ABSTRACT

Title of Document: ECOPHYSIOLOGY OF MICROBIAL COMMUNITIES ASSOCIATED WITH MARINE SPONGES *IRCINIA STROBILINA* AND *MYCALE LAXISSIMA*

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Marine sponges are hosts to many microorganisms that can constitute up to 60% of the sponge biomass. Complex symbiotic interactions may exist between sponges and associated microorganisms. My primary goal was to develop a model sponge system for laboratory studies of complex symbioses between bacteria and marine invertebrates. Two sets of aquaculture systems were designed in order to optimize the conditions for culturing marine sponges *Mycale laxissima* and *Ircinia strobilina.* Bacterial communities associated with the sponges were characterized using culture-based and molecular techniques. There was a substantial change in the diversity and composition of bacterial communities upon transfer into aquaculture. This work shows a potential for maintaining healthy marine sponges in closed aquaculture systems, a necessary foundation for using sponges in aquaculture as a laboratory model.

There is a limited knowledge of the contributions of bacteria to sponges, and thereby indirectly to the coral reef ecosystem. I examined the role of sponge-associated bacteria in nitrogen fixation. Nitrogen fixation was demonstrated in sponges using nitrogen isotopic composition. The potential for nitrogen fixation by symbionts was assessed by amplification of *nifH* gene fragments from total DNA and RNA extracted from sponges. Diverse *nifH* genes were detected, and gene expression studies proved that *nifH* genes were expressed in sponge-associated bacteria. These *nifH* transcripts were closely related to cyanobacterial *nifH* genes. This is the first demonstration of the expression of bacterial genes in sponge symbionts. Cyanobacterial symbionts are likely fixing nitrogen and provide fixed nitrogen to their hosts.

Quorum sensing in sponges is hypothesized to play a role in colonization by symbionts and the regulation of symbiosis between sponges and associated bacteria. Alpha- and gamma-proteobacterial isolates were tested for the production of acyl homoserine lactones (AHLs) using diffusion bioassays coupled with thin-layer chromatography fractionation. Isolates affiliated with the *Silicibacter-Ruegeria* subgroup of the *Roseobacteria* clade were the main producers of AHLs. These findings demonstrate that sponge associated bacteria show a high incidence of production of complex suites of AHLs involved in cell signaling.

This research contributes to the field of sponge microbiology by advancing the understanding of the roles of symbionts and interactions with their hosts.

ECOPHYSIOLOGY OF MICROBIAL COMMUNITIES ASSOCIATED WITH MARINE SPONGES *IRCINIA STROBILINA* AND *MYCALE LAXISSIMA*

By

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DEDICATION

I dedicate my thesis to my family especially my mother, Faten Mohamed, and my husband, Tamer Abdelgawad for their endless love and support.

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The past four years and a half have been an exciting experience. Working at COMB exposed me to many scientific backgrounds and cultures. Most importantly, this has been a great experience on the personal level where I met many great people who helped starting my life in the United States and supported me throughout my PhD years.

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STATEMENT OF CONTRIBUTION

Venkateswara Rao (University of Mississippi) performed the LC-MS analysis on extracts of wild and aquacultured *I. strobilina* samples.

Scott McIntosh designed and implemented the recirculating aquaculture system.

Elisha Cicirelli (Indiana University) performed the overlay and thin-layer chromatographic assays detect the production of AHLs in *Proteobactria* associated with sponges.

Jinjun Kan performed the proteomics analysis of the AHL-producing roseobacters.

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Chapter 1. Introduction and Literature Review

1.1. Marine sponges

1.1.1. Background

Marine sponges (Porifera) are considered the evolutionarily oldest multicellular animals with a fossil record dating back to ca. 580 million years (Li *et al.*, 1998). They are highly successful reef building organisms and have possessed a conserved fundamental body plan for over 500 million years (Qun *et al.*, 2007). The presence of sponges in the late Proterozoic era, the period of Earth's history that began 2.5 billion years ago and ended 543 million years ago, has been predicted by molecular and morphological phylogenies (Wainright *et al.*, 1993; Müller *et al.*, 1994). Recent models to establish congruence between the fossil record and molecular clocks suggest the origination of sponge-like organisms in the early to middle Neoproterozoic era, between ~1,000 and ~660 million years ago (Qun *et al.*, 2007).

Biomineralization is an important feature of metazoan animals whereby they can build skeletal structures such as bones in mammals, calcium carbonate skeletons in corals and siliceous or calcite spicules in many species of sponges. A key process in the evolution of complex life was the development of the ability to catalyze the hydration of CO_2, a chemical reaction that is catalyzed by the metalloenzyme carbonic anhydrase. This enzyme functions in several metabolic processes such as secretion of calcium carbonate skeletons, pH regulation, carbon fixation, and

transportation of ions across membranes (Henry, 1996). Jackson *et al.* (2007) recently showed that sponges have inherited the precursor to α-carbonic anhydrases, a gene family that is important for many physiological processes including photosynthesis and biomineralization, from the last common ancestor of Metazoa. They used a living fossil of the coralline Indo-Pacific sponge *Astrosclera willeyana* and a paleogenomic approach including gene and protein expression techniques and phylogeny. Schröder *et al.* (2007) found that the freshwater sponge *Lubomirskia baicalensis* contained an interferon (INF)-antiviral protection system. This finding is very important for understanding the evolution of metazoan immune systems to protect against virus load. Until this study, INFs have been found only in vertebrates where they display antiviral, cell growth regulatory, and immunomodulatory activities (Stark *et al.*, 1998). Phylogenetic analyses of genes found in sponges as well as higher animals, including humans, indicate that sponges are likely the evolutionary oldest metazoans (Taylor *et al*., 2007c).

1.1.2. Taxonomy of sponges

Phylum Porifera is unique and comprises the most primitive Metazoa. The classification of Porifera is still unclear especially at the lower taxonomic levels. It has been challenging due to the high phenotypic plasticity originating from the lack of basic organ and tissue organization. Even the same species can show different growth forms and colors depending on the environmental conditions (Hooper and Van Soest, 2002; McDonald *et al.*, 2002). About 6000 species have been described in a wide range of marine and freshwater habitats, and the total number of extant sponge

species has been estimated at 15,000 (Bergquist, 1978; Hooper and Van Soest, 2002).

The phylum Porifera has been divided into three classes, the Calcarea, the Hexactinellida and the Demospongiae, based on skeletal chemistry. All sponges in these classes consist of a colony of cells loosely held together in a fibrous or glassy skeleton. The calcareous sponges have skeletons composed entirely of calcium carbonate spicules. They are common in shallow temperate and tropical waters. There are about 400 described species. The Hexactinellida sponges are known as the glass sponges. They have skeletons comprised of six-rayed siliceous spicules, and they are most common in deep and inaccessible waters. There are about 600 described species. The Demospongiae is the largest class; it contains approximately 95% of marine and fresh water sponges. The sponges of this class have skeletons composed of spongin fibers alone or in combination with siliceous spicules. Collagen filaments and proteinaceous spongin fibers occur in most families and provide additional skeletal support (Bergquist, 1978; Hooper and Van Soest, 2002).

1.1.3. Sponge cells: structure and function

Sponges are multicellular sessile animals. The body of sponges lacks basic organ and tissue organization. Instead, the body plans and structures are based on complex water-canal and skeletal systems. A generalized body plan is composed of an outer thick epithelium called the pinacoderm and the internal choanoderm layer, sandwiching the intermediate mesohyl layer (Fig. 1.1). The pinacoderm is made up of a single cell type of pinacocyte cells. This epithelium is perforated with inhalant openings known as ostia that are lined with the same pinacocytes (Bergquist, 1978).

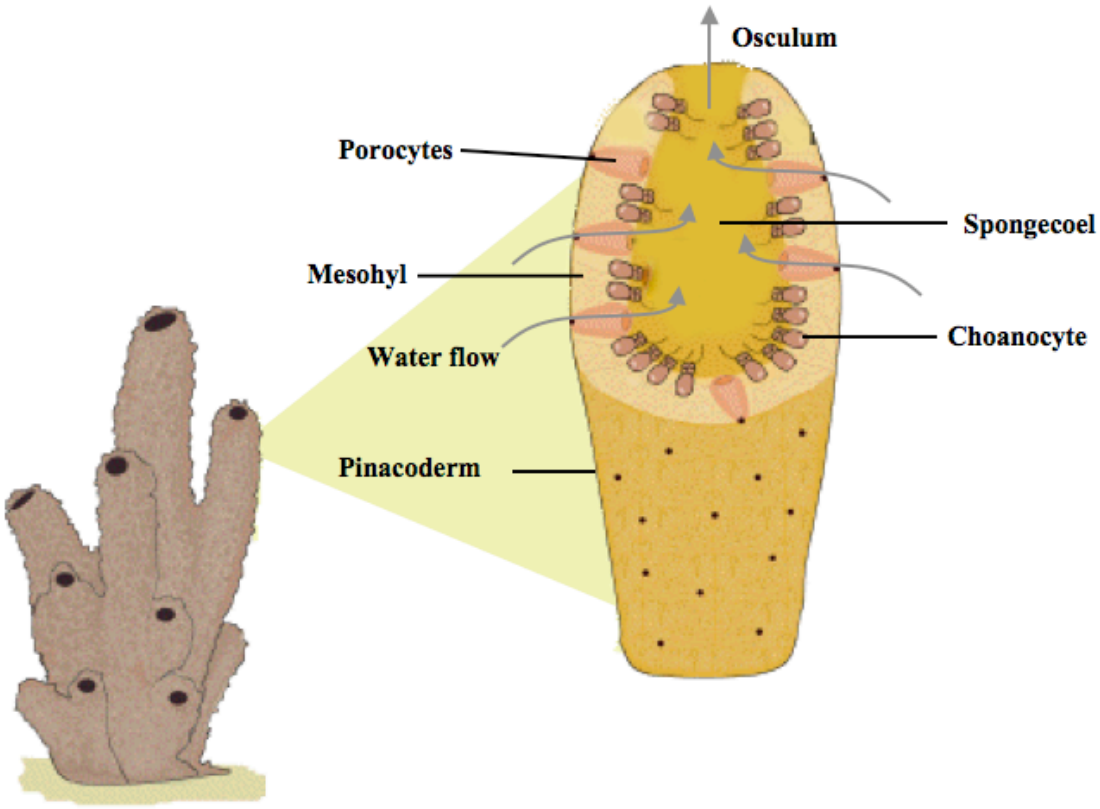


Fig. 1.1. Schematic diagram of a sponge. Gray arrows indicate the water flow through the sponge. Adapted with permission from (http://biology.unm.edu/ccouncil/Biology_203/Images/SimpleAnimals/sponge.gif).

The internal choanoderm consists of a layer of choanocyte cells that line the internal choanocyte chambers and are linked by a system of water canals. Choanocytes consist of a cell body with a single long flagellum surrounded by microvillae. They form an elaborate aquiferous system consisting of a single flagellated layer inside the sponge and actively pump in water in a current that passes in one direction through the sponge body. The average number of choanocyte chambers per cubic millimeter of sponge tissue is 7000-8000, with each chamber pumping approximately 1200 times

its own volume of water each day (Wilkinson, 1992). Between the pinacoderm outer layer and the internal choanoderm is a third region, the mesohyl. It is an extensive later of connective tissue, which contains some mobile cells such as archaeocytes and some skeletal materials such as spongin, collagen and sponge fibers (Bergquist, 1978).

Sponges are filter feeders and actively pump huge amounts of water every day. They can filter up to 24 m^2/kg/day (Hentschel *et al.*, 2002). They pump water via flagellated choanocytes through ostia and the water then passes into the canals to reach choanocyte chambers. Choanocytes' flagellae beat from base to tip, driving water from the choanocyte chamber out of the exhalent pores or oscula, creating a current (Harrison and Cowden, 1976; Simpson, 1984). The water expelled from the oscula is essentially sterile (Wehrl *et al.*, 2007), with food particles including bacteria and microalgae having been captured by both phagocytic pinacocyte cells, which line the inhalant canals, and choanocytes. Food particles are transferred to the mesohyl, where they are digested via phagocytosis by another group of sponge cells, the archaeocytes. Archaeocytes are mobile, amoeboid cells, and they are capable of moving through sponge tissues and differentiating into any of the other sponge cells (Bergquist, 1978).

1.1.4. Reproduction of marine sponges

Sponges reproduce asexually and sexually. Levi (1957) divided the Demospongiae according to reproductive characteristics. Hooper *et al*. (1992) challenged this classification since it was based only on reproductive characteristics without

considering phylogenetic analyses. Several methods of asexual reproduction are found in sponges including formation of internal and external buds or the formation of complex gemmules (Bergquist, 1978). Asexual budding is frequently a response to damage, which gives sponges remarkable regeneration properties in addition to their reproductive function. Broken fragments of an adult sponge can regenerate by a complete reorganization of body tissue and grow into new sponges (Harrison and Cowden, 1976).

The absence of gonads makes it difficult to understand sexual reproduction in sponges. The majority of sponges are hermaphroditic, producing sperms and eggs at different times or in some cases housing both sperm and egg cells within the same adult. The mesohyl tissue and choanocyte chambers are usually interspersed with reproductive elements at different developmental stages (Bergquist, 1978). Gamete production and embryogenesis take place within the endosome and mesohyl tissue. Choanocytes can transform to become male sex cells, or oocytes. Spermatogenesis occurs within specialized cysts, which are densely packed and separated from the mesohyl by a single layer of follicle cells (Bergquist, 1978). Fertilization of oocytes occurs externally in the water column or by sperm brought into sponge in choanocytes via the inhalant water current. Although the mechanisms for sexual reproduction in many species have not been extensively studied, studies of a haplosclerid demosponge suggest that developmental events are very similar to those observed in cnidarians (Leys and Degnan, 2002).

Sponge larvae are free-swimming and come in a variety of forms. The mechanism by which the larvae are expelled from their follicles in the mesohyl to the

excurrent canals is not clear. All larvae are covered with flagellated cells, which allow them to be motile. During metamorphosis they attach to a substrate and the larvaes' external flagellated cells migrate internally during invagination where they develop collars and become the characteristic choanocytes (Bergquist, 1978).

1.2. Sponges as sources of marine natural products

1.2.1. Bioactive compounds from sponges

Marine sponges are sessile invertebrates that have developed effective strategies to protect themselves against viruses, bacteria and eukaryotic predators. One of these defense mechanisms is the production of secondary metabolites functioning as antibiotics, antifungal compounds and compounds that prevent predation or fouling (Sarma *et al.*, 1993; Proksch, 1994; Osinga *et al.*, 2001). Sponges are known as prolific sources of bioactive compounds that can potentially be used to treat various human diseases (Faulkner *et al.*, 1999; Faulkner, 2000). The medicinal value of marine invertebrates has attracted many researchers and led to the development of a new research field of marine natural products in the early 1970s. This resulted in the isolation of more than 10,000 new compounds over the last 30 years with a continuous steady arithmetic increase in the marine natural products observable since 1965 (Blunt *et al.*, 2007).

The isolation of bioactive metabolites from sponges, including a number of structurally unusual and biologically interesting compounds with a wide range of activity, has been extensively reviewed (Kobayashi and Ishibashi, 1993; Lee *et al.*,

2001; Faulkner, 2002; Hildebrand *et al.*, 2004; Piel *et al.*, 2004; Schirmer *et al.*, 2005; Schmidt *et al.*, 2005). The discovery in the 1950s of the two nucleosides spongothymidine and spongouridine in the Caribbean sponge *Tethya crypta* (Bergmann and Burke, 1955) inspired the development of a series of synthetic nucleosides that are now commercially available (Newman and Cragg, 2004a). These include Ara-A (Vidarabine®, Vidarabin Thilo®), an anti-viral drug active against the *Herpes simplex encephalitis* virus (Field and De Clercq, 2004), and Ara-C (Cytarabine, Alexan®, Udicil®), an anti-cancer drug (Scheuer, 1996).

Several bioactive compounds from sponges have passed the preclinical stage of the drug testing process (Newman and Cragg, 2004a). One of the significant sponge-derived metabolites in phase I of clinical trials since 2004 is the polyketide discodermolide from the Carribbean sponge *Discoderma dissoluta* (Gunasekera *et al.*, 1990; Gunasekera *et al.*, 2002). Discodermolide has been shown to inhibit the proliferation of human cells by arresting the cell cycle in G2- and M-phase. The mechanism of action of discodermolide is similar to that of the major anticancer drug paclitaxel (Taxol®). However, discodermolide binds microtubules with an even higher affinity than paclitaxel (ter Haar *et al.*, 1996). Discodermolide proved to be too toxic at the therapeutic dosages required for effective immune system suppression and development as a potential anticancer agent was initiated (Mita *et al.*, 2004). The drug has since proven very potent against certain cancers, and development efforts are ongoing. Another sponge-derived anticancer compound in phase I of clinical trials is the glycolipid KRN7000 from the marine sponge *Agelas mauritianus* (Hayakawa *et al.*, 2003). Halichondrin B, an unusual polyether macrolide originally

discovered from the Japanese marine sponge *Halichondria okadai*, is highly promising as an anticancer agent (Hart *et al.*, 2000). In total, there are currently 39 drugs derived from marine invertebrates in clinical trials, and ten of these are derived from sponges (Newman and Cragg, 2004b).

1.2.2. Bioactive compounds from bacterial symbionts of sponges

Marine sponges harbor microorganisms including bacteria, cyanobacteria and fungi within their extra and intra-cellular spaces (Vacelet and Donadey, 1977; Wilkinson, 1992) (see section 1.3, symbionts of marine sponges). *Actinobacteria* isolated from sponges have been sources of novel bioactive compounds. Examples of bioactive compounds are urauchimycins and antimycin antibiotics from a *Streptomyces* sp. that was isolated from an unidentified sponge (Imamura *et al.*, 1993). A *Microbacterium* sp. isolated from the sponge *Halichondria panicea* was the source of four glycoglyceropeptides, one of which showed antitumor activities (Wicke *et al.*, 2000).

The variability in chemical yield and composition from morphologically identical sponge species collected from the same place and time suggests a microbial origin of some compounds that are isolated from sponges (Brantley *et al.*, 1995). The isolation of manzamine alkaloids from many unrelated and geographically diverse sponge species suggests a microbial origin for this important antimalarial compound (Hill, 2004). Hill (2004) suggested that not only do symbionts produce manzamines but also it is possible that they play a significant role in the bioconversion of manzamines to produce the large number of manzamine alkaloids found in sponges. Hill *et al.* (2007) discovered the bacterial origin of an antimalarial lead, manzamine A. It is

produced by a *Micromonospora* sp. strain M42 from the Indonesian sponge *Acanthostrongylophora*.

Circumstantial evidence for the microbial origin of a sponge-derived compound may be supplied by the structure of the compound (Kobayashi, 2000). The Philippines sponge *Theonella swinhoei* contains the antifungal cyclic peptide theopalauamide and the cytotoxic macrolide swinholide A. Theopalauamide was detected in a fraction obtained by differential centrifugation. This fraction mostly contained a filamentous deltaproteobacterium that has been assigned to "*Candidatus* Entotheonella palauensis" (Schmidt *et al.*, 2000). This bacterium has not yet been cultured. Swinholide A was detected in a fraction containing unicellular bacteria. Cellular fractionation can be used to investigate the original source of bioactive compounds. However, many bacteria are closely attached to sponge cells, and it is difficult to obtain bacteria-free fractions. Also, co-localization of a compound and a particular cell type is not irrefutable evidence that those cells are producing the compound of interest. In some cases, compounds may be produced by other cells and then sequestered by the cells in which the compound is co-localized. In order to unambiguously assign the production of bioactive compounds to symbionts rather than sponge cells, it is necessary to culture the bacterium producing the compound and demonstrate production by the bacterial culture (Faulkner, 1999).

Strong evidence was provided for a bacterial producer of the onnamides and theopederins found in the sponge *T. swinhoei* (Piel *et al.*, 2004). The onnamides and theopederins are polyketides that structurally resemble pederin, the defensive polyketide in *Paederus fuscipes* beetles. Genes closely resembling those encoding

pederin were found in the complex metagenome of *T. swinhoei* and had characteristic prokaryotic signatures (Piel *et al.*, 2004). Many of the bioactive compounds from sponges belong to the polyketide natural product group. Since the enzymes catalyzing complex polyketide biosynthesis, termed type I polyketide synthases (PKSs) (Staunton *et al*., 2001), are so far known exclusively from microorganisms, it has long been suspected that bacteria are the true producers of these compounds. Piel *et al*. (2004) provided a strong support for this theory by cloning PKS genes for the biosynthesis of antitumor polyketides of the onnamide series from *T. swinhoei* and traced them back to a prokaryote.

Ilan and coworkers (2000) located latrunculin B, a toxin from the Red Sea sponge *Negombata magnifica* in sponge cells, suggesting that sponge cells may produce this compound. In addition, bacterial symbionts were poorly labeled by a latrunculin B-specific antibody (Gillor *et al.*, 2000). However, it remains a possibility that latrunculin B is produced by symbiotic bacteria and later translocated to sponge cells or that the compound is produced by endosymbiotic bacteria within the sponge cells. A bacterial source for latrunculin B is suggested by its polyketide structure that indicates it is derived from a complex polyketide biosynthesis pathway typical of prokaryotes and has not been found in metazoans. As discussed above, when the compound is being localized within sponge cells, it is difficult to eliminate the possibility that symbiotic bacteria are the main producers.

1.2.3. Supply problem and solutions

The issue of whether a compound of interest is produced by the sponge or by

symbiontic bacteria associated with the sponge has important practical implications. The low yield of sponge-derived bioactive compounds, known as the supply problem, is a major obstacle limiting successful transition of marine invertebrates-derived compounds through clinical studies and into commercial production (Munro *et al.*, 1999; Osinga *et al.*, 1999b). An example is discodermolide, where the marine sponge *D. dissoluta* contains only 0.002% of discodermolide (Paterson and Florence, 2003). Harvesting the sponge from the environment generally will not provide a reliable, large-scale supply and can eventually lead to the extinction of the particular sponge species. The relatively simple structure of discodermolide allowed chemical synthesis to overcome the supply problem and the various synthetic approaches have been reviewed by Paterson and Florence (2003). Chemical synthesis is not feasible for many marine natural compounds due to their structural complexity, which make synthesis too difficult or expensive (Sipkema *et al.*, 2005). Several techniques are available for the production of large quantities of sponge biomass needed for the extraction of bioactive metabolites that are not viable candidates for total synthesis. Such techniques include sponge farming (Duckworth and Battershill, 2003a,b) and sponge cell culture.

Culturing of sponges is an alternative route for scaling up the production of bioactive compounds in cases where sponges are the main producers of bioactive metabolites, microbial origin of the compounds is unknown or cultivation of the producer bacterium is not achievable. Sponge culture methods include: (i) mariculture (Battershill and Page, 1996), (ii) cultivation of sponge explants under controlled or semi-controlled environmental conditions (Duckworth *et al.*, 1997), and

(iii) *in vitro* cultures, under totally controlled conditions, of explants, primmorphs (cell aggregates derived from cell suspension in which cell proliferation occurs) or dissociated cells (Pomponi and Willoughby, 1994; Müller *et al.*, 1999; Osinga *et al.*, 1999a). Considerable effort into large-scale mariculture of *Lissodendoryx* sp., a sponge that produces larger amounts of halichondrian B than the sponge in which the compound was first found, has been made by research groups in New Zealand (Dumdei *et al.*, 1998; Munro *et al.*, 1999). Mariculture is used mainly for the production of commercial bath sponges. This approach has the advantage of cost efficiency and the provision of natural environmental conditions required for sponge growth. However, changes in the marine environment such as storms, predation and infection cannot be predicted.

In vitro sponge cell culture studies have been based on suspended sponge cells (Pomponi, 2006) or primmorphs (Sipkema *et al.*, 2003; Müller *et al.*, 2004a). An advantage of primmorphs is that they contain symbiotic microorganisms, which allows for the production of bioactive metabolites from the sponge host and/or the associated microbe. Müller *et al.* (2000) showed that primmorphs from *Dysidea avara* grown in a bioreactor produced the secondary metabolite avarol, which has antitumor, antibacterial and antiviral activities. Single *D. avara* cells did not produce avarol, suggesting that the symbiotic bacteria present in the primmorphs may play a role in the production of this compound or that only intact primmorphs are capable of synthesis of the compound.

In most of the sponge cell culture studies, primary cell cultures were successfully obtained but it was challenging to maintain continuously dividing cell lines, keep

cells axenic (free of bacteria, yeast and fungi) and decrease cell mortality (De Caralt *et al.*, 2007). De Caralt *et al.* (2007) recommended the development of cell cultures from immortalized sponge stem cells. This can be achieved by concentrating on reducing the high apoptosis activity of sponge cells in addition to focusing on the strong capability of sponge cells to divide. They proposed sponge embryos, highly proliferative cells that are capable of differentiating into all cell types present in an adult sponge, and sponge larvae as new promising sources of stem cells.

When bioactive compounds are produced by a sponge-associated bacterium, options for production of these compounds include isolation and cultivation of the producer bacterium. Using molecular approaches such as the transfer of symbiont biosynthetic genes into fast growing cultivable bacteria such as *Escherichia coli* has been explored recently (Fortman and Sherman, 2005; Piel, 2006). However, the current results are not conclusive since the large number of genes involved in sponge metabolic pathways complicates this process (Schmidt, 2005). Understanding these metabolic pathways will greatly improve the efficiency of heterologous expression of biosynthetic pathways, thus the production of target metabolites (De Caralt *et al.*, 2007).

Culturing the entire sponge and its microbial consortia in a contained rather than open-water aquaculture systems is an option. The advantage of this strategy is better control of environmental conditions such as temperature, light and food supply and possibly precursors of important bioactive metabolites. There is little information on the microbiology of cultured sponges. In order to examine the potential of cultivation of marine sponges as a solution for the supply problem, it is crucial to determine

whether their microbial communities change upon culturing. This is particularly important if symbionts are the producers of important bioactive compounds since removing the sponge from its natural environment might lead to the loss of these symbionts. If the sponge is the main producer of bioactive metabolites, associated microbes might be still of direct importantance directly by providing metabolite precursors or indirectly by affecting the health of their hosts (Hill, 2004).

1.3. Symbionts of marine sponges

1.3.1. Diversity of sponge-associated microbes

Sponges can be described as microbial fermenters (Taylor *et al.*, 2007a) that harbor diverse and complex assemblages of microorganisms including heterotrophic bacteria, cyanobacteria, facultative anaerobes, unicellular algae, and Archaea (Webster *et al.*, 2001b; Thacker and Starnes, 2003; Montalvo *et al.*, 2005; Hentschel *et al.*, 2006; Hill *et al.*, 2006; Taylor *et al.*, 2007b). Sponge-associated microbes can constitute up to 60% of the sponge biomass (Vacelet, 1975; Vacelet and Donadey, 1977; Wilkinson, 1978b; Hentschel *et al.*, 2006). As proposed by Taylor *et al.* (2007), the term "symbiont" is used here to describe these sponge-associated bacteria. The broadest definition of "symbiont" is used to refer to two different organisms that are found in a stable relationship, living together for a long period, without either necessarily benefiting its partner.

In the past, characterization of the microbial communities of sponges relied on their observation within sponge tissue using electron microscopy and culture-based

studies. Both approaches were severely limited. Early electron microscopy studies showed two different types of sponge-bacteria relationships (Vacelet and Donadey, 1977). The first is characterized by the presence of large densities of bacteria represented by few morphotypes. This was observed in dense thick-walled sponges with relatively well-developed mesohyl and small choanocyte chambers. The second type of relationship was characterized by low bacterial densities dominated by one or two morphotypes. This was observed in sponges with well-developed aquiferous systems and low density of mesohyl (Vacelet and Donadey, 1977). It was therefore hypothesized that the type of relationship is related to the sponge morphology and development of the aquiferous system. Taylor *et al.* (2007b) described the presence of three broad types of microbial associates in sponges, based on the pioneering research on sponge symbionts: (i) abundant populations of sponge-specific microbes in the sponge mesohyl, (ii) small populations of specific intracellular bacteria, and (iii) populations of nonspecific bacteria resembling those in the surrounding seawater. It is likely that bacteria within the mesohyl have a strong association with the sponge host and hence play a physiological role, while the non-specific bacteria and those in sponge cells are used as food (Wilkinson, 1978c).

Early and on-going culture-based studies have resulted in the isolation of many groups of bacteria from sponges. Members of bacterial phyla *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, *Planctomycetes*, *Proteobacteria* and *Verrucomicrobia* from marine sponges have been isolated in pure culture [for example, (Burja and Hill, 2001; Kim *et al.*, 2005; Montalvo *et al.*, 2005; Enticknap *et al.*, 2006; Lee *et al.*, 2006)]. The number of bacterial strains obtained using culture-

dependent techniques is very small compared to the total diversity present in sponges. In many studies, less that 1% of environmental microorganisms can be cultured using standard techniques (Hugenholtz *et al.*, 1998). This situation has improved in the last few years due to the advancement of cultivation techniques. Montalvo and Hill were able to isolate over 400 morphotypes from the marine sponge *Xestospongia muta*. These bacteria fell into three phyla: *Proteobacteria*, *Actinobacteria* and Firmicutes, with putative novel species and genera within *Gammaproteobacteria* (Montalvo and Hill, 2007). There have been many recent approaches to increase the culturable microbial diversity (Nichols, 2007). The more recent culturing techniques combine high throughput cultivation with simulation of natural growth conditions including use of natural media such as seawater or synthetic media with low concentrations of nutrients that mimick the oligotrophic conditions of the samples (Bruns *et al.*, 2002; Connon and Giovannoni, 2002). Other methods include extinction and dilution culturing (Button *et al.*, 1993), the extension of incubation times (Davis *et al.*, 2005), the addition of cell-to-cell signaling compounds to culture media (Bruns *et al.*, 2002), and employing diffusion via devices such as the environmental growth chamber, where microorganisms are incubated in contact with their natural environment (Kaeberlein *et al.*, 2002). Other methods include dialysis membranes to deliver environmental growth factors to microorganisms during incubation (Ferrari *et al.*, 2005).

The advancement of molecular techniques such as 16S rRNA gene-based community analysis and fluorescent *in situ* hybridization (FISH) has increased our knowledge of the diversity of microorganisms associated with marine sponges.

Taylor *et al.* (2007b) recently conducted an extensive phylogenetic analysis of ~ 1500 sponge-derived 16S rRNA gene sequences available in the GenBank database at the time of the study (28 February 2006), in addition to 184 bacterial and archaeal 16S rRNA gene sequences contributed by his group and that of Hentschel (2002). Sponge-derived sequences in the GenBank database included sequences from pure isolates, 16S rRNA gene clone libraries and excised denaturing gradient gel electrophoresis (DGGE) bands. They used the ARB software package to compile and analyze all of the sequence data. This meta-analysis revealed sequences belonging to 16 bacterial phyla. They proposed two new bacterial phyla, the phylum *Lentisphaerae* and candidate phylum TM6, each of which was represented by a single 16S rRNA gene sequence from the marine sponges *Plakortis* sp. and *Anthochartacea*, respectively. The rest of the phyla listed in Taylor's review are: *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Deinococcus-Thermus*, *Firmicutes*, *Gemmatimonadetes*, *Nitrospira*, *Planctomycetes*, candidate phylum *Poribacteria*, *Proteobacteria* (*Alpha-*, *Beta-*, *Delta-*, and *Gammaproteobacteria*), *Spirochaetes*, *Verrumicrobia* and *Chlorobi*. Although sequences from the *Chlorobi* (green sulfur bacteria) phylum have not been recovered from sponges, positive FISH signals were visualized in tissues of *Rhopaloeides odorabile* with a specific probe for this phylum (Webster *et al.*, 2001b). Fieseler *et al.* (2004) described the novel candidate phylum *Poribacteria*, which is moderately related to the *Planctomycetes, Verrucomicrobia* and *Chlamydiae*, in different sponges from different geographical locations.

The two major archaeal lineages *Crenarchaeota* and *Euryarchaeota*, are also

present in sponges (Taylor *et al.*, 2007b). Preston *et al.* (1996) were the first to report the presence of a symbiotic psychrophilic crenarchaeon, *Cenarchaeum symbiosum*, in the marine sponge *Axinella mexicana* using 16S rRNA analysis. Archaeal sequences belonging to *Crenarchaeota* and *Euryarchaeota* with higher representation of *Crenarchaeota* have been reported from several marine sponges (Preston *et al.*, 1996; Webster *et al.*, 2001a; Holmes and Blanch, 2007). Lipid biomarkers suggested the presence of both phyla of Archaea in the deep-water Arctic sponge *Tentorium semisuberites* (Pape *et al.*, 2006). Molecular analyses have revealed that at least 16 bacterial and archaeal phyla are present in sponges (Taylor *et al.*, 2007b). Considering that only a small number of sponge species have been studied to date, the diversity is likely to increase even further. Sponges are clearly hosts to a remarkable diversity of symbiotic bacteria and Archaea. More effort on culturing these bacteria is required to provide information that cannot be obtained directly from sequencing efforts alone, about bacterial communities.

1.3.2. Specificity of sponge-bacterium associations

Early researchers studying sponge microbiology, including Wilkinson and Vacelet (Vacelet and Donadey, 1977; Wilkinson, 1978c,1984), observed that marine sponges host specific microbes. Evidence for specificity of sponge-microbe association is: (i) many sponges harbor massive consortia of microorganisms within the mesohyl matrix, which are phylogenetically distinct from those in the adjacent environments of seawater and sediments, and (ii) the presence of sponge-specific 16S rRNA gene sequence clusters.

The presence of sponge-specific bacteria that are different than those in the water column was inferred from my work (Chapters 2 and 3) and by others (Santavy and Colwell, 1990; Taylor *et al.*, 2005; Hill *et al.*, 2006). Members of the candidate phylum *Poribacteria* were found in several sponges from diverse geographical locations but never in surrounding seawater or sediment (Fieseler *et al.*, 2004). Moreover, Taylor *et al.* (2007) found that few of the > 1,000 16S rRNA sequences obtained from the Sargasso Sea metagenome (Venter *et al.*, 2004) were closely related to the sponge specific clusters. This further supports the concept of "sponge-specific bacteria".

Hentschel *et al.* (2002) defined sponge-specific clusters based on these criteria: (i) the cluster has three or more 16S rRNA sequences, (ii) the sequences of sponge-associated bacteria are more similar to each other than to sequences from non-sponge sources, and (iii) these sequences are found in different host species or in the same species from different geographical locations. A nember of monophyletic groups were found in this study including *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Nitrospira*, and *Proteobacteria* (*Alpha*, *Delta*, and *Gammaproteobacteria*). In addition, a group of closely related alphaproteobacteria has been found to be ubiquitous in many sponges from different oceans (Webster and Hill, 2001; Enticknap *et al*., 2006). In the recent study by Taylor *et al.* (2007b), they further confirmed the presence of sponge specific clusters. Approximately one-third (32%) of all sponge-derived bacterial and archaeal sequences included in their analysis fell into such clusters with an increase to 42% if sequences derived from cultured isolates are excluded. Sponge-specific clusters were found mainly in phyla

Cyanobacteria, *Chloroflexi* and *Actinobacteria* (particularly in the sub-class *Acidimicrobiaceae*). An interesting observation in their study was that many sponge-derived sequences formed clusters with coral-derived sequences, particularly for the *Acidobacteria*, *Deltaproteobacteria, Gemmatimonadetes* and *Nitrospira* (Taylor *et al.*, 2007b).

1.4. Roles of bacterial and archaeal symbionts

1.4.1. Overview

Sponges are filter feeders that take up microorganisms from seawater and digest them by phagocytosis. At the same time, the sponge-associated microbes are present as exosymbionts in the outer layers of sponges or as intercellular endosymbionts within the mesohyl tissue. Intracellular endosymbionts can exist in sponge cells or within the nuclei of sponge cells as intranuclear symbionts (Fuerst *et al.*, 1999; Lee *et al.*, 2001). Because sponge-bacteria interactions are widely distributed and sometimes host specific, it is believed that specific symbiotic interactions exist between sponges and microorganisms (Arillo *et al.*, 1993; Oren *et al.*, 2005).

A group of closely related *Alphaproteobacteria*, affiliated with *Pseudovibrio denitrificans*, are believed to be true symbionts due to this compelling evidence: (i) Enticknap *et al.* (2006) demonstrated the association of these alphaproteobacterial symbionts with larvae of the marine sponge *Mycale laxissima*, (ii) these symbionts were ubiquitous in many sponges in several oceans, and (iii) a member of this group (NW001) dominated the microbial community of the Great Barrier Reef sponge *R.*

odorabile independent of seasonal and spatial sampling variations (Webster and Hill, 2001) was never detected in the surrounding seawater.

Cyanobacteria have received attention as sponge symbionts since they are believed to significantly enhance the growth of their hosts via photosynthesis and nitrogen fixation (Wilkinson and Fay, 1979; Borowitzka *et al.*, 1988; Wilkinson *et al.*, 1999). At least 26 of the 72 recognized Demospongiae families host cyanobacterial symbionts (Diaz and Ward, 1999). Usher *et al.* (2004) looked at the biodiversity and biogeographic distribution of unicellular cyanobacterial symbionts in sponges from Australia and the Mediterranean. They observed the presence of three types of cyanobacterial symbionts: (i) diverse symbionts that had host specific associations and comprised at least four closely related species of *Synechococcus*, (ii) a fifth symbiont related to *Oscillatoria spongiarum* that was associated only with one sponge from Australia, and (iii) one symbiont "*Candidatus* Synechococcus spongiarum", that was found in diverse sponge genera in the Mediterranean sea, Indian, Pacific, and Southern oceans and was not restricted in host association and distribution. This symbiont was also absent in the surrounding *Synechococcus*- rich water column at Fremantle, Australia. Moreover, *Ca.* S. spongiarum formed one of the largest sponge-specific clusters in Taylor's recent study (2007b). This cluster contained sequences from 21 sponges located around the world. This indicates that *Ca.* S. spongiarum is a true sponge symbiont. It is also possible that this symbiont co-evolved with a sponge host given the fact that it was detected in diverse sponge species in different geographical locations.

The uncultivated crenarchaeon *C. symbiosum* is also believed to be a true

symbiont of *A. mexicana* for to the following reasons: (i) it was the sole archaeon in *A. mexicana*, comprising up to 65% of prokaryotic cells with this sponge, (ii) it was present in all 23 individuals of *A. mexicana* collected from the Californian coast over a 9-month period (Preston *et al.*, 1996), (iii) constant and high levels of *C. symbiosum* rRNA were detected in *A. mexicana* maintained in aquaculture for more than a year (Preston *et al.*, 1996), and (iv) there was a consistent and stable association of the sponge host and its single archaeal symbiont in three Mediterranean axinellid sponges (*Axinella damicornis*, *Axinella verrucosa*, and *Axinella* sp.). The archaeal symbionts in all cases were related to *C. symbiosum* (Margot *et al.*, 2002).

1.4.2. Mode of acquisition of symbionts

The mode whereby sponges acquire their symbionts is still unclear. Wilkinson (1984) suggested the association of symbiotic bacteria in ancestral sponges prior to sponge evolution. Such symbiosis was possibly maintained via vertical transmission. Vertical transmission is considered a key factor in the evolution of symbiosis away from parasitism and towards mutualism (Yamamura, 1993; Herre *et al.*, 1999). It can happen via sponge larvae or during the process of asexual reproduction (Hill, 2004). Vertical transmission via asexual reproduction may allow for the transmission of multiple bacteria and permit transmission of a greater diversity than the more stringent transmission through the germ line (Hentschel *et al.*, 2002). Vertical transmission of symbionts has now been demonstrated in several studies and provides strong evidence for specific sponge-microbe symbioses. Usher *et al.* (2005) reported vertical transmission of the cyanobacterial symbiont *Ca.* S. spongiarum in the

Australian sponge *Chondrilla australiensis* via eggs and sperms and the presence of biparental mechanisms of transmission. Enticknap *et al.* (2006) isolated an alphaproteobacterium and showed that is vertically transmitted via sponge larvae of the marine sponge *M. laxissima*. Diverse groups of microbes were shown to be vertically transmitted in *Corticium* sp. (Sharp *et al.*, 2007) and *Ircinia felix* (Schmitt *et al.*, 2007). Vertical transmission of symbionts has been speculated for *Poribacteria* symbionts (Taylor *et al.*, 2007b).

Horizontal transmission is another mode for acquisition of symbionts in sponges. A 1-kg sponge is capable of filtering up to 24,000 liters of seawater per day (Vogel, 1977). Hill (2004) suggested that a 1-kg sponge is capable of ingesting 2.4×10^{13} bacterial cells/day, assuming a typical bacterial density for the tropical coral reef environment of about 10^6 cells/ml. This filtration capacity provides a huge supply of bacteria that can potentially be sequestered within sponge tissues (Hill, 2004). It is likely that some sponge-specific bacteria are initially acquired by filtration from the oligotrophic water column where they exist in low abundance. Sponge tissues then provide increased nutrient availability and a safe habitat. Recognition of symbionts versus food particles has been proven previously (Wilkinson, 1984). In favor of this scenario is that the vertically-transmitted alphaproteobacterial symbiont in *M. laxissima* was closely related to bacteria isolated from seawater (Enticknap *et al.*, 2006). It is possible that in some cases, a symbiont may be both vertically and horizontally transmitted. It is difficult to exclude the possibility of horizontal transmission of a symbiont because of the very large volume of water filtered by sponges.

1.4.3. Physiology of sponge symbionts

Understanding the metabolic capabilities of the bacteria within sponges is crucial for understanding the symbiotic relationships between bacteria and sponges. It is still unclear why sponges harbor microorganisms. One important role of sponge symbionts may be participation in the host's chemical defense mechanism against pathogens, predators and biofouling. The role of symbiotic bacteria in production of bioactive compounds was discussed in the Section 1.2.2 "Bioactive compounds from bacterial symbionts of sponges". Although it is reasonable to assume that bioactive compounds produced by symbionts play a role in the defense of sponges, there is no experimental evidence that supports this hypothesis. The most obvious potential explanation might be that microorganisms provide food or other useful metabolic products to their host.

1.4.3.1. Carbon

The growth of beneficial microorganisms has been termed 'gardening' or 'farming' and may occur frequently in sponges (Osinga *et al.*, 2001). An example of gardening is the relationship between sponges and phototrophic microorganisms such as cyanobacteria (Wilkinson, 1978a). Translocation of photosynthate mainly in the form of glycerol has been shown from cyanobacterial symbionts to marine sponges (Wilkinson, 1979). Wilkinson (1983) showed that the primary production of the cyanobacterial symbionts was higher than the total respiration of the sponge-symbiont consortium. It was estimated that 80% of the total energy requirement of the sponge

Phyllospongia lamellose was produced by its phototrophic symbionts (Cheshire *et al.*, 1997).

1.4.3.2. Nitrogen

The nitrogen cycle in sponges can comprise many chemical transformations (Fig. 1.2). A role for sponge symbionts in nitrogen fixation has been postulated. Many sponges grow in tropical reef environments where concentrations of fixed nitrogen in the surrounding water are generally very low (Wilkinson *et al.*, 1999). In cases where sponges obtain photosynthetically derived carbohydrates from cyanobacterial symbionts, these nutrients are rich in carbon but devoid of nitrogen (Wilkinson and Fay, 1979; Wilkinson, 1983). Without an added source of nitrogen, sponge growth rates could be reduced due to limited amino acid biosynthesis and subsequently protein synthesis. There has been much speculation in the past thirty years regarding the role of cyanobacterial symbionts in nitrogen fixation in marine sponges. In a study of sponge-cyanobacterial symbioses by Wilkinson and Fay (1979), nitrogenase activity was detected using the acetylene reduction assay in two sponges that harbored cyanobacteria but not in a third sponge species lacking cyanobacteria. The three sponges were collected from a coral reef in the Red Sea. This was the first demonstration of nitrogenase activity in an animal with symbiotic cyanobacteria. Nitrogenase activity was attributed to symbiotic cyanobacteria rather than other bacteria, for the following reasons: (i) nitrogenase activity was evident in *Siphonochalina tabernacula* and *T. swinhoei,* which harbor symbiotic cyanobacteria, and was absent in *Inodes erecta,* which lacks cyanobacteria, (ii) more ethylene was

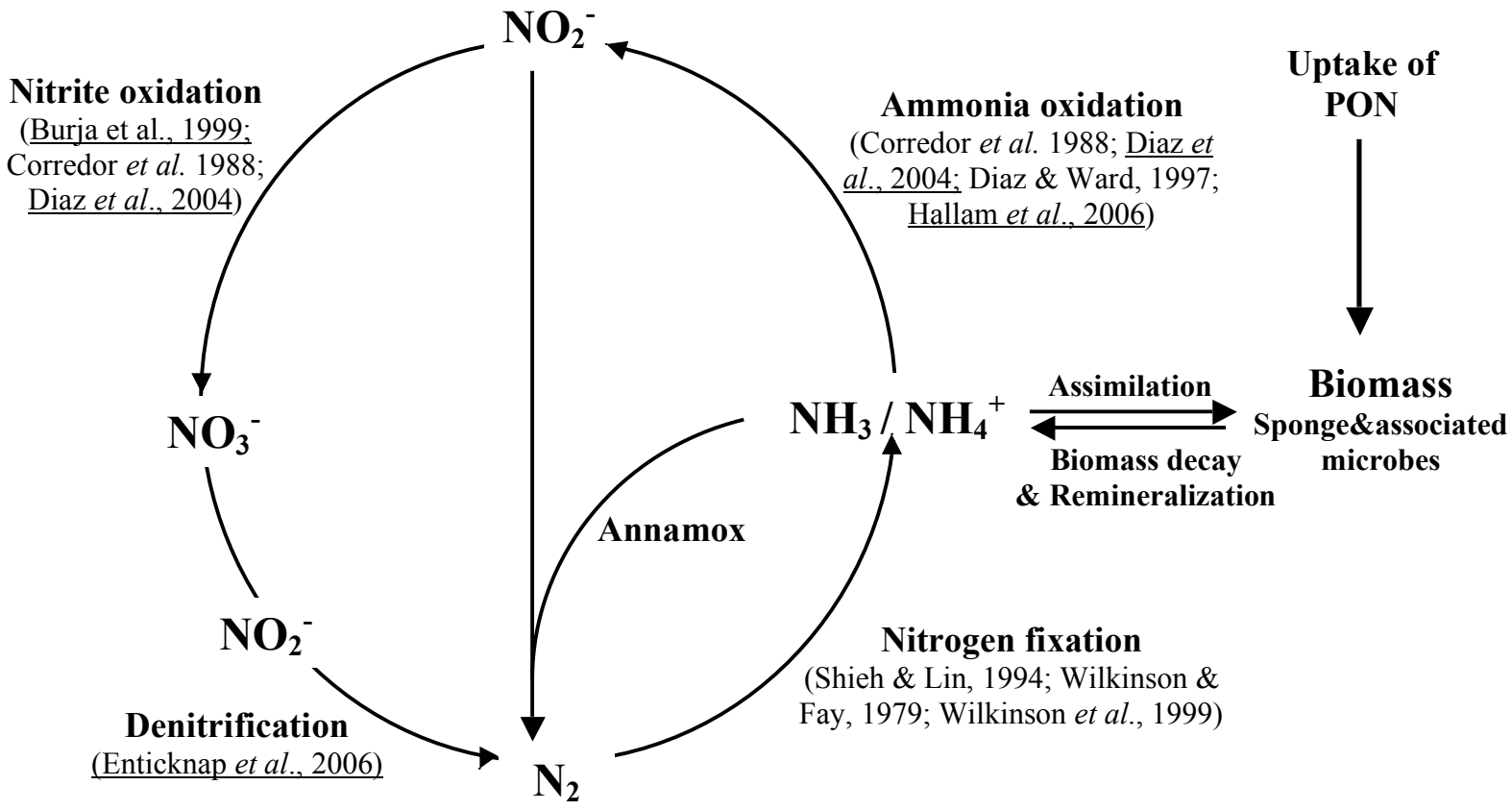


Fig. 1.2. Nitrogen cycle in marine sponges. Underlined references indicate that there was no experimental demonstration for the corresponding process and evidence was limited to the presence of bacteria or genes generally implicated in the process. Annomox, anaerobic ammonia oxidation, has not yet been shown in sponges. Adapted from Taylor *et al*., 2007.

produced by the ectosome of *T. swinhoei*, which contained cyanobacteria, (iii) nitrogenase activity was higher in illuminated tissue than in tissue incubated in the dark, and (iv) the amounts of ethylene produced in *S. tabernacula* and *T. swinhoe* were comparable although the total bacterial population in *T. swinhoe* was many times larger than that in *S. tabernacula* and similar to that in *I. erecta*. A subsequent study by Wilkinson *et al.* (1999) provided stronger evidence for nitrogen fixation in the Indo-Pacific coral reef sponge *Callyspongia muricina.* Using stable isotope analysis, they demonstrated the incorporation of the stable isotope $^{15}N_2$ into various amino acids in *C. muricina*. They related this activity to cyanobacterial symbionts since nitrogen fixation was observed in whole sponges and cyanobacterial cell preparations. Wilkinson and Fay (1979) did not exclude the possibility of participation by other bacterial symbionts in the observed nitrogenase activity and they suggested that synergistic nitrogen fixation might occur in sponges when cyanobacterial and heterotrophic bacteria are closely associated. The role for bacterial symbionts of sponges in nitrogen fixation is discussed in more detail in Chapter 4.

