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Activity profiles for marine sponge-associated bacteria obtained by 16S rRNA vs 16S rRNA gene comparisons

Janine Kamke, Michael W Taylor and Susanne Schmitt

School of Biological Sciences, The University of Auckland, Auckland, New Zealand

The phylogenetic diversity of microorganisms in marine sponges is becoming increasingly well described, yet relatively little is known about the activities of these symbionts. Given the seemingly favourable environment provided to microbes by their sponge hosts, as indicated by the extra-ordinarily high abundance of sponge symbionts, we hypothesized that the majority of sponge-associated bacteria are active *in situ*. To test this hypothesis we compared, for the first time in sponges, 16S rRNA gene- vs 16S rRNA-derived bacterial community profiles to gain insights into symbiont composition and activity, respectively. Clone libraries revealed a highly diverse bacterial community in *Ancorina alata*, and a much lower diversity in *Polymastia* sp., which were identified by electron microscopy as a high- and a low-microbial abundance sponge, respectively. Substantial overlap between DNA and RNA libraries was evident at both phylum and phylotype levels, indicating *in situ* activity for a large fraction of sponge-associated bacteria. This active fraction included uncultivated, sponge-specific lineages within, for example, *Actinobacteria*, *Chloroflexi* and *Gemmatimonadetes*. This study shows the potential of RNA vs DNA comparisons based on the 16S rRNA gene to provide insights into the activity of sponge-associated microorganisms.

Introduction

Many marine sponges harbour dense and diverse microbial communities of considerable ecological and biotechnological importance (Hentschel *et al.*, 2006; Taylor *et al.*, 2007b). These communities, which can include bacteria, archaea and eukaryotic microorganisms, are often quite specific to sponges, with many microbial phylotypes appearing to live exclusively within sponge hosts and not in the surrounding seawater (Hentschel *et al.*, 2002; Taylor *et al.*, 2007b; Schmitt *et al.*, 2008). Although our understanding of the microbial diversity in sponges is rapidly improving, much remains unknown about the activity of these microbes (Taylor *et al.*, 2007a). Specific microbially mediated processes within sponges, such as photosynthesis, sulphate reduction, nitrogen fixation and nitrification, have been quantified and in many cases the relevant microbes have been identified (Wilkinson and Fay, 1979; Wilkinson, 1983; Diaz and Ward, 1997; Wilkinson *et al.*, 1999; Hoffmann *et al.*, 2005, 2009; Hallam *et al.*, 2006; Bayer *et al.*, 2008; Mohamed *et al.*, 2008a,

2009; Steger *et al.*, 2008). These studies, which have utilized methods such as isotope enrichments, metagenomics and functional gene analyses, have extended our knowledge of symbiont function in sponges, yet they remain focused on specific processes or particular functional groups of organisms. What is lacking to date is a community-wide assessment of microbial activity in marine sponges. This would be useful, as identification of those sponge-associated microbes that are active *in situ* is an important step towards elucidating their ecological role and contribution to the host.

All living organisms contain ribosomal RNA, and these molecules have become the gold standard for microbial ecology and taxonomy (Ludwig and Schleifer, 1999; Tringe and Hugenholtz, 2008). The analysis of 16S rRNA genes through clone libraries and fingerprinting approaches such as denaturing gradient gel electrophoresis (DGGE) has greatly extended our knowledge about the phylogenetic richness of sponge-associated bacteria and archaea (Webster *et al.*, 2001, 2004; Hentschel *et al.*, 2002; Taylor *et al.*, 2004, 2007b; Holmes and Blanch, 2006; Longford *et al.*, 2007; Schmitt *et al.*, 2007, 2008; Thiel *et al.*, 2007; Mohamed *et al.*, 2008b; Zhu *et al.*, 2008). Researchers in other systems have taken this approach one step further, yielding insights into both richness and activity by comparing 16S rRNA gene- and 16S rRNA-derived sequences,

Correspondence: MW Taylor, School of Biological Sciences, The University of Auckland, Private Bag 92019, Auckland 1142, New Zealand. E-mail: mw.taylor@auckland.ac.nz

respectively (Moeseneder et al., 2001, 2005; Winter et al., 2001; Troussellier et al., 2002; Mills et al., 2005; Gentile et al., 2006; Martinez et al., 2006; Brinkmann et al., 2008; McIlroy et al., 2008; West et al., 2008; Rodriguez-Blanco et al., 2009). In general, cellular concentrations of rRNA are correlated with growth rate and activity (DeLong et al., 1989; Poulsen et al., 1993), hence—with acknowledgement of certain caveats (e.g., for ammonia-oxidizing bacteria; Morgenroth et al., 2000)—the rRNA itself can yield useful information about which community members are active.

In this study we investigated bacterial community composition (16S rRNA gene) and activity (16S rRNA) in two marine sponges from north-eastern New Zealand. Clone libraries, generating a total of 313 sequences, were constructed from the high-microbial-abundance sponge *Ancorina alata* (Demospongiae: Astrophorida: Ancorinidae) and the low-microbial-abundance sponge *Polymastia* sp. (Demospongiae: Hadromerida: Polymastiidae). The existence of both high- and low-microbial-abundance sponges is well documented (Vacelet and Donadey, 1977; Reiswig, 1981; Hentschel et al., 2006; Weisz et al., 2008), although the exact reasons for these differences in microbial loads are uncertain. In addition to the well-characterized taxa, such as the *Alpha*- and *Gammaproteobacteria*, activity was inferred for uncultivated, sponge-specific lineages within phyla, including the *Gemmatimonadetes*, *Chloroflexi* and a taxon of uncertain affiliation related to the sponge-specific 'Poribacteria' (Fieseler et al., 2004). Moreover, our results show the potential of rRNA gene vs rRNA comparisons to provide insights into the activity of sponge-associated microorganisms.

