environmental microbiology reports

Environmental Microbiology Reports (2017) **9**(6), 750–755 doi:10.1111/1758-2229.12591

# Redefining the sponge-symbiont acquisition paradigm: sponge microbes exhibit chemotaxis towards host-derived compounds

**Jessica Tout,[1] Carmen Astudillo-García,[2] Michael W. Taylor,[2] Gene W. Tyson,[3] Roman Stocker,[4] Peter J. Ralph,[1] Justin R. Seymour[1] and Nicole S. Webster[3,5]***

[1]*Plant Functional Biology & Climate Change Cluster, University of Technology Sydney, NSW, Australia.*

[2]*School of Biological Sciences, University of Auckland, New Zealand.*

[3]*Australian Centre for Ecogenomics, School of Chemistry and Molecular Biosciences, University of Queensland, St Lucia, QLD, Australia.*

[4]*Department of Civil, Environmental and Geomatic Engineering, Institute of Environmental Engineering, ETH Zurich, 8093 Zurich, Switzerland.*

[5]*Australian Institute of Marine Science, Townsville, QLD, Australia.*

## Summary

**Marine sponges host stable and species-specific microbial symbionts that are thought to be acquired and maintained by the host through a combination of vertical transmission and filtration from the surrounding seawater. To assess whether the microbial symbionts also actively contribute to the establishment of these symbioses, we performed *in situ* experiments on Orpheus Island, Great Barrier Reef, to quantify the chemotactic responses of natural populations of seawater microorganisms towards cellular extracts of the reef sponge *Rhopaloeides odorabile*. Flow cytometry analysis revealed significant levels of microbial chemotaxis towards *R. odorabile* extracts and 16S rRNA gene amplicon sequencing showed enrichment of 'sponge-specific' microbial phylotypes, including a cluster within the *Gemmatimonadetes* and another within the *Actinobacteria*. These findings infer a potential mechanism for how sponges can acquire bacterial symbionts from the surrounding environment and suggest an active role of the symbionts in finding their host.**

Received 21 November, 2016; revised 30 August, 2017; accepted 31 August, 2017. *For correspondence. E-mail: n.webster@aims.gov.au; Tel. +61 747534151; Fax +61 747725852.



## Introduction

Sponges contain microbial symbionts from all three domains of life – Bacteria, Archaea and Eukarya – which can comprise up to 35% of a sponge's biomass and are essential for host fitness and survival [reviewed in (Webster and Taylor, 2012)]. Molecular surveys have revealed that many sponge-associated microbes occur exclusively within sponges (Hentschel *et al*., 2002; Taylor *et al*., 2007b) or are exceptionally rare in the surrounding coral reef environment (Taylor *et al*., 2013; Thomas *et al*., 2016). These microbes often form monophyletic 'sponge-specific' 16S rRNA gene sequence clusters (Hentschel *et al*., 2002; Taylor *et al*., 2007a, Simister *et al*., 2012). The occurrence of these 'sponge-specific' bacteria in phylogenetically distant sponges from geographically isolated locations, coupled with the rarity of these bacteria in the surrounding environment, has led to interest in the evolutionary mechanisms that have maintained these complex and diverse symbioses (Webster and Thomas, 2016).

Previous research has indicated that sponges likely acquire their symbionts via the dual mechanisms of microbial filtration from the surrounding seawater (Reiswig, 1971; Webster *et al*., 2010) and vertical transmission from parent to offspring (Schmitt *et al*., 2007; Sharp *et al*., 2007; Webster *et al*., 2010). However, it has remained unclear whether these microbial associations are solely controlled by the sponge or if the microbes are also capable of actively seeking out their hosts on coral reefs. For instance, it has recently been shown that coral-associated microbes display high levels of chemotaxis to chemicals released from the coral holobiont (Garren *et al*., 2014; Tout *et al*., 2015). Here we propose that chemotaxis may also be involved in the formation of 'sponge-specific' microbial interactions and we assessed this using the model Great Barrier Reef sponge *Rhopaloeides odorabile*, which hosts a highly diverse and stable microbial community that is critical in regulating host health (Webster and Hill, 2001; Webster *et al*., 2001a,b; 2008; 2011; Fan *et al*., 2012). We assessed whether natural populations of coral reef bacteria exhibit chemotaxis towards cellular extracts of *R. odorabile* and examined whether previously identified

sponge symbionts, which are rare or undetectable in seawater, are among these chemotactic microbes.

