

ORIGINAL ARTICLE

Competitive strategies differentiate closely related species of marine actinobacteria

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Although competition, niche partitioning, and spatial isolation have been used to describe the ecology and evolution of macro-organisms, it is less clear to what extent these principles account for the extraordinary levels of bacterial diversity observed in nature. Ecological interactions among bacteria are particularly challenging to address due to methodological limitations and uncertainties over how to recognize fundamental units of diversity and link them to the functional traits and evolutionary processes that led to their divergence. Here we show that two closely related marine actinomycete species can be differentiated based on competitive strategies. Using a direct challenge assay to investigate inhibitory interactions with members of the bacterial community, we observed a temporal difference in the onset of inhibition. The majority of inhibitory activity exhibited by *Salinispora arenicola* occurred early in its growth cycle and was linked to antibiotic production. In contrast, most inhibition by *Salinispora tropica* occurred later in the growth cycle and was more commonly linked to nutrient depletion or other sources. Comparative genomics support these differences, with *S. arenicola* containing nearly twice the number of secondary metabolite biosynthetic gene clusters as *S. tropica*, indicating a greater potential for secondary metabolite production. In contrast, *S. tropica* is enriched in gene clusters associated with the acquisition of growth-limiting nutrients such as iron. Coupled with differences in growth rates, the results reveal that *S. arenicola* uses interference competition at the expense of growth, whereas *S. tropica* preferentially employs a strategy of exploitation competition. The results support the ecological divergence of two co-occurring and closely related species of marine bacteria by providing evidence they have evolved fundamentally different strategies to compete in marine sediments.

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Introduction

Molecular analyses reveal extraordinary levels of bacterial diversity in ocean environments (Sogin *et al.*, 2006; Huber *et al.*, 2007). This diversity creates a paradox in terms of how a limited range of resources can support unexpectedly large numbers of species, as has long been observed among phytoplankton (Hutchinson, 1961). The majority of bacterial diversity observed in nature falls into closely related groups of sequences that have been described as microdiverse sequence clusters (Acinas *et al.*, 2004). The ecological implications of this microdiversity remain largely unknown, yet are crucial for understanding community structure and function (Koeppel *et al.*, 2008). Among examples where phylogenetic diversity has been linked to ecological differentiation, it has been possible to

identify functional traits that distinguish related groups of bacteria (Ferris *et al.*, 2003; Sikorski and Nevo, 2005; Johnson *et al.*, 2006) including links between sympatric speciation, competition-dispersal tradeoffs (Yawata *et al.*, 2014) and resource partitioning (Hunt *et al.*, 2008; Oakley *et al.*, 2010). However, the delineation of fundamental units of diversity that maintains species-like properties and the ecological traits that make them distinct remains a major challenge in the field of microbial ecology (Fraser *et al.*, 2009).

Resource competition is thought to be a major driver of evolutionary diversification (Svanbäck and Bolnick, 2007). Two competitive strategies by which organisms compete for resources are exploitation competition, which is characterized by rapid nutrient utilization, and interference competition, which occurs when one organism directly harms another. Each strategy involves tradeoffs in energy investment that are manifested as differences in growth rates and reproduction (Nicholson, 1954; Case and Gilpin, 1974; Little *et al.*, 2008). Among bacteria, there is evidence that exploitation competition drives diversification in lab cultures (Hibbing *et al.*, 2010),

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whereas interference competition is possibly best known in regards to suppressive soils (Weller *et al.*, 2002) and has been proposed as a mechanism to explain the high levels of diversity observed in temporally constant and spatially homogenous environments (Czárán *et al.*, 2002). Chemically mediated interference competition has been linked to improved fitness in biofilm-forming bacteria (Rao *et al.*, 2005; Shank *et al.*, 2011) and shown to affect community structure in hypersaline mats (Long *et al.*, 2013) and freshwater sediments (Pérez-Gutiérrez *et al.*, 2013), with theoretical models supporting the hypothesis that antibiotic production can improve fitness and stimulate biodiversity (Little *et al.*, 2008; Hibbing *et al.*, 2010). In the marine environment, it has been shown that interference competition is greater among *Vibrio* populations than within them (Cordero *et al.*, 2012) and more common among particle-associated bacteria than those that are free living (Long and Azam, 2001). However, the extent to which bacteria employ interference vs exploitation competition in nature remains unknown due to a poor understanding of the spatiotemporal dynamics of microbial interactions and a lack of suitable methodologies for characterizing these processes. Furthermore, microbes experience other metabolic tradeoffs (Litchman *et al.*, 2007; Flamholz *et al.*, 2013) that complicate efforts to link phylogeny with specific functional traits. Although new genomic and spectral-imaging techniques have provided improved methods to address the ecology of both cultured and uncultured microbes (Watrous *et al.*, 2012; Hugoni *et al.*, 2013; Ottesen *et al.*, 2013; Rinke *et al.*, 2013), competitive interactions among groups of closely related bacteria remain largely unknown.

As a major source of biologically active natural products (Berdy, 2005), actinomycetes represent a particularly interesting subject for chemical ecology studies. They are common inhabitants of complex environments such as terrestrial soils and marine sediments, where it has long been hypothesized that the secondary metabolites they produce mediate interactions with competing microbes (Williams *et al.*, 1989; Jarvis, 1995). The actinomycete genus *Salinispora* is readily cultured from marine sediments (Jensen *et al.*, 2005; Mincer *et al.*, 2005) and has proven to be a useful model to address bacterial biogeography (Jensen and Mafnas, 2006), species concepts (Jensen, 2010), and the evolution of secondary metabolism (Freel *et al.*, 2011; Ziemert *et al.*, 2014). It is comprised of three closely related species, *Salinispora arenicola*, *Salinispora tropica* and *Salinispora pacifica* (Maldonado *et al.*, 2005; Ahmed *et al.*, 2013), which share 99% 16 S ribosomal RNA gene sequence identity (Jensen and Mafnas, 2006), thus placing them within a micro-diverse sequence cluster (Acinas *et al.*, 2004). The cells form branching filaments that develop into a mycelium and produce dormant, non-motile spores that are broadly distributed over large geographic

areas (Jensen *et al.*, 2005). Existing evidence suggests that *S. tropica* and *S. pacifica* are geographically isolated (Mincer *et al.*, 2005; Freel *et al.*, 2012), whereas the co-occurrence of both species with the more cosmopolitan and abundant species *S. arenicola* has been used as evidence for ecological divergence (Jensen and Mafnas, 2006). Although patterns of secondary metabolite production (Jensen *et al.*, 2007) and biosynthetic gene cluster distribution (Penn *et al.*, 2009; Ziemert *et al.*, 2014) have been linked to *Salinispora* species-level divergence, functional support for sympatry has remained elusive.

