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Effects of Actinomycete Secondary Metabolites on Sediment Microbial Communities

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ABSTRACT Marine sediments harbor complex microbial communities that remain poorly studied relative to other biomes such as seawater. Moreover, bacteria in these communities produce antibiotics and other bioactive secondary metabolites, yet little is known about how these compounds affect microbial community structure. In this study, we used next-generation amplicon sequencing to assess native microbial community composition in shallow tropical marine sediments. The results revealed complex communities comprised of largely uncultured taxa, with considerable spatial heterogeneity and known antibiotic producers comprising only a small fraction of the total diversity. Organic extracts from cultured strains of the sediment-dwelling actinomycete genus *Salinispora* were then used in mesocosm studies to address how secondary metabolites shape sediment community composition. We identified predatory bacteria and other taxa that were consistently reduced in the extract-treated mesocosms, suggesting that they may be the targets of allelopathic interactions. We tested related taxa for extract sensitivity and found general agreement with the culture-independent results. Conversely, several taxa were enriched in the extract-treated mesocosms, suggesting that some bacteria benefited from the interactions. The results provide evidence that bacterial secondary metabolites can have complex and significant effects on sediment microbial communities.

IMPORTANCE Ocean sediments represent one of Earth's largest and most poorly studied biomes. These habitats are characterized by complex microbial communities where competition for space and nutrients can be intense. This study addressed the hypothesis that secondary metabolites produced by the sediment-inhabiting actinomycete *Salinispora arenicola* affect community composition and thus mediate interactions among competing microbes. Next-generation amplicon sequencing of mesocosm experiments revealed complex communities that shifted following exposure to *S. arenicola* extracts. The results reveal that certain predatory bacteria were consistently less abundant following exposure to extracts, suggesting that microbial metabolites mediate competitive interactions. Other taxa increased in relative abundance, suggesting a benefit from the extracts themselves or the resulting changes in the community. This study takes a first step toward assessing the impacts of bacterial metabolites on sediment microbial communities. The results provide insight into how low-abundance organisms may help structure microbial communities in ocean sediments.

KEYWORDS chemical ecology, marine sediments, microbial communities, secondary metabolites

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Ocean sediments represent one of Earth's largest biomes, yet we know relatively little about microbial diversity and function in these habitats. While extensive sequence data have been obtained from the Global Ocean Survey (1, 2), Tara Oceans (3), and the International Census of Marine Microbes (ICoMM) (4), these efforts have been heavily biased in favor of water column samples. By comparison, marine sediments remain poorly studied in terms of microbial taxonomic and functional diversity (5). Ocean sediments harbor microbial cell counts that can exceed those of seawater by three orders of magnitude (6–10), with diversity estimates consistently among the highest of all studied environments (11, 12). These communities commonly include taxa like *Planctomycetales*, *Firmicutes*, and *Verrucomicrobiales* (7, 9, 13), which are generally minor components of seawater communities. Microbial diversity in marine sediments has been shown to vary with environmental parameters such as seasonality (9) and anthropogenic contamination (14–16); however, the main drivers of community composition remain largely unknown (9, 16). The structured nature of sediments provides opportunities for the development of microenvironments in which bacteria interact and compete using mechanisms such as the production of allelopathic secondary metabolites. While such competitive interactions have been addressed with marine bacteria (17, 18), it remains unknown how competitive interactions structure sediment microbial communities.

The competitive strategies employed by bacteria range from rapid growth and mineral chelation to the production of allelopathic secondary metabolites (19–21). In a few well-studied examples, such as the production of antibiotics by symbionts to protect their hosts from pathogens (22, 23), the ecological functions of antagonistic compounds have been identified. Similarly, allelopathic bacteria in suppressive soils and the plant rhizosphere have been shown to play important roles in plant survival and health (24, 25). In the marine environment, bacterial antagonism is widely recognized and thought to be more frequent among particle- and surface-associated bacteria than among free-living bacteria (17, 26, 27). Antibiotic production in seawater is known to be more prevalent among taxa like the *Gammaproteobacteria* (17, 26, 28) and can define ecologically distinct populations (29). However, the role of antibiotics in competition remains poorly understood (19, 29, 30), with even less known about how these compounds structure microbial communities.

Bacteria in the order *Actinomycetales* constitute a minor component of sediment communities (5, 7, 31–36), yet decades of culturing efforts have shown that they persist in most well-sampled sediments (37–40). These bacteria are well known for secondary metabolite production (41), and their mycelial growth form creates the potential for the formation of large networks (42) that have yet to be spatially characterized. Antibiotic production in actinomycetes has been linked to nutrient sensing and morphological differentiation (43), and their biosynthetic potential is much larger than laboratory observations suggest (44, 45). While the natural cues that trigger the production of most secondary metabolites remain unknown, many of these compounds are potent antibiotics and thus have the potential to affect members of the community with which they interact.

The marine actinomycete genus *Salinispora* provides a useful model to address the ecological roles of bacterial secondary metabolites. It is comprised of three named species, *Salinispora arenicola*, *Salinispora tropica*, and *Salinispora pacifica* (46, 47), which are well delineated despite sharing 99% 16S rRNA gene sequence identity (48). Notably, these bacteria are a rich source of secondary metabolites (49), which have proven to be key phenotypic (50) and genotypic (45) features that differentiate the species. Secondary metabolism also distinguishes the competitive strategies employed by *S. arenicola* and *S. tropica*; in particular, the production of rifamycin antibiotics by *S. arenicola* contributes to its broad inhibitory capacity compared to that of the faster-growing *S. tropica* (18). With ~10% of the genome of *S. arenicola* devoted to secondary metabolism (45, 51) and the potent bioactivity of its natural products, this species was an obvious choice to explore the effects of secondary metabolites on the sediment microbial community.

