Supplementary Figures S19–21). While citrinadin A has potent activity against leukemia cell lines,^{32,33} its functional role in an ecological environment remains unknown.

Most microbes produce siderophores to transport critical, solubilized iron into the cell.³⁴ When *P. citrinum* was grown in the dark either alone or adjacent to OT59, more significant production of desferrichrome, a fungal hydroxamate siderophore, was observed than when grown in the light (Supplementary Figures S8, S22). Incubation of alteramide A adjacent to *P. citrinum* in the dark (Figure 1c, column iv) led to a higher concentration of the fungal siderophore at the interface, indicating some directional effect of the microbial metabolite. Since iron is often a limiting nutrient, especially in

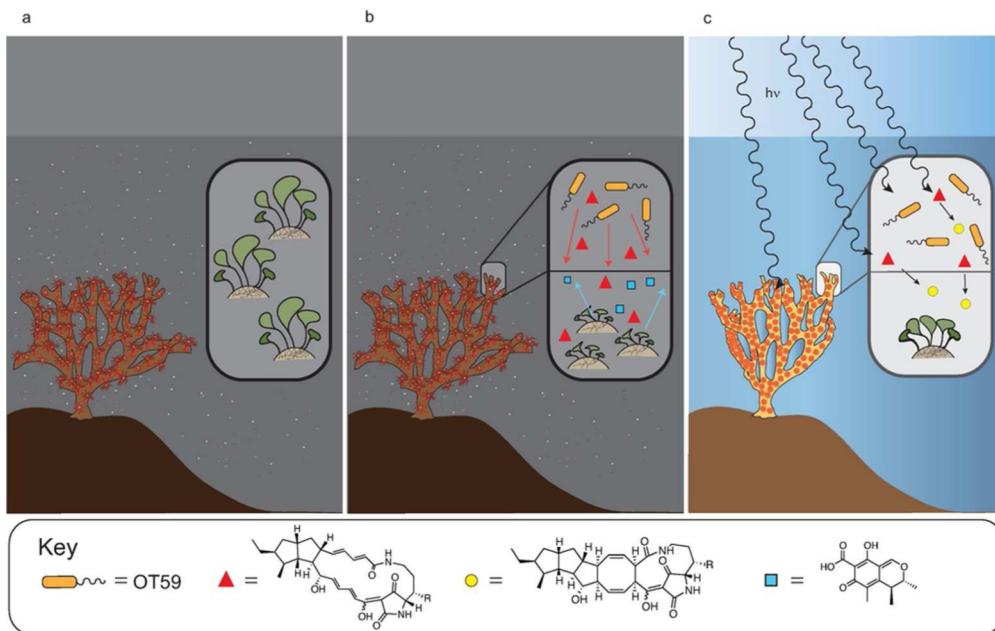


Figure 4. Hypothesized light-dependent role of a coral-associated microbe *Pseudoalteromonas*. (a) Nighttime: most corals open their polyps to feed (plankton pictured), which increases the surface area of the coral and potential encounters with microbes including pathogenic fungi (shown in insert). (b) *Pseudoalteromonas* spp. associated with corals secrete antifungal alteramides (triangle) in the dark that inhibit the growth of *P. citrinum* and various other fungal species. This in turn will lead to an increased secretion of mycotoxin citrinin (square) by *P. citrinum*. (c) Daytime: polyps on corals are closed, and coral-associated *Pseudoalteromonas* is exposed to light, leading to lower amounts of the antifungal alteramides (triangle), which are photochemically inactivated (circle).

the marine environment,³⁵ microorganisms will compete to acquire this metal through various mechanisms including increased siderophore production.

The alteramide molecular species responsible for the antifungal activity of OT59 belong to a family of polycyclic tetramic macrolactams produced by gene clusters present in *Pseudoalteromonas* and many other bacteria including *Streptomyces* from which we and others also isolated alteramides^{27,36,37} (Supplementary Methods and NMR Figures). *Pseudoalteromonas* species have been isolated from many aqueous localities,³⁸ and we expect the antifungal effects of light-sensitive alteramides to play a role in other biological niches besides Panamanian corals. The observed model microbial interactions between two coral microbes suggests corals may have symbiotic associations with their microbiota to produce antifungal agents in a light-dependent manner (Figure 4). We further propose that antifungal agents produced in the dark reduce the chance of infections during feeding when corals are more exposed. Finally, we anticipate that other coral-associated microbial metabolites exhibit light-modulated biological activity that contributes to the ability of coral microbiota to combat infections.

METHODS

Materials. All chemicals used for YES and M1 media, Universal MALDI matrix, citrinin, sephadex-LH20, and Amberlite XAD-16 were purchased from Sigma-Aldrich except for Instant Ocean aquarium salts (Pentair Aquatic Eco-Systems, Inc.). J. T. Baker organic solvents were purchased from Avantor Performance Materials, Inc. Iron-free ferrichrome was purchased from Santa Cruz Biotechnology Inc.

