

SPONGE-ASSOCIATED BACTERIA: SPECIFICITY, DIVERSITY AND ANTIMICROBIAL POTENTIAL



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SUMMARY

In the present study sponge-bacteria associations as well as the antimicrobial potential of sponge-associated bacteria was investigated. Culture independent methods were applied to examine diversity, specificity and temporal consistency of sponge-associated bacterial communities while culture-based methods were used to address the antimicrobial potential of bacteria associated with different Mediterranean sponges. For molecular studies, *Chondrilla nucula* and *Tethya aurantium* were used as model organisms for sponges containing an apparent high number of associated bacteria ('bacteriosponges') and sponges without apparent associated bacteria ('bacteria-free' sponges), respectively. Stable and specific bacterial associations were demonstrated for both sponges suggesting that associations between sponges and bacteria are a common occurrence and 'bacteria-free' sponges might not exist.

Sponge-microbe associations are proposed as 'microbial consortia inhabiting the micro-environment sponge'. The association of *Bdellovibrio*-like *Deltaproteobacteria* as well as putatively ammonia-oxidizing archaea with *C. nucula* indicate a high complexity of possible interactions not only between sponge and associated bacteria but also within the associated bacterial community. High sequence similarities between sponge-derived and seawater-derived 16S rDNA sequences reflect filter-feeding by sponges and emphasise the need for comparative controls. However, distinct bacterial communities associated with two different sponge-species living in close proximity in their natural habitat demonstrate low impact of the ambient seawater for sponge-bacteria associations. The bacterial populations in the two model organisms differed in community structure and diversity. Bacterial sequences obtained from *C. nucula* were affiliated to other sponge-derived sequences in large parts (79%), while only a minor fraction (22%) of *T. aurantium*-associated phylotypes shared similarity with known sponge-associated bacteria. Further, comparison of cortex and endosome revealed spatial differences in the *T. aurantium*-associated bacterial community.

Antimicrobially active bacteria were isolated from different sponges, demonstrating that cultivable sponge-associated bacteria exhibit a high potential for the search of new antimicrobial substances with biotechnological application. The cultivable bacterial communities associated with at least six different sponges were found to be dominated by a single alphaproteobacterial phylotype revealing antimicrobial activity. Kind of association, specificity as well as way of acquisition is still uncertain and critically discussed in this study.

ZUSAMMENFASSUNG

In der vorliegenden Arbeit wurden Assoziationen mariner Bakterien mit Schwämmen und das antimikrobielle Potential dieser schwamm-assoziierten Bakterien untersucht. Diversität, Spezifität und Stabilität der Assoziationen wurden mithilfe molekularbiologischer Methoden untersucht, während das antimikrobielle Potential assoziierter Bakterien verschiedener Mittelmeerschwämme mittels Isolationsmethoden bestimmt wurde. Am Beispiel der Mittelmeerschwämme *Chondrilla nucula* und *Tethya aurantium* wurden assoziierte bakterielle Gemeinschaften sogenannter 'Bakterienschwämme' und 'bakterien-freier' Schwämme studiert. Es wurden stabile, spezifische bakterielle Assoziationen in beiden Schwämmen nachgewiesen. Dies deutet darauf hin, dass Schwämme unter natürlichen Bedingungen regelmäßig mit Bakterien in Assoziation leben und es keine 'bakterien-freien' Schwämme gibt.

Schwämme können als bakterieller Lebensraum bezeichnet werden und fungieren nicht ausschließlich als Partner oder Wirt spezifischer Assoziationen. Sowohl *Bdellovibrio*-ähnliche Deltaproteobakterien als auch möglicherweise Ammonium-oxidierende Archaea wurden in Assoziation mit *C. nucula* nachgewiesen und deuten auf eine komplexe bakterielle Gemeinschaft hin, die eine Vielzahl verschiedener Interaktionen nicht nur zwischen Schwamm und Bakterium, sondern auch zwischen assoziierten Mikroorganismen beinhaltet. Da Schwämme filtrierende Tiere sind, dringen Bakterien des Umgebungswassers mit dem Wasser in das Kanalsystem der Schwämme ein. Jedoch weisen die bakteriellen Gemeinschaften unterschiedlicher Schwämme des gleichen Habitats deutliche Unterschiede auf. Dies deutet darauf hin, dass die gefundenen bakteriellen Gemeinschaften schwammart-spezifisch sind und der Einfluss des umgebenden Meerwassers gering ist. Die bakteriellen Gemeinschaften der untersuchten Schwämme unterscheiden sich sowohl in der Zusammensetzung als auch in der Diversität spezifisch assoziierter Bakterien. In *C. nucula* weisen 79% der bakteriellen Sequenzen Ähnlichkeit zu bekannten schwamm-assoziierten Bakterien auf, während es in *T. aurantium* nur 22% sind. Ferner wurde in *T. aurantium* eine räumliche Verteilung der bakteriellen Gemeinschaft innerhalb des Schwammes nachgewiesen.

Schwamm-assoziierte Bakterien stellen ein hohes Potential zur Isolierung neuer Substanzen für die biotechnologische Nutzung dar. Antimikrobiell wirksame Bakterien wurden aus unterschiedlichen Mittelmeerschwämmen als dominante Arten der kultivierbaren bakteriellen Gemeinschaft isoliert. In mindestens sechs unterschiedlichen Schwämmen dominierte ein einziger Phylotyp aus der Gruppe der Alphaproteobakterien mit

antimikrobiellen Eigenschaften die assoziierte Gemeinschaft. Art der Assoziation, Spezifität und Art der Aufnahme in den Schwamm sind noch unbekannt und werden in dieser Arbeit kritisch diskutiert.

GENERAL INTRODUCTION

THEORETICAL BACKGROUND

Sponges

Sponges (phylum Porifera, Latin: ‘pore bearer’) live primarily in marine environments, but also inhabit freshwater habitats such as lakes and rivers, as well as fjords and estuaries (Frost 1991). Sponges are benthic, sessile and filter feeding, multicellular organisms; phylogenetically belonging to the first true metazoans. They are regarded as the oldest animal phylum with recent representatives. Sponges already existed millions of years before the so called ‘Cambrian Explosion’, when most of the recent animals evolved. Fossil records of all classes of the Porifera document their existence at least since the early Cambrian (Reitner and Mehl 1996, Reitner and Mehl 1995). Fossils of whole sponges (Fig. 1) were found in ancient rocks dated back to the early Cambrian 580 million years ago (Li *et al.* 1998), while the oldest spicules with demosponge affinities were found in approx. 750 million years old Noon Day Dolomite in Nevada (Reitner and Wörheide 2002). Furthermore, chemo-fossil records of specific C30 steranes (24-isopropylcholestanes), which are used as sponge biomarkers, were found in 1,8 billion years old stromatolites (McCaffrey *et al.* 1994, Moldowan 1994) and indicate the existence of sponges - or their ancestors - in the Early Proterozoic.

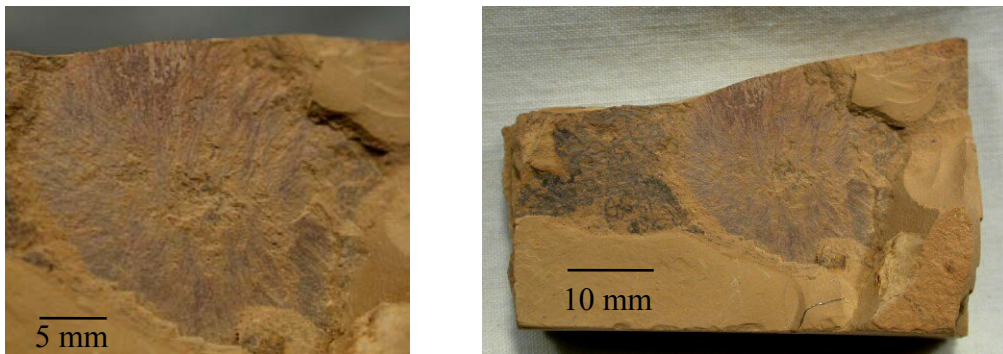


Fig. 1: A fossil of the demosponge *Hazelia* sp. from the early Cambrian found in Chengjiang, China, dated approx. 525 million years ago (www.fossilmuseum.net)

Apart from the debate on monophyly of the Porifera (Borchiellini *et al.* 2001, Schutze *et al.* 1999, Zrzavy *et al.* 1998, Reitner and Mehl 1996), the phylum is generally accepted to be divided into three taxonomic classes, characterised by the composition of their spicules (Fig. 2). The Calcarea (calcareous sponges) contain calcareous spicules, while the Hexactinellida (glass sponges) are characterised by siliceous spicules of hexactine structure.

The third class, Demospongiae (demosponges) contains the majority of the estimated 15,000 sponge species living today (Hooper and van Soest 2002). In general, demosponges have a mineral skeleton composed of siliceous spicules. However, the class further includes several lineages, like the common bath sponge, which have no skeleton at all.

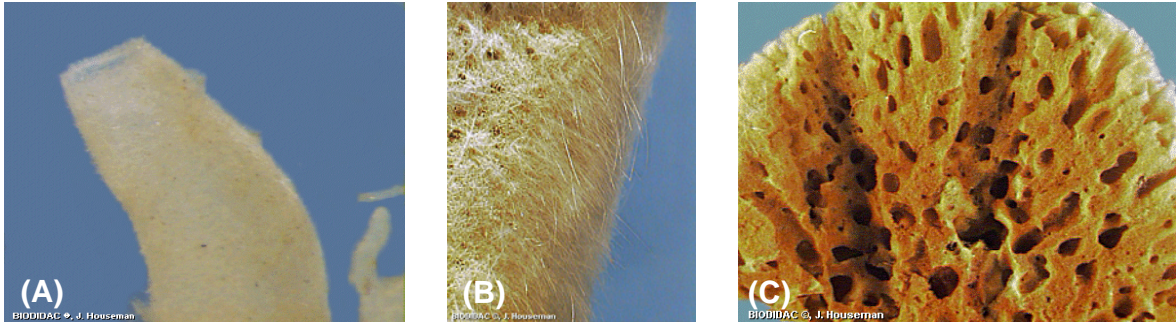


Fig. 2: Preserved sponge specimens with their characteristic spicules of the classes (A) Calcarea (calcareous spicules), (B) Hexactinellida (siliceous spicules of hexactine structure) and (C) Demospongiae (no spicules). (© BIODIDAC, <http://biodidac.bio.uottawa.ca>)

Sponges display a great morphological variability. Dimensions, shapes and colours vary from species to species as well as at intra-specific level depending on environmental and/or physiological conditions (Brümmer *et al.* 2003, Kaestner 1980, Bergquist 1978). Sponges feature a quite simple anatomy of water channels and chambers. This aquiferous system is supported by a skeleton of spicules embedded in the mesohyl, a glycosidic matrix containing several cell types, which is coated by an external cell layer called pinacoderm (Fig. 3). Although lacking real tissues or organs, sponges can be differentiated into an outer region, called the cortex and an inner region, the endosome (=choanosome), in which the choanocyte chambers are located (Brümmer *et al.* 2003). Due to the lack of real tissues and organs, with all the physiological actions occurring at cellular level, sponges can be considered as ‘colonies’ of genetically related protozoans or ‘cell societies’ (Rasmont 1979).

Morphologically sponges are divided into three main groups called asconoid, syconoid and leuconoid, referring to the complexity of their aquiferous systems (Fig. 4). Asconoid sponges have an ovate structure with thin body walls, an osculum on top and an inner cavity (spongocoel or atrium) lined by choanocytes (flagellated cells, Fig. 3 B) (Fig. 4 A). The syconoid morphology is characterised by a thickened, folded body wall forming rounded flagellated chambers (choanocyte chambers, Fig. 3 C) (Fig. 4 B). The leuconoid type has the most complex aquiferous system. These sponges show no spongocoel but a cellular matrix (the mesohyl), wherein chambers and canals are embedded (Fig. 4 C) (Bergquist 1978).

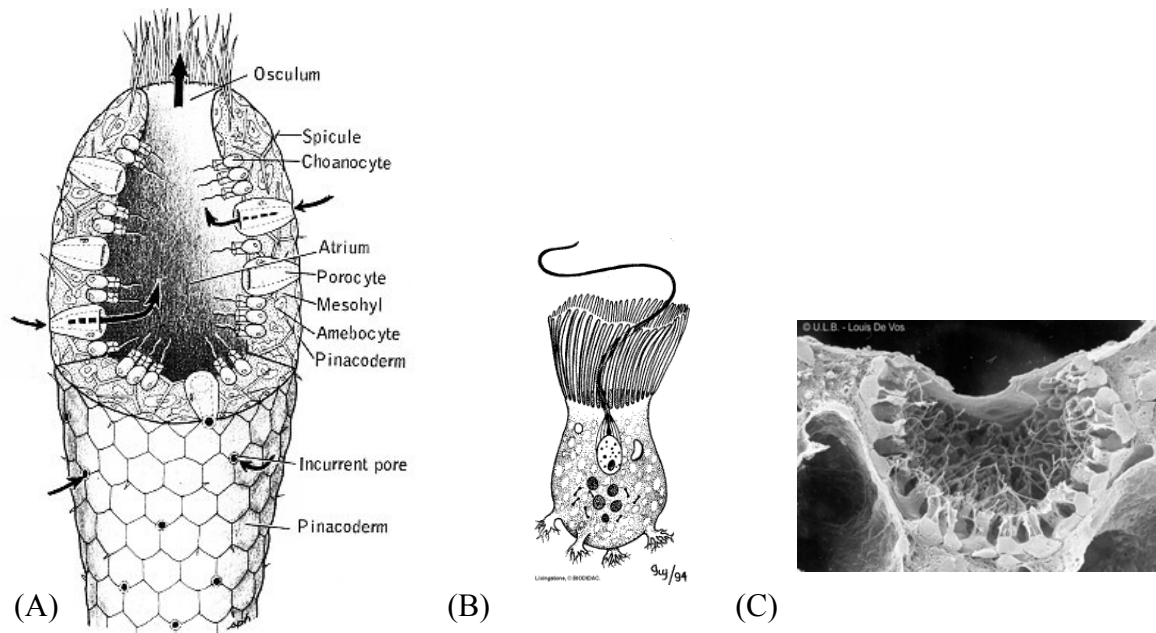


Fig. 3: Schematic picture of (A) a partly cross-sectioned asconoid sponge and (B) a choanocyte; (C) an electron microscopic picture of a choanocyte chamber. ((A) Berczi *et al.* 2005; (B) © BIODIDAC, <http://bioididac.bio.uottawa.ca>; (C) © ULB - Louis de Vos)

The aquiferous system provides the basis of filtration and nutrition uptake. In the filter feeding process the ambient water enters the sponge through pores at the outer surface and is pumped through the channel system by the action of the flagellated choanocytes. Bacteria, single-celled algae and other organic particles as well as dissolved nutrients are removed from the water by translocation into the mesohyl interior. Digestion is performed by amoeboid archaeocytes that move freely through the extracellular matrix of the sponge. The water leaves the sponge through the osculum essentially sterile (Reiswig 1973).

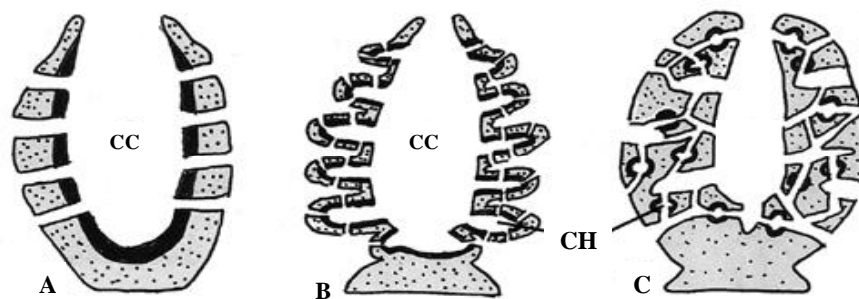


Fig. 4: Schematic drawing of (A) asconoid, (B) syconoid and (C) leuconoid sponges. Black areas indicate choanocytes. CC: Central chamber; CH: Choanocyte chambers (Storch and Welsch 1999)

Additionally to bacteria functioning as food particles, prokaryotes have also been reported to live in association with sponges (for review see Hentschel *et al.* 2006). Though bacteria seem to be the most common associates of sponges, these animals have also been reported to live in association with several other micro- and macroorganisms, such as micro- and macroalgae (Frost *et al.* 1997, Rützler 1990, Saller 1989, Wilkinson 1987, Reisser 1984,

Vacelet 1982), yeasts (Maldonado *et al.* 2005) as well as polychaetes and prosobranchs (Kunzmann 1996).

Biotechnological potential of sponges and sponge-associated bacteria

Sponges are a rich source of bioactive compounds. To date, more than 5300 different substances, with antiinflammatory, antitumoral, immunosuppressive or neurosuppressive, antiviral, antimalarial, antibiotic, or antifouling activity have been isolated from marine sponges; and hundreds of new compounds are discovered every year. These bioactive sponge-derived substances represent a remarkable chemical diversity: unusual nucleosides, bioactive terpenes, sterols, cyclic peptides, alkaloids, fatty acids, peroxides, and amino acid derivatives isolated from sponges have been described (for review see Sipkema *et al.* 2005).

Some sponge-derived metabolites reveal striking similarity to known microbial metabolites (Faulkner *et al.* 1993). Furthermore, structurally related or identical compounds have been reported from taxonomically distinct marine invertebrates or from individuals collected in certain geographic locations. Thus, it was hypothesised that many marine invertebrate natural products may be of microbial origin (Faulkner *et al.* 1993).

The isolation of sponge-associated bacteria producing bioactive metabolites originally isolated from sponges strongly supports the hypothesis of the microbial origin of the compounds formerly ascribed to sponges (Oclarit *et al.* 1994, Shigemori *et al.* 1992, Stierle *et al.* 1988). However, a specific association between sponges and those bacteria has not been demonstrated. Additionally, algal origin of sponge-ascribed substances was demonstrated. Okadaic acid, first found in the sponges *Halichondria okadai* and *Halichondria melanodocia*, is produced by a dinoflagellate (Yasumoto *et al.* 1987, Tachibana *et al.* 1981). Again, it is not known whether the toxin is accumulated by the sponge from the water column, from digested algal cells or from algal symbionts (Faulkner *et al.* 1993).

Due to the close dependency of the partners in an obligate mutual association (refer to paragraph 'Sponge-bacteria associations: obligate mutual symbioses?', p. 8), the isolation of the involved microorganism often fails. Therefore, cellular localisation studies have become a model of evaluating invertebrate-microbial associations and the production of microbial derived natural products in sponges. Faulkner *et al.* (1993) argued that if a secondary metabolite is clearly associated with a particular cell type, it is most probably synthesised by that cell type. In addition to the localisation of the substance, a reasonable relationship between the quantity of an isolated metabolite and the biomass of the producing organism is

needed to prove the microbial origin of a sponge-derived substance. Cell separation and localisation of the metabolite suggested a microbial origin for sponge-derived compounds in the sponges *Dysidea herbacea* and *Theonella swinhoei* (Bewley *et al.* 1996, Unson *et al.* 1994, Unson and Faulkner 1993). However, two bromated sponge metabolites, aérothionin and homoaérothionin, were localised within spherulous cells in the mesohyl of the marine sponge *Aplysina fistularis* and not in the associated bacteria (Thompson 1985).

These results indicate sponges as well as associated bacteria as sources of bioactive compounds. Therefore, one of the major goals of biotechnological sponge research is to find appropriate culture conditions for associated microorganisms that produce pharmacologically active metabolites. In view of the fact that sponge aquaculture is still in its infancy and little success has been reported in growing sponge cells in culture media (Faulkner *et al.* 2000), sponge-derived natural products are only available in low amounts and pharmaceutical use would put high harvesting pressure on marine sponge communities. In contrast, mass fermentation of the bioactive associate can provide a renewable resource and thus preserve natural populations of the sponges.

SPONGE-ASSOCIATED BACTERIA

Sponge-bacteria associations: obligate mutual symbioses?

The term ‘symbiosis’ was introduced by DeBary (1879) as a ‘cohabitation of dissimilarly named organisms’. It can be classified as mutualism (both species benefit), commensalism (one species benefits, the other is not harmed) or parasitism (one species benefits, the other is harmed) and further be differentiated by the dependency of the partners as obligate (the partners cannot live without each other) or facultative (the partners can live separately). ‘Symbiosis’ (association) always occurs between members of different species regardless of their taxonomic order. For example, bacteria can interact with other bacteria or archaea as well as with eukaryotes. Possible benefits of a mutual symbiosis are the supply of e.g., food, protection, cleaning, transportation or a combination of these factors. A mutual symbiosis consequently leads to a mutual dependence (obligation) which is reflected by adaptation of the metabolisms of the two partners (DeBary 1879). The terminology used in the literature is not consistent and ‘symbiosis’ is often used as a synonym for (sometimes obligate) mutualism (Goff 1982). To avoid confusion, here the term ‘association’ is used for a cohabitation of non-related species with unknown interaction and dependency between the partners.

Bacteria have been observed in the reproductive stages of sponges, such as the oocyte stage (Usher *et al.* 2001, Vacelet *et al.* 1995, Gallissian and Vacelet 1976, Levi and Levi 1976), which generally is considered as an indicator for an obligate association. A further important indicator of permanent or even obligate association is host specificity. Recently, the question of host specificity and stableness of sponge-bacteria association was addressed by 16S rDNA- and rpoB-based denaturing gradient gel electrophoresis (DGGE) (Taylor *et al.* 2004). The stability and structure of the associated bacterial community of three co-occurring sponge species from temperate Australian waters was studied over a period of five seasons. By the use of 16S rDNA DGGE and sequencing of excised bands Taylor *et al.* (2004) identified three types of sponge-associated bacteria: ‘specialists’ – found on only one host species, ‘sponge-associates’ – found on multiple host species but not in seawater and ‘generalists’ from multiple host species and seawater. Only one of the three sponge species investigated, *Cymbastela concentrica*, was shown to harbour a stable bacterial community differing significantly from that of ambient seawater over the whole studying time (Taylor *et al.* 2004).

In most cases, the exact nature of the sponge-bacteria association is uncertain. Based upon a variety of methods different possible sponge-microbe interactions have been discussed. Cyanobacterial associates, for example, supply the sponges with organic material derived from photosynthesis (Cheshire and Wilkinson 1991, Wilkinson 1983, Sará 1971), nitrogenous metabolites from nitrogen-fixation (Shieh and Lin 1994, Wilkinson and Fay 1979), as well as with reduction equivalents (Arillo *et al.* 1993). Further they may provide protection from excessive illumination for the sponge and, perhaps, a larger quantity of O₂ (Sará 1971). Additionally, the associated cyanobacteria may contribute to host nutrition through extracellular lysis and phagocytosis (Wilkinson 1980a, Wilkinson and Garrone 1980, Wilkinson 1978a). Even production of antimicrobial substances (Flowers *et al.* 1998) by associated cyanobacteria was demonstrated. In return the photosynthetic associate benefits from CO₂ production by the host sponge, preventing a carbon limitation for its autotrophic life (Wilkinson and Garrone 1980). These mutual effects and the vertical transmission of cyanobacterial symbionts in the marine sponge *Chondrilla australiensis* (Usher *et al.* 2001) indicate an obligate mutual character of sponge-cyanobacteria associations. In the deep-sea sponge *Cladorhiza* sp. methane-oxidation by associated bacteria has been demonstrated (Vacelet *et al.* 1995). Putative nutritional dependency of the sponge on the associated methane-oxidizing bacteria indicates a mutual obligate association.

Commensalistic or mutualistic associations may be characterised by metabolic interactions such as the use of a metabolic end- or by-product of one associate as energy- or

nutrient-source by the other associate. As demonstrated in previous studies, nitrogen-fixing cyanobacteria may provide sponges with N-containing metabolites (Shieh and Lin 1994, Wilkinson and Fay 1979). Supplementary, sponges may release nitrogen in form of ammonia, which can further be used by associated organisms, e.g., algae (Davy *et al.* 2002). Further, nitrate release was observed in different sponge species of the Caribbean Sea. Nitrate production by eukaryotic organisms has never been demonstrated; thus the released nitrate is unlikely to be a sponge product and conversion of released ammonia to nitrate by associated bacteria (nitrification) can be assumed (Diaz and Ward 1997, Corredor *et al.* 1988). However, up to now only in one study ribosomal DNA sequences related (97%) to an ammonia-oxidizing bacterium (AOB) (*Nitrosococcus mobilis*) were obtained from a nitrate-releasing sponge (Diaz 1997). For long, the ability of ammonia-oxidation, the first step in nitrification, was thought to be restricted to a few groups within the *Beta*- and *Gammaproteobacteria*. However, molecular evidence for ammonia-oxidizing archaea has emerged (Könneke *et al.* 2005, Schleper *et al.* 2005, Venter *et al.* 2004), and very recently, the sponge-associated archaeon, Candidatus *Cenarchaeum symbiosum*, was shown to contain the putative ammonia-monooxygenase-encoding genes (*amoA*) (Hallam *et al.* 2006) indicating also archaea-mediated nitrification in sponges.

Microscopic and culture-based studies

Bacteria serve as food source for sponges, being taken up by filtration and ingested by phagocytosis. Additionally, bacteria are observed in the mesohyl of many sponges. Sponges containing high amounts of bacteria (up to 40% of the total volume; (Santavy *et al.* 1990, Wilkinson 1978c, Vacelet and Donadey 1977, Vacelet 1975) have been termed 'bacteriosponges' (Reiswig 1981). Studies of the associations of sponge and bacteria by light and electron microscopy demonstrated the presence of large numbers of bacteria within the sponge partly being morphologically different to those from the ambient seawater (Wilkinson 1978a, b, c, Vacelet and Donadey 1977, Vacelet 1975, 1971, 1970). These microscopic studies also gave insight into distribution and localisation of bacteria within sponges. Bacteria are present extracellularly within the mesohyl, intracellularly inside the vacuoles of archeocytes which are referred to as bacteriocytes (Simpson 1984) and even in the nucleus of sponge cells (Friedrich *et al.* 1999, Vacelet 1975, 1970). Electron microscopic studies provide information about localisation, form and number of the bacteria, as well as cell wall type (Gram-positive or Gram-negative) and internal membrane structures such as thylakoide systems. Because of their

characteristic internal thylakoid systems cyanobacteria were quite easily recognised within sponges (Hinde *et al.* 1994, Simpson 1984, Wilkinson 1983, Wilkinson and Fay 1979).

As predicted from these morphologic studies, a great variety of different physiological and phylogenetic groups of bacteria were isolated from sponges. By the use of different cultivation methods a broad diversity of chemoheterotrophic bacteria were selectively isolated from marine sponges (Olson *et al.* 2002, Webster and Hill 2001, Burja *et al.* 1999, Wilkinson *et al.* 1981). Additional to phototrophic cyanobacteria, different physiological bacterial groups such as non-oxygenic phototrophic bacteria (Imhoff and Trüper 1976), nitrogen-fixing bacteria (Shieh and Lin 1994), methane-oxidizing bacteria (Vacelet *et al.* 1996) and anaerobic bacteria (Wilkinson 1978a) were isolated from marine sponges. In these studies possible benefits for the sponge have been discussed, but obligate mutual association was not demonstrated.

First support for the idea of bacteria being specifically associated with members of the phylum Porifera was given by the isolation of phenotypically similar bacteria from nine different sponges from the Mediterranean Sea and the Great Barrier Reef, which were not obtained from the surrounding seawater (Wilkinson *et al.* 1981). Specificity of those sponge-bacteria associations was deduced from immunological cross-reactions. ‘Specifically’ sponge-associated bacteria (not found in the ambient seawater) were tested against immune sera from other ‘specific’ and ‘non-specific’ strains. Agglutination reactions were only found between ‘sponge-specific’ isolates indicating possible common ancestry (Wilkinson *et al.* 1981). The authors suggested that those associations are obligate mutual associations which may exist since the Precambrian, before the evolution of the extant sponge orders and classes took place (Wilkinson 1984). However, the phenotypic characterisation of the sponge associated bacteria was not specific enough for an identification of the bacteria on species level.

With the development of molecular methods, cultured sponge-associated bacteria were additionally phylogenetically classified based on their 16S rDNA sequences. Similar to the phenotypic study, comparison of different culture-based studies on 16S rDNA level revealed a cluster of closely related *Alphaproteobacteria* obtained from sponges of different taxonomic and geographic origin (Lafi *et al.* 2005, Hentschel *et al.* 2001, Webster and Hill 2001) suggesting sponge-specificity of these bacterial associates.

Culture-independent DNA-based studies

Isolation of bacteria is always highly selective due to media and culture conditions used. Therefore, isolation-based studies provide only limited information concerning the diversity of sponge-associated bacterial communities. Even with a combination of different culture methods the bacterial recoverability from environmental samples is estimated to be in a range of only 0.01% to 12.5% of the existing community (Olson *et al.* 2000). Cloning and sequencing of amplified 16S rDNA from extracted genomic DNA of sponge samples allows identification of bacteria present in the environmental sample without need for cultivation. Most recent studies approach the question of sponge-bacteria association using culture-independent molecular methods (Taylor *et al.* 2005, Burja and Hill 2001, Webster and Hill 2001, Webster *et al.* 2001b, Burja *et al.* 1999, Althoff *et al.* 1998, Hinde *et al.* 1994). In all cases in which a sponge-associated bacterial community was studied using both isolation as well as culture-independent methods, the two methods revealed different pictures: comparison of the 16S rDNA sequences of isolated bacteria and 16S rDNA clone sequences obtained from several species of the deep-water sponge *Discodermia* sp. showed no overlap (Lopez *et al.* 1999). The alphaproteobacterium strain NW001 clearly dominated the culturable community of *R. odorabile*. Additionally, the strain was visualized within the sponge mesohyl localised around the choanocyte chambers by fluorescent *in-situ* hybridisation (FISH) (Webster and Hill 2001) but was not retrieved in a culture independent approach using analysis of a 16S rDNA clone library (Webster *et al.* 2001b). Hentschel *et al.* (2001) isolated an alphaproteobacterium with antimicrobial activity from *Aplysina aerophoba* and *Aplysina cavernicola* closely related to strain NW001, which was not detected in both *A. aerophoba* and *A. cavernicola* by fluorescence *in-situ* hybridisation (Friedrich *et al.* 1999). Selectivity of either method has to be kept in mind when interpreting either isolation-based or culture-independent studies. Additional to the selectivity of isolation-methods, the PCR step prior to cloning and sequencing is known to bias the results of 16S rDNA cloning. Therefore, lack of detection of a bacterium in either way does not necessarily imply absence from the sponge-associated community.

However, phylogenetic comparison of 16S rDNA retrieved from different sponges revealed unexpected conformity (Hentschel *et al.* 2002). 190 16S rDNA sequences obtained from several individuals of three different sponge species and different locations were analysed. The phylogenetic comparison resulted in fourteen monophyletic clusters belonging to at least seven different bacterial divisions. The clusters were characterised by sequences either obtained from different sponge species or the same species sampled in different geographic regions and comprised 70% of all sponge-derived 16S rDNA sequences publicly

available at that time (Hentschel *et al.* 2002). Monophyletic clustering hints towards common ancestry and thus a uniform sponge-associated bacterial community was proposed (Hentschel *et al.* 2002). However, monophyletic clustering does not necessarily indicate mutual association or actually the necessity of host contact (obligation).

AIM OF THE THESIS

The present study has been accomplished as part of the German Federal Ministry of Education and Research project 'Kompetenz-Zentrum BIOTECmarin', which aims on sponge ecology and the production of natural products from Porifera and their associated microorganisms (www.biotecmarin.de). In the search for new potentially pharmaceutical agents, marine invertebrates and especially sponges turned out to be a rich source of biologically active substances. In the meantime several studies deal with the biotechnological application of biologically active compounds from marine invertebrates. The isolation of promising new substances from sponges involves the risk of a strong harvesting pressure and the exploitation of the marine resources with serious consequences for the biodiversity of the ecosystem. BIOTECmarin focuses on the sustainable use of sponges as natural resources for biologically active compounds.

The aim of the study was to investigate the nature of sponge-bacteria association and, as for some sponge-derived compounds microbial origin has been proposed, the potential of sponge-associated bacteria in the search for new bioactive substances. Instead of focusing on one specific sponge, we aimed to study several different sponge species concerning their biotechnological potential. The isolation of biologically active bacteria from sponges further raises the question of kind and specificity of the putative association as well as of a possible ecological role of bioactive bacteria in sponges. As isolation-methods always selectively limit the recovery of specific bacterial strains from an environmental sample, culture-independent molecular methods were used to study the diversity and specificity of sponge-associated bacterial communities. Two sponge species sharing the same habitat were chosen to specify the impact of the ambient seawater for the sponge-bacteria associations. Furthermore, one species known to harbour high amounts of associated bacteria as well as one species with low bacterial abundance was chosen to address the question of the lack of specifically associated bacteria in some sponges. By the comparison of the bacterial community associated with one sponge species from different Mediterranean locations the specificity of sponge-bacteria associations was addressed.

STUDY OBJECTS

Chondrilla nucula

Chondrilla nucula (Demospongiae, Tetractinomorpha, Chondrosida, Chondrillidae) is commonly found in the Mediterranean Sea and harbours a high number of intra- and extracellular bacteria. Among them, cyanobacteria occupy mainly the cortical region conferring the characteristic brown-olive green colour of the sponge. Specimens grown in or transplanted into caves lose their cyanobacteria, become white and eventually undergo metabolic



Fig. 5: *Chondrilla nucula* in-situ photograph at the Limski kanal

collapse indicating an obligate mutual association (Ariño *et al.* 1993, Gaino *et al.* 1977). The cyanobacterium *Candidatus Synechococcus spongiarum* (Usher *et al.* 2004c) associated with the species *C. nucula* and *Chondrilla australiensis* is transmitted vertically (Usher *et al.* 2001) supporting the idea of an obligate mutual sponge-cyanobacteria association. The same cyanobacterial species inhabit different colour-morphotypes of *C. australiensis* (Usher *et al.* 2004a) suggesting reflection of cyanobacterial photoacclimation by means of phycoerythrin level by the sponge colour also for *C. nucula*. Very recently, the unusual association of vertically transmitted yeasts has been demonstrated for *C. nucula* specimens from different geographic origin (Maldonado *et al.* 2005).

C. nucula is characterised by a thick encrusting growth, a lobate form (1 cm to 4.5 cm thick), with a smooth surface as well as by a cartilaginous and firm consistency. Species of the genus *Chondrilla* are abundant in reefs in both tropical and temperate oceans of the world (Usher *et al.* 2004b). They own a relative simple skeleton and few spicule types and are not easy to distinguish from each other. Using classical taxonomic and morphological criteria *C. nucula* was thought to be cosmopolitan (Carballo *et al.* 2003, Boury-Esnault 2002). Recent molecular studies, however, hint towards *C. nucula* occurring only in the Mediterranean Sea (Usher *et al.* 2004b, Klautau *et al.* 1999), where the original holotype described by Schmidt (1862) was collected. However, re-classification of *C. nucula* from geographic regions other than the Mediterranean Sea has not been addressed yet. In the Northern Adriatic Sea *C. nucula*

has been recorded almost everywhere by several authors (Brümmer *et al.* 2004, Novosel *et al.* 2002, Müller *et al.* 1984, Rützler 1967). In the Limski kanal nearby Rovinj (Croatia) *C. nucula* was found to be one of the most common species of the sponge fauna (Sidri 2004). Abundance of the sponge was correlated with light exposure and depth as well as with substrate. The optimum light exposure of the sponge did not correspond to the maximal light availability (Sidri 2004), possibly due to the strong competition with algae in the surface waters (Rützler 1965). As for most sponges, the type of substrate plays an important role for the distribution of *C. nucula*. For settlement and growth the sponge depends on stable hard substrate, preferably rocky walls; but also small stones or shells provide a possible substrate. However, *C. nucula* is reported to be adapted to unstable conditions, like muddy bottoms, by its ability to change into a round, nodular shape and thus being able to persist and roll around freely (Sidri 2004). Furthermore, *C. nucula* owns a remarkable reorganization plasticity (Sidri 2004). It can drop part of its body in order to colonize new substrate (Gaino and Pronzato 1983) and also shows negative geotaxis capabilities (Pronzato 2002). Finger-like structures which act like 'pseudopodia' are used for the attachment to the substrate and to trap sand grains and shells to stabilize its position (Sidri 2004). With these attributes the sponge is additionally able to crawl on the substrate and to climb surfaces, 'running' several centimetres in few weeks, when conditions are not optimal for its survival (Bond and Harris 1988).

In nature *C. nucula* is always observed with a smooth and clean surface even in environments with high sedimentation rates. A recent study showed that the sponge is able to remove particles from its surface by trapping them into a mucous substance that is constantly shed from the surface (Sidri 2004). Occasionally *C. nucula* acts as an epibiont of other organisms such as macroalgae, molluscs and other sponge species; but *C. nucula* is never overgrown by other organisms itself (Sidri 2004). Antimicrobial, antiviral and cytotoxic activities from *C. nucula* extracts were detected by several authors (Sepcic *et al.* 1997, Opric *et al.* 1996, Schmitz 1994, Schröder *et al.* 1990). It was proposed that the active substances, concentrated mainly on the surface and in the mucus, are responsible for *C. nucula* being never overgrown by other organisms (Sidri 2004).

C. nucula has been discussed to be a suitable candidate as a bioremediator (Milanese *et al.* 2003). Unlike other sponges, *C. nucula* is easy to farm (Pronzato *et al.* 1999) and produces lectines as well as antiviral and cytotoxic compounds (Opric *et al.* 1996, Schmitz 1994, Schröder *et al.* 1990). Recently it was shown that *C. nucula* has high bacterial retention rates of $6-7.4 \times 10^6$ cells *E. coli* hour⁻¹ cm⁻¹ at a relatively low filtration rate of 0.2-1.4ml h⁻¹ cm⁻¹ (Milanese *et al.* 2003).