In this study, we assessed the effects of *S. arenicola* and *S. tropica* on the growth of a diverse collection of co-occurring environmental bacteria in an effort to determine the extent to which secondary metabolites mediate competitive interactions. Direct challenge assays revealed distinct temporal patterns in the onset of growth inhibition, with *S. arenicola* employing interference competition mediated by antibiotic production and the relatively fast growing *S. tropica* preferentially employing exploitation competition. Although these two mechanisms of competition are well known to occur among plants, the results provide evidence that competitive strategies represent functional traits that can be used to distinguish between closely related yet ecologically distinct populations of bacteria.

Materials and methods

Sediment collection and processing

Sediment samples were collected via SCUBA at depths from 3 to 16 m in July 2012 during a research cruise aboard the R/V Walton Smith (U Miami). Individual sediment samples (5–10 g per sample) were collected from the sediment surface to depths of ca. – 3 cm using sterile Whirl-Pak bags (Nasco, Ft. Atkinson, WI, USA). Locations included sites off Miami and the Dry Tortugas in the United States and Cancún, Cozumel, Akumal, and Banco Chinchorro in the Mexican Caribbean. All samples were processed immediately aboard ship using two methods: drying and stamping for selective actinomycete cultivation (Mincer *et al.*, 2002) and serial dilution and plating for the general cultivation of marine bacteria. For the latter, ca. 1 g of sediment was serially diluted 1:1, 1:10 and 1:100 in sterile seawater, vortex mixed, and 50 µl of the supernatant inoculated onto agar media and spread with a sterile glass rod. Three types of media were used for both methods: (1) 25% marine agar (9.3 g Marine Broth Difco 2216, 16-g agar, 750-ml 0.2-µm filtered seawater, 250-ml deionized water), (2) seawater-agar (16-g agar, 1-l 0.2-µm filtered seawater) and (3) 25% A1 (2.5-g starch, 1-g yeast extract, 0.5-g peptone, 16-g agar, 750-ml 0.2-µm filtered seawater, 250-ml deionized water).

Strain isolation

Salinispora strains were recognized based on colony morphology (Mincer *et al.*, 2002) and repeatedly transferred onto new agar media until pure cultures were obtained as evidenced by uniform colony morphology. The collection of sediment-derived bacteria used in the direct challenge assays were purified in a similar manner and selected to represent a diverse range of colony morphologies and pigmentation. All strains were maintained on medium A1 prepared with 75% artificial seawater (22 g/l Instant Ocean, United Pet Group, Cincinnati, OH, USA), grown with shaking in A1 without agar (hereafter 'A1') and cryopreserved at -80°C with 10% glycerol.

DNA extraction, PCR and 16 S ribosomal RNA gene sequencing

All strains presumed to be actinomycetes based on morphology were grown in 7-ml A1 for 3–10 days while shaking at 160 rpm. DNA was extracted from 1 ml of the resulting culture according to the DNeasy protocol (Qiagen Inc., Valencia, CA, USA) with previously described changes (Gontang *et al.*, 2007). For all other strains, colony PCR was performed by suspending a single colony in 5- μl dimethyl sulfoxide and using 1 μl as the PCR template. 16 S ribosomal RNA PCR primers are described in Supplementary Table S4. Each PCR consisted of a 25 μl mixture containing 10 \times PCR Buffer (Applied Biosciences, Foster City, CA, USA), 2.5 mM MgCl_2 (Applied Biosciences), 0.7% dimethyl sulphoxide, 10 mM dNTPs, 1.5 U AmpliTaq Gold DNA Polymerase (Applied Biosciences) and 10 μmol of each primer. PCR thermocycling conditions were as follows: 5 min of initial denaturation at 95°C followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min. Sequencing was performed by SeqXcel, Inc. (San Diego, CA, USA). Sequences were submitted to the National Center for Biotechnology Information Basic Local Alignment Search Tool (BLASTN) and identified based on the taxonomic assignment of the closest Basic Local Alignment Search Tool match.

Direct challenge assays

Salinispora cultures were inoculated from frozen stocks into 25 ml A1. Cultures were shaken at 160 rpm for 6 days (*S. tropica*) or 11 days (*S. arenicola*) after which 60 μl was transferred by pipet to square 150 \times 150 mm petri plates containing 100 ml of A1 agar media. The cultures were spread with a sterile loop down the center of the plate to create a narrow (ca. 3–5 mm) lawn of growth and allowed to grow for 7 days (*S. tropica*) or 10 days (*S. arenicola*), based on the time required to reach confluence. These incubation periods correspond to the entry of liquid cultures into stationary phase

(Supplementary Figure S1). Environmental isolates were then inoculated in triplicate from established plate colonies to within 1–2 mm of the *Salinispora* lawn (cross-streaking) with up to 50 perpendicular inoculations made per plate using sterile toothpicks (replicates inoculated onto different plates). Growth of the test strains was considered inhibited if a clearing zone ≥ 5 mm was observed in the area adjacent to the *Salinispora* lawn in at least two of three replicate assays. The pH within the zones of inhibition was tested in comparison with a medium control using pH test strips (Micro Essential Laboratories Inc., New York, NY). Given that antibiotic production can be time-dependent (Bibb, 1996), a second series of assays was performed in which strains from both *Salinispora* spp. were allowed to grow on agar plates for 23 days prior to adding the bacterial strains. Only strains that showed no evidence of inhibition in the initial assays were tested at this second time point.

Interference vs exploitation competition assays

Follow-up assays were performed to distinguish between interference competition (the production of diffusible growth inhibitors) and exploitation competition (nutrient depletion) as the source of the growth inhibition detected in the direct challenge assays. Agar diffusion assays were performed in triplicate using two *S. arenicola* (CNY-679 and CNY-685) and two *S. tropica* (CNY-678 and CNY-681) strains. These strains were grown in the same manner used in the direct challenge assays, after which 1 cm^2 agar blocks were cut from the area immediately adjacent to the culture and placed over a freshly inoculated lawn of an environmental isolate that previously tested positive for growth inhibition. The lawns were periodically checked (1–10 days) for zones of inhibition surrounding the agar block and scored as positive when a clear zone (≥ 5 mm) was observed. The blocks were visually assessed throughout the tests to be free of *Salinispora* colonies, which are easily recognized based on morphology, so that any observed inhibition could be linked to the diffusion of compounds that were transferred with the agar blocks. All strains that were not inhibited in the agar diffusion assay were further analyzed to determine whether the cause of the activity observed in the direct challenge assay was due to iron depletion. In this case, the direct challenge assays were repeated using standard A1 and A1 supplemented with FeSO_4 (10 $\mu\text{g ml}^{-1}$ final concentration). If growth was inhibited on A1 but not on iron-replete A1, the inhibition was attributed to iron depletion.