Collection, Isolation, and Identification of *Pseudoalteromonas OT59*. The octocoral *Leptogorgia alba* was collected using SCUBA at 12 m deep near Otoque Island (Pacific Ocean, Panama, Aug 2009). Coral was rinsed with autoclaved seawater, and a small portion of the coral mucus was inoculated directly on agar plates with seawater-based

nutrient medium (potato starch (10 g), yeast extract (4 g), peptone (2 g), agar (18 g), cyclohexamide (0.1 g) in 1 L of natural seawater). Bacteria were isolated at RT over a period of 1 month. Strain OT59 was further isolated and successively replated until a pure strain was obtained (Supplementary Figure S1a).

The coral specimen was identified as *Leptogorgia alba* based on its morphology and SEM micrographs of the coral sclerites.³⁹ Taxonomic identification of the bacterial strain OT59 used PCR amplification and nucleotide sequencing of 16S rRNA and cpn60 gene using GenBank and RDP databases (partial 16S rRNA >99% homology with most *Pseudoalteromonas* species and partial cpn60 gene sequences ~93% homology with *P. piscicida*). Phylogenetic reconstruction, using GenBank and cpnDB reference sequences, indicated that the OT59 sequence diverged sufficiently from other *Pseudoalteromonas* species considering this strain as *Pseudoalteromonas* sp. (Supplementary Figure S10). Reference specimen of the coral (GLOT-120209-02) and the bacterial strain (OT59) are deposited at the INDICASAT's CBDD.

Collection, Isolation, and Identification of *P. citrinum*. A diseased octocoral *Eucinea* sp. was collected using SCUBA at 6 m depth from Isla Grande in Colon (Caribbean of Panama, September 2011) and transported to the laboratory within 1 h of collection. The coral was rinsed with autoclaved seawater and disinfected with 70% ethanol, and small pieces were placed on plates with Peptone Yeast Glucose (PYG) agar (1 L marine water, peptone (1.25 g), yeast extract (1.25 g), glucose (3.0 g), agar (20 g) supplemented with 100 mg mL⁻¹ of penicillin and streptomycin). Fungi were isolated at RT over 1 month. *P. citrinum* was further isolated and successively replated until a pure strain was obtained (Supplementary Figure S1b). Fungal spore stocks were generated by inoculating the fungal slants on M1 agar (potato starch (10 g), yeast extract (4 g), peptone (2 g), seasalts (28 g), 1 L deionized water) at 28 °C for 7 d. Sterile water was added on top of the fungal colonies, diluted to 20% glycerol, and stored in aliquots at -80 °C (colony forming units (CFU) 5.5 × 10⁶ mL⁻¹) without tween detergent.

The coral specimen was identified as *Eucinea* sp. based on its morphological characteristics and following Bayer.⁴⁰ Taxonomic identification using PCR amplification and fungal internal transcribed spacer (ITS) sequencing indicated >99% similarity to *Penicillium*

citrinum using BLAST. Reference specimen of the coral (GLIC-280911-10) and the fungal strain (*P. citrinum*) are deposited at the INDICASAT's CBDD.

Other Fungal Strains. For sources of other fungal strains used in this study, see Moree et al.⁴¹

Fungal spore stocks were generated by inoculating the fungal slants on YES agar⁴² (terrestrial strains) or M1 agar (marine strains) at 28 °C for 7 d as described for *P. citrinum*. (CFU between 7 and 8 × 10⁶ mL⁻¹).

Preparation of Bacterial and Fungal Samples for MALDI-IMS and Extract Analysis. OT59 was grown from a single colony in M1 liquid media overnight ($OD_{600} = 1.007$), diluted to a 20% glycerol stock, and stored in aliquots at -80 °C (CFU 1.05×10^8 mL⁻¹).

OT59 (1 μL) was inoculated in a 5 mm streak at a 5 mm distance from a spot of *P. citrinum* inoculum (1 μL) on M1 agar (10 mL) in 100 o.d. × 25 mm Petri dishes. Similarly, OT59 and *A. fumigatus* were co-inoculated. All organisms were also inoculated on separate Petri dishes as controls. Samples were incubated for 48 h at 30 °C in the dark, under constant light exposure (149 μE m⁻² s⁻¹) or at 12 h intervals alternating dark vs light exposure (149 μE m⁻² s⁻¹) using a TL 70 Phosphor manufactured by Philips (PHIL-F32T8/TL741 NEW ALTO) as the light source.