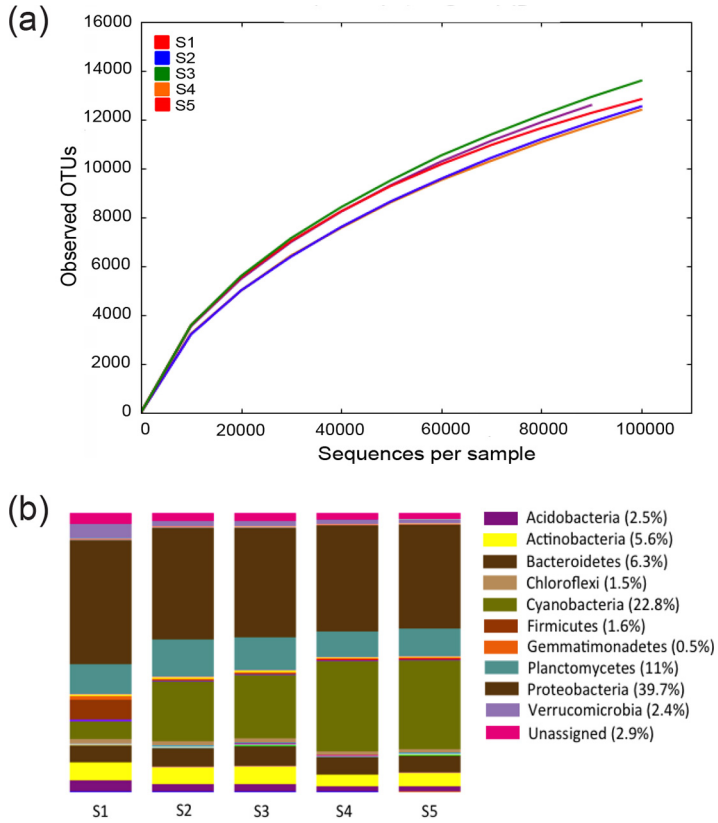

FIG 1 Sediment microbial diversity in five replicate samples (S1 to S5). (a) Rarefaction curves measuring the number of observed OTUs clustered at 99% 16S rRNA sequence identity with respect to sequencing depth. (b) Phylum-level community composition.

In this study, we tested the hypothesis that secondary metabolites from marine sediment-dwelling bacteria affect microbial community composition. The first goal was to assess microbial diversity in shallow tropical sediments using next-generation sequencing technology. We then tested for shifts in this baseline community following exposure to *S. arenicola* secondary metabolites in a series of mesocosm experiments. Finally, we used cultured strains to test hypotheses about which taxa may be inhibited based on the results from the mesocosm studies.

RESULTS

Sediment communities. Five replicate sediment samples were collected from a 1-m^2 sand patch at a depth of 1 m on a reef slope off Viti Levu, Fiji. Bacterial diversity was assessed using next-generation 16S rRNA amplicon sequencing and found to be consistently high, ranging from 12,400 to 13,600 operational taxonomic units (OTUs) at 99% sequence identity with no sign of saturation in the rarefaction curves (Fig. 1). The phylum-level diversity in four of the five samples (S2 to S5) was highly consistent while sample S1 was enriched in *Firmicutes* (6.9% of the population compared to under 0.5% on average for S2 to S5) and *Verrucomicrobia* (5.1% compared to 1.6%), and reduced in *Cyanobacteria* (6.2% compared to 27%) relative to levels in the other samples (Fig. 1). On average for all samples, approximately 40% of the bacterial community was comprised of *Proteobacteria*, with *Gamma-* and *Alphaproteobacteria* representing the largest subphyla (data not shown). Other well-represented phyla included *Cyanobacteria* (22.8%), *Planctomycetes* (11.0%), *Bacteroidetes* (6.3%), and *Actinobacteria* (5.6%). The archaeal phylum *Euryarchaeota* was also detected in all samples, at proportions ranging from 0.1 to 0.3%. These results are comparable to those from similar studies of coastal sandy sediments (7, 32). The genus *Salinispora* was detected in all five sediment samples at abundances between 0.0008% and 0.036% of the total community. *Salinis-*

pora species could not be resolved using the region of the 16S rRNA gene sequenced. Between 2.2% and 3.8% of the reads in each sample could not be classified at the domain level using the Silva database (http://www.arb-silva.de) (52).

Mesocosm diversity. We established a mesocosm protocol to test for the effects of *Salinispora* extracts on sediment community composition. We first compared the native sediment analyses described above to time point 0 (T0) mesocosms (frozen immediately after inoculation) to test for the effects of sample handling on diversity. The T0 mesocosm samples included five medium controls and nine *Salinispora* treatments (five from strain CNY-679 and four from strain CNS-820) (see Table S1 in the supplemental material). Community composition at the phylum level was generally comparable between the treated and control T0 mesocosm samples (Fig. S1). Compared to the native sediments (S1 to S5), however, the relative contribution of cyanobacteria in the mesocosms was significantly reduced (Mann-Whitney U test; $P = 0.001$), suggesting that sample handling had an impact on photosynthetic prokaryotes. The exception was native sample S1, which, as previously noted, was enriched in *Firmicutes* and depleted of cyanobacteria relative to the other four native sediments. At the phylum level, the starting mesocosm communities (T0) were representative of the communities seen in the native samples.

Mesocosm time course. We next used mesocosms to compare the effects of organic extracts from *S. arenicola* strains CNY-679 and CNS-820 on sediment microbial communities. Because the time frame of any potential effects was not known, we performed a preliminary time series from 0 h to 5 days using extracts from the medium control and CNY-679. Results from beta diversity analyses performed using QIIME (53) suggested that the *S. arenicola* treatment community began changing at the first time point, while the medium extract control community remained relatively stable until time point 3 (72 h; T3), after which there was a large shift (Fig. S2). We therefore chose to compare the effects of *Salinispora* extracts and medium controls at T3 for both strains. The results were analyzed individually to test for differences between stains and collectively to determine trends associated with each species. Four samples were not included due to insufficient numbers of high-quality reads resulting in the analysis of 26 treatment and control mesocosms (Table S1).

Effects of extracts. A comparison of the *S. arenicola* T3 treatment mesocosms with the T3 medium controls revealed no significant difference in average alpha diversity measurements (Mann-Whitney U test; $P = 0.46$) although the rarefaction curves did not plateau when rarefied to 90,000 reads (Fig. 2). The medium control mesocosms were more similar to each other and the *S. arenicola* CNY-679 treatments (Bray-Curtis dissimilarity values, 0.55 and 0.54, respectively) than to the *S. arenicola* CNS-820 treatments (Bray-Curtis, 0.68) (Table S4). In general, *S. arenicola* CNS-820 treatments showed the highest level of dissimilarity among replicates and were less similar to the other conditions.