This study was conducted in July 2013 at Orpheus Island (18°35.5959′S, 146°28.9559′E) on the Great Barrier Reef, Australia. To assess microbial chemotaxis towards extracts of the sponge *R. odorabile*, we used a microfluidic-based *in situ* chemotaxis assay (ISCA) (Tout *et al.*, 2015) (Supporting Information), whereby the strength of microbial chemotaxis was assessed using cytometric cell counts and the composition of the chemotactic microbes was determined using 16S rRNA gene amplicon sequencing.

To prepare sponge cellular extracts, three replicate *R. odorabile* samples were used. A 1 $cm^3$ section of tissue was excised from each sponge and homogenized in 10 ml of 0.2 μm-filtered seawater (FSW) using a mortar and pestle. The tissue homogenates were carefully filtered through 0.2 μm syringe filters (Millipore) to remove microbial cells and subsequently filtered through Amicon Ultra 0.5 3 Kda filters (Millipore) to remove any remaining DNA. To ensure all microbial DNA had been removed from the sponge extracts, PCR reactions were performed as described in Fig. 2 and no positive amplificates were obtained. The FSW controls were prepared by filtering 50 ml of reef water through a 0.2 μm syringe filter (Millipore). The Marine Broth (MB) positive controls were prepared by mixing a 1% MB (Difco) solution and filtering through a 0.2 μm syringe filter (Millipore).

Across all ISCAs the chemotactic response of the natural microbial community to the cellular extracts of *R. odorabile* was significantly greater than to the FSW and even the MB positive controls ($I_c = 5.7 \pm 1.3$, $P < 0.05$; Fig. 1, Supporting Information Table S1). While the community composition (OTUs defined at 97% sequence similarity) of chemotactic bacteria responding to the *R. odorabile* extracts was not significantly different from the FSW controls (Fig. 2A, $P > 0.05$; Supporting Information Table S2), we identified an elevated presence of 'sponge-specific' sequences within the *R. odorabile* extract samples. A total of 56 sequence matches to previously defined 'sponge-specific' sequence clusters were observed (Supporting Information Table S3), with 96% of these occurring within the *R. odorabile* cellular extracts (Fig. 2B). Of these, 66% were affiliated to the *Gemmatimonadetes* cluster SC67 and 30% to the *Actinobacteria* cluster SC22 [as defined by (Simister *et al.*, 2012)]. The *R. odorabile* microbiome has previously been described using full length clone sequencing and 454 sequencing of the 16S rRNA gene and was found to be dominated by *Proteobacteria*, *Acidobacteria*, *Choloroflexi*, *Actinobacteria* and *Gemmatimonadetes* (Webster *et al.*, 2001a,b). While the chemotactic microorganisms that fell into sponge-specific sequence clusters were not identical matches to published *R. odorabile* sequences, the assignment to *Actinobacteria* and *Gemmatimonadetes* sponge-specific lineages is nonetheless consistent with the previously described microbial community of *R. odorabile*. The most abundant bacterial families responding to cellular extracts of *R. odorabile* were the *Sphingomonadaceae, Rhodobacteraceae* and *Piscirickettsiaceae*, which represented 12%, 9% and 8% of the total community, respectively (Fig. 2A, Supporting Information Table S4). The primary drivers of the differences between the communities responding to *R. odorabile* extracts and to the FSW control were members of the *Piscirickettsiaceae, Sphingomonadaceae* and *Vibrionaceae* (each contributed 2% to the total dissimilarity), which were all more abundant in the *R. odorabile* cellular extracts than in the FSW controls (Fig. 2A, Supporting Information Table S5).

