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The role of inter-species interactions in *Salinispora* specialized metabolism

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Abstract

Bacterial genome sequences consistently contain many more biosynthetic gene clusters encoding specialized metabolites than predicted by the compounds discovered from the respective strains. One hypothesis invoked to explain the cryptic nature of these gene clusters is that standard laboratory conditions do not provide the environmental cues needed to trigger gene expression. A potential source of such cues is other members of the bacterial community, which are logical targets for competitive interactions. In this study, we examined the effects of such interactions on specialized metabolism in the marine actinomycete *Salinispora tropica*. The results show that antibiotic activities and the concentration of some small molecules increase in the presence of co-occurring bacterial strains relative to monocultures. Some increases in antibiotic activity could be linked to nutrient depletion by the competitor as opposed to the production of a chemical cue. Other increases were correlated with the production of specific compounds by *S. tropica*. In particular, one interaction with a *Vibrio* sp. consistently induced antibiotic activity and was associated with parent ions that were unique to this interaction, although the associated compound could not be identified. This study provides insight into the metabolomic complexities of bacterial interactions and baseline information for future genome mining efforts.

INTRODUCTION

Bacteria in the order *Actinomycetales*, commonly known as actinomycetes, produce the vast majority of microbially derived bioactive small molecules [1]. These compounds, also known as specialized or secondary metabolites, include clinically relevant cytotoxins, immunosuppressants and antibiotics [1, 2]. Despite many decades of bioactive compound discovery in both academia and industry, the ecological roles of actinomycete specialized metabolites remain largely unknown [3, 4]. Understanding why these compounds are produced in nature has potential benefits for future discovery efforts, as well as the field of chemical ecology. This argument is supported by recent genome mining efforts, which reveal considerably more genetic potential for specialized metabolite production than the actinomycete compounds discovered to date would suggest [5, 6]. The rich biosynthetic potential detected in genome sequences raises the possibility that traditional laboratory cultivation techniques do not provide the environmental cues needed for the production of many specialized metabolites.

There is growing interest in exploiting the ecological functions of specialized metabolites as a method to facilitate their discovery. One potential function is allelopathy, which has been established as an important driver of competitive interactions among bacteria [7, 8]. In the case of soil actinomycetes, antibiotics have been shown to mediate antagonistic interactions among co-occurring populations [9]. However, antibiotic production is generally under strict regulatory control [10, 11] as there is little ecological rationale for the constitutive production of energetically expensive and toxic metabolites. Co-culture has become a popular method to induce specialized metabolite production [12], leading to the isolation of new compounds from fungi [13–15], phytoplankton [16–18] and bacteria, including actinomycetes [19–22]. In some cases, physical contact is required for induction [20, 23], while in others a chemical elicitor is sufficient to effect changes in specialized metabolism [16, 24, 25]. However, the ecological relevance of induction is often speculative as most producer and challenge organisms were isolated from different environments and therefore unlikely to interact in nature.

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Abbreviations: ELSD, evaporative light scattering detector; ESI, electrospray ionization; MS/MS, mass spectrometry/mass spectrometry (tandem mass spectrometry); TFA, trifluoroacetic acid; UHPLC, ultra-high performance liquid chromatography.

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One supplementary table and five supplementary figures are available with the online version of this article.

Identifying ecologically relevant microbial interactions is further complicated by a poor understanding of community dynamics. Deep sequencing has shown that actinomycetes usually constitute only a small fraction of soil and sediment communities [26–32], and thus the extent to which they interact with other taxa remains unknown. However, the relative abundances of taxa often considered 'rare' can fluctuate over time [33], and such taxa have been shown to be important for structuring bacterial communities in soils [34] and other environments [35]. Furthermore, the ability of actinomycetes to form dormant spores adds an element of complexity when assessing the ecological relevance of chemical interactions. In a previous study, *Salinispora tropica* strains consistently inhibited bacteria in a competitive interaction assay via the production of a diffusible compound [36]. This made *S. tropica* an ideal choice for testing the hypothesis that antibiotic production was induced in response to these interactions.

Here we confirmed previously reported antagonistic interactions between *S. tropica* and co-occurring marine bacteria [36]. We expanded on these results by documenting numerous examples where antibiotic activities increased in response to competitive interactions relative to monocultures. While induction has often been attributed to chemical signals from competing microbes [20, 22, 37], our results show that what appears to be induction can often be linked to temporal changes in compound production. We observed a complex pattern of metabolite production that varies by interaction and metabolite. These results add to the growing body of evidence that co-cultivation can induce compound production and provide insight into the ecological functions of specialized metabolites.

METHODS

Competitive interaction assays

Twelve bacterial strains were selected as challenge organisms in cross-streak assays based on results from a previous study addressing antagonism among *Salinispora* spp. [36]. The strains represented a mix of those whose growth was inhibited (eight) or unaffected (four) following interactions with *S. tropica* CNY-681 (Table 1). Triplicate cross-streak assays were performed as previously reported [36] to confirm the responses of these strains and provide material for mass spectrometry analysis. In all cases, *S. tropica* CNY-681 starter cultures were prepared by inoculating a frozen glycerol stock into 50 ml A1 medium [10 g starch, 4 g yeast extract, 2 g peptone, 22 g Instant Ocean Sea Salt (Instant Ocean Spectrum Brands, Blacksburg, VA, USA), 1 l deionized water; hereafter 'A1'] and allowing it to grow at room temperature with shaking at 200 r.p.m. for seven days. For the assays, A1 agar plates (16 g l^{-1} agar, 100×100 mm) were inoculated with 50 µl of the starter culture, which was spread in a vertical line down the middle of the plate using a sterile loop. These plates were incubated at room temperature for six days before cross-streaking overnight cultures of the 12 challenge strains (50 ml A1, inoculated from frozen glycerol stocks) with a sterile cotton swab perpendicular to the *S. tropica* culture. Triplicate agar plates containing monocultures of *S. tropica* CNY-681 and the 12 challenge strains were also prepared. For each competitive interaction assay, inhibition was defined as a zone of no growth that measured 5 mm or more from the CNY-681 culture. Only strains for which all three replicates generated a zone of inhibition were considered positive.