Materials and methods

Sponge sampling
Small samples were taken from three individuals of each of the sponges *A. alata* and *Polymastia* sp. (both class Demospongiae). Sampling was carried out by SCUBA diving at depths of 3–10 m at Mathesons Bay (36°18′S, 174°47′E) and Jones Bay (36°23′S, 174°49′E), northeastern New Zealand, in November/December 2008. Tissue samples were transferred into RNAlater (Applied Biosystems/Ambion, Foster City, CA, USA), then transported to the laboratory on ice before freezing at −80 °C. Samples were subsequently freeze-dried and stored again at −80 °C. Tissue samples for electron microscopy were cut into small pieces of about 1 mm³, fixed in 2.5% glutaraldehyde double-distilled water, and kept overnight at 4 °C before processing.

Transmission electron microscopy (TEM)
Fixed sponge samples (three individuals per sponge) were washed five times in cacodylate buffer (50 mM, pH 7.2), fixed in 2% osmium tetroxide for 90 min, washed five times in double-distilled water,

and incubated overnight in 0.5% uranyl acetate. After dehydration in an ethanol series (30%, 50%, 70%, 90%, 96%, and three times at 100% for 30 min each), samples were incubated three times for 30 min in 1 × propylene oxide, maintained overnight in 1:1 (vol/vol) propylene oxide-Epon 812 (EM bed-812, Electron Microscopy Science, Hatfield, PA, USA), incubated twice for 2 h in Epon 812, and finally embedded in Epon 812 for 48 h at 60 °C. Samples were then sectioned with an ultra-microtome (Leica, Wetzlar, Germany; EM UC6) and examined by TEM (Philips CM12, Fei Company, Hillsboro, OR, USA).

Nucleic acid extraction and construction of 16S rRNA gene/rRNA clone libraries
Total DNA and RNA were co-extracted from 5–6 mg of freeze-dried sponge tissue using the AllPrep DNA/RNA mini kit (Qiagen, Hilden, Germany). Extractions were performed separately for all three individuals of both species (*A. alata* and *Polymastia* sp.). The RNA extract was subsequently purified by DNA digestion for 60 min using RQ1 RNase-free DNase (Promega, Madison, WI, USA) and RNA was reverse-transcribed into cDNA using random hexamers in the SuperScript III First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Afterwards, DNA, cDNA and RNA were PCR-amplified with the universal bacterial primers 616V (5′-AGAGTTTGATYMTGGCTC-3′) and 1492R (5′-GGYTACCTTGTTACGACTT-3′) (Kane et al., 1993; Juretschko et al., 1998), spanning a ∼1500 bp region of the 16S rRNA gene. The RNA template served as a control in the PCR and did not give any products. Cycling conditions on a T1 Thermocycler (Biometra, Goettingen, Germany) were as follows: initial denaturing step at 95 °C for 5 min, 30 cycles of denaturing at 95 °C for 1 min, primer annealing at 54 °C for 1 min and elongation at 72 °C for 90 s, followed by a final extension step at 72 °C for 10 min. The PCR products from each sponge species representing the respective DNA and cDNA fractions were pooled and ligated into the pGEM-T-easy vector (Promega) and clone libraries were constructed according to the manufacturer's instructions (four libraries in total). Pooling of PCR products was justified given that corresponding denaturing gradient gel electrophoresis profiles based on DNA and RNA revealed high inter-individual similarities (data not shown). Clones that contained an insert (as evaluated by blue/white colony screening) were grown overnight at 37 °C. After a lysis step for 30 min at 94 °C, a PCR was performed with the vector-specific primers PGEM-F and PGEM-R (Aislabie et al., 2009), to determine which clones contained a correct-sized insert. Cycling conditions were the same as described above. In total, 375 (192 for *Polymastia* sp. and 183 for *A. alata*) PCR products were sequenced by Macrogen (Seoul, Korea), and, after the removal of poor quality sequences and chimeric sequences

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Statistical and phylogenetic analyses

The nonparametric richness estimator Chao1, used to evaluate how much of the richness in each library was sequenced, was calculated at different operational taxonomic unit (OTU) thresholds using DOTUR implented in Mothur (Schloss and Handelsman, 2005). Preliminary phylogenetic affiliations were obtained for all DNA- and cDNA (RNA)-derived sequences using the NCBI's BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences from A. alata and Polymastia sp., as well as their closest relatives identified by BLAST, were aligned using the web-based SINA aligner, then imported into a 16S rRNA ARB-SILVA database (Ludwig et al., 2004; Pruesse et al., 2007) (version 96, containing 271 543 bacterial rRNA sequences) for subsequent manual refinement of the alignment (inspection of the automatic alignment by eye and manual corrections where appropriate using the editor tool in the ARB software package). Maximum likelihood, maximum parsimony and neighbor-joining trees were calculated in ARB using long (⩾1200 bp) sequences only. Shorter sequences were added using the parsimony interactive tool in ARB without altering the tree topology. Phylogenetic consensus trees, using the maximum likelihood tree as a backbone, were manually constructed (Ludwig et al., 1998). Maximum parsimony bootstraps (1000 resamplings) were performed to further assess the stability of observed branching patterns.

