Exploiting the Diverse Microbial Ecology of Marine Sponges

A Thesis presented to the National University of Ireland for the Degree of Doctor of Philosophy

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For Daniel,

My inspiration

Declaration:

I, the undersigned, hereby declare that the work herein is my own and that it has not been submitted for any other degree, either at University College Cork, or elsewhere.

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Stephen Jackson

Abstract

Marine sponges (phylum *Porifera*) are the oldest extant metazoan animals on earth today and they host large populations of symbiotic microbes: *Bacteria*, *Archaea* and unicellular *Eukaryota*. Those microbes play various ecological functions which are essential to the health of the host. Their functions include carbon, nitrogen and sulfur cycling as well as defence of the host through the production of bioactive secondary metabolites which protect against infection and predation. The diversity of sponge-associated microbes is remarkable with thousands of OTUs reported from individual sponge species. Amongst those populations are sponge-specific microbes which may be specific to sponges or specific to sponge species.

Sponges are a source of a vast array of chemical entities with many bioactive properties of interest to industry and pharmacology. While marine natural product discovery concerns many animal phyla, *Porifera* account for the largest proportion of novel compounds. Evidence suggests that many of these compounds of interest are the products of symbiotic microbes.

Descriptions of sponge-associated microbial community structures has been greatly advanced by the development of next-generation sequencing technologies while the discovery and exploitation of sponge derived biocatalysts and bioactive compounds has increased due to developments in sequence-based and function-based metagenomics.

Here we use pyrosequencing to describe the bacterial communities associated with two shallow, temperate water sponges namely *Raspailia ramosa* and *Stelligera stuposa* from Irish coastal waters and to describe the bacterial and archaeal communities from three individuals of a single sponge species (*Inflatella pellicula*) from two different depths in cold, deep waters in the Atlantic Ocean in Irish waters, including at a depth of 2900 m, a depth far greater than that of any previous sequence-based sponge-microbe investigation. We identified diverse microbial communities in all sponges and the presence of sponge-specific taxa recruiting to previously described sponge-specific clusters and also to novel sponge-specific clusters. We also identified archaeal communities which dominated sponge-microbe communities. We demonstrate that sponge-associated microbial communities differ from ambient seawater communities indicating host selection processes.

We used sequence-based metagenomic techniques to identify genes of potential industrial and pharmacological interest in the metagenomes of various sponge species and function-based metagenomic screening in an attempt to identify lipolytic and antibacterial activities from metagenomic clones from the metagenome of the marine sponge *Stelletta normani*.

In addition we have cultured many diverse bacterial species from sponge tissues, many of which display antimicrobial activities against clinically relevant bacterial and yeast test strains. Other isolates represent novel species in the genus *Maribacter* and require emendments to the description of that genus.

Chapter 1

Literature Review

1.1 Marine sponges

Marine sponges (phylum *Porifera*) are the oldest extant metazoan animals (Figure 1.1) with the oldest fossils dating back to ~630 million years (Maloof *et al*., 2010). Sponges are globally distributed (Figure 1.2) and are important members of all benthic communities. Sponges have been reported to be more abundant (area coverage/biomass/volume) than other benthic organisms (Meesters *et al*., 1991) with increased relative abundances with increasing depth and also sponge species diversity often outnumbering all other benthic species combined (Meesters *et al*., 1991; Diaz & Rützler, 2001). Sponges play vital roles in marine nutrient cycling as important sources of dissolved inorganic nitrogen (DIN), mediated by nitrifying endosymbiotic microbes resulting in high concentrations (40 µM) of nitrate near the ocean floor (Diaz & Ward, 1997). Sponges are also important sinks and sources of particulate organic carbon (POC), dissolved organic carbon (DOC) and dissolved organic nitrogen (DON) (Diaz & Ward, 1997).

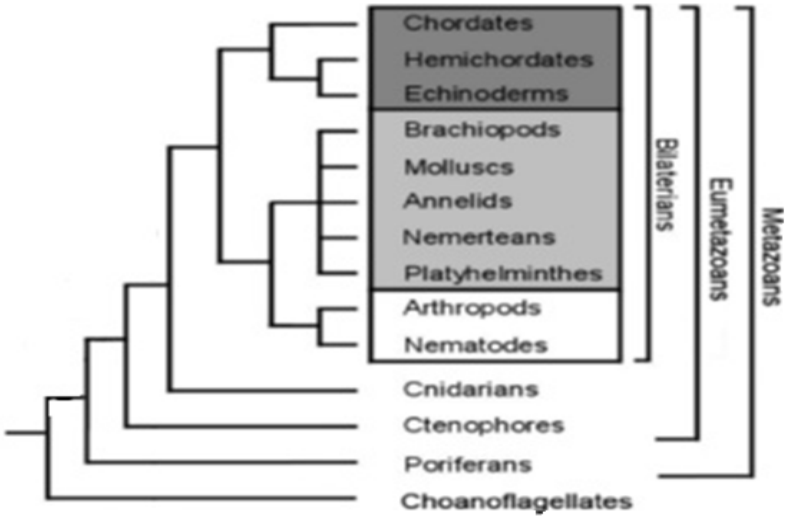

Figure 1.1: Phylogeny of metazoa, adapted from Degnan *et al*., 2005.

The World Porifera Database (van Soest *et al*., 2012) currently lists > 8,370 valid sponge species, which are distributed amongst 680 genera in four distinct classes; *Calcarea*, *Hexactinellida*, *Demospongiae* and the recently recognised *Homoscleromorpha* (Gazave *et al*., 2010). *Demospongiae* is by far the largest class, comprising ~83% of valid species (van Soest *et al*., 2012b). Almost all sponges are found in seawater, however, one suborder of *Demospongiae* (*Spongillina*) comprising ~250 species are freshwater sponges (van Soest *et al*., 2012b).

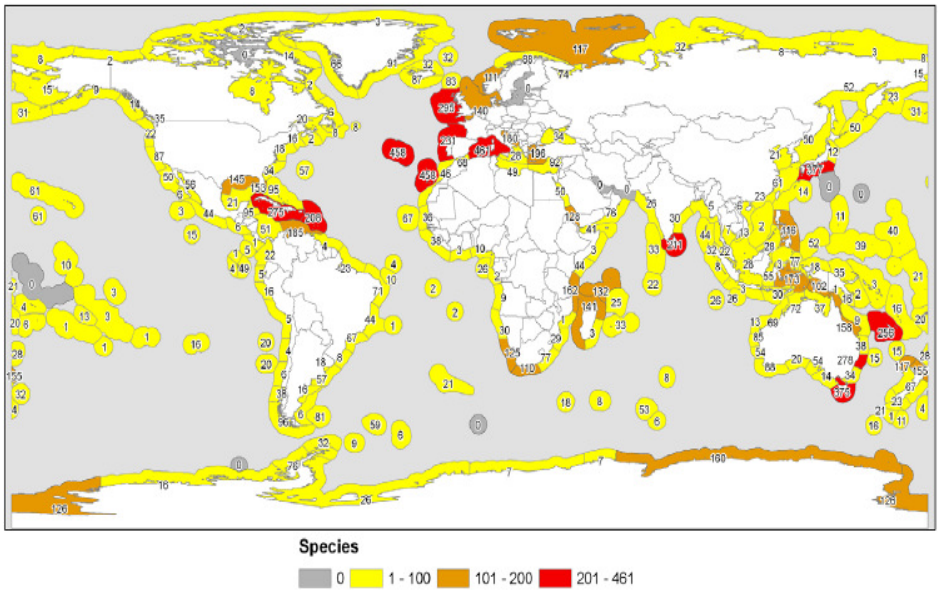

Figure 1.2: Global distribution of marine sponges (van Soest *et al*., 2012b).

1.1.1 Sponge anatomy and physiology

1.1.1.1 Sponge skeletons

Porifera exhibit a wide range of morphologies, from encrusting, through branching to barrel types. Sponge skeletal systems are comprised of spicules which may be calcareous, composed of calcium carbonate ($CaCO_3$); siliceous, composed of silicon dioxide (SiO_2) or spongin – a collagenous protein (Figure 1.3).

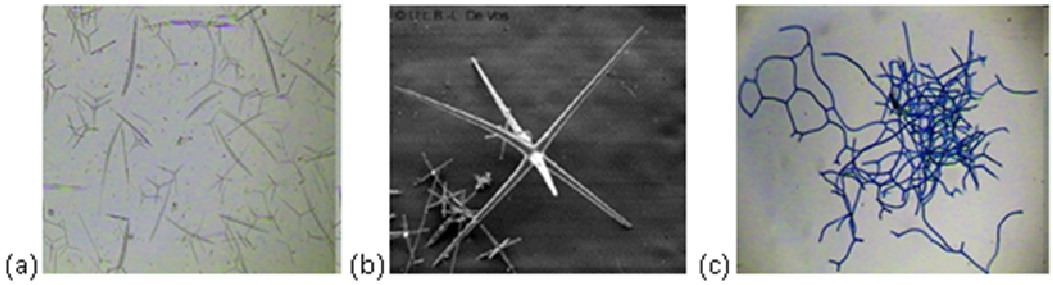

Figure 1.3: Sponge skeletal components (a) calcareous spicules, (b) siliceous spicules, (c) spongin (http://www.okc.cc.ok.us/biologylabs/documents/Porifera_Cnidaria/Porifera.htm).

The class *Calcarea* have calcareous spicules, *Hexactinellida* have siliceous spicules, *Demospongia* and *Homoscleromorpha* can be spiculate, with a combination of siliceous spicules and spongin, or aspiculate which contain spongin skeletons.

1.1.1.2 Sponge cell types

The sponge body is composed of very few differentiated cell types. The sponge epidermis (pinacoderm) is composed of pinacocyte cells interspersed with porocyte cells, which form a porous aquiferous system throughout the sponge body. Choanocyte cells line choanosome 'chambers', where these flagellated cells, through a whipping action, create a water current which flows from outside the sponge body, through ostia – pores in the pinacoderm, through the sponge aquiferous system and is expelled through the osculum (Figure 1.4). Choanocytes also produce spermatocytes for sexual reproduction. The sponge body is composed of a mesohyl – collagenous material through which archaeocytes travel. These totipotent cells play a role in phagocytosis of food and can also differentiate into oocytes for sexual reproduction or gemmules for asexual reproduction. Pinacocyte cells are also capable of digesting food particles while sclerocyte cells produce and excrete spicules.

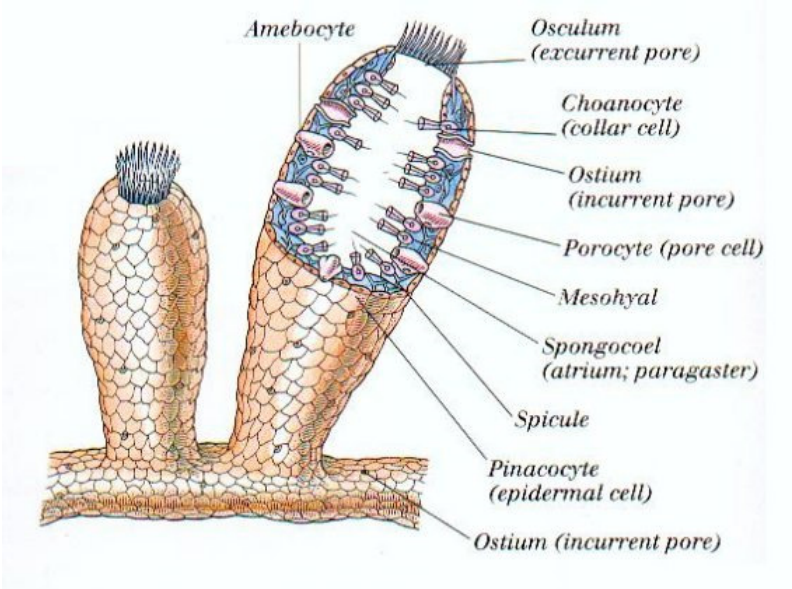

Figure 1.4: Anatomy of a marine sponge (http://universe-review.ca/R10-33-anatomy.htm#sponges).

1.1.1.3 Sponge physiology

Sponges can reproduce either sexually or asexually. Sexual reproduction is achieved through the differentiation of archaeocyte cells to oocytes which are released into the water column. When the eggs enter the aquiferous canals of a sponge of the same species they are transported to the choanosome where they are engulfed by choanocytes, fertilization occurs, the eggs develop and the larva is released to the water column where the motile organism searches for a suitable settlement site. In asexual reproduction gemmules, aggregates of archaeocytes and spicules are detached by the water current and settle in a dormant state until a suitable attachment site and favourable growth conditions are found. Dormant gemmules are known to be able to survive stresses such as extreme cold or lack of oxygen (Bergquist, 1998).

Sponges do not have distinct systems or organs; with the aquiferous system serving the role which is analogous to the circulatory, digestive and excretory systems found in higher metazoans. Most adult sponges are sessile filter-feeding animals that filter bacteria, micro-eukaryotes and particulate matter from ambient seawater which they pump through the canal systems in their bodies. Oxygen is delivered to cells by diffusion, food is engulfed and digested by phagocytosis in the mesohyl and metabolic waste is removed in the constant water current throughout the body. Sponges can pump remarkable volumes of seawater through their bodies with reports of 24,000 L kg^{-1} day^{-1} in some sponge species (Taylor *et al*., 2007). Some sponges (~120 species) do not possess the aquiferous canal systems and thus are not filter feeders. Instead they are carnivorous, capturing prey on 'hooks' on the outer surface of the body where specialised cells migrate to the captured prey and phagocytize and digest the food prey. Carnivorous sponges have to date only been found in-the deep sea (van Soest *et al*., 2012).

Sponges do not possess adaptive immunity though innate immunity featuring an interferon-like 2'-5' adenylate-synthetase system, a variable immunoglobin-like system and LPS activated kinase cascades are all present (Müller & Müller, 2003) and compounds with anti-microbial and anti-inflammatory properties have been extracted from sponge tissues. The primary producer of sponge-derived secondary metabolites is however still quite unclear though with many of these sponge derived

compounds strongly resembling compounds that are produced by microbes (Hentschel *et al*., 2012).

1.2 Sponge associated microorganisms

Marine sponges (*Porifera*) are host to microbes from all domains of life; *Eukarya* (Baker *et al*., 2008; Cerrano *et al*., 2004), *Archaea* (Margot *et al*., 2002; Webster *et al*., 2004) and *Bacteria* (Taylor *et al*., 2007). Viruses and bacteriophages have also been detected in sponge tissues (Lohr et al., 2005; Harrington *et al*., 2012). These close and consistent associations are thought to be based on various symbiotic relationships including commensalist and mutualist (Wilkinson, 1983) as well as parasitic (Bavestrello *et al*., 2007). Microbes are also a significant food source for marine sponges (Reiswig, 1975) which, as sessile animals, must derive their nutrition by active filter-feeding from ambient seawater. This water filtering activity results in a remarkable enrichment of microbes in sponge tissues where 10^8-10^{10} bacteria per gram wet weight have been recorded (Lee *et al*., 2009). This is orders of magnitude more than in the surrounding water (10^6 ml^{-1}). Much research interest has focused on the bacterial associates of marine sponges since the early work of Clive Wilkinson (Wilkinson, 1978) and Jean Vacelet (Vacelet & Donadey, 1977) in the 1970s showed that bacteria comprise a significant proportion of sponge tissues.

1.2.1 Sponge associated bacteria

1.2.1.1 Culture dependent analyses

Bacterial associates of sponges have been investigated through both culture-dependent and culture-independent methods. Culture isolation from sponges is, like all other source environments, hampered by 'the great plate anomaly' where less than 1% of taxa observed through other methods, have proved amenable to laboratory culture through traditional or innovative means (Hentschel *et al*., 2012).

Researchers have used a wide range of culture conditions (media/ incubation temperatures) in attempts to access as wide a variety of bacterial diversity as possible (Kanagasabhapathy *et al*., 1996; Kennedy *et al*., 2008; Flemer *et al*., 2011; Gopi *et al*., 2012; Hentschel *et al*., 2001; Lee *et al*., 2009; Margassery *et al*., 2012;

Muscholl-Silberhorn *et al*., 2008). Others have targeted the isolation of particular taxa of interest (Abdelmohsen *et al*., 2010; Hoffmann *et al*., 2010; Jiang *et al*., 2007; O'Halloran *et al*., 2011; Phelan *et al*., 2012; Radwan *et al*., 2010; Santos *et al*., 2010; Schneemann *et al*., 2010; Sun *et al*., 2010; Zhang *et al*., 2006; Zhang *et al*., 2008; Zhu *et al*., 2008; Webster *et al.,* 2001; Xi *et al*., 2012). In addition a number of innovative culture isolation methods have been employed including- in the spirit of Winogradsky, manipulation of bacterial communities through antibiotic administration prior to isolation (Richardson *et al*., 2012), or imaginative approaches of liquid culturing and floating-filter culturing methodologies have been used (Sipkema *et al*., 2011)

Despite these efforts the same bacterial phyla repeatedly appear following culture isolations, with members of only seven bacterial phyla (*Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Planctomycetes*, *Verrucomicrobia*, *Cyanobacteria* and *Bacteroidetes*) (Taylor *et al*., 2007) to date being isolated in culture from sponge tissues; despite the observation that >30 phyla or candidate phyla can be found in close association with sponges through molecular methods (Hentschel *et al*., 2012). Notwithstanding this, diverse novel bacterial taxa are regularly isolated from sponge species worldwide (Table 1.1).

1.2.1.2 Culture independent analyses

1.2.1.2.1 Microscopy

The presence of bacteria in the mesohyl of sponges was first confirmed by Lévi and Porte in the early 1960s (Wilkinson, 1978) using an electron microscope (EM). Subsequently, EM studies reported various cell types, including *Cyanobacteria*, in sponge tissues (Vacelet, 1971) and later still dense bacterial cell populations in sponge mesohyl tissues (Vacelet and Donadey, 1977) were estimated to comprise 30% of the sponge biomass. Magnino *et al*. used scanning electron microscopy (SEM) to report, in 1999, the presence of unicellular cyanobacteria and non-photosynthetic filamentous cyanobacteria in the tissues of *Theonella swinhoei* (Magnino *et al*., 1999).

Genus/species	Phylum	Host	Reference
Desulfoluna spongiiphila	*Proteobacteria (δ)*	*Aplysina aerophoba*	Ahn *et al*., 2009
Kangiella spongicola	*Proteobacteria (γ)*	*Chondrilla nucula*	Ahn *et al*., 2010
Fulvitalea axinellae	*Bacteroidetes*	*Axinella verrucosa*	Haber *et al*., 2012
Spongiibacter marinus	*Proteobacteria (γ)*	*Haliclona* sp.	Graeber *et al*., 2008
Spongiispira norvegica	*Proteobacteria (γ)*	*Isops phlegraei*	Kaesler *et al*., 2008
Rubritalea squalenifaciens	*Verrucomicrobia*	*Halichondria okadai*	Kasai *et al*., 2007
Planococcus plakortidis	*Firmicutes*	*Plaktoris simplex*	Kaur *et al*., 2012
Streptomyces tateyamensis	*Actinobacteria*	*Haliclona* sp.	Khan *et al*., 2010
Winogradskyella poriferorum	*Bacteroidetes*	*Lissodendoryx isodictyalis*	Lau *et al*., 2005
Fabibacter halotolerans	*Bacteroidetes*	*Tedania ignis*	Lau *et al*., 2006
Roseivirga spongicola	*Bacteroidetes*	*Tedania ignis*	Lau *et al*., 2006
Stenothermobacter spongiae	*Bacteroidetes*	*Lissodendoryx isodictyalis*	Lau *et al*., 2006b
Gillisia myxillae	*Bacteroidetes*	*Myxilla incrustans*	Lee *et al*., 2006
Shewanella irciniae	*Proteobacteria (γ)*	*Ircinia dendroides*	Lee *et al*., 2006b
Thalassococcus halodurans	*Proteobacteria (α)*	*Halichondria panicea*	Lee et al., 2007
Marinobacter xestospongiae	*Proteobacteria (γ)*	*Xestospongia testudinaria*	Lee *et al*., 2012
Leptobacterium flavescens	*Bacteroidetes*	*Clathria eurypa*	Mitra *et al*., 2009
Salegentibacter agarivorans	*Bacteroidetes*	*Artemisina* sp.	Nedashkovskaya *et al*., 2006
Endozoicomonas numazuensis	*Proteobacteria (γ)*	*Haliclona* sp.	Nishijima *et al*., 2011
Tsukamurella spongiae	*Actinobacteria*	?	Olson *et al*., 2007
Pseudovibrio axinellae	*Proteobacteria (γ)*	*Axinella dissimilis*	O'Halloran *et al*., 2012
Mycobacterium poriferae	*Actinobacteria*	*Halichondria bowerbanki*	Padgitt & Moshier, 1987
Saccharopolyspora cebuensis	*Actinobacteria*	*Haliclona* sp.	Pimentel-Elardo *et al*., 2008
Streptomyces axinellae	*Actinobacteria*	*Axinella polypoides*	Pimentel-Elardo *et al*., 2009
Pseudomonas pachastrellae	*Proteobacteria (γ)*	*Pachastrella sp.,*	Romanenko *et al*., 2005
Lysobacter spongiicola	*Proteobacteria (γ)*	*Pachastrella* sp.	Romanenko *et al*., 2008
Rubritalea marina	*Verrucomicrobia*	*Axinella polypoides*	Scheuermeyer *et al*., 2006
Marinoscillum pacificum	*Bacteroidetes*	?	Seo *et al*., 2009
Vibrio caribbeanicus	*Proteobacteria (γ)*	*Scleritoderma cyanea*	Hoffmann *et al*., 2012
Shewanella spongiae	*Proteobacteria (γ)*	?	Yang *et al*., 2006
Spongiibacterium flavum	*Bacteroidetes*	*Halichondria oshoro*	Yoon & Oh, 2012
Rubritalea spongiae	*Verrucomicrobia*	?	Yoon *et al*., 2007
Aquimarina spongiae	*Bacteroidetes*	*Halichondria oshoro*	Yoon *et al*., 2010
Formosa spongicola	*Bacteroidetes*	*Hymeniaciden flavia*	Yoon and Oh., 2010

Table 1.1: Novel bacteria isolated from marine sponges

The development of fluorescence *in situ* hybridisation (FISH) allowed subsequent investigators to identify particular bacterial taxa and their spatial distribution within sponge tissues by designing probes to target particular 16S rRNA genes. This allowed for the identification of *Cyanobacteria* (Ridley *et al*., 2005; Pfannkuchen *et*

al., 2010) *Actinobacteria*, γ- and β-*Proteobacteria*, *Bacteroidetes* and *Planctomycetes* (Webster *et al*., 2001) in sponges and also demonstrated the vertical transmission of eubacteria and archaea in sponge larvae (Sharp *et al*., 2007).

1.2.1.2.2 16S rRNA clone libraries

The development of the polymerase chain reaction (PCR) along with molecular cloning techniques allowed, for the first time; very detailed descriptions of the species' composition of unculturable sponge-associated bacterial communities as well as explorations of other aspects of sponge microbial ecology to be undertaken. The bacterial community structures in many sponges have to date been elucidated (Webster *et al*., 2004; Erwin *et al*., 2011; Cassler *et al*., 2008; Kennedy *et al* 2008b; Zhu *et al*., 2008; Hardoim *et al*., 2009; Sipkema *et al*., 2009; Wang *et al*., 2009; Radwan *et al*., 2010; Brück *et al*., 2012). In addition both inter- and intra- sponge species microbial community comparisons have been performed (Hentschel *et al*., 2002; Lee *et al*., 2009; Montalvo *et al*, 2011). The structures of communities within taxa of particular interest, have been examined including: *Actinobacteria* (Sun *et al*., 2010) *Chloroflexi* (Schmitt *et al*., 2011) and *Cyanobacteria* (Webb & Maas, 2002; Usher *et al*., 2004; Ridley *et al*., 2005; Steindler *et al*., 2005). Differences in community profiles between inner and outer sponge tissues have also been explored (Thiel *et al*., 2007; Sipkema & Blanch, 2010; Gerçe *et al*., 2011). Cloning of 16S rRNA genes has led to the discovery of a novel candidate bacterial phylum, *Poribacteria* (Fiesler *et al*., 2004), which is common to many sponge species (Lafi *et al*., 2009) but almost exclusively known from sponges.

These investigations have spanned a large range of sponge species from all of the worlds' oceans (Table 1.2). The sequencing of 16S rRNA clone libraries led to the identification of 16 bacterial phyla or candidate phyla (*Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Deinococcus-Thermus*, *Firmicutes*, *Gemmatimonadetes*, *Lentisphaerae*, *Nitrospira*, *Planctomycetes*, *Poribacteria, Proteobacteria* [α-, β-, δ- and γ-], *Spirochaetes*, TM6 and *Verrucomicrobia*) which have been found in close association with sponges (Taylor *et al*., 2007). Subsequently, sequencing of sponge-derived DGGE bands (Hardoim *et al*., 2009) added the phyla *Aquificae*, *Deferribacteres*, *Dictyoglomi* and the candidate phylum TM7 to the list of taxa found in association with sponges.

Sponge species	Reference	Sponge species	Reference	Sponge species	Reference
Agelas oroides	Gerçe *et al*., 2011	*Halichondria panicea*	Lee *et al*., 2009	*Raspailia topsenti*	Schmitt *et al*., 2012
Amphimedon sp.	Radwan *et al*., 2010	*Haliclona (? gellius)*	Sipkema *et al*., 2009	*Rhabdastrella globostellata*	Lafi *et al*., 2009
Ancorina alata	Kamke *et al*., 2010; Schmitt *et al*., 2012	*Haliclona foraminosa*	Lee *et al*., 2009	*Rhopaloides odorabile*	Webster *et al*., 2001; Hentschel *et al*., 2003
		Haliclona rufescens	Lee *et al*., 2009	*Smenospongia aurea*	Fiesler *et al*., 2004
Aplysina aerophoba	Fiesler *et al*., 2004; Hentschel *et al*., 2002; Hentschel *et al*., 2003; Steindler *et al*., 2005; Usher *et al*., 2004	*Haliclona* sp.	Steindler *et al*., 2005; Usher *et al*., 2004	*Sphaerotylus antarcticus*	Webster *et al*., 2004
Aplysina archeri	Steindler *et al*., 2005	*Homaxinella balfourensis*	Webster *et al*., 2004	*Spheciospongia floridae*	Steindler *et al*., 2005
Aplysina fistularis	Fiesler *et al*., 2004; Lafi *et al*., 2009	*Hymeniacidon perleve*	Sun *et al*., 2010	*Stelleta kallitetilla*	Steindler *et al*., 2005
Aplysina fulva	Hardoim *et al*., 2009	*Hyrtios erectus*	Radwan *et al*., 2010	*Stelleta maori*	Schmitt *et al*., 2012
Aplysina insularis	Fiesler *et al*., 2004	*Ircinia fasciculata*	Erwin *et al*., 2011; Erwin *et al*., 2012	*Stelleta pudica*	Steindler *et al*., 2005
Aplysina lacunose	Fiesler *et al*., 2004	*Ircinia felix*	Steindler *et al*., 2005	*Stylinos sp.*	Taylor *et al*., 2004
Axinella polypoides	Gerçe *et al*., 2011	*Ircinia oros*	Erwin et a., 2011; Erwin *et al*., 2012	*Stylissa carteri*	Giles *et al*., 2012
Callyspongia sp.	Taylor *et al*., 2004	*Ircinia variabilis*	Erwin *et al*., 2011; Erwin *et al*., 2012; Steindler *et al*., 2005	*Suberites zeteki*	Zhu *et al*., 2008
Callyspongia vaginalis	Giles *et al*., 2012	*Kirkpatrickia variolosa*	Webster *et al*., 2004	*Svenzea zeai*	Steindler *et al*., 2005
Candidaspongia flabellata	Steindler *et al*., 2005	*Lamellodysidea chlorea*	Ridley *et al*., 2005	*Terpios hoshinota*	Tang *et al*., 2011
Carteriospongia foliascens	Steindler *et al*., 2005	*Lamellodysidea herbacea*	Ridley et sl., 2005	*Tethya calaforniana*	Sipkema & Blanch, 2010
Chondrilla australiensis	Steindler *et al*., 2005; Usher *et al*., 2004	*Latrunculia apicalis*	Webster *et al*., 2004	*Tethya* sp.	Gerçe *et al*., 2011
Chondrilla nucula	Steindler *et al*., 2005; Thiel *et al*., 2007b	*Lendenfeldia chondrodes*	Ridley *et al*., 2005	*Tethya stolonifera*	Schmitt *et al*., 2012
Chondrilla sp.	Usher *et al*., 2004	*Mycale acerata*	Webster *et al*., 2004	*Theonella conica*	Steindler *et al*., 2005
Chondrilla sp.	Steindler *et al*., 2005	*Mycale adhaerens*	Lee *et al*., 2009	*Theonella swinhoei*	Hentschel *et al*., 2002; Hentschel *et al*., 2003; Steindler *et al*., 2005
Chondrosia reniformis	Gerçe *et al*., 2011	*Mycale armata*	Usher *et al*., 2004	*Theonella swinhoei*	Lafi *et al*., 2009
Cinachyra sp.	Khan *et al*., 2011	*Mycale hentscheli*	Webb & Maas, 2002	*Tsitsikamma favus*	Walmsley *et al*., 2012
Clathria pennata	Lee *et al*., 2009	*Mycale loveni*	Lee *et al*., 2009	*Ulosa sp.*	Khan *et al*., 2011
Craniella austrialiensis	Li *et al*., 2006	*Myxilla intruscans*	Lee *et al*., 2009	*Verongula gigantean*	Fiesler *et al*., 2004
Crella cyathophora	Giles *et al*., 2012	*Niphates digitalis*	Giles *et al*., 2012	*Vetulina* sp.	Cassler *et al*., 2006
Cribochalena vasculum	Steindler *et al*., 2005	*Oscarella lobularis*	Gerçe *et al*., 2011	*Xestospongia hispida*	Lee *et al*., 2009
Cymbastela concentrica	Taylor *et al*., 2004; Taylor *et al*., 2005	*Petrosia ficiformis*	Gerçe *et al*., 2011; Steindler *et al*., 2005; Usher *et al*., 2004	*Xestospongia muta*	Montalvo *et al*., 2011; Steindler *et al*., 2005
Cymbastela marshae	Usher *et al*., 2004	*Petrosia* sp.	Steindler *et al*., 2005; Khan *et al*., 2011	*Xestospongia proxima*	Steindler *et al*., 2005
Discodermia dissoluta	Brück *et al*., 2012	*Phakella fusca*	Han *et al*., 2012	*Xestospongia testudinaria*	Montalvo *et al*., 2011
Dysidea avara	Gerçe *et al*., 2011	*Phyllospongia papyracea*	Ridley *et al*., 2005		
Dysidea granulosa	Gopi *et al*., 2012	*Polymastia* sp.	Kamke *et al*., 2010		
Gelliodes carnosa	Li *et al*., 2011	*Polymastia* sp.	Schmitt *et al*., 2012	*Pseudoceratina fistularis*	Lafi *et al*., 2009
Geodia sp.	Gerçe *et al*., 2011	*Pseudoaxinella tubulosa*	Steindler *et al*., 2005		

Table 1.2: Sponge species from which bacterial 16S rRNA gene clone libraries have been reported.

1.2.1.2.3 Pyrosequencing

Next generation sequencing has had a profound effect on microbial ecology studies. The technology allows for the generation of hundreds of thousands of sequencing reads from metagenomic DNA samples. Barcoding of samples allows for the pooling and parallel processing of samples and so very robust and comprehensive descriptions of bacterial community structures from diverse sources have been generated. The large datasets generated by pyrosequencing analyses have allowed for the identification of members of the 'rare-biosphere' (Sogin *et al*., 2006). Also, more accurate descriptions of community structures and rank-abundance profiles of bacterial communities from a huge diversity of biomes have been described.

Various aspects of human associated microbial communities have been reported including: the gut (elderly – [Kraneveld *et al*., 2012; O'Toole *et al*., 2012], infant – [Fouhy *et al*., 2012]), skin (Blaser *et al*., 2012), mouth (Alcaraz *et al*., 2012), disease associated (pulmonary disease – [Cabrera-Rubio et a., 2012], cirrhosis – [Bajaj *et al*., 2012], intestinal disease – [Ukhanova *et al*., 2012], cystic fibrosis – [Delhaes *et al*., 2012] and the healthy (Ling *et al*., 2012).

Soil-associated bacterial communities from forest (Hartmann *et al*., 2012), agricultural (Shange *et al*., 2012) and contaminated soils (Ge *et al*., 2012) have been described. Aquatic bacterial consortia from lakes (Campbell & Kirchman *et al*., 2012; Lin *et al*., 2012), seawater (Ray *et al*., 2012 and hydrothermal vents (Sylvan *et al*., 2012) have also been reported. The bacterial communities associated with a wide range of terrestrial animals including (chicken [Lei *et al*., 2012], cow [Machado *et al*., 2012], dog [Garcia-Mazcorro *et al*., 2012], horse [Shepherd *et al*., 2012], mosquito [Boissière *et al*., 2012], honey bee [Sabree *et al*., 2012], beetle [Mattila *et al*., 2012], fleas and ticks [Hawlena *et al*., 2012]) and marine animals (fish [van Kessel *et al*., 2012], squid [Collins *et al*., 2012], corals [Lee *et al*., 2012b; Morrow *et al*., 2012] and a marine polychaete [Neave *et al*., 2012]) have also been described.

The same is true for marine sponges. A recent review of publicly available sponge-associated 16S rRNA sequences (Simister *et al*., 2012) analysed a dataset of ~7,500 sequences. However, pyrosequencing analyses have generated >700,000 sponge-derived bacterial 16S rRNA gene sequences which were not included in that study. These datasets have investigated various aspects of sponge-bacterial associations,

including bacterial community structures (Webster *et al*., 2010; Jackson *et al*., 2012; Trindade-Silva *et al*., 2012), seasonal variations in community composition (White *et al*., 2012), bacterial-archaeal relative abundances (Lee *et al*., 2011), vertical symbiont transmission (Webster *et al*., 2010) and core, variable and species-specific bacterial communities from a range of sponge species (Schmitt *et al*., 2012). These pyrosequencing studies have thus far investigated 15 sponge species (Table 1.3) and have led to the identification 35 bacterial phyla or candidate phyla which have been found in close association with sponges. Taxa identified in sponges for the first time by pyrosequencing include BRC1, *Chlamydiae*, *Fibrobacteres*, *Fusobacteria*, *Tenericutes* and WS3 (Webster *et al*., 2010), *Chlorobi*, *Chrysiogenetes*, OD1, *ε-Proteobacteria* and *Thermodesulfobacteria* (Lee *et al*., 2011), OP10, OS-K (Schmitt *et al*., 2012) and *Thermotogae*, *Elusimicrobia* and *Synergistetes* (Trindade-Silva *et al*., 2012). Many of these extra taxa are amongst the rarest members of the sponge-associated communities. Highly diverse communities described at genus, family, order and class levels have been described with ~3,000 OTUs (95% sequence identity) reported from the marine sponge *Rhopaloides odorabile* (Webster *et al*., 2010).

Sponge species	**Reference**	**Sponge species**	**Reference**
Ianthella basta	Webster *et al*., 2010	*Aplysina aerophoba*	Schmitt *et al*., 2012
Ircinia ramosa	Webster *et al*., 2010	*Aplysina cavernicola*	Schmitt *et al.,* 2012
Rhopaloides odorabile	Webster *et al*., 2010	*Ircinia variabilis*	Schmitt *et al.*, 2012
Hyrtios erectus	Lee *et al*., 2011	*Petrosia ficiformis*	Schmitt *et al*., 2012
Stylissa carteri	Lee *et al*., 2011	*Pseudocorticium jarrei*	Schmitt *et al*., 2012
Xestospongia testudinaria	Lee *et al*., 2011	*Axinella corrugata*	White *et al*., 2012
Raspailia ramosa	Jackson *et al*., 2012	*Arenosclera brasiliensis*	Trindade-Silva *et al*., 2012
Stelligera stuposa	Jackson *et al*., 2012		

Table 1.3: Sponge species from which pyrosequencing of bacterial 16S rRNA genes has been reported.

The utility of pyrosequencing has allowed for the launch of two ambitious projects, The Earth Microbiome Project (Gilbert *et al*, 2010) and the Human Microbiome Project (Huttenhower *et al*., 2012) where consortia from around the world are attempting to document the bacterial diversity of (a) the entire planet and (b) the human.

1.2.2 Sponge associated archaea

Archaea were first reported in association with marine sponges in 1996 (Preston *et al*., 1996) when *Cenarchaeum symbiosum* was found in the tissues of *Axinella mexicana*. It was subsequently found that *C. symbiosum* was consistently found in sponges of the family *Axinellidae* (Margot *et al*., 2002). Many reports of sponge associated archaea followed (Webster *et al*., 2001; Lee *et al*., 2003; Webster *et al*., 2004; Pape *et al*., 2006; Holmes & Blanch, 2007; Meyer & Kuever *et al*., 2008; López-Legentil *et al*., 2010; Turque *et al*., 2010; Liu *et al*., 2011; Radax *et al*., 2012) and included studies which demonstrated the vertical transmission of archaea in sponge larvae suggesting a close co-evolutionary relationship (Sharp *et al*., 2007; Steger *et al*., 2008).

Lee and colleagues used pyrosequencing to determine the relative abundances of bacteria and archaea in sponges from the Red Sea (Lee *et al*., 2011). Relative abundances of archaea ranged from 4-28% in different sponges and comprised almost exclusively *Crenarchaeota*.

1.2.3 Sponge associated Eukaryota

1.2.3.1 Sponge associated fungi

In recent years the relative paucity of information regarding sponge-associated fungi has partly been addressed. A number of research groups have begun to focus on the diversity and pharmacological potential of sponge-associated fungi (Wei *et al*., 2009; Abdel-Lateff *et al*., 2009; Zhang *et al*., 2009; Paz *et al*., 2010; Wiese *et al*., 2011; Chu *et al*., 2011; Ding *et al*., 2011; Zhou *et al*., 2011). Fungi from 32 orders, from three phyla (*Ascomycota* [22 orders], *Basidiomycota* [8 orders], *Zygomycota* [2

orders]), representing >120 genera have to date been found in or on sponges (Höller *et al*., 2000; Yu *et al*., 2012). At least 18 orders of fungi have been isolated in culture (Yu *et al*., 2012). Many of the fungi reported are closely related to terrestrial species though members of marine-fungal clades (Gao *et al*., 2008) have been reported. In particular *Penicillium* sp. and *Aspergillus* sp. have been found to be common in marine sponges. While these reports have come from a diverse range of sponge species from around the world, vertical transmission of fungal symbionts has been reported in three sponge species. Maldonado and colleagues used Transmission Electron Microscopy (TEM) to observe the close association of a filamentous fungus with sponge oocytes (Maldonado *et al*., 2005) while Rozas and co-workers cultured 6 fungal species from in vitro cultures of sponge primmorphs and single cells (Rozas *et al*., 2011). These reports suggest that fungi may in fact be true sponge symbionts and thus might have an important role in host physiology.

1.2.3.2 Sponge associated diatoms

Diatoms have long been known to be associated with marine sponges (Cox & Larkum, 1983), but their precise role in sponge tissues is as yet unclear. Parasitism has been suggested (Bavastrello *et al*., 2000; Cerrano *et al*., 2004) as diatoms invading and damaging sponge pinacocytes has been observed. Mutualism is also possible. As organisms which are important in photosynthesis in marine ecosystems, diatoms found growing within sponge tissues, may provide photosynthates for the host or may help to strengthen the spiculate skeleton (Totti *et al*., 2005) in return for a growth niche. Other evidence points to diatoms as a food source for sponges (Gaino *et al*., 1994; Cerrano *et al*., 2004).

1.2.3.3 Sponge associated dinoflagellates

Dinoflagellates of the genus *Symbiodinium* form close symbiotic relationships with many marine animals but are most commonly known in corals where nutrient exchange between the partners has been demonstrated (Weisz *et al*., 2010). Four distinct clades of *Symbiodinium* spp. have been reported in close association with sponges (Hill *et al*., 2011). These symbioses are almost exclusively known from the *Clionaidae* family of sponges, notable exceptions being the symbioses with a

Haliclona sp. sponge (Garson *et al*., 1998) and an *Anthosigmella* sp. (Hill *et al*., 1996). *Cliona* spp. display variable morphologies and the encrusting phenotype is a boring, bioeroding sponge which grows on and kills corals (Xavier *et al*., 2011). It was thought that symbiotic *Symbiodinium* sp. may have been acquired from the coral, however Schönberg and colleagues identified genetically unrelated dinoflagellates in sponges and in the sponge-invaded coral species (Schönberg & Loh, 2005), suggesting a distinct sponge-dinoflagellate symbiotic partnership.

1.2.3.4 Other sponge associated eukaryota

Other eukaryotes have been reported to be present in close association with sponges. Polychaetes (annelid worms) and shrimp were reported from Caribbean sponges (Duffy, 1992). *Ophiuroidea* (brittle stars), *Cnidaria* (sessile *Anthozoa*), *Turbellaria* (flatworms), *Nemertinia* (ribbon worms), *Sipuncula* (sipunculid worms), *Polychaeta*, *Mollusca*, *Crustacea*, *Pycnogondia* (sea spiders), *Echinodermata* (sea cucumbers), *Ascidiacea* (sea squirts) and *Pisces* (fish) have all been observed in association with a Brazilian sponge (*Zygomycale parishii*) over a 5 year study period (Duarte & Nalesso, 1996). *Ophiuroidea* were also found to be consistently associated with sponges but the authors suggest that this relationship is species-specific between *Callyspongia vaginalis* and *Ophiothrix lineata* (Henkel and Pawlik, 2004). Although many of these phyla are known parasites, their precise roles within their sponge hosts are as yet not known. A mutualist relationship between a sponge (*Halichondria panicea*) and a scallop (*Chlamys varia*) has however been reported where the sponge obtains increased suspended nutrients while the scallop gains protection from predation (Forrester, 1979).

1.2.4 Sponge-specific microorganisms

In 2002, Hentschel and colleagues performed a meta-analysis of all publically available (n = 190) sponge-derived 16S rRNA gene sequences (Hentschel *et al*., 2002). The analysis included 5 sponge species from different geographical regions as follows: Mediterranean Sea (France, Israel and Croatia), Red Sea, North Pacific (Japan and USA), Australian waters (Davies Reef) and from the Philippine Sea

(Palau). Phylogenetic analyses of these sequences revealed monophyletic clusters of sponge-derived sequences more closely related to each other than to sequences of the same taxa derived from non-sponge sources. This led Hentschel to frame the hypothesis of sponge-specific microbes and to speculate on the evolutionary establishment of those clusters. That study established that 14 monophyletic sequence clusters from 7 bacterial phyla, representing 70% of all sponge-derived sequences, were 'sponge-specific.' Hentschel went on to define sponge-specific to apply to groups of at least 3 sequences which are (i) recovered from different sponge species and/or from individuals of the same species from different geographic locations, (ii) more closely related to each other than to sequences from non-sponge sources and (iii) cluster together independently of the tree-building algorithm used.

By 2006, ~1,700 sponge derived 16S rRNA sequences were publically available and Taylor and colleagues endeavoured to determine whether the sponge-specific microbe hypothesis could still be supported (Taylor *et al*., 2007). They reported that 32% of all sponge-derived sequences from at least 10 bacterial phyla and also from a major archaeal lineage (*Crenarchaeota*) recruited to sponge-specific clusters. These sponge-specific clusters included 100% (n = 21) of all sequences, then available, from the putatively sponge-specific candidate phylum *Poribacteria*. High proportions of sponge derived sequences from *Chloroflexi* (62%), *Cyanobacteria* (79%), *Nitrospira* (57%), and *Spirochaetes* (67%) were classified as sponge-specific. Notable proportions of sequences from *Actinobacteria* (38%), *Gemmatimonadetes* (25%) and β-/γ- *Proteobacteria* (34%) were assigned to sponge-specific clusters. Conversely, only 5% of *Acidobacteria* sequences, 9% of *Firmicutes* sequences and 0% of *Bacteroidetes* sequences were determined to be sponge-specific. Approximately one quarter of sponge-derived archaeal 16S rRNA sequences were defined as sponge-specific.

Although many sponge-specific clusters withstood Taylors' rigorous analysis, an approximate nine-fold increase in the number of sponge-derived sequences analysed, combined with a concomitant increase in the numbers of non-sponge derived sequences from which to draw comparison, led to an approximate halving (32%) of the proportion of sponge-derived sequences being classed as sponge-specific.

Simister and colleagues revisited the issue in 2011 (Simister *et al*., 2011). By this time the number of publicly available (non-pyrosequencing) sponge-derived 16S rRNA sequences had risen to ~7,500. In their analysis they found that 27% of sponge-derived sequences were assigned to sponge-specific clusters from 14 bacterial phyla and one major archaeal lineage (*Thaumarchaeota*). In keeping with Taylors' findings, large proportions of sponge derived *Chloroflexi* (61%), *Cyanobacteria* (53%), *Nitrospirae* (39%) and *Spirochaetes* (92%) were classified as sponge-specific. The low abundance detection of *Poribacteria* in seawater resulted in 79% of the 170 sponge-derived *Poribacteria* being described as sponge-specific. Simister *et al*. also reported high proportions of sponge-derived *Acidobacteria* (57%), β- *Proteobacteria* (55%), *Deinococcus-Thermus* (53%), TM6 (43%) and TM7 (67%) sequences in sponge-specific clusters. Intermediate proportions of *Actinobacteria* (21%), *Gemmatimonadetes* (36%), γ- (20%) and δ- (33%) *Proteobacteria* appear sponge-specific. Low proportions of *Firmicutes* (3%), *Bacteroidetes* (6%) and *Planctomycetes* (7%) recruit to sponge-specific clusters. From the domain *Archaea*, 41% of sponge-derived 16S rRNA gene sequences fell into four distinct clusters of sponge-specific taxa.

While the study by Simister and colleagues analysed a dataset of ~7,500 sponge-derived sequences, they like the previous Taylor study, only considered relatively long sequencing reads. The emergence of pyrosequencing however has contributed ~700,000 sponge-derived 16S rRNA sequences to public databases. These reads vary in length from 50-60 bp (Webster *et al*., 2010) up to an average 430 bp (Jackson *et al*., 2012). Despite not being considered in the meta-analyses Webster, Jackson and Lee (Lee *et al*., 2011) assigned pyrosequencing data sequence reads to previously described and new sponge-specific clusters. Webster and co-workers assigned 13.3% (n = 52,270) of their sequences to sponge-specific clusters, Lee and colleagues analysed >110,000 sponge-derived sequences and reported that 36-65% of sequences from sponge individuals could be assigned to previously described sponge-specific clusters. Jackson and colleagues analysed ~26,000 sequences from two sponge species and reported that 2.8% of reads from one sponge and 26% from the other sponge appeared to be sponge-specific.

1.3 Symbiotic functions of sponge-associated microbes

1.3.1 Methods to elucidate sponge symbiont functions

The detection of microbial biomarker gene sequences from sponge metagenomes has led to speculation about the possible symbiotic functional roles of those taxa. Known physiological functions of microbes may be used to predict possible functions but empirical conclusions cannot be drawn from phylogenetic biomarker data analyses. In addition, these predictions can only be made for microbes which have been cultured and from which physiological characterizations have been elucidated. Other methods used to determine sponge symbiont functions include genome reconstruction (Liu *et al*., 2011b), single-cell genomics (Hallam *et al*., 2006; Siegl *et al*., 2011), metatranscriptomics (Kamke *et al*., 2012; Radax *et al*., 2012b), shotgun cloning and sequencing of sponge metagenomic DNA (Thomas *et al*., 2010), shotgun pyrosequencing (Trindade-Silva *et al*., 2012) and the targeted PCR amplification of functional genes from sponge metagenomes (Schirmer *et al*., 2005; Kim & Fuerst, 2006; Fiesler *et al*., 2007; Bayer *et al*., 2008; Kennedy *et al*., 2008b; Mohamed *et al*., 2008; Mohamed *et al*., 2010; Han *et al*., 2012; Yang & Li, 2012).

A recent example of a successful shotgun sequencing based approach has been the genome reconstruction of an unidentified δ-proteobacterium from shotgun sequence data from the sponge *Cymbastela concentrica* (Liu *et al*., 2011b). The application of single-cell genomics has been used to make predictions about sponge symbiont functions (Kamke *et al*., 2012) from uncultured microbes. The genome of *Cenarchaeum symbiosum* derived from the marine sponge *Axinella mexicana*, was sequenced following cell enrichment and differential centrifugation (Hallam *et al*., 2006). Siegl and colleagues used fluorescence activated cell sorting (FACS) to obtain single cells of Poribacteria from the sponge *Aplysina aerophoba* for genome sequencing (Siegl *et al*., 2011).

Kamke and colleagues compared the presence of 16S rRNA genes with the presence of 16S rRNA in two sponge species (*Ancorina alata* and *Polymastia* sp.) to determine which taxa were active in the holobiont (Kamke *et al*., 2010). Pyrosequencing of cDNA has recently been used by Radax and co-workers to elucidate the diversity and abundance of actively transcribed genes from the sponge *Geodia barretti* (Radax *et al*., 2012b); while shotgun approaches (cloning – Thomas

et al., 2010; pyrosequencing – Trindade-Silva *et al*., 2012) have identified functional genes in the sponges *Cymbastela concentrica* and *Arenosclera brasiliensis*, respectively.

Researchers have also targeted functional genes of particular interest for PCR amplification and sequencing, with genes involved in ammonia-oxidation, nitrification and putative host defence in particular being targeted. Ammonia-oxidation (*amoA*) genes have been noted in the metagenomes of *Aplysina aerophoba* (Bayer *et al*., 2008), *Ircinia strobilina*, *Mycale laxissima* (Mohamed *et al*., 2010) and *Phakiella fusca* (Han *et al*., 2012). Nitrification genes (*nirS*) have been amplified from the sponge *Astrosclera willeyana* (Yang & Li, 2012). Genes involved in the production of bioactive secondary metabolites which may contribute to sponge defence have also been targeted. Polyketide synthase (PKS) genes have been noted from the sponges *Pseudoceratina clavata* (Kim & Fuerst, 2006), *Discodermia dissoluta* (Schirmer *et al*., 2005), *Theonella swinhoei*, *Aplysina aerophoba* (Fiesler *et al*., 2007) and *Haliclona simulans* (Kennedy *et al*., 2008b).

1.3.2 Discrimination between food microbes and symbiotic microbes

A long standing question in the sponge microbiology area has been how sponges discriminate between food and symbionts when both occur in the sponge mesohyl. Genomic, metagenomic and metatranscriptomic analyses have identified factors which may play crucial roles in the symbiosis of sponge and microbe. These include factors associated with cell recognition, adhesion and signalling. Gene transcripts for cell recognition factors [Polycystic Kidney Domain-like (PKD)] have been identified in the *Geodia barretti* metatranscriptome (Radax *et al*., 2012b) while Ig-like domain protein encoding gene sequences were found in the genome of 'candidatus' *Poribacteria* (Siegl *et al*., 2010). Adhesion related genes (ankyrin repeat, tetratrico peptide repeat, fibronectin type III and laminin-G domain proteins) were also noted in the genomes of sponge-derived *Poribacteria* (Siegl *et al*., 2010) and δ-proteobacteria (Liu *et al*., 2011b) and adhesion related gene transcripts (ankyrin repeat domain proteins, tetratrico repeat domain proteins, Ton B-dependent receptors and collagen binding surface proteins) were observed from the metatranscriptome of *Cymbastela concentrica* (Thomas *et al*., 2010) and *Geodia barretti* (Radax *et al*., 2012b). Cell signalling related protein transcripts were also noted by Radax and

colleagues. However signalling related gene sequences were reported to be under-represented in the genome of the sponge derived δ-proteobacterium when compared to the genome of a related non-symbiotic δ-proteobacterium (Liu *et al*., 2011b).