Table 1. Competitive interaction (CI) and disc diffusion assay results

Twelve strains were tested for growth inhibition when inoculated adjacent to *S. tropica* CNY-681 in a CI assay. Inhibitory interactions are scored positive (+), while interactions that did not result in growth inhibition are scored negative (−). Disc diffusion assays were used to test organic extracts derived from a monoculture of *S. tropica* CNY-681 (M), the competitive interaction assay plates (C), and the pure compound lomaiviticin C (1 mg ml^{-1}) (L). CI extracts were assessed for increased production (induction) of lomaiviticin (Lom) and sioxanthin (Siox) relative to monocultures (+, increased yield; −, no change in yield).

Strain	GenBank accession #	Inhibition				Induction	
		CI	M	C	L	Lom	Siox
Vibrio sp. CUA-759	KJ732853.1	+	+	+	−	+	+
Loktanella pyoseonensis CUA-786	KJ732874.1	+	+	+	−	+	+
Streptomyces sp. CUA-789	KJ732877.1	+	−	−	−	+	−
Rhodococcus sp. CUA-806	KJ732890.1	+	−	−	−	+	+
Psychrobacter sp. CUA-827	KJ732906.1	+	−	−	−	+	+
Serinicoccus sp. CUA-874	KJ732932.1	+	−	−	−	−	−
Micrococcus sp. CUA-879	KJ732935.1	+	+	+	−	−	−
Pseudoalteromonas sp. CUA-898	KJ732954.1	+	−	−	−	−	+
Erythrobacter citreus CUA-812	KJ732894.1	−	−	−	−	−	+
Alteromonas sp. CUA-818	KJ732900.1	−	−	−	−	−	−
Paracoccus homiensis CUA-883	KJ732939.1	−	−	−	−	+	+
Ruegeria lacuscaerulensis CUA-829	KJ732907.1	−	−	−	−	−	−

Organic extracts and disc-diffusion assays

Organic extracts were generated from *S. tropica* CNY-681 monocultures, challenge strain monocultures and competitive interaction assays in which the challenge strain was inhibited. The interaction assay plates were extracted after the growth of the challenge strain reached confluence, which was either one or two days following inoculation, depending on the strain. The extractions were performed by removing the agar media around the *S. tropica* cultures using a sterile scalpel (Fig. S1, available in the onine verion of this article), cutting it into ~0.5 cm^2 pieces, adding it to approximately 100 ml ethyl acetate in an Erlenmeyer flask and shaking at 200 r.p.m. for 2 h. The total volume of agar extracted from the triplicate plates was approximately 50 ml as measured by volume displacement. The agar was collected by filtration and extracted a second time (1 h) using an equal volume of ethyl acetate. Extracts were combined, dried by rotary evaporation and weighed. *S. tropica* CNY-681 and challenge strain monocultures were extracted using the same protocol.

Disc diffusion assays were used to determine whether the *S. tropica* CNY-681 extracts inhibited the growth of the challenge strains. In brief, overnight cultures of each challenge strain were inoculated onto duplicate A1 agar plates using a sterile cotton swab and spread to create a lawn. These plates were allowed to air dry for ~30 min. The extracts were re-suspended in methanol at 5 mg ml^{-1} and 10 µl applied to a sterile paper disc, and the solvent allowed to evaporate for ~15 min. A solvent control disc was similarly prepared. Each disc was placed onto the bacterial lawn using sterile tweezers. An antibiotic disc containing 5 µg ciprofloxacin (BD, Sparks, MD) was used as a positive control. The *S. tropica* metabolite lomaiviticin C (provided by S. Herzon) was also tested at 1 mg ml^{-1} using this method.

Time course experiments

Two experiments were conducted to assess changes in *S. tropica* CNY-681 compound production over time. In both experiments, CNY-681 monocultures were grown on A1 plates as described above. In the first experiment (Time Course 1), single monoculture plates were extracted on days 7, 9, 10, 13–16, 17, 20–23, 27 and 29, with day 7 corresponding to the addition of the challenge strains in the competitive interaction assays. The second experiment (Time Course 2) was optimized for the most informative time frame (days 4, 7, 8, 10, 12, 14 and 16) and included nine replicate plates at each time point. The nine plates were pooled into groups of three to generate triplicate extracts for each time point. All extracts were analysed using HPLC and tandem mass spectrometry (MS/MS) molecular networking as described below.