C. nucula, as a common Mediterranean sponge, was chosen as a representative for 'bacteriosponges', containing a high number of bacteria within its mesohyl. Contemplating the question of specificity of sponge-bacteria associations, specimens from two different locations within the Mediterranean Sea were sampled.

Tethya aurantium

Tethya aurantium (Porifera, Demospongiae) has been observed in European waters (Mediterranean and North-European) as well as in the Western Atlantic and the Eastern Atlantic coast and the Indian Ocean. It is characterised by a thick and well developed cortex, an orange colour and a generally globular shape. Bacteria are observed in the sponge mesohyl only in low abundance and *T. aurantium* has been thought to lack the presence of associated bacteria (U. Hentschel, pers. communication). However, the related species *Tethya seychellensis* harbours an associated green-algae, which is possibly provided with light energy for photosynthesis by the radiate spicule bundles functioning as a natural light conductor, similar to fibre-optic systems (Gaino and Sará 1994).



Fig 6: *Tethya aurantium* in-situ photograph

The cosmopolite genus *Tethya* is generally recognised as a member of the family Tethyidae in the order Hadromerida. However its taxonomic position is still disputed and it has been suggested that *Tethya* should be phylogenetically classified with the Tetractinellida (van Soest 1987). The genus *Tethya* is characterised by a generally globular shape and a radiate structure determined by the strongyloxeas which run in bundles from the centre to the periphery of the sponge. The shape sometimes may be irregularly rounded, hemispherical and even encrusting. Members of the genus *Tethya* generally contain a well developed ectosome or cortex. The cortex surrounds the endosome (choanosome), which contains the choanocyte chambers, and may display varied structure and width. The cortex protects the choanosome against high intensities of hydrodynamism and light and can be used as a taxonomical characteristic feature (Sará 1987). According to Burton (1928) the cortex developed as a protective device for shallow water habitats and is found to be thicker and well structured in

species living in shallow waters where currents and light intensities are rather high while species living in more protected habitats display a thin, nearly indistinct cortex (Sar 1987).

Phylogenetically, *Tethya* with its radiate structure appears to have been derived from deep water ancestors without a cortex (Burton 1928). Therefore, species with less developed cortices may be considered as more primitive. *Tethya* species in general, in comparison with other sponges, may be considered as specialists: all reproduce through oviparity and are characterised by slow growth, definitive shape, complexity of structure and adaptations.

T. aurantium Pallas 1766 and *T. citrina* Sar and Melone 1965 were formerly considered as the same species, called *T. lyncurium* Linn 1767. However, a comparative study of the closely related species revealed that they differ strongly with respect to width and structure of the cortex: *T. citrina* displays a thin cortex while *T. aurantium* possesses a thick and well structured cortex. Comparison between the ecological requirements and distribution of *T. aurantium* and *T. citrina* in the Mediterranean waters revealed that, where the two species coexist in parapatry, they inhabit different niches with regard to current and light. Habitats in which *T. citrina* is found are characterised by low currents as well as low illumination. *T. aurantium* generally inhabits areas more exposed to light with stronger currents in depth of 1-40 m while *T. citrina* possesses a thinner cortex and is found in sheltered places mainly in the depth of 0-1 m or more than 40 m (Sar 1987). Furthermore, *T. citrina*, with a thinner and softer cortex, shows a greater capability of contraction and expansion.

T. aurantium was chosen to study the sponge-associated bacterial community of sponges harbouring low amounts of bacteria and the local distribution within the sponge.

SAMPLING SITES

The Limski kanal

The Limski kanal (Canal di Lemme or Limsky channel) harbours a very diverse sponge fauna. The community of 42 different sponge species is dominated by the species *Chondrilla nucula* and further includes three species of the genus *Tethya*: *T. aurantium* Pallas 1766, *T. citrina* Sar and Melone 1965 and *T. limski* Mller and Zahn 1968 (Brmmer *et al.* 2004). The high sponge diversity in the Limski kanal displays about 21% of the species described for the Adriatic Sea (Brmmer *et al.* 2004). The semi-closed fjord-like bay nearby Rovinj (Istrian Peninsula, Croatia) lies along an east-west axis and has a length of approximately 11 km, a maximum width of about 650 m and a maximum depth of 32 m. At its maximum depth the

Limski kanal is characterised by a flat muddy bottom. The sides are mainly detritic with rocky walls and cliffs towards the surface. The sedimentation rate is very high and especially the east of the channel bottom consists of mainly fine particles (Zavodnik 1971). The water exchange is rapid with a main outward current on the surface and income current in the depth (Kuzmanovic 1985).

The bay originated during the Oligocene due to the erosion of Foiba River which scavenged the area around it. During the Holocene the sea level raised and penetrated through the valley produced by the Foiba river resulting in the channel bay (Vatova 1928).

For its high sponge diversity the Limski kanal was chosen as sampling site to compare the sponge-associated bacterial communities of different species inhabiting the same habitat. Thus specificity and influence of ambient seawater was addressed by the experimental design.

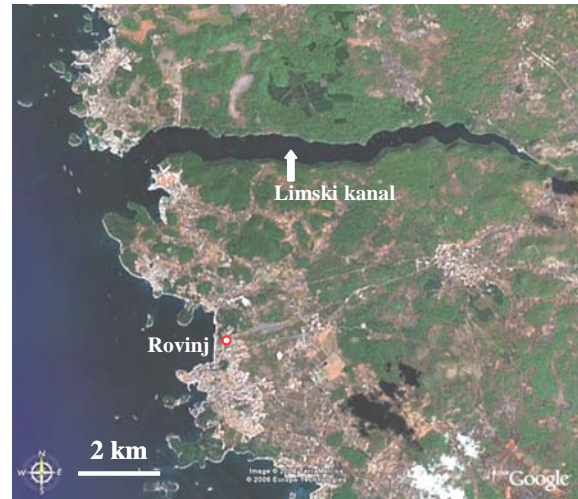


Fig. 7: Map of the Adriatic sampling site, Limski kanal, north off Rovinj at the Croatian Coast (© Google Earth)

The Portofino Reserve



Fig. 8: Map of the Ligurian Sea sampling site, Portofino Reserve off the Italian Coast (© Google Earth)

The natural park of Portofino Marine Reserve displays a high diversity of natural macro- and microenvironments, which favours the development of a rich and diversified benthic fauna including sponges (e.g., *C. nucula*; Sidri *et al.* 2005, Milanese *et al.* 2003). It is located in the Ligurian Sea, nearby Genoa (Italy) and includes the peninsula of Portofino Promontory, its coast and the surrounding marine area. Promontory of Portofino juts out into the sea for more than 3 kilometres and extends

itself along the coast for about 13 kilometres. The coastal line is characterised by a rocky spur of about 200 m and by bays covered with beaches formed by stream deposits. The southern

coast consists of the so-called ‘puddinga di Portofino’, a characteristic Oligocene puddingstone with mainly calcareous clasts. It is dominated by submerged cliffs rapidly reaching 40-50 metres of depth. On the contrary, the eastern and western coasts are made of limestone from Mt. Antola outcrops with stratified sedimentary rocks. Due to the geomorphological features of the Promontory an underwater environment very rich in ravines, roofs, and small caves has evolved. Moreover, an east-west directed current with an average superficial speed of 25 cm/sec, ensures a good water exchange, which prevents the formation of polluted backwater near the coast (www.riservaportofino.it).

Portofino Reserve was chosen as second sampling site for *C. nucula* due to its distinct water mass compared to the Adriatic Sea. Thus the specificity of the *C. nucula*-associated bacterial community was addressed by comparison of samples from two locations (Limski kanal and Portofino Reserve).

ORGANISATION OF THE THESIS

This thesis is divided into different chapters approaching the different topics of the study. After the general introduction, chapter I and II deal with the bacterial community associated with the sponges as determined by isolation-independent methods. While in chapter I the bacterial community associated with *Chondrilla nucula*, as representative of a ‘bacteriosponge’, is described by molecular methods and by the comparison of two sponge specimens obtained from distinct Mediterranean regions. Chapter II demonstrates that sponge-associated bacterial community exists in sponges containing a rather low amount of bacteria in its mesohyl, i.e., *Tethya aurantium*. Chapter III and IV approach the antimicrobial potential of sponge-associated cultivable bacteria. First evidence of cultivable antimicrobial active bacteria in sponges is demonstrated in chapter III, while chapter IV demonstrates the wide distribution of one antimicrobial active sponge-associated alphaproteobacterium in Porifera and discusses its possible ecological role.

The different chapters are presented in form of manuscripts for publication, followed by a general discussion as well as cited literature and appendix. The manuscripts are submitted as the following titles:

- Spatial distribution of sponge-associated bacteria in the Mediterranean sponge *Tethya aurantium*
- Sponge-specific bacterial associations in the Mediterranean sponge *Chondrilla nucula* (Demospongiae, Tetractinomorpha)

- Phylogenetic identification of bacteria with antimicrobial activities isolated from Mediterranean sponges
- Abundance, bioactivity and ecological relevance of cultured sponge-associated bacteria from the Mediterranean Sea.

CHAPTER I

SPONGE-SPECIFIC BACTERIAL ASSOCIATIONS OF THE MEDITERRANEAN SPONGE *CHONDRILLA NUCULA* (DEMOSPONGIAE, TTRACTINOMORPHA)

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MICROBIAL ECOLOGY

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ABSTRACT

A stable and specific bacterial community was shown to be associated with the Mediterranean sponge *Chondrilla nucula*. The associated bacterial communities were demonstrated to be highly similar for all studied specimens regardless of sampling time and geographical region. Additionally, analysis of 16S rDNA clone libraries revealed three constantly *C. nucula*-associated bacterial phylotypes belonging to the *Acidobacteria*, the *Gamma*- and *Deltaproteobacteria* present in sponge specimens from two Mediterranean regions with distinct water masses (Ligurian Sea and Adriatic Sea). For the first time, candidate division TM7 bacteria were found in marine sponges. A major part (79%) of the *C. nucula*-derived 16S rDNA sequences were closely related to other sponge-associated bacteria. Phylogenetic analysis identified fourteen 16S rRNA gene sequence clusters, of which seven consisted of exclusively sponge-derived sequences while the other seven clusters contained additional environmental sequences. This study adds to a growing database on the stability and variability of microbial consortia associated with marine sponges.

INTRODUCTION

Sponges are sessile filter feeders which pump large amounts of water through their aquiferous channel system (Reiswig 1973). Bacteria, single-celled algae and other food particles are removed from the water by phagocytosis into the mesohyl interior and digested by amoeboid archaeocytes that move freely through the extracellular matrix of the sponge. The phylum Porifera (sponges) is divided into three taxonomic classes: the Calcarea (calcareous sponges), the Hexactinellida (glass sponges) and the Demospongiae (demosponges), the latter containing the majority of sponges living today (Hooper and van Soest 2002). *Chondrilla nucula* (Demospongiae, Tetractinomorpha, Chondrosida, Chondrillidae) is a thick (1-4.5 cm), encrusting sponge with a brown-olive green colour. It has a lobate form with a smooth surface and a cartilaginous and firm consistency. Species of the genus *Chondrilla* are abundant in reefs in both tropical and temperate oceans of the world (Usher *et al.* 2004b). Sponges of this genus possess a relative simple skeleton and few spicule types and are not easy to distinguish. Using classic taxonomical and morphological criteria, *C. nucula* was thought to be cosmopolitan (Carballo *et al.* 2003). Recent molecular studies, however, hint towards *C. nucula* occurring only in the Mediterranean Sea (Usher *et al.* 2004b, Klautau *et al.* 1999), where the original holotype described by Schmidt (Schmidt 1862) was collected. In the Northern Adriatic Sea *C. nucula* is commonly found as recorded by several authors (Brümmer *et al.* 2004, Novosel *et al.* 2002, Müller *et al.* 1984).

Bacteria serve as a food source for sponges. Additionally bacteria also live associated with many sponges. They have been found to be present mainly within the mesohyl. Since the 1970s associations of sponges and bacteria have been studied systematically by microscopic methods and on the basis of bacterial cultures (Webster and Hill 2001, Wilkinson *et al.* 1981, Wilkinson 1978a, b, Vacelet and Donadey 1977, Vacelet 1971). More and more studies using culture-independent molecular methods have been approaching the question of sponge-microbe association in recent years (Burja and Hill 2001, Webster *et al.* 2001a, Webster *et al.* 2001b, Althoff *et al.* 1998). Phylogenetic studies of 16S rDNA retrieved from sponges give indications for a specific bacterial community associated with these animals (Hentschel *et al.* 2002).

Microscopic and culture-based studies revealed different bacterial morphotypes in *C. nucula* (Wilkinson *et al.* 1981, Gaino *et al.* 1977). Cyanobacteria occupy mainly the cortical region conferring the characteristic colour. Specimens grown or transplanted into caves loose their cyanobacteria, become white and eventually undergo metabolic collapse (Arillo *et al.* 1993, Gaino *et al.* 1977). Based on 16S rDNA sequence similarity, cyanobacteria associated with *C. nucula* and *Chondrilla australiensis* were identified as the same species ‘Candidatus *Synechococcus spongiarum*’ (Usher *et al.* 2004c). In *C. australiensis* the cyanobacterial symbiont was shown to be transferred vertically via sponge eggs (Usher *et al.* 2001). Variation in the colour of *C. australiensis* apparently is not due to the presence of different cyanobacteria but rather due to photoacclimation of the associated cyanobacteria (Usher *et al.* 2004a).

We report here on the first culture-independent genetic analysis of the bacterial community associated with *C. nucula* from the Mediterranean Sea. Sponges from two different geographical locations (Ligurian Sea and Adriatic Sea) were compared to address specificity of the associated bacterial community. A stable bacterial community including specifically sponge-associated groups as well as unspecific groups were identified.

MATERIALS AND METHODS

Sampling sites

The Limski kanal is a semi-closed fjord-like bay in the Adriatic Sea near Rovinj (Istrian Peninsula, Croatia) (N45°7'; E13°43') (Fig. I-1). It extends along an east-west axis, with an approximate length of 11 km, a maximum width of about 650 m and a maximum depth of 32 m. The bottom is generally muddy, with insular sites comprised of stones and shallow rocky slopes along the shore. It is characterised by a very high sedimentation rate and a rapid water exchange (Kuzmanovic 1985). In a recent study 42 sponge species were found in the Limski kanal with *Chondrilla nucula* being one of the most common species (Brümmer *et al.* 2004).

The natural park of Portofino Marine Reserve is located in the Ligurian Sea, nearby Genoa (Italy) (N44° 18'; E09°13') (Fig. I-1). It includes the peninsula of Portofino Promontory, its coast and the surrounding marine area. Portofino Promontory protrudes into the sea for more than 3 km. The geomorphological features of the Promontory have determined an underwater environment very rich in ravines, roofs, and small caves that favour the development of a rich and very diverse benthic fauna (www.riservaportofino.it).

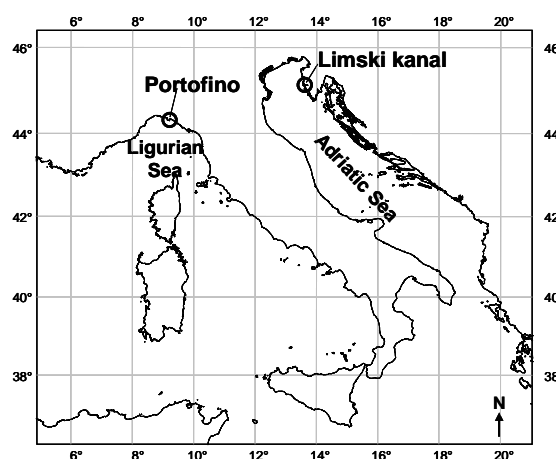


Fig. I-1: Map of sampling sites in the Mediterranean Sea. *Chondrilla nucula* sponge samples were taken at the coast off Portofino (Ligurian Sea, Italy) in August 2002 and along the Limski kanal (Adriatic Sea, Croatia) in April 2003, June 2004 and May 2005.

Sponge sampling

Apparently healthy specimens of the Mediterranean sponge *C. nucula* were sampled by SCUBA diving. In April 2003, June 2004 and May 2005 *C. nucula* specimens were collected in the Limski kanal in a depth of 5 to 15 m. The sponges were placed into sterile plastic bags, cooled on ice and transported immediately to the laboratory. In the laboratory the sponges were washed in filter-sterilised seawater (0.2 µm pore size, Sartorius) and cut. The sponge samples were frozen in liquid nitrogen and stored at -80°C.

Samples of seawater surrounding the sponge were taken at different sampling sites in the Linski kanal, Croatia. An autoclaved glass bottle (Duran, Schott) was filled with 1L of seawater at the sampling site by SCUBA diving. The water was cooled on the way back to the laboratory and filtered through a cellulose-acetate filter (0.2 µm pore size, Sartorius) immediately. The filter was frozen in liquid nitrogen and stored at -80°C until further investigation.

For comparison, *C. nucula* was sampled in the Ligurian Sea in August 2002. The specimen was sampled by SCUBA diving in the natural park of Portofino Marine Reserve at the coast off Italy (Fig. I-1) in a depth of approximately 25 m and was treated as described above.

DNA extraction

Genomic DNA from sponge tissue and filter was extracted and purified using the 'DNA mini Spin Column DNA Purification Kit' tissue protocol and 'genomic DNA purification' protocol (GLuniversal).

PCR and cloning procedure

Amplification of rDNA was performed using puReTaq™Ready-To-Go™PCR Beads (Amersham Biosciences) with the eubacterial primers 27f and 1492r (Lane 1991). The PCR conditions were: initial denaturation (2 min at 94°C) followed by 30 cycles of primer annealing (40 sec at 50°C), primer extension (90 sec at 72°C), and denaturation (40 sec at 94°C), a final primer annealing (1 min at 42°C) and a final extension (5 min at 72°C). For amplification of vector inserts the primers M13f (5'-GTA AAA CGA CGG CCA G-3') and M13r (5'-CAG GAA ACA GCT ATG AC-3') were used. The PCR conditions were identical to those used for 27f/1492r.

DGGE-PCR was conducted using the primers 342-GCf and 534r (Muyzer *et al.* 1993). Temperature profile was as follows: initial denaturation (2 min at 94°C) followed by 15 'touchdown'-cycles of primer annealing (40 sec at 65-50°C), primer extension (40 sec at 72°C), and denaturation (40 sec at 94°C) as well as additional 20 cycles of primer annealing (40 sec at 50°C), primer extension (90 sec at 72°C), and denaturation (40 sec at 94°C), a final primer annealing (1 min at 42°C) and a final extension (5 min at 72°C).

DNA was purified using the High Pure PCR Product Purification Kit (Roche). DNA was ligated into pCR®4-TOPO® vector and transformed into One Shot® Competent *E. coli*

cells using the TOPO TA[®] Cloning Kit (Invitrogen). Inserts were amplified as described above using the M13f/M13r primer set.

All PCR products were checked for correct length on a Tris-borate-EDTA (TBE) agarose gel.

Denaturing gradient gel electrophoresis (DGGE)

DGGE was conducted in double gradient gels containing a linear 6-8% polyacrylamid (acrylamid:bis-acrylamid ratio of 37.5:1) and a 50-80% denaturing gradient (Petri and Imhoff 2001). 100% corresponded to 7 M urea and 40% deionised formamide (Abrams and Stanton 1992). The gel was run in a Tris-EDTA-acetic acid buffer (10 mM Tris, 5 mM acetic acid, 5 mM EDTA, titrated to pH 7.5). Running conditions were 75V for 15 hours. The gel was dyed in 1x SYBR Gold stain (Invitrogen) in Tris-acetate-EDTA (TAE) buffer and photographed.

Cluster analysis

For statistical comparison of the DGGE banding patterns, a similarity cluster analysis (Clarke and Warwick 1994) and analysis of similarity (ANOSIM) (Clarke 1993) were performed using the program PRIMER 5 (PRIMER-E Ltd.) version 5.2.2. Similarity was calculated using the Bray-Curtis index and cluster analysis was done with complete linkage. Subsequently one-way ANOSIM with all possible permutations was performed. In accordance with the PRIMER-manual (Clarke and Gorley 2001) ANOSIM R-values >0.75 were interpreted as well separated; R>0.5 as separated, but overlapping and R<0.25 as barely separable at all. Replicate samples were grouped according to source 'sponge' or 'seawater' as factors for the analyses.

Sequencing and phylogenetic analysis

Sequencing was performed using the ABI PRISM[®] BigDye[™] Terminator Ready Reaction Kit (Applied Biosystems) and a ABI PRISM[®] 310 Genetic Analyser (Perkin Elmer Applied Biosystems) with the M13f and M13r sequencing primers and the 16S rDNA-specific primers 342f (Lane 1991), 534r (Muyzer *et al.* 1993), 790f (5'-GAT ACC CTG GTA GTC C-3'), 907f (5'-GTC AAT TCC TTT GAG TTT-3'), 1093f (5'-TCC CGC AAC GAG CGC AAC CC-3') and 1093r (5'-GGG TTG CGC TCG TTG CGG GA-3'). Sequence data were

edited with Lasergene Software SeqManTMII (DNAS^Tar Inc.) and checked for possible chimeric origins by using the program CHECK_CHIMERA (<http://35.8.164.52/html/index.html>) of the Ribosomal Database Project (<http://rdp.cme.msu.edu/index.jsp>) (Maidak *et al.* 1999). Next relatives were determined by comparison to 16S rRNA genes in the GenBank database using BLAST (Basic Local Alignment Search Tool) searches (Altschul *et al.* 1990) and the Sequence Match program of the Ribosomal Database Project (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp). The sequences were aligned using the FastAlign function of the alignment editor implemented in the ARB software package (www.arb-home.de) (Ludwig *et al.* 2004) and refined manually. For phylogenetic calculations the PhyML software (Guindon and Gascuel 2003) and the online version of PhyML (Guindon *et al.* 2005) were used. Sequences with similarities of >99% were considered as one phylotype. For each phylotype one representing sequence from each specimen was used in phylogenetic calculations. Phylogenetic trees were calculated using the Maximum Likelihood (ML) method (Felsenstein 1981) with GTR model and estimated proportion of invariable sites as well as the gamma distribution parameter using near full length sequences (>1200bp). Calculated trees were imported into ARB and short sequences added subsequently to the trees without changing their topologies by use of the ARB parsimony method. Position and bootstrap values of short sequences (<1200bp) were additionally determined by phylogenetic analysis (ML, 100 bootstraps) including all sequences. Sequences have been submitted to the EMBL database (accession numbers AJ850091-AJ850100; AM259899-AM259940).

Electron microscopy

Sponge samples were prepared for scanning electron microscopy by fixation with 1% glutaraldehyde in seawater, replacement of water with an ethanol series and subsequent critical-point-drying. After mounting samples were sputtered with Au/Pd and observed with a Zeiss DSM 940 scanning electron microscope.

RESULTS

Microscopic studies

Electron micrographs showed high numbers of bacteria in the *Chondrilla nucula* sponge mesohyl (Fig. I-2 B+C). At least 5-6 morphological types of rods and cocci, differing in size and shape, were detected. One type of slender rods (0.5-0.6 x 2.0-4.0 μm ; Fig. I-2 C) was very abundant in the tissue, and also large rods with cell diameters of 1.2 to 1.5 μm (Fig. I-2 B) were frequently observed. Dividing cells of several morphological types was observed to occur regularly (Fig. I-2 B).

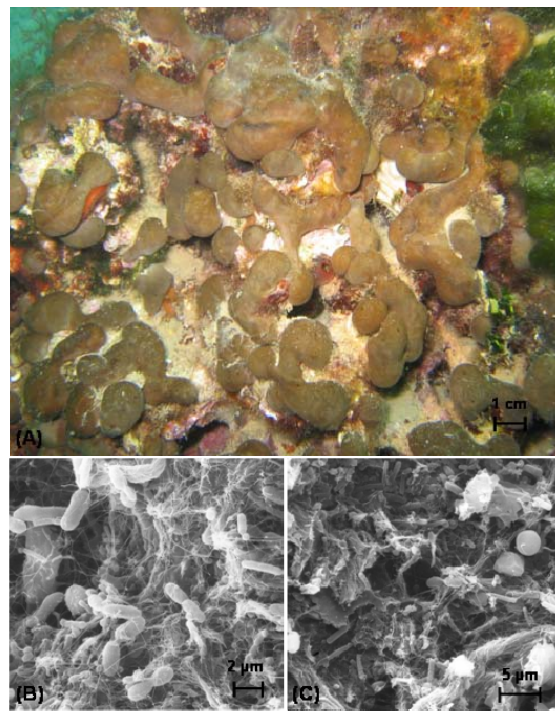


Fig. I-2: *Chondrilla nucula*. (A) *In-situ* photograph (B+C) electron microscopic pictures (REM) of bacteria in the sponge mesohyl

DGGE

Electron micrographs showed high numbers of bacteria in the *C. nucula* sponge mesohyl (Fig. I-2 B+C). The sponge-associated microbial community was apparently different from that of the surrounding seawater as shown by DGGE banding patterns (Fig. I-3). ANOSIM with all possible permutations confirmed the obtained clusters to be well separated ($R > 0.75$). In addition, DGGE banding patterns demonstrated a highly stable community composition in different individuals originating from the same geographical region in the year

2005 (data not shown). High similarity was also demonstrated between consecutive years as well as between the two locations within the Mediterranean Sea (Fig. I-3). Apart from slight differences, several bands were found consistently in all sponge specimens and were absent from the seawater samples.

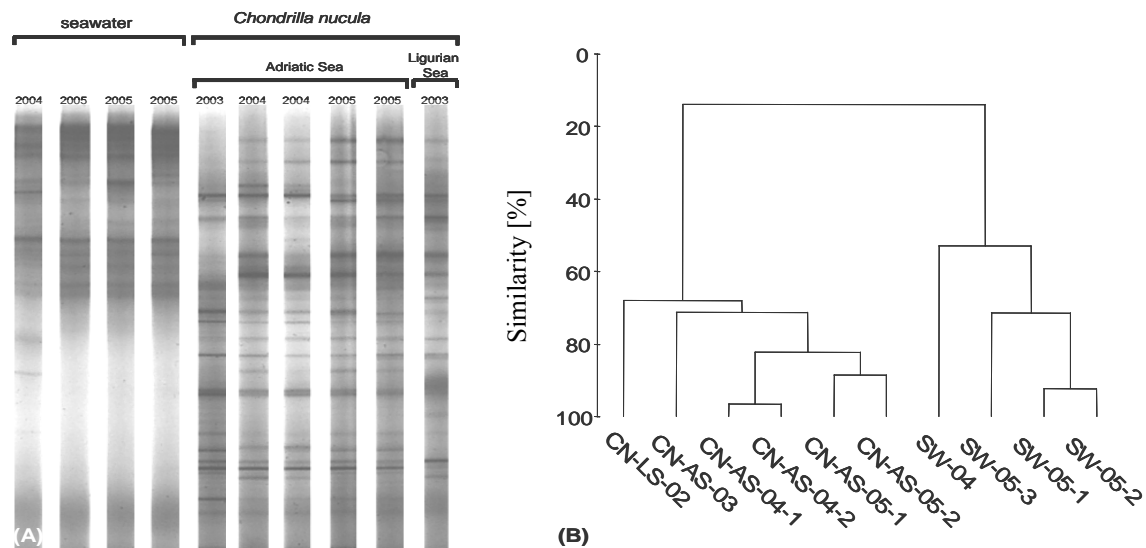


Fig. I-3: DGGE banding patterns of amplified 16S rDNA fragments obtained from *Chondrilla nucula* (CN) specimens and surrounding seawater (A). Banding patterns for sponges sampled in different years (02=2002; 03=2003; 04=2004; 05-1 and 05-2=2005 (two different specimens)) and/or different Mediterranean regions (Ligurian Sea: LS and Adriatic Sea: AS) show high similarity compared to seawater (SW), as demonstrated by cluster analysis (B). Clusters in dendrogram were confirmed to be well separated by ANOSIM method.

Phylogenetic analysis

Altogether 22 phylotypes were identified on the basis of 16S rDNA sequences obtained from *C. nucula* of the Adriatic Sea (AS, 34 sequences) and the Ligurian Sea (LS, 18 sequences). These sequences were distributed among six taxonomically validated bacterial divisions and the candidate division TM7 (Table I-1). The 16S rDNA sequences obtained from *C. nucula* were in large parts (79%) affiliated to other sponge-derived sequences available from databases. These sequences were assigned to fourteen phylogenetic clusters (Sponge-associated bacterial 'SAB'-clusters) within the *Gamma*- and *Deltaproteobacteria*, the *Actinobacteria*, the *Acidobacteria*, the *Bacteroidetes*, the *Gemmatimonadales* and the *Chloroflexi* (Fig. I-4 A+B). Four of the SAB-clusters found in *C. nucula* (*SAB-acido-I*, *SAB-gemmatimona-I*, *SAB-bacteroid-I* and *SAB-actino-I*) comprise sponge-specific clusters previously found in *Aplysina aerophoba*, *Theonella swinhoei* and *Rhopaloeides odorabile* ('Acido-II', 'uncertain-I', 'Bacteroid-I' and 'Actino-II' described by Hentschel *et al.* 2002).

Phylogenetic analysis demonstrated that *C. nucula* from both habitats harbour *Gamma*- and *Deltaproteobacteria*, *Actinobacteria*, *Acidobacteria* and *Chloroflexi* (Table I-1, Fig. I-4). Within the

Delta- and *Gammaproteobacteria*, as well as in the *Acidobacteria*, sequences obtained from *C. nucula* of both geographical regions shared >99% similarity (*Chondrilla nucula*-clusters: *CN-delta*, *CN-gamma*, *CN-acido*, Fig. I-4 A+B). *Alphaproteobacteria* and representatives of an uncultured cluster of sponge-derived sequences with previously uncertain affiliation (Hentschel *et al.* 2002) were present in *C. nucula* from the Ligurian Sea, but were not identified in sponges from the Adriatic Sea. In contrast, members of the *Bacteroidetes*, the candidate division TM7 and the *Gemmatimonadales* were detected only in the specimen from the Adriatic Sea (Fig. I-4 B, Table I-1).

Two clone sequences of *Acidobacteria* were found to be present in sponges from both Mediterranean regions. They share >99% sequence similarity and form a tight phylogenetic cluster, *CN-acido*, which is closely related to other sponge-derived sequences (>98% similarity) within group IV of the *Acidobacteria* (Quaiser *et al.* 2003) (Fig. I-4 B). *CN-acido* is part of the sponge-associated bacterial cluster *SAB-acido-I* (corresponding to 'Acido-II'; Hentschel *et al.* 2002).

Three different phylotypes of *Gammaproteobacteria* were associated with *C. nucula*, represented by 22 clone sequences (Fig. I-4 A). Half of all *C. nucula*-derived *Gammaproteobacteria* share >99% sequence identity (*CN-gamma*) and were found in *C. nucula* from both regions. One additional clone sequence more distantly related to cluster *CN-gamma* (95%) was obtained from *C. nucula* in the Ligurian Sea. *CN-gamma* is distantly related (<89%) to other sponge-associated bacteria and clusters monophyletically with sequences obtained from *Phyllospongia papyraceae* (Ridley *et al.* 2005) and *Rhopaloides odorabile* (Webster *et al.* 2001b) (cluster *SAB-gamma-II*, Fig. I-4 A). Eight additional clone sequences from *C. nucula* (AS) were related to other sponge-associated bacteria (clusters *SAB-gamma-I* and *SAB-gamma-III*, Fig. I-4 A). Within *SAB-gamma-I* the sponge-derived bacterium Candidatus *Spongiobacter nickelotolerans*, (unpublished, EMBL database entry AB205011) is the next cultivated relative to a cluster of six *C. nucula*-associated bacterial sequences (represented by clone CN34v, Fig. I-4 A), which underlines the specific association between *Gammaproteobacteria* of this cluster with sponges.

Table I-1: List of all *Chondrilla nucula* derived 16S rDNA clone sequences used for phylogenetic analysis. Next relative was obtained by BLAST search (Altschul *et al.* 1990). Sequence length refers to the first clone in the group. Numbers in brackets resemble the number of clones represented.

clone	source	length [nt]	next relative	Accession		identity	phylogenetic affiliation
				no.	overlap [bp]		
CN25v [1]	Adriatic Sea	1481	<i>Enterobacter</i> sp. B901-2	AB114268	1483	99%	<i>Gammaproteobacteria</i>
CN34v [6]	Adriatic Sea	1448	<i>Spongibacter nickelotolerans</i>	AB205011	1238	97%	<i>Gammaproteobacteria</i>
CN59v [1]	Adriatic Sea	1486	Arabian seawater clone A714013	AY907800	1492	95%	<i>Gammaproteobacteria</i>
CN33v [2]	Adriatic Sea	1443	unc. sponge associated bacterium clone E01-9c-26	AJ581351	1356	94%	<i>Gammaproteobacteria</i>
CN63v [5]	Adriatic Sea	1443	Arabian seawater clone A314008	AY907757	502	91%	<i>Gammaproteobacteria</i>
SLIVK05v [6]	Ligurian Sea	1447	Arabian seawater clone A314008	AY907757	502	91%	<i>Gammaproteobacteria</i>
SLIVK07 [1]	Ligurian Sea	1449	Arabian seawater clone A314008	AY907757	502	91%	<i>Gammaproteobacteria</i>
SLIHK01 [1]	Ligurian Sea	449	alpha proteobacterium clone CD204C11	DQ200432	458	93%	<i>Alphaproteobacteria, Rhodobacteriaceae</i>
SLIHK12 [1]	Ligurian Sea	533	unc. sponge associated bacterium clone Cnuc17	DQ079033	421	95%	<i>Alphaproteobacteria, Rhodobacteriaceae</i>
CN28v [1]	Adriatic Sea	1302	Uncultured bacterium clone 300C-C03	AY662023	568	85%	<i>Proteobacteria</i>
CN51v [4]	Adriatic Sea	1430	marine delta proteobacterium clone HMMVPog-22	AJ704689	676	88%	<i>Deltaproteobacteria, Bacteriovorax</i>
SLIHK17v [1]	Ligurian Sea	1367	bacterium clone SS-3	AY945894	676	87%	<i>Deltaproteobacteria, Bacteriovorax</i>
CN91v [1]	Adriatic Sea	1490	uncultured gamma proteobacterium R25	AF333536	871	95%	<i>Deltaproteobacteria</i>
SLIHK02 [1]	Ligurian Sea	518	unc. sponge associated bacterium clone JAWS5	AF434982	425	94%	<i>Deltaproteobacteria</i>
CN77v [1]	Adriatic Sea	1474	<i>Ornithobacterium rhinotracheale</i> D95-36425	U87100	1256	89%	<i>Bacteroidetes, Flavobacteriaceae</i>
CN31v [3]	Adriatic Sea	1488	unc. sponge associated bacterium clone TK44	AJ347046	1385	96%	<i>Bacteroidetes, Crenothricaceae</i>
CN82v [2]	Adriatic Sea	887	<i>Gemmatimonadetes</i> clone Dover77	AY499877	993	93%	<i>Gemmatimonadales</i>
CN45v [1]	Adriatic Sea	1473	unc. sponge associated bacterium clone TK81	AJ347051	1384	96%	<i>Acidobacteria</i>
SLIHK07v [1]	Ligurian Sea	1456	unc. sponge associated bacterium clone TK81	AJ347051	1387	96%	<i>Acidobacteria</i>
CN81v [1]	Adriatic Sea	1471	uncultured High G+C Gram-positive bacterium Sva0996	AJ241005	1395	93%	<i>Actinobacteria, Acidimicrobiaceae</i>
SLIVK06v [3]	Ligurian Sea	1414	unc. sponge associated bacterium clone PAUC32f	AF186411	1415	98%	<i>Actinobacteria, Acidimicrobiaceae</i>
CN72v [1]	Adriatic Sea	1491	<i>Chloroflexi</i> clone SAR269	AY534090	1448	95%	<i>Thermomicrobia, Chloroflexaceae</i>
SLIVK08 [2]	Ligurian Sea	547	unc. sponge associated bacterium clone Dd-sp-U-7	AY897091	322	95%	<i>Thermomicrobia, Chloroflexaceae</i>
CN69v [1]	Adriatic Sea	1443	<i>Chloroflexi</i> soil clone AKYH973	AY921926	723	88%	<i>Thermomicrobia, Chloroflexaceae</i>
CN94v [3]	Adriatic Sea	1429	uncultured bacterium SBR2013	AF269000	749	88%	TM7-division

One cluster of almost identical sequences obtained from *C. nucula* (AS+LS) was only distantly related (<88%) to known sequences in the EMBL databases, but could be affiliated to the *Deltaproteobacteria* (CN-delta, Fig. I-4 A) with *Bdellovibrio bacteriovorus* as the next described relative. Other *Deltaproteobacteria* from *C. nucula* with affiliation to sponge-derived sequences were found in clusters *SAB-delta-I* and *SAB-delta-II* (Fig. I-4 A).

Representatives of a sponge-specific cluster of previously uncertain affiliation ('uncertain-I'; Hentschel *et al.* 2002) were present in *C. nucula* from the Adriatic Sea. Here, the cluster was phylogenetically assigned to the division *Gemmatimonadetes* and is named *SAB-gemmatimona-I* (Fig. I-4 B).

The bacterial phylum *Bacteroidetes* was represented by four *C. nucula*-derived 16S rDNA sequences obtained from the Adriatic Sea specimen (Fig. I-4 B). Three of these clones (represented by sequence CN31v, Fig. I-4 B) were related to other sponge-associated bacteria, forming cluster *SAB-bacteroid-I*. This sponge-associated bacterial cluster includes the sponge-specific cluster 'Bacteroid-I' (Hentschel *et al.* 2002) but does not comprise sponge-associated bacteria exclusively.