Results

Examination of sponge mesohyl (three individuals per species) by TEM revealed the presence of large numbers of extracellular, morphologically diverse microorganisms within the sponge A. alata, whereas very few microbial cells were seen in Polymastia sp. (Figure 1). We thus deem A. alata to be a 'high-microbial-abundance' sponge (sensu; Hentschel et al., 2006), whereas Polymastia sp. is a 'low-microbial-abundance' sponge.

A total of 313 sequences was obtained from the sponges A. alata and Polymastia sp., all of which were of bacterial origin. Of the 157 sequences from A. alata, 78 were derived from the 16S rRNA gene and 79 from 16S rRNA. For Polymastia sp. we obtained 85 16S rRNA gene sequences and 71 sequences from 16S rRNA. Bacterial phylum-level richness was much higher in the A. alata libraries, with members of eight phyla (including three Proteobacteria classes) recovered from the DNA fraction, and seven of these also occurring in the RNA fraction (Figures 2–5, Supplementary Figures 1–4). When a 99% sequence similarity threshold was used to define an OTU, 43 OTUs were found in the A. alata DNA-derived library, whereas 31 OTUs were found when a 95% OTU definition was applied. The Chao1 estimates of total OTU richness were 73 and 35 for the 99% and 95% OTUs, respectively. In the A. alata RNA library, thirty-eight 99% OTUs were identified (Chao1 estimate = 74), whereas thirty 95% OTUs were found (Chao1 = 45). The Polymastia sp. DNA library comprised only two bacterial phyla, with three represented in the RNA library (Figures 2–5, Supplementary Figures 1–4). Forty-five 99% OTUs were found in the DNA library (Chao1 = 279), with twenty-two 95% OTUs recovered (Chao1 = 37). In the RNA library from Polymastia sp., sixteen 99% OTUs were found (Chao1 = 21), compared with thirteen 95% OTUs (Chao1 = 15).

At the phylum level, the compositions of the DNA- and RNA-derived libraries from A. alata were very similar (Figure 2). The most abundant taxa in both libraries were the class Gammaproteobacteria (21% of DNA clones and 34% of RNA clones) and the Chloroflexi-affiliated organisms (32% of DNA clones and 20% of RNA clones). Other taxa represented in both libraries were the Acidobacteria,
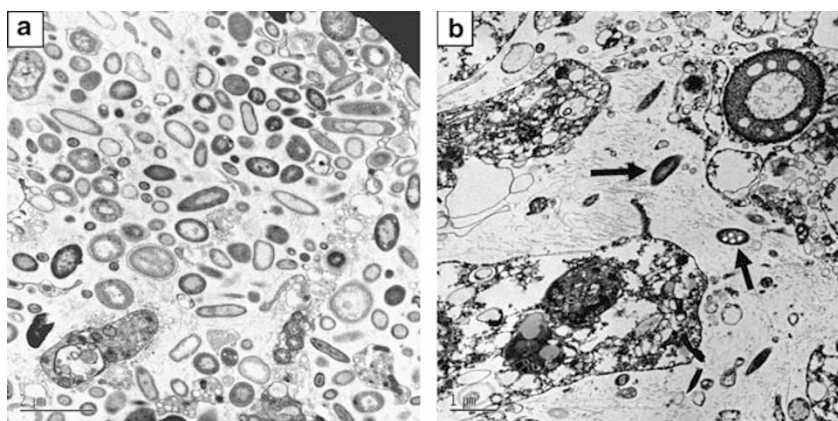

Figure 1 Transmission electron micrographs of mesohyl tissue from the marine sponges (**a**) Ancorina alata and (**b**) Polymastia sp. Note the much higher abundance of microbial cells in A. alata. Arrows in (**b**) indicate microbial cells.
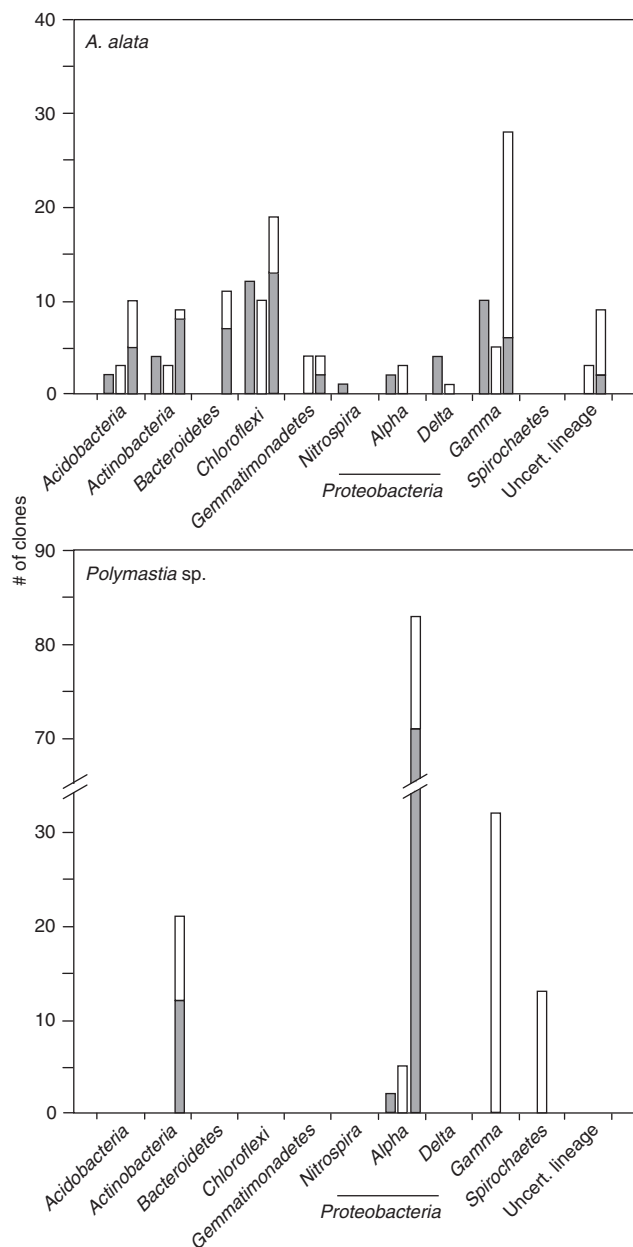
Figure 2 Phylogenetic distribution of bacteria clones recovered from the DNA and RNA libraries from the marine sponges *Ancorina alata* (top) and *Polymastia* sp. (bottom). For each bacterial phylum, up to three bars are shown on the graphs. The leftmost (grey) bar indicates the number of clones representing phylotypes recovered exclusively from the DNA-derived library; the centre (white) bar indicates clones recovered exclusively from the RNA-derived library; the rightmost bar indicates clones recovered from both the DNA- and RNA-derived libraries (DNA, grey, RNA, white).