Sponges were also found to both assimilate and release dissolved inorganic nitrogen (DIN: ammonia, nitrite and nitrate) as well as dissolved and particulate organic nitrogen (DON and PON, respectively) (Diaz and Ward, 1997; Pile, 1997). In a study by Diaz and Ward (1997), the nitrification capacity was investigated in tropical sponges and was related to the association between sponges and bacteria. Higher rates were observed in sponges that harbored cyanobacteria. A clear difference in the levels of DIN accumulation was detected between three species that

possess cyanobacteria endosymbionts (ten times higher) and the one species that lacks them. In a recent study by Jiménez and Ribes (2007), when they evaluated the nitrogen ingestion from particulate organic matter and DIN excretion by Mediterranean sponges, there was an imbalance with higher nitrogen release than uptake in most of the sponges studied. They suggested that this imbalance might be due to the presence of an additional nitrogen source, such as nitrogen fixation by sponge-associated cyanobacteria.

16S rRNA genes from ammonia oxidizing betaproteobacteria (Diaz *et al.*, 2004) and nitrite oxidizing bacteria of the genus *Nitrospira* (Hentschel *et al.*, 2002) were recovered from sponges. Interestingly, the *amoA* gene encoding ammonia monooxygenase A, an enzyme required for ammonia oxidation, was found in the genome of the symbiotic archaeon *C. symobiosum* (Hallam *et al.*, 2006). It is possible that both Archaea and bacteria are capable of ammonia oxidation in marine sponges, but whether archaeal or bacterial symbionts are the key ammonia-oxidizers is not yet clear.

Denitrification by sponge-associated microbes has not yet been reported. The alphaproteobacterium that was found to be widely distributed and vertically transmitted (Enticknap *et al.*, 2006) is closely related to the marine denitrifier *Pseudovibrio dentirificans*, and some of these strains test positive for denitrification using a biochemical assay (Taylor *et al.*, 2007b). It is possible that sponges, as efficient filter feeders, can in some cases provide denitrifying populations with PON. Heterotrophy of the sponge *Halichondria cymaeformis* on ultraplankton was shown to yield enough nitrogen for the sponge and its macroalgal symbiont (Davy *et al.*, 2002;

Pile *et al.*, 2003). Analysis of genes encoding essential enzymes in the denitrification process and examination of the expression of these genes could give insights into this metabolism in bacteria associated with sponges. This process could be confirmed by isolation of symbionts that are shown to be capable of denitrification.

1.4.3.3. Sulfur

Sulfate reducing and oxidizing bacteria have been found in 16S rRNA gene libraries of sponges (Imhoff and Trüper, 1976). Green sulfur bacteria (*Chlorobi*) were detected in *R. odorabile* by FISH studies (Webster *et al.*, 2001b). Sulfate-reducing bacteria were detected using FISH in the Mediterranean sponges *Chondrosia reniformis* and *Petrosia ficiformis* (Manz *et al.*, 2000) (Schumann-Kindell *et al.*, 1997) as well as the cold water sponge *Geodia barretti* (Hoffmann *et al.*, 2005; Hoffmann *et al.*, 2006). Hoffmann *et al.* (2005) showed the presence of anoxic zones within sponge tissues especially during periods of low pumping activity, using microelectrodes. This further suggests the possibility of anaerobic sulfate-reduction metabolism within sponges. The presence of anaerobic bacterial metabolic activity in sponges indicates the presence of anaerobic niches within sponge tissues. This is consistent with respiratory depletion of oxygen in the water pumped through sponge tissue, leading to anaerobic environments in the interior tissues. Obtaining pure cultures of sponge-associated microbes will help understanding the physiological characteristics of sponge-associated microorganisms and thus their roles in the major nutrient cycles within sponges.

1.4.4. Host-symbiont interactions

1.4.4.1. Recognition mechanisms between sponges and their bacterial symbionts

The fossil record provides evidence for ancient close associations of sponges and microorganisms (Brunton and Dixon, 1994; Oloriz *et al.*, 2003; Soja *et al.*, 2003). The potential for host-symbiont co-evolution, particularly between sponges and their cyanobacterial symbionts, is supported by vertical transmission of symbionts between sponge generations. One of the interesting aspects of sponge-microbe interactions is the presence of symbionts within phagocytic archaeocytes in the mesohyl. Wilkinson *et al.* (1984) fed tritium-labeled bacteria from sponges and seawater to a sponge. The sponge symbionts passed through uneaten while the seawater bacteria were largely consumed. They proposed two mechanisms, either symbionts were selectively recognized by sponges or they had extracellular capsules to avoid detection by sponge cells. This is consistent with the recent work by the Hentschel group where *A. aerophoba* was shown to consume seawater bacteria faster than sponge symbionts (Wehrl *et al.*, 2007). These studies suggest the presence of special recognition mechanisms whereby sponge hosts are capable of recognizing bacterial symbionts in the bulk supply of bacteria in the surrounding seawater and vice versa.

In a study by Kelman *et al.* (2001), they found that the marine sponge from the Red Sea, *Amphimedon viridis*, produced pyridinium alkaloids. These bioactive alkaloids were highly active against eight strains of bacteria isolated from the surrounding seawater. Interestingly, six bacterial strains associated with *A. viridis* resisted these compounds. This selective toxicity again indicates a special

recognition and interaction between a sponge and its symbionts.

Interestingly, certain genes encoding cell surface, regulatory, or defense mechanisms found in *C. symbiosum* were absent from its free-living relatives, suggesting that these genes could be involved specifically with the establishment and maintenance of the symbiosis between this archaeon and the host sponge *A. mexicana* (Hallam *et al.*, 2006).

Studies of the Adriatic sponge *Suberites domuncula* demonstrated immune defense responses against Gram-negative bacteria via production of antibacterial compounds (Böhm *et al.*, 2001; Wiens *et al.*, 2005) and Gram-positive bacteria via endocytosis and release of lysozyme (Thakur *et al.*, 2005). Müller *et al.* (2004b) studied the mechanism of association of *S. domuncula* and its alphaproteobacterial symbiont SB2. SB2 grew preferentially on minimal medium supplemented with the aromatic compound protocatechchuate rather than glucose. The authors detected tyrosinase activity that is required for the breakdown of protocatechchuate and expression of tyrosinase-encoding gene in *S. domuncula*, as well as bacterial genes responsible for the utilization of protocatechchuate in SB2, and they were all maximal under aerated conditions. In addition, SB2 symbionts were lost from the surface layer of the sponge under low oxygen conditions, which indicates a role for oxygen level in regulating these symbionts in *S. domuncula*.

1.4.4.2. Quorum sensing

The term ‘quorum sensing’ describes chemical communication between bacteria, and involves producing, releasing and responding to autoinducers, hormone-like

molecules, in a population-dependant manner (Fuqua *et al.*, 2001; Waters and Bassler, 2005). Gram-negative *Proteobacteria* produce a group of well-studied autoinducers known as *N*-acyl homoserine lactones (AHLs). Quorum sensing was first discovered in the bioluminescent marine bacterium *Vibrio fischeri* (Nealson *et al.*, 1970). *V. fischeri* colonizes the light organs of several marine fishes and squids, and controls light production through expression of an operon of bioluminescence genes (*luxICDABEG)*. The *lux* operon is regulated through the production of the signaling molecule *N*-3-oxo-hexanoyl-L-homoserine lactone (Eberhard *et al.*, 1981). Quorum sensing has been shown to play an integral role in the regulation of a variety of cellular processes, including, but not limited to, biofilm formation, exoenzyme production, synthesis of virulence factors, antibiotic production, and conjugal plasmid transfer (Fuqua and Greenberg, 2002).

All forms of higher life involve interactions between eukaryotes and prokaryotes. Sponges are the most primitive multicellular eukaryotic organisms and provide an excellent model system for understanding evolution of complex symbiotic interactions. Sponges harbor dense and complex assemblages of microbial populations, which create an environment highly conducive to microbial interactions such as quorum sensing. Quorum sensing might regulate some of the important roles of sponge endosymbionts such as the production of bioactive secondary metabolites. It is also possible that it plays an important role in colonization of sponges by dense bacterial populations. One appealing scenario is that sponge-specific bacteria are initially acquired from the oligotrophic water column where they exist in low abundance. In sponge tissues, the bacteria encounter favorable survival conditions

such as availability of nutrients and grow to achieve high population densities that allow for a bacterial quorum.

The production of AHL molecules by sponge-associated bacteria was first reported by Taylor *et al.* (2004a). They identified two AHL-producing bacteria; a gammaproteobacterium, affiliated with *Vibrio campellii*, and an alphaproteobacterium within the *Roseobacter* clade. A sponge isolate from the *Roseobacter* clade (within the TM1040 cluster) was capable of producing 10 distinct cyclic dipeptides. Cyclodipeptides constitute one of the families of cell-cell signaling compounds and may have some role to play in sponge-bacteria interactions (Mitova *et al.*, 2004). Another intriguing study was the stimulation of the growth of a marine gammaproteobacterium isolated from marine sponge *Jaspis joinstoni* under iron-limited conditions with trace amounts of exogenous siderophore from an alphaproteobacterium, and a quorum-sensing chemical signal (Guan *et al.*, 2000). Quorum sensing may play a major role in regulating aspects of sponge-microbe interactions and/or microbial community structure. Quorum sensing in sponge bacterial symbionts is discussed in detail in Chapter 5.

1.5. Focus and objectives

My research is designed to understand the diversity and the roles of bacterial communities associated with marine sponges *I. strobilina* and *M. laxissima.* My research not only provides a better understanding of the genetic diversity of bacteria associated with marine sponges, but also provides new insights into understanding the

roles of these symbionts that moves the field of sponge microbiology forward. In addition, my research provides an improved understanding of the role of sponge symbionts in the marine and especially the oligotrophic tropical reef nitrogen budget.

The primary objectives of this research are:

1. To characterize the bacterial communities associated with *M. laxissima* sponges collected from the wild and maintained in aquaculture, and surrounding seawater using culture-dependent and molecular techniques.
2. To compare the bacterial communities between wild sponges and those maintained in aquaculture systems and between sponges and surrounding water samples.
3. To correlate bacterial and chemical diversities of *I. strobilina* on transfer into aquaculture.
4. To demonstrate nitrogen fixation in sponges using nitrogen isotopic composition.
5. To investigate the diversity and expression of *nifH* genes of bacterial populations associated with *I. strobilina* and *M. laxissima.*
6. To culture nitrogen fixing bacteria carrying *nifH* genes.
7. To test the production of *N*-acyl homoserine lactone signaling compounds by bacterial symbionts associated with *I. strobilina* and *M. laxissima.*

The microbial diversity present in many sponges is remarkable and spans all three domains of life, with at least 18 bacterial and archaeal phyla (Taylor *et al.*, 2007b). There is a growing knowledge of this diversity based on culture-dependent and independent techniques. Current challenges in sponge symbiont microbiology were

the topic of discussion in a recent roundtable session "Marine sponges as microbial fermenters", held under the auspices of the 11th International Symposium on Microbial Ecology, Vienna, Austria (Taylor *et al.*, 2007a). The lead scientists of sponge microbiology concluded that although there is currently a great knowledge of microbial diversity in sponges, there is a limited knowledge of symbiont function and sponge-microbe interactions.

Two sets of aquaculture systems were designed in order to optimize the conditions for culturing two marine sponges, *I. strobilina* and *M. laxissima.* My primary objective is to develop a model sponge system in aquaculture, which can be used as a powerful tool to test the following hypotheses: (i) some of the microbial communities associated with marine sponges are sponge-specific thus they are stable when sponges are transferred into aquaculture (Objectives 1 and 2 in Chapters 2 and 3), (ii) quorum sensing plays a role in the interactions between bacteria and sponge hosts (Objective 7 in Chapter 5), and (iii) aquaculture can provide a solution for the supply problem of sponge-derived bioactive compounds (Objective 3 in Chapter 3).

Important questions in my research are: how are sponges obtaining their nitrogen in the oligotrophic coral reef environment and is nitrogen fixation a possible role for associated microbes? Two possibilities are that sponges obtain their nitrogen primarily from the organic nitrogen present in the bacteria that they filter out of the water column, or that their nitrogen is primarily supplied from nitrogen-fixing symbiotic bacteria within the sponges. My hypothesis is that bacterial symbionts fix nitrogen and provide nitrogen to their sponge hosts. To test this hypothesis, I first investigated nitrogen fixation in marine sponges using the stable nitrogen isotopic

composition. I then used molecular techniques to detect the diversity and expression of nitrogen fixation genes in bacteria associated with sponges (Objectives 4, 5, and 6 in Chapter 4). Since sponges are efficient filter feeders, the possibility exists that *nifH* genes recovered from the sponge-hosted community could include genes from planktonic nitrogen-fixing bacteria trapped from the ambient water by filtration. To exclude this possibility, I also investigated the diversity of *nifH* genes in two seawater samples collected from the same site as our sponges.

There are two well-known systems of eukaryote-bacterial symbioses and interactions: the bioluminescent squid, *Euprymna scolopes* and the marine heterotrophic bacteria, *Vibrio fischeri* (Visick *et al.*, 2000), and aphids and the obligate bacterial symbiont *Buchnera aphidicola* (Dale and Moran, 2006). Studying the two systems was facilitated by the simplicity of their endosymbiotic populations. This simple host-symbiont interaction is not expected to be the norm in nature (Taylor *et al.*, 2007a). The complex and diverse microbial communities hosted by marine sponges indicate that prokaryote-eukaryote associations in the oceans are probably complex and involve more that one symbiont. Sponges are potential models to study symbiosis. I hypothesize that quorum sensing may play a major role in regulating aspects of host interactions and/or community structure. One important goal in my research was to detect whether sponge-associated bacteria are capable of producing *N*-acyl homoserine lactone signal molecules (Objective 7 in Chapter 5). Although this does not directly test my hypothesis, it lays the groundwork for future studies of quorum sensing as a key factor in the interactions between sponge hosts and their symbionts as well as between symbiont populations.

Chapter 2. Changes in Bacterial Communities of the Marine Sponge *Mycale laxissima* on Transfer into Aquaculture

2.1. *Abstract*

The changes in bacterial communities associated with the marine sponge *M. laxissima* on transfer to aquaculture were studied using culture-based and molecular techniques. *M. laxissima* was maintained alive in flow-through and closed recirculating aquaculture systems for one year and two years, respectively. The bacterial communities associated with wild and aquacultured sponges as well as the surrounding water were assessed using 16S rRNA gene clone library analysis and denaturing gradient gel electrophoresis. Bacterial richness and diversity were measured using DOTUR computer software and clone libraries were compared using S-LIBSHUFF. DGGE analysis revealed that the diversity of the bacterial community of *M. laxissima* increased when sponges were maintained in aquaculture and that these bacterial communities were markedly different than those of the surrounding water for both wild and aquacultured sponges. Clone libraries of bacterial 16S rRNA from sponges confirmed that the bacterial communities changed during aquaculture. These communities were significantly different than those of seawater and aquarium water. The diversity of bacterial communities associated with *M. laxissima* increased significantly in aquaculture. My work shows that it is important to monitor changes in bacterial communities when examining the feasibility of growing sponges in aquaculture systems because these communities may change. This could have implications for the health of sponges or for production of bioactive compounds by sponges in cases where these compounds are produced by symbiotic bacteria rather

than by the sponges themselves. This work contributes to development of *M. laxissima* as a model system for laboratory studies of complex symbioses between bacteria and marine invertebrates.

2.2. Introduction

The taxonomy of the marine sponge *M. laxissima* is class Demospongiae Sollas 1885, order Poecilosclerida Topsent 1928, family Mycalidae Lundbeck 1905, *Mycale* (*Arenochalina*) *laxissima* (Duchassaing and Michelotti, 1864). *M. laxissima* is identified by being tubular to globular, solitary or in clusters. It has 1-2 cm thin walls and is up to 50 cm tall. Another characteristic is the presence of a 3-6 cm wide large pseudoscule with a transparent membrane. *M. laxissima* is dark wine red to black with a spiny surface. It is tough but compressible and releases sticky mucus when squeezed. It is distributed in reefs and mangrove peat banks, occasionally on roots (Collin *et al.*, 2005; Enticknap *et al.*, 2006).

Marine sponges have been recognized as hosts for many microorganisms. Sponges are filter-feeders; numerous tiny pores on the surface allow water to enter and circulate through a series of aquiferous channels where microorganisms and organic matter are filtered out (Lee *et al.*, 2001). In addition to those bacteria serving as a food source, the sponge microbial community is comprised of a transient seawater population that is coincidentally present in the sponge, microbes that grow in the mesohyl and symbionts that live inside the sponge cells.

Sponges are known as prolific sources of bioactive compounds that can

potentially be used to treat various human diseases (Faulkner *et al.*, 1999; Faulkner, 2002). *In situ* cultivation of marine sponges (mariculture) and cell culture approaches have been explored as possibilities for large-scale production of sponge derived compounds (Munro *et al.*, 1999; Duckworth, 2001; Osinga *et al.*, 2003; van Treeck *et al.*, 2003b; Zhang *et al.*, 2003; Müller *et al.*, 2004a; Sipkema *et al.*, 2005). One promising strategy is the *ex situ* culture of sponges in closed or semi-closed systems. Aquaculture in tanks might be preferable to *in situ* mariculture because it is reliable and provides the possibility of switching from seasonal growth to continuous growth during the year (Duckworth and Battershill, 2003a; Sipkema *et al.*, 2005). Also, maintenance of marine sponges in aquaculture provides a potential model to study sponge-microbe interactions. However, very little is known about the optimal environmental conditions and ecological needs of sponges and this makes the optimization of sponge growth and compound production a difficult process (Osinga *et al.*, 1999b; van Treeck *et al.*, 2003b; Duckworth and Battershill, 2003b).

In order to examine the potential of *ex situ* cultivation of marine sponges as a solution for the supply problem, it is crucial to determine whether the microbial communities change upon culturing. *M. laxissima* was chosen as the model sponge due to its high capability of adapting to aquaculture conditions compared to other sponges from the same reef environment examined in preliminary trials. Also *M. laxissima* is a representative of the genus *Mycale* that is of considerable interest as a source of metabolites with a wide range of bioactivities including significant cytotoxicity, antiviral, antitumor and antimitotic activities (Perry *et al.*, 1988; Fusetani *et al.*, 1991; Northcote *et al.*, 1991; Thompson *et al.*, 1994; Matsunaga *et al.*,

1998a; Matsunaga *et al.*, 1998b; Rzasa *et al.*, 1998; West *et al.*, 2000a; West *et al.*, 2000b; Hood *et al.*, 2001; Pattenden *et al.*, 2004; Nishimuraa *et al.*, 2005; Phuwapraisirisan *et al.*, 2005; Tsukamoto *et al.*, 2005). A number of these cytotoxins have been isolated from *Mycale hentscheli* (Bergquist and Fromount, 1988) from New Zealand waters. Mycalamide D was isolated from *M. hentscheli* collected from the northeast coast of the North Island (West *et al.*, 2000a). Immunosuppressant and apoptotic activities have been reported for the secondary metabolite pateamine A from *M. hentscheli* (Northcote *et al.*, 1991; Rzasa *et al.*, 1998; Hood *et al.*, 2001; Pattenden *et al.*, 2004). A new oxazole-containing proteasome inhibitor, secomycalolide A, together with known mycalolide A and 30-hydroxymycalolide A, were isolated from a sponge in the genus *Mycale* and demonstrated a proteasome inhibitory effect (West *et al.*, 2000b; Tsukamoto *et al.*, 2005). Perloruside A, the most recent cytotoxic metabolite, was isolated from *M. hentscheli* collected from the North coast of the South Island. It is a microtubule stabilizer with potency and mode of action similar to that of the major anticancer drug paclitaxel (Taxol®) and epothilones (Page *et al.*, 2005).

The first objective of this study was to characterize the microbial communities associated with wild and aquacultured *M. laxissima* sponges using culture-dependent and molecular techniques. The second objective was to compare the bacterial communities between wild sponges and those in aquaculture systems. The following questions were addressed: 1. Is there a sponge-specific community in *M. laxissima* that is different from that of the bacterioplankton in the water column? and 2. Does the transfer of the sponges into aquaculture cause a change in the bacterial

community?

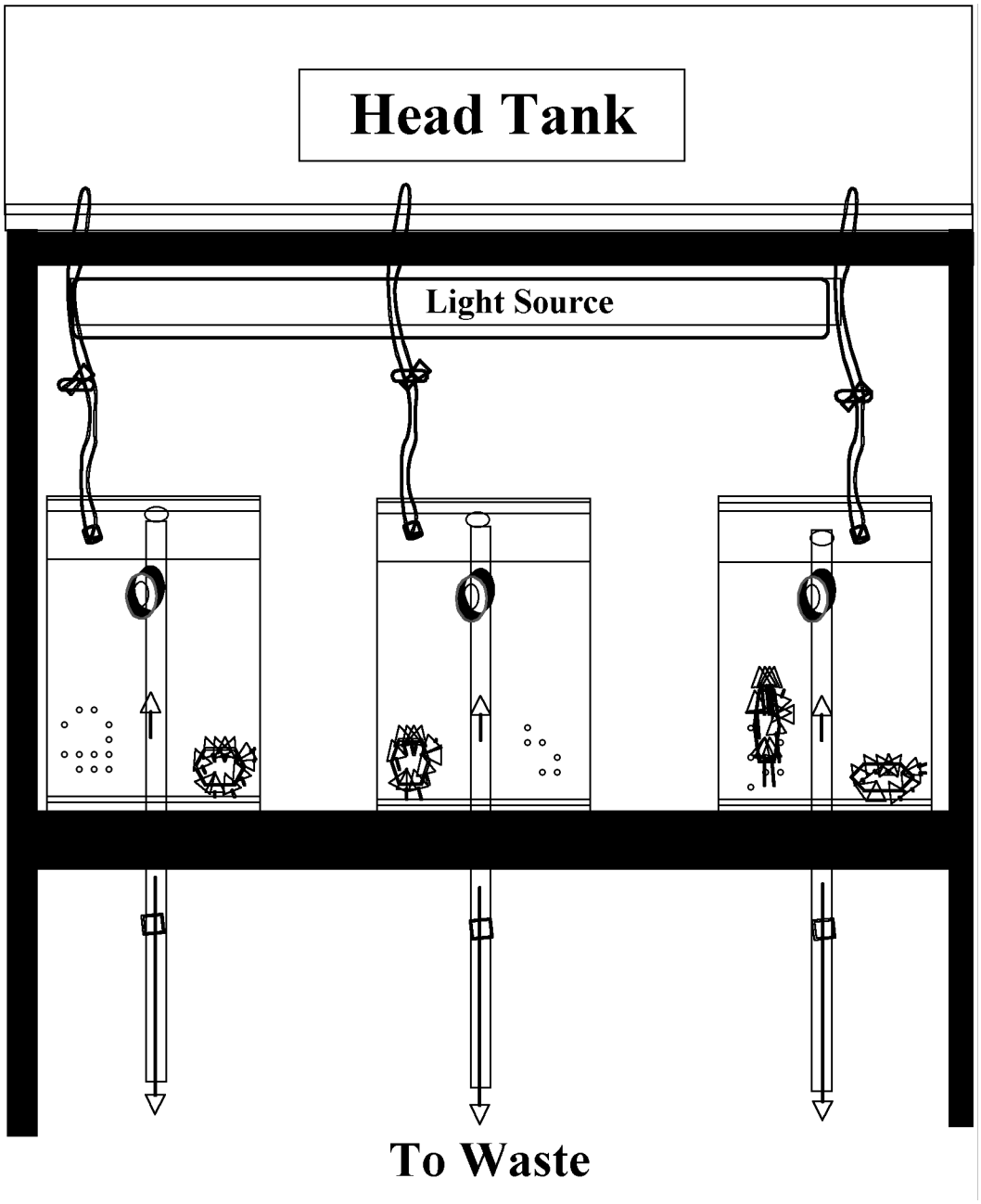
2.3. Materials and methods

Sponge collection and taxonomic identification. Individual *M. laxissima* sponges were collected by SCUBA at Conch Reef, Key Largo, Florida in July 2001 and June 2004 in water depths of ca. 15 meters (latitude 24°57.11′N, longitude 80°27.57′W). Water salinity was 36 ppt and temperature was 26.7˚C. Chemically characterized voucher specimens were registered with the Natural History Museum (formerly the British Museum of Natural History) (Enticknap *et al.*, 2006). Water samples were collected near the sponges at a depth of ca. 15 m in sterile 20-liter containers and 15-20 l were filtered through 0.22-µm-pore-size Sterivex filters (Millipore) for each water sample. Sterivex filters were frozen immediately and stored at -20˚C for isolation of nucleic acids. Sponge samples and Sterivex filters were transferred to Baltimore on dry ice and stored at -80˚C. The sponges collected for aquaculture were transported in containers filled with aerated seawater. The time from collection to release in aquaria was generally less than 48 h. Water (50% volume) was changed every 4-6 h and was kept aerated by battery operated air pumps and airstones.

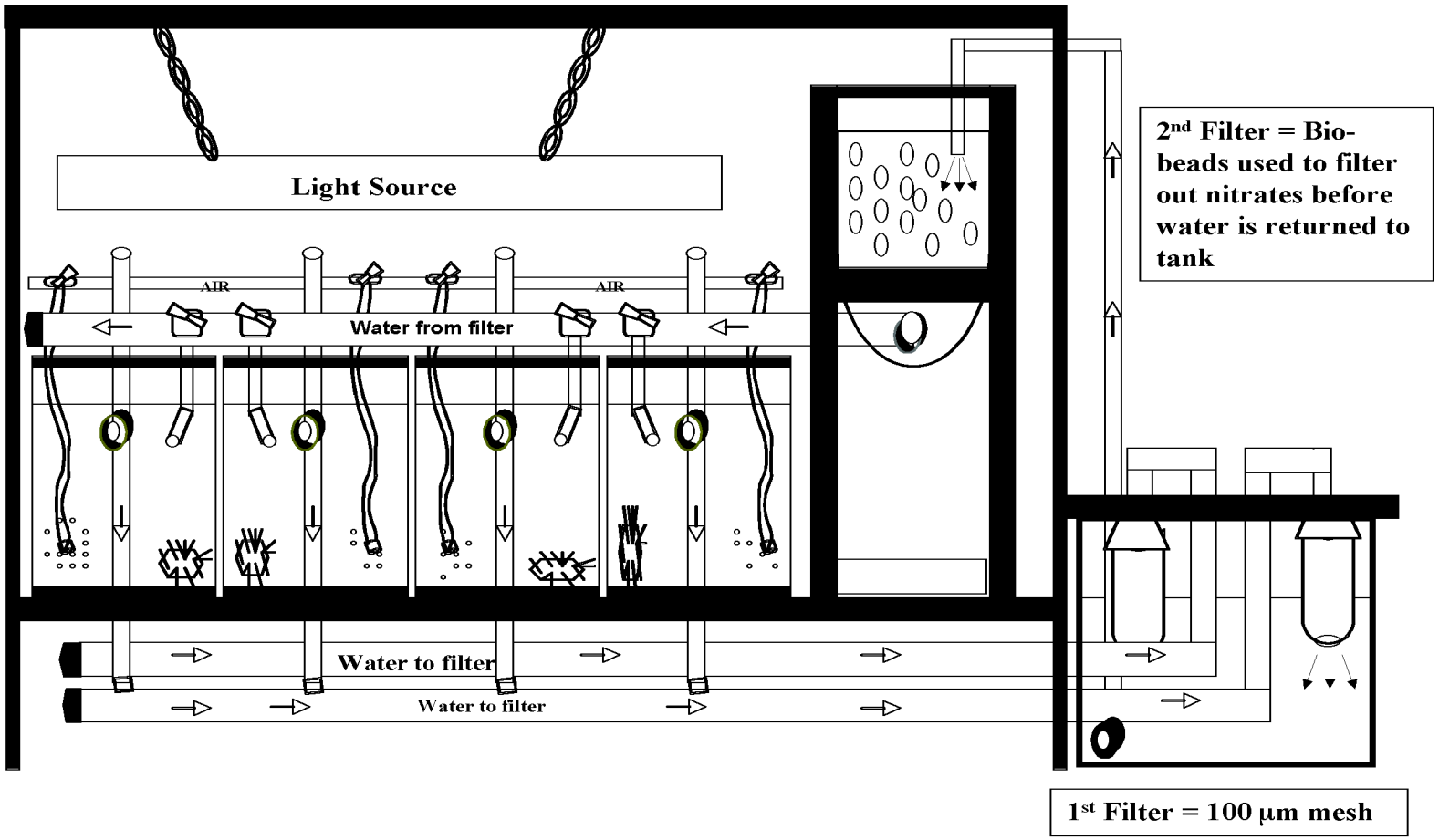
Sponge aquaculture. Two different aquaculture systems were designed: **A. Flow-through system.** A closed flow-though system was constructed using a 360-liter head tank and three 80-liter fish tanks fitted with small external filters and siphons (Fig. 2.1). The flow rate was controlled using a valve set to 4 l per hour giving a turnover rate of 2.4 times every 24 hours.

Fig. 2.1. Two aquaculture systems: **A.** Flow-through system and **B.** Recirculating system.

A.



B.


Light Source
AIR
AIR
Water from filter
Water to filter
Water to filter
2nd Filter = Bio-beads used to filter out nitrates before water is returned to tank
1st Filter = 100 µm mesh

The constant influx of new sterile artificial seawater and removal of old salt water helps maintain water quality. Sponges were fed the microalga *Nanochloropsis* sp. with addition of 40 ml of a 4×10^6 cells ml^{-1} culture every two to three days. Four sponge individuals were collected in 2001. One was processed as a wild sponge. It was processed for microbiology in the field and a specimen of the sponge was frozen for DNA extraction. One individual was kept in this aquaculture system for six months, and two for two years. The health of the aquacultured sponges was monitored visually during this period and digital images were taken. After six months, one sponge individual was sacrificed and processed immediately for microbiology. Specimens were stored at -80˚C for isolation of nucleic acids. The procedure was repeated for the two remaining sponges after two years in the aquaculture system. At two years, four liters of water were filtered through Sterivex filters for isolation of nucleic acids from bacteria in the aquaculture tank water.

B. Recirculating system. A large-scale recirculation aquaculture system was designed and constructed to house the 2004 sponge collection (Fig. 2.1). The 800-liter system was constructed using four 80-liter tanks and two 160-liter tanks. These tanks were drilled with a 1.5-cm hole about 9.5-cm from the bottom, and bulkheads were attached. Bulkheads were connected with 1.5-cm PVC pipe to allow water to drain from the tanks. These pipes emptied into a sump where the waste passed through a 100-µm mesh bag filter. A protein skimmer was installed in the sump to remove organic material from the water. From the sump, the water was pumped into a biofilter and an algal turf scrubber. The system had actinic and 10,000 K lights set on a normal daily light regime. Five sponges were collected in 2004. Three were

processed as wild sponges. Two were maintained in the recirculating aquaculture system for three months where their health condition was inspected visually during this period.

Sponge processing for isolation of culturable bacteria. Immediately after collection of sponges, samples were rinsed thoroughly three times with sterile artificial seawater (ASW) to remove any transient bacteria, algae or mucus attached to the surface of the sponge. Sponge tissue (1 cm^3) was ground in ASW and ten fold serial dilutions were plated on Difco marine agar 2216 (BD Biosciences, Franklin Lakes, NJ, USA). Plates were incubated at 30˚C for a week. Serial dilutions of water samples were processed similarly for bacterial isolation.

Determination of culturable and total bacterial counts. After one week of incubation of marine agar plates, the plate counts were determined. To determine the total bacterial counts, a defined volume of the tissue homogenate was fixed with 37 % (w/v) paraformaldehyde to a final concentration of 2-4% (w/v) and stored at 4^oC until use. DAPI (4,6-diamidino-2-phenylindole) was added to the fixed samples to a final concentration of 20-µg ml^{-1} as described by Porter and Feig (1980). A volume of 10 ml of DAPI-stained homogenates was filtered under slow vacuum onto a 25 mm diameter, 0.1-µm polycarbonate membrane (GE Osmonics, Minneapolis) that was supported with a 45 µm GF-F type membrane (Whatman International Ltd, Maidstone, England). The filters were air dried and then mounted with immersion oil onto a microscope slide. Bacterial numbers were determined using an epifluorescent microscope (Axioplan Microscope, Zeiss, Germany). I processed three, two and one independent samples from wild, 3-month and nine-month sponges, respectively. For

each sample, an average bacterial number was determined by counting 10 fields.

Identification of isolates by 16S rRNA gene sequence analysis. A representative of each bacterial morphotype was selected for further purification and sequencing. Single pure colonies of each isolate were transferred to 20 ml of marine broth 2216 (BD BioSciences) and incubated overnight at 30˚C in a shaking incubator 100 rpm. DNA was extracted from these isolates using the Ultra-Clean microbial kit (MoBio Laboratories, Carlsbad, CA, USA). Isolates were stored at -80˚C in marine broth 2216 supplemented with 30% glycerol to a final volume of 2 ml and 15% glycerol. The 16S rRNA gene was PCR-amplified using universal primers 27F and 1492R (Lane, 1991) as described in Enticknap *et al*. (2006). The genomic DNA (15-40 ng) was added to 49 µl PCR mixtures containing 5 µl of 10 × High Fidelity Buffer, 1 µl of a mix of deoxynucleotide triphosphates (2.5 mM each), 2 µl of $MgSO_4$, 38.5 µl of PCR-water, 1 µl of 10 µM 27F, 1 µl of 10 µM 1492R, and 0.2 µl of Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen Life Technologies). The temperature-cycling conditions were as follows: Pre-incubation at 94°C 5 min, followed by 30 cycles of 94°C for 30 sec, 48°C for 2 min, and 72°C for 1.5 min. Cycling was followed by 7 min incubation at 72°C. For every PCR experiment, negative (no DNA) controls were included. Amplification products were visualized by agarose gel electrophoresis.

DNA extraction from sponges and surrounding water samples. Freeze-dried sponge tissue (1 cm^3) was ground using a sterile mortar and pestle. Total genomic DNA was extracted using the method described by Pitcher *et al*. (1989). The protocol was modified for sponge tissues (Enticknap *et al.*, 2004). DNA was extracted from

the filters obtained from seawater and aquarium water samples using the protocol described by Somerville *et al*. (1989). Detailed protocols are given in Appendix B.

Denaturing gradient gel electrophoresis (DGGE) of bacterial communities. A 195 bp-region corresponding to positions 341 and 534 in the variable V3 region of 16S rRNA gene of *Escherichia coli* was PCR-amplified from genomic DNA extracted from sponges and water samples using P2 and P3 primers (Muyzer *et al.*, 1993). DGGE was performed using a DCode system (Bio-Rad, Hercules, CA, USA) on a 6% (w/v) polyacrylamide gel with a denaturing gradient of 40-70% in 1 × TAE. A detailed protocol is given in (Appendix B). Electrophoresis was performed for 17 h at 60 V and 60˚C. Gels were stained in a staining bath of Syber green in 1 × TAE and visualized with the Typhoon 9410 image system (Amersham Biosciences, Piscataway, NJ, USA).

PCR amplification of genomic DNA, cloning and sequencing. 16S rRNA gene fragments were PCR- amplified from the total genomic DNA isolated from sponge and water samples using the same protocol described for culturable isolates. Cycling conditions were as described previously in the section of the identification of isolates, but the PCR reaction was terminated after 15, 20, 25, 30 cycles with 30 cycles for the negative control sample. Amplification products were visualized by agarose gel electrophoresis. Visible bands of approximately 1,500-bp from the reactions with the least number of cycles were cut and gel purified using QIAquick gel extraction kit. Corresponding areas from the negative control samples were excised and taken through the cloning procedure to provide strict negative controls. Purified PCR products were ligated into pCR-XL-TOPO vector and transformed into OneShot TOP

10 chemically competent *E. coli* cells using the TOPO XL PCR Cloning Kit (Invitrogen Life Technologies, Carlsbad, CA, USA). Plasmid DNA was isolated from individual clones and purified using Agencourt® SprintPrep® 384 HC kit (Agencourt Bioscience, Beverly, MA, USA). Purified plasmids were sequenced with M13 forward (-20) primer (5' GTTGTAAAACGACGGCCAGT 3'). M13 reverse (-20) primer (5' CACAGGAAACAGCTATGACC 3') was used for sequencing of reversed clones.

Phylogenetic analysis. 16S rRNA gene sequences from isolates were analyzed using BLASTn tool at the National Center of Biotechnology Information and then imported to ARB software (Ludwig *et al.*, 2004) to align homologous regions of 16S rRNA gene sequences from different isolates. The aligned sequences were analyzed by distance matrix methods available in ARB. Multiple alignments were checked manually and improved by ARB editor tool. Isolates were presumptively identified according to the identity of the closest well-described cultured relative in the top BLAST hits. 16S rRNA gene sequences from clone libraries were edited using PreGap4 and Gap4 from the Staden Package and analyzed initially using BLAST tool to aid selection of the closest reference sequences. Chimeric sequences were identified using CHECK_CHIMERA program of the Ribosomal Database Project (Maidak *et al.*, 1999). Phylogenetic analyses of clone libraries were performed using the ARB software package and sequences were aligned using the PT-server with a dataset containing the nearest relative matches. Trees were constructed using the neighbor-joining (Jukes-Cantor correction) (Saitou and Nei, 1987) algorithms implemented in ARB based on 642 nucleotide alignment positions corresponding to

positions 8 to 650 in the *E. coli* 16S rRNA gene (NCBI accession no. AJ567617). The robustness of the inferred trees topologies was evaluated after 1,000 bootstrap replicates of the neighbor-joining data. Bootstrap values were generated using Phylip (Felsenstein, 2004).

Statistical analyses of clone libraries and estimation of microbial diversity. S-LIBSHUFF V1.22 was used to compare libraries statistically (Schloss *et al.*, 2004). It compares more than two libraries at once with the same distance matrix to determine whether two libraries were drawn from the same population. DOTUR V1.53 (distance-based OTU and richness) (Schloss and Handelsman, 2005) was used to assign sequences to operational taxonomic units (OTUs) and to calculate collector's curves for observed unique OTUs, Chao1, ACE richness estimators. Shannon and Simpson's diversity indices were also calculated (Hill, 1973). Rarefaction analysis was done to determine the number of observed OTUs as a function of the distance between sequences and the number of sequences sampled. The rarefaction curves data was obtained using DOTUR.

Nucleotide sequence accession numbers. 16S rRNA gene sequences from isolates were submitted to GenBank under accession no. EF629829 to EF629882. 16S rRNA gene sequences from clone libraries were submitted to GenBank under accession no. EF629883 to EF630353.

2.4. Results

Maintenance of *M. laxissima* sponges in two aquaculture systems. Individuals of

M. laxissima were successfully maintained in two aquaculture systems, a flow-through system and a closed recirculating system (Fig. 2.1). Sponge health was assessed visually by observing size, color and appearance of necrotic spots. No significant growth of sponges was observed in either aquaculture system. Sponges maintained integrity and showed no necrosis or fouling although there was some color change from black to grey, possibly indicating loss of dark-pigmented cyanobacteria. Sponges remained viable throughout the study period as shown by a sponge cell aggregation assay (Bagby, 1972; Müller *et al.*, 1974; Blumbach *et al.*, 1998). Manually dispersed sponge tissue reaggregates spontaneously when the sponge cells were alive. Digital images were taken routinely.

Bacterial enumeration. Total (DAPI stained) and culturable (plate) bacterial counts were determined for samples from wild *M. laxissima* sponges and sponges maintained in a closed aquaculture system for one month and three months. For the three wild sponges, the mean and standard error of total counts was $1.1\pm4.4 \times 10^9$ cells ml^{-1} and the mean of plate counts was $4.0\pm1.5 \times 10^6$ colony forming units (CFU) ml^{-1}. For the two one-month individuals, the mean of total counts was 8.0×10^8 cells ml^{-1} and the mean of plate counts was $2.4\pm1.6 \times 10^7$ CFU ml^{-1}. For the two three-month individuals, the total count was $4.3\pm0.6 \times 10^9$ cells ml^{-1} and the plate count was 3.0×10^7 CFU ml^{-1}. Based on these counts, the percentage of culturable bacteria in the sponge samples ranged from 0.23% to 1.8%, indicating the importance of assessing these communities by using molecular techniques. Using more cultivation media, longer incubation time, or a wide range of temperature and oxygen conditions are examples of approaches that can be expected to increase the percentage of

culturable bacteria recovered from the sponges.

DGGE. DGGE analysis revealed that bacterial communities varied substantially between wild sponges and surrounding seawater and between aquacultured sponges and aquarium water under both aquaculture conditions (Fig. 2.2). DGGE banding patterns were generally consistent between sponges sampled at the same time point. Marked differences in overall DGGE patterns indicate that bacterial communities associated with *M. laxissima* were clearly different from those in the surrounding water. A few DGGE bands were shared between both sponge and water samples suggesting the commonality of some bacteria. The diversity of the microbial community associated with *M. laxissima,* assessed by the number of bands present in DGGE*,* increased in both aquaculture systems.

Phylogenetic analysis of 16S rRNA gene clone libraries. In order to determine the stability of the microbial communities upon transfer of the *M. laxissima* into aquaculture, seven 16S rRNA gene clone libraries were generated. One clone library from a representative sponge was constructed at each selected time point, described in detail below. This was based on the general consistency of DGGE banding patterns between individuals sampled at the same time point (Fig. 2.2). DOTUR was used to assign sequences to OTUs based on the genetic distance between sequences. Additional clone libraries were constructed from 16S rRNA genes amplified from bacteria in the seawater surrounding *M. laxissima* in the wild and in the flow-through aquaculture system.

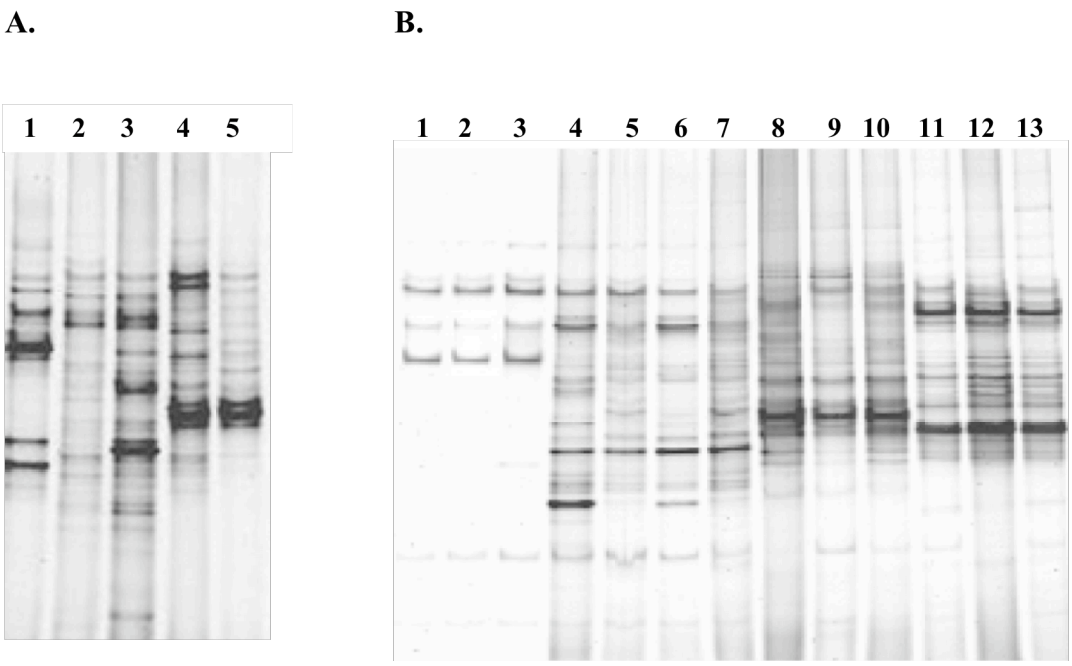
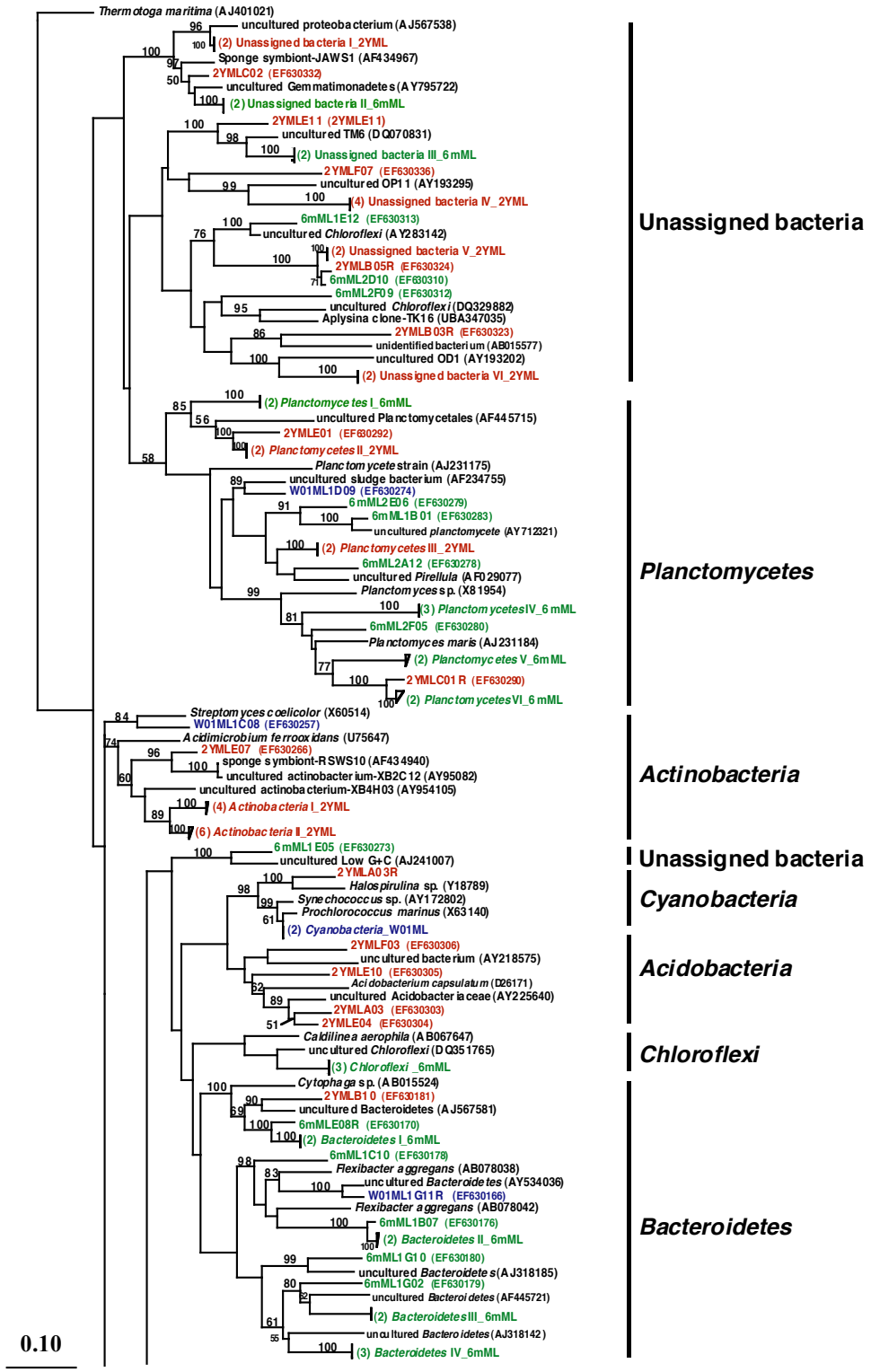


Fig. 2.2. Denaturing gradient gel electrophoresis (DGGE) fingerprints of the bacterial communities associated with *M. laxissima* individual sponges from: **A.** Flow-through aquaculture system including wild (lane 1), six-month aquacultured (lane 2), two-year aquacultured sponges (lane 3), 2001-seawater (lane 4) and 2004-seawater (lane 5) samples. **B.** Recirculating system including wild individuals (lanes 1-3), one-month (lanes 4 and 5), three-month aquacultured (lanes 6 and 7) sponges, seawater samples from the surrounding vicinity of freshly collected *M. laxissima* (lanes 8, 9, and10) and aquarium water samples from the recirculating system (lanes 11,12, and 13). The denaturing gradient was from 40% to 70%.