In *C. nucula* from both Mediterranean habitats *Actinobacteria* affiliated with the previously described sponge-specific cluster 'Actino-II' (Hentschel *et al.* 2002) were found. Together, these sequences form the sponge-associated bacterial cluster *SAB-actino-I* (Fig. I-4 B). The *C. nucula*-derived *Actinobacteria* in *SAB-actino-I* obtained from both Mediterranean regions share low sequence similarity of only 93% and therefore certainly belong to different species or even genera.

Four *C. nucula*-derived sequences could be assigned to the *Chloroflexi*. Three of them showed affiliation to sequences previously found in sponges (clusters *SAB-chloroflexi-I* and *SAB-chloroflexi-II*, Fig. I-4 B). The unspecific sponge-cluster *SAB-chloroflexi-I* comprises sponge-associated bacteria from *C. nucula* from the Ligurian Sea (this study) and from the Caribbean Sea (Hill *et al.* 2006), from *Discoderma dissoluta* (Schirmer *et al.* 2005), *Theonella swinhoei* (Hentschel *et al.* 2002) and also coral-associated clone sequences (unpublished, EMBL entry AF365719). *SAB-chloroflexi-II* was found in *C. nucula* from the Adriatic Sea as well as in an unidentified marine sponge (unpublished, EMBL entry AY046066).

A cluster of three almost identical clone sequences distantly related (88%) to uncultured representatives of candidate division TM7 was retrieved from *C. nucula* (AS) (Fig. I-4 B). Members of the recently recognised candidate division TM7 have been shown to be present in aquatic habitats including seawater and deep-sea sediments, but have not been described from sponges before.

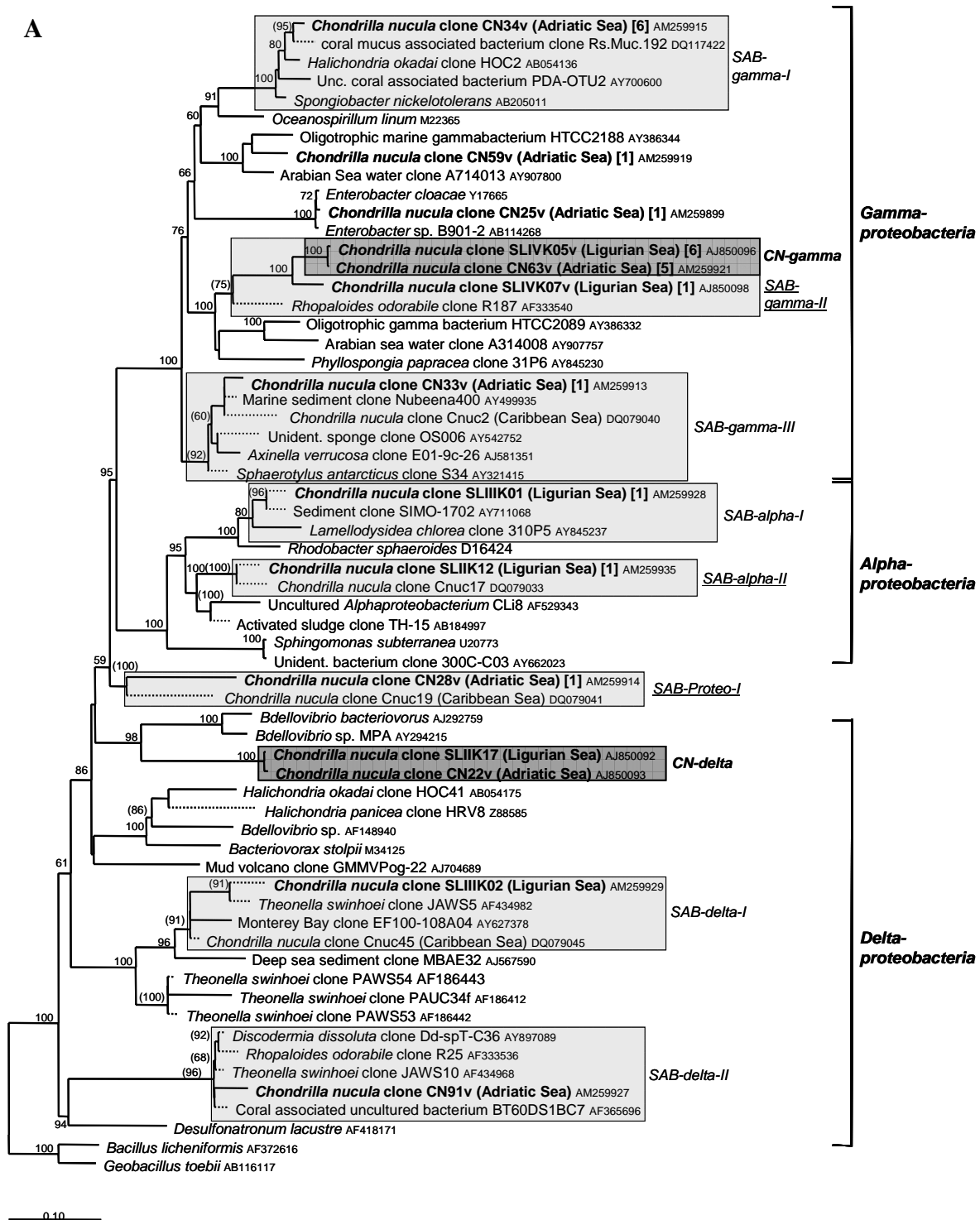
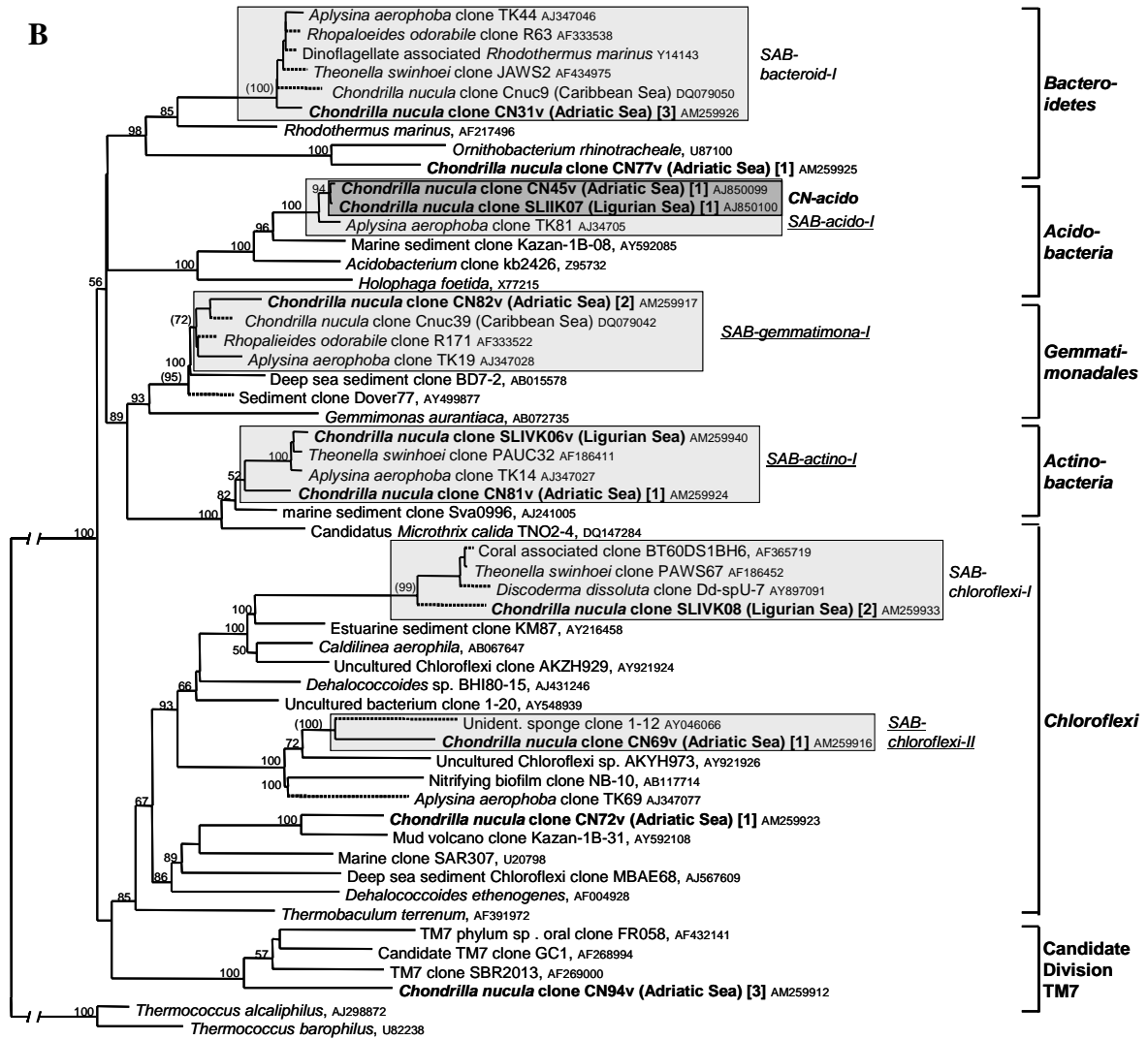


Fig. I-4: Phylogenetic tree of 16S rDNA sequences obtained from *Chondrilla nucula*-associated bacteria and relatives belonging to *Proteobacteria* (A) and several other bacterial divisions (B). Sequences obtained from *C. nucula* in this study are written in bold letters. Sponge clones ‘CN’ were obtained from the Adriatic Sea specimen whereas ‘SL’ clones were obtained from the Ligurian Sea specimen. Each sequence represents one phylotype. Numbers of clones represented by the sequence are given in brackets. Sponge-associated bacterial (SAB-) clusters are framed and lightly highlighted, *C. nucula* specific (CN-) clusters are framed and highlighted darker. Clusters including exclusively sponge-derived sequences are underlined. Dotted tree-branches indicate partial sequences (<1200bp). Numbers at knots represent bootstrap values (100 replicates done). Bootstrap values shown in parenthesis refer to calculations including short sequences. The bar represents 0.1 substitutions per alignment position.



DISCUSSION

Sponge specificity

Most of the 16S rDNA sequences (79%) obtained from the marine sponge *Chondrilla nucula* were related to other sponge-derived sequences, suggesting common sponge-associated bacterial groups. In previous studies phylogenetically related bacteria were found in sponges of distant taxonomic and geographic origin, and the existence of a highly diverse yet specifically sponge-associated bacterial community was proposed (Hentschel *et al.* 2006, Sfanos *et al.* 2005, Imhoff and Stöhr 2003, Thiel and Imhoff 2003, Hentschel *et al.* 2002, Wilkinson *et al.* 1981). However, with the increasing number of bacterial sequences obtained from marine environments available in the databases, sequences from marine habitats other than sponges are found to cluster with phylotypes formerly considered to be sponge-specific. Only four of the fourteen phylogenetic clusters of sponge-associated bacteria in this study are strictly confined to sponges (*SAB-actino-I*, *SAB-acido-I*, *SAB-gemmatimona-I* and *SAB-gamma-I*, Fig. I-4 A+B). Seven *SAB*-clusters include sequences obtained from other marine organisms or sediment and cannot be considered to be strictly sponge-specific. Clusters *SAB-alpha-II*, *SAB-chloroflexi-II* and *SAB-proteo-I* consist of only few sequences sharing low similarity. Sponge-specificity is still uncertain for those groups. For other sponge-associated bacterial clusters, a stable association with less specificity regarding the host-organisms is assumed.

C. nucula specificity and biogeography

Sponges are filter-feeders and retain bacteria from filtered seawater; therefore not all bacterial sequences present in 16S rDNA gene libraries obtained from sponges can be expected to be specifically associated. On the other hand, associations of bacteria with sponges may be specific even on a sponge species level. Using DGGE methods, sponge-associated bacteria were classified as (1) ‘specialists’, (2) ‘sponge-associates’ (3) ‘generalists’, depending on their presence in (1) one host species only, (2) several sponges species, but absent in seawater or (3) several sponge hosts and seawater (Taylor *et al.* 2004). The proportion of the community resembling these types (‘specialists’, ‘sponge-associates’ and ‘generalists’) differs between sponge species (Taylor *et al.* 2004). The associated bacterial community was demonstrated to be specific and stable in *Cymbastela concentrica*, whereas the bacterial community associated with *Callyspongia* sp. and *Stylinos* sp. from the same habitat were highly influenced by seawater bacteria (Taylor *et al.* 2004).

In this study, a stable bacterial community was shown to be associated with *C. nucula* by constant DGGE banding patterns of different sponge individuals with only few variant bands regardless of time and origin (Ligurian Sea and Adriatic Sea, Fig. I-1 and I-3). Furthermore, a comparison with DGGE banding patterns of other sponges from the same habitat, e.g., *Tethya aurantium*, *Cacospongia* sp. and *Chondrosia reniformis* (data not shown), revealed a high number of bands constantly present in *C. nucula* exclusively, but absent from the seawater and other sponges. Those *C. nucula*-specific DGGE bands hint towards a high fraction of 'specialists' or 'sponge-associates' little influenced by the geographical region and surrounding seawater (Fig. I-3). Additionally analysis of 16S rDNA gene libraries revealed three tight bacterial clusters present in *C. nucula* specimens from both Mediterranean regions (*CN-acido*, *CN-gamma* and *CN-delta*, Fig. I-4) but absent in *Tethya aurantium* from the same Adriatic Sea habitat (Thiel *et al.* in press). The two habitats which were compared in this study are characterised by distinct water masses. The Mediterranean topography as well as the water circulation forced by wind stress, freshwater and heat fluxes prevents a water exchange between the Ligurian Sea and the Adriatic Sea (Milot 1999). Thus the particularly high sequence similarities (>99%) of bacteria present in sponge specimens of both regions and the absence from surrounding seawater imply a specific association to sponges or even *C. nucula* for the corresponding bacteria of clusters *CN-gamma*, *CN-delta* and *CN-acido* (Fig. I-4 A+B).

CN-acido and *CN-gamma* are closely related to other sponge-derived sequences and belong to two of the four sponge-specific monophyletic clusters (*SAB-acido-I* and *SAB-gamma-II*), indicating a stable and highly sponge-specific association between these bacteria and *C. nucula*. High sequence similarity (98%) to an uncultured *Aplysina aerophoba*-associated *Acidobacterium* sp. (clone TK81) implies a specific association of closely related *Acidobacteria* with different sponges. In the marine habitat, *Acidobacteria* have so far only been detected by culture-independent methods, e.g., in sponges and marine sediments (Olson and McCarthy 2005, Lopez-Garcia *et al.* 2003, Hentschel *et al.* 2002). Therefore, no information regarding physiology and metabolism of those marine *Acidobacteria* is available. Even for terrestrial strains the genetic and metabolic diversity of *Acidobacteria* is still largely undescribed (Barns *et al.* 1999, Hugenholtz *et al.* 1998). The type of association and the ecological function of *Acidobacteria* specifically associated with sponges will be an interesting field for further studies.

A stable and highly specific association of *Gammaproteobacteria* with marine sponges is indicated for cluster *CN-gamma*, present in *C. nucula* from both Mediterranean regions. *CN-gamma* shows close phylogenetic relation to other sponge-associated bacteria with distinct monophyletic clustering of sponge-derived sequences (bootstrap value of 100 for cluster

SAB-gamma-II, Fig. I-4 A), suggesting sponge-specificity for this cluster. The closest relative to cluster *SAB-gamma-II* in culture is an obligatory oligotrophic marine bacterium with growth optimum at 16°C (Cho and Giovannoni 2004). It is the only cultured representative of a widely distributed clade of marine bacterioplankton (Fuchs *et al.* 2005). The phylogenetic distance between the sponge-associated bacteria of cluster *SAB-gamma-II* and this planktonic gammaproteobacterium is relatively large (91%), which indicates the level of a new genus or even family for the sponge-specific bacteria of cluster *SAB-gamma-II*.

In contrast, *CN-delta* represents a putative sponge symbiont with no known relatives. Similarity to other sequences in the databases was low (<88%) for all known phylogenetic groups. By phylogenetic analysis *CN-delta* could be assigned to the *Deltaproteobacteria* with phylogenetic relation to *Bdellovibrio*, a small motile bacterium which preys on other Gram-negative bacteria (Jurkevitch 2000). So far, no members of the genus *Bdellovibrio* have been obtained from sponges. However, Wilkinson observed *Bdellovibrio*-like bacteria infecting unicellular symbiotic cyanobacteria in coral reef sponges (Wilkinson 1979). Neither the infected cyanobacteria nor the *Bdellovibrio*-like bacterium could be cultured, hence the studies on the interaction could not be further pursued (Wilkinson 1979). Possible interactions of sponge-associated bacteria, not only with the sponge-hosts but also with other bacteria open a new perspective for sponges as microhabitats.

In addition to the phylotypes described above, a highly diverse bacterial community was demonstrated to be present in *C. nucula*. The majority of sequences belonged to bacterial phyla obtained from sponges before. For the first time, we found members of the novel candidate division TM7 associated with sponges. Candidate division TM7 has been detected in a variety of terrestrial, clinical and aquatic habitats including seawater and deep-sea sediments but has not been reported from marine sponges before (Donachie *et al.* 2004, Brinig *et al.* 2003, Lopez-Garcia *et al.* 2003, Hugenholtz *et al.* 2001). So far, candidate division TM7 includes no pure-culture representatives (Ferrari *et al.* 2005, Hugenholtz *et al.* 2001). Molecular approaches including fluorescence-*in-situ*-hybridisation (FISH) revealed that members of candidate division TM7 show sheathed filament morphology and a Gram-positive cell envelope ultrastructure (Hugenholtz *et al.* 2001). For the lack of pure-cultures, no information concerning physiology and metabolism is available.

A recent study addressed the bacterial diversity associated with *C. nucula* originating from the Caribbean Sea (Hill *et al.* 2006). Sponge-taxonomic studies based on molecular methods, however, revealed noteworthy differences between *C. nucula* specimens from the Mediterranean Sea and from other oceans (Usher *et al.* 2004b, Klautau *et al.* 1999) indicating

that they might be different species. In our study we identified sequences sharing >99% sequence similarity in both Mediterranean regions while sequences obtained from *C. nucula* specimens from the Caribbean Sea share <96% to Mediterranean *C. nucula*-derived sequences. Thus *C. nucula* from the Mediterranean and the Caribbean Sea seem to harbour different bacterial species, limiting the specificity of the associated bacterial community to *C. nucula* from the Mediterranean Sea. However, *C. nucula* from both geographical regions (Mediterranean Sea and Caribbean Sea) show noticeable overlap in their associated bacterial communities in terms of bacterial groups (members of sponge-associated clusters *SAB-alpha-II*, *SAB-proteo-I*, *SAB-delta-I*, *SAB-gemmatimona-I* and *SAB-bacteroid-I*, Fig. I-4), indicating common ancestry of the associated bacteria.

Sponges as micro-environments

Within the Mediterranean Sea, *C. nucula* harbours a specific and stable bacterial community little influenced by ambient seawater. A high number of specifically associated bacteria were shown by DGGE banding patterns and at least three constantly associated phylotypes were identified by 16S rDNA sequencing. The bacterial community associated with the sponge was highly diverse as shown by 16S rDNA sequencing. A specific and stable association with the sponge was demonstrated for members of phylogenetically unrelated bacterial groups with putatively different metabolic functions. Thus, adaptation to distinct ecological niches and possible interactions within the microbial community (e.g., bacterial predator-prey relationships as indicated by the existence of *Bdellovibrio*-like *Deltaproteobacteria* in the sponge) are assumed for members of the associated community. Additional to a high diversity of bacteria, other micro- and macroorganisms (i.e., archaea, yeasts, algae as well as polychaetes and prosobranchs) have been found in association with sponges (Holmes and Blanch 2006, Maldonado *et al.* 2005, Kunzmann 1996). This suggests that sponges as opposed to being 'only' a partner of specific (two-partners-) associations can be referred to as 'micro-environments' providing a broad variety of ecological niches. Thus, further studies and ecological models on sponge-associated bacterial communities should not only focus on the sponge-microbial interactions, but also include interactions within the microbial community. Factors controlling sponge-associated microbial communities could include the impact of bacterial viruses, surface characteristics and chemical factors (e.g., natural antibiotics) as well as other microbial population interactions. In fact, there may be a great variety of inter-microbial relations within the sponge, ranging from mutualistic to parasitic, which might open new and highly promising research fields.

CHAPTER II

SPATIAL DISTRIBUTION OF SPONGE-ASSOCIATED BACTERIA IN THE MEDITERRANEAN SPONGE *TETHYA AURANTIUM*

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ABSTRACT

For the first time local distribution of the bacterial community associated with the marine sponge *Tethya aurantium* Pallas 1766 was studied. Distinct bacterial communities inhabit endosome and cortex. Clear differences in the associated bacterial populations were demonstrated by DGGE and analysis of 16S rDNA clone libraries. Specifically associated phylotypes were identified for both regions: a new phylotype of *Flexibacteria* was only recovered from the sponge cortex, while *Synechococcus* spp. were present mainly in the sponge endosome. Light conduction via radiate spicule bundles conceivably facilitates the unusual association of cyanobacteria with the sponge endosome. Furthermore, a new monophyletic cluster of sponge-derived 16S rDNA sequences related to the *Betaproteobacteria* was identified using analysis of 16S rDNA clone libraries. Members of this cluster were specifically associated with both cortex and endosome of *T. aurantium*.

INTRODUCTION

The phylum Porifera contains an estimated 15,000 species in three taxonomic classes: Calcarea (calcareous sponges), Hexactinellida (glass sponges) and Demospongiae (demosponges) (Hooper and van Soest 2002). As sessile filter feeding organisms sponges pump large amounts of water through their aquiferous channel system. They take up bacteria, single-celled algae and other food particles from the filtered water by phagocytosis within the choanocyte chambers which are located within the inner part of the sponge, the endosome (= choanosome). The endosome is protected against strong currents and high light intensities by an outer region, the cortex (or ectosome) (Sar 1987), which is also used as a taxonomic criterion. As a protective device for shallow water habitats (Burton 1928), the cortex is found to be particularly thick and well structured in species living in shallow waters with strong currents and high light intensities. In contrast, species living in more protected habitats display a thin, almost indistinct cortex (Sar 1987).

Tethya aurantium is characterised by a globular shape and a thick and well developed cortex, clearly differentiated from the endosome by texture and colour of the tissue (Fig. II-1 B). In the Mediterranean Sea *T. aurantium* co-occurs with the very similar species of *T. citrina*, but inhabits different niches and can be distinguished by the development of its cortex. *T. aurantium* generally inhabits areas that are more exposed to light and current in depths of 1-40 m, while *T. citrina* prefers more sheltered places and possesses a thinner cortex compared to *T. aurantium* (Sar 1987)

Associations between microorganisms and sponges have been systematically studied using microscopy and isolation methods since the 1970s (Lafi *et al.* 2005, Webster and Hill 2001, Manz *et al.* 2000, Vacelet and Donadey 1977, Vacelet 1975, 1971, 1970). These studies showed that bacteria are abundant within the mesohyl of sponges and can form up to 40% of the sponge volume (Wilkinson 1978c). More recent studies on sponge-microbe associations were based mainly on culture independent molecular methods (Burja and Hill 2001, Webster and Hill 2001, Webster *et al.* 2001b, Burja *et al.* 1999, Althoff *et al.* 1998, Hinde *et al.* 1994). Comparison of 16S rDNA clone libraries obtained from several sponges of different geographical origin revealed unexpected conformity between the different sponge species and a uniform sponge-associated bacterial community was proposed (Hentschel *et al.* 2002).

The aim of this study was to characterise the microbial community associated with *T. aurantium*. We demonstrate specific differences between bacteria associated with cortex and endosome. Furthermore, we report on a new phylogenetic cluster of sponge-associated *Betaproteobacteria* and a possible association of *T. aurantium* with cyanobacteria.

MATERIALS AND METHODS

Sampling sites

The Limski kanal is a semi-closed fjord-like bay in the Adriatic Sea near Rovinj (Istrian Peninsula, Croatia) (N45°8'; E13°44'). It extends along an east-west axis, with an approximate length of 11 km, a maximum width of about 650 m and a depth of up to 32 m. The bottom is generally muddy, with insular sites comprised of stones and shallow rocky slopes along the sides. It is characterised by a very high sedimentation rate and a rapid water exchange (Kuzmanovic 1985). In a recent study 42 sponge species were found in the Limski kanal including three species of the genus *Tethya*: *T. aurantium* Pallas 1766, *T. limski* Müller & Zahn 1969 and *T. citrina* Sará & Melone 1965 (Brümmer *et al.* 2004).

Sponge sampling

Specimens of *T. aurantium* were collected by SCUBA diving from a depth of 5-15 m in April 2003, June 2004 and May 2005. The sponges were collected into sterile plastic bags, cooled in an isolation box, immediately transported to the laboratory, and processed within 3 hours. In the laboratory the sponges were washed carefully three times in filter-sterilised seawater (0.2 µm pore size, Sartorius) prior to cutting. The sponges were separated into cortex

and endosome sections and washed again separately in sterile seawater. Tissues were cut into small pieces of approximately 20-30 mg each, frozen in liquid nitrogen, and kept frozen (-80°C) until further investigation.

Ambient seawater was collected into sterile glass bottles (Duran, 1 L) prior to sponge sampling. The water was cooled on the way back to the laboratory and immediately filtered through a cellulose-acetate filter (0.2 µm pore size, Sartorius). The filters were put into a cryovial, frozen in liquid nitrogen, and stored at -80°C until further investigation.

Electron microscopy

Sponge samples were prepared for scanning electron microscopy by fixation with 1% glutaraldehyde in seawater, replacement of water with an ethanol series and subsequent critical-point-drying. After mounting samples were sputtered with Au/Pd and observed with a Zeiss DSM 940 scanning electron microscope.

DNA extraction

Genomic DNA was extracted and purified using the QIAGEN DNeasy® Tissue Kit following the manufacturer's protocol for Gram-positive bacteria and animal tissue.

PCR and cloning procedure

Amplification of ribosomal DNA was performed using puReTaq™Ready-To-Go™PCR Beads (Amersham Biosciences). For amplification of the nearly complete 16S rRNA gene the eubacterial primers 27f and 1492r (Lane 1991) were used. The conditions for this PCR were: initial denaturation (2 min at 94°C) followed by 30 cycles of primer annealing (40 sec at 50°C), primer extension (90 sec at 72°C) and denaturation (40 sec at 94°C), a final primer annealing (1 min at 42°C) and a final extension phase (5 min at 72°C). PCR products were checked for correct length on a 1% Tris-Borate-EDTA (TBE) agarose gel (1% agarose, 8.9 mM Tris, 8.9 mM borate, 0.2 mM EDTA), stained with ethidium bromide and visualized under UV-illumination.

DNA was purified using the High Pure PCR Product Purification Kit (Roche) prior to ligation into pCR®4-TOPO® vector and transformation into One Shot® Competent *E. coli* cells using the TOPO TA® Cloning Kit (Invitrogen). Inserts were amplified as described above using the M13f/M13r primer set (M13f: 5'-GTA AAA CGA CGG CCA G-3' / M13r:

5'-CAG GAA ACA GCT ATG AC-3') (0.1 μ M each). Correct insert size was verified using agarose gel electrophoresis.

PCR for denaturing gradient gel electrophoresis (DGGE) was performed using the primers 342-GCf and 534r (Muyzer *et al.* 1993). Temperature profile was as follows: initial denaturation (2 min at 94°C) followed by 15 cycles of touchdown primer annealing (40 sec at 65°C to 50°C), primer extension (1 min at 72°C), and denaturation (40 sec at 94°C), additional 20 cycles of primer annealing (40 sec at 50°C), primer extension (1 min at 72°C), and denaturation (40 sec at 94°C), and a final primer annealing (1 min at 42°C) with a final extension phase (5 min at 72°C). PCR products were checked for correct length on a 2% TBE-agarose gel. Excised DGGE bands (s. below) were re-amplified using primers GC/M (5'-GGGGGCAGGGGGGC-3') and 534r (Muyzer *et al.* 1993) as described above with 50°C annealing temperature and 30 cycles.

Denaturing gradient gel electrophoresis (DGGE)

DGGE was conducted in a double gradient gel (Petri and Imhoff 2001), containing a linear 6-8% polyacrylamid (acrylamid : bisacrylamid ratio 37,5:1) and a 50-80% denaturing gradient (100% corresponds to 7 M urea / 40% deionised formamide). The gel was run in a Tris-EDTA-acetic acid (TAE) buffer (10 mM Tris, 5 mM acetic acid, 5 mM EDTA, titrated to pH 7.5) at a voltage of 80V for 15 h. The gel was stained in 1x SYBR Gold (Invitrogen) (Tuma *et al.* 1999) in TAE and documented digitally.

Bands exclusively present in sponge samples were cut out and DNA was extracted for sequencing. Excised bands were transferred into 50 μ l of molecular grade water, crushed with sterile pistils and incubated overnight at 4°C. The supernatant (1 μ l) was used as template for re-amplification with primers GC/M and 534r as described above and sequenced.

Cluster analysis

For statistical comparison of the DGGE banding patterns, similarity cluster analysis (Clarke and Warwick 1994) and analysis of similarity (ANOSIM) (Clarke 1993) were performed using the program PRIMER 5 v5.2.2 (PRIMER-E Ltd.). Similarity was calculated using the Bray-Curtis index and cluster analysis was conducted with complete linkage. Subsequently one-way ANOSIM with all possible permutations was performed. In accordance with the PRIMER-manual (Clarke and Gorley 2001) ANOSIM R-values >0.75 were interpreted as well separated; R>0.5 as overlapping, but clearly different and R<0.25 as barely

separable at all. Replicate samples were grouped according to source (e.g., endosome, cortex, seawater) as factors for the analyses.

Sequencing and phylogenetic analysis

After verification of correct insert size, clones (for each sample 29-41) were sequenced using the ABI PRISM® BigDye™ Terminator Ready Reaction Kit (Applied Biosystems) and an ABI PRISM® 310 Genetic Analyser (Perkin Elmer Applied Biosystems). Sequence primers used were: plasmid primers M13f and M13r as well as the 16S rDNA-specific primers 342f (Muyzer *et al.* 1993), 534r (Muyzer *et al.* 1993), 790f (5'-GAT ACC CTG GTA GTC C-3'), 907f (5'-GGC AAA CTC AAA GGA ATT GAC-3'), 1093f (5'-TCC CGC AAC GAG CGC AAC CC-3') and 1093r (5'-GGG TTG CGC TCG TTG CGG GA-3'). Sequence data were edited with Lasergene Software SeqMan™II (DNASTar Inc.) and checked for possible chimeric origin using the program CHECK_CHIMERA (<http://35.8.164.52/html/index.html>) of the Ribosomal Database Project (<http://rdp.cme.msu.edu/index.jsp>) (Maidak *et al.* 1999). Putative chimeric sequences were removed from phylogenetic analyses. Next relatives were determined by comparison to 16S rRNA genes in the NCBI GenBank database using BLAST (Basic Local Alignment Search Tool) searches (Altschul *et al.* 1990) and the RDPII Sequence Match Program (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp). Sequences were aligned using the FastAlign function of the alignment editor implemented in the ARB software package (www.arb-home.de) (Ludwig *et al.* 2004) and refined manually employing secondary structure information. For phylogenetic calculations the PhyML software (Guindon and Gascuel 2003) as well as the online version of PhyML (Guindon *et al.* 2005) were used. Trees were calculated by Maximum Likelihood (ML) method (Felsenstein 1981) using the GTR model and estimated proportion of invariable sites as well as the Gamma distribution parameter with near full length sequences (≥ 1000 bp/ ≥ 1200 bp for tree Fig. II-4 B/C, respectively). Calculated trees were imported into ARB and short sequences were subsequently added by use of the ARB parsimony method without changing tree topologies. Phylogenetic positions of short sequences (< 1000 bp/ < 1200 bp) were additionally verified by phylogenetic analysis (ML, 100 bootstraps) including full and partial sequences.

Nucleotide sequence accession numbers

The 16S rDNA sequences obtained in this study have been deposited in the EMBL database. They have been assigned accession numbers AM259730-AM259769, AM259770-

AM259831 and AM259832-AM259898 for the sequences obtained from seawater, endosome and cortex, respectively.

Diversity estimation

Sequences with similarities >99.0% were defined as one phylotype, i.e., operational taxonomic unit (OTU). The proportion of prokaryotic diversity represented by the clone libraries was estimated by rarefaction analysis combined with non-linear regression, and by calculation of the chao1 estimator as proposed by Kemp & Aller (2004). Rarefaction analysis calculations were performed applying the algorithm described by Hurlbert (1971) with the program aRarefactWin (<http://www.uga.edu/~strata/Software.html>). Rarefaction curves were plotted and regressions performed using two different equations:

$$\text{regression (1)} \quad y_1 = a_1(1 - e^{-b_1/x}),$$

$$\text{regression (2)} \quad y_2 = a_2(1 - e^{-b_2/x^c}),$$

with sample size x , observed number of OTU y , and the number of OTU to be expected with infinite sample size a (i.e., total diversity) (Koellner *et al.* 2004). Equation (1) is the most common regression approach for rarefaction analysis and is used by many authors (e.g., Yakimov *et al.* 2006, Webster *et al.* 2004). However, curves derived from regression (1) exhibited an apparent misfit to the data points of the rarefaction analysis. The increase of the regression curves was too steep at small OTU numbers and curves flattened visually too early at large sample sizes. The expected underestimation of the maximum species number proposed a modification of regression (1), namely equation (2). To prove this, we developed an algorithm to simulate sampling of specimens for rarefaction analysis that has the potential to produce datasets ranging from highly diverse (each OTU occurring only once or twice) to almost uniform (one or two abundant OTU): a cohort of random integer numbers k_i ($i \in \mathbb{N}$; $1 \leq i \leq 50$) were created with the equation $k_i = 5 \cdot \Gamma_{\alpha_i}(z_i)$; Γ is the standard Gamma function of z_i (randomized; $0 \leq z < 1$) with shape parameter α_i (randomized; $0.1 \leq \alpha_i < 0.6$). Of this cohort, n numbers k were taken so that $\sum_{i=1}^n k_i \leq s$ (randomized; $30 \leq s < 70$). This procedure is tantamount to sampling up to s specimens (i.e., clones) representing n OTU of abundance k_i . The randomized Gamma function causes the distribution of k_i to be skewed more or less to the right, resembling abundance proportions in most natural communities. The simulated datasets were subjected to rarefaction analysis, and the resulting rarefaction curves were fitted by regressions (1) and (2), respectively. From both equations, the asymptote a and the PRESS (Predicted Residual Error Sum of Squares) statistic were obtained. PRESS is a gauge of how

well a regression predicts new data. The smaller the PRESS statistic, the better is the predictive ability of the regression. To test whether the two equations differ statistically from each other, we calculated the ratios $A = a_2/a_1$ and $P = \text{PRESS}_1/\text{PRESS}_2$. If there is one highly abundant OTU ($k_i \geq 20$) beside many OTU of low abundance ($k_i < 3$) in the sampling datasets, the rarefaction curve tends to converge to a line without apparent limit. This occurred three times with our simulated datasets with $a_2 \gg a_1$ ($10^4 < a_2 < 10^7$; $6 < a_1 < 40$). For these cases, ratio A was not calculated. Transformed ratios $A' = \ln(\ln(A+1/100))$ and $P' = \ln(P+1/100)$ did not show significant deviations from the normal distribution (Shapiro-Wilk W test). A' and P' were tested against the null hypotheses $\bar{A}' = -4.61$ ($\bar{A} = 1$) and $\bar{P}' = 0.01$ ($\bar{P} = 1$), respectively.

SigmaPlot v6.0 (SPSS) was used for plotting and regression analysis. Statistical tests were performed with Statistica v6.1 (StatSoft).

RESULTS

Electron microscopy

Tethya aurantium cortex and endosome (Fig. II-1) samples were studied separately and sponge samples from consecutive years were compared. Electron micrographs revealed large differences between cortex and endosome. Only low numbers of bacteria were associated with the sponge in the cortex region while bacteria were fairly abundant in the endosome of the sponge (Fig. II-1 C-E). Different morphotypes, especially a high abundance of rod shaped bacteria, were found associated with the sponge endosome.

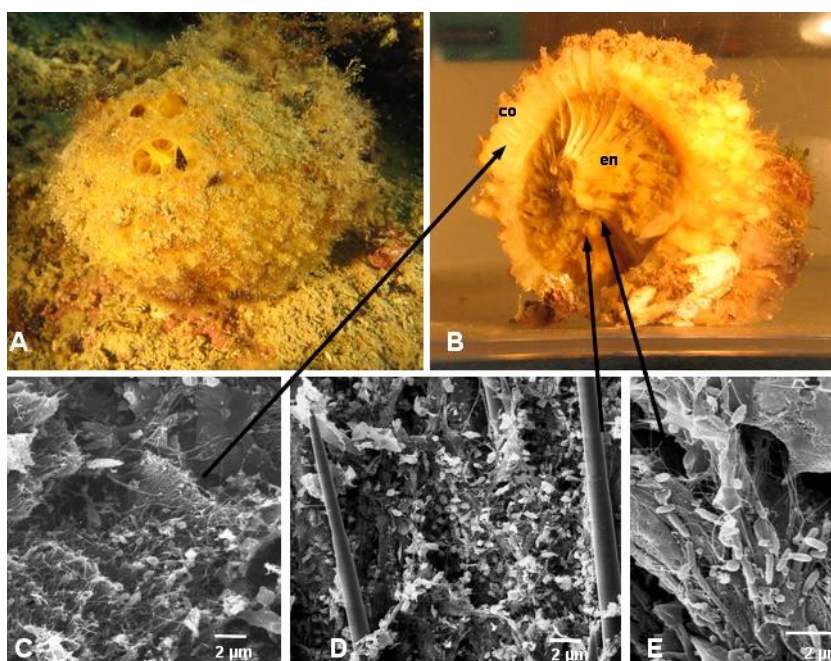


Fig. II-1: Photograph of the Mediterranean sponge *Tethya aurantium* Pallas *in-situ* (A). A cross section (B) shows the morphologically different regions endosome (en) and cortex (co). Electron microscopic photography shows no apparent accumulation of bacteria in the cortex (C) and a moderate number of different bacterial morphotypes within the endosome (D+E).