Proteobacteria represented the most abundant phylum in all samples, comprising on average 61.1% of the sequence reads (Fig. 2). The phylum *Planctomycetes* was the second-most abundant, averaging 10% of the reads. We then used the linear discriminant analysis (LDA) effect size (LEfSe) algorithm (54) to determine which taxa were differentially distributed between the medium controls and treated mesocosms. This algorithm identifies differentially abundant taxa between sample conditions, or classes, by emphasizing both statistical significance and biological relevance. We identified several taxa reduced in the *S. arenicola* treatments, including the phyla *Firmicutes* and "*Candidatus* Gracilibacteria" (Fig. 3 and Fig. S3). Upon closer examination, most of the inhibition observed at the phylum level could be linked to specific genera or families. These included the genus *Alteromonas* within the *Gammaproteobacteria* and the genera *Erythrobacter*, *Ruegeria*, and *Shimia* within the *Alphaproteobacteria* (Fig. 4). These include predatory bacteria related to the genera *Enhygromyxa* (order *Myxococcales*) (55), *Saprospira*, and *Bacteriovorax* (Fig. 4) although only the CNS-820 extract had a significant effect on *Enhygromyxa* spp. Several other taxa were also more sensitive to

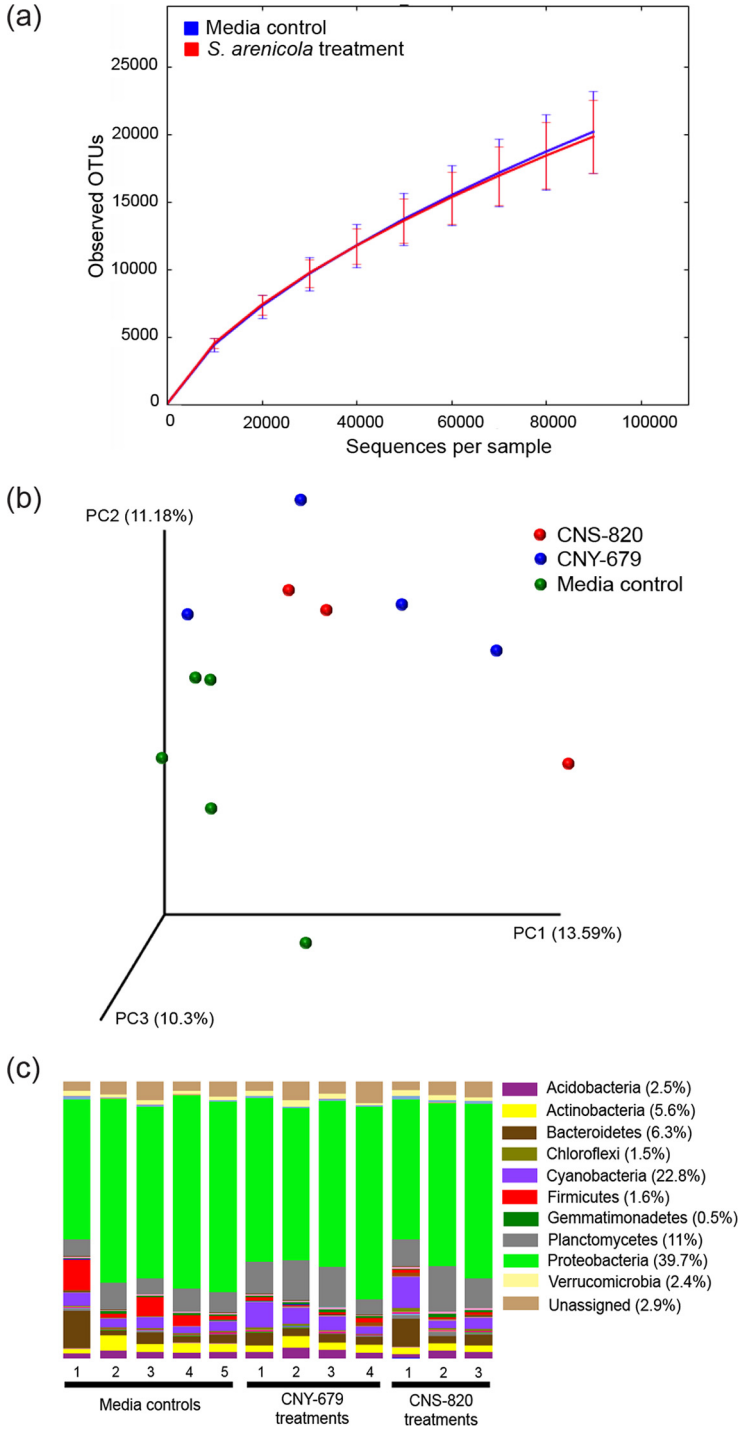

FIG 2 Alpha and beta diversity plots for T3 mesocosm samples. (a) Rarefaction curves showing average observed OTUs for *S. arenicola* treatments and medium controls with respect to sequencing depth. (b) Three-dimensional principal component (PC) analysis plot of treatment and control mesocosm communities. (c) Phylum-level community composition.

extracts of CNS-820 than CNY-679. Some taxa were enriched in the treated mesocosms compared to levels in the medium controls. These included the phyla *Chloroflexi* and "*Candidatus* Latescibacteria," as well as several clades within the *Planctomycetes* and *Acidobacteria* (Fig. 3 and Fig. S3). An unexpected result was the depletion of many taxa in the medium controls. This may be due to the extraction of toxic components from