Vibrio sp. interaction

The competitive interaction assay between *Vibrio* sp. CUA-759 and *S. tropica* CNY-681 was scaled up to investigate the potential production of a chemical elicitor by CUA-759. Monoculture and cross-streak assay plates were prepared as described above, with a total of six plates for each of the following three conditions: *S. tropica* CNY-681 monoculture, CNY-681 and *Vibrio* sp. CUA-759 cross-streak and CNY-681 monoculture treated with CUA-759 cell-free supernatant. Cell-free supernatant was prepared from the same CUA-759 culture used for the cross-streak plates by centrifuging 1 ml at 10 000 r.p.m. for 3 min then passing the supernatant through a 0.2 µm syringe filter (Whatman). From this supernatant, 200 µl were applied to each of three plates using a pipette to evenly distribute the liquid adjacent to the previously inoculated *S. tropica* culture. CNY-681 was allowed to grow for six days before cross-streaking with an overnight culture of CUA-759, or adding the cell-free supernatant. The cultures were then allowed to grow for one additional day and zones of inhibition documented. The plates inoculated with cell-free supernatant were monitored to confirm there was no growth of CUA-759. The plates were pooled into groups of three to generate duplicate samples, which were extracted twice with ethyl acetate and tested in a disc-diffusion assay as described above.

HPLC analysis

All extracts were re-suspended in methanol at 4 mg ml^{-1} and run on an Agilent 1100 HPLC connected to a Shimadzu low-temperature Evaporative Light Scattering Detector (ELSD) and Ultra Violet (UV) light detector. For each sample, 15 µl were injected onto a C18 Kinetex column (Phenomenex) with a flow rate of 0.7 ml min^{-1} under the following LC run conditions: 1–2 min: 10 % acetonitrile [0.1 % trifluoroacetic acid (TFA)] in H_2O (0.1 % TFA), 2–13 min: 10–100 % acetonitrile (0.1 % TFA), 13–15 min: 100 % acetonitrile (0.1 % TFA). Both UV and ELSD data were analysed using the program ChemStation (Agilent). UV profiles for each peak were compared to an in-house library of *Salinispora* spp. metabolites and authentic standards. Integrated ELSD peak areas were used to compare changes in compound production among time-course and interaction samples.

For Time Course 1, compound yields were monitored based on area under the curve for individual peaks in the HPLC trace. To determine proportional differences in compound production during Time Course 2, the highest observed yield was divided by the starting value (day 7). The maximum yield was not necessarily observed at the same time for each peak. Values were log-transformed because of the large spread of the data, with positive values indicating increased production. For decreases in peak area, absolute values were log-transformed and converted to a negative value.

Figs 2, 3 and 4 were generated using IPython (Oliphant 2007) and the Seaborn (https://stanford.edu/~mwaskom/software/seaborn/index.html) and Bokeh (https://bokeh.pydata.org) Python visualization libraries.

MS/MS molecular networking

HPLC-mass spectrometry was used to compare samples and support compound identification. Chromatographic

separations were achieved at 30 °C using an Agilent 1290 Infinity Ultra-High Performance Liquid Chromatography (UHPLC) system (Agilent) with a flow rate of 0.5 ml min^{-1} and a 1.7 µm C18 (50×2.1 mm) Kinetex UHPLC column (Phenomenex). The mobile phase gradient was 0–0.5 min 5 % B, 0.5–8 min 5–100 % B, 8–11 min 100 % B, 11–11.5 min 100–5 % B, 11.5–12 min 5 % B, where solvent A was water with 0.1 % formic acid (v/v) and solvent B was acetonitrile with 0.1 % formic acid (v/v). Mass spectra were obtained on a MicroTOF Q II high-resolution mass spectrometer (Bruker Daltonics) equipped with an electrospray ionization (ESI) source in positive mode with mass range 100–2000 *m/z*. Before each run, the instrument was externally calibrated to 1.0 ppm mass accuracy with ESI-L Low Concentration Tuning Mix (Agilent). During the run, hexakis (1 h,1H,2H-difluoroethoxy)phosphazene (Synquest Laboratories), *m/z* 622.029509, was used as an internal calibrant. Instrument parameters were as follows: nebulizer gas (N_2) pressure, 2 bar; capillary voltage, 4500 V; ion source temperature, 200 °C; dry gas 9 l min^{-1}; spectra rate, 2 Hz. MS/MS fragmentation of the five most abundant ions per spectrum was performed with adaptive collision energy and acquisition time based on precursor ion properties. With a total cycle time of 3 s, ions were actively excluded from reselection after three spectra and released after 20 s. An exclusion list was used to prevent sampling of the lock mass. All UPLC-MS analyses were controlled by Hystar and Otof Control software packages (Bruker Daltonics). Data are publicly stored in MassIVE under IDs MSV000080240 and MSV000080116.

High-resolution MS/MS data were used to generate molecular networks [38]. Networks were created for the Time Course 1 and competitive interaction samples (GNPS job: https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=7c0abe9a07524a-d88a19e73b6e12d100), and for the *Vibrio* sp. CUA-759 CI experiment (GNPS job: https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=e96b04f91e2b4fd7b4d465894c59f712). Raw data files were converted to mzXML format and analysed using the Global Natural Products Social molecular networking database (GNPS) [39], which provided the following automated description of the networking parameters: 'A molecular network was created using the online workflow at GNPS. The data were filtered by removing all MS/MS peaks within ± 17 Da of the precursor *m/z*. MS/MS spectra were window filtered by choosing only the top six peaks in the ± 50 Da window throughout the spectrum. The data were then clustered with MS-Cluster with a parent mass tolerance of 2.0 Da and a MS/MS fragment ion tolerance of 0.5 Da to create consensus spectra. Consensus spectra that contained fewer than two spectra were discarded. A network was then created where edges were filtered to have a cosine score >0.7 and more than six matched peaks. Edges between two nodes were kept in the network if, and only if, each of the nodes appeared in each other's respective top 10 most similar nodes. The spectra in the network were then searched against the GNPS spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a cosine score >0.7 and at least six matched peaks.' Networks were visualized using Cytoscape [40].

