RESEARCH ARTICLE

Competitive interactions between sponge-associated bacteria

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One sentence summary: A subpopulation of sponge-associated Pseudovibrio spp. specifically inhibits potentially pathogenic Bacillus spp. and likely influence the composition of the sponge microbiome.

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ABSTRACT

The diversity of the microbial communities associated with marine sponges has been extensively studied, but their functioning and interactions within the sponge holobiont are only recently being appreciated. Sponge-associated microorganisms are known for the production of a range of inhibitory metabolites with biotechnological application, but the ecological role that these compounds remains elusive. In this work, we explore the competitive interactions between cultivated sponge-associated bacteria to inspect whether bacteria that produce antimicrobial activities are able to inhibit potentially pathogenic bacteria. We isolated a Bacillus sp. bacterium with sponge-degrading activity, which likely has a negative impact on the host. This bacterium, along with other sponge isolates from the same genus, was found to be inhibited by a subpopulation of closely related sponge-derived Pseudovibrio spp. In some Pseudovibrio strains, these inhibitory activities were correlated with the genetic capacity to produce polyketides, such as erythronolide. Our observations suggest that antagonistic activities likely influence the composition of the sponge microbiome, including the abundance of bacteria that can be harmful to the host.

Keywords: antagonistic interactions; bacterial competition; sponge microbiome; spongin-degrading bacteria; Pseudovibrio; Bacillus

INTRODUCTION

Nearly all internal and external surfaces of multicellular organisms are colonised by bacteria, forming dynamic and resilient microbial communities of variable complexity (Fischbach and Segre 2016). Within the marine environment, sponges represent true hotspots for microbial diversity, with individual sponge species harbouring complex, yet specific communities encompassing several hundreds of different taxa (Thomas et al. 2016). This microbial diversity provides a wide range of metabolic and biochemical features and affords an abundant potential for ‘goods and services’ to be exchanged within the sponge holobiont (Hillman and Goodrich-Blair 2016). This chemical richness has been extensively explored for pharmaceutical and biotechnological applications (Indraningrat, Smidt and Sipkema 2016). However, its true ecological function remains largely elusive, with only a few studies lending an environmental point of view to the chemical and biological interactions of the sponge microbiota (Mangano et al. 2009; Laport et al. 2016).

Densely populated microbial communities, such as the ones found in sponges, can produce antimicrobial substances up to millimolar concentrations, which very likely alter the physiology of neighbouring cells and/or induce transcriptional responses (Fischbach and Segre 2016). Such antagonistic interactions among bacteria inhabiting the same niche confer a competitive advantage for nutrients and space, thus acting as an...
effective population control (Hentschel et al. 2001). The study of these competitive relationships offers a window into the processes that operate in complex natural microbial ecosystems and may ultimately dictate the structure of the sponge holobiont.

In the present work, we investigated the antagonistic interactions between bacteria previously isolated from three marine sponge species, Cymbastela concentrica, Scopalina sp. and Tedania sp., collected along the coast of Sydney, Australia. These sponges have been shown to harbour diverse microbial communities and hold several metabolic features for symbiosis (Thomas et al. 2010). Previous studies on cultured bacteria from C. concentrica revealed an array of compounds with interesting antimicrobial activities (Penesyan et al. 2011) and the presence of collagenolytic enzymes (Yung, Kjelleberg and Thomas 2011). Given that collagen is an important component of the spongion fibres that form the sponge skeleton (Simpson 1984), this latter activity can have a negative impact on the host. Considering these observations and the requirement for sponge holobionts to form stable relationships, we hypothesised that the sponge microbiota forms a network of competitive and synergistic interactions, with some of its members producing antibiotic compounds able to inhibit otherwise potentially damaging bacteria.