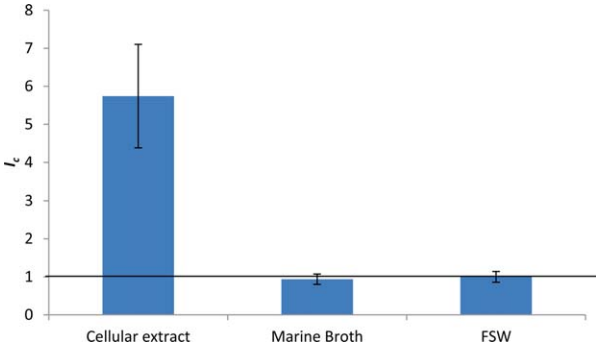
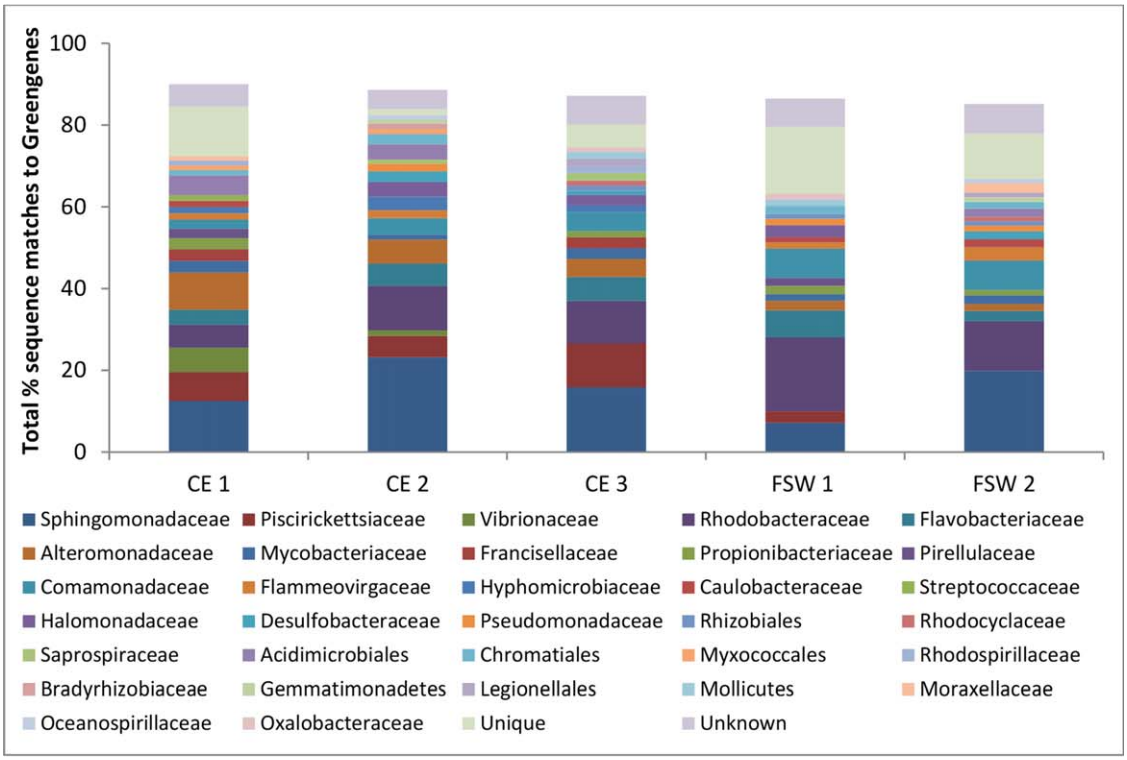
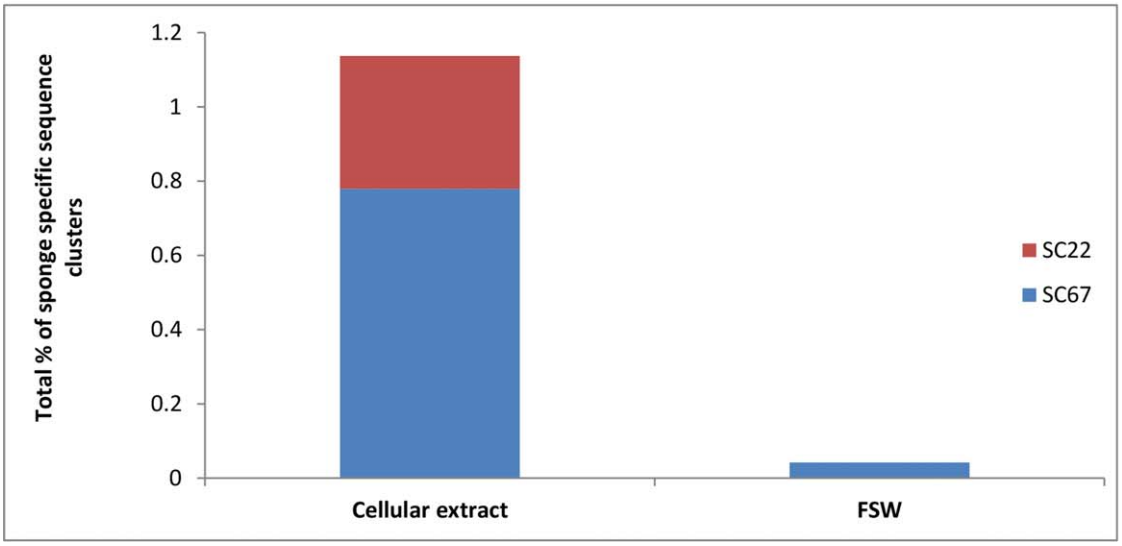