RESULTS

Competitive interaction assays and extract inhibitory activity

S. tropica strain CNY-681 was previously shown to inhibit the growth of marine bacteria in a competitive interaction (CI) assay [36]. Since none of the compounds reported from this species are known to possess antibiotic properties [41], we selected for further study eight interactions where the competing strain was inhibited and four controls where no inhibition was observed [36]. The previously reported sensitivities of the 12 strains to CNY-681 were successfully reproduced in the CI assay (Table 1). We next tested whether growth inhibition could be reproduced in an organic extract generated from a monoculture of *S. tropica* CNY-681. If active, this would suggest that the inhibition was not induced, but instead due to a diffusible molecule that was also produced in the absence of a challenge strain. When tested in a disc-diffusion assay, the CNY-681 monoculture extract inhibited three of the eight strains that were inhibited in the CI assays (Table 1).

Despite extensive efforts, it was not possible to identify the chemical nature of this inhibition. There are many possible explanations for the lack of inhibition by the monoculture extract against five of the strains that were sensitive in the CI assays. These include the possibility that the active compounds were induced in the CI assays and not produced in the *S. tropica* CNY-681 monoculture. It is also possible that the activities are associated with multiple compounds, some of which were not extracted with the methods employed. To test for the former, we generated extracts directly from the zones of inhibition observed in the eight positive CI assays. Here again, the same five strains that were insensitive to the monoculture extracts were also unaffected by extracts from the zones of inhibition. These results suggest that the activities observed against these five strains in the CI assays were not due to an induced, diffusible molecule (Table 1).

Extract composition

To further explore compound induction, independent of antibiotic activity, we next compared extracts generated from monocultures of *S. tropica* CNY-681 and each of the 12 competitor strains to those derived from the CI assays. No peaks were unique to the CI assays, indicating that no detectable compounds were produced only in the presence of a challenge strain. In total, 20 peaks were identified in the *S. tropica* CNY-681 monoculture and the CI assays but not in the challenge strain monocultures. Among these, we identified two compounds previously reported from *S. tropica*: the cytotoxic agent lomaiviticin C [42] (Figs 1 and S2) and the carotenoid pigment sioxanthin (Figs 1 and S3) [41]. When tested against all 12 strains at 1 mg ml^{-1}, lomaiviticin C produced no growth inhibition (Table 1), making this

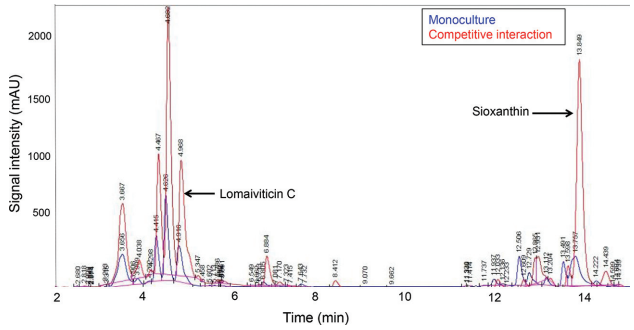

Fig. 1. Changes in compound production in response to competitive interaction. HPLC traces (ELSD detection) of the *S. tropica* CNY-681 monoculture extract (blue) and the extract generated from the competitive interaction assay with CUA-827 (red). Both samples were extracted at the same time point. Arrows indicate peaks corresponding to lomaiviticin C and sioxanthin.

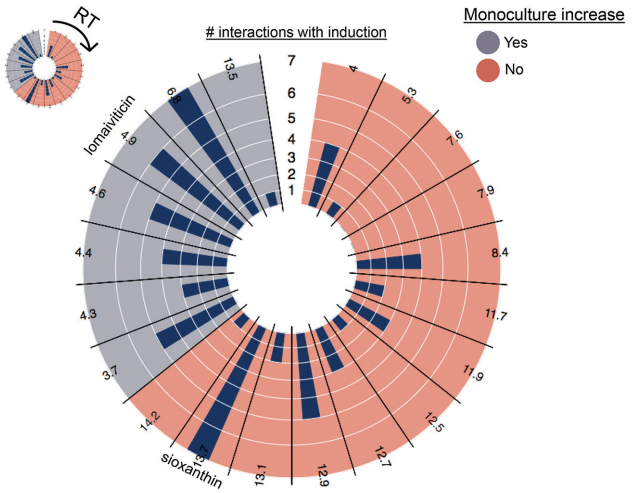

Fig. 2. Effects of competitive interactions and culture age on compound production. Each wedge of the circle represents one of the 20 peaks identified in the HPLC trace (labelled by retention time in min). Only peaks observed in the *S. tropica* CNY-681 monoculture or CI assays are reported. Grey wedges indicate peaks that increased over time (Time Course 1) in the monoculture, while orange wedges indicate peaks that remained the same. The blue bars represent the number of CI assays out of 12 in which an increase in that peak was observed. The sioxanthin peak increased in 7 of the 12 CI assays, but not over time in the CNY-681 monoculture. RT, retention time.

compound an unlikely source of the antibiotic activity. Sioxanthin is unstable and difficult to isolate [43] and thus could not be tested.