Actinobacteria, *Bacteroidetes*, *Gemmatimonadetes*, *Alpha-* and *Deltaproteobacteria*, and a lineage of uncertain affiliation that seems to fall within the *Planctomycetes–Verrucomicrobia–Chlamydiae* (PVC) superphylum (Wagner and Horn, 2006). The only major taxon to be recovered from only one library was the *Nitrospirae*, for which a single sequence was found in the DNA-derived library. Phylogenetic

consensus trees were constructed for all obtained sequences, and examination of these reveals the extent of overlap between DNA and RNA libraries at the phylotype level as indicated by clusters A1–A16 in Figures 3–5 and Supplementary Figures 1–4. For example, concordance between the libraries was high within the *Actinobacteria* (Figure 3), in which both DNA and RNA clones (cluster A1) occurred within a sponge-specific cluster. In addition, there was an RNA phylotype without a corresponding DNA sequence, and DNA phylotypes with no matching RNA sequences. Similar results were evident for the other bacterial phyla (Figures 4 and 5, Supplementary Figures 1–4), in which there were typically some overlapping DNA and RNA sequences but also examples of DNA or RNA sequences on their own.

The *Polymastia* sp. clone libraries were much less diverse than those of *A. alata* (Figure 2). The DNA library was dominated (84% of clones) by a single *Alphaproteobacteria* lineage (Supplementary Figure 2), with the remaining sequences falling elsewhere within the *Alphaproteobacteria* and within one *Actinobacteria* lineage (Figure 3). Both the *Alphaproteobacteria* (cluster P2; Supplementary Figure 2) and *Actinobacteria* (cluster P1; Figure 3) were also represented in the RNA-derived library, although the *Alphaproteobacteria* comprised only 24% of the sequenced clones. Although absent from the DNA library, the *Gammaproteobacteria* were well represented in the RNA library, with several phylotypes together making up 45% of the recovered clones (Supplementary Figure 1). *Spirochaetes* were also abundant in the RNA library, comprising 18% of clones (Supplementary Figure 4), but were absent from the DNA library.

Consistent with previous studies, many of the sequences obtained in this study fell into monophyletic, sponge-specific sequence clusters (Hentschel et al., 2002). These occurred to varying extents in all recovered bacterial phyla, although some clusters within the *Chloroflexi* (Figure 4), *Gemmatimonadetes* (Figure 5), *Nitrospirae* and *Deltaproteobacteria* (both Supplementary Figure 4) also contained at least one coral-derived sequence.