1.3.3 Nutrient cycling in sponges

It is thought that sponge endosymbiotic microbes play crucial roles in carbon, nitrogen and sulfur cycling (Taylor *et al*., 2007).

1.3.3.1 Carbon cycling in sponges

Carbon cycling in sponges occurs through autotrophic (chemotrophic and phototrophic) or heterotrophic activities. The presence of large populations of photosynthetic microbes (cyanobacteria and zooxanthellae) in sponges has been shown to contribute to host nutrition through the production of photosynthates, with the transfer of carbon from symbiont to host being observed (Wilkinson, 1979; Freeman & Thacker, 2011). Illumination has been shown to play an important role in sponge distribution and growth rates. Sponges hosting large cyanobacterial populations (*Pericharax heteroraphis*, *Jaspis stellifera* and *Neofibularia irata*) have been observed to grow only at depths of less than 15m where sunlight can penetrate, enabling photosynthesis (Wilkinson, 1978). Differential growth rates were observed in clionaid sponges, which host photosynthetic *Symbiodinium* spp., while naturally illuminated or kept in darkness, indicating the contribution of photosynthesis to sponge growth (Rosell & Uriz, 1992).

Chemotrophy related genes have been reported from the genomes of sponge derived *Poribacteria* (Siegl *et al*., 2010) and from the sponge derived archaeon *Cenarchaeum symbiosum* (Hallam *et al*., 2006). Siegl and colleagues reported genes of the Wood–Ljungdahl carbon assimilation pathway in *Poribacteria* while Hallam and co-workers reported genes from the 3-hydroxypropionate pathway in *C. symbiosum*. Evidence for the presence of genes or enzymes of the Wood-Ljungdahl pathway were also reported from the metagenomes of *Cymbastela concentrica* (Thomas *et al*., 2010) and *Arenosclera brasiliensis* (Trindade-Silva *et al*., 2012) as well as from the metatranscriptome of *Geodia barretti* (Radax *et al*., 2012b). Trindade-Silva and colleagues have also reported genes from the reductive citric acid cycle in the *A. brasiliensis* metagenome.

Heterotrophic carbon cycling occurs through the filter feeding activities of sponges and phylogenetic biomarker genes from methanotrophic microbes have also been detected in sponges and it is thought that they may contribute to carbon cycling (Webster *et al*., 2010; Lee *et al*., 2011; Jackson *et al*., 2012).

1.3.3.2 Nitrogen cycling in sponges

As with terrestrial systems, nitrogen is a major limiting factor for all of life in marine ecosystems. The cycling of nitrogen from nitrogen gas (N_2) through inorganic (nitrate [NO_3^-], nitrite [NO_2^-], ammonium [NH_4^{+}]) and organic forms (e.g. proteins, amino acids and nucleotides) is highly complex in the ocean (Gruber, 2008). The importance of marine sponges to benthic ecosystems suggests that nitrogen cycling in sponges plays a major role in the nitrogen budget of those habitats.

1.3.3.2.1 Nitrogen fixation

Biological nitrogen fixation is the principal source of fixed nitrogen in the marine environment and is mediated in large part by phototrophic microorganisms such as cyanobacteria (Gruber, 2008). Nitrogen fixation, via nitrogenase activity, was first reported in sponges in the 1970s (Wilkinson & Fay 1979). Nitrogen fixing *Vibrionaceae* have been reported in association with *Halichondria* sp. by Shieh and colleagues (Shieh & Lin., 1994) while stable isotope analysis by Wilkinson and co-workers showed the incorporation of ^{15}N into amino acids in the sponge *Callyspongia muricina* (Wilkinson *et al*., 1999).

It has been demonstrated that low ^{15}N:^{13}N ratio in sponges is inversely correlated with bacterial diversity in sponges (Weisz *et al*., 2007). Low levels of ^{15}N is indicative of biological nitrogen fixation and Weisz and colleagues measured low ^{15}N ratios in sponges (*Ircinia felix* and *Aplysina cauliformis*) with highly diverse associated bacterial communities, as determined by microscopy (TEM) and DGGE; while higher ratios of ^{15}N were present in a sponge (*Niphates erecta*) with low microbial abundance and diversity.

In 2008, Mohamed and colleagues used PCR to identify *nifH* genes related to α- (*Methylocystis* sp.), δ- (*Desulfovibrio* sp.) and γ- (*Azotobacter* sp.) proteobacterial and cyanobacterial (*Tolypothrix* sp., *Leptolyngbya* sp.) genes and to archaeal (*Methanosarcina* sp.) genes in the sponges *Ircinia strobilina* and *Mycale laxissima*

(Mohamed *et al*., 2008). The *nifH* gene encodes nitrogenase reductase, a key enzyme in nitrogen fixation. That study also showed for the first time the active expression of *nifH* in sponges through reverse transcriptase PCR (RT-PCR). The latest evidence for nitrogen fixation in sponges comes from Liu and co-workers who described the partial genome reconstruction of a nitrogen fixing bacterium (*Mesorhizobium* sp.) from shotgun Sanger sequencing data in the sponge *Cymbastela concentrica* (Liu *et al*., 2012).

Thus, mounting evidence suggests that nitrogen fixation by sponge symbiotic microbes occurs in sponge tissues and thus may play a major role in marine ecosystem nitrogen budgets.

1.3.3.2.2 Nitrification

The second step in the nitrogen cycle is the aerobic oxidation of ammonium (NH_4^+) to nitrate (NO_3^-). This biological process is performed by ammonia oxidising bacteria (AOB) and ammonia oxidising archaea (AOA) (Purkhold *et al*., 2000). The two step process is mediated by the oxidation of ammonium to hydroxylamine (NH_2OH) by ammonia monooxygenase followed by the oxidation of hydroxylamine to nitrate by hydroxylamine oxidoreductase in bacteria. For archaeal nitrifiers hydroxylamine oxidoreductase homologs have not yet been described and so an alternative process in archaea has been suggested (Junier *et al*., 2010). Genome sequence data of a nitrifying archaeon (*Nitrosopumilus maritimus*) suggests hydroxylamine oxidation may occur via multicopper oxidases (Walker *et al*., 2010). Nitrate is subsequently oxidised to nitrite. The gene which encodes ammonia monooxygenase (*amoA*) is used as a biomarker for both function and taxonomic surveys. Global diversity of nitrifying microorganisms is thought to be limited to two monophyletic clades of bacteria (one clade of *γ-Proteobacteria* and one clade of *β-Proteobacteria*) and to *Crenarchaeota* (Purkhold *et al*., 2000).

In sponges, ammonia is a toxic metabolic waste product and the role of nitrifying symbionts may be crucial to sponge health. Evidence of nitrification in sponges comes from a number of different sources including: direct measurements of nitrite/nitrate excretion (Corredor *et al*., 1988; Diaz & Ward, 1997; Jiménez & Ribes, 2007; Bayer *et al*., 2008; Hoffmann *et al*., 2009; Schläppy *et al*., 2010; Ribes *et al*., 2012), PCR mediated bacterial (Meyer & Kuever, 2008; Bayer *et al*., 2008) and

archaeal *amoA* gene amplification (Steger *et al*., 2008; Meyer & Kuever, 2008; Bayer *et al*., 2008; Steger *et al*., 2008; Hoffmann *et al*., 2009) in sponges, *amoA* gene transcription in *Xestospongia muta* via RT-PCR (López-Legentil *et al*., 2010), metatranscriptomic detection of 16S rRNA transcripts from known nitrifying taxa and mRNA transcripts of *amoA* and nitrite oxidoreductase genes in *G. barretti* (Radax *et al*., 2012b) and from genome analysis from the sponge derived archaeon *C. symbiosum* (Hallam *et al*., 2006).

Corredor and colleagues provided the first evidence of nitrification in sponges when reporting the large release of nitrate from *Chondrilla nucula*, the first time nitrate excretion from any animal has been recorded (Corredor *et al*., 1988). Similar experiments later showed nitrate excretion by *C. nucula*, *Pseudoaxinella zeai*, *Oligoceras violacea* (Diaz & Ward, 1997), *Axinella polypoides*, *Ircinia oros* (Jiménez & Ribes, 2007), *Aplysina aerophoba* (Jiménez & Ribes, 2007; Bayer *et al*., 2008), *Geodia barretti* (Hoffmann *et al*., 2009), *Chondrosia reniformis* (Jiménez & Ribes, 2007; Schläppy *et al*., 2010; Ribes *et al*., 2012), *Dysidea avara* (Jiménez & Ribes, 2007; Schläppy *et al*., 2012) and *Agelas oroides* (Jiménez & Ribes, 2007; Ribes *et al*., 2012). Interestingly, Ribes and colleagues reported no nitrate excretion from *Dysidea avara* and suggested seasonal differences for this contradiction to the findings of Schläppy and colleagues. Ribes and co-workers also reported that different taxa were responsible for nitrification in *A. oroides* and *C. reniformis*. It is clear from these studies that nitrification is an important symbiotic function in marine sponges.

1.3.3.2.3 Denitrification

The nitrogen cycle is completed by the reduction of nitrite to dinitrogen gas via nitric oxide (NO) and nitrous oxide (N_20) or via NO and hydrazine (N_2H_4). Alternatively NO_2 can be reduced to ammonium. Genes encoding enzymes which mediate denitrification (e.g. nitrite reductase, nitrous oxide reductase) are found in diverse microbial phyla (Zumft, 1997).

Denitrification and anaerobic ammonia oxidation (anammox) have been reported in *Geodia barretti* (Hoffmann *et al*., 2009) as well as 16S sequences related to denitrifiers and the amplification of *nirS* (nitrite reductase). Schläppy and colleagues also reported denitrification in *Chondrosia reniformis* and *Dysidea avara* but could

not detect anammox activity in either of these sponges (Schläppy *et al*., 2010). Siegl and colleagues reported the presence of nitrite reductase and nitric oxide reductase genes in the genome of the sponge derived 'candidatus' *Poribacteria* (Siegl *et al*., 2010). Liu and co-workers combined metagenomic and metaproteomic methods to report 16S sequences related to the denitrifying *Nitratireductor* sp., a nitrate reductase gene cluster and expressed nitrate reductase proteins (NarG and NarY) in *Cymbastela concentrica* (Liu *et al*., 2012).

Complete cycling of nitrogen in sponges has been demonstrated as well as elements involved in nitrogen assimilation (Hentschel *et al*., 2012) and genes related to aspects of the nitrogen cycle have also been reported from sponge larvae (Steger *et al*., 2008), which is strongly indicative of both true symbiotic relationships and vital ecological functioning.

1.3.3.3 Sulfur cycling in sponges

Sulfur comprises ~1% of the dry weight of living organisms as a constituent of amino acids (cysteine and methionine), co-enzymes (e.g. co-enzyme A [CoA]), in metalloproteins and in ligands (e.g. cytochrome oxidase c) (Sievert *et al*., 2007). However, animals are dependent on microbial transformations of sulfur (sulfur oxidation/ sulfur and sulfate reduction). Diverse bacterial taxa mediate these transformations in assimilatory and dissimilatory processes which are vital to both life and biogeochemical cycling.

Anaerobic green sulfur bacteria - *Chlorobium* sp. (Eimhjellen, 1967), and purple sulfur bacteria – *Chromatium* sp., *Ectothiorhodospira* sp. (Imhoff & Trüper, 1976) and *Thiocystis* sp. (Eimhjellen, 1967) when isolated in culture from sponges in the 1960s and 1970s gave the first indication that sulfur cycling may be occurring in sponges and also that microaerobic and anaerobic microenvironments existed within sponge tissues.

Subsequently, Hoffmann and colleagues monitored oxygen gradients in the tissues of *Geodia barretti*, measured sulfate reduction in that sponge, demonstrated biomass transfer from bacteria to sponge cells and used FISH to map the spatial distribution of sulfate reducing taxa in the sponge (Hoffmann *et al*., 2005). These elegant experiments confirmed sulfur cycling symbioses between microbes and sponges.

Similarly, the spatial distribution of *Desulfovibrionaceae* in the sponge *Chondrosia reniformis* has been reported (Manz *et al*., 2000).

Genomic analyses of sponge derived microbes have resulted in the identification of biotin and thiamine synthesis genes in the genome of *Cenarchaeum symbiosum* (Hallam *et al*.,2006), sulfatase genes in the genome of 'candidatus' *Poribacteria* (Siegl *et al*., 2010) and glutathione transport genes in the genome of a sponge associated δ-proteobacterium (Liu *et al*., 2011b). These analyses further demonstrated the potential for sulfur cycling and assimilation in sponge tissues. In metagenomic analyses Thomas *et al*. reported the metagenome of the *Cymbastela concentrica* to be enriched for glutathione S transferase genes when compared to planktonic seawater communities but a comparative under-representation of sulfate permeases in the sponge was also observed (Thomas *et al*., 2010). Trindade-Silva and colleagues noted abundant dimethyl sulfoxide (DMSO) reductase genes in the metagenome of *Arenosclera brasiliensis* (Trindade-Silva *et al*., 2012). While in a metatranscriptomic study Radax and co-workers noted a highly transcribed iron-sulfur binding domain protein in *Geodia barretti* (Radax *et al*., 2012b).

Diverse sulfur metabolizing taxa have been reported in association with sponges where comprehensive community structure analyses have been determined by pyrosequencing (Table 1.4). Notable amongst these studies is the relative abundances of these taxa in individual sponge species. *Chloroflexi* comprise up to 6.5% of the *Ircinia ramosa* bacterial community and up to 11% of the *Rhopaloides odorabile* community (Webster *et al*., 2010). The same phylum comprises up to ~35% of the microbial communities of *Hyrtios erectus* and *Xestospongia testudinaria* (Lee *et al*., 2011). *Ectothiorhodospiraceae* account for up to 7% of the *R. odorabile* community (Webster *et al*., 2010), ~5% of the cohort from *Raspailia ramosa* and ~34% of the *Stelligera stuposa* bacterial associates (Jackson *et al*., 2012). Such abundances indicate the importance of sulfur metabolising symbionts to their sponge hosts. Also of note is the abundant detection of *Chloroflexi* and *Ectothiorhodospiraceae* in the larvae of *R. odorabile* (Webster *et al*., 2010), which is indicative of vertical transmission of these symbionts.

Reference	Sponge species	Phototrophic sulfur oxidisers	Chemolithotrophic sulfur oxidisers	Sulphur reducers	Sulphate reducers
Webster *et al.*, 2010	*Ianthella basta*	*Rhodobacter, Ectothiorhodospiraceae, Chloroflexi*			
	Ircinia ramosa	*Rhodobacter, Ectothiorhodospiraceae, Chloroflexi*			
	Rhopaloides odorabile	*Rhodobacter, Rhodomicrobium, Chromatiaceae, Ectothiorhodospiraceae, Chloroflexi*	*Paracoccus, Thiomicrospira*	*Desulfuromonas, Desulfobacterium*	
Lee *et al*., 2011	*Hyrtios erectus*	*Chlorobi, Chloroflexi, Ectothiorhodospiraceae*		*Thermoproteales*	*Desulfovibrio*
	Stylissa carteri	*Chlorobi, Chloroflexi*	*Arcobacter*	*Thermoproteales, Thermoplasmatales*	*Desulfobacter*
	Xestospongia testudinaria	*Chlorobi, Chloroflexi*		*Thermoproteales*	
Jackson *et al*., 2012	*Raspailia ramosa*	*Chromatiacaea, Chloroflexi, Ectothiorhodospiraceae*	*Paracoccus, Arcobacter, Sulfurimonas, Sulfurovum*	*Desulfuromonas*	*Desulfovibrio, Desulfonema, Desulfosarcina*
	Stelligera stuposa	*Ectothiorhodospiraceae*			
Schmitt *et al*., 2012	*Aplysina aerophoba*	*Chloroflexi , Ectothiorhodospiraceae*			
	Aplysina cavernicola	*Chloroflexi, Ectothiorhodospiraceae*			
	Ircinia variabilis	*Chloroflexi, Ectothiorhodospiraceae*			
	Petrosia ficiformis	*Chloroflexi, Ectothiorhodospiraceae*			
	Pseudocorticium jarrei	*Chloroflexi*			
White *et al*., 2012	*Axinella corrugata*	*Ectothiorhodospiraceae*			*Desulfovibrio, Desulfobacter*
Trindade-Silva *et al*., 2012	*Arenosclera brasiliensis*	*Chlorobi, Chloroflexi, Rhodocyclales*	*Aquificae*		

Table 1.4: Sulfur metabolizing taxa reported from marine sponges by pyrosequencing of 16S rRNA genes.

Other sulfur cycling taxa have been reported at low abundances but a recent study demonstrated that a sulfate reducing species, present at just 0.006% relative abundance in a peat soil, was responsible for a considerable amount of sulfate reduction in that soil and therefore, though such taxa can be uncommon the physiological contribution to the community functioning cannot however be underestimated (Pester *et al*., 2010).

1.3.4 Other putative symbiosis factors

Genomic, metagenomic and metatranscriptomic studies have identified other factors with possible roles in the symbiotic partnerships between sponges and microbes. Transposable insertion elements have been identified in the metagenome of *Cymbastela concentrica* (Thomas *et al*., 2010) and transposase gene transcripts were reported from the metatranscriptome of *Geodia barretti* (Radax *et al*., 2012). These elements are thought to play roles in microbial genomic rearrangements and streamlining to help with adaptation to a symbiotic lifestyle (Thomas *et al*., 2010). Factors with possible roles in the maintenance of a symbiotic relationship including tetracycline resistance genes and multidrug resistance protein genes were found in the genome of a sponge associated unidentified δ-proteobacterium (Liu *et al*., 2011b) while clustered regularly interspaced short palindromic repeat (CRISP) gene sequences with possible roles in resistance to viral infection were found in the metagenome *C. concentrica* (Thomas *et al*., 2010). Genes and gene transcripts involved in the biosynthesis of essential vitamins (B_2 or B_{12}) have been noted in the genomes of *Cenarchaeum symbiosum* (Hallam *et al*., 2006), 'candidatus' *Poribacteria* (Siegl *et al*., 2010) and a sponge associated δ-proteobacterium (Liu *et al*., 2011b), in the metagenome of *C. concentrica* (Thomas *et al*., 2010) and in the metatranscriptome of *G. barretti* (Radax *et al*., 2012b). This suggests that symbiotic microbes may be an important source of these essential vitamins for their hosts.

Sponges and sponge associated microbes have also been noted to be a remarkably rich sources of various classes of chemicals with a wide range of bioactive properties and are thought to potentially play important roles in sponge host defence from infection and predation (Taylor *et al*., 2007).

1.4 Pharmacological potential of marine sponges

Extensive research into marine sponges and marine sponge associated microbes has primarily been driven due to the pharmacological potential of diverse chemical entities with wide ranging biological activities being discovered from marine environments (Blunt *et al*., 2010). Physico-chemical properties of the marine environment (pH, pressure, temperature, osmolarity) mean that bioactive substances produced in that environment may have sufficiently different properties to terrestrially produced products to make them of interest for novel drug discovery (Thakur *et al*., 2005). The search for novel drugs has involved many phyla of marine invertebrates but the phylum *Porifera* has proved the most promising (Figure 1.5) (Leal *et al*., 2012). As sessile filter feeders, sponges, with no adaptive immunity, rely on a barrage of chemical entities to defend against infection, parasitism and disease and also to gain a competitive advantage (Thakur *et al*., 2005).

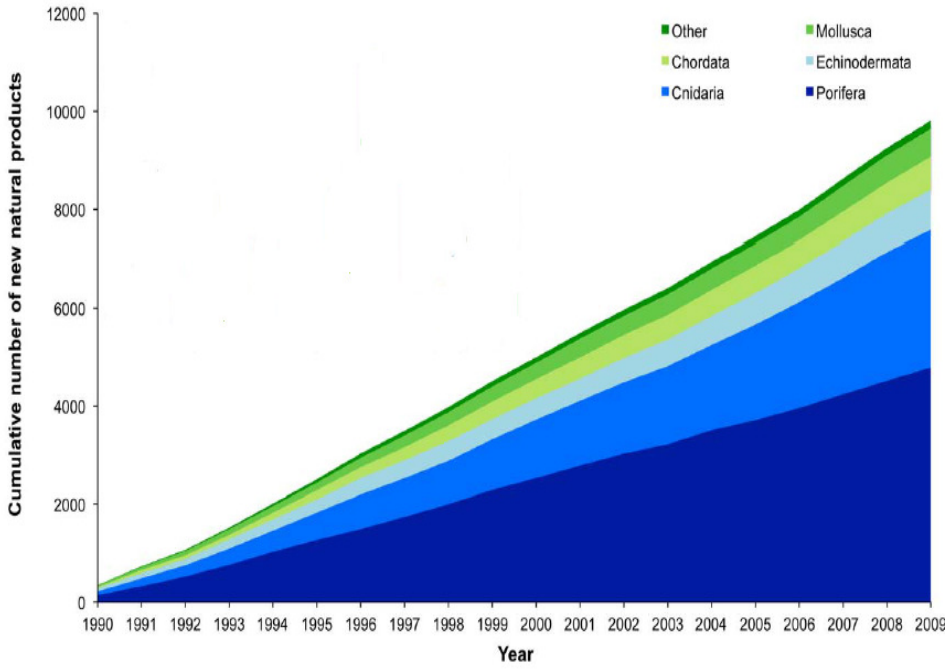

Figure 1.5: Marine natural product discovery from marine phyla from 1990-2009. (Other phyla include *Annelida*, *Arthropoda*, *Brachiopoda*, *Hemichordata*, *Platyhelminthes* and *Bryozoa*). Adapted from Leal *et al*., 2012.

The diverse range of chemical classes with bioactive properties obtained from sponges and sponge derived microbes include alcohols (Bugni *et al*., 2004),

alkaloids (Table 1.5), amino acid derivatives (Clark *et al*., 2001; Aiello *et al*., 2010; de Madeiros *et al*., 2012), aromatic compounds (Dai *et al.*, 2010), fatty acids (Tachibana *et al*., 1981; Aratake *et al*., 2009; Keffer *et al*., 2009), lactones (Namikoshi *et al*., 2004; Sirirak *et al*., 2012; Zhang *et al*., 2012), peptides (Table 1.6), polyacetylenes (Ankisetty & Slattery, 2012; Lee *et al*, 2012c), polyketides (Table 1.7), quinones and quinolones (Bultel-Poncé *et al*., 1999; Lucas *et al*., 2003; Davis *et al*., 2012; Kumar *et al.*, 2012), sphingolipids (Ando *et al*., 2010; Yoo *et al*., 2012), sterols (Rudi *et al*., 2004; Yu *et al*., 2006; Guo *et al*., 2012), terpenes and terpenoids (Table 1.8). These bioactivities have been identified from bacterial or fungal isolates from sponges or from aqueous or organic extracts from the sponge tissues. In many cases the bioactive compounds have been identified, purified and characterised.

Reference	Sponge species	Compound	Target of activity
Ang *et al*., 2000	*Haliclona* sp.	Manzamine A	*Plasmodium berghei*
Chang *et al*., 2003	*Monanchora* sp.	Crambescidin 826	HIV
Endo *et al*., 2004	*Agelas* sp.	Nagelamides A-H	Gram positive bacteria
Hassan *et al*., 2004	*Leucetta chagosensis*	Naamine G	*Cladosporium herbarum*
Zhang *et al*., 2008	*Halichondria panicea*	Circumdatin I	UV-A protectant
Yasuda *et al*., 2009	*Agelas* sp.	Nagelamide O	Gram positive bacteria
Carroll *et al*., 2010	*Ianthella flabelliformis*	Bastadin 25	δ-opoid receptor
Regalado *et al*., 2010	*Pandaros acanthifolium*	Pandaroside G	*Trypanosoma brucei rhodesiense*
Yang *et al*., 2010	*Hyattella* sp.	Psammaplysin G	*Plasmodium falciparum*
Dyshlovoy *et al*., 2012	*Aaptos aaptos*	Aaptamine	NT2 (embryonal carcinoma) cells
Liu *et al*., 2012b	*Aaptos suberitoides*	Suberitines B & D	P388 (lymphoblastic) cells
Yamazaki *et al*., 2012	*Haliclona* sp.	Papuamine & Haliclonadiamine	MCF-7 (breast), LNCap (prostate), Caco-2 (colon) and HCT-15 (colon) cells
Yang *et al*., 2012	Agelas mauritiana	Ageloxime B	MRSA

Table 1.5: Examples of sponge derived alkaloids with bioactive properties.

Compounds and activities against important human infections and diseases have been reported. Important bioactive compounds which have been reported include anti-bacterial compounds (including anti-MRSA and anti-tuberculosis) (Table

1.9), anti-fungal compounds (Table 1.5), anti-parasitic compounds (including anti-malarial) (Tables 1.5-1.8), anti-viral compounds (including anti-HIV) (Tables 1.5, 1.6 & 1.8), anti-coagulant compounds (Carroll *et al*., 2002; Carroll *et al*., 2004), anti-helminth compounds (Capon *et al*., 2004), anti-biofouling compounds (Devi *et al*., 1998; Sera *et al*., 2002; Hellio *et al*., 2006), anti-inflammatory compounds (Tables 1.7 & 1.8), neuromodulatory compounds (Capon *et al*., 2004b; Carroll *et al*., 2010; Zhang *et al.,*2012), a UV-A protectant compound (Zhang *et al*., 2008) and a large array of cytotoxic compounds with potential uses as anti-cancer drugs (Tables 1.5-1.8).

Reference	Sponge species	Compound	Target of activity
Rashid *et al*., 2000	*Haliclona nigra*	Haligramides A & B	cytotoxic
Sera *et al*., 2002	*Haliclona sp.*	Haliclonamides C, D & E	*Mytilus edulis galloprovincialis*
Pabel *et al*., 2003	*Aplysina aerophoba*	lipopeptides	*S. aureus, E.coli, Vibrio sp., C. albicans*
Oku *et al*., 2004	*Neamphius huxleyi*	Neamphamide A	HIV
Plaza *et al*., 2007	*Siliquariaspongia mirabilis*	Mirabamides A-D	HIV
Plaza *et al*., 2009	*Siliquariaspongia mirabilis*	Celebesides A-C & Theopapuamides B-D	HIV
Williams *et al*., 2009	*Eurypon laughlini*	Rolloamides A & B	cytotoxic
Pimentel-Elardo *et al*., 2010	*Tedania* sp.	Valinomycin	*Leishmania major*
Zhang *et al*., 2010	*Phakellia fusca*	Phakellistatins 15-18	P388 (lymphoblastic) cells
Chu *et al*., 2011	*Holoxea* sp.	L-Trp-L-Phe	cytotoxic
Kimura *et al*., 2012	*Discodermia calyx*	Calyxamides A & B	P388 (lymphoblastic) cells
Rabelo *et al*., 2012	*Cinachyrella apion*	Lectin	HeLa cells
Sorres *et al*., 2012	*Pipestela candelabra*	Pipestelides A-C	cytotoxic

Table 1.6: Examples of sponge derived peptides with bioactive properties.

Reference	Sponge species	Compound	Target of activity
Piel *et al*., 2004	*Theonella swinhoei*	Theopederin	Anti-tumour
Johnson *et al*., 2007	*Cacospongia mycofijiensis*	Fijianolide	Anti-tumour
Plaza *et al*., 2008	*Siliquariaspongia mirabilis*	Mirabilin	Anti-tumour
Ankisetty *et al*., 2010	*Plaktoris halichondrioides*	? aromatic compounds	Anti-inflammatory
Fattorusso *et al*., 2010	*Plakortis* cfr. *simplex*	Manadoperoxides A-D	*Plasmodium falciparum*
Feng *et al*., 2010	*Plaktoris* sp.	Plaktoride Q	*Trypanosoma brucei brucei*
Jiménez-Ribero *et al*., 2010	*Plaktoris halichondrioides*	Plaktoride J	*Plasmodium falciparum*
Schneemann *et al*., 2010	*Halichondria panicea*	Mayamycin	Anti-cancer, anti-bacterial

Table 1.7: Examples of sponge derived polyketides with bioactive properties.

Reference	Sponge species	Compound	Target of activity
Lucas *et al*., 2003	*Dysidea* sp.	Bolinaquinone	Anti-inflammatory
Posadas *et al*., 2003	*Fasciospongia cavernosa*	Cacospongionolide B	Anti-inflammatory
Wonganuchitmeta *et al*., 2004	*Brachiaster* sp.	12-deacetoxyscalarin 19-acetate	*M. tuberculosis*
Zhang *et al*., 2009	*Stelletta* sp.	sesquiterpenoids	Anti-inflammatory
Chao *et al*., 2010	*Negombata corticata*	Negombatoperoxides	cytotoxic
Hirashima *et al*., 2010	*Rhabdastrella globostellata*	Isomalabaricane	cytotoxic
Orhan *et al*., 2010	*Ircinia* sp.	Dorisenone D	*Trypanosoma* sp.
Park *et al*., 2010	*Phorbas gukulensis*	Gukulenins A & B	cytotoxic
Chang *et al*., 2012	*Hippospongia* sp.	Hippospongide A	cytotoxic
Chanthathamrongsiri *et al*., 2012	*Stylissa* cf. *massa*	8-isocyano-15-formamidoamphilect-11	*Plasmodium falciparum*
Diyabalanage *et al*., 2012	*Carteriospongia flabellifera*	Flabelliferans A& B	cytotoxic
Li *et al*., 2012	*Xestospongia testudinaria*	Aspergiterpenoid A	Bacteria
Gupta *et al*., 2012	*Clathria compressa*	Clathric acid	Gram positive bacteria
Salam *et al*., 2012	?	Manoalide	Hepatitis C
Wang *et al*., 2012	*Phorbas* sp.	Phorbasone A	Anti-inflammatory

Table 1.8: Examples of terpene/terpenoids compounds from marine sponges with bioactive properties

Reference	Sponge species	Source of activity	Target of activity
Monks *et al*., 2002	*Haliclona* aff *tubifera*	Organic extract	*E. coli, S. aureus, S. epidermis*
Pabel *et al*., 2003	*Aplysina aerophoba*	*Bacillus* sp.	*E. coli, S. aureus*
	Melophlus sarassinorum	Melophlins (tetramic acids)	*S. aureus, B. subtilis*
Wonganuchitmeta *et al*., 2004	*Brachiaster* sp.	Heteronemin (sesterterpene)	*M. tuberculosis*
Endo *et al*., 2004	*?*	Nagelamides (alkaloids)	Gram positive bacteria
Namikoshi *et al*., 2004	*Luffariella* sp.	Manoalides	*S. aureus*
Thakur e*t al*., 2005	*Suberites domuncula*	*α-Proteobacteria*	*S. aureus, S. epidermis*
Baker *et al*., 2008	*Haliclona simulans*	*Penicillium* sp.	*B. subtilis; S. aureus*
		Pezizomycotina sp.	
		Hypocreales spp.	
		Phaeosphaeriaceae sp.	
Kennedy *et al*., 2008	*Haliclona simulans*	*Pseudoalteromonas* sp., *Halomonas* sp., *Psychrobacter* sp.,	*B. cereus, B. subtilis, E. coli,* MRSA
Keffer *et al*., 2009	*Siliquariaspongia* sp.	Motualevic acid	MRSA
Schneemann *et al*., 2010	*Halichondria panicea*	Myamycin (polyketide)	MRSA
		Microbacterium sp.	*S. aureus; E. faecalis*
		Rhodococcus sp.	*S. aureus*
		Streptomyces sp.	*S. aureus*
		Micromonospora sp.	*S. aureus; E. faecalis*
Jiménez-Romero *et al*., 2010	*Plakortis halichondrioides*	Plaktoride J (lactone)	*M. tuberculosis*
Abdelmohsen *et al*., 2010	?	*Dietzia* sp.	*S. aureus*
Devi *et al*., 2010	*Halichondria* sp.	*Bacillus licheniformis*	*P. aeruginosa, S. aureus, V. cholerae,* MRSA
El-Amraoui *et al*., 2010	*Cliona viridis*	Ethanol extracts	*E. coli; B. subtilis; P. flourescens; S. aureus*
	Haplosclerida spp.		
	Cliona celata		
	Ircinia dendroides		
	Haliclona mediterranea		
	Haliclona viscosa		
O'Halloran *et al*, 2011	*Axinella dissimilis, Polymastia boletiformis, Haliclona simulans*	*Pseudovibrio* spp.	MRSA
Flemer *et al*., 2011	*Suberites carnosus*	*Arthrobacter* sp., *Pseudovibrio* spp., *Spongiobacter* spp.	*E. coli; B. subtilis; S. aureus*
Kumar *et al*., 2012	*Hippospongia* sp.	Epi-ilimaquinone	MRSA
Ankisetty & Slattery, 2012	*Xestospongia* sp.	Methanol extracts	*P. aeruginosa, M. intracellulare*
Gopi *et al*., 2012	*Dysidea granulosa*	*Acinetobacter calcoaceticus*	*A. hydrophila, V. alginolyticus, V. parahaemolyticus*
Gupta *et al*., 2012	*Clathria compressa*	Organic extract	*Gram positive bacteria*
Marinho *et al*., 2012	*Petromica citrina*	Aqueous extract	*S. aureus, S. epidermidis, E. faecalis*
Yang *et al*., 2012	*Agelas mauritiana*	Ageloxime B (alkaloid)	MRSA

Table 1.9: Examples of anti-bacterial activities from marine sponge aqueous or organic extracts, bacterial or fungal isolates from sponges or from compounds purified from sponges, bacterial or fungal extracts.

1.5 Exploiting the pharmacological potential of marine sponges

Although many novel bioactive compounds have been, and continue to be, isolated from sponges and their symbiotic microbes these compounds are produced naturally in minute quantities and the utility of these compounds to the pharmaceutical industry is therefore somewhat limited (Gulder & Moore, 2009). When halichondrins were isolated from the marine sponge *Halichondria okadai* (Hirata & Uemura, 1986), they were identified as very potent anti-tumour compounds with enormous clinical potential. However, it was estimated that one tonne of sponge biomass would need to be harvested to obtain 300 mg of a mixture of the halichondrin analogues (Proksch *et al*., 2003). With 1-5 kg of the drug potentially required annually for treating cancer patients, natural harvest was obviously unrealistic.

To help overcome the supply problem the biosynthetic origin of bioactive chemical entities is an important consideration. Bacteria have long been used for industrial production of food products (Raspor & Goranovic, 2008; Prevost *et al*., 1985), biopolymers (Rehm, 2010) and antibiotics (Tamehiro *et al*., 2003). Systems and tools for manipulation of bacteria for industrial purposes are long established. Where marine natural products are of bacterial origin, industrial and biotechnological manipulations offer hope for natural compound production in sufficient quantities for clinical trials. In some cases, evidence such as molecular architectures, suggest that bioactive compounds from sponges may in fact be secondary metabolite products of symbiotic bacteria (Waters *et al*., 2010; Hentschel *et al*., 2012).

The extensive search for pharmaceutical products from marine sponges has led to some success stories. The nucleosides Ara-A (Acyclovir) and Ara-C (Cytarabine) from the sponge *Cryptotethya crypta* are commercially available as antiviral and anti-tumour drugs, respectively (Sashidhara *et al.*, 2009). The chemical synthesis of Halichondrin B (Eribulin) has been achieved and was recently approved for breast cancer treatment (Jain & Cigler, 2012; Pean *et al*., 2012). At the time of writing, the synthetic tripeptide Hemiasterlin first identified in the marine sponge *Cymbastela* sp. had entered phase I clinical trials for cancer treatment (Waters *et*

al., 2010) while a derivative of the hydroxamic acid, psammaplin (Panobinostat [LBH-589]), from the sponge *Psammaplysina* sp. is in phase II clinical trials

(http://clinicaltrials.gov/search/intervention=lbh-589).

1.6 Metagenomic strategies for the discovery and production of novel industrial and pharmacological products

The term 'metagenome' was first coined by Handelsman and colleagues (Handelsman *et al*., 1998) when they used it to describe the collective genomes of soil microbes. Metagenomic analyses involve describing the sequence based or function based characteristics of a metagenome. Where gene sequences of particular interest are known, primers for PCR or probes for hybridisation can be designed to investigate a metagenome for the presence of desired genes (Kennedy *et al*., 2010). Where investigations are focusing on genes and gene products where sequences are not known a functional metagenomics approach is possible (Brady *et al*., 2007). This involves the extraction of total DNA from the metagenome of choice, fractionating the DNA to provide DNA fragments large enough to include complete gene clusters and operons and cloning the large fragments via bacterial artificial chromosomes (BACs) or fosmids into a heterologous host such as *E. coli* (Figure 1.6).

Generation of large libraries of these clones allows for the high-throughput functional screening of the libraries for desired functions, by culturing the clones on media incorporating appropriate substrates to reveal phenotypic functions (Handelsman, 2004).

Large insert BAC and fosmid clone libraries have to date been constructed from a variety of different environmental niches including: marine plankton (Suzuki *et al*., 2001), seawater (Cottrell et al., 1999; Béjà *et al*., 2000; DeLong *et al*., 2006; Woebken *et al*., 2007; Martinez *et al*., 2010), from sediment (Nesbø *et al*, 2005; Lee *et al*., 2006c; Hardeman & Sjoling, 2007; Huang *et al*., 2009), from a hydrothermal chimney biofilm (Brazelton & Baross, 2009), from soil (Henne *et al*., 2000; Rondon *et al*., 2000; Brady et al., 2001; Wang *et al*., 2000; Entcheva *et al*., 2001; MacNeil *et al*., 2001; Gillespie *et al*., 2002; Courtois *et al*., 2003) and

also from the metagenome of marine sponges (Schirmer *et al*., 2005; Kim & Fuerst, 2006; Chen *et al*., 2006; Fiesler *et al*., 2007; Okamura *et al*., 2010; Abe *et al*., 2012; Pimentel-Elardo *et al*., 2012; Selvin *et al*., 2012).

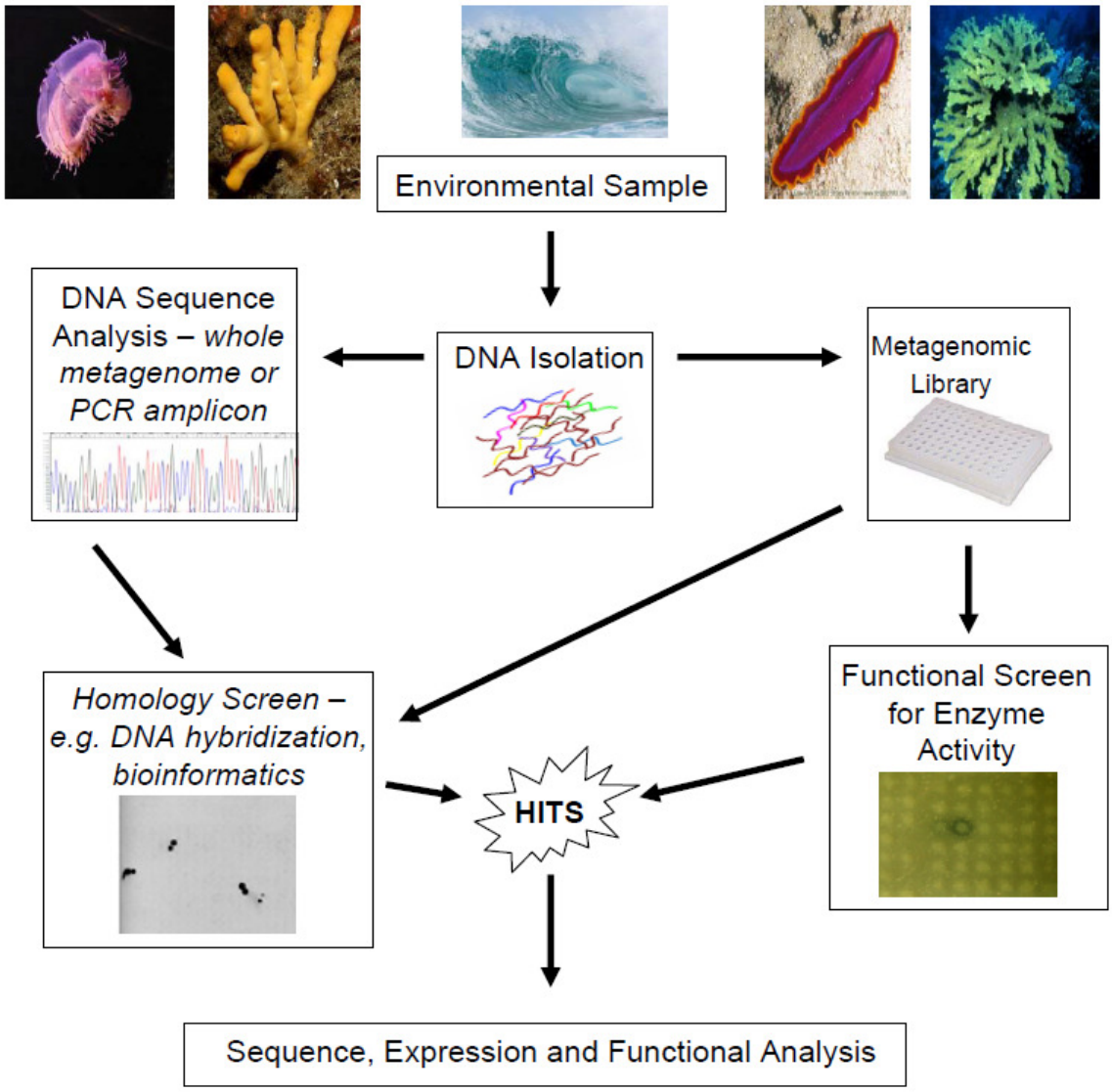

Figure 1.6: Sequence based and function based metagenomics (Kennedy *et al*., 2010).

Clone libraries from soil metagenomes have led to the discovery of novel antibiotic compounds and antimicrobial activities (Henne *et al*., 2000; Wang et al., 2000: Brady et al., 2001; Mac Neil et al., 2001: Gillespie et al., 2002: Courtois et al., 2003), while marine sponge derived large insert metagenomic

clone libraries have led to the identification of novel polyketide synthase (PKS) genes from the sponges *Discodermia dissoluta* (Schirmer *et al*., 2005), *Pseudoceratina clavata* (Kim & Fuerst, 2006), *Theonella swinhoei* and *Aplysina aerophoba* (Fiesler *et al*., 2007); together with novel non-ribosomal peptide synthase (NRPS) genes from the sponges *Haliclona okadai* (Abe *et al*., 2012) and *A. aerophoba* (Pimentel-Elardo *et al*., 2012). Antimicrobial activity has also been noted from a clone from the metagenome of *Gelliodes gracilis* (Chen *et al*., 2006). With respect to novel biocatalysts, a novel esterase has been discovered from the metagenome of *Hyrtios erectus* (Okamura *et al*., 2010) and a novel lipase was isolated and biochemically characterised from a *Haliclona simulans* clone library (Selvin *et al*., 2012).

1.6.1 Problems associated with large insert metagenomic clone libraries

Several problems hamper the discovery of novel genes and gene products from metagenomic clone libraries. These include the choice of heterologous host, detection of activities and appropriate screens for the detection of activities, which can all prove to be problematic.

E.coli is the heterologous host of choice in most cases (Ekkers *at al*., 2012), with Uchiyama and colleagues having reported that ~40% of foreign genes are expressed in *E. coli*. However the expression of foreign genes can be impeded by host codon usage preferences, problems with gene promoter recognition, transcription initiation factors, improper protein folding and the inability to export gene products from the host cell (Ekkers *et al*., 2012). In addition expression of foreign gene products can sometimes be toxic to the heterologous host (Uchiyama & Miyazaki, 2009). The abundance of genes of interest in the source environment and the cloned insert size and library size also has an effect on the probability of cloning particular genes (Uchiyama & Miyazaki, 2009). Ekkers and colleagues have described the ‘great screen anomaly’, where gene and product discovery from clone libraries is disappointingly low compared to what might be expected (Ekkers *et al*., 2012).

Efforts to increase the rate of gene and product discovery can possibly be improved by the use of multiple heterologous host expression systems. Shuttle vectors that can be transformed from *E. coli* to hosts such as *Streptomyces* or

Pseudomonas may increase the chances of heterologous expression (Ekkers *et al*., 2012). Enhanced detection methods such as the inclusion of reporter genes (e.g. green fluorescent protein (GFP), β-lactamase or tetracycline resistance on vectors may allow for detection of activities which is below detection thresholds from phenotypic assays alone (Uchiyama & Miyazaki, 2009). Uchiyama and colleagues also suggest that improvements in synthetic biology can lead to the design and synthesis of novel genes based on gene sequences in curated databases which may then be cloned into expression systems. Finally, the design of novel functional screens to detect activities of interest will be required if functional based metagenomic approaches are to lead to an increased discovery of genes and gene products of industrial or pharmaceutical interest (Steele *et al*., 2009).

1.7 Summary

Marine sponges host a remarkable diversity of symbiotic microorganisms. These symbionts appear to play vital physiological roles in the host, including cycling of vital nutrients – carbon, nitrogen and sulfur, and may also play an important role in host defence through the production of bioactive secondary metabolites of varied chemical classes, which in themselves may display wide ranging activities of biotechnological interest. The vast genetic diversity associated with individual sponges can be exploited through culture dependent and culture independent techniques. Exploitation of sponge associated microbial genes has led to the development of commercially available pharmaceutical products while others are in clinical trials. Increased efforts to sample, characterize, analyse and screen sponge derived microbial products offers hope for the development of many more such products for the marketplace.

1.8 References

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Chapter 2

Diverse and distinct sponge-specific bacterial communities in sponges from a single geographical location in Irish waters and antimicrobial activities of sponge isolates

Part of this chapter has been published in [Jackson SA, Kennedy J, Morrissey JP, O'Gara F, and Dobson ADW. (2012). Pyrosequencing reveals diverse and distinct sponge-specific microbial communities in sponges from a single geographical location in Irish waters. *Microbial Ecology* 64(1): 105-116.]

2.1 Abstract

Marine sponges are host to numerically vast and phylogenetically diverse symbiotic bacterial populations, with 35 major phyla or candidate phyla to date having been found in close association with sponge species worldwide. Analyses of these microbial communities have revealed many sponge-specific novel genera and species. These endosymbiotic microbes are believed to play significant roles in sponge physiology including the production of an array of bioactive secondary metabolites. Here, we report on the use of culture-based and culture-independent (pyrosequencing) techniques to elucidate the bacterial community profiles associated with the marine sponges *Raspailia ramosa* and *Stelligera stuposa* sampled from a single geographical location in Irish waters and with ambient seawater. We also report antimicrobial activities from bacterial isolates from these sponges. To date little is known about the microbial ecology of sponges of these genera. Culture isolation grossly underestimated sponge-associated bacterial diversity. Four bacterial phyla (*Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria*) were represented amongst ~200 isolates, compared with ten phyla found using pyrosequencing. Twenty bacterial isolates displayed antimicrobial activity against bacteria or yeasts Long average pyrosequencing read lengths of ~430b (V1-V3 region of 16S rRNA gene) allowed for robust resolution of sequences to genus level. 2,109 bacterial OTUs, at 95% sequence similarity, from 10 bacterial phyla were recovered from *R. ramosa*, 349 OTUs were identified in *S. stuposa* representing 8 phyla, while 533 OTUs from 6 phyla were found in surrounding seawater. Bacterial communities differed significantly between sponge species and the seawater. Analysis of the data for sponge-specific taxa revealed that 2.8% of classified reads from the sponge *R. ramosa* can be defined as sponge-specific while 26% of *S. stuposa* sequences represent sponge-specific bacteria. Novel sponge-specific clusters were identified. The majority of previously reported sponge-specific clusters (e.g. *Poribacteria*) were absent from these sponge species. This deep and robust analysis provides further evidence that the microbial communities associated with marine sponge species are highly diverse and divergent from one another and appear to be host selected through as yet unknown processes.

2.2 Introduction

Marine sponges (phylum: *Porifera*) host significant microbial populations which may be symbiotic (Wilkinson, 1983), pathogenic (Bavestrello *et al*., 2000), a food source (Reiswig, 1975) or transient. In some sponges, up to 30% of total biomass can comprise endosymbiotic microorganisms (Wilkinson, 1978). Symbiotic microbes may play important physiological roles in sponges. Associated cyanobacteria may supply photosynthates and fixed nitrogen (Wilkinson, 1978b) sulphur oxidising bacteria may remove sponge metabolic waste products (Webster *et al*., 2001) while proteobacteria and actinobacteria may produce bioactive secondary metabolites which supplement the host immune defences (Hentschel *et al*., 2001). This complex microbiota makes marine sponges of particular interest to microbial ecology studies and also offers a potentially invaluable source of novel genes and gene products for biotechnological applications.

Sponge-microbe associations have to date been studied using both culture-dependent and culture-independent techniques. As is common with other environments the vast majority of bacteria present in sponge tissues have not as yet been cultivated. Early culture-independent ecological investigations used transmission electron microscopy to observe diverse cell types in sponge tissues (Vacelet & Donadey, 1977; Wilkinson, 1978). Subsequently, fluorescence *in situ* hybridisation studies have been used to identify numerous bacterial phyla closely associated with sponges (Sharp *et al*., 2007). Other culture-independent studies employed PCR amplification of bacterial 16S rRNA genes directly from sponge metagenomic DNA followed by denaturing gradient gel electrophoresis (Usher *et al*., 2004; Lee *et al*., 2007) or restriction fragment length polymorphism analyses (Lee *et al*., 2009; Zhang *et al*., 2006). Cloning and sequencing of 16S rRNA genes has also been used in many microbial diversity investigations from a wide range of sponge species (Cassler *et al*., 2008; Kennedy *et al*., 2008b; Lafi *et al*., 2009; Montalvo *et al*., 2005; Ridley *et al*., 2005; Sipkema *et al*., 2009; Webb & Maas, 2002; Webster *et al*., 2001; Webster *et al*., 2004). Recently pyrosequencing of PCR amplicon libraries from metagenomic sources has allowed for deeper insights into environmental microbial community structures,

negating the requirement for a cloning step and providing numbers of sequencing reads orders of magnitude greater than was previously possible. This is also true for sponge metagenomic samples, with recent studies identifying remarkable levels of bacterial diversity associated with sponges from Australian Waters (Webster *et al*., 2010) from the Red Sea (Lee *et al*., 2011, Schmitt *et al*., 2011), from the Indian and Pacific Oceans, the Caribbean and Mediterranean Seas (Schmitt *et al*., 2011), Brazilian waters (Trindade-Silva *et al*., 2012) and from the Atlantic Ocean off the coast of Florida, USA (White *et al*., 2012). Members of 35 bacterial phyla or candidate phyla have been reported from sponges in these analyses, with up to ~3000 bacterial OTUs at 95% sequence similarity, reported in association with a single sponges individual (Webster *et al*., 2010).

Culture-dependent studies of marine sponge-associated microorganisms have attempted to access maximum cultivable diversity through use of different isolation media (Kennedy *et al*., 2008; Sipkema *et al*., 2011) or have targeted particular groups for isolation. Members of the phyla *Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Planctomycetes, Proteobacteria* and *Verrucomicrobia* have been isolated in growth culture from sponge species (Taylor *et al*., 2007). Several researchers have targeted the isolation of member species of taxa such as *Actinomycetes* and *Streptomycetes* in attempts to access the metabolic capabilities of these groups. This strategy has led to the isolation of several novel actinobacterial species (Abdelmohsen *et al*., 2010; Olsen *et al*., 2007; Padgitt & Moshier, 1987). Similar studies have also led to the isolation of novel bacterial genera and species from other phyla, with novel *α-Proteobacteria* (Lee *et al*., 2007), *γ- Proteobacteria* (Hentschel *et al*., 2001; Lee *et al*., 2006b; Romanenko *et al*., 2005; Romanenko *et al*., 2008), *Bacteroidetes* (Lau *et al*., 2005; Lau *et al*., 2006; Lau *et al*., 2006b; Lee *et al*., 2006) and *Verrucomicrobia* (Scheuermayer *et al*., 2006; Yang *et al*., 2010) being cultured from sponge tissues. There is growing evidence that monophyletic bacterial lineages have co-evolved with their sponge hosts to form sponge-specific clades which are more similar to each other than to similar taxa from non-sponge sources (Lee *et al*., 2011; Taylor *et al*., 2007; Webster *et al*., 2010).