Time course experiments

We next assessed relative changes in peak height in the CI assays relative to the *S. tropica* CNY-681 monoculture. In total, 18 of 20 peaks increased in at least one of the CI assays (Fig. 2). However, one possible reason for induction of antibiotic activity is a higher rate of nutrient depletion when a second organism is added to the assay, which may accelerate the growth cycle of *S. tropica*. To address this concept, we asked whether compound levels similar to those observed in the CI assays were observed over time in a CNY-681 monoculture (Time Course 1). Of the 18 peaks that increased in the CI assays, seven also increased over time in the *S. tropica* culture (Fig. 2), suggesting these changes are a normal part of the CNY-681 growth cycle. Eleven peaks increased in the CI assays but not in the time course study (Fig. 2). These increases were observed with anything from one to seven of the CI assay strains (Fig. 2), suggesting they may be examples of induction due to biotic interactions. The peaks that increased in the CI assays but could not be linked to the normal growth cycle of strain CNY-681 included sioxanthin, which increased in seven of the CI assays.

A closer analysis of lomaiviticin and sioxanthin production over time showed two different patterns. Lomaiviticin levels increased up to day 10, when they exceeded those observed in two of the CI assays (Fig. 3a). There was no evidence of increased antibiotic activity in the CI assays associated with the highest levels of lomaiviticin production (e.g. CUA-883 and CUA-827), further decoupling this compound from the observed activity. In contrast, sioxanthin levels remained low throughout the monoculture time course yet increased by an order of magnitude in all of the CI assays (Fig. 3b). In two interactions (CUA-759 and CUA-827), sioxanthin levels exceeded 30 000 mAUs compared to a maximum of ca. 1000 in the time course (Fig. 3b). These increases were not associated with increased antibiotic activity (Table 1), suggesting that, like lomaiviticin, sioxanthin is not responsible for the observed growth inhibition. Nonetheless, these results clearly demonstrate that sioxanthin production increases in response to competitive interactions with co-occurring marine bacteria.

We next examined the relative scale of the peak increases among the CI assays. Although sioxanthin showed the most consistent increase in production in response to competitors, unidentified peaks at 11.7, 11.9 and 13.1 min showed the highest fold-increase in production when challenged with certain strains (Fig. 4). Three peaks (5.3, 7.6 and 12.7 min) decreased over time in the *S. tropica* CNY-681 monoculture, while only one peak decreased in the CI assays (6.8 min, CUA-812) (Fig. 4).

MS/MS networking of interaction and time course samples

Tandem mass spectrometry (MS/MS) molecular networking was used to further assess differences in the metabolites produced in the CI assays relative to the *S. tropica* CNY-681 monoculture. Molecular networking provides a rapid method to identify known compounds (de-replicate) and distinguish among different chemical analogues that have similar retention times and UV absorption spectra [38, 39, 44]. The network revealed two molecular families (clusters of nodes) seen exclusively in extracts from the CI assays