Discussion

This study represents the first community-wide approach to investigating bacterial activity in marine sponges. Previous studies have provided valuable information on specific metabolic processes and/or the organisms involved, but to gain a broader picture of microbial diversity and activity within any system it is essential to consider the whole community. By comparing 16S rRNA gene and 16S rRNA profiles of bacterial identity and activity, respectively, we were able to provide insights into the *in situ* activity of uncultivated, sponge-specific bacterial lineages. Taxa such as the *Actinobacteria*,
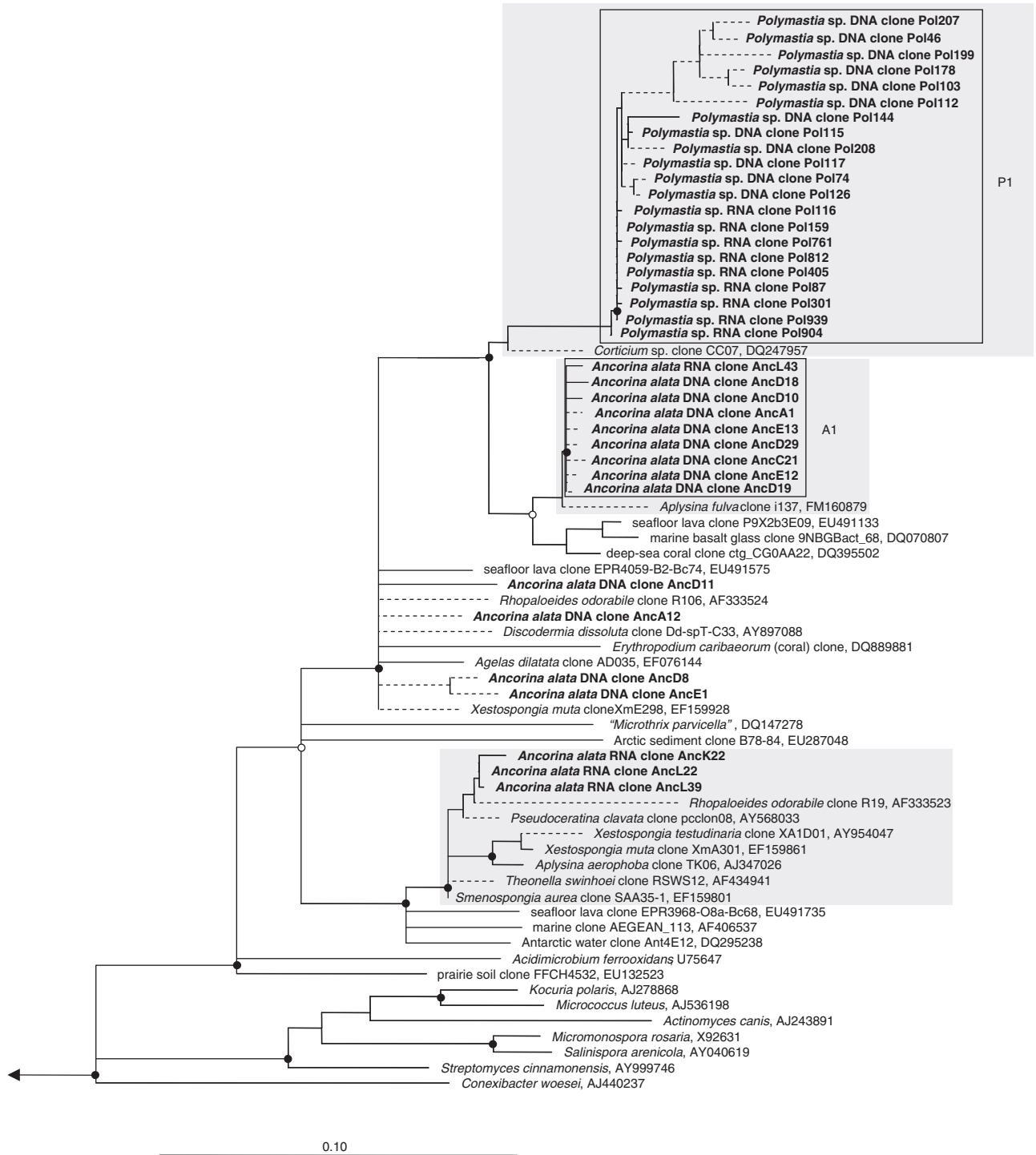
Figure 3 16S rRNA-based phylogenetic consensus tree of sponge-associated *Actinobacteria*. Sequences obtained during this study from the New Zealand sponges *Ancorina alata* or *Polymastia* sp. are shown in bold, and their clone names are labelled with either DNA or RNA. Polytomies indicate that respective branching order could not be unambiguously resolved using different treeing methods. Dashed lines represent short sequences (<1200 bp) that were added using the parsimony interactive tool in ARB. Shaded boxes represent sponge-specific monophyletic clusters, as defined previously (Hentschel *et al.*, 2002). Boxes labelled with 'A' for *A. alata* or 'P' for *Polymastia* sp. signify monophyletic groupings ('phylotypes') of DNA- and RNA-derived clones from the respective sponge species. Filled circles indicate bootstrap support of ≥90%, and open circles represent ≥75% support. The outgroup (not shown) consisted of a range of sequences representing other bacterial phyla. Scale bar signifies 10% sequence divergence.