A. Flow-through system. In this system, six-month and two-year aquacultured *M. laxissima* were compared to *M. laxissima* collected from the wild in 2001. Clone libraries, designated W01ML and 6mML were constructed from the corresponding one wild sponge and one sponge maintained in aquaculture for six months. Two sponge samples were processed at the 2-year time point for DGGE analysis and culturing of isolates. However, a clone library (designated 2YML) was constructed from the bacterial community in only one sponge, judged to be representative of communities in both sponges based on the similarity of the DGGE banding patterns. A total of 119 16S rRNA gene clones were analyzed from the wild sponge. This generated 67 unique operational taxonomic units (OTUs), which fell within seven bacterial phyla (*Alpha-, Gamma-* and *Deltaproteobacteria*, *Actinobacteria, Bacteroidetes*, *Cyanobacteria* and *Planctomycetes*). A total of 85 16S rRNA gene clones were analyzed from the six-month aquacultured sponge. This generated 75 unique OTUs, which fell into eight phyla (*Alpha-, Gamma-* and *Deltaproteobacteria*, *Bacteroidetes*, *Caldithrix, Chloroflexi*, *Planctomycetes* and unassigned bacteria). A total of 47 16S rRNA gene clones were analyzed from the two-year aquacultured sponge. This generated 35 unique OTUs, which fell into eight phyla (*Alpha-* and *Gammaproteobacteria*, *Acidobacteria, Actinobacteria, Bacteroidetes*, *Cyanobacteria*, *Planctomycetes* and unassigned bacteria). A phylogenetic tree showing relationships between sequences from these three libraries is shown in Fig. 2.3.

A total of 38 16S rRNA gene clones were analyzed from bacteria in the seawater. This library was designated WW01 and comprised 31 unique OTUs, which fell into eight phyla (*Alpha-* and *Gammaproteobacteria*, *Actinobacteria*, *Bacteroidetes*,

A.


Thermotoga maritima (AJ401021)
uncultured proteobacterium (AJ567538)
(2) Unassigned bacteria I_2YML
Sponge symbiont-JAWS1 (AF434967)
2YMLC02 (EF630332)
uncultured Gemmatimonadetes (AY795722)
(2) Unassigned bacteria II_6mML
2YMLE11 (2YMLE11)
uncultured TM6 (DQ070831)
(2) Unassigned bacteria III_6mML
2YMLF07 (EF630336)
uncultured OP11 (AY193295)
(4) Unassigned bacteria IV_2YML
6mML1E12 (EF630313)
uncultured Chloroflexi (AY283142)
(2) Unassigned bacteria V_2YML
2YMLB05R (EF630324)
6mML2D10 (EF630310)
6mML2F09 (EF630312)
uncultured Chloroflexi (DQ329882)
Aplysina clone-TK16 (UBA347035)
2YMLB03R (EF630323)
unidentified bacterium (AB015577)
uncultured OD1 (AY193202)
(2) Unassigned bacteria VI_2YML
(2) Planctomycetes I_6mML
uncultured Planctomycetales (AF445715)
2YMLE01 (EF630292)
(2) Planctomycetes II_2YML
Planctomycete strain (AJ231175)
uncultured sludge bacterium (AF234755)
W01ML1D09 (EF630274)
6mML2E06 (EF630279)
6mML1B01 (EF630283)
uncultured planctomycete (AY712321)
(2) Planctomycetes III_2YML
6mML2A12 (EF630278)
uncultured Pirellula (AF029077)
Planctomyces sp. (X81954)
(3) Planctomycetes IV_6mML
6mML2F05 (EF630280)
Planctomyces maris (AJ231184)
(2) Planctomycetes V_6mML
2YMLC01R (EF630290)
(2) Planctomycetes VI_6mML
Streptomyces coelicolor (X60514)
W01ML1C08 (EF630257)
Acidimicrobium ferrooxidans (U75647)
2YMLE07 (EF630266)
sponge symbiont-RSWS10 (AF434940)
uncultured actinobacterium-XB2C12 (AY95082)
uncultured actinobacterium-XB4H03 (AY954105)
(4) Actinobacteria I_2YML
(6) Actinobacteria II_2YML
6mML1E05 (EF630273)
uncultured Low G+C (AJ241007)
2YMLA03R
Halospirulina sp. (Y18789)
Synechococcus sp. (AY172802)
Prochlorococcus marinus (X63140)
(2) Cyanobacteria_W01ML
2YMLF03 (EF630306)
uncultured bacterium (AY218575)
2YMLE10 (EF630305)
Acidobacterium capsulatum (D26171)
uncultured Acidobacteriaceae (AY225640)
2YMLA03 (EF630303)
2YMLE04 (EF630304)
Caldilinea aerophila (AB067647)
uncultured Chloroflexi (DQ351765)
(3) Chloroflexi _6mML
Cytophaga sp. (AB015524)
2YMLB10 (EF630181)
uncultured Bacteroidetes (AJ567581)
6mMLE08R (EF630170)
(2) Bacteroidetes I_6mML
6mML1C10 (EF630178)
Flexibacter aggregans (AB078038)
uncultured Bacteroidetes (AY534036)
W01ML1G11R (EF630166)
Flexibacter aggregans (AB078042)
6mML1B07 (EF630176)
(2) Bacteroidetes II_6mML
6mML1G10 (EF630180)
uncultured Bacteroidetes (AJ318185)
6mML1G02 (EF630179)
uncultured Bacteroidetes (AF445721)
(2) Bacteroidetes III_6mML
uncultured Bacteroidetes (AJ318142)
(3) Bacteroidetes IV_6mML
0.10
Unassigned bacteria
Planctomycetes
Actinobacteria
Unassigned bacteria
Cyanobacteria
Acidobacteria
Chloroflexi
Bacteroidetes

B.

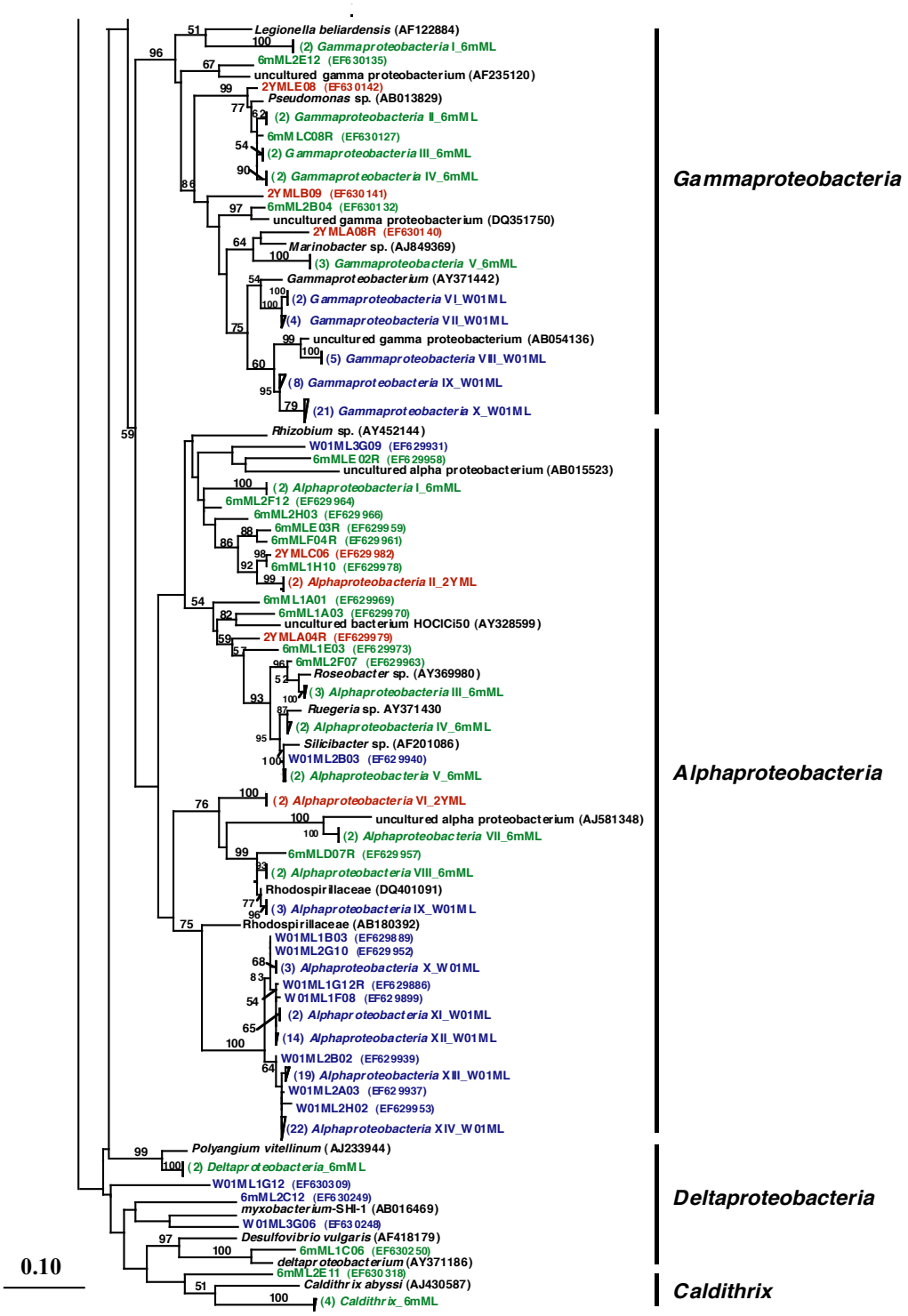

Legionella beliardensis (AF122884)
(2) Gammaproteobacteria I_6mML
6mML2E12 (EF630135)
uncultured gamma proteobacterium (AF235120)
2YMLE08 (EF630142)
Pseudomonas sp. (AB013829)
(2) Gammaproteobacteria II_6mML
6mMLC08R (EF630127)
(2) Gammaproteobacteria III_6mML
(2) Gammaproteobacteria IV_6mML
2YMLB09 (EF630141)
6mML2B04 (EF630132)
uncultured gamma proteobacterium (DQ351750)
2YMLA08R (EF630140)
Marinobacter sp. (AJ849369)
(3) Gammaproteobacteria V_6mML
Gammaproteobacterium (AY371442)
(2) Gammaproteobacteria VI_W01ML
(4) Gammaproteobacteria VII_W01ML
uncultured gamma proteobacterium (AB054136)
(5) Gammaproteobacteria VIII_W01ML
(8) Gammaproteobacteria IX_W01ML
(21) Gammaproteobacteria X_W01ML
Gammaproteobacteria
Rhizobium sp. (AY452144)
W01ML3G09 (EF629931)
6mMLE02R (EF629958)
uncultured alpha proteobacterium (AB015523)
(2) Alphaproteobacteria I_6mML
6mML2F12 (EF629964)
6mML2H03 (EF629966)
6mMLE03R (EF629959)
6mMLF04R (EF629961)
2YMLC06 (EF629982)
6mML1H10 (EF629978)
(2) Alphaproteobacteria II_2YML
6mML1A01 (EF629969)
6mML1A03 (EF629970)
uncultured bacterium HOCICi50 (AY328599)
2YMLA04R (EF629979)
6mML1E03 (EF629973)
6mML2F07 (EF629963)
Roseobacter sp. (AY369980)
(3) Alphaproteobacteria III_6mML
Ruegeria sp. AY371430
(2) Alphaproteobacteria IV_6mML
Silicibacter sp. (AF201086)
W01ML2B03 (EF629940)
(2) Alphaproteobacteria V_6mML
(2) Alphaproteobacteria VI_2YML
uncultured alpha proteobacterium (AJ581348)
(2) Alphaproteobacteria VII_6mML
6mMLD07R (EF629957)
(2) Alphaproteobacteria VIII_6mML
Rhodospirillaceae (DQ401091)
(3) Alphaproteobacteria IX_W01ML
Rhodospirillaceae (AB180392)
W01ML1B03 (EF629889)
W01ML2G10 (EF629952)
(3) Alphaproteobacteria X_W01ML
W01ML1G12R (EF629886)
W01ML1F08 (EF629899)
(2) Alphaproteobacteria XI_W01ML
(14) Alphaproteobacteria XII_W01ML
W01ML2B02 (EF629939)
(19) Alphaproteobacteria XIII_W01ML
W01ML2A03 (EF629937)
W01ML2H02 (EF629953)
(22) Alphaproteobacteria XIV_W01ML
Alphaproteobacteria
Polyangium vitellinum (AJ233944)
(2) Deltaproteobacteria_6mML
W01ML1G12 (EF630309)
6mML2C12 (EF630249)
myxobacterium-SHI-1 (AB016469)
W01ML3G06 (EF630248)
Desulfovibrio vulgaris (AF418179)
6mML1C06 (EF630250)
deltaproteobacterium (AY371186)
6mML2E11 (EF630318)
Deltaproteobacteria
Caldithrix abyssi (AJ430587)
(4) Caldithrix_6mML
Caldithrix
0.10

Fig. 2.3. Rooted neighbor-joining phylogenetic tree of partial 16S rRNA gene sequences of clones that were recovered from flow-through system including wild (prefixed W01ML, presented in blue), six-month aquacultured (prefixed 6mML, presented in green) and two-year (2YML, presented in red) aquacultured *M. laxissima* sponges. Bootstrap confidence values > 50% are shown at the nodes. The tree was constructed using ARB. The tetragons represent clones that are > 99.5% similar (Table A.1, Appendix A); the numbers listed in bold before the group names indicate the numbers of clones. *T. maritima* was used as the outgroup in the analysis. Scale bar indicates 0.10 substitutions per nucleotide position. Reference sequences are shown with GenBank accession numbers listed after each sequence name. Major bacterial groups found in both libraries are indicated in bold on the right hand side of the tree.

Cyanobacteria, *Planctomycetes*, uncultured TM7, and *Verrucomicrobia*). A phylogenetic tree showing sequences from the seawater library is shown in Fig. 2.4. A total of 51 16S rRNA gene clones were analyzed from the aquarium water generating 40 unique OTUs, which fell within five phyla (*Alpha*- and *Gammaproteobacteria*, *Bacteroidetes*, *Cyanobacteria* and *Planctomycetes*). A phylogenetic tree showing sequences from the aquarium water library is shown in Fig. 2.5.

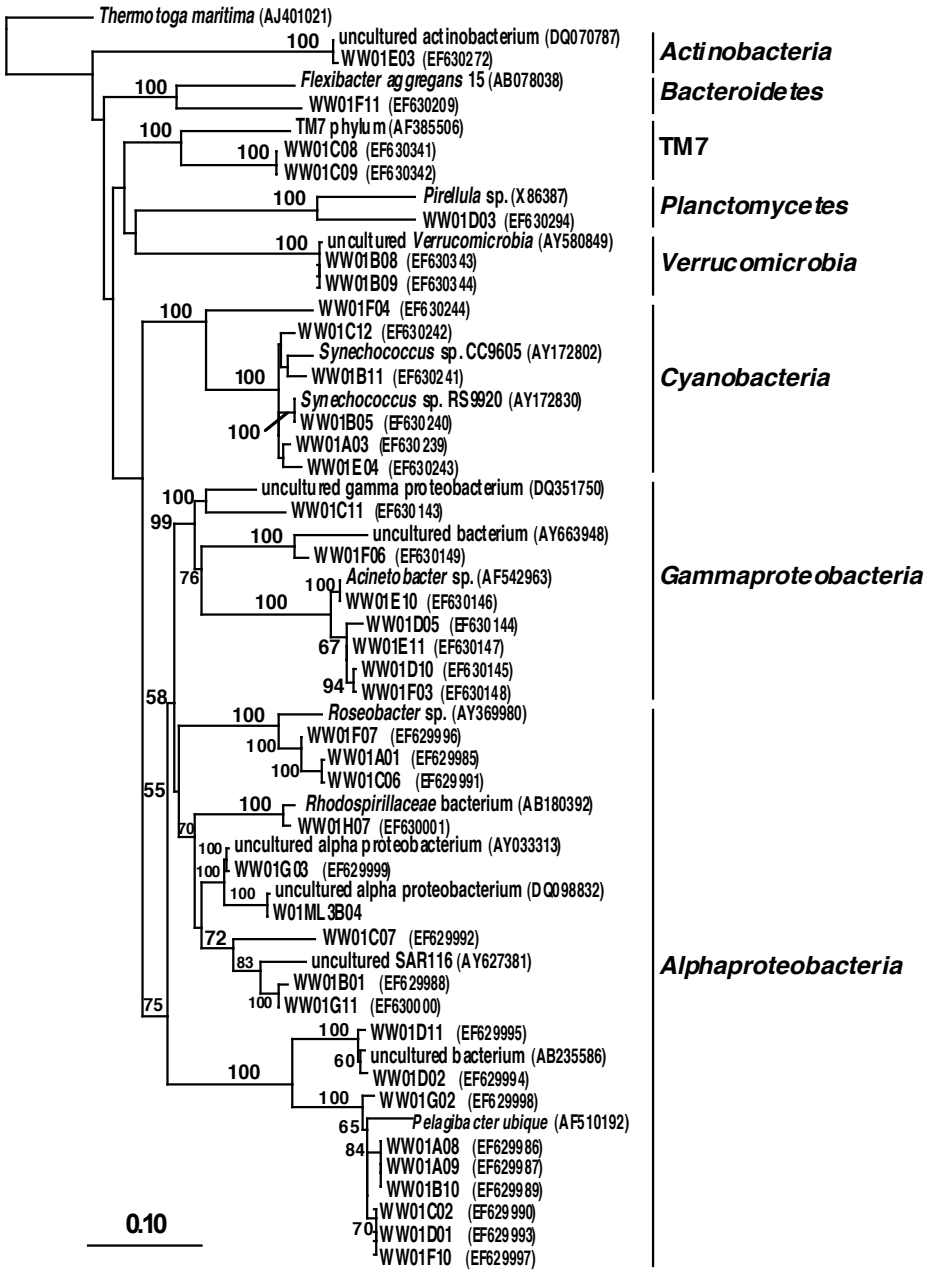


Fig. 2.4. Rooted neighbor-joining phylogenetic tree of partial 16S rRNA gene sequences of clones that were recovered from seawater sample collected in vicinity of wild sponges at Key Largo (prefixed WW01).

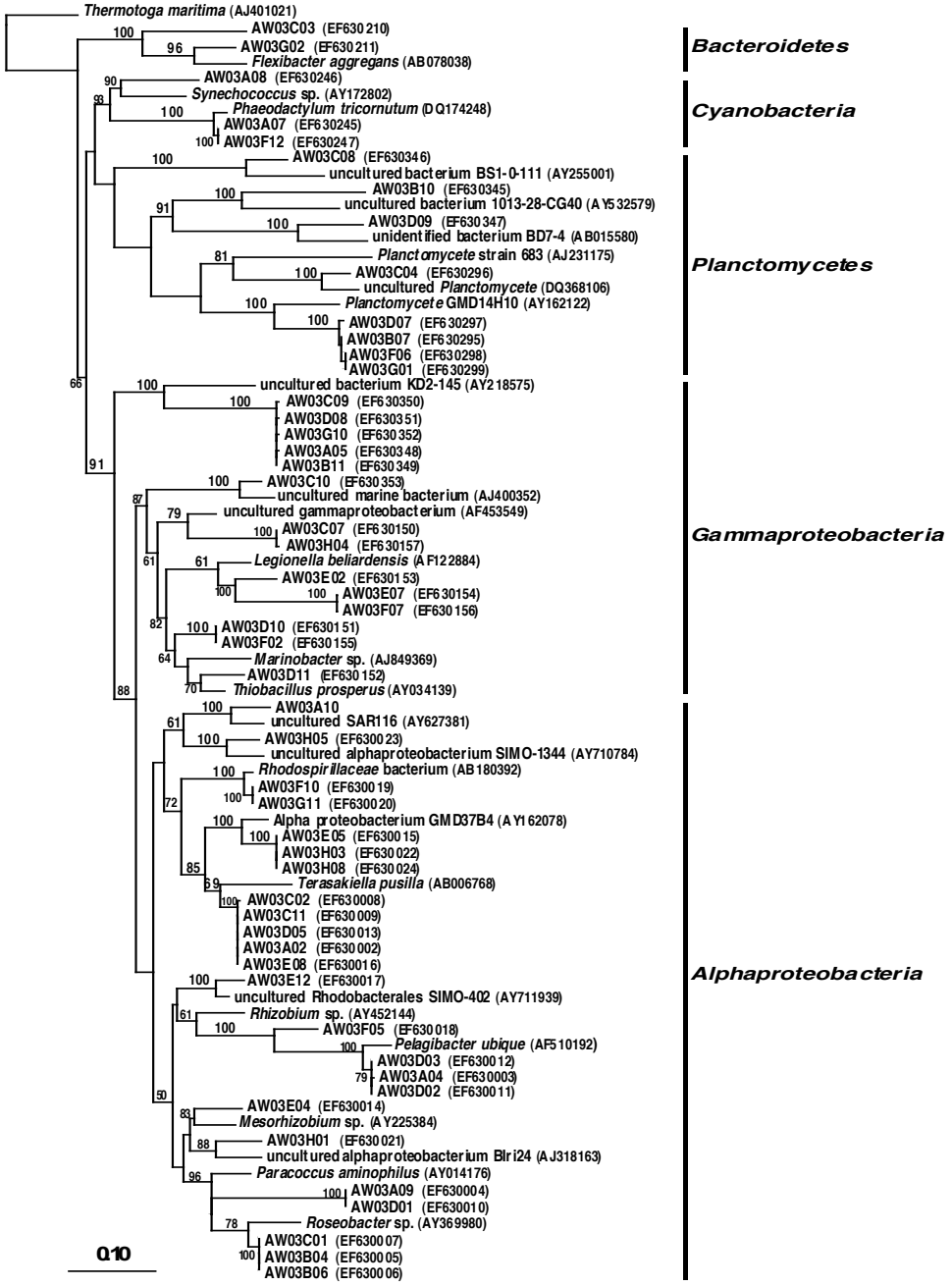


Fig. 2.5. Rooted neighbor-joining phylogenetic tree of partial 16S rRNA gene sequences of clones that were recovered from water from the flow-through aquaculture system (prefixed AW03).

B. Recirculating system. Two sponges were compared in this analysis, *M. laxissima* collected from the wild in 2004 and *M. laxissima* maintained in the recirculating aquaculture system for one month. A clone library designated 1m04AML was constructed from a sample of one representative sponge maintained for one month in aquaculture. This sample was regarded as a representative sample based on the similarity of the DGGE banding patterns of the two one-month aquacultured sponges (Fig. 2.2). A total of 67 16S rRNA gene clones were analyzed from the wild sponge. This generated 59 unique OTUs, which fell into ten bacterial phyla (*Alpha*-, *Gamma*- and *Deltaproteobacteria*, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes, Cyanobacteria*, *Planctomycetes*, uncultured TM7 and unassigned bacteria). An equal number of 16S rRNA gene clones were analyzed from the one-month aquacultured sponge. This generated 52 unique OTUs, which fell into eight phyla (*Alpha*-, *Gamma*- and *Deltaproteobacteria*, *Acidobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Planctomycetes* and unassigned bacteria). A phylogenetic tree showing comparisons of sequences from these two libraries is shown in Fig. 2.6. The distribution of OTUs within the major phylogenetic groups detected in sponges from both aquaculture systems is shown in Fig. 2.7.

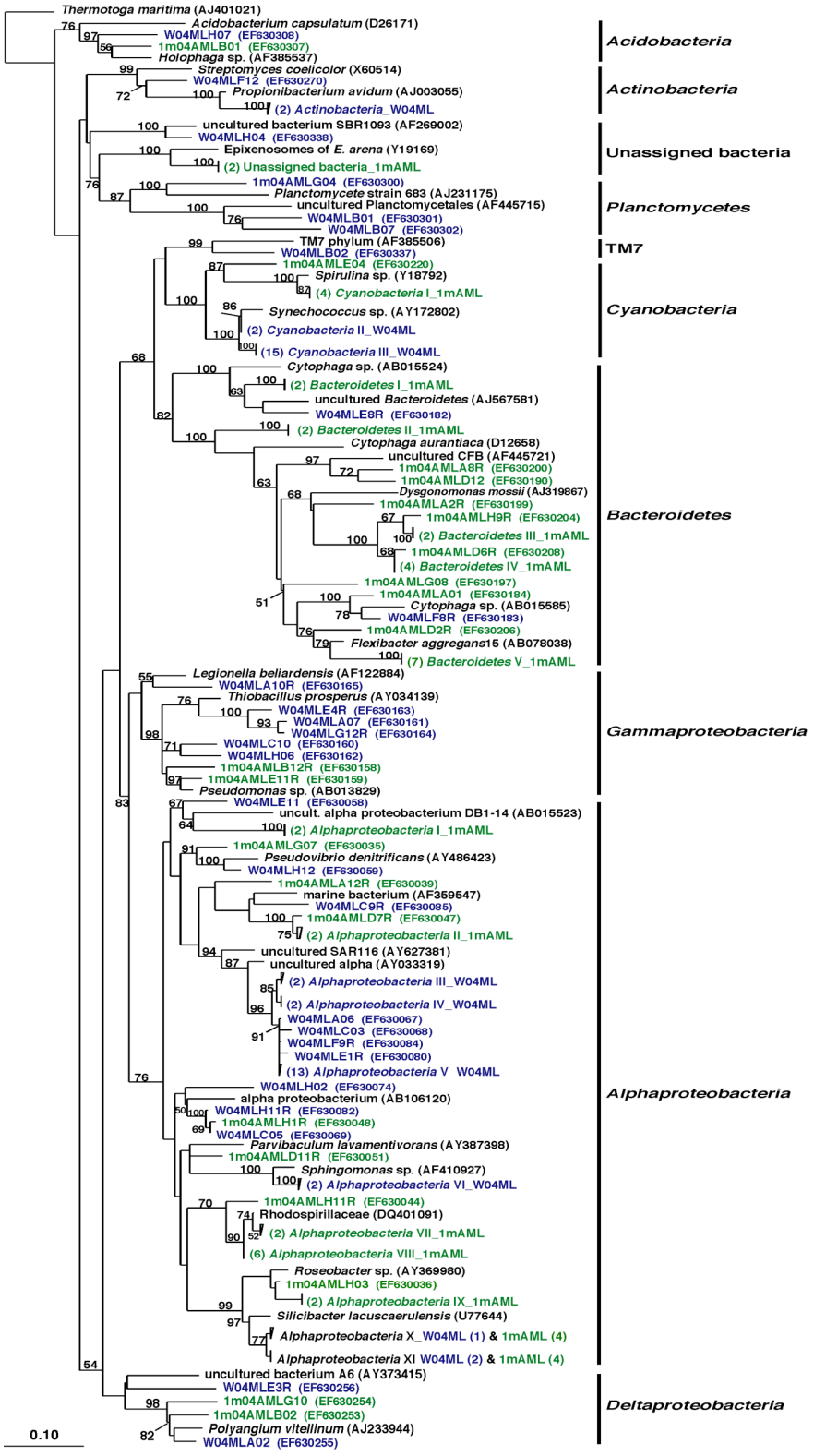
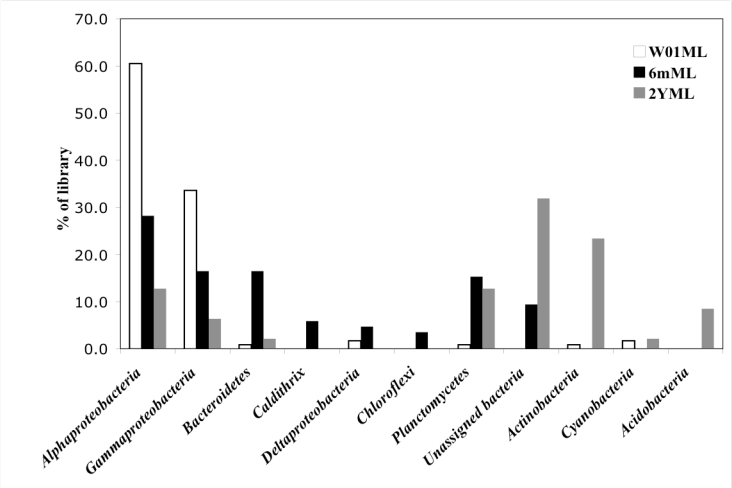

Thermotoga maritima (AJ401021)
Acidobacterium capsulatum (D26171)
W04MLH07 (EF630308)
1m04AMLB01 (EF630307)
Holophaga sp. (AF385537)
Acidobacteria
Streptomyces coelicolor (X60514)
W04MLF12 (EF630270)
Propionibacterium avidum (AJ003055)
(2) Actinobacteria_W04ML
Actinobacteria
uncultured bacterium SBR1093 (AF269002)
W04MLH04 (EF630338)
Epixenosomes of E. arena (Y19169)
(2) Unassigned bacteria_1mAML
Unassigned bacteria
1m04AMLG04 (EF630300)
Planctomycete strain 683 (AJ231175)
uncultured Planctomycetales (AF445715)
W04MLB01 (EF630301)
W04MLB07 (EF630302)
Planctomycetes
TM7 phylum (AF385506)
W04MLB02 (EF630337)
TM7
1m04AMLE04 (EF630220)
Spirulina sp. (Y18792)
(4) Cyanobacteria I_1mAML
Synechococcus sp. (AY172802)
(2) Cyanobacteria II_W04ML
(15) Cyanobacteria III_W04ML
Cyanobacteria
Cytophaga sp. (AB015524)
(2) Bacteroidetes I_1mAML
uncultured Bacteroidetes (AJ567581)
W04MLE8R (EF630182)
(2) Bacteroidetes II_1mAML
Cytophaga aurantiaca (D12658)
uncultured CFB (AF445721)
1m04AMLA8R (EF630200)
1m04AMLD12 (EF630190)
Dysgonomonas mossii (AJ319867)
1m04AMLA2R (EF630199)
1m04AMLH9R (EF630204)
(2) Bacteroidetes III_1mAML
1m04AMLD6R (EF630208)
(4) Bacteroidetes IV_1mAML
1m04AMLG08 (EF630197)
1m04AMLA01 (EF630184)
Cytophaga sp. (AB015585)
W04MLF8R (EF630183)
1m04AMLD2R (EF630206)
Flexibacter aggregans15 (AB078038)
(7) Bacteroidetes V_1mAML
Bacteroidetes
Legionella beliardensis (AF122884)
W04MLA10R (EF630165)
Thiobacillus prosperus (AY034139)
W04MLE4R (EF630163)
W04MLA07 (EF630161)
W04MLG12R (EF630164)
W04MLC10 (EF630160)
W04MLH06 (EF630162)
1m04AMLB12R (EF630158)
1m04AMLE11R (EF630159)
Pseudomonas sp. (AB013829)
Gammaproteobacteria
W04MLE11 (EF630058)
uncult. alpha proteobacterium DB1-14 (AB015523)
(2) Alphaproteobacteria I_1mAML
1m04AMLG07 (EF630035)
Pseudovibrio denitrificans (AY486423)
W04MLH12 (EF630059)
1m04AMLA12R (EF630039)
marine bacterium (AF359547)
W04MLC9R (EF630085)
1m04AMLD7R (EF630047)
(2) Alphaproteobacteria II_1mAML
uncultured SAR116 (AY627381)
uncultured alpha (AY033319)
(2) Alphaproteobacteria III_W04ML
(2) Alphaproteobacteria IV_W04ML
W04MLA06 (EF630067)
W04MLC03 (EF630068)
W04MLF9R (EF630084)
W04MLE1R (EF630080)
(13) Alphaproteobacteria V_W04ML
W04MLH02 (EF630074)
alpha proteobacterium (AB106120)
W04MLH11R (EF630082)
1m04AMLH1R (EF630048)
W04MLC05 (EF630069)
Parvibaculum lavamentivorans (AY387398)
1m04AMLD11R (EF630051)
Sphingomonas sp. (AF410927)
(2) Alphaproteobacteria VI_W04ML
1m04AMLH11R (EF630044)
Rhodospirillaceae (DQ401091)
(2) Alphaproteobacteria VII_1mAML
(6) Alphaproteobacteria VIII_1mAML
Roseobacter sp. (AY369980)
1m04AMLH03 (EF630036)
(2) Alphaproteobacteria IX_1mAML
Silicibacter lacuscaerulensis (U77644)
Alphaproteobacteria X_W04ML (1) & 1mAML (4)
Alphaproteobacteria XI W04ML (2) & 1mAML (4)
Alphaproteobacteria
uncultured bacterium A6 (AY373415)
W04MLE3R (EF630256)
1m04AMLG10 (EF630254)
1m04AMLB02 (EF630253)
Polyangium vitellinum (AJ233944)
W04MLA02 (EF630255)
Deltaproteobacteria
0.10

Fig. 2.6. Rooted neighbor-joining phylogenetic tree of partial 16S rRNA gene sequences of clones that were recovered from wild (prefixed W04ML, shown in blue) and one-month (prefixed 1m04AML, shown in green) aquacultured *M. laxissima* sponges from the recirculating aquaculture system. Bootstrap confidence values > 50% are shown at the nodes. The tree was constructed using ARB. The tetragons represent clones that are > 99.5% similar (Table A.2, Appendix A); the numbers listed in bold before the group names indicate the numbers of clones. *T. maritima* was used as the outgroup in the analysis. Scale bar indicates 0.10 substitutions per nucleotide position. Reference sequences are shown with GenBank accession numbers listed after each sequence name. Major bacterial groups found in both libraries are indicated in bold on the right hand side of the tree.

A.



B.

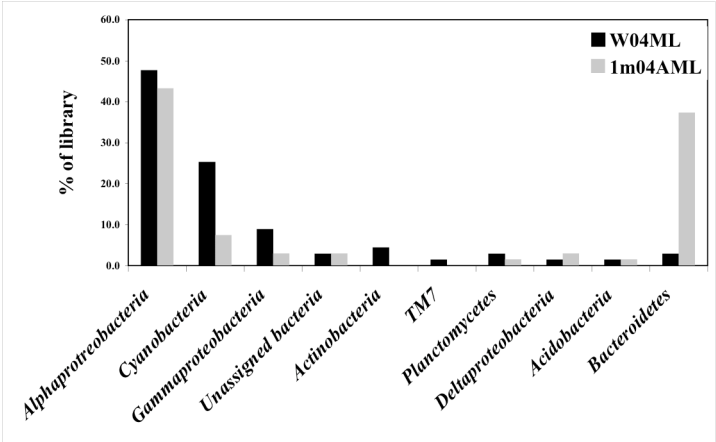


Fig. 2.7. Distribution of bacterial 16S rRNA gene sequences within the phylogenetic groups detected in the clone libraries from individual *M. laxissima* individuals from: **A.** Flow-through system including wild *M. laxissima* (W01ML), six-month (6mML) and two-year (2YML) aquacultured sponges and **B.** Recirculation system including wild *M. laxissima* (W04ML) and one-month (1m04AML) aquacultured sponges.

Phylogenetic analysis of isolates. Culturing techniques were used to isolate heterotrophic bacteria from sponge samples. Alpha- and gammaproteobacterial isolates dominated the culturable bacterial assemblages under these conditions in wild sponges and seawater samples (Table 2.1). Isolates affiliated with *Acidobacteria* were isolated only from sponges maintained under flow-through aquaculture conditions. In the recirculating system, a diverse assemblage of isolates affiliated with *Alphaproteobacteria* and the *Bacteroidetes* group was obtained from all sponges with an increase in the number of culturable *Gammaproteobacteria* from sponges maintained for three months.

Rarefaction analysis. Rarefaction curves at the estimated phylum level (distance= 0.20) reached saturation for all of the seven libraries suggesting that the sampling effort was sufficient to reveal all phyla present in the samples. Only the clone library of the 2001 wild sponge reached saturation at the estimated species level (distance= 0.03). Further sampling from the other six libraries may have revealed more diversity at the species level. Bacterial species richness in sponges maintained in both aquaculture systems was greater than in sponges collected from the wild, indicated by steeper inclines in rarefaction curves (Fig. 2.8).

Statistical analysis of bacterial diversity. The computer program LIBSHUFF was used to compare libraries of wild and aquacultured sponges. This program is designed to compare undersampled 16S rRNA gene libraries. Evolutionary distances were calculated using the neighbor-joining algorithm in ARB. In the flow-through system, libraries of wild, six-month and two-year aquacultured sponges were significantly different at the 99% confidence level ($P \leq 0.01$). Similarly, libraries

Table 2.1. 16S rRNA gene sequence similarities of isolates from sponges and surrounding water samples. A. Flow-through system including wild sponges collected in 2001, sponges maintained in aquaculture for two years, seawater collected from the vicinity of freshly collected sponges (seawater 2001) and seawater collected from the aquaculture tanks in 2003 (aquarium water), and B. Recirculating aquaculture system including wild sponges collected in 2004 and sponges maintained in aquaculture for one month and three months, respectively. GenBank accession numbers are given after the name of each isolate.

A.

Source	**Isolate,** NCBI accession no.	**Phylum**	**Closest cultured organism**	**% Identity**
Wild *M. laxissima* (2001)	**KLH10**, EF629829	*Alphaproteobacteria*	*Stappia* sp. M8, AY307927	99%
	KLH11, EF629830	*Alphaproteobacteria*	*Ruegeria* sp. AS-36, AJ391197	98%
Two-year *M. laxissima*	**N2yML1**, EF629831	*Bacteroidetes*	*Flexibacteraceae* bacterium UST030701-097, DQ080995	95%
	N2yML2, EF629832	*Gammaproteobacteria*	*Xanthomonas* sp. ML-122, AF139997	87%
	N2yML3, EF629833	*Alphaproteobacteria*	*Mesorhizobium* sp. BNC1, CP000390	96%
	N2yML4, EF629834	*Acidobacteria*	*Holophaga foetida* strain TMBS4-T, X77215	80%
	N2yML5, EF629835	*Alphaproteobacteria*	*Rhodospirillaceae* bacterium CL-UU02, DQ401091	89%
	N2yML6, EF629836	*Alphaproteobacteria*	*Mesorhizobium* sp. NH-14, AB196496	95%
Seawater (2001)	**SWKLH6**, EF629837	*Alphaproteobacteria*	*Sulfitobacter* sp. KMM 3457, AY682197	99%
	SWKLH7, EF629838	*Alphaproteobacteria*	*Erythrobacter* sp. JL-378, DQ285076	100%
	SWKLH8, EF629839	*Alphaproteobacteria*	*Erythrobacter* sp. JL1020, DQ985038	100%
	SWKLH14, EF629840	*Alphaproteobacteria*	*Roseobacter* sp. RED68, AY136132	97%
	SWKLH15, EF629841	*Gammaproteobacteria*	*Pseudoalteromonas* sp. S511-1, AB029824	99%
	SWKLH16, EF629842	*Gammaproteobacteria*	*Shewanella putrefaciens*, U91549	95%
Aquarium water	**N03AW1**, EF629843	*Bacteroidetes*	*Cytophaga* sp. J18-M01, AB017046	97%
	N03AW2, EF629844	*Alphaproteobacteria*	*Roseobacter* sp. DSS-8, AF098493	98%
	N03AW3, EF629845	*Acidobacteria*	*Holophaga foetida* strain TMBS4-T, X77215	67%
	N03AW4, EF629846	*Alphaproteobacteria*	*Roseobacter* sp. JL-126, AY745859	99%

B.

Source	**Isolate,** NCBI accession no.	**Phylum**	**Closest cultured organism**	**% Identity**
Wild *M. laxissima* (2004)	**JE022**, DQ097257	*Alphaproteobacteria*	*Pseudovibrio denitrificans*, AY486423	99%
	JE023, DQ097258	*Alphaproteobacteria*	*Pseudovibrio denitrificans*, AY486423	100%
	JE025, DQ097259	*Alphaproteobacteria*	*Pseudovibrio denitrificans*, AY486423	99%
	N04ML1, EF629847	*Bacilli*	*Bacillus cereus*, AY689066	99%
	N04ML2, EF629848	*Alphaproteobacteria*	*Silicibacter* sp. JC1077, AF201086	99%
	N04ML4, EF629849	*Alphaproteobacteria*	*Ruegeria* sp. AS-36, AJ391197	98%
	N04ML5, EF629850	*Alphaproteobacteria*	*Ruegeria atlantica*, AB255399	98%
	N04ML6, EF629851	*Alphaproteobacteria*	*Ruegeria* sp. AS-36, AJ391197	98%
	N04ML7, EF629852	*Bacteroidetes*	*Flavobacteriaceae* bacterium LA8, AF513435	95%
	N04ML8, EF629853	*Bacteroidetes*	*Flavobacteriaceae* bacterium LA8, AF513435	95%
	N04ML9, EF629854	*Alphaproteobacteria*	*Ruegeria* sp. AS-36, AJ391197	98%
	N04ML10, EF629855	*Bacteroidetes*	*Flexibacteraceae* bacterium UST030701-097, DQ080995	95%
	N04ML11, EF629856	*Alphaproteobacteria*	*Silicibacter* sp. JC1077, AF201086	99%
One-month *M. laxissima*	**N1mML3**, EF629857	*Bacteroidetes*	*Flavobacteriaceae* bacterium KE2-02, AJ784113	93%
	N1mML4, EF629858	*Bacteroidetes*	*Flexibacteraceae* bacterium UST030701-097, DQ080995	93%
	N1mML5, EF629859	*Alphaproteobacteria*	*Silicibacter* sp. E932, AY369990	98%
	N1mML6, EF629860	*Alphaproteobacteria*	*Stappia* sp. M8, AY307927	98%
	N1mML7, EF629861	*Alphaproteobacteria*	*Silicibacter* sp. E923, AY369990	99%
	N1mML8, EF629862	*Alphaproteobacteria*	*Silicibacter* sp. E923, AY369990	100%
	N1mML9, EF629863	*Alphaproteobacteria*	*Ruegeria atlantica*, DQ888840	98%
	N1mML10c, EF629864	*Bacteroidetes*	*Flexibacteraceae* bacterium UST030701-097, DQ080995	95%

Table 2.1.B, continued.

Source	Isolate	Phylum	Closest cultured organism	% Identity
	N1mML11, EF629865	*Alphaproteobacteria*	*Ruegeria atlantica*, DQ888840	98%
	N1mML12, EF629866	*Bacteroidetes*	*Flavobacteriaceae* bacterium UST030701-097, AF513435	94%
	N1mML13, EF629867	*Gammaproteobacteria*	*Oceanospirillum beijerinckii*, AB006760	89%
	N1mML14, EF629868	*Alphaproteobacteria*	*Ruegeria atlantica*, DQ888840	99%
Three-month *M. laxissima*	**N3mML1**, EF62986	*Bacteroidetes*	*Flexibacteraceae* bacterium UST030701-097, DQ080995	94%
	N3mML2, EF629870	*Alphaproteobacteria*	*Ruegeria* sp. N286, AY369984	98%
	N3mML3, EF629871	*Alphaproteobacteria*	*Silicibacter* sp. E923, AY369990	99%
	N3mML4, EF629872	*Alphaproteobacteria*	*Silicibacter* sp. E923, AY369990	99%
	N3mML5, EF629873	*Alphaproteobacteria*	*Ruegeria* sp. N354, AY371430	98%
	N3mML6, EF629874	*Bacteroidetes*	*Flexibacteraceae* bacterium UST030701-097, DQ080995	94%
	N3mML7, EF629875	*Alphaproteobacteria*	*Silicibacter* sp. E923, AY369990	99%
	N3mML8, EF629876	*Alphaproteobacteria*	*Roseivivax* sp. K376, AY368571	100%
	N3mML9, EF629877	*Gammaproteobacteria*	*Vibrio neptunius*, AY620979	99%
	N3mML11, EF629878	*Gammaproteobacteria*	*Vibrio* sp. R-14968, AJ316168	99%
	N3mML12, EF629879	*Alphaproteobacteria*	*Pseudovibrio denitrificans*, AY486423	100%
	N3mML13, EF629880	*Gammaproteobacteria*	*Vibrio* sp. R-14968, AJ316168	99%
	N3mML14, EF629881	*Gammaproteobacteria*	*Pseudoalteromonas ruthenica*, AY723742	100%
	N3mML15, EF629882	*Gammaproteobacteria*	*Ferrimonas futtsuensis*, AB245515	98%

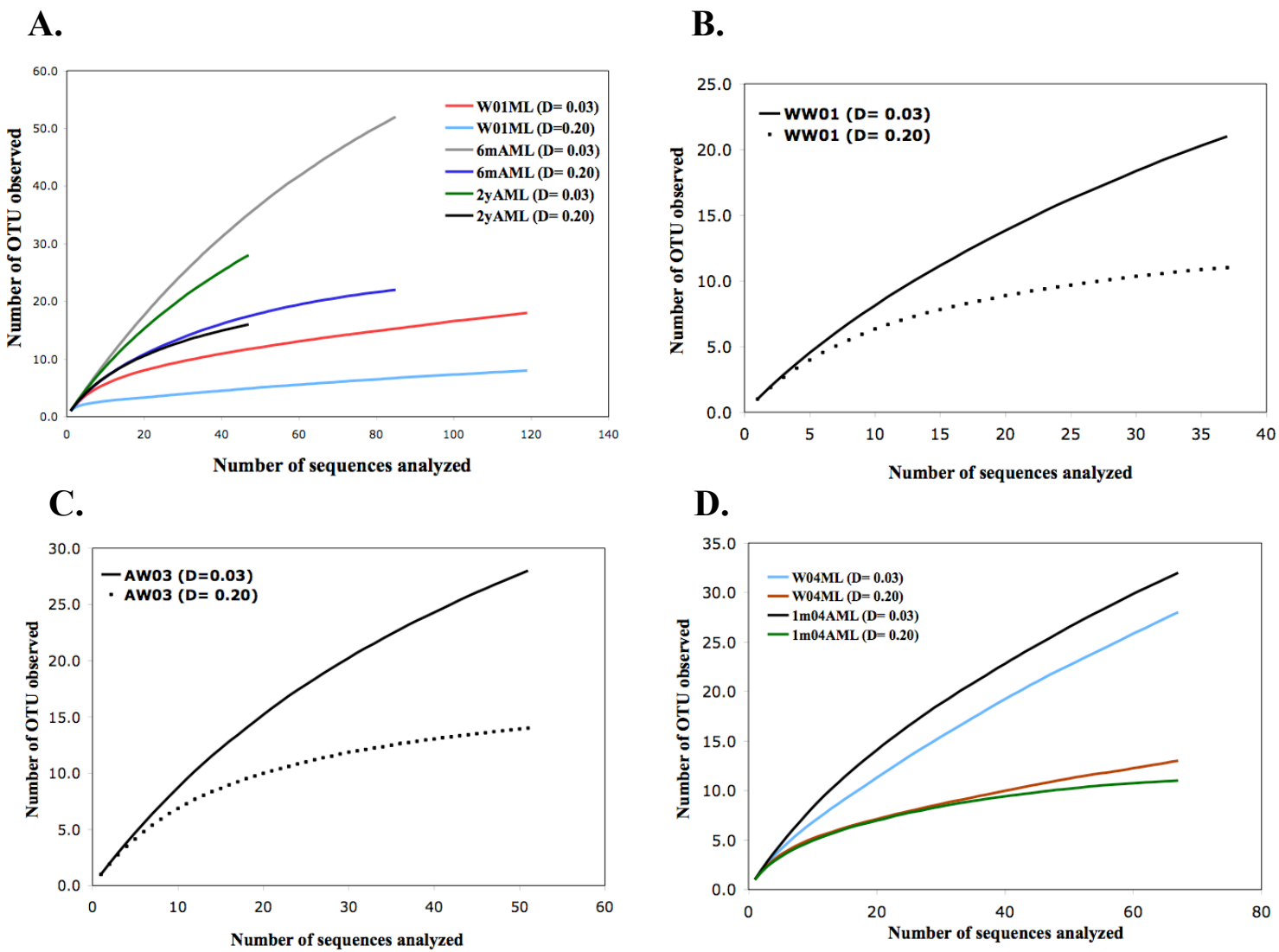

A.
B.
C.
D.
Number of OTU observed
Number of sequences analyzed
W01ML (D= 0.03)
W01ML (D=0.20)
6mAML (D= 0.03)
6mAML (D= 0.20)
2yAML (D= 0.03)
2yAML (D= 0.20)
WW01 (D= 0.03)
WW01 (D= 0.20)
AW03 (D=0.03)
AW03 (D= 0.20)
W04ML (D= 0.03)
W04ML (D= 0.20)
1m04AML (D= 0.03)
1m04AML (D= 0.20)

Fig. 2.8. Rarefaction curves indicating relative richness within bacterial 16S rRNA gene clone libraries of: **A.** Flow through system including wild *M. laxissima* (W01ML) and six-month (6mML) and 2-year (2YAML) aquacultured *M. laxissima*, **B.** Seawater collected in 2001 (WW01), **C.** Aquarium water collected low-through aquaculture system in 2003 (AW03), and **D.** Recirculating system including wild *M. laxissima* (W04ML) and one-month (1m04AML) aquacultured *M. laxissima.* OTUs are defined at estimated distances at both phylum (D= 0.20) and species level (D= 0.03).

from the sponge maintained for one month in the recirculating system was significantly different from the wild sponge 2004 (99% confidence level). Additional measures of diversity and richness were obtained (Table 2.2). These indices were calculated using DOTUR. The input files were in the form of distance matrices generated by using ARB. DOTUR uses the furthest- neighbor method to collapse similar sequences into groups at arbitrary levels of taxonomic similarity and then computes the Shannon, Chao, and ACE statistics for that taxonomic level (Schloss and Handelsman, 2005). Maintenance of *M. laxissima* in the flow-through system increased bacterial richness at both phylum and species levels. This was consistent with the higher number of OTUs observed using rarefaction analyses. The values of Shannon and Simpson indices were higher for aquacultured sponges than sponges collected from the wild. The richness and diversity estimates slightly changed after *M. laxissima* was kept for one month in the recirculating system.