Growth rates

Salinispora growth rates were determined by changes in liquid culture dry weight biomass over time. *Salinispora* cultures were inoculated from

frozen bacterial stocks (1.8 ml) into 50 ml A1. Following 7 days of growth (25 °C, 160 rpm), 1-ml of this culture was inoculated into each of 24 glass tubes containing 10 ml A1 and shaken at 160 rpm (25 °C). On days 0, 3, 6, 9, 12, 15 and 18, triplicate tubes were filtered onto pre-weighed 47-mm glass fiber filters (Pall Corporation, Ann Arbor, MI, USA), dried overnight (32 °C) and weighed. Cell mass was calculated as mg dry weight per ml and growth curves generated by plotting the log of cell mass vs time. Growth rates were calculated as the change in biomass over time during the exponential phase of growth.

Chemical extractions and disc-diffusion assays

S. arenicola CNY-679 and *S. tropica* CNY-678 were grown on A1 agar plates for 10 and 23 days, respectively. Cell-free agar adjacent to *Salinispora* growth was removed and cut into small pieces using a sterile scalpel and extracted using methanol (500 ml, 160 rpm, 2 h). The volume of agar extracted was measured by solvent displacement. The extract was filtered (0.45 µm Whatman), dried *in vacuo*, dissolved in ca. 10 ml of water and extracted with an equal volume of ethyl acetate. The ethyl acetate layer was separated, filtered (0.45 µm Whatman), dried under N₂, and weighed. A1 agar media control extracts were similarly prepared. Extracts were dissolved in methanol at 1×, 10× and 100× volumetrically equivalent concentrations with 1× equal to the extract being dissolved in a volume of solvent equivalent to the volume of agar extracted. Extracts were tested for antibiotic activity against environmental isolates using standard disc-diffusion assays. For these assays, 15-µl of *Salinispora* extract, media extract or solvent controls (MeOH) were added to paper discs, allowed to dry and placed onto A1 agar plates, along with an antibiotic control disc (5 µg ciprofloxacin; BD, Sparks, MD, USA), onto which a bacterial strain had been inoculated as per the agar diffusion assays. Zones of inhibition were recorded as the diameter of clear halos surrounding the discs. Thirteen of the strains showing sensitivity to *S. arenicola* CNY-679 culture extracts were similarly tested for sensitivity to commercially available rifamycin SV (Sigma-Aldrich, St Louis, MI, USA) at concentrations of 0.01, 0.1, and 1 mg ml⁻¹.

Liquid chromatography-tandem mass spectrometry and MALDI-TOF imaging mass spectrometry

High-resolution liquid chromatography-tandem mass spectrometry was performed using an Agilent 6530 Accurate Mass Q-TOF coupled to an Agilent 1260 LC system (Santa Clara, CA, USA) as described in the Supporting Information. Imaging mass spectrometry was performed on agar plates prepared as per the direct challenge assays using *S. arenicola* CNY-679 and *Kytococcus* sp. CUA-766. The latter

was chosen based on the consistently large zones of inhibition observed in the direct challenge assays with all four *S. arenicola* strains. The interaction zone, along with monocultures of both strains and a medium control, were processed for MALDI-based imaging mass spectrometry in positive mode using a Microflex Bruker Daltonics mass spectrometer as described in the Supporting Information.

Statistical analyses

A non-parametric PERMANOVA analysis was used to test for correlations between growth inhibition and the taxonomy of the strains used in the direct challenge assays (Anderson, 2001). This test was performed using the 'adonis' function provided by the vegan package (Oksanen *et al.*, 2014) and run in the statistical program R (R Core Team). Welch's two-sample *t*-tests (Welch, 1947) were performed in R to test for significant differences between growth rates ($n=4$ for each species), average zones of inhibition ($n=4$ for each species) and average percentage of strains inhibited ($n=12$ for *S. arenicola*, $n=13$ for *S. tropica*).

Genome sequencing and analysis

All genome sequences were generated as previously described (Ziemert *et al.*, 2014) according to the guidelines of the Department of Energy Joint Genome Institute. Twenty-four genomes (Supplementary Table S3) were downloaded from the Joint Genome Institute website and submitted to antiSMASH for identification of genes associated with secondary metabolism (Medema *et al.*, 2011). Gene clusters linked to rifamycin biosynthesis were submitted to NaPDoS (Ziemert *et al.*, 2012) to confirm their identity based on a phylogenetic analysis of the associated ketosynthase domains. To assess the potential for siderophore biosynthesis, previously identified siderophore gene clusters (Supplementary Table S5) were extracted from the closed genomes of *S. tropica* CNB-440 and *S. arenicola* CNS-205 (Penn *et al.*, 2009) using Geneious Pro 5.5.9 (created by Biomatters, available at <http://www.geneious.com>). These gene clusters were used as queries against a database created from 24 *Salinispora* genomes (12 *S. arenicola* and 12 *S. tropica*) using Multi-GeneBlast 1.1.13 (Medema *et al.*, 2013). Genome sequences were considered to contain a gene cluster if the sequence coverage and identity values were > 50% to the query sequences.

Results

Strain isolation and identification

A total of 289 sediment samples were collected from 22 locations and processed for the selective cultivation of actinomycetes. These efforts yielded 22 *S. arenicola* and four *S. tropica* strains from seven locations ranging from the Dry Tortugas to the

Yucatán peninsula. These results provide further support for the co-occurrence of the two *Salinispora* species (Jensen and Mafnas, 2006) and the higher relative abundance of *S. arenicola* (Mincer et al., 2005). Based on 16S ribosomal RNA gene sequencing, the *Salinispora* strains all belong to previously identified sequence types (Freel et al., 2013). In addition, 127 taxonomically diverse marine bacteria were isolated from the same habitats that yielded the *Salinispora* strains (Supplementary Table S1). These bacteria were assigned to 23 families in four phyla (Supplementary Table S2) and used in a direct challenge assay designed to detect the ability of established *Salinispora* cultures to inhibit the growth of potential competitors. The genus *Bacillus* comprised the largest number of strains (32) followed by *Streptomyces* (phylum Actinobacteria; 12 strains), *Erythrobacter* (subphylum alphaproteobacteria; 11 strains) and the genera *Pseudoalteromonas* and *Microbulbifer* (both subphylum gammaproteobacteria; six strains). Forty-five strains had no matches at 100% identity to sequences in GenBank suggesting they have not previously been cultured (Supplementary Table S1).