The aims of this study are: (1) to compare the bacterial communities of two temperate water sponges, *Raspailia ramosa* (Montagu, 1818) and *Stelligera*

stuposa (Ellis and Solander, 1786), from a single geographical location. This will be accomplished by deep sequencing of 16S rRNA genes; (2) to compare these to similar studies on sponges from tropical waters; (3) by culture isolation to determine if the abundant phylotypes from each sponge species are cultivable; and (4) to identify antimicrobial activities from marine sponge isolates.

R. ramosa and *S. stuposa* are particularly abundant species from depths of 6-24 m, amongst a notably diverse sponge community, in Lough Hyne (Bell & Barnes, 2000). The success of these species in a highly competitive habitat of almost 60 sponge species makes them an interesting research focus.

2.3 Materials and Methods

2.3.1 Sponge Sampling

Sponge sampling was performed at the beginning of winter (November) 2008 at Lough Hyne Marine Nature Reserve (N 51°30′, W 9°18′) by SCUBA diving at a depth of 15-20 m. Lough Hyne has an unusual tide flow system and is noted for harbouring a highly diverse population of sponges (Bell & Barnes, 2000). The marine sponges, *Raspailia ramosa* (Class *Demospongiae*; Order *Poecilosclerida*; Family *Raspailiidae*) and *Stelligera stuposa* (Class *Demospongiae*; Order *Hadromerida*; Family *Hemiasterellidae*) were collected within a few meters of each other by excision of a piece (1-5 g) of sponge tissue *in situ* at similar depths. Sponge species were identified by Bernard Picton (Ulster Museum) and Christine Morrow (Queens University Belfast). Seawater was collected from the sponge sampling site simultaneously. Sponge samples were rinsed in sterile artificial seawater (ASW) to remove exogenous materials. ASW is derived from a commercial synthetic ion and mineral formulation (Instant Ocean – Aquatic Eco-Systems, Inc., Apopka, FL, USA) and is commonly used in aquaria. A sample was removed for immediate microbial culturing and the remainder was placed in sterile plastic Ziploc bags and stored on dry ice for transport and then frozen at -80°C. Seawater was stored on dry ice for transport and then stored at 4°C.

2.3.2 Culture Isolation

2.3.2.1 General Isolation

Sponge tissue was weighed, rinsed with sterile artificial seawater and macerated with a sterile razor blade. The macerated tissue was placed in a tube with sterile glass beads and vortexed. Sterile artificial seawater was added and the samples were again vortexed for 2 min. Dilution series' were performed to 10^{-5} with sterile ASW and 100 µl of each dilution was spread plated onto each of three growth media:

starch-yeast-peptone seawater agar (SYP-SW): 1% (*w/v*) starch, 0.4% (*w/v*) yeast extract, 0.2% (*w/v*) peptone, 3.33% (*w/v*) artificial sea salts - Instant Ocean (Aquatic Eco-Systems Inc., Apopka, FL, USA), 1.5% (*w/v*) agar; **modified marine agar (MMA)**: 0.005% (*w/v*) yeast extract, 0.05% (*w/v*) tryptone, 0.01% (*w/v*) β- glycerol phosphate disodium salt, pentahydrate ($C_3H_7Na_2O_6P \cdot 5H_2O$), 3.33% (*w/v*) artificial sea salt (Instant Ocean), 1.5% (*w/v*) agar, and **chitin agar**: 4% (*v/v*) colloidal chitin, 1.5% (*w/v*) agar.

Culture plates were incubated at 18°C in an attempt to isolate mesophilic phylotypes and thus ensure that the widest range of diversity was obtained. Colonies were picked from the master growth plates and isolated as axenic cultures by successive re-streaking on fresh media until pure cultures were obtained. Colonies were chosen to represent the widest range of diversity possible as adjudged by colony characteristics such as colour, morphology and growth rate.

2.3.2.2 Targeted isolation

A second isolation strategy was employed to target possible antibiotic producing bacteria. Sponge tissues (*R. ramosa*) were macerated and serial diluted as described above and 100 µl of each dilution was spread on each of seven growth different media. Additionally, aliquots of the serial diluted sponge homogenates were heat treated by incubating for 55°C for 6 min and then spread on each of seven growth media as before. The media used were:

(1) starch-yeast-peptone seawater agar plus nalidixic acid: 1% (*w/v*) starch, 0.4% (*w/v*) yeast extract, 0.2% (*w/v*) peptone, 3.33% (*w/v*) artificial sea salts, 0.001% (*w/v*) nalidixic acid;

(2) starch-yeast-peptone seawater agar plus rifampicin: 1% (*w/v*) starch, 0.4% (*w/v*) yeast extract, 0.2% (*w/v*) peptone, 3.33% (*w/v*) artificial sea salts, 0.0005% (*w/v*) rifampicin;

(3) actinomycete isolation agar: 0.2% (*w/v*) sodium caseinate, 0.4% (*w/v*) sodium propionate [$Na(C_2H_5COO)$], 0.01% (*w/v*) magnesium sulfate ($MgSO_4$), 0.01% (*w/v*) asparagine, 0.05% (*w/v*) dipotassium phosphate (K_2HPO_4), 0.0001% (*w/v*) ferrous sulfate ($FeSO_4$), 1.5% (*w/v*) agar, 0.5% (*v/v*) glycerol;

(4) actinomycete isolation agar plus seawater: 0.2% (*w/v*) sodium caseinate, 0.4% (*w/v*) sodium propionate [$Na(C_2H_5C00)$], 0.01% (*w/v*) magnesium sulfate ($MgSO_4$), 0.01% (*w/v*) asparagine, 0.05% (*w/v*) dipotassium phosphate (K_2HPO_4), 0.0001% (*w/v*) ferrous sulfate ($FeSO_4$), 1.5% (*w/v*) agar, 0.5% (*v/v*) glycerol, 3.33% (*w/v*) artificial sea salts;

(5) starch casein nitrate agar: 1% (*w/v*) starch, 0.2% (*w/v*) dibasic potassium phosphate, 0.2% (*w/v*) potassium nitrate (KNO_3), 0.2% (*w/v*) sodium chloride (NaCl), 0.03% (*w/v*) casein, 0.05% (*w/v*) magnesium sulfate ($MgSO_4$), 0.001% (*w/v*) ferrous sulfate ($FeSO_4$), 1.5% (*w/v*) agar;

(6) starch casein nitrate agar plus seawater: 1% (*w/v*) starch, 0.2% (*w/v*) dibasic potassium phosphate (K_2HPO_4), 0.2% (*w/v*) potassium nitrate (KNO_3), 0.2% (*w/v*) sodium chloride (NaCl), 0.03% (*w/v*) casein, 0.05% (*w/v*) magnesium sulfate ($MgSO_4$), 0.001% (*w/v*) ferrous sulfate ($FeSO_4$), 1.5% (*w/v*) agar, 3.33% (w/v) artificial sea salts;

(7) raffinose histidine agar: 1% (*w/v*) raffinose, 0.1% (*w/v*) L-histidine, 0.05% (*w/v*) magnesium sulfate ($MgSO_4$), 0.001% (*w/v*) ferrous sulfate ($FeSO_4$), 2% (*w/v*) agar, 0.0001% (*w/v*) nalidixic acid, 0.0001% (*w/v*) cycloheximide, 0.00025% (*w/v*) nystatin. Culture plates were incubated at 18°C for four weeks. Colonies were picked from master growth plates and subcultured until pure cultures were obtained.

2.3.3 Phylogenetic Analysis of Cultured Isolates

Cultured isolates were analysed by PCR amplification of 16S rRNA genes, sequencing of amplified genes and BLAST analyses of obtained sequences.

DNA templates for PCR Template DNA was obtained by addition of 25 µl of glycerol stock culture to 100 µl TE buffer followed by incubation at 98°C for 10 min. The lysed cells were pelleted by centrifugation at 1,400 g. The resultant supernatant served as template DNA for PCR.

16S rRNA PCR Each 30 µl PCR reaction comprised 1X reaction buffer, 0.2 mM dNTPs, 0.5 µM forward primer 27f (5'-GAGTTTGATCCTGGCTCAG-3'), 0.5 µM reverse primer 1492r (5'-GGTTACCTTGTTACGACTT-3'), 1 U Taq polymerase (5 U/µl), 1.0 µl template DNA, sdH_2O.

PCR Cycle Conditions Cycle conditions comprised initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 s, primer annealing at 50°C for 30 s and extension at 72°C for 2 min. A final extension at 72°C for 10 min followed (Lane, 1991). PCR amplicons were analysed by electrophoresis on 1% agarose gels.

Sequencing 16S rRNA PCR amplicons were sequenced by capillary electrophoresis, single extension sequencing (Macrogen Inc., Korea), using 3730xl DNA Analyser.

Sequence Data Analysis Sequences were edited manually using FinchTV 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com). Sequences were dereplicated using FastGroupII (http://biome.sdsu.edu/fastgroup/) (Yu *et al*., 2006). Sequence alignment and tree construction were performed using Mega version 4 (http://www.megasoftware.net/) (Tamura *et al*., 2007). Alignment was performed with ClustalW and tree construction was by neighbour joining method (Saitou & Nei, 1987) and included bootstrap tests (Felsenstein, 1985). The evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the datasets (complete deletion option). Reference sequences

were downloaded from the Ribosomal Database Project (release 10, update 13) (http://rdp.cme.msu.edu/).

2.3.4 Antimicrobial assays

Deferred antagonism assays were performed with all bacterial isolates from the marine sponges *R. ramosa* and *S. stuposa.* A panel of test strains was used: *Escherichia coli* NCIMB 12210, *Bacillus subtilis* IE32 and *Staphylococcus aureus* NCIMB 9518, *Candida albicans* Sc5314, *Candida glabrata* CBS138, *Saccharomyces cerevisiae* BY4741 and *Kluyveromyces marxianus* CBS86556. Sponge isolates were spotted to SYP-SW agar plates and incubated for 24-48 hr. Bacteria test strains were grown overnight in 5 ml Luria Bertani (LB) broth, the overnight culture was added to 50 ml LB broth and incubated shaking until it reached an OD_{600nm} 0.8. The culture was diluted 1/1000 with soft LB agar [2% (*w/v*) LB powder (Sigma), 0.5% (*w/v*) agar]. The test cultures were poured over the sponge isolates and incubated at 18°C for 24-36 hr. For yeast test cultures, overnight cultures were grown in yeast-peptone-dextrose agar (YPD) [1% (*w/v*) yeast extract, 2% (*w/v*) peptone, 2% (*w/v*) D-glucose, 1.5% (*w/v*) agar]. Overlays were poured with soft YPD – 0.7% (*w/v*) agar. A zone of inhibition of the test strain around a sponge isolate colony was determined to be an antimicrobial producing strain.

2.3.5 Metagenomic DNA Extraction from Sponges

Sponge tissue was weighed and ground to a fine powder under liquid N_2 in a sterile mortar with a sterile pestle. The ground sponge tissue was suspended in lysis buffer (100 mM Tris, 100 mM EDTA, 1.5 M NaCl (*w/v*), 1% CTAB (*w/v*), 2% SDS (*w/v*)) – adapted from Brady, 2007. Metagenomic DNA was then extracted as described by Kennedy *et al.*, 2008b. DNA was analysed by gel electrophoresis and quantified using a spectrophotometer (NanoDrop ND-1000). The DNA solutions were stored at -20°C.

2.3.6 Metagenomic DNA Extraction from Seawater

Seawater was filtered through a sterile 0.45 µm filter membrane (Whatman, Austin, TX, USA) under vacuum. DNA was then extracted using WaterMaster DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA) according

to the manufacturer's instructions. The DNA was analysed by gel electrophoresis. The DNA solutions were stored at -20°C.

2.3.7 PCR Amplicon Library Preparation for Pyrosequencing

PCR amplicon libraries of the V1-V3 region of the 16S rRNA genes from metagenomic DNA preparations from (1) *R. ramosa*, (2) *S. stuposa* and (3) seawater were prepared. The PCR primers used, 63f and 518r were adapted for pyrosequencing by addition of adapter sequences and multiplex identifier (MID) sequences (see Table 2.1) which allowed for the mixing and parallel sequencing of the samples.

PCR for pyrosequencing Each 50 µl reaction comprised 1X buffer, 0.1 mM dNTPs, forward primer 63f* [5'-GCCTAACACATGCAAGTC-3'] (0.5 µM), reverse primer 518r* [5'-ATTACCGCGGCTGCTGG-3'] (0.5 µM), 2 U Taq polymerase, 2.0 µl template DNA, 30.0 µl sdH_2O. Template DNA was metagenomic DNA extracted from (1) *R. ramosa*, (2) *S. stuposa* and (3) seawater. (Asterisk denotes primer adapted for pyrosequencing as per Table 2.1).

	Primer	Adapter	MID	Template specific sequence
Raspailia ramosa	forward	CGTATCGCCTCCCTCGCGCCATCAG	ACGAGTGCGT	GCCTAACACATGCAAGTC (63f)
	reverse	CTATGCGCCTTGCCAGCCCGCTCAG	ACGAGTGCGT	ATTACCGCGGCTGCTGG (518r)
Stelligera stuposa	forward	CGTATCGCCTCCCTCGCGCCATCAG	ACGCTCGACA	GCCTAACACATGCAAGTC (63f)
	reverse	CTATGCGCCTTGCCAGCCCGCTCAG	ACGCTCGACA	ATTACCGCGGCTGCTGG (518r)
seawater	forward	CGTATCGCCTCCCTCGCGCCATCAG	AGACGCACTC	GCCTAACACATGCAAGTC (63f)
	reverse	CTATGCGCCTTGCCAGCCCGCTCAG	AGACGCACTC	ATTACCGCGGCTGCTGG (518r)

Table 2.1: Primer sequences for the amplification of the V1-V3 regions of the bacterial 16S rRNA genes modified with adapter and multiplex identifier (MID) barcodes.

PCR Cycle Conditions Cycle conditions comprised initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 92°C for 60 s, primer annealing at 55°C for 60 s and extension at 72°C for 60 s. A final extension at 72°C for 10 min followed (El-Fantroussi *et al*., 1999). Three individual PCR reactions were performed for each sample. The PCR amplicon libraries were purified using Qiagen (Qiagen Ltd., UK) PCR purification kit as per the manufacturer's instructions. The DNA concentration of each resultant solution was quantified on NanoDrop. To minimise the effects of PCR bias on results equimolar amounts of each of the 3 individual amplicon libraries were pooled for each of the samples. Amplicon libraries were sequenced on the GS FLX Titanium platform (454 Life Sciences) at the University of Liverpool, UK.

2.3.8 Pyrosequencing Data Analysis

Sequencing reads were quality-filtered in the Ribosomal Database Project (Release 10) pyrosequencing pipeline (http://pyro.cme.msu.edu/). Reads with ambiguous bases *N* were removed, primer sequences were trimmed, sequence reads shorter than 100 bases and reads with average quality score <20 were discarded. Replicate sequences were removed using the Dereplicate tool. Sequences were clustered by complete-linkage clustering. Sequences were aligned using the secondary structure Infernal Aligner algorithm (Nawrocki & Eddy, 2007). Sequences were assigned to taxa using naïve Bayesian rRNA classifier using a confidence threshold of 50% (Wang *et al*., 2007). Shannon and Chao1 indices and rarefaction curves were obtained using the RDP tools. Sponge specific cluster analysis was performed by aligning sequences to the complete datasets used by Taylor *et al*, 2007, followed by phylogenetic tree building using neighbour joining, maximum likelihood and minimum evolution algorithms.

Accession Numbers The 16S rRNA gene sequences for the isolates were deposited in GenBank under the accession numbers JF820664-JF820814. The pyrosequencing reads were deposited to the NCBI Sequence Read Archive under the accession number SRA035391.

2.4 Results

2.4.1 Culture isolation

In the general isolation strategy, partial 16S rRNA sequences were obtained for 123 bacterial isolates from *Raspailia ramosa* and for 82 isolates from *Stelligera stuposa*. Phylogenetic analyses identified members of 4 phyla [*Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria* (*α*- and *γ*- classes)] associated with each sponge species. The community profiles show similarities at the genus level within the *Firmicutes* and *Proteobacteria* but are dissimilar within the phyla *Actinobacteria* and *Bacteroidetes* (Figure 2.1). Both cohorts are dominated by *γ-Proteobacteria* (71% of *R. ramosa* isolates and 54% of *S. stuposa* isolates – Figure 2.2). The dominant phylotypes of this class, from both sponges, are close relatives of *Pseudoalteromonas* sp., *Vibrio* sp. and *Halomonas* sp. Seven genera of *γ-Proteobacteria* were isolated from *R. ramosa* while six genera of *γ-Proteobacteria* were isolated from *S. stuposa*. Three genera (*Pseudoalteromonas*, *Shewanella*, *Halomonas*) were isolated from both sponges. Four genera (*Colwellia*, *Vibrio*, *Aliivibrio*, *Microbulbifer*) were unique to *R. ramosa* while 3 genera (*Glaciecola*, *Alteromonas*, *Acinetobacter*) were unique to *S. stuposa.* The *α–Proteobacteria* cultured from *S. stuposa* are most closely related to the common marine genera *Roseobacter* spp., and *Ruegeria* spp. while the *R. ramosa* derived *α–Proteobacteria* are almost exclusively *Pseudovibrio* spp. Amongst the *Firmicutes* isolates *Staphylococcus* spp. and *Bacillus* spp. were isolated from both sponges. A *Microbacterium* sp. isolate was obtained from *S. stuposa*. Five genera of *Bacteroidetes* were isolated from each sponge species though only one genus (*Cellulophaga*) was common to both sponges. Three actinobacterial genera were isolated from *R. ramosa* and five actinobacterial genera were obtained from *S. stuposa* with only two genera (*Micrococcus*, *Arthrobacter*) common to both sponges. For the targeted isolation strategy, partial 16S rRNA sequences were obtained for 33 isolates (Figure 2.3). ~85% of these isolates were from the phylum *Firmicutes* (14 x *Bacillus* spp., 12 x *Staphylococcus* spp. and 2 *Paenibacillus* spp.). Other isolates were related to *Tetrathiobacter* sp. (*β-Proteobacteria*) and *Pantoea* sp. (*γ-Proteobacteria*).

(a)
Micrococcus luteus strain Z05 GU947857
WH009143s
WH009048s
WH009138s
J2153c
Micrococcus yunnanensis strain CAIM 334 HM583989
J2016s
Arthrobacter rhombi strain 11-2 FJ795684
WH009006s
WH009003s
WH009004s (3)
WH009001s
WH009059s
Arthrobacter gandavensis OS-44.c2 AM237357
WH009008s
J2064s
Curtobacterium sp. 124NP18 AB242692
J2029m
Brachybacterium sp. 1-2 FJ795650
WH009010s
WH009009s
Brevibacterium antiquum isolate KFC-74 EF459545
Actinobaculum massiliense CIP 107404 AF487679
J2095c
Isoptericola sp. JSM 077025 FJ237392
J2056s
J2008s
Actinobacteria
J2049s
Microbacterium testaceum Rp10621 HM032882
J2129s
Bacillus mycoides REG150 GQ844974
J2009s
J2046s
Bacillus pumilus strain MPK HM851283
J2094c
WH009142s
J2048s
J2015s
Staphylococcus succinus SV17 GU143799
WH009155s (2)
WH009140s
Staphylococcus sp. CTDB5 GQ169062
WH009141s
J2004s
J2052s
J2089m
J2031s
Staphylococcus sp. 09BS3-3 HM565997
Firmicutes
Algoriphagus sp. AP40p EU374904
WH009051s
WH009018s
J2022s
Owenweeksia hongkongensis AB125062
Aquimarina muelleri strain KMM 6021 AY608407
WH009122m
Marine bacterium KMM 3937 AF536386
WH009150s
Flavobacterium gelidilacus strain R-8899 NR 025538
WH009022s
Joostella marina strain En5 EF660761
J2115m
Cytophaga sp. RP8 EU375128
J2051s
J2117m
Antarctic bacterium C65SC20c1
WH009170s
WH009172s
Cellulophaga sp. SW265 AF493680
WH009166s
J2024s
Cellulophaga pacifica strain:KMM 3915 AB100842
J2146c
J2164c
Maribacter sp. MED381 EU253579
Zobellia laminariae strain KOPRI 22206 EU000235
WH009065c
WH009158s
Zobellia russellii strain KMM 3677 NR 024828
J2109m
Cellulophaga sp. RE2-13 AF539758
Bacteroidetes
Sulfolobus solfataricus DSM 1616 X90478
Archaea
0.05
(b)
WH009031s
J2021s
J2119m
Pseudoalteromonas sp. DG1628 EU239912
Pseudoalteromonas sp. IE1-2 EF089559
J2042s
J2125s
J2128s
J2118m
WH009128m
J2020s
WH009047s (30)
J2053s
WH009174s
WH009102c (9)
WH009023s (2)
WH009118s
J2158c
Pseudoalteromonas sp. QY202 GQ202280
J2134c
J2057s
J2002s
J2127s
WH009105c (2)
J2044s
WH009030s (3)
Pseudoalteromonas sp. Ld19 FJ96944
WH009167s
Pseudoalteromonas sp. SE014 FJ889540
Pseudoalteromonas sp. PA1 EU768826
J2017s
WH009082c
Pseudoalteromonas sp. SEM6 AB274762
J2001s
WH009173s
Marine gamma proteobacterium O1K AF488782
Marine bacterium Tw-4 AY028199
J2142m
WH009050s
WH009163s(4)
WH009040s
WH009119m
Colwellia sp. KMD002 EU599214
J2072c
Colwellia sp. BSi20435 EF639383
WH009076c
Marine sponge bacterium PLATEcassta-(1)-53 EU346584
J2136m
J2107m
Glaciecola chathamensis S18K5 AB247624
J2108m
Alteromonas sp. S2985 FJ457282
J2067c
WH009061s
J2018s
WH009028s
Antarctic bacterium TB 35 EU237129
Aliivibrio logei isolate AV02/2007 EU257755
WH009014s
WH009019s
Vibrio vulnificus
WH009111m (8)
WH009039s
J2104m
Vibrio sp. B109 FN295823
J2132c
J2041s
Vibrio sp. KV040308-5 EU862332
WH009056s
WH009024s
J2114m
WH009027s
Shewanella oneidensis AB447987
WH009149s
WH009151s
WH009131s
Shewanella sp. E505-6 FJ169982
WH009132s
Shewanella piezotolerans WP3 AJ551090
J2011s
J2066c
WH009038s
J2028s
WH009012s
Shewanella sp. B246 FN295778
J2082m
J2135c
J2101c
J2147c
J2163c
J2045s
J2088m
J2043s
WH009071c
Cobetia sp. RP38 EU375099
WH009157s
Halomonas sp. HM5 EU768828
J2087m
J2007s
Acinetobacte johnsonii CTDJ1 GQ169068
J2005s
WH009176s
Microbulbifer salipaludis SM-1 NR 025232
WH009175s
Pseudomonas sp. UC14a AM180745
J2152c
J2054s
J2060s
γ-Proteobacteria
WH009103c
WH009046s
WH009063c
WH009094c
Pseudovibrio sp. Ca31 EU862084
J2099c
Marine sponge bacterium FILTEROTU1 EU346387
J2100c
Pseudovibrio sp. MOLA 511 AM990736
J2103c
J2102c
WH009125m
Pseudovibrio sp. PV1 EU768842
WH009104c
Alpha proteobacterium GWS-TZ-H112 AY332195
WH009113m
WH009113m(2)
J2121m
Roseobacter sp. ARCTIC-P4 AY573044
J2151c
Roseobacter sp. SOVoc28 AM709732
J2006s
J2058s
Rhodobacteraceae bacterium P92 EU195947
J2110m
Ruegeria sp. R214E7 FJ357642
J2111m
Roseobacter sp. P123 EU195952
Sulfolobus solfataricus
α-Proteobacteria
0.05

Figure 2.1: Neighbour-joining phylogenetic trees [(a) - *Actinobacteria*, *Bacteroidetes* and *Firmicutes*, (b) *Proteobacteria*] of bacterial isolates from the marine sponges *R. ramosa* and *S. stuposa*. ●-denotes *S. stuposa* isolate ◊-denotes *R. ramosa* isolate. Numbers in parentheses represent numbers of replicate isolates. Only isolates from the general isolation strategy are included.

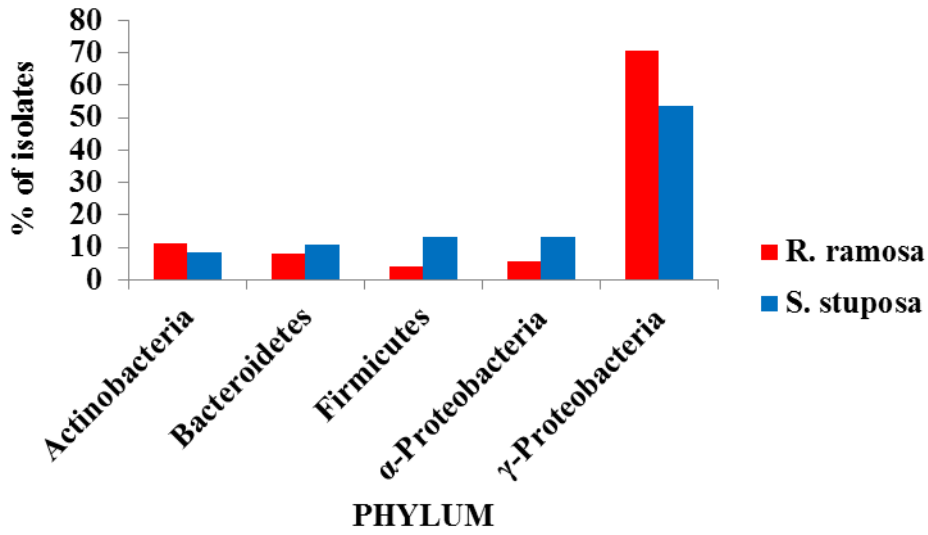

Figure 2.2: Percentage of bacterial isolates from the marine sponges *R. ramosa* and *S. stuposa* by phylum. Only isolates from the general isolation strategy are included.

2.4.2 Antimicrobial assay

Antimicrobial activities against one or more test strains were noted from 20 sponge isolates (Table 2.2). While ~3% of isolates from the general isolation strategy displayed antimicrobial activity, ~42% of isolates from the targeted isolation strategy showed antimicrobial activities. Three isolates (*Bacillus* sp., *Tetrathiobacter* sp., *Staphylococcus* sp.) showed strong inhibitory activity against *Candida glabrata*. One unidentified isolate (WH018scsh40) inhibited all yeast test strains tested (Figure 2.4).

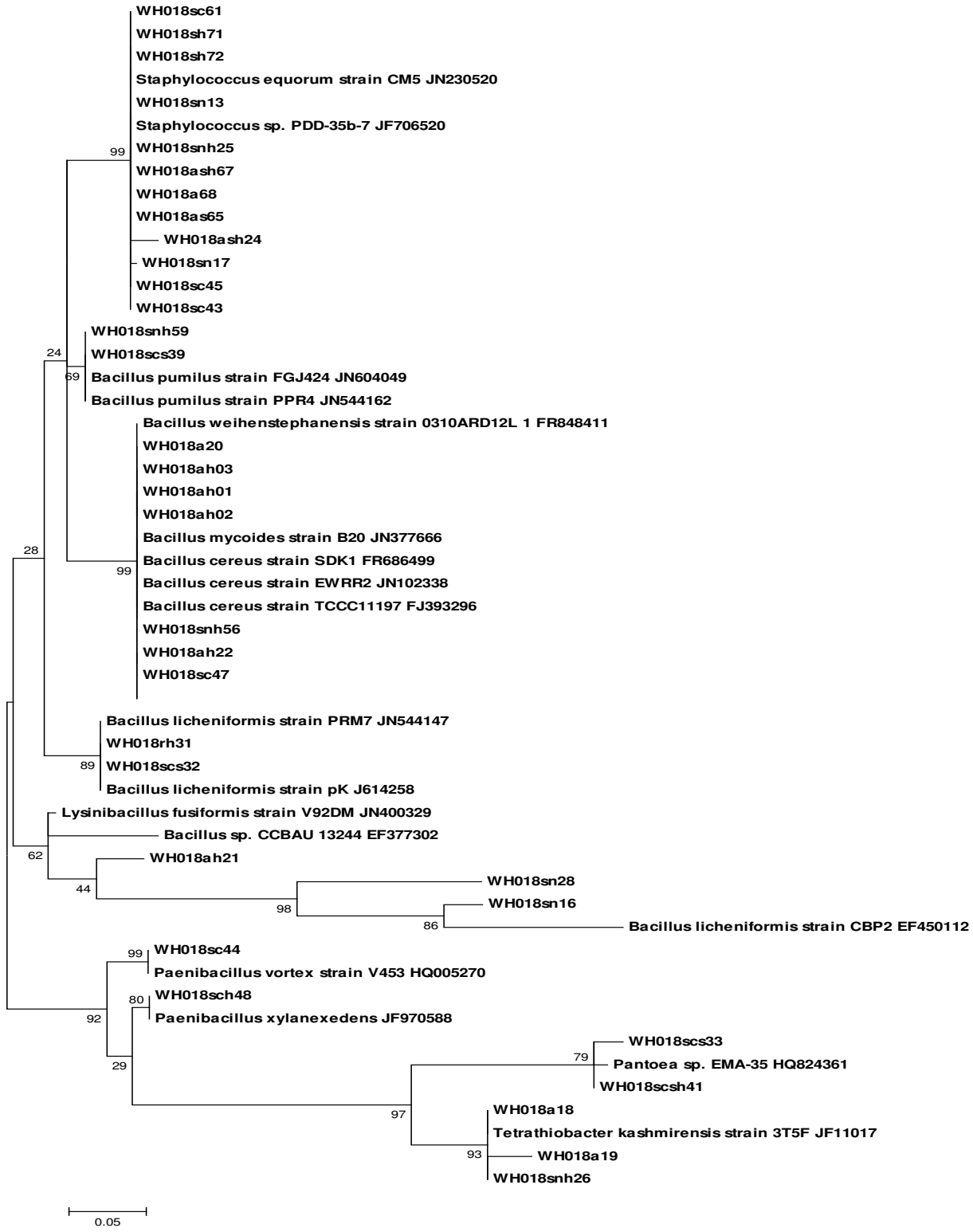

Figure 2.3: Neighbour joining phylogenetic tree of bacterial isolates from the targeted isolation strategy. Sponge isolates are identified by the prefix –WH018'.

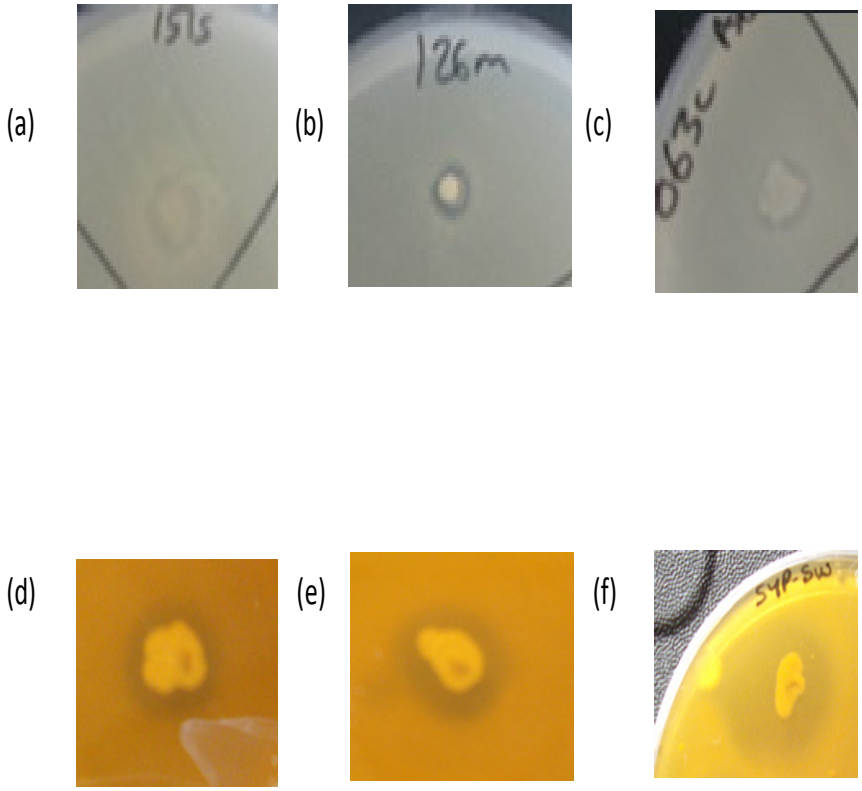

Figure 2.4: Examples of antimicrobial activities of sponge isolates. (a) WH009151s V *E. coli*, (b) WH009126m V *S. aureus*, (c) WH009063c V *E. coli*, (d) WH018sccsh40 V *C. glabrata*, (e) WH018scsh40 V *K. marxianus* (f) WH018scsh40 V *S. cerevisiae*.

Isolate		*E.coli*	*S.aureus*	*C.glabrata*	*C.albicans*	*K.marxianus*	*S.cerevisiae*
WH009063c	*Pseudovibrio sp.*	X					
WH009094c	*Pseudovibrio sp.*	X					
WH009103c	*Pseudovibrio sp.*	X					
WH009126m	*Pseudoalteromonas sp.*		X				
WH009151s	*Shewanella sp.*	X					
WH009171s	*Pseudoalteromonas sp.*	X					
WH018ah01	***Bacillus sp.***				**XXX**		
WH018ah02	***Bacillus sp.***				**X**		
WH018ah03	***Bacillus sp.***				**X**		
WH018ash04	***Bacillus sp.***				**X**		
WH018snh08	***Bacillus sp.***	**X**					
WH018a18	***Tetrathiobacter sp.***				**X**		
WH018a20	***Bacillus sp.***				**XX**		
WH018ah21	***Bacillus sp.***	**X**					
WH018snh26	***Tetrathiobacter sp.***				**XXX**		
WH018scs33	***Pantoea sp.***				**X**		
WH018scsh40	***?***			**X**		**XX**	**XX**
WH018ah58	***?***				**XXX**		
WH018sh71	***Staphylococcus sp.***				**XXX**		
WH018sh73	***Pantoea sp.***			**XX**			

Table 2.2: Antimicrobial activities of sponge isolates against bacterial and yeast test strains as determined by the deferred antagonism assay. Isolates in **bold** text indicate isolates from the targeted isolation strategy. X – denotes moderate inhibition of the test strain, XX – denotes intermediate inhibition of the test strain, XXX – denotes strong inhibition of the test strain.

2.4.3 Pyrosequencing

A combined total of ~70,000 raw bacterial 16S rRNA tag sequences comprising over 20 million bases were obtained from pyrosequencing. After quality filtering 14,146 sequence reads from *R. ramosa* with an average length 420 bp, 12,099 sequences of average length 437 bp from *S. stuposa* and 12,126 sequences of average length 369 bp from seawater were analysed. The number of OTUs in each sample was determined and Shannon and Chao1 diversity indices were calculated (Table 2.3).

Rarefaction curves at 5% sequence dissimilarity for all three samples showed some levelling off indicating that the libraries were representative and that the estimations of microbial diversity were likely to be accurate (Figure 2.5a). Rank abundance curves indicated that the majority of the sequences belonged to rare species although differences in the slope indicated that the microbial community associated with *S. stuposa* had lower evenness than *R. ramosa* (Figure 2.5b).

	No. of reads	**No. of reads after quality filtering**	**Average sequence length**	**No. of OTU's (97% sequence identity)**	**No. of OTU's (95% sequence identity)**	**Chao1 richness (95% sequence identity)**	**Shannon Index (95% sequence identity)**
R. ramosa	24,433	14,146	420	3,013	2,109	3,466	5.49
S. stuposa	26,918	12,099	437	570	349	581	2.94
Seawater	18,271	12,126	369	1,380	533	730	4.17

Table 2.3: Analysis of 16S rRNA (V1-V3) pyrosequencing reads from the marine sponges *R. ramosa*, *S. stuposa* and from seawater. Chao1 species richness and Shannon diversity indices were calculated at 95% sequence identity.

Taxonomic classifications of sequences resulted in 98% of *R. ramosa*-derived sequences being classified, ~96.75% of *S. stuposa*-derived reads classified and 99.8% of seawater-derived reads classified [Supplementary Table S2.1 (see Appendix)]. Distinct differences between all three datasets were evident. Very high levels of diversity were noted from the *R. ramosa* sequences with 3,013 unique reads at 97% sequence similarity and 2,109 unique reads at 95% sequence similarity. Ten bacterial phyla [*Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Deferribacteres*, *Firmicutes*, *Nitrospira*, *Proteobacteria* (*α-, β-, γ-, δ-* and *ε-* classes) and TM7] were observed in *R. ramosa*-derived reads. From the sponge *S. stuposa,* much lower diversity was evident with 570 unique reads seen at 97% sequence identity and 349 unique sequences at 95% sequence similarity. Sequences representing eight bacterial phyla [*Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, *Nitrospira*, *Proteobacteria* (*α-, β-, γ-* and *δ-* classes) and TM7] were recovered from *S. stuposa*.

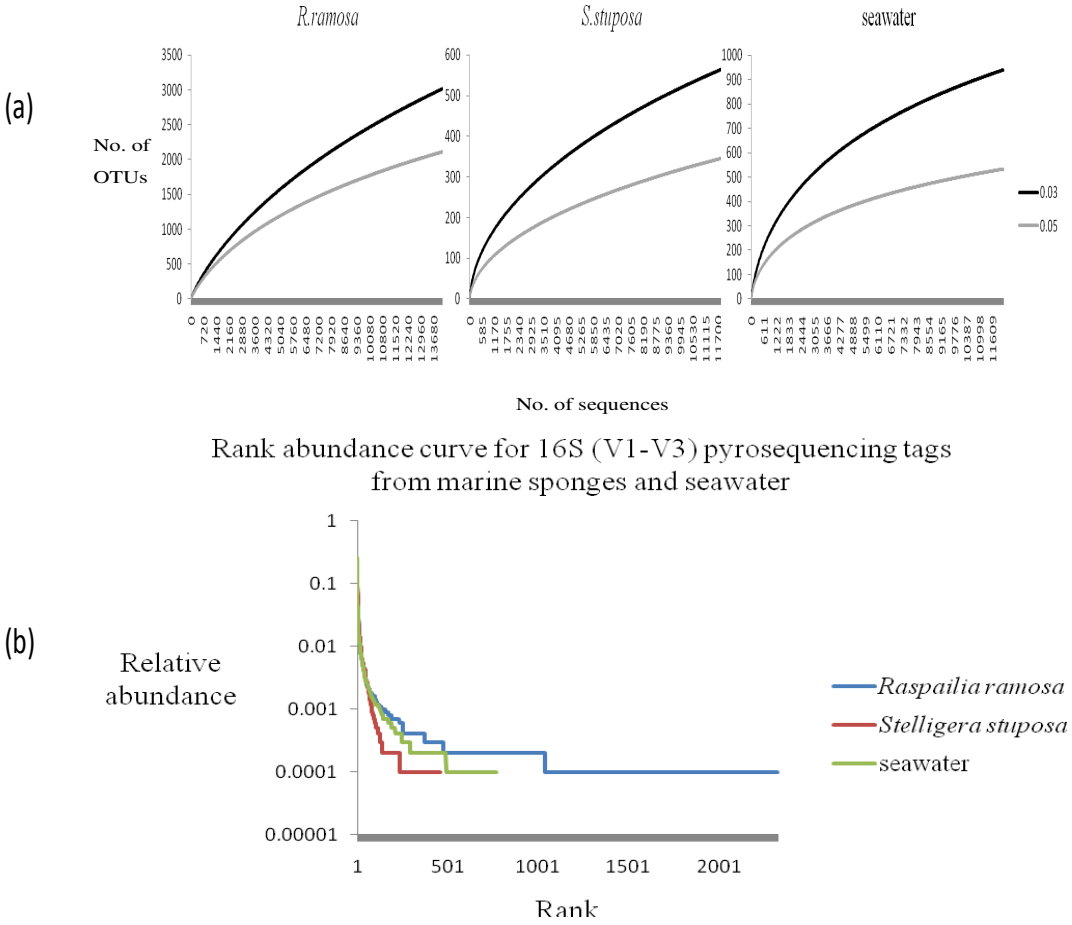

Figure 2.5: (a) Rarefaction curves and (b) rank abundance curves for marine sponge and seawater derived pyrosequencing reads.

The bacterial diversity observed in seawater was lower than for either of the two sponges when phylum level analysis was examined. Members of six bacterial phyla [*Actinobacteria*, *Bacteroidetes, Cyanobacteria, Firmicutes*, *Nitrospira* and *Proteobacteria* (*α-, γ-*, and *ε-* classes)] were represented. However, more OTUs at 97% sequence identity (1,380) and at 95% sequence identity (533) were noted in seawater when compared to *S. stuposa* (570 and 349 respectively). These diversity levels are reflected in the Shannon diversity indices calculated for each sample (Table 2.2). Chao1 species richness estimates predict 3,466 OTUs at 95% sequence identity for *R. ramosa* suggesting that 40% of the diversity present was not sampled.

Similarly, ~40% of OTUs from *S. stuposa* were not sampled relative to Chao1 estimates of 581 OTUs at 95% similarity. Chao1 estimates for seawater (730) suggest that greater than 75% of OTUs (95% identity) present in seawater were sampled here. Rarefaction curves for each sample (Figure. 2.5a) reflect these estimates and also show the differences in evenness of the microbial communities. The microbial community associated with *R. ramosa* is the most diverse with many species present at relatively low abundance; for *S. stuposa* the community is less diverse with a greater proportion of the community consisting of dominant clusters.

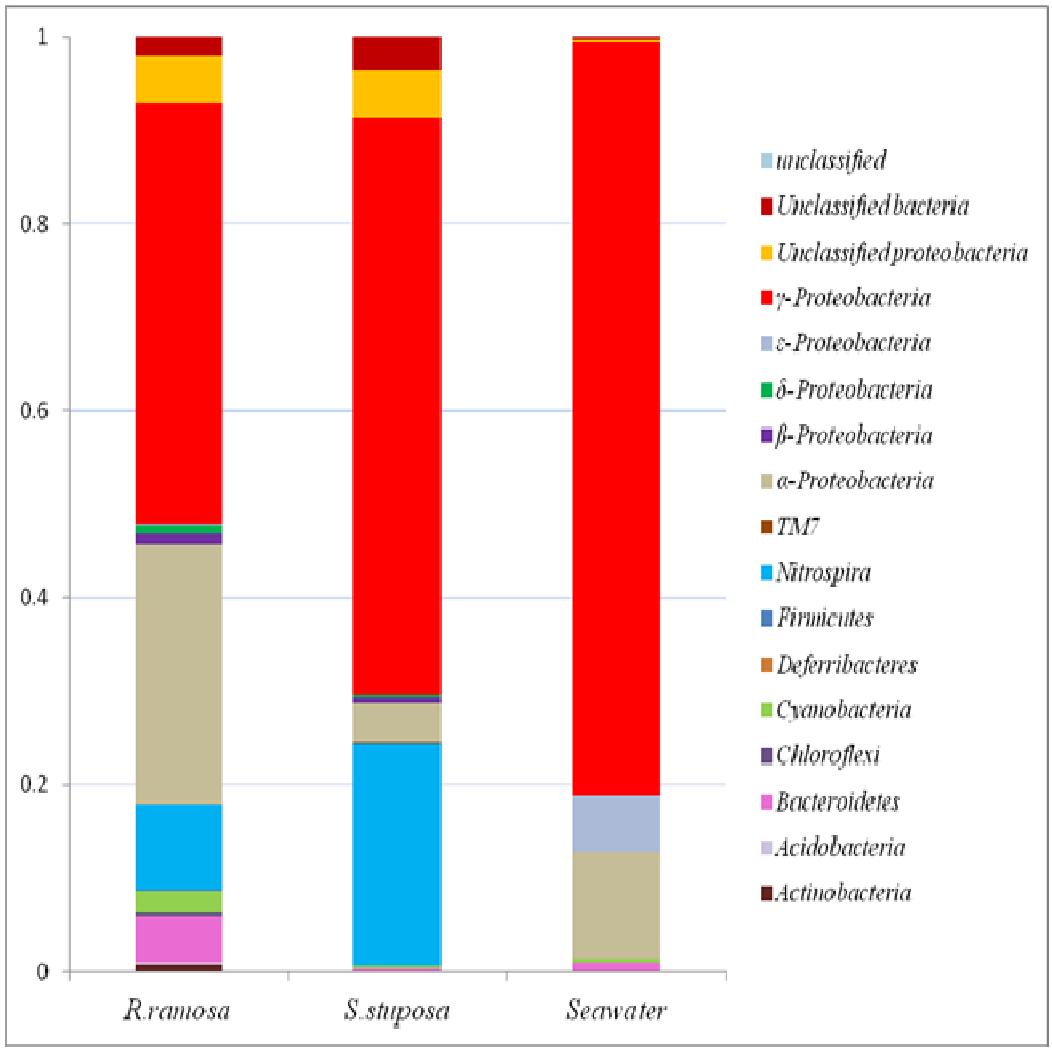

Figure 2.6: Relative abundance of 16S tag sequences by phylum from marine sponges and seawater.

At the phylum level *Proteobacteria* dominated in both sponges (Figure 2.6), making up 78% and 71% of classified reads from *R. ramosa* and *S. stuposa*, respectively. The next most abundant phylum for both sponges was the *Nitrospira*. This phylum accounts for 9.16% of *R. ramosa*-derived sequences and ~24% of S. *stuposa*-derived reads [including all of *S. stuposa* cluster 1 (Ssc1 – Figure 2.8)] while <0.01% of seawater-derived sequences belong to the phylum *Nitrospira. Bacteroidetes* accounted for a significant proportion (5%) of *R. ramosa* sequences but only 0.2% of *S. stuposa* sequences and 0.9% of sequences from seawater. *Cyanobacteria* and *Actinobacteria* were also more abundant in *R. ramosa* (2.4% and 0.7%) than *S. stuposa* (0.3% and 0.03%). More rarely found phyla were *Firmicutes* (in both sponges), *Chloroflexi* and *ε-Proteobacteria* (unique to *R. ramosa*); *Acidobacteria* and TM7 (both sponges) and *Deferribacteres* (only in *R. ramosa*). Low-abundance δ-*Proteobacteria* were found in both sponges but were absent from seawater. The only δ-proteobacterial order (*Myxococcales*) found in *S. stuposa* was also found in *R. ramosa*. β-*Proteobacteria* were also found at low abundance in both sponges but not in seawater. Amongst the *γ-Proteobacteria* low numbers of *Vibrionales* and *Xanthomonadales* were observed in both sponges; *Xanthomonadales* were present at low abundance in seawater also but *Vibrionales* completely dominated the seawater with more than half of all seawater-derived tag sequences recruiting to this order. *Alteromonadales*, *Enterobacteriales* and *Pseudomonadales* were also found at low abundance in *S. stuposa* but were more common in *R. ramosa*. Low abundance *Thiotrichales* and *Legionellales* were identified to be associated with *R. ramosa,* but these orders were absent from *S. stuposa*.


Mycale sp. clone AJ292193
Mycale sp. clone AJ292192
Mycale sp. clone AJ292194
Mycale sp. clone AJ292195
Geminocystis hermandii PCC 6308 AB039001
Cyanobacterium stanieri PCC 7202 AF132782
Antarctic cyanobacterium clone AY151723
Chamaesiphon subglobosus PCC 7430 AY170472
Anabaena cylindrica NIES19 AF247592
Nostoc commune AB101003
Synechocystis trididemni AB011380
Prochloron sp. X63141
Lyngbia sp. NIH309 AY049752
Lynbia sp. VP417b AY049751
Dysidea herbacea isolate AY599503
Dysidea herbacea isolate AY599502
Dysidea herbacea isolate AY599501
Leptolyngbya foveolarum X84808
Theonella sp. clone AF434966
Theonella sp. clone AF434965
Lendenfeldia chondrodes clone AY845228
Lendenfeldia chondrodes clone AY845229
Lendenfeldia chondrodes clone AY845227
Acaryochloris marina MBIC11017 AY163573
Thermosynechococcus elongatus BP-1 AP005376
Trichodesmium contortum AF013028
Microcoleus glaciei UTCC 475 AF218374
Microcoleus vaginatus PCC 9802 AF284803
Oscillatoria agardhii AB074507
Lyngbya aestuarii PCC 7419 AB039013
Oscillatoria coallinae X84812
Gloeobacter violaceus PCC 7421 AF132790
diseased coral clone AY038527
Phyllospongia papyracea isolate AY615507
Lamellodysidea chlorea isolate AY615504
Phyllospongia papyracea isolate AY615508
Phyllospongia papyracea isolate AY615509
Lendenfeldia chondrodes isolate AY615505
Lendenfeldia chondrodes isolate AY615506
Lamellodysidea herbacea isolate AY615503
Lamellodysidea herbacea isolate AY615502
Lamellodysidea herbacea isolate AY615501
Discodermia dissoluta clone AY897085
Mycale laxissima clone DQ098848
Mycale laxissima clone DQ098847
Cuscuta platyloba AJ401355
Chlorella vulgaris D11347
Chlamydomonas reinhardtii J01395
Gloeothece membranacea PCC 6501 X78680
Antarctic lichen clone AY250870
Synechococcus sp. IR11 AF448079
Antthamnion sp. X54299
Asterionellopsis glacialis AJ536455
Candidaspongia flabellata associated diatom AF369650
Skeletonema costatum X82154
Candidaspongia flabellata associated diatom AF369649
Raspailia_cluster_388(4)
Cymbastela concentrica clone AY942768
Cymbastela concentrica clone AY942758
Cymbastela concentrica clone AY942770
Cymbastela concentrica clone AY942766
Cymbastela concentrica clone AY942759
novel sponge specific cluster
Sympcola sp. VP377 AF306497
Pylaiella littoralis X14873
Ochrosphaera neapolitan X80390
Emiliania huxleyi X82156
Synechococcus sp. PCC 7002 AJ000716
Oscillatoria rosea IAM M-220 AB003164
Halospirulina tepeticola Y18791
Microcystis aeruginosa NIES89 U03403
sponge isolate AF295635
Anacystis nidulans X03538
Prochlorothrix hollandica AJ007907
Halothece sp. PCC 7418 AJ000708
Aplysina aerophoba clone AJ347083
Antho chartacea clone EF076240
Aplysina aerophoba clone AJ347056
Synechococcus sp. PCC 7918 AF216947
Synechococcus sp. WH 5701 AY172832
Candidaspongia flabellata isolate AF369648
Cymbastela concentrica clone AY942769
Antho chartacea clone EF076234
sponge clone AY542772
Synechococcus sp. WH 8012 AF539812
coral associated clone AY700638
uncultured cyanobacterium AF268237
Synechococcus sp. RS9910 AY172820
Synechococcus sp. A315003 AY125375
sponge clone AY542755
sargasso sea clone AACY01068573
Synechococcus sp. MW10#1 AY151248
Prochlorococcus marinus MIT9313 BX548175
Theonella swinhoei clone AF186426
Theonella swinhoei clone AF186425
Discodermia dissoluta clone AY897071
Prochlorococcus marinus DQ366714
Xestospongia proxima clone AY701315
marine clone DQ300610
Prochlorococcus marinus subsp. pastoris AF180967
uncultured marine clone AF382128
Synechococcus sp. Oli31 AY172810
Synechococcus sp. WH 8101 AF001480
Synechococcus sp. HOS AF448064
sponge clone AY542768
Mycale laxissima clone DQ098845
Mycale laxissima clone DQ098844
Mycale laxissima clone DQ098833
Mycale laxissima clone DQ098830
Prochlorococcus marinus NATL2A CP000095
marine clone DQ300603
Borrelia burgdorferi U03396
Streptomyces griseus M76388
Bacteroides fragilis M61006
Bifidobacterium bifidus S83624
Agrobacterium tumefaciens D14504
Pseudomonas aeruginosa M34133
Chlamidya trachomatis M59178
0.05

Figure 2.7: Maximum Likelihood phylogenetic tree of bacteria from the phylum *Cyanobacteria* including a cluster (4 sequences) derived from the marine sponge *Raspailia ramosa* (Rrc388 – *Raspailia ramosa* cluster 388) forming a monophyletic novel sponge specific cluster.