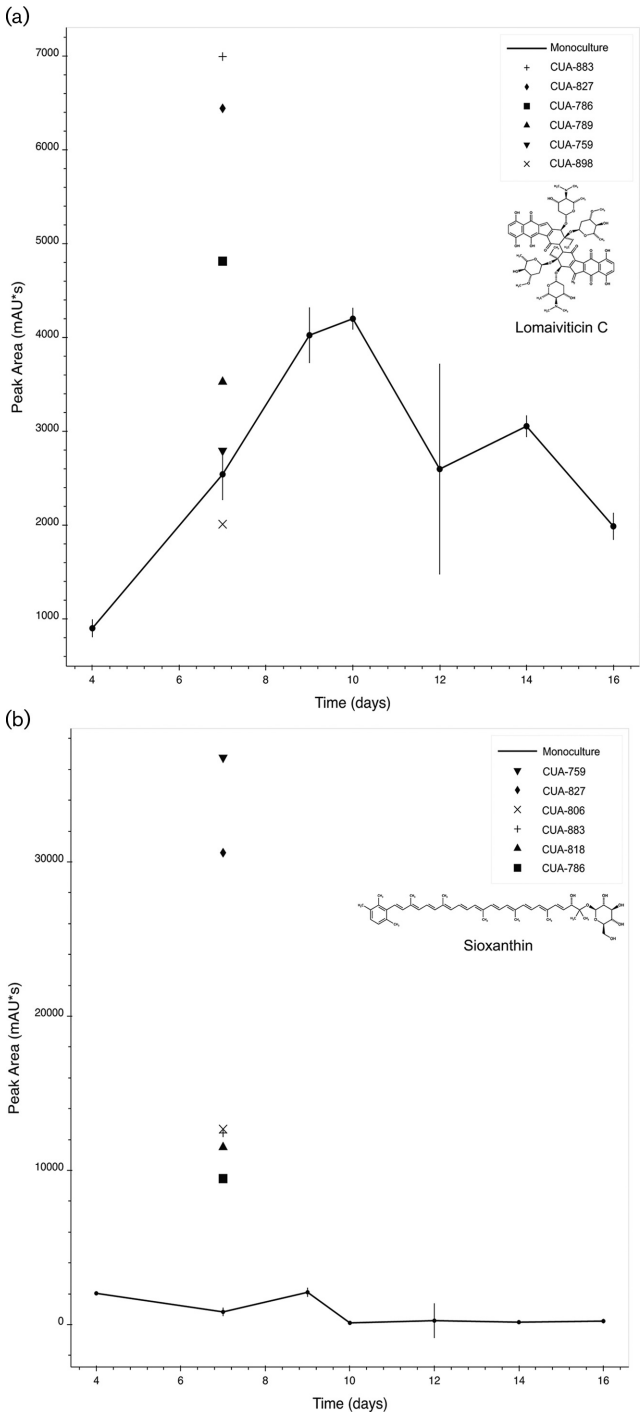

Fig. 3. Comparisons of lomaiviticin (a) and sioxanthin (b) production in the CI assays relative to *S. tropica* CNY-681 monoculture (Time Course 2). The mean values and standard deviations for each set of triplicate monoculture samples are shown, and the interaction values displayed as points on day 7. The *X*-axes correspond to the day the sample was extracted. The lines show relative metabolite production at each time point in the monoculture. The symbols at day 7 show the relative yields for each of the interactions listed in the legend. Error bars represent the standard deviations of the biological replicates.

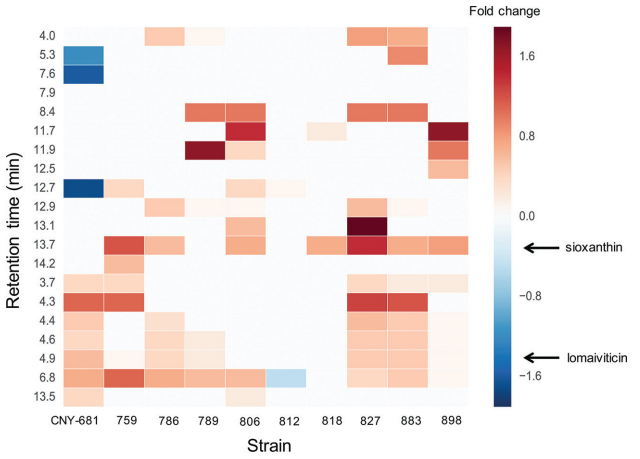

Fig. 4. Heat map of log-transformed ELSD peak integration results comparing the monoculture time course experiment to each CI assay. Each row corresponds to a peak in the HPLC trace. The 'CNY-681' column shows the maximum peak area seen at any time during the *S. tropica* monoculture time course (Time Course 2). Colours correspond to the fold change in peak area. Arrows indicate the peaks associated with sioxanthin and lomaiviticin.

(Fig. S4). The nodes in cluster 1 were associated largely with two interactions, those with *Paracoccus homiensis* CUA-883 and *Psychrobacter* sp. CUA-827 (Table S1). The cluster 2 nodes were observed almost entirely in the interaction with *Pseudoalteromonas* sp. CUA-898. Efforts are under way to isolate and identify these compounds.

Additionally, molecular networking of the *S. tropica* time course and CI assay extracts revealed the presence of lomaiviticin analogues. Lomaiviticin C was present in all time course samples and most CI assays, while lomaiviticin D and an unidentified analogue were identified only at day 12 and onward and lomaiviticin E only at days 14 and 16 (Fig. 5). In addition, we identified analogues of lymphostin that did not match MS data from any published compounds. The lymphostin pyrroloquinone alkaloids are potent immunosuppressants, many of which are produced by *S. tropica* [45]. Neolymphostins A and D were identified along with an unidentified analogue, with neolymphostin D seen in most monoculture time points and several co-cultures and neolymphostin A seen only at day 15 and onwards (Fig. 5). The unidentified analogue was seen at day 14 and onwards.

Vibrio sp. interaction and induction

While there was no chemical evidence of compound induction due to competitive interactions, results from the CI assays revealed significantly greater antibiotic activity (P=0.008) resulting from the interaction of *S. tropica* CNY-681 with *Vibrio* sp. CUA-759 (Fig. S5). A smaller increase in activity was also seen in extracts treated with a cell-free supernatant, although those effects were not significant (P=0.09) (Fig. S5). HPLC data from the CUA-759