Direct challenge assays

Four *S. arenicola* and four *S. tropica* strains were tested at two time points in a direct challenge assay designed to detect their ability to inhibit the growth of potential bacterial competitors (Figure 1). In total, growth inhibition was detected in 671 (38%) of the 1769 interactions tested. The vast majority of the 127 strains tested (119 or 93%) were sensitive to at

least one *Salinispora* strain indicating the genus has the capacity to inhibit a broad spectrum of bacteria. The overall levels of inhibition were similar for the two species, with *S. arenicola* inhibiting on average slightly fewer strains (82) than *S. tropica* (87) when both time points were considered (Table 1). However, the potency of the inhibition, as measured by the average size of the zones of inhibition produced by each *Salinispora* strain (Supplementary Figure S1), was significantly greater for *S. arenicola* ($P=0.008$, Welch's two-sample t -test).

The patterns of inhibition varied both within and between *Salinispora* species (Figure 2). A PERMANOVA analysis showed no correlation between the taxonomy of the potential competitors and the likelihood they would be inhibited ($P>0.05$ for all taxonomic levels). The percentages of environmental isolates inhibited by all four *Salinispora* strains of either species were roughly the same (Supplementary Figure S2). However, in cases where bacteria were inhibited by at least one *Salinispora* strain, they were inhibited by all four strains in 42% of the cases for *S. tropica* relative to 28% for *S. arenicola*. This difference may largely be attributable to *S. arenicola* strain CNY-694, which grew poorly relative to the other strains (see below) and inhibited only 34 of the strains against which it was tested relative to an average of 98 for the other three *S. arenicola* strains (Table 1). An antiSMASH analysis of the secondary metabolite gene clusters in CNY-694 revealed a biosynthetic potential that is equivalent to other *S. arenicola* strains for which genome sequences were analyzed (Supplementary Table S3).

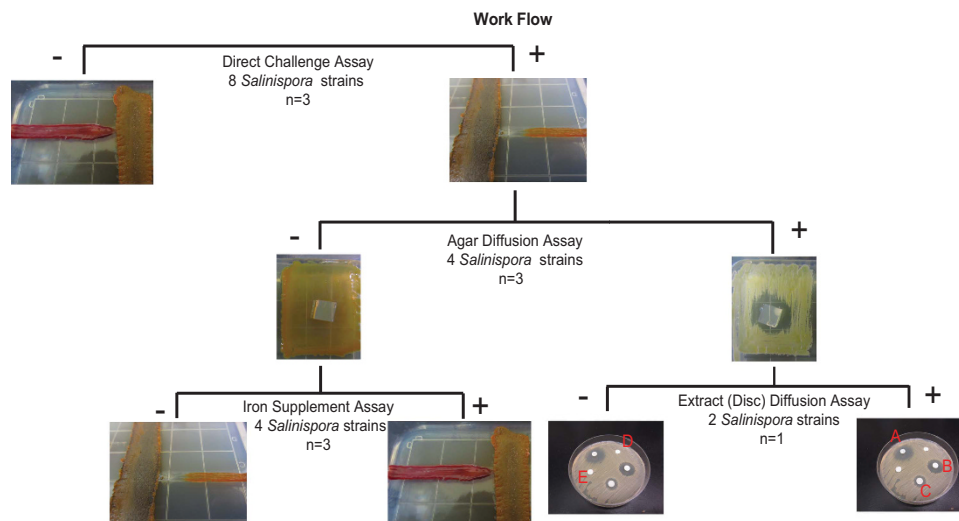


Figure 1 Workflow. A direct challenge assay was used to detect the ability of established *Salinispora* cultures to inhibit the growth of co-occurring bacterial strains. All strains that were inhibited in the direct challenge assay (+) were tested further in an agar diffusion assay to determine whether the activity was due to a diffusible molecule. A positive result (+) was recorded when growth of the test strain was inhibited around the agar block but not around a medium control. Organic extracts generated from similar agar blocks were then tested against the sensitive strains in a disk-diffusion assay to determine whether the activity was organic soluble. Active organic extracts (a–c) were identified based on the detection of zones of inhibition around the discs. Strains that were inhibited in the direct challenge assay but not in the agar diffusion assay were tested further to determine whether the inhibition was due to iron depletion. Iron depletion was identified as the source of the inhibition when growth was restored on iron-supplemented media (+).

Table 1 Results from the direct challenge assay for all 8 *Salinispora* strains at each time point and for both time points combined

Species	Strain	10-day time pt		23-day time pt		Combined time pts	
		# Tested	# Inhibited (%)	# Tested	# Inhibited (%)	# Inhibited	% Inhibited
<i>S. arenicola</i>	CNY-679	127	59 (47)	68	39 (31)	98	77
<i>S. arenicola</i>	CNY-680	127	83 (65)	44	23 (18)	106	84
<i>S. arenicola</i>	CNY-685	127	58 (46)	69	31 (24)	89	79
<i>S. arenicola</i>	CNY-694	127	21 (17)	106	13 (10)	34	27
	Avg.±SD		55 ± 26 (44 ± 20)		27 ± 11 (21 ± 9)	82 ± 33	65 ± 26

Species	Strain	7-day time pt		23-day time pt		Combined time pts	
		# Tested	# Inhibited (%)	# Tested	# Inhibited (%)	# Inhibited	% Inhibited
<i>S. tropica</i>	CNY-678	127	14 (11)	113	59 (47)	73	57
<i>S. tropica</i>	CNY-681	127	20 (16)	107	80 (63)	100	79
<i>S. tropica</i>	CNY-682	127	26 (20)	101	71 (56)	97	76
<i>S. tropica</i>	CNY-684	127	21 (17)	106	58 (46)	79	62
	Avg.±SD		20 ± 5 (16 ± 4)		67 ± 10 (53 ± 8)	87 ± 13	69 ± 10

Only strains that were not inhibited at the first time point were repeated at the second time point.