METHODS
Isolation and identification of spongin-degrading bacteria

To isolate spongin-degrading bacteria, frozen microbial cell suspensions prepared from each of the three specimens of the marine sponges Scopalina sp., Tedania sp. and Cymbastela concentrica according to the methodology described by Esteves et al. (2016) were slowly thawed on ice and diluted to 10^{-2} with sterile calcium magnesium-free sea water (CMFSW; 25 g L^{-1} NaCl, 0.8 g L^{-1} KCl, 1 g L^{-1} Na_{2}SO_{4} and 0.04 g L^{-1} NaHCO_{3}) to prevent cell aggregation (Jumbiatt, Schlup and Burger 1980). A spongion slice (2–3 mm thick), which was cut from a commercial natural sponge, was autoclaved and incubated in the thawed microbial dilution overnight with shaking and at room temperature (20–23 °C). After incubation, each spongion slice was washed three times in CMFSW for 10 min with shaking at room temperature and placed on a 0.8% (w/v) gellan-gum plate prepared in CMFSW and amended with 10% (v/v) sponge extract obtained as described by Esteves et al. (2016). Briefly, to obtain this sponge extract, sponge specimens of the same species were pooled and homogenised in 10 mL of sterile natural seawater (NSW) per gram of sponge sample. This mixture was then centrifuged and the supernatant was filtered through a series of vacuum filtration steps using decreasing pore sizes (12, 3 and 0.2 µm).

After a long-term incubation (5 months) at 18 °C, colonies of potential spongin-degrading bacteria were observed. These colonies were then transferred to 15 mL of Marine Broth 2216 (BD Difco) diluted 1× with sterile NSW and incubated for 8 days at room temperature with shaking. After this incubation, a dense bacterial culture was observed for only one colony morphotype, it and other sponge-derived Bacillus strains (Table S1, Supporting Information) were tested for their sponge-degrading activity using the C. concentrica skeleton cleaned as described above. A colony of each tested isolate was inoculated into 15 mL of marine broth. Given that the presence/absence of the sponge skeleton could affect bacterial growth, two sets of tubes were prepared: in one set, a piece of cleaned sponge was added to the sponge skeleton was added to the tube at the time of inoculation, and in the other set, the sponge was added after 2 days of incubation, when a dense bacterial culture was observed. Liquid cultures were then incubated for a total of 9 days at room temperature with shaking. A control tube, consisting of 15 mL of sterile marine broth with an added piece of sponge, was included in the assay. This assay was repeated in three independent experiments over time.

Antagonistic assays and PKS gene screening

Two independent antagonistic activity assays were performed using a double-layer method as described by Esteves et al. (2013). Briefly, stationary-phase cultures of test strains (Tables S2 and S3, Supporting Information) were spotted in triplicate marine agar plates and incubated at 18 °C for 24–48 h. Colonies were then overlaid with soft marine agar (7.5 g L^{-1} agar) seeded with 1% of a stationary-phase culture of the indicator strain. Plates were incubated at 18 °C, and zones of inhibition were recorded after 2 days. Antagonistic activity was considered positive when the median of the size of the inhibition halo in the triplicates was ≥0.5 mm.

In the first antagonistic screening (Table S2), a total of 107 sponge-derived bacteria previously isolated according to the methodology described by Esteves et al. (2016) were used as test strains. These isolates were assigned to different bacterial phyla (Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria) and 34 bacterial genera. Indicator strains of this isolate, named AU673, were prepared in 3 mL marine broth at 18 °C with shaking until a dense culture was formed and aliquoted in 20% glycerol at –80 °C.

Bacterial identification was performed based on amplification and sequencing of the 16S rRNA gene using the universal primers F27 and R1492, according to the methodology described by Esteves et al. (2016). The resulting 16S rRNA gene sequence for isolate AU673 was deposited in the EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/ena) under accession number LN878593.
Figure 1. Optimal 16S rRNA gene maximum-likelihood (ML) tree for Bacillus spp. isolated from C. concentrica, Scopalina sp. and Tedania sp. (highlighted in bold and underlined). The sponge-degrading Bacillus sp. is highlighted in grey. ML bootstrap values (>70%) and Bayesian posterior probabilities (>0.90) are shown above and below branches, respectively. The tree is rooted with genus Staphylococcus (Firmicutes, Staphylococcaceae).