2.5 Discussion

2.5.1 Isolated Bacteria

The phylogeny of the cultured isolates showed broad similarities to previously reported studies with the four bacterial phyla isolated here being regularly cultured from sponges (Taylor *et al*., 2007). All of the proteobacterial genera isolated in culture were represented in the pyrosequencing dataset from the sponge from which they were cultivated this was not the case for the *Actinobacteria* or *Firmicutes* isolates, many of which were members of genera that were not detected by pyrosequencing. While it is well known that much of the sponge microbiota is currently inaccessible by culture-dependent methods (Sipkema *et al*., 2011), it would also appear that bacteria accessible by culturing approaches are likewise not detected by culture-independent approaches. Similar findings were noted by Sun and colleagues (Sun *et al*., 2010) and by Zhang and co-workers (Zhang *et al*., 2006). Both groups targeted Actinomycetes for isolation from marine sponges. Sun and co-workers also constructed a 16S rDNA clone library while Zhang and colleagues performed RFLP analysis. Both groups identified cultured isolates which were absent from their culture-independent analyses. Whether this is due to extreme low abundance or methodological bias is currently unknown.

Members of the genera *Pseudoalteromonas* and *Pseudovibrio* were isolated from both sponge species and were also detected by pyrosequencing from both sponges. Previously, sponge-derived *Pseudovibrio* spp. isolates have displayed strong antimicrobial activities (Kennedy *et al*., 2008; O'Halloran *et al*., 2011) while other α-proteobacterial isolates, *Ruegeria* spp. and *Roseobacter* spp. have been implicated in signalling processes in sponges through the production of quorum-sensing molecules (Mohamed *et al*., 2008). Sponge-associated *Actinobacteria* are of particular interest due to the propensity of terrestrial members of this phylum to

produce bioactive secondary metabolites. *Arthrobacter* spp. were isolated in culture from both sponges here. Members of this genus are very common in soil and can metabolise toxic heavy metals and pesticides (Megharaj *et al*., 2003). Similarly, *Micrococcus* spp. were isolated from both sponges and this genus also includes species which harbour pesticide-degrading gene products (Sims *et al*., 1986).

The targeted isolation strategy was used in an attempt to obtain antibiotic producers such as *Streptomyces* sp. Although, no actinobacteria were in fact isolated the relative number of isolates displaying antimicrobial activity was an order of magnitude greater than that observed from the general isolation method used here. *Bacillus* sp. and *Staphylococcus* sp. were isolated by both strategies but *Paenibacillus* sp., *Tetrathiobacter* sp. and *Pantoea* sp. were only seen from the targeted approach. It is clear that different culture isolation strategies result in different phylotypes being obtained and to ascertain the full cultivable bacterial diversity of a sponge associated bacterial community a wide range of disparate isolation conditions are required.

2.5.2 Antimicrobial activities

Antibacterial activities were observed from eight sponge isolates, seven isolates inhibited *E. coli* and one isolate inhibited *S. aureus* (Table 2.2). The sponge isolates exhibiting these activities are most closely related to *Pseudovibrio* sp., *Pseudoalteromonas* sp., *Shewanella* sp. and *Bacillus* sp. *Pseudovibrio* sp. isolates from other sponge species have been noted to display inhibitory activity against important clinical pathogens such as MRSA (O'Halloran *et al*., 2011). Sponge derived *Pseudoalteromonas* sp., and *Bacillus* sp., have also previously been reported to display antimicrobial activities (Flemer *et al*., 2011). *Shewanella* spp. are known to produce antibiotic compounds also (Shnit-Orland *et al*., 2007).

Yeast test strains were inhibited by 14 of the sponge isolates. Half of those isolates were closely related to *Bacillus* sp., with phylogenetic analysis suggesting that these isolates may represent at least two different species within the genus (Figure 2.2). Other isolates exhibiting antimicrobial activities were close relatives of *Staphylococcus* sp., *Tetrathiobacter* sp., *Pantoea* sp. as well as two unidentified isolates. The mechanism of the antimicrobial activities being displayed by these isolates is as yet unknown. Furthermore, it is unclear whether the antimicrobial

compounds are produced *in vivo* in the sponge host or what ecological roles they may play, if any. It has however been suggested that production of antimicrobial compounds by sponge symbiotic bacteria plays roles in chemical defence of the host against infection and predation (Taylor *et al*., 2007).

2.5.3 Pyrosequencing

Phylum level analysis in this study reveals much lower diversity than has been noted in some previous sponge pyrosequencing studies (Lee *et al*., 2011; Webster *et al*., 2010). Those studies identified 26 and 23 bacterial phyla associated with sponges from Red Sea and Australian waters respectively. However, analysis of OTUs at 95% sequence similarity reveals levels of species diversity approaching what was noted by Webster despite the disparity in numbers of sponge-derived sequence reads analysed (~51,000 obtained here versus ~250,000 by Webster and colleagues). While Lee and co-workers identified ~850 bacterial OTUs (95% sequence similarity) in association with a single sponge species and Webster *et al* noted ~3,000 OTU's in a single species, ~ 2,100 bacterial OTU's were found here in the most diverse sponge (*R. ramosa)* community. This is in contrast to *S. stuposa* where 349 bacterial OTU's were noted. Chao1 estimates for the *R. ramosa* community (3,466 OTUs) at 95% sequence identity, though much higher than any previous report for marine sponges, reflects the data of Lee *et al*. where a similar proportion (~60%) of the community was represented relative to Chao1 estimates. Other sponge pyrosequencing studies have reported 14 (Trindade-Silva *et al*., 2012), 18 (White *et al*., 2012) and 8-15 (Schmitt *et al*., 2011) bacterial phyla associated with different sponge species. These findings echo Schmitt and colleagues findings that bacterial communities associated with sponges are largely species-specific. Their analyses revealed that >72% of OTUs were species–specific in five sponges which they examined while 26% of OTUs were common two-to-four of those sponges and only 2% of OUTs were found in all 5 sponge species. In this study ~13% of classified genera were found in both sponge species.

2.5.4 Community analysis

Genus level and cluster analyses of classified sequences reveal that different phylotypes dominate each of the communities. The largest cluster from the *R. ramosa* derived sequences aligned to the ubiquitous *SAR11* clade of *α-Proteobacteria*. The genus *Pelagibacter* accounts for 10% of all classified sequences from that sponge, the most common identified genus. This compares to 0.5% of reads from *S. stuposa* and 0.97% of seawater-derived reads identified as *SAR11*. *Nitrospirae* account for a large proportion of sequences from both sponges (9.1% of *R. ramosa* sequences, ~24% of *S. stuposa* sequences) but are scarce in seawater (one sequence read). *Nitrospira* is the most common identified genus from *S. stuposa*. *Nitrospiraceae* have been commonly detected in other sponge species, however the levels found here are significantly higher than other pyrosequencing studies that showed levels of 0.01% to 3% among several sponge species (Lee *et al*., 2011; Webster *et al*., 2010).

A large number of *S. stuposa* tag sequences were classified as purple sulfur bacteria from the family *Ectothiorhodospiraceae* (34% of reads) with a further 4.4% of reads being classified as members of the same order (*Chromatiales*). This family of bacteria also appear to constitute a significant proportion of the microbiota of *R. ramosa* with 4.3% of classified reads recruiting to the *Ectothiorhodospiraceae* and a further 0.7% to other *Chromatiales* families. Within other sponge species the presence of significant numbers of purple sulfur bacteria have been found in *Haliclona simulans* from the west coast of Ireland, where 44% of clones recruited to the *Ectothiorhodospiraceae* (Kennedy *et al*., 2011b). Webster and co-workers have also reported high levels of *Ectothiorhodospiraceae* ranging from ~0.4% to >5% among different sponge species (Webster *et al*., 2010). The high levels of this group of bacteria within both sponges and their absence from the surrounding seawater implies a significant role in sponge biology. The *Ectothiorhodospiraceae* are typically sulfur-oxidising anaerobic phototrophs, although the role of these bacteria in sponge biology is as yet unclear. The order *Rhodobacterales* from the α- class of Proteobacteria accounted for 9% of *R .ramosa* derived sequences, 10% of seawater sequences but just 0.5% of sequences from *S. stuposa*. A large cluster from *S. stuposa* aligns to the order *Oceanospirillales* of *γ-Proteobacteria*. While 5% of *S. stuposa* sequences recruit to this order, only 0.1% of *R. ramosa* reads and 0.9% of

seawater reads recruit to *Oceanospirillales*. Within *Oceanospirillales* the most abundant genus present in *S. stuposa* is the *Endozoicomonas* which constitutes 5% of the classified reads. A small proportion (<0.1%) of *R. ramosa* tag sequences recruit to this genus while one tag sequence from seawater represents this genus, suggesting that this may be a sponge species-specific symbiont. Members of this genus are mostly associated with marine animals (sponges, corals, marine slugs) while the nearest related genera are mostly found in saline or hypersaline aquatic environments or in sea sediment (Kurahashi & Yokota, 2007; Yang *et al*., 2010). The order *Flavobacteriales* from the phylum *Bacteroidetes* is abundant in *R. ramosa* (4.9% of reads) but only accounts for 0.26% of *S. stuposa* sequences and 0.72% of reads from seawater. Many genera from the *Flavobacteriaceae* family are present with no dominant clusters. *Flavobacteriaceae* have been identified as an important environmental reservoir for β-lactamase genes (Naas *et al*., 2003). *Alteromonadales* from *γ-Proteobacteria* also constitute a significant proportion of the *R. ramosa* community (3% of sequences) and account for 19% of sequences from seawater but only 0.12% of *S. stuposa* sequences recruit to that order. The *R. ramosa* derived sequences include seven reads and the *S. stuposa* derived sequences include one sequence recruited to the candidate division TM7. Prior to pyrosequencing technology, few TM7 sequences were reported from marine sponges. Three TM7 sequences were reported from *Chondrilla nucula* (Taylor *et al*., 2007) through cloning experiments. Lately, through pyrosequencing, low abundance TM7 sequences were found in various sponge species. Webster reported TM7 sequences derived from *Ianthella basta* and also from sponge larvae (*Rhopaloeides odorabile*) (Webster *et al*., 2010). Lee and colleagues report TM7 sequences associated with four *Hyrtios erectus* individuals, with *Stylissa carteri* and also with two *Xestospongia testudinaria* individuals (Lee *et al*., 2011). Regular identification of sponge-associated TM7 sequences due to deeper sequencing suggests that members of this division may be widespread in sponges at very low abundance. Many *γ-Proteobacterial* sequences from all three samples remained unclassified at lower taxonomic levels. These include 32% of all *R. ramosa* sequences, 17% of all *S. stuposa* sequnces and 1% of sequences from seawater. The seawater tag sequences are completely dominated by the common marine order of *γ-Proteobacteria, Vibrionales*. More than 55% of sequences from seawater recruit to the order *Vibrionales*; this compares to 0.4% of *R. ramosa* tag sequences and 0.03% of *S.*

stuposa sequences. More than 98% of the seawater sequences belong to the phylum *Proteobacteria*, the other dominant groups being 9% *Rhodobacteraceae* and ~13% *Pseudoalteromonas*. In comparable studies, Lee *et al*. found >90% of pyrosequencing reads from seawater aligning to three bacterial phyla (*Proteobacteria*, *Cyanobacteria* and *Bacteroidetes*) (Lee *et al*., 2011) while Webster and co-workers reported that 90–95% of pyrosequencing reads belong to the same three phyla (Webster *et al*., 2010). The filter pore size used for DNA extraction in this study may have allowed more diminutive cells to pass through, thereby affecting the seawater community profile. However, the three phyla which dominate in water from the Red Sea (Lee *et al*., 2011) and Australian waters (Webster *et al*., 2010) also account for >99% of tag sequences from Lough Hyne.

2.5.5 Sponge-Specific Phylotypes

One of the most striking features of sponge microbial ecology is the identification of sponge-specific phylotypes as defined by Hentschel (Hentschel *et al*., 2002). Numerous sequence clusters identified in this study can be classified as sponge-specific. From the sponge *R. ramosa* 17 sequence clusters, representing 2.8% of quality-filtered reads, constitute 2 novel sponge-specific clusters. One cluster of four sequences represents a novel sponge-specific cluster in the phylum *Cyanobacteria* (Figure 2.7) while 16 *R. ramosa*-derived clusters representing 391 sequences represent a novel sponge-specific cluster in the *α*- class of Proteobacteria (Figure 2.9). From *S. stuposa*, 18 sequence clusters representing 26% of sequences from that sponge align to 'sponge cluster 23' in the phylum *Nitrospira* (Figure 2.8) using the cluster numbering system used by Webster (Webster *et al*., 2010). Notable sponge-specific clusters from numerous phyla (*Chloroflexi*, *Bacteroidetes*, *Gemmatimonadetes*, *Verrucomicrobia*, *Planctomycetes*, *Lentisphaerae*, *Poribacteria*, *Spirochaetes, γ-Proteobacteria*) identified in other sponge species were absent from the sponges examined here. In addition, *Poribacteria*-specific PCR primers failed to amplify a product from metagenomic DNAs of the sponges examined here (data not shown).

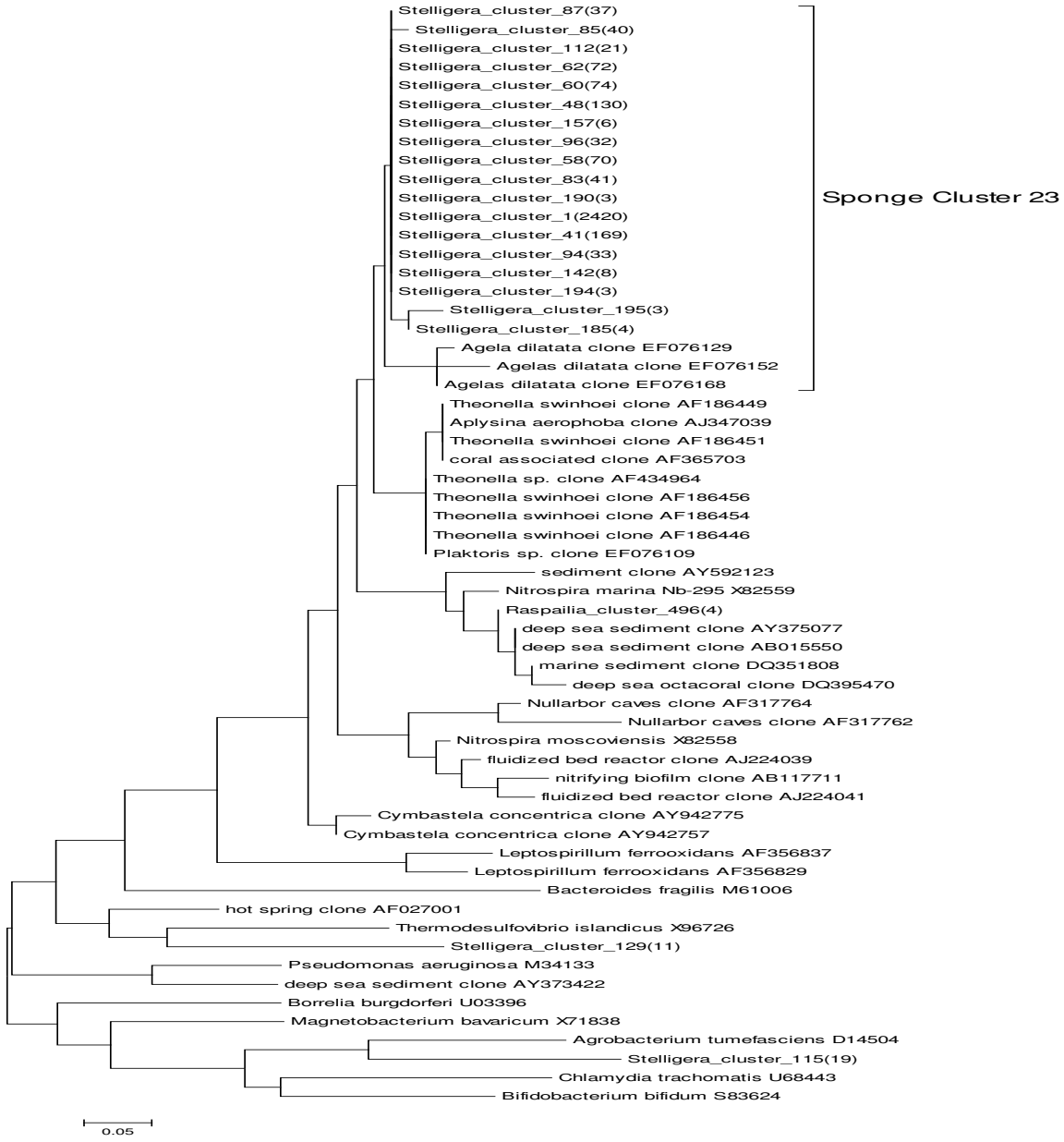

Figure 2.8: Maximum Likelihood phylogenetic tree of bacteria from the phylum *Nitrospira* including 18 clusters (3,166 sequences) derived from the marine sponge *Stelligera stuposa* recruiting to sponge cluster 23 according to the numbering system of Webster *et al*, 2010.

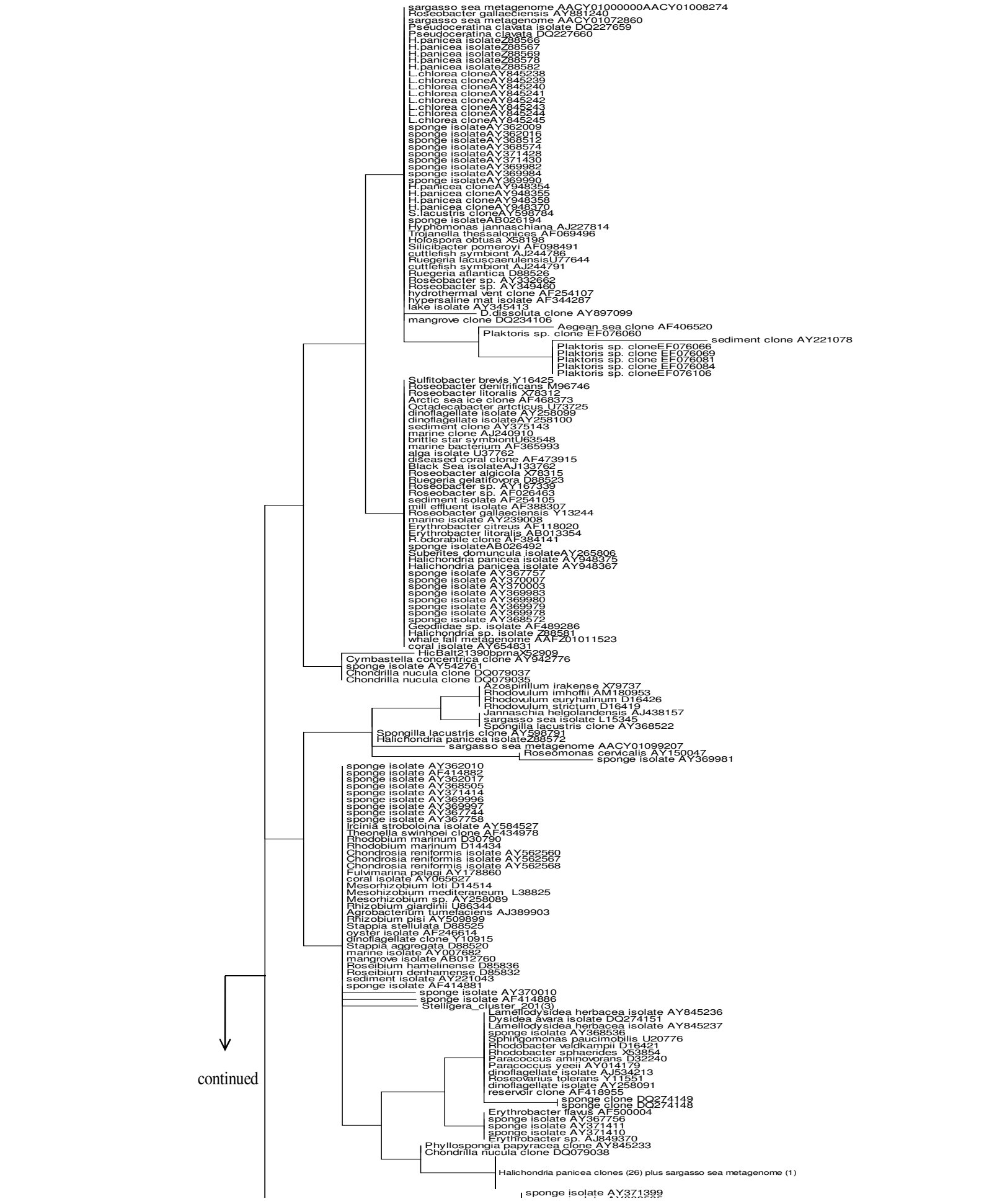
sargasso sea metagenome AACY01000000AACY01008274
Roseobacter gallaeciensis AY881240
sargasso sea metagenome AACY01072860
Pseudoceratina clavata isolate DQ227659
Pseudoceratina clavata DQ227660
H.panicea isolateZ88566
H.panicea isolateZ88567
H.panicea isolateZ88569
H.panicea isolateZ88578
H.panicea isolateZ88582
L.chlorea cloneAY845238
L.chlorea cloneAY845239
L.chlorea cloneAY845240
L.chlorea cloneAY845241
L.chlorea cloneAY845242
L.chlorea cloneAY845243
L.chlorea cloneAY845244
L.chlorea cloneAY845245
sponge isolateAY362009
sponge isolateAY362016
sponge isolateAY368512
sponge isolateAY368574
sponge isolateAY371428
sponge isolateAY371430
sponge isolateAY369982
sponge isolateAY369984
sponge isolateAY369990
H.panicea cloneAY948354
H.panicea cloneAY948355
H.panicea cloneAY948358
H.panicea cloneAY948370
S.lacustris cloneAY598784
sponge isolateAB026194
Hyphomonas jannaschiana AJ227814
Trojanella thessalonices AF069496
Holospora obtusa X58198
Silicibacter pomeroyi AF098491
cuttlefish symbiont AJ244786
Ruegeria lacuscaerulensisU77644
cuttlefish symbiont AJ244791
Ruegeria atlantica D88526
Roseobacter sp. AY332662
Roseobacter sp. AY349460
hydrothermal vent clone AF254107
hypersaline mat isolate AF344287
lake isolate AY345413
D.dissoluta clone AY897099
mangrove clone DQ234106
Aegean sea clone AF406520
Plaktoris sp. clone EF076060
sediment clone AY221078
Plaktoris sp. cloneEF076066
Plaktoris sp. clone EF076069
Plaktoris sp. clone EF076081
Plaktoris sp. clone EF076084
Plaktoris sp. cloneEF076106
Sulfitobacter brevis Y16425
Roseobacter denitrificans M96746
Roseobacter litoralis X78312
Arctic sea ice clone AF468373
Octadecabacter arcticus U73725
dinoflagellate isolate AY258099
dinoflagellate isolateAY258100
sediment clone AY375143
marine clone AJ240910
brittle star symbiontU63548
marine bacterium AF365993
alga isolate U37762
diseased coral clone AF473915
Black Sea isolateAJ133762
Roseobacter algicola X78315
Ruegeria gelatifovora D88523
Rosebacter sp. AY167338
Roseobacter sp. AF026463
sediment isolate AF254105
mill effluent isolate AF388307
Roseobacter gallaeciensis Y13244
marine isolate AY239008
Erythrobacter citreus AF118020
Erythrobacter litoralis AB013354
R.odorabile clone AF384141
sponge isolateAB026492
Suberites domuncula isolateAY265806
Halichondria panicea isolate AY948375
Halichondria panicea isolate AY948367
sponge isolate AY367757
sponge isolate AY370007
sponge isolate AY370003
sponge isolate AY369983
sponge isolate AY369980
sponge isolate AY369979
sponge isolate AY369978
sponge isolate AY368572
Geodiidae sp. isolate AF489286
Halichondria sp. isolate Z88581
whale fall metagenome AAFZ01011523
coral isolate AY654831
HicBalt21390bprnaX52909
Cymbastella concentrica clone AY942776
sponge isolate AY542761
Chondrilla nucula clone DQ079037
Chondrilla nucula clone DQ079035
Azospirillum irakense X79737
Rhodovulum imhoffii AM180953
Rhodovulum euryhalinum D16426
Rhodovulum strictum D16419
Jannaschia helgolandensis AJ438157
sargasso sea isolate L15345
Spongilla lacustris clone AY368522
Spongilla lacustris clone AY598791
Halichondria panicea isolateZ88572
sargasso sea metagenome AACY01099207
Roseomonas cervicalis AY150047
sponge isolate AY369981
sponge isolate AY362010
sponge isolate AF414882
sponge isolate AY362017
sponge isolate AY368505
sponge isolate AY371414
sponge isolate AY369996
sponge isolate AY369997
sponge isolate AY367744
sponge isolate AY367758
Ircinia stroboloina isolate AY584527
Theonella swinhoei clone AF434978
Rhodobium marinum D30790
Rhodobium marinum D14434
Chondrosia reniformis isolate AY562560
Chondrosia reniformis isolate AY562567
Chondrosia reniformis isolate AY562568
Fulvimarina pelagi AY178860
coral isolate AY065627
Mesorhizobium loti D14514
Mesohizobium mediteraneum L38825
Mesorhizobium sp. AY258089
Rhizobium giardinii U86344
Agrobacterium tumefaciens AJ389903
Rhizobium pisi AY509999
Stappia stellulata D88525
oyster isolate AF246614
dinoflagellate clone Y10915
Stappia aggregata D88520
marine isolate AY007682
mangrove isolate AB012760
Roseibium hamelinense D85836
Roseibium denhamense D85832
sediment isolate AY221043
sponge isolate AF414881
sponge isolate AY370010
sponge isolate AF414886
Stelligera_cluster_201(3)
Lamellodysidea herbacea isolate AY845236
Dysidea avara isolate DQ274151
Lamellodysidea herbacea isolate AY845237
sponge isolate AY368536
Sphingomonas paucimobilis U20776
Rhodobacter veldkampii D16421
Rhodobacter sphaerides X53854
Paracoccus aminovorans D32240
Paracoccus yeeii AY014179
dinoflagellate isolate AJ534213
Roseovarius tolerans Y11551
dinoflagellate isolate AY258091
reservoir clone AF418955
sponge clone DQ274149
sponge clone DQ274148
Erythrobacter flavus AF500004
sponge isolate AY367756
sponge isolate AY371411
sponge isolate AY371410
Erythrobacter sp. AJ849370
Phyllospongia papyracea clone AY845233
Chondrilla nucula clone DQ079038
Halichondria panicea clones (26) plus sargasso sea metagenome (1)
sponge isolate AY371399
continued

Figure 2.9 continued

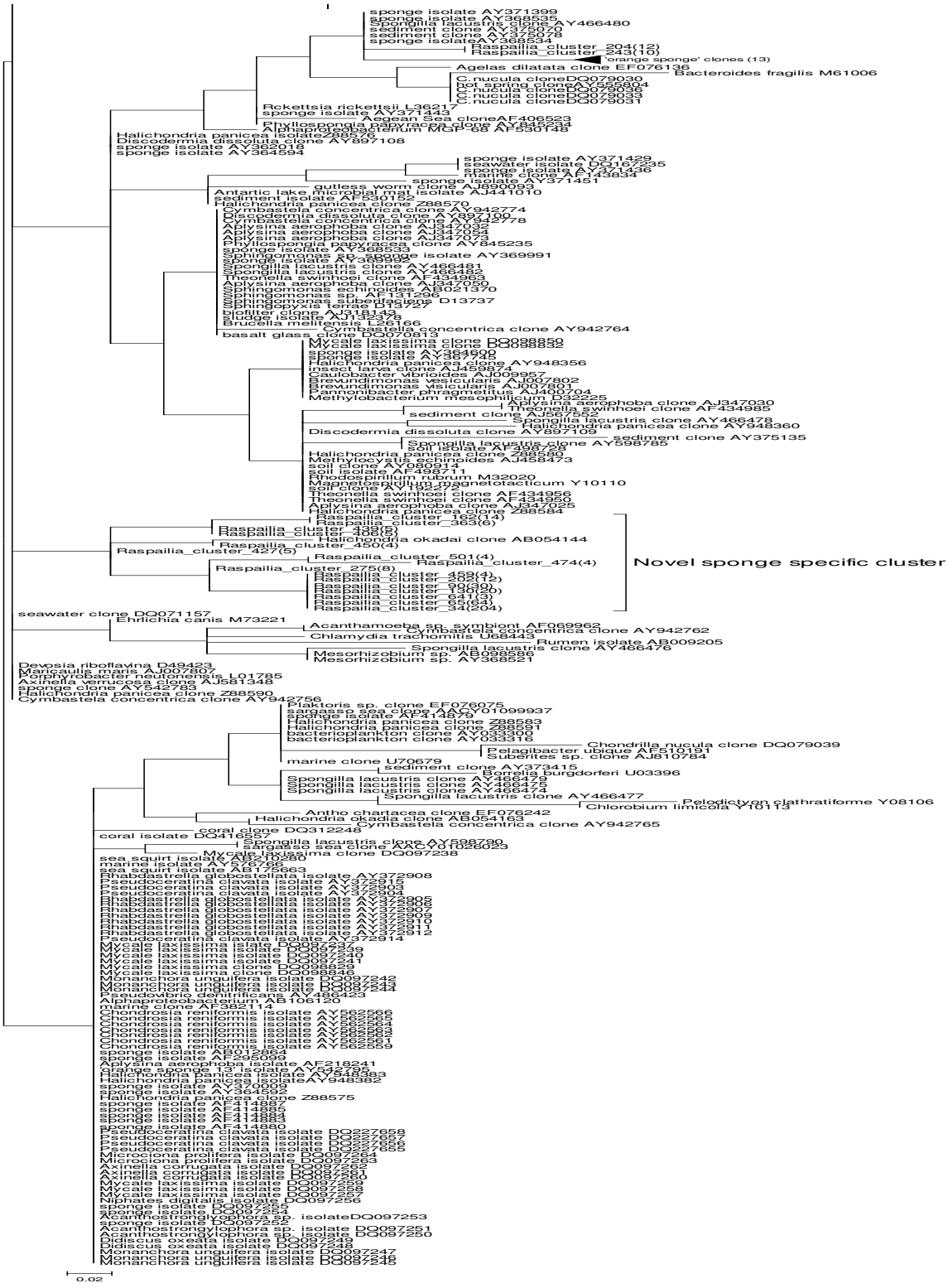

Figure 2.9: Maximum Likelihood phylogenetic tree of bacteria from the α- class of Proteobacteria including 16 clusters (391 sequences) derived from the marine sponge *Raspailia ramosa* forming a monophyletic novel sponge specific cluster.

2.5.6 Linking Taxonomy to Function

The composition of the microbial communities, present within each sponge is likely to reflect the metabolic roles of these bacteria. The community associated with *S. stuposa* appears to be much less diverse than that associated with *R. ramosa*; with approximately 62% of the total microbial community being made up of three distinct bacterial groups, the order *Chromatiales* (33%), the genus *Nitrospira* (24%), and the genus *Endozoicomonas* (5%). Within the more complex microbiota of *R. ramosa* the most abundant of these three groups also make up a significant portion of the microbial population with ~9% *Nitrospira* and 5% *Chromatiales* present, implying an important, if not fundamental, role in the biology of these sponge species. Taxonomic biomarker genes cannot be used to identify symbiotic roles for bacterial communities but some inferences can be made based on known physiological and metabolic capabilities of particular phylotypes. Cyanobacterial photosynthesis may be an important source of carbon for many sponges (Taylor *et al*., 2007) and were present in both of these sponges. Bacterial groups involved in all steps of nitrogen metabolism, N_2 fixation (*Rhizobia* sp.), ammonia oxidation *Nitrospira* sp., *Nitrosomonas* sp.), nitrite and nitrate reduction (*Flavobacterium* sp.) were also associated with both sponge species. Finally sulphur metabolising bacterial groups were also evident associated with sponges. Sulfur-oxidising (*Ectothiorhodospiraceae* and *Sulfurovum* sp.) and sulfide-oxidising bacteria (*Arcobacter* sp.) were present in *R. ramosa* as were sulfate-reducing phylotypes *Desulfovibrio* sp. and *Desulfuromonas* sp., while in *S. stuposa* sulfur metabolism may chiefly involve the dominant *Ectothiorhodospiraceae*. The abundance of both of these sponge species in the same ecological niche (Bell & Barnes, 2000) suggests that, although in some cases different bacterial groups appear to perform similar symbiotic roles for each individual host, the difference in complexity between the microbial communities does not alter the success of these sponges in that habitat.

2.6 Conclusion

Different pyrosequencing studies have targeted various regions of the 16S rRNA gene for amplification (Lee *et al*., 2011; Webster *et al*., 2010) and no standard has emerged yet. However, with increasing read-lengths obtainable, it has been shown that sequences spanning a variable region and a hypervariable region of the 16S gene can provide the most robust taxonomic classification of sequences (Kim *et al*., 2010; Wang *et al*., 2007; Wommack *et al*., 2008). For this reason, we targeted the V1-V3 region, and the average sponge-derived sequence lengths obtained here (~430b) resulted in the majority of quality-filtered sequence reads spanning the full length of the variable regions being targeted. However, it has been shown that intrinsic pyrosequencing errors can result in diversity estimates which are orders of magnitude higher than the actual diversity levels (Kunin *et al*., 2010). Pyrosequencing of 16S rRNA amplicon libraries generated from the sponge metagenome has provided a detailed insight into the composition of the sponge associated cohorts. Clear differences in community profiles, when compared to seawater-derived data, show that the major proportion of sponge-associated bacteria is not incidental or transient, as most OTUs identified in the sponge hosts were not present in seawater. This was also shown in other deep sequencing studies comparing sponges to seawater (Lee *et al*., 2011; Webster *et al*., 2010). Host selection is remarkably divergent. Of the 10 bacterial phyla identified in *R. ramosa*, two are absent from *S. stuposa* and four were not found in the surrounding seawater. Also evident are the differences between the microbial communities associated with these sponges and other sponges that have been studied by deep sequencing approaches. While similarities are present, as illustrated by the analysis of sponge-specific clusters; what is perhaps more clear are the differences in the microbial populations between sponge species, with many sponge-specific groups being absent from these species.

It is clear from the deep analysis of the microbiota of *S. stuposa* and *R. ramosa*, the first temperate sponge species studied in this way that the cosmopolitan nature of sponge-microbial associations are to varying degrees both sponge-specific and species-specific. The symbiotic roles attributed to bacteria within sponge tissues are performed in some cases by similar phylotypes that seem to be almost universally

present within sponges and across habitats (e.g. *Cyanobacteria*, *Nitrospira*) and in other instances by dissimilar populations (e.g. sulfur metabolism). As deep sequencing approaches are applied to additional sponge species from varied habitats, and more sponge-specific clusters are identified; more detailed patterns of sponge-microbial interactions will emerge. The challenge that this data presents is in linking our increasingly in-depth knowledge of sponge-microbial phylogeny to informed approaches to study sponge-microbial physiology and reveal the biochemical roles of the microbial consortia.

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Chapter 3

Archaea Dominate the Microbial Communities in the Marine Sponge *Inflatella pellicula* in the Deep Sea as Revealed by Pyrosequencing

3.1 Abstract

Microbes associated with marine sponges play significant roles in host physiology. Remarkable levels of microbial diversity have been observed in sponges worldwide through culture-dependent and culture-independent studies. Most studies have focused on the structure of the bacterial communities in sponges and have involved sponges sampled from shallow waters. Here we used pyrosequencing to compare the bacterial and archaeal communities associated with three individuals of the marine sponge *Inflatella pellicula* from the deep-sea, one individual from a depth of 780 m and two individuals from 2900 m, a depth which far exceeds any previous sequence-based report of sponge-associated microbial communities. Sponge-microbial communities were also compared to the microbial communities in seawater from concomitant depths. Although the sponges from 2900 m hosted similar communities, clear differences between the sponge-associated community from 780 m and the sponge communities from the greater depth were apparent. The seawater communities did not resemble the sponge communities. *Archaea* were remarkably dominant in the sponge-associated communities. *Thaumarchaeota* comprised large proportions of the sponge-associated cohorts and occurred in increased abundance with increased sampling depth. While *Archaea* comprised ~11.3-36.6% of seawater communities their abundance in sponges ranged from ~43-72.5%. *Euryarchaeota* which were the dominant archaeal phyla in seawater were rare in sponges. Bacterial communities associated with these sponge samples are less diverse and less even than in any other sponge species investigated to date by pyrosequencing. Sponges hosted 9-12 bacterial phyla, fewer than was found in seawater (13 and 15 phyla). Deep-sea sponge microbial communities appear to differ greatly from sponge-microbe communities from shallow waters.

3.2 Introduction

Marine sponges (*Porifera*) are host to microbes from all domains of life; *Eukarya* (Baker *et al*., 2008; Cerrano *et al*., 2004), *Archaea* (Margot *et al*., 2002; Webster *et al*., 2004) and *Bacteria* (Taylor *et al*., 2007). These close and consistent associations are thought to be based on various symbiotic relationships; commensalist, mutualist (Wilkinson, 1983) and parasitic (Bavestrello *et al*., 2007). Microbes are also a

significant food source for marine sponges (Reiswig, 1975) which, as sessile animals, must derive their nutrition by active filter-feeding from ambient seawater. Much research interest has focused on the bacterial associates of marine sponges since the early work of Clive Wilkinson (Wilkinson, 1978) and Jean Vacelet (Vacelet & Donadey, 1977) in the 1970s showed that bacteria comprise significant proportions of sponge tissues. Progressive advances in technologies in molecular biology have shown that enormous levels of bacterial diversity inhabit sponge tissues. Members of 35 major bacterial phyla or candidate phyla (Schmitt et al., 2011) as well as archaea (Taylor *et al*., 2007) and eukaryotic microbes (fungi and diatoms) have been detected in sponge tissues through culture isolation (Kennedy *et al*., 2008), microscopy; TEM (Vacelet & Donadey, 1977) and FISH (Sharp *et al*., 2007) and molecular investigations; DGGE (Usher *et al*., 2004), RFLP (Zhang *et al*., 2006), PCR (Sipkema *et al*., 2009) and latterly pyrosequencing (Webster *et al*., 2010; Lee *et al*., 2011; Schmitt *et al*., 2011; Jackson *et al*., 2012; White *et al*., 2012). Numerous sponge families, genera and species from tropical, temperate and polar waters have to date been investigated. These studies have revealed inter- and intra-species similarities and differences, with apparent sponge-specific taxa (Hentschel *et al*., 2002), which despite being derived from disparate sponge species and distant biogeographic regions are more closely related to each other than to similar taxa from non-sponge habitats. Recently massively parallel pyrosequencing has enabled very detailed descriptions of sponge-associated microbial communities, generating sequence datasets many orders of magnitude greater than was previously possible. This has enabled the discovery of low-abundance members of these microbial communities and a more complete and accurate description of the structures and stability of the highly complex resident symbiont communities. Few studies to date have considered the relative abundance of *Archaea* in sponge-associated microbial communities. However, Lee and colleagues (Lee *et al*., 2011) showed that *Archaea* comprise significant proportions (ranging from 4-28%) of the microbial communities inhabiting various individuals of three sponge species from the Red Sea. Such significant levels of *Archaea* within sponge tissues suggest that they may play important roles in host physiology, particularly as they have been shown to be of ecological importance in nitrogen cycling (Koenneke *et al*., 2005).

Here we use pyrosequencing to describe the archaeal and bacterial communities associated with the sponge *Inflatella pellicula* (Schmidt, 1875) from the deep ocean. The marine sponge *I. pellicula* has to date only been found in cold and deep waters below 200 m and has been found in the North Atlantic and North Pacific oceans. Three individuals are compared, one sampled from a depth of 780 m and two individuals sampled from a single location at a depth of 2900 m. We also compare the sponge-derived cohorts to those of seawater sampled from both depths.

The objectives of the work presented in this chapter are: (1) to elucidate the microbial community structures associated with the marine sponge *I. pellicula*, (2) to compare the sponge-associated communities to those of ambient seawater, and (3) to determine if deep-sea sponge-associated microbial structures resemble those of shallow water sponges.

3.3 Materials and Methods

3.3.1 Sampling

Sponges and seawater were sampled using the Irish research vessel, *RV Celtic Explorer* and the remotely operated vehicle (ROV), *Holland I* from the Atlantic Ocean in Irish waters as per Table 3.1. One individual of the marine sponge *Inflatella pellicula* (Class *Demospongiae*; Order *Poecilosclerida*; Suborder *Myxillina*; Family *Coelospheridae*) was sampled at a depth of 780 m while two individuals of the same species were obtained from a single location at a depth of 2900 m. Sponges were immediately rinsed with sterile artificial seawater, placed in sterile Ziploc bags and then frozen at -80°C until ready for use. Artificial seawater comprised 33.3g/L Instant Ocean, (Aquarium Systems – Blacksburg, VA, USA), a defined ion and mineral formulation commonly used in aquaria. Seawater (30L) was collected at the same depths as the sponge sampling depths and immediately filtered through 0.2 µm membrane filters (Whatman – Austin, TX, USA) and the filters were stored in sterile tubes at -80°C until ready for use.

Sample	Depth (m)	GPS Location	Temperature (°C)
Seawater	780	N54° 00' 03" W12° 18' 36"	9.9
Inflatella pellicula	780	N54° 00' 03" W12° 18' 36"	9.9
Seawater	2900	N54° 14' 31" W12° 41' 38"	2.76
Inflatella pellicula	2900	N54° 14' 31" W12° 41' 38"	2.76
Inflatella pellicula	2900	N54° 14' 31" W12° 41' 38"	2.76

Table 3.1: Sampling of sponges and seawater from the Atlantic Ocean in Irish waters.

3.3.2 Metagenomic DNA Extraction from Sponges

Sponge tissues were weighed and finely ground under liquid N_2 with a sterile mortar and pestle. The ground tissues were suspended in lysis buffer [100 mM Tris, 100 mM EDTA, 1.5 M NaCl (*w/v*), 1% CTAB (*w/v*), 2% SDS (*w/v*)] - adapted from Brady, 2007. Metagenomic DNA was then extracted as previously described (Kennedy *et al*., 2008b). DNA solutions were analysed by gel electrophoresis, quantified by spectrophotometry (NanoDrop ND-1000 – Wilmington, DE, USA) and then stored at -20°C.

3.3.3 Metagenomic DNA Extraction from seawater

DNA was extracted from filters using WaterMaster DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions and stored at -20°C.

3.3.4 PCR Amplicon Library Preparation for Pyrosequencing

PCR amplicon libraries of the V5-V6 region of 16S rRNA genes were prepared from *I. pellicula* and seawater metagenomic DNAs. Universal primers U789f (5'-TAGATACCCSSGTAGTCC-3') and U1068r (5'-CTGACGRCRGCCATGC-3') (Lee *et al*., 2011), targeting both bacteria and archaea, were adapted for pyrosequencing by the addition of sequencing adapters and multiplex identifier (MID) sequences as per Table 3.2. Each 50 µl PCR reaction comprised 1X buffer, 0.2 mM dNTPs, 0.1 µM of each primer, 2U Taq polymerase, ~10 ng template DNA and sdH_2O. PCR cycle conditions comprised initial denaturation at 94°C for 5 min followed by 26 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s

and extension at 72°C for 45 s. A final extension 72°C for 6 min was added (Lee *et al*., 2011). To minimise PCR bias three individual reactions were performed per template and equimolar amounts of PCR products from each of the three reactions were pooled for pyrosequencing. PCR products were purified using Qiagen PCR Purification Kit (Qiagen Ltd., UK) as per the manufacturer's instructions. Barcoded samples were pooled and sequenced on GS FLX Titanium platform (454 Life Sciences) at the University of Liverpool, Centre for Genomic Research, Liverpool, UK.

Sample	Primer	Adapter	Multiplex Identifier (MID)	Template specific primer
SW780m (seawater 780m)	Forward	CGTATCGCCTCCCTCGCGCCATCAG	ACGAGTGCGT	TAGATACCCSSGTAGTCC (U789f)
	Reverse	CTATGCGCCTTGCCAGCCCGCTCAG	ACGAGTGCGT	CTGACGRCRGCCATGC (U1068r)
SW2900m (seawater 2900m)	Forward	CGTATCGCCTCCCTCGCGCCATCAG	ACGCTCGACA	TAGATACCCSSGTAGTCC (U789F)
	Reverse	CTATGCGCCTTGCCAGCCCGCTCAG	ACGCTCGACA	CTGACGRCRGCCATGC (U1068r)
Ip780m (*I.pellicula* 780m)	Forward	CGTATCGCCTCCCTCGCGCCATCAG	TAGTATCAGC	TAGATACCCSSGTAGTCC (U789f)
	Reverse	CTATGCGCCTTGCCAGCCCGCTCAG	TAGTATCAGC	CTGACGRCRGCCATGC (U1068r)
Ip2900mA (*I.pellicula* 2900m)	Forward	CGTATCGCCTCCCTCGCGCCATCAG	TCTCTATGCG	TAGATACCCSSGTAGTCC (U789F)
	Reverse	CTATGCGCCTTGCCAGCCCGCTCAG	TCTCTATGCG	CTGACGRCRGCCATGC (U1068r)
Ip2900mB (*I.pellicula* 2900m)	Forward	CGTATCGCCTCCCTCGCGCCATCAG	TGATACGTCT	TAGATACCCSSGTAGTCC (U789f)
	Reverse	CTATGCGCCTTGCCAGCCCGCTCAG	TGATACGTCT	CTGACGRCRGCCATGC (U1068r)

Table 3.2: Primer design for pyrosequencing of 16S rRNA (V5-V6) genes from archaea and bacteria in sponges and seawater.

3.3.5 Pyrosequencing Data Analysis

Primer adapter and MID sequences were removed from all reads and reads were filtered for quality using the Ribosomal Database Project (RDP) -Release 10.29, Pyrosequencing Pipeline (http://rdp.cme.msu.edu/). Reads with ambiguous bases 'N', average quality score <20 or shorter than 100 bp were discarded from further

analysis. Individual sample libraries were aligned using the INFERNAL aligner (Nawrocki & Eddy, 2007). OTUs were determined using the RDP clustering tool (complete linkage clustering). Taxonomic classifications were determined using the 'Classifier' tool (naïve Bayesian rRNA classifier- Wang *et al.*, 2007) at 50% confidence threshold by comparing to the database of 2320464 rRNA sequences. Rarefaction curves were generated from data obtained from the 'Rarefaction' tool; diversity indices (Shannon index & Chao1 species estimator) were obtained using the relevant tools at sequence similarities of 95%. Rank-abundance curves were derived from cluster analysis results. Unclassified sequences were further investigated using BLAST analyses (Altschul *et al.*, 1990) at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Representative sequences from sponge derived sequence clusters of identical reads (0% distance) were extracted, analysed by BLAST and used to generate phylogenetic trees. Sequence alignment and tree construction were performed using MEGA version 5 (http://www.megasoftware.net/) (Tamura *et al.*, 2011). Alignment was performed with ClustalW and tree construction was by Neighbour-Joining (Saitou & Nei, 1987) method. Reference sequences were downloaded from the RDP database. All sequence data is publicly available on MG-RAST (ID no.s 4497997.3, 4497995.3, 4497996.3, 4497999.3, 4497998.3). (http://metagenomics.anl.gov/).

3.4 Results

3.4.1 Sequencing

Pyrosequencing of 16S rRNA genes from archaea and bacteria from three individual sponges of the same species (*I. pellicula*) was performed. One individual was sampled from a depth of 780 m (*I. pellicula* 780m) while the other sponges were sampled from a single location at a depth of 2900 m (*I. pellicula* 2900m sample A and *I. pellicula* 2900m sample B). Sequencing was also performed from seawater, one sample for each sampling depth. The five combined samples yielded ~46300 raw 16S rRNA sequence reads, of which ~43600, comprising >12.2 million bp were included in the final analysis after quality filtering (Table 3.3). Sponge-derived datasets combined accounted for ~ 24800 reads. Average sequence lengths varied from 280bp for samples from 780 m to 277bp for samples from 2900 m.

Sample	No. of reads	No .of reads after quality filtering	No. of bacterial phyla	No. of OTUs (97%)	No. of OTUs (95%)	Shannon Index	Chao1
Seawater 780m	6350	5961	13	817	561	4.89	812
Seawater 2900m	13577	12849	15	1508	1026	4.79	1769
I. pellicula 780m	10211	9537	9	327	203	2.16	361
I. pellicula 2 900mA	6540	6088	11	368	289	1.96	592
I. pellicula 2 900mB	9688	9179	12	446	340	2.17	654

Table 3.3: Analysis of 16S rRNA gene (V5-V6) pyrosequencing reads from three individuals of the marine sponge *I. pellicula* from two different sampling depths and from seawater sampled at the same depths. Shannon indices and Chao1 estimates were calculated at sequence similarities of 95%.

3.4.2 Sequence Classification

Greater than 99.99% of quality filtered sequence reads were assigned to domains, *Archaea* or *Bacteria*. However, ~9% of all sequences could not be assigned to phyla. The majority (>75%) of sequences not assigned to domains derived from a single sample (*I. pellicula* 780m) and all sponge-derived unclassified reads shared homology with host mitochondrial DNA sequences as determined by BLAST searches.

3.4.3 Relative Abundances of Archaea and Bacteria

Archaeal sequences were more abundant in sponges than in seawater (Figure 3.1) and were more abundant in sponges sampled at 2900 m than in the sponge sampled at 780 m. While the relative abundances of archaeal sequencing reads in the samples from 780 m (~36.6% in seawater, 43% in *I. pellicula*) were comparable, major differences were seen in the relative abundance of archaeal reads in samples from 2900 m (11.3% in seawater, 72.6% and 60.3% in sponges).