Chloroflexi, *Gemmatimonadetes* and *Acidobacteria* contain large sponge-specific clusters from diverse host sponges; yet, a failure to obtain most of these organisms in pure culture has led to a paucity of information about their activities and likely function within the host.
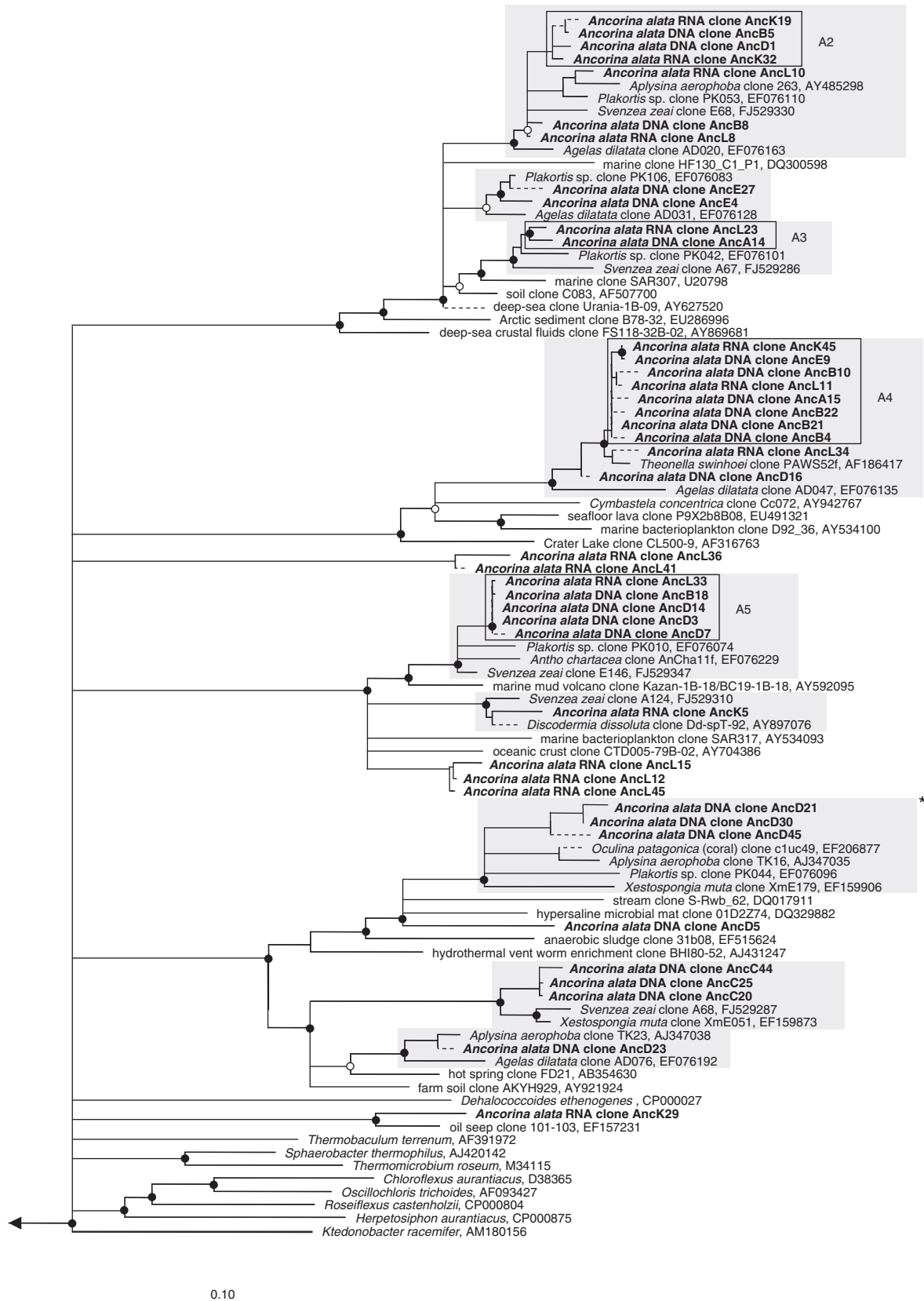
Figure 4 16S rRNA-based phylogenetic consensus tree of sponge-associated *Chloroflexi* and affiliated organisms. Details are the same as those provided for Figure 3, with the following addition: boxes with an asterisk represent monophyletic clusters containing sponge- and coral-derived sequences.
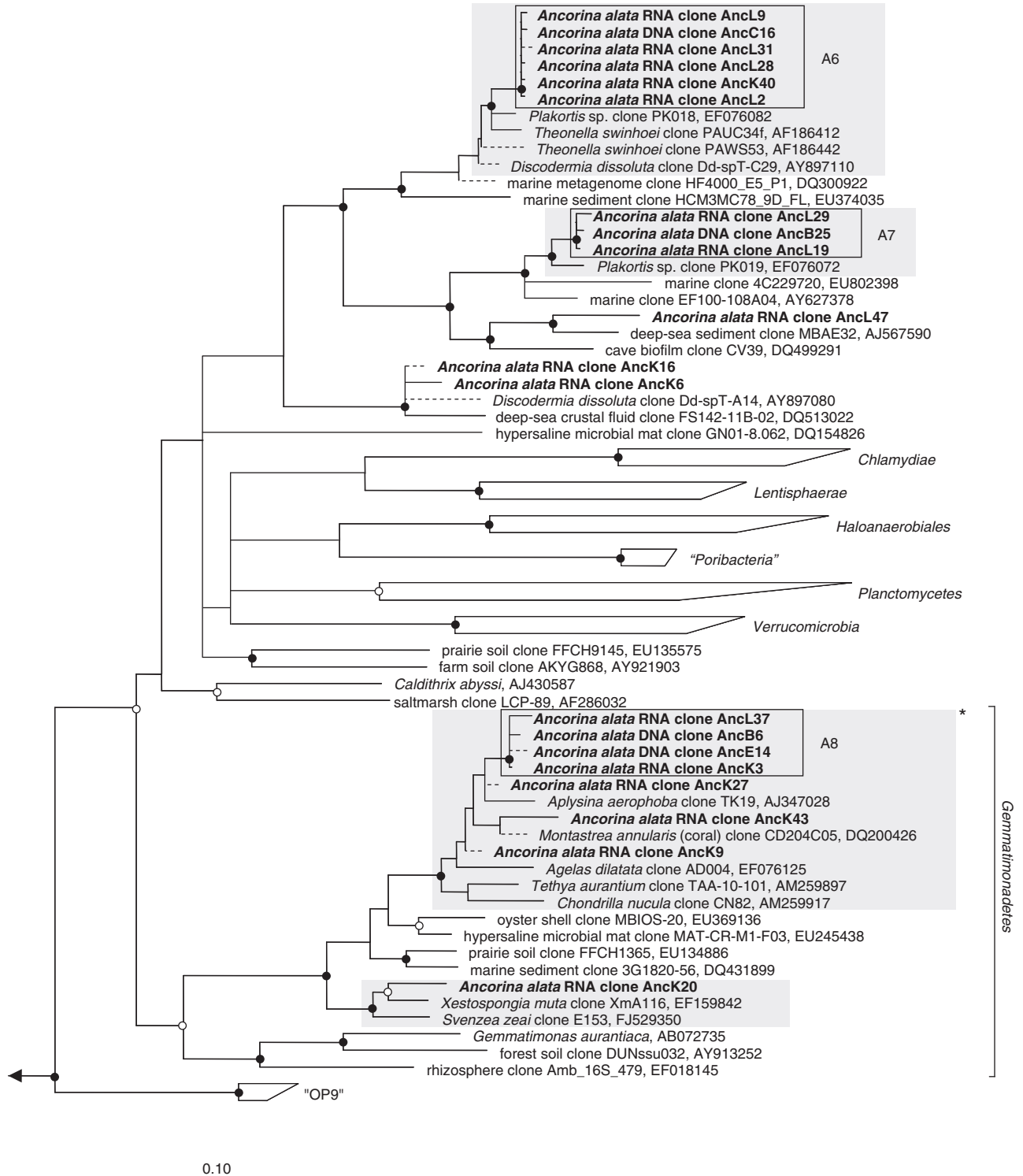
Figure 5 16S rRNA-based phylogenetic consensus tree of sponge-associated *Gemmatimonadetes* and a lineage of uncertain affiliation related to the *Planctomycetes–Verrucomicrobia–Chlamydiae* (PVC) superphylum (Wagner and Horn, 2006). Details are the same as those provided for earlier figures, and some clusters (not containing sequences from this study) are grouped (shown as wedges) in the interests of saving space.