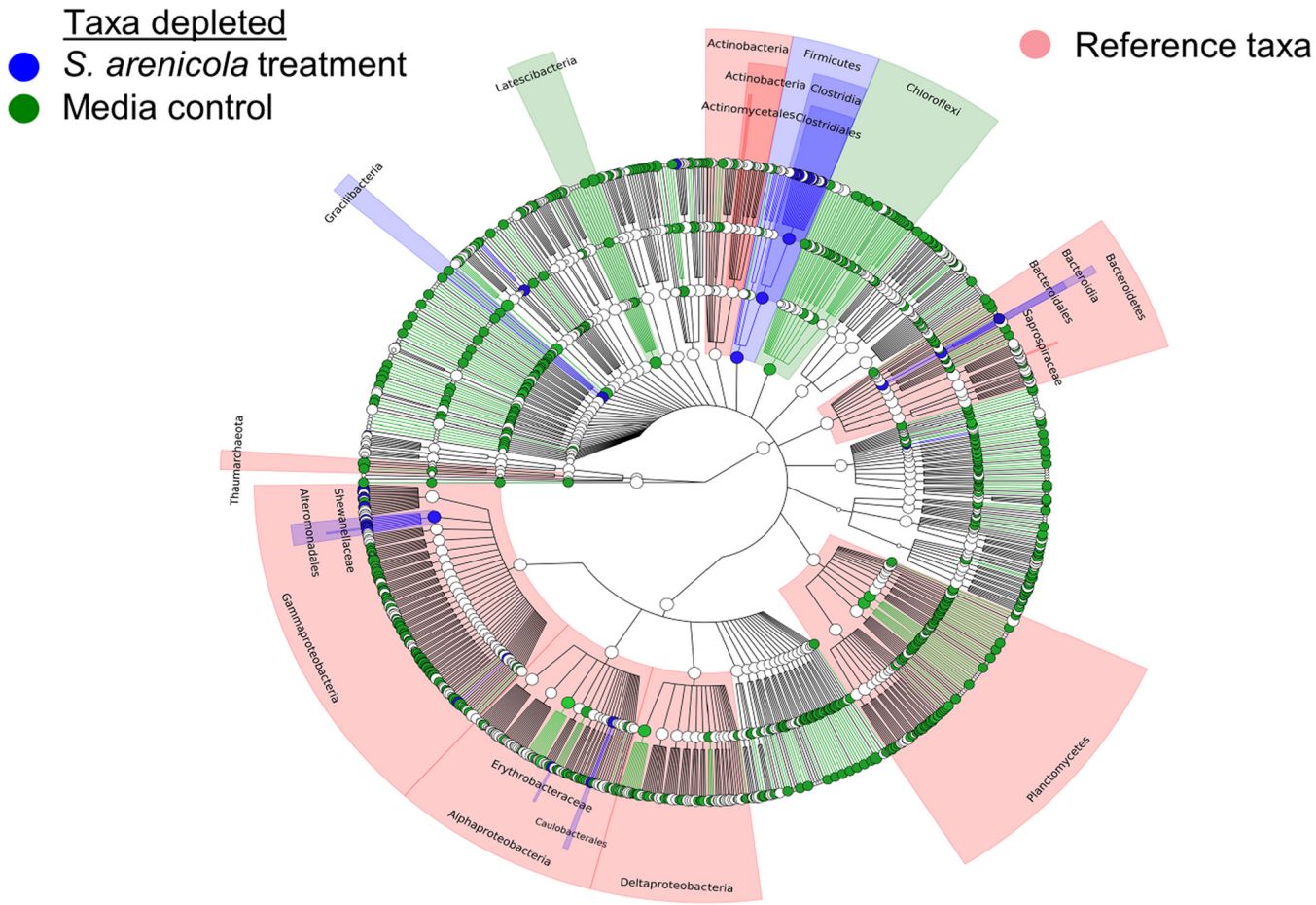

FIG 3 Phylogenetic representation of family-level differences between T3 treatment and control mesocosm communities. Blue and green nodes indicate clades that were depleted in the *S. arenicola* treatments or medium controls, respectively. Clades are shaded when they are also annotated with the taxon name. Unaffected clades that were annotated for taxonomic reference are shaded red. For example, the phylum *Bacteroidetes* was not significantly depleted under either condition, and so its background is shaded red; however the family *Bacteroidia* within this phylum was significantly depleted in the *S. arenicola* treatments, and so the nodes and corresponding shading for this family are blue.

medium ingredients, such as yeast extract (56), and may have resulted in the underestimation of the inhibitory effects of the *Salinispora* extracts.

***S. arenicola* extract sensitivity assays.** We next tested if changes in mesocosm community composition could be used to predict taxa that are sensitive to *S. arenicola* secondary metabolites. To test this hypothesis in culture-based assays, we selected 10 strains representing bacteria that were either depleted or unchanged in relative abundance in the *S. arenicola* treatment mesocosms (Table 1). When the strains were tested for sensitivity to *S. arenicola* extracts, five out of eight were inhibited by extracts from both *S. arenicola* strains at a concentration of 1 mg/ml, while another two were sensitive only to the CNS-820 extract at this concentration (Table 1). Seven out of eight strains of this same group were also sensitive to both extracts at 10 mg/ml. While these concentrations were higher than those used in the mesocosm studies, only 10 μl was added to the discs, and thus only 10 or 100 μg of extract was tested. The predatory *Myxococcus xanthus* strain, our closest cultured relative to the genus *Enhygromyxa*, was inhibited by the CNS-820 extract at 1 mg/ml and by the CNY-679 extract at 10 mg/ml (Table 1). The results were mixed for the two representatives of taxa that were unaffected in the mesocosms, with *Vibrio* sp. strain CUA-833 completely resistant to the extracts while *Labrenzia* sp. strain CUA-809 was sensitive to both extracts at 1 mg/ml.

Organic extracts. Tandem mass spectrometry (MS/MS)-based analyses visualized as molecular networks (57) led to the identification of known metabolites, including rifamycins, saliniketals, and staurosporine, in the extracts of both CNY-679 and CNS-820

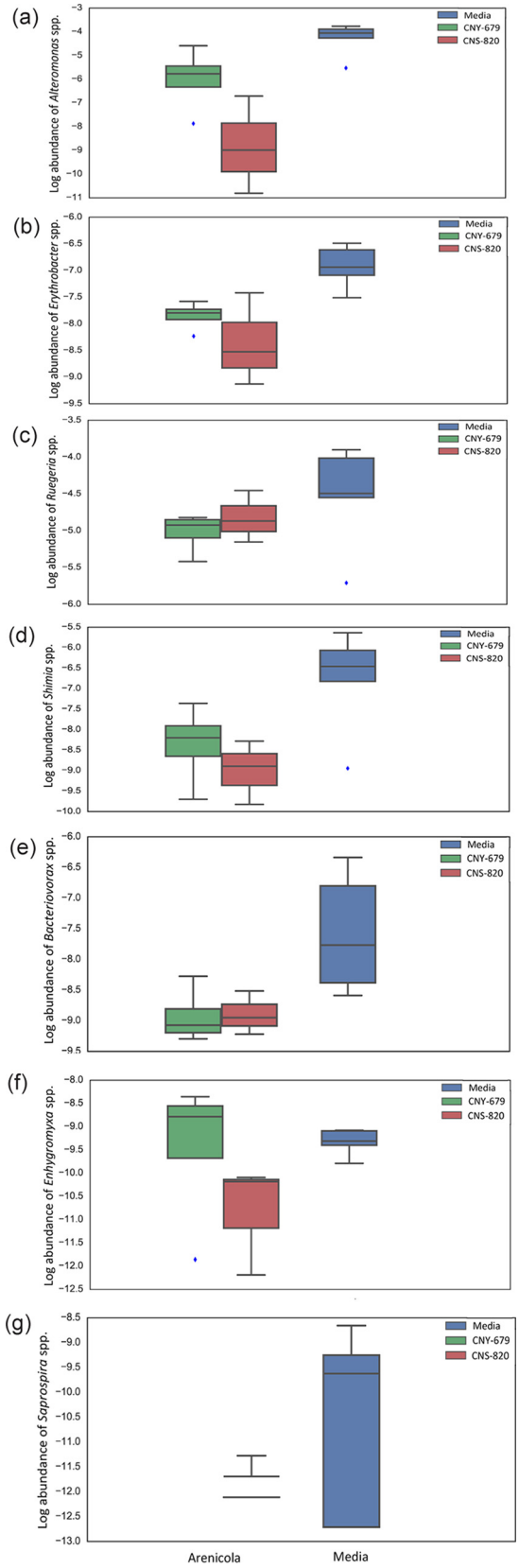

FIG 4 Box plots showing relative abundances of genera depleted in the *S. arenicola* treatment mesocosms. Values have been normalized and log transformed. Boxes show the quartiles of the data set, and whiskers show the remaining distribution, with outliers represented by blue diamonds. Genera are as identified on the *y* axes.