**Fig. 1.** Accumulation of bacteria in response to chemoattractants. Chemoattractants included (i) 3 Kda filtered *R. odorabile* cellular extracts, (ii) 0.2 μm-FSW controls and (iii) 0.2 μm-filtered 1% MB nutrient controls. Three replicate ISCAs were deployed onto the Orpheus Island reef flat at 8 m water depth in a region devoid of sponges. For each ISCA deployment, 7 replicates of each chemoattractant were randomly distributed across the ISCA with 4 subsequently used for flow cytometry and 3 used for DNA extraction. A background seawater sample (1l) was also collected from the same location prior to ISCA deployments for 16S rRNA gene sequencing. Over the course of the 4 h deployment, the chemoattractant diffuses into the external environment, creating a chemical gradient in the surrounding seawater (Berg, 1993) that triggers the migration of chemotactic bacteria into the wells. The accumulation of bacteria was assessed by flow cytometry as described in (Tout *et al.*, 2015) and expressed in terms of a Chemotactic Index, $I_c$ (mean ± standard deviation), with $I_c$ calculated as the number of cells responding to the chemoattractant relative to the number of cells responding to the FSW control. Responses above $I_c = 1$ represent statistically significant positive chemotaxis. Vertical bars represent mean ± SD ($n = 4$).

The important role of chemotaxis in structuring host-bacterial associations within the marine environment is becoming increasingly apparent (Banin *et al.*, 2001; Rosenberg *et al.*, 2007; Meron *et al.*, 2009; Garren *et al.*, 2014; Tout *et al.*, 2014; 2015). The results presented here are the first observations of chemotaxis by sponge symbionts towards sponge-derived compounds. Importantly, previously defined 'sponge-specific' bacteria were not detected by amplicon sequencing of the




background coral reef seawater, which is consistent with previous reports that these microorganisms are exceptionally rare outside of sponge hosts (Taylor *et al.*, 2013). However, environmentally rare 'sponge-specific' bacteria were present in the *R. odorabile* extract treatment, indicating that they exhibited chemotaxis towards sponge-derived chemicals, highlighting a potential new mechanism for the establishment of sponge-bacteria associations.