Table 2.2. Richness and diversity estimates for bacterial 16S rRNA gene clone libraries from wild and aquacultured *M. laxissima* from both aquaculture systems and surrounding water samples. 80% identity (D=0.20) was estimated as the phylum level distance and 97% identity (D=0.03) was estimated as the species level distance.

Sample	Flow-through system						Recirculating system				Water samples			
	2001-wild (n*=119)		Six-month (n*=85)		Two-year (n*=47)		2004-wild (n*=67)		One-month (n*=67)		Seawater (n*=37)		Aquarium (n*=51)	
	0.20	0.03	0.20	0.03	0.20	0.03	0.20	0.03	0.20	0.03	0.20	0.03	0.20	0.03
Richness‡	8	18	22	52	16	28	13	28	11	32	11	21	14	28
ACE†	14	37	28	89	22	60	29	71	13	77	13	46	17	48
Chao1§	10	36	26	85	23	50	24	66	11	59	12	34	16	45
Shannon¶	0.97	2.2	2.6	3.8	2.4	3.1	1.9	2.6	1.8	3.1	2.2	2.8	2.4	3.1
1/ Simpson††	2.2	6.3	9.3	71.4	9.7	30	5.2	8.3	4.2	21.7	8.6	20	10.8	33.3

* Number of gene sequences analyzed.

‡ Richness based on observed unique operational taxonomic units.

† Abundance coverage Estimator – nonparametric statistical prediction of total richness of different OTUs based on distribution of abundant (>10) and rare (≤ 10) OTUs.

§ Nonparametric statistical predictions of total richness of OTUs based on distribution of singletons and doubletons.

¶ Shannon diversity index. Higher number represents more diversity.

†† Reciprocal of Simpson's diversity index- higher number represents more diversity.

2.5. *Discussion*

Two aquaculture systems were used to examine the feasibility of maintaining the marine sponge *M. laxissima* in *ex situ* closed systems under controlled environmental conditions with ecological parameters similar to those in the sponges' natural habitat. The aquaculture of sponges in closed or semi-closed systems is a promising strategy to overcome the supply problem for sponge-derived compounds. This strategy offers good control of environmental conditions such as light levels and periods, temperature, food supply and possible precursors of important secondary metabolites (Sipkema *et al.*, 2005). However, aquaculture of marine sponges in completely closed systems is still challenging (Belarbi *et al.*, 2003; de Caralt *et al.*, 2003; Mendola, 2003; Osinga *et al.*, 2003; Duckworth *et al.*, 2003c). Sponges generally do not have fast growth rates and growth has rarely been obtained in aquaculture systems (Sipkema *et al.*, 2005). It was therefore not surprising that sponges did not grow under aquaculture conditions. Further optimization of the aquaculture system is required for it to be useful in terms of production of sponge biomass for harvesting natural products. *M. laxissima* showed high capability of adapting to aquaculture conditions compared to other sponges from the same reef environment examined in preliminary trials. *M. laxissima* was successfully maintained in the flow-through system for two years. The recirculating system was designed to give a well-controlled steady-state system with no reliance on a continual input of fresh seawater. My study is one of few reports to monitor the microbial communities associated with marine sponges in aquaculture (N. M. Mohamed, V. Rao, M. T. Hamann, M. Kelly,

and R. T. Hill, submitted for publication, L. T. Isaacs, J. Kan, L. Nguyen, P. Videau, M. A. Anderson, T. L. Wright, and R. T. Hill, submitted for publication, (Friedrich *et al.*, 2001; Hoffmann *et al.*, 2006). Hoffmann *et al.* (2006) used fluorescent *in situ* hybridization to study the stability and specificity of microbes associated with the marine cold-water sponge *Geodia barretti* during cultivation for eight months in open recirculation system. They suggested that the explants, which survived aquaculture conditions, have developed effective buffer systems to prevent infection by foreign sulfate reducing bacteria during the critical phase of cultivation. In agreement with my study, members of *Alpha* and *Gammaproteobacteria* were maintained during the period of cultivation (Fig. 2.7). Friedrich *et al.* (2001) found that a large fraction of microbial community of the Mediterranean sponge *Aplysina aerophoba* remained stable by starvation of sponges or antibiotic exposure over the eleven days in recirculating seawater aquariums.

The marine sponge *M. laxissima* harbors a diverse group of bacteria. Analyses of 16S rRNA gene clone libraries revealed the presence of clones affiliated with *Cyanobacteria* [<10% of the library of wild sponge collected in 2001(W01ML) and 25% of the library of the sponge collected in 2004 (W04ML)]. Cyanobacterial symbionts were also detected in aquacultured sponges in both systems. *Cyanobacteria* were previously detected in the larvae of *M. laxissima* (Enticknap *et al.*, 2006) and in the New Zealand sponge, *M. hentscheli* (Webb and Maas, 2002). This indicates that *Cyanobacteria* may be important for the life cycle and/or the nutrition of sponge hosts of the genus *Mycale*.

Changes in the microbial community associated with *M. laxissima* on maintenance in the flow-through system included the presence of clones from *Actinobacteria* that were highly enriched after two years in the aquaculture system (Figs. 2.5 and 2.7). *Actinobacteria* were not detected after 6 months in aquaculture. This may be due to their absence or a decrease in abundance to numbers below the detection limit. *Acidobacteria* were detected only from a sponge maintained for two years in this system. This was consistent with the culture-based approach where an isolate (N2yML4) affiliated with *Acidobacteria* was recovered from the two-year aquacultured sponge. Interestingly, sequences affiliated with *Acidobacteria* were not detected in the library of the aquarium water and are therefore absent or present at concentrations below detection limit. This indicates that these strains might be sponge-specific and have increased in numbers from levels undetectable in the wild sponges to detectable numbers after long-term maintenance in aquaculture. After acclimation to aquaculture environmental conditions, the growth of some bacteria may be favored, resulting in these groups becoming major components of the microbial communities of aquacultured sponges. Changes in the microbial community associated with *M. laxissima* on maintenance in the recirculating system included a decrease in the dominance of *Alpha-* and *Gammaproteobacteria* as was seen in sponges maintained in the flow-through system (Figs. 2.6 and 2.8). *Actinobacteria* were not detected in aquaculture and the *Bacteroidetes* group was significantly enriched.

The library representing bacterial communities found in wild sponges was significantly different from the library of the bacterial community from the

surrounding seawater based on LIBSHUFF results. In addition, there were marked differences in overall DGGE patterns of bacterial communities associated with *M. laxissima* and those in the surrounding water. This suggests that the bacterial community associated with wild *M. laxissima* is sponge-specific rather than simply comprising a transient population from the water column. After maintenance in aquaculture for two years, the bacterial community in an aquacultured sponge was different than the library of bacterial community in the aquaculture system water. This suggests that *M. laxissima* maintains a distinct bacterial community different from that in the surrounding water filtered by the sponges, both in the wild and in aquaculture. This is consistent with other reports (Wilkinson, 1978a; Santavy *et al.*, 1990; Hentschel *et al.*, 2002; Taylor *et al.*, 2004b; Taylor *et al.*, 2005; Taylor *et al.*, 2007b) showing that sponges harbor different bacteria than those in the surrounding water but this is the first time that this has been shown for sponges maintained in aquaculture systems. Limitations of the DGGE analyses are that only a limited number of samples were available for analysis and that the identity of bands were not confirmed by sequence analysis. It is therefore possible that in some cases, bands with the same migration could have originated from different organisms.

The total number of OTUs in bacterial communities was calculated using non-parametric estimators. Chao1 richness estimates were based on singletons and doubletons as described by Chao (1984) while ACE (abundance-base coverage estimator) was based on the distribution of abundant (>10) and rare (≤ 10) species. Shannon and the reciprocal of Simpson's indices were used as diversity indices. Higher numbers indicate greater diversity. Small sample size may affect the

performance of diversity estimators. I predict, based on the rarefaction analyses that indicate further sampling from almost all libraries would reveal more diversity at the species level (Fig. 2.8), that the diversity that was observed is an underestimate and additional sampling would lead to an increased estimate of total diversity.

The richness and the diversity of the bacterial communities increased in the flow-through aquaculture system. This was somewhat unexpected, as we had anticipated a decrease in the bacterial diversity on long-term maintenance of sponges in aquaculture systems. A loss in bacterial diversity may have had adverse consequences for sponge health and for the production of bioactive compounds such as antibiotics, antifungal and antifouling compounds by bacterial symbionts. An increase in the bacterial diversity of the sponge-associated communities raises the interesting possibility that additional novel bacteria could be cultured from aquacultured sponges compared with wild sponges, although it has not yet shown that these novel cultured bacteria are sources of new bioactive compounds. Maintenance of sponges in aquaculture may provide a means for assessing new culturable bacterial diversity from sponges. This is supported by my successful isolation of a putative *Acidobacterium* strain (N2yML4) from *M. laxissima* after maintenance of this sponge in aquaculture. To my knowledge, *Acidobacteria* have not previously been cultivated from marine sponges. This strain is a potential candidate for a genomics approach that may reveal aspects of its metabolic capabilities and importance for the sponge host. I was successful in culturing several additional novel strains that were distantly (≤95% 16S rRNA gene identity) related to previously cultured strains with sequences deposited in GenBank. These included four *Flavobacteriaceae*, six

Flexibacteriaceae, two *Gammaproteobacteria*, and two *Alphaproteobacteria* (Table 2.1). All of these strains warrant description as new species or genera.

M. laxissima was maintained alive in closed aquaculture systems. The bacterial community of *M. laxissima* changed substantially on transfer into aquaculture. Based on results from both flow-through and recirculating systems, there was a permanent component of the bacterial community that was present in wild sponges and was maintained in sponges in aquaculture. This fraction included members of *Alpha-*, *Gamma- and Deltaproteobacteria*, *Bacteroidetes* and *Planctomycetes*. This suggests that specific strains in this stable component may be essential for the health of the sponge and possibly play essential symbiotic roles such as the production of antifouling agents and antimicrobial agents that prevent the growth of pathogenic bacteria in aquacultured sponges.

In conclusion, my study highlights the potential of developing a model system of sponges in closed aquaculture systems. Manipulative studies of this model can be used to understand sponge-bacterium symbiotic relationships. An example is colonization assays of sponges in aquaculture using specific symbionts.

Chapter 3. Monitoring Bacterial Diversity and Metabolite Production of the Marine Sponge *Ircinia strobilina* on Transfer into Aquaculture

3.1. Abstract

Marine sponges in the genus *Ircinia* are known to be good sources of secondary metabolites with biological activities. A major obstacle in the development of sponge-derived metabolites is the difficulty in ensuring an economic, sustainable supply of the metabolites. A promising strategy is the *ex situ* culture of sponges in closed or semi-closed aquaculture systems. In this study, the marine sponge *I. strobilina* (Order Dictyoceratida: Family Irciniidae), collected from the wild and maintained for a year in a recirculating aquaculture system, was used. Microbiological and molecular community analyses were performed on freshly collected sponges and sponges maintained in aquaculture for three months and nine months. Chemical analyses were performed on wild collected sponges and individuals maintained in aquaculture for three months and one year. Denaturing gradient gel electrophoresis was used to access the complexity and monitor changes of the microbial communities associated with *I. strobilina*. Culture-based and molecular techniques showed an increase in the *Bacteroidetes, Alpha-* and *Gammaproteobacteria* components of the bacterial community in aquaculture. Populations affiliated with *Beta-* and *Deltaproteobacteria, Clostridia* and *Planctomycetes* emerged in sponges maintained in aquaculture. The diversity of bacterial communities increased on transfer into aquaculture. Analysis of primary and secondary metabolites from the organic extracts of sponges collected from the wild and compared to those maintained in aquaculture showed no significant change

in the production of the major metabolites by liquid chromatography coupled with time-of-flight mass spectrometry.

3.2. Introduction

Several techniques may produce large quantities of sponge biomass needed for the extraction of bioactive metabolites that are not viable candidates for a total synthesis. Such techniques include sponge farming (Duckworth and Battershill, 2003a,b) and primmorph systems that are aggregates of sponge cells that still contain bacteria (reviewed by Müller *et al.*, 2004). The NOMATEC (Novel Marine Technologies) project showed that *Ircinia variabilis* is suitable for mariculture (van Treeck *et al.*, 2003a). Further, De Rosa *et al.* (2001) reported the development of cell cultures from *Ircinia muscarum*. There were major differences in the composition of secondary metabolites between the wild sponge and its cell cultures, with lower concentration of lipids and loss of sterols and volatile compounds in cell cultures (De Rosa *et al.*, 2002).

Aquaculture in tanks might be preferable to open-water mariculture because it is reliable and inexpensive and it is also possible to shift from seasonal growth to continuous growth during the year (Osinga *et al.*, 1999b; Duckworth and Battershill, 2003a,b; Duckworth *et al.*, 2003c; Sipkema *et al.*, 2005). *Ircinia strobilina* (Lamarck, 1816) was chosen for this study as a representative of the genus *Ircinia* (Order Dictyoceratida: Family Ircinidae) whose species are very rich sources of secondary metabolites with a variety of biological activities and structural classes

(Faulkner *et al.*, 2000). *I. strobilina* contains variabilin, a furanosesterterpene that has been identified as a fish feeding deterrent (Epifanio *et al.*, 1999). A diversity of metabolites has been found from other sponges in the genus *Ircinia* and includes other sesterterpenes (Alfano *et al.*, 1979; Buchanan *et al.*, 2001; Martínez *et al.*, 2001; Tziveleka *et al.*, 2002; Issa *et al.*, 2003; Yang *et al.*, 2003; Rifai *et al.*, 2005) in addition to variabilin from *I. strobilina* as well as alkaloids, macrolides and a ceramide (Fig. 3.1). The compounds ircinal A and B (precursors of antimalarial manzamine alkaloids) were isolated from the Okinawan marine sponge *Ircinia* sp. (Kondo *et al.*, 1992). Ircinamine, an alkaloid with moderate activity against the murine leukemia cell line P388, was purified from a marine *Ircinia* sp. (Kuramoto *et al.*, 2004). Tedanolide C, cytotoxic macrolide was isolated from the Papua New Guinea sponge *Ircinia* sp. (Chevallier *et al.*, 2006). Other compounds of potential biomedical importance from the genus *Ircinia* include two murine and human cancer cell growth inhibitors, irciniastatin A and B (Pettit *et al.*, 2004) and a ceramide (Zhang *et al.*, 2005).

To examine the potential of *ex situ* culture of sponges in closed aquaculture systems, it is crucial to determine whether the microbial communities change upon transfer into aquaculture. The aim of this study was to address two questions: 1. Does transferring the sponge into aquaculture affect the stability of sponge-microbe associations? and 2. Are changes in the microbial communities correlated with changes in the chemistry of the sponge?

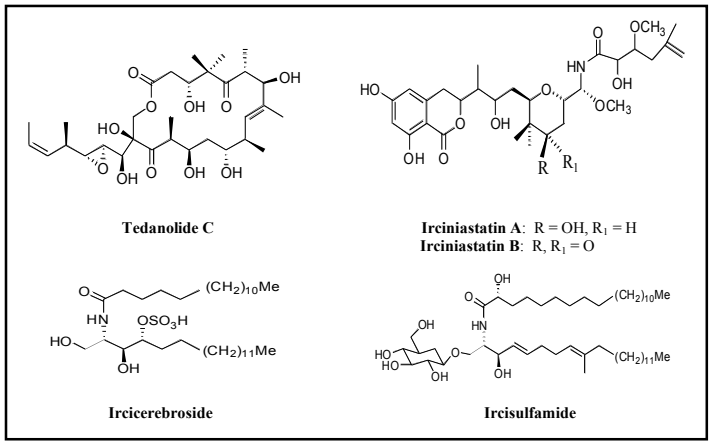


Fig. 3.1. Selected compounds isolated from *Iricinia* sp.

3.3. Materials and methods

Sponge and water sample collection. The marine sponge *I. strobilina* was collected by SCUBA at Conch Reef, Key Largo, Florida in June 2004 at a depth of ca. 18 m. Voucher samples were preserved in 70% ethanol immediately after collection for taxonomic identification. Samples were frozen for later molecular and chemical characterization. Three water samples were collected from the vicinity of the sponge in sterile 20-liter containers and filtered through 0.22-µm pore-size Sterivex filters (Millipore, Billerica, MA, USA). Sterivex filters were frozen immediately and stored them at -20˚C for isolation of nucleic acids. Four individuals of *I. strobilina* were collected for the aquaculture study and kept them in containers filled with seawater that was replaced every 2-6 hours during the road transportation from Florida to Baltimore. The sponges were kept aerated by battery operated air pumps and airstones. Voucher specimens were examined histologically by Michelle Kelly (National Centre for Aquatic Biodiversity and Biosecurity, National Institute of

Water and Atmospheric Research (NIWA) Ltd., Auckland, New Zealand) using light microscopy of thin sections for taxonomic identification of marine sponge *I. strobilina.* In life, the sponge forms a squat rubbery mass with distinctive webs extending between large blunt conules set well apart on the sponge surface. The oscules are typically grouped together on the apex of the sponge. In reefal areas with high current activity, sponges are taller and more cylindrical with oscules raised on an apical ridge. The sponge is dark brownish black under full illumination; shaded portions are cream. The consistency is tough and spongy and it is very difficult to tear or cut; on cutting the sponge emits a fetid odour. The primary fibers are large and coarse trellises with only rare connecting fibers.

Sponge aquaculture. The same recirculating aquaculture system described in Chapter 2 was used to house the collected *I. strobilina* sponges. Four *I. strobilina* sponges collected in 2004 were maintained in this aquaculture system for one year. Sponges were inspected visually during this period to monitor their health. Viability assays (Müller *et al.*, 1974; Blumbach *et al.*, 1998) were used to check that the sponges were alive immediately before sacrificing them for microbiological studies. Manually dispersed sponge tissue reaggregates spontaneously indicating viability of sponge cells. Two sponges and then one sponge were sacrificed after three months and nine months, respectively.

Sponge processing for isolation of culturable bacteria. Immediately after collection of the sponges from the field and harvesting from the aquaculture system, the sponge samples were processed following the same procedure described in Chapter 2.

Determination of culturable and total bacterial counts. Plate counts and total counts were determined following the same procedure described in Chapter 2.

Identification of isolates by 16S rRNA gene sequence analysis. All cultured bacteria were classified according to their morphology and a representative of each colony type was selected for sequencing. Single pure colonies of each isolate were transferred to 20 ml of marine broth and incubated them overnight at 30˚C in a shaking incubator. DNA extraction, cryopreservation, and PCR-amplification of bacterial 16S rRNA, were done following the same procedure described in Chapter 2.

DNA extraction from sponges and aquarium water samples. Details are given in Chapter 2 and Appendix B.

PCR amplification of genomic DNA, cloning and sequencing. 16S rRNA gene fragments were PCR- amplified from the total genomic DNA isolated from sponges collected from the wild and maintained in aquaculture, purified PCR products, and cloned purified products using the same general protocol described in Chapter 2. Purified plasmid DNA from individual clones was sequenced with M13 universal forward and reverse sequencing primers.

Phylogenetic analysis. Phylogenetic analyses of clone libraries were performed using ARB as described in Chapter 2. Sequences of three libraries were determined using the PT-server with a dataset containing the nearest relative matches. This database was supplemented with relevant environment sequences that were submitted recently in GenBank. These sequences were selected based on the top BLAST hits of the clone sequences. Phylogenetic trees including novel sequences and reference taxa were constructed using the neighbor-joining (Jukes-Cantor correction) (Saitou and

Nei, 1987) implemented in ARB. The robustness of the inferred trees topologies was evaluated after 1,000 bootstrap replicates of the neighbor-joining data. Phylip was used to generate bootstrap values (Felsenstein, 2004). Partial sequences (about 500-600 bp) were analyzed with the BLAST algorithm for initial identification. The identification of partial sequences was confirmed by adding them to a tree consisting of full-length sequences without changing the tree topology by using the ARB parsimony interactive method.

Estimation of microbial diversity and statistical analysis of clone libraries. I used the same software as in Chapter 2. S-LIBSHUFF (Schloss *et al.*, 2004) was used to compare the libraries statistically and DOTUR (distance-based OTU and richness) (Schloss and Handelsman, 2005) was used to assign sequences to operational OTU, calculate collector's curves for observed unique OTUs, Chao1 and ACE richness estimators, and Shannon and Simpson's indices.

DGGE. The 195 bp-region corresponding to positions 341 and 534 in the 16S rRNA gene of *E. coli* was amplified from genomic DNA extracted from sponges and water samples using P2 and P3 primers (Muyzer *et al.*, 1993). DGGE was performed following the same procedure in Chapter 2. DGGE gels were run twice to confirm the reproducibility of the overall pattern.

Profiles of small molecules. Three *I. strobilina* individuals were used as control samples whereas test samples comprised two *I. strobilina* individuals maintained for three months and one individual maintained for one year in the aquaculture system. Two grams of frozen sponge tissues were lyophilized and extracted with ethanol. The dried ethanol extract (100 mg) was dissolved in methanol and passed through a C18

column. My collaborators at the University of Mississippi carried out LC-MS analysis on a Bruker microTOF^{TM} with electrospray ionization. Sample solutions were prepared in methanol and subjected to LCMS analysis using reverse-phase C8 column (Phenomenex, Torrance, CA, USA; 5 μm, 4.6 × 150 mm) eluting at 0.4 ml/minute flow rate with 15 min linear gradient from 20% to 100% phase B. Phase A was water and phase B was acetonitrile. ESI-MS of the samples (elutes) was carried out in a positive mode on a mass spectrometer equipped with an electrospray ion source and a microTOF^{TM} data system.

Nucleotide sequence accession numbers. 16S rRNA gene sequences from isolates were submitted to GenBank under accession numbers EF629549 to EF629580. 16S rRNA gene sequences from clone libraries were submitted to GenBank under accession numbers EF629581 to EF629828.

3.4. Results

Bacterial enumeration. Total (DAPI stained) and culturable (plate) bacterial counts were determined for samples from wild *I. strobilina* sponges and sponges maintained in a closed aquaculture system for three months and nine months. For the three wild sponges, the mean and standard error of total counts was $2.8\pm0.4 \times 10^9$ cells ml^{-1} and the mean of plate counts was $1.1\pm0.4 \times 10^6$ colony forming units (CFU) ml $^{-1}$. For the two three-month individuals, the mean of total counts was $5.8\pm0.8 \times 10^9$ cells ml^{-1} and the mean of plate counts was $9.7\pm3.3 \times 10^6$ CFU ml^{-1}. For the nine-month sponge, the total count was 7.3×10^9 cells ml^{-1} and the plate count was 8.0×10^5 CFU ml^{-1}. Based on these counts, the percentage of culturable bacteria in the sponge samples

ranged from 0.01% to 0.17%, indicating the importance of assessing these communities by using molecular techniques.

Phylogenetic analysis of bacterial isolates. Traditional culturing techniques were used to isolate heterotrophic bacteria. Ten strains were characterized by 16S rRNA sequence analysis from wild sponges and sponges maintained for three months in aquaculture and 12 strains were characterized from sponges maintained for nine months. Alpha- and gammaproteobacterial strains dominated wild sponges and those maintained in aquaculture under the culture conditions used in this study (Fig. 3.2). For sponges in aquaculture, the abundances of bacterial groups belonging to *Alpha*- and *Gamma*-subdivisions of the *Proteobacteria* remained high. However, bacteria belonging to *Bacteroidetes* appeared only in the culturable bacterial communities of sponges maintained in aquaculture.

Denaturing gradient gel electrophoresis (DGGE). 16S rRNA DGGE was used to analyze total bacterial communities present in sponges. Bacterial communities varied substantially between wild sponges and surrounding seawater and between sponges in aquaculture and surrounding water in the aquaculture system, respectively (Fig. 3.3). This indicates that the sponges harbor different assemblages of bacteria from those found in the surrounding water. The diversity of the microbial community, inferred by the complexity of the banding patterns, increased upon maintenance of *I. strobilina* in aquaculture for three months. A replicate three-month sample gave a similar banding pattern to the three-month sample shown in Fig. 3.3. The community diversity then decreased in the sample from the sponge maintained for nine months in aquaculture, reverting to a similar pattern (judged on the basis of

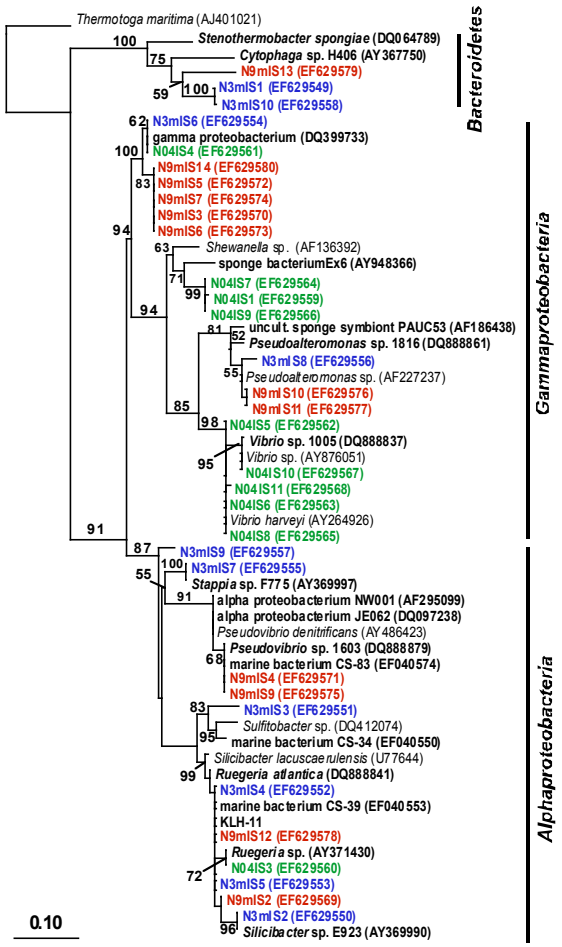


Fig. 3.2. Rooted neighbor-joining phylogenetic tree of partial 16S rRNA gene sequences of bacterial isolates that were recovered from *I. strobilina* sponges collected from the wild (prefixed NMM04IS, displayed in green) and maintained for three months (prefixed NMM3mAIS, displayed in blue) and nine months (prefixed NMM9mIS, displayed in red) in a recirculating aquaculture system. Bootstrap confidence values > 50% are shown at the nodes. *Thermatoga maritima* was used an outgroup in this analysis. Scale bar indicates 0.10 substitutions per nucleotide position. Bacteria isolated from different marine sponges are shown in bold with GenBank accession numbers listed after each sequence name.

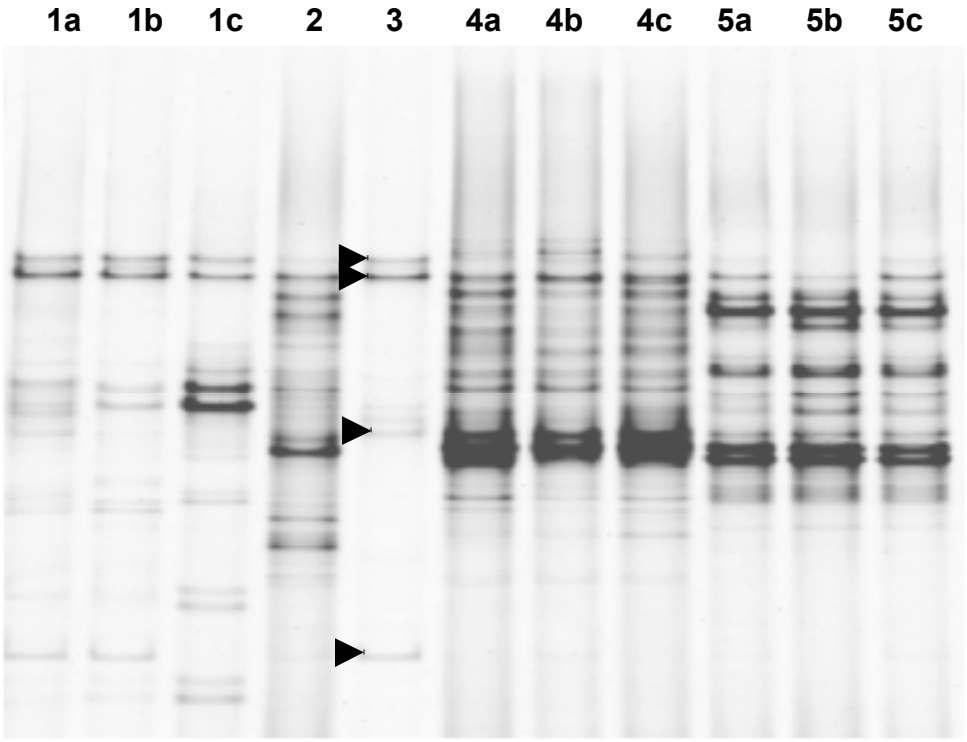


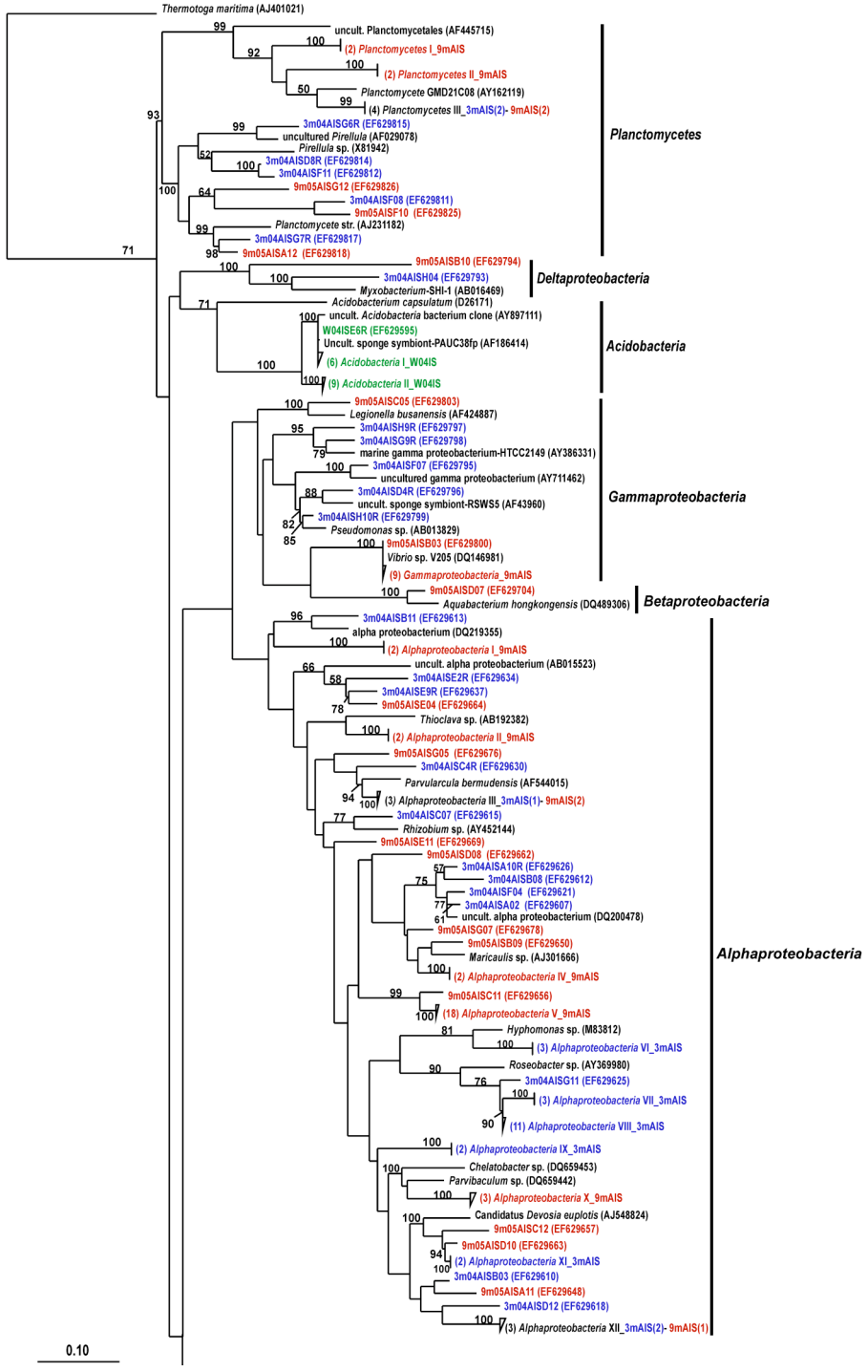
Fig. 3.3. Denaturing gradient gel electrophoresis fingerprint of the bacterial communities associated with *I. strobilina* sponges collected from the wild (lanes 1a-c), and maintained for three months (lane 2) and nine months (lane 3) in a recirculating aquaculture system. Fingerprints of bacterial communities in seawater (lanes 4a-c) and water samples from the aquaculture system (lanes 5a-c) are also shown. The denaturing gradient was 40% to 70%.

the four dominant bands in the nine-month sample, marked by black arrows in Fig. 3.3, to that of the wild sponge, although this should be interpreted with caution, because the weak banding pattern of the nine-month sponge might be due to poor PCR amplification (Lane 3, Fig. 3.3) and only a single sponge was processed after nine months in aquaculture so it was not possible to obtain a replicate for this time-

point. DGGE data convincingly demonstrate that the bacterial communities of sponge-associated bacteria in wild sponges differ from those in the surrounding seawater and the water in aquaculture tanks (Fig. A.1, Appendix A). Although the DGGE data are not conclusive alone in concluding that shifts occurred in sponge-associated bacterial communities during aquaculture, the trends observed by DGGE are consistent with the findings from statistical analysis of clone library data.

Phylogenetic analysis of 16S rRNA gene clone libraries. To determine the stability of the microbial community upon transfer of the *I. strobilina* sponge into aquaculture, 16S rRNA gene clone libraries were generated from community DNA obtained from a representative wild sponge and sponges maintained for three months and nine months in aquaculture. In total, 270 clones of bacterial 16S rRNA gene fragments from the three libraries were sequenced. After elimination of a small number of chimeric clones from each library, I analyzed 100, 74 and 74 clones from the wild, three-month and nine-month libraries, respectively. The 16S rRNA gene clones of the wild sponge corresponded to 35 unique OTUs, which fell in the following five bacterial lineages: *Acidobacteria, Actinobacteria*, *Bacteroidetes, Chloroflexi*, and *Cyanobacteria* (Fig. 3.4). The 16S rRNA gene clones of the three-month sponge corresponded to 48 unique OTUs that encompassed the following eight bacterial lineages: *Bacteroidetes*, *Clostridia*, *Cyanobacteria*, *Planctomycetes, Proteobacteria (Alpha-*, *Gamma-*, and *Deltaproteobacteria*), and unassigned bacteria (Fig. 3.4). The 16S rRNA gene clones of the nine-month sponge corresponded to 47

A.


Thermotoga maritima (AJ401021)
uncult. Planctomycetales (AF445715)
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(2) Planctomycetes II_9mAIS
Planctomycete GMD21C08 (AY162119)
(4) Planctomycetes III_3mAIS(2)- 9mAIS(2)
3m04AISG6R (EF629815)
uncultured Pirellula (AF029078)
Pirellula sp. (X81942)
3m04AISD8R (EF629814)
3m04AISF11 (EF629812)
9m05AISG12 (EF629826)
3m04AISF08 (EF629811)
9m05AISF10 (EF629825)
Planctomycete str. (AJ231182)
3m04AISG7R (EF629817)
9m05AISA12 (EF629818)
Planctomycetes
9m05AISB10 (EF629794)
3m04AISH04 (EF629793)
Myxobacterium-SHI-1 (AB016469)
Deltaproteobacteria
Acidobacterium capsulatum (D26171)
uncult. Acidobacteria bacterium clone (AY897111)
W04ISE6R (EF629595)
Uncult. sponge symbiont-PAUC38fp (AF186414)
(6) Acidobacteria I_W04IS
(9) Acidobacteria II_W04IS
Acidobacteria
9m05AISC05 (EF629803)
Legionella busanensis (AF424887)
3m04AISH9R (EF629797)
3m04AISG9R (EF629798)
marine gamma proteobacterium-HTCC2149 (AY386331)
3m04AISF07 (EF629795)
uncultured gamma proteobacterium (AY711462)
3m04AISD4R (EF629796)
uncult. sponge symbiont-RSWS5 (AF43960)
3m04AISH10R (EF629799)
Pseudomonas sp. (AB013829)
9m05AISB03 (EF629800)
Vibrio sp. V205 (DQ146981)
(9) Gammaproteobacteria_9mAIS
Gammaproteobacteria
9m05AISD07 (EF629704)
Aquabacterium hongkongensis (DQ489306)
Betaproteobacteria
3m04AISB11 (EF629613)
alpha proteobacterium (DQ219355)
(2) Alphaproteobacteria I_9mAIS
uncult. alpha proteobacterium (AB015523)
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3m04AISE9R (EF629637)
9m05AISE04 (EF629664)
Thioclava sp. (AB192382)
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3m04AISC4R (EF629630)
Parvularcula bermudensis (AF544015)
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3m04AISC07 (EF629615)
Rhizobium sp. (AY452144)
9m05AISE11 (EF629669)
9m05AISD08 (EF629662)
3m04AISA10R (EF629626)
3m04AISB08 (EF629612)
3m04AISF04 (EF629621)
3m04AISA02 (EF629607)
uncult. alpha proteobacterium (DQ200478)
9m05AISG07 (EF629678)
9m05AISB09 (EF629650)
Maricaulis sp. (AJ301666)
(2) Alphaproteobacteria IV_9mAIS
9m05AISC11 (EF629656)
(18) Alphaproteobacteria V_9mAIS
Hyphomonas sp. (M83812)
(3) Alphaproteobacteria VI_3mAIS
Roseobacter sp. (AY369980)
3m04AISG11 (EF629625)
(3) Alphaproteobacteria VII_3mAIS
(11) Alphaproteobacteria VIII_3mAIS
(2) Alphaproteobacteria IX_3mAIS
Chelatobacter sp. (DQ659453)
Parvibaculum sp. (DQ659442)
(3) Alphaproteobacteria X_9mAIS
Candidatus Devosia euplotis (AJ548824)
9m05AISC12 (EF629657)
9m05AISD10 (EF629663)
(2) Alphaproteobacteria XI_3mAIS
3m04AISB03 (EF629610)
9m05AISA11 (EF629648)
3m04AISD12 (EF629618)
(3) Alphaproteobacteria XII_3mAIS(2)- 9mAIS(1)
Alphaproteobacteria
0.10

B.

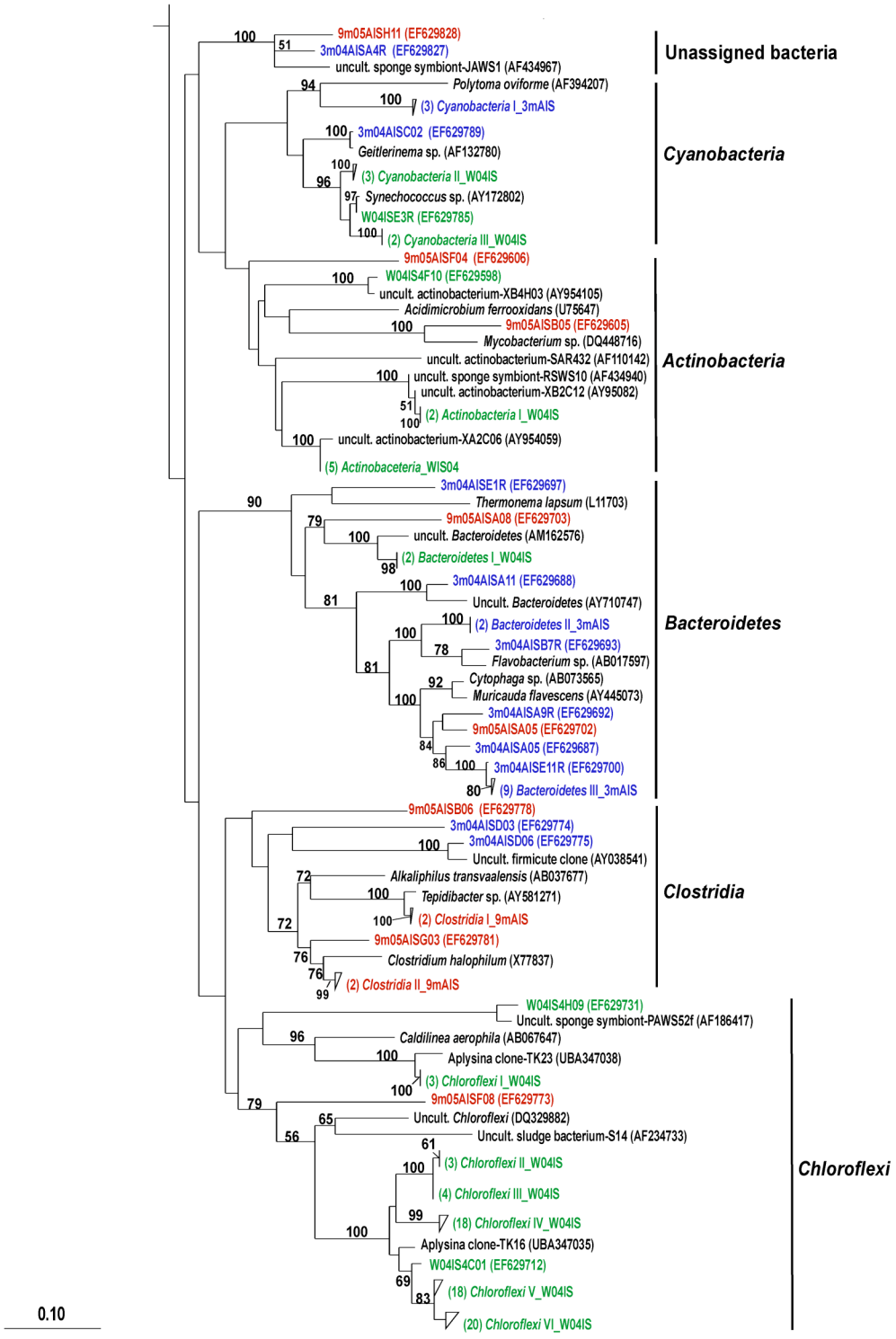

9m05AISH11 (EF629828)
3m04AISA4R (EF629827)
uncult. sponge symbiont-JAWS1 (AF434967)
Unassigned bacteria
Polytoma oviforme (AF394207)
(3) Cyanobacteria I_3mAIS
3m04AISC02 (EF629789)
Geitlerinema sp. (AF132780)
(3) Cyanobacteria II_W04IS
Synechococcus sp. (AY172802)
W04ISE3R (EF629785)
(2) Cyanobacteria III_W04IS
Cyanobacteria
9m05AISF04 (EF629606)
W04IS4F10 (EF629598)
uncult. actinobacterium-XB4H03 (AY954105)
Acidimicrobium ferrooxidans (U75647)
9m05AISB05 (EF629605)
Mycobacterium sp. (DQ448716)
uncult. actinobacterium-SAR432 (AF110142)
uncult. sponge symbiont-RSWS10 (AF434940)
uncult. actinobacterium-XB2C12 (AY95082)
(2) Actinobacteria I_W04IS
uncult. actinobacterium-XA2C06 (AY954059)
(5) Actinobaceteria_WIS04
Actinobacteria
3m04AISE1R (EF629697)
Thermonema lapsum (L11703)
9m05AISA08 (EF629703)
uncult. Bacteroidetes (AM162576)
(2) Bacteroidetes I_W04IS
3m04AISA11 (EF629688)
Uncult. Bacteroidetes (AY710747)
(2) Bacteroidetes II_3mAIS
3m04AISB7R (EF629693)
Flavobacterium sp. (AB017597)
Cytophaga sp. (AB073565)
Muricauda flavescens (AY445073)
3m04AISA9R (EF629692)
9m05AISA05 (EF629702)
3m04AISA05 (EF629687)
3m04AISE11R (EF629700)
(9) Bacteroidetes III_3mAIS
Bacteroidetes
9m05AISB06 (EF629778)
3m04AISD03 (EF629774)
3m04AISD06 (EF629775)
Uncult. firmicute clone (AY038541)
Alkaliphilus transvaalensis (AB037677)
Tepidibacter sp. (AY581271)
(2) Clostridia I_9mAIS
9m05AISG03 (EF629781)
Clostridium halophilum (X77837)
(2) Clostridia II_9mAIS
Clostridia
W04IS4H09 (EF629731)
Uncult. sponge symbiont-PAWS52f (AF186417)
Caldilinea aerophila (AB067647)
Aplysina clone-TK23 (UBA347038)
(3) Chloroflexi I_W04IS
9m05AISF08 (EF629773)
Uncult. Chloroflexi (DQ329882)
Uncult. sludge bacterium-S14 (AF234733)
(3) Chloroflexi II_W04IS
(4) Chloroflexi III_W04IS
(18) Chloroflexi IV_W04IS
Aplysina clone-TK16 (UBA347035)
W04IS4C01 (EF629712)
(18) Chloroflexi V_W04IS
(20) Chloroflexi VI_W04IS
Chloroflexi
0.10

Fig. 3.4. Rooted neighbor-joining phylogenetic tree of partial 16S rRNA gene clones that were recovered from *I. strobilina* sponges collected from the wild (prefixed W04IS in green) and maintained for three months (prefixed 3m04AIS in blue) and nine months (prefixed 9m05AIS in red) in the aquaculture system. Bootstrap confidence values > 50% are shown at the nodes. The tetragons represent clones that are ≥ 99.5% similar. Composition of each of these groups is shown in (Table A.3, Appendix A). The numbers listed in bold before the group names indicate the numbers of clones. *T. maritima* was used as an outgroup. Scale bar indicates 0.10 substitutions per nucleotide position. Reference sequences are shown in bold with GenBank accession numbers listed after each sequence name.