RESULTS AND DISCUSSION

The addition of a natural sponge skeleton to the microbial cell suspensions of three different sponge species, namely Cymbastela concentrica, Scopalina sp. and Tedania sp., was only successful in the isolation of sponge-derived bacteria from Scopalina sp. This resulted in the isolation of one strain, isolate AU673, with sponge-degrading activity and forming peach pink colonies. 16S rRNA gene sequencing yielded a 1429 bp sequence (accession nr. LN878593) showing 99% similarity to other Bacillus strains isolated from the marine environment. Isolate AU673 shared 91%–97% 16S rRNA gene sequence similarity with other Bacillus spp. that were previously isolated from C. concentrica, Scopalina sp. and Tedania sp. (Esteves et al. 2016) and which span across several different species (Fig. 1).

The sponge-degrading properties of Bacillus sp. AU673 and other sponge-derived Bacillus spp. (Table S1) were investigated using a cleaned skeleton of C. concentrica, a sponge material closest to what these bacteria are likely to be exposed in their natural environmental conditions. It was observed that the co-inoculation of the culture media with sponge material could sometimes inhibit bacterial growth, precluding any conclusions regarding sponge degradation activity. However, if a dense bacterial culture was already established, the addition of the sponge skeleton did not seem to affect bacterial growth. For this reason, two sets of tubes were prepared: one set with co-inoculation of bacteria and sponge skeleton and another set with addition of sponge skeleton to the pre-incubated bacterial culture.

Of the eight sponge-derived Bacillus spp. tested, AU29 and AU40 showed mild and inconsistent sponge-degrading activity, with big pieces of non-degraded sponge still visible after 9 days of incubation (Fig. 2). For isolate AU40, the co-inoculation of bacteria and sponge strain seemed to affect bacterial growth, with a much less dense culture being formed and no sponge degradation observed after incubation. This inhibition activity may be due to the presence of metabolites that were not efficiently removed during the sponge cleaning process or a susceptibility of isolate AU40 to the sponge skeleton itself. Isolate AU29 showed opposite results, with sponge degradation occurring...
Figure 2. Sponge degradation assay for *Bacillus* spp. isolated from sponge species *Scopalina* sp., *Tedania* sp. and *C. concentrica*. Bottom row: simultaneous inoculation of bacteria and sponge material; top row: addition of sponge piece after 2 days of inoculation. Mid-left: control tube with a piece of sponge added to sterile marine broth.

Figure 3. Binary map of the antagonistic activity screening of several sponge-derived bacterial isolates against environmentally relevant indicator strains *Bacillus* sp. AU673, *Vibrio* sp. AU189 and *Micrococcus* sp. PT110. In brackets, n stands for the number of isolates tested that presented the same antagonistic phenotype.

only in the co-inoculated tube. However, no difference in bacterial growth was observed between the co-inoculated and the pre-incubated tubes, indicating a possible loss of degradation activity at later stages of bacterial growth. Only isolate AU673 showed strong and consistent degradation activity with sponge pieces being completely reduced to fine particles in both pre-incubated and co-inoculated tubes (Fig. 2). These results were consistent and reproducible across the three independent experiments performed over time.

To identify environmentally relevant antagonistic relationships within the cultivable sponge microbiota, a broad antagonistic screening was performed, testing 107 sponge isolates against three potentially pathogenic sponge bacteria (Table S2). In this first antagonistic activity screening assay, four bacterial genera—*Streptomyces*, *Aquimarina*, *Pseudovibrio* and *Pseudoalteromonas*—showed to be antagonistic against at least one of the indicator strains. Isolates of the genus *Pseudovibrio* were particularly active against the sponge-degrading isolate AU673, with 48% of the tested *Pseudovibrio* spp. isolates showing inhibitory activity (Fig. 3). One *Pseudovibrio* isolate (AU243) inhibited *Vibrio* sp. AU189, but no isolate was found to inhibit both bacteria, which indicates that different groups of *Pseudovibrio* isolates have distinct inhibitory spectra. No activity by *Pseudovibrio* was found against *Micrococcus* sp. PT110, which was in contrast to other groups of antagonistic bacteria (e.g. *Streptomyces* and *Pseudoalteromonas*) that inhibited both Gram-positive bacteria.