Marine sponges produce a wide spectrum of bioactive molecules (Mehbub *et al.*, 2014), which can be actively secreted from the sponge into the surrounding seawater. For instance, *Geodia barretti* secretes the brominated cyclopeptide barrettin into the surrounding seawater in sufficient concentrations to completely inhibit the settlement of fouling barnacles (Sjögren *et al.*, 2004). This secretion of molecules would generate chemical gradients that could stimulate chemotaxis towards the

**Fig. 2.** A. Taxonomic composition of bacteria responding to the ISCA deployments. To identify the composition of the chemotactic microbes, samples from 3 replicate wells per ISCA were pooled and DNA was extracted from the sponge extract and FSW ISCA samples using the Ultra Clean microbial DNA isolation kit (Mo Bio, Carlsbad, CA). DNA was additionally extracted from the bulk 1l seawater sample to screen for the presence of sponge-specific microbial OTUs in the surrounding reef seawater. Extracted DNA was amplified using the 16S primers 803F (5′-ATTAGATACCCTGGTAGTC-3′) and 1392R (5′-ACGGGCGGTGTGTRC-3′) under the following cycling conditions: 95°C for 3 min; 25 cycles of 95°C for 30 s, 55°C for 45 s and 72°C for 90 s; followed by a final extension at 72°C for 10 min. Amplicons were sequenced using the 454 GS-FLX pyrosequencing platform (Roche) at the Australian Centre for Ecogenomics (University of Queensland, Australia). 16S rRNA gene sequences were analysed using the QIIME pipeline (Kuczynski *et al*., 2011; Caporaso *et al*., 2010) and data were submitted to the sequence read archive at NCBI under the accession SUB1639379. Bar charts summarize the bacterial taxonomy at the family level (data are averages of $n = 3$ ISCAs/treatment). The microbial community identified in the FSW control is representative of organisms that swam into this treatment as a consequence of random motility, rather than chemotaxis due to the lack of any chemical gradient. Thus, this sample provides an overview of the motile, but not necessarily chemotactic proportion of the community. B. Composition of chemotactic 'sponge-specific' sequence clusters in the cellular extract of *R. odorabile* and FSW control. A representative sequence (average sequence length of 505 bp) of each identified OTU was taxonomically assigned (using a BLAST search) with a curated SILVA 16S rRNA database containing 173 previously identified bacterial sponge-specific clusters (SC) and 32 sponge/coral-specific clusters (SCC) (Simister *et al*., 2012). For each BLAST search, the 10 best hits were aligned to determine sequence similarities. A sequence was assigned to an SC/SCC when it was more similar to the sequences comprising that cluster than to other sequences outside the cluster and the similarity to this sequence was at least 75% (Taylor *et al*., 2013).

sponge and is likely further facilitated by diffusion, as these basal marine invertebrates lack true tissues as barriers to the external environment. Therefore, while the complete compliment of chemical compounds released from sponges into the surrounding seawater remains to be defined, we envisage that a substantial proportion of the molecules present within our sponge extract will be released into the surrounding seawater, providing potential cues for chemotactic migration towards the surface of the sponge.