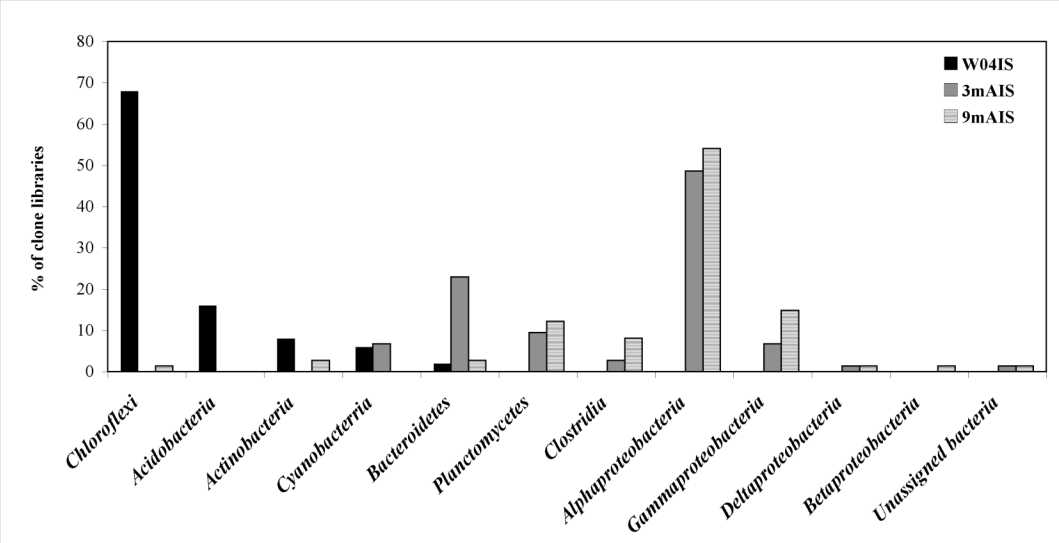


Fig. 3.5. Distribution of bacterial 16S rRNA gene clones from *I. strobilina* sponges collected from the wild (W04IS) and maintained for three months (3mAIS) and nine months (9mAIS) in the aquaculture system within the major phylogenetic groups detected in the three libraries. Percentages of each group were determined from sequence data.

unique OTUs, which fell in the following ten bacterial lineages: *Actinobacteria, Bacteroidetes*, *Chloroflexi*, *Clostridia, Planctomycetes*, *Proteobacteria* (*Alpha-*, *Beta-*, *Gamma-*, and *Deltaproteobacteria*), and unassigned bacteria (Fig. 3.4). The relative distribution of the major phylogenetic groups from each clone library is shown in Fig. 3.5.

Rarefaction analysis. Rarefaction analysis was performed to determine whether the total diversity of bacterial communities was well represented by the number of clones sequenced in each library. The rarefaction curves were obtained with DOTUR using 10,000 random iterations. For clones from wild *I. strobilina*, the rarefaction curves at the phylum level (distance= 0.24) and species level (distance= 0.03) reached saturation, indicating sufficient sampling of this clone library (Fig. 3.6). On the other hand, the rarefaction curves of the three-month and nine-month sponges reached saturation at the phylum level, where richness reached an asymptotic maximum, but not at the species level, indicating that further sampling of the clone library would have revealed additional OTUs and diversity. The wild sponge had lower bacterial richness compared to sponges maintained in aquaculture especially at the species level. Maintaining *I. strobilina* in aquaculture clearly increased the bacterial richness, demonstrated by steeper rarefaction curves and high numbers of OTUs compared with those from wild sponges.

Statistical analysis of bacterial diversity. Additional measures of diversity and richness were obtained with the statistical richness estimators and diversity indices shown in Table 3.1. These indices were calculated using DOTUR. The input files were in the form of distance matrices generated by ARB. The total number of OTUs

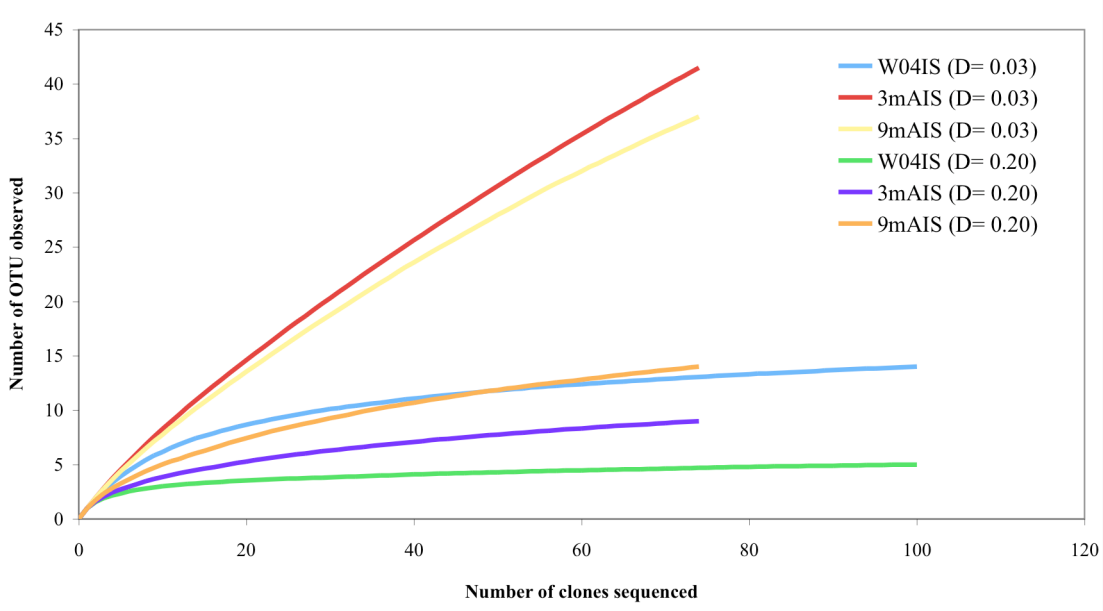


Fig. 3.6. Rarefaction curves indicating relative richness within bacterial 16S rRNA gene clones of *I. strobilina* sponges collected from the wild and maintained for three months and nine months in the aquaculture system. Operational taxonomic units were defined at estimated distances at both phylum (D= 0.20) and species (D= 0.03) levels.

in a bacterial population was calculated using non-parametric estimators. Chao1 richness estimates were based on singletons and doubletons as described by Chao (1984), while ACE (abundance-base coverage estimator) was based on the distribution of abundant (>10) and rare (≤ 10) species. Shannon and the reciprocal of Simpson's were used as diversity indices, where higher numbers indicates greater diversity. Consistent with the rarefaction curves, both statistical indices suggested that the community diversity of sponges maintained in aquaculture was higher than that of wild sponge. LIBSHUFF was used to quantitatively compare the three

libraries. Evolutionary distances were calculated using the neighbor-joining algorithm in ARB and the three libraries were significantly different ($P<0.0001/0.0100$).

Table 3.1. Richness and diversity estimates for bacterial 16S rRNA gene clone libraries from *I. strobilina* samples collected from the wild and maintained in aquaculture at specific distance (D) cutoff values.

Sample	Wild *I. strobilina* (n*= 100)		Three-month *I. strobilina* (n*= 74)		Nine-month *I. strobilina* (n*= 74)	
	0.20	**0.03**	**0.20**	**0.03**	**0.20**	**0.03**
Richness‡	7	14	12	41	14	37
ACE†	8	15	17	200	21	130
Chao1§	7	14	17	107	19	67
Shannon¶	1.2	2.2	1.8	3.3	1.8	3.1
1/ Simpson††	2.3	8.3	4	17.8	3.8	12.8

* Number of 16S rRNA gene sequences analyzed.

‡ Richness based on observed unique operational taxonomic units.

† Abundance coverage Estimator – nonparametric statistical prediction of total richness of different OTUs based on distribution of abundant (>10) and rare (≤ 10) OTUs.

§ Nonparametric statistical predictions of total richness of OTUs based on distribution of singletons and doubletons.

¶ Shannon diversity index- higher number represents more diversity.

†† Reciprocal of Simpson's diversity index- higher number represents more diversity.

Liquid chromatography and mass spectrometry (LC-MS) profiles of small molecules. Overall profiles of small molecules extracted from sponge samples by using organic solvents were determined in order to detect any gross shifts in chemistry of sponges on transfer into aquaculture. LC-MS analysis was performed by my collaborators of the University of Mississippi to determine whether gross changes in overall metabolic profiles of *I. strobilina* occurred upon transfer into aquaculture. Minor changes in profiles of small molecules were observed in the sponges maintained in aquaculture when compared to control wild samples but overall patterns remained consistent, indicating no major shift in the profiles of secondary metabolites (Fig. 3.7).

3.5. Discussion

I. strobilina was maintained alive in an aquaculture system for one year. The growth of the sponges was observed visually after three and nine months. Growth rates of the sponges were not quantified but significant growth was not visually apparent. The health of sponges was assessed visually and judged to be good because no necrosis was observed and all four *Ircinia* individuals remained unfouled for the entire course of the study. In addition, when sponges were removed for analysis, the

area beneath and immediately adjacent to each sponge was unfouled, whereas the rest of the sediment in tanks was covered by a thin algal film. All sponges in aquaculture

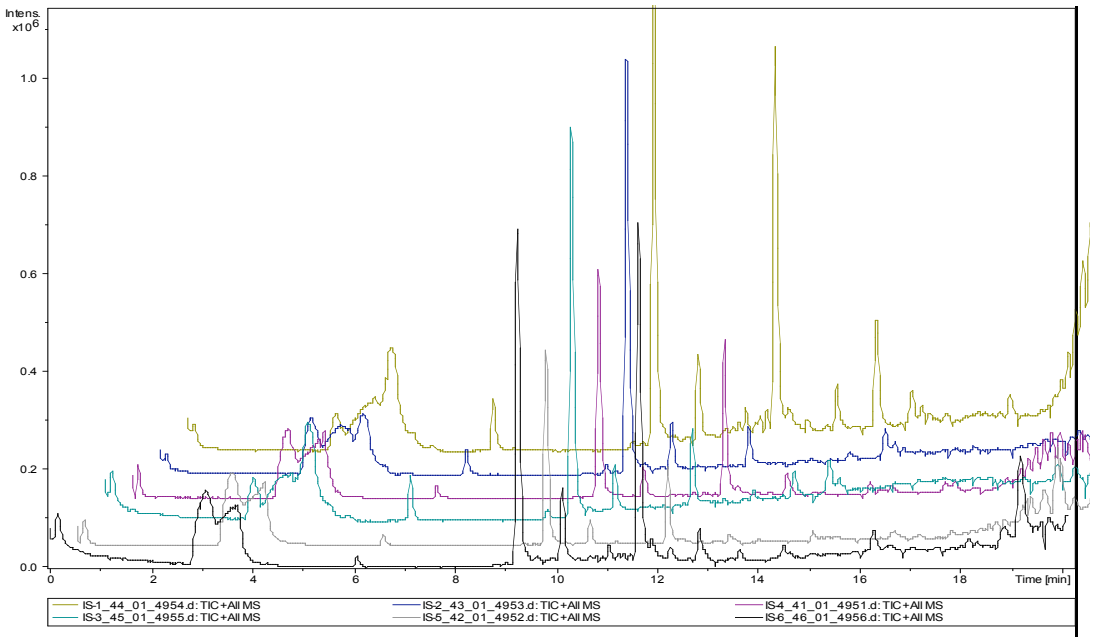


Fig. 3.7. Stacked ion chromatograms of organic extracts of *I. strobilina* sponges. IS1-3 (shown in yellow, blue and green, respectively) represent three sponge individuals collected from the wild, IS4-5 (shown in pink and gray, respectively) represent two sponge individuals maintained for three months in the aquaculture system and IS6 (shown in black) represents a sponge maintained for one year in the aquaculture system.

also retained the black coloration present in sponges in the wild. Phylogenetic and statistical analyses of the small subunit rRNA gene libraries were used to monitor structural shifts in the microbial communities associated with *I. strobilina* following

cultivation in aquaculture. These systematic analyses can help identify changes of the bacterial communities that result from aquaculture. Bacterial communities associated with *I. strobilina* had the following characteristics:

(i) They were different from bacterioplankton communities found in the surrounding seawater. DGGE analysis of sponges maintained in aquaculture and the surrounding water indicate substantial differences in these bacterial communities. This was confirmed by community analysis of these samples by 16S rRNA genes sequencing studies. The data for bacterial community analysis of the *I. strobilina* samples are presented here and those for bacterial community analysis of the surrounding water are shown in Chapter 2.

(ii) Isolates from wild sponges included isolates found only in marine sponges (Webster and Hill, 2001; Hentschel *et al.*, 2002; Enticknap *et al.*, 2006). Based on the presence of sponge-associated bacteria in the top 25 BLAST hits of the 16S rRNA gene sequences of isolates, a comparison was made between a subset of bacteria isolated from *I. strobilina* and the surrounding seawater. Sixty percent of the top BLAST hits of isolates from *I. strobilina* were to bacteria found only in sponges. On the other hand, bacteria from the seawater had only 16% of top BLAST hits matching sponge bacteria.

(iii) Total communities included representatives primarily clustered within the *Acidobacteria*, *Cyanobacteria, Actinobacteria, Bacteroidetes* and *Chloroflexi* groups. Interestingly, a large number of the clones detected in the sponge collected from the wild were affiliated with uncultured *Chloroflexi* (59% of the clone library).

(iv) 16S rRNA gene clones related to *Actinobacteria* were no longer detectable in

sponges maintained for three months in aquaculture and then were detected again in the nine-month sponge. Since *Actinobacteria* associated with marine sponges may be sources of bioactive compounds, the retrieval of actinobacterial diversity may play a role in the production of specific bioactive compounds that are derived from this group.

Both culture-based and molecular techniques showed an increase in the *Bacteroidetes* community in aquaculture with the highest representation in the three-month sponge. Populations affiliated with *Planctomycetes, Alpha-*, *Gamma-* and *Deltaproteobacteria* and *Clostridia* emerged in aquaculture. This indicates that adaptation to aquaculture conditions favored the abundance of these populations. These increasingly large populations may have originally existed in lower abundance in the wild sponge or have been acquired from the surrounding water in the aquaculture system. The diversity of the bacterial community associated with *I. strobilina* increased in aquaculture. Based on the statistical analyses of clone libraries, a higher bacterial diversity was present in the three-month sponge samples than in wild sponges which, is possibly due to the perturbation of the bacterial community associated with the sponge when it was first transferred to aquaculture. On the basis of all five statistical tests used to compare clone libraries at the species level, the bacterial community diversity from the sponge maintained for nine months in aquaculture was intermediate between wild and three-month sponges. This may indicate an acclimation in this bacterial community after the longer nine-month period in aquaculture. Statistical analyses revealed that a significant shift in the population composition in the bacterial communities occurred in aquaculture in

sponges maintained for both three and nine months, compared to that in the wild sponge.

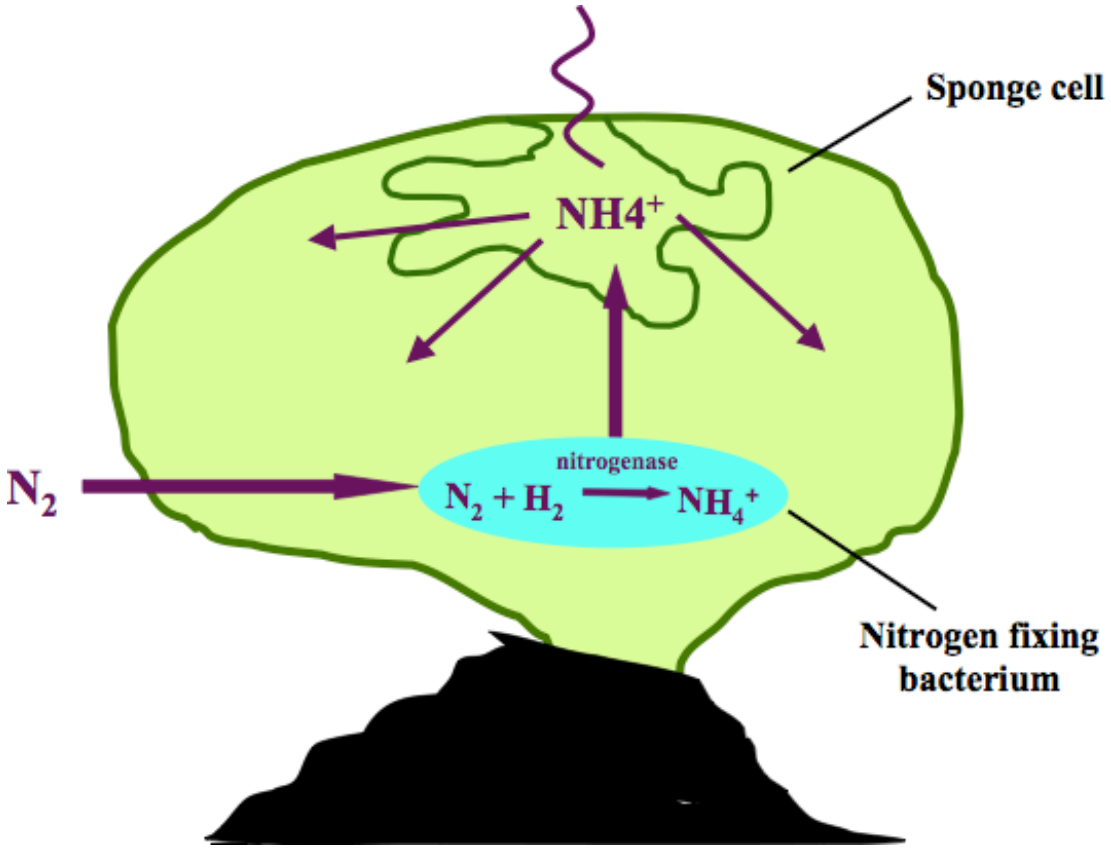
Metabolomics, the study of the non-proteinaceous, endogenously synthesized small molecules present in an organism, is an emerging strategy in drug discovery and development (Dunn and Ellis, 2005; Harrigan and Yates, 2006). The combination of chromatography followed by mass spectrometry allows the separation of individual metabolites and their identification based on mass. The change in the metabolome of an organism can be used to understand what has changed in the system. Applying this tool to my study shows that the environmental stress following the transfer of *I. strobilina* into aquaculture produced no detectable effect on the overall profile of small molecules associated with the sponges. The LC-MS chemical fingerprinting revealed no major changes in the natural product profiles of *I. strobilina* although the composition of bacterial communities changed substantially following transfer into aquaculture. This suggests that bacterial symbionts associated with *I. strobilina* may not be involved in the production of the major metabolites or that these metabolites are produced by a stable bacterial fraction that was maintained in aquaculture. Candidates are members of *Actinobacteria*, *Bacteroidetes* and *Chloroflexi.* In this latter case, the stability of the metabolites in aquaculture may imply that these symbionts constitutively produce essential metabolites.

In conclusion, my findings highlight the importance of monitoring the microbial communities associated with marine sponges when maintaining sponges in aquaculture systems by showing that profound changes may occur in these microbial communities. In this study, concomitant changes in the overall chemical profile of

the sponge were not detected. Additional studies of this type are needed to determine on a case-by-case basis whether changes in sponge-associated microbial communities are linked with changes in overall chemical profiles or specific compounds of interest.

The survival of *I. strobilina* in aquaculture makes it another potential candidate in addition to *M. laxissima*, for the development of sponge model systems to study symbiotic relationships of sponges and associated microorganisms.

Chapter 4. Diversity and Expression of Nitrogen Fixation Genes in Bacterial Symbionts of Marine Sponges



Schematic representation of nitrogen fixation in a sponge where bacterial symbionts fix nitrogen and provide fixed nitrogen to their sponge host.

4.1. Abstract

Marine sponges contain complex assemblages of bacterial symbionts, the roles of which remain largely unknown. Diverse bacterial *nifH* genes were identified within sponges and found that *nifH* genes are expressed in sponges. This is the first demonstration of the expression of any bacterial gene within a sponge. Two sponge species *Ircinia strobilina* and *Mycale laxissima* were collected from Key Largo, Florida. $\delta^{15}N$ values from sponge samples indicated that biological nitrogen fixation was likely a major contributor to the nitrogen budget of *I. strobilina.* Higher $\delta^{15}N$ values for *M. laxissima* suggest that seawater dissolved inorganic nitrogen, dissolved and particulate organic nitrogen, and biological nitrogen fixation may all be important in satisfying the sponge's nitrogen requirement. The potential for nitrogen fixation by symbionts was assessed by amplification of *nifH* gene fragments from total DNA and RNA. Diverse *nifH* genes affiliated with *Proteobacteria* and *Cyanobacteria* were detected, and gene expression studies proved that *nifH* genes similar to those from cyanobacteria were expressed. The *nifH* genes amplified from surrounding seawater were similar to those of *Trichodesmium* and clearly different from the cyanobacterial *nifH* genes detected in the two sponges. This study enhances our understanding of the role of bacterial symbionts in sponges and suggests that the provision of fixed nitrogen is a means by which bacterial symbionts benefit sponges in nutrient-limited coral reef environments. Nitrogen fixation by sponge symbionts is likely an important source of new nitrogen to the entire reef environment that heretofore has been neglected in reef nutrient budgets.

4.2. Introduction

Higher invertebrates are known to benefit from the metabolic capabilities of nitrogen-fixing bacteria. For example, the hindgut of wood-feeding termites is colonized by flagellate protozoa (Honigberg, 1970; Berchthold *et al*., 1999), which facilitate digestion of lignocellulose (Brune, 2003). The carbon-rich but nitrogen-poor nature of the termite diet requires nitrogen from other sources (Breznak, 2000). This is thought to be provided by intracellular nitrogen fixing bacteria associated with termite gut flagellates (Stingl *et al*., 2005).

Interactions of bacteria with plants are the most common symbiotic association for nitrogen assimilation. Diverse bacteria are involved in these associations, including Gram-negative proteobacteria such as *Rhizobia* sp. and *Burkholderia* sp., Gram-positive *Frankia* sp. (Benson and Silvester, 1993) and filamentous or unicellular cyanobacteria (Rai *et al*., 2000). The symbiotic relationship between non-photosynthetic proteobacteria of the order *Rhizobialses* with plants is mutualistic. The rhizobia-legume symbiosis is characterized by typical root- nodule structures of the plant host, which are colonized by the endosymbiotic rhizobia, known as bacteroids (Parniske, 2000). The nodulated plant roots supply the bacteria with carbon compounds and obtain fixed nitrogen from the bacteroids in return. The nodule formation is a highly regulated and complex process driven by both partners. Free-living rhizobia enter the plant root epidermis and stimulate nodule formation by reprogramming root cortical cells. For the establishment of symbiosis, the plant partner secretes flavonoids (Bladergroen and Spaink, 1998) and the subsequent induction of bacterial nodulation (*nod*) genes (Goethals *et al*., 1992). The Nod-

factors play a role in the formation of the nodule, a complex structure optimized for the requirements of both partners (van Brussel *et al*., 1992). In the nodule, bacteroids are localized in membrane bound vesicles. Nitrogenase activity is protected by the spatial separation of the bacteroids inside the nodule structure and special oxygen-scavenging leghemoglobin that is synthesized in the nodules (de Billy *et al*., 1991). The rhizobia-legume symbiosis is not a permanent or obligate relationship since both partners can live and propagate autonomously and each host generation has to be populated by a new strain of free-living rhizobia.

Symbiosis between autotrophic and heterotrophic organisms has been recognized in environments where dissolved nutrients and particulate organic matter are limited such as coral reefs (Muscatine and Porter, 1977). Sponges that grow on photosynthetically derived carbohydrates, which are rich in carbon but devoid of nitrogen (Wilkinson and Fay, 1979; Wilkinson, 1983), may encounter nitrogen limitation. It is therefore appealing to hypothesize that marine sponges provide an ecological niche to nitrogen-fixing bacteria.

Nitrogen fixation was demonstrated in marine sponges in earlier studies. Wilkinson and Fay (1979) did not exclude the possibility of participation of other bacterial symbionts in the observed nitrogenase activity and they suggested that synergistic nitrogen fixation might occur in sponges when cyanobacterial and heterotrophic bacteria are closely associated. Wilkinson *et al.* (1999) showed nitrogen fixation in the Indo-Pacific coral reef sponge *Callyspongia muricina* with incorporation of the $^{15}N_2$ into the amino acids glutamine, glutamate and aspartate. It

is worth mentioning that the authors had variable results when they measured N_2 fixation in 23 sponge species using acetylene reduction assay.

Isotopic nitrogen composition was my method of choice for for detection of nitrogen fixation in sponges since the stable isotope $^{15}N_2$ supplementation and ethylene reduction methods have been problematic in sponges (Wilkinson *et al*. 1999). Compositions are reported in δ-notation as the "permil, ‰" deviation of a sample from a standard according to the following equation:

$$\delta^{15}N = \frac{(R_{sample} - R_{std})}{R_{std}} * 1000$$

R_{sample} = $^{15}N/^{14}N$ ratio in sample and R_{std} = $^{15}N/^{14}N$ ratio in standard

At Conch Reef, there are four potential sources of nitrogen for the sponges. The particulate organic nitrogen that is filtered out by the sponges has an isotopic composition around +4 to +5 ‰. Ambient nitrate is supplied mainly by tidally driven incursions of the Florida Current, and this nitrate is around +5 ‰. Nitrogen in sewage treatment plant effluent is restricted largely to Florida Bay. It has a higher distinctive isotopic composition that would be easily detected. Biological nitrogen fixation (BNF) on the other hand results in biomass that is isotopically light, with values around 0 ‰ or slightly lower. In a comparison of three sponges from the Florida Keys, the two species that had large and diverse microbial communities had low $\delta^{15}N$ values whereas the sponge with a low bacterial biomass had higher $\delta^{15}N$

values, suggesting a microbial input of nitrogen in those sponges containing prolific bacteria (Weisz *et al.*, 2007).

BNF, the reduction of atmospheric nitrogen gas to biologically active ammonium, is catalyzed by the nitrogenase enzyme complex that exists in a limited but phylogenetically diverse distribution of prokaryotes (Postgate, 1982; Young, 1992). Nitrogenase is composed of two proteins: an iron containing dinitrogenase reductase encoded by the *nifH* gene and a molybdenum-iron containing dinitrogenase, composed of alpha and beta subunits encoded by *nifD* and *nifK* genes. The multi-subunit structure coordinates FeS clusters and the MoFe cofactor (Paerl and Zehr, 2000). The dinitrogenase serves as the substrate-binding site, which is reduced by the dinitrogenase reductase through a series of single electron transfers. Some microorganisms have alternative nitrogenases with vanadium instead of molybdenum in the cofactor, which are encoded by *vnfH* genes, or only iron, encoded by *anfH* genes (Paerl and Zehr, 2000). *nifH* is highly conserved among diverse microorganisms and the relationship based on the divergence of this gene resembles the 16S rRNA phylogenetic tree (Hennecke *et al.*, 1985; Young, 1992). It has been extensively used as molecular marker to assess the diversity of nitrogen-fixing bacteria in aquatic and terrestrial environments (Zehr *et al.*, 1998; Zehr *et al.*, 2003; Man-Aharonovich *et al.*, 2007).

Stable isotope compositions have been used to investigate nutritional sources in many marine symbioses (Muscatine and Kaplan, 1994; Kline and Lewin, 1999; Lovell *et al.*, 2001). Nitrogen isotopes provide information on sources and transformation processes of nitrogen in different environments (Montoya *et al.*,

2002). Biomass produced by planktonic autotrophic diazotrophs is isotopically depleted relative to average oceanic combined nitrogen (Carpenter *et al.*, 1997; Montoya *et al.*, 2002). Stable nitrogen isotope analysis was utilized to identify sponges species most likely to harbor nitrogen-fixing symbionts. The diversity and expression of *nifH* genes were then investigated in sponge species targeted by the stable isotope screening.

4.3. Materials and methods

Sample collection. Four different sponge species were colleted from Conch Reef, Key Largo, Florida (latitude 24°57.11′N, longitude 80°27.57′W): *I. strobilina, M. unguifera, M. laxissima* and *X. muta* at a depth of ca. 20 m in summer 2004 and 2005. Water salinity was 36 ppt and temperature was 26-27˚C. The sponges were collected by SCUBA and were frozen immediately. Sponge samples for RNA extraction were stored in RNA*later* RNA stabilization solution (Qiagen, Valencia, CA, USA). Two water samples were collected from the vicinity of the sponges.

Measurement of stable isotope composition. Stable isotope composition was used to demonstrate nitrogen fixation in sponges for two reasons. First, Wilkinson *et al.* (1999) reported technical difficulty and ambiguous results of measuring nitrogen fixation in sponges using acetylene reduction assay. Second, I was unsuccessful in demonstrating nitrogen fixation using a $^{15}N_2$ enrichment approach. *M. unguifera* was used as a model sponge and the experiment was conducted in small jars filled with seawater saturated with $^{15}N_2$. There was no incorporation of the isotopic nitrogen in the sponge tissue, likely because the experiment was conducted in small jars that did

not allow for efficient water pumping. This possibly stressed the sponge and did not allow it to function properly.

Three independent individual sponges were processed for each species from each year. Samples were lyophilized, ground to a fine powder, and homogenized. Samples (ca. 1.0 mg) were packed in tin capsules and analyzed for nitrogen isotope ratios by continuous flow isotope ratio mass spectrometry at the UC Davis Stable Isotope Facility. Samples were combusted to CO_2 and N_2 at 1000 °C in an on-line elemental analyzer (ANCA-GSL, PDZ Europa, Crewe, UK), separated in a He carrier stream on a Carbosieve G column (Supelco, Bellefonte, PA, USA), and introduced to a 20-20 isotope ratio mass spectrometer (PDZ Europa). Nitrogen isotope compositions are reported as $\delta^{15}N$ values relative to atmospheric nitrogen (0.00‰). Sample isotope ratios were compared to a pure N_2 gas reference cylinder, and the system was calibrated using multiple runs of a powdered working standard (ammonium sulfate and sucrose mixture with $\delta^{15}N$ vs. air of 1.33 ‰). The working standard was periodically calibrated against international isotope standards (IAEA N1 and N3).

Genomic DNA extraction. One cm^3 of freeze-dried sponge tissue was ground using a sterile mortar and pestle. DNA was extracted using the bead beater method described in Chapter 2 and Appendix B. Some of the DNA samples were prepared using TissueLyser system (Qiagen) and AllPrep DNA/RNA mini kit (Qiagen).

Recovery of nucleic acids from water samples. DNA was extracted from Sterivex filters using the same protocol described in Chapter 2 and Appendix B.

PCR amplification, cloning, and sequencing of *nifH* genes. An internal

fragment of the nitrogenase (*nifH*) gene (ca. 360 bp) was amplified with a nested PCR experiment. First round nested PCR reactions were performed with HPLC-purified primers nifH32F and nifH623R designed by Steward *et al*. (2004) and the second round PCR reactions were performed with internal HPLC-purified primers nifH1 and nifH2 designed by Zehr and McReynolds (1989). PCR was carried out as described by Steward *et al.* (2004). A detailed protocol is described in Appendix B. Two negative control samples were used to test for false amplification of contaminants because of the nested PCR approach. The first control contained 1 µl PCR water and the second contained 2 µl of the first round PCR control. Amplification products were analyzed by electrophoresis in 1.5% (w/v) agarose gels in 1 × TAE buffer. PCR products of the appropriate size and corresponding positions of the negative controls were gel purified using QIAquick gel extraction kit (Qiagen). Amplification products were analyzed by electrophoresis in 1.5% (w/v) agarose gels in 1 × TAE buffer. PCR products were excised and gel purified using QIAquick gel extraction kit (Qiagen). The corresponding positions of the negative controls were also excised. PCR products were ligated into PCR-XL-TOPO vector and transformed into OneShot TOP10 chemically competent *Escherichia coli* cells using the TOPO XL PCR Cloning Kit (Invitrogen Life Technologies, Carlsbad, CA, USA). Plasmid DNA was isolated from individual clones and purified using Agencourt® SprintPrep® 384 HC kit (Agencourt Bioscience, Beverly, MA, USA). Purified plasmids were sequenced with M13 forward (-20) primer (5' GTTGTAAAACGACGGCCAGT 3'). Nested PCR proved to be more efficient and less affected by sample inhibition compared to single-stage PCR in the case of sponge DNA. *nifH* genes were amplified from seawater

samples in a single stage PCR using HPLC-purified nifH1 and nifH2 primers in contrast to desalted primers (Fig. A.2, Appendix A).

RNA extraction and RT-PCR. Total RNA was extracted from sponge samples collected in 2005 using the TissueLyser system (Qiagen) and the RNeasy® minikit (Qiagen). RNA samples were purified using RNAeasy mini-spin column and DNA was digested by adding RNAase-free DNAase (Qiagen) to RNAeasy mini columns. Reverse transcription reactions were performed using nifH3 primer and ThermoScript™ RT-PCR system (Invitrogen) as described previously by Zani *et al.* (2000). After reverse transcription, *nifH* genes were amplified in cDNA samples using nested PCR. HPLC purified nifH3 and nifH4 (100 μM each) were used in the first round of nested PCR (Zani *et al.*, 2000) and HPLC purified nifH1 and nifH2 primers (100 μM each) were used in the second round PCR (Zehr and McReynolds, 1989). I used the same negative controls mentioned in the previous section plus two more controls. The first additional control contained RT reaction without RNA and the second used a direct nested PCR of the RNA samples without the reverse transcription step. Clone libraries from RT-PCR amplified *nifH* cDNA of *I. strobilina* and *M. laxissima* were constructed as described in the previous section.

Phylogenetic analysis of *nifH* gene clones. *nifH* gene sequences from the clone libraries were edited and the vector sequences were clipped using PreGap4 and Gap4 from the Staden package (http://sourceforge.net/projects/staden/) and analyzed initially using the BLASTn tool to aid the selection of the closest reference sequences. Sequences were imported into ARB (Ludwig *et al.*, 2004), which was used to align homologous regions of the *nifH* gene clones and the nearest relative

matches. This database was supplemented with relevant environmental sequences that had been submitted recently to GenBank. These sequences were selected based on the top BLAST hits of the clone sequences. Multiple alignments were checked manually and improved by the ARB editor tool and the aligned sequences were analyzed by the distance matrix methods available in ARB. Neighbor-joining trees (Jukes-Cantor correction) (Saitou and Nei, 1987) were constructed based on 303-nucleotide alignment positions corresponding to positions 139 to 442 in the *Anabaena variabilis* strain ATCC 29413 *nifH* gene (GenBank accession no. U49859) and the robustness of the inferred tree topologies was evaluated after 100 bootstrap replicates of the neighbor-joining data. Bootstrap values were generated using Phylip (Felsenstein, 2004).

Culturing sponge-associated bacteria carrying *nifH* genes. Forty proteobacterial isolates were screened for the *nifH* gene using the same protocol described for the amplification of the *nifH* genes from sponge total DNA.

I used a nitrogen-free glucose (NFG) medium, used previously to isolate heterotrophic nitrogen-fixing bacteria from sponges (Shieh and Lin, 1994), to enrich for nitrogen-fixing bacteria. Sponge tissue (1 cm^3) was rinsed with sterile seawater and then homogenized in 10 ml of NaCl-Tris buffer (30 g NaCl and 0.24 g Tris in 1 liter of deionized water, pH 8.0) containing 2 ppm Tween 80. The sponge homogenate was serially diluted in NaCl-Tris buffer and 1 ml of each dilution was added to a tube containing 5 ml NFG media. NFG liquid media consisted of 4 parts: (i) basal medium (25 g NaCl, 5 g $MgSO_4.7H_2O$, 10 mg $CaCl_2$, 7.3 mg Na_2MoO_4, 6.1 g Tris and 800 ml deionized water), adjusted to pH 8.0, (ii) 5 g glucose in 100 ml

deionized water, and (iii) 0.2 g K_2HPO_4 dissolved in 50 ml deionized water, and (iv) 13.5 mg $FeCl_3.6H_2O$ dissolved in 50 ml deionized water (Shieh and Lin, 1992). The four parts were autoclaved separately and combined after cooling at room temperature. The tubes were incubated in anaerobic jars at 25°C and air was replaced by nitrogen gas. Growth was measured using the absorbance at 600 nm. After one month, 100 µl of each 10^0 dilution of each of the four sponges were plated in duplicates on NFG agar. The plates were incubated at 25°C under anaerobic and microaerophilic conditions. After several subculturings on NFG agar media, growing colonies were plated on marine agar.

4.4. Results

Nitrogen isotope composition. $\delta^{15}N$ values were determined for four different sponge species of the class Demospongiae (Fig. 4.1). These measured values were used as a first order screen to determine sponges that likely derived a substantial portion of their nitrogen from BNF. BNF results in $\delta^{15}N$ values around 0 to -2 ‰ (Carpenter *et al.*, 1997; Montoya *et al.*, 2002), close to that of atmospheric nitrogen dissolved in sea water [0.6 ‰, (Sigman and Casciotti, 2001)] and significantly lighter than dissolved inorganic nitrogen supplied to the outer Florida Keys reef tract [4 to 5 ‰, (Leichter *et al.*, 2007)]. Low $\delta^{15}N$ values of *I. strobilina* (0.98 ± 0.20 ‰ [1 SD] in 2004 and -0.24 ± 0.65 ‰ in 2005) implicated BNF. For *M. unguifera*, $\delta^{15}N$ values were 3.23 ± 0.43 ‰ in 2004 and 4.08 ± 0.19 ‰ in 2005. For *M. laxissima*, $\delta^{15}N$ values were 3.51 ± 0.61 ‰ in 2004 and 3.61 ± 0.24 ‰ in 2005. For *X. muta*,

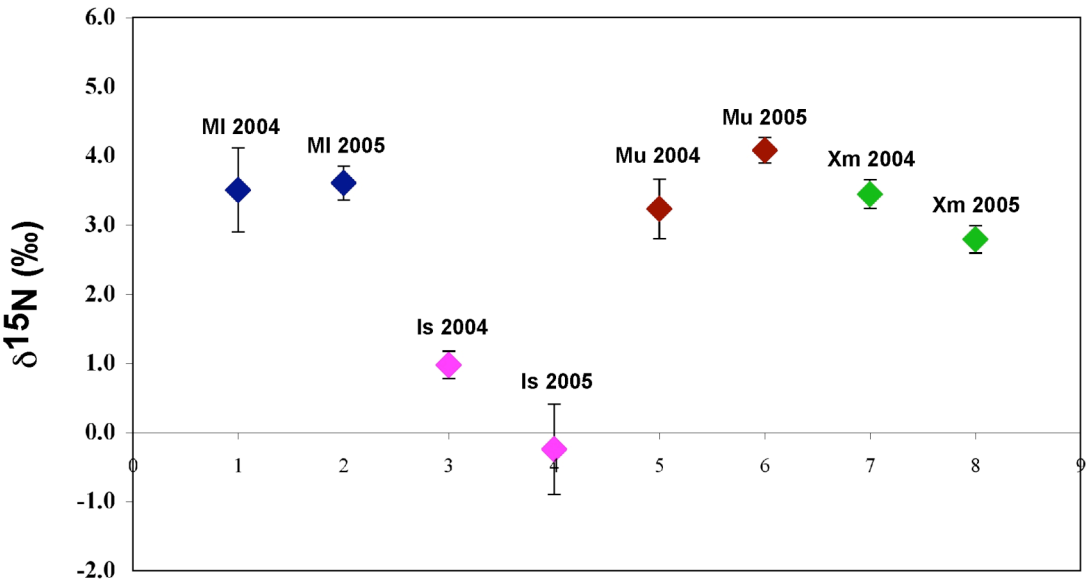


Fig. 4.1. $\delta^{15}N$ values of sponges: *I. strobilina* [Is, in pink], *M. unguifera* [Mu, in red], *M. laxissima* [Ml, in blue] and *X. muta* [Xm, triangle in green]. Each point represents mean (± 1 S.D.) of single analyses on three separate sponge specimens.

$\delta^{15}N$ values were 3.45 ± 0.21 ‰ in 2004 and 2.79 ± 0.20 ‰ in 2005. Based on the $\delta^{15}N$ measurements, I chose *I. strobilina* with an isotopic signal suggestive of BNF and *M. laxissima* as a representative of the sponges with the higher $\delta^{15}N$ values, for a comparative investigation of the diversity and expression of *nifH* genes.

***nifH* gene sequence analysis**. Nitrogenase genes were amplified from marine sponges, *M. laxissima* and *I. strobilina* and surrounding seawater from Key Largo, Florida, using nested PCR and universal primers for the nitrogenase iron protein gene (*nifH*) (Zehr and McReynolds, 1989). The *nifH* gene clone sequences from the sponges and seawater samples were compared to each other and with sequences in the GenBank databases, and their phylogenetic relationships were investigated. Most

nifH gene clone sequences had < 90% identity, and many < 80%, to the nearest environmental clones, underscoring the novelty of sponge-associated *nifH* gene sequences (Table 4.1).

The largest assemblage of *nifH* clones fell into the previously described cluster I, which consists of *nifH* from conventional eubacterial Mo-Fe operons and some *vnfH* (Chien and Zinder, 1996). Cluster I includes *nifH* sequences from *Alpha*-, *Beta*-, and *Gammaproteobacteria*, *Frankia* spp. and *Cyanobacteria*. The sponge-associated bacterial *nifH* sequences fell into ten groups within Cluster I (Fig. 4.2 and Table 4.1). They were similar to *nifH* gene sequences of *Azotobacter chroococum* (group 1), *Methylocystis* sp. (group 2), *Rhizobium* sp. (group 3), *Leptolyngbya* sp. (group 4, 6 and 7), *Myxosarcina* sp. (group 9) and *Tolypothrix* sp. (group 10).

A second, smaller assemblage of *nifH* gene sequences showed phylogenetic relatedness to the anaerobic group of Cluster III (Chien and Zinder, 1996), which includes *nifH* sequences from diverse anaerobes such as *Clostridia* (Gram-positive), sulphate reducers (*Deltaproteobacteria*) and *Methanosarcina barkeri* 227. The Cluster III sponge-associated bacterial *nifH* sequences fell into three major groups similar to *nifH* sequence of *Desulfovibrio vulgaris* (groups 11 and 12) and *Desulfovibrio salexigens* (group 13) (Fig. 4.3 and Table 4.1).

***nifH* diversity in the surrounding seawater.** 16 *nifH* gene clones were recovered from two different seawater samples. They were more than 98% identical to each other and fell into one cluster that was similar to the marine cyanobacterium,

Table 4.1. Closest matches of sponge-associated microbial *nifH* genes on the basis of BLAST analysis.

Group	No. of clones[1]	% Identity[2]	Source	Closest environmental *nifH* sequences and source[3]	% Identity[4]
1	32	94-99%	*I. strobilina,* DNA	Amazon river, clone NAtnifH_05, DQ481454; (*Azotobacter Chroococum*, AY351672)	77-80%
2	5	98-99%	*M. laxissima*, DNA	Gramineous crop, *Stenotrophomonas maltophilia* isolate KNUC170, DQ431165; (*Methylocystis* sp.*,* AF378718)	98-100%
3	29	97-100%	*I. strobilina,* DNA	Sargasso Sea, plankton, meso- to abyssopelagic, clone19216A5, DQ481295; (*Rhizobium* sp., M16710)	98-100%
4	40	87-99%	*M. laxissima*, DNA	Thermal springs, thermophilic cyanobacterium isolate tBTRCCn 24, DQ471425; (*Leptolyngbya* sp.*,* AY768415)	81-86%
5	76	> 95%	*I. strobilina* (45) *& M. laxissima* (31), RNA	Marine Bahamian stromatolites, Highborne Cay, clone HB 0898, AF227937; (*Lyngbya lagerheimii,* L15550)	85-87%
6	3	87-98%	*I. strobilina,* DNA	Marine Bahamian stromatolites, Highborne Cay, clone HBC2C2, AF227932; (*Leptolyngbya* sp.*,* AY768415)	80-88%
7	13	94-99%	*I. strobilina,* DNA	Marine Bahamian stromatolites, Highborne Cay, clone HBC2C2, AF227932; (*Leptolyngbya* sp.*,* AY768415)	84-89%
8	8	> 98%	*I. strobilina,* RNA	*Myxosarcina* sp., U73133	>98%
9	8	82-99%	*M. laxissima*, DNA	Great Barrier Reef, Heron Reef Lagoon, clone 20107A08, EF174708; (*Myxosarcina* sp., U73133)	82-98%
10	18	95-99%	*I. strobilina,* DNA	*Tolypothrix* sp., AY768421	96-97%
11	13	> 99%	*M. laxissima*, DNA	Wood feeding termite *Microcerotermes crassus* gut, clone McE13, AB273246; (*Desulfovibrio Vulgaris*, AY040514).	78%
12	16	96-98%	*I. strobilina,* DNA	Eastern Mediterranean Sea, clone JUL_H01_DNA_B07, EF568471; (*Desulfovibrio Vulgaris*, AY040514).	76-77%
13	14	> 97%	*I. strobilina,* DNA	Great Barrier Reef, Heron Reef Lagoon, clone 20125A28, EF174836; (*Desulfovibrio salexigens,* AF227926)	81-82%
SW	16	> 98%	Seawater, DNA	*Trichodesmium thiebautii,* U23507	96-97%

[1] No of clones in each group.

[2] Percent identity of clones in each group to each other.

[3] The nearest cultured relative is given in parenthesis in cases where the closest relative is an environmental clone.

[4] Percent identity of clones in each group to the closest *nifH* sequenece in the GenBank database.