Cortex- and endosome-specific bacteria

DGGE banding patterns clearly showed the presence of different bacterial communities in endosome and cortex of *T. aurantium* (Fig. II-2). Additionally, both parts of the sponge differ in their DGGE banding patterns from surrounding seawater samples. Bacterial phylotypes specifically associated with distinct sponge regions were represented by DGGE bands that were exclusively present in all endosome or cortex samples, respectively, but not found in seawater (Fig. II-2 A). Other bands were found in all sponge samples (Fig.

II-2 A). While the patterns in sub-samples of endosome and cortex of one individual were apparently identical, slight variations were found between endosome as well as cortex samples from consecutive years (2003-2005) (Fig. II-2 B+C). Nevertheless, bacterial communities inhabiting the same sponge region (endosome or cortex) were more similar compared to populations of different regions (Fig. II-2 A+B). In contrast to the sub-samples of a sponge individual (2005), which showed identical DGGE banding patterns, seawater samples from different near-by locations varied between each other to some extent (Fig. II-2 C). The clusters of DGGE banding patterns of endosome, cortex, and seawater samples, respectively, were confirmed to be well separated by ANOSIM (R-value >0.75).

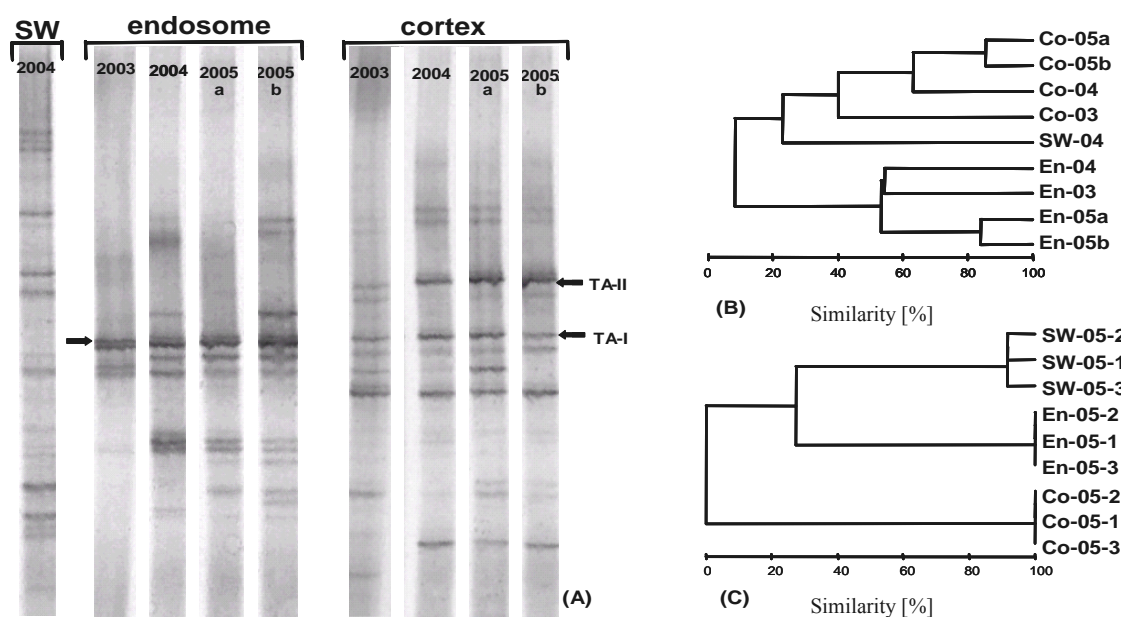


Fig. II-2: (A) Denaturing gradient gel electrophoresis (DGGE) banding patterns of amplified bacterial DNA extracted from *Tethya aurantium* Pallas cortex region (2003, 2004 and 2005-1/-2), endosomal region (2003, 2004 and 2005-1/-2) and surrounding seawater (SW). Specialized bands, which are not present in seawater, occur in all sponge samples (TA-I) and in the cortex only (TA-II). (B+C) Dendrograms of similarity cluster analysis with DGGE banding patterns of amplified bacterial DNA extracted from *T. aurantium* cortex (co) and endosomal (en) samples. Comparison of sponge individuals from 2003, 2004 and 2005 and surrounding seawater (B) show that, although not identical, a clear clustering of the banding patterns is seen. Regardless of the year, samples of endosomal regions are more similar to each other than to the cortex samples of the same individual. Replicate subsamples from one individual (May 2005) (C) show identical banding patterns and precise differences between endosome and cortex, as well as to several nearby seawater samples which differ to some extent.

Methods of diversity estimation

Comparison of the two regressions (Table II-1) showed that rarefaction analysis using regression (2) resulted in higher estimated maximum OTU numbers a_2 as compared to a_1 of regression (1). a_2 is, in most cases, well comparable to richness estimation using the non-parametric estimator chao1, as proposed by Kemp & Aller (2004) (Table II-1). Equality of parameters a_1 and a_2 and the statistics PRESS₁ and PRESS₂ of the two regressions could be

rejected on a highly significant level ($p < 10^{-6}$) in both cases. We can therefore state that (i) equation (2) fits significantly better than equation (1) as a regression for fitting rarefaction curves and predicting total diversity and that (ii) the total diversity a_1 calculated with the conventional equation (1) is systematically lower than a_2 calculated with regression (2).

Table II-1: Observed and estimated total bacterial diversity of phylotypes (OTU) in the different sponge and seawater samples. Rarefaction analysis and the non-parametric richness estimator chao1 were used for diversity estimation. Rarefaction analysis was conducted with a commonly used regression (1) and a modified regression (2). In all cases regression (2) showed higher r^2 values and a higher expected diversity than regression (1). Except for the seawater clone library, chao1 coverage resembles about total coverage estimated by the use of regression (2). Total diversity was best covered by the analysed clones for the clone library from the sponge cortex 2004 (76-103% depending on method used).

clone library	n	OTU	rarefaction analysis (RA)						chao1	
			regression (1)			regression (2)			S_{chao1}	C_{chao1}
			a_1	r^2_{RA1}	C_{RA1}	a_2	r^2_{RA2}	C_{RA2}		
seawater 2004	41	27	46.2	0.99976	58%	56.5	0.99993	48%	88.2	31%
sponge cortex total	66	30	41.1	0.99816	73%	67.7	0.99996	44%	59.0	51%
Sponge cortex 2003	37	26	49.5	0.99992	52%	56.6	0.99998	46%	60.0	43%
sponge cortex 2004	29	6	5.8	0.97321	103%	7.9	0.99814	76%	6.7	90%
spong endosome total	65	21	23.0	0.99246	91%	36.1	0.99954	58%	35.5	59%
sponge endosome 2003	33	13	14.8	0.99599	88%	20.5	0.99968	63%	20.6	63%
sponge endosome 2004	32	9	9.1	0.99010	99%	11.8	0.99920	76%	12.4	73%

n = number of clones in clone library

OTU = number of phylotypes/OTU in clones library

a = asymptote of regression equation, displays estimated total diversity

r^2 = square rule of correlation-coefficient / explained variance

C = OTU/estimated diversity = coverage of clone library

^a Regressions were extrapolated from the analytical rarefaction curves determined by the algorithm described by Hurlbert (1971)

^b Regression (1) $y_1 = a_1(1 - e^{-bx_1})$

^c Regression (2) $y_2 = a_2(1 - e^{-bx_2})^c$

Diversity of sponge-associated bacteria

A total of 171 clone sequences were obtained (29-41 sequences for each clone library). Observed numbers of OTU and the total diversity estimated by rarefaction analyses revealed high variability in the sponge-associated bacterial community (i) between sponge endosome and cortex, (ii) between sponge and ambient seawater and (iii) between the different sampling times. In the sponge samples from June 2004, in cortex and endosome, 6 and 9 OTU were identified, respectively (Table II-1). In contrast, seawater collected at the same time displayed 27 identified OTU (Table II-1). *T. aurantium* sampled in April 2003 displayed a higher diversity. 26 and 13 OTU were identified in cortex and endosome, respectively (four fold higher compared to cortex 2004). The total number of OTU obtained from the sponge cortex (30) was higher than each of the values calculated individually for 2003 (26) and 2004 (6).

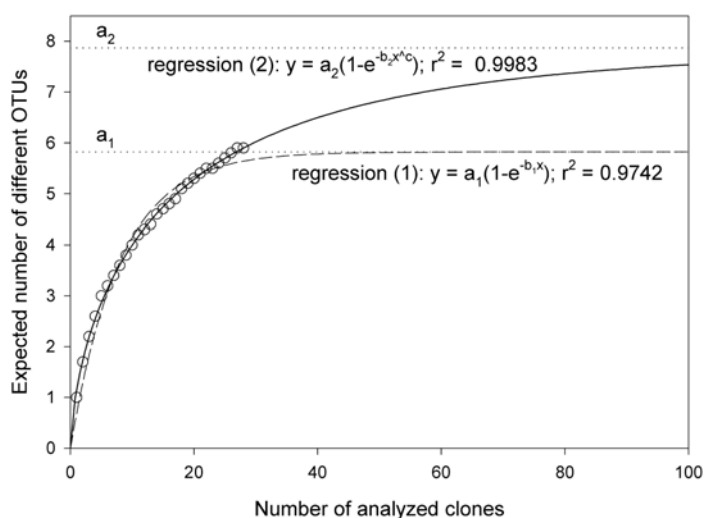


Fig. II-3: Analytic rarefaction curve plotted exemplarily for one sponge-derived 16S rDNA clone library (sponge cortex 2004). The expected number of OTU as determined by the analytical algorithm described by Hurlbert (1971) was plotted against the number of analysed clones (circles). Extrapolated regression curves (solid line and dashed line) are shown for the different regression equations (1) and (2). The expected total diversity determined by the asymptotes (a_1/a_2) is indicated by dotted lines. Regression (1), which has been used in former studies (e.g., Yakimov *et al.* 2006, Webster *et al.* 2004), results in a lower expected total diversity compared to regression (2) ($a_1 < a_2$) with also lower values for the non-linear coefficient of determination ($r_1^2 < r_2^2$).

Applying rarefaction analysis, regression (2) always showed higher r^2 values and led to a higher estimated total diversity (Fig. II-2 and II-3, Table II-1). According to regression (1), the total bacterial diversity seems to be well covered by the gene libraries obtained from the sponge sampled in June 2004 (88-103%). However, rarefaction analysis using regression (2) demonstrated that these portions are overestimated under regression (1). When applying regression (2), approximately 48-76% of the diversity seems to be covered by the clone libraries in this study (Table II-1). Only three phylotypes were obtained from endosome or cortex in both years. The majority of the sequences were found only in one of the years. Thus the total diversity of endosome- and cortex-associated sequences (as determined by rarefaction analysis), respectively, is much higher if both years are included than if each year is considered separately. Those differences in the diversity possibly demonstrate annual and seasonal variation in the bacterial communities.

Phylogenetic analysis

A high phylogenetic diversity was observed for the 171 bacterial sequences obtained from both sponge samples and surrounding seawater, including members of the *Alpha*-, *Beta*-, *Gamma*- and *Deltaproteobacteria*, *Bacteroidetes*, *Verrucomicrobia*, *Planctomycetales*, *Gemmatimonadales*, *Acidobacteria*, *Actinobacteria*, *Spirochaeta* and *Cyanobacteria* (Table II-2). However, the majority of

sequences were affiliated with the *Proteobacteria* (38%), the *Cyanobacteria* (27%) and the *Bacteroidetes* (25%).

Some phylotypes were found in *T. aurantium* from both years. They form monophyletic clusters i) related to the *Betaproteobacteria* (*Tethya-I*, Fig. II-4 A), ii) affiliated with the *Cyanobacteria* (*Tethya-II*, Fig. II-4 B), and iii) within the *Bacteroidetes* (*Tethya-III*, Fig. II-4 C). Within each of these clusters, sequences share >99% similarity. Of special interest is cluster *Tethya-I*, which is represented by 26 clone sequences (19%) and was found in the cortex and endosome of all *T. aurantium* individuals. *Tethya-I* forms a monophyletic cluster with sponge-derived 16S rDNA sequences from different sponges from Antarctic (Webster *et al.* 2004) and Australian waters (Taylor *et al.* 2004), as well as the Mediterranean Sea (Althoff *et al.* 1998). This sponge-specific monophyletic cluster is related to the *Betaproteobacteria* and branches deeply (dotted frame in Fig. II-4 A). In addition, the DGGE band TA-I, assigned to the sponge specific cluster *Tethya-I*, was unique to all *T. aurantium* samples but was not found in seawater. Moreover, no clone sequences belonging to the *Betaproteobacteria* were obtained from seawater in this study.

Within the *Cyanobacteria*, three phylotypes comprising 30 *Synechococcus* spp. sequences (22%) were obtained from *T. aurantium*-derived clone libraries. One of the phylotypes was found repeatedly in consecutive years (*Tethya-II*, Fig. II-4 B). *Synechococcus* spp. sequences were found mainly in the endosome samples (Table II-2), but not in the surrounding seawater. All cyanobacteria-like sequences obtained from the surrounding seawater belonged to chloroplasts of different algae. Only one chloroplast sequence was also found in the sponge cortex in April 2003.

Within the *Bacteroidetes* one bacterial cluster (*Tethya-III*) of *Flexibacteriaceae* was repeatedly found in *T. aurantium* (Fig. II-4 C, Table II-2). The sequences of the cluster shared highest similarity with *Microscilla furvescens* (AB078079, 91%) and were found in the cortex only. Additionally, DGGE band TA-II, present exclusively in cortex samples and absent from endosome and seawater, was assigned to this cluster (Fig. II-4 C). A further cluster of *T. aurantium*-derived *Flexibacteriaceae* was found in both endosome and cortex in June 2004 samples (Fig. II-4 C). Similarity between the clusters was 90%. *Flexibacteriaceae* related (92%) to putative vertically transmitted sponge-symbionts (Enticknap *et al.* 2006) were found in the sponge cortex from 2004 only (TAA-5-15v, Fig. II-4 C). All other *T. aurantium*-derived sequences within the *Bacteroidetes* belonged to the *Flavobacteriaceae* and *Saprospiraceae*, regardless of their origin from sponge cortex or endosome (Fig. II-4 C). All sequences showed a high

In total 30 *T. aurantium*-derived 16S rDNA sequences (22%) were closely related to other sponge-derived 16S rDNA sequences. 26 of these belonged to cluster *Tethya-I*, the remaining four sequences were affiliated to the *Gemmatimonadales*, the *Gammaproteobacteria*, the *Actinobacteria* and the *Acidobacteria*. Clone TAA-10-101v, obtained from *T. aurantium* cortex in 2003, clustered monophyletically with the sponge-specific cluster ‘uncertain-I’ described by Hentschel *et al.* (2002) within the *Gemmatimonadales* (represented by sponge clone TK19, Table II-2). Clone TAA-10-78v from the *T. aurantium* cortex (April 2003) is related to sequences obtained from sponges as well as other marine habitats within the *Gammaproteobacteria* (data not shown). BLAST search results showed closest relation to sponge-associated bacteria (Table II-2), but phylogenetic analysis could not identify monophyly of the sponge-derived gammaproteobacterial sequences. TAI-2-153v, representing two sequences obtained from the sponge endosome in 2004, was most similar to the *Phyllospongia papyracea*-associated *Gammaproteobacteria* clone 34P16 (Ridley *et al.* 2005) (sequence similarity of 86%, Table II-2). Again, phylogenetic analysis did not support monophyletic clustering of the sponge-derived sequences. *Actinobacteria* as well as *Acidobacteria* derived from sponges have been reported previously (Kim *et al.* 2005, Schirmer *et al.* 2005, Imhoff and Stöhr 2003, Hentschel *et al.* 2002) and sponge-specific clusters have been described (Hentschel *et al.* 2002). *T. aurantium*-derived sequences TAI-8-67v and TAA-10-43v showed similarities of 84-95% to different sponge-associated *Actinobacteria* sequences but did not group with any of the three described sponge-specific *Actinobacteria* clusters (Hentschel *et al.* 2002). TAA-10-23v, which represents two clones obtained from the *T. aurantium* cortex, showed <90% sequence similarity to the sponge-specific cluster *Acido-I* (Hentschel *et al.* 2002) and does not cluster monophyletically with it.

Several sequences obtained from *T. aurantium* were not specifically related to sequences found in sponges, but were either closely related to seawater-derived bacteria or to bacteria associated with other marine macroorganisms (Brown *et al.* 2005, Cho and Giovannoni 2004, Vergin *et al.* 1998) (i.e., TAI-8-61v, TAI-8-64v, TAI-8-20v (*Gammaproteobacteria*) and TAI-2-130v, TAA-10-09v (*Planctomycetales*), Table II-2). Bacteria affiliated with the *Gammaproteobacteria* (TAU-7-100v/TAI-8-75p) and the *Flavobacteriaceae* (*Bacteroidetes*) (TAI-2-145v/TAU-7-02v and TAI-2-81v/TAU-7-69) were found in both *T. aurantium* endosome and seawater in this study. No sequence from the sponge cortex showed similarity to seawater-derived sequences obtained in this study.

Alphaproteobacteria were exclusively found in the *T. aurantium* cortex and in ambient seawater in June 2004 (Table II-2). The sponge-derived sequences were related to bacterioplankton-derived sequences (Suzuki *et al.* 2001), coral mucus-associated bacteria (Koren *et al.*, unpublished, GenBank Acc. AY654769) and a filamentous bacterium from a wastewater treatment plant (Levantesi *et al.* 2004). *Alphaproteobacteria* closely related to those found in seawater in this study (TAU-7-44p and TAU-7-38) had been obtained from sponges (*Halichondria panicea* and *Halichondria okadae*) previously (Althoff *et al.* 1998; Okano *et al.* unpublished, GenBank Acc. AB054143).

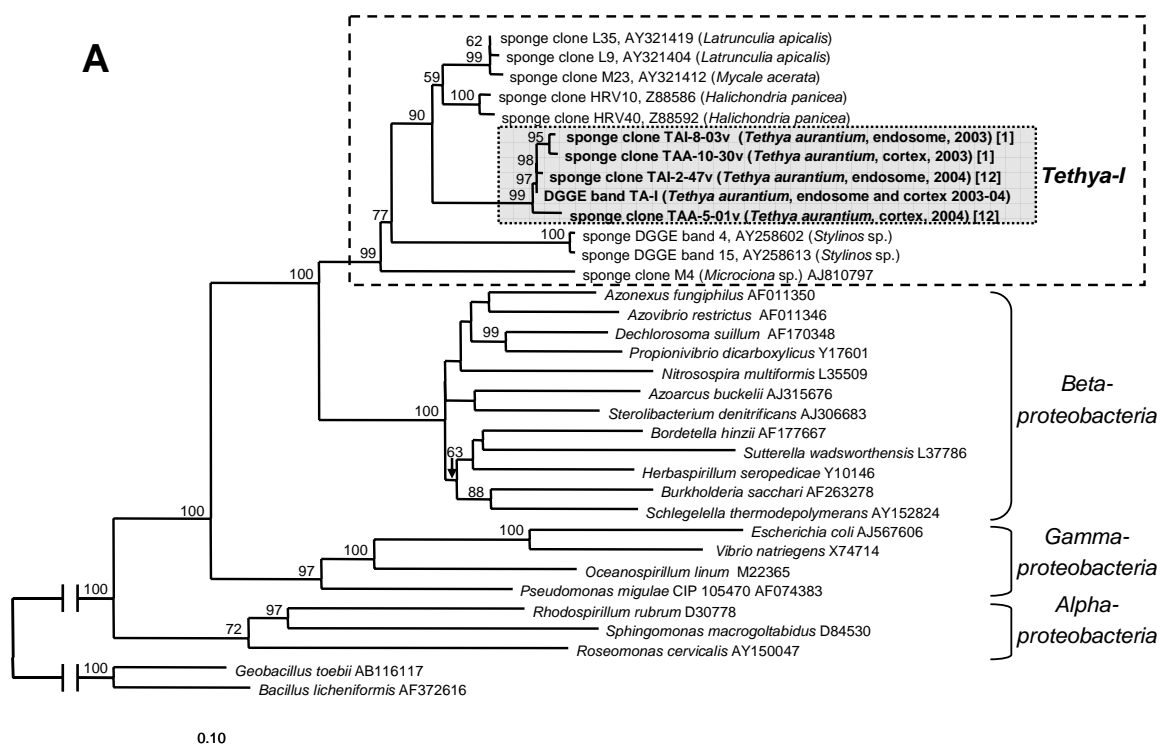
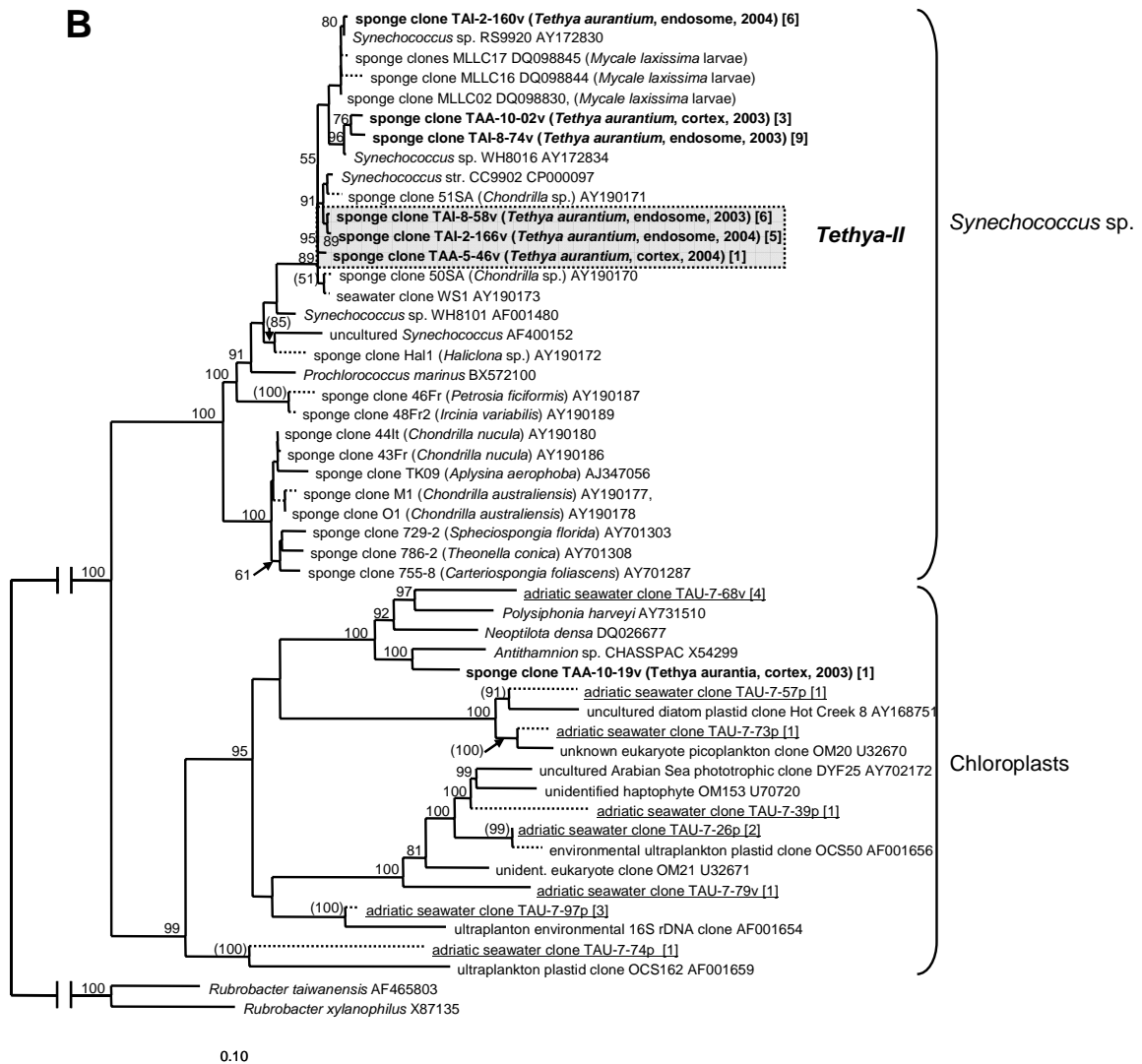
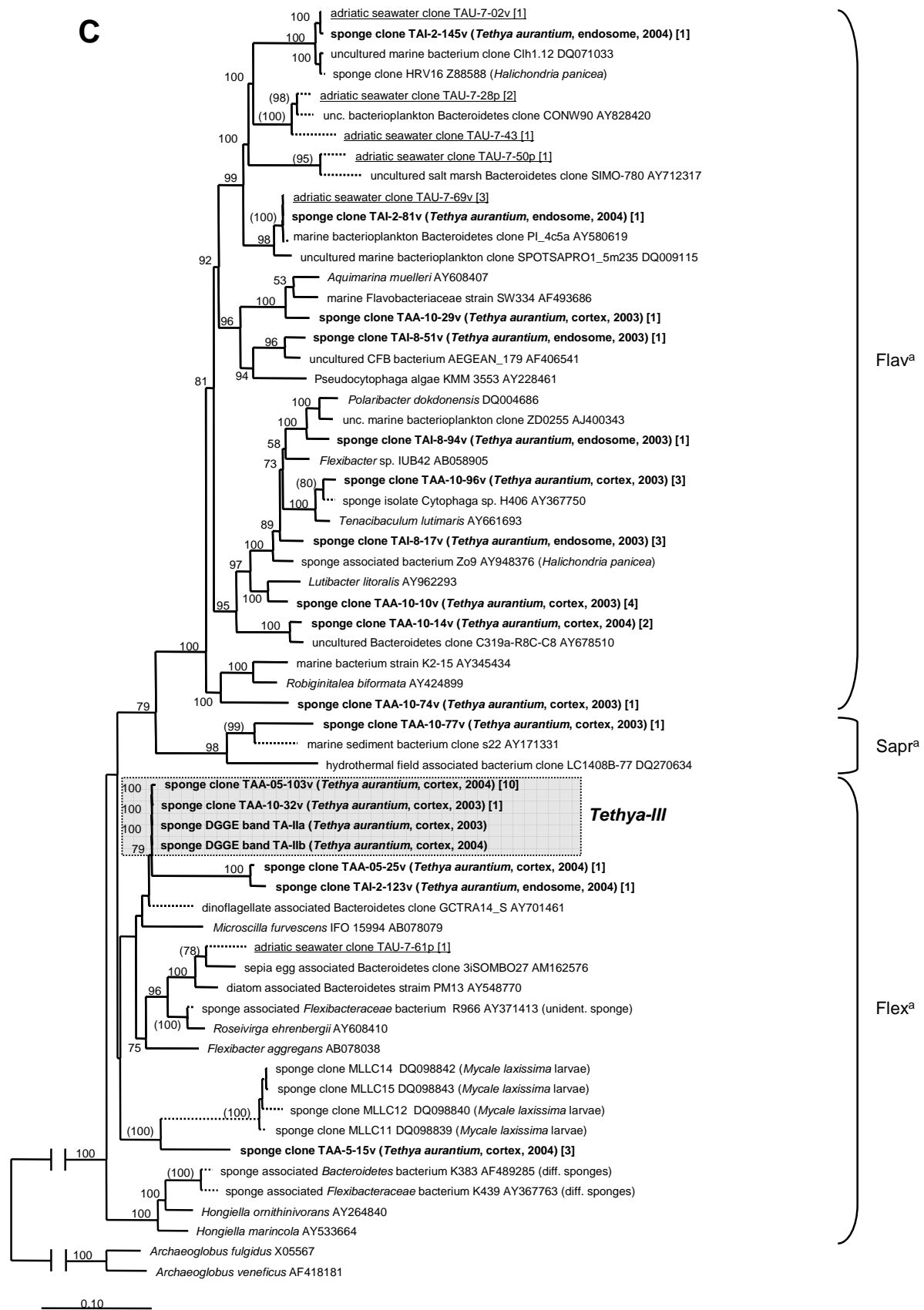


Fig. II-4: Phylogenetic trees constructed of 16S rDNA sequences related to the *Betaproteobacteria* (A), *Cyanobacteria* (B) and the *Bacteroidetes* (C). Sequences obtained from *Tethya aurantium* are printed in bold letters. Adriatic seawater clone sequences are underlined. Numbers of represented clones in each OTU are given in parenthesis behind the clone names. Clusters of *Tethya aurantium*-associated bacterial phylotypes found in both years (*Tethya-I*, *Tethya-II* and *Tethya-III*) are framed and highlighted. The new sponge-specific cluster related to the *Betaproteobacteria* is framed with a dotted line (not highlighted) (Fig. II-4 A). All trees were generated using the Maximum Likelihood method. Tree (A) is based on sequences of 500-1500bp length. The DGGE sequence (<500) was added without changing the tree topology using the parsimony method in ARB. Trees (B) and (C) are based on almost complete sequences (≥ 1000 bp Fig. II-4 B, ≥ 1200 bp Fig. II-4 C). Partial sequences (<1000/<1200 bp) were added without changing the tree topology using the parsimony method in ARB and are indicated by dashed branches. Phylogenetic positions of partial sequences were verified by calculation of all length sequences separately. 100 fold bootstrapping analysis was conducted. Values equal or greater 50 are shown. Bootstrap values in parenthesis are referring to tree calculations including short sequences. The scale bars indicate the number of substitutions per nucleotide position.





^a Flav = Flavobacteriaceae, Sapr = Saprospiraceae, Flex = Flexibacteriaceae

DISCUSSION

Although sponges do not possess organs or real tissues, cortex and endosome are clearly differentiated with respect to structure and function. For the first time, our studies on *Tethya aurantium* revealed that they also differ in their bacterial communities. Distinct phylotypes, represented by DGGE bands and 16S rDNA clone sequences, were affiliated with the different regions of the sponge.

T. aurantium supports a relatively low diversity of specifically associated bacteria. Only three bacterial phylotypes were found in sponge specimens from both years. For diversity estimation we applied rarefaction analysis in combination with two non-linear regressions. The commonly used regression (1) was shown to systematically underestimate the total diversity, while regression (2) results in estimates comparable to chao1. Regression (2) was demonstrated to be significantly better suited as a regression for fitting rarefaction curves and predicting total diversity, and therefore is recommended to be used for diversity estimation of clone libraries in future studies. Under application of the more conservative regression (2), rarefaction analysis displays only 2 (cortex) and 4 (endosome) expected additional bacterial phylotypes (OTU) not identified in this study for the *T. aurantium* specimen from 2004.

One characteristic cluster closely related to the *Betaproteobacteria* (cluster *Tethya-I*) is associated with both endosome and cortex of *T. aurantium*. *Betaproteobacteria*, with the exception of ammonium oxidisers (Voytek and Ward 1995), are not abundant in open-oceans (Giovannoni and Rappé 2000), but are characteristic for freshwater habitats (Schweitzer *et al.* 2001, Methe *et al.* 1998) and have also been observed in coastal waters (Fuhrman and Ouverney 1998, Rappé *et al.* 1997). Nonetheless, *Betaproteobacteria*-affiliated bacteria were previously found in sponges by culture-independent methods (Taylor *et al.* 2004, Webster *et al.* 2004, Thoms *et al.* 2003, Webster *et al.* 2001b, Althoff *et al.* 1998). In *Rhopaloides odorabile*, they were located intracellularly in some cases, as demonstrated by FISH (Webster *et al.* 2001b). *Tethya-I* clusters monophyletically with those other sponge-derived sequences (Taylor *et al.* 2005, Taylor *et al.* 2004, Webster *et al.* 2004, Althoff *et al.* 1998), forming a sponge-specific monophyletic cluster (Fig. II-4 A). Our studies indicate that this cluster branches deeply within the *Betaproteobacteria*, but exact phylogenetic affiliation remains uncertain. Phylogenetic analysis using the backbone tree and the parsimony method implemented in the ARB program (Ludwig *et al.* 2004) demonstrated a consistent affiliation with the *Betaproteobacteria*, though within this group the phylogenetic position of the *Tethya-I* cluster largely depends on the (DNA)-filter used. On the other hand, complete phylogenetic calculations including a reasonable number of representatives of the *Beta*-, *Gamma*- and *Alphaproteobacteria*, using the

Maximum Likelihood method (Felsenstein 1981), placed the sponge-specific cluster outside the known *Betaproteobacteria* (Fig. II-4 A).

Finding of *Tethya-I*-related sequences in *Halichondria panicea* from the Adriatic Sea but not in individuals from the North or the Baltic Sea (Althoff *et al.* 1998) possibly indicates a synergistic sponge-microbe association. Yet, the occurrence of members of the *Betaproteobacteria*-related sponge-specific cluster is not limited to the Mediterranean Sea, as formerly unaffiliated sequences obtained from the Antarctic sponges *Latrunculia apicalis* and *Mycale acerata* (Webster *et al.* 2004) can now be assigned to the cluster. Since *Betaproteobacteria* are abundant in freshwater habitats (Schweitzer *et al.* 2001, Methe *et al.* 1998) a freshwater origin cannot be excluded for the *T. aurantium*-derived sequences obtained from the river fed Limski kanal. However, no *Betaproteobacteria* were found in the seawater surrounding the *T. aurantium* habitat and furthermore no freshwater-derived bacteria closely related to the *Tethya-I* cluster have been described.

Cyanobacterial associations in sponges have been known for many years. Within the *Synechococcus* group one specifically sponge-associated cluster and several additional sponge-associated cyanobacteria have been identified (Steindler *et al.* 2005). Besides symbiotic associations, the genus *Synechococcus* is also known as a member of marine picoplankton communities and serves as food for different filter feeding animals, including sponges (Pile *et al.* 1996). For *T. aurantium* no cyanobacterial associations have been reported in literature. Interestingly, we found *Synechococcus* spp. sequences composing a major part of the endosome-derived clone library (n=26, 39%) (Fig. II-4 B, Table II-2). They represent three closely related phylotypes, only distantly related to the sponge-specific group of *Synechococcus* sp. strains. They show high sequence similarity to several cultured and uncultured *Synechococcus* strains, also including sponge and sponge-larvae associated uncultured strains obtained from *Chondrilla* sp. and *Mycale laxissima* (Enticknap *et al.* 2006, Usher *et al.* 2004a). The close relation to putative vertically transmitted sponge-associated *Synechococcus* strains indicates constant and obligate association to sponges. However, unlike the *T. aurantium*-derived sequences in this study, the closely related cyanobacterial symbionts from *Chondrilla* sp. were also found in seawater. It was hypothesised that these cyanobacteria have become symbiotic with sponges relatively recently (Usher *et al.* 2004a). Due to the close phylogenetic relationship to planktonic *Synechococcus* strains, a seawater origin cannot be excluded for the *T. aurantium*-associated strains either. Cyanobacteria in sponges have generally been found in the thin (few mm) outer tissue regions, where light energy is available for photosynthesis. The endosome of *T. aurantium* is covered by a thick and dense cortex region, but spicule bundles might function as a natural light

conductor, similar to fibre-optic systems, as it was postulated for the growth of the sponge-associated green alga *Ostreobium* sp. in *Tethya seychellensis* (Gaino and Sará 1994).

A specifically cortex-associated bacterial cluster was identified within the division *Bacteroidetes*, affiliated with the family *Flexibacteriaceae* (clone cluster *Tethya-III* and DGGE band TA-II, Fig. II-2). While several previous studies demonstrated *Bacteroidetes* belonging to the family *Flavobacteriaceae* to be associated with sponges (Lafi *et al.* 2005, Webster *et al.* 2004, Webster *et al.* 2001b), only very recently sponge-associated *Flexibacteriaceae* have been obtained from sponge larvae by culture-independent methods (Enticknap *et al.* 2006). These putative vertically transmitted sponge symbionts are closely related to sequences obtained from *T. aurantium* cortex in 2004 and distantly related to cluster *Tethya-III* found in both years. Cluster *Tethya-III* has been observed in sponges for the first time in this study. The bacteria were not detected in the ambient seawater and in the sponge endosome. Due to the occurrence in the sponge cortex exclusively and presence in specimens of consecutive years as well as the phylogenetic relatively large distance (<91%) to known sequences, we assume specific association between the *Microscilla*-like bacteria and *T. aurantium*.

As sponges are filter feeding animals, a seawater origin of sponge-derived bacterial sequences cannot be excluded despite repeated washing steps in sterile seawater prior to DNA-extraction. Several sequences obtained from seawater in this study share high similarity with clone sequences found in both *T. aurantium* and other sponges. Thus some of the endosome-associated bacterial sequences may solely resemble DNA of ambient seawater bacteria ingested in the choanocyte chambers. Additionally, the high variability of different phlotypes observed implies either seasonal differences in the sponge-associated bacterial communities or possibly reflects seasonal microbial population dynamics in the ambient seawater. The similarity between the sponge endosome-associated bacterial community and the ambient seawater bacterioplankton as demonstrated in DGGE and phylogenetic analysis again emphasises the need to differentiate between sponge-specific and merely ingested bacteria.