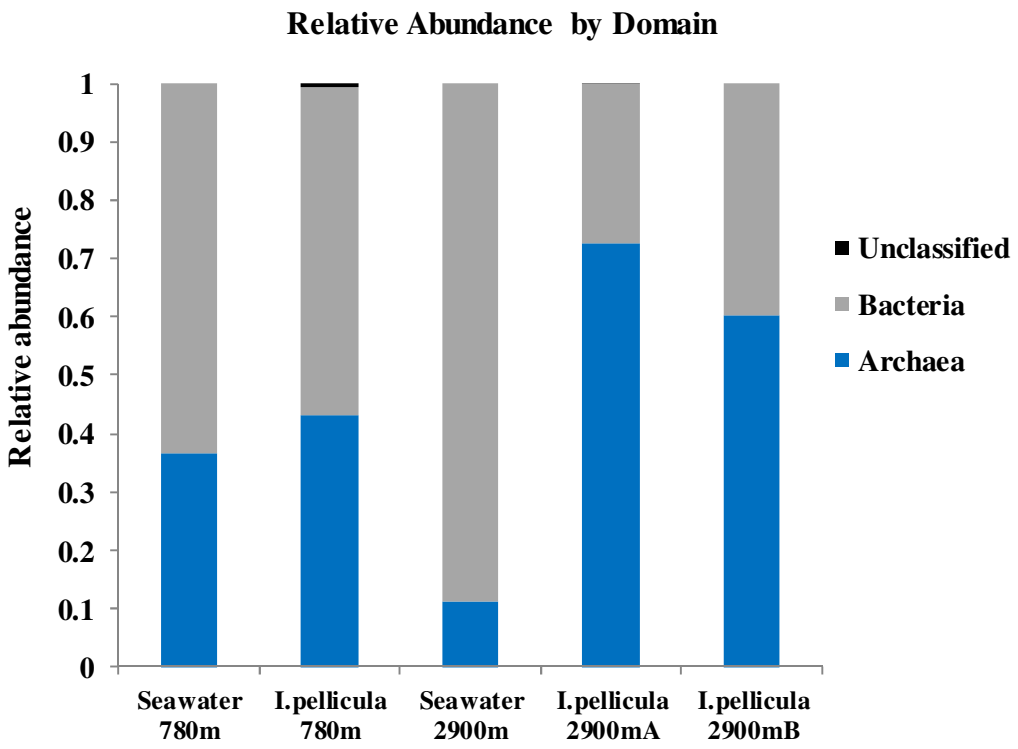

Figure 3.1: Relative abundance of sponge and seawater associated microbes by domain.

3.4.4 Sponge and seawater from 780 m

Archaea comprised a large proportion of the sequencing reads from both the seawater (36.6%) and sponge (~43%) sampled at 780 m, but the communities were dissimilar. From seawater 30.5% of all sequence reads (83% of archaeal reads) recruit to the phylum *Euryarchaeota* while from the sponge 0.43% of all reads (~1% of archaeal reads) were classified as *Euryarchaeota*. Members of 13 bacterial phyla were found in seawater from 780 m compared to 9 bacterial phyla from *I. pellicula* from the same depth (Figures 3.2a & 3.2b). *Spirochaetes* and *Nitrospira* were detected in the sponge but were absent from the seawater. Five phyla (*Elusimicrobia*, *Chlamydiae*, *Lentisphaerae*, *Cyanobacteria/Chloroplast* and *Deinococcus-Thermus*) were found in seawater from this depth but not in the sponge from 780 m.

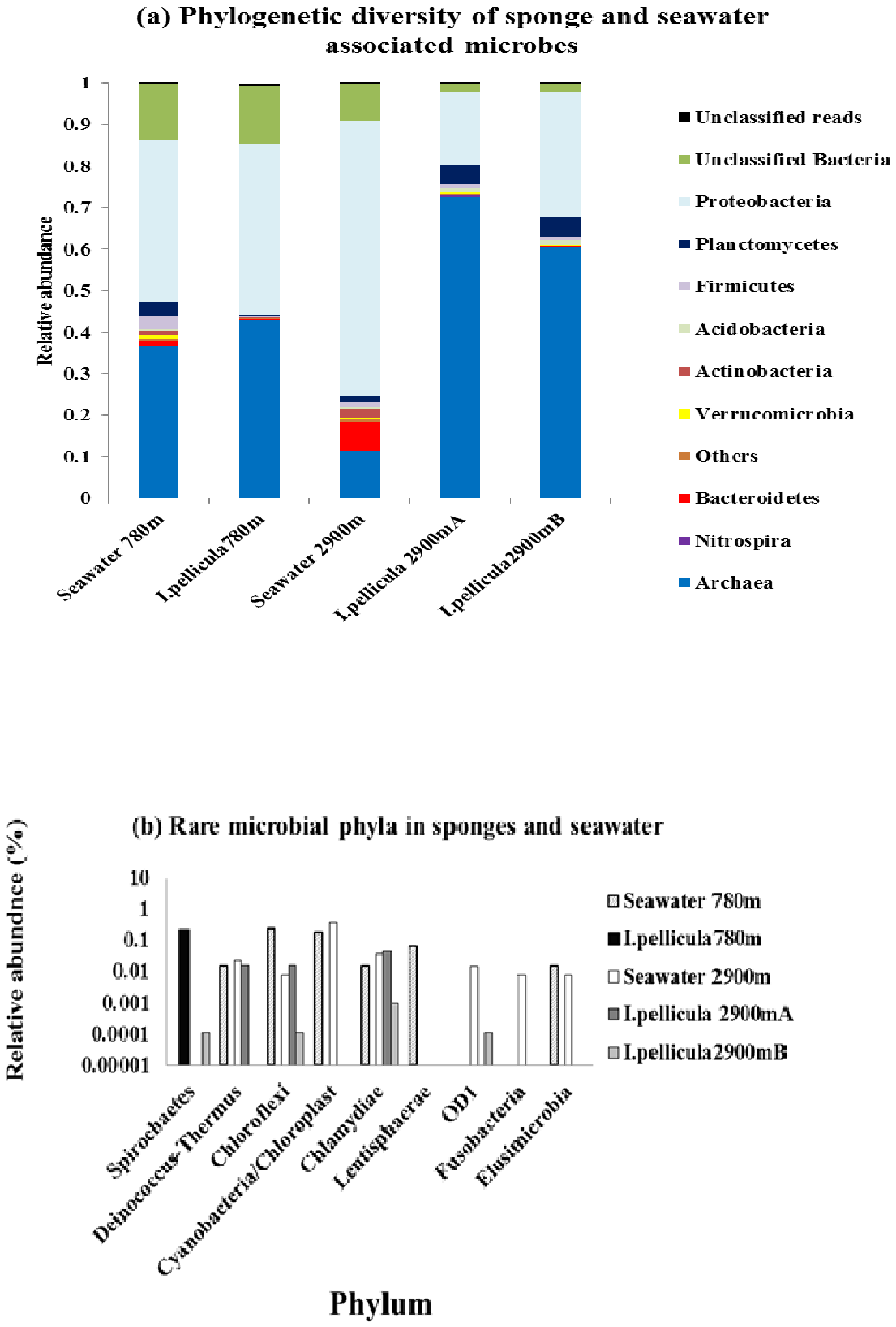

Figure 3.2: Phylogenetic affiliations of pyrosequencing reads assigned to (a) abundant microbial phyla. 'Others' in (a) represent (b) - rare microbial phyla associated with marine sponges and seawater.

The number of OTUs found in seawater was much larger than the sponge-associated cohort. At 95% sequence identity 203 OTUs (56% of the community when compared to Chao1 species richness estimator predictions of 361) were identified in the sponge while 561 OTUs were present in the seawater (70% of the Chao1 estimate of 812) (Table 3.3). At 97% sequence identity 327 OTUs were identified in the sponge compared with 817 OTUs in the seawater. Rarefaction curves (Figure 3.3) show that the seawater community is better represented in the data than is the sponge community. Shannon diversity indices of 4.89 (seawater) and 2.16 (sponge) reflect the difference in observed diversity levels. Rank-abundance curves (Figure 3.4) show that the seawater community is much more even than the sponge community, with the steepness of the curve showing that the major proportion of the sponge community is composed of a few dominant phylotypes.

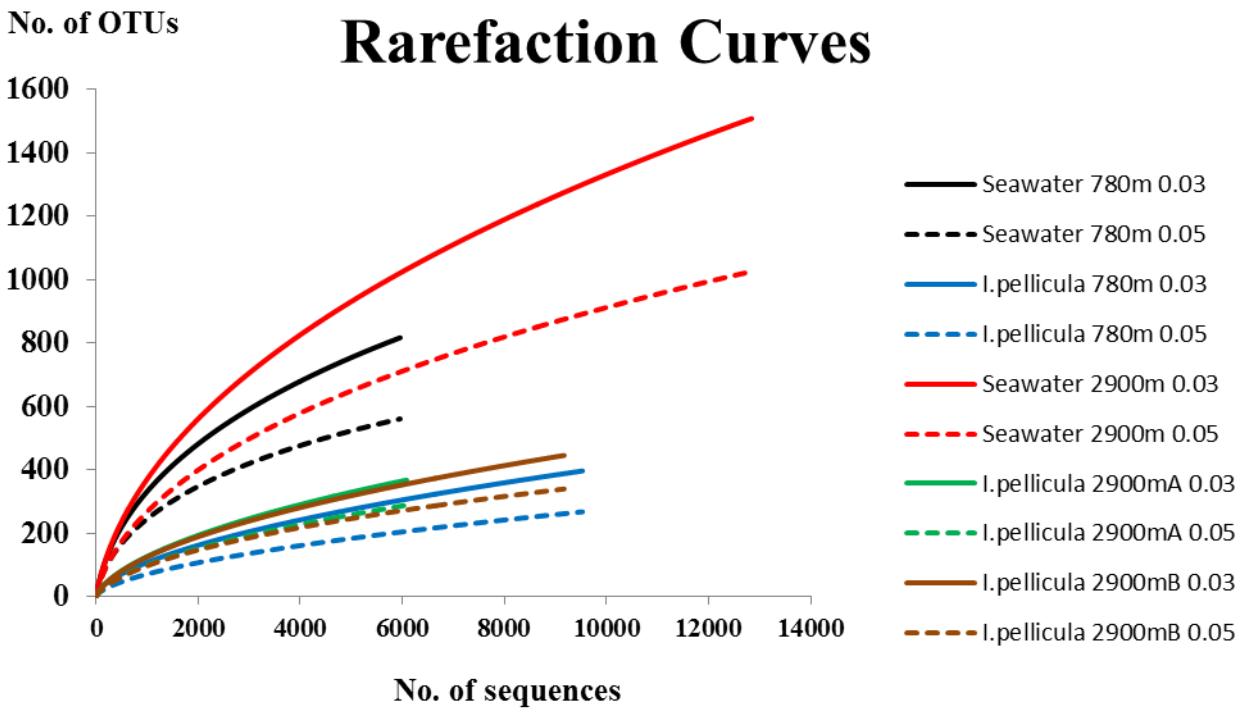

Figure 3.3: Rarefaction curves for sponge and seawater microbial communities.

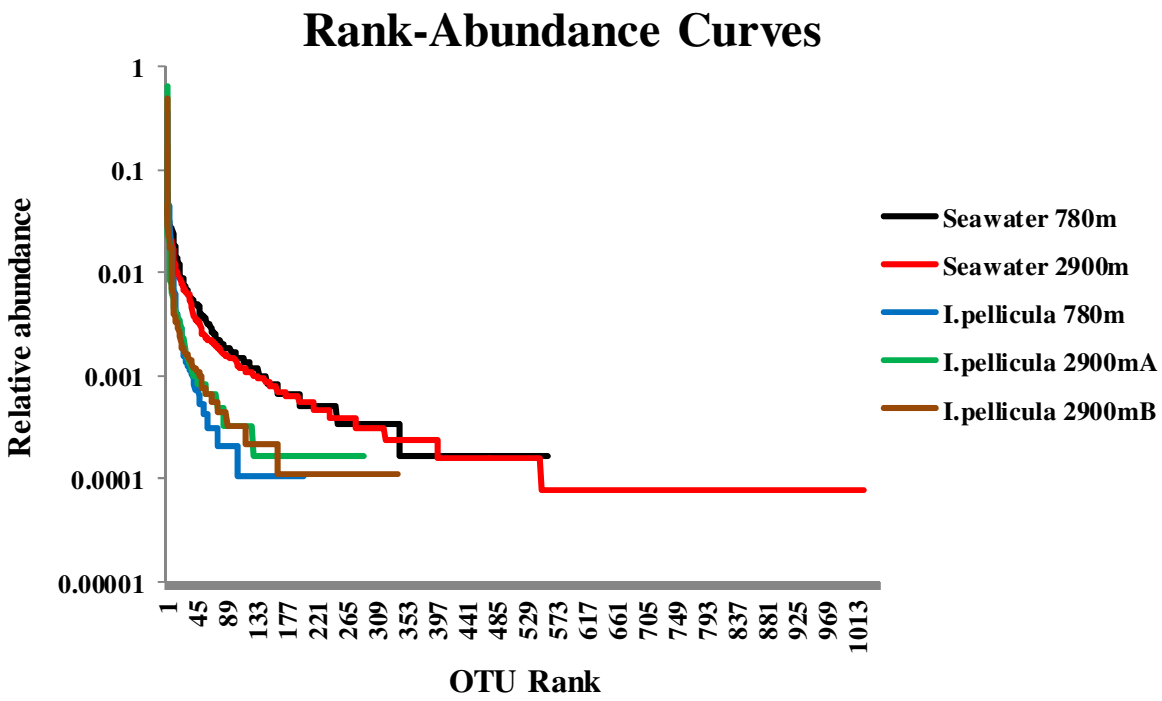

Figure 3.4: Rank-abundance curves for sponge and seawater associated microbial communities.

A notable proportion of sequence reads from each microbial community was not classified to phylum level (14.8% of sponge derived reads, 14% of seawater reads). Most sequences were assigned to two taxa, one archaeal and one bacterial. *Archaea* and *Proteobacteria* combined accounted for 70% of sequences (81% of classified sequences) from seawater while *Archaea* and *Proteobacteria* combined comprised 82% of sequence reads (97% of classified reads) from the sponge (Figure 3.2a). Proteobacterial reads comprised 40% of sequences from both sponge and seawater. A large number of proteobacterial sequences from seawater (31.6% of proteobacterial reads, 12.4% of total reads) were not classified below the phylum level. From the sponge just 1.76% of total sequence reads (4.33% of proteobacterial reads) were not assigned to a class. Gamma-Proteobacteria comprised the most abundant proteobacterial class from both samples (14% of all seawater derived sequences, 37% of sponge derived reads). While ~10% of sequences from seawater recruited to the α- class of *Proteobacteria*, less than 1% of sponge derived sequences were assigned to this class (Supplementary Table S3.1, see Appendix). Delta-Proteobacterial sequences were more abundant in seawater (~2% of reads) than in

the sponge (0.03%), as were β-Proteobacterial sequences (0.43% of the seawater community, 0.02% of sponge derived reads).

Minor but significant proportions of sponge-associated sequences were assigned to the phyla *Planctomycetes* (0.5% of reads), *Euryarchaeota* (0.43% of reads) and *Spirochaetes* (0.23%) while rarer phyla encountered in the sponge included *Firmicutes*, *Bacteroidetes*, *Nitrospira*, *Actinobacteria*, *Acidobacteria* and *Verrucomicrobia* (Figure 3.3a). In the seawater 3% of sequences were classified as *Planctomycetes*, where other notable phyla were *Firmicutes* (3%), *Bacteroidetes*, *Actinobacteria* and *Verrucomicrobia* (1% of reads each) (Figure 3.3a). Bacterial phyla detected at low abundance in seawater but not in the sponge were *Chloroflexi*, *Cyanobacteria/Chloroplast*, *Elusimicrobia*, *Chlamydiae*, *Lentisphaerae* and *Deinococcus-Thermus* (Figure 3.3b).

3.4.5 Sponge and seawater from 2900 m

Archaeal sequences were highly abundant in both the sponges from 2900 m (60.3 & 72.6% of reads) but much less so in the seawater sample (11.3% of reads) from that depth. Sequences representing 15 bacterial phyla or candidate phyla were found in seawater from 2900 m while only 11 and 12 bacterial phyla were found in sponges from this depth (Figures 3.2a & 3.2b). No bacterial phylum which was absent from seawater was found to be common to both sponge individuals from this depth. *Spirochaetes* were found in one *I. pellicula* individual but in neither the other sponge from this depth nor the seawater from this depth. *Fusobacteria*, *Elusimicrobia* and *Cyanobacteria* were found in seawater while not found in either sponge individual from 2900 m.

Microbial diversity in sponges was much lower than in seawater. Seawater contained 1026 OTUs (95% sequence similarity) while sponges hosted 289 (*I. pellicula* 2900m sample A) and 340 (*I. pellicula* 2900m sample B) OTUs. Chao1 species richness estimates, calculated at 95% sequence identity (Table 3.3) suggest that ~49-52% of the sponge communities and 58% of the seawater community were sampled in this study. These estimates are reflected in rarefaction curves (Figure 3.3) where curves do not significantly plateau. High levels of microbial diversity in seawater are reflected in Shannon Index values (Table 3.3) while relatively low diversity levels in sponges are also shown by Shannon Index values. Rank-abundance curves (Figure

3.4) show that the seawater community is more even than that of either sponge individual.

While a large proportion of seawater sequences (9.35% of reads) were not classified below the domain level, just 2.76% (*I. pellicula* 2900m sample A) and 3.5% (*I. pellicula* 2900m sample B) of reads from sponges were not assigned to phyla. Within the domain archaea, seawater and sponge communities differed greatly. The phylum *Euryarchaeota* comprised 6.5% of all sequences from seawater (57.8% of archaeal sequences) whereas this phylum only comprised 0.57% and 0.16% of all sequences from the sponges (0.79% and 0.27% of archaeal sequences). *Proteobacteria* was the dominant microbial phylum in seawater, represented by 66.35% of sequences. Proteobacterial abundance in sponges differed greatly with ~18% (*I. pellicula* 2900m sample A) and 30.4% (*I. pellicula* 2900m sample B) of sequences assigned to that phylum. Within the sponge proteobacterial cohorts, most reads were not classified below the phylum level. Gamma-Proteobacteria was the most abundant class of Proteobacteria in seawater and in both sponge individuals. This class was represented by ~40% of all seawater derived sequences but just 3.73% (*I. pellicula* 2900m sample A) and 4.33% (*I. pellicula* 2900m sample B) of sponge derived sequences. Alpha-Proteobacteria also comprised a notable proportion of seawater sequences but was rarer in sponges. Delta-Proteobacteria comprised 3.6% of the seawater community but only 0.33% (*I. pellicula* 2900m sample A) and 0.29% (*I. pellicula* 2900m sample B) of the sponge communities. Low abundance reads from the β- class of Proteobacteria were present in all three communities.

Bacteroidetes were abundant in seawater but less so in sponges. *Planctomycetes* were more abundant in sponges than in seawater. *Firmicutes* were present at similar abundances in all three samples. Three bacterial phyla (*Fusobacteria*, *Elusimicrobia* and *Cyanobacteria*) were found to be very rare in seawater and absent from both sponge individuals. *Spirochaetes* and the candidate phylum OD1 were found at low abundance in one sponge (*I. pellicula* 2900m sample A) but absent from the other sponge and the seawater from this depth. While *Nitrospira* sequences were more abundant in sponges than seawater (0.31% and 0.21% of sequences versus <0.01% of sequences), *Actinobacteria* were more abundant in seawater (2% of seawater sequences versus 0.03% and 0.06% of sponge sequences). *Acidobacteria* (1% of reads) and *Verrucomicrobia* (0.19% of reads) were found at identical relative

abundances in both sponge individuals and compared to 0.44% (*Acidobacteria*) and 0.65% (*Verrucomicrobia*) of seawater derived sequences. Low abundance *Chloroflexi* and *Chlamydiae* were common to all three samples while rare *Deinococcus-Thermus* sequences were found in seawater and one of the two sponge individuals (Figure 3.2b).

3.4.6 Phylogeny of Clustered Sponge Sequences

As there is a dearth of reference sequences of good length and quality, as well as few cultured isolates from newly recognised Archaeal phyla *Thaumarchaeota*, *Nanoarchaeota* and *Korarchaeota* from which to compare sequences, the RDP classifier tool appears to incorrectly classify archaeal sequence reads. In addition many bacterial sequences although classified as bacterial and/or proteobacterial the RDP classifier failed to provide any deeper taxonomic information for those sequence reads. BLAST analysis was used in an attempt to overcome these limitations. To gain further insight into the phylogeny of the most important sponge associates, representative sequences from all clusters of identical sequences (0% distance) which comprised at least 0.5% of the entire sponge community were investigated by BLAST searches to find the closest known relatives. Bootstrap-consensus neighbor-joining phylogenetic trees (Figures 3.5 & 3.6) show those relationships.

The archaeal sponge clusters represent ~58% (*I. pellicula* 780m), ~74% (*I. pellicula* 2900m A) and 75% (*I. pellicula* 2900m B) of the archaeal fraction from each respective sample and these sequence clusters fall exclusively into the phylum *Thaumarchaeota*. All sponge-derived sequence clusters form part of a polyphyletic clade which branches entirely separately from the publicly available sequenced genomes from the phylum *Thaumarchaeota*, *Cenarchaeum symbiosum* and *Nitrosopumilus maritimus* (Figure 3.5).

The majority of the bacterial sponge clusters fall into two distinct clades within the class γ-*Proteobacteria* (Figure 3.6). However, these clades are distinct and separate from the recognised orders within this class. One clade is closely related to uncultured γ- proteobacterial sequences from deep sea sediment, and from sponges. The other clade is closely related to a coral derived gene sequence (Figure 3.6).

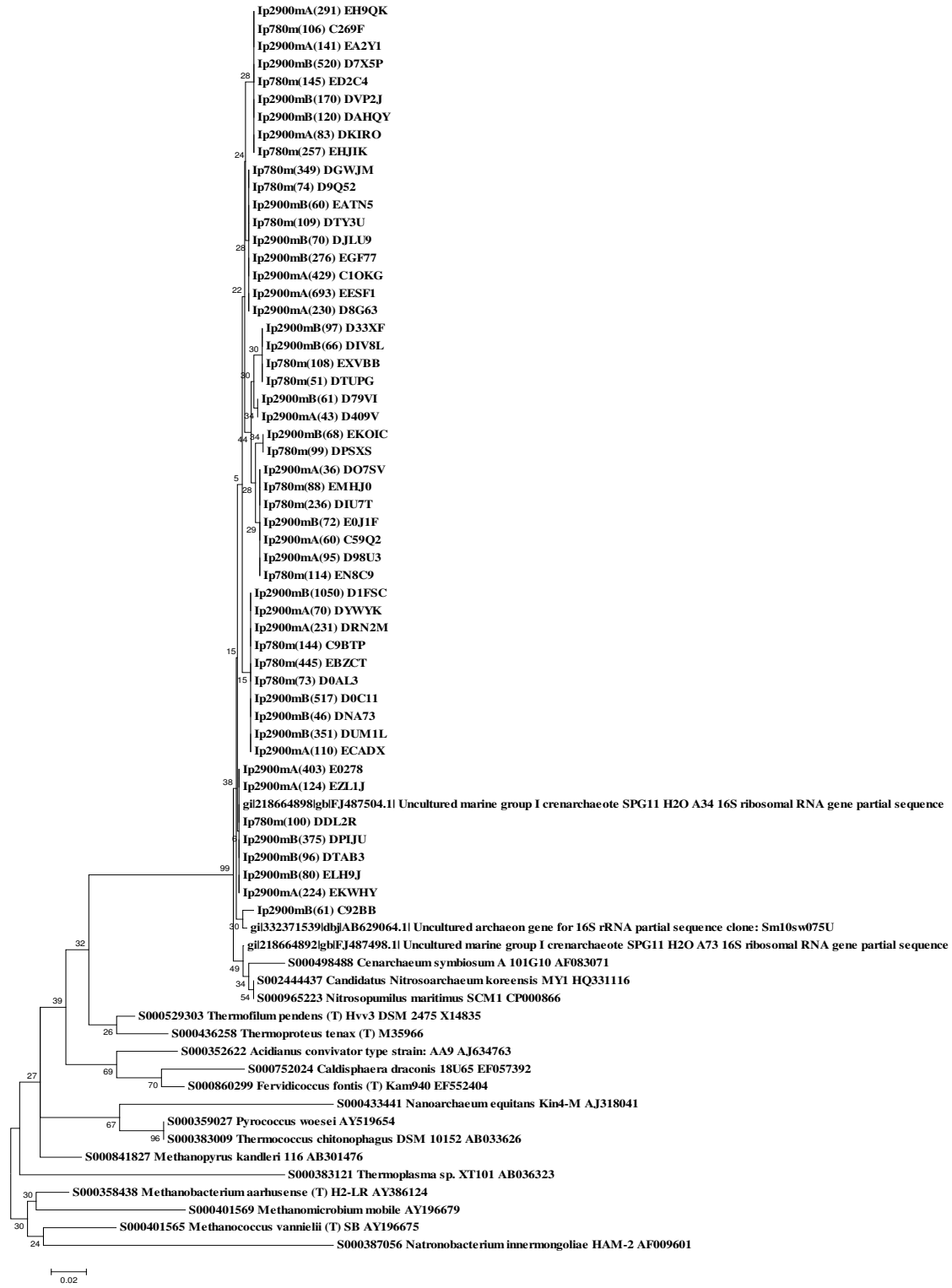

Figure 3.5: Bootstrap-consensus neighbor-joining phylogenetic true of archaeal sequence clusters comprising >0.5% of sequences from three individuals of the marine sponge *I. pellicula* in the phylum *Archaea*. Numbers in parentheses represent the number of sequences in that cluster.

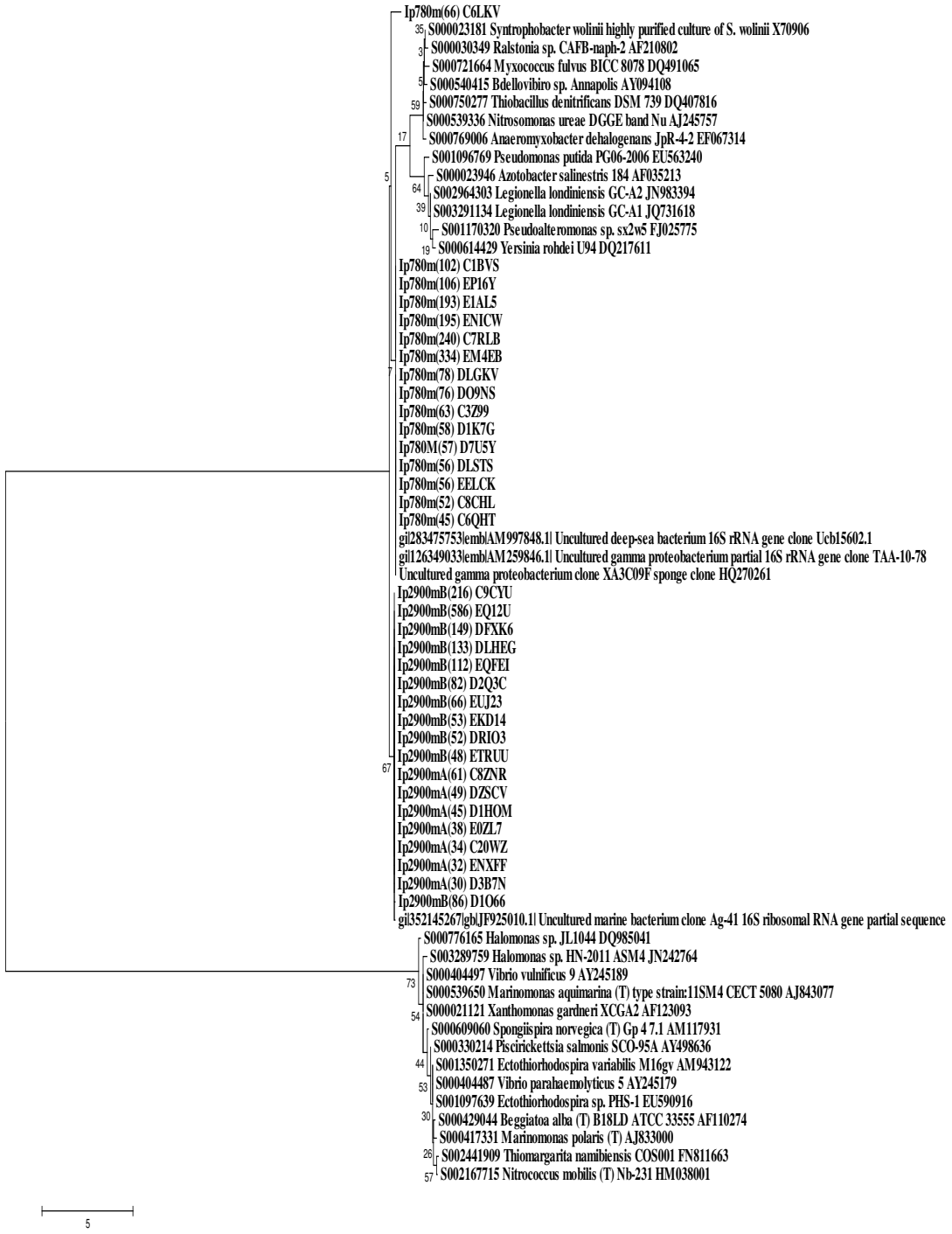

Figure 3.6: Bootstrap-consensus neighbor-joining phylogenetic tree of sponge derived sponge bacterial sequence clusters comprising >0.5% of sequences from three individuals of the sponge *I. pellicula*. Numbers in parentheses represent the number of sequences in that cluster.

3.5 Discussion

3.5.1 Context of this study

Descriptions of sponge-associated microbes now span more than four decades (Vacelet, 1971). Initially, microscopy and culture isolation were used. Subsequently, the advent of molecular techniques led to the identification of latent sponge endosymbionts through PCR amplification and sequencing of 16S rRNA genes directly from sponge metagenomic DNA. This led to the identification of members of 16 bacterial phyla or candidate phyla and two major archaeal phyla up to 2007 (Taylor *et al*., 2007). However, those labour intensive and costly methods meant that prior to 2010 the largest sponge derived 16S rRNA clone library contained fewer than 600 clones (Webster *et al*., 2010). This resulted in the most abundant sponge-associated taxa being over-represented in analyses with more rare phylotypes being overlooked.

Since the development of pyrosequencing, more comprehensive descriptions of sponge microbial communities have appeared in the scientific literature (Webster *et al*., 2010; Lee *et al*., 2010; Schmitt *et al*., 2011; Jackson *et al*., 2012; White *et al*., 2012). These studies when combined have contributed >700000 sponge-derived 16S rRNA sequences to public databases, orders of magnitude more than the ~7500 sequences recently studied in a 'sponge-specific cluster' analysis (Simister *et al.,* 2012). Consequently, at least 32 bacterial phyla and two archaeal divisions have now been found in sponge tissues worldwide, including rare but recurring phyla. Half of the detected phyla had not been reported upon in sponges prior to pyrosequencing studies.

In addition, these studies have described various ecological aspects of sponge-microbe associations, including bacterial community structures (Webster *et al*., 2010; Jackson *et al*., 2012); bacterial-archaeal relative abundances (Lee *et al*., 2011); core, variable and species-specific communities (Schmitt *et al*., 2011), vertical symbiont transmission (Webster and colleagues) and seasonal variations in bacterial community structures (White *et al*., 2012). Whereas a diverse range of 15 different sponge species have been examined in the aforementioned studies, all sampling was performed by SCUBA diving up to depths of 20 m. Other studies have reported

microorganisms and microbial communities from sponges in deeper waters (Brück *et al*., 2008 -550 ft; Cassler *et al*., 2008 – 212 m; Brück *et al*., 2010 -300 m; Nishijima *et al*., 2010 – 686 m; Meyer & Kuever, 2008 – 1127 m). No study has however, to date, reported on sponge-associated bacterial and archaeal relative abundances in such comprehensive datasets such as is reported here from such extreme ocean depths (up to 2900 m). Furthermore, prior to this study, the microbial ecology of the deep-sea marine sponge *Inflatella pellicula* was not known.

Caution is required when drawing conclusions about microbial community structures from deep-sequencing datasets. Species variations in 16S rRNA gene copy number result in differences between sequencing-read abundance and taxon cell abundance. However, these data can still reveal abundant and rare community members.

The unique profile of sequence abundances recruiting to domains *Bacteria* and *Archaea* are assumed to be robust as the primers and other experimental parameters applied here have been previously applied to sponge communities from shallow waters in the Red Sea (Lee *et al*., 2011) where Archaeal relative abundances were much lower than what is reported here. Also, parallel studies in our laboratory (unpublished data) revealed Archaeal relative abundances ranging from 4-28% in three other sponge species, which suggests no primer bias exists. Interestingly, those sponges, sampled at 3 different depths, in deep waters, also showed a positive correlation between sampling depth and archaeal relative abundance.

3.5.2 Archaeal Relative Abundance and Diversity

Archaea were first reported from marine sponges in 1996 (Preston *et al*., 1996). Subsequently Margot and colleagues (Margot *et al*., 2002) reported a consistent association between *Axinellidae* and *Cenarchaeum symbiosum*. Many subsequent reports of sponge associated *Archaea* (Webster *et al*., 2001; Lee *et al*., 2003; Pape *et al*., 2006; Holmes & Blanch, 2006) include a study (Sharp *et al*., 2007) where vertical transmission of *Archaea* to sponge-host larvae was observed, indicating a close symbiotic relationship.

In our study, archaeal relative abundances differed noticeably in seawater from different depths and also in sponges from different depths. While *Archaea* were rarer in seawater with increasing depth (Figure 3.1), they were more abundant in sponges

with increasing depth. Although *Archaea* comprised less than half of the sponge community from 780 m they accounted for the major proportion of the sponge communities (up to ~72%) from 2900 m. A previous study by Pape and colleagues, using lipid biomarker analysis, reported that 79-90% of microbes inhabiting deep sea sponges (*Tenturium semisuberites*, sampled at depths of 2340 m & 2440 m) were *Archaea* (Pape *et al*., 2006). This far exceeds what has been reported by Lee and colleagues who found archaeal relative abundances ranging from 4-28% in different sponge species from shallow waters in the Red Sea (Lee *et al.*, 2011) or a study by Han and colleagues who found archaeal relative abundance of ~5% in a shallow water sponge (*Phakiella sp*., sampled at a depth of 20 m) from the South China Sea (Han *et al*., 2012). Archaea were rare or absent in different seawater samples from the Red Sea but significant populations were found here, with archaeal sequences accounting for more than one third of the seawater community from 780 m and more than one in ten of all reads from 2900 m. Similar to the findings of Lee and co-workers, vastly different archaeal populations appear to inhabit sponges compared to seawater. We noted that non-Euryarchaeotal archaea dominate the sponge communities but formed a lesser proportion of the seawater archaeal cohorts which were dominated by *Euryarchaeota*. This may be reflective of the sampling depths, as Galand and colleagues observed *Euryarchaeota* dominating archaeal communities in deep waters (1000m) in the Arctic Ocean, but that *Crenarchaeota* were dominant in surface waters (Galand *et al*, 2009).

3.5.3 Bacterial Diversity

About 2% of the 2900 m sponge-derived sequences that were classified in the domain *Bacteria* could not be assigned to phyla. Contrastingly, 9-14% of sequences from Seawater 780 m, Seawater 2900 m and *I. pellicula* 780 m though classified as *Bacteria*, were not assigned to phyla. The utility of the RDP classifier tool for assigning phyla to reads of this length from this 16S region (V5-V6) has been previously demonstrated (Lee *et al*., 2011) and thus the unclassified bacterial cohorts found here may represent previously unencountered taxa. This possibility is supported by BLAST analysis and tree-building using representative sequences of clustered sponge-derived sequences. That analysis shows that the sponge sequence clusters, although recruiting to the gamma class of proteobacteria, do not branch with

any recognised order within the class but are related to sequences of unknown genera from other marine sources – sediments, sponges and corals.

Proteobacteria was the dominant bacterial phylum in all samples but was less abundant in the deeper water sponges than in the sponge from 780 m. Although similar proportions of the Seawater 780 m and *I. pellicula* 780 m communities comprised *Proteobacteria*, 12% of the seawater cohort and 1.7% of the sponge community classified as *Proteobacteria* were not classified below phylum level. From the deeper samples large proportions of proteobacterial sequence reads (Seawater 2900m [10.6%]; *I. pellicula* 2900m sample A [11.9%]; *I. pellicula* 2900m sample B [24%]) were not classified below phylum level. The relative abundance of γ-Proteobacterial sequences in the deeper water sponges is one of the most striking features of this study. While this proteobacterial class represents 14% (Seawater 780 m) and 40% (Seawater 2900 m) of the seawater communities and 40% of the *I. pellicula* 780m community they accounted for just 3.7% and 4.3% of the deeper water sponge assemblages. Proteobacterial reads from the α- and δ- classes were clearly orders of magnitude more abundant in seawater than in sponges while β-Proteobacterial abundances across the samples showed no clear pattern (Supplementary Table S3.1, see Appendix).

All other bacterial phyla combined comprised notable proportions of the seawater communities (10% and 13%) but only a minor fraction of the *I. pellicula* 780 m community (1.2%). In contrast, both sponges from 2900 m hosted ~7% of non-proteobacterial bacteria, most of which were *Planctomycetes*, *Acidobacteria* and *Firmicutes*, which were present in similar proportions in both sponge individuals. Surprisingly, *Cyanobacteria*/*Chloroplast* sequences were found in both seawater samples, far below the photic zone. These taxa were however absent from sponges in this study.

Spirochaetes is the only bacterial phylum found in sponges but absent from seawater (Figure 3.2). *Bacteroidetes*, *Verrucomicrobia*, *Actinobacteria* and *Nitrospira* were found in all three sponges and also in both water samples apart from *Nitrospira* which was not found in the water from 780 m.

Five bacterial phyla (*Chlamydiae*, OD1, *Chloroflexi*, *Deinococcus-Thermus* and *Spirochaetes* were noted in at least one sponge but were not present in all three. Of

these, *Chlamydiae* and *Chloroflexi* were found in both sponges from 2900 m but were absent from the sponge from 780 m while none was absent at 2900 m but present at 780 m. The influence of depth and/or temperature was notable when sponge communities were compared. Sequencing reads from the bacterial taxa Gp10 (phylum *Acidobacteria*), *Selenomonadales* (phylum *Firmicutes*), *Halovibrio*, *Pseudoalteromonadales* and *Xanthomonadales* (γ-*Proteobacteria*) were all found at 2900 m but not at 780 m. Sequence reads classified as Gp6 (phylum *Acidobacteria*), *Pasteuria* (phylum *Firmicutes*), *Phycisphaera* and *Blastospirellula* (phylum *Planctomycetes*) were more abundant at 2900 m than at 780 m. Conversely, *Myxococcales* (δ-*Proteobacteria*), *Alteromonadaceae*, *Hahellaceae* and *Chromatiales* (γ-*Proteobacteria*) sequences were more abundant at 780m than at 2900 m.

3.5.4 Functional Capabilities of Sponge Symbionts

It is well established that sponges host diverse microbes which are transmitted horizontally from ambient seawater and vertically through sponge larvae (Sharp *et al*., 2007; Webster *et al*., 2010). Sponge associated microbes have also been shown to perform significant physiological roles within sponge tissues, including nutrient exchange (Wilkinson, 1983), provision of fixed nitrogen (Wilkinson, 1978b) and host metabolic waste processing (Webster *et al*., 2001; Hentschel *et al*., 2012). Furthermore, it is widely believed that bioactive secondary metabolite production by sponge associated *Actinobacteria* may play a role in host defence (Hentschel *et al*., 2001).

Many recent studies have linked the presence of *Archaea* in sponge tissues through 16S rRNA analyses, to ammonia-oxidation through PCR amplification of archaeal *amoA* genes (Meyer & Kuever, 2008; Steger *et al*., 2008; Hoffmann *et al*., 2009; Liu *et al*., 2011; Radax *et al*., 2012). Ammonia-oxidising bacteria (AOB) and nitrite-oxidising bacteria (NOB) have also been reported in sponge tissues (Bayer *et al*., 2008). Though it is tempting to speculate about the functional role of microbes through 16S rRNA surveys, further studies will be required to determine which taxa are transient or resident, a food source or a mutualist symbiont and which taxa are metabolically active and which are dormant. Nevertheless, ammonia-oxidising *Archaea* (*Thaumarchaeota*), AOB (*Nitrosomonas* and *Nitrospira* [β-*Proteobacteria*];

Nitrosococcus [γ-*Proteobacteria*]), NOB (*Nitrospira* [phylum *Nitrospira*]), sulphur-metabolising *Archaea* (*Desulfurococcaceae*) and Bacteria (*Paracoccus* [α-*Proteobacteria*]; *Ectothiorhodospiraceae* [γ-*Proteobacteria*]) were all found in sponge tissues here, where the ammonia-oxidising *Thaumarchaeota* formed an especially abundant group. The sulfur-metabolising genus, *Endozoicomonas,* which has previously been shown to form significant proportions of sponge-associated bacterial communities (Jackson *et al*., 2012), was found here in all three sponge samples but was absent from seawater and as this genus is known to be almost exclusively associated with various marine invertebrates a true symbiotic relationship is suggested.

3.6 Conclusions

The sponge-microbial communities reported here are by a considerable distance the deepest water sponges yet investigated through rRNA gene sequencing. Microbial communities in deep-sea sponges appear to be host selected and strongly influenced by sampling depth, presumably due to increased pressure, decreased temperature or both.

Archaea account for remarkable proportions of the sponge-associated communities. *Euryarchaeota* dominate the archaeal fraction of the seawater communities while sponge-archaeal assemblages are almost exclusively *Thaumarchaeota*. Gamma-Proteobacteria, which almost invariably dominate sponge-microbial communities in other studies, and comprise ~38% of the *I. pellicula* community from 780 m, only account for ~3.7-4.3% of the sponge-associates from 2900 m.

Sampling sponges from such extreme depths is logistically difficult and so only one sponge individual from 780 m was available for this study. Replicate samples would be needed to draw strong conclusions from the apparent differences in community structures between the sponges from the different depths.

Broad phylogenetic similarities between all three sponge-microbial communities suggest that this sponge species has a well-established and consistent host-selected microbiota. Rarefaction analysis reveals that despite the many thousands of sequence

reads obtained the communities were not fully represented and a deeper sequencing effort would be required for a more comprehensive view of the community structures. Deep-sea sponges examined here show a very different profile to previously examined shallow water sponges and we suggest that future attempts to describe sponge-microbial communities should not overlook the archaeal fraction, which has been shown to be significant, especially in the deep-sea, and must be presumed to play an important role in sponge host physiology.

3.7 Acknowledgements

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Chapter 4

“Mining” the Metagenomes of Marine Sponges

4.1 Abstract

Marine sponges (phylum *Porifera*) are an abundant reservoir of marine natural products. The primary producers of such natural products are often symbiotic microorganisms. Accessing the biosynthetic machinery responsible for the production of biocatalytic enzymes and bioactive compounds of pharmaceutical interest is hampered by the recalcitrant nature of the majority of microbes to laboratory culture. Metagenomic strategies have been developed to attempt to access such latent genes. Sequence-based investigations can identify the presence of genes of interest in a metagenome while function-based methods are available to probe metagenomes for activities of interest. As many microbial enzymes are employed in a multitude of industries the search for enzymes with enhanced characteristics is an ongoing endeavour. Similarly, the emergence of multi-drug resistant human pathogens necessitates the discovery and development of novel antimicrobial compounds. The metagenomes of marine sponges has offered great promise in the search for these new chemical entities. Here we used sequence-based metagenomic strategies to identify potential polyketide synthase (PKS), non-ribosomal peptide synthetase (NRPS) and laccase genes in the metagenomes of sponges from coastal waters and from the deep-sea in Irish waters. We also employ function-based strategies to identify lipolytic and antibacterial activities from cloned DNA from sponge metagenomes. Diverse PKS and NRPS gene fragments were identified in the metagenome of *Raspailia ramosa* including genes closely related to genes responsible for the production of known antimicrobial compounds but also gene fragments only distantly related to known genes. We have also identified possible laccase genes in the metagenome of *Stelletta normani*. A large insert fosmid clone library was constructed from the metagenome of same sponge. Clones from this library displayed lipase activities and antibacterial activity. Preliminary sequence analysis of the cloned inserts suggests lipase genes from diverse bacterial taxa were cloned while insert of the antibacterial clone is likely of actinobacterial origin with similarities to genes involved in polyketide or non-ribosomal peptide production.

4.2 Introduction

The term ‘metagenome’ was coined in the late 1990s by Jo Handelsman and colleagues in reference to the total genetic material of a soil derived microbial community (Handelsman *et al*., 1998). There, the authors described methods to potentially access biosynthetic resources from microorganisms without the necessity to isolate those organisms in pure culture. Only a minor fraction of microbes have thus far proven amenable to culture under laboratory conditions and the latent majority are likely to possess diverse genetic capabilities and previously inaccessible biosynthetic capabilities. Such biosynthetic genes offer great promise for the industrial biotechnological and pharmacological sectors. Metagenomics has developed over the last decade and a half, whereby sophisticated sequence led and function led tools and systems have been developed to investigate and exploit the metagenomes of diverse environmental sources such as soils (Henne *et al*., 2000; Rondon *et al*., 2000), aquatic sources [pond water (Ranjan *et al*., 2005), soda lakes (Rees *et al*., 2003; Kim *et al*., 2006), coastal and estuarine waters (Cottrell *et al*., 1999), deep-sea hypersaline basin (Ferrer *et al*., 2005), hydrothermal vent (Brazelton & Baross, 2009)], sediments (Huang *et al*., 2009; Kim *et al*., 2009), animal guts [cow (Ferrer *et al*., 2005b), insects (Healy *et al*., 1995; Piel *et al*., 2002), bryozoan (Hildebrand *et al*., 2004), and marine sponges (Abe *et al*., 2012; Bayer *et al*., 2012; Fiesler *et al*., 2007; Chen *et al*., 2006; Kim & Fuerst, 2006; Okamura *et al*., 2010; Pimentel-Elardo *et al*., 2012; Schirmer *et al*., 2005; Selvin *et al*., 2012).

Gene and enzyme discovery, from extreme environments in particular, offers hope that ecological evolutionary pressures of those environments have produced novel biocatalysts and bioactive compounds sufficiently different to known entities to be of great interest to industry or the biopharmaceutical sector. Indeed, functional screening of metagenomic clone libraries has led to the discovery of novel xylanases (Brennan *et al*., 2004), hydrolases (Ferrer *et al*., 2005), lipases (Lee *et al*., 2006; Selvin *et al*., 2012), amylases (Yun *et al*., 2004) and also antimicrobial compounds (Gillespie *et al*., 2002; MacNeil *et al*., 2001).

Here we screen the metagenomes of sponges, using a sequence-based strategy, for the presence of polyketide synthase (PKS), non-ribosomal synthetase (NRPS) and

laccase genes and function-based strategies for lipolytic, laccase and antimicrobial activities of interest.

Type I Polyketide synthase (PKS) genes are often contiguous genetic elements responsible for multi-modular enzyme suites which act iteratively to produce a wide diversity of often complex secondary metabolites which can display antimicrobial, anti-cancer, anti-parasitic and immuno-modulatory activities (Sherman *et al*., 2005). The emergence of antibiotic resistance in human pathogens makes the search for novel therapeutic drugs particularly urgent and the discovery of novel polyketide synthase genes can aid in that search. Similarly non-ribosomal synthetase (NRPS) genes are known to produce compounds of clinical interest (e.g. vancomycin, gramicidin) and the discovery of novel NRPS genes may lead to new drug-lead compounds.

Laccases (benzenediol: oxygen oxidoreductase EC 1.10.3.2) are multi-copper enzymes which to date have been mostly studied from fungi but which have been found in all domains of life (Ausec *et al*., 2011). In nature, laccases play roles in lignin degradation, melanin production and pigment production (fungal laccases) (Galhaup & Haltrich, 2001), lignin biosynthesis (plant laccases) (Giardina *et al*., 2010), spore-coat pigment production and copper homeostasis (bacterial laccases) (Roberts *et al*., 2002). In industry laccases have many uses including paper production, ethanol production, wine clarification, bioremediation (hydrocarbon pollutant and pesticide degradation) and dye reduction for industrial waste processing or for use in the textile industry (Mayer & Staples, 2002). For industrial biotechnological purposes heterologous expression of bacterial laccases could prove useful as eukaryotic genes are much less amenable to manipulation and over-expression.

Lipases (EC 3.1.1.3) are lipolytic enzymes which are classified by their substrate specificity. They hydrolyse triacyl glycerol to glycerol and synthesise acylglycerols (Selvin *et al*., 2012). Industrial uses of lipases include biodiesel production, fine chemical synthesis, food flavouring, cosmetic production and herbicide production (Jaeger & Eggert, 2002). Expanded enzyme activity parameters may prove useful in those industrial processes. For example, thermostable lipases (Kumari & Gupta,

2012) and cold-active, halotolerant lipases (Selvin *et al*., 2012) have recently been described and may be of use for specific industrial applications.

The physico-chemical properties of marine environments (temperature, pressure, osmolarity) coupled with the enormous microbial diversity levels associated with marine sponges (Webster *et al*., 2011, Jackson *et al*., 2012) make sponge metagenomes a potentially promising source in the search for novel genes, gene products and bioactivities.

4.3 Materials and Methods

4.3.1 Sponge Sampling

The marine sponge *Raspailia ramosa* (Class *Demospongiae*; Order *Poecilosclerida*; Family *Raspailiidae*) was collected from Lough Hyne Marine Nature Reserve, West Cork, Ireland (N 51°30', W9°18') by SCUBA diving at depths of 15-20 m. The marine sponge *Stelletta normani* (Class *Demospongiae*; Order *Astrophorida*; Family *Ancorinidae*) was collected from a depth of 1348 m in the Atlantic Ocean in Irish waters (N54° 06' W12°55') using the remotely operated vehicle (ROV) *Holland I* aboard the Irish research vessel *RV Celtic Explorer*. The sponges were rinsed with sterile artificial seawater (ASW) to remove exogenous materials, placed in sterile Ziploc bags and stored on dry ice for transport and subsequently stored at -80° C until ready for use. Artificial seawater was prepared (3.33% *w/v*) from Instant Ocean (Aquatic Eco-Systems, Inc., Apopka, FL, USA), a mineral and ion formulation commonly used in aquaria.

4.3.2 Metagenomic DNA Extraction from Sponges

Sponge tissues were ground to a fine powder under liquid nitrogen (N_2) with a sterile mortar and pestle. The ground tissue was suspended in lysis buffer [100 mM Tris, 100 mM EDTA, 1.5 M NaCl (*w/v*), 1% CTAB (*w/v*), 2% SDS (*w/v*)] - adapted from Brady, 2007. DNA was then extracted as described by Kennedy *et al*., 2008. DNA was analysed by gel electrophoresis and quantified using a spectrophotometer

[NanoDrop ND-1000 (NanoDrop, Wilmington, DE, USA)]. The DNA solutions were stored at −20°C.

4.3.3 Polymerase Chain Reactions (PCR)

4.3.3.1 Polyketide Synthase (PKS) PCR

Polyketide synthase gene fragments were PCR amplified from the metagenome of *R. ramosa*. Degenerate primers were used to target the ketosynthase domains of Type I PKS genes. PCR reactions comprised 1X buffer, 0.2 mM dNTPs, 0.1 µM primers [MDPQQRf 5'-RTYGAYCCNAGCAICG-3'; HGTGTr 5'-VGTNCCNGTGCCRTG-3' (Kim & Fuerst, 2006)], ~10 ng template DNA, 1 U Taq polymerase, sdH_2O. A touchdown PCR cycle was employed and comprised: (**1**) 95° C initial denaturation for 5 min, (**2**) 95° C denaturation for 1 min, (**3**) 60° C annealing for 1 min, minus 2° C per cycle, (**4**) 72° C primer extension for 1 min, **(5)** go to (2) 10 times, **(6)** 95° C denaturation for 1 min, **(7)** 40° C annealing for 1 min, **(8)** 72° C extension for 1 min, **(9)** go to (6) 39 times, **(10)** 72° C final extension for 10 min. PCR amplicons were analysed by gel electrophoresis.

4.3.3.2 Non-Ribosomal Peptide Synthetase (NRPS) PCR

Non-ribosomal peptide synthetase gene fragments were PCR amplified from the metagenome of the marine sponge *R. ramosa.* Degenerate primers were used to target the adenylation domain of potential NRPS genes. PCR reactions comprised 1X reaction buffer, 0.2 mM dNTPs, 4 µM primers: MTF2 [5'-GCNGGYGGYGCNTAYGTNCC-3' and MTR 5'- CCNCGDATYTTNACYTG-3' (Neilan *et al*., 1999)], ~10 ng template DNA, 1.5 U Taq polymerase, sdH_2O. PCR cycle conditions comprised initial denaturation at 94° C for 2 min, followed by 35 cycles of denaturation at 93° C for 10 s, annealing at 52° C for 20 s and extension at 72° C for 1 min, with a final extension 72° C for 10 min (Vizcaíno *et al*., 2005). Reaction products were analysed by gel electrophoresis.