The concept of sponges hosting specific and stable symbiont populations that provide benefit to the host was originally proposed by Vacelet and Donadey (1977) and subsequently validated by extensive molecular research [reviewed in (Hentschel *et al*., 2012; Webster and Taylor, 2012; Webster and Thomas, 2016)]. For example, members of the *Piscirickettsiaceae* and *Sphingomonadaceae*, which were the most differentially represented taxa responding to the sponge extract and the FSW control in this study, are known to form intimate associations with marine sponges (Thomas *et al*., 2010) and have been linked by functional metagenomics to the production of enzymes involved in the vitamin B12 synthesis pathway (Thomas *et al*., 2010). The theory of sponge symbiont specificity was expanded by the discovery that sponges host microbes falling within monophyletic 'sponge-specific' 16S rRNA gene sequence clusters (Hentschel *et al*., 2002; Taylor *et al*., 2007a,; Simister *et al*., 2012). These 'sponge-specific' sequence clusters are defined as groups of 16S rRNA gene sequences that share greater similarity to each other than to sequences from non-sponge sources, are derived from at least two or more sponge species (or the same species from at least two different locations) and are supported by at least three independent phylogenetic tree-building algorithms (Hentschel *et al*., 2002). The observed chemotaxis of microbes within the *Gemmatimonadetes* and *Actinobacteria* 'sponge-specific' 16S rRNA gene sequence clusters provides initial evidence that some sponge symbionts may actively find their sponge host on coral reefs using chemotaxis. These microbial phyla are known to associate with a diverse range of sponges (Taylor *et al*., 2007b) and are highly active within their respective hosts (Kamke *et al*., 2010), yet have been shown to be rare in the surrounding seawater on the Great Barrier Reef (Webster *et al*., 2010; Bourne *et al*., 2013).

This study illustrates that chemotaxis may underpin the establishment of some sponge-bacterial associations. Future studies that further refine the specific chemical cues derived from different sponge hosts and explore the mechanisms of chemoattractant secretion are likely to reveal further patterns of sponge symbiont attraction. Our observations indicate that the behavior of individual microbes can potentially underpin the establishment of an important animal-microbe symbiosis and adds to the growing evidence that chemotaxis is an ecologically important phenotype in the marine environment.

## Acknowledgements

We thank Steve Whalan and Muhammad Abdul Wahab for their assistance in the field. NSW and JRS were funded through Australian Research Council Future Fellowships FT120100480 and FT130100218 respectively. ISCA technology was funded through Australian Research Council Grant DP110103091 to JRS, GWT and RS and the Gordon and Betty Moore Foundation Grant #3801 to JRS, RS and GWT. Field support for ISCA deployments was provided by the Human Frontiers in Science Program award no. RGY0089 to JRS and RS. JT was supported by a postgraduate award from the Department of Environmental Science and Climate Change Cluster at the University of Technology Sydney. The authors have no conflict of interest.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Table S1.** $I_c$ data were tested for normality using the Kolmogorov-Smirnov test and Levene's test was used to test for homogeneity of variance. A one-way ANOVA was used to determine differences between the FSW control and the sponge cellular extract (FSW*cellular extract). All analyses were performed using Minitab software (version 15.1.0.0 2006, Pennsylvania, USA). One-way fixed factor ANOVA to determine chemotactic response using an ISCA containing cellular extracts of *R. odorabile*, FSW control and MB control. Table relates to data presented in Fig. 1.

**Table S2.** PERMANOVA analysis based on a weighted Unifrac distance matrix and 9999 permutations.

**Table S3.** Previously identified sponge-specific sequence clusters that were detected among the OTUs in this study; taxonomy matches were generated by comparing the sequences with BLASTn to the Greengenes database in QIIME. The *e* value cutoff was assigned at 0.000001, and the minimum query coverage was set at 60%.

**Table S4.** SIMPER analysis of chemotactic families responding to the cellular extract of *R. odorabile* with an average similarity of 49.66% across all three ISCA deployments on Orpheus Island

**Table S5.** SIMPER analysis of chemotactic families contributing to dissimilarities between the cellular extracts of *R. odorabile* and the FSW control used in ISCA deployments on Orpheus Island with an average dissimilarity of 52.43%.