Hentschel *et al.* (2002) found 70% of all sponge-derived sequences clustering together in different phylogenetic clades. In contrast, a minor fraction (22%) of the *T. aurantium*-derived sequences was closely related to other sponge-associated bacteria. Apart from cluster *Tethya-I* related to the *Betaproteobacteria*, only two additional sequences obtained from *T. aurantium* cluster with other known sponge-derived sequences. Monophyly of the sponge-specific cluster indicates common ancestry for members of this group. Further, limitation of these bacteria to associations with the phylum Porifera can be hypothesised. However, since

abundance and diversity of bacteria associated with different sponges highly depends on the sponge species and possibly on seasonal influences, a uniform specifically sponge-associated bacterial community as proposed by Hentschel *et al.* (2002), probably does not exist.

We conclude a specific association of both the *Betaproteobacteria*-related cluster *Tethya-I* and the *Flexibacteriaceae* cluster *Tethya-III* with the sponge *T. aurantium*. The new cluster of specifically associated *Flexibacteriaceae* was so far exclusively found in *T. aurantium* and its presence in sponges of other taxonomic affiliation and geographical regions is still to be investigated. The unusual association of *Synechococcus* sp. strains with the *T. aurantium* endosome and the putative light conduction by sponge spicule bundles will be further studied in future research.

CHAPTER III

PHYLOGENETIC IDENTIFICATION OF BACTERIA WITH ANTIMICROBIAL ACTIVITIES ISOLATED FROM MEDITERRANEAN SPONGES

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ABSTRACT

Bacteria were isolated from Mediterranean sponges and specifically selected according to their antimicrobial activity. They were classified on the basis of partial 16S rDNA sequences as members of distinct clusters of *Alpha*- and *Gammaproteobacteria*. The action spectrum of antimicrobial activities against target organisms was related to the phylogenetic position of the isolates.

STUDY, RESULTS AND DISCUSSION

Sponges are known to be a rich source of bioactive secondary products of biotechnological interest, showing antiviral, antitumor, antimicrobial or general cytotoxic properties. Some of the isolated substances from sponges have striking structural similarities to metabolites of microbial origin (Proksch *et al.* 2002). Accumulated evidence suggests that microorganisms could well be the true source of at least some of these metabolites (Imhoff and Stöhr 2003, Flowers *et al.* 1998, Bewley *et al.* 1996). This offers the possibility to use the sponge-associated bacteria for the production of biological active substances instead of the sponge itself. Because bacteria quite rapidly produce high amounts of biomass, biological active secondary products can easily be produced in large amounts on a biotechnological scale without the necessity to harvest or cultivate the sponge.

The aim of this study was to isolate and characterise bacteria with antimicrobial activities from various marine sponges. Different sponge species were collected from shallow coastal habitats of the Mediterranean Sea (up to approx. 15 m depth) by SCUBA diving. To test for antimicrobial activity in these sponges, sponge pieces were applied to agar media (TSB3) inoculated with different indicator organisms (*Escherichia coli*, *Staphylococcus lentus*, *Candida glabrata*, *Bacillus subtilis*, *Mycobacterium phlei*) and incubated for 24 hours at room temperature. Specimens of *Halichondria panicea*, *Ircinia fasciculata*, *Axinella polypoides*, and *Acanthella* spec. showed activity against at least two out of five test organisms and were chosen for isolation of bacteria. In addition, a *Suberites domuncula* specimen was included in these studies. Samples of the sponges were homogenized by using an Ultraturrax® T25 (IKA® Werke, Germany), diluted in appropriate steps up to a dilution of 10^{-4} in sterile seawater and plated onto fifteen different agar media, varying in salinity, nutrient composition and concentration. The agar plates were incubated at room temperature until visible colonies had formed and then were subjected to tests for antibiotic activities. Bacterial strains showing activity against at least one of the indicator organisms were isolated and further investigated.

Isolates with antibiotic activity were identified on the basis of 16S rDNA gene sequences. PCR of pure cultures was performed using the general eubacterial primer set 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTACGACTT-3'). The PCR product was cleaned using the QIAquick purification kit (QIAGEN Germany) and partly sequenced. The sequencing was performed using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and an ABI 310 analyser (Perkin Elmer Applied Biosystems, Foster City, USA). Primary sequence data (350-400bp) were aligned and phylogenetically classified by using the ClustalX program. The phylogenetic tree was generated using the Neighbor-Joining method with 100 resamplings in bootstrap analysis.

Most of the isolates with antimicrobial activity were affiliated with distinct eubacterial groups within the *Alpha*- and *Gammaproteobacteria* (Fig. III-1). Bacteria of other phylogenetic groups including Gram-positive bacteria were not obtained in this study. Thus, the phylogenetic diversity of the obtained antimicrobial active isolates was rather limited, even though a number of different media were used for their isolation. Similar observations were made earlier (Hentschel *et al.* 2001). In particular one group of closely related strains was dominant. Comparison of 16S rDNA sequences using BLAST (Altschul *et al.* 1990) showed highest sequence similarities for one of the clusters to unnamed *Alphaproteobacteria* (98-99% to strain MBI3368) previously isolated from sponges by other research groups (Olson *et al.* 2002, Hentschel *et al.* 2001). Similarity to other described *Alphaproteobacteria* is well below 90%.

A second group of bacteria isolated from the sponges was affiliated with the *Gammaproteobacteria* and related to marine *Vibrio* species and *Pseudoalteromonas* species, respectively. Quite interestingly, the groups of *Alphaproteobacteria* and *Gammaproteobacteria* did not occur within one and the same sponge. All antibiotic active isolates from *Halichondria panicea* and *Axinella polypoides* were *Alphaproteobacteria*, but none was related to the *Gammaproteobacteria*. The isolates from *Acanthella* spec., *Suberites domuncula* and *Ircinia fasciculata* were *Gammaproteobacteria*.

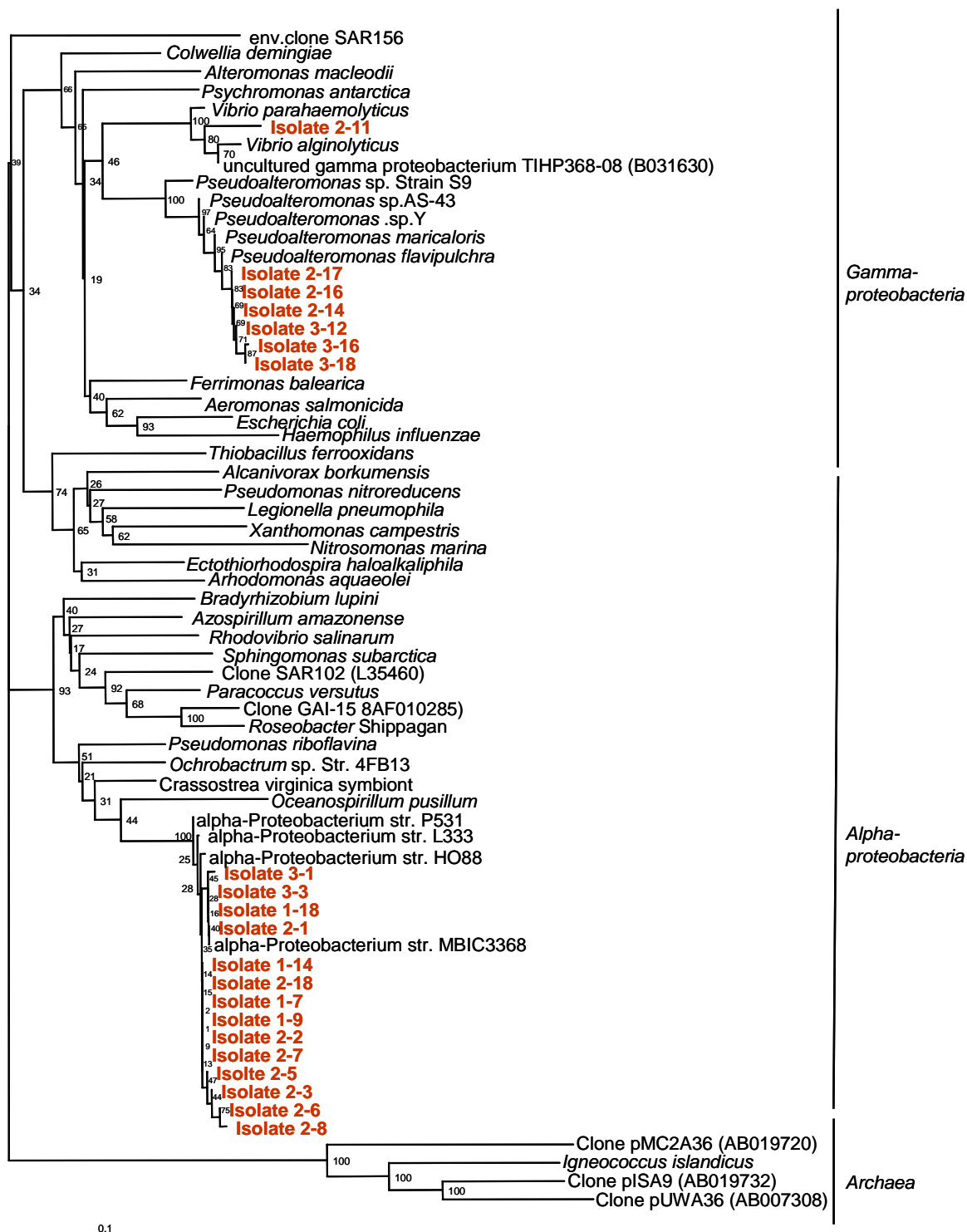


Fig. III-1: Phylogenetic tree of representative *Alpha*- and *Gammaproteobacteria* based on 16S rDNA sequences, including sequences of new bacterial isolates of sponges from Mediterranean habitats. Phylogenetic calculations are based on the Neighbor-Joining method with 100 bootstrap replicates (bootstrap values given at the nodes). The bar indicates a phylogenetic distance of 10% sequence difference.

CHAPTER IV

ABUNDANCE, BIOACTIVITY AND ECOLOGICAL RELEVANCE OF CULTURED SPONGE-ASSOCIATED BACTERIA FROM THE MEDITERRANEAN SEA

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ABSTRACT

In several Mediterranean sponge species the most abundant cultured bacterium (15-74% of the complete colony forming units (CFU)) is an alphaproteobacterium with a remarkable antimicrobial activity. Though the activity is weak and unstable, gets easily lost during cultivation, and is mainly directed against Gram-negative bacteria, it is shown by up to 10^6 cells per gram sponge wet-weight. The bioactivity, the high abundance and its worldwide uniform occurrence in sponges implies an important ecological role of this bacterium within the sponge habitat. Other bioactive bacteria, first of all from the genus *Bacillus*, were regularly found, too, while actinomycetes known for their production of bioactive substances were present in very low abundance.

INTRODUCTION

Sponges represent one of the oldest groups of multicellular organisms tracing back to the Precambrian. Because of their sessile lifestyle they largely rely on chemical weapons in defence against potential predators. Therefore, a highly versatile spectrum of bioactive compounds optimised in half a billion years of evolution is to be expected. Indeed, a lot of novel natural products originating from sponges have been isolated in recent years (Faulkner 2002). However, the biosynthetic pathways responsible for their production remain largely unknown, and in most cases it is not even clear if the substance is produced by the sponge itself or by microorganisms tightly associated with it (Haygood *et al.* 1999):

Microorganisms play a central role in sponge biology; they serve as food particles ingested in large amounts by these powerful filter feeders or as symbiotic or commensal partners settling in the mesohyl and other tissue-equivalents intercellularly, or even survive within the cells (Friedrich *et al.* 1999, Vacelet and Donadey 1977). Several observations support the idea that bacteria synthesise sponge-specific compounds either completely or in the form of precursors completed subsequently by sponge metabolism (Proksch *et al.* 2002, Schmidt *et al.* 2000, Flowers *et al.* 1998, Bewley *et al.* 1996, Unson *et al.* 1994). In terms of biotechnological and commercial applications bacteria have the great advantage of being available in any amount by fermentation once they have been brought into pure culture, while most sponge species are not accessible by mass cultivation procedures such as aqua farming. Apart from the advantage bioactive compounds might have for the sponge there is little information on the importance of bacteria producing such substances within the sponge ecosystem.

The recent progress in cultivation-independent microbiological research (Head *et al.* 1998, Amann *et al.* 1995) has provided a fascinating insight into the complex microbial communities of sponges. They differ largely from the bacterial population of the surrounding water in quantitative and qualitative terms, but may be surprisingly constant for a given sponge species despite its complexity (Steindler *et al.* 2005, Taylor *et al.* 2004, Imhoff and Stöhr 2003, Hentschel *et al.* 2002, Webster *et al.* 2001b). In contrast, cultured bacteria are rarely considered for quantitative ecological investigations (Enticknap *et al.* 2006, Webster and Hill 2001, Olson *et al.* 2000, Santavy *et al.* 1990), but isolated rather in search of novel drugs, of degrading enzymes, or of new species (Sfanos *et al.* 2005, Lang *et al.* 2004, Ahn *et al.* 2003, Thakur *et al.* 2003, Hentschel *et al.* 2001, Perovic *et al.* 1998).

The quantitative and qualitative data presented here combine both aspects and support the idea that also the cultured fraction of sponge-associated bacteria is specific to the host and not only concentrated from the surrounding seawater by filtration of food particles. In addition, the antimicrobial activity displayed by this group is likely to have an ecological dimension.

MATERIALS AND METHODS

Sampling of sponges and environmental water

Single individuals of ten Mediterranean sponge species (*Chondrosia reniformis*, *Axinella polypoides*, *Chondrilla nucula*, *Tethya aurantium*, *Clathrina clathris*, *Agelas oroides*, *Petrosia ficiformis*, *Ircinia* sp., *Suberites domuncula*, *Acanthella acuta*) were collected from coastal waters in the Adriatic Sea near Rovinj, Croatia, by SCUBA diving. The specimens were manually removed from three different sampling sites (Limski kanal, Banjole, St. Giovanni) at depths of 2 to 20 m. Environmental water representing each location was taken prior to sponge sampling and filled up in sterilised bottles (1 L, Duran™ Schott). To remove loosely associated microorganisms from inner and outer sponge surfaces and enable the digestion of non-adapted bacteria the sponges were kept for 3 x 30 min in 1 L each of filter-sterilised (0.2 µm pore size, Sartorius) seawater at nearly ambient conditions (i.e., with respect to temperature and water origin) in separate containers.

Processing of samples

Small pieces of tissue representing the complete sponge were cut off with sterile scalpels, weighed, and homogenized in 5 ml of sterile habitat water by use of an Ultraturrax (IKA T25 basic; 30 sec, max. speed; steel tip sterilised with 70% ethanol and washed with filtered seawater immediately before use). Dilutions from 10^0 to 10^{-3} were made of sponge samples and habitat water using autoclaved environmental water and 50 μ l aliquots plated on various solid media in two parallels.

Growth media

Nine different media supplemented with 1.5% agar were used for isolation: i) HSPC agar ('semi-synthetic poly-carbon'): a defined phosphate-/bicarbonate-buffered medium containing mixtures of salts, trace elements, vitamins, low-molecular-weight carbon sources, and amino acids (Imhoff and Stöhr, unpublished); ii) GPYNS agar: 0.1% Glucose; 0.05% Bacto Pepton (Becton Dickinson, BD), 0.01% yeast extract (BD), 2.5% artificial sea salts (Tropic Marin); iii) Chitin agar: 0.1% K_2HPO_4 , 0.1% NH_4Cl , 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% $NaCl$, 0.011% $CaCl_2 \cdot 2H_2O$, 0.0001% $FeCl_3 \cdot 6H_2O$, chitin from crab shells (Sigma); iv) Actinomycete isolation agar (Difco; only used for spore-forming isolates); v) FS agar: Baltic Sea water from Kiel bight (Förde) supplemented with 1.5% $NaCl$. These media were prepared about two weeks prior to sampling.

The remaining media were prepared freshly the day before use. They consisted of 1.5% agar in Mediterranean habitat water (HW) taken from the seawater pipe system of Ruder Boskovic Institute (Rovinj) and were supplemented with yeast extract (Bacto™, Becton-Dickinson) in the following concentrations: vi) none (HW0%); vii) 0.01% (HW0.01%); viii) 0.1% (HW0.1%); and ix) 1% (HW1%).

For agar diffusion assays 3% Tryptic Soy Broth (Difco) was used corresponding to 10% of the concentration proposed by the manufacturer and supplemented with 1% $NaCl$ (subsequently called 'TSB3/1'). 0.7% agar was added to create soft agar (see below).

Handling of isolates

The plates were incubated at room temperature (16-20°C) for several weeks and colonies repeatedly counted to obtain the complete CFU (colony forming units) titre including slowly growing or metabolically discriminated species. For reasons of reliability, only dilutions resulting in approx. 50 to 400 colonies per plate were chosen for statistic evaluation (though

colonies from other dilutions were counted for control purposes, too). Colonies were selected by optical differentiation at 15-fold magnification (Binocular M8, Wild), picked with sterile pins and tipped on TSB3/1-plates according to a grid scheme with 88 numbered fields (5 parallels). Colonies were purified from selected grids on individual agar plates by standard procedures.

Selection of spore-forming microorganisms

To selectively favour spores by killing vegetative cells an aliquot each of the sponge macerate and its 1:10 dilution, respectively, was treated with phenol (final concentration of 1.5%). After one hour of incubation the samples were plated on the four HW media and on actinomycete isolation agar.

In a separate approach, residual sponge macerates were pooled and vacuum-filtered through a 0.2 μm sterile filter to remove the liquid. Similarly, 10 L of habitat water was filtered in two parallels. The filters were air-dried in sterile Petri-dishes for three days, and then rinsed in 5 ml of autoclaved habitat water for one hour, diluted from 10^0 to 10^{-4} and plated as 50 μl aliquots to the media also used for the phenol-treated samples.

Testing for antimicrobial activity

Four of the five grid plates were overlaid with 0.7% soft agar containing one of the following indicator strains: *Escherichia coli* K12 (DSM 498), *Bacillus subtilis* (DSM 347), *Staphylococcus lentus* (DSM 6672), or *Candida glabrata* (DSM 6425). Overnight cultures of these strains grown in TSB3/1 broth were diluted 1:100 in sterilised soft agar kept at 45°C and cast on the grid plates carefully enough to minimize the spreading of colony-derived cells over neighbouring grids. After overnight incubation (or two days for *C. glabrata*, respectively) the plates were checked for growth inhibition zones around the colonies. The activity was considered to be weak if the inhibition zone was i) <1 mm wide, ii) <10% of the colony diameter, or iii) opaque due to an only partially inhibited growth.

To judge the diversity of sponge-specific isolates, the morphotypes of colonies derived from contiguous sectors of mother plates were compared optically and by checking the consistency with a sterile pin. The abundance of morphotypes found at least three times (within about 100 isolates available per sponge) was calculated for each sample.

Phylogenetic analysis

Genomic DNA from purified isolates was extracted as follows: colony material corresponding to approximately 10^8 cells was removed from the plates with a platinum loop, suspended in 100 μ l DNA-free water and supplemented with lysozyme (10 μ g/ml). After three cycles of freezing (15 min. at -20°C) and thawing (at room temperature) the cell debris was removed by centrifugation and the supernatant directly used for PCR.

Amplification of 16S rDNA was performed using puReTaqTMReady-To-GoTMPCR beads (Amersham Biosciences) with the eubacterial primers 27f and 1492r (Lane 1991). The PCR conditions were: initial denaturation (2 min at 94°C) followed by 25 cycles of primer extension (90 sec at 72°C), primer annealing (40 sec at 50°C) and denaturation (40 sec at 94°C), a final primer annealing (1 min at 42°C) and a final extension (5 min at 72°C).

Purification of PCR products and determination of sequences using the 16S rDNA-specific primers 342f, and 534r (Muyzer *et al.* 1993, Lane 1991) was done at the Institute for Clinical Molecular Biology (Universitätskliniken Schleswig-Holstein in Kiel). Sequences of approx. 400-450 bp were obtained for all PCR products using the 534r primer. For 13 representative PCR products the sequence was prolonged to >1000 bp by use of 342f.

Sequence data was edited with Lasergene Software SeqManTMII (DNASTar Inc.) and next relatives determined by comparison to 16S rRNA genes in the NCBI GenBank database using BLAST (Basic Local Alignment Search Tool) searches (Altschul *et al.* 1990). The sequences were aligned using the FastAlign function of the alignment editor implemented in the ARB software package (www.arb-home.de) (Ludwig *et al.* 2004) and refined manually. Phylogenetic calculations were carried out with the PhyML software (Guindon and Gascuel 2003) using the Maximum Likelihood (ML) method (Felsenstein 1981).

Nucleotide sequences

Sequences obtained in this study were submitted to the GenBank database (accession numbers DQ888826 - DQ888887).

RESULTS

In May 2005, ten different Mediterranean sponge species were collected to compare and quantify their specific microbial communities. Investigation was restricted to the cultured fraction of microorganisms accessible to activity screening. In order to expand this fraction as far as possible, nine different types of media were used. After the removal of loosely associated (sea water-derived) microorganisms (see Material and Methods) the microbial community was isolated and allowed to form colonies under these diverse growth conditions.

Dependence of isolation efficiency on media

The resulting CFU titres as calculated for one gram wet weight of sponge tissue varied considerably in a range between 9.5×10^4 (*C. reniformis*) and 6.3×10^6 CFU (*T. aurantium*) corresponding to a 100-fold to 8,300-fold excess over the habitat water (7.6×10^2 - 9.5×10^2 CFU/ml; Table IV-1). When considering the culture conditions, the individual differences with respect to the optimal medium varied from sponge to sponge. However, when looking at the average preferences irrespective of an individual sample, some general rules can be deduced (Fig. IV-1): by far the best yields were obtained with HW0.01% consisting of original habitat water slightly enriched with 0.01% yeast extract (YE). This preference extends to pure habitat water without additives. Increasing the YE concentration to 0.1% reduced the CFU titre by nearly a factor of 2, while 1% YE inhibited the major part of the microorganisms as counts dropped to 16% of the value obtained in media with 0.01% YE (Fig. IV-1).

The average efficiency of GPYNS and HSPC agar is comparable to HW0.1%. In several cases, however, these media induce swarming of some bacteria making the plates useless for counting or colony picking. A dramatic reduction of CFU numbers was observed for chitin and FS agar (Fig. IV-1).

Table IV-1: CFU titre (per g wet weight) obtained from various sponge and water samples on different media. The highest titre for each sample is printed in bold type.

Agar	habitat water 1	habitat water 2	habitat water 3	<i>Chondrosia reniformis</i>	<i>Axinella polytipoides</i>	<i>Chondrilla nucula</i>	<i>Tethya aurantium</i>	<i>Clathrina clathris</i>	<i>Agelas oroides</i>	<i>Ircinia</i> sp.	<i>Petrosia ficiformis</i>	<i>Suberites domuncula</i>	<i>Acanthella acuta</i>
HW0%	6.8E+02	3.7E+02	n.d.	9.5E+04	1.0E+05	2.2E+06	4.1E+06	3.7E+05	5.1E+05	2.1E+06	1.6E+06	5.4E+05	2.1E+05
HW0.01%	9.5E+02	7.6E+02	8.6E+02	6.3E+04	1.7E+05	4.6E+06	6.3E+06	2.0E+05	3.7E+05	1.2E+06	7.1E+05	1.1E+06	2.1E+05
HW0.1%	5.5E+02	4.6E+02	n.d.	1.1E+05	2.3E+05	1.5E+06	1.3E+06	4.1E+05	4.1E+05	1.1E+06	2.1E+06	4.3E+05	2.0E+05
HW1%	2.0E+02	2.5E+02	n.d.	5.8E+04	3.7E+04	5.8E+04	3.9E+05	1.6E+05	4.8E+04	1.1E+06	1.9E+05	1.5E+05	9.9E+04
FS	3.0E+02	3.0E+01	n.d.	6.3E+02	9.2E+03	7.3E+03	1.2E+05	8.5E+04	0.0E+00	5.6E+04	0.0E+00	2.7E+04	5.3E+04
HSPC	5.3E+02	4.1E+02	n.d.	9.4E+04	1.5E+05	2.0E+06	3.1E+06	4.5E+05	2.9E+05	1.7E+06	4.9E+05	6.2E+05	1.8E+05
GPY	5.1E+02	4.0E+02	n.d.	1.0E+05	1.5E+05	2.4E+06	8.9E+05	3.1E+05	2.1E+05	1.6E+06	1.3E+05	1.8E+06	2.1E+05
Chitin	7.0E+01	4.0E+01	n.d.	6.3E+02	0.0E+00	1.5E+04	1.4E+04	2.8E+03	1.0E+03	1.7E+04	1.5E+04	2.7E+04	5.0E+02
average	4.7E+02	3.4E+02	n.d.	6.6E+04	1.1E+05	1.6E+06	2.0E+06	2.5E+05	2.3E+05	1.1E+06	6.5E+05	5.9E+05	1.5E+05

High colony numbers, of course, may be due to the selective proliferation of one or a few strains while other bacteria even might be suppressed. However, when plates were incubated for a prolonged time (up to several weeks) and colonies checked by optical magnifying (stereo microscope), colonies on poor media appeared more diverse than on rich media (not shown). The additional colonies not observed on rich media remained very small and were largely resistant to further cultivation via transfer to fresh plates of the same or different type.

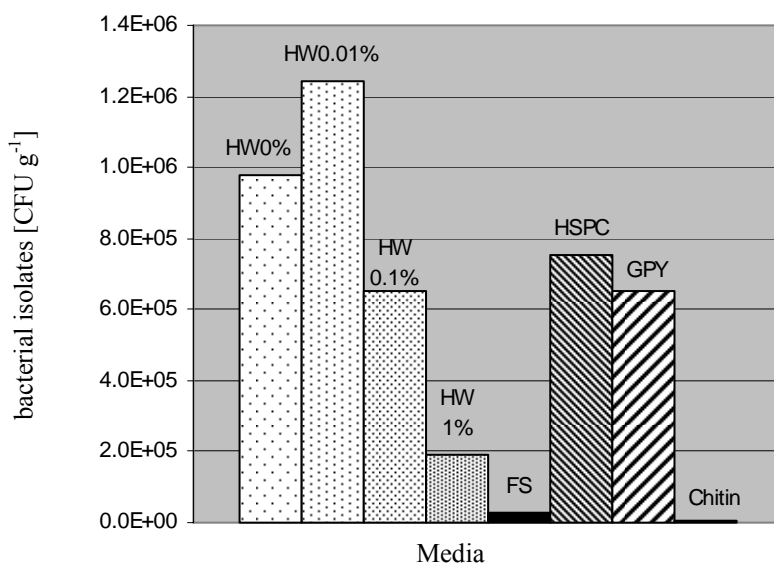


Fig. IV-1: Bacterial CFU titre per gram wet weight sponge tissue obtained on different types of media as averaged for the total of samples. Media abbreviations are consistent with Material and Methods paragraph.

Assessment of spore-forming isolates

With respect to the production of bioactive secondary products spore-forming microorganisms play a fundamental role. Two approaches were launched for selective isolation of spore formers. These approaches took advantage of the high resistance of spores to both toxic chemical agents (here: phenol) and desiccation (see Materials and Methods).

The yield of colonies after spore-selection was very low and only in three sponges, *A. polypoides*, *T. aurantium*, and *S. domuncula*, between 3 and 33 colonies per plate were found, which numerically corresponded to <1% of the complete population of cultured microorganisms (not shown). No colonies were found on actinomycete isolation agar. The morphological characteristics of the colonies imply that great part of the phenol-resistant bacteria belonged to the *Bacillus* group while no typical actinomycete colony was detected. After several months of storage the ‘mother’ plates still available from primary isolation were rechecked and about 100 belatedly grown colonies with potential actinomycete morphology picked. Only one of the selected strains turned out to represent a *Streptomyces* isolate.

Bioactivity of isolates

A selection of 2562 colonies derived from all samples was isolated by transfer to new plates (five parallels) (see Materials and Methods); of which 1866 formed visible colonies. The colonies were overlaid with indicator strains to test for antimicrobial activity. Two independent criteria were used for the selection of colonies: i) in order to cover a great variety of strains, colonies were picked from all types of media according to their unique appearance in terms of colony morphology and colour; ii) for a quantitative evaluation of the populations all colonies from contiguous sectors of mother plates were picked irrespective of their appearance; to guarantee an optimal comparability of samples the HW0.01% plates were arbitrarily selected as the standard source. A total of 569 strains (30.5% of all sub-cultured colonies) were active against at least one of the test strains. Excluding weak activities (as defined in Materials and Methods) the number of active strains was reduced to 283 (15.2% of all sub-cultured colonies). The relative abundance of active strains was different in distinct sponge species, ranging from 0% for *P. ficiformis* to 70% in the case of *C. reniformis* (Fig. IV-2). The absolute abundance could sum up to 1.1×10^6 CFU per gram wet weight as calculated for *T. aurantium* (Fig. IV-2, label of the bars).

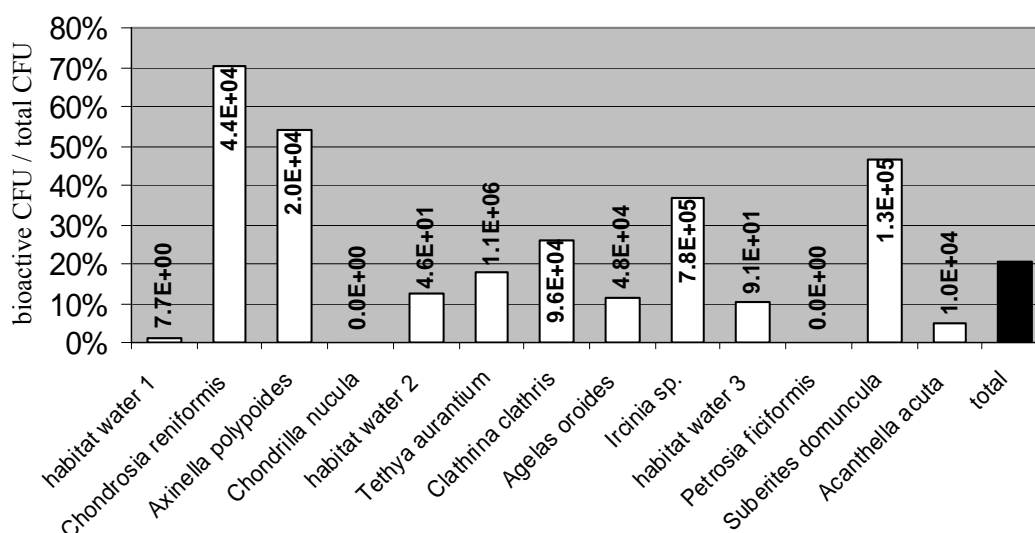


Fig. IV-2: Relative abundance of bioactive bacteria isolated from various sponge and habitat water samples. The absolute abundance (CFU g⁻¹ wet weight sponge tissue) is given on each bar.

Activity range of bioactive isolates

The most frequently inhibited target was *E. coli* as a representative of the Gram-negative bacteria (Fig. IV-3). This activity was displayed by >75% of all bioactive isolates, while the two Gram-positive test strains were inhibited to a much lower extent (38% for *B. subtilis* and 22% for *S. lentus*). Only 5% of the bioactive isolates inhibited the yeast test

strain, *C. glabrata* (Fig. IV-3). However, this ratio is valid only for primary testing after a single transfer of colonies from the mother plates to assay plates. When activity testing is repeated with subsequently purified strains or even chemical extracts thereof results shift to an over-proportional inhibition of Gram-positive test strains while activity against *E. coli* gets largely lost (Fig. IV-4). Possible explanations for this paradox will be discussed below.

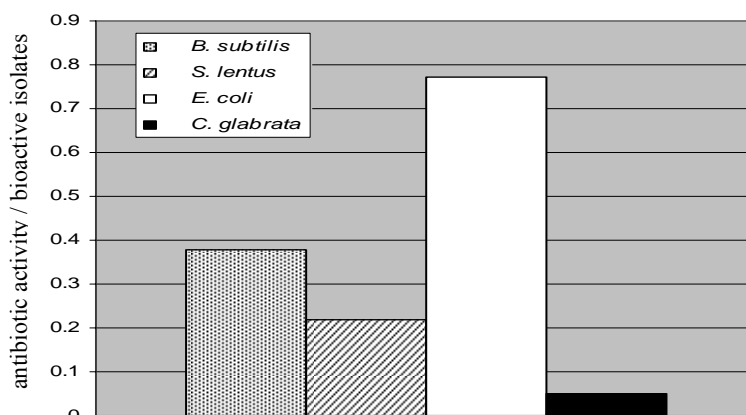


Fig. IV-3: Specific antibiotic activity against four test strains relative to the total number of active isolates.

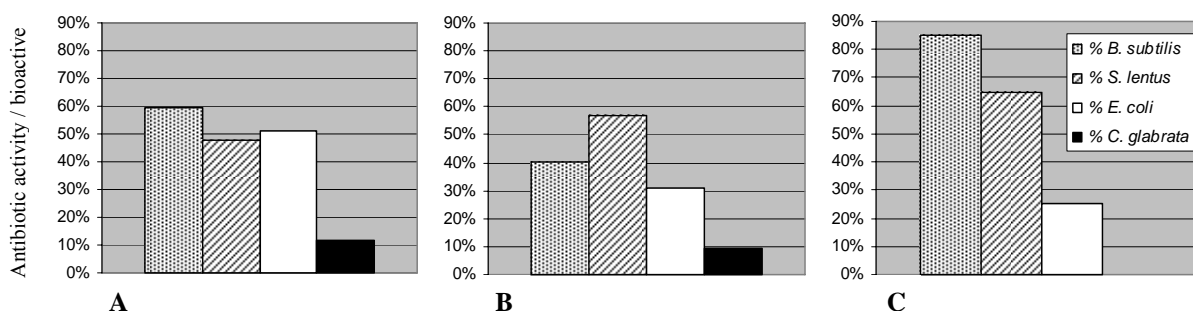


Fig. IV-4: Change of overall specific activity in the course of strain selection and retesting. A) primary selection of active isolates (n = 163 strains); B) second overlay of selected isolates with test strains (n = 42 strains); C) Activity of extracts from selected isolates (n = 20 strains). (The numerical reduction of active strains is due to a successive loss of activities.)

Frequencies and activities of dominant species

As shown in Table IV-2, 16 to 74% of the bacterial strains derived from individual sponges could be assigned to a single morphotype. For habitat water samples 1 and 3 no dominant isolate could be determined, while for *C. nucula* the survival rate of transferred colonies was too poor to allow a statement. In general, the frequencies of the second-most prominent morphotypes in the sponges were below 15% (except for *C. clathris* with 23%).

Interestingly, with respect to the abundance of antibiotically active isolates, the dominant morphotype always showed a striking bioactivity (Table IV-2), though this activity was weak and unstable as it got lost after repeated transfers of the strains to fresh media. In

absolute numbers, this homogenous population of a single active morphotype may sum up to 8×10^5 individuals g^{-1} sponge tissue (*T. aurantium*, $>10^5$ for *Ircinia* sp. and *S. domuncula*, too; Table IV-2). Except for the habitat water samples, the activity was most frequently and sometimes exclusively directed against Gram-negative bacteria (test organism *E. coli*) (Table IV-3). Part of the second-most and (if available) third-most frequent morphotypes were active, too (Table IV-2), but with a less consistent activity range (not shown).

Table IV-2: Relative abundance of the most (a), second-most (b) and third-most frequent (c) bacterial morphotypes obtained from the different sponges and their antibiotic activity (irrespective of specificity) relative to their total number within a given sample.

Sample	Activity (total)	Morphotype a	Activity (a)	Morphotype b	Activity (b)	Morphotype c	Activity (c)
habitat water 2	13%	26%	38%	24%	11%	-	-
<i>Chondrosia reniformis</i>	70%	74%	89%	9%	20%	-	-
<i>Axinella polypoides</i>	54%	31%	100%	15%	50%	-	-
<i>Tethya aurantium</i>	18%	39%	38%	7%	0%	-	-
<i>Clathrina clathris</i>	26%	42%	48%	23%	19%	-	-
<i>Agelas oroides</i>	12%	17%	42%	5%	0%	-	-
<i>Ircinia</i> sp.	37%	46%	70%	6%	17%	3%	33%
<i>Petrosia ficiformis</i>	0%	19%	0%	10%	0%	6%	0%
<i>Suberites domuncula</i>	46%	50%	75%	5%	0%	4%	40%
<i>Acanthella acuta</i>	5%	32%	12%	6%	0%	-	-
average	29%	36%	55%	11%	11%	6%	41%

Table IV-3: Specific activity of the most frequent morphotype relative to its total abundance (a given strain may be active against more than one test strain).

Sample	% <i>B. subtilis</i>	% <i>S. lentus</i>	% <i>E. coli</i>	% <i>C. glabrata</i>
habitat water 2	5%	19%	14%	19%
<i>Chondrosia reniformis</i>	29%	2%	67%	0%
<i>Axinella polypoides</i>	0%	0%	100%	0%
<i>Tethya aurantium</i>	14%	21%	31%	0%
<i>Clathrina clathris</i>	2%	0%	40%	0%
<i>Agelas oroides</i>	5%	0%	37%	0%
<i>Ircinia</i> sp.	53%	38%	88%	3%
<i>Petrosia ficiformis</i>	0%	0%	0%	0%
<i>Suberites domuncula</i>	23%	7%	68%	0%
<i>Acanthella acuta</i>	6%	3%	9%	0%
average	18%	10%	48%	3%

Identification of dominant and bioactive species

When the dominant bacteria from different sponges were compared among each other a striking overall similarity was observed, represented by brownish-white, semi-transparent, protruding, and round-edged colonies with a rubber-like consistency. To verify their taxonomic relatedness a selection of purified isolates from several samples was submitted to partial 16S rDNA analysis, followed by BLAST search of GenBank database and phylogenetic tree calculation using the Maximum Likelihood algorithm. The dominant strains of *A. polypoides* and *P. ficiformis* morphologically corresponded to those of the other sponges but did not survive the sub-culturing procedure; therefore, no sequence data are available for these two sponges.