TABLE 1 Strains tested for sensitivity to *S. arenicola* CNY-679 and *S. arenicola* CNS-820 culture extracts

		Extract sensitivity by strain and treatment[b]					
		CNS-820			CNY-679		
Genus	Test strain[a]	Control	1 mg/ml	10 mg/ml	Control	1 mg/ml	10 mg/ml
Ruegeria	*Ruegeria* sp. strain CUA-829	Y	Y	Y	Y	Y	Y
Erythrobacter	*Erythrobacter* sp. strain CUA-862	Y	Y	Y	Y	N	Y
Erythrobacter	*Erythrobacter* sp. strain CUA-812	Y	N	N	Y	N	N
Alteromonas	*Alteromonas* sp. strain CUA-818	Y	Y	Y	Y	Y	Y
Alteromonas	*Alteromonas* sp. strain CUA-848	Y	Y	Y	Y	Y	Y
Shimia	*Shimia* sp. strain CUA-847	Y	Y	Y	Y	Y	Y
Enhygromyxa	*Myxococcus xanthus* DK1622	Y	Y	Y	N	N	Y
Saprospira	*Saprospira grandis*	Y	Y	Y	Y	N	Y
Labrenzia	*Labrenzia* sp. strain CUA-809*	N	Y	Y	N	Y	Y
Vibrio	*Vibrio* sp. strain CUA-759*	N	N	N	N	N	N

[a]Seven strains were chosen as representatives of taxa that were inhibited in the mesocosms while two strains (*) represented taxa that were unchanged in relative abundances between the treatment and control mesocosms.
[b]Y, inhibition; N, no inhibition.

(Fig. 5; Table S3). All of these compounds have been previously reported from *S. arenicola* (49), and all three metabolite groups contained strain-specific analogs. Out of 119 bacterial metabolites, 55 were present in the extracts of both *S. arenicola* strains, while 24 were unique to *S. arenicola* CNS-679, and 40 were unique to *S. arenicola* CNS-820. There were two clusters with five or more nodes that were exclusively seen in the *S. arenicola* extracts but could not be matched to known compounds. From these clusters, eight nodes were unique to the CNS-820 extract, and three were unique to CNY-679, with the remaining nodes seen in both extracts. Additionally, there were eight clusters containing between two and four nodes that were not observed in the medium and did not match any known compounds. Among the known compounds identified, rifamycins possess antibiotic activity targeting RNA polymerase (58), while the staurosporines are cytotoxic inhibitors of protein kinase (59).

DISCUSSION

The first aim of this study was to gain a better understanding of microbial diversity in near-shore marine sediments. Although next-generation sequencing technologies have been applied to many different environments, relatively few studies have addressed microbial communities in tropical sediments. This lack of attention is surprising, given that these environments adjoin important coastal habitats, including coral reefs, sea grass beds, and mangrove forests. Moreover, shallow sediments are known to harbor bacteria such as actinomycetes that are enriched in the production of secondary metabolites, with numerous compounds reported over the last few decades (39, 60, 61).

We analyzed five replicate sediment samples from a Fijian shallow reef habitat to generate baseline data describing microbial diversity in this near-shore environment. To minimize next-generation sequencing biases and platform-specific errors (62), we applied rigorous quality controls (QCs) at every step of sample preparation, sequencing, and data analysis. Environmental DNA was subjected to a minimum number of amplification cycles to minimize PCR errors. We used the most up-to-date Ion Torrent chip and Hi-Q Sequencing kit, which feature significant quality improvements over previous chips and chemistries (63). We further adopted strict sequence quality filters, including an average Q-score of 28. We sequenced to a minimum depth of 100,000 reads per sample and clustered OTUs at 99% 16S rRNA sequence identity to maximize the resolution of the data. Alpha diversity estimates revealed that this level of sequencing was insufficient for a comprehensive portrayal of the community as observed OTUs did not reach saturation (Fig. 1). Replication within a small area showed fine-scale heterogeneity in the sediment community, with four of the five samples showing similar phylum-level composition while the fifth was more similar to the starting conditions for the mesocosm experiments (see Fig. S2 in the supplemental material). This outlier was enriched in a phylum that included spore-forming bacteria and contained fewer

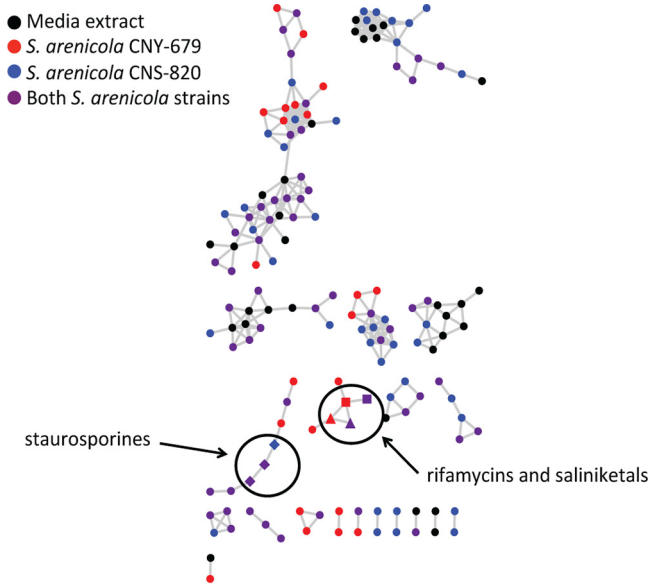

FIG 5 Molecular network of MS/MS data from the three extracts used in the mesocosm experiments: medium control, *S. arenicola* CNS-820, and *S. arenicola* CNY-679. Each node represents a parent ion that fragmented to produce MS/MS data. Closely related nodes (cosine value of 0.6) are connected by an edge. Nodes denote ions present according to the color legend on the figure. Clusters with nodes that matched known compounds are circled, and compounds are indicated as follows: triangles, rifamycin analogs; squares, saliniketal analogs; diamonds, staurosporine analogs.

cyanobacterial sequences, suggesting that it may have originated from farther below the sediment surface. These results reflect the complexity of marine sediments and the challenges associated with replicate sampling. Furthermore, a recent study on the fidelity of universal PCR primers demonstrated that certain components of the community were likely missed (64). Notably, up to 3.8% of the reads in each of our samples could not be classified at the phylum level, suggesting that marine sediments harbor major microbial taxa that have yet to be classified.