4.3.3.3 Laccase PCR

Laccase gene fragments were amplified from the metagenome of *S. normani*. Degenerate primers were used to target the conserved copper binding domains of

laccase genes. PCR reactions comprised 1X reaction buffer, 0.2 mM dNTPs, 2 µM primers [Cu1AF 5'-ACMWCBGTYCAYTGGCAYGG-3' and Cu4R 5'-GRCTGTGGTACCAGAANGTNCC-3' (Ausec *et al*., 2011)], ~50 ng template DNA, 1 U Taq polymerase, sdH_2O. PCR cycle conditions comprised initial denaturation 94° C for 3 min followed by 30 cycles of denaturation at 94° C for 30 s, annealing at 48° C for 30 s and extension at 72° C for 1 min. A final extension of 72° C for 5 min followed (Ausec *et al*., 2011). PCR products were analysed by gel electrophoresis.

4.3.4 Cloning and Sequencing of PCR Amplicons

4.3.4.1 Cloning PCR Products

PCR products of the expected size (PKS: ~700 bp, NRPS: ~1 Kbp: Laccase: ~1.2 Kbp) were excised from agarose gels and purified using Qiagen Gel Extraction Kit (Qiagen Ltd, Crawley, West Sussex, UK) according to the manufacturer's instructions. Purified PCR products were cloned using Qiagen PCR Cloning Kit according to the manufacturer's instructions. Briefly, PCR products were ligated to the Qiagen pDrive vector, transformed into Qiagen EZ Competent Cells and plated on Luria Bertani agar plates containing IPTG, X-Gal and kanamycin and transformants were chosen by blue/white selection.

4.3.4.2 M13 PCR

Template DNA for M13 PCR was obtained by lysing colonies of transformants by suspending in 100 µl TE buffer and incubating at 98° C for 10 min, followed by centrifugation at 2500 g for 5 min. Supernatants served as template DNA for PCR. M13 PCR reactions comprising of 1X reaction buffer, 0.2 mM dNTPs, 4 µM primers (M13f 5'-GTAAAACGACGGCCAGT-3' and M13r 5'-GTTTTCCCAGTCACGAC-3'), 1 U Taq polymerase, template DNA (variable concentration) and sdH_2O. PCR cycle conditions comprised initial denaturation at 94° C for 5 min, followed by 35 cycles of denaturation at 94° C for 30 s, annealing at

50° C for 30 s and extension at72° C for 90 sec. A final extension at 72° C for 10 min followed. PCR products were analysed by gel electrophoresis.

4.3.4.3 Sequencing and Analysis of Cloned PCR Products

M13 PCR products were excised from agarose gels and purified using Qiagen PCR Purification Kit according to the manufacturer's instructions. Purified PCR products were sequenced by Macrogen Inc., (Macrogen Inc., Seoul, Korea) by capillary electrophoresis, single extension sequencing using a 3730xl DNA Analyser. Partial gene sequences were manually edited for quality using FinchTV (Geospiza, Inc., Seattle, WA, USA; http://www.geospiza.com). Sequences were translated using ExPASy Bioinformatics Resource Portal, hosted by the Swiss Institute for Bioinformatics (http://www.expasy.org/). Translated sequences were investigated using the Domain Enhanced Lookup Time Enhanced Basic Local Alignment Search Tool (DELTA-BLAST) on GenBank at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignment and tree construction were performed using *Mega* version 5 (http://www.megasoftware.net/) (Tamura *et al*, 2011). Alignment was performed with MUSCLE (Edgar *et al*., 2004) and tree construction was by Unweighted Pair Group Method with Arithmetic mean (UPGMA) (Sneath and Sokal, 1973) or Maximum likelihood (Zuckerlandl and Pauling, 1965) methods and included bootstrap tests (1000 replicates) (Felsenstein, 1985). Evolutionary distances were computed using the Poisson correction method (Zuckerlandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site.

4.3.5 Large Insert Metagenomic Clone Library Construction

4.3.5.1 Insert Preparation - DNA Fractionation

A large insert fosmid clone library was constructed from metagenomic DNA extracted from the marine sponge *S. normani*. Crude DNA preparations were fractionated by pulse-field gel electrophoresis (PFGE). The DNA was warmed to ~50° C and carefully loaded to a well of an agarose gel which was then electrophoresed for 16 h with initial switching time of 1 s, final switching time of 25 s, a gradient of 6 V/cm and an included angle of 120°. For size selection, DNA

standard marker ladders were loaded to the gel [1kb plus (Thermo Fisher Scientific, Waltham, MA, USA) and Lambda Ladder PFG Marker (NEB, Ipswich, MA, USA)] and electrophoresed adjacent to the sponge DNA. A gel slice corresponding to ~30-50 Kbp DNA was excised from the gel as described by Brady, 2007. DNA was electro-eluted from the gel by electrophoresis for 3 h at 80 V in 14000MWCO BioDesign Dialysis Tubing (Fisher Scientific). DNA was concentrated by centrifugation in VivaSpin20 50000 MWCO spin columns (Sigma Aldrich, Arklow, Ireland). DNA was analysed by gel electrophoresis and quantified using NanoDrop.

4.3.5.2 Insert Preparation

Size-fractionated, concentrated DNA was blunt-ended in an 80 µl reaction comprising ~2.5 µg of DNA, 1X end repair buffer, 0.25 mM dNTPs, 1 mM ATP, end-repair enzyme mix [End-It End Repair Kit (Epicentre Biotechnologies, Madison, WI, USA)] and sdH_2O according to the manufacturer's instructions. The reaction was incubated at room temperature for 45 min, the enzymes were inactivated by incubating at 70° C for 10 min and the DNA was precipitated using sodium acetate and isopropanol. The blunt ended, purified DNA was dissolved in TE buffer and stored at -20° C until ready for use.

4.3.5.3 Vector Preparation

The cloning vector pCCERI-1Fos (Figure 4.1) is a modified version of the pCC-1Fos vector (Epicentre Biotechnologies). The modification allows for conjugation of the vector to multiple heterologous hosts.

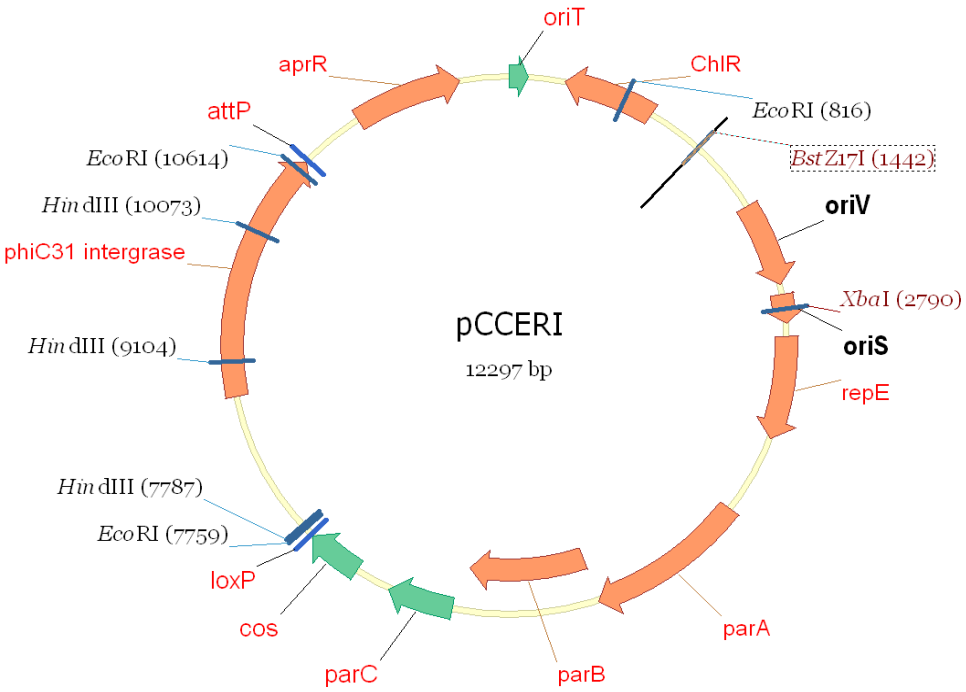

Figure 4.1: Cloning vector pCCERI-1Fos.

The vector was prepared for cloning by digestion to linearize, blunt-ending and dephosphorylating the ends. The vector was incubated at 37° C with the restriction enzyme BstZ17I (NEB) according to the manufacturer's instructions. After the digestion, antarctic alkaline phosphatase [AAP – (NEB)] was added and the incubation was continued. The phosphatase was inactivated by incubating at 70° C for 10 min and the restriction enzyme was inactivated by phenol extraction.

4.3.5.4 Ligation, Phage Packaging & Transfection

The blunt-ended DNA insert was ligated to the digested vector in a 20 µl reaction comprising 3.0 µl insert DNA (120 ng/µl), 4.0 µl digested vector (150 ng/µl), 2.0 µl T4 Ligase buffer, 1.0 µl T4 Ligase (NEB), 2.0 µl polyethylene glycol (PEG) and 8.0 µl sdH_2O. The reaction mixture was incubated overnight (~16 h) at 4° C.

The ligated product was packaged in λ Phage Packaging Extracts (Epicentre Biotechnologies) according to the manufacturer's instructions. In brief, phage packaging extract (~25 µl) was thawed on ice; ~0.2 µg of vector/insert was added and gently pipetted to mix. The solution was incubated at 30° C for 90 min. A further 25 µl packaging extract was thawed and added to the reaction, the mixture was incubated for a further 90 min at 30° C and was then diluted to 500 µl with

phage dilution buffer (10 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$). Chloroform (25 µl) was added the solution was vortexed and then stored at 4° C until ready for use.

TransforMax EPI300 *E. coli* cells (genotype: *F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ⁻ rpsL (Str^R) nupG trfA dhfr*) were grown in LB broth supplemented with 10 mM $MgSO_4$, 0.2% maltose, 12.5 µg/ml chloramphenicol and 50 µg/ml kanamycin. Cells were transfected by adding 10 µl of the packaging reaction mixture to 100 µl of competent cells. The reaction was incubated for 20 min at room temperature and then at 37° C for 75 min. The reaction efficiency was titrated thus: serial dilutions of the transfection reaction were performed using phage dilution buffer and 100 µl of each dilution was spread to LB agar plates supplemented with 50 µg/ml kanamycin. Subsequently the reaction was scaled up to obtain as many as clones as possible.

Clones was generated, picked, arrayed and replicated using a QPix 2^{XT} robotic system (Genetix, Hampshire, UK).

4.3.6 Clone Library Functional Screening

4.3.6.1 Antimicrobial Activity Screening

The clone library was screened for antimicrobial activities using a deferred antagonism assay. The test strains used were *Escherichia coli* NCIMB 12210, *Bacillus subtilis* IE32, *Staphylococcus aureus* NCIMB 9518, *Pseudomonas aeruginosa* POA1, *Candida albicans* Sc5314, *Candida glabrata* CBS138, *Saccharomyces cerevisiae* BY4741 and *Kluyveromyces marxianus* CBS86556. Clones were arrayed to Q-Trays (Genetix) on LB agar supplemented with 0.01% arabinose (*w/v*) and grown for 24 h at 37° C. Bacterial test strains were grown overnight at 37° C, shaking at 200 rpm, in 5 ml LB broth. The overnight cultures were diluted to 50 ml with LB broth and then grown until an OD_{600nm} 0.8-1.0 was reached. The culture was then diluted 1:100 with soft LB agar [0.5% agar (*w/v*)] and carefully poured over the clones arrayed on Q-Trays which had been exposed to UV light for 1 min. Q-Trays were then incubated at 28° C for up to seven days and inspected daily for zones of inhibition. Yeast test strains were grown overnight in 5

ml yeast-peptone-dextrose broth (YPD) [1% yeast extract (*w/v*), 2% peptone (*w/v*), 2% D-glucose (*w/v*)]. The overnight cultures were diluted to 50 ml with YPD broth and then grown until an OD_{600nm} 0.8-1.0 was reached. The culture was then diluted 1:50 with soft YPD agar [0.5% agar (*w/v*)] and carefully poured over the clones arrayed on Q-Trays (Genetix) which had been exposed to UV light for 1 min. Q-Trays were then incubated at 25° C for up to seven days and inspected daily for zones of inhibition.

4.3.6.2 Laccase Activity Screening

The clone library was screened for laccase activities. Three substrates were used for the screen: (1) Remazol Brilliant Blue R [RBBR ($C_{22}H_{16}N_2Na_2O_{11}S_3$)] (Sigma), (2) Guiacol [$C_6H_4(OH)(OCH_3)$] (Sigma) and (3) 2'2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) [($C_{18}H_{18}N_4O_6S_4$)] (Sigma).

(1) RBBR: The clones were arrayed to Q-Trays on LB agar supplemented with 0.04% RBBR, 250 µM copper chloride ($CuCl_2$), 50 µg/ml kanamycin, 12.5 µg/ml chloramphenicol and 0.01% arabinose (*w/v*).

(2) Guiacol: A 0.1 M stock solution of guiacol was prepared in a 0.1 M phosphate buffer pH 6.0. The clones were arrayed to Q-Trays on LB agar supplemented with 0.01% guiacol, 250 µM $CuCl_2$, 50 µg/ml kanamycin, 12.5 µg/ml chloramphenicol and 0.01% arabinose (*w/v*).

(3) ABTS: A 10 mM solution of ABTS was prepared in 10 mM sodium acetate. Clones were arrayed to Q-Trays on LB supplemented with 1 mM ABTS, 250 µM $CuCl_2$, 50 µg/ml kanamycin, 12.5 µg/ml chloramphenicol and 0.01% arabinose (*w/v*).

All plates for laccase screens were incubated at 37 ° C for 48 h and then at 25° C for a further 5 days. Plates were regularly examined for the appearance of halos around colonies.

4.3.6.3 Lipase Activity Screening

Metagenomic clones were screened for lipase activities by arraying on Q-Trays on LB agar supplemented with 1% tributyrin (Sigma), 50 µg/ml kanamycin, 12.5 µg/ml chloramphenicol and 0.01% arabinose (*w/v*). Q-Trays were incubated for 7 days at

37° C and examined daily for the appearance of halos around colonies. Clones producing halos were further investigated to determine if the observed activities were lipase activities or esterase activities by plating on LB agar supplemented with 50 µg/ml kanamycin, 12.5 µg/ml chloramphenicol, 0.01% arabinose (*w/v*), 0.001% rhodamine B (*w/v*) and 1% olive oil (*v/v*). The plates were incubated at 37° C for 3 days and examined daily for the appearance of halos around the clone colony.

4.3.7 Fosmid Analysis

A selection of fosmids from clones which produced 'hits' in any of the functional screens were investigated by end-sequencing. Clones were grown overnight in LB broth supplemented with 0.01% arabinose. Fosmids were extracted and purified using a Qiagen Miniprep Kit (Qiagen) according to the manufacturer's instructions. Fosmid end-sequencing was performed by Beckman Coulter Genomics (UK) using the sequencing primers ERI-1f (5'-ACGTTCCGCCATTCCTATG-3') and ERI-1r (5'-AACCTTCGTGTAGACTTCGG-3').

4.4 Results

4.4.1 Polyketide Synthase Genes

Partial putative polyketide synthase genes were cloned from the metagenome of *R. ramosa*. Blast analyses of 5 translated cloned sequences and tree building indicated that two distinct types of PKS were cloned (Figure 4.2).

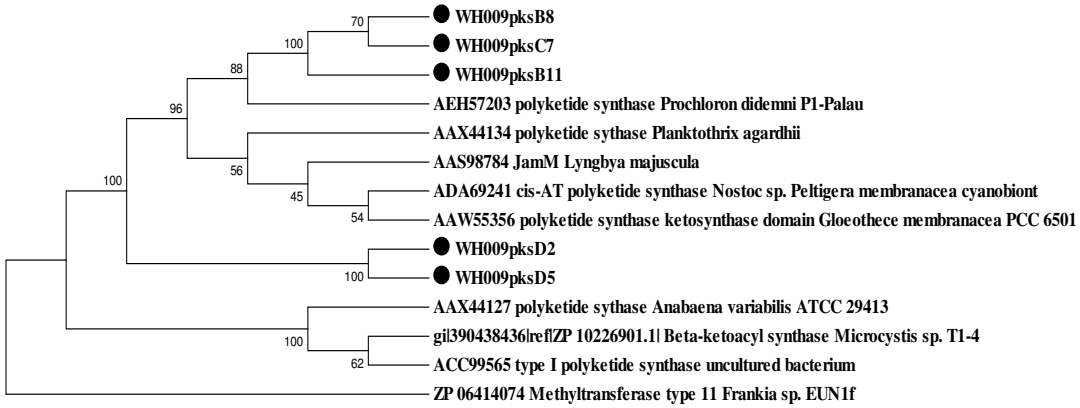

Figure 4.2: UPGMA bootstrap-consesus tree showing the evolutionary relationship of inferred amino acid sequences from translated putative PKS gene fragments cloned from the metagenome of the marine sponge *R. ramosa*. Included are the closest BLAST relatives and a methyltransferase outgroup. '●' – denotes sponge derived sequences.

One tree branch included two highly similar ketosynthase gene fragments which were only distantly related (40-45% amino acid sequence identity) to previously known genes. The closest known related sequences derive from cyanobacterial (*Anabaena* sp., *Microcystis* sp.) PKS genes. The other clade includes 3 sequences more related to each other than to any other known gene sequence, with significant amino acid sequence homology (58-73% similar) to known ketosynthase genes, including genes involved in the biosynthesis of Jamaicamide (Edwards *et al*., 2004), a neurotoxic polyketide from *Lyngbya majuscula*. In all cases a highly conserved cysteine residue (residue 104, Figure 4.3) at the enzyme active site is present.

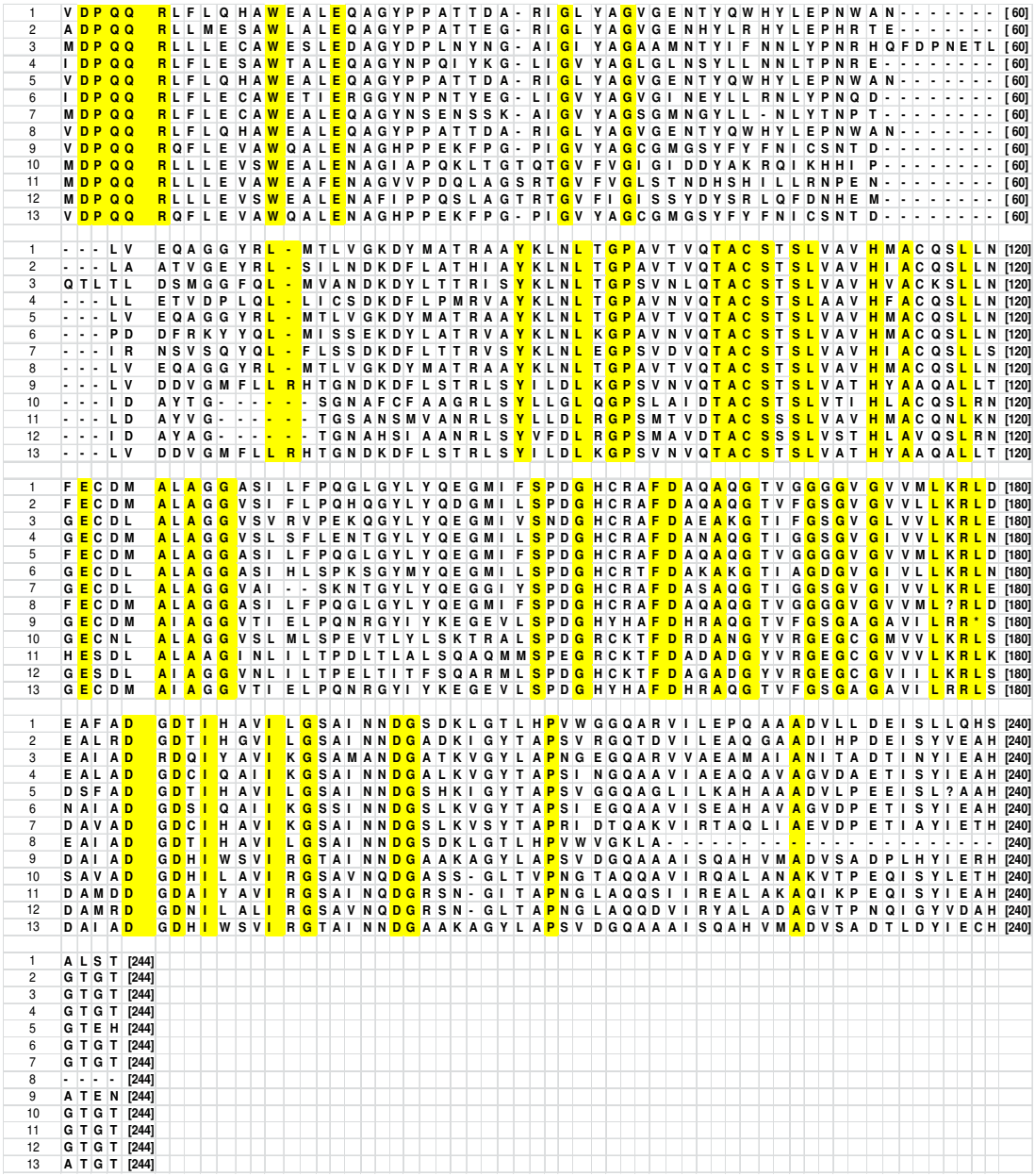

Figure 4.3: Alignment of inferred amino acid sequences of cloned putative PKS gene fragments from the metagenome of the marine sponge *R. ramosa* with the closest known BLAST relatives. Conserved residues are highlighted in yellow. (1) WH009pksB8 (2) *Prochloron didemni* P1-Palau polyketide synthase (3) *Planktothorix agardhii* polyketide synthase (4) *Nostoc* sp. cis-AT polyketide synthase (5) WH009pksB11 (6) JamM *Lyngbya majuscula* (7) *Gloeothece memranacea* polyketide synthase ketosynthase domain (8) WH009pksC7 (9) WH009pksD2 (10) *Anabaena variabilis* polyketide synthase (11) *Microcystis* sp. β-ketoacyl synthase (12) type I polyketide synthase, uncultured bacterium (13) WH009pksD5. Sponge derived sequences are denoted 'WH009'.

4.4.2 Non-Ribosomal Peptide Synthetase Genes

Partial putative NRPS gene sequences were cloned from the metagenome of the marine sponge *R. ramosa*. BLAST analyses and tree building (Figure 4.4) revealed that the 32 cloned partial genes were highly diverse with many sharing homology (amino acid sequence identities ranging from 40-99%) with genes known to be involved in antimicrobial compound biosynthesis. Cloned products were related in varying degrees to gene products from at least 21 bacterial genera from 4 bacterial phyla.

Partial genes identified here which are related to known antimicrobial biosynthetic genes shared high homology (86-98% amino acid sequence identity) with those known genes. Three cloned partial genes were related to biosynthetic genes involved in the production of the topical antibiotic Gramicidin. One sponge derived gene sequence was similar to the biosynthetic genes responsible for the production of the cytotoxic cyanobacterial product, Microcystin. Other genes identified shared significant homology with genes involved in the biosynthesis of antibacterial (Fusariscidin) and antifungal (Bacillomycin L, Mycosubtilin) compounds.

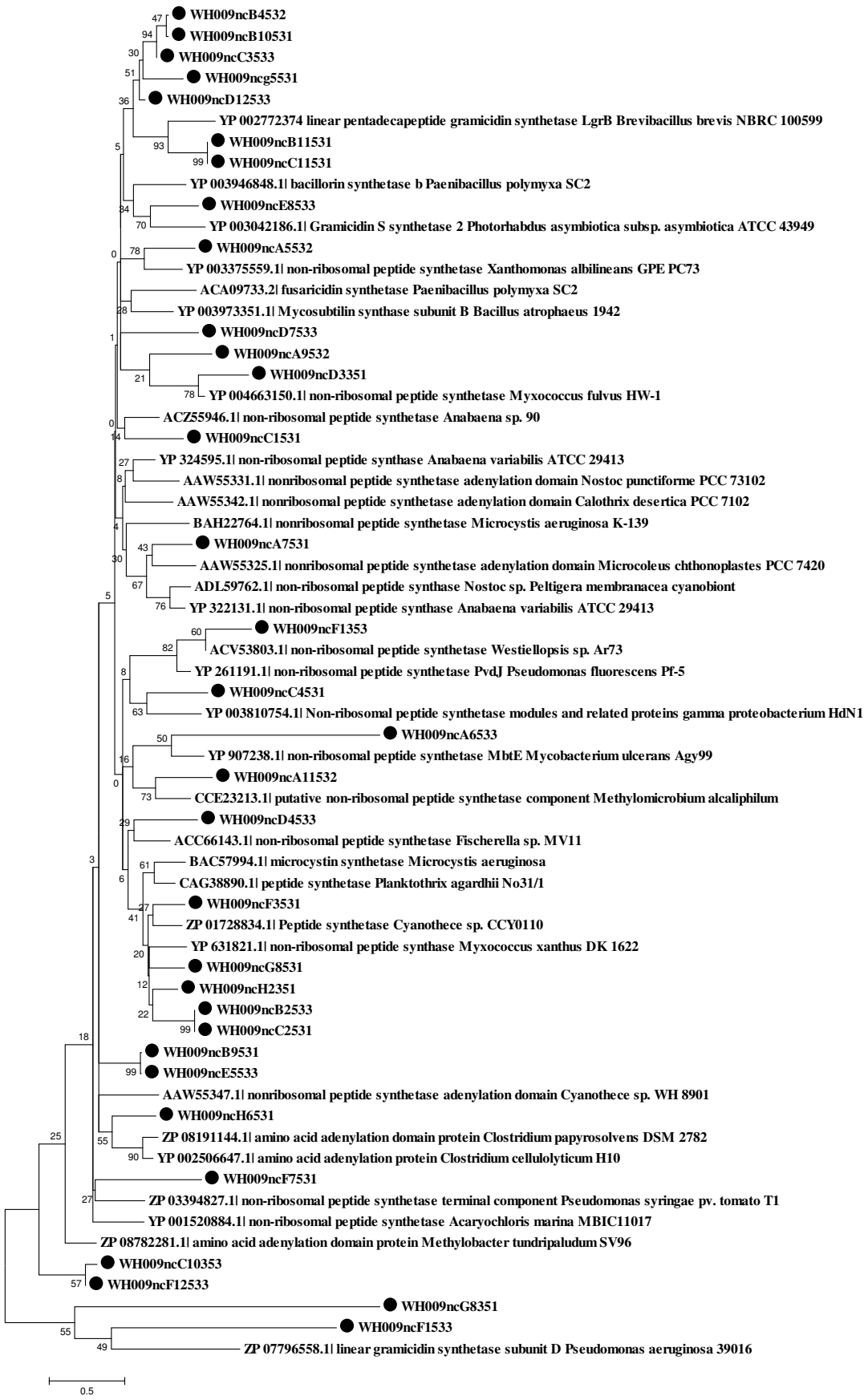
WH009ncB4532
WH009ncB10531
WH009ncC3533
WH009ncg5531
WH009ncD12533
YP 002772374 linear pentadecapeptide gramicidin synthetase LgrB Brevibacillus brevis NBRC 100599
WH009ncB11531
WH009ncC11531
YP 003946848.1| bacillorin synthetase b Paenibacillus polymyxa SC2
WH009ncE8533
YP 003042186.1| Gramicidin S synthetase 2 Photorhabdus asymbiotica subsp. asymbiotica ATCC 43949
WH009ncA5532
YP 003375559.1| non-ribosomal peptide synthetase Xanthomonas albilineans GPE PC73
ACA09733.2| fusaricidin synthetase Paenibacillus polymyxa SC2
YP 003973351.1| Mycosubtilin synthase subunit B Bacillus atrophaeus 1942
WH009ncD7533
WH009ncA9532
WH009ncD3351
YP 004663150.1| non-ribosomal peptide synthetase Myxococcus fulvus HW-1
ACZ55946.1| non-ribosomal peptide synthetase Anabaena sp. 90
WH009ncC1531
YP 324595.1| non-ribosomal peptide synthase Anabaena variabilis ATCC 29413
AAW55331.1| nonribosomal peptide synthetase adenylation domain Nostoc punctiforme PCC 73102
AAW55342.1| nonribosomal peptide synthetase adenylation domain Calothrix desertica PCC 7102
BAH22764.1| nonribosomal peptide synthetase Microcystis aeruginosa K-139
WH009ncA7531
AAW55325.1| nonribosomal peptide synthetase adenylation domain Microcoleus chthonoplastes PCC 7420
ADL59762.1| non-ribosomal peptide synthase Nostoc sp. Peltigera membranacea cyanobiont
YP 322131.1| non-ribosomal peptide synthase Anabaena variabilis ATCC 29413
WH009ncF1353
ACV53803.1| non-ribosomal peptide synthetase Westiellopsis sp. Ar73
YP 261191.1| non-ribosomal peptide synthetase PvdJ Pseudomonas fluorescens Pf-5
WH009ncC4531
YP 003810754.1| Non-ribosomal peptide synthetase modules and related proteins gamma proteobacterium HdN1
WH009ncA6533
YP 907238.1| non-ribosomal peptide synthetase MbtE Mycobacterium ulcerans Agy99
WH009ncA11532
CCE23213.1| putative non-ribosomal peptide synthetase component Methylomicrobium alcaliphilum
WH009ncD4533
ACC66143.1| non-ribosomal peptide synthetase Fischerella sp. MV11
BAC57994.1| microcystin synthetase Microcystis aeruginosa
CAG38890.1| peptide synthetase Planktothrix agardhii No31/1
WH009ncF3531
ZP 01728834.1| Peptide synthetase Cyanothece sp. CCY0110
YP 631821.1| non-ribosomal peptide synthase Myxococcus xanthus DK 1622
WH009ncG8531
WH009ncH2351
WH009ncB2533
WH009ncC2531
WH009ncB9531
WH009ncE5533
AAW55347.1| nonribosomal peptide synthetase adenylation domain Cyanothece sp. WH 8901
WH009ncH6531
ZP 08191144.1| amino acid adenylation domain protein Clostridium papyrosolvens DSM 2782
YP 002506647.1| amino acid adenylation protein Clostridium cellulolyticum H10
WH009ncF7531
ZP 03394827.1| non-ribosomal peptide synthetase terminal component Pseudomonas syringae pv. tomato T1
YP 001520884.1| non-ribosomal peptide synthetase Acaryochloris marina MBIC11017
ZP 08782281.1| amino acid adenylation domain protein Methylobacter tundripaludum SV96
WH009ncC10353
WH009ncF12533
WH009ncG8351
WH009ncF1533
ZP 07796558.1| linear gramicidin synthetase subunit D Pseudomonas aeruginosa 39016
0.5

Figure 4.4: Maximum likelihood bootstrap-consensus tree showing the evolutionary relationships between inferred amino acid sequences of partial putative NRPS gene sequences cloned from the metagenome of the marine sponge *R. ramosa* and their closest BLAST relatives. '●' – denotes sponge derived sequences.

4.4.3 Laccase genes

Partial putative laccase gene sequences were cloned from the metagenome of the marine sponge *S. normani*. Two gene fragments were obtained from the sponge; they were more similar to each other than to any other bacterial laccase gene as determined by DELTA-BLAST searches. One (BD243A_12353) shared a maximum amino acid homology of 44% with the closest known protein sequence (CumA gene from *Oligotropha carboxidovorans*). The other (BD243a_B11351) was a maximum of 23% similar to a known protein sequence (multicopper oxidase from *Enterobacter mori*). Nonetheless, conserved copper binding domains (His-Cys-His or His-X-His, where X can be a variable residue) which are characteristic of laccase enzymes were present in both of the cloned genes (Figure 4.6). Figure 4.5 shows the evolutionary relationships of the sponge derived putative laccases and the closest related known laccase proteins.

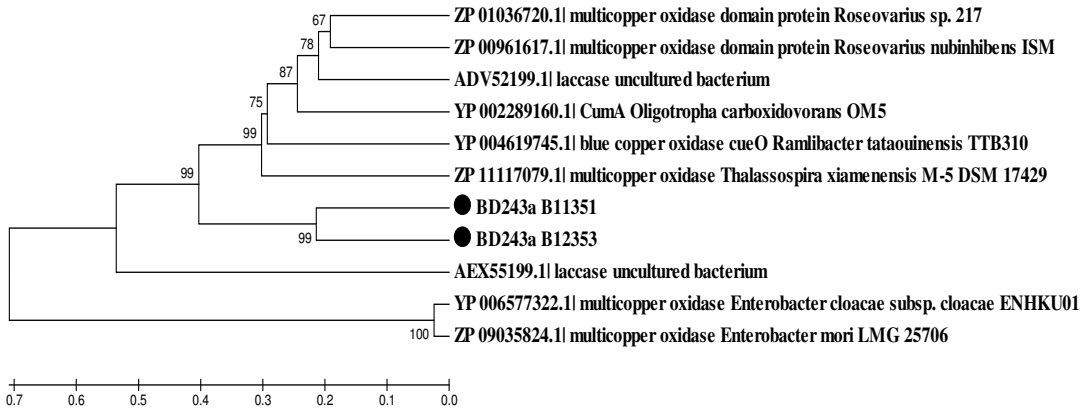

Figure 4.5: Bootstrap-consensus UPGMA tree showing the evolutionary relationships between the inferred amino acid sequences of partial putative laccase genes cloned from the metagenome of the marine sponge *S. normani* with the closest known protein sequences as determined by DELTA-BLAST. '●' – denotes sponge derived sequences.

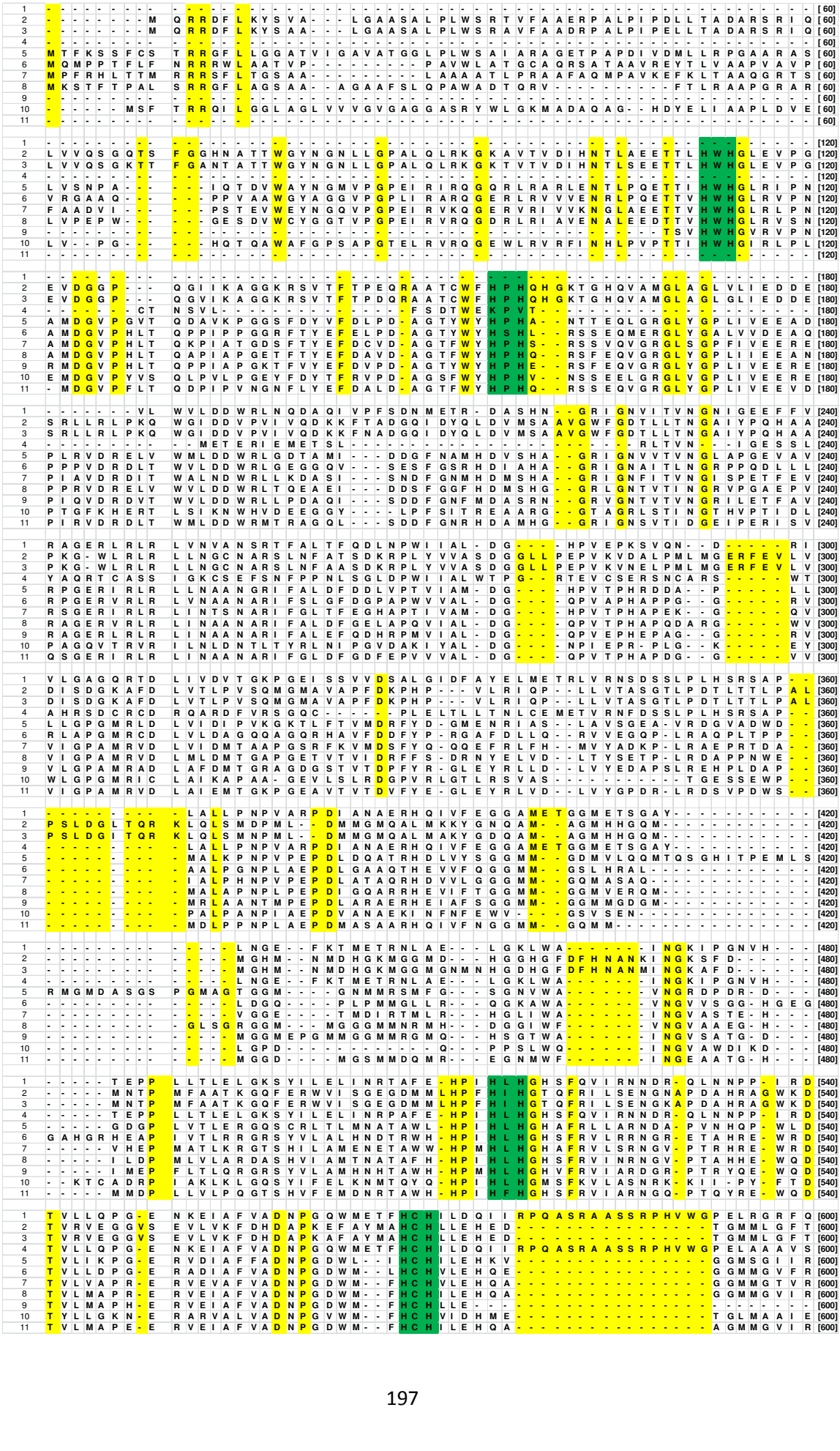

Figure 4.6: Alignment of inferred amino acid sequences from cloned putative laccase genes from the metagenome of the marine sponge *S. normani* and closest related BLAST relatives. Conserved copper binding motifs (His-Cys-His or His-X-His where X can be a variable residue) are highlighted in green while other conserved residues are highlighted in yellow. (1) BD243a_B11351 (2) *Enterobacter cloacae* multicopper oxidase (3) *Enterobacter mori* multicopper oxidase (4) BD243a_B12353(5) *Thalassospira xiamenensis* multicopper oxidase (6) *Ramlibacter tatouinensis* blue copper oxidase (7) CumA *Oligotropha carboxidovorans* (8) *Roseovarius* sp. multicopper oxidase (9) laccase uncultured bacterium (10) laccase uncultured bacterium (11) *Roseovarius nubinhibens* multicopper oxidase.

4.4.4 Functional screening of clone library.

A large insert fosmid metagenomic clone library was generated from *S. normani* DNA. The library comprised ~11500 clones. The library was screened for laccase activities using three different indicator substrates (RBBR, ABTS and guiacol). However, no laccase activities were observed under the experimental conditions employed.

The library was also screened for putative lipase activities. Ten clones were observed to produce halos on tributyrin agar on Q-Trays (Figure 4.7). The activities were confirmed by re-plating the relevant clones on identical media on petri dishes (Figure 4.8). The lipolytic activities were further assayed on media containing olive oil to determine if the observed activity was putatively lipase or esterase activity. No halos were observed on the olive oil media indicating that the activities observed were from lipase enzymes. One of the active clones (SN29K8) displayed particularly strong activity in the assay (Figure 4.9).

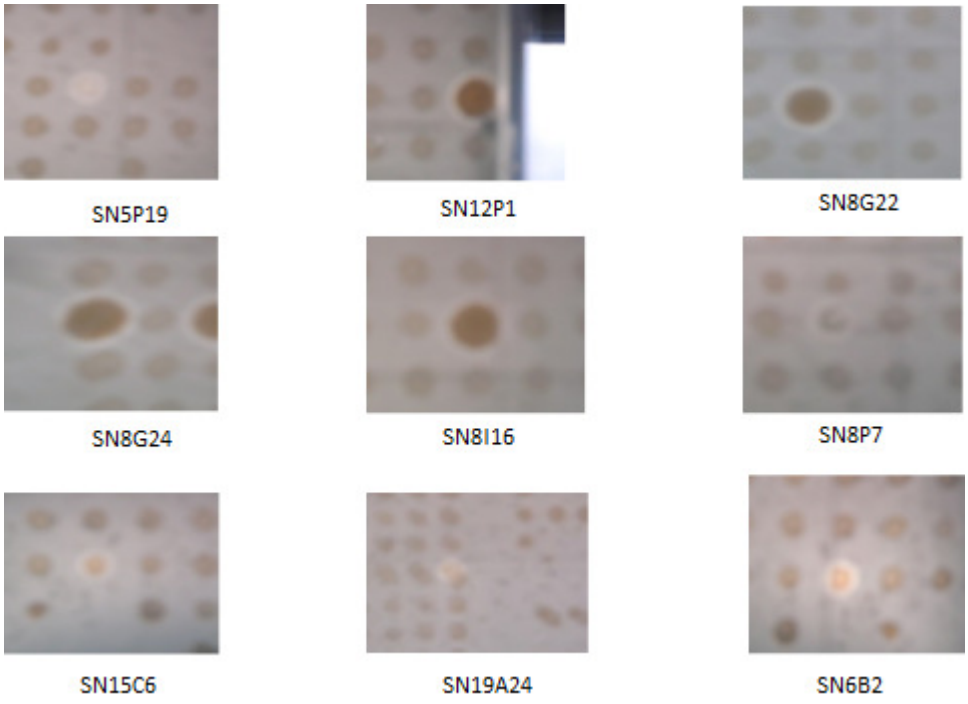

Figure 4.7: Examples of lipolytic activities of metagenomic clones on tributyrin agar.

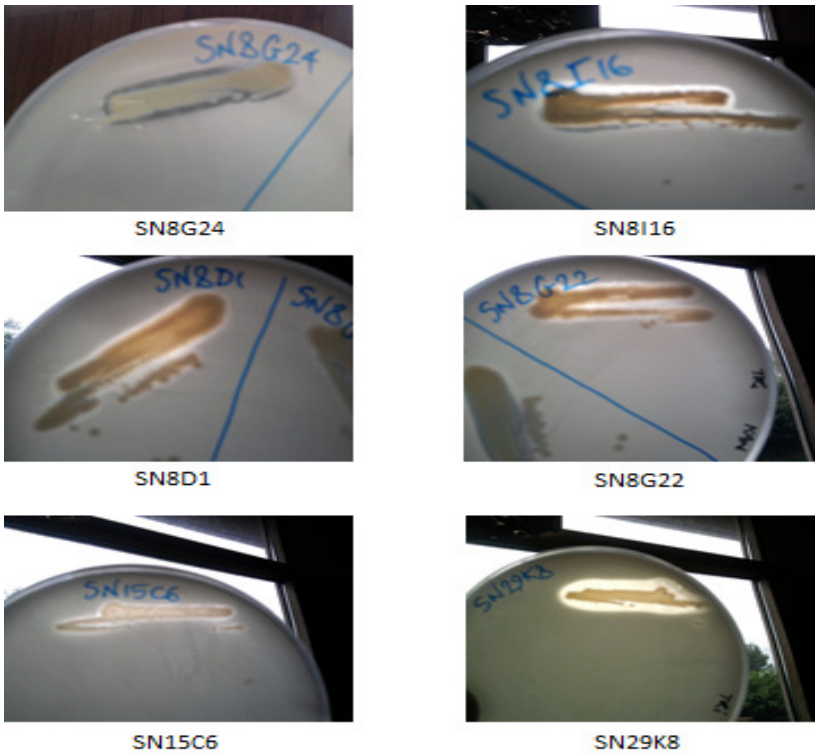

Figure 4.8: Examples of confirmed lipolytic activities of metagenomic clones on tributyrin agar.

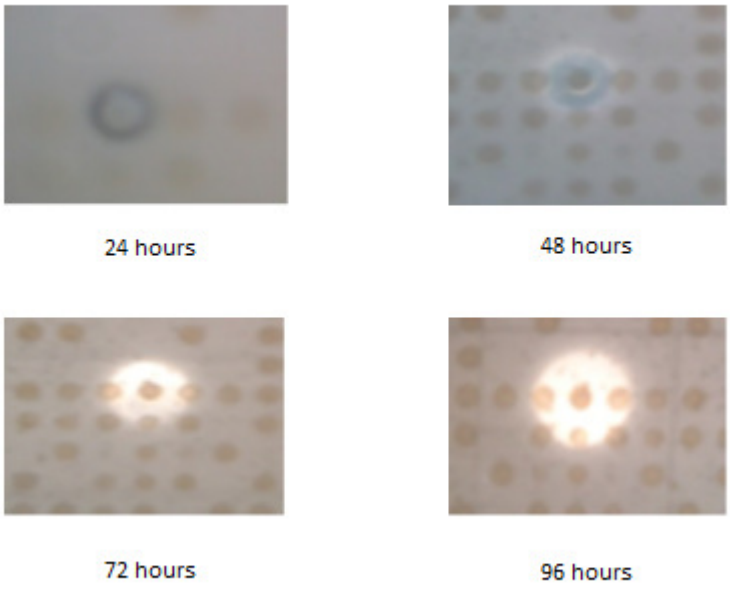

Figure 4.9: Lipolytic activity of a hyper-producing metagenomic clone (SN29K8) on tributyrin agar.

The metagenomic clone library was screened for antimicrobial activities against a panel of clinically relevant prokaryotic and eukaryotic test organisms. One clone (SN5P15) appeared to display "mild" antibacterial activity against *Pseudomonas aeruginosa*. The activity was limited to the area of the agar plate directly above the metagenomic clone (Figure 4.10).

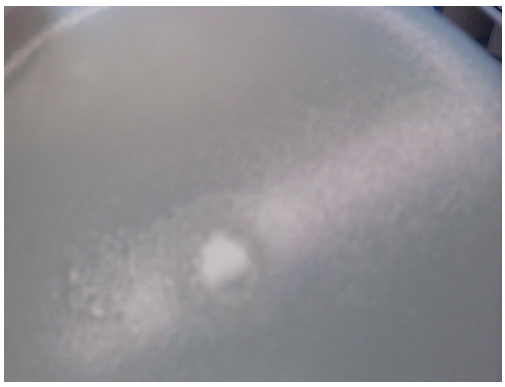

Figure 4.10: Antibacterial activity of a metagenomic clone (SN5P15) against *P. aeruginosa*.

4.4.5 Fosmid end-sequencing

End-sequencing was performed on a selection of fosmids of clones showing functional activities in an attempt to gain insight into taxonomy of the microbial source of the fosmid inserts. Sequences were analysed by BLASTx.

Lipase producing clone SN6B2: The forward reaction sequence (959 bp) shared homology with transcriptional regulator domain proteins from members of the phylum Chloroflexi (*Roseiflexus* sp., *Caldilinea* sp., *Oscillochloris* sp.). Sequence homologies were in the range 50-53%. The reverse reaction sequence (924 bp) was more similar to dehydratase proteins from α-Proteobacteria (*Bradyrhizobium* sp., *Parvibaculum* sp.) with sequence identities in the range 34-49%.

Lipase producing clone SN12P1: Blast analysis of the forward sequencing reaction product (999 bp) indicated that the insert was of α-proteobacterial origin. The closest BLAST relatives were nucleotidyl transferase proteins from *Zymomonas* sp., *Tistrella* sp. and *Sinorhizobium* sp. *Proteobacteria* with sequence identities of 58-65%.

Lipase producing cone SN15C6: The cloned insert was of uncertain origin as ambiguous BLAST results matched the forward sequence (1036 bp) to δ-*Proteobacteria* and *Bacteroidetes* genes with 25-29% sequence identity to ABC transporter genes while the reverse reaction sequence (1075 bp) was most similar (55-57% sequence identity) to transamidase genes from *Firmicutes* (*Desulfotomaculum* spp.).

Lipase producing clone SN19A24: The forward sequence reaction (1021 bp) shared homology (48-65% sequence identity) with amidase genes from a range of taxa (*Actinobacteria*, *Firmicutes*, β-*Proteobacteria*). The reverse reaction sequence (973 bp) was most related to hypothetical protein gene sequences from *Chloroflexi* and δ-*Proteobacteria* with sequence homologies ranging from 40-48%.

Lipase producing clone SN29K8: The forward reaction sequence (885 bp) was almost exclusively related to genes from δ-*Proteobacteria* (*Desulfobacter* sp., *Desulfobacula* sp., *Geobacter* sp.). The closest related sequences (49-53% sequence identity) relate to genes with roles in DNA replication or transposase protein gene sequences.

Antibacterial clone SN15P5: The 1013 bp sequence from the reverse sequencing reaction appears to be of Actinobacterial origin with homologies ranging from 80-100% with adenylate-forming domain (AFD) protein genes and acyl-activating protein (AAE) enzyme genes from *Streptomyces* sp. and *Salinispora* sp.

4.5 Discussion

4.5.1 PKS and NRPS

The marine sponge *Raspailia ramosa* hosts a diverse bacterial community with >3000 OTUs (97% sequence identity) from ten bacterial phyla reported (Jackson *et al*., 2012). Many bioactive secondary metabolites and bioactivities have also been reported from sponges of this genus (Cerda-García-Rojas & Faulkner, 1995; Yosief *et al*., 2000; Rangel *et al*., 2001; Monks *et al*., 2002; Capon *et al*., 2004; Rudi *et al*., 2004; Rudi *et al*., 2004b; Saludes *et al*., 2007; Wojnar & Northcote, 2011). We have also identified antimicrobial activities from cultured isolates from *R. ramosa* (see Chapter 2). These data combined suggest a high likelihood that the metagenome of this sponge may be a good source of potentially exploitable bioactivity.

Given that a wide variety of diverse and potentially novel PKS genes have previously been reported from the metagenomes of a wide range of marine sponges (Schirmer *et al*., 2005; Kim & Fuerst, 2006; Fiesler *et al*., 2006; Kennedy *et al*., 2008) it is perhaps not surprising that we succeeded in cloning 5 putative PKS gene fragments from the *R. ramosa* metagenome using degenerate primers targeting β-ketosynthase gene fragments. Approximately 20% of the inferred amino acid residues were conserved when aligned to the closest known PKS gene fragments (Figure 4.3) including a conserved cysteine residue at the enzyme active site. Despite this the gene fragments amplified here displayed ≤ 45% homology to known genes. It is not clear whether the sequence identity distances confer significant protein structure differences on the gene products or what the biological function of the gene products are likely to be, but it is reasonable to speculate that such sequence differences may reflect an ability to produce novel products.

A remarkably diverse number of NRPS genes fragments were also cloned from the *R. ramosa* metagenome. Thirty-two individual cloned sequences were obtained

which represent a wide variety of bacterial taxa and align to 14 separate clades in phylogenetic trees (Figure 4.4). Many of the cloned gene fragments are related to genes which are known to produce the antimicrobial peptides Gramicidin, Fusariscidin, Bacillomycin and Mycosubtilin while others are deeply branching and only distantly related to any known NRPS genes [e.g. WH009ncA6533, WH009ncG8351 and WH009ncF1533 – (Figure 4.4)]. As no NRPS gene fragment was cloned twice it is possible that the full diversity of such genes in the sponge metagenome may not yet have been identified. The majority (~90%) of NRPS gene fragments identified here share high homology (≥ 85%) with known genes including high sequence identities with the genes identified in antimicrobial compound production. This suggests that the known antibiotics or compounds which are very similar may be produced in the sponge host. This may imply a significant ecological function possibly a supplement to host defence mechanisms.

The high levels of bacterial diversity associated with *R. ramosa* together with the identification of diverse biosynthetic gene fragments of interest and the previous isolation of bacteria showing antimicrobial activities from *R. ramosa* make this species of particular research interest.