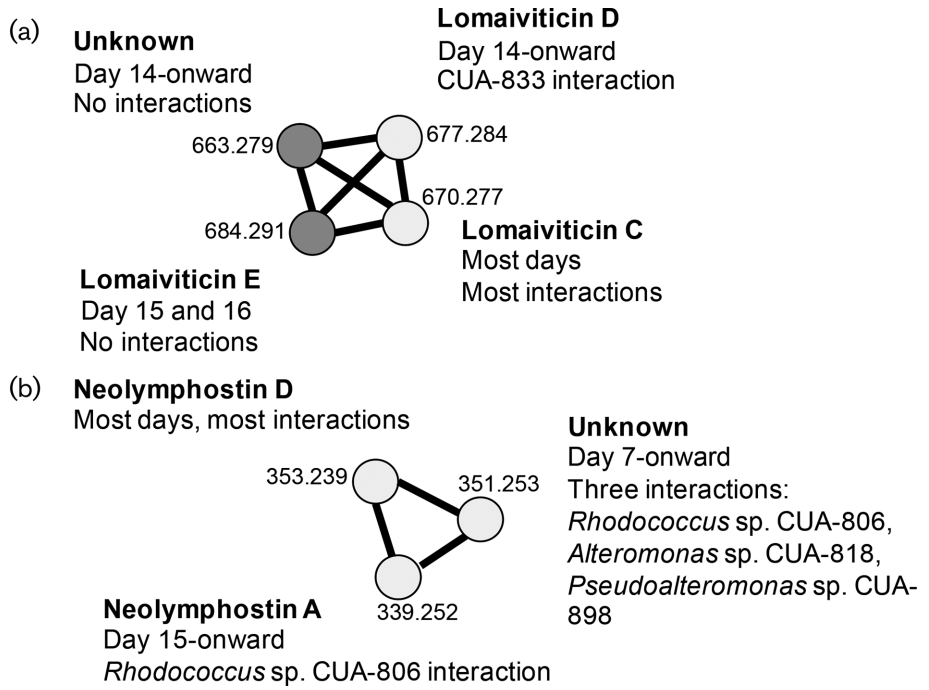

Fig. 5. An MS/MS molecular network generated from all Time Course 1 and interaction extracts revealed a number of molecular families that included known compounds. Nodes are labelled with parent ion mass, compound identity if known and extract in which they were observed (monoculture or interaction). White nodes were seen in at least one interaction, while grey nodes were seen only in monocultures. (a) Three of the four nodes matched known lomaiviticin analogues while the fourth likely represents a related and hitherto unidentified analogue. (b) Two of the three nodes matched known lymphostin analogues, while a third likely represents a related and hitherto unidentified analogue.

CI assay showed no peaks unique to the CI extract. However, three peaks (sioxanthin at 12.7 min and two unidentified peaks at 13.7 and 14.2 min, respectively) increased in the CI assay but not in the *S. tropica* monoculture time course (Fig. 3). One additional unidentified peak (at 6.8 min) showed a greater increase in the CI assay than in the time course (Fig. 3). We attempted bioassay-guided fractionation to isolate peaks associated with this antibiotic activity, but these efforts failed due to a loss of activity (data not shown).

We then turned to mass spectrometry to look for induced molecules. The MS/MS molecular network generated from plates treated with CUA-759 cell-free supernatant showed a molecular family containing nodes that were also seen in the CUA-759 CI assays but not in any of the monocultures. These included one identified as the siderophore desferrioxamine E (Fig. 6). Five out of 12 nodes in this family were associated exclusively with the interactions, suggesting induction of siderophores or related molecules as a result of the interaction.

DISCUSSION

Interspecies interactions have been employed to induce novel compound production or increase the yield of known compounds [46–48]. Proponents of this approach draw on growing evidence that adding biotic competitors or antagonists to standard culture conditions can alter the specialized metabolome of producers [16, 17, 20–22, 49]. However, little is known about the specific interaction dynamics that lead to induction, or how it relates to simple concepts like nutrient depletion or the growth phase of the producing organism. The marine actinomycete *S. tropica* is an excellent model to study chemically mediated allelopathy. It produces several bioactive specialized metabolites, none of which have been reported to possess antibiotic activity [50]. Nonetheless, recent genomic analyses have shown that only six of the 18 biosynthetic gene clusters in the *S. tropica* CNY-681 genome have been linked to their products [6], suggesting considerable unrealized biosynthetic potential that may include the production of hitherto unidentified antibiotics. Combined with prior evidence that this species has the capacity to inhibit co-occurring bacteria [36], we chose strain CNY-681 to test the hypothesis that antimicrobial specialized metabolites are induced via competitive interactions.

Among the eight environmental strains that were inhibited by strain CNY-681 in the CI assays, only three were sensitive to extracts generated from the CI assay plates (Table 1). This suggests that a majority of the inhibitory events were either due to factors other than antibiotic production, or that these strains were sensitive to compounds that were not

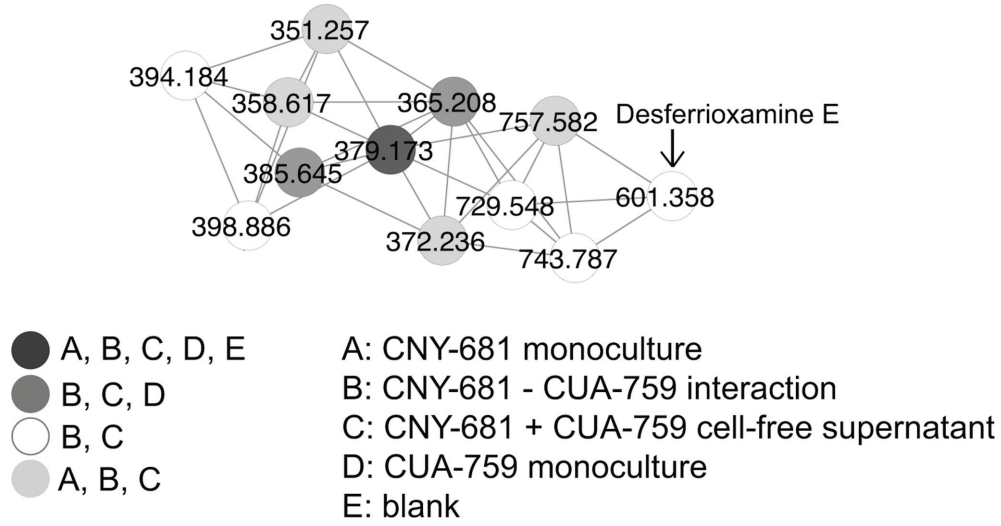

Fig. 6. A molecular family from the MS/MS molecular network generated from the *S. tropica* CNY-681–*Vibrio* sp. CUA-759 interaction and cell-free supernatant experiment. Nodes are coloured based on their observed presence in one or more extracts. An ion with *m/z* 601.358 was identified as the siderophore desferrioxamine E and was observed in only the interaction and cell-free supernatant treatment extracts.hin.