Bacterial 16S rRNA gene vs 16S rRNA comparisons in sponges

Earlier studies have successfully used the 16S rRNA gene vs rRNA approach to investigate the activity of, for example, marine plankton, sediment and gas hydrate communities (Moeseneder *et al.*, 2001, 2005; Mills *et al.*, 2005; Gentile *et al.*, 2006; Rodriguez-Blanco *et al.*, 2009). Similar to earlier studies, we

found substantial overlap between the DNA- and RNA-derived libraries (clusters A1–16 and P1 and 2 in Figures 3–5, and Supplementary Figures 1–4), but also many cases in which a particular DNA or RNA sequence occurred alone (e.g., both phenomena can be seen for the *Actinobacteria* in Figure 3). Each of these cases can be readily explained—or at least speculated upon (Moeseneder *et al.*, 2005). First, phylotypes from the same sponge species represented in both DNA and RNA libraries are clearly present and—as indicated by the detection of their rRNA—are presumably active as well. This is the case for, for example, cluster A1 in Figure 3. On this same tree, the *A. alata* DNA clones AncD11, AncA12, AncD8 and AncE1 may represent abundant bacteria and/or those that contain multiple 16S rRNA gene operons. The absence of matching clones in the RNA library implies that the bacteria represented by these sequences may have only low activity. RNA clones without corresponding DNA clones (e.g., AncK22, AncL22 and AncL39 in Figure 3) may represent bacteria that are uncommon but metabolically highly active. It is worth noting here that a conservative approach was taken, with any inferences about bacterial activity remaining strictly qualitative.

Discrepancies between DNA- and RNA-derived libraries can often be explained within a biological context, as discussed above. However, it is also worthwhile to consider methodological factors that could potentially contribute to such variation among libraries. Most critically, the entire approach is based on the use of rRNA as a proxy for activity. Although it is now known that ammonia-oxidizing bacteria retain appreciable cellular concentrations of rRNA even during idle periods when activity is expected to be minimal (Morgenroth *et al.*, 2000), it is widely accepted that in most bacteria rRNA levels are typically correlated with cellular growth rate and activity (DeLong *et al.*, 1989; Poulsen *et al.*, 1993). The approach therefore seems valid for most bacteria in most situations, although further investigation of host-associated microbes is required to establish definitively the relationship between rRNA level and activity for symbionts. In addition, small cells are likely to have a lower ribosomal RNA content when compared with larger cells, even though they could be metabolically more active. Another point to consider is that a specific sequence could be detected in one (i.e., either DNA or RNA) library but missed in the corresponding library because of insufficient numbers of clones being sequenced. Thus, a difference between DNA and RNA libraries would seem to be present, when in fact there is none. Given the considerable overlap between our DNA- and RNA-derived libraries, we do not believe this to be a major factor in our case, although this does depend on the phylotype definition used. If an exclusively monophyletic grouping of DNA- or RNA-derived sequences is used to define a phylotype, then concordance between the libraries is high. However, one can also consider the case in which phylotypes are defined based on a—somewhat arbitrary—sequence similarity threshold (e.g., 99% may represent 'species', whereas 95% may approximate 'genus' level). Using a 95% threshold, observed OTU richness was almost as high in the *A. alata* DNA library as the total richness predicted by the Chao1 estimator, suggesting that further sequencing of this library would have revealed very few additional genera. Predictably, the observed and predicted OTU richness values diverge more when a more stringent (99%) OTU threshold is used. Similar results can be seen for other libraries, indicating that further sequencing could potentially eliminate some of the observed differences between DNA- and RNA-derived libraries. Irrespective of this, the overall conclusion, that a large fraction of sponge-associated bacteria are active *in situ*, is not affected. What is worth noting is the seemingly low coverage of the DNA-derived library from *Polymastia* sp. The observed number of 99% OTUs in this library was 45, whereas an OTU richness of 279 was estimated by the Chao1 analysis. Interestingly, only twenty-two 95%-OTUs were found, whereas the corresponding Chao1 estimate fell to only 37. The vast majority of sequences in this library fall into one monophyletic cluster within the *Alphaproteobacteria*, with a minimum pairwise similarity of 93.7%; thus, there is evidently a high level of microdiversity ('species-level') in this cluster, but almost all sequences belong to the same 'genus' (95%).