As shown in Table IV-4 and Figure IV-5, all most frequent sponge isolates identified by sequence analysis belonged to the *Alphaproteobacteria* and - with the exception of *T. aurantium* - to a sponge-specific cluster related to *Pseudovibrio denitrificans* (designated 'Aplysina sponge isolate MBIC3368' in Table IV-4) including formerly collected isolates from a variety of sponge species (see Discussion). For the high degree of similarity (99-100%) these isolates can be looked upon as identical or closely related strains of a single species. In *T. aurantium* the dominant species was 100% identical to another alphaproteobacterium, *Ruegeria atlantica*, unrelated to the sponge-specific cluster mentioned above (<89% identity).

Table IV-4: Phylogenetic position of most (a) and second-most (b) frequent bacterial isolates and 16S rDNA sequence similarity to their closest relative as determined by BLAST search.

Sample	p ¹	n ²	next relative in database	subdivision	Accession no.	similarity
<i>Chondrosia reniformis</i>	a	8	<i>Aplysina</i> sponge isolate MBIC3368	Alphaproteobacteria	AF218241	100%
<i>Clathrina clathris</i>	a	2	<i>Aplysina</i> sponge isolate MBIC3368	Alphaproteobacteria	AF218241	100%
<i>Agelas oroides</i>	a	2	<i>Aplysina</i> sponge isolate MBIC3368	Alphaproteobacteria	AF218241	100%
<i>Ircinia</i> sp.	a	2	<i>Aplysina</i> sponge isolate MBIC3368	Alphaproteobacteria	AF218241	100%
<i>Suberites domuncula</i>	a	4	<i>Aplysina</i> sponge isolate MBIC3368	Alphaproteobacteria	AF218241	100%
<i>Tethya aurantium</i>	a	3	<i>Ruegeria atlantica</i>	Alphaproteobacteria	AJ968649	100%
Habitat water 2	a	2	<i>Pseudoalteromonas marina</i>	Gammaproteobacteria	AY563032	98.3/ 100%
<i>Clathrina clathris</i>	b1	1	<i>Pseudoalteromonas marina</i>	Gammaproteobacteria	AY563032	99.6%
<i>Clathrina clathris</i>	b2	1	uncultured <i>Gammaproteobacterium</i>	Gammaproteobacteria	AJ561154	99.6%
<i>Acanthella acuta</i>	b	2	<i>Pseudoalteromonas atlantica</i>	Gammaproteobacteria	X82134	99.8%
<i>Agelas oroides</i>	b1	1	<i>Sphingomonas</i> sp. GC14	Gammaproteobacteria	AY690679	98.4%
<i>Agelas oroides</i>	b2	1	<i>Kytococcus sedentarius</i>	Actinobacteria	AJ717359	99.6%
<i>Suberites domuncula</i>	b	2	uncultured <i>Microbacterium</i>	Actinobacteria	AY569297	99.1%
<i>Ircinia</i> sp.	b1	1	<i>Roseobacter</i> sp. WHOI	Alphaproteobacteria	AY349460	99.3%
<i>Ircinia</i> sp.	b2	1	<i>Vibrio splendidus</i>	Gammaproteobacteria	AJ874367	100%
Habitat water 2	b	3	<i>Vibrio splendidus</i>	Gammaproteobacteria	AJ874367	98.4-99.2%

¹p = abundance of morphotype: a = most frequent, b = second-most frequent

²n = absolute number of representative sequences

For the second-most frequent isolates the assignment was not as uniform as for the dominant isolates (see Table IV-4). In most sponges it was represented by *Gammaproteobacteria* belonging to the genera *Pseudoalteromonas* or *Vibrio*, while for *S. domuncula* it belonged to the *Actinobacteria* (*Microbacterium*). For *A. oroides*, *Ircinia* sp., and *C. clathris* the morphologically identical strains selected for sequencing turned out to be unrelated (morphotypes ‘b1’ and ‘b2’ in Table IV-4); therefore, a decision as to the second-most frequent isolate cannot be made.

In the case of habitat water, the cultured bacterioplankton was very diverse without clear dominance of a definite morphotype, except for one sample (‘seawater 2’ from Limski kanal). In contrast to the sponge samples, this most frequent isolate was *Pseudoalteromonas marina*, while the second-most frequent isolate corresponded to *Vibrio splendidus*. Both species were represented by two different strains, since the corresponding 16S rDNA sequences were not identical (Table IV-4). Interestingly, the second-most frequent isolates from *A. acuta*, *C. clathris* and *Ircinia* sp. were highly similar to the *Pseudoalteromonas* isolated from seawater, and the second-most frequent isolate of *Ircinia* sp. additionally corresponded to *Vibrio splendidus* representing the second-most frequent habitat water isolate.

Additional 21 bacterial strains selected for reproducible antibiotic activity (instead of morphotypes) were submitted to 16S rDNA analysis, too (Fig. IV-5). Two of them belonged to the sponge-specific *Alphaproteobacteria* cluster described above. 13 strains turned out to represent a variety of *Bacillus* species with diverse activity spectra, but no specific association

between *Bacillus* spp. and sponge species could be deduced. The remaining isolates belonged to the *Gammaproteobacteria* (4 *Pseudomonas* sp. and 2 *Alteromonas* sp.) (Fig. IV-5).

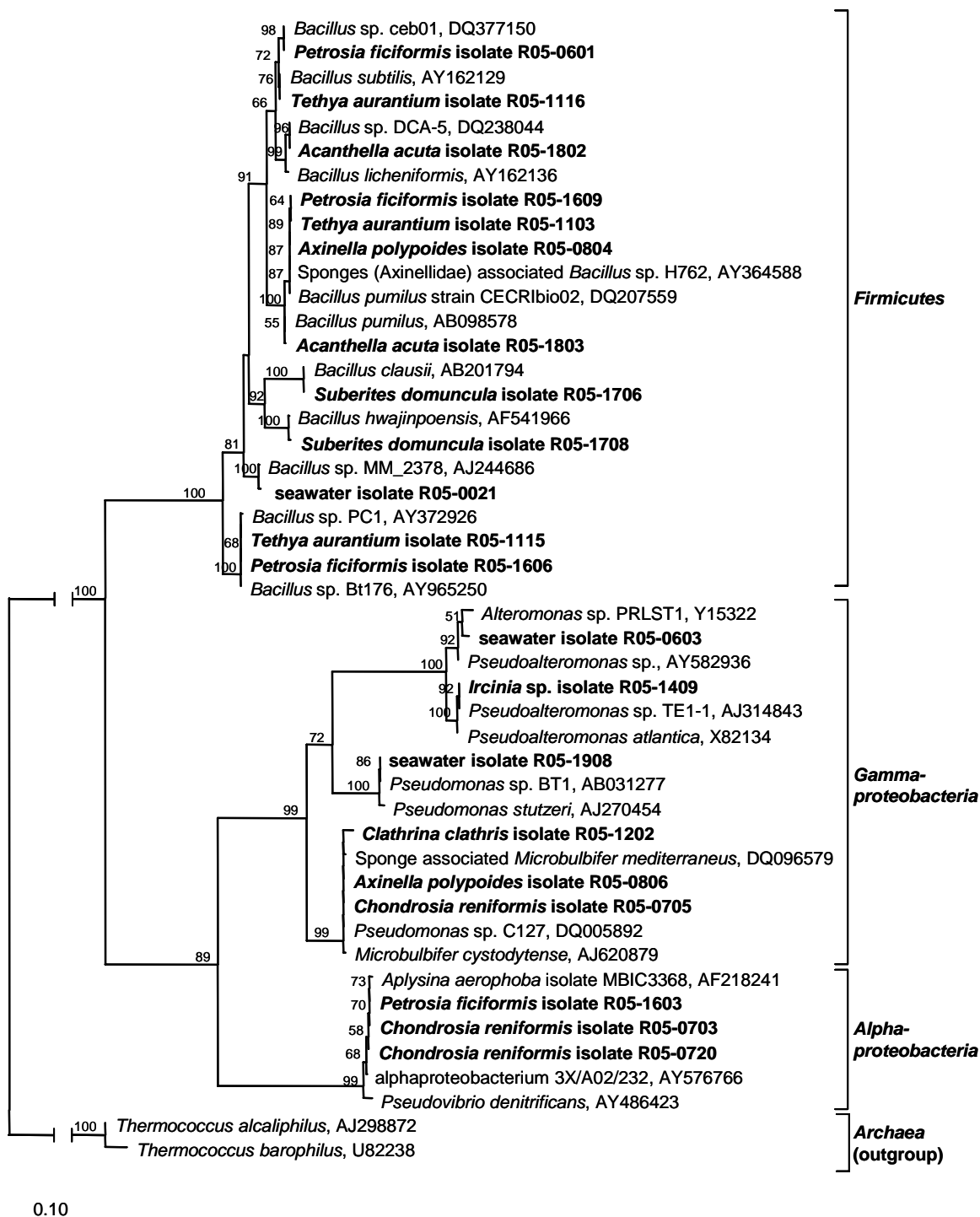


Fig. IV-5: Phylogenetic tree of bacterial isolates with reproducible antimicrobial activity obtained from sponges and habitat water. The strains isolated in this study are highlighted in bold type. Sponge origins of affiliated reference strains are indicated. Maximum Likelihood calculation relies on partial sequences > 400 to 1100 bp of the 5'-end from the 16S rDNA gene. Numbers at nodes represent bootstrap values; only values equal or greater 50 are shown (100 replicates done). The scale bar indicates the number of substitutions per nucleotide position.

DISCUSSION

In the present study the cultured microbial communities isolated from ten different Mediterranean sponge species were qualitatively and quantitatively checked for media preference, dominant species and antimicrobial activity. We found a single alphaproteobacterium to dominate the cultured community of at least six of the sponges. The high abundance of this isolate, its worldwide occurrence, its obvious restriction to the phylum Porifera (see below) and its striking antimicrobial activity indicate a specific ecological role within the sponges. The prominent role of this alphaproteobacterium in sponge biology raises the questions of i) the specificity of the association (obligate or facultative), ii) the kind of association (mutualistic, neutral, commensal, or parasitic), and iii) the way of acquisition (vertical or horizontal transfer).

In the past years the *Pseudovibrio denitrificans*-related alphaproteobacterium has been obtained worldwide from a great variety of sponges and in several cases was found to dominate the cultured microbial sponge community (Enticknap *et al.* 2006, Lafi *et al.* 2005, Webster and Hill 2001). As can be concluded from these studies and databases relying on 16S rDNA sequences it is largely absent from seawater or macroorganisms other than sponges. Though the distance to *P. denitrificans* is only 1-2% on 16S rDNA level, the differences are significant since typical 'signature nucleotides' within the conserved 16S RNA secondary structure are affected (not shown). The alphaproteobacterium strain NW001 was recognised as the dominant cultured species of the Great Barrier Reef sponge *Rhopaloeides odorabile* and quantified to >60% of the cultured bacterial community for 44 sponge specimens from distant geographical locations (Webster and Hill 2001). NW001 was lacking only from two out of these 44 sponge individuals; both were severely diseased, but it remained unclear if necrosis and fouling were a consequence or rather a cause of this loss. Nevertheless, the association seems to be highly stable in healthy sponge individuals. FISH and gradient centrifugation demonstrated NW001 to be tightly attached to sponge cells, in particular to those surrounding the choanocyte chambers, where it might contribute to sponge nutrition (Webster and Hill 2001).

A NW001-related alphaproteobacterium (isolate MBIC3368 used as reference in Table IV-3) was isolated from the Mediterranean sponge *Aplysina aerophoba*, but according to DGGE results was not present in a constantly high abundance (Thoms *et al.* 2003); it could not be detected at all by FISH (Friedrich *et al.* 1999). Recently, a survey on the large collection of deep-water microorganisms available at the Harbor Branch Oceanographic Institution

revealed that 376 out of 2273 strains (16.5%) correspond to the NW001 clade of *Alphaproteobacteria* (Sfanos *et al.* 2005). These strains derived from a great variety of geographic regions, but their occurrence apparently is restricted to the phylum Porifera, where it was found in any taxonomic order within the Demospongiae.

Interestingly, *Tethya aurantium* deviates from the other sponges in that its dominant alphaproteobacterium, *Ruegeria atlantica*, is unrelated to the NW001 type. Analysis of 16S rDNA from the clone library of exactly this *T. aurantium* specimen (Thiel *et al.*, in press) shows that *Alphaproteobacteria* can be found only in low abundance within the cortex (and not at all in the mesohyl) of the sponge, and that these *Alphaproteobacteria* correspond to neither the NW001 type nor *R. atlantica*. These observations further underline the discrepancy between cultivation-dependent and cultivation-independent approaches in the analysis of the composition of sponge-derived (and other) bacterial communities.

The mode of transmission of the alphaproteobacterial associate from one sponge generation to the next is still unclear. The NW001-type alphaproteobacterium is distributed worldwide as a taxonomically uniform species; this fact implies an effective horizontal transfer through the seawater and a permanent mixing of the bacterial population. Further, it has been detected in Mediterranean surface waters very recently, suggesting a planktonic life for the alphaproteobacterium and a horizontal transfer between the sponges (Agogue *et al.* 2005). In contrast, a vertical transfer of NW001-type *Alphaproteobacteria* via gametes was recently shown for *Mycale laxissima* (Enticknap *et al.* 2006). The authors argued that presence of the alphaproteobacterium in seawater possibly results from release from diseased, damaged or dead sponge (Enticknap *et al.* 2006). However, if vertical transfer was the exclusive mode of distribution and occurrence of the sponge-associate in surface waters only caused by release from a necrotic or dead sponge, it should result in a co-evolution of the host/symbiont pair and concomitantly in a genotypic separation from unrelated sponge/bacteria pairs. However, evolutionary separation obviously takes place for the sponges exclusively while NW001 remains largely unaltered irrespective of host species or geographical region. A combination of both horizontal and vertical transfer therefore is assumed, possibly indicating an early stage in establishing an obligate symbiosis.

The claim of a specific ecological role of the dominant *Alphaproteobacteria* in the sponge host is further supported by their antibiotic activity (Table IV-2), which in view of their high abundance may have inhibitory effects in the sponge itself. These findings quantitatively confirm previous observations that individual NW001 type isolates from sponges *A. aerophoba* and *Suberites domuncula* show antimicrobial activity (Thakur *et al.* 2005, Thiel and Imhoff 2003,

Hentschel *et al.* 2002). In terms of activity, the dominant *R. atlantica* isolate of *T. aurantium* is comparable to the NW001-related *Alphaproteobacteria*. Interestingly, an active *Ruegeria* sp. isolate producing cyclic dipeptides was obtained from *S. domuncula* previously (Mitova *et al.* 2004). Irrespective of the bioactive compound and the taxonomic position of its producer, the overproportional activity of the most abundant isolates may be an immediate result of the inhibition of the (cultured) competitors in the host environment (the effects on the uncultured bacterial community and vice versa remain unclear).

The marine bacterioplankton is dominated by Gram-negative bacteria (Giovannoni and Rappé 2000). From this point of view it is conceivable that the test strain *E. coli* representing the Gram-negative bacteria is the preferred target of the antibiotic activity. Surprisingly, only part of the closely related or even identical isolates was recognised as active and with a variable activity range (the Gram-positive test strains in some cases are inhibited along with *E. coli*; not shown). Obviously, activities are highly unstable since the same strain may be once active but completely inactive in a subsequent experiment. This metabolic instability also could explain the paradox why activities against *E. coli* and other Gram-negative bacteria are underrepresented in strain collections of bioactive marine microorganisms: during strain purification and assay reproduction the *Alphaproteobacteria* get lost or are sorted out, while easily manageable candidates such as bacilli persist; this could be clearly observed in the course of the present study, too (Fig. IV-4).

The fluctuation of secondary metabolism may be extended to the complete metabolism: the described *Alphaproteobacteria* develop well on mother plates inoculated with original sponge material and may form large colonies after overnight incubation of freshly inoculated daughter plates; more often, however, they fail to grow at all or develop only weakly after repeated colony transfers. This unsteady behaviour possibly indicates a successive depletion of (a) sponge-derived growth factor(s) on cultivation media, comparable to strain SB2, which shares 98.5% identity with NW001 and settles the surface of *S. domuncula* (Müller *et al.* 2004); here, the usage of sponge-derived protocatechuate was shown.

Typical bioactive microorganisms such as the spore-forming streptomycetes and bacilli do not seem to play an important role in the analyzed sponges. Various species of the genus *Bacillus*, many of them with antibiotic activity, could be regularly found in nearly all sponges and seawater samples (Fig. IV-5). In terms of quantity, however, they do not belong to the dominant microbial community. Actinomycetes were largely absent, even when enrichment procedures like growth on special actinomycete media or selection for spores by desiccation or chemical treatment were used.

The relative quantities of specific isolates were deduced from the number of colonies with identical properties determined by optical means. This practicable approach appears quite reliable at least for the most frequent isolates, as from the 25 random colonies also checked by 16S rDNA sequencing none contradicted the optical judgement. The cultivable community obtained from the habitat water samples was dominated by members of the *Gammaproteobacteria* (mainly *Pseudoalteromonas* sp.), which were also found in moderate abundance in some sponge species indicating that these bacteria are quite resistant to digestion. Whether they play an ecological role within the autochthonous bacterial community of the sponges remains unclear.

The colony yield as well as the diversity of isolates judged from the variability of colony morphologies is largely influenced by the medium. Generally - though there are deviations for individual sponges (Table IV-1) - yields are best when original habitat water in combination with low nutrient supply is used. In contrast, medium containing seawater other than habitat water, such as Baltic Sea water (with a salt concentration adapted to Mediterranean conditions by addition of 1.5% NaCl), causes an extreme drop with respect to both criteria. Similarly, a nutrient supply exceeding 0.01% YE considerably reduces the yield, too. Nutrient excess obviously is a severe stress factor, comparable to oxidative stress investigated by Olson *et al.* (2000) who showed that the addition of oxygen detoxifying compounds or enzymes (sodium pyruvate, catalase) enhance the cultivation efficiency of sponge-derived bacteria.

Unlike for colony yields, the type of medium does not seem to play a significant role for the selection of bioactive strains, as was concluded from a comparative evaluation of the isolation media (not shown). A possible exception may be represented by the media based on habitat water. Here, a slightly enhanced all-over rate of bioactive strains could be observed, which in this case was independent of the YE concentration (not shown).

As outlined above, one common sponge-associated alphaproteobacterium clearly dominates the cultured bacterial community of many sponges world-wide. The striking bioactivity of this interesting alphaproteobacterial cluster, as quantitatively analyzed here for the first time, now further supports the ecological dimension of its ubiquity and abundance in sponges. With a future shift of the scientific focus to the physiology (primary and secondary metabolism) of this important bacterium profound insights into both microbial and sponge ecology can be expected.

GENERAL DISCUSSION

Sponges have been known to live in association with bacteria for a long time. Different metabolic and physiological groups, including chemoheterotrophic bacteria, (Chapter III+IV, Montalvo *et al.* 2005, Webster *et al.* 2001b, Olson *et al.* 2000), as well as phototrophic bacteria (Chapter II, Steindler *et al.* 2005, Diaz and Ward 1999, Wilkinson 1980a) have been found in association with sponges and often have been referred to as symbiotic. The terms ‘association’ and ‘symbiosis’, however, are often used inconsistently in literature and despite its definition by DeBary (1879) ‘symbiosis’ is often used as a synonym for ‘mutualism’ (Goff 1982). Thus, in order to avoid confusion, here the term ‘association’ is used for spatial co-existence of different organisms with unknown or uncertain interaction and dependency between the partners; the term ‘symbiosis’ is used only with further classification regarding specific interaction.

Possible mutual benefits for either associate have been discussed for sponge-bacteria associations. For example, bacteria producing antimicrobial substances may potentially contribute to the chemical defence of the sponges (Chapter IV), while bacteria utilize and possibly depend on sponge metabolites as carbon or energy source (Chapter IV). In the association with nitrogen-fixing cyanobacteria sponges benefit from an additional supply with nitrogen (Shieh and Lin 1994, Wilkinson and Fay 1979), as well as from fixed carbon and additional O₂ from photosynthesis (Ariello *et al.* 1993, Wilkinson and Garrone 1980, Wilkinson and Vacelet 1979). Another benefit of cyanobacterial association for the sponge may be protection from excessive illumination (Sara 1971), whereas CO₂ production from the host prevents carbon limitation for the photosynthetic associate (Wilkinson and Garrone 1980). In case of the deep-sea sponge *Cladorhiza* sp. the sponge does not only benefit from but even nutritionally relies on symbiotic methane-oxidizing bacteria in order to be able to inhabit hydrothermal vent fields (Vacelet *et al.* 1995).

When contemplating sponge-bacteria associations it has to be taken into account that sponges also have been found in association with other organisms. *Chondrilla nucula*, for example, harbours an associated, putatively ammonia-oxidizing crenarchaeon (Appendix A), and further sponge-archaea associations have been reported for sponges of different taxonomic and geographic origin (Lee *et al.* 2003, Margot *et al.* 2002, Preston *et al.* 1996). Furthermore, eukaryotic microscopic and macroscopic organisms, i.e., a broad diversity of algae (Frost *et al.* 1997, Rutzler 1990, Saller 1989, Wilkinson 1987, Reisser 1984, Vacelet 1982) as well as non-phototrophic micro- and macroorganisms, e.g., yeasts (Maldonado *et al.* 2005),

polychaetes and prosobranchs (Kunzmann 1996) live associated with sponges. However, a mutual benefit for the sponge host has only been demonstrated for some sponge-algae associations (Hill 1996, Rosell and Uriz 1992, Wilkinson 1992, Frost and Williamson 1980, Wilkinson 1980b, Muscatine *et al.* 1967).

Concerning the classification of sponge-bacteria associations, the specificity of the association needs to be addressed. Specificity and consistency over time usually implies commensal or mutual benefits. However, regarding the specificity of sponge-bacteria associations it has to be taken into consideration that sponges are powerful filter feeders. They take up bacteria from the ambient seawater by filtration and great caution is therefore necessary in order to separate associated bacteria from seawater-derived organisms. Even after repeated washing in sterile-filtered seawater, specificity and constant association is not assured for all bacterial sequences obtained (Chapter II).

Wilkinson *et al.* (1981) isolated phenotypically similar bacteria from different sponges. The authors deduced specificity of the bacteria to the phylum Porifera based on their absence from ambient water and immunological cross-reactions which were only positive between the 'specific' sponge associated bacteria. Later, with the invention of molecular methods, i.e., identification of microorganisms based on their ribosomal DNA sequences, more precise taxonomic and phylogenetic classification as well as detection of uncultured sponge-associated bacteria became possible. Comparison of 16S rDNA clone libraries obtained from several sponge species of different geographical origin revealed unexpected conformity between the different sponge species and a uniform sponge-associated bacterial community was proposed (Hentschel *et al.* 2002). However, ambient seawater was not examined in that study and as the number of bacterial sequences available in the databases increases, also sequences from other marine habitats than sponges are found to cluster with phlotypes previously thought to be sponge-specific. In this study, in spite of the high percentage (79%) of bacterial sequences in *C. nucula* showing affinity to other sponge-derived sequences, only four monophyletic clusters were confirmed as sponge-specific (Chapter I). Additionally, associations of bacteria can be specific to either a restricted group of sponges (e.g., genus or family) or one single sponge species. In this study, both general sponge-specific bacterial phlotypes as well as bacteria specific to a restricted group of sponges or possibly the species *C. nucula* solely, were obtained (Chapter I). Specificity of sponge-bacteria associations are further supported by the fact that different sponges, despite of sharing the same habitat and ambient seawater, harbour different bacterial phlotypes of which none were found in the ambient seawater (Chapter II, Appendix B-5). Moreover, specificity may be extended to the spatial distribution within one sponge

individual. For example, distinct bacterial populations were detected associated with *Tethya aurantium* endosome and cortex (Chapter II).

Sponge-bacteria associations have been studied since the 1970s using different methods. At the beginning of sponge research only a limited range of methods, e.g., microscopy, isolation in fluid and solid media and metabolic tests, was available and identification of associated bacteria purely relied upon morphological features. With the introduction of molecular methods like 16S ribosomal DNA analysis by Woese (1987), identification of uncultured bacteria associated with sponges became possible and since then many bacterial communities associated with sponges were described based on 16S rDNA clone libraries. However, the information of ribosomal sequences is limited to their phylogenetic relation within the tree of life. No metabolic or physiological conclusions can be drawn from it directly. Only for few exceptions in which a certain characteristic trait is restricted to a phylogenetic group of organisms, metabolic assumptions can be inferred from ribosomal DNA sequences. For identification of metabolic traits in isolates and environmental samples further molecular methods are available. Analysis of functional genes, e.g., *amo*-genes for ammonia-oxidation and *nif*-genes for N-fixation as well as metagenomic approaches facilitate the study of metabolic possibilities of uncultured organisms. For example, detection of the gene for a putative archaeal ammonia-monooxygenase gene (*amoA*), which is related with the ability of ammonia-oxidation, indicated the sponge-associated archaeon to be involved in nitrate-production within *C. nucula* (Appendix A). However, molecular tools will never compensate the need for pure culture isolates in microbiological research, but they disclose a glimpse into the great variety and the fascinating high diversity of yet uncultured organisms associated with marine sponges. In order to understand the nature of a sponge-bacteria association, metabolic and physiological information about the partners are needed. However, a mutual symbiosis consequently leads to a mutual dependence (obligation) which is reflected by adaptation of the metabolisms of the two partners (DeBary 1879). Thus, the dependency of one or both partners in an obligate mutual symbiosis often prevents isolation of the symbiont in pure cultures. Therefore physiological studies on obligate symbionts are rare. In only a few cases cultivation of the associate is possible and specific adaptations to the host environment can be studied. For example, green algae isolated from sponges and ciliates, referred to as 'endosymbiotic' by the authors, differ from their free-living relatives by excretion of mono- and disaccharides and an unusual high oxygen production at low light intensities (Reisser 1984).

Studies concerning sponge-bacteria association usually focus on associations including only one specific group of associated organisms, e.g., sponge-cyanobacteria associations, and possible benefits for only these groups are considered. For a comprehensive and global model of sponges as microenvironments, though, the whole associated community including a complexity of possible interactions has to be kept in mind. Regarding the great diversity of different micro- and macroorganisms that live associated with sponges, also interactions between different species within the sponge associated community have to be taken into account. For example, *Bdellovibrio*-like *Deltaproteobacteria* (Chapter I), found in sponges for the first time, raise the question of possible predatory interactions between distinct bacterial members of the sponge-associated community within *Chondrilla nucula*.

As sessile filter feeding organisms, sponges largely depend on chemical defence to protect themselves from potential threats, like pathogens, epibionts and predators; and they have been known as a rich source of biological active secondary metabolites. For the given reasons this great diversity of highly active substances makes sponges especially interesting for the search of new drugs (Sipkema *et al.* 2005, Jha and Zi-rong 2004, Kuramoto *et al.* 2004, Haefner 2003, Munro *et al.* 1999). A high diversity of new compounds has been isolated from marine sponges already and plenty new ones are discovered every year (Sipkema *et al.* 2005). Interestingly, some of the sponge-derived substances show remarkable similarity with microbial metabolites implying associated microbes to be the true producers (Faulkner *et al.* 2000, Faulkner *et al.* 1993) and, in some cases, the secondary products originally isolated from sponges could be assigned to associated bacteria (Schmidt *et al.* 2000, Bewley *et al.* 1996, Jayatilake *et al.* 1996, Oclarit *et al.* 1994, Unson *et al.* 1994, Shigemori *et al.* 1992). Sponges are extremely slow-growing and not easy to cultivate (Osinga *et al.* 1999). Additionally, bioactive substances often are produced only in very small amounts. Unreasonably large quantities of sponge biomass would be needed to comply with the requirements on the drug market. The isolation of bioactive sponge-associated bacteria (Chapter III+IV), however, enables biotechnological use of the products without the high harvesting pressure on sponge populations and may further provide insights in the ecology of chemical defence of sponges mediated by associated bacteria.

CONCLUSION

In this study it was shown that cultivable sponge-associated bacteria display a high potential for the search of new antimicrobial substances and biotechnological use. Further, the study has led to a deeper insight into the ecology of sponge-microbe associations. Sponge-bacteria associations are specific and differ in distinct sponge species inhabiting the same environment as well as in spatial distribution within the sponges. Also sponges containing small numbers of bacteria were shown to contain specific bacterial associates, possibly distinct to those associated with bacteria-rich sponges ('bacteriosponges'). Thus, no 'bacteria-free' sponges seem to exist. In general, sponges provide different micro-niches for associated microbes, e.g., low-oxygen and anoxic regions (Hoffmann 2003) and harbour a highly complex community of specifically associated bacteria, with an unknown number of species spanning interactions and benefits for the involved organisms. The production of antimicrobial active substances by associated bacteria possibly contributes to the chemical defence of the sponge. Regarding the micro-niches and the high diversity of sponge-associated organisms, as well as the complexity of possible interactions, it might be suitable to refer to sponges as micro-habitats or micro-environments, providing ecological niches for associated micro- and macroorganisms rather than just as associate in a (two-partners-) association (host). Due to the limitation of each method used for sponge-microbe associations, only a combined approach using culture-based methods as well as culture-independent molecular tools will lead to a comprehensive understanding of the highly complex system of sponges as natural micro-environments. Many questions are still unresolved and sponges and their associated communities still provide a fascinating area for future research.

LITERATURE

- Abrams E.S. and Stanton V.P. (1992) Use of denaturing gradient gel-electrophoresis to study conformational transitions in nucleic-acids. *Methods in Enzymology*, **212**, 71-104.
- Agogue H., Casamayor E.O., Bourrain M., Obernosterer I., Joux F., Herndl G.J. and Lebaron P. (2005) A survey on bacteria inhabiting the sea surface microlayer of coastal ecosystems. *FEMS Microbiology Ecology*, **54**, 269-280.
- Ahn Y.B., Rhee S.K., Fennell D.E., Kerkhof L.J., Hentschel U. and Haggblom M.M. (2003) Reductive dehalogenation of brominated phenolic compounds by microorganisms associated with the marine sponge *Aplysina aerophoba*. *Applied and Environmental Microbiology*, **69**, 4159-4166.
- Althoff K., Schütt C., Steffen R., Batel R. and Müller W.E.G. (1998) Evidence for a symbiosis between bacteria of the genus *Rhodobacter* and the marine sponge *Halichondria panicea*: harbor also for putatively toxic bacteria? *Marine Biology*, **130**, 529-536.
- Altschul S.F., Gish W., Miller W., Myers E.W. and Lipman D.J. (1990) Basic local alignment search tool. *Journal of Molecular Biology*, **215**, 403-410.
- Amann R.I., Ludwig W. and Schleifer K.H. (1995) Phylogenetic identification and *in-situ* detection of individual microbial cells without cultivation. *Microbiological Reviews*, **59**, 143-169.
- Ariño A., Bavestrello G., Burlando B. and Sará M. (1993) Metabolic integration between symbiotic cyanobacteria and sponges - a possible mechanism. *Marine Biology*, **117**, 159-162.
- Barns S.M., Takala S.L. and Kuske C.R. (1999) Wide distribution and diversity of members of the bacterial kingdom *Acidobacterium* in the environment. *Applied and Environmental Microbiology*, **65**, 1731-1737.
- Berczi I., Bertók L. and Chow D.A. (2005) Host defense: an interaction of neuroendocrine, metabolic and immune mechanisms in the interest of survival. In: L. Bertók and D.A. Chow (Eds). *NeuroImmune Biology*. Elsevier Science, 3-25.
- Bergquist P.R. (1978) *Sponges*. Hutchinson and Company, London: 268pp
- Bewley C.A., Holland N.D. and Faulkner D.J. (1996) Two classes of metabolites from *Theonella swinhoei* are localized in distinct populations of bacterial symbionts. *Experientia*, **52**, 716-722.
- Bond C. and Harris A.K. (1988) Locomotion of sponges and its physical mechanism. *Journal of Experimental Zoology*, **246**, 271-284.
- Borchiellini C., Manuel M., Alivon E., Boury-Esnault N., Vacelet J. and Le Parco Y. (2001) Sponge paraphyly and the origin of Metazoa. *Journal of Evolutionary Biology*, **14**, 171-179.
- Boury-Esnault N. (2002) Order Chondrosida BOURY-ESNAULT & LOPÉS 1985 Family Chondrillidea GREY 1872. In: N.J.A. Hooper and R.W.M. van Soest (Eds). *Systema Porifera: a guide to the classification of sponges*. Kluwer Academic Plenum Publishers, New York: 291-297.

- Brinig M.M., Lepp P.W., Ouverney C.C., Armitage G.C. and Relman D.A. (2003) Prevalence of bacteria of division TM7 in human subgingival plaque and their association with disease. *Applied and Environmental Microbiology*, **69**, 1687-1694.
- Brown M.V., Schwalbach M.S., Hewson I. and Fuhrman J.A. (2005) Coupling 16S-ITS rDNA clone libraries and automated ribosomal intergenic spacer analysis to show marine microbial diversity: development and application to a time series. *Environmental Microbiology*, **7**, 1466-1479.
- Brümmer F., Nickel M. and Sidri M. (2003) Porifera (Schwämme). In: R. Hofrichter (Eds). *Das Mittelmeer*. Spektrum Akademischer Verlag, Heidelberg: 302-381.
- Brümmer F., Calcinaï B., Götz M., Leitemann F., Nickel M., Schillak L., Sidri M. and Zucht W. (2004) Overview on the sponge fauna of the Limski kanal, Croatia, Northern Adriatic Sea. *Bollettino dei Musei e degli Istituti Biologici dell'Università di Genova*, **68**, 219-227.
- Burja A.M., Webster N.S., Murphy P.T. and Hill R.T. (1999) Microbial symbionts of Great Barrier Reef sponges. *Memoirs of the Queensland Museum*, **44**, 63-75.
- Burja A.M. and Hill R.T. (2001) Microbial symbionts of the Australian Great Barrier Reef sponge, *Candidaspongia flabellata*. *Hydrobiologia*, **461**, 41-47.
- Burton M. (1928) A comparative study of the characteristics of shallow-water and deep-sea sponges, with notes on their external form and reproduction. *Journal of the Quekett Microscopical Club, Series 2*, **16**, 49-70.
- Carballo J.L., Gomez P., Cruz-Barraza J.A. and Flores-Sanchez D.M. (2003) Sponges of the family Chondrillidae (Porifera, Demospongiae) from the Pacific coast of Mexico, with the description of three new species. *Proceedings of the Biological Society of Washington*, **116**, 515-527.
- Cheshire A.C. and Wilkinson C.R. (1991) Modelling the photosynthetic production by sponges on Davies Reef, Great Barrier Reef. *Marine Biology*, **109**, 13-18.
- Cho J.C. and Giovannoni S.J. (2004) Cultivation and growth characteristics of a diverse group of oligotrophic marine *Gammaproteobacteria*. *Applied and Environmental Microbiology*, **70**, 432-440.
- Clarke K.R. (1993) Non-parametric multivariate analysis of changes in community structure. *Australian Journal of Ecology*, **18**, 117-143.
- Clarke K.R. and Warwick R.M. (1994) Similarity-based testing for community pattern - the 2-way layout with no replication. *Marine Biology*, **118**, 167-176.
- Clarke K.R. and Gorley R.N. (2001) *PRIMER v5: User manual/tutorial*, PRIMER-E. Plymouth UK: 91pp
- Corredor J.E., Wilkinson C.R., Vicente V.P., Morell J.M. and Otero E. (1988) Nitrate release by Caribbean reef sponges. *Limnological Oceanography*, **33**, 144-120.
- Davy S.K., Trautman D.A., Borowitzka M.A. and Hinde R. (2002) Ammonium excretion by a symbiotic sponge supplies the nitrogen requirements of its rhodophyte partner. *Journal of Experimental Biology*, **205**, 3505-3511.
- DeBary A. (1879) *Erscheinung der Symbiose*. Verlag KJ Trübner, Strassburg: 30pp
- Diaz M.C. (1997) Molecular detection and characterization of specific bacterial groups associated with tropical sponges. *8th International Coral Reef Symposium*, **2**, 1399-1402.