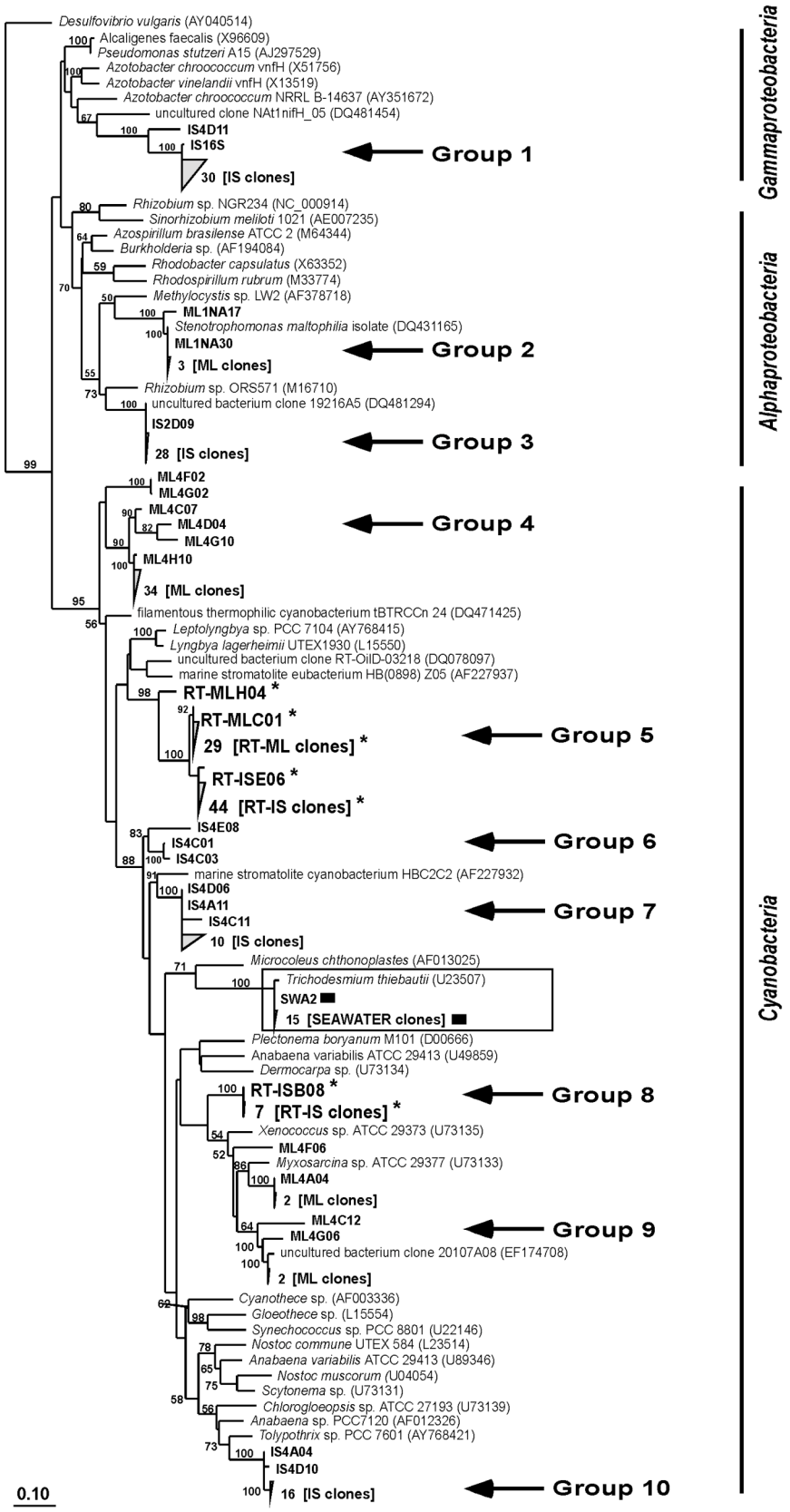

Desulfovibrio vulgaris (AY040514)
Alcaligenes faecalis (X96609)
Pseudomonas stutzeri A15 (AJ297529)
Azotobacter chroococcum vnfH (X51756)
Azotobacter vinelandii vnfH (X13519)
Azotobacter chroococcum NRRL B-14637 (AY351672)
uncultured clone NAt1nifH_05 (DQ481454)
IS4D11
IS16S
30 [IS clones]
Group 1
Gammaproteobacteria
Rhizobium sp. NGR234 (NC_000914)
Sinorhizobium meliloti 1021 (AE007235)
Azospirillum brasilense ATCC 2 (M64344)
Burkholderia sp. (AF194084)
Rhodobacter capsulatus (X63352)
Rhodospirillum rubrum (M33774)
Methylocystis sp. LW2 (AF378718)
ML1NA17
Stenotrophomonas maltophilia isolate (DQ431165)
ML1NA30
3 [ML clones]
Group 2
Rhizobium sp. ORS571 (M16710)
uncultured bacterium clone 19216A5 (DQ481294)
IS2D09
28 [IS clones]
Group 3
Alphaproteobacteria
ML4F02
ML4G02
ML4C07
ML4D04
ML4G10
ML4H10
34 [ML clones]
Group 4
filamentous thermophilic cyanobacterium tBTRCCn 24 (DQ471425)
Leptolyngbya sp. PCC 7104 (AY768415)
Lyngbya lagerheimii UTEX1930 (L15550)
uncultured bacterium clone RT-OilD-03218 (DQ078097)
marine stromatolite eubacterium HB(0898) Z05 (AF227937)
RT-MLH04 *
RT-MLC01 *
29 [RT-ML clones] *
Group 5
RT-ISE06 *
44 [RT-IS clones] *
IS4E08
IS4C01
IS4C03
Group 6
marine stromatolite cyanobacterium HBC2C2 (AF227932)
IS4D06
IS4A11
IS4C11
10 [IS clones]
Group 7
Microcoleus chthonoplastes (AF013025)
Trichodesmium thiebautii (U23507)
SWA2
15 [SEAWATER clones]
Plectonema boryanum M101 (D00666)
Anabaena variabilis ATCC 29413 (U49859)
Dermocarpa sp. (U73134)
RT-ISB08 *
7 [RT-IS clones] *
Group 8
Xenococcus sp. ATCC 29373 (U73135)
ML4F06
Myxosarcina sp. ATCC 29377 (U73133)
ML4A04
2 [ML clones]
ML4C12
ML4G06
uncultured bacterium clone 20107A08 (EF174708)
2 [ML clones]
Group 9
Cyanothece sp. (AF003336)
Gloeothece sp. (L15554)
Synechococcus sp. PCC 8801 (U22146)
Nostoc commune UTEX 584 (L23514)
Anabaena variabilis ATCC 29413 (U89346)
Nostoc muscorum (U04054)
Scytonema sp. (U73131)
Chlorogloeopsis sp. ATCC 27193 (U73139)
Anabaena sp. PCC7120 (AF012326)
Tolypothrix sp. PCC 7601 (AY768421)
IS4A04
IS4D10
16 [IS clones]
Group 10
Cyanobacteria
0.10

Fig. 4.2. Phylogenetic tree of Cluster I *nifH* gene (includes *nifH* sequences from *Alpha*-, *Beta*- and *Gammaproteobacteria*, *Frankia* spp., and *Cyanobacteria*) showing the relative positions of the major groups of *nifH* gene clones retrieved from the microbial communities of *M. laxissima* [ML, in bold], *I. strobilina* [IS, in bold] and seawater [SW, boxed]. The depth and width of polygons reflect the branching lengths of the clones within the groups. The number and origin of clones within each polygon is shown in bold. "*" Symbol indicates RT-PCR clones recovered from the mRNA in the microbial communities of sponges. The tree was inferred using the neighbor-joining method implemented in ARB based on 303-nucleotide alignment positions corresponding to positions 139 to 442 in the *A. variabilis* strain ATCC 29413 *nifH* gene (GenBank accession no. U49859). Bootstrap values above 50 from 100 resamplings are shown at each node. The outgroup used to root the tree was *Desulfovibrio vulgaris* (GenBank accession no. AY040514). The scale indicates the number of nucleotide substitutions per site.

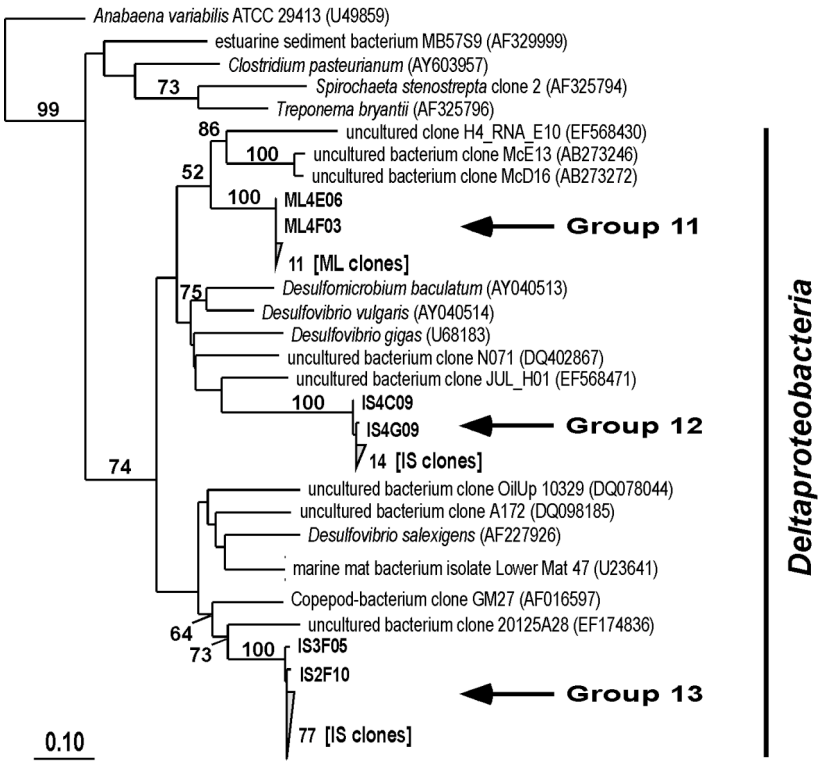


Fig. 4.3. Phylogenetic tree of Cluster III *nifH* gene [includes *nifH* sequences from diverse anaerobic bacteria such as *Clostridia* (Gram-positive), sulphate reducers (*Deltaproteobacteria*) *and Methanosarcina barkeri*] showing the relative positions of the major groups of *nifH* gene clones retrieved from the microbial communities of *M. laxissima* [ML, in bold], *I. strobilina* [IS, in bold]. The depths and widths of polygons reflect the branching lengths of the clones within the groups. The number and origin of clones within each polygon is shown in bold. The tree was inferred using neighbor-joining method implemented in ARB based on 303-nucleotide alignment positions corresponding to positions 139 to 442 in the *Anabaena variabilis* strain ATCC 29413 *nifH* gene (GenBank accession no. U49859). Bootstrap values above 50 from 100 resamplings are shown at each node. The outgroup used to root the tree was *A. variabilis*. The scale indicates the number of nucleotide substitutions per site.

Trichodesmium thiebautii (Fig. 4.2 and Table 4.1).

***nifH* gene expression.** Transcribed *nifH* mRNA in the microbial communities was analyzed using reverse transcriptase PCR (RT-PCR). No amplification was detected in the RNA sample of *M. laxissima* without the RT reaction step (Fig. 4.4). Although all RNA preparations were treated with RNAase-free DNAase, there was a faint band in the RNA sample of *I. strobilina*, which might indicate slight DNA contamination of this RNA preparation. However, the thickness and the size of the cDNA band suggest that *nifH* genes were mainly amplified from *nifH* mRNA of *I. strobilina*. In addition, *nifH* gene clones recovered from cDNA of *I. strobilina* (Groups 5 and 8) were distant from those amplified from DNA (Groups 5, 7, and 10), which further confirms that the source of these clones is cDNA and not contaminating DNA in the RNA preparation of *I. strobilina*. Therefore, RT-PCR could be used specifically to detect *nifH* transcripts in the total RNA pool extracted from sponges. Clone libraries were constructed from *nifH* cDNA amplified by RT-PCR from total RNA isolated from *M. laxissima* and *I. strobilina*. I recovered 84 clones from the RT-PCR clone libraries that clustered into two main groups (Groups 5 and 8) (Fig. 4.2). Group 5 contained *nifH* gene clones from both *M. laxissima* and *I. strobilina* similar to *nifH* sequence of *Lyngbya lagerheimii.* Group 8 contained 8 clones from *I. strobilina* similar to *nifH* sequence of *Myxosarcina* sp.

Culturing sponge-associated bacteria carrying *nifH* genes. A collection of 40 strains cultured from sponges were screened for the presence of *nifH* and two of 40 strains contained *nifH* genes, both closely related to *nifH* genes of *Stenotrophomonas maltophilia* KNUC170, DQ431165 (Fig. 4.5). Both isolates fell in group 2,

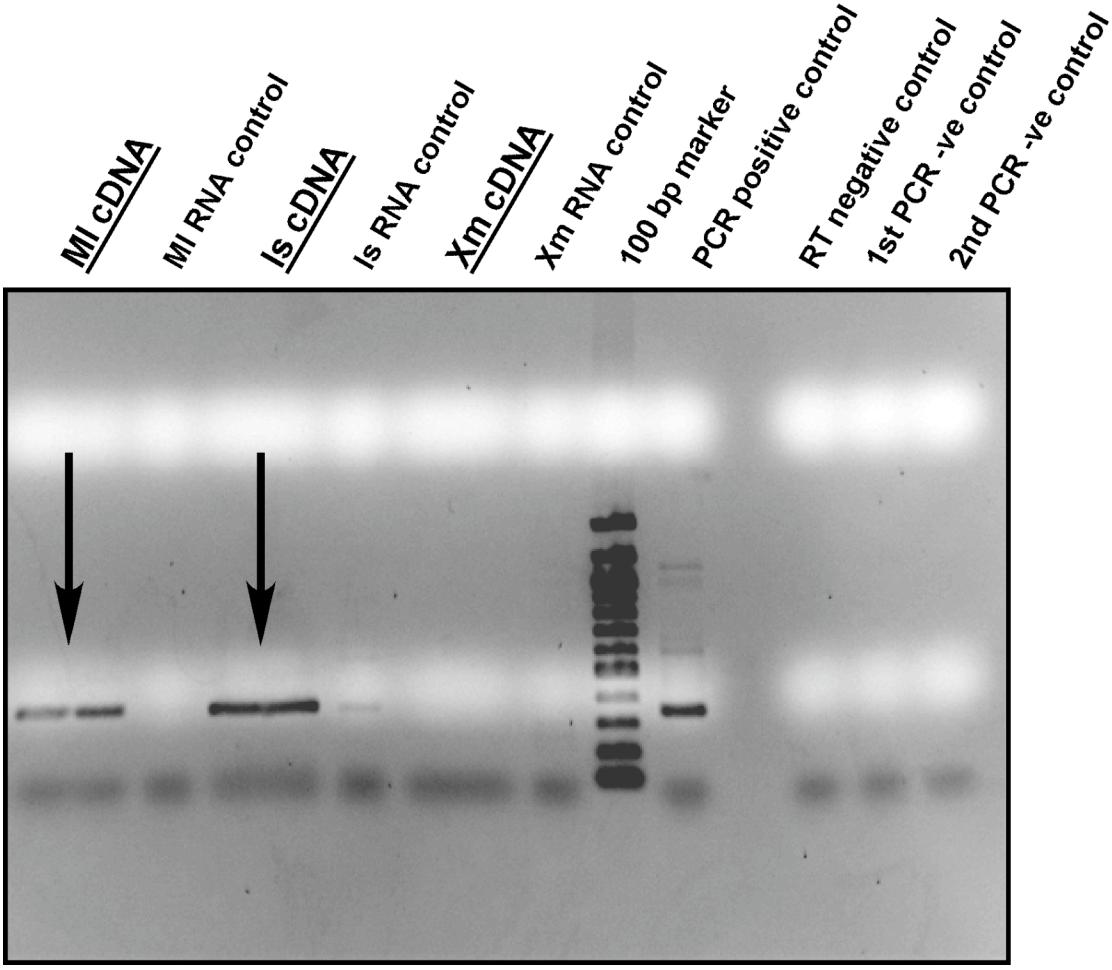


Fig. 4.4. RT-PCR of sponge samples: *M. laxissima* [Ml cDNA], *I. strobilina* [Is cDNA] and *X. muta* [Xm cDNA]. Direct nested PCR was performed for each sponge RNA sample to test for DNA contamination [RNA control]. PCR positive control was *Sinorhizobium meliloti* strain 1021 DNA. RT and PCR negative controls included RT control (amplification of *nifH* genes in RNA samples without the RT reaction step**)**, first round nested PCR control and second round nested PCR control (PCR reaction containing no DNA). Dark arrows refer to the bands corresponding to the cDNA *nifH* PCR products of *M. laxissima* and *I. strobilina* that were used to construct the clone libraries.

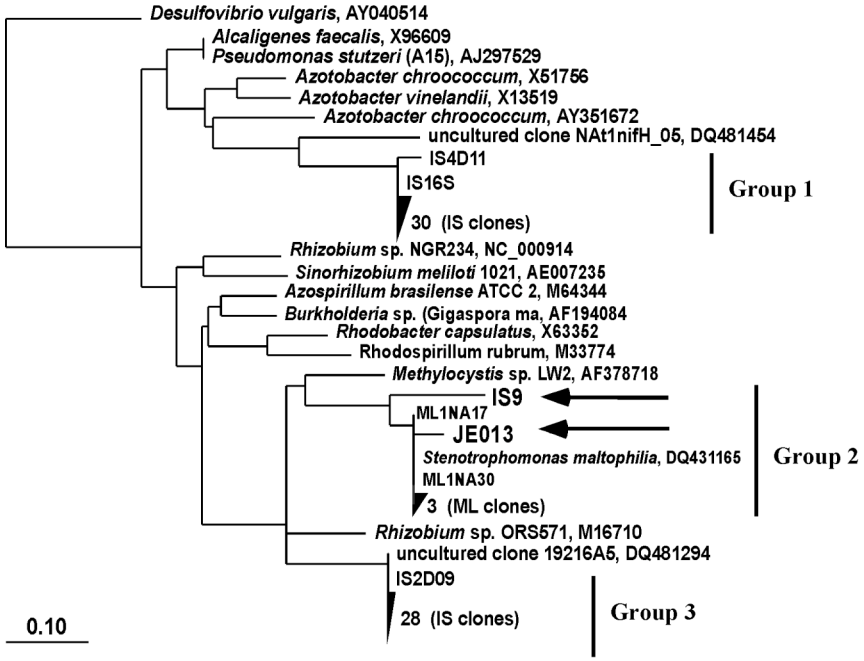


Fig. 4.5. Phylogenetic tree of groups 1, 2 and 3 of Cluster I *nifH* genes (Fig. 4.2). The arrows indicate the two nitrogen fixing *Proteobacteria*, JE013 and IS9.

Cluster I (Fig. 4.2). Isolate JE013 is an alphaproteobacterium closely related to the ubiquitous sponge isolate NW001, which is in turn closely related to *Pseudovibrio denitrificans* (Webster and Hill, 2001). Isolate IS9 is a gammaproteobacterium isolated from *I. strobilina* closely related to *Shewanella* sp. on the basis of 16S rRNA gene sequence analysis. There was some growth of bacteria in the NFG liquid media indicated by increasing absorbance at 600 nm. Two pure isolates were obtained and identified as *Halomonas* and *Citrobacter* sp. based on 16S rRNA gene analysis.

4.5. Discussion

Bioavailable nitrogen concentrations in coral reef environments are very low

(Wilkinson and Fay, 1979). Nitrogen has been found as the primary limiting nutrient in the marine environment (Thomas, 1971; Capone and Carpenter, 1982). Nitrate and ammonia concentrations were measured in the Florida Keys National Marine Sanctuary, including Conch Reef, between March 1995 and September 2002. Median NO_3^- and NH_4^+ concentrations were 0.09, 0.30, respectively. NH_4^+ was the dominant DIN species in most of the samples (~70%). DIN comprised a small fraction (4%) of the total nitrogen pool with total organic nitrogen making up the bulk (median 10.3 μM) (Boyer and Jones, 2003).

Biological nitrogen fixation by sponge symbionts would provide a competitive advantage to host sponges in nitrogen-stressed ecosystems, and such a relationship (commensalism or mutualism) could prove to be a quantitatively important source of fixed nitrogen for reef ecosystems. Sponges in which BNF supplies a significant portion of the fixed nitrogen demand should have a characteristic nitrogen isotope composition. Isotopic analyses on *Trichodesmium* colonies actively engaged in nitrogen fixation consistently exhibit $\delta^{15}N$ values between 0 to -2 ‰ [e.g., (Carpenter *et al.*, 1997; Montoya *et al.*, 2002)], bracketing the range of isotopic compositions typically assumed for marine BNF (Sigman and Casciotti, 2001). However, the nitrogen cycle within sponges can include a variety of chemical transformations, which exhibit a range of isotopic fractionations (Sigman and Casciotti, 2001). Alternate combinations of processes could result in sponge $\delta^{15}N$ values that are similar to that produced by BNF, e.g., preferential uptake of isotopically light nitrate from the ambient water (Montoya *et al.*, 2002), preferential remineralization of isotopically light nitrogen from DON and PON sources (Macko and Estep, 1984), and

additional fractionation of ammonium through preferential uptake of isotopically light ammonium from the remineralized pool (Hoch *et al.*, 1992). I therefore used $\delta^{15}N$ values as a means to identify sponges with isotopic compositions consistent with a BNF source, an approach also adopted recently by Weisz *et al.* (2007).

The replicate analyses on each sponge species showed consistent results for the three specimens collected during each season. Results from the 2004 and 2005 samples were also consistent for a single species. These results indicate that overall nitrogen metabolism for each sponge species and its associated microbial community is a robust characteristic of individual sponges.

I. strobilina was distinct among the species that were sampled in possessing $\delta^{15}N$ values that are consistent with BNF as a major source of nitrogen. The mean $\delta^{15}N$ values for *I. strobilina* in 2004 and 2005 (0.98 ‰ and -0.24 ‰, respectively) were lower compared to $\delta^{15}N$ of *M. unguifera, M. laxissima,* and *X. muta* ($\delta^{15}N$ values within the range from 2.79 to 4.05 ‰). These three sponges had $\delta^{15}N$ values that approach the 4 to 5 ‰ values typical of the nitrate supplied to the mid and outer Florida shelf from the Florida Current (Leichter *et al.*, 2003; Leichter *et al.*, 2007). The nitrogen isotope compositions of these three sponge species could be slightly influenced by nitrogen fixation, but not to the same extent as with *I. strobilina.* A recent study by Weisz *et al.* (2007) also reports nitrogen isotope compositions from sponges in the Florida Keys including specimens collected at Conch Reef. The one sponge they analyzed from the genus *Ircinia* (*I. felix)* gave the lowest nitrogen isotope compositions of any sponges at each of the two sites they analyzed. If these nitrogen isotope compositions represent BNF in both *Ircinia* species, then this suggests that

nitrogen metabolism of a sponge and its associated microbial community could be uniform at the sponge genus level.

Here, I present the first evidence of the presence and expression of nitrogenase genes in marine sponges, *I. strobilina* and *M. laxissima*. The detected sequences clustered among those of *Alpha*- and *Gammaproteobacteria*, *Cyanobacteria* and *Desulfovibrio* spp. (Figs. 4.2 and 4.3). All the sequences were from the previously described cluster I and cluster III (Chien and Zinder, 1996). Obtaining diverse *nifH* sequences suggests that the low $\delta^{15}N$ values for *I. strobilina* (mean value < 1.0 ‰) and even *M. laxissima* (mean value < 4.0‰) are the result of nitrogenase activity within the sponges. The *nifH* sequences obtained in this study clustered more closely to each other and had the greatest similarity to unidentified bacterial *nifH* sequences amplified from environmental samples. The low identity scores of the sponge symbiont *nifH* sequences with well-described and known *nifH* sequences (generally < 90% identity) indicate the novelty of these sequences. This suggests that the sponge-associated nitrogen-fixing bacteria are clearly different than nitrogen-fixing bacteria in other environments. The *nifH* sequences from a single sponge species formed clusters that did not overlap with sequences from the other species. Thus it appears that *nifH* genes derive from diazotrophic communities that are host specific and are not generally distributed throughout different species collected from the same site.

Diverse *nifH* genes detected in sponges were closely related to cyanobacterial *nifH* genes mainly those of *Lyngbya* spp. Marine cyanobacteria are producers of secondary metabolites such as antiproliferative, cytotoxic and neurotoxic compounds.

Filamentous cyanobacteria such as *Lyngbya* sp. have been prolific sources of novel natural products with therapeutic and biotechnological potential. It was hypothesized that many sponge metabolites are derived from metabolic processes of associated symbiotic cyanobacteria (Ramaswamy *et al.,* 2006).

Nitrogen fixation is an energy intensive process. It is estimated that nitrogen-fixing bacteria require at least 1 g of glucose to fix 10-20 mg of molecular nitrogen (Postgate, 1982). For this reason, heterotrophic nitrogen-fixing bacteria inhabiting marine environments would not be active under free-living conditions because of the limited organic carbon availability. According to several reports, the restriction of heterotrophic nitrogen fixation in marine ecosystems to sediments is due to insufficient organic substrate in the water column (Maruyama *et al.*, 1974; Herbert, 1975; Zuberer and Silver, 1978). Developing symbiotic relationships with sponges would provide substrate and energy to drive nitrogenase activity.

The presence of anaerobic *nifH* genes in sponges is consistent with respiratory depletion of oxygen in the water pumped through sponge tissue, leading to anaerobic environments in the interior tissues. Anaerobic bacterial metabolic activity has been demonstrated previously in sponges (Hoffmann *et al.*, 2005) and anaerobic *nifH* sequences have been found in other invertebrates such as copepods (Zehr *et al.*, 1998) and termite guts (Braun *et al.*, 1999). Sequences from Cluster II, which includes non-Mo, non-V *anfH* genes as well as nitrogenases from some archaea were not detected in mRNA and genomic DNA of sponges' microbial communities. Since I used universal degenerate primers that are commonly used to amplify bacterial as well archaeal *nifH* genes, this suggests a relative low abundance or absence of

diazotrophic archaea in sponges.

In this study, I examined *nifH* gene expression and detected active *nifH* transcribing populations in marine sponges. Transcribed *nifH* mRNA in the microbial communities was analyzed using RT-PCR. Sequences recovered from RNA by RT-PCR yielded two phylogenetic groups that were similar to cyanobacterial *nifH* genes (group 5 and 8). The domination of *nifH* expression by cyanobacterial *nifH* genes suggests that nitrogen-fixing conditions existed for cyanobacterial symbionts at the time of sampling (daytime). The similarity of *nifH* mRNA sequences recovered from *M. laxissima* to those recovered from *I. strobilina* in group 5 suggests the presence of one group of specialized cyanobacterial symbionts that are responsible for nitrogen fixation in both sponges. The genotype detected in group 5 was not observed in *nifH* PCR clone libraries from DNA samples, suggesting that these cells were present at low concentration compared to other diazotrophs. Differences between DNA and RNA derived *nifH* gene clones have been reported previously in studies of *nifH* genes in cyanobacterial mats from Guerrero Negro in Mexico (Omoregie *et al.*, 2004), the eastern Mediterranean Sea (Man-Aharonovich *et al.*, 2007) and Lake George in New York (Zani *et al.*, 2000).

Nitrogen isotope compositions indicate that BNF is likely a more significant contributor to the nitrogen budget of *I. strobilina* than to *M. laxissima*. However, the *nifH* gene expression study showed that cyanobacterial *nifH* genes were expressed in both sponges. Heavier nitrogen isotope compositions do not rule out the possibility of nitrogen fixation in *M. laxissima*. The redox cycling of nitrogen in sponges is complex, and a variety of nitrogen transformations has been observed.

Remineralization of nitrogen rich particulate organic matter or the removal of nitrate by denitrification may more heavily influence the nitrogen isotopic composition in *Mycale*. Interestingly alphaproteobacteria, closely related to *Pseudovibrio denitrificans* on the basis of 16S rRNA, have been isolated from *M. laxissima.* They also have been shown to be vertically transmitted via the larvae (Enticknap *et al*., 2006).

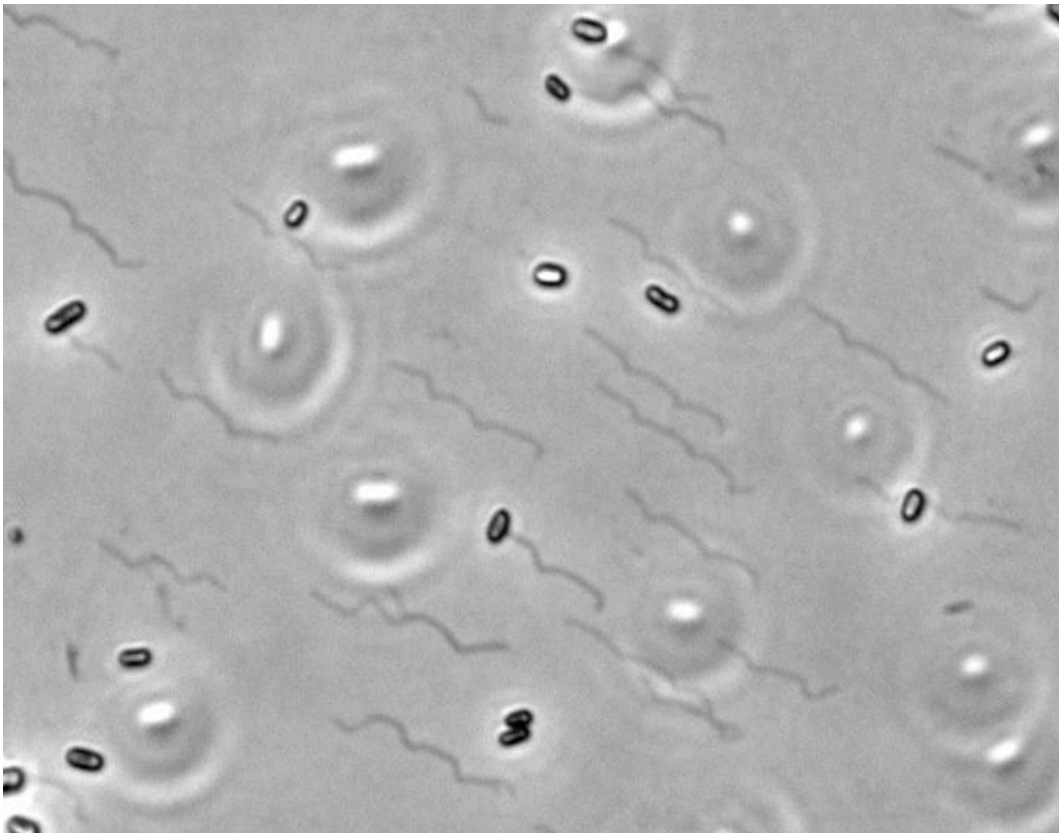
Since sponges are efficient filter feeders, the possibility exists that *nifH* genes recovered from the sponge-hosted community could include genes from planktonic nitrogen-fixing bacteria trapped from the ambient water. The diversity of *nifH* genes was investigated in two seawater samples collected from the same site as sponges. The *nifH* sequences obtained from seawater were distinct from those from the two sponges, suggesting that the *nifH* gene sequences found in sponge samples are derived from symbiotic bacteria within the sponges rather than bacteria filtered out of the water column.

Two of the forty examined proteobacterial isolates carried *nifH*. One approach to identify culturable bacteria carrying *nifH* is simply to continue this screening effort. However, enrichment experiments targeting nitrogen-fixing bacteria should be the primary approach. My results showed that NFG media supported growth of nitrogen-fixing bacteria as indicated by increasing absorbance at 600 nm. Two pure isolates were obtained and identified as *Halomonas* and *Citrobacter* spp. Although intial screening was negative for *nifH* in these strains, nitrogen fixation should be further investigated by standard acetylene reduction assays. Culturing nitrogen-fixing cyanobacteria is important and comparing their *nifH* gene sequences with those

obtained from clones would reveal whether isolates include representatives carrying the major groups of *nifH* genes found in the analysis of cloned *nifH* gene sequences.

In conclusion, phylogenetic analysis of *nifH* sequences obtained by PCR and RT-PCR showed that nitrogen-fixing bacteria were present within sponges and were expressing *nifH* genes. The abundance and diversity of cyanobacterial *nifH* genes and transcripts indicate that cyanobacterial symbionts are an important group responsible for nitrogen fixation in *I. strobilina* and *M. laxissima*. Since sponges have significant coverage and constitute a major portion of the biomass of many coral reefs, sponge associated nitrogen fixation likely contributes significantly to the nitrogen budget of coral reefs. My work provides the first evidence for the expression of a bacterial gene within sponges. It also reveals on the genomic level that nitrogen fixation is a metabolic capability of sponge symbionts, playing an important role in the complex symbioses of sponge-hosted microbial communities.

Chapter 5. Diversity and Quorum Sensing Signal Production of *Proteobacteria* Associated with Marine Sponges



Alphaproteobacterium strain KLH11 isolated from the marine sponge *M. laxissima*. Strain KLH11 produces acyl homoserine lactone signaling molecules. Kindly provided by Elisha Cicirelli.

5.1. *Abstract*

Marine sponges are hosts to diverse and dense bacterial communities and thus provide a potential environment for quorum sensing. Quorum sensing, a key factor in cell-cell communication and bacterial colonization of higher animals, might be involved in the symbiotic interactions between bacteria and their sponge hosts. Given that marine *Proteobacteria* are known to produce *N*-acyl homoserine lactone (AHL) signal molecules, the production of AHLs by *Alpha*- and *Gammaproteobacteria* isolated from marine sponges *M. laxissima* and *I. strobilina* and the surrounding water column was tested. Three different AHL biodetection systems were used in diffusion assays: *Chromobacterium violaceum, Agrobacterium tumefaciens* and *Sinorhizobium meliloti* with optimal sensitivity to short chain (C4-C6), moderate chain (C8-C12) and long chain (≥ C14) AHLs, respectively. Thirteen of 23 isolates from *M. laxissima* and five of 25 isolates from *I. strobilina* were found to produce AHLs. Signals were detected from two of eight proteobacterial strains from the water column. Thin-layer chromatographic assays based on the *A. tumefaciens* reporter system were utilized to determine the AHL profiles of the positive isolates. The types and amounts of AHLs synthesized varied considerably among the strains. Small ribosomal rRNA gene sequencing revealed that the AHL-producing alphaproteobacterial isolates were mainly from the *Silicibacter-Ruegeria* subgroup of the *Roseobacter* clade. Two dimensional gel electrophoresis (2DGE)-based proteomic analyses were congruent with phylogenetic relationships but provided higher resolution to differentiate these closely related AHL-producing strains.

5.2. Introduction

Quorum sensing allows bacteria to communicate and regulate gene expression in a population density dependent manner through the accumulation of signals, which are often diffusible (Fuqua and Greenberg, 2002); (Waters and Bassler, 2005). Quorum sensing, historically called autoinduction, was discovered from studies of the symbiotic association between the bioluminescent marine heterotrophic *Gammaproteobacterium*, *Vibrio fischeri* and certain marine fishes and squids (Nealson *et al.*, 1970; Eberhard *et al.*, 1981).

The *V. fischeri* autoinducer was purified and chemically identified in 1981 as 3-oxo-*N*-(tetrahydro-2-oxo-3-furanyl) hexanamide, or *N*-3-(oxohexanoyl) homoserine lactone (3-oxo-C6-HSL) (Eberhard *et al.*, 1981). The nomenclature of acyl HSLs follows the following scheme: (i) the prefix denotes the substituent at the third carbon, 3-oxo for an oxygen, 3-OH for a hydroxy, and no prefix for fully reduced, (ii) C followed by the length of the acyl chain, and (iii) HSL for homoserine lactone. Unsaturated bonds in the acyl chain are indicated as Δ, followed by the first bonded position in the chain.

V. fischeri has provided a general model for acyl HSL-type quorum sensing particularly through studies of its symbiosis with the Hawaiian bobtail squid, *Euprymna scolopes* (Visick *et al.*, 2000). In 1983, the *V. fischeri* bioluminescence (*lux*) genes were isolated and expressed in *E. coli*, which was not only bioluminescent, but exhibited cell density-dependent regulation (Engebrecht *et al.*, 1983). Molecular genetic studies of the cloned *lux* locus indicated the presence of two genes, designated as *luxR* and *luxI* (Fuqua, 2000). These genes are necessary for

the autoinduction of bioluminescence. The *luxR* gene is essential for response to 3-oxo-C6-HSL and encodes a transcriptional regulates that activates expression of the *lux* operon. The *luxI* gene is required for 3-oxo-C6-HSL synthesis. The *lux* operon starts with *luxI* followed by the structural genes responsible for bioluminescence (*luxICDABEG*). *luxR* is physically linked to *luxI* and it is expressed divergently from the *lux* operon.

At low cell densities as in seawater, a small amount of 3-oxo-C6-HSL is synthesized by limiting pools of LuxI. The signal molecule does not accumulate due to the passive diffusion across the bacterial envelope. Colonization of the light organ provides *V. fischeri* with a nutrient-rich environment. As the bacterium multiplies and increases in number, the relative concentration of 3-oxo-C6-HSL increases as well. At a specific threshold concentration, the signal binds to the LuxR receptor protein, presumably resulting in conformational change that results in LuxR-dependent activation of the *lux* operon promoter and this leads immediately to an increase of bioluminescence (Fig. 5.1). Elevated transcription of the *luxI* gene causes an increase in 3-oxo-C6-HSL synthesis in a positive feedback loop (Fuqua, 2000).

Common quorum-sensing signals among the gram-negative *Proteobacteria* are the *N*-acyl homoserine lactones (AHLs). Synthesis of AHLs, with a range of acyl chain lengths from C4 to C18, and with different chemistries at the third carbon of this chain (3-oxo, 3-hydroxy, or fully reduced), has been reported in diverse *Proteobacteria*. Likewise, many of these bacteria also encode homologues of *luxI* and *luxR* and are known to employ quorum sensing gene regulation (Gray and Garey, 2001).

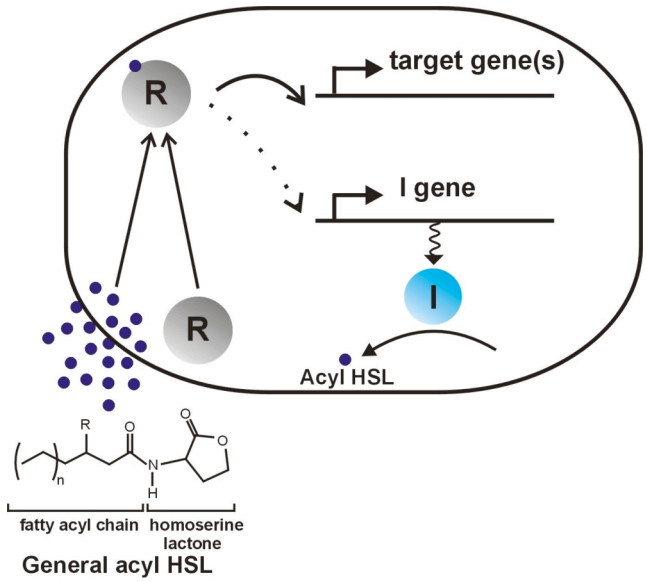


Fig. 5.1. General model for acyl HSL quorum sensing. LuxR-type protein and LuxI protein are represented by spheres labeled as R and I, respectively. The acyl HSL is represented as a filled circle. Filled arrows indicate noncovalent association and catalysis. The dashed arrow indicates that the activation of I gene expression is variable between different bacteria. Squiggles indicate expression and translation of I gene. The acyl HSL present in the figure is a general structure, R can be H, OH, or O, and N equals the number of carbons 0-10 (Fuqua, 2000). This figure was used without modification from Fuqua, 2000.

Despite the intense study of AHL signaling in *V. fischeri* and related marine vibrios, there is only limited information on AHL-dependent regulation in other marine bacteria. Recently, a mixture of long and short chain AHLs were chemically characterized for a collection of marine alphaproteobacterial isolates (Wagner-Döbler *et al.*, 2005). Gram and colleagues (2002) found that three *Roseobacter* isolates from marine snow produced compounds capable of activating an AHL-responsive

biological reporter. In a separate study, the cultivation efficiency of heterotrophic bacteria from the Baltic Sea was enhanced on addition of cyclic AMP and AHLs, suggesting a broad role in adaptation of marine bacteria to conditions in laboratory culture (Bruns *et al.*, 2002). AHLs can also affect the behavior of marine eukaryotes. For example, bacterial biofilms that produce AHLs stimulate the settlement of motile zoospores of the marine alga *Ulva intestinalis*, and the inhibition of AHL signaling can block this settlement (Joint *et al.*, 2002).

The cell-cell communication afforded by AHLs often comes into play in the bacterial colonization of metazoan organisms (Passador *et al.*, 1993; Zhang *et al.*, 1993; Ruby, 1996; Fuqua *et al.*, 2001). Many pathogens utilize AHLs to modulate specific aspects of infection. Likewise, symbiotic microbes often utilize AHL quorum sensing. AHLs have an important role in the development of symbiotic root nodules on leguminous plants by rhizobia (Oldroyd *et al.*, 2005). Based on these and many similar examples, it is appealing to hypothesize that AHLs produced by sponge-associated bacteria might be involved in regulating symbiotic interactions with their hosts. Quorum sensing might regulate colonization of the sponge by the bacteria or some of the important interactions among sponge microbiota, such as the production of bioactive compounds. In a small-scale screen of several marine invertebrates, Taylor *et al*. (2004) reported production of AHLs by sponge-associated bacteria. One AHL-producing bacterial isolate from the sponge *Cymbastela concentrica* collected off of the coast of southeastern Australia was found to be a member of the *Roseobacter-Ruegeria* marine bacterial subgroup.

The first objective of this study was to survey the production of AHLs by *Proteobacteria* from the shallow water marine sponges, *M. laxissima and I. strobilina.* The second objective was to determine the phylogeny of AHL-producing bacteria utilizing 16S rRNA gene sequencing. Finally, a proteomics approach was used to investigate fine-scale diversity among more closely related AHL-producing proteobacterial isolates.

Proteomics is defined as the analysis of microbial proteome. Proteome is the total protein complement able to be encoded by a given genome (Wasinger *et al.*, 1995). It is a technique that documents and analyses the proteins expressed in biological samples. Proteomic analysis includes the classic two-dimensional gel electrophoresis (2DGE)-based approach and non-gel based approach. In 2DGE, proteins are separated based on their isoelectric points (pI) and molecular wights (MW). In the first dimension, high voltage power enforces the individual protein species to migrate until they reach their neutral pH point (pI). Proteins with same pI are further separated based on MW in the second dimension (Fichmann and Westermeier, 1999). Proteins spots of interest can be excised from the gel and characterized by methods such as mass spectrometry.

5.3. Materials and methods

Cultivation of bacteria from sponge tissue of *M. laxissima* and *I. strobilina* (Chapters 2 and 3) yielded a collection of bacterial isolates from the groups *Alpha-* and *Gammaproteobacteria* and *Bacterioidetes*. Initial screening of the isolates showed that a portion of the proteobacterial isolates might produce AHLs. Based on these

preliminary findings, we focused on alpha- and gammaproteobacterial isolates for the detection of AHL-signaling molecules.

Bioassays for AHL synthesis. In collaboration with Dr. Clay Fuqua/Elisha Cicirelli (Department of Biology, Indiana University), my isolates were tested for the production of AHLs with a series of bioreporter systems, each with different optimal sensitivities to short chain, moderate chain and long chain AHLs (Table 5.1). The At-AHL reporter expresses a *lacZ* fusion most strongly in response to medium chain length AHLs (C6-C12, although weakly to C4), with limited distinction of AHLs carrying hydrogen, hydroxyl or carbonyl as the R-group at the β carbon. The Cv-AHL derivative produces the purple pigment violacein in response to fully reduced short chain AHLs (C4-C6 side chains) with a hydrogen as the R-group at the β carbon. The Sm-AHL reporter also expresses a *lacZ* fusion in response to long chain AHLs (C14-C18) with or without unsaturated bonds in the side chains, and with hydrogens and carbonyls as the R group (hydroxyls have not been tested) (McClean *et al.*, 1997; Zhu *et al.*, 2003; Llamas *et al.*, 2004). Preliminary screening protocols involved cultivation of sponge isolates on Marine Agar and incubation at 28˚C for up to 7 days until a healthy big colony size or patch of growth was obtained. In most cases standard 10 cm Petri dishes were used, although the assay was occasionally scaled up to 15 cm plates. An overlay of soft agar was either inoculated directly with the AHL reporter strain (At-AHL and Sm-AHL), or this strain (Cv-AHL) was gently spread on the overlay surface. Cleavage of X-Gal (5-bromo-4-chloro-3-indolyl-galactopyranoside) (Promega, Madison, WI, USA) and blue staining (At-AHL and

Sm-AHL) or purple pigmentation (Cv-AHL) within the agar overlay in a diffusible ring around a colony was measured and scored as AHL production.

To prepare cells for the bioassays, the At-AHL reporter strains were grown to mid-exponential phase (optical density at 600nm, OD_{600} = 0.6). Cells were harvested by centrifugation and the pellet was thoroughly washed and resuspended with sterile deionized distilled water, followed by a final centrifugation and resuspension in 30% glycerol to a final OD_{600} of 12. For the overlay assays, 100 ml of AT minimal medium (Tempé *et al.*, 1977) soft agar (0.6%) with glucose as a carbon source (0.5 %) and ammonium sulfate (15mM) as a nitrogen source, and 40 µg/ml X-Gal, was inoculated with 1 ml of the concentrated At-AHL reporters. Marine Agar 2216 plates with colonies were overlaid with 20-25 ml of the ATGN agar and incubated at 28˚C overnight.

Sm-AHL cells were grown in LB (supplemented with 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$), and the cells were prepared in a similar manner to the At-AHL strains as described previously (Llamas *et al.*, 2004). For overlays, 0.6% TY agar was supplemented with 2.5 mM $CaCl_2$, 2.5 mM $MgSO_4$, 40 µg/ml X-gal, and 1 ml of concentrated Sm-AHL cells. Marine agar plates were overlaid with 25 ml of the inoculated TY soft agar and incubated at 28˚C overnight.

Due to salt intolerance the Cv-AHL assays were performed slightly differently. Marine Agar 2216 plates with fully grown colonies were overlaid with 25 ml of 0.6% TY (Tryptone Yeast Extract) agar and this was allowed to solidify. Following this step, overnight cultures of CV026 grown in Luria-Bertani (LB) broth were centrifuged and concentrated 6–fold in fresh LB followed by spread plating of

approximately 100 µl of the suspension per plate. Plates were incubated at 28˚C overnight.

TLC profiling of AHLs. My collaborators in Indiana University investigated TLC profiles of the produced AHLs. Five ml MB 2216 cultures were grown to an optical density at OD_{600} of 1.5 – 2.0, followed by extraction with an equal volume of dichloromethane. Culture pH was monitored and was within the range of 7.6±0.2 at the time of harvesting (sterile Marine Broth 2216 is pH 7.6±0.2). Following centrifugation, the organic phase was removed and allowed to evaporate in a fume hood. Extracts were concentrated 1000 fold and normalized to an OD_{600} of 1.5 and resuspended in a final volume of approximately 5 µl of acidified (0.01%) ethyl acetate and loaded onto a C18 RP-TLC plate (Mallinckrodt Baker, Phillipsburg, NJ, USA). TLC plates were developed in a 60% methanol water mobile phase, dried, and overlaid with 100 ml of 0.6% ATGN media supplemented with 40 µg/ml X-gal and 1 ml of an OD_{600} = 12.0 suspension of the highly sensitive At-AHL reporter. TLC plate overlays were placed in a sealed container and incubated at 28˚C for 16-24 hrs.

Protein extraction. Nine isolates belonging to the SR subgroup were chosen for proteomic analysis. They included KLH11, N04ML2, N04ML4, N04ML5, N04ML6, N04ML9 and N04ML11 from *M. laxissima* and N04IS3 and N05IS9 from *I. strobilina*. The growth curve of each isolate was monitored by absorbance (OD_{600}) and samples (1.8 ml) were harvested at mid-exponential phase for proteomic analysis (Fig. A.3, Appendix A). Cells were centrifuged at 10,600 × g for 3 min and rinsed with washing buffer (Tris-Cl 10 mM, Sucrose 250 mM, pH 7.6). Pellets were stored at -80˚C until further processing. My collaborators at COMB (Feng Chen and Jinjun

Kan) performed the protein extraction. Frozen cells were thawed on ice and resuspended with 0.5 ml of extraction buffer, consisting of 0.01 M Tris-HCl, pH 7.4, 1 mM EDTA, 7 M urea and 2 M thiourea, 10% (v/v) glycerol, 2 % CHAPS, 0.2 % amphylotes, 0.002 M tributyl phosphine (TBP), DNase (0.1 mg/ml), RNase (0.025 mg/ml) and proteinase inhibitor cocktail (CalBiochem, San Diego, CA, USA). TBP, DNase, RNase and proteinase inhibitor cocktail were freshly prepared and added to the extraction buffer prior to the experiment. Samples were incubated on ice and vortexed for 15 sec every 5 min. Cellular debris was removed by centrifugation (10,600 × g, 4˚C for 5 min). Protein concentration was estimated by measuring the absorbance at 280 nm using a spectrophotometer (Bio-Rad, Hercules, CA, USA).

Isoelectric focusing (IEF) and SDS-PAGE. The first dimension separation of proteins (ca. 200 µg each sample) was conducted in the immobilized pH gradient strips (11 cm, pH 4-7) on a Bio-Rad Protean IEF Cell system (Bio-Rad, Hercules, CA, USA). The IEF program was run at 250 V for 20 min followed with a linear ramp to 8000 V for 2.5 hr, and 8000 V for a total 40,000 V/hr with a rapid ramp. After the first dimension, the IEF strips were equilibrated in freshly made Buffer 1 (6 M urea, 2% SDS, 0.05 M Tris/HCl pH 8.8, 50% glycerol) and Buffer 2 (6 M urea, 2% SDS, 0.375 M Tris/HCl pH 8.8, 20% glycerol and 0.5 g iodoacetamide) (Bio-Rad), respectively. The second dimension of 2D-PAGE were performed using 8-16% gradient precast polyacrylamide gels (Bio-Rad) following the manufacturer's instructions. Gels were stained with SYPRO Ruby (Bio-Rad) after electrophoresis and scanned using a Typhoon 9410 gel imager (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) with 488 nm excitation and emission filter 610 BP30.

Image analyses. My collaborators at COMB analyzed the 2-DGE images using the ImageMaster software (GE Healthcare Bio-Sciences) following the manufacturer's instructions. The gel images were imported into the workspace designated for analysis. Since the gel images acquired with the Typhoon 9410 were already calibrated, no intensity calibration was necessary. Spot detection parameters including smooth, saliency and minimum area were adjusted and optimized in order to detect all the real spots and filter out the noise. Gel images were compared using the total density in gel method for spot quantification. A single landmark was annotated and used for image automatic matching procedure. Matches were edited manually to eliminate poor quality matches. Pairwise similarities were obtained based on the percentage of spots that were matched, which was calculated as follows: Percent matches = $2m/(n_g + n_m)$, where m was the total number of matches between the gel and the master image (reference gel), and n_g is the number of spots in the gel and n_m is the number of spots in the Master. The pairwise similarities were further used to construct the distance matrix for MDS analysis.

Nonmetric multidimensional scaling (MDS). MDS is a commonly used analytical method to describe changes in bacterial communities over time or space (van Hannen *et al.*, 1999). It arranges the bacterial communities in two or three dimensions to identify community patterns and help to explain observed relationships. The MDS analysis was performed using the SAS System (SAS Institute, Cary, NC, USA). The relationship between any two isolates is reflected by the relative distance between them in two-dimension MDS plots. Bacterial isolates with more similar proteome patterns are plotted closer, while isolates with less similar

proteome patterns are further apart. The stress value (measure of goodness-of-fit) was recorded. In general, stress value less than 0.1 indicated a good ordination with little risk of misinterpretation of data (Clarke, 1993).

Characterization of AHL-producing roseobacters. Six AHL-producing roseobacters from *M. laxissima*, N04ML2, N04ML4, N04ML5, N04ML6, N04ML9 and N04ML11 were characterized using API 20NE strips (bioMérieux, Paris, France).