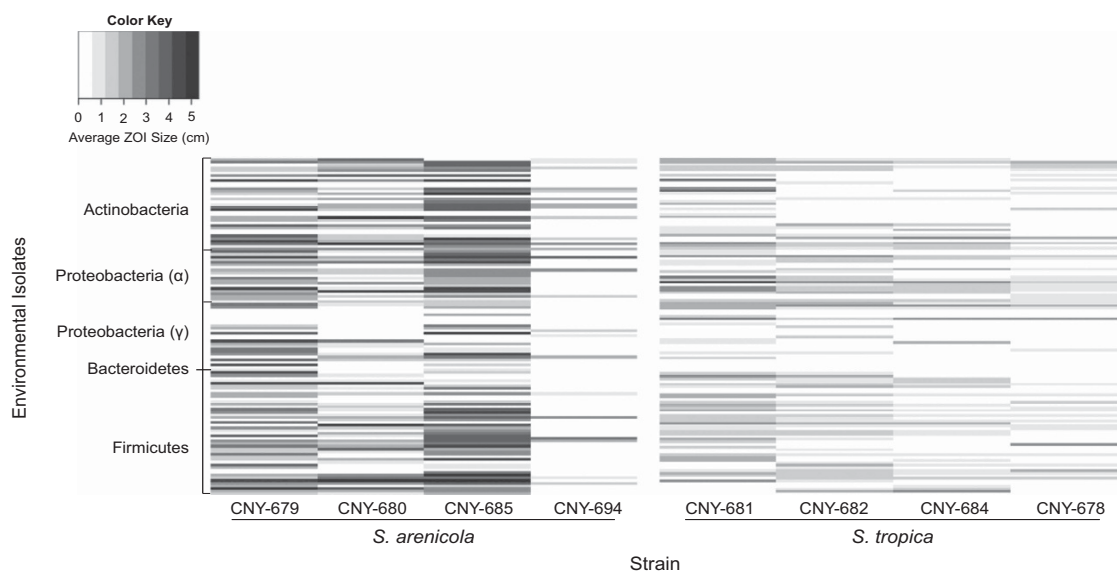


Figure 2 Growth inhibition observed in the direct challenge assays over both time points. Each row corresponds to one bacterial strain tested against eight *Salinispora* strains. The bacteria are grouped by phyla, with the phylum Bacteroidetes represented by only one strain. The color intensity represents the average size (cm) of the zone of inhibition (ZOI) for triplicate assays.

Temporal variability

Although the total number of inhibitory interactions recorded for each *Salinispora* species was similar, there was a major difference in the temporal onset of the activities (Figure 3, Supplementary Figure S3). Most notably, *S. arenicola* exhibited on average 55 inhibitory interactions at the first time point relative to 20 for *S. tropica* (Table 1). The trend of greater activity at the first time point was also observed for *S. arenicola* strain CNY-694 despite the relatively low number of bacteria inhibited. To determine whether these patterns were consistent features of the two *Salinispora* species, eight additional *S. arenicola* and nine additional *S. tropica* strains

were tested against a subset of 30 bacteria chosen to represent a range of the taxa encompassed by the original 127 strains (Supplementary Table S1). When the data from all assays were combined, *S. arenicola* remained significantly more active at the early time point (*t*-test; $P=0.02$), whereas there was no difference between the two species when the averages of the combined time points were compared (Figure 3) (*t*-test; $P=0.49$). Given that growth inhibition in direct challenge assays can be caused by factors such as nutrient depletion in addition to the production of inhibitory compounds, we next sought to distinguish between these two possibilities in an effort to better categorize the observed activities.

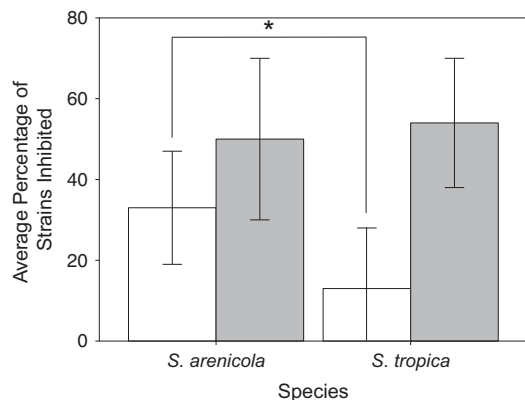


Figure 3 Average percentage of strains inhibited by 12 *S. arenicola* and 13 *S. tropica* strains. White bars represent the results for the first time point (7 and 10 days for *S. tropica* and *S. arenicola*, respectively). Gray bars represent the combined percentage of strains inhibited at the first and second (23 days for both *Salinispora* species) time points. There was a significant difference between the average percentage of strains inhibited at the first time point by each species (*).

Interference vs exploitation competition

Growth inhibition due to the production of antibiotics or other inhibitory substances can be defined as interference competition, whereas inhibition owing to nutrient depletion can be considered exploitation competition (Hibbing *et al.*, 2010). Agar diffusion assays were performed to determine whether the activities detected in the direct challenge assays could be linked to the presence of diffusible molecules (Figure 1) and thus indicative of antibiotic production. Agar taken at both time points from areas adjacent to two *S. arenicola* and two *S. tropica* strains was tested against all environmental isolates that showed sensitivity to these strains in the direct challenge assays. Together, these isolates represent 103 (95%) and 94 (87%) of the bacteria that displayed sensitivity to any of the *S. arenicola* or *S. tropica* strains, respectively. At the first time point, the inhibition generated by *S. arenicola* strains CNY-679 and CNY-685 was diffusible in 72% and 40% of the tests performed, respectively. In comparison, the inhibition generated by *S. tropica* strains CNY-678 and CNY-681 was diffusible in 9% and 14% of the assays, respectively (Figure 4). When the combined time points were considered, the inhibition generated by *S. arenicola* was diffusible in 51% of the assays compared with 20% for *S. tropica*. We next asked if the diffusible activities could be extracted with an organic solvent (Figure 1). At 10× concentration, an extract generated from *S. arenicola* strain CNY-679 inhibited 32 of the 44 strains that were inhibited in the agar diffusion assay (Table 2). In contrast, an extract prepared from *S. tropica* strain CNY-678 did not exhibit any inhibition, even when tested at 100× concentration.

In cases where the inhibitory activities could not be linked to a diffusible molecule, an iron

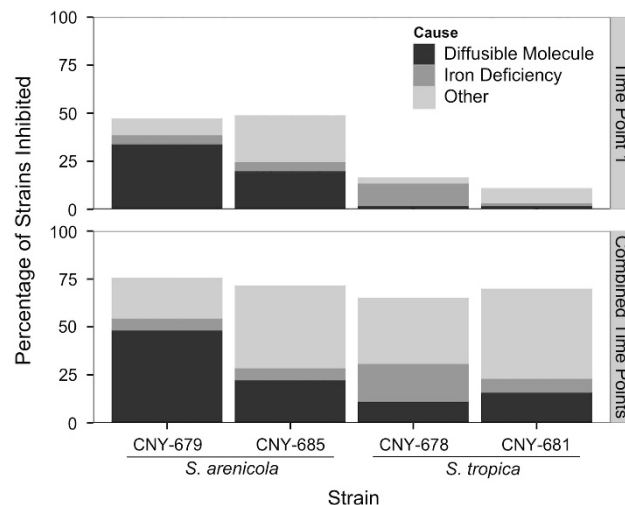


Figure 4 Sources of growth inhibition. The top panel presents the results from the first time point (7 days for *S. tropica*, 10 days for *S. arenicola*), whereas the bottom panel presents cumulative results from the first and second time points (23 days for both species). Sensitive isolates were tested in an agar diffusion assay to determine whether activity could be linked to a diffusible molecule. If negative, further tests were performed to determine whether growth was restored when excess iron was added to the medium, in which case the activity was attributed to iron depletion. If both assays were negative, growth inhibition was attributed to 'other' sources. Each *Salinispora* strain was tested against the environmental isolates they inhibited in the direct challenge assays.