To study the effect of alteramide A on *P. citrinum*, a solution of 10 μg of alteramide A in MeOH was applied to a paper disk (6 mm diameter), air-dried, and placed at a 5 mm distance from a 1 μL inoculum of *P. citrinum* on M1 agar in a Petri dish. All sample manipulations were carried out under minimal light exposure and incubated for 48 h at 30 °C in the dark. Sample preparation and MALDI-IMS was carried out as previously described.⁴¹

For extract analysis, 6 spots of OT59 (1 μL/spot), 6 spots of *P. citrinum* (1 μL/spot), and 6 interactions of *P. citrinum* at a 5 mm distance from OT59 were inoculated on separate M1 agar plates (10 mL) and incubated at 30 °C for 48 h. At four different locations in the colonies hole punches (diameter of 2 mm) were taken and extracted with 300 μL of nBuOH for 4 h. For the interaction of OT59 and *P. citrinum* hole punches were taken at the interface. After centrifugation the supernatant was diluted with spray solvent, analyzed by mass spectrometry, and subjected to molecular MS/MS generation by Cytoscape as previously described.⁴¹

Isolation of Antifungal Compounds from OT59 (No Light Exposure). Seventy-five Petri dishes containing M1 agar were each inoculated with OT59 ($OD_{600} = 1.03$ average) in 15 streaks of 1 cm and incubated for 3 days at 30 °C. The agar was cut and extracted twice with 250 mL of EtOAc/MeOH (2/1) and gently shaken (1.5 h at 30 °C). Extracts were filtered and concentrated by rotary evaporation, redissolved in MeOH, filtered, and reconcentrated. After repeating twice, ~2 g of crude material in MeOH was subjected to three rounds of column chromatography on Sephadex LH20 (MeOH eluent, 0.2 mL/min). Of 18 fractions containing alteramides, the 6 middle fractions most enriched for the alteramides were purified by HPLC (gradient 10% to 100% MeCN/H₂O (0.1% TFA) in 26 min with a 2.0 mL min⁻¹ flow rate, Agilent Infinity 1260 HPLC, Xterra C-18 150 mm × 10 mm, 5 μm column). Yields for Alteramide A, B, and C were 1.9 mg, 0.8 mg, and <0.3 mg, respectively. Exposure of alteramide A and B (500 μg) in MeOH (500 μL) to indirect sunlight (6 h) yielded quantitative conversion to the intramolecular cyclized alteramides 5 and 6.

HPLC Comparison of OT59 Grown in Dark vs Light. Two samples of OT59 were inoculated in a 1 cm streak on M1 agar and grown for 48 h at 30 °C under dark and constant light exposure (149 μE m⁻² s⁻¹). OT59 colonies grown in the dark and in the light were punched out with the underlying agar, extracted with nBuOH, and analyzed by HPLC (2-step gradient of 40% to 55% MeCN/H₂O (0.1% TFA) in 30 min to 99.9% MeCN/0.1% TFA in 10 min at a 1.0 mL min⁻¹ flow rate, Luna C-18, 250 mm × 4.6 mm, 5 μm).

Bioassay. Fungal growth inhibition by OT59 was assessed by spreading fungal spore stock (20 μL) on M1 agar (4 mL) plates in 50 o.d. × 25 mm Petri dishes using glass beads and letting the spore solution air-dry. OT59 was inoculated in the middle (2 μL). After incubation at 30 °C for 48 h either in the dark or light at 149 μE m⁻²

s⁻¹, diameters of clearance zones were measured. Inhibition of fungal growth by purified alteramides was assessed by inoculating *P. citrinum* (30 μL) in a lawn on M1 agar and placing 6 mm paper disks treated with either control or different doses of alteramides. After incubation for 5 d at 30 °C in the dark, diameters of clearance zones were measured.

NMR Measurements. NMR spectra were recorded on a Bruker Avance III 600 MHz NMR with 1.7 mm Micro-CryoProbe at 298 K (600 and 150 MHz for ¹H and ¹³C NMR, respectively), using standard pulse sequences (Bruker), or on a Varian Unity 500 MHz spectrometer (500 and 125 MHz for ¹H and ¹³C NMR, respectively). Data were analyzed using the Topspin 2.1 software. For NMR acquisition on the 600 MHz, 200–300 μg of alteramide dissolved in 50 μL of CD₃OD were used. For NMR acquisition on the 500 MHz, 600–800 μg of alteramide was dissolved in 200 μL of CD₃OD, and a SHIGEMI NMR tube assembly was employed.

ASSOCIATED CONTENT

Supporting Information

Photographs of corals and fungal inhibition; HPLC and UV data; additional MALDI-IMS data; MS/MS spectra, conformational analyses; NMR tables and spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Accession Codes

Accession numbers for *Pseudoalteromonas* sp. strain OT59 deposited in GenBank are KF880834 and KF880835, and for *P. citrinum* strain the number is KJ154959. Data files used for the molecular network have been uploaded to Global Natural Products Social Molecular Networking (<http://gnps.ucsd.edu>), accession number MSV000078621.

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Notes

The authors declare no competing financial interest.

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