- Diaz M.C. and Ward B.B. (1997) Sponge-mediated nitrification in tropical benthic communities. *Marine Ecology Progress Series*, **156**, 97-107.
- Diaz M.C. and Ward B.B. (1999) Perspectives on sponge-cyanobacterial symbioses. *Memoirs of the Queensland Museum*, **44**, 154.
- Donachie S.P., Hou S., Lee K.S., Riley C.W., Pikina A., Belisle C., Kempe S., Gregory T.S., Bossuyt A., Boerema J., Liu J., Freitas T.A., Malahoff A. and Alam M. (2004) The Hawaiian Archipelago: a microbial diversity hotspot. *Microbial Ecology*, **48**, 509-520.
- Enticknap J.J., Kelly M., Peraud O. and Hill R.T. (2006) Characterization of a culturable alphaproteobacterial symbiont common to many marine sponges and evidence for vertical transmission via sponge larvae. *Applied and Environmental Microbiology*, **72**, 3724-3732.
- Faulkner D.J., He H.-Y., Unson M.D., Bewley C.A. and Garson M.J. (1993) New metabolites from marine sponges: are symbionts important? *Gazzetta Chimica Italiana*, **123**, 301-307.
- Faulkner D.J., Harper M.K., Haygood M.G., Salomon C.E. and Schmidt E.W. (2000) Symbiotic bacteria in sponges: sources of bioactive substances. In: N. Fusetani (Eds). *Drugs from the Sea*. Karger, Basel: 107-119.
- Faulkner D.J. (2002) Marine natural products. *Natural Product Reports*, **19**, 1-48.
- Felsenstein J. (1981) Evolutionary trees from DNA-sequences - a maximum-likelihood approach. *Journal of Molecular Evolution*, **17**, 368-376.
- Ferrari B.C., Binnerup S.J. and Gillings M. (2005) Microcolony cultivation on a soil substrate membrane system selects for previously uncultured soil bacteria. *Applied and Environmental Microbiology*, **71**, 8714-8720.
- Flowers A.E., Garson M.J., Webb R.I., Dumdei E.J. and Charan R.D. (1998) Cellular origin of chlorinated diketopiperazines in the dictyoceratid sponge *Dysidea herbacea* (Keller). *Cell and Tissue Research*, **292**, 597-607.
- Friedrich A.B., Merkert H., Fendert T., Hacker J., Proksch P. and Hentschel U. (1999) Microbial diversity in the marine sponge *Aplysina cavernicola* (formerly *Verongia cavernicola*) analyzed by fluorescence *in-situ* hybridization (FISH). *Marine Biology*, **134**, 461-470.
- Frost T., Graham L., Elias J., Haase M., Kretchmer D. and Kranzfelder J. (1997) A yellow-green algal symbiont in the freshwater sponge, *Corvomeyenia everetti*: convergent evolution of symbiotic associations. doi:10.1046/j.1365-2427.1997.00254.x. *Freshwater Biology*, **38**, 395-399.
- Frost T.M. and Williamson C.E. (1980) In situ determination of the effect of symbiotic algae on the growth of the freshwater sponge *Spongilla lacustris*. *Ecology*, **61**, 1361-1370.
- Frost T.M. (1991) Porifera. In: J.H. Thorp and A.P. Covich (Eds). *Ecology and Classification of North American Freshwater Invertebrates*. Academic Press, Inc., New York: 95-124.
- Fuchs B.M., Woebken D., Zubkov M.V., Burkill P. and Amann R. (2005) Molecular identification of picoplankton populations in contrasting waters of the Arabian Sea. *Aquatic Microbial Ecology*, **39**, 145-157.

- Fuhrman J.A. and Ouverney C.C. (1998) Marine microbial diversity studied via 16S rRNA sequences: cloning results from coastal waters and counting of native archaea with fluorescent single cell probes. *Aquatic Ecology*, **32**, 3-15.
- Gaino E., Pansini M. and Pronzato R. (1977) Aspects of association between *Chondrilla nucula* Schmidt (Demospongiae) and its symbiotic extracellular microorganisms (Bacteria and Cyanophyceae) in natural and experimental conditions. *Cahiers De Biologie Marine*, **18**, 303-310.
- Gaino E. and Pronzato R. (1983) Electron-microscope study of the filament of the stretched forms on *Chondrilla nucula* Schmidt (Porifera, Demospongiae). *Annales des Sciences Naturelles. Zoologie*, **5**, 221-234.
- Gaino E. and Sará M. (1994) Siliceous spicules of *Tethya seychellensis* (Porifera) support the growth of a green-alga - a possible light conducting system. *Marine Ecology Progress Series*, **108**, 147-151.
- Gallissian M.F. and Vacelet J. (1976) Ultrastructure de quelques stades de l'ovogenese de spongiaires du genre *Verongia* (Dictyoceratida). *Annales des Sciences Naturelles. Zoologie*, **18**, 381-404.
- Giovannoni S.J. and Rappé M.S. (2000) Evolution, diversity, and molecular ecology of marine prokaryotes. In: D.L. Kirchman (Eds). *Microbial Ecology of the Oceans*. Wiley-Liss, New York: 47-84.
- Goff L.J. (1982) Symbiosis and parasitism - another viewpoint. *Bioscience*, **32**, 255-256.
- Guindon S. and Gascuel O. (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology*, **52**, 696-704.
- Guindon S., Lethiec F., Duroux P. and Gascuel O. (2005) PHYML Online - a web server for fast maximum likelihood-based phylogenetic inference. *Nucleic Acids Research*, **33**, W557-W559.
- Haefner B. (2003) Drugs from the deep: marine natural products as drug candidates. *Drug Discovery Today*, **8**, 536-544.
- Hallam S.J., Mincer T.J., Schleper C., Preston C.M., Roberts K., Richardson P.M. and DeLong E.F. (2006) Pathways of carbon assimilation and ammonia oxidation suggested by environmental genomic analyses of marine Crenarchaeota. *PLoS Biology*, **4**, 520-536.
- Haygood M.G., Schmidt E.W., Davidson S.K. and Faulkner D.J. (1999) Microbial symbionts of marine invertebrates: opportunities for microbial biotechnology. *Journal of Molecular Biotechnology*, **1**, 33-43.
- Head I.M., Saunders J.R. and Pickup R.W. (1998) Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. *Microbial Ecology*, **35**, 1-21.
- Hentschel U., Schmid M., Wagner M., Fieseler L., Gernert C. and Hacker J. (2001) Isolation and phylogenetic analysis of bacteria with antimicrobial activities from the Mediterranean sponges *Aplysina aerophoba* and *Aplysina cavernicola*. *FEMS Microbiology Ecology*, **35**, 305-312.
- Hentschel U., Hopke J., Horn M., Friedrich A.B., Wagner M., Hacker J. and Moore B.S. (2002) Molecular evidence for a uniform microbial community in sponges from different oceans. *Applied and Environmental Microbiology*, **68**, 4431-4440.

- Hentschel U., Usher K.M. and Taylor M.W. (2006) Marine sponges as microbial fermenters. *FEMS Microbiology Ecology*, **55**, 167-177.
- Hill M., Hill A., Lopez N. and Harriott O. (2006) Sponge-specific bacterial symbionts in the Caribbean sponge, *Chondrilla nucula* (Demospongiae, Chondrosida). *Marine Biology*, **148**, 1221-1230.
- Hill M.S. (1996) Symbiotic zooxanthellae enhance boring and growth rates of the tropical sponge *Anthosigmella varians* forma *varians*. *Marine Biology*, **125**, 649-654.
- Hinde R., Pironet F. and Borowitzka M.A. (1994) Isolation of *Oscillatoria spongelliae*, the filamentous cyanobacterial symbiont of the marine sponge *Dysidea herbacea*. *Marine Biology*, **119**, 99-104.
- Hoffmann F. (2003) Microbial sulfate reduction in the tissue of the cold-water sponge *Geodia barretti*. PhD Thesis. Georg-August-University of Göttingen, Germany
- Holmes B. and Blanch H. (2006) Genus-specific associations of marine sponges with group I crenarchaeotes. *Marine Biology*, 1-14.
- Hooper N.J.A. and van Soest R.W.M. (2002) *Systema Porifera: a guide to the classification of sponges*. Kluwer Academic/Plenum Publishers, New York: 1707pp
- Hugenholtz P., Goebel B.M. and Pace N.R. (1998) Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *Journal of Bacteriology*, **180**, 4765-4774.
- Hugenholtz P., Tyson G.W., Webb R.I., Wagner A.M. and Blackall L.L. (2001) Investigation of candidate division TM7, a recently recognized major lineage of the domain bacteria with no known pure-culture representatives. *Applied and Environmental Microbiology*, **67**, 411-419.
- Hurlbert S. (1971) The nonconcept of species diversity: a critique and alternative parameters. *Ecology*, **52**, 577-586.
- Imhoff J.F. and Trüper H.G. (1976) Marine sponges as habitats of anaerobic phototrophic bacteria. *Microbial Ecology*, **3**, 1-9.
- Imhoff J.F. and Stöhr R. (2003) Sponge-associated bacteria: general overview and special aspects of bacteria associated with *Halichondria panicea*. In: W.E.G. Müller (Eds). *Sponges (Porifera)*. Springer Verlag, Berlin Heidelberg: 35-56.
- Jayatilake G.S., Thornton M.P., Leonard A.C., Grimwade J.E. and Baker B.J. (1996) Metabolites from an Antarctic sponge-associated bacterium, *Pseudomonas aeruginosa*. *Journal of Natural Products*, **59**, 293-296.
- Jha R.K. and Zi-rong X. (2004) Biomedical compounds from marine organisms. *Marine Drugs*, **2**, 123-146.
- Jurkevitch E. (2000) The genus *Bdellovibrio*. In: M. Dworkin, S. Flakow, E. Rosenberg, K.H. Schleifer and E. Stackebrandt (Eds). *The Prokaryotes*. Springer Verlag, New York: <http://141.150.157.117:8080/prokPUB/chaprender/jsp/showchap.jsp?chapnum=8327>.
- Kaestner A. (1980) *Lehrbuch der speziellen Zoologie*. Fischer Verlag, Jena: 318pp
- Kemp P.F. and Aller J.Y. (2004) Estimating prokaryotic diversity: when are 16S rDNA libraries large enough? *Limnology and Oceanography - Methods*, **2**, 114-125.

- Kim T.K., Garson M.J. and Fuerst J.A. (2005) Marine actinomycetes related to the *Salinospora* group from the Great Barrier Reef sponge *Pseudoceratina clavata*. *Environmental Microbiology*, **7**, 509-518.
- Klautau M., Russo C.A.M., Lazoski C., Boury-Esnault N., Thorpe J.P. and Sole-Cava A.M. (1999) Does cosmopolitanism result from overconservative systematics? A case study using the marine sponge *Chondrilla nucula*. *Evolution*, **53**, 1414-1422.
- Koellner T., Hersperger A.M. and Wohlgemuth T. (2004) Rarefaction method for assessing plant species diversity on a regional scale. *Ecography*, **27**, 532-544.
- Könneke M., Bernhard A.E., de la Torre J.R., Walker C.B., Waterbury J.B. and Stahl D.A. (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature*, **437**, 543-546.
- Kunzmann K. (1996) Associated fauna of selected sponges (Hexactinellida and Demospongiae) from the Weddel Sea, Antarctica. *Reports on Polar Research*. Alfred-Wegener-Institut für Polar- und Meeresforschung, Bremerhafen, Germany: 93pp
- Kuramoto M., Arimoto H. and Uemura D. (2004) Bioactive alkaloids from the sea: a review. *Marine Drugs*, **2**, 39-54.
- Kuzmanovic (1985) Preliminarna istrazivanja dinamike vodenih masa Limskog Kanala (Završni Izvještaj). Rep. "Institut Ruder Boskovic", Rovinj, Croatia,
- Lafi F., Garson M. and Fuerst J. (2005) Culturable bacterial symbionts isolated from two distinct sponge species (*Pseudoceratina clavata* and *Rhabdastrella globostellata*) from the Great Barrier Reef display similar phylogenetic diversity. *Microbial Ecology*, **50**, 213-220.
- Lane D.L. (1991) 16S/23S rRNA sequencing. In: E. Stackebrandt and M. Goodfellow (Eds). *Nucleic acid techniques in bacterial systematics*. John Wiley & Sons, New York: 115-175.
- Lang S., Beil W., Tokuda H., Wicke C. and Lurtz V. (2004) Improved production of bioactive glucosylmannosyl-glycerolipid by sponge-associated *Microbacterium* species. *Marine Biotechnology*, **6**, 152-156.
- Lee E.Y., Lee H.K., Lee Y.K., Sim C.J. and Lee J.H. (2003) Diversity of symbiotic archaeal communities in marine sponges from Korea. *Biomolecular Engineering*, **20**, 299-304.
- Levantesi C., Beimfohr C., Geurkink B., Rossetti S., Thelen K., Krooneman J., Snaidr J., van der Waarde J. and Tandoi V. (2004) Filamentous *Alphaproteobacteria* associated with bulking in industrial wastewater treatment plants. *Systematic and Applied Microbiology*, **27**, 716-727.
- Levi C. and Levi P. (1976) Embryogenesis of *Chondrosia reniformis* nardo - oviparous demosponge - transmission of symbiotic bacteria. *Annales des Sciences Naturelles - Zoologie et Biologie Animale*, **18**, 367-380.
- Li C.W., Chen J.Y. and Hua T.E. (1998) Precambrian sponges with cellular structures. *Science*, **279**, 879-882.
- Lopez J.V., McCarthy P.J., Janda K.E., Willoughby R. and Pomponi S.A. (1999) Molecular techniques reveal wide phyletic diversity of heterotrophic microbes

- associated with *Discodermia* spp. (Porifera: Demospongiae). *Memoirs of the Queensland Museum*, **44**, 329-341.
- Lopez-Garcia P., Duperron S., Philippot P., Foriel J., Susini J. and Moreira D. (2003) Bacterial diversity in hydrothermal sediment and epsilonproteobacterial dominance in experimental microcolonizers at the Mid-Atlantic Ridge. *Environmental Microbiology*, **5**, 961-976.
- Ludwig W., Strunk O., Westram R., Richter L., Meier H., Yadhukumar, Buchner A., Lai T., Steppi S., Jobb G., Forster W., Brettske I., Gerber S., Ginhart A.W., Gross O., Grumann S., Hermann S., Jost R., Konig A., Liss T., Lussmann R., May M., Nonhoff B., Reichel B., Strehlow R., Stamatakis A., Stuckmann N., Vilbig A., Lenke M., Ludwig T., Bode A. and Schleifer K.H. (2004) ARB: a software environment for sequence data. *Nucleic Acids Research*, **32**, 1363-1371.
- Maidak B.L., Cole J.R., Parker C.T., Garrity G.M., Larsen N., Li B., Lilburn T.G., McCaughey M.J., Olsen G.J., Overbeek R., Pramanik S., Schmidt T.M., Tiedje J.M. and Woese C.R. (1999) A new version of the RDP (Ribosomal Database Project). *Nucleic Acids Research*, **27**, 171-173.
- Maldonado M., Cortadellas N., Trillas M.I. and Rutzler K. (2005) Endosymbiotic yeast maternally transmitted in a marine sponge. *Biological Bulletin*, **209**, 94-106.
- Manz W., Arp G., Schumann-Kindel G., Szewzyk U. and Reitner J. (2000) Widefield deconvolution epifluorescence microscopy combined with fluorescence in situ hybridization reveals the spatial arrangement of bacteria in sponge tissue. *Journal of Microbiological Methods*, **40**, 125-134.
- Margot H., Acebal C., Toril E., Amils R. and Puentes J.L.F. (2002) Consistent association of crenarchaeal *Archaea* with sponges of the genus *Axinella*. *Marine Biology*, **140**, 739-745.
- McCaffrey M.A., Moldowan J.M., Lipton P.A., Summons R.E., Peters K.E., Jeganathan A. and Watt D.S. (1994) Paleoenvironmental implications of novel C-30 steranes in precambrian to cenozoic age petroleum and bitumen. *Geochimica Et Cosmochimica Acta*, **58**, 529-532.
- Methe B.A., Hiorns W.D. and Zehr J.P. (1998) Contrasts between marine and freshwater bacterial community composition: analyses of communities in Lake George and six other Adirondack lakes. *Limnology and Oceanography*, **43**, 368-374.
- Milanese M., Chelossi E., Manconi R., Sará A., Sidri M. and Pronzato R. (2003) The marine sponge *Chondrilla nucula* Schmidt, 1862 as an elective candidate for bioremediation in integrated aquaculture. *Biomolecular Engineering*, **20**, 363-368.
- Millot C. (1999) Circulation in the Western Mediterranean Sea. *Journal of Marine Systems*, **20**, 423-442.
- Mitova M., Tommonaro G., Hentschel U., Muller W.E.G. and De Rosa S. (2004) Exocellular cyclic dipeptides from a *Ruegeria* strain associated with cell cultures of *Suberites domuncula*. *Marine Biotechnology*, **6**, 95-103.
- Moldowan J.M. (1994) Molecular fossil evidence for late proterozoic-early paleozoic environments. *Terra Nova Abstract Supplement*, **3**, 4.
- Montalvo N.F., Mohamed N.M., Enticknap J.J. and Hill R.T. (2005) Novel actinobacteria from marine sponges. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology*, **87**, 29-36.

- Müller W.E.G., Zahn R.K., Kurelec B. and Müller I. (1984) A catalogue of the sponges near Rovinj. *Thalassia Jugoslavika*, **20**, 13-23.
- Müller W.E.G., Grebenjuk V.A., Thakur N.L., Thakur A.N., Batel R., Krasko A., Muller I.M. and Breter H.J. (2004) Oxygen-controlled bacterial growth in the sponge *Suberites domuncula*: toward a molecular understanding of the symbiotic relationships between sponge and bacteria. *Applied and Environmental Microbiology*, **70**, 2332-2341.
- Munro M.H.G., Blunt J.W., Dumdei E.J., Hickford S.J.H., Lill R.E., Li S.X., Battershill C.N. and Duckworth A.R. (1999) The discovery and development of marine compounds with pharmaceutical potential. *Journal of Biotechnology*, **70**, 15-25.
- Muscatine L., Karakashian S.J. and Karakashian M.W. (1967) Soluble extracellular products of algae symbiotic with a ciliate, a sponge and a mutant hydroid. *Comparative Biochemistry and Physiology*, **20**, 1-12.
- Muyzer G., Dewaal E.C. and Uitterlinden A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S ribosomal RNA. *Applied and Environmental Microbiology*, **59**, 695-700.
- Novosel M., Bakran-Petricioli T., Pozar-Domac A., Kruzic P. and Radic I. (2002) The benthos of the northern part of the Velebit Channel (Adriatic Sea, Croatia). *Natura Croatica*, **11**, 387-409.
- Oclarit J.M., Okada H., Ohta S., Kaminura K., Yamaoka Y., Iizuka T., Miyashiro S. and Ikegami S. (1994) Anti-bacillus substance in the marine sponge, *Hyatella* species produced by an associated *Vibrio* species bacterium. *Microbios*, **78**, 7-16.
- Olson J.B., Lord C.C. and McCarthy P.J. (2000) Improved recoverability of microbial colonies from marine sponge samples. *Microbial Ecology*, **40**, 139-147.
- Olson J.B., Harmody D.K. and McCarthy P.J. (2002) *Alphaproteobacteria* cultivated from marine sponges display branching rod morphology. *FEMS Microbiology Letters*, **211**, 169-173.
- Olson J.B. and McCarthy P.J. (2005) Associated bacterial communities of two deep-water sponges. *Aquatic Microbial Ecology*, **39**, 47-55.
- Opric M.M., Poznanovic S., Kljajic Z., Sladic D., Pupic G., Perunovic B. and Gasic M.J. (1996) Labelling of breast carcinoma, thyroid carcinoma and melanoma with manno- and galacto-specific lectins from marine invertebrates. *European Journal of Histochemistry*, **40**, 211-218.
- Osinga R., Tramper J. and Wijffels R.H. (1999) Cultivation of marine sponges. *Marine Biotechnology*, **1**, 509-532.
- Perovic S., Wichels A., Schutt C., Gerds G., Pahler S., Steffen R. and Muller W.E.G. (1998) Neuroactive compounds produced by bacteria from the marine sponge *Halichondria panicea*: activation of the neuronal NMDA receptor. *Environmental Toxicology and Pharmacology*, **6**, 125-133.
- Petri R. and Imhoff J.F. (2001) Genetic analysis of sea-ice bacterial communities of the Western Baltic Sea using an improved double gradient method. *Polar Biology*, **24**, 252-257.

- Pile A.J., Patterson M.R. and Witman J.D. (1996) *In situ* grazing on plankton <10 µm by the boreal sponge *Mycale lingua*. *Marine Ecology Progress Series*, **141**, 95-102.
- Preston C.M., Wu K.Y., Molinski T.F. and DeLong E.F. (1996) A psychrophilic crenarchaeon inhabits a marine sponge: *Cenarchaeum symbiosum* gen nov, sp, nov. *Proceedings of the National Academy of Sciences of the United States of America*, **93**, 6241-6246.
- Proksch P., Edrada R.A. and Ebel R. (2002) Drugs from the seas - current status and microbiological implications. *Applied Microbiology and Biotechnology*, **59**, 125-134.
- Pronzato R., Bavestrello G., Cerrano C., Magnino G., Manconi R., Pantelis J., Sará A. and Sidri M. (1999) Sponge farming in the Mediterranean Sea: new perspectives. *Memoirs of the Queensland Museum*, **44**, 485-491.
- Pronzato R. (2002) A climber sponge. *Bollettino dei Musei e degli Istituti Biologici dell'Università di Genova*, **66-67**, 159.
- Quaiser A., Ochsenreiter T., Lanz C., Schuster S.C., Treusch A.H., Eck J. and Schleper C. (2003) Acidobacteria form a coherent but highly diverse group within the bacterial domain: evidence from environmental genomics. *Molecular Microbiology*, **50**, 563-575.
- Rappe M.S., Kemp P.F. and Giovannoni S.J. (1997) Phylogenetic diversity of marine coastal picoplankton 16S rRNA genes cloned from the continental shelf off Cape Hatteras, North Carolina. *Limnology and Oceanography*, **42**, 811-826.
- Rasmont R. (1979) Les sponges: des metazoaires at des societes de cellules. In: C. Levi (Eds). *Biologie des spongiaeres*. Colloques Internationaux du Centre National de la Recherche Scientifique, Paris: 21-29.
- Reisser W. (1984) The taxonomy of green algae endosymbiotic in ciliates and a sponge. *British Phycological Journal*, **19**, 309-318.
- Reiswig H.M. (1973) Water transport, respiration and energetics of 3 tropical marine sponges. *Journal of Experimental Marine Biology and Ecology*, **14**, 231-249.
- Reiswig H.M. (1981) Partial carbon and energy budgets of the bacteriosponge *Verongia fistularis* (Porifera: Demospongiae) in Barbados. *Marine Ecology*, **2**, 273-293.
- Reitner J. and Mehl D. (1995) Early paleozoic diversification of sponges: new data and evidences. *Geologisch-paläontologische Mitteilungen, Innsbruck*, **20**, 335-347.
- Reitner J. and Mehl D. (1996) Monophyly of the Porifera. *Verhandlungen des naturwissenschaftlichen Vereins in Hamburg*, **36**, 5-32.
- Reitner J. and Wörheide G. (2002) Non-lithistid fossil demospongiae - origins of their palaeobiodiversity and highlights in history of preservation. In: J.N.A. Hooper and R.W.M. van Soest (Eds). *Systema Porifera*. Kluwer, New York: 52-68.
- Ridley C.P., Faulkner D.J. and Haygood M.G. (2005) Investigation of *Oscillatoria spongeliae*-dominated bacterial communities in four dictyoceratid sponges. *Applied and Environmental Microbiology*, **71**, 7366-7375.
- Rosell D. and Uriz M.J. (1992) Do associated *Zooxanthellae* and the nature of the substratum affect survival, attachment and growth of *Cliona viridis* (Porifera, Hadromerida) ? - an experimental approach. *Marine Biology*, **114**, 503-507.

- Rützler K. (1965) Substratstabilität im marinen Benthos als ökologischer Faktor, dargestellt am Beispiel adriatischer Porifera. *Internationale Revue der Gesamten Hydrobiologie*, **50**, 281-292.
- Rützler K. (1967) Liste und Verteilung der Poriferen aus der Umgebungs von Rovinj. *Thalassia Jugoslavika*, **3**, 79-87.
- Rützler K. (1990) Associations between Caribbean sponges and photosynthetic organisms. In: K. Rützler (Eds). *New perspectives in sponge biology*. Smithsonian Institute, Washington, DC: 455-471.
- Saller U. (1989) Microscopical aspects on symbiosis of *Spongilla lacustris* (Porifera, Spongillidae) and green algae. *Zoomorphology*, **108**, 291-296.
- Santavy D.L., Willenz P. and Colwell R.R. (1990) Phenotypic study of bacteria associated with the Caribbean sclerosponge, *Ceratoporella nicholsoni*. *Applied and Environmental Microbiology*, **56**, 1750-1762.
- Sará M. (1987) A study on the genus *Tethya* (Porifera Demospongiae) and new perspectives in sponge systematics. In: N. Boury-Esnault (Eds). *Taxonomy of Porifera*. Springer-Verlag, Berlin Heidelberg: 205-225.
- Sará M. (1971) Ultrastructural aspects of the symbiosis between two species of the genus *Aphanocapsa* (Cyanophyceae) and *Ircinia variabilis* (Demospongiae). *Marine Biology*, **11**, 214-221.
- Sará M. (1987) A study on the genus *Tethya* (Porifera Demospongiae) and new perspectives in sponge systematics. In: J. Vacelet and N. Boury-Esnault (Eds). *Taxonomy of Porifera*. Springer, Berlin: 205-225.
- Schirmer A., Gadkari R., Reeves C.D., Ibrahim F., DeLong E.F. and Hutchinson C.R. (2005) Metagenomic analysis reveals diverse polyketide synthase gene clusters in microorganisms associated with the marine sponge *Discodermia dissoluta*. *Applied and Environmental Microbiology*, **71**, 4840-4849.
- Schleper C., Jurgens G. and Jonscheit M. (2005) Genomic studies of uncultivated archaea. *Nature Reviews Microbiology*, **3**, 479-488.
- Schmidt E.W., Obratsova A.Y., Davidson S.K., Faulkner D.J. and Haygood M.G. (2000) Identification of the antifungal peptide-containing symbiont of the marine sponge *Theonella swinhoei* as a novel delta-proteobacterium, "Candidatus *Entotheonella palauensis*". *Marine Biology*, **136**, 969-977.
- Schmidt O. (1862) *Die Spongien des Adriatischen Meeres*. Verlag Wilhelm Engelmann, Leipzig: 88pp
- Schmitz F.J. (1994) Cytotoxic compounds from sponges and associated microfauna. In: R.W.M. van Soest (Eds). *Sponges in time and space*. Rotterdam: 485-498.
- Schröder H.C., Kljajic Z., Weiler B.E., Gasic M., Uhlenbruck G., Kurelec B. and Müller W.E.G. (1990) The galactose-specific lectin from the sponge *Chondrilla nucula* displays anti-human immunodeficiency virus activity *in vitro* via stimulation of the (2'-5')oligoadenylate metabolism. *Antiviral Chemistry & Chemotherapy*, **1**, 99-105.

- Schutze J., Krasko A., Custodio M.R., Efremova S.M., Muller I.M. and Muller W.E.G. (1999) Evolutionary relationships of Metazoa within the eukaryotes based on molecular data from Porifera. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **266**, 63-73.
- Schweitzer B., Huber I., Amann R., Ludwig W. and Simon M. (2001) *Alpha*- and *Betaproteobacteria* control the consumption and release of amino acids on lake snow aggregates. *Applied and Environmental Microbiology*, **67**, 632-645.
- Sepcic K., Batista U., Vacelet J., Macek P. and Turk T. (1997) Biological activities of aqueous extracts from marine sponges and cytotoxic effects of 3-alkylpyridinium polymers from *Reniera Sarai*. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology*, **117**, 47-53.
- Sfanos K., Harmody D., Dang P., Ledger A., Pomponi S., McCarthy P. and Lopez J. (2005) A molecular systematic survey of cultured microbial associates of deep-water marine invertebrates. *Systematic and Applied Microbiology*, **28**, 242-264.
- Shieh W.Y. and Lin Y.M. (1994) Association of heterotrophic nitrogen-fixing bacteria with a marine sponge of *Halichondria* sp. *Bulletin of Marine Science*, **54**, 557-564.
- Shigemori H., Bae M.A., Yazawa K., Sasaki T. and Kobayashi J. (1992) Alteramide-a, a new tetracyclic alkaloid from a bacterium *Alteromonas* sp. associated with the marine sponge *Halichondria okadaei*. *Journal of Organic Chemistry*, **57**, 4317-4320.
- Sidri M. (2004) *Chondrilla nucula* (Porifera, Demospongiae): an example of successful plasticity. Ecological and morphological aspects. PhD Thesis. University of Stuttgart, Germany
- Sidri M., Milanese M. and Bummer F. (2005) First observations on egg release in the oviparous sponge *Chondrilla nucula* (Demospongiae, Chondrosida, Chondrillidae) in the Mediterranean Sea. *Invertebrate Biology*, **124**, 91-97.
- Simpson T.L. (1984) *The cell biology of sponges*. Springer Verlag, New York: 662pp
- Sipkema D., Franssen M.C.R., Osinga R., Tramper J. and Wijffels R.H. (2005) Marine sponges as pharmacy. *Marine Biotechnology*, **7**, 142-162.
- Steindler L., Huchon D., Avni A. and Ilan M. (2005) 16S rRNA phylogeny of sponge-associated cyanobacteria. *Applied and Environmental Microbiology*, **71**, 4127-4131.
- Stierle A.C., Cardellina J.H. and Singleton F.L. (1988) A marine *Micrococcus* produces metabolites ascribed to the sponge *Tedania ignis*. *Experientia*, **44**, 1021.
- Suzuki M.T., Beja O., Taylor L.T. and DeLong E.F. (2001) Phylogenetic analysis of ribosomal RNA operons from uncultivated coastal marine bacterioplankton. *Environmental Microbiology*, **3**, 323-331.
- Tachibana K., Scheuer P.J., Tsukitani Y., Kikuchi H., Vanengen D., Clardy J., Gopichand Y. and Schmitz F.J. (1981) Okadaic acid, a cyto-toxic polyether from 2 marine sponges of the genus *Halichondria*. *Journal of the American Chemical Society*, **103**, 2469-2471.
- Taylor M.W., Schupp P.J., Dahllorf I., Kjelleberg S. and Steinberg P.D. (2004) Host specificity in marine sponge-associated bacteria, and potential implications for marine microbial diversity. *Environmental Microbiology*, **6**, 121-130.

- Taylor M.W., Schupp P.J., de Nys R., Kjelleberg S. and Steinberg P.D. (2005) Biogeography of bacteria associated with the marine sponge *Cymbastela concentrica*. *Environmental Microbiology*, **7**, 419-433.
- Thakur A.N., Thakur N.L., Indap M.M., Pandit R.A., Datar V.V. and Muller W.E.G. (2005) Antiangiogenic, antimicrobial, and cytotoxic potential of sponge-associated bacteria. *Marine Biotechnology*, **7**, 245-252.
- Thakur N.L., Hentschel U., Krasko A., Pabel C.T., Anil A.C. and Muller W.E.G. (2003) Antibacterial activity of the sponge *Suberites domuncula* and its primmorphs: potential basis for epibacterial chemical defense. *Aquatic Microbial Ecology*, **31**, 77-83.
- Thiel V. and Imhoff J.F. (2003) Phylogenetic identification of bacteria with antimicrobial activities isolated from Mediterranean sponges. *Biomolecular Engineering*, **20**, 421-423.
- Thiel V., Neulinger S.C., Staufenberger T., Schmaljohann R. and Imhoff J.F. (in press) Spatial distribution of sponge-associated bacteria in the marine sponge *Tethya aurantium*. *FEMS Microbiology Ecology*,
- Thompson J.E. (1985) Exudation of biologically-active metabolites in the sponge *Aplysina fistularis* .1. Biological Evidence. *Marine Biology*, **88**, 23-26.
- Thoms C., Horn M., Wagner M., Hentschel U. and Proksch P. (2003) Monitoring microbial diversity and natural product profiles of the sponge *Aplysina cavernicola* following transplantation. *Marine Biology*, **142**, 685-692.
- Tuma R.S., Beaudet M.P., Jin X.K., Jones L.J., Cheung C.Y., Yue S. and Singer V.L. (1999) Characterization of SYBR gold nucleic acid gel stain: a dye optimized for use with 300-nm ultraviolet transilluminators. *Analytical Biochemistry*, **268**, 278-288.
- Unson M.D. and Faulkner D.J. (1993) Cyanobacterial symbiont biosynthesis of chlorinated metabolites from *Dysidea herbacea* (Porifera). *Experientia*, **49**, 349-353.
- Unson M.D., Holland N.D. and Faulkner D.J. (1994) A brominated secondary metabolite synthesized by the cyanobacterial symbiont of a marine sponge and accumulation of the crystalline metabolite in the sponge tissue. *Marine Biology*, **119**, 1-11.
- Usher K.M., Kuo J., Fromont J. and Sutton D.C. (2001) Vertical transmission of cyanobacterial symbionts in the marine sponge *Chondrilla australiensis* (Demospongiae). *Hydrobiologia*, **461**, 9-13.
- Usher K.M., Fromont J., Sutton D.C. and Toze S. (2004a) The biogeography and phylogeny of unicellular cyanobacterial symbionts in sponges from Australia and the Mediterranean. *Microbial Ecology*, **48**, 167-177.
- Usher K.M., Sutton D.C., Toze S., Kuo J. and Fromont J. (2004b) Biogeography and phylogeny of *Chondrilla* species (Demospongiae) in Australia. *Marine Ecology Progress Series*, **270**, 117-127.
- Usher K.M., Toze S., Fromont J., Kuo J. and Sutton D.C. (2004c) A new species of cyanobacterial symbiont from the marine sponge *Chondrilla nucula*. *Symbiosis*, **36**, 183-192.
- Vacelet J. (1970) Description de cellules a bactéries intranucléaires chez des éponges *Verongia*. *Journal de Microscopie*, **9**, 333-346.

- Vacelet J. (1971) Étude en microscopie électronique de l'association entre une cyanophycée chroococcale et une éponge du genre *Verongia*. *Journal de Microscopie*, **12**, 363-380.
- Vacelet J. (1975) Étude en microscopie électronique de l'association entre bactéries et spongiaires du genre *Verongia* (Dictyoceratida). *Journal de Microscopie et de Biologie Cellulaire*, **23**, 271-288.
- Vacelet J. and Donadey C. (1977) Electron microscope study of the association between some sponges and bacteria. *Journal of Experimental Marine Biology and Ecology*, **30**, 301-314.
- Vacelet J. (1982) Algal-sponge symbioses in the coral reefs of New Caledonia: a morphological study. *Proceedings of the 4th International Coral Reef Symposium*, **2**, 713-719.
- Vacelet J., Boury-Esnault N., Fiala-Medioni A. and Fisher C.R. (1995) A methanotrophic carnivorous sponge. *Nature*, **377**, 296-296.
- Vacelet J., Fiala-Medioni A., Fisher C.R. and Boury-Esnault N. (1996) Symbiosis between methane-oxidizing bacteria and a deep-sea carnivorous cladorhizid sponge. *Marine Ecology Progress Series*, **145**, 77-85.
- van Soest R.W.M. (1987) Phylogenetic exercises with monophyletic groups of sponges. In: J. Vacelet and N. Boury-Esnault (Eds). *Taxonomy of Porifera*. Springer, Berlin: 227-241.
- Vatova A. (1928) Compendio della flora e fauna del Mare Adriatico presso Rovigno. *Memoria Regio Comitato Talassografico Italiano*, **143**, 1-614.
- Venter J.C., Remington K., Heidelberg J.F., Halpern A.L., Rusch D., Eisen J.A., Wu D.Y., Paulsen I., Nelson K.E., Nelson W., Fouts D.E., Levy S., Knap A.H., Lomas M.W., Nealson K., White O., Peterson J., Hoffman J., Parsons R., Baden-Tillson H., Pfannkoch C., Rogers Y.H. and Smith H.O. (2004) Environmental genome shotgun sequencing of the Sargasso Sea. *Science*, **304**, 66-74.
- Vergin K.L., Urbach E., Stein J.L., DeLong E.F., Lanoil B.D. and Giovannoni S.J. (1998) Screening of a fosmid library of marine environmental genomic DNA fragments reveals four clones related to members of the order *Planctomycetales*. *Applied and Environmental Microbiology*, **64**, 3075-3078.
- Voytek M. and Ward B. (1995) Detection of ammonium-oxidizing bacteria of the beta-subclass of the class *Proteobacteria* in aquatic samples with the PCR. *Applied and Environmental Microbiology*, **61**, 1444-1450.
- Webster N.S. and Hill R.T. (2001) The culturable microbial community of the Great Barrier Reef sponge *Rhopaloeides odorabile* is dominated by an alphaproteobacterium. *Marine Biology*, **138**, 843-851.
- Webster N.S., Webb R.I., Ridd M.J., Hill R.T. and Negri A.P. (2001a) The effects of copper on the microbial community of a coral reef sponge. *Environmental Microbiology*, **3**, 19-31.
- Webster N.S., Wilson K.J., Blackall L.L. and Hill R.T. (2001b) Phylogenetic diversity of bacteria associated with the marine sponge *Rhopaloeides odorabile*. *Applied and Environmental Microbiology*, **67**, 434-444.