Given that *Pseudovibrio* was the most abundant genus (n = 27) in our isolate collection and their high frequency of bioactive isolates (n = 12) against *Bacillus* sp., we performed a second screening using the 13 *Pseudovibrio* spp. isolates showing activity in the first screening and the 8 sponge-associated *Bacillus* spp. tested for sponge-degrading activity (Table S3). Seven out of the eight *Bacillus* spp. isolates were inhibited, including strains that did not show any sponge-degrading activity (Fig. 4). Three different groups of *Pseudovibrio* spp. could be identified based on their activity spectrum. The first group consisted of strains AU6, AU9, AU12, AU59, AU62, AU92 and AU134 that had no inhibitory activity in the second antagonism screening. Such a loss of activity has been previously observed for other sponge-associated *Pseudovibrio* spp., where antimicrobial activity was unstable and easily lost during cultivation (Muscholl-Silberhorn, Thiel and Imhoff 2008). The second group of *Pseudovibrio* isolates constituted of strains AU26, AU46, AU53, AU74 and AU243, which showed high specificity as they only inhibited strain AU673. The third group had only one strain, AU185, and showed a broader inhibitory activity against six *Bacillus* spp. isolates (Fig. 4).

To understand how these different specificities come about, we then aimed to identify the potential chemical nature of the inhibitory activities. PKS are known for their involvement in the synthesis of natural products, including compounds with potent antimicrobial activities (Staunton and Weissman 2001). In a previous study, PKS genes were detected in 73 sponge-associated...
Figure 4. Bipartite network of antagonistic interactions between Pseudovibrio spp. (blue dots) and Bacillus spp. (orange dots) sponge-associated bacteria. Arrows indicate the direction of the inhibition (test strain towards indicator strain).

Table 1. Antimicrobial activity against sponge-derived Bacillus sp. and PKS gene screening of sponge-associated Pseudovibrio spp. isolates.

| Isolate | Bioactivity | PKS | BLASTx result | % Similarity | Sequence length(bp) |
|---------|-------------|-----|---------------|--------------|-------------------|
| AU62    | –           | –   | –             | –            | –                 |
| AU53    | +           | –   | –             | –            | –                 |
| AU59    | –           | –   | –             | –            | –                 |
| AU46    | +           | –   | –             | –            | –                 |
| AU134   | –           | –   | –             | –            | –                 |
| AU92    | –           | –   | –             | –            | –                 |
| AU46    | –           | –   | –             | –            | –                 |
| AU93    | –           | –   | –             | –            | –                 |
| AU12    | –           | –   | –             | –            | –                 |
| AU74    | +           | +   | ACI32870 PKS/FAZ ketosynthase domain (Pseudovibrio PV2) | 88 | 657 |
| AU9     | –           | –   | –             | –            | –                 |
| AU26    | +           | +   | ADY17935 ketosynthase domain protein, partial (Pseudovibrio sp. Ad30) | 99 | 604 |
| AU185   | ++          | +   | KZL24059 Erythronolide synthase, modules 3 and 4 (Pseudovibrio sp. Ad37) | 99 | 592 |
| AU243   | ++          | +   | ADY17935 ketosynthase domain protein, partial (Pseudovibrio sp. Ad30) | 99 | 607 |

Bioactivity was categorised into non-active (–), specific (+) if inhibition was observed against a single Bacillus sp. strain and broad spectrum (+++) if antagonism against two or more Bacillus spp. strains was detected.

*Pseudovibrio* spp. (O’Halloran et al. 2011), and genes coding for these enzymes have been found in the metagenomes of *C. concentrica* and *Scopalina* sp. (Woodhouse et al. 2013). We therefore hypothesised that the antibacterial activity detected in our *Pseudovibrio* spp. isolates could also be related to polyketide production. A genetic screen for PKS genes showed that the antagonistic activities observed were not directly correlated with the presence of PKS genes. Some of the inhibitory *Pseudovibrio* spp. isolates did not show the presence of PKS genes, although all isolates with PKS genes were shown to be antagonistic against Bacillus spp. (Table 1).