Table 2 Number of strains inhibited by a diffusible molecule and organic extract

Inhibitory factor	Strain extracted	
	<i>S. arenicola</i> CNY-679	<i>S. tropica</i> CNY-678
Diffusible molecule	44 (35)	12 (9)
MeOH extract ($\geq 1 \times$)	11	0
MeOH Extract ($\geq 10 \times$)	32	0
MeOH Extract (100×)	42	0

For the diffusible-molecule test, percentage of total test panel is also included in parentheses. Cell-free areas adjacent to *Salinispora* cultures growing on agar media were extracted from *S. arenicola* and *S. tropica* strains and tested at three volumetric concentrations (1×, 10× and 100×) against all environmental isolates that were inhibited in the agar diffusion assays. Extractions were performed at the time point that generated the most activity in the direct challenge assays (10 days for *S. arenicola*, 23 days for *S. tropica*).

supplementation assay was performed to determine whether iron depletion was the cause of the inhibition (Figure 1). On average over both time points, 9% of the inhibition generated by *S. arenicola* could be linked to iron depletion compared with 20% for *S. tropica* (Figure 4). The remaining inhibitory activities (on average, 40% and 60% for *S. arenicola* and *S. tropica*, respectively) were ascribed to 'other' causes. There were no measurable differences in pH between the zones of inhibition and media controls

(data not shown) suggesting that a change in pH was not the cause of the 'other' inhibition.

Growth rates

To explore the hypothesis that *S. tropica* preferentially employs exploitation competition as a competitive strategy, the growth rates of the two species were assessed. Growth curves generated for all eight strains revealed that *S. tropica* has faster growth rates as determined by changes in dry weight biomass over time, which were significantly different between the two species (Figure 5; *t*-test, $P=0.02$). In addition to faster growth rates, cellular biomass was greater on average in stationary phase *S. tropica* cultures (mean = $4.13 \pm \text{SD } 1.11 \text{ mg ml}^{-1}$) than in stationary phase *S. arenicola* cultures (mean = $2.98 \pm \text{SD } 1.19 \text{ mg ml}^{-1}$) (Supplementary Figure S4). *S. arenicola* CNY-694, which produced relatively few inhibitory interactions, also produced the least biomass per volume of any strain (Supplementary Figure S4).

Bioinformatic analyses

A prior bioinformatic analysis revealed that *S. arenicola* maintains a larger number of polyketide synthase and non-ribosomal peptide synthetase biosynthetic gene clusters than *S. tropica* (Ziemert *et al.*, 2014). To more broadly assess the secondary metabolite potential of the two species, genome sequences from 12 strains of each species (including two *S. tropica* and three *S. arenicola* strains used in the direct challenge assays) (Supplementary Table S1) were analyzed for the presence of secondary metabolite gene clusters using antiSMASH, which can identify pathway types other than polyketide synthase and non-ribosomal peptide synthetase including those associated with lantibiotic and terpene biosynthesis (Medema *et al.*, 2011).

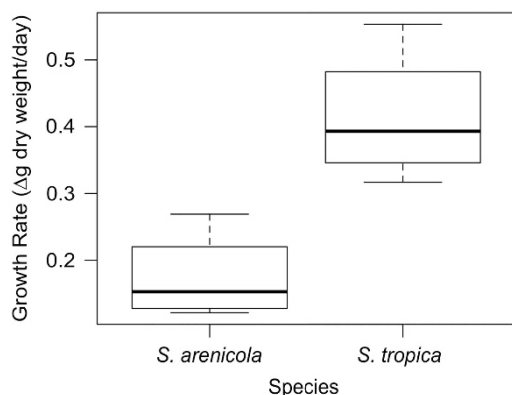


Figure 5 *Salinispora* growth rates. For each box, the dark horizontal bar represents the median value for the change in dry weight/day of the filtered cell biomass collected during exponential growth phase. The box edges represent the upper and lower quartiles of the data, and the whiskers represent the minimum and maximum values. The plot contains data from four strains of each species grown in triplicate.

In addition, the genome sequences were investigated for gene clusters previously linked to siderophore biosynthesis in *Salinispora* spp. (Penn *et al.*, 2009; Roberts *et al.*, 2012). On average, 31.3 secondary metabolite gene clusters were identified in the *S. arenicola* strains in comparison with 23.1 for *S. tropica* (Supplementary Table S3). These results conform well with the 31 and 19 clusters detected in an earlier analysis of two closed *S. arenicola* and *S. tropica* genomes, respectively (Penn *et al.*, 2009). The presence of the rifamycin (*rif*) gene cluster (Floss and Yu, 2005) was confirmed in all of the *S. arenicola* strains, whereas none of the pathways detected in *S. tropica* could be linked to the production of a known antibiotic. Although the total number of gene clusters is greater in *S. arenicola*, the trend is reversed when it comes to siderophore biosynthesis. In an earlier report, both *S. arenicola* CNS-205 and *S. tropica* CNB-440 were identified as possessing two siderophore-related gene clusters, one predicted to encode desferrioxamines (*des*) and a second (*sid2*) related to the gene cluster for yersiniabactin biosynthesis. However, two additional siderophore gene clusters (*sid3* and *sid4*) were also identified in *S. tropica* CNB-440 (Penn *et al.*, 2009). The production of desferrioxamines B and E was later confirmed for both *S. tropica* and *S. arenicola* and linked to the *des* locus (Roberts *et al.*, 2012). A MultiGeneBlast analysis revealed that all 12 *S. tropica* strains for which genome sequences are available possess all four of the siderophore gene clusters, whereas the 12 *S. arenicola* genomes chosen for this study only possess the *des* and *sid2* clusters (Supplementary Table S3).