One notable finding was the prevalence of largely uncultured taxa, including the phyla *Verrucomicrobia*, *Acidobacteria*, and *Planctomycetes*. *Verrucomicrobia* are nearly ubiquitous in soil (65) and include free-living and symbiotic representatives, as well facultative anaerobes and methanotrophs (66, 67). In the marine environment, they are found in the water column and sediments (68–70), and different lineages appear to have different habitat preferences (70). Likewise, *Acidobacteria* are found in high abundance in soils, sediments, and deep-sea marine environments. They have few cultured representatives and play unknown roles in these ecosystems (71, 72). The phylum *Planctomycetes* comprised up to 13.3% of the sediment community and includes the lineage responsible for anaerobic ammonia oxidation (73, 74). These bacteria play important roles in carbon, nitrogen, and sulfur cycling and have been studied for their unusual features, including the lack of peptidoglycan and intracellular compartmentalization (75). Additionally, the persistence of the *Euryarchaeota* across all five replicates is evidence that this least-understood domain of life is an ecologically important component of sediment communities. Conversely, the rarity of actinomycetes is surprising, given the relative ease with which they can be cultured from these types of sediments (36, 42). We detected extremely low numbers of *Streptomyces* and *Salinispora* spp., with three sediment samples yielding no *Streptomyces* sequences. These results may either indicate sediment heterogeneity or simply show that some rare taxa go undetected in complex communities at a sequencing depth of 100,000 reads.

The ultimate goal of this study was to address the effects of bacterial secondary metabolites on community structure. While the ecological functions of bacterial sec-

ondary metabolites remain poorly understood, even less is known about how these compounds may help structure bacterial communities. In this case, we selected the sediment-inhabiting marine actinomycete *S. arenicola* because it is a rich source of secondary metabolites and has been shown to employ interference competition as a competitive strategy (18). Although this species may be a rare member of the bacterial community, its mycelial growth form and ability to secrete biologically active secondary metabolites suggest that it could have a major impact on localized community structure, thereby contributing to sediment microbial heterogeneity. We extracted the *Salinispora* cultures using ethyl acetate since most compounds isolated to date from this genus are ethyl acetate soluble. However, these extracts likely underestimate the effects of *Salinispora* metabolites on community composition as more polar compounds were likely missed.

The mesocosm experiments were designed to simulate natural conditions while retaining the ability for replicate sampling and the control of experimental variables. Nevertheless, taking sediment from an open system and establishing mesocosms unavoidably altered community composition, as was observed by the depletion of cyanobacteria relative to what was observed in the native sediments. Additionally, it is unknown whether the extract concentrations in the treated mesocosms were ecologically relevant. Given that the metabolite concentrations experienced by bacteria in nature remain unknown, our goal was to select test concentrations that would provide some insight into the types of bacteria that could be affected but that remained below the levels typically used to screen for antibiotic activity. There has been much debate on the natural function of compounds like antibiotics (76–78), with evidence suggesting that sublethal concentrations can act as signaling molecules rather than chemical weapons (30, 79–82). Despite these caveats, we found consistent effects on the microbial communities under treatment conditions and used these to generate testable hypotheses about the targeted taxa. In the future, it would be useful to test a range of extract concentrations to better understand the levels that must be reached in nature to achieve biological relevance.

The observation that several taxa were significantly depleted in the treated mesocosms provides evidence that microbial secondary metabolites can affect sediment microbial communities. The chemical repertoire of *S. arenicola* is large and includes the rifamycins (49), a group of ansamycin antibiotics with activity against Gram-positive bacteria (83). Rifamycin production has been shown to play a role in the competitive strategy of *S. arenicola*, which contrasts with the lack of production of any known antibiotics by the closely related species *S. tropica* (18). While many *S. arenicola* compounds have biological activity, their ecological functions remain unknown. Nevertheless, it is unsurprising that many taxa appeared to be consistently inhibited by the secondary metabolites present in the treatment mesocosms. More challenging to explain is the prevalence of taxa that were significantly enriched in the *S. arenicola* treatments, including the phyla *Chloroflexi* and "*Candidatus* Latescibacteria" (Fig. 3 and Fig. S3). Ecological cascades resulting from the inhibition of certain taxa, like predatory bacteria, could result in the enrichment of the bacteria on which they prey. Another possible explanation for the clades enriched in the treated mesocosms is that when strains occupying certain niches are inhibited, their competitors can bloom. Teasing apart such fine-scale interactions was beyond the scope of this study; nevertheless, it is clear that complex ecological dynamics were at play in these mesocosms.

Notably, many of the inhibitory effects were more pronounced in the mesocosms treated with extracts from CNS-820 than in those treated with CNY-679. The extract molecular network showed nodes unique to both *S. arenicola* strains (Fig. 5), which is not surprising, given the genetic diversity associated with secondary metabolism in this species (45, 84). However, almost twice as many nodes were unique to CNS-820, suggesting that the chemical repertoire of this strain may exceed that of CNY-679 and contribute to its apparent greater inhibitory capacity. The discrepancy between the activities of the two strains may also have biased the overall LDA effect size analysis,

which addressed the differences in taxa between the control mesocosms and the combined *S. arenicola* treatments (Fig. 3).

Although most of the significantly affected taxa remain uncultured, we identified several with cultured representatives that could be tested as a follow-up to the results observed in the mesocosm studies. These included two genera of predatory bacteria, *Bacteriovorax* and *Saprospira*, and the bacteriolytic genus *Enhygromyxa*. *Bacteriovorax* spp. are found in animal gut and marine environments; until recently, they were thought to prey exclusively on Gram-negative bacteria, but new evidence suggests that they may also be capable of preying on Gram-positive bacteria (85). *Saprospira* spp. are filamentous, gliding bacteria in the phylum *Bacteroidetes* that have been isolated from marine and freshwater environments. They use direct contact to trap their prey, which include Gram-negative bacteria as well as cyanobacteria (86) and microeukaryotes like diatoms (87). Because we were unable to secure a strain of *Enhygromyxa* sp. for follow-up assays, we tested *Myxococcus xanthus*, another predatory member of the order *Myxococcales*. *Myxococcus* spp. have been isolated from marine and terrestrial environments and are characterized by their complex social behavior (88). They prey on taxonomically diverse bacteria, including the actinomycete *Streptomyces coelicolor*, whereupon *M. xanthus* induces higher production of the antibiotic actinorhodin and triggers aerial mycelium production (89). Precedence therefore exists for chemical defense by actinomycetes in response to bacterial predation. Combining these observations with our data showing inhibition of three predatory taxa in the *S. arenicola* treatment mesocosms, we were strongly motivated to include predatory strains in our extract sensitivity assays.