4.5.2 Laccase genes and clone library screening

The marine sponge *Stelletta normani* was sampled from an extreme environment (from the deep ocean at a depth of ~1350 m) where cold temperatures and high pressures prevail. It is likely that microbial products produced in this environment are evolutionarily adapted to that environment and are thus likely to have characteristics dissimilar to their terrestrial counterparts. Furthermore, microbial taxa from such environments may be only distantly related to previously encountered taxa (see Chapter 3) and consequently may possess genes and produce gene products with high degrees of novelty. For that reason this sponge species was chosen for the construction of a large insert metagenomic library for functional screening. A sequence based investigation was also performed to determine if laccase genes were present in the metagenome. Two distinct laccase gene fragments were successfully cloned from metagenomic DNA. Analysis of the inferred amino acid sequences of those cloned genes revealed putative laccase genes with four conserved copper-

binding domains. When the evolutionary relationships between the *S. normani* derived inferred amino acid sequences and the closest known protein sequences are considered (Figure 4.6) only ~8% of the aligned residues are conserved across all the proteins examined here. Few residues outside of the copper-binding motifs are conserved. When compared only to the individual closest known protein sequence, one cloned laccase gene here, shared only 44% amino acid sequence homology with its closest relative while the other was only 23% similar to its closest relative. BLAST analyses of our cloned gene fragments suggest that they are proteobacterial in origin. One cloned sequence aligns exclusively to laccases from α-*Proteobacteria* (*Oligotropha* sp., *Roseovarius* sp., *Ramlibacter* sp.) while the other was more similar to laccases from γ-*Proteobacteria* (*Enterobacter* spp.).The sponge was sampled from an extreme environment, the cold, deep-sea with high pressure. The environmental source may have resulted in the evolution of proteins with secondary and tertiary structures significantly different to those of their terrestrial homologues where optimal enzyme activities are presumed to diverge from those of their terrestrial origin. These properties make these biocatalysts of great interest to industry.

Despite the detection of laccase genes in the metagenome of *S. normani* no laccase activities were observed from a clone library constructed from the metagenome of this sponge. The library was however quite modest in size – 11500 clones with average insert size of ~40 Kbp – comprising ~460 Mbp of DNA. Others studies have shown that bacterial DNA is preferentially cloned over sponge DNA (Schirmer *et al*., 2005). Thus our library comprises the equivalent of approximately 230 bacterial genomes, assuming an average genome size of 2 Mbp (Gilbert & Dupont, 2011). It is clear that a much larger clone library would be required to achieve coverage which would be sufficient to reasonably expect to capture a low abundance gene sequence on a single cloned insert. Even assuming that a gene of interest was captured on a single cloned insert many obstacles to achieving detectable gene expression exist. In this case however the likelihood of achieving expression of an α-proteobacterial gene in an *E. coli* host is more reasonable than attempting to express a product from a more distantly related taxon.

The library was also screened for lipolytic activities and ten active clones were identified. The lipolytic activities were determined to be due to lipase enzymes as opposed to esterases as no activities were noted when the clones were plated on olive

oil containing media. End-sequencing of the cloned fosmid inserts was used to attempt to determine the taxonomic affiliation of the sources of the cloned genes. Some sequences proved ambiguous with sequence similarities to diverse unrelated taxa while others seemed to be conclusively from a particular taxon (e.g. *Chloroflexi*). Thus it appears that lipases from a wide range of phyla are likely to have been cloned. It will be necessary to sequence the entire fosmids to further elucidate the likely source of the inserts. This will be necessary also to confirm the presence of lipase gene sequences. Subsequent protein purification and enzyme biochemical characterisation can determine whether or not the enzymes possess characteristics of potential commercial interest.

One clone in particular (SN29K8) showed remarkable activity and was identified as a hyper-producing clone. The observed activity was quite noticeably being expressed shortly after incubation (<12 hours) and was sustained over a number of days (Figure 4.9). Further characterization of the fosmid insert, followed by biochemical characterisation of the purified protein is perhaps warranted as given the source of the metagenomic DNA from which the library was constructed, this particular cloned gene may possess future commercial potential.

Screening of the metagenomic clone library for antimicrobial activities was performed using a panel of clinically relevant prokaryotic and eukaryotic test strains. Activity was noted from one clone. The inhibition of the test strain (*P. aeruginosa*) was limited to the area of the plate directly above the clone and did not appear particularly potent under the assay conditions employed. However, the indication is that a complete operational gene cluster is likely to have been captured on a single cloned insert, and this is not trivial. For all the promise metagenomics offers, few useful antimicrobial compounds have to date been derived from libraries such as this. Sequence analysis of the fosmid insert indicates that the DNA fragment is of actinobacterial origin with significant homology to genes from *Streptomyces* sp. and *Salinispora* sp. Further, the end sequences show high sequence homology with proteins [adenylate forming domain (AFD) proteins and acyl activating enzyme (AAE)] possibly associated with NRPS, PKS or hybrid PKS-NRPS gene clusters. The opportunity exists to shuttle the fosmid to alternative heterologous expression hosts to determine if expression of the bioactive compound can be increased.

In addition, opportunities also exist to design probes based on PCR amplified laccase genes discovered here, whereupon the library can be probed for the presence of the full length laccase genes. In this manner it can be determined if the genes have been cloned even though detectable expression has not been achieved.

The results presented here clearly identify the considerable promise of metagenomic technologies and techniques to discover and exploit novel genes and gene products with potential commercial value. However much work remains to be done to determine if the enzymes and compounds discovered here can realize that potential.

4.6 Acknowledgements

We wish to thank Bernard Picton (Ulster Museum) and Rob McAllen [University College Cork – (UCC)] for collection and identification of *R. ramosa* sponge samples. We also thank Burkhardt Flemer (UCC) and Robert Phelan (UCC) for collecting *S. normani* sponge samples. We acknowledge Christine Morrow (Queens University Belfast) for identification of *S. normani*. Thanks are also due to cruise chief scientist Louise Allcock as well as the captain and crew of the *RV Celtic Explorer* for facilitating the deep-sea sponge sampling.

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Chapter 5

Maribacter spongiicola sp. nov., and *Maribacter vaceletii* sp. nov., isolated from marine sponges and emended description of the genus *Maribacter*

5.1 Abstract

Diverse bacterial populations are commonly found in close association with marine sponges (phylum *Porifera*) (Taylor *et al*., 2007) and are suspected to play symbiotic roles vital to the host (Hentschel *et al*., 2012). Bacterial phylotypes have in some cases been confirmed as sponge specific and sponge species specific (Hentschel *et al.,* 2002; Taylor *et al*., 2007; Schmitt *et al*., 2012). In a project aimed at discovering novel bioactive secondary metabolites from sponge associated bacteria, we isolated two strains of *Maribacter* spp. bacteria from marine sponges collected from Irish waters. These two non-motile Gram-negative rod-shaped bacterial strains, W13M1a^T and W15M10^T, were isolated from the marine sponges *Suberites carnosus* and *Leucosolenia* sp. respectively. Phylogenetic analyses placed these strains within the genus *Maribacter* in the *Flavobacteriaceae* family of *Bacteroidetes*. Phenotypic properties along with phylogenetic analyses suggest that these strains represent two novel species of the genus *Maribacter*.

5.2 Introduction

Culture-dependent and culture-independent investigations have identified dense and diverse bacterial populations in the tissues of marine sponges (Wilkinson, 1978; Taylor *et al*., 2007; Webster *et al*., 2010). While recent culture-independent studies have used pyrosequencing to identify remarkable levels of bacterial diversity associated with individual sponge species (Webster *et al*., 2010; Lee *et al*., 2010; Jackson *et al*., 2012), only a fraction of that diversity is amenable to lab culture thus far. Members of 35 bacterial phyla or candidate phyla have been found in association with sponges but only 7 phyla have to date been cultured (Taylor *et al*., 2007).

Nevertheless, many novel bacterial genera and species have been isolated in lab culture from sponges (Lau *et al*., 2005; Scheuermayer *et al*., 2006; Lee *et al*., 2007; Graeber *et al*., 2008; Pimentel-Elardo *et al*., 2009; Ahn *et al*., 2010; Nishijima *et al*., 2011; O'Halloran *et al*., 2012). Amongst these novel isolates are members of the phylum *Bacteroidetes* (Lau *et al*., 2006; Lau *et al*., 2006b; Lee *et al*., 2006; Mitra *et*

al., 2009; Seo *et al*., 2009; Yoon *et al*., 2010; Yoon & Oh, 2010; Haber *et al*., 2012; Yoon & Oh, 2012).

Within the phylum *Bacteroidetes*, the genus *Maribacter* was established in 2004 by Nedashkovskaya *et al*. The genus currently contains ten recognised species which form a monophyletic clade within the family *Flavobacteriaceae* (Bernardet *et al*., 2002). To date, members of the genus are exclusively known from marine habitats; seawater (*Maribacter aquivivus*, *Maribacter orientalis* [Nedashkovskaya *et al*., 2004], *Maribacter dokdonensis* [Yoon *et al*., 2005], *Maribacter forsetii* [Barbeyron *et al*., 2008]), sediment (*Maribacter sedimenticola* [Nedashkovskaya *et al*., 2004], *Maribacter arcticus* [Cho *et al*., 2008]) and alga (*Maribacter ulvicola* [Nedashkovskaya *et al*., 2004], *Maribacter polysiphoniae* [Nedashkovskaya *et al*., 2007], *Maribacter antarcticus* [Zhang *et al*., 2009], *Maribacter stanieri* [Nedashkovskaya *et al*., 2010]).

Isolation of culturable bacteria from marine sponges for the purpose of novel bioactive compound discovery was performed and here we report on the phenotypic and phylogenetic characteristics of two novel sponge-derived *Maribacter* species, one (W13M1a^{T}) isolated from the marine sponge *Suberites carnosus*, the other (W15M10^{T}) isolated from the marine sponge *Leucosolenia* sp.

5.3 Materials & Methods

5.3.1 Sampling & Culture Isolation

Sponge species were collected by SCUBA diving at a depth of 15m in Lough Hyne Marine Nature Reserve, Co. Cork, Ireland (N51°30′, W 9°18′). Sponge samples were rinsed in sterile artificial seawater (ASW), a solution prepared from a commercial synthetic ion and mineral formulation (Instant Ocean - Aquatic Eco-Systems, Inc., Apopka, FL, USA)) to remove exogenous materials. Sponge tissues (~1 g) were weighed, macerated with sterile razor blades, suspended in ASW in sterile tubes with glass beads (3mm), vortexed for 2 mins then serial diluted to 10^{-6}. Dilutions were spread to Modified Marine Agar (MMA [0.005% (*w/v*) yeast extract, 0.05% (*w/v*)

tryptone, 0.01% (*w/v*) *β*-glycerol phosphate disodium salt, pentahydrate, 3.33% (*w/v*) artificial sea salt (Instant Ocean – Aquatic Eco-Systems Inc., Apopka, FLA, USA), 1.5% (*w/v*) agar]). Culture plates were incubated at 18°C for ~4 weeks. Colonies were selected based on morphology to access as much diversity as possible, sub-cultured to ensure axenic cultures and stored in glycerol stocks at -80°C.

5.3.2 Phylogenetic Analysis

Phylogenetic analysis was performed by PCR amplification and sequencing of 16S rRNA genes. Template DNA was obtained by suspending a colony in 100 µl TE buffer and incubating at 98°C for 10 mins, the lysed cells were centrifuged at 1400 g to pellet cell debris and the resultant supernatant served as template DNA for PCR. Each 30-µl PCR reaction comprised 1X reaction buffer, 0.2 mM dNTPs, 0.5 µM forward primer 27f (5′-AGAGTTTGATCCTGGCTCAG-3′), 0.5 µM reverse primer 1492r (5′-GGTTACCTTGTTACGACTT-3′) (Lane, 1991), 1 U Taq polymerase (5 U/µl), 1.0 µl template DNA, sdH_2O. PCR cycle conditions comprised initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 s, primer annealing at 50°C for 30 s and extension at 72°C for 2 min. A final extension at 72°C for 10 min followed. PCR amplicons were analysed by electrophoresis on 1% agarose gels. PCR amplicons were sequenced by capillary electrophoresis, single extension sequencing (Macrogen Inc., Korea), using 3730xl DNA Analyser. Phylogeny of sequences was determined by BLAST analysis (Altschul *et al*., 1997). Sequence alignment and tree-building were performed using MEGA5 (Tamura *et al*., 2011). Sequences were aligned to reference sequences using ClustalW (Thompson *et al*., 1994) and tree-building was performed using Minimum Evolution (Rzhetsky & Nei, 1992), Maximum Likelihood (Tamura & Nei, 1993) and Neighbour Joining methods (Saitou & Nei, 1987). Bootstrap tests (Felsenstein, 1985) were performed 1000 times.

5.3.3 Phenotypic & Biochemical Characterisation

Colony morphology was observed after growth on 2216 agar plates (Difco) [0.5% peptone (*w/v*); 0.1% yeast extract (*w/v*); 0.01% ferric citrate (*w/v*); 1.945% sodium chloride (NaCl) (*w/v*); 0.88% magnesium chloride ($MgCl_2$) (*w/v*); 0.324% sodium sulfate (Na_2SO_4) (*w/v*); 0.018% calcium chloride ($CaCl_2$) (*w/v*); 0.0055% potassium chloride (KCl) (*w/v*); 0.0016% sodium bicarbonate ($KHCO_3$) (*w/v*); 0.0008% potassium bromide (KBr) (*w/v*); 0.0034% strontium chloride ($SrCl_2$) (*w/v*); 0.0022% boric acid (H_3BO_3) (w/v); 0.0004% sodium silicate (Na_2SiO_3) (*w/v*); 0.00024% sodium fluoride (NaF) (*w/v*); 0.00016% ammonium nitrate (NH_4NO_3) (*w/v*); 0.0008% disodium phosphate (Na_2HPO_4) (*w/v*) 1.5% agar (w/v)].

Flexirubin type determination was performed by spreading a fresh colony on a glass slide and flooding the slide with 20% potassium hydroxide (KOH) (Bernardet *et al*., 2002). Motility was assessed by phase contrast microscopy.

Catalase activity was determined by adding a drop of hydrogen peroxide (H_2O_2) to a colony on a 2216 agar plate and observing for the evolution of gas. Oxidase activity was determined by smearing a colony, on a piece of filter paper in a glass petri dish, with Kovac's reagent. The reaction was monitored for the development of a blue colouration.

The temperature growth range was determined by inoculating 2216 plates (DIFCO) and incubating at different temperatures ranging from 4°C to 45°C. Culture plates were examined periodically for up to 6 weeks. The salinity range for growth was assessed by adding different concentrations (0-10% *w/v*) of sodium chloride (NaCl) to Zobell agar plates [0.5% peptone (*w/v*); 0.1% yeast extract (*w/v*); 0.01% iron sulfate ($FeSO_4$) (*w/v*); 1.5% agar (*w/v*)]. Culture plates were incubated at 25°C for 14 days. The pH growth range was assessed by inoculating marine Cytophaga broth [0.1% tryptone (w/v); 0.05% (w/v); 0.05% yeast extract (w/v); 0.02% sodium acetate (w/v)] supplemented with 4% artificial sea salts (Sigma) at pHs ranging from 4-11. Oxygen requirement was determined by inoculating 2216 agar plates and incubating at 25°C in an anaerobic jar for 14 days.

DNase activity was investigated by inoculating DNase plates (Oxoid) to which 3ml of 33.3% (*w/v*) artificial sea salts was added followed by incubation at 25°C for 7 days. After incubation the plates were flooded with 1N HCl.

Hydrolysis of starch was determined by growth on 2216 agar plates supplemented with 1% (*w/v*) starch at 25°C for 9 days. The culture plates were then flooded with iodine. Agar hydrolysis was assessed by examining cultures grown on 2216 agar plates at 25°C for 7 days. Tween hydrolysis was assessed by inoculating 2216 agar plates supplemented with 1% Tween 80 followed by incubation at 25°C for 9 days. Plates were examined daily for the appearance of a halo of precipitation around colonies.

Acid production from glucose and starch were investigated. Modified slopes of ammonium salt sugars (0.1% diammonium phosphate [$(NH_4)_2HPO_4$] (*w/v*); 0.02% KCl (*w/v*); 0.02% magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) (*w/v*); 0.02% yeast extract (*w/v*); 0.2% Bromocresol purple (*w/v*); 1.5% agar were used. For both tests the media was supplemented with 4% (*w/v*) artificial sea salts (Instant Ocean). For the glucose test the media was supplemented with 50% glucose (*w/v*) and for the starch test 50% starch (*w/v*) was added to the media. The slopes were inoculated with a broth culture and incubated at 25°C for 28 days. The slopes were periodically examined for growth and acid production as indicated by a colour change from purple to yellow.

Resistance to Penicillin G was assessed. Broth cultures were spread to 2216 agar plates. A disc of Penicillin G (1 µg) (MAST, Reinfeld, Germany) was placed on the surface of the plates which were then incubated at 25°C for 7 days. Plates were examined daily for the appearance of a zone of inhibition.

The fatty acid compositions were determined using the Sherlock Microbial Identification System (MIDI – Microbial ID Inc., Newark, DE, USA) from cultures grown on 2216 media.

Biochemical profiles were obtained using AP1 20 E, API 20NE and API ZYM kits following the manufacturer's instructions (Biomérieux) except that incubations were

performed at 25°C and as after 24 hours no significant colour changes were observed the API strips were incubated and read after 48 and 72 hours.

5.4 Results

5.4.1 Phylogenetic Analysis

The neighbour joining tree analysis (Figure 5.1) indicated that strains W13M1a^T and W15M10^T were new species in the genus *Maribacter,* from the *Flavobacteriaceae* family of *Bacteroidetes,* and this was supported by other tree building methods (Figures 5.2 & 5.3). The near full-length (1332 bp) 16S rRNA sequence for strain W13M1a^T shared 96.5 % identity with *M. forsetii* KT02ds18-6^T and 96.1 % identity with *M. aquivivus* KMM 3949^T, the closest related type strains. The near full length (1331 bp) 16S rRNA sequence for strain W15M10^T shared 98.3 % sequence identity with *M. sedimenticola* KMM 3903^T and with *M. forsetii* KT02ds18-6^T the closest related type strains.

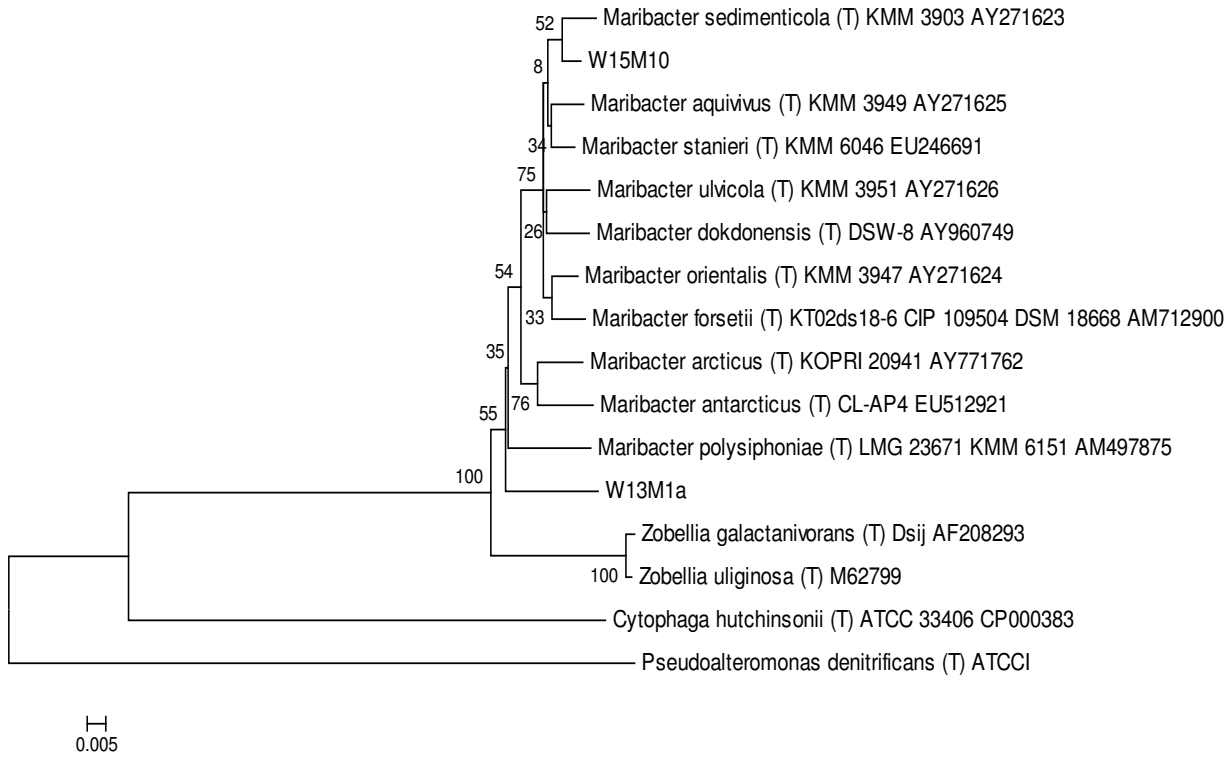

Figure 5.1: Neighbour joining bootstrap-consensus (1000 iterations) phylogenetic tree of *Maribacter* spp. type strains, novel species (*W13M1a & W15M10*) and outgroup reference taxa.

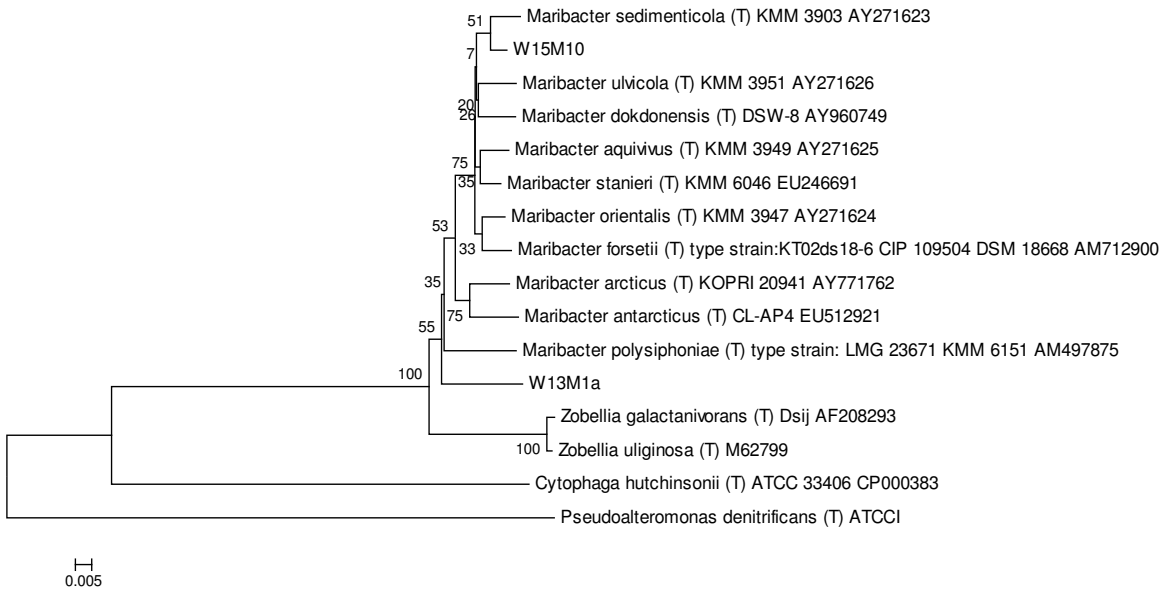

Figure 5.2: Minimum evolution bootstrap-consensus (1000 iterations) phylogenetic tree of *Maribacter* type strains, novel species and reference outgroup taxa.

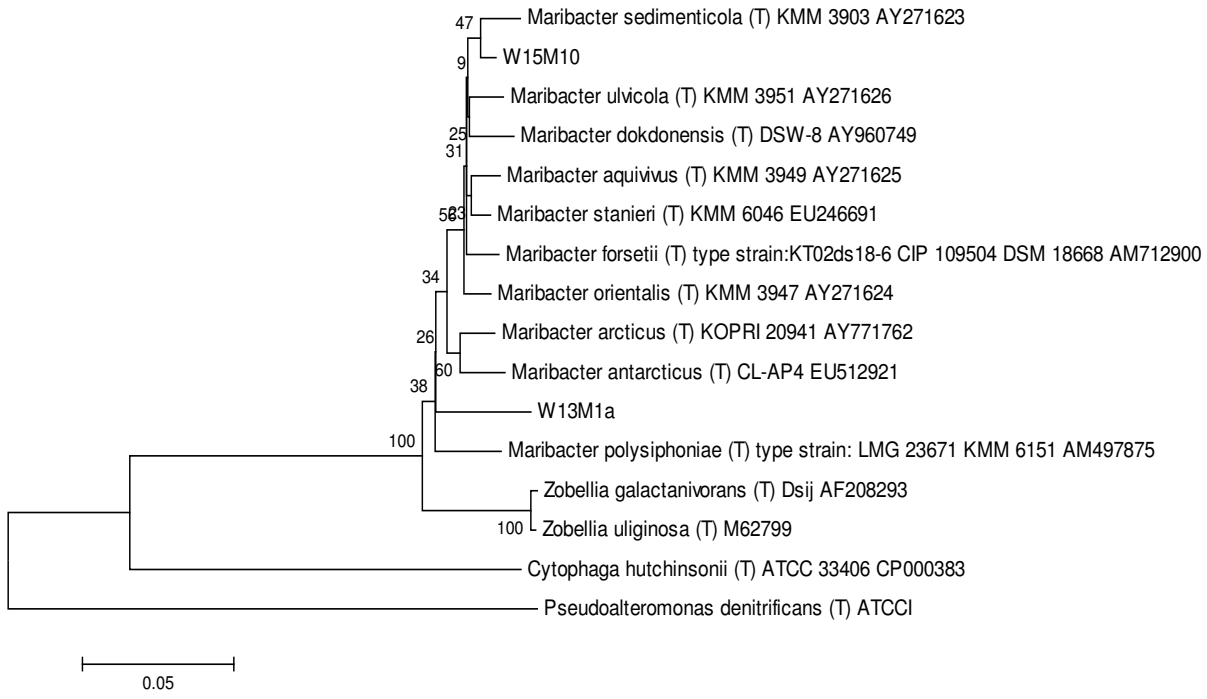

Figure 5.3: Maximum likelihood bootstrap-consensus (1000 iterations) phylogenetic tree of *Maribacter* type strains, novel species and reference outgroup taxa.

5.4.2 Biochemical Characterisation

The main fatty acids in both strains were iso-$C_{15:0}$, iso-$C_{15:1}$ G, $C_{16:1}$, iso-$C_{17:1}\omega 9c$ and iso-$C_{15:0}$ 3-OH though the composition in each strain differed (Table 5.1).

Fatty Acid	1	2	3	4	5	6	7	8	9	10	11
$C_{13:0}$	-	-	-	-	-	-	-	-	-	-	0.4
$C_{13:1}$	0.6-1.0	0.7	1.1	-	-	0.3	-	1.1	-	-	0.41
iso-$C_{14:0}$	-	-	-	-	0.4	-	1.4	-	-	-	-
$C_{14:0}$	0.6	0.5	1	0.9	0.4	0.5		0.9		0.98	0.37
$C_{14:1}\omega 5$	-	-	1	-	-	-	-	0.9	-	-	-
iso-$C_{15:0}$	10.4-14.0	20.5	12.3	19.7-20.3	11.9-12.5	10.6	15.4	13.6	12.5	8.24	15.74

anteiso-$C_{15:0}$	2.4-2.5	1.2	1.3	0.3-0.4	1	2.3	7.6	1.9	9	2.74	-
iso-$C_{15:1}$ G	15.2-16.0	16.9	13.6	15.7-16.1	11.1-11.7	10.1	9	18.9	8	12.11	16.97
$C_{15:0}$	6.1-9.9	6.3	14.5	3.5-3.8	14.7-15.5	12.3	11	8.1	3.9	-	-
$C_{15:1}\omega6c$	1.6-2.1	1.7	4.8	0.6	2.8	2.5	3.9	1.6	-	0.62	1.62
$C_{15:0}$ 2-OH	-	-	-	-	-	-	-	-	-	1	-
iso-$C_{16:1}$ H	0-0.2	-	-	-	0.7-0.9	-	1.4	-	-	-	-
iso-$C_{16:0}$	0.4-0.7	1.1	0.7	-	1.6-1.7	0.3	1.8	0.3	1	1.3	0.65
$C_{16:0}$	0.6	1	0.5	1.3-1.4	1	1.2	1..0	1		1.7	2.55
$C_{16:1}$	-	-	-	-	-	-	-	-	-	13.62	13.7
$C_{16:1}$ 2-OH	-	-	-	-	-	-	-	-	-	3.03	-
iso-$C_{17:0}$	-	-	-	-	-	-	-	-	-	-	0.5
anteiso-$C_{17:0}$	-	-	-	-	-	-	-	-	-	-	1.52
iso-$C_{17:1}\omega5c$	-	-	1.4	-	-	-	-	1.2	-	-	-
iso-$C_{17:1}\omega9c$	7.5-8.6	2.3	2.2	2.0-2.2	4.1-5.0	4	5.4	2.2	7.9	2.94	5.57
$C_{17:1}\omega8c$	0-0.3	-	-	-	0.8	-	1.2	-	-	-	0.97
$C_{17:1}\omega6c$	0.9-1.0	0.5	1.7	0.2	1.1-1.2	1.3	1.4	0.5	-	-	2.41
iso-$C_{15:0}$ 3-OH	3.5-3.6	5.4	3.2	5	3.7-3.8	2.9	5	4.1	2.8	7.54	8.58
$C_{15:0}$ 3-OH	-	2.4	2.3	1.4	1.4-1.5	1.5	2.1	1.5	14.8	-	-
iso-$C_{16:0}$ 3-OH	0.7-0.9	1.7	2.5	1.0-1.1	5.9-6.8	2.1	9.1	1.7	4	6.63	2.65
$C_{16:0}$ 3-OH	0.9-1.2	2.2	2.9	5.4-5.5	1.8-2.2	3	0.5	3.7	1.5	4.79	1.76
$C_{17:0}$ 3-OH	-	-	-	-	-	-	-	-	-	-	0.51
iso-$C_{17:0}$ 3-OH	11.1-11.9	20.4	11.6	28.6-29.2	18.3-19.7	18.8	7.2	14.5	19.1	30.66	22.29
$C_{17:0}$ 2-OH	0.3-0.4	-	-	-	0-0.3	-	1.4	-	3.8	2.1	-
iso-$C_{18:1}$	-	-	-	-	-	2.4	-	-	-	-	-
$C_{18:1}\omega5c$	-	-	-	-	-	-	-	-	-	-	0.45
Summed feature 3	11.4-14.8	5.8	12.9	8.4-8.6	10.1-11.0	11.4	10.1	12.2	-	-	-
Summed feature 4	0-1.2	-	-	-	-	-	-	-	-	-	-
unknown fatty acid	-	-	-	-	-	-	-	-	2.8	-	-

Table 5.1: Percentage whole-cell fatty acids of species of the genus *Maribacter*. 1. *Maribacter stanieri* KMM 6046^T; 2. *Maribacter sedimenticola* KMM 3903^T; 3. *Maribacter aquivivus* KMM 3949^T; 4. *Maribacter dokdonensis* KCTC 12393^T; 5. *Maribacter forsetii* KT02ds18-6^T; 6. *Maribacter orientalis* KMM 3947^T; 7. *Maribacter polysiphoniae* KMM 6151^T; 8. *Maribacter ulvicola* KMM 3951^T; 9.

Maribacter arcticus KOPRI 20941^T ; 10. W15M10^T ; 11. W13M1a^T. Summed feature 3 & summed feature 4 comprise fatty acids that were not separated as detailed in Nedashkovskaya *et al*., 2010. Data for columns 1-9 are taken from Nedashkovskaya *et al*., 2010.

5.4.3 Phenotypic Characterisation

Strains W13M1a^T and W15M10^T were heterotrophic, Gram-negative, orange pigmented non-motile organisms whose growth is strictly aerobic. Many phenotypic and biochemical features previously described for the genus *Maribacter* were observed in these strains (Table 5.2), however some important differences were observed which require an emended description of the genus. These strains were catalase positive, oxidase positive and alkaline phosphatase positive and hydrolysed Tween 80, consistent with the previous description of this genus (Nedashkovskaya *et al*., 2010). These strains did not reduce nitrate, could not hydrolyse agar or gelatin and did not produce acid from glucose or starch. However, when sea salts were added to glucose strain W15M10^T produced acid and when sea salts were added to starch strain W13M1a^T produced acid. Contrary to previous descriptions of the genus, these strains were non-motile and a major fatty acid in both was $C_{16:1}$, a fatty acid not reported in other *Maribacter* spp. type strains. Strain W13M1a^T produced indole after 72 hours incubation, was arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase positive as well as producing flexirubin type pigment. This strain does not grow below 10°C.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Flexirubin type pigment	-	-	-	-	-	-	-	-	-	-	+
Motility	+	+	+	+	+	+	+	+	+	-	-
Temperature range for growth (°C)	4-35	4-30	4-30	4-35	4-32	4-32	4-41	4-33	4-32	4-30	10-30
Salinity range for growth	1.5-8	1-7	1-7	1-10	0-9	1-5	1-8	1-6	1-4	1-6	2-5*
Nitrate reduction	+	+	+	-	-	-	-	+	-	-	-
β-Galactosidase activity	+	+	-	-	+	+	+	-	+	+	-
Acetoin production	w	-	+	ND	ND	-	w	-	-	+	+†
Arginine dihydrolase	-	-	-	-	-	-	-	-	-	-	+†
Lysine decarboxylase	-	-	-	-	-	-	-	-	-	-	+†
Ornithine decarboxylase	-	-	-	-	-	-	-	-	-	-	+†
Tryptophan deaminase	-	-	-	-	-	-	-	-	-	+†	+†
Indole production	-	-	-	-	-	-	-	-	-	-	+†
Hydrolysis of;											
Agar	-	+	-	+	-	-	-	+	+	-	-
Gelatin	+	+	-	-	-	+	+	-	-	-	-
Starch	-	-	-	-	-	-	+	+	-	+	-
DNA	v	+	ND	ND	-	-	-	-	-	-	-
Tween 80	-	+	-	+	+	-	+	+	+	+	+
Acid production from:											
D-Glucose	+	-	-	+	+	+	+	-	+	-	-
D-Glucose plus sea salts	?	?	?	?	?	?	?	?	?	+	-
Starch	-	-	-	-	-	-	+	+	-	-	-
Starch plus sea salts	?	?	?	?	?	?	?	?	?	+	-
Utilization of:											
L-Arabinose	-	-	-	+	ND	+	+	+	-	-	-
D-Glucose, D-mannose	+	+	-	+	+	+	+	-	+	-	-
Mannitol	-	-	-	+	-	-	+	-	-	-	-
Citrate	+	-	-	-	ND	-	-	-	-	+	+

Table 5.2: Phenotypic characteristics of species of the genus *Maribacter*. 1. *Maribacter stanieri* KMM 6046^T; 2. *Maribacter sedimenticola* KMM 3903^T ; 3. *Maribacter aquivivus* KMM 3949^T ; 4. *Maribacter dokdonensis* KCTC 12393^T ; 5. *Maribacter forsetii* KT02ds18-6^T ; 6. *Maribacter orientalis* KMM 3947^T ; 7. *Maribacter polysiphoniae* KMM 6151^T ; 8. *Maribacter ulvicola* KMM 3951^T ; 9. *Maribacter arcticus* KOPRI 20941^T ; 10. W15M10^T ; 11. W13M1a^T. All strains are positive for oxidase, catalase and alkaline phosphatase activities. All strains are negative for hydrolysis of urea and H_2S production. All data for columns 1-9 are taken from Nedashkovskaya *et al*., 2010. w, weakly positive; ND, not detected; +, positive; -, negative; *, no growth with added NaCl but growth observed with added sea salts (Instant Ocean); †, production only observed after 48 or 72 hours.

5.5 Description of novel Maribacter spp. sponge isolates and emended description of the genus Maribacter

5.5.1 Emended description of the genus *Maribacter* Nedashkovskaya *et al*., 2004 emend. Nedashkovskaya *et al*., 2010

The description of the genus proposed here is based on the original description (Nedashkovskaya *et al*., 2004) including emendments (Barbeyron *et al*., 2008; Nedashkovskaya *et al*., 2010) except that these strains are non-motile, strain W13M1a^T produced flexirubin type pigment and indole, was arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase positive. Both strains described here are also tryptophan deaminase positive.

5.5.2 Description of *Maribacter spongiicola* sp. nov.

Maribacter spongiicola [spon.gi.i.co'la. L. n. gen. *spongia* sponge; L. suff. *-cola* (from L. n. *incola*) inhabitant, dweller; N.L. n. *spongiicola* sponge inhabitant] for which strain W15M10^T is proposed as the type strain is a heterotrophic, strictly aerobic, salt-requiring, Gram-negative, non-motile rod-shaped cell, ~0.44-0.69 µM wide and ~8.0-12.3 µM long (Figure 5.4).

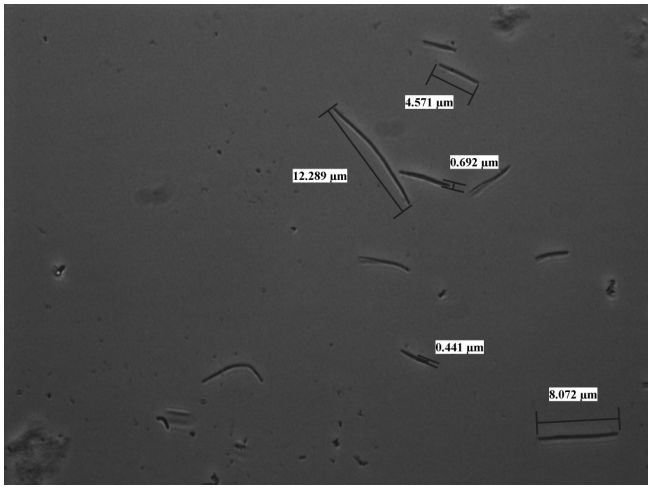

Figure 5.4: Microscopic examination of the novel strain W15M10

When growing on 2216 agar (Difco), colonies were entire, convex, circular, smooth and shiny. The orange pigment was non-diffusible. The main fatty acids were iso-$C_{15:0}$, iso-$C_{15:1}$ G, $C_{16:1}$, iso-$C_{17:1}\omega 9c$ and iso-$C_{15:0}$ 3-OH. The strain is catalase positive, oxidase positive and alkaline phosphatase positive. Growth was observed in the range of 4-30°C and at pH's ranging from 6-10. Optimum growth occurred at 25-30°C and at pH 6-7. The strain hydrolysed starch with acid production only when sea salts were added. Similarly acid production from glucose only occurred when sea salts were added. The strain hydrolyses Tween 80 and easculin, is Penicillin G resistant and displays β-galactosidase activity. Assimilation of glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, maltose, potassium gluconate, capric acid, adipic acid, malate, trisodium citrate and phenylacetic acid were all observed. The strain utilized citrate and produced acetoin. No nitrite reduction, nitrate reduction, indole production or H_2S production were observed. Urease activity was noted. Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase activities were absent. Tryptophan deaminase activity was observed after 72 hours. The main distinguishing characteristics which differentiate this strain from other *Maribacter* spp. are the content of the $C_{16:1}$ fatty acid (13.62%) and the lack of motility. The type strain was isolated from the marine sponge *Leucosolenia* sp. collected from Lough Hyne Marine Nature Reserve, Co. Cork, Ireland.

5.5.3 Description of *Maribacter vaceletii* sp. nov.

Maribacter vaceletii (va'sel.et.i N.L. gen. n. *vaceletii* named to acknowledge the work of Jean Vacelet in the field of sponge-microbiology) for which strain W13M1a^T is proposed as the type strain is a heterotrophic, strictly aerobic, salt-requiring, Gram-negative, non-motile rod-shaped cell, ~0.5-0.54 µM wide and ~3.0-3.3 µM long (Figure 5.5).

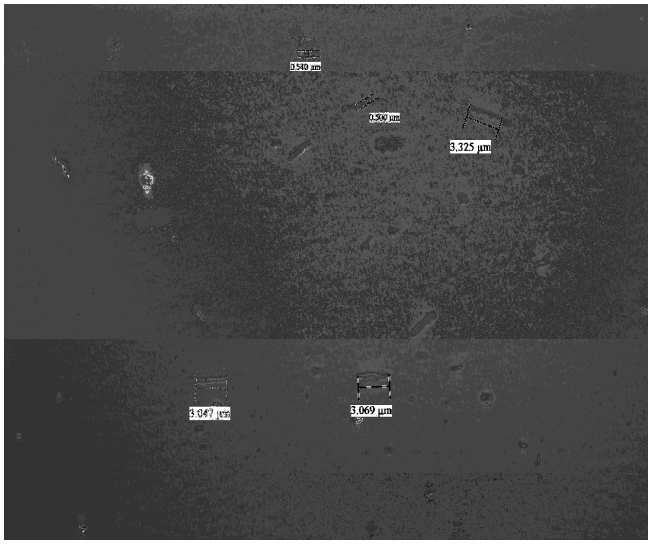

Figure 5.5: Microscopic examination of cells of the novel strain W13M1a.

When growing on 2216 agar (Difco), colonies were entire, convex, circular, smooth and shiny with an orange pigment which was non-diffusible. The main fatty acids were iso-$C_{15:0}$, iso-$C_{15:1}$ G, $C_{16:1}$, iso-$C_{17:1}\omega 9c$ and iso-$C_{15:0}$ 3-OH. The strain is catalase positive, oxidase positive and alkaline phosphatase positive. Growth occurred at 10-30°C with an optimum growth temperature of 25°C. Growth occurred at pH 6-9 with optimum growth pH of 6-7. This strain did not hydrolyse starch, DNA, casein, gelatine or agar, did hydrolyse Tween 80 and easculin and was Penicillin G resistant. The strain did not reduce nitrite or nitrate, did not produce H_2S and was urease negative. The strain did not display β-galactosidase activity when diluted in 0.85% saline but did when diluted in 33.3% Instant Ocean. Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase activities were all observed as was indole production. Assimilation of glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, maltose, potassium

gluconate, capric acid, adipic acid, malate, trisodium citrate and phenylacetic acid were not observed. The main distinguishing characteristics which differentiate this strain from previously described *Maribacter* spp. is the presence of a flexirubin type pigment, lack of motility, the high percentage of the $C_{16:1}$ fatty acid (13.7%), lack of growth below 10°C and indole production. The strain was isolated from the marine sponge *Suberites carnosus* sampled from Lough Hyne Marine Nature Reserve, Co. Cork, Ireland.

Marine sponges have proved to be, and continue to be, a good source of novel bacterial genera and species. For the purposes of biodiscovery and biotechnology the genetic capabilities of novel species may be crucial to the development and commercialisation of novel industrial enzymes or pharmaceutical products. For this reason intensification of culture isolation efforts through traditional or innovative methods should not be dismissed.

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6.0 General Discussion

Research interest in the area of the microbial ecology of marine sponges has grown substantially in recent years largely due to the fact that a wide variety of natural products with interesting bioactive properties have been discovered from sponge tissues and from sponge-associated microorganisms (Blunt *et al*., 2010). Sponges have been shown to host remarkable levels of diverse microbes from all domains of life. *Archaea* (Sharp *et al*., 2007), *Eukaryota* (Maldonado *et al*., 2005), and *Bacteria* (Webster *et al*., 2010) have not only been shown to form consistent associations with sponge hosts but have also been demonstrated to be vertically transmitted from adult sponges to sponge larvae. Such associations are strongly indicative of well-established and ecologically important symbioses. In the case of the sponge host, as sessile animals with rudimentary immune systems, an endosymbiotic microbial population which produces biologically active secondary metabolites to protect against infection or predation, the benefit of such a relationship is clear. For the microbial communities, an environmental growth niche enriched in nutrients is highly desirable and the marine sponge provides an ideal habitat.

Marine sponges are the oldest extant metazoan animals (Maloof *et al*., 2010) and so the established symbiotic communities, in co-evolutionary relationships, may also represent the oldest extant tri-partite symbioses between all three kingdoms of life.

Many aspects of sponge microbial ecology are quite remarkable. Firstly, the levels of bacterial species diversity associated with some sponge species rivals the diversity observed in the human-gut microbiome. Secondly, sponge-specific microbial taxa have been reported where those taxa have only to date been found in sponges (e.g. *Spongiispira*), are almost exclusive to sponges (e.g. *Poribacter*) or share phylogenetic relationships in which the taxa are more closely related to other sponge associates than to similar taxa from non-sponge sources, despite often being found in unrelated sponge species or in related species from distant biogeographical locations (Hentschel *et al*., 2002; Taylor *et al*., 2007). Additionally, novel microbial taxa and putative novel taxa whose 16S rRNA gene sequences do not resemble those of known phylotypes are regularly identified in associations with sponges.

Prior to next generation sequencing technologies, descriptions of sponge-microbial communities were somewhat hampered by the practical limits of 16S rRNA clone library sizes, imposed by the labour intensiveness of those techniques. For that reason the largest 16S rRNA dataset from a single sponge species contained fewer than 600 sequences (Webster *et al.,* 2010). This was clearly insufficient to accurately describe sponge-associated community structures, particularly as pyrosequencing technologies which have now been successfully employed have resulted in the description of sponge-associated bacterial communities comprising thousands of OTUs (97% sequence identity) (Webster *et al*., 2010; Lee *et al*., 2011; Jackson *et al*., 2012). Pyrosequencing studies have to date resulted in the identification of 15 bacterial phyla or candidate phyla associated with sponges which were not found using more traditional analyses, increasing the total number of sponge-associated bacterial phyla to 35. Deep-sequencing strategies have also led to the identification of large numbers of bacterial sequence reads which cannot be classified at the phylum level [e.g. sponge associated unidentified lineage (SAUL)] (Schmitt *et al*., 2011). There are several possible reasons for this. Firstly, relatively short pyrosequencing reads are more difficult to classify than near full length 16S rRNA sequences. Secondly, sequence databases used to compare and classify sequences are often deficient in good quality near full length 16S sequences from rare taxa from unusual environments. Finally, these sequences may actually belong to taxa previously unknown to science. Considering the source habitats and ancient evolutionary symbioses the latter is a real possibility.

Accurate descriptions of microbial community structures and taxon relative abundances by deep sequencing also face other problems also. Often, biases in PCR amplicon library generation are unknown. Primer biases, template-specific biases and PCR cycle condition biases are also likely to exist and can lead to preferential amplification of certain sequences over others. In addition, 16S gene-copy number variation between bacterial species can lead to the an over-estimation of relative abundances of taxa with higher gene-copy numbers. Despite these limitations, sequencing read relative abundances can be reported and used as a proxy for species relative abundances and though not sufficient to accurately describe community

structures may still be useful for sequencing read-abundance comparisons between sponge individuals or between sponge species. An additional consideration is the 16S gene region targeted for amplification in pyrosequencing studies. No standard has emerged. In sponge derived datasets alone the V1-V3 (Jackson *et al*., 2012), V2 (White *et al*., 2012), V3 (Schmitt *et al*., 2011), V5-V6 (Lee *et al*., 2011) and V6 regions (Webster *et al*., 2010) have all been targeted. These variances add an additional level of complexity with respect to comparisons between studies. One notable study shows that if sequence reads cover a variable region in addition to a hypervariable region of the 16S rRNA gene, better classification of sequence reads is achieved (Kim *et al*., 2011). For that reason in this work we targeted the V1-V3 region (V1 & V2 - variable, V3 - hypervariable) for amplification in our pyrosequencing study (Chapter 2, this thesis). We achieved relatively long sequence read-lengths (average ~430bp) from sponges using this strategy. This allowed us to successfully classify >96.5% of sponge derived pyrosequencing reads at least to the phylum level. However, a large proportion of sequences assigned to the class γ-*Proteobacteria* could not be classified below the class level and can reasonably be expected to represent novel taxa.

While bacterial diversity in sponges has been extensively studied, archaeal diversity and fungal diversity in sponges has been relatively overlooked. However, the recognition that sponge-associated fungi in particular can produce antimicrobial compounds has intensified research in this area (Baker *et al*., 2008; Wei *et al*., 2009; Wiese *et al*., 2011). Hence, >120 fungal genera from three phyla have been isolated in culture from sponges. Similarly, the recognition that archaea are consistently associated with many sponges has increased interest in the diversity, abundance and function of these symbionts in the host. *Archaea* are recognised as important ammonia-oxidisers in marine environments and the contribution of archaea to nitrogen cycling in sponge tissues is likely to be vital to sponge health.

We were particularly interested in whether or not sponge-microbial communities from extreme environments resembled those of sponges from less extreme habitats such as shallow waters and tropical waters. We used pyrosequencing to investigate the community structures of three sponge individuals of the species *Inflatella*

pellicula. One individual was sampled from a depth of 780 m while the other two were sampled from a depth of 2900 m. Prior to this study (see Chapter 3) no sequence based study had been reported for sponges from such extreme ocean depths. We hypothesised that archaea, as extremophiles, may form significant proportions of those microbial communities. For that reason we chose to employ universal PCR primers to concurrently investigate the bacterial and archaeal communities in *I. pellicula.* Notwithstanding the caveats associated with the interpretation of pyrosequencing datasets mentioned earlier, we noted remarkable levels of archaeal relative abundances (~43 - ~73%) in deep-sea sponges. We found increased archaeal relative abundances with increasing depth. Assuming no biases between sponge library amplicons and parallel seawater library amplicons, generated from seawater sampled from the sponge sampling sites, the sponges were clearly enriched for *Archaea*. The 780 m seawater community comprised ~11% *Archaea*, while the sponge from that depth hosted ~43% *Archaea*. The seawater from 2900 m contained ~36% *Archaea* while sponges from that depth hosted between ~60% and ~72% *Archaea*. The paucity of good quality Archaeal 16S rRNA gene sequences in reference databases, particularly from the newly proposed archaeal phyla, *Thaumarchaeota*, *Korarchaeota* and *Nanoarchaeota*, make gaining insights into the phylogeny of our sponge derived sequences more difficult. BLAST analyses however, suggest that the majority of our sequences align to the phylum *Thaumarchaeota*. The bacterial community profiles which were observed in the deep-sea sponges were diverse with up to 12 phyla being noted from the individual with highest diversity. The two individuals from 2900 m hosted communities more similar to each than to the individual from 780 m suggestive of a possible depth-specific influence.

In our pyrosequencing studies firm conclusions about host-associated community profiles are hampered by the lack of replicate samples for statistical comparisons. Degrees of sampling difficulty are responsible for these shortcomings. In the case of *Raspailia ramosa* and *Stelligera stuposa* (Chapter 2), sampling was performed from a protected marine nature reserve and as such over-sample volumes are restricted.

For the sponge *Inflatella pellicula*, sampling from extreme ocean depths using a remotely operated vehicle, is in effect opportunistic.

Despite the fact that 35 bacterial phyla or candidate phyla have been reported in association with sponges, members of just 7 phyla have been isolated in laboratory culture. Although increased efforts in genome sequencing and shotgun metagenomic sequencing studies continue to hint at the biochemical capabilities of uncultured taxa, culture isolation is still the gold standard for determining the actual phenotypic and biochemical activities of bacteria. While some researchers are employing innovative culture isolation strategies to attempt to grow recalcitrant organisms (Sipkema *et al*., 2011) others are targeting particular groups for isolation (Hoffmann *et al*., 2010; Phelan *et al*., 2012). Although these efforts have led to the isolation of new species, the phylum level diversity of sponge-associated bacterial isolates has thus far not increased. In truth, innovation and imagination are required in attempts to overcome this problem and such luminaries as Sergei Winogradsy and Martinus Beijerinck should be looked upon to inspire microbiologists to systematically devise methods to address the 'great plate count anomaly'.