The broad-spectrum antimicrobial activity of isolate AU185, shown to inhibit six of the eight tested Bacillus isolates (Fig. 4), is potentially related to the production of erythronolide (Table 1), a macrolide antibiotic synthesised by the PKS enzyme erythronolide synthase. This enzyme has also been identified in the genome of another sponge-associated *Pseudovibrio* sp. (Romano et al. 2016). It is interesting to note that strain AU185 did not inhibit the sponge-degrading isolate AU673, suggesting that this strain has some kind of resistance mechanism against the antibiotic.
For isolates AU243, AU26 and AU74, the PKS genes found could not be linked to a known polyketide molecule(s) thus precluding a correlation with their observed inhibitory activities. In isolates AU53 and AU46, which were active against the sponge-degrading bacterium AU673, no PKS type I gene was detected, suggesting that the antimicrobial compound(s) is (are) either produced by a PKS of a different type or are of non-polyketide nature. Previous studies have shown that Pseudovibrio spp. from sponges and seaweed produce a range of other antimicrobial compounds, such as phenol and tropodithietic acid (Penesyan et al. 2011) and toxin-like proteins (Romano et al. 2016). Whether our isolates produce these compounds and these metabolites are responsible for their antimicrobial activities remains to be tested.

The distribution of antimicrobial properties and presence of PKS genes in the Pseudovibrio isolates used in this study were not apparently correlated with their phylogenetic relationship (Fig. 5), highlighting limitations of a 16S rRNA-based analysis to predict functional properties for this genus. These limitations have also been observed for Pseudovibrio spp. derived from other sponge species and from disparate geographical locations. Using random amplification of polymorphic DNA, O’Halloran et al. (2011) identified 33 different Pseudovibrio spp. strains clustering in the same monophyletic group at the 16S rRNA level. Esteves et al. (2013) isolated 125 Pseudovibrio spp. representatives from north-eastern Atlantic Ircinidae sponges, covering 49 different BOX-PCR genotypes that shared 99% or more 16S rRNA gene sequence similarity. Such higher resolution phylogenetic markers might reveal a better correlation between evolutionary relationships and phenotypic properties in the future.

Within the sponge host, diverse patches and microniches can be found due to local and/or temporal variations (Fieseler et al. 2016), highlighting limitations of a 16S rRNA-based analysis to predict functional properties for this genus. These limitations have also been observed for Pseudovibrio spp. derived from other sponge species and from disparate geographical locations. Using random amplification of polymorphic DNA, O’Halloran et al. (2011) identified 33 different Pseudovibrio spp. strains clustering in the same monophyletic group at the 16S rRNA level. Esteves et al. (2013) isolated 125 Pseudovibrio spp. representatives from north-eastern Atlantic Ircinidae sponges, covering 49 different BOX-PCR genotypes that shared 99% or more 16S rRNA gene sequence similarity. Such higher resolution phylogenetic markers might reveal a better correlation between evolutionary relationships and phenotypic properties in the future.

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potent antimicrobial properties have been extensively documented (Crowley et al. 2014 and references therein). 

Pseudovibrio spp. have also been detected in sponge larvae indicating likely vertical transmission (Enticknap et al. 2006). Recent genomic studies have further revealed a versatile metabolism (Bondarev et al. 2013), diverse genomic features linked to symbiosis (Romano et al. 2016) and a lifestyle allowing for host switching (Alex and Antunes 2015). These previous findings integrate well with our present observations on the antagonistic activity of sponge-associated 
Pseudovibrio spp. towards potentially harmful Bacillus spp., their diversified chemical arsenal and their respective relative abundances found within the host. Altogether, these results suggest that 
Pseudovibrio spp. is an ideal candidate for a true sponge symbiont, with a potential role in host defence and thus ultimately playing a preponderant part in structuring sponge-associated microbial communities. Results from inhibitory assays among cultivated bacteria in laboratory systems do provide indications on microbial interactions occurring in the natural environment. Further studies on these interactions at the organismal level will be crucial to understand the context of these behaviours, their positive or negative contribution to host health, and to broaden our knowledge on the dynamics and structure of the marine sponge microbiome.

SUPPLEMENTARY DATA
Supplementary data are available at FEMSEC online.

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