Chemical analyses

To determine whether any known antibiotics were produced by either *Salinispora* species, organic culture extracts of *S. tropica* CNY-678 and *S. arenicola* CNY-679 were examined by liquid chromatography-tandem mass spectrometry and the results compared with the AntiMarin database (Blunt *et al.*, 2006) and an in-house database of previously identified *Salinispora* secondary metabolites. The *S. arenicola* extract contained the antibiotic rifamycin S (Supplementary Figure S5), which belongs to a class of compounds previously reported from this species (Kim *et al.*, 2006; Jensen *et al.*, 2007). In addition, a parent ion that matched rifamycin S was observed directly in a zone of inhibition between *S. arenicola* CNY-679 and *Kyococcus* sp. CUA-766 using MALDI-TOF imaging mass spectrometry (Figure 6). Thirteen strains showing sensitivity to *S. arenicola* culture extracts were tested for sensitivity to commercially available rifamycin SV (the quinone analog of rifamycin S) and found to be sensitive at concentrations between $10\text{--}100 \mu\text{g ml}^{-1}$ (data not shown), which is comparable to the MIC-50 values reported against a variety of enteropathogens (Farrell *et al.*, 2011).

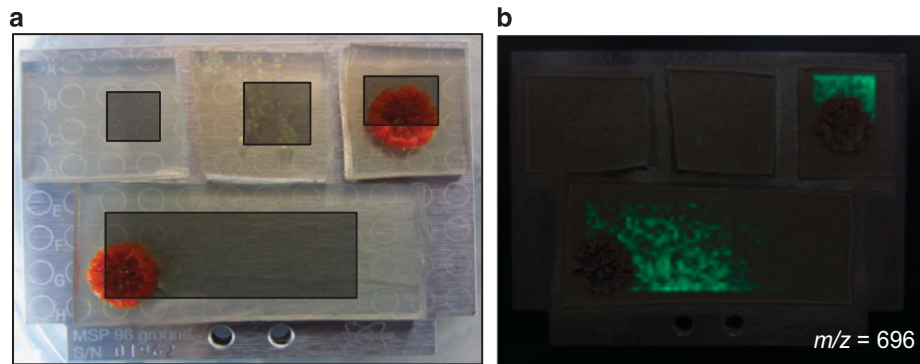


Figure 6 MALDI-TOF imaging mass spectrometry of an *S. arenicola* CNY-679 interaction with *Kytococcus* sp. CUA-766. (a) MALDI plate setup with control samples in the top row, from left to right: media blank, *Kytococcus* sp. CUA-766 monoculture, *S. arenicola* CNY-679 monoculture. The sample in the bottom row contains the zone of inhibition between the two strains. The gray boxes define the areas chosen for imaging. (b) Spatial distribution of the m/z 696 ion, an exact match to rifamycin S (M+H), shown in green surrounding the *S. arenicola* colony and diffusing outwards toward the inhibited bacterial strain. This ion was observed in the *S. arenicola* monoculture but not in the medium blank or the *Kytococcus* sp. monoculture.

These results provide evidence that the antibiotic rifamycin S is, at least in part, associated with the inhibitory activities generated by *S. arenicola*. Compounds in this class have been similarly linked to the activities observed between sponge-derived *S. arenicola* strains and *Mycobacterium* spp. (Izumi et al., 2010). No known antibiotics were detected in the *S. tropica* extract.

Discussion

Molecular surveys have revealed that bacteria exhibit extraordinary levels of phylogenetic diversity (Rappe and Giovannoni, 2003). Although the existence of this diversity is widely appreciated, establishing links between the clades observed in phylogenetic trees and the ecological and evolutionary processes that create and maintain them remains one of the great challenges in microbial ecology (Fuhrman, 2009; Cordero and Polz, 2014). It has been proposed that fine-scale phylogeny can be used to delineate bacteria into ecologically cohesive units or ‘ecotypes’ (Cohan, 2002) and that in the few examples available, ecological populations represent ‘gene flow units’ for which genome wide rates of homologous recombination are much greater within than between clusters (Cordero and Polz, 2014). A multilocus sequence analysis of *Salinispora* species supports the description of *S. tropica* and *S. arenicola* in accordance with what appear to be natural barriers to recombination (Freel et al., 2013). Although there are many processes that can account for these barriers (Cohan, 2002; Acinas et al., 2004; Thompson et al., 2005; Fraser et al., 2007; Hellweger et al., 2014), ecological differentiation has been proposed as the mechanism driving diversification between *S. arenicola* and the two sister species with which it co-occurs (Jensen et al., 2007; Freel et al., 2013). To date, the major adaptive traits that distinguish the three species relate to secondary metabolite production (Jensen et al., 2007), but

experimental evidence linking these differences to divergent ecological strategies has been lacking. Here we provide evidence that *S. tropica* and *S. arenicola* employ fundamentally different competitive strategies that are mediated in part by secondary metabolites, with the former investing in growth or exploitation competition and the latter in interference competition via the production of antibiotics and at the expense of growth. The results create a link between the fine-scale phylogenetic relationships maintained by these two lineages and functional traits that establish them as distinct ecotypes.

The two *Salinispora* species exhibited similar overall levels of growth inhibition against a diverse panel of co-occurring marine bacteria. However, temporal differences in the onset of inhibition suggested there could be fundamental differences in the mechanisms by which these activities were generated. Subsequent tests made it possible to distinguish between inhibition due to the production of allelopathic molecules and that caused by other factors, including nutrient depletion. The observation that *S. arenicola* generated more than twice the number of inhibitory activities linked to the production of diffusible substances relative to *S. tropica* provided the initial line of evidence that these two species differentially invest in interference and exploitation competition, respectively. Additional evidence comes from the identification of the antibiotic rifamycin S in *S. arenicola* culture extracts and directly in the zones of inhibition, whereas no antibiotic activities were detected in the *S. tropica* culture extracts. *S. arenicola* also averaged 31 gene clusters related to secondary metabolism relative to 23 for *S. tropica*, indicating greater genetic investment in this functional trait. The distinction between interference and exploitation competition was further supported by the significantly faster growth rates recorded for *S. tropica*. One notable result is the large number of activities that could not be linked to diffusible molecules or iron limitation (Figure 4). These activities require further study and may be

linked to the depletion of nutrients other than iron including carbon and nitrogen sources. The prevalence of non-diffusible growth inhibition suggests that exploitation competition may be widely overlooked as the source of growth inhibition in direct challenge assays. This is not to imply, however, that *S. tropica* does not also engage in interference competition via the production of inhibitory compounds; they were simply not detected in the assays employed.