Nucleotide sequence accession numbers. 16S rRNA gene sequence fragments from AHL-producing isolates were submitted in the GenBank and accession numbers are given in Figs. 5.4 and 5.5.

5.4. Results

Identification of AHL activities in bacteria isolated from *M. laxissima* and *I. strobilina*. Cultivable *Proteobacteria* from *M. laxissima* and *I. strobilina* were analyzed by bioassays using three different AHL-reporter bacteria (Table 5.1). *Chromobacterium violaceum* strain 026 (Cv-AHL) was used, it cannot synthesize AHLs, but responds to the presence of short chain AHLs (C4-C6) by producing violacein, a purple pigment (McClean *et al.*, 1997). A sensitive and broader spectrum AHL-responsive reporter derived from *Agrobacterium tumefaciens* was also utilized and described as an ultrasensitive AHL reporter (At-AHL) (Zhu *et al*., 2003). The

Table 5.1. AHL reporter systems

Name	Bacteria (Plasmids)	Cognate[1]	Effective Range[2]	Reference
At-AHL	*A. tumefaciens* KYC55 (pJZ372)(pJZ384)(pJZ410)	3O-C8	C4-C14 (H, O, OH)	Zhu *et al*. 2003
Cv-AHL	*C. violaceum* 026	C6	C4-C6 (H)	McClean *et al*. 1997
Sm-AHL	*S. meliloti* (pJnSinR)	3O-C18	C16-C20 (O)	Llamas *et al*. 2004

[1] AHL produced by cognate AHL synthase to which reporter has greatest sensitivity.

[2] Range of AHL side chain length to which the reporter will detectably respond (specificity for R-group at β position).

At-AHL reporter responds efficiently to AHLs with acyl chain lengths of C6-C14, with decreasing response to AHLs shorter and longer than its cognate ligand 3-oxo-octanoyl-L-homoserine lactone (3OC8-HSL). Finally, a *Sinorhizobium meliloti* AHL reporter (Sm-AHL), which responds to long chain AHLs was also used (Llamas *et al.*, 2004) (Tables 5.1 and 5.2, and Fig. 5.1). These AHL-responsive biodetection systems were utilized to score the sponge isolates for production of AHL-type inducers. In marine agar overlay assays with the At-AHL reporter in large Petri dishes (150 × 15 mm), three distinct "activation" phenotypes were defined (Fig. 5.1): very strong activation (an activation zone radius greater than 2.5 cm of activity), moderate activation (a radius of 0.5-2.5 cm) and minimal activation of the reporter (a radius less than or equal to 0.5 cm). Due to the overall weak coloration imparted by the Sm-AHL biosensor, its activity was recorded as relative intensity of positive

Table 5.2. AHL production by 20 bacterial strains isolated from: seawater collected in 2001 (SWKLH8); *M. laxissima* collected in 2001 (KLH11), 2004 (N04ML) and 2005 (N05ML); and *I. strobilina* collected in 2004 (N04IS) and 2005 (N05IS).

Phylum	Isolate	% Identity[1]	Bioassays[2,3]		AHL Profile[4]
			At-AHL	Sm-AHL	
Alphaproteobacteria					
AJ391197, *Ruegeria* sp. AS-36	KLH11	98%	++	++	3
AJ391197, *Ruegeria* sp. AS-36	N04ML4	98%	++	++	3
AJ391197, *Ruegeria* sp. AS-36	N04ML6	98%	++	++	3
AJ391197, *Ruegeria* sp. AS-36	N04ML9	98%	++	++	3
AJ391197, *Ruegeria* sp. AS-36	N04IS3	98%	++	+	3
AJ391197, *Ruegeria* sp. AS-36	N05ML9	98%	++	+	4
AF201086, *Silicibacter* sp. JC1077	N04ML2	99%	++	++	3
AF201086, *Silicibacter* sp. JC1077	N05ML8	99%	++	+	4
AF201086, *Silicibacter* sp. JC1077	N04ML11	99%	++	+	4
AF201086, *Silicibacter* sp. JC1077	N05IS9	98%	++	++	5
AB255399, *Ruegeria atlantica*	N04ML5	98%	+++	+	6
AY486423, *Pseudovibrio denitrificans*	N05ML11	100%	+	-	-
DQ285076, *Erythrobacter* sp.	SW-KLH7	100%	+	-	N.D.[5]
DQ985038, *Erythrobacter* sp.	SW-KLH8	100%	+	-	12
Gammaproteobacteria					
X74701, *Vibrio diazotrophicus*	N05ML4	98%	++	-	9
X74701, *Vibrio diazotrophicus*	N05ML5	98%	++	-	9
DQ212914, *Thalassomonas agarivorans*	N05ML13	95%	+	-	-
DQ317675, *Vibrio* sp. FLLU3	N05IS8	99%	-	+	-
AB205011, *Spongiobacter nickelotolerans*	N05IS14	94%	+	-	-
AJ316168, *Vibrio* sp. R-14968	N05IS15	98%	++	-	10

[1] Percent identity to the closest well described taxa. GenBank accession numbers are listed before each reference sequence.

[2] Overlay bioassays using sensor strains: *A. tumefaciens* (At-AHL), and *S. meliloti* (Sm-AHL).

[3] AHL production activity is presented as: +++ = radius > 2.5 cm of activity, ++ = radius of 0.5 to 2.5 cm of activity and + = radius of < 0.5 cm of activity. Activation of the *S. meliloti* reporter strain is presented as relative intensity of positive strains. ++ = strongest intensity, + = roughly 50% of the intensity of strongest producing strains.

[4]AHL profile as assessed by thin-layer chromatography bioassay. Numbers are lane numbers referring to AHL profiles shown in Fig. 5.3.

[5] N.D. – not determined.

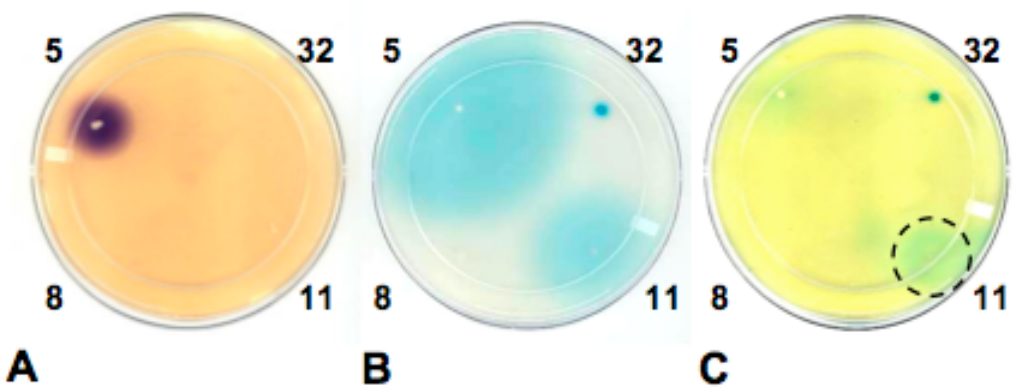


Fig. 5.2. Example overlay marine agar bioassays for AHL activity. Isolates are indicated around each plate; 8- N04ML8 (AHL negative), 5- N04ML5 (AHL+), 32- N05ML13 (weak AHL+) and 11- KLH11 (AHL+). Reporter strains, (A) Cv-AHL, (B) At-AHL and (C) Sm-AHL. Figure provided by Elisha Cicirelli and Clay Fuqua.

strains (++ indicating strongest intensity, + indicating roughly 50% of the intensity of strongest producing strains). Twenty-three proteobacterial isolates from *M. laxissima* were assayed for AHL production and 13 of the 23 strains (57%) activated the At-AHL reporter, varying from less than 0.5 cm to greater than 2.5 cm for the radius of the activation zone. One of the 13 strains, N04ML5, an *Alphaproteobacterium* from *M. laxissima* displayed the strongest AHL activity, activating all three reporter strains including the Cv-AHL reporter (Fig. 5.2). As further confirmation of AHL production by the sponge isolates, the *S. meliloti* (Sm-AHL) reporter detected long chain AHL production in 11 of the 13 AHL positive strains (Fig. 5.2.C and Table 5.2). Four of the 25 proteobacterial strains isolated from *I. strobilina*, activated the At-AHL reporter in overlay assays (Table 5.2). Two of the strains, which activated

the At-AHL reporter, were also able to activate the Sm-AHL reporter (N04IS3 and N05IS9), indicating long chain AHL production. One strain N05IS8, a *Gammaproteobacterium* from *I. strobilina* activated only the Sm-AHL reporter, indicating primarily long chain AHL production, outside the detection range of the other AHL biosensors. Of the eight proteobacterial isolates from the surrounding water column assayed for AHL production, two *Alphaproteobacteria* SWKLH7 and SWKLH8, activated the At-AHL reporter.

Profiling of AHL production by thin-layer chromatography (TLC) and bioassays. Reverse phase (RP) TLC was utilized to determine the signal molecule profiles of AHL^+ isolates that tested positive in the plate-based overlay assays. A comparison of the AHL profiles revealed several common patterns (Table 5.2). A representative of each observed unique AHL profile from *Alpha-* and *Gammaproteobacteria* is included in Fig. 5.3. Isolate KLH11 (Fig. 5.3, Lane 3) from *M. laxissima* produced greater than six different spots of activity, ranging in migration from those that do not move from the site of application (long chain, non-polar), to those that migrated the furthest (short, highly polar molecules), with significantly higher Rf values than the *N*-butanoyl homoserine lactone (C4-HSL) standard. KLH11 shared a nearly identical AHL profile with five other alphaproteobacterial strains (N04ML2, N04ML4, N04ML6, N04ML9, and N04IS3). N04ML5 and N05IS9, *Alphaproteobacteria* from *M. laxissima* and *I. strobilina*, respectively, had very distinct profiles. In comparison to KLH11, N05IS9 produced two prominent activities that had similar Rf values to 3O-C6 and 3O-C8 AHL standards (Fig. 5.3, Lane 5). The AHL profiles of strains N04ML11 (Lane 4) and

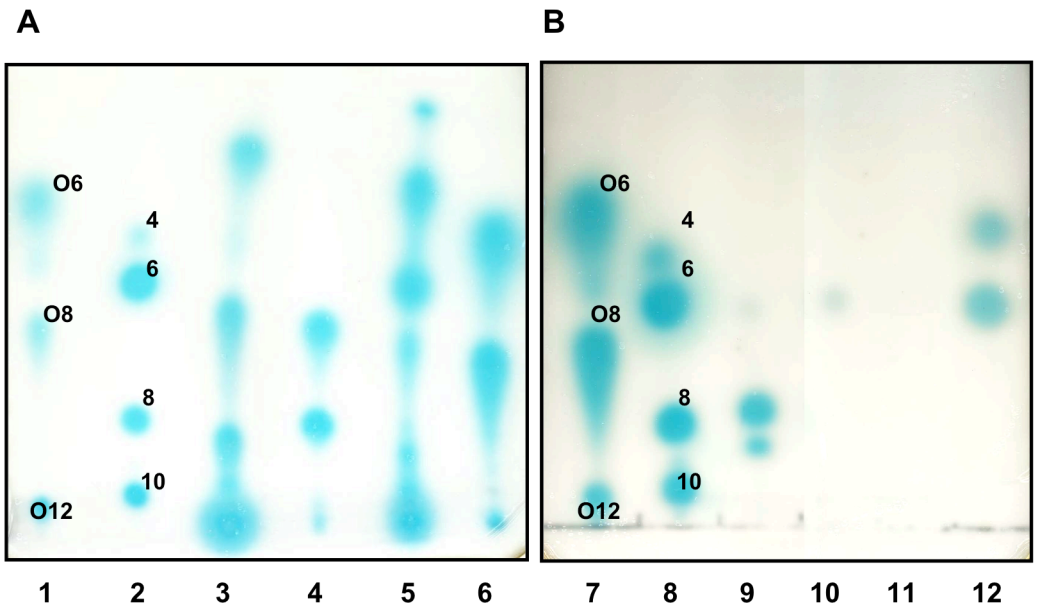


Fig. 5.3. RP-TLC profiling of AHL+ isolates. TLC plates were overlaid with At-AHL reporter strain. Mixtures of synthetic 3-oxo-AHL and fully reduced AHLs standards were run on each plate (labeled on plate), Lanes 1 and 7, 2 and 8, respectively. Plate A represents alphaproteobacterial strains; Lanes 3-6 are KLH11, N04ML11, N05IS9 and N04ML5, respectively. Lane 6 is a 2000-fold dilution of N04ML5 extract. Plate B (Lanes 9 and 10) represents gammaproteobacterial strains; Lanes 9 and 10 are N05ML4 and N05IS15, respectively. Lane 11 is a marine broth control and Lane 12 is AHL^{+} water column isolate (SWKLH8). AHL standard concentrations are: Fully reduced, C4, 1 mM; C6, 500 µM; C8, 50 nM; C10, 125 µM and 3-oxo derivatives, 3-oxo-C6, 50 nM; 3-oxo-C8, 42 nM; 3-oxo-C12, 68 µM. Figure provided by Elisha Cicirelli and Clay Fuqua.

N04ML5 (Lane 6) were clearly distinct from each other, but shared an apparent lack of AHL activities that migrated with high Rf values on RP-TLC. The *Gammaproteobacteria* produced moderate chain length AHLs, lacking the nonpolar activities with low Rf values observed for the sponge-associated *Alphaproteobacteria* (Fig. 5.3B). Analysis of the AHL^+ isolates from the water column resulted in AHL profiles different from those of any of the sponge isolates.

Phylogenetic analysis and diversity of AHL-producing isolates. A phylogenetic tree of partial 16S rRNA gene sequences was constructed for AHL-producing proteobacterial strains (Fig. 5.4). Eleven AHL^+ alphaproteobacterial isolates fell within the *Silicibacter-Ruegeria* (SR) subgroup of the *Roseobacter* clade and three isolates were in genera (*Pseudovibrio* and *Erythrobacter*) that are distinct from this subgroup (Table 5.2 and Fig. 5.4). *N*-acyl homoserine lactone-producing gammaproteobacterial isolates fell within the genera *Vibrio, Thalassomonas and Spongiobacter.* 16S rRNA sequence analysis indicated that SWKLH7 and SWKLH8, the AHL^+ seawater isolates, shared greatest similarity to an *Alphaproteobacterium* species in the genus *Erythrobacter*. Nine *Roseobacter* isolates were chosen to represent the AHL-producing SR subgroup for further phylogenetic analysis including full 16S rRNA phylogenetic analysis and proteomics. A phylogenetic tree of the full 16S rRNA gene sequences of the nine SR isolates is shown in Fig. 5.5. KLH11, N04ML9, N04ML6, and N04ML4 shared 99-100% identity and formed a cluster (cluster 1), which was closely related to the roseobacters from *I. strobilina*, N04IS3 and N05IS9. N04ML2 and N04ML11 (cluster 2) were 99.2% identical and clustered with *Silicibacter* sp. JC1077 (NCBI accession no. AF201086, 99.8-99.9%

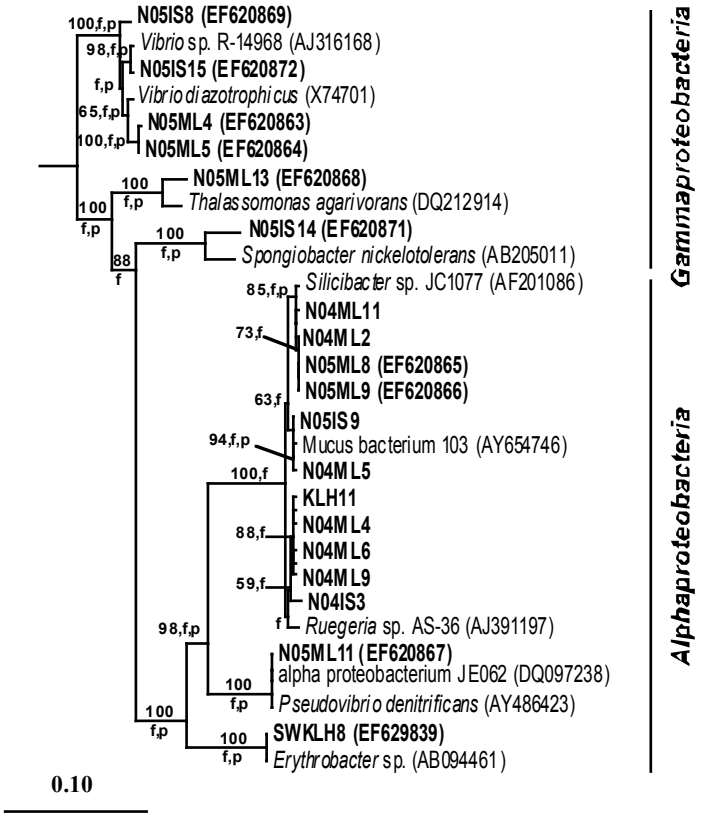


Fig. 5.4. Rooted neighbor-joining tree of partial 16S rRNA gene sequences of AHL-producing *Alphaproteobacteria* and *Gammaproteobacteria* from *M. laxissima*, collected in 2001 (KLH11), 2004 (N04ML) and 2005 (N05ML), *I. strobilina*, collected in 2004 (N04IS) and 2005 (N05IS), and seawater (SWKLH8). Bootstrap confidence values > 50% are shown at the nodes. Ca. 500 bp were used in the phylogenetic analysis and branches indicated by f and p were found using Fitch-Margoliash and maximum parsimony methods, respectively. The outgroup used in this analysis was *Escherichia coli* BL21 (NCBI accession no. AJ605115). Scale bar indicates 0.10 substitutions per nucleotide position. GenBank numbers are not given for partial 16S rRNA sequences from nine *Roseobacter* isolates; these GenBank numbers for almost complete 16S rRNA sequences are provided in Fig. 5.5.

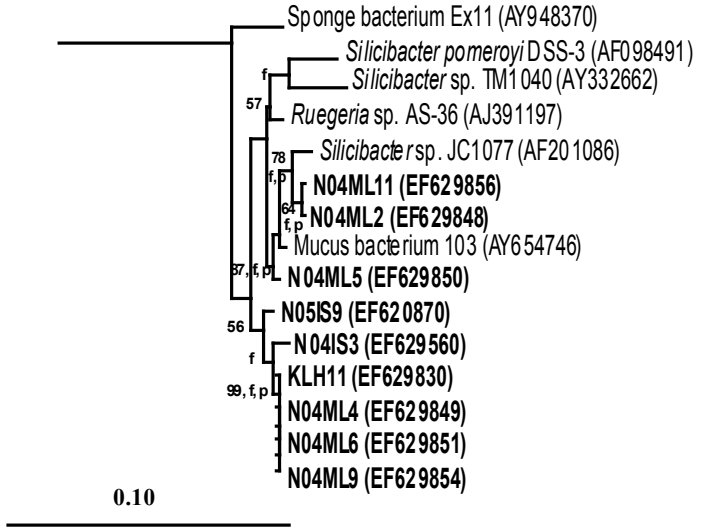


Fig. 5.5. Rooted neighbor-joining tree of almost complete 16S rRNA gene sequences of AHL-producing SR roseobacters from *M. laxissima* and *I. strobilina.* Bootstrap confidence values > 50% are shown at the nodes. Ca. 900 bp were used in the phylogenetic analysis and branches indicated by f and p were found using Fitch-Margoliash and maximum parsimony methods, respectively. The outgroup used in this analysis was *Agrobacterium* sp. PNS-1 (NCBI accession no. AY762361). Scale bar indicates 0.10 substitutions per nucleotide position.

identity). N04ML5 was notably distinct with 98.9% identity to cluster 1 and 98.6% identity to cluster 2.This strain was closely related to a SR-type bacterium isolated from coral mucus (NCBI accession no. AY654746, 99.3% identity). It is important to note that 100% of the sponge-associated SR-type *Roseobacteria* were AHL^+, while two roseobacters isolated from the water column did not produce detectable AHLs.

Biochemical characterization of *Roseobacter* strains. API 20NE strips were used for biochemical characterization of six AHL^+ SR strains from *M. laxissima.* API

20NE profiles were identical for strains, N04ML2, N04ML4, N04ML6, N04ML9, and N04ML11. The strains were positive for β-galactosidase, negative for denitrification, indole production, glucose acidification, arginine dihydrolase, urease, esculin hydrolysis, gelatin hydrolysis and oxidase and negative for assimilation of the following sugars: glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, maltose, potassium gluconate, capric acid, malic acid, trisodium citrate, and phenylacetic acid. N04ML5 was different than the other strains in being positive for nitrate reduction, urease and esculin hydrolysis and weakly positive for arginine dihydrolase.

Proteome pattern analyses. Proteomic analysis was used to investigate fine-scale diversity among the more closely related AHL-producing alphaproteobacterial isolates KLH11, N04ML9, N04ML4, N04IS3, N05IS9, N04ML5, N04ML2, N04ML11 and *S. pomeroyi* DSS-3. As a first step, protein expression profiles of KLH11 at three different growth stages (early and mid exponential, and stationary phases) were compared (Fig. 5.6), to understand how functional proteins vary at the different growth stages. Although some modulation of protein levels was apparent at different growth periods, the 2DGE images at the three different stages shared higher than 90% similarity. Based on these results, samples were acquired at mid-exponential phase for other bacterial strains because functional proteins at this phase were more uniformly expressed and produced a greater number of spots compared to the other time points. The reproducibility of the 2-DGE system was tested and triplicates yielded highly reproducible results (>95% similarity, data not shown). The protein profiles of nine AHL-producing SR roseobacters were different to each other

(Fig. A.4, Appendix A), but could be compared using the ImageMaster software. Multi-dimensional scaling (MDS) analysis showed that some bacterial strains (i.e. N04ML4 and N04ML9; N04ML2 and N04ML11) share more similar 2-DGE patterns, while others are more distinct from each other (Fig. 5.7). The relatedness of these nine isolates based on the MDS analysis supported the branch points and general topology of the 16S rRNA gene phylogeny shown in Fig. 5.5. For example, N04IS3 and N05IS9 were close to KLH11, N04ML9 and N04ML4, while N04ML5 was more similar to N04ML2 and N04ML11 (Fig. 5.7).

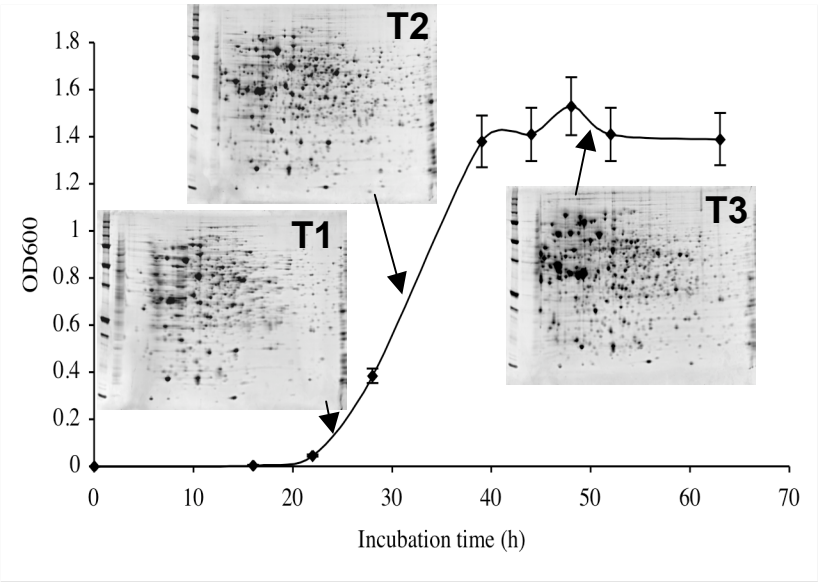


Fig. 5.6. Proteomes of KLH11 at three different growth stages, T1, T2, and T3 which correspond to early exponential, mid-exponential and stationary phases, respectively. Figure provided by Jinjun Kan and Feng Chen.

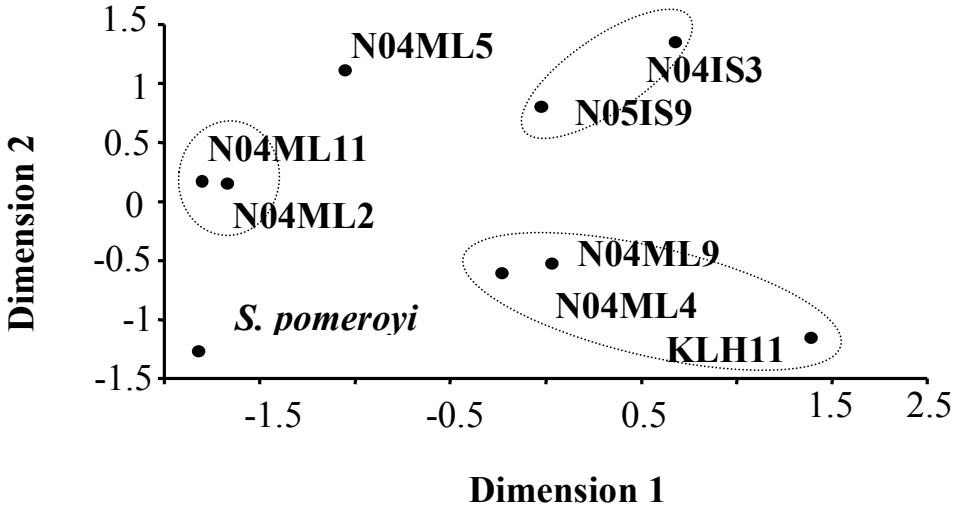


Fig. 5.7. Two-dimensional MDS plots showing the relative relationships of the nine *Roseobacter* strains based on their protein expression patterns. Bacterial isolates are indicated next to each point. Stress = 0.039. Figure provided by Jinjun Kan and Feng Chen.

5.5. Discussion

The dense bacterial community harbored within the sponge mesohyl lends itself to interbacterial communication mechanisms. The prevalence of sponge-associated proteobacteria in this environment suggested that AHL-based signaling systems might be important. It is likely that some sponge-specific bacteria are initially acquired from the oligotrophic water column where they exist in low abundance. Sponge tissues provide increased nutrient availability relative to bulk seawater, and the colonizing bacteria can grow to achieve high population densities that allow for a bacterial quorum. Opportunities for competition and collaborative metabolism are also provided within the sponge.

I cultivated 48 individual proteobacterial isolates from sponge tissues, 38% (18 total) of which were AHL^+. Among the bacteria tested, AHL production was more frequently observed for the *Proteobacteria* associated with *M. laxissima* than those with *I. strobilina*, although the sample sizes were not sufficient to establish this as a definitive trend. Of the AHL^+ bacteria, 67% were *Alphaproteobacteria* and 33% were *Gammaproteobacteria*. The AHL^+ gammaproteobacterial isolates included taxa closely related to species of *Vibrio, Thalassomonas* and *Spongiobacter.* It is not surprising that AHL^+ *Vibrio* spp. were identified, but to my knowledge this is the first report suggesting that bacteria related to *Thalassomonas* and *Spongiobacter* produce AHL type activities. Alphaproteobacterial isolates were predominantly from the *Silicibacter-Ruegeria* subclade of *Roseobacter* clade (11 strains). One *Alphaproteobacterium* from *M. laxissima* that resulted in a weak response in the AHL reporter bacteria was from the genus *Pseudovibrio* (N05ML11). Most of the AHL^+ bacteria were detected with the At-AHL reporter, the most sensitive and least specific AHL bio-detection system available, while a subset of these were positive for long chain AHL synthesis in the Sm-AHL assay. A single *Alphaproteobacterium* (N04ML5) was found to produce AHLs detected by the Cv-AHL system (Fig. 5.2A), specific for short chain AHLs, and the least sensitive biodetection strain that was used. Proteobacterial isolates from the water column adjacent to the site of sponge collection were also characterized and eight isolates were tested. Two isolates identified from the genus *Erythrobacter* (SWKLH7 and SWKLH8) proved to be AHL^+. Two water column strains SWKLH6 and SWKLH14 fell within the *Roseobacter* clade, based on 16S rRNA sequence, but both of these strains were

negative for AHL production. Although far larger data sets are required to test the idea, our observations bear facile similarity to reports for plant-associated pseudomonads in which host-associated bacteria are disproportionately AHL^+ relative to those in bulk soil (Elasri *et al.*, 2001).

Although all of the AHL^+ sponge-associated isolates within the *Silicibacter-Ruegeria* subclade were similar at the 16S rRNA sequence level, a surprising range of AHL profiles was observed following comparison of the strains with RP-TLC (Fig. 5.2A). Not only did the set of AHLs synthesized vary among the strains, but also some of the isolates produced vastly different amounts of the signals. The AHL profiles obtained for the alphaproteobacterial isolates were highly complex, often exhibiting 4-6 or more species separated on the TLC plate. On the other hand, the profiles obtained for the signal positive gammaproteobacterial isolates were similar and fairly simple with 1-2 primary species, generally in the medium chain length range (Fig. 5.3B). It is important to recognize that this analysis will only detect AHLs synthesized under laboratory conditions. There is ample evidence that AHL synthesis can be tightly regulated by environmental conditions including host released cues and nutritional status. Our findings may therefore be an underrepresentation of the true prevalence of AHL synthesis.

In a small-scale survey of several marine sponges, an *Alphaproteobacterium* that produced AHL signal molecules was isolated from the marine sponge *Cymbastela concentrica* (Taylor *et al.*, 2004a). Strikingly, this single AHL^+ isolate from an Australian sponge falls within the same *Silicibacter-Ruegeria* group as the dominant AHL^+ microbes I have isolated from the Florida sponges *M. laxissima* and *I.*

strobilina. Synthesis of AHLs is apparently a prevalent characteristic of the broader group of *Alphaproteobacteria* within the *Roseobacter* clade (Wagner-Döbler *et al.*, 2005). As with the rhizobia, terrestrial plant-associated *Alphaproteobacteria*, the production of long chain AHLs is often observed in the *Silicibacter-Ruegeria* group. I hypothesize that AHL-based signaling in those *Silicibacter-Ruegeria* bacteria associated with marine sponges controls functions that have significance to the sponge-microbe interaction and also the structure of the polymicrobial community within the sponge (Fig. 5.8).

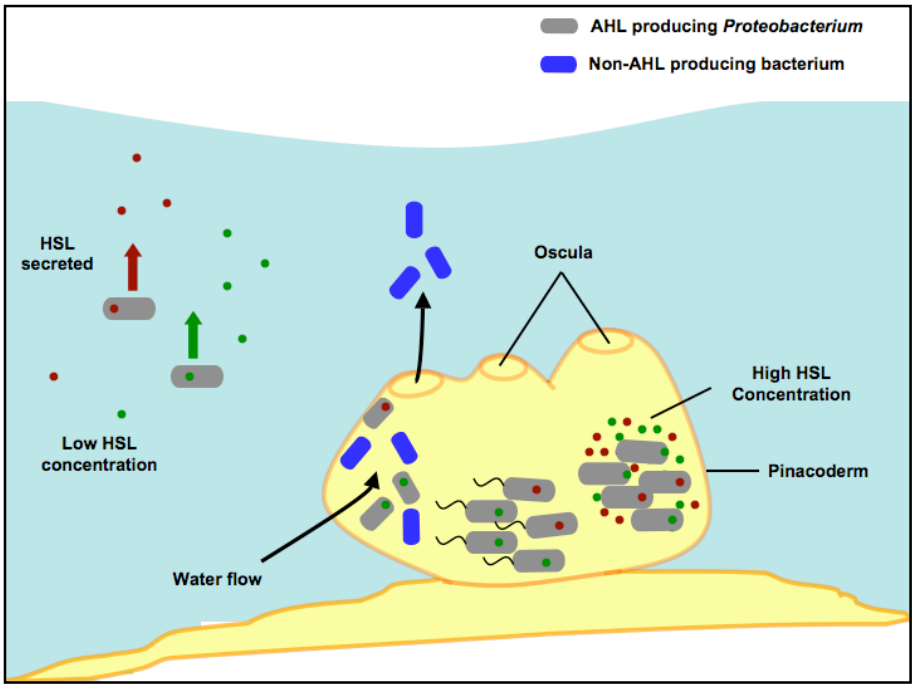


Fig. 5.8. Schematic representation of quorum sensing in a sponge where changes in flagellar gene expression in proteobacterial symbionts induce colonization of the sponge.

Differentiation of closely related microbial species is not straightforward. Although identical 16S rRNA gene sequences were found among actinobacterial strains isolated from different thermal niches in temperate, subtropical, and tropical freshwater habitats, the isolates had sequence differences (Hahn and Pockl, 2005). Differences were observed at zero to five positions in a 2,310 nucleotide fragment of the ribosomal operon, including part of the intergenic spacer upstream of the 16S rRNA gene, the complete 16S rRNA gene, the complete 16S-23S internal transcribed spacer, and a short part of the 23S rRNA gene. Thus, bacterial species characterization cannot solely rely on sequence analysis of a single gene marker. The ecology of closely related bacterial species should be considered not only at phylogenetic and genetic levels, but also at metabolic and functional levels. Our results suggest that species or strain-specific protein expression profiles provide high resolution for closely related bacterial strains. The nine AHL-producing SR bacteria examined shared greater than 98% identity in terms of their 16S rRNA gene sequences, and they would be considered as the same species [>97%, (Rossello-Mora and Amann, 2001)]. Some strains (i.e. KLH11, N04ML9, and N04ML4) could not be resolved based on the 16S rRNA phylogeny, but metabolic or functional differences were observed based on the AHL profiles or proteome patterns. These results are congruent with previous studies in which protein profiles could be used to resolve and identify closely related *Ferroplasma* isolates (Dopson *et al.*, 2004), and even for phylogenetic studies (Navas and Albar, 2004). In this study, all the bacterial strains were cultivated and harvested under the same growth conditions with identical culture

media and therefore, the protein profiles should reflect real differences in protein expression between strains.

In conclusion, isolates from the *Silicibacter-Ruegeria* subclade within the *Alphaproteobacteria* are the dominant cultivatable producers of AHLs in marine sponges *M. laxissima* and *I. strobilina*. This is consistent with previous studies showing the production of AHLs by members of the *Roseobacter* clade from the marine environment (Wagner-Döbler *et al.*, 2005). All the *Roseobacter* isolates included in the proteomic analyses are AHL producers. The 2DGE reference maps for AHL producers can be generated as a reference standard for AHL-producing species recognition. Characteristic protein spots shared by all the AHL producers might be used as "AHL indicators" to differentiate these strains from non-AHL producers. More importantly, characterization of unique signature proteins for AHL positive strains or species may hold a key to understanding the mechanisms of the AHL production pathway. Quorum sensing regulatory genes including AHL synthases have been isolated and directed mutant strains of AHL-producing SR bacteria have been generated (E.M. Cicirelli, J. Herman, N.M. Mohamed, M.E.A. Churchill, R.T. Hill and C. Fuqua, unpublished). The proteomes of these mutants are under comparison with wild type SR bacteria. Sponge colonization assays will also be carried out using wild type and mutant strains. These future studies will begin to provide an understanding of the functional roles of different proteins in AHL production and regulation in the sponge environment, phenotypic targets of this regulation, and symbiotic interactions between sponges and their bacterial communities.

Chapter 6. Summary and Future Directions

My research was part of a large study of the microbial communities of marine sponges of the class Demospongiae in Key Largo, Florida, funded by the National Science Foundation Microbial Observatories Program.

I used culture-based techniques and 16S rRNA based molecular techniques including clone library construction and DGGE to characterize the bacterial communities associated with the sponges *I. strobilina* and *M. laxissima*. The comprehensive molecular techniques revealed that each harbored a different, highly diverse bacterial community and both communities were distinct from that of the surrounding seawater (Chapters 2 and 3). The bacterial community associated with *M. laxissima* was dominated by *Proteobacteria* (*Alpha-* and *Gammaproteobacteria*). Interestingly, a large number of the clones detected in *I. strobilina* were affiliated with uncultured *Chloroflexi* (59% of the clone library). Although overall bacterial communities were different, some bacterial groups, including members of *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Cyanobacteria*, were found in both sponge species. The *Cyanobacteria* are common group of sponge symbionts that may be important for the nutrition of the sponge hosts. *Actinobacteria* have a track record of production of bioactive compounds. This suggests that different sponge species may select for similar groups of bacteria due to the sponges' specific ecological need in their environment.

My work shows that the bacterial communities in *I. strobilina* and *M. laxissima* are comprised of sponge specific bacteria and likely include some symbionts that are benefiting the sponge. Evidence for symbionts is:

(i) Bacterial communities in sponges were significantly different from bacterioplankton communities found in the surrounding seawater. This was based on the statistical comparison of the 16S rRNA libraries of *M. laxissima* and surrounding seawater (Chapter 2).

(ii) DGGE banding patterns of both wild *I. strobilina* and *M. laxissima* were clearly different than the surrounding seawater (Chapters 2 and 3).

(iii) DGGE analysis of sponges maintained in aquaculture and the surrounding water indicated substantial differences in their bacterial communities, which was confirmed by community analysis of these samples via 16S rRNA genes sequencing studies (Chapter 2).

(iv) Isolates from *I. strobilina* and *M. laxissima* included isolates found only in marine sponges, consistent with previous reports for wild sponges but shown here for the first time for sponges maintained in aquaculture systems,

(v) Cyanobacterial *nifH* genes detected in sponges were different than those in the surrounding seawater (Chapter 4).

(vi) *Proteobacteria* isolated from *I. strobilina* and *M. laxissima* produced signaling compounds in contrast to *Proteobacteria* isolated from the surrounding seawater, which generally tested negative for the production of AHLs (Chapter 5).

Two sets of aquaculture systems were designed in order to optimize the conditions for culturing two marine sponges, *I. strobilina* and *M. laxissima.* Both

sponges were more capable of adapting to recirculating aquaculture conditions compared to other sponges from the same reef environment. This is important since these sponges can be used to develop a laboratory system to study complex symbioses between bacteria and sponges. Maintaining sponges in aquaculture provided a means for culturing novel bacteria. An *Acidobacterium* strain was successfully isolated from aquacultured *M. laxissima*. I also successfully cultured several putative novel strains. These included four *Flavobacteriaceae*, six *Flexibacteriaceae*, two *Gammaproteobacteria*, and two *Alphaproteobacteria*. All of these strains warrant description as new species or genera (Chapters 2 and 3).

Molecular techniques revealed the presense of amazing diversity of bacteria in *Mycale* and *Ircinia* sponges (Chapters 2 and 3). The bacterial community of *I. strobilina* was dominated by *Chloroflexi* (Chapter 3). *Chloroflexi*, known as green nonsulfur bacteria, are a phylum of photoheterotrophs that produce energy through photosynthesis (Hugenholtz and Stackebrandt, 2004). Members of *Chloroflexi* are facultative anaerobes and they make up the bulk of the filamentous anoxygenic phototrophs. *Chloroflexi*-related sequences have been identified in geothermal, soil, freshwater, marine, wastewater, and subsurface environments (Morris *et al*., 2004). The few cultivated representatives exhibit a diverse range of phenotypes, including anoxygenic photosynthesis (Keppen *et al*., 2000), thermophilic organotrophy (Jackson *et al*., 1971), and chlorinated hydrocarbon reduction (*Dehalococcoides ethenogenes*) (Maymo-Gatell *et al*., 1997). *Chloroflexi* in sponges might be playing a role in anoxygenic photosynthesis as an important source of energy without the

release of oxygen, which is inhibitory to nitrogenase enzymes of other associated nitrogen fixing symbionts.

Proteobacteria (*Alpha- and Gammaproteobacteria)* are important groups in sponges. In my studies, they were found both in wild sponges and those maintained in aquacultures systems (Chapters 2 and 3). A novel type of rhodopsin, known as proteorhodopsin, functions as a light driven proton pump, was identified on a genomic fragment linked to the 16S rRNA gene of an uncultivated marine *Gammaproteobacterium* (Beja *et al*., 2000). This phototrophy in *Proteobacteria* might play an important role in microbial processes in marine sponges. Many *Alphaproteobacteria* isolated from *M. laxissima* and *I. strobilina* sponges were affiliated with *Roseobacter*. In addition to the production of a suite of signaling molecules (Chapter 5), it has been suggested that *Roseobacter* bacteria play a major role in the sulfur cycle in the ocean. They benefit significantly from association with dimethylsulfoniopropionate (DMSP)-producing dinoflagellates because of the high metabolic rate at which *Roseobacter* can degrade them.

Bacteroidetes are among the phyla that were highly enriched in aquaculture (Chapters 2 and 3). Phylum *Bacteroidetes*, formerly known as the *Cytophaga-Flavobacteria-Bacteroides* (CFB), is among the major taxa of marine heterotrophic bacterioplankton, mainly found on marine snow particles. They also represent a significant part of free-living microbial assemblages in nutrient-rich microenvironments. Their abundance and distribution pattern in combination with enzymatic activity studies has led to predict that organisms of this group play a role for degradation of high molecular weight compounds in both the dissolved and

particulate fraction of the marine organic matter pool. Bauer *et al*. (2006) suggested a clear adaptation of this marine *Bacteroidetes* representative to the degradation of high molecular weight organic matter based on the functional analysis of the predicted proteome. *Bacteroidetes* may have an important role in the carbon cycle of marine sponges.

Acidobacteria was found in *I. strobilina* (Chapter 3) and enriched significantly when *M. laxissima* was transferred to the flow-through aquaculture system (Chapter 2). *Acidobacteria* form a novel phylum of bacteria that is consistently detected in many different habitats around the globe by 16S rDNA-based molecular surveys. As implied by their name they are acidophilic. A known member of this group is *Acidobacterium capsulatum* ATCC 51196. It is a high G + C, acidophilic, Gram-negative rod originally isolated from acid mine drainage (Kishimoto *et al*., 1991). Culture-independent microbial community analyses and metagenomic libraries of soil revealed that the *Acidobacteria* are ubiquitous and abundant across a wide variety of soil types, suggesting that they play important roles in soil microbial communities (Barns *et al*., 1999; Liles *et al*., 2003). The ecological role of *Acidobacteria* in marine sponges is unclear.

Various roles have been ascribed to symbiotic bacteria in sponges, but there is little direct experimental data elucidating the contributions of the bacteria to the sponge, and thereby indirectly to the coral reef ecosystem. The concentrations of nitrogen in coral reef environments are low. A role of sponge symbionts in nitrogen fixation has been experimentally demonstrated in the past. In Chapter 4, I examined the role of bacteria associated with sponges in nitrogen fixation using nitrogen

isotopic composition and molecular techniques. There are three possible mechanisms for how sponges obtain their nitrogen: (i) They obtain nitrogen from dissolved inorganic nitrogen and dissolved organic nitrogen in the surrounding seawater, (ii) They obtain their nitrogen from the organic nitrogen present in the bacteria that they filter out of the water column, and (iii) Their nitrogen is supplied from nitrogen-fixing symbiotic bacteria within the sponges. My results support the presence of symbiotic bacteria that fix nitrogen and provide fixed nitrogen to the sponge host. This is the first evidence of nitrogen fixation in marine sponges using molecular techniques. *nifH* genes recovered from sponges were clearly distinct from *nifH* genes of bacteria in the surrounding water column. This excludes the possibility that sponges obtain their nitrogen by filtering nitrogen-fixing bacteria from the water column. I showed the expression of *nifH* genes in bacteria within sponges, which is the first demonstration of the expression of any bacterial genes within sponges. Cyanobacterial symbionts are an important group responsible for nitrogen fixation in *I. strobilina* and *M. laxissima*. This is supported by the detection of *Cyanobacteria* in the bacterial communities of both sponges (Chapters 2 and 3).

Unique nitrogen-fixing symbionts may have intimate and ancient associations with their sponge hosts, and the importance of these symbionts for their hosts means that they may be vertically transmitted between sponge generations. Several studies have demonstrated the importance of vertical transmission in structuring and maintaining symbiotic bacterial communities in sponges (Usher *et al.*, 2005; Enticknap *et al.*, 2006; Schmitt *et al.*, 2007; Sharp *et al.*, 2007). Usher *et al.* (2005) reported vertical transmission of the cyanobacterial symbiont *Candidatus*

Synechococcus spongiarum in the marine sponge *Chondrilla australiensis* via biparental mechanisms. Cyanobacterial symbionts in the sponge *Diacarnus erythraenus* were identified in both adult sponges and their larvae (Oren *et al.*, 2005). In a study by Enticknap *et al.* (2006), *Cyanobacteria* were a major fraction of the bacterial community transferred in *M. laxissima* larvae. I hypothesize that vertical transmission of these cyanobacterial symbionts is related to their importance in nitrogen fixation.

In Chapter 5, quorum sensing was hypothesized to play a major role in regulating aspects of host interactions and/or community structure. In collaboration with Dr. Clay Fuqua, the production of AHLs was tested by *Alpha*- and *Gammaproteobacteria* isolated from marine sponges *I. strobilina* and *M. laxissima*. Isolates from the *Silicibacter-Ruegeria* subclade within the *Alphaproteobacteria* are the dominant cultivatable producers of AHLs in both sponges. AHL- producing roseobacters are more abundant in *M. laxissima* compared to *I. strobilina*. This is consistent with the domination of culturable bacteria and clone libraries of *M. laxissima* by *Proteobacteria* (Chapter 2). An intriguing finding is that the domination of proteobacterial symbionts slightly decreased but was not lost when *M. laxissima* was transferred into the two aquaculture systems (Chapter 2). This may indicate the symbiotic nature and the importance of members of *Proteobacteria* to sponge hosts. In the case of *I. strobilina,* both *Alpha*- and *Gammaproteobacteria* emerged and were significantly enriched upon the transfer of the sponge to the recirculating aquaculture system. It is appealing to hypothesize that quorum sensing is a key factor for the symbiosis between sponges and their proteobacterial isolates (Chapter 4).

There was a striking diversity of AHL production among closely related roseobacters isolated from *M. laxissima*. This indicates that each microbe might have a unique relationship with the sponge microbial community and with the sponge host itself. It is worth mentioning that one *Roseobacter* strain from *I. strobilina* is closely related to several roseobacters from *M. laxissima* (~99% 16S rRNA identity) and had the same AHL profile. The production of similar AHLs by closely related strains from two different sponge species provides indirect evidence of the symbiotic nature of these AHL-producing bacteria since two different sponges have the same bacteria, which is presumably performing the same function.

Our collaborators at Indiana University have recently identified two discrete quorum sensing loci (*luxI* and *luxR* homologues) in a *Roseobacter* isolate from *M. laxissima* designated KLH11 (E.M. Cicirelli, J. Herman, N.M. Mohamed, M.E.A. Churchill, R.T. Hill and C. Fuqua, manuscript in preparation). Expression of these genes in *E. coli* led to the production of AHLs. Interestingly, they obtained *Roseobacter* AHL^- mutants that are non-motile and aflagellate in standard laboratory swim agar. One possible scenario is that the sponge larvae inherit a single strain or a group of roseobacters from the parent sponge, and this constitutes the early colonizers. Upon release of the larvae in the water column, roseobacters produce signaling compounds. When these AHLs accumulate, they allow for a bacterial quorum that induces the expression of flagellar genes, thus the motility of other *Proteobacteria* in the water column, and attracts them to colonize the larvae tissues and this constitutes the secondary colonizers. It is also possible that quorum sensing is a mechanism whereby adult sponges select their symbionts from the huge bacterial

load provided by filtration of large volumes of water every day. Quorum sensing induced motility may help these symbionts in initial invasion of the sponge tissue and in subsequent dispersal throughout the sponge. The detection of quorum sensing compounds in bacteria isolated from both sponges, combined with its possible regulation of the sponge-microbe interaction and/or microbe-microbe interactions within the sponge environment, indeed makes our sponges excellent model system for understanding complex symbiotic interactions.

My dissertation work supports previous concepts such as the occurrence of sponge-specific bacteria. It provides new insights into understanding the roles of these symbionts, especially in the nitrogen budget of sponges. This helps move the field of sponge microbiology from the current level of knowledge of the diversity of bacterial communities to a new stage of understanding symbiont function and sponge-microbe interactions.

My research as well as our collaborators' work lay the groundwork for future studies of quorum sensing as a key factor in the interactions between sponge hosts and their symbionts, as well as between symbiont populations. This includes sponge colonization assays using wild type and mutant strains.

To further understand the role of sponge symbiont in nitrogen fixation, it is important to cultivate representative bacteria carrying *nifH* genes, especially those affiliated with *Cyanobacteria*. It is important to localize nitrogenase gene expression using techniques such as mRNA FISH to detect bacterial phylotypes that actively transcribe *nifH* genes. It will be useful to link nitrogen fixation activity to the identity of the microorganisms *in situ*. This can be done by combining rRNA and mRNA

FISH. The nitrogenase enzyme can be visualized directly in thin sections of sponges by immunogold probing using anti *nifH* polyclonal antibodies. This approach can also enable identification of cells of specific bacterial groups such as *Cyanobacteria* that contain nitrogenase enzymes.