The culture-based sensitivity tests were generally consistent with the results predicted by the mesocosm data, with seven out of eight strains showing sensitivity to at least one of the two *S. arenicola* extracts (Table 1). More strains were sensitive to the lower extract concentration of CNS-820, supporting the trend seen in the mesocosm results. Coupled with the differences detected in the MS/MS data, this observation indicates that the two *S. arenicola* strains likely vary in their production of inhibitory compounds. Given the different isolation locations of the two strains (CNY-679, Mexico; CNS-820, Fiji), it is likely that the secondary metabolome of each strain has evolved independently to gain the highest competitive advantage in the location-specific community. Although rifamycins and saliniketals were seen in extracts of both strains, there were several clusters in the network that contained nodes generated from unidentified parent masses. Given the apparent difference in inhibitory activities, *S. arenicola* CNS-820 warrants further chemical study. Furthermore, it will be important to determine the MICs of the extracts against the test strains to better understand the relationships between these activities and the results observed in the mesocosm studies.

In conclusion, we found that marine actinomycete secondary metabolites had consistent effects on microbial community structure in mesocosm experiments. The results provide evidence that rare members of the community can regulate predator-prey dynamics and that bioactive metabolites can influence community composition. The extensive diversity seen in these sediments and the dominance of uncultivated taxa underscore the need for further research targeting this major, yet poorly studied, biome.

MATERIALS AND METHODS

Sediment collection. Six surface sediment samples (upper 1 to 2 cm) from within a 1-m² area were collected by hand in sterile Whirl-Pak bags (Nasco) in August 2014 from a reef flat off Viti Levu, Fiji (Votua Reef; 18°13.049′S, 177°42.968′E) and transported to a nearby field station. Five samples (S1 to S5) were immediately frozen at −20°C. The sixth sample (S6) was used to establish the mesocosm experiments.

Sediment processing and DNA extraction. Samples S1 to S5 were transported on dry ice back to the Scripps Institution of Oceanography, where they were kept frozen until DNA was extracted using a combined chemical and physical lysis protocol as previously described (90). Briefly, ca. 1 g of wet sediment was combined with 10% SDS and sodium phosphate buffer in a tube containing ceramic beads and subjected to vigorous bead beating using a Fast-Prep instrument. The supernatant was combined with sodium acetate (pH 5.3) before being subjected to a phenol-chloroform-isoamyl alcohol (25:24:1)

partition. The aqueous layer was column purified twice using spin columns (Qiagen), and the final product was resuspended in 100 μl of Tris-EDTA (TE) buffer (pH 8).

Library preparation and sequencing. DNA samples were diluted to 4 ng/μl and used as the template for 16S rRNA gene amplification with the primers B341F (CCTACGGGNGGCWGCAG) and B785R (GACTACHVGGGTATCTAATCC) (91) modified with Ion Torrent adaptor sequences and barcodes specific to the individual sample. An initial PCR was performed in quadruple 25-μl reaction mixtures with the following components: 13.3 μl of Milli-Q H_2O, 2.5 μl of 10× HiFi buffer, 1 μl of the deoxynucleoside triphosphate (dNTPs; 4 mM), 0.8 μl of bovine serum albumin (BSA), 0.8 μl of $MgSO_4$ (50 mM), 1.25 μl of B341F, 1.25 μl of B785R, 0.1 μl of Platinum Taq Hi-Fi (Invitrogen), and 4 μl of DNA. Thermocycling conditions were 94°C for 1 min, followed by either 16, 20, 24, or 30 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 1 min, with a final extension at 68°C for 1 min. Each reaction was run on a 0.9% agarose gel, and the minimum cycle number necessary for sufficient amplification was visually determined and used for subsequent PCR runs in triplicate for each sample. The replicate PCR products were combined, run on a 0.9% agarose gel, and purified using a gel extraction kit according to the manufacturer's instructions (Qiagen). Sets of six samples were combined in equal concentrations for multiplexed Ion Torrent sequencing.

An Ion PGM Hi-Q OT2 kit (Thermo Fisher Scientific) was used for sample preparation with an Ion OneTouch 2 system with a modified thermoprofile that included extended cycling parameters for longer extension times and an increased denaturation temperature. Sequencing was performed using an Ion Torrent Personal Genome Machine with an Ion PGM Hi-Q sequencing kit (Thermo Fisher Scientific), according to standard protocol, on a 318v2 sequencing chip (Thermo Fisher Scientific). Sequence data for each sample were retained if at least 100,000 reads were generated (see Table S1 in the supplemental material).

Sequence data processing. Raw sequence data were demultiplexed and quality filtered using the software Quantitative Insights Into Microbial Ecology (QIIME) (53). Reads were filtered under the following parameters: average quality control (QC) score of 28, no homopolymers longer than 6 bases, and no primer mismatches. Sequences were clustered into OTUs at 99% identity using uclust, and taxa were assigned using PyNast (92) with the most current Silva database (52). The resulting OTUs were analyzed for alpha and beta diversity using QIIME. Pairwise Bray-Curtis dissimilarity values were calculated between replicates of each sample group, and the average values were used to determine within-group dissimilarity. Average values were also calculated between all sample group pairs (medium control and CNY-679 treatment, medium control and CNS-820 treatment, and the two *S. arenicola* treatments) to determine between-group dissimilarity. All principal coordinates analysis (PCoA) plots were generated using the unweighted Unifrac metric in EMPeror (93).

Mesocosm experimental setup. *S. arenicola* strains isolated from the Mexican Caribbean (CNY-679) and Fiji (CNS-820) (NCBI Sequence Read Archive [SRA] accession numbers SRP054153 and SRP054138, respectively) were selected to generate organic extracts for the mesocosm studies. Each strain was cultured in 1 liter of autoclaved A1M1 medium (5 g of starch, 2 g of yeast extract, 2 g of peptone, 22 g of Instant Ocean, 1 liter of deionized water). Strains were inoculated from frozen cultures into 25 ml of A1M1 medium, scaled up to 1 liter, and extracted using 1 liter of ethyl acetate after 10 days of growth at room temperature with shaking at 200 rpm. The organic phase was separated and concentrated to dryness under vacuum using rotary evaporation. An uninoculated A1M1 medium control extract was similarly prepared. Culture and medium control extracts were resuspended in methanol, filtered (0.2-μm-pore-size filter; Whatman), and added to 20-ml scintillation vials that were first autoclaved for 30 min and then UV sterilized for 10 min to degrade residual DNA. The amount of extract added was equivalent to that obtained from 20 ml of culture or uninoculated medium (Table S2). The solvent was evaporated under N_2, and the vials were capped and transported to the experimental site.