As mentioned, novel bacterial species are regularly isolated from sponge tissues (e.g. Lee *et al*., 2006; Romanenko *et al*., 2008; Yoon *et al*., 2010 O'Halloran *et al*., 2012). We also describe here the isolation of two novel species in the genus *Maribacter* (Chapter 5). The characterisation of these species necessitates emendments to the description of the genus and thus highlights the importance of such studies. Apart from minor differences in the biochemical repertoire of the species described here and other members of the genus, the major difference noted is that the sponge derived species are non-motile while all other species described in the genus are motile. This difference may reflect the lifestyle of the sponge associates where one may speculate that genome-streaming of the endosymbionts has led to the loss of motility from an ancestral motile phenotype.

Sponge-derived microbial isolates are a promising source of antimicrobial activities. Such activities have been reported from diverse bacteria (Kennedy *et al*., 2008; Santos *et al*., 2010; Gopi *et al*., 2012) and fungi (Höller *et al*., 2000; Baker *et al*.,

2008). It is widely believed that the production of bioactive secondary metabolites by sponge symbionts plays a role in host defence (Hentschel *et al*., 2012). We have also noted antimicrobial activities from bacterial sponge isolates also (see Chapter 2). Initially we adapted a general culture isolation approach in attempts to access as much phylogenetic diversity as possible and when those isolates were screened for antimicrobial activities we found that ~3% of isolates displayed activities against *E. coli* or *S. aureus*. Subsequently, we used a targeted isolation approach which was intended to isolate actinobacteria, as members of that phylum are known to be prolific producers of antimicrobial compounds. This approach failed to yield actinobacteria but instead enriched almost exclusively for spore-formers mostly from the phylum *Firmicutes* (e.g. *Bacillus* spp.). When cultures from the targeted isolation approach were tested for antimicrobial activities ~42% of these isolates displayed inhibitory activity against one or more bacterial or yeast test strains. Therefore, in the search for novel bioactive compounds from cultured isolates a targeted approach may prove more fruitful.

The antimicrobial activities noted by us were displayed in a deferred antagonism assay only. Much work remains to be done if any of the compounds responsible for the activities are to be exploited. Initially, it needs to be determined if the compounds of interest can be extracted from culture broth. Well diffusion assays from crude culture supernatants and from aqueous and organic extracts from culture supernatants are recommended. Subsequently, should bioactivities still be observed, fractionation of the solutions will be required to begin to elucidate the structures, properties and potential novelty of such compounds.

Perhaps just as interesting as the microbial diversity in sponges, is the question of what the functions of those symbionts are. The abundance and diversity of sponges in benthic habitats makes them important in biogeochemical and nutrient cycling in aquatic communities. Investigations targeting functional genes such as *amoA* [ammonia-oxidation (Bayer *et al*., 2008; Mohamed *et al*., 2010)], *nirS* [nitrification (Yang & Li, 2012) and secondary metabolite production genes [PKS (Kim & Fuerst, 2006; Fiesler et al., 2007)] in sponge metagenomes have been performed. While the detection of these genes in sponge metagenomes only hints at biological activities *in*

situ, other studies employed metatranscriptomic strategies (Kamke *et al*., 2010; Radax *et al*., 2012) to demonstrate active transcription of functional genes involved in carbon, nitrogen and sulfur cycling in sponge tissues.

We employed sequence guided strategies to identify laccase genes, PKS genes and NRPS genes in the metagenomes of sponges. Ultimately the detection of those genes is used as a starting point to access and exploit those genes. However, despite cloning laccase gene fragments from the metagenome of *Stelletta normani* we were unable to identify laccase activities in functional assays from a metagenomic clone library. Considering the number of clones the likelihood that we have captured the full laccase gene on an individual cloned DNA fragment is quite low. This is not yet certain however and the opportunity exists to design hybridisation probes where the library can be investigated further for the presence of those gene sequences.

We noted abundant and diverse genes involved in the production of secondary metabolites (PKS and NRPS) in the metagenome of *Raspailia ramosa.* Ideally we would have liked to access those genes for further investigations and possible exploitation. Disappointingly, our *R. ramosa* DNA resource was limited and attempts to construct a large insert clone library for functional screening ultimately failed. Sponges of the genus *Raspailia* have been shown to offer great potential for the discovery of novel bioactive compounds with interesting properties. Compounds with anti-cancer [Asmarines A-I (Yosief *et al*., 2000; Rudi *et al*., 2004; Rudi *et al*., 2004b)] and neuropharmacological [Esmodil (Capon *et al*., 2004)] properties and compounds with possible uses in diabetes treatments (Saludes *et al*., 2007) have previously been identified from *Raspailia* species sponges. The diversity and abundance of NRPS genes in particular identified in the metagenome of *R. ramosa* indicates that a further sampling effort to obtain sponge tissues from this species would prove warranted and may offer considerable promise.

The field of metagenomics has opened up new avenues in the areas of ecology and biotechnology. Prior to the developments of metagenomic techniques global microbial diversity was hugely underestimated and the exploitation of microbes for industrial or pharmaceutical purposes was limited to species which could be cultured

in laboratories. The Nobel Prize winning microbiologist Selman Waksman famously stated: "*There is no field of human endeavour, whether it be in industry or agriculture, or in the preparation of food or in connection with the problems of shelter or clothing, or in the conservation of human and animal health and the combating of disease, where the microbe does not play an important and often dominant role*." It is thus self-evident that the exploitation of the diverse genetic resources hosted by microbes is essential to many aspects of human society. In this regard the vast majority of those genetic resources were up until recently both unknown and inaccessible. Great progress has however been made where cloning and heterologous expression of genes from often unknown microbial sources has led to the discovery of novel enzymes and bioactive molecules which are of commercial interest. Functional screening of clone libraries constructed from DNA from the metagenomes of soils, sediments, water and animals have all yielded products of interest. Some examples of enzymes of industrial interest which have been discovered through functional screens of metagenomic libraries include an esterase from marine sediment (Park *et al*., 2007), chitinases from estuarine water (Cottrell *et al*., 1999) and amidases from marine sediment (Gabor *et al*., 2004). Examples of some compounds of pharmaceutical interest which have been discovered include, from soils [Violacein – (Brady *et al*., 2001); Turbomycin (Gillespie *et al*., 2002)], from a beetle (*Paederus fuscipes*) endosymbiont [Pederin – (Piel, 2002)] and from a bryozoan (*Bugula simplex*) [Bryostatin – (Hildebrand *et al*., 2004)].

Metagenomic libraries from marine sponges for their part have yielded halogenases (Bayer *et al*., 2012), esterases (Okamura *et al*., 2010), lipases (Selvin *et al*., 2012) and an antitumour compound [Onnamide – (Piel *et al*., 2004)] amongst others. The recognition that marine sponges are the most prolific source of novel marine natural products (Leal *et al*., 2012) indicates that continued efforts to clone and screen metagenomic DNA from sponges may lead to many more discoveries of industrially relevant or pharmacologically interesting products in the future. Apart from screening libraries, strategies to overcome problems associated with heterologous gene expression in traditional clone library hosts must be tackled.

Functional screening of a library constructed from metagenomic DNA from the sponge *Stelletta normani* resulted in the identification of many putative lipase genes, with ten such lipolytic clones being identified. One of these clones produces lipolytic activity of note – with activity being observable shortly after incubation on appropriate media and being sustained over a number of days. The reason for the high expression levels is as yet unknown. Initial sequence analysis of the fosmid insert suggests that the DNA is of δ-proteobacterial in origin. Complete sequencing of the fosmid is warranted to determine the gene sequence as is protein purification and subsequent enzyme characterisation. The fosmid sequence may reveal whether strong transcriptional promoters are associated with the DNA insert while enzyme characterisation will provide information as to whether or not the product has commercial value, by possessing novel biochemical properties such as cold-adaptation, salt tolerance or broad range pH tolerances.

Antibacterial activity was observed from a library clone. Activity was observed against *P. aeruginosa.* Initial sequence analysis of the fosmid insert shows some homology with PKS and NRPS gene modules. Again complete sequencing of the fosmid is recommended. The implication of the observed activity is that a complete gene cluster has been cloned on a single insert. However, as the activity was not pronounced further investigations are required to determine the potential commercial potential of the gene product. Conjugation to an alternative heterologous expression host may see increased expression. Alternatively, different culture conditions to those used here (e.g. plating at different pHs) may improve expression levels.

In summary, marine sponges host numerous and diverse microbial symbionts whose ecological functions are vital to the host as well as to the marine ecosystem as a whole. Despite great progress in the understanding of microbial diversity in sponges we are only beginning to discover and understand their symbiotic functions. These diverse microbial communities possess a vast genetic resource which is as yet largely under-explored and under-exploited.

We have demonstrated that sponge-microbial communities in sponge species, about which little was known prior to these investigations, harbour communities and taxa

of intrinsic interest, including as of yet unidentified microbial taxa which may represent phylotypes previously unknown. We have described the microbial community in a sponge (*I. pellicula*) from a sampling depth much deeper than any previous sequence-based sponge-microbe investigation. We have isolated bacterial species which display antimicrobial activities against clinically relevant human pathogenic bacterial and yeast species. Additionally we have identified genes in the metagenomes of sponges which may be of industrial interest as well as cloning sponge metagenomic DNA which exhibits lipolytic activity of note and antibacterial activity which warrants further investigation.

Much future work derives from these studies. The antimicrobial compounds from the sponge isolates and from the metagenomic clone demand further analyses while the activity of one lipolytic clone shows initial promise and should be further investigated. The metagenomic clone library is a valuable resource and screening for other enzymatic activities may yield new activities while conjugation of the library into alternative expression hosts may yield additional novel activities, which were heretofore not detected in the heterologous *E. coli* host system employed in this study.

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7.0 Appendix

Phylum	Class	Sub-class	Order	Suborder	Family	Genus	Raspailia ramosa	Stelligera stuposa	seawater
Acidobacteria	Acidobacteria Gp6					Acidobacteria Gp6	0.007		
	Acidobacteria Gp9					Acidobacteria Gp9	0.007		
	Acidobacteria Gp10					Acidobacteria Gp10	0.099		
	Acidobacteria Gp22					Acidobacteria Gp22	0.042	0.017	
	Acidobacteria Gp23					Acidobacteria Gp23	0.007		
	Holophagae		Acanthopleuribacterales		Acanthopleuribacteraceae	Acanthopleuribacter	0.007		
			Unclassified Holophagae				0.007		
	Unclassified Acidobacteria						0.021	0.008	
Actinobacteria	Actinobacteria	Acidomicrobidae	Acidomicrobiales	Acidomicrobinae	Iamiaceae	Iamia	0.035		
					Acidomicrobiaceae	Unclassified Acidomicrobiaceae		0.008	
					Acidomicrobidae incerta sedis	Ilumatobacter	0.276	0.017	
					Unclassified Acidomicrobidae		0.17		
					Unclassified Acidomicrobinae		0.035		
		Actinobacteridae	Actinomycetales	Streptomycineae	Streptomycetaceae	Streptomyces	0.007		
				Propionibacterinae	Propionibacteraceae	Propionibacterium	0.042	0.008	
				Micrococcineae	Microbacteriaceae	Cryobaterium	0.007		
						Unclassified Microbacteriaceae	0.014		
					Intrasporangiaceae	Unclassified Intrasporangiaceae	0.035		
					Unclassified Micrococcineae		0.028		
				Corynebacterinae	Mycobacteriaceae	Mycobacterium	0.014		
					Corynebacteriaceae	Corynebacterium	0.021		0.016
					Dietziaceae	Dietzia	0.007		

					Unclassified Corynebacterinae		0.007		
				Unclassified Actinomycetales				0.008	
			Unclassified Actinobacteridae						
		Unclassified Actinobacteria							
Bacteroidetes					Bacteroidetes incerta sedis	Marinifilum	0.014		
	Bacteroidia		Bacteroidales		Unclassified Bacteroidales		0.007		
	Sphingobacteria		Sphingobacteriales		Flammeovirgaceae	Persicobacter	0.007		
						Marinoscillum	0.021		
						Fabibacter		0.017	
						Unclassified Flavobacteraceae			
					Cytophagaceae	Cytophaga		0.008	
					Unclassified Sphingobacteriales		0.007		
	Flavobacteria		Flavobacteriales		Cryomorphaceae	Owenweeksia	0.042		
						Brumimicrobium			0.008
						Fluviicola	0.042		0.066
						Sediminitomix	0.007		
						Crocinitomix	0.021	0.008	
						Unclassified Cryomorphaceae	0.64	0.008	0.206
					Flavobacteriaceae	Winogradskyella	0.13	0.008	0.066
						Capnocytophaga		0.008	
						Polaribacter	0.1	0.008	0.033
						Ulvibacter	0.34	0.017	0.041
						Maribacter	0.05	0.025	0.008

						Leptobacterium	0.007		
						Dokdonia	0.007		0.008
						Croceitalea	0.007		
						Aquimarina	0.014		
						Croceibacter	0.007		
						Eudoraea	0.035		
						Lacinutrix	0.042		
						Costeronia	0.007		
						Persicivigra	0.007		
						Kordia	0.014		
						Bizionia	0.021		
						Pibocella	0.064		
						Gaetbulibacter	0.184		0.008
						Psychoserpens	0.2		
						Zobellia	0.007		
						Tenacibaculum	0.07		0.008
						Algibacter	0.184		
						Joostella	0.007		
						Mariniflexile	0.007		
						Mesoflavibacter	0.021		
						Lutimonas	0.007		
						Gilvibacter	0.028		
						Gramella			0.025
						Marixanthamonas			0.008
						Formosa	0.007		

						Krokinobacter			0.016
						Unclassified Flavobacteriaceae	2.18	0.041	0.371
					Unclassified Flavobacteriales		0.45	0.041	0.082
	Unclassified Bacteroidetes						0.078		
Chloroflexi	Anaerolineae		Anaerolineales		Anaerolineaceae	Bellilinea	0.007		
						Unclassified Anaerolineaceae	0.007		
	Caldilineae		Caldilineales		Caldilineaceae	Caldilinea	0.205		
	Unclassified Chloroflexi						0.014		
Cyanobacteria	Cyanobacteria				Family IV	GpIV	0.007		
					Family VIII	GpVIII	0.014		
					Family II	GpIIa	1.14		
			Chloroplast		Chloroplast	Chlorophyta	0.05		0.008
						Bacillariophyta	1	0.141	0.478
						Cryptomonadaceae	0.028	0.141	0.008
						Unclassified Chloroplast	0.134		
					Unclassified Cyanobacteria		0.028		
Deferribacteres	Deferribacteres		Deferribacterales		Deferribacterales incerta sedis	Caldithrix	0.007		
Firmicutes	Bacilli		Bacillales		Staphylococcaceae	Staphylococcus	0.007		
					Paenibacillaceae	Paenibacillus	0.014		
					Bacillaceae	Unclassified Bacillaceae	0.007		
			Lactobacillales		Camobacteriaceae	Granulicatella	0.014		
					Leuconostocaceae	Leuconostoc	0.007		
	Clostridia		Clostridiales		Incertae sedis XI	Anaerococcus	0.007		0.008
					Peptostreptococcaceae	Sporacetigenium	0.007		

					Lachnospiraceae	Roseburia	0.021		
					Unclassified Clostridiales			0.008	
			Unclassified Clostridia				0.007	0.008	
	Unclassified Firmicutes						0.021		
Nitrospira	Nitrospira		Nitrospirales		Nitrospiraceae	Nitrospira	9.15	23.92	0.008
						Unclassified Nitrospiraceae	0.014		
TM7						TM7 Incerta sedis	0.05	0.008	
Proteobacteria	α-Proteobacteria		Snaethiellales		Snaethiallaceae	Snaethiella	0.014		
			Sphingomonadales		Erythrobacteraceae	Croceicoccus	0.035		
						Erythrobacter			0.008
						Altererythrobacter	0.028		
						Unclassified Erythrobacteraceae			
					Sphyngomonadaceae	Sphyngosinicella		0.008	
						Novosphingobium	0.007		
						Sandarakinorhabdus	0.007		
						Sphingopyxis	0.007		
						Blastomonas		0.017	
						Unclassified Sphyngomonadaceae	0.014		0.008
					Unclassified Sphyngomonadales		0.007		
			Kordimonadales		Kordimonadaceae	Kordimonas	0.057		0.008
			Rhizobiales		Brucellaceae	Daeguia	0.007		
					Cohaesibacteraceae	Cohaesibacter	0.007		
					Aurantimonadaceae	Martelella	0.007		
					Phyllobacteriaceae	Defulvibacter	0.007		

						Hoeflea	0.035		
						Unclassified Phyllobacteriaceae	0.057		
					Rhodobiaceae	Roseospirillum	0.014		
						Afifella	0.007		
						Parvibaculum	0.007		
					Hyphomicrobiaceae	Cucumibacter	0.007		
						Prosthecomicrobium	0.007		
						Filmomicrobium	0.057		
						Unclassified Hyphomicrobiaceae	0.212		
					Beijerinckiaceae	Unclassified Beijerinckiaceae		0.017	
					Unclassified Rhizobiales		0.93	0.041	
			Rhodospirillales		Rhodospirillaceae	Rhodovibrio	0.007		
						Fodinicurvata	0.007		
						Nisaea	0.502	0.066	0.082
						Pelagibus	0.424	0.05	
						Unclassified Rhodospirillaceae	0.608		0.016
					Unclassified Rhodospirillales		0.042	0.017	
			Caulobacterales		Caulobacteraceae	Caulobacter	0.007		
						Brevundimonas	0.007		0.074
						Unclassified Caulobacteraceae			
					Hyphomonadaceae	Henriciella	0.028		
						Hellea	0.028		
						Hirschia	0.014	0.008	
						Robiginitomaculum	0.007		
						Unclassified Hyphomonadaceae	0.021		

			Rhodobacterales		Rhodobacteraceae	Pseudovibrio	0.042		
						Silicibacter	0.021		
						Paracoccus	0.014		
						Octadecabacter	0.014		0.016
						Ponticoccus	0.014		
						Citreicella	0.007		
						Oceanicola	0.007		
						Tateyamaria	0.007		
						Donghicola	0.078		
						Maritimibacter	0.007		
						Sulfitobacter	0.134	0.008	0.041
						Phaeobacter	0.014		
						Pelagicola	0.007		0.025
						Roseobacter	0.014		0.008
						Seohaeicola	0.007		
						Nautella	0.021		
						Pseudorugeria	0.014		
						Maribius	0.021		
						Albidovulum	0.014		
						Wenxinia	0.007	0.008	
						Leisingera	0.007		
						Ahrensia	0.007		
						Tropicimonas	0.028		
						Lutimaribacter	0.064		
						Thalassobius	0.283	0.008	0.223

						Roseicyclus	0.007		
						Ruegeria	0.028	0.008	
						Jannaschia	0.22	0.008	0.033
						Thalassococcus	0.071		0.016
						Roseovarius	0.375		0.181
						Nereida	0.071		0.033
						Thalassobacter	0.325		0.206
						Loktanella	0.269	0.017	0.082
						Pseudorhodobacter			0.008
						Shimia	0.042	0.008	0.008
						Marivita	0.057	0.008	0.115
						Unclassified Rhodobacteraceae	6.93	0.438	8.89
			Rickettsiales		Rickettsiaceae	Orientia	0.007		
						Unclassified Rickettsiaceae			
					SAR11	Pelagibacter	9.97	0.521	0.973
					Unclassified Rickettsiales		0.028		
			Parvularculales		Parvularculaceaea	Parvularcula		0.248	
			Unclassified α-Proteobacteria				5.06	2.273	0.223
	β-Proteobacteria		Nitrosomonadales		Nitrosomonadaceae	Nitrospira	0.007		
			Burkholderiales		Alcaligenaceae	Achromobacter	0.007		
					Comamonadaceae	Delftia	0.007	0.017	

						Acidovorax	0.007		
					Unclassified Burkholderiales		0.007		
			Unclassified β-Proteobacteria				0.94	0.636	
	δ-Proteobacteria		Desulfovibrionales		Desulfovibrionaceae	Desulfovibrio	0.007		
			Bdellovibrionales		Bacteriovoracaceae	Bacteriovorax	0.035	0.124	
						Unclassified Bacteriovoracaceae		0.017	
					Bdellovibrionaceae	Bdellovibrio	0.021		
			Desulfuromonadales		Desulfuromonadaceae	Desulfuromonas	0.007		
					Unclassified Desulfuromonadales		0.007		
			Desulfobacterales		Desulfobulbaceae	Desulfopila	0.028		
						Unclassified Desulfobulbaceae	0.007		
					Desulfobacteraceae	Desulfosarcina	0.007		
						Desulfonema	0.021		
						Unclassified Desulfobacteraceae	0.085		
					Unclassified Desulfobacterales				
			Myxococcales	Nannocystineae	Haliangiaceae	Haliangium	0.007		
					Nannocystaceae	Enhygromyxa	0.085	0.017	
						Plesiocystis	0.028	0.017	
						Unclassified Nannocystaceae	0.028	0.008	
				Sorangiineae	Polyangiaceae	Chondromyces	0.021		
						Sorangium	0.014		
						Byssovorax	0.007		
						Unclassified Polyangiaceae	0.021		
				Cystobacterinae	Cystobacteraceae	Unclassified Cystobacteraceae	0.014		

					Unclassified Myxococcales		0.035		
			Unclassified δ-Proteobacteria				0.48	0.107	0.008
	ε-Proteobacteria		Campylobacterales		Helicobacteraceae	Sulfurovum	0.028		
						Sulfurimonas	0.05		
					Campylobacteraceae	Arcobacter	0.05		0.437
						Unclassified Campylobacteraceae	0.028		0.025
					Unclassified Campylobacterales				0.536
			Nautiliales		Nautiliaceae	Nitratifactor			0.008
						Unclassified Nautiliaceae			0.115
			Unclassified ε-Proteobacteria						0.85
	γ-Proteoacteria		Legionellales		Coxiellaceae	Aquicella	0.007		
			Vibrionales		Vibrionaceae	Aliivibrio	0.021		0.561
						Photobacterium	0.035		0.305
						Vibrio	0.113	0.017	48.3
						Listonella	0.057		6.91
						Enterovibrio			0.008
						Unclassified Vibrionaceae	0.021		1.451
			Alteromonadales		Moritellaceae	Moritella	0.021		0.033
					Shewanallaceae	Shewanella	0.064	0.008	0.157
					Ferrimonadaceae	Ferrimonas	0.007		0.033
						Paraferrimonas	0.007		0.61
						Unclassified Ferrimonadaceae			2.66
					Colwelliaceae	Thalassomonas	0.007		0.115
						Colwellia	0.085		1.37

						Unclassified Colwelliaceae	0.014		0.033
					Pseudoalteromonadaceae	Pseudoalteromonas	0.3	0.05	12.93
						Unclassified Pseudoalteromonadaceae			0.016
					Alteromonadaceae	Aestuariibacter	0.021	0.008	
						Microbulbifer	0.007		0.008
						Marinimicrobium	0.007		
						Glaciecola	0.021	0.041	0.454
						Haliela	1.06	0.083	0.066
						Alteromonas			0.025
						Aliagarivorans			0.016
						Agarivorans			0.033
						Bowmanella			0.008
						Unclassified Alteromonadaceae	0.127	0.008	0.041
					Psychromonadales	Psychromonas			0.016
					Unclassified Alteromonadales		0.064		1.517
			Enterobacteriales		Enterobacteriaceae	Serratia	0.007		
						Yersinia	0.057		
						Salmonella	0.057		
						Tatumella	0.021		
						Enterobacter	0.156		
						Escherichia/Shigella	0.163		
						Citrobacter		0.008	
						Unclassified Enterobacteraceae	0.184		0.008
			Thiotrichales		Piscirickettsiaceae	Cycloclasticus	0.007		
						Unclassified Piscirickettsiaceae	0.007		

					Thiotricaceae	Leucothrix	0.078		0.008
						Unclassified Thiotricaceae	0.007		
					Unclassified Thiotrichales				
			Pseudomonadales		Pseudomonadaceae	Cellvibrio	0.007		
						Pseudomonas	0.014	0.008	0.066
						Unclassified Pseudomonadaceae			
					Pseudomonadales incerta sedis	Dasania	0.233	0.008	
					Moraxellaceae	Acinetobacter	0.007		
						Unclassified Moraxellaceae			
			Chromatiales		Granulosicoccaceae	Granulosicoccus	0.007		
					Ectothiorhodospiraceae	Thioalkalispira	0.007		
						Thioalkalivibrio	0.007		
						Thiohalospira	0.021	0.008	
						Natronocella	0.007		
						Ectothiorhodosinus	0.035	0.017	
						Methylohalomonas*	3.74	0.083	
						Ectothiorhodospira		0.018	
						Unclassified Ectothiorhodospiraceae	0.45	32.88	0.008
					Chromatiaceae	Rheinheimera			0.025
						Unclassified Chromatiaceae	0.057		
					Unclassified Chromatiales		0.643	4.26	
			Oceanospirillales		Oceanospirillaceae	Marinimonas	0.007		0.57
						Oceanospirillum	0.007		0.199
						Amphritea	0.014		0.025
						Neptunomonas	0.057		0.1

						Neptuniibacter	0.035		0.05
						Thalassolituus	0.007		0.016
						Oceaniserpentilla			0.008
						Oleispira			0.132
						Reinekea		0.008	
						Unclassified Oceanospirillaceae	0.035	0.066	0.1
					Halomonadaceae	Modicisisalibacter	0.014		
						Camimonas	0.007		
						Zymobacter	0.007		0.033
						Halomonas			0.008
						Unclassified Halomonadaceae	0.163		0.05
					Hahellaceae	Endozoicomonas	0.092	4.81	0.008
						Unclassified Hahellaceae	0.014	0.5	
					Oceanospirillales incerta sedis	Spongiispira			0.016
					Unclassified Oceanospirillales		0.403	0.397	0.1
			Xanthomonadales		Xanthomonadaceae	Stenotrophomonas	0.007	0.025	0.016
						Unclassified Xanthomonadaceae	0.007	0.041	
					Sinobacteraceae	Singularimonas			0.008
			Aeromonadales		Aeromonadaceae	Unclassified Aeromonadaceae			0.008
			γ-Proteobacteria incerta sedis			Thiohalomonas	0.092		
						Simiduia	0.014	0.025	0.173
						Gilvimarinus	0.014		0.008
						Sedimenticola	0.085		
						Spongiibacter	0.014		0.008
						Thiohalophilus	0.092		

						Unclassified γ-Proteobacteria incerta sedis		0.033	0.124
			Unclassified γ-Proteobacteria				31.83	17.05	1.105
	Unclassified Proteobacteria						5.06	3.53	0.272
Unclassified Bacteria							1.95	3.53	0.19
Unclassified root							0.042	0.025	0.025

Supplementary Table S2.1: Relative abundance (by percentage) of 16S V1-V3 454 tag sequence reads from marine sponges and seawater at all taxonomic levels to genus level.

Domain	Phylum	Class	Subclass	Order	Suborder	Family	Genus	SW780m	Ip780m	SW2900m	Ip2900mA	Ip2900mB
Archaea	Crenarchaeota/Thaumarchaeota*	?		?		?	?	5.87	42.054	4.428	71.27	58.718
	Euryarchaeota	Halobacteria		Halobacteriales		Halobacteriaceae	Unclassified Halobacteriaceae	0.067	0	0.015	0	0.021
		Methanomicrobia		Unclassified Methanomicrobia				0.016	0	0.023	0	0.01
				Methanosarcinales		Metherthmicoccaceae	Methermicoccus	0	0	0	0	0.021
		Thermoplasmata		Thermoplasmatales		Thermoplasmataceae	Thermoplasma	0.117	0	0	0	0
						Thermoplasmatales incertae sedis	Thermogymnomonas	3.120	0	0.824	0	0
						Unclassified Thermoplasmatales		23.284	0.01	4.646	0	0.021
						Ferroplasmaceae	Ferroplasma	0.0167	0	0	0	0
							Unclassified Ferroplasmaceae	0.402	0	0.007	0	0
						Unclassified Ferroplasmatales		0	0	0	0	0
		Unclassified Euryarchaeota						3.455	0.419	1.019	0.574	0.087
	Unclassified Archaea							0.285	0.534	0.342	0.722	1.459
Bacteria	Elusimicrobia	Elusimicrobia		Elusimicrobiales		Elusimicrobiaceae	Elusimicrobium	0.016	0	0.007	0	0
	Fusobacteria	Fusobacteria		Fusobacteriales		Fusobacteriaceae	Psychrilyobacter	0	0	0.007	0	0
	Nitrospira	Nitrospira		Nitrospirales		Nitrospiraceae	Unclassified Nitrospiraceae	0	0	0.007	0.065	0.054
							Nitrospira	0	0.052	0	0.246	0.152
	Chlamydiae	Chlamydiae		Chlamydiales		Unclassified Chlamydiales		0.016	0	0.015	0.016	0.065
						Parachlamydiaceae	Parachlamidya	0	0	0.015	0.032	0.032
							Unclassified Parachlamydiaceae	0	0	0.007	0	0
	Lentisphareae	Lentisphareae		Unclassified Lentisphareae				0.067	0	0	0	0
	Bacteroidetes	Sphingobacteria		Sphingobacteriales		Chitinophagaceae	Sediminibacterium	0.05	0	0.0311	0	0

							Gracilimonas	0	0.01	0	0	0
						Saprospiraceae	Lewinella	0.016	0.01	0.334	0	0
							Unclassified Saprospiraceae	0	0	0.023	0.016	0.01
						Flammeovirgaceae	Unclassified Flammeovirgaceae	0.016	0.02	0.101	0	0.021
							Reichenbachiella	0	0	0.007	0	0
						Rhodothermaceae	Rhodothermus	0	0	0.007	0	0
							Unclassified Rhodothermaceae	0	0	0	0	0.01
						Cytophagaceae	Spirosoma	0	0	0.007	0	0
						Unclassified Sphingobacteriales		0.05	0.031	0.155	0.032	0.01
		Bacteroidia		Bacteroidales		Prevotellaceae	Prevotella	0	0	0.023	0	0
		Flavobacteria		Flavobacteriales		Cryomorphaceae	Crocinitomix	0.016	0.01	0.023	0	0.021
							Wandonia	0.016	0	0.07	0	0
							Brumimicrobium	0.067	0	0.49	0	0
							Fluviicola	0.016	0	0.054	0	0
							Lishizhenia	0.134	0	0.07	0	0.01
							Owenweeksia	0	0.01	0.062	0	0
							Unclassified Cryomorphaceae	0.184	0	0.879	0.016	0
						Flavobacteriaceae	Winogradskyella	0.033	0	0.007	0	0
							Meridianimaribacter	0.016	0.01	0.038	0	0
							Gilvibacter	0.05	0.02	0.342	0.131	0
							Unclassified Flavobacteriaceae	0.251	0	2.148	0.065	0
							Aestuariicola	0	0	0.007	0	0
							Maribacter	0	0	0.031	0.016	0
							Kordia	0	0	0.062	0	0
							Tenacibaculum	0	0	0.023	0	0

							Lacinutrix	0	0	0.077	0	0
							Tamlana	0	0	0.046	0.016	0
							Psychroserpens	0	0	0.007	0	0
							Formosa	0	0	0.07	0	0
							Lutibacter	0	0	0.116	0	0
							Aquimarina	0	0	0.054	0	0
							Polaribacter	0	0.01	0.093	0	0
							Joostella	0	0	0.007	0	0
							Ulvibacter	0	0	0.933	0	0
							Gaetbulibacter	0	0	0	0.016	0
						Unclassified Flavobacteriales		0.15	0	0.428	0	0
		Unclassified Bacteroidetes						0.067	0	0.132	0	0.01
	Cyanobacteria/Chloroplast	Chloroplast				Chloroplast	Bacillariophyta	0.117	0	0.186	0	0
		Cyanobacteria				Family II	GpIIa	0	0	0.015	0	0
							Bangiophyceae	0.016	0	0.007	0	0
							Unclassified Chloroplast	0.050	0	0.054	0	0
				Unclassified Cyanobacteria/Chloroplast				0	0	0.023	0	0
	Chloroflexi	Anaerolineae		Anaerolineales		Anaerolinaceae	Unclassified Anaerolinaceae	0.067	0	0.007	0	0
							Levilinea	0	0	0.007	0	0
						Unclassified Chloroflexi		0.184	0	0.093	0.016	0.01
	Deinococcus-Thermus	Deinococci		Deinococcales		Trueperaceae	Truepera	0.016	0	0	0	0
							Thermus	0	0	0.023	0	0
						Unclassified Deinococcales		0	0	0	0.016	0
	Verrucomicrobia	Verrucomicrobiae		Verrucomicrobiales		Rubritaleaceae	Rubritalea	0.1	0	0.023	0	0
						Verrucomicrobiaceae	Unclassified Verrucomicrobaceae	0.1	0	0.023	0.032	0.021

							Persicirhabdus	0	0	0.007	0	0
							Roseibacillus	0	0	0	0.016	0.01
						Unclassified Verrucomicrobiales		0.033	0	0.023	0	0
		Unclassified Verrucomicrobia						0	0	0	0.016	0
		Opitutae		Opitutales		Opitutaceae	Alterococcus	0.016	0	0.023	0	0
				Puniceicoccales		Puniceicoccaceae	Coraliomargarita	0	0	0.015	0	0
		Subdivision3					Subdivision3 incertae sedis	0.251	0.01	0.311	0.131	0.119
		Subdivision5					Subdivision5 incertae sedis	0	0	0.007	0	0
		Unclassified Verrucomicrobia						0.318	0.041	0.21	0	0.043
	OD1						OD1 incertae sedis	0	0	0.015	0	0.01
	Actinobacteria	Actinobacteria	Actinobacteridae	Actinomycetales	Propionibacterineae			0.05	0	0.217	0	0
					Actinomycinae			0	0	0.007	0	0
					Corynibacterinae			0	0	0.07	0	0.01
					Micrococcineae			0	0	0.225	0	0
					Unclassified Actinomycetales			0	0	0.07	0	0
				Bifidobacteriales		Bifidobacteriaceae		0	0	0.007	0	0
			Acidimicrobidae	Acidimicrobiales	Acidimicrobineae			1.107	0.031	1.424	0	0.032
			Coriobacteridae	Coriobacteriales	Coriobacterinae			0	0	0.015	0	0
						Unclassified Rubrobacteridae		0	0	0	0.016	0
			Unclassified Actinobacteria					0	0	0.007	0.016	0.021
	Acidobacteria	Acidobacteria Gp21					Gp21	0.016	0	0.077	0.082	0.108
		Acidobacteria Gp26					Gp26	0.033	0	0.007	0.016	0
		Acidobacteria Gp6					Gp6	0.083	0.031	0.21	0.328	0.544
		Acidobacteria Gp2					Gp2	0	0	0.007	0	0

		Acidobacteria Gp22					Gp22	0	0	0.007	0.049	0.01
		Acidobacteria Gp3					Bryobacter	0	0	0.015	0	0.01
		Acidobacteria Gp10					Acidobacteria Gp10	0	0	0	0.131	0.119
		Holophagae		Holophagales		Holophagaceae	Unclassified Holophagaceae	0	0	0.007	0.01	0
				Unclassified Holophagae				0	0	0.007	0	0
		Unclassified Acidobacteria						0.251	0.041	0.101	0.344	0.294
	Firmicutes	Clostridia		Clostridiales		Unclassified Clostridiales		0.033	0	0.007	0.016	0
						Ruminococcaceae	Ethanoligenens	0	0	0.007	0	0
						Clostridiales Incertae Sedis IV	Mahella	0	0	0.007	0	0
						Clostridiales Incertae Sedis XI	Peptoniphilus	0	0	0.007	0	0
							Anaerococcus	0	0	0.015	0	0
						Lachnospiraceae	Lachnospiraceae incertae sedis	0	0	0.007	0	0
							Unclassified Lachnospiraceae	0	0	0.054	0	0
				Unclassified Clostridia				0	0	0.015	0.082	0
						Clostridiales Incertae Sedis XIII	Anaerovorax	0	0	0.007	0	0
		Negativicutes		Selenomonadales		Acidaminicoccaceae	Phascolarctobacterium	0	0	0.007	0	0
						Veillonellaceae	Unclassified Veillonellaceae	0	0	0.015	0	0
						Unclassified Selenamonadales		0	0	0.007	0.049	0.01
		Bacilli		Bacillales		Pasteuriaceae	Pasteuria	0.067	0.041	0.171	0.558	0.588
						Staphylococcaceae	Staphylococcus	0	0	0.202	0	0
							Unclassified Staphylococcaceae	0	0	0.007	0	0
						Bacillaceae 1	Geobacillus	0	0	0.062	0	0
							Anoxybacillus	0	0	0.054	0	0
							Unclassified Bacillaceae 1	0	0	0.007	0	0
						Bacillales Incertae Sedis XII	Exiguobacterium	0	0	0.007	0	0

						Bacillales Incerta Sedis XI	Gemella	0	0	0.015	0	0
						Unclassified Bacillales		0	0	0.007	0	0
								0	0	0	0	0
				Lactobacillales		Aerococcaceae	Facklamia	0	0	0.031	0	0
						Carnobacteriaceae	Carnobacterium	0	0	0.007	0	0
							Granulicatella	0	0	0.007	0	0
						Streptococcaceae	Streptococcus	0	0	0.085	0	0
						Unclassified Lactobacillales		0	0	0.007	0	0
						Unclassified Bacilli		0	0	0.007	0	0
		Unclassified Firmicutes						3.103	0.115	0.443	0.459	0.239
	Spirochaetes	Spirochaetes		Spirochaetales		Leptospiraceae	Leptonema	0	0.01	0	0	0.01
						Spirochaetaceae	Spirochaeta	0	0.199	0	0	0
						Unclassified Spirochaetales		0	0.02	0	0	0
	Planctomycetes	Phycisphaerae		Phycisphaerales		Phycisphaeraceae	Phycisphaera	0.469	0.146	0.568	0.804	1.067
		Planctomycetacia		Planctomycetales		Planctomycetaceae	Planctomyces	0.251	0	0.015	0.032	0
							Blastopirellula	0.771	0.073	0.179	2.118	2.113
							Schlesneria	0	0	0.007	0	0.01
							Rhodpirellula	0.285	0.062	0.062	0.082	0.152
							Unclassified Planctomycetaceae	1.107	0.199	0.373	0.722	0.686
		Unclassified Planctomycetes						0.234	0.052	0.194	0.476	0.501
	Proteobacteria	β-Proteobacteria		Methylophilales		Methylophilaceae	Methylotenera	0.15	0.01	0.054	0	0.01
							Methylobacillus	0	0	0.015	0	0
				Neisseriales		Neisseriacea	Neisseria	0.016	0	0.007	0	0
							Kingella	0	0	0.007	0	0
				Nitrosomonadales		Nitrosomonadaceae	Unclassified Nitrosomonadacea	0.05	0	0.007	0.032	0.01

							Nitrosomonas	0	0	0	0	0.01
							Nitrospira	0	0.01	0.015	0	0.021
				Burkholderiales		Burkholderiales incertae sedis	Aquabacterium	0.083	0	0.046	0	0
							Tepidimonas	0	0	0.07	0	0
						Comamonadaceae	Pelomonas	0.05	0	0.007	0	0
						Burkholderiaceae	Burkholderia	0	0	0.007	0	0
							Ralstonia	0	0	0.015	0	0
						Alcaligenaceae	Bordatella	0	0	0.007	0	0
						Unclassified Burkholderiales		0	0	0	0.065	0.01
				Hydrogenophilales		Hydrogenophilaceae	Hydrogenophilus	0	0	0.046	0	0
						Oxalobacteraceae	Massilia	0.083	0	0.007	0	0
							Unclassified Oxalobacteraceae	0	0	0.007	0	0
				Unclassified β-Proteobacteria				0	0	0	0.082	0.283
		δ-Proteobacteria		Desulfuromonadales		Geobacteraceae	Geoalkibacter	0.016	0	0.007	0	0
							Geothermobacter	0	0	0.015	0	0
						Unclassified Desulfuromonadales		0	0	0.007	0	0
				Bdellovibrionales		Bacteriovoraceae	Bacteriovorax	0.05	0	0.046	0	0
							Peredibacter	0.016	0	0.031	0.016	0
							Unclassified Bacteriovoracaeae	0.033	0	0.023	0	0
				Desulfobacterales		Desulfobacteraceae	Unclassified Desulfobacteraceae	0.016	0	0.0466	0	0
							Desulfofrigus	0	0	0.007	0	0
						Desulfobulbaceae	Unclassified Desulfobulbaceae	0.016	0	0	0	0.021
						Unclassified Desulfobacterales		0	0	0.007	0	0
				Myxococcales	Nannocystineae	Haliangiaceae		0.05	0	0.007	0	0
						Nannocystaceae		0.067	0	0.031	0	0

						Unclassified Nannocystineae		0	0	0	0.016	0.01
					Sorangiineae	Polyangiaceae		0	0	0.007	0	0
						Unclassified Myxococcales		0.067	0	0	0.016	0
				Syntrophobacterales		Syntrophobacteraceae	Desulfofacinum	0	0	0.007	0	0
						Unclassified Syntrophobacterales		0	0	0.007	0	0
				Unclassified δ-Proteobacteria				1.694	0.031	3.369	0.279	0.261
		γ-Proteobacteria		Vibrionales		Vibrionaceae	Photobacterium	0.016	0	0.015	0	0
							Vibrio	0	0	0.303	0	0.01
							Unclassified Vibrionaceae	0	0	0.007	0	0
				Enterobacteriales		Enterobacteriaceae	Escerichia/Shigella	0.033	0	0	0	0
							Salmonella	0	0	0.015	0	0
							Providencia	0	0	0.007	0	0
							Citrobacter	0	0	0.031	0	0
							Klebsiella	0.016	0	0	0	0
							Unclassified Enterobacteriaceae	0.016	0.01	0.015	0	0
				Legionellales		Coxiellaceae	Coxiella	0.016	0	0.038	0	0.01
							Unclassified Coxiellaceae	0	0	0.007	0	0
				Alteromonadales		Ferrimonadaceae	Paraferrimonas	0.016	0	0	0	0
						Shewanellaceae	Shewanella	0.033	0	0.031	0	0.021
						Moritellaceae	Moritella	0.1	0	0.085	0	0
							Unclassified Moritellaceae	0	0	0	0.016	0
						Colwelliaceae	Thalassomonas	0.05	0	0.202	0	0
							Colwellia	0.234	0.031	1.035	0	0
							Unclassified Colwellaceae	0	0	0.046	0	0
						Alteromonadales incertae sedis	Teredinibacter	0.016	0	0	0	0

						Pseudoalteromonadaceae	Pseudoalteromonas	0.318	0.01	19.822	0	0
							Unclassified Pseudoalteromonadaceae	0.05	0	0.023	0	0
						Psychromonadaceae	Psychromonas	0	0	0.015	0	0
						Alteromonadaceae	Glaciecola	0.05	0	2.778	0	0
							Alteromonas	0.1	0	0.132	0	0
							Marinobacter	0	0	0.062	0	0
							Haliea	0.05	2.831	0.038	0	0
							Melitea	0.033	0.01	0.07	0	0.01
							Unclassified Alteromonadaceae	0.201	0.041	0.303	0	0
						Unclassified Aleromonadales		0.033	0.02	0.116	0	0
				Oceanospirillales		Alcanivoracaceae	Alcanivorax	0.033	0	0	0	0
						Oceanospirillales incertae sedis	Spogiispira	0	0	0.031	0	0
						Halomonadaceae	Halomonas	0.033	0	0.062	0	0
							Cobetia	0	0	0.015	0	0
							Halovibrio	0	0	0.412	0.032	0.043
							Unclassified Halomonadaceae	0	0	0.007	0	0
						Hahellaceae	Kistimonas	0	0	0.007	0	0
							Endozoicomonas	0	0.429	0	0.032	0.021
							Unclassified Hahellaceae	0	0.157	0	0	0.01
						Oleiphilaceae	Oleiphilus	0	0	0.007	0	0
						Oceanospirillaceae	Bermanella	0.016	0	0	0	0
							Amphritea	0	0	0.023	0	0
							Oceanospirillum	0	0	0.015	0	0
							Neptunibacter	0	0	0.023	0	0
							Thalassolituus	0.033	0	0.031	0	0

							Oleispira	0.016	0	0.101	0	0
							Marinomonas	0.016	0	0.007	0	0
							Nitrincola	0.033	0	0.015	0	0
							Balneatrix	0	0	0	0.016	0
							Unclassified Oceanospirillaceae	0.033	0.031	0.132	0.032	0.01
						Unclassified Oceanospirillales		0.234	0.167	0.459	0.295	0.108
				Gammaproteobacteria incertae sedis			Umboniibacter	0.016	0	0.023	0	0
							Arenicella	0.033	0	0.303	0.065	0.01
							Marinicella	0	0	0.031	0	0
							Gilvimarinus	0	0	0.007	0	0
							Methylohalomonas	0	0	0.007	0	0
							Spongiibacter	0	0.01	0.077	0	0
							Eionea	0.016	0	0	0	0
							Congregibacter	0	0	0	0	0.01
							Thiohalobacter	0.15	0	0	0	0
							Unclassified Gammaproteobacteri a incertae sedis	0.15	0.083	0.21	0.049	0.501
				Pseudomonadales		Pseudomonadaceae	Rhizobacter	0.016	0	0	0	0
							Cellvibrio	0	0	0.007	0	0
							Serpens	0	0	0.023	0	0
							Azomonas	0	0	0.023	0	0
							Pseudomonas	0	0	0.537	0	0
							Unclassified Pseudomonadaceae	0	0	0.809	0	0
						Pseudomonadales incerta sedis	Dasania	0.083	0	0.163	0	0
						Unclassified Pseudomonadales		0	0	0.023	0	0
						Moraxellaceae	Acinetobacter	0.117	0	0.124	0.016	0

							Psychrobacter	1.593	0	2.225	0	0
							Unclassified Moraxellaceae	0	0	0.007	0	0.01
				Thiotrichales		Thiotrichales incerta sedis	Fangia	0.067	0	0.054	0	0.032
							Unclassified Thiotrichales incertae sedis	0	0	0	0	0.01
						Piscirickettsiaceae	Unclassified Piscirickettsiaceae	0.033	0	0.007	0	0
				Chromatiales		Chromatiaceae	Nitrosococcus	0.016	0	0	0	0
							Unclassified Chromatiales	0.268	5.819	0.062	0	0.043
						Ectothiorhodospiraceae	Unclassified Ectothiorhodospiraceae	0	0	0.031	0	0
						Granulosicoccaceae	Granulosicoccus	0	1.677	0.007	0	0
				Xanthomonadales		Xanthomonadaceae	Lysobacter	0	0	0.007	0	0
							Stenotrophomonas	0	0	0.007	0	0
							Luteibacter	0	0	0.015	0	0
							Thermomonas	0	0	0.077	0	0
							Xanthomonas	0	0	0.054	0	0
							Unclassified Xanthomonadaceae	0	0	0.077	0.213	0.294
						Unclassified Xanthomonadales		0	0	0.023	0.032	0.098
				Unclassified γ-Proteobacteria				9.629	26.63	8.366	2.48	3.061
		α-Proteobacteria		Kordiimonodales		Kordiimonadaceae	Kordimonas	0.033	0	0.17	0.016	0.021
				Rhodobacterales		Rhodobacteraceae	Phaeobacter	0.016	0	0.287	0.016	0
							Marivita	0.05	0	0.038	0	0
							Litorimicrobium	0.067	0	0.023	0	0
							Loktanella	0.033	0	0.07	0	0
							Jannaschia	0.016	0	0	0	0
							Thalassobius	0.016	0	0.046	0	0

							Vadicella	0.167	0.01	0	0	0
							Pelagicola	0.184	0	0	0	0
							Shimia	0.067	0.01	1.12	0	0.01
							Sulfitobacter	0.117	0	0.038	0	0
							Pseudoruegeria	0.536	0.031	0.015	0	0
							Roseovarius	0	0	0.007	0	0.01
							Roseisalinus	0	0	0.007	0	0
							Rhodobaca	0	0	0.046	0	0
							Rhodobacter	0	0	0.007	0	0
							Paracoccus	0	0	0.015	0	0
							Ruegeria	0	0	0.147	0	0
							Vadicella	0	0	0.077	0	0
							Pelagicola	0	0	0.98	0	0
							Unclassified Rhodobacteraceae	0.788	0.020	0.965	0.098	0.043
							Thalassobacter	0	0	0.062	0	0.043
							Antarctobacter	0	0	0	0.016	0
				Sphingomonadales		Erythrobacteraceae	Erythrobacter	0.033	0	0.287	0	0
							Porphyrobacter	0.016	0	0.038	0	0
						Sphingomonadaceae	Sphingomonas	0.05	0	0.038	0	0
							Sphingopyxis	0	0	0.07	0	0
				Caulobacterales		Hyphomonadaceae	Hellea	0.016	0	0.225	0.098	0
							Hyphomonas	0.016	0	0	0	0
							Maricaulis	0	0	0.116	0	0
							Unclassified Hyphomonadaceae	0.016	0	0.007	0.016	0
						Caulobacteraceae	Brevundimonas	0	0	0.007	0	0
							Caulobacter	0	0	0.023	0	0

				Rhizobiales		Aurantimonadaceae	Aurantimonas	0.016	0	0.023	0	0
						Bradyrhizobiaceae	Bradyrhizobium	0.016	0	0	0	0
						Phyllobacteriaceae	Chelativorans	0.033	0	0	0	0
							Aquamicrobium	0	0	0	0.016	0
							Aminobacter	0	0	0	0.032	0.01
							Unclassified Phyllobacteriaceae	0.603	0.02	0.163	0	0
						Methylocystaceae	Terasakiella	0.822	0.01	0.124	0	0.021
						Unclassified Rhizobiales		0.201	0	0.241	0.032	0.043
						Hyphomicrobiaceae	Unclassified Hyphomicrobiaceae	0	0	0.007	0	0
						Rhodobiaceae	Unclassified Rhodobiaceae	0	0	0.023	0	0
						Unclassified Rhizobiales		0	0.01	0	0.032	0
				Rhodospirillales		Acetobacteraceae	Stella	0.016	0	0	0	0
							Unclassified Acetobacteraceae	0.033	0	0.007	0	0
						Rhodospirillaceae	Thalassobaculum	0.05	0	0.007	0	0
							Oceanobaculum	0.083	0	0.015	0	0
							Inquilinus	0	0.01	0	0	0
							Rhodovibrio	0	0.01	0	0	0
							Skermanella	0	0	0.007	0	0
							Pelagibius	0.033	0.02	0.015	0.032	0
							Unclassified Rhodospirillaceae	0.687	0.377	0.63	0.049	0.021
						Unclassified Rhodospirillales		2.298	0.125	3.478	0.82	0.66
				Sneathiellales		Snaethiellaceae	Snaethella	0	0	0.038	0	0.01
				Rickettsiales		Rickettsiaceae	Orientia	0	0.01	0.007	0	0
				Parvularculales		Parvularculaceae	Parvularcula	0	0	0	0.065	0
				Alphaproteobacteria incertae sedis		Unclassified Alphaproteobacteria incertae sedis		0	0	0.007	0	0

				Unclassified α-Proteobacteria				3.153	0.272	2.077	0.049	0.337
		Unclassified Proteobacteria						12.414	1.761	10.6	11.892	24.218
	Unclassified Bacteria							13.739	14.281	9.012	2.036	2.037
Unclassified								0.0167	0.702	0.031	0.114	0.032

Supplementary Table S3.1: Detailed classification of bacteria and archaea by percentage, at all taxonomic levels, for pyrosequencing reads (16S rRNA; V5-V6 region) from marine sponges and from seawater. SW780m = seawater sampled at a depth of 780m; SW2900m = seawater sampled at a depth of 2900m; Ip780m = *Inflatella pellicula* sampled at 780m; Ip2900mA and Ip2900Mb = *Inflatella pellicula* sampled at a depth of 2900m. * Denotes non- euryarchaeotal archaeal sequences. Although the RDP Classifier classifies these reads as *Crenarchaeota*, BLAST analyses suggest these sequences recruit to the phylum *Thaumarchaeota*.

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