extracted with the solvents used. A comparison of monoculture and CI assay extracts revealed several interactions that contained higher levels of two known *S. tropica* compounds, the cytotoxic glycoside lomaiviticin and the carotenoid pigment sioxanthin, which gives *Salinispora* spp. its characteristic orange colour. These included one CI assay in which the test strain was not inhibited by the live *S. tropica* culture (Table 1), providing the first evidence that neither of these compounds was associated with the observed antibiotic activity. Notably, lomaiviticin levels increased over time in the monoculture, peaking around 10 days after the agar plate was inoculated (Fig. 3a). The maximum levels seen in the monoculture approached those seen in the CI assays, and in some cases exceeded them. This suggests that the induction of lomaiviticin production may not be linked to any specific signals received from the environmental strain but instead by an accelerated growth cycle, likely in response to nutrient depletion. Thus, adding the environmental strain may in effect speed up the growth cycle of *S. tropica* CNY-681 by increasing the rate at which nutrients are consumed. The absence of antibiotic activity by lomaiviticin also supports the idea that increased production is a general stress response to nutrient depletion or other changes to the medium produced by the co-cultures. However, a different pattern was seen for sioxanthin, which was present in several CI culture extracts at concentrations an order of magnitude higher than those observed in the monoculture (Fig. 3b). This provides compelling evidence for up-regulation of this carotenoid pigment in the presence of other bacteria.

Results from the *S. tropica* CNY-681 time-course monoculture experiments revealed a complex response in the secondary metabolome with respect to time. Six of the 20 peaks in the HPLC trace increased with time, which corresponds to well-established observations that actinomycete specialized metabolism is linked to the growth cycle [11, 51]. All of these peaks also increased in at least one interaction (Fig. 2), suggesting that many cases of induction in response to co-culture may not be due to a chemical signal produced by, or biotic interaction with, the competing strain. Interestingly, while lymphostin and lomaiviticin were produced throughout the monoculture time course, we observed different analogues at later time points (Fig. 5). These differences, rather than higher production of the original analogue, may also have contributed to the increase in HPLC peak areas. Taken together, these results make clear that the chemical response of *S. tropica* CNY-681 to biotic interactions is highly nuanced.

We were interested in determining the source of the antibiotic activity seen in the CI assays. Lomaiviticin C was eliminated as a candidate since it was inactive in all of the sensitivity tests. The carotenoid sioxanthin was among the compounds that showed the most dramatic increase in the CI assays without similarly increasing over time in the monocultures (Figs 3b and 4). Although carotenoids are thought to act as antioxidants, there is also precedence for them to have antibiotic activity [52–54]. Unfortunately, sioxanthin was not available for antibiotic activity testing. However, there were several other unidentified peaks seen in the HPLC trace that could be associated with the antibiotic activity, most of which had uninformative UV spectra. Attempts were made at bioassay-guided fractionation of an extract generated from a large-scale CNY-681 culture, but activity was lost during the fractionation process (data not

shown). Further work is needed to identify the source of antibiotic activity produced by *S. tropica*.

The increased activity observed in the CI assay with *Vibrio* sp. CUA-759 relative to the *S. tropica* CNY-681 time-course monoculture extracts provided an opportunity to examine more closely the possibility of induction, as well as the production, of a potential inductive signal by *Vibrio* sp. The addition of a cell-free supernatant from a liquid culture of *Vibrio* sp. produced extracts that were, on average, more active than the monoculture extracts, although the results were not significant (Fig. S5). If CUA-759 produces a chemical cue that induces antibiotic production, it may do so more efficiently on solid media than when grown in liquid. MS/MS molecular networking revealed a molecular family that contained a desferrioxamine siderophore in extracts generated from both the CI assay and following the addition of cell-free CUA-759 supernatant (Fig. 6). Increased production of an iron-chelating molecule in the presence of a bacterial competitor makes ecological sense, as the rate at which iron is depleted from the growth medium will likely increase. Indeed, this phenomenon has been observed among cultured assemblages of marine bacteria [55] and by the soil actinomycete *Streptomyces coelicolor* [56]. Desferrioxamines E and B have been reported from *S. tropica* [57], raising the possibility that the complex molecular family observed (Fig. 6) includes additional compounds in this series.

In summary, this study demonstrates that changes in specialized metabolite production in response to competitive interactions are often reproduced during the normal growth cycle of a strain. Furthermore, the responses of a single strain varied widely when challenged with different competitors, demonstrating the potential complexities of microbial interactions in nature. Deep amplicon sequencing has uncovered incredible diversity at the community level [58], and genome sequencing has revealed considerable population-level diversity in specialized metabolite biosynthetic potential [59]. The competitor-specific responses in specialized metabolite production observed here add a new level of complexity to the interpretation of this diversity and potential opportunities for specialized metabolite discovery.

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Conflicts of interest
PCD is an advisor to Sirenas, a biotechnology company that uses marine resources for therapeutic potential.

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