Within 1 h of collection, approximately 3 g of sediment (sample S6) and 3 ml of sterile-filtered (0.2-μm-pore-size filter) seawater were added to vials that contained either treatment (*Salinispora* culture extracts) or A1M1 medium control extracts. Final extract concentrations for each vial were between 0.25 and 0.3 mg/ml for the *S. arenicola* treatments and 0.1 mg/ml for the medium controls (see Table S2). Vials were loosely capped to ensure gas exchange and incubated at room temperature (~25°C).

Mesocosm sample processing and data analysis. Five replicate treatment mesocosms containing extracts from each of the two *Salinispora* species and five medium extract controls were frozen (−20°C) immediately following the addition of the sediment and seawater to the vials (0 h; T0) and again after 6 h, 24 h, 72 h, and 5 days (T1 to T4, respectively). All mesocosm samples were processed and analyzed as described above for the native sediment samples.

To characterize differences in community composition between treatment and control mesocosms, the LDA effect size (LEfSe) algorithm was used (54) (https://huttenhower.sph.harvard.edu/galaxy/root). The results from the five replicate native (untreated) sediment samples (S1 to S5) were combined to assess microbial diversity at the sampling site.

The mesocosm samples were classified as either control or *S. arenicola*, and a subgrouping within *S. arenicola* was classified as either CNY-679 or CNS-820 to designate the appropriate strain. Mesocosm communities were considered significantly affected by the treatments (either enriched or inhibited) if the alpha value for either the factorial Kruskal-Wallis test (class level) or the pairwise Wilcoxon test (subclass level) was ≤0.05. The threshold on the logarithmic LDA score for discriminative features was 2.0. The strategy for multiclass analysis was all-against-all. The LEfSe results were visualized using GraPhlAn (94). Box plots for each significantly affected taxon were generated using IPython and the Python visualization library Seaborn (http://seaborn.pydata.org). A Mann-Whitney U test was performed to test for significant differences in the cyanobacterial components of the mesocosm T0 communities and the native sedi-

ments. *Cyanobacteria* community percentages from all mesocosm T0 samples were grouped together and compared to those of all native sediment samples using the SciPy stats library in IPython (95). The same test was used to determine differences in alpha diversity measurements as represented by the number of OTUs observed in the *S. arenicola* treatment mesocosms and the medium control mesocosms.

Bacterial sensitivity assays. Ten bacterial strains were selected to test for sensitivity to *S. arenicola* culture extracts based on their relationships to taxa that were significantly affected in the mesocosm studies. Eight of these strains were isolated from tropical sediments as part of a previous study (18) and grown on A1 agar medium (10 g of starch, 4 g of yeast extract, 2 g of peptone, 22 g of Instant Ocean, 16 g of agar, 1 liter of deionized water). *Myxococcus xanthus* DK1622 (GenBank accession number CP000113) was kindly provided by Rolf Mueller (Saarland University) and grown on CTT agar (96), and *Saprospira grandis* (ATCC 23124) was purchased from the ATCC and grown on RL1 agar (2 g of yeast extract, 3 g of peptone, 0.5 g of KNO_3, 1 ml of trace elements [2.85 g of H_3BO_3, 1.8 g of $MnCl_2{\cdot}4H_2O$, 1.36 g of $FeSO_4$, 1.77 g of sodium tartrate, 26.9 mg of $CuCl_2{\cdot}2H_2O$, 20.8 mg of $ZnCl_2$, 40.4 mg of $CoCl_2{\cdot}6H_2O$, 25.2 mg of $Na_2MoO_4{\cdot}2H_2O$, 1 liter of deionized H_2O], 5 ml of vitamin B_{12}, 16 g of agar, and 1 liter of filtered seawater, pH 7).

All of the above strains were tested for sensitivity to extracts of *S. arenicola* strains CNY-679 and CNS-820 generated as previously described. The extracts were tested at 1 and 10 mg/ml in methanol by applying 10 μl to paper discs, allowing the solvent to evaporate, and placing the discs onto petri plates containing agar seeded with the test strains. These plates were prepared using 50 ml of A1 medium containing 0.8% agar, which was cooled to 65°C before the addition of 5 ml of overnight culture of the test strain. All tests were performed in triplicate and included solvent-only and ciprofloxacin (5 μg) (Fisher Scientific) controls. Results were checked for 1 to 4 days depending on the growth rate of the strain, and activity is reported as the diameters of zones of clearing around each disc.

MS/MS molecular networking. Extracts were dissolved in MeOH at a final concentration of 1.0 mg/ml and injected onto an Agilent 6530 Accurate-Mass quadrupole time of flight (Q-TOF) spectrometer coupled to an Agilent 1260 liquid chromatography (LC) system. The gradient LC conditions were 1 to 5 min with 10% MeCN in H_2O, 5 to 26 min with 10 to 100% MeCN, and 26 to 30 min with 100% MeCN, all run with 0.1% trifluoroacetic acid (TFA) on a Phenomenex Kinetex C_{18} reversed-phase high-performance LC (HPLC) column (internal diameter, 2.6 mm; 100 by 4.6 mm). The divert valve was set to waste for the first 5 min. Q-TOF MS settings were as follows: positive ion mode mass range of 300 to 2,500 *m/z*, MS scan rate of 1/s, MS/MS scan rate of 5/s, fixed collision energy of 20 eV, source gas temperature of 300°C, gas flow of 11 liters/min, and nebulizer at 45 pounds per square inch gauge (psig). Scan source parameters were as follows: capillary voltage (VCap), 3,000; fragmentor, 100 V; skimmer1, 65 V; octopole radio-frequency (RF) peak, 750 V. The MS was auto-tuned using Agilent tuning solution in positive mode before each measurement. LC (photodiode array detection [DAD]) data were analyzed with ChemStation software (Agilent), and MS data were analyzed with MassHunter software (Agilent). High-resolution MS/MS data were used to generate molecular networks. The Global Natural Products Social molecular networking database (GNPS) (57) dereplication tool was used to identify known compounds with a cosine value cutoff of 0.6 and to format MS/MS data for network visualization in Cytoscape (97). Network nodes with parent ions matching known compounds in the database are listed in Table S3.

Accession number(s). All sequence data from this study were submitted to the NCBI Sequence Read Archive (SRA) under accession number SRP080800.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02676-16.

TEXT S1, PDF file, 0.6 MB.

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