We used clone libraries to provide the highest degree of phylogenetic information (through the generation of full-length 16S sequences) while demonstrating the effectiveness of the combined DNA vs RNA approach for sponges. However, other techniques are more appropriate in situations requiring high sample numbers, for example when examining biological and/or environmental variability. Fortunately, the DNA vs RNA approach is equally applicable to high-throughput community fingerprinting techniques such as denaturing gradient gel electrophoresis (Winter *et al.*, 2001; Troussellier *et al.*, 2002), terminal restriction fragment length polymorphism (T-RFLP) (Moeseneder *et al.*, 2001) and single-stranded conformational polymorphism (SSCP) (West *et al.*, 2008; Rodriguez-Blanco *et al.*, 2009). It could also be used in conjunction with next-generation sequencing methods such as tag pyrosequencing (Sogin *et al.*, 2006; Huse *et al.*, 2008). Our laboratory is currently investigating this for sponges, with the aim of detecting bacterial activity in both rare and abundant community members.

New insights into the in situ *activity of sponge-associated bacteria*
Marine sponges fall into one of two main categories with respect to their associated microorganisms. High-microbial-abundance sponges contain very

dense communities of diverse microorganisms. In these sponges, microbes occur at densities of up to 10^{10} cells per gram wet weight of sponge and their collective biomass may rival that of the host sponge cells (Vacelet, 1975; Friedrich *et al.*, 2001). Our TEM data indicate that the New Zealand sponge *A. alata* belongs to this category (Figure 1A). Our second sponge, *Polymastia* sp., exhibited much lower densities of microbes in the mesohyl (Figure 1B) and therefore seems typical of a low-microbial-abundance sponge, which tend to have microbial densities of 10^{5}–10^{6} cells per gram, similar to that of seawater (Hentschel *et al.*, 2006). In addition, *A. alata* and *Polymastia* sp. also differ in the fact that the latter sponge has a much lower bacterial sequence richness, with only three phyla detected compared with eight in *A. alata* (Figure 2).

Within each sponge, the DNA and RNA libraries showed considerable overlap, indicating that a substantial fraction of the bacterial community within both sponges was physiologically active. This is evident at the levels of both phylum and specific sequence types, with overlapping phylotypes marked by boxes in Figures 3–5 and Supplementary Figures 1–4. In *Polymastia* sp., corresponding DNA and RNA phylotypes were found in two major clusters, one within the *Actinobacteria* (P1, Figure 3) and the other within the *Alphaproteobacteria* (P2, Supplementary Figure 2). Interestingly, about one-third of all RNA clones, and almost all DNA clones, fell within these two clusters. Phylotypes P1 and P2 might therefore represent true symbionts of *Polymastia* sp., although this needs to be confirmed by future studies. In contrast, many remaining RNA clones, mainly within *Gammaproteobacteria* and *Spirochaetes*, could represent highly active bacteria serving as food or being contaminants from seawater. Many of these clones cluster with sequences from non-sponge sources (Figures 3–5, Supplementary Figures 1–4). In *A. alata*, corresponding DNA and RNA phylotypes were distributed among seven different phyla, thus encompassing almost the entire bacterial diversity found in this sponge. Activity was therefore not restricted to a specific phylogenetic group of bacteria, but rather to a phylogenetically complex bacterial community.

Many of the detected phylotypes in *A. alata* (including those found at both DNA and RNA levels) were similar to sequences derived from other sponges, or even fell within monophyletic sponge-specific clusters. Much recent attention has been focused on the phenomenon that even distantly related sponges from different oceans share a subset of their microbial communities that is not found outside sponge hosts (Hentschel *et al.*, 2002; Taylor *et al.*, 2007b). This is particularly the case for the high-microbial-abundance sponges, which contain numerous lineages that are apparently absent from other environments. However, despite this interest, there is still little known about the nature of these

symbioses. The function of sponge-specific microbes has only been determined for certain microbial groups that are responsible for, for example, photosynthesis, nitrification or sulphate reduction in sponges (Wilkinson, 1983; Hoffmann *et al.*, 2005; Bayer *et al.*, 2008). This study suggests that in fact many of the sponge-specific symbionts are active within their respective host sponges.

The activity patterns described here for microbial consortia in *A. alata* and *Polymastia* sp. represent only a snapshot at a single time point. Microbial activities may be heavily influenced by host biology and other environmental factors. For example, the pumping activity of a sponge influences the oxygenation of its mesohyl matrix (Hoffmann *et al.*, 2008). The mesohyl of the Mediterranean sponge *Aplysina aerophoba* was well oxygenated while the sponge was pumping water through its body, but became anoxic minutes after pumping ceased (Hoffmann *et al.*, 2008). It is easily conceivable that in the first circumstance aerobic microbes are active, whereas under anoxic conditions anaerobic microbes would be more active. Additional, chemical and physical factors that might influence the activity of sponge-associated microbes include changes in temperature, salinity, light or turbulence. The combined 16S rRNA and 16S rRNA gene approach offers a way to evaluate activity changes within the overall sponge microbial community, whereas analyses of mRNA could give more insights into which specific pathways are being affected.

Concluding remarks

In this study we have successfully shown the application of the 16S rRNA gene vs rRNA approach to marine sponge-associated bacteria. In the process, we were able to provide the first insights into the *in situ* activity of uncultivated, sponge-specific bacterial lineages. There is a compelling argument for including both rRNA gene and rRNA analyses in future investigations of sponge-associated micro-organisms, as the combined approach allows identification of phylotypes that would remain hidden when DNA or RNA clones are examined in isolation. Furthermore, it helps us to tackle one of the key focal points for sponge microbiology research (Taylor *et al.*, 2007a), the challenge of elucidating symbiont activity and function.

Acknowledgements

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