Sponges have significant coverage and biomass in marine communities such as coral reefs (Wilkinson, 1983; 1987). Nitrogen fixation by sponge symbionts is likely an important source of new nitrogen to the entire reef environment. Future studies including measurement of nitrogen fixation rates in sponges harboring nitrogen-fixing symbionts and the biomass of these sponges will help determine the contribution of marine sponges to the Conch Reef nitrogen budget. Reliable estimation of cyanobacterial populations and isolation of cyanobacteria in pure culture and testing them for nitrogen fixation will help establish the ecological importance of nitrogen fixation in marine sponges in different reef environments.

My work provides an excellent resource of several candidate bacteria for genomic approaches such as AHL-producing *Roseobacteria*, nitrogen-fixing *Proteobacteria* and the novel *Acidobacterium* strain that was cultivated from aquacultured *M. laxissima*. Genome sequencing projects of sponges and their symbionts and integrating their genomes as well as metagenomic approaches might help our understanding of many missing aspects of sponge-microbe interactions such as: **1.** Did sponges and their symbionts co-evolve together? **2.** Is it an ancient obligate association? **3.** What makes a bacterium a food particle and what makes it a symbiont? **4.** What is the mechanism whereby sponges recognize and select their symbionts versus pathogens and food particles from the water filtered out everyday,

5. How do sponges regulate the growth of their symbionts? and **6.** Is quorum sensing playing a role in the colonization process and in the regulation of the interactions between sponges and their symbionts? For example, comparison of the genome sequences of bacteria that are sponge symbionts and close relatives that are free living may help to locate "symbiosis islands" (Finan, 2002) that harbor genes encoding mechanisms whereby the symbionts recognize sponge cells.

Comparison of the genomes of sponge symbionts to the genomes of close relatives can also provide insights into their metabolic capabilities and the nature of the symbiotic relationship of these symbionts with their host sponge. An example of the use of comparative genomics is the comparison of the genomes of both uncultured archaeon *C. symbiosum* and *Nitrosopumilus maritimus*, the first cultivated nonthermophilic crenarchaeon. Hallam *et al*. (2006) suggested one possible interaction between the sponge host and its archaeon, consistent with the *C. symbiosum* genome complement and the nitrifying phenotype of *N. maritimus* is the removal of nitrogenous host-waste products such as ammonia and urea. This could simultaneously fuel the symbiont's respiratory energy metabolism and might even provide new carbon to the host, via archaeal chemolithotrophic CO_2 fixation, and subsequent symbiont–host carbon exchange (Hallam *et al.*, 2006).

The availability of genomes of both sponge hosts and their symbionts can be helpful in sheding the light on the biological nature of the sponge-microbe symbiosis. An example is the aphid-bacteria symbiosis. The genome of the pea aphid symbiont, the gammaproteobacterium *Buchnera* sp., revealed some important insights into the biological nature of this symbiosis (Shigenobu *et al*., 2000). Aphids feed by sucking

the sugar-rich phloem sap of higher plants. Phloem is generally poor in amino acids and, to compensate for that, many aphid species harbor endosymbiotic bacteria. Earlier work had shown that the association between the bacterial symbionts and the aphid lies in amino acid production by endosymbionts (Lai *et al*., 1994). However, the *Buchnera* genome encoded 54 genes involved in amino acid synthesis but only for the synthesis of essential amino acids, that is those that the aphids are unable to synthesize themselves (Shigenobu *et al*., 2000). Genes for the synthesis of non-essential amino acids were missing altogether in the *Buchnera* genome. This indicated that endosymbiotic bacteria provide essential amino acids to the aphid and in turn, obtain their non-essential amino acids from their hosts.

Appendix A. Supplemental Information

Table A.1. Number and names of clones in each bacterial group tetragon from the phylogenetic tree of the 16S rRNA gene clones from wild (W01ML), six-month (6mML) and two-year (2YML) aquacultured *M. laxissima* from the flow-through aquaculture system (Fig. 2.3). GenBank accession numbers are given in parenthesis. (*) Number of similar clones that share ≥ 99.5% similarity (Chapter 2).

Group name	No*	Clones
Unassigned bacteria I_2YML	2	**2YMLA07** (EF630327), **2YMLA07R** (EF630322)
Unassigned bacteria II_6mML	2	**6mML1F03** (EF630314), **6mML1F04** (EF630315)
Unassigned bacteria III_6mML	2	**6mML1G05** (EF630316), **6mML2E02** (EF630311)
Unassigned bacteria IV_2YML	4	**2YMLA01** (EF630325), **2YMLA05** (EF630326), **2YMLB12** (EF630331), **2YMLC03** (EF630333)
Unassigned bacteria V_2YML	3	**2YMLB04** (EF630328), **2YMLF05** (EF630335)
Unassigned bacteria VI_2YML	2	**2YMLB07** (EF630329), **2YMLB11** (EF630330)
***Planctomycetes* I_6mML**	2	**6mML1A04** (EF630282), **6mML2H11** (EF630281)
***Planctomycetes* II_2YML**	2	**2YMLA02R** (EF630288), **2YMLB08R** (EF630289)
***Planctomycetes* III_2YML**	2	**2YMLA04** (EF630291), **2YMLF06** (EF630293)
***Planctomycetes* IV_6mML**	3	**6mMLC03R** (EF630275), **6mMLF01R** (EF630276), **6mMLF02R** (EF630277)
***Planctomycetes* V_6mML**	2	**6mML1D03** (EF630284), **6mML1F01** (EF630287)
***Planctomycetes* VI_6mML**	2	**6mML1D11** (EF630285), **6mML1E11** (EF630286)
***Actinobacteria* I_2YML**	4	**2YMLB01R** (EF630259), **2YMLF08** (EF630267), **2YMLB03** (EF630260), **2YMLF09** (EF630268)

Table A.1, continued.

Group name	No*	Clones
***Actinobacteria* II_2YML**	6	**2YMLA01R** (EF630258), **2YMLC04** (EF630261), **2YMLD04** (EF630262), **2YMLD11** (EF630263), **2YMLE03** (EF630264), **2YMLE05** (EF630265)
***Calditrhrix*_6mML**	4	**6mMLF05R** (EF630317), **6mMLF02** (EF630319), **6mMLG04** (EF630320), **6mMLG08** (EF630321)
***Deltaproteobacteria*_6mML**	2	**6mML1G11** (EF630252), **6mML2C07** (EF630251)
***Cyanobacteria*_W01ML**	2	**W01ML2A07** (EF630216), **W01ML3G02** (EF630215)
***Chloroflexi*_6mML**	3	**6mML2F04** (EF630212), **6mML1C03** (EF630213), **6mML1E04** (EF630214)
***Bacteroidetes* II_6mML**	2	**6mML1B05** (EF630175), **6mML2A07** (EF630173)
***Bacteroidetes* III_6mML**	2	**6mML1C08** (EF630177), **6mML2F11** (EF630174)
***Bacteroidetes* IV_6mML**	3	**6mMLC07R** (EF630167), **6mMLE01R** (EF630168), **6mMLE06R** (EF630169)
***Gammaproteobacteria* I_6mML**	2	**6mML1E02** (EF630138), **6mML2D08** (EF630134)
***Gammaproteobacteria* II_6mML**	2	**6mML2C01** (EF630133), **6mML2G12** (EF630137)
***Gammaproteobacteria* III_6mML**	2	**6mML1H11** (EF630139), **6mML2G11** (EF630136)
***Gammaproteobacteria* IV_6mML**	2	**6mMLC04R** (EF630126), **6mMLE04R** (EF630131)
***Gammaproteobacteria* V_6mML**	3	**6mMLD01R** (EF630128), **6mMLD04R** (EF630129), **6mMLD05R** (EF630130)
***Gammaproteobacteria* VI_W01ML**	2	**W01ML3A02** (EF630100), **W01ML3A03** (EF630101)
***Gammaproteobacteria* VII _W01ML**	4	**W01ML2C07** (EF630118), **W01ML2D03** (EF630119), **W01ML2D08** (EF630120), **W01ML2F07** ((EF630123)
***Gammaproteobacteria* VIII_W01ML**	5	**W01ML2H01** (EF630125), **W01ML3F04** (EF630112), **W01ML1A09** (EF630090), **W01ML3D03** (EF630106), **W01ML3E05** (EF630109)
***Gammaproteobacteria* IX_W01ML**	8	**W01ML3F11** (EF630114), **W01ML1H11R** (EF630087), **W01ML1A07** (EF630089), **W01ML1D06** (EF630095), **W01ML3C10** (EF630104), **W01ML3D10** (EF630108), **W01ML1A12** (EF630091), **W01ML2G09** (EF630124)
***Gammaproteobacteria* X_W01ML**	21	**W01ML3B10** (EF630103), **W01ML2E07** (EF630122), **W01ML1E11** (EF630097), **W01ML1H09R** (EF630086), **W01ML1A03** (EF630088), **W01ML1B11** (EF630093), **W01ML1D04** (EF630094), **W01ML1E09** (EF630096), **W01ML1F03** (EF630098), **W01ML1H10 (**EF630099), **W01ML3B09** (EF630102), **W01ML3C12** (EF630105), **W01ML3D07** (EF630107), **W01ML3E06** (EF630110), **W01ML3E09** (EF630111), **W01ML3F09** (EF630113), **W01ML3H08** (EF630115), **W01ML3H11** (EF630116), **W01ML2C02** (EF630117), **W01ML2E02** (EF630121), **W01ML1B02** (EF630092)

Table A.1, continued.

Group name	No*	Clones
***Alphaproteobacteria* I_6mML**	2	**6mML1F08** (EF629975), **6mML1F11** (EF629976)
***Alphaproteobacteria* II_2YML**	2	**2YMLD08** (EF629983), **2YMLD09** (EF629984)
***Alphaproteobacteria* III_6mML**	3	**6mML2H07** (EF629968), **6mML2H04 (**EF629967), **6mMLC05R (**EF629955)
***Alphaproteobacteria* IV_6mML**	2	**6mML2G03** (EF629965), **6mMLD03R** (EF629956)
***Alphaproteobacteria* V_6mML**	2	**6mML2B0**5 (EF629962), **6mMLE05R** (EF629960)
***Alphaproteobacteria* VI_2YML**	2	**2YMLA06** (EF629980), **2YMLA10** (EF629981)
***Alphaproteobacteria* VII_6mML**	2	**6mML1C04** (EF629971), **6mML1F12** (EF629977))
***Alphaproteobacteria* VIII_6mML**	2	**6mML1D04** (EF629972), **6mML1E10** (EF629974)
***Alphaproteobacteria* IX_W01ML**	3	**W01MLB01** (EF629888), **W01ML1G07** (EF629903), **W01ML1H03** (EF629907)
***Alphaproteobacteria* X_W01ML**	3	**W01ML1F09R** (EF629883), **W01ML1D02** (EF629895), **W01ML3E11** (EF629925)
***Alphaproteobacteria* XI_W01ML**	2	**W01ML1G08** (EF629904), **W01ML2B06** (EF629942)
***Alphaproteobacteria* XII_W01ML**	14	**W01ML1B06** (EF629890), **W01ML1E03** (EF629897), **W01ML1G04** (EF629902)**, W01MLF1H08** (EF629910), **W01ML3A07** (EF629912), **W01ML3F05** (EF629926), **W01ML3G05** (EF629929), **W01ML3G08** (EF629930), **W01ML3H12** (EF629936), **W01ML2E05** (EF629944), **W01ML2E06** (EF629945), **W01ML2F04** (EF629947), **W01ML2F11** (EF629950), **W01ML3E01** (EF629921)
***Alphaproteobacteria* XIII_W01ML**	19	**W01ML3E07** (EF629923), **W01ML3B02** (EF629914), **W01ML2H05 (**EF629954), **W01ML1G11** (EF630166), **W01ML3A10** (EF629913), **W01ML3B03 (**EF629915), **W01ML3B04 (**EF629916), **W01ML3B12** (EF629917), **W01ML3C01 (**EF629918), **W01ML3E10** (EF629924), **W01ML3H05 (**EF629934), **W01ML2B04 (**EF629941), **W01ML3H09** (EF629935), **W01ML2E01 (**EF629943), **W01ML2E11 (**EF629946), **W01ML1C06 (**EF629892), **W01ML1D01 (**EF629894), **W01ML2F06 (**EF629948), **W01ML2F08** (EF629949)
***Alphaproteobacteria* XIV_W01ML**	22	**W01ML3G11** (EF629933), **W01ML2A05** (EF629938), **W01ML2G07** (EF629951), **W01ML3G10** (EF629932), **W01ML3F10** (EF629928), **W01ML3F07** (EF629927), **W01ML3E04** (EF629922), **W01ML3D11** (EF629920), **W01ML3C09** (EF629919), **W01ML3A01** (EF629911), **W01ML1H05** (EF629909), **W01ML1H04** (EF629908), **W01ML1G09** (EF629884), **W01ML1G02** (EF629901), **W01ML1F11** (EF629900), **W01ML1E06** (EF629898), **W01ML1C10** (EF629893), **W01ML1B08 (**EF629891), **W01ML1A04** (EF629887), **W01ML1D10** (EF629896), **W01ML1G09R** (EF629884), **W01ML1G10** (EF629885)

Table A.2. Number and names of clones in each bacterial group tetragon in the phylogenetic tree of the 16S rRNA gene clones from wild (W04ML) and one-month aquacultured (1mAML) *M. laxissima* from the recirculating aquaculture system (Fig. 2.6). GenBank accession numbers are given in parenthesis. (*) Number of similar clones that share ≥ 99.5% similarity (Chapter 2).

Group name	No*	Clones
***Actinobacteria*_W04ML**	2	**W04MLE10** (EF630269), **W04MLG11R** (EF630271)
Unassigned bacteria_1mAML	2	**1m04AMLB11** (EF630339), **1m04AMLD04** (EF630340)
***Cyanobacteria* I_1mAML**	4	**1m04AMLB07** (EF630217), **1m04AMLB08** (EF630218), **1m04AMLC12** (EF630219), **1m04AMLH06** (EF630221)
***Cyanobacteria* II_W04ML**	2	**W04MLB09** (EF630223), **W04MLD8R** (EF630237)
***Cyanobacteria* III_W04ML**	15	**W04MLB12** (EF630227), **W04MLG10** (EF630226), **W04MLC06** (EF630229), **W04MLE12** (EF630228), **W04MLC11R** (EF630231), **W04MLF06** (EF630230), **W04MLC12R** (EF630232), **W04MLD7R** (EF630233), **W04MLD11R** (EF630234), **W04MLF2R** (EF630235), **W04MLD9R** (EF630238), **W04MLF3R** (EF630236), **W04MLA09** (EF630222), **W04MLB10** (EF630224), **W04MLE09** (EF630225)
***Bacteroidetes* I_1mAML**	2	**1m04AMLA03** (EF630185), **1m04AMLF08** (EF630194)
***Bacteroidetes* II_1mAML**	2	**1m04AMLF06** (EF630193), **1m04AMLG01** (EF630195)
***Bacteroidetes* III_1mAML**	2	**1m04AMLB4R** (EF630201), **1m04AMLB9R** (EF630202)
***Bacteroidetes* IV_1mAML**	4	**1m04AMLB10** (EF630187), **1m04AMLD09** (EF630188), **1m04AMLF12R** (EF630203), **1m04AMLG02** (EF630196)
***Bacteroidetes* V_1mAML**	7	**1m04AMLB06** (EF630186), **1m04AMLD10** (EF630189), **1m04AMLE12** (EF630191), **1m04AMLF04** (EF630192), **1m04AMLH08** (EF630198), **1m04AMLE8R** (EF630205), **1m04AMLE9R** (EF630207)

Table A.2, continued.

Group name	No*	Clones
***Alphaproteobacteria* I_1mAML**	2	**1m04AMLC6R** (EF630049), **1m04AMLD05** (EF630030)
***Alphaproteobacteria* II_1mAML**	2	**1m04AMLA07** (EF630025), **1m04AMLD03** (EF630029)
***Alphaproteobacteria* III_W04ML**	2	**W04MLB11** (EF630057), **W04MLF05** (EF630073)
***Alphaproteobacteria* IV_W04ML**	2	**W04MLD01** (EF630064), **W04MLD02** (EF630061)
***Alphaproteobacteria* V_W04ML**	13	**W04MLG09** (EF630054), **W04MLE5R** (EF630081), **W04MLA11R** (EF630078), **W04MLA8R** (EF630077), **W04MLD06** (EF630072), **W04MLD03** (EF630071), **W04MLC07** (EF630070), **W04MLA05** (EF630066), **W04MLF01** (EF630065), **W04MLC01** (EF630063), **W04MLE02** (EF630062), **W04MLA01** (EF630060), **W04MLH08** (EF630055)
***Alphaproteobacteria* VI_W04ML**	2	**W04MLD12R** (EF630079)**, W04MLH05** (EF630076)
***Alphaproteobacteria* VII_1mAML**	2	**1m04AMLH12** (EF630037), **1m04AMLH7R** (EF630041)
***Alphaproteobacteria* VIII_1mAML**	6	**1m04AMLE01** (EF630031), **1m04AMLF01** (EF630032), **1m04AMLA4R** (EF630038), **1m04AMLD1R** (EF630042), **1m04AMLC9R** (EF630053), **1m04AMLH4R** (EF630052)
***Alphaproteobacteria* IX_1mAML**	2	**1m04AMLC1R** (EF630043), **1m04AMLH2R** (EF630050)
***Alphaproteobacteria* X_W04ML (1) & 1mAML (4)**	5	**1m04AMLC05** (EF630027), **1m04AMLC08** (EF630028), **1m04AMLG06** (EF630034), **1m04AMLG05** (EF630033), **W04MLH03** (EF630075)
***Alphaproteobacteria* XI_W04ML (2) & 1mAML (6)**	6	**1m04AMLA10** (EF630026), **1m04AMLB3R** (EF630040), **1m04AMLC2R** (EF630045), **1m04AMLG12R** (EF630046), **W04MLA12** (EF630056), **W04MLD10R** (EF630083)

Table A.3. Number and names of clones in each bacterial group tetragon in the phylogenetic tree (Fig. 3.4) of the 16S rRNA gene clones from *I. strobilina* sponges collected from the wild (W04IS) and maintained for three months (3m04AIS) in the aquaculture system. GenBank accession numbers are given in parenthesis. (*) Number of similar clones that share ≥ 99.5% similarity (Chapter 3).

Group name	No*	Clones
***Planctomycetes* I_9mAIS**	2	**9m05AISD09** (EF629821), **9m05AISE09** (EF629823)
***Planctomycetes* II_9mAIS**	2	**9m05AISB07** (EF629819), **9m05AISC08** (EF629820)
***Planctomycetes* III_3mAIS(2)-9mAIS(2)**	4	**3m04AISB2R** (EF629813), **3m04AISF1R** (EF629816), **9m05AISD12** (EF629822), **9m05AISF05** (EF629824)
***Acidobacteria* I_W04IS**	6	**W04IS5C06** (EF629592), **W04IS4E06** (EF629587), **W04IS4A02** (EF629581), **W04IS4E05** (EF629586), **W04IS5A01** (EF629591), **W04ISE5R** (EF629594)
***Acidobacteria* II_W04IS**	9	W04IS4G08 **(EF629589)**, W04IS4B10 **(EF629583)**, W04IS4C07 **(EF629584)**, W04IS4C12 **(EF629585)**, W04IS4A07 **(EF629582)**, W04IS4E12 **(EF629588)**, W04IS4H01 **(EF629590)**, W04ISE2R **(EF629593)**, W04ISG6R **(EF629596)**
***Gammaproteobacteria* I_9mAIS**	9	**9m05AISB08** (EF629801), **9m05AISD05** (EF629805), **9m05AISE06** (EF629806), **9m05AISE12** (EF629807), **9m05AISF07** (EF629808), **9m05AISG08** (EF629809), **9m05AISH07** (EF629810), **9m05AISB11** (EF629802), **9m05AISC06** (EF629804)
***Alphaproteobacteria* I_9mAIS**	2	**9m05AISD03** (EF629659), **9m05AISF06** (EF629672)
***Alphaproteobacteria* II_9mAIS**	2	**9m05AISA03** (EF629643), **9m05AISG06** (EF629677)
***Alphaproteobacteria* III_3mAIS(1)-9mAIS(2)**	3	**3m04AISB04** (EF629611), **9m05AISC04** (EF629653), **9m05AISA09** (EF629647)
***Alphaproteobacteria* IV_9mAIS**	2	**9m05AISF09** (EF629673), **9m05AISG10** (EF629680)

Table A.3, continued.

Group name	No*	Clones
***Alphaproteobacteria* V_9mAIS**	18	**9m05AISD06** (EF629661), **9m05AISA06** (EF629645), **9m05AISA07** (EF629646), **9m05AISC10** (EF629655), **9m05AISE05** (EF629665), **9m05AISG11** (EF629681), **9m05AISA04** (EF629644), **9m05AISB04** (EF629649), **9m05AISB12** (EF629651), **9m05AISD02** (EF629658), **9m05AISE07** (EF629666), **9m05AISF01** (EF629670), **9m05AISF11** (EF629674), **9m05AISG04** (EF629675), **9m05AISG09** (EF629679), **9m05AISH12** (EF629682), **9m05AISC09** (EF629654), **9m05AISE10** (EF629668)
***Alphaproteobacteria* VI_3mAIS**	3	**3m04AISE12** (EF629620), **3m04AISB5R** (EF629628), **3m04AISF12** (EF629622)
***Alphaproteobacteria* VII_3mAIS**	3	**3m04AISE7R** (EF629636), **3m04AISD10** (EF629617), **3m04AISH5R** (EF629639)
***Alphaproteobacteria* VIII_3mAIS**	11	**3m04AISB01** (EF629609), **3m04AISC6R** (EF629631), **3m04AISC9R** (EF629632), **3m04AISG02** (EF629623), **3m04AISE6R** (EF629635), **3m04AISH3R** (EF629638), **3m04AISH6R** (EF629640), **3m04AISH7R** (EF629641), **3m04AISA06** (EF629608), **3m04AISC01** (EF629614), **3m04AISE08** (EF629619)
***Alphaproteobacteria* IX_3mAIS**	2	**3m04AISA12R** (EF629627), **3m04AISD02** (EF629616)
***Alphaproteobacteria* X_9mAIS**	3	**9m05AISC02** (EF629652), **9m05AISE08** (EF629667), **9m05AISF03** (EF629671)
***Alphaproteobacteria* XI_3mAIS**	2	**3m04AISG10** (EF629624), **3m04AISH12R** (EF629642)
***Alphaproteobacteria* XII_3mAIS(2)-9mAIS(1)**	3	**3m04AISC10R** (EF629633), **3m04AISB12R** (EF629629), **9m05AISD04** (EF629660)
***Cyanobacteria* I_3mAIS**	4	**3m04AISB09** (EF629788), **3m04AISG4R** (EF629791), **3m04AISC08** (EF629790), **3m04AISF10R** (EF629792)
***Cyanobacteria* II_W04IS**	3	**W04IS4G07** (EF629782), **W04IS5C09** (EF629783), **W04IS5C10** (EF629784)
***Cyanobacteria* III_W04IS**	2	**W04ISG3R** (EF629786), **W04ISG4R** (EF629787)

Table A.3, continued.

Group name	No*	Clones
***Actinobacteria* I_W04IS**	2	**W04IS5B10** (EF629600), **W04IS5C11** (EF629601)
***Actinobacteria* II_W04IS**	5	**W04ISA6R** (EF629603), **W04ISB6R** (EF629604), **W04IS4E07** (EF629597), **W04IS4F11** (EF629599), **W04ISC3R** (EF629602)
***Bacteroidetes* I_W04IS**	2	**W04ISC2R** (EF629683), **W04ISG1R** (EF629684)
***Bacteroidetes* II_3mAIS**	2	**3m04AISA03** (EF629686), **3m04AISD9R** (EF629695)
***Bacteroidetes* III_3mAIS**	9	**3m04AISC11** (EF629690), **3m04AISA01** (EF629685), **3m04AISC05** (EF629689), **3m04AISA8R** (EF629691), **3m04AISB10R** (EF629694), **3m04AISD11R** (EF629696), **3m04AISE10R** (EF629698), **3m04AISF9R** (EF629701), **3m04AISG5R** (EF629699)
***Clostridia* I_9mAIS**	2	**9m05AISA02** (EF629776), **9m05AISC07** (EF629779)
***Clostridia* II_9mAIS**	2	**9m05AISA10** (EF629777), **9m05AISF12** (EF629780)
***Chloroflexi* I_W04IS**	3	**W04IS5A09** (EF629734), **W04IS5C04 (EF629740), W04IS5E04** (EF629744)
***Chloroflexi* II_W04IS**	3	**W04IS5B07** (EF629736), **W04IS5C02** (EF629739), **W04ISB1R** (EF62974)
***Chloroflexi* III_W04IS**	4	**W04IS5B12** (EF629738), **W04ISB4R** (EF629752), **W04ISE1R** (EF629756), **W04ISA5R** (EF629762)
***Chloroflexi* IV_W04IS**	18	**W04IS4A09** (EF629706), **W04IS4B06** (EF629711), **W04IS4C02** (EF629713), **W04IS4D08** (EF629720), **W04IS5A02** (EF629733), **W04IS5C07** (EF629741), **W04IS5D01** (EF629742), **W04IS5E12** (EF629745), **W04ISE4R** (EF629757), **W04ISF1R** (EF629758), **W04ISH1R** (EF629760), **W04ISH2R** (EF629761), **W04ISC6R** (EF629764), **W04ISD5R** (EF629765), **W04ISD6R** (EF629766), **W04ISH5R** (EF629770), **W04ISH6R** (EF629771), **W04IS5C5RF (**EF629772)
***Chloroflexi* V_W04IS**	18	**W04IS4A05** (EF629705), **W04IS4A10** (EF629707), **W04IS4A11** (EF629708), **W04IS4B01** (EF629709), **W04IS4B04** (EF629710), **W04IS4C05** (EF629715), **W04IS4C08** (EF629716), **W04IS4C09** (EF629717), **W04IS4D07** (EF629719), **W04IS4D10** (EF629721), **W04IS4E04** (EF629722), **W04IS4E11** (EF629723), **W04IS4F01** (EF629724), **W04IS4G10** (EF629729), **W04IS5B06** (EF629735), **W04ISD2R** (EF629754), **W04ISF3R** (EF629759), **W04ISB5R** (EF629763)
***Chloroflexi* VI_W04IS**	20	**W04IS4C03** (EF629714), **W04IS4C10** (EF629718), **W04IS4F02** (EF629725), **W04IS4F04** (EF629726), **W04IS4F06** (EF629727), **W04IS4F12** (EF629728), **W04IS4H06** (EF629730), **W04IS4H10** (EF629732), **W04IS5B09** (EF629737), **W04IS5D12 (EF629743), W04ISA2R** (EF629746), **W04ISA3R** (EF629747), **W04ISA4R** (EF629748), **W04ISB2R** (EF629750), **W04ISB3R** (EF629751), **W04ISC1R** (EF629753), **W04ISD4R** (EF629755), **W04ISF5R** (EF629767), **W04ISF6R** (EF629768), **W04ISG5R** (EF629769)

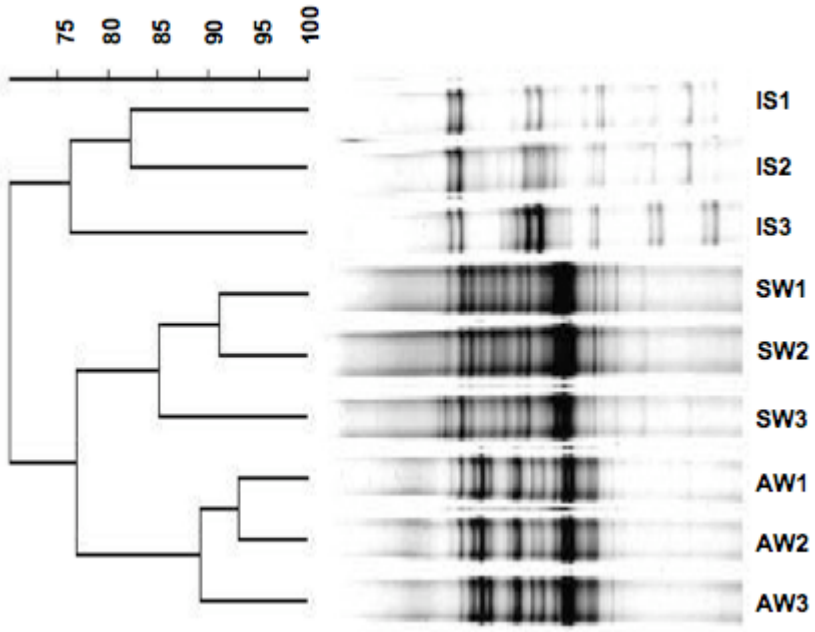


Fig. A.1. Dendrogram constructed from DGGE profiling of PCR-amplifed 16S rRNA of bacterial communities associated with three different individuals of *I. strobilina* collected from the wild (Lanes IS1-3), three replicate seawater samples (SW1-3) and three replicate water samples from the aquaculture system (AW1-3). Jukes and Cantor's model was used for distance calculation, and UPGMA was used for dendrogram construction (Chapter 3).

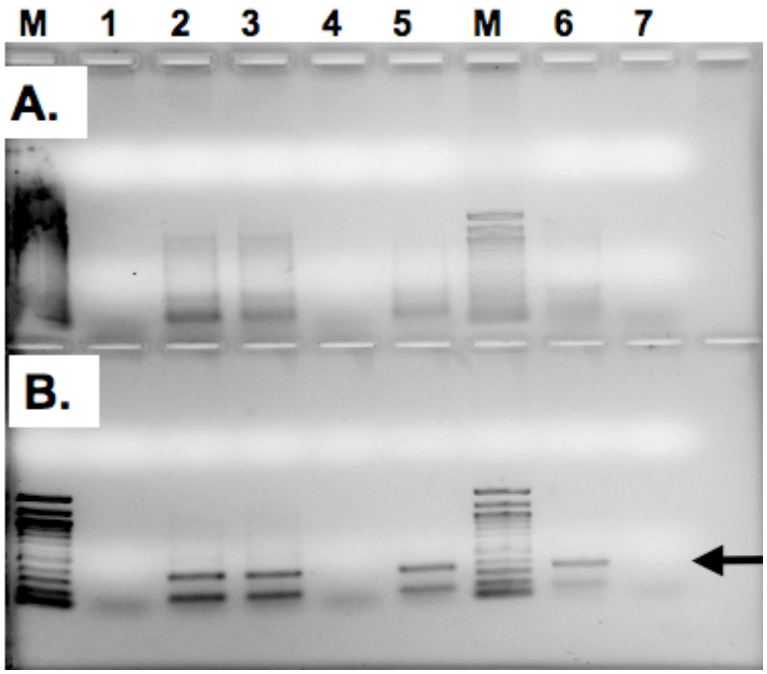


Fig. A.2. 1.2% agarose gel electrophoresis image of PCR amplification of *nifH* genes from the two seawater samples SWA and SWB. Lanes1, 2 and 3 are undiluted DNA, 1:10 and 1:100 diluted DNA of SWA, respectively. Lanes 4, 5 and 6 are undiluted DNA, 1:10 and 1:100 diluted DNA of SWB, respectively. Lane 7 is a negative control. M is a 100 bp marker. Panel A indicates direct PCR with desalted primers and panel B indicates direct PCR with HPLC-purified primers. The arrow indicates the bands corresponding to the *nifH* PCR products of the right size (~360 bp) (Chapter 4). The lower smaller bands are non-specific amplification products.

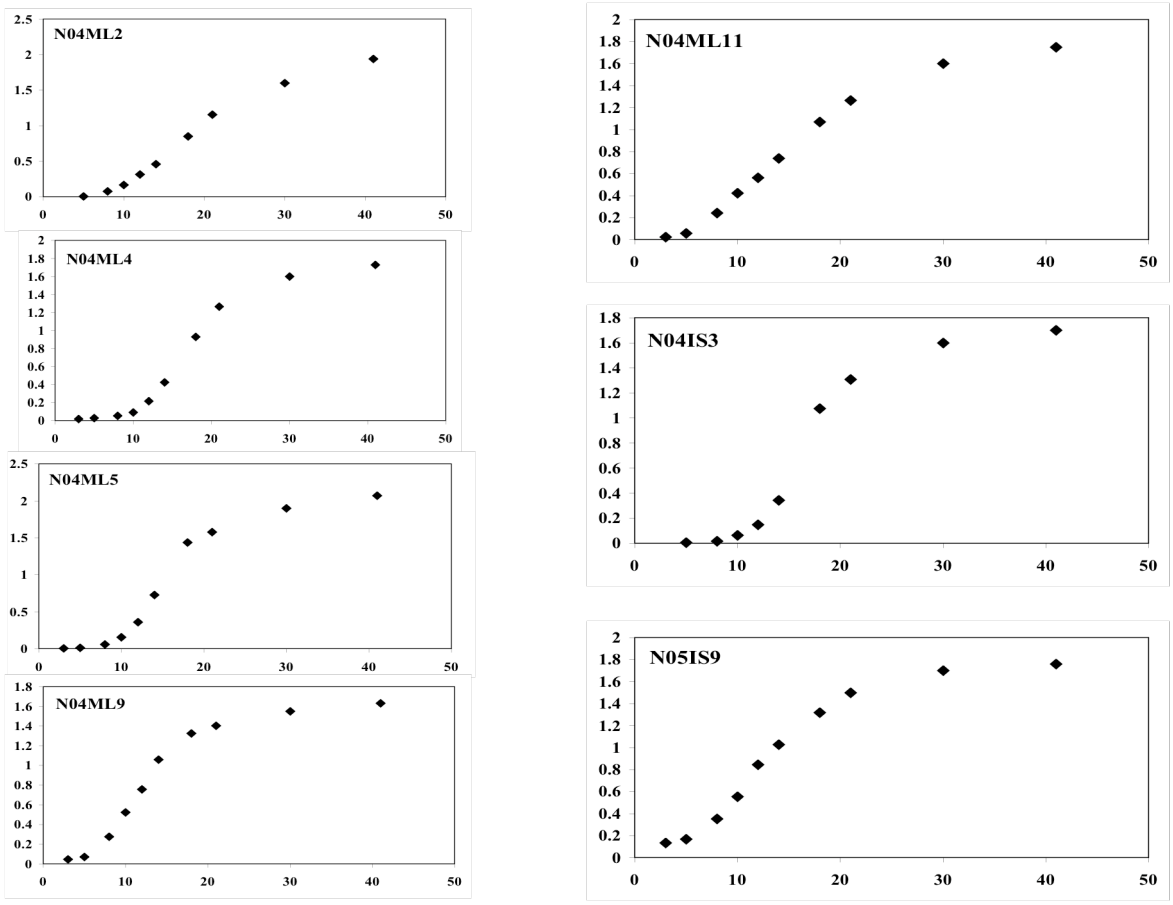


Fig. A.3. Growth curves of nine AHL-producing roseobacters (Chapter 5). X axis = h and y axis = OD_{600}.

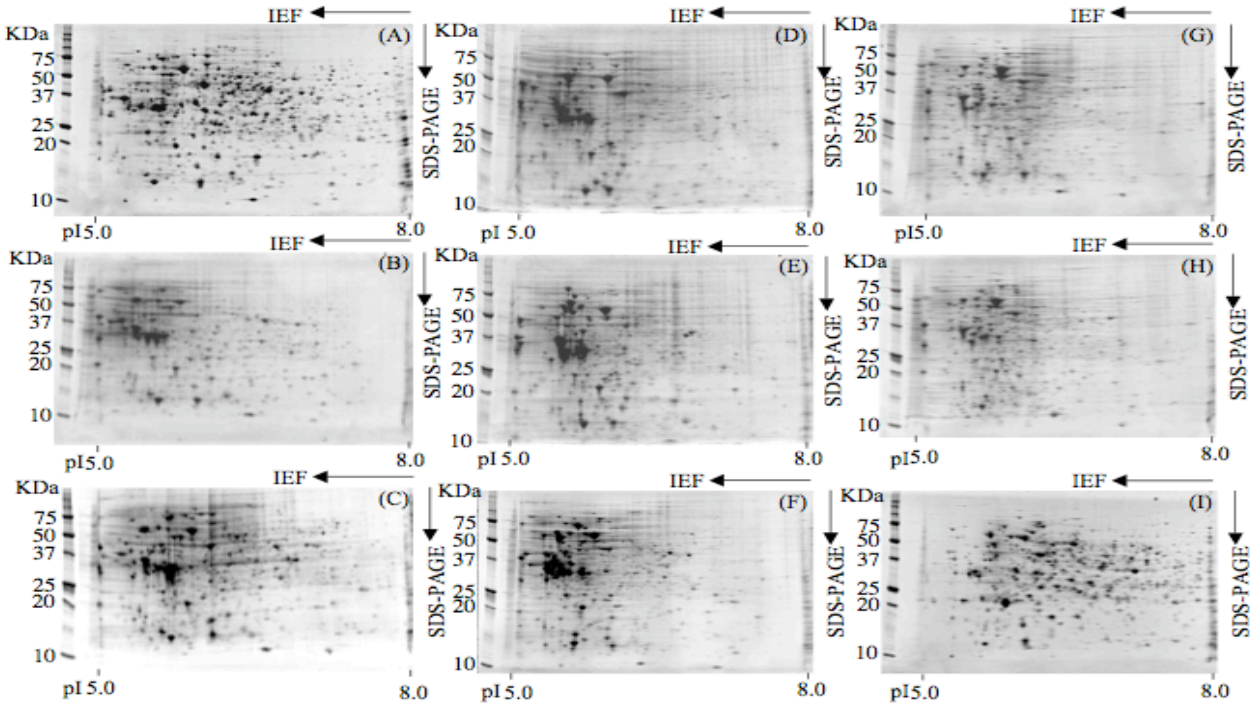


Fig. A.4. 2DGE protein patterns of soluble proteins from AHL-producing SR roseobacters isolated from *M. laxissima* and *I. strobilina*. (A) KLH11, (B) N04ML9, (C) N04ML4, (D) N04IS3, (E) N05IS9, (F) N04ML5, (G) N04ML2, (H) N04ML11 and (I) *S. pomeroyi* DSS-3 (Chapter 5).

Appendix B. Protocols

I. Extraction of DNA from sponge tissues using bead beater method

Adapted from "Pitcher, D. G., N. A. Saunders, R. J. Owen. 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Letters Appl. Micro. **8**:151-156".

1. Take a vertical cross section 1 cm^3 of the sponge.
2. Freeze-dry the sponge tissue and grind it to fine powder.
3. Sterilize Zirconia/silica beads by autoclaving. Add the sponge powder into the bead beater chamber. Add an equal volume of 0.1 mm and 1 mm Zirconia/Silica beads to fill half of the chamber. Cover the beads and tissue with TE buffer. Cool the bead chamber by surrounding with crushed ice.
4. Run bead beater for 3 × 30 sec cycles with one minute in between cycles. Pipette the liquid out of chamber into 50 ml conical tubes.
5. While keeping sample on ice, add an equal volume of guanidium thiocyanate buffer and mix gently (60 g of guanidium thiocyanate in 20 ml of 100 mM EDTA heat to 65ºC, cool, add water to a final volume of 100 ml and then filter sterilize the buffer).
6. Add 10 M ammonium acetate to a 2.5 M final concentration.
7. Add double volume of phenol (pH 6.0) and centrifuge at 10000×g for 15 min. Transfer aqueous phase to a new tube.

8. Add equal volume of chloroform/ isoamylalcohol (24:1) and centrifuge at 10000×g for 10 min. Transfer aqueous phase to a new tube.
9. Add equal volume of chloroform/ isoamylalcohol (24:1) and centrifuge at 10000×g for 10 min.
10. Transfer the supernatant to sterile 15 ml conical tubes.
11. Precipitate with half volume of isopropanol. Store tube at -20°C overnight.
12. Pellet at 13000×g for 20 min.
13. Wash the pellet twice with 70% ethanol.
14. Air-dry the pellets overnight or in Speedyvac.
15. Resuspend DNA in PCR water and store at -20°C.

II. Isolation of nucleic acids from marine water samples by using Sterivex filters

Adapted from "Somerville, C. C., I. T. Knight, W. L. Straube, and R. R. Colwell**.** 1989. Simple, rapid method for direct isolation of nucleic acids from aquatic environments. Appl. Environ. Microbiol. **55:**548-554".

A. Filtration procedure

Materials:

1. Sterivex-GS 0.22 μm filter, Type SVGS01015 (Millipore Corp., Bedford, MA, USA).
2. Peristaltic pump
3. Sterile Teflon pipe

4. SET butter (20% glucose, 50 mM EDTA, and 50 mM Tris-HCl pH 7.6)
5. 3 ml syringe (luer lok)
6. 10 ml syringes (luer lok)
7. 10 ml plastic pipettes
8. 2 l measuring cylinder

Protocol:

1. Connect Sterivex filter to luer lock fitting on the outlet of sterile Teflon pipe fitted to the peristaltic pump.
2. Insert sterile 10 ml plastic pipette into pipe inlet and place in water sample. Sterile techniques are required.
3. Filter appropriate quantity of water at approximately 100 ml/min. Determine the volume of water filtered with the measuring cylinder.
4. Remove Sterivex, blow out remaining water off the filter using a sterile 10 ml syringe and flush gently with 5 ml SET buffer using another sterile 10 ml syringe.
5. Blow out SET buffer with a 10 ml sterile syringe and cap both ends of Sterivex filter. A disposable 3 ml syringe (luer lok) is useful for this procedure. The exit can be capped with the trimmed syringe tip and the entrance port can be capped with the luer end of the syringe.
6. Store filter at -20°C.

B. Nucleic acids recovery

Materials:

1. SET buffer
2. Lysozyme (fresh preparation of 5 mg/ml in 10 mM Tris-HCL pH 8.0, 1 mM EDTA, and 10 mM NaCl)
3. Proteinase K (20 mg/ml in deionized water)
4. 3 ml syringe (luer lok)
5. 15 ml sterile Corex tube
6. Phenol stabilized in TE buffer pH 7.8-8.0
7. Phenol:chloroform:isoamyl alcohol (25:24:1)
8. Chloroform
9. 5 M NaCl
10. 100% Ethanol
11. 70% Ethanol
12. TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)

Protocol:

1. Thaw the Sterivex filter and add 1.8 ml SET buffer into the inlet of the filter unit with a 25-gauge needle and 3 ml syringe.
2. Add 62 μl lysozyme solution and incubate on ice for 15 min.
3. Add 16 μl 25% SDS and incubate with slow shaking for 1 h at room temperature.
4. Add 50 μl proteinase K and incubate with slow shaking at least 2 h at room temperature.
5. Use a 3 ml syringe on inlet port and withdraw lysate.
6. Add 1 ml SET buffer to empty filter, recap and shake gently for 5 minutes.
7. Pull off the rinse and pool with the rest of lysate.

8. Put the lysate in sterile 15 ml Corex tube.
9. Do a phenol/chloroform extraction (equal volume).
10. Do a chloroform extraction (equal volume). Repeat steps 8 and 9 if necessary.
11. Add 2 vol 100% ethanol, and store at -20°C overnight.
12. Spin down precipitate at 13000×g for 30 minutes.
13. Wash the pellet twice with 70% ethanol. Spin at 13000×g for 5 minutes.
14. Wash the pellet with 100% ethanol. Spin at 13000×g for 5 minutes.
15. Air-dry overnight at room temperature or in Speedyvac.
16. Resuspend in 200 μl TE buffer and store at -20°C.

III. Denaturing gradient gel electrophoresis

System: Dcode Universal Mutant Detection System (Bio-Rad).

Solutions: Acrylamide stock solutions for 6% acrylamide gel.

1. 0% Denaturing acrylamide

    15 ml Acrylamide solution

    2 ml 50 × TAE

    83 ml milli Q water.

2. 100% Denaturing acrylamide

    15 ml Acrylamide solution

    2 ml 50 × TAE

    40 ml Demonized formamide

    42 g Urea

Milli Q water to 100 ml

Dissolve in 50°C water bath.

Store in side arm vacuum flask covered in foil at 4°C.

Both solutions need to be de-gassed before each use for at least 15 minutes.

3. 10% Ammonium persulphate

   0.1 g Ammonium persulfate

   Milli Q water to 1 ml

4. TEMED

5. 6 × Gel loading dye

6. 1 × TAE buffer (~ 7 l)

Casting and electrophoresis:

1. Clean glass plate with ethanol and assemble gel sandwich, lock into position on casting tray.
2. Make Hi and Low denaturing concentrations of acrylamide solution in separate 50 ml tubes.

   Hi denaturing solution (70%)

   8.4 ml of 100% Acrylamide solution

   3.6 ml of 0% Acrylamide solution

   Low denaturing solution (40%)

   4.8 ml of 100% Acrylamide solution

   7.2 ml of 0% Acrylamide solution.

3. In a separate 50 ml tube, add 5 ml of 0% acrylamide for the formation of the stacking layer.

4. Close the tap, which connects the chambers to the tubing and close the channel between the chambers. Ensure that the pump is working and going in the correct direction.

5. Add 24 µl of ammonium persulphate solution to the Hi and Low denaturing solutions.

6. Add 9 µl of TEMED to the Hi and Low denaturing solutions, mix and transfer into chambers of gradient former.

7. Turn on the magnetic stirrer and open the channel between the chambers.

8. Open the tap to the tubing.

9. Turn on the pump.

10. Let the acrylamide settle for a few minutes between the plates and tap out any bubbles that may have formed.

11. To form the stacking layer, add 24 µl of ammonium persulphate and 9 µl of TEMED to the 0% acrylamide solution and mix well.

12. Using a 1 ml pipette apply this solution to the top of the gradient gel.

13. Once the stacking layer is 3-4 mm from the top of the plates gently insert the comb, making sure that there are no bubbles between or underneath wells. Leave gel to set for at least 3 hours.

14. Fill the electrophoresis tank with 7 l of 1 × TAE buffer and heat the buffer 60ºC.

15. Add the 5µl gel loading buffer to 50µl PCR products and load them on the gel.

16. Run the gel at 60 V for 16-18 h.

IV. Amplification of *nifH* genes from the total DNA of sponge samples using a nested PCR approach

Adapted from "Zehr, J. P., and L. A. McReynolds**.** 1989. Use of degenerate oligonucleotides for amplification of the nifH gene from the marine cyanobacterium *Trichodesmium thiebautii*. Appl. Environ. Microbiol. **55:**2522-2526" and "Steward, G. F., J. P. Zehr, R. Jellison, J. P. Montoya, and J. T. Hollibaugh**.** 2004. Vertical distribution of nitrogen-fixing phylotypes in a meromictic, hypersaline lake. Microb. Ecol. **47:**30-40".

Primers (HPLC-purified):

1. nifH32F (5̀-TGAGACAGATAGCTATYTAYGGHAA-3̀).
2. nifH623R (5̀-GATGTTCGCGCGGCACGAADTRNATSA-3̀).
3. nifH1 (5̀-TGYGAYCCNAARGCNGA-3̀).
4. nifH2 (5̀-ADNGCCATCATYTCNCC-3̀).

Master mix for the first round PCR reactions:

10 × High Fidelity PCR Buffer	5 µl
50 mM $MgSO_4$	2 µl
10 mM dNTPs	1 µl
nifH32F (100 pm/ µl)	1 µl
nifH623R (100 pm/ µl)	1 µl
PCR H_2O	37.8 µl
Platinum ®*Taq* polymerase High Fidelity	0.2 µl

Add 1 µl of (DNA/ cDNA) to 49 µl of the master mixture.

Master mixture for the second-round PCR reactions:

10 × High Fidelity PCR Buffer	5 µl
50 mM $MgSO_4$	2 µl
10 mM dNTPs	1 µl
nifH1 (100 pm/ µl)	1 µl
nifH2 (100 pm/ µl)	1 µl
PCR H_2O	37.8 µl
Platinum ®*Taq* polymerase High Fidelity	0.2 µl

Add 2 µl of the first PCR reaction amplicons to 48 µl of the master mix.

PCR temperature cycling conditions:

In the first-round PCR, the cycling conditions were as follows: pre-denaturation step at 94°C 5 min, followed by 30 cycles performed at 94°C for 1 min, 50°C for 1 min,

and 72°C for 1 min. Cycling was followed by 7 min extension 72°C. The conditions were the same for the second-round PCR except that the annealing temperature was 57°C.

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