Although *S. arenicola* maintains a larger number of gene clusters devoted to secondary metabolism, this trend is reversed when it comes to siderophore biosynthesis. Siderophores are an important mechanism by which bacteria acquire growth-essential iron (Neilands, 1995) and their production has been linked to antagonism in *Vibrio* (Pybus *et al.*, 1994) and *Pseudomonas* spp. (Simões *et al.*, 2008). Although siderophores are secreted secondary metabolites, it can be argued they have a functional role in exploitation competition as opposed to more traditional allelopathic agents such as antibiotics, which function in interference competition. *S. tropica* genome sequences maintain two gene clusters predicted to encode siderophore biosynthesis (*sid3* and *sid4*) that are not found in *S. arenicola* (Supplementary Table S3 and S5), suggesting an additional investment in iron uptake by this species. Although the inactivation of key genes in the *S. tropica* *sid3* and *sid4* clusters did not affect growth in iron-limited media (Roberts *et al.*, 2012), these pathways may have a role in the acquisition of other limiting metals (Bellenger *et al.*, 2008) or be regulated by factors other than iron limitation, and thus enhanced siderophore production could help support higher growth rates in this species. The conservation of *sid3* and *sid4* among all 12 *S. tropica* strains for which genome sequences were available suggests that there are strong selective pressures to maintain the functions provided by the products of these pathways. The observation that secondary metabolites can have a role in exploitation competition by facilitating nutrient acquisition as well as interference competition via antibiotic production emphasizes the importance of distinguishing between these two competitive mechanisms when addressing the ecological functions of secondary metabolites.

Considerable intra-specific variation was observed in the inhibitory activities generated by both species. This supports a growing body of evidence in which inhibitory activities are strain-specific (Grossart *et al.*, 2004; Rypien *et al.*, 2010; Long *et al.*, 2013) or occur at low frequencies within a population (Vetsigian *et al.*, 2011). It also supports the concept that secondary metabolite gene cluster evolution is a dynamic process (Medema *et al.*, 2014) with high levels of plasticity within a single species (Ziemert *et al.*, 2014). One rationalization for this plasticity is that the targets of the antibiotics produced by any one strain may matter less than the ability of the population as a

whole to minimize the total number of competitors. Intra-specific variability also provides a rational approach to ensure that at least some individuals will remain competitive as new challengers are encountered either when spores are dispersed or when new resources become available at an existing site. It also provides an effective strategy to avoid resistance, as in the application of combinatorial drug therapy, and the subsequent need to enter into a co-evolutionary arms race (Kinkel *et al.*, 2013). Regardless of the ecological benefits, this variability can likely be linked to recent horizontal gene transfer events and the concept that bacteria frequently 'sample' gene clusters from the local gene pool (Ziemert *et al.*, 2014), with conservation observed for only those clusters whose products provide sufficient selective advantage (Jensen *et al.*, 2007).

The bacteria used in the direct challenge assays were all heterotrophic and originated from similar, if not the same, sediment samples as the *Salinispora* isolates, thus making them potential competitors. As with any culture-based study, these strains represent only a small fraction of the total bacterial community and do not include classes such as the Deltaproteobacteria and the Planctomycetacia, which can represent major components of sediment communities (Schauer *et al.*, 2010). Nonetheless, these strains encompass a wide range of taxonomic diversity, including common marine families such as the Rhodobacteraceae and Pseudoalteromonadaceae (Acinas *et al.*, 2004; Gilbert *et al.*, 2012), and share similar growth requirements with *Salinispora* spp., further supporting their ecological relevance. Although colony growth on agar plates is not the natural state of sediment bacteria, the encroachment of an established colony by competing bacteria represents a scenario that may be important in dictating defensive strategies in marine sediments. Structured habitats such as sediments are prime locations for interference competition as has been suggested for colicinogenic bacteria grown in soft agar matrices (Chao and Levin, 1981). The mycelium growth form exhibited by *Salinispora* spp. may facilitate the accumulation of antibiotics at concentrations that achieve functional levels in the surrounding micro-environment. However, translating the results obtained here to what occurs in nature will require additional studies aimed toward gaining a spatial context for competitive interactions and more accurate estimates for *in situ* compound concentrations.

Salinispora strains largely occur as dormant spores and represent relatively rare members of the sediment microbial community (Mincer *et al.*, 2005). Thus, it is possible for actively growing, localized populations to originate from individual spores. This may help explain the apparent lack of 'social cheaters' benefiting from the production of antibiotics by con-specifics, as has been reported in marine Vibrionaceae (Cordero *et al.*, 2012). The consistent production of antibiotics in the rifamycin class by *S. arenicola* (Jensen *et al.*, 2007) suggests there are

strong selective pressures to maintain the functions of specific compounds and that non-producers or potential cheaters are rapidly selected against. The large diversity of *Salinispora* secondary metabolites that do not possess antibiotic activity (Fenical and Jensen, 2006) suggests that many of these compounds provide ecological functions other than allelopathy.

Methodological limitations and a poor understanding of the scale at which microbial interactions occur have hampered ongoing efforts to resolve genetic diversity in the context of ecological interactions. Advances in microscopy and mass spectrometry have enhanced our ability to observe physical interactions within limited microbial consortia (Orphan *et al.*, 2001; Malfatti *et al.*, 2010; Valm *et al.*, 2011) but the detailed visualization of interactions in complex environments like sediments remains out of reach. The imaging mass spectrometry technique applied here provided clear evidence that rifamycins were present in the zones of inhibition between *Salinispora* strains and potential bacterial challengers. The ability to resolve specific compounds in a more natural setting provides expanded opportunities to decipher their roles in mediating interactions among microbes, including those that occur at sub-lethal concentrations (Jauri *et al.*, 2013).

The resource availability hypothesis states that natural selection favors slow growth rates and high levels of defense in low resource habitats (Coley *et al.*, 1985). Although the genus *Salinispora* belongs to a larger group of actinomycetes that are widely recognized for slow growth and secondary metabolite production, it is interesting that, within this broader ecological strategy, lineages can be identified that preferentially invest in growth or defense. Although preliminary evidence indicates that *S. tropica* is the less abundant of the two species, higher growth rates and more effective nutrient acquisition suggest it may experience ephemeral blooms in response to nutrient pulses, a concept that can be tested in future studies. Such fine-scale differentiation also provides evidence for co-evolutionary strategies within a microdiverse cluster. Synergistic evolution has been suggested to boost the ecological success of co-occurring species, particularly those that employ secondary metabolites as a form of chemical defense (Cordero and Polz, 2014). Although the extensive arsenal of secondary metabolites available to these two species suggest their ecological functions are more complex than simply distinguishing between two competitive strategies, the results provide the first experimental evidence for ecological divergence in the genus *Salinispora*.

Conflict of Interest

The authors declare no conflict of interest.

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