- Webster N.S., Negri A.P., Munro M.M.H.G. and Battershill C.N. (2004) Diverse microbial communities inhabit Antarctic sponges. *Environmental Microbiology*, **6**, 288-300.
- Wilkinson C.R. (1978a) Microbial Associations in Sponges. I. Ecology, Physiology and Microbial Populations of Reef Sponges. *Marine Biology*, **49**, 161-167.
- Wilkinson C.R. (1978b) Microbial Associations in Sponges. II. Numerical Analysis of Sponge and Water Bacterial Population. *Marine Biology*, **49**, 169-176.
- Wilkinson C.R. (1978c) Microbial associations in sponges. III. Ultrastructure of the *in-situ* association in coral reef sponges. *Marine Biology*, **1978**, 177-185.
- Wilkinson C.R. (1979) Bdellovibrio-like parasite of cyanobacteria symbiotic in marine sponges. *Archives of Microbiology*, **123**, 101-103.
- Wilkinson C.R. and Fay P. (1979) Nitrogen-fixation in coral-reef sponges with symbiotic cyanobacteria. *Nature*, **279**, 527-529.
- Wilkinson C.R. and Vacelet J. (1979) Transplantation of marine sponges to different conditions of light and current. *Journal of Experimental Marine Biology & Ecology*, **37(1)**, 91-104.
- Wilkinson C.R. (1980a) Cyanobacteria symbiotic in marine sponges. In: W. Schwemmler and H. Schenk (Eds). *Endocytobiology I: Endosymbiosis and Cell Biology*. Walter de Gruyter & Co, Berlin: 553-563.
- Wilkinson C.R. (1980b) Nutrient translocation from green algal symbionts to the fresh-water sponge *Ephydatia fluviatilis*. *Hydrobiologia*, **75**, 241-250.
- Wilkinson C.R. and Garrone R. (1980) Nutrition of marine sponges. Involvement of symbiotic bacteria in the uptake of dissolved carbon. In: D.C. Smith (Eds). *Nutrition in lower metazoan*. Oxford: 157-161.
- Wilkinson C.R., Nowak M., Austin B. and Colwell R.R. (1981) Specificity of bacterial symbionts in Mediterranean and Great Barrier reef sponges. *Microbial Ecology*, **7**, 13-21.
- Wilkinson C.R. (1983) Phylogeny of bacterial and cyanobacterial symbionts in marine sponges. In: W. Schwemmler and H.E.A. Schenk (Eds). *Endocytobiology II : Intracellular space as oligogenetic ecosystem*. Walter de Gruyter & Co, Berlin New York: 993-1002.
- Wilkinson C.R. (1984) Immunological evidence for the precambrian origin of bacterial symbioses in marine sponges. *Proceedings of the Royal Society of London. Series B: Biological Sciences (London)*, **220**, 509-517.
- Wilkinson C.R. (1987) Significance of microbial symbionts in sponge evolution and ecology. *Symbiosis*, **4**, 135-146.
- Wilkinson C.R. (1992) Symbiotic interactions between marine sponges and algae. In: W. Reisser (Eds). *Algae and Symbiosis: Plants, Animals, Fungi, Viruses. Interactions Explored*. Biopress LTD, England, 112-151.
- Woese C.R. (1987) Bacterial Evolution. *Microbial Reviews*, **51**, 221-271.
- Yakimov M.M., Cappello S., Crisafi E., Tursi A., Savini A., Corselli C., Scarfi S. and Giuliano L. (2006) Phylogenetic survey of metabolically active microbial communities associated with the deep-sea coral *Lophelia pertusa* from the Apulian plateau, Central Mediterranean Sea. *Deep-Sea Research Part I: Oceanographic Research Papers*, **53**, 62-75.

- Yasumoto T., Seino N., Murakami Y. and Murata M. (1987) Toxins produced by benthic dinoflagellates. *Biological Bulletin*, **172**, 128-131.
- Zavodnik D. (1971) Contribution to the dynamics of benthic communities in the region of Rovinj (Northern Adriatic). *Thalassia Jugoslavika*, **7**, 447-514.
- Zrzavy J., Mihulka S., Kepka P., Bezdek A. and Tietz D. (1998) Phylogeny of the Metazoa based on morphological and 18S ribosomal DNA evidence. *Cladistics - the International Journal of the Willi Hennig Society*, **14**, 249-285.

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INDIVIDUAL SCIENTIFIC CONTRIBUTION TO MULTIPLE-AUTHOR PUBLICATIONS

The chapters of this thesis are partly published (Chapter III), accepted for publication (Chapter II) or submitted (Chapters I and IV) papers of multiple authorships. This list serves as a clarification of personal contributions on each publication.

Chapter I: Sponge-specific bacterial associations in the Mediterranean sponge *Chondrilla nucula* (Demospongiae, Tetractinomorpha)

Sampling at the Adriatic Sea site and most of the experimental work was done by Vera Thiel under supervision of Johannes F. Imhoff. Sven Leininger contributed by sponge sampling at the Ligurian Sea site as well as part of the experimental work (16S rDNA cloning) of the Ligurian Sea sample under supervision of Franz Brümmer. REM-microcopy was done by Rolf Schmaljohann. Evaluation of data, phylogenetic analyses and preparation of the manuscript was done by Vera Thiel. All co-authors contributed to the manuscript by critical revision.

Chapter II: Spatial distribution of sponge-associated bacteria in the Mediterranean sponge *Tethya aurantium*

Sampling of the sponges was done by Vera Thiel, Sven C. Neuling and Tim Staufenberger. Experimental work was done by Vera Thiel and Tim Staufenberger under supervision of Johannes F. Imhoff. All microscopic work was done by Rolf Schmaljohann. Sven C. Neuling conducted statistical analyses of the clone libraries (rarefaction analyses). Evaluation of the data, phylogenetic analysis and preparation of the manuscript was done by Vera Thiel. All co-authors contributed by critical revision of the manuscript.

Chapter III: Phylogenetic identification of bacteria with antimicrobial activities isolated from Mediterranean sponges

Sampling, experimental work as well as evaluation of data, phylogenetic analysis and preparation of the manuscript was done by Vera Thiel under supervision of Johannes F. Imhoff.

Chapter IV: Abundance, bioactivity and ecological relevance of cultured sponge-associated bacteria from the Mediterranean Sea

Sampling was done by Vera Thiel, Sven C. Neulinger and Tim Staufenger. Experimental work was done by Albrecht Muscholl-Silberhorn and Vera Thiel. Phylogenetic analysis was done by Vera Thiel. Evaluation of the data and preparation of the manuscript was done by Albrecht Muscholl-Silberhorn in close collaboration with Vera Thiel. Johannes F. Imhoff contributed by critical revision of the manuscript.

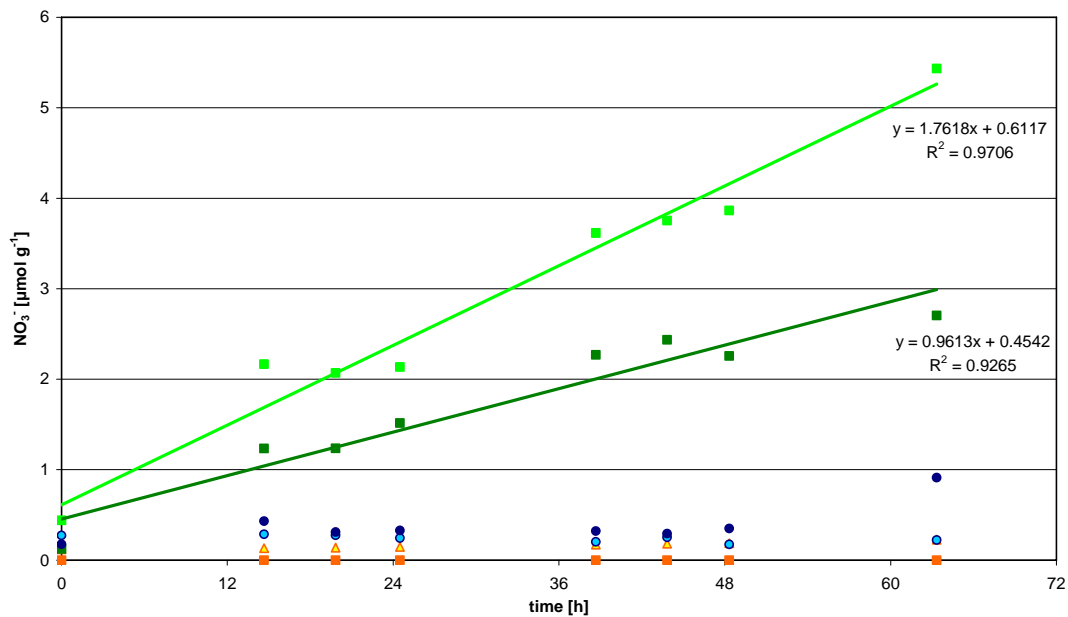
ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbstständig und ohne unerlaubte Hilfe angefertigt habe und dass sie nach Form und Inhalt meine eigene Arbeit ist. Sie wurde keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt. Dies ist mein einziges und bisher erstes Promotionsverfahren. Die Promotion soll im Fach Mikrobiologie erfolgen. Des Weiteren erkläre ich, dass ich Zuhörer bei der Disputation zulasse.

(Vera Thiel)

APPENDIX A

The marine sponge *Chondrilla nucula* has been shown to release great amounts of nitrate (Appendix A-1). Nitrification, the microbiological stepwise oxidation of ammonium to nitrate, is assumed to be the origin of the nitrate-production in the sponge (Corredor *et al.* 1988, Diaz and Ward 1997). Ammonia-oxidation, the first step in the biological process of nitrification, was long believed to be restricted to bacteria of the Beta- and Gamma- subdivision of the *Proteobacteria*. Only the very recent isolation of *Nitrosopumilus maritimus*, expanded the group of ammonia-oxidizing organisms to non-thermophilic archaea and correlated the ability of ammonia-oxidation with the presence of putative ammonium-mono-oxygenase (AMO) encoding genes (Könneke *et al.* 2005). Preliminary results indicate the association of a non-thermophilic archaeon with the marine sponge *C. nucula*, and the presence of archaeal *amoA* genes in the sponge (Appendix A-(2-5)). The sponge-associated archaeon is believed to be involved in the sponge mediated nitrification process.



Appendix A-1: Sponge mediated nitrate-production in *Chondrilla nucula* measured as nitrate in surrounding seawater [μmol] * g⁻¹ sponge biomass (ww). Sponge specimens, either detached from their natural substrate (● ■), or on their natural substrate, i.e., rock (○ □), each one parallel with (○ ●) or without (□ ■) addition of the nitrification inhibitor allythiourea (ATU). Seawater alone (■), as well as the sponge *Tethya* sp. (▲) served as control.

Appendix A-2: FASTA EMBL database search results for bacterial amoA sequence obtained from *Chondrilla nucula*

SUBMISSION PARAMETERS			
Title	CN AOB amoA AS	Database	uniprot
Sequence length	151	Sequence type	p
Program	fasta	Version	3.4t25 Sept 2, 2005
Expectation upper value	10	Matrix	BL50
Sequence range	1-	Number of scores	50
Number of alignments	50	Word size	2
Open gap penalty	-10	Gap extension penalty	-2

UNIPROT entry	Name	Length [AS]	Identity [%]	Similar [%]	Overlap [AS]	E()
UNIPROT:Q66V31_9PROT	Ammonia monooxygenase [Fragment]	162	96.667	99.333	150	8.3e-61
UNIPROT:Q66V28_9PROT	Ammonia monooxygenase [Fragment]	162	95.333	99.333	150	1.3e-60
UNIPROT:Q6V3K8_9PROT	Ammonia monooxygenase subunit A	150	95.333	99.333	150	1.7e-60
UNIPROT:Q66V37_9PROT	Ammonia monooxygenase [Fragment]	163	95.333	99.333	150	2.5e-60
UNIPROT:Q6V3L6_9PROT	Ammonia monooxygenase subunit A	150	95.333	99.333	150	2.8e-60
UNIPROT:Q6V3J6_9PROT	Ammonia monooxygenase subunit A	150	96.000	98.667	150	2.8e-60
UNIPROT:Q66UY5_9PROT	Ammonia monooxygenase [Fragment]	164	96.000	98.667	150	4.7e-60
UNIPROT:Q6V3J4_9PROT	Ammonia monooxygenase subunit A	150	96.000	98.667	150	6.1e-60
UNIPROT:Q5ICL7_9PROT	Ammonia monooxygenase subunit A	163	94.667	99.333	150	7.6e-60
UNIPROT:Q4VP17_9BACT	Ammonia monooxygenase [Fragment]	161	94.667	98.667	150	1e-59
UNIPROT:Q66V13_9PROT	Ammonia monooxygenase [Fragment]	163	94.667	98.667	150	1e-59
UNIPROT:Q66UX5_9PROT	Ammonia monooxygenase [Fragment]	163	94.667	98.667	150	1e-59
UNIPROT:Q6V3N2_9PROT	Ammonia monooxygenase subunit A	150	94.667	98.667	150	1.1e-59
UNIPROT:Q66V15_9PROT	Ammonia monooxygenase [Fragment]	162	94.667	98.667	150	1.2e-59
UNIPROT:Q66UX2_9PROT	Ammonia monooxygenase [Fragment]	163	94.667	98.667	150	1.2e-59
UNIPROT:Q66V21_9PROT	Ammonia monooxygenase [Fragment]	163	94.667	98.667	150	1.2e-59
UNIPROT:Q6V3K3_9PROT	Ammonia monooxygenase subunit A	150	94.000	98.667	150	1.3e-59
UNIPROT:Q5IFW0_9PROT	Ammonia monooxygenase subunit A	149	96.644	98.658	149	1.6e-59
UNIPROT:Q6V3D9_9PROT	Ammonia monooxygenase subunit A	150	94.000	98.667	150	2.1e-59

Appendix A-3: FASTA UNIPROT database search results for putative archaeal *amoA* aminoacid (AS) sequence obtained from *Chondrilla nucula*

SUBMISSION PARAMETERS			
Title	AOA amoA CN AS	Database	uniprot
Sequence length	203	Sequence type	p
Program	fasta	Version	3.4t25 Sept 2, 2005
Expectation upper value	10	Matrix	BL50
Sequence range	1-	Number of scores	50
Number of alignments	50	Word size	2
Open gap penalty	-10	Gap extension penalty	-2

UNIPROT entry	Name	Length [AS]	Identity [%]	Similar [%]	Overlap [AS]	E()
UNIPROT:Q2LDV9_9CREN	Putative ammonia monooxygenase subunit a	203	98.507	100.000	201	2.5e-79
UNIPROT:Q2LDW0_9CREN	Putative ammonia monooxygenase subunit a	204	98.507	100.000	201	2.5e-79
UNIPROT:Q3LZS6_9CREN	Putative ammonia monooxygenase subunit a	201	97.015	100.000	201	3.1e-78
UNIPROT:Q3I2S8_9CREN	Putative ammonia monooxygenase subunit a	202	97.980	100.000	198	7.6e-78
UNIPROT:Q3I2T0_9CREN	Putative ammonia monooxygenase subunit a	202	97.980	100.000	198	2.5e-77
UNIPROT:Q3I2S9_9CREN	Putative ammonia monooxygenase subunit a	202	97.475	100.000	198	2.9e-77
UNIPROT:Q3I2H1_9CREN	Putative ammonia monooxygenase subunit a	202	97.475	100.000	198	5.3e-77
UNIPROT:Q0QMD9_9CREN	Putative ammonia monooxygenase subunit a	202	97.475	100.000	198	5.3e-77
UNIPROT:Q3I245_9CREN	Putative ammonia monooxygenase subunit a	202	96.970	100.000	198	6.1e-77
UNIPROT:Q3I2R3_9CREN	Putative ammonia monooxygenase subunit a	202	97.475	100.000	198	7.1e-77
UNIPROT:Q3I2F2_9CREN	Putative ammonia monooxygenase subunit a	202	97.475	100.000	198	8.3e-77
UNIPROT:Q0QMD4_9CREN	Putative ammonia monooxygenase subunit a	202	97.475	99.495	198	8.3e-77
UNIPROT:Q0QMI4_9CREN	Putative ammonia monooxygenase subunit a	202	96.970	99.495	198	8.3e-77
UNIPROT:Q3I2S4_9CREN	Putative ammonia monooxygenase subunit a	202	96.465	100.000	198	1.3e-76
UNIPROT:Q3I2S7_9CREN	Putative ammonia monooxygenase subunit a	202	97.475	99.495	198	1.5e-76
UNIPROT:Q3I2T6_9CREN	Putative ammonia monooxygenase subunit a	202	96.465	99.495	198	1.5e-76
UNIPROT:Q0QME1_9CREN	Putative ammonia monooxygenase subunit a	202	96.465	100.000	198	1.5e-76

Appendix A-4: FASTA EMBL database search results for archaeal 16S rDNA sequence obtained from *Chondrilla nucula* (929nt)

SUBMISSION PARAMETERS			
Title	CN_SAA_E_16S	Database	empro
Sequence length	930	Sequence type	n
Program	fasta	Version	3.4t25 Sept 2, 2005
Expectation upper value	10	Sequence range	1-
Number of scores	50	Number of alignments	50
Word size	6	Open gap penalty	-14
Gap extension penalty	-4	Histogram	false

EMBL entry	Name	Length [nt]	Identity [%]	Similar [%]	Overlap [nt]	E()
EM PRO: DQ085097	Candidatus Nitrosopumilus maritimus	1650	95.160	95.160	909	1.1e-52
EM PRO: AF083071	Cenarchaeum symbiosum strain A hypothetical protein 01 gene, complete cds; 23S ribosomal RNA and 16S ribosomal RNA genes, complete sequence; glutamate-1-semialdehyde aminotransferase (gsat) and hypothetical protein 02 genes, complete cds	32998	91.694	91.694	927	7.6e-51
EM PRO: AF083072	Cenarchaeum symbiosum strain B histone H1 DNA binding protein (hc2), hypothetical protein, lysyl tRNA synthetase (syk), and hypothetical protein 01 genes, complete cds; 23S ribosomal RNA and 16S ribosomal RNA genes, complete sequence	42432	91.694	91.694	927	8.4e-51
EM PRO: U51469	Cenarchaeum symbiosum small subunit ribosomal RNA gene sequence	1474	91.667	91.667	924	1.7e-49
EM PRO: AF421159	Crenarchaeote symbiont of Axinella sp. 16S ribosomal RNA gene, partial sequence	1305	90.868	90.868	887	1.9e-45
EM PRO: AF420236	Crenarchaeote symbiont of Axinella damicornis 16S ribosomal RNA gene, partial sequence	1305	90.868	90.868	887	1.9e-45
EM PRO: AF420237	Crenarchaeote symbiont of Axinella verrucosa 16S ribosomal RNA gene, partial sequence	1286	91.696	91.696	867	5.7e-45

```

      10      20      30      40      50      60      70      80      90      100     110     120     130
C. nucula clone SAA-E  TTCCCGTTGATCCTGCCGGACCTGACTGCTATCGGATTGATACTAAGCCATGCGAGTCATTGTAGTAAACAAGGCAGACGGCTCAGTAAACGCGTAGTCAAACCTAACTATGGACGGGAATAACCTCGGGAAACTGA
C. symbiosum          .....TTGATCNTGCCGGACCTGACTGCTATCGGATTGATACTAAGCCATGCGAGTCATTGTAGCAATAACAAGGCAGACGGCTCAGTAAACGCGTAGTCAACTAACCTATGGACGGGATTAACCTCGGGAAACTGA
Axinella sp. symbiont .....ACTAAGCCATGCGAGTCATTGTAGCAATAACAAGGCAGACGGCTCAGTAAACGCGTAGTCAACCTAACCTTGACAGCGGGGATTAACCTCGGGAAACTGA
N. maritimus          .....ACCTGACTGCTATCGGATTGATACTAAGCCATGCGAGTCATTGTAGTAAACAAGGCAGACGGCTCAGTAAACGCGTAGTCAAACCTAACCTATGGACGGGAATAACCTCGGGAAACTGA

      160     170     180     190     200     210     220     230     240     250     260     270     280
C. nucula clone SAA-E  ATAGAACATATAGCCTGGAAATGGTTTATGTTTAAATGATTTATCGCCGTAGGATGGGACTCGGCCATCAGTTTGTTGGTGAGGTAATGGCCCAACAGACGATTAACAGGTACGGGCTCTGAGAGGAGTAGCCCGG
C. symbiosum          ATAGGCCACTATAGCCTGGAAATGGTTTGGGCCAAATGATTTATCGCCGTAGGATGGGACTCGGCTCTCAGCTTGTTGGTGAGGTAATGGCCCAACAGGCTATAACAGATACGGGCTCTGAGAGGAGAGGCCCGG
Axinella sp. symbiont ATAGGTCATGGTGCCTGGAAATGGCCATGGCTCAAATGATTTATCGCTGCAGGATGGGACTCGGCCATCAGCTTGTTGGTGAGGTAATGGCCCAACAGGCTATAACAGGTACGGGCTCTGAGAGGAGAGGCCCGG
N. maritimus          ATAGAACACTATACCTGGAAATGGTTTGTGTTCCAAATGATTTATCGCCGTAGGATGGGACTCGGCCATCAGTTTGTTGGTGAGGTAATGGCCCAACAGACTATAACAGGTACGGGCTCTGAGAGGAGTAGCCCGG

      310     320     330     340     350     360     370     380     390     400     410     420     430
C. nucula clone SAA-E  AGACACGGACCCAGGCCCTATGGGGCGCAGCAGGCGAGAAAACTTTGCAATGTGCGAAAGCAGCACAAGGTTAATCCGAGTGGTTTCTGATAAAGAGACCTTTTGTGGTCTTAATACACCGATGAATAAGGGGTGG
C. symbiosum          AGACACGGACCCAGGCCCTATGGGGCGCAGCAGGCGAGAAAACTTTGCAATGTGCGAAAGCAGCACAAGGTTAATCCGAGTGGTTTCTGCTAAAAGAAATCTTTTACCGGCTCTAAAACACCACCGGCAATAAGGGGTGG
Axinella sp. symbiont AGACACGGACCCAGGCCCTATGGGGCGCAGCAGGCGAGAAAACTTTGCAATGTGCGAAAGCAGCACAAGGTTAATCCGAGTGGTCTCTGCTAAAAGAGACCTTTTGTAGTCTTAAGACCTTTTGTAGTCTAAAAGCACTGGCGAATAAGGGGTGG
N. maritimus          AGACACGGACCCAGGCCCTATGGGGCGCAGCAGGCGAGAAAACTTTGCAATGTGCGAAAGCAGCACAAGGTTAATCCGAGTGGTTTGTCTAAAAGAACTTTTGTAGTCTTAAGACCTTTTGTGAATAAGGGGTGG

      460     470     480     490     500     510     520     530     540     550     560     570     580
C. nucula clone SAA-E  GTCAGCCGCCCGGTTAAAACCCAGCACCTCAAGTGGTCAGGATGATTATTGGGCCTAAAGCATCCGTAGCCGGCTCTGTAAGTTTTCCGGTTAAATCTGTATGCTCAACATACAGGCTGCCGGGAATACTGCAAGCTAG
C. symbiosum          GTCAGCCGCCCGGTTAAAACCCAGCACCTCAAGTGGTCAGGAGGATTAATTGGGCCTAAAGCATCCGTAGCCCTGCCGTGTAAGTTTTCCGGTTAAATCTATGCGCTCAACGCATAGGCTGCCGGAAATACTGCACGGCTAG
Axinella sp. symbiont GTACAGCCGCCCGGTTAAAACCCAGCACCTCAAGTGGTCAGGAGGATTAATTGGGCCTAAAGCATCCGTAGCCGGCCGTGTAAGTTTTCCGGTTAAATCCACGTGCTCAACACGTGGGCCCGGAAATCTGCACGGCTAG
N. maritimus          GTCAGCCGCCCGGTTAAAACCCAGCACCTCAAGTGGTCAGGATGATTATTGGGCCTAAAGCATCCGTAGCCGGCTCTGTAAGTTTTCCGGTTAAATCTGTACGCTCAACGTACAGGCTGCCGGGAATACTGCAGAGCTAG

      610     620     630     640     650     660     670     680     690     700     710     720     730
C. nucula clone SAA-E  GTAGACGGTACTCCGTAGGAAGGGGTAAAATCCTGTGATCTATTGATGACCCTGTGGCGAAGGCCGTTACTACAGAACACGTCCTCCGACGGTGAGGGATGAAAAGCTGGGGGAGCAAAACCGGATTAGATACCCGGGTAGT
C. symbiosum          GTAGACGGTACTCCGTAGGAAGGGGTAAAATCCTTTGATCTATCGATGACCCTGTGGCGAAGGCCGTTACTAGAACACGTCCTCCGACGGTGAGGGATGAAAAGCTGGGGGAGCAAAACCGGATTAGATACCCGGGTAGT
Axinella sp. symbiont GTAGACGGTACTCCGTAGGAAGGGGTAAAATCCTGTGATCTATTGATGACCCTGTGGCGAAGGCCGTTACTAGAACATGTCACGACGGTGAGGGATGAAAAGCTGGGGGAGCAAAACCGGATTAGATACCCGGGTAGT
N. maritimus          GTAGACGGTACTCCGTAGGAAGGGGTAAAATCCTTTGATCTATTGATGACCCTGTGGCGAAGGCCGTTACTACAGAACACGTCCTCCGACGGTGAGGGATGAAAAGCTGGGGGAGCAAAACCGGATTAGATACCCGGGTAGT

      760     770     780     790     800     810     820     830     840     850     860     870     880
C. nucula clone SAA-E  CTATGCAAACTCAATGATGCATTGAA-AGTTAGAAATTTCTAACTCGTGCAATGCAAGTGTTCAGGGAAGCCGTTAAGTTTGCCGCTGGGAAGTACGTACGCAAGTATGAAACTTAAAGGAATTGGCGGGGAGCAC
C. symbiosum          CAATGCAAACTCAGTGATGCATTGGTGAAGAC-----CAGTGCAGTGCCGACGGAAGCCGTTAAGTTTGCCGCTGGGAAGTACGTACGCAAGTATGAAACTTAAAGGAATTGGCGGGGAGCAC
Axinella sp. symbiont CTATGCAAACTCAGTGATGCATTGGC-C-TTT- GC-----CAGTGCAGTGTCGACGGAAGCCGTTAAGTTTGCCGCTGGGAAGTACGTACGCAAGTATGAAACTTAAAGGAATTGGCGGGGAGCAC
N. maritimus          CTATGCAAACTCAGTGATGCATTGGCTTGTGG-C-----CAATGCAAGTGTTCAGGGAAGCCGTTAAGTTTGCCGCTGGGAAGTACGTACGCAAGTATGAAACTTAAAGGAATTGGCGGGGAGCAC

      910     920
C. nucula clone SAA-E  AGCATGCGGTTCAATTGGAGTCAACGCC
C. symbiosum          AGC.TGCGGTTCAATTGGAGTCAACGCN
Axinella sp. symbiont AGCCTGCGGTTCAATTGGAGTCAACGCC
N. maritimus          AGCCTGCGGTTCAATTGGAGTCAACGCC

```

Appendix A-5: sequence alignment of archaeal 16S rDNA sequences obtained from the marine sponges *Chondrilla nucula* (clone SAA-E), *Axinella cf. mexicana* (*C. symbiosum*) and *Axinella* sp.(symbiont) as well as from the ammonium-oxidizing marine archaeon *Nitrosopumilus maritimus*.

APPENDIX B

List of presented posters

Part of this thesis has been presented as posters at following symposia:

- **Vera Thiel and Johannes F. Imhoff:** *Phylogenetic identification of bacteria with antimicrobial activities isolated from different Mediterranean sponges.* International Symposium 'Marine Biotechnology: Basics and Application'. February 2003 in Sevilla, Spain
- **Vera Thiel and Johannes F. Imhoff:** *Bacterial diversity in the marine sponge *Chondrilla nucula*.* Annual meeting of the 'Vereinigung für Allgemeine und Angewandte Mikrobiologie, VAAM', March 2004 in Braunschweig, Germany
- **Vera Thiel, Sven Leininger, Franz Brümmer and Johannes F. Imhoff:** *Sponge specific bacterial clusters in *Chondrilla nucula* from two different locations in the Mediterranean Sea.* 10th International Symposium on Microbial Ecology ISME-10, August 2004 in Cancún, Mexico
- **Vera Thiel, Tim Staufenberger and Johannes F. Imhoff:** *Spatial distribution of sponge associated bacteria within *Tethya aurantium*.* Annual meeting of the 'Vereinigung für Allgemeine und Angewandte Mikrobiologie, VAAM', March 2006 in Jena, Germany
- **Vera Thiel, Johannes F. Imhoff:** *Sponge-associated bacterial diversity differs significantly between sponge species at the same location in the Adriatic Sea.* 11th International Symposium on Microbial Ecology ISME-11. August 2006 in Vienna, Austria



Phylogenetic identification of bacteria with antimicrobial activities isolated from different Mediterranean sponges

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Fig. 1: *Acanthella acuta*

Introduction

Sponges are known to be a rich source of bioactive secondary products of biotechnological interest, showing antiviral, antitumor, antimicrobial or general cytotoxic properties. Some of the isolated substances from sponges have striking structural similarities to metabolites of microbial origin [1, 2]. Accumulated evidence suggests that microorganisms could well be the true source of these metabolites. This offers the possibility to use not the sponge but the bacteria associated to the sponge for production of biological active substances. Because bacteria quite rapidly produce high amounts of biomass, biological active secondary products can easily be produced in large amounts on a biotechnological scale



Fig. 2: Schwamm-Gemeinschaft mit *Ircinia spec.*

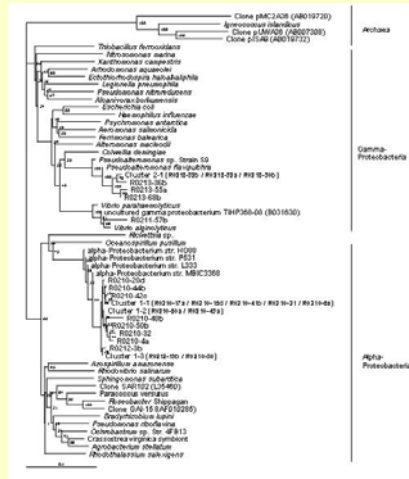


Figure 3. Phylogenetic tree of representative α - and γ -Proteobacteria based on 16S rDNA sequences, including sequences of new isolates of sponges from Mediterranean habitats.

Approach

The aim of this study was to isolate and characterize bacteria with antimicrobial activities from various marine sponges. Different sponge species were collected from coastal habitats of the Mediterranean Sea by SCUBA diving. To test for antimicrobial activity in these sponges, sponge pieces were applied to agar media inoculated with different indicator organisms and incubated for 24 hours at room temperature. Specimen of *Halichondria panicea*, *Ircinia fasciculata*, *Axinnella polypoides*, and *Acanthella spec.* showed activity against at least two out of five test organisms and were chosen for isolation studies. Additionally, a *Suberites domuncula* specimen was chosen. Samples of the sponges were homogenized and diluted in sterile seawater and plated onto fifteen different agar media, varying in salinity, nutrient composition and concentration. The agar plates were incubated at room temperature until visible colonies had formed and then were subjected to tests for antibiotic activities. Bacterial strains showing activity against at least one of the indicator organisms were isolated and further investigated. Isolates with biological activity were identified on the basis of 16S rDNA gene sequences. The phylogenetic tree was generated using the neighbor joining method with 100 resamplings in bootstrap analysis.



Fig. 4: *Halichondria panicea*



Fig. 5: Inhibition zone

Results and discussion

Most of the isolates with antimicrobial activity were affiliated with distinct eubacterial groups within the α - and γ -Proteobacteria (Figure 3). Bacteria of other phylogenetic groups including Gram-positive bacteria were not obtained in this study. Thus, the phylogenetic diversity of the obtained antimicrobial active isolates was rather limited, even though a number of different media were used for their isolation. Similar observations were made by Hentschel et al. (2001). In particular one group of closely related strains was dominant. Comparison of 16S rDNA sequences using the BLAST method showed highest sequence similarities for one of the clusters to unnamed α -Proteobacteria (approx. 98% to strain MBI3368) previously isolated from sponges by other research groups [3, 4]. Similarities to other described α -Proteobacteria is well below 90%. A second group of strains was affiliated with the γ -Proteobacteria, marine *Vibrio* and *Pseudomonas* strains, respectively. Quite interestingly, the groups of α - and γ -Proteobacteria were not found within one and the same sponge. All biological active isolates from *Halichondria panicea* and *Axinnella polypoides* were related to the α -Proteobacteria, but none to the γ -Proteobacteria. The isolates from *Acanthella spec.*, *Suberites domuncula* and *Ircinia fasciculata* belong to the γ -subgroup of the Proteobacteria. A relation between the antimicrobial activity of the isolates and the phylogenetic relationship was observed indicating a high inhibition of closely related strains.

References

- [1] Proksch P, Edrada RA, Ebel R. Appl Microbiol Biotechnol 2002; 59:125-134.
- [2] Imhoff JF and Stöhr R. In Müller WEG, editor. Molecular Marine Biotechnology, Vol. 1, Springer, 2003; (in press).
- [3] Hentschel U, Schmid M, Wagner M, Fieseler L, Gernert C, Hacker J. FEMS Microbiology Ecology 2001; 35:305-312.
- [4] Olson JB, Harmody DK, McCarthy PJ. FEMS Microbiol Lett. 2002; 211:169-73.



Acknowledgements

We gratefully acknowledge help of R. Batel and the personnel at the Ruder Boskovic Institut (Rovinj, Croatia) during sampling of the sponges. We thank F. Brümmer and W. Zucht for identification of the sponges. This work was supported by the bmb+f as part of the Kompetenzzentrum BIOTECmarin (grant no. 03F0345B)

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Bacterial diversity in the marine sponge *Chondrilla nucula*

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Abstract

Sponges are known to contain large numbers of bacteria located mostly extracellular enclosed within the mesohyl. It is assumed that symbiotic interactions exist between sponges and microorganisms and sponge-specific phylogenetic clusters have been found [1]. Even though symbiotic cyanobacteria have been reported for *Chondrilla australiensis* [2] the bacterial community composition has not been studied for this species.

In the presented study the bacterial community associated to the Mediterranean sponge *Chondrilla nucula* was examined by DGGE and 16S rDNA cloning experiments. 37 clone sequences belonging to 14 different phylogenetic clusters have been obtained. Clone sequences were compared with sponge derived sequences available in the databases from other studies. Sequences belonging to different subgroups of the *Proteobacteria*, the *Chloroflexi*, *Actinobacteria* and *Bacteroidetes* as well as an undefined sponge-associated group were found. Within these groups a clustering with other sponge-derived sequences was observed and it is concluded that specific sponge-associated bacterial groups exist.

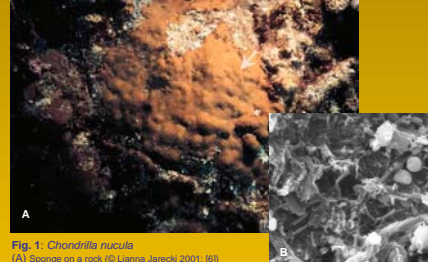


Fig. 1: *Chondrilla nucula*
(A) Sponge on a rock (© Lienna Jarecki 2001; [6])
(B) REM picture of mesohyl with bacteria (Dr. Rolf Schmaljohann; IFM-GEOMAR)

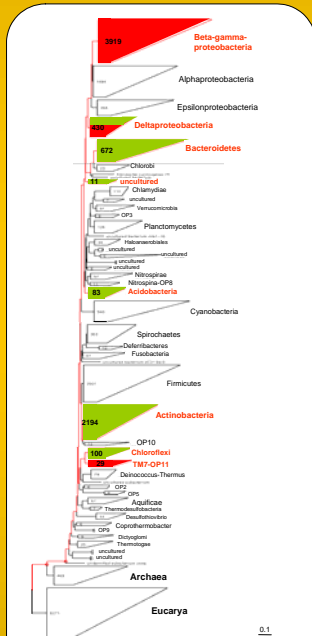


Fig. 2: Phylogenetic rDNA tree (Max. Parsimony, ARB version Beta 2003_08_22). Colored groups include phylogenetic cluster of sponge-derived 16S rDNA sequences from *C. nucula* only (red) or *C. nucula* sequences clustering with sequences obtained from other sponges (green)

Results

A high number of bands were discovered in DGGE gels (data not shown). 37 16S rDNA clone sequences belonging to 14 different phylogenetic clusters (Fig. 2) were recovered from the cloning experiment. Additional sponge-derived 16S rDNA sequences from *Aplysina aerophoba*, *Theonella swinhoei* and *Rhopaloides odorabile* [1] were included into the phylogenetic analysis for comparison. Figure 2 provides an overview of the phylogenetic positions of the clone sequences obtained from the Mediterranean *Chondrilla nucula* specimen in this study.

The majority of the *C. nucula* derived sequences belonged to the *Gammaproteobacteria* (n=16; 43%). Sequences affiliated with the *Deltaproteobacteria* (n=7; 19%), the *Bacteroidetes* (n=4; 11%), the *Acidobacteria* (n=2; 5%), *Actinobacteria* (n=2; 5%), the *Chloroflexi* (n=2; 5%), the TM7-OP11 group (n=3; 8%) and a group of uncultured bacteria with uncertain affiliation (n=2; 5%) were also abundant. Within these groups a clustering with other sponge-derived sequences was observed in some but not all cases.

Clustering of clones with other sponge-derived sequences was observed for 6 (*Rhodothermus*, *Chloroflexi*, *Acidobacteriaceae*, *Acidimicrobiaceae* (Fig. 4), *Deltaproteobacteria* and an uncultured group) of the 14 phylogenetic clusters. Sequences not clustering with other sponge-derived sequences belonged to phylogenetic groups of *Gammaproteobacteria*, *Chloroflexi*, TM7-OP11, *Deltaproteobacteria* (Fig. 3) and the *Flavobacteria*. From this data together with former publications it can be concluded that specific sponge-associated bacterial groups exist. Studies to determine whether the phylogenetic clusters not yet associated with bacteria in sponges are also found in other sponge specimens are in progress

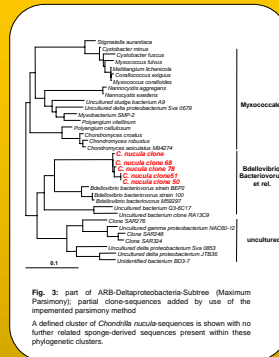


Fig. 3: part of ARB-Deltaproteobacteria-Subtree (Maximum Parsimony), partial clone-sequences added by use of the implemented parsimony method
A defined cluster of *Chondrilla nucula*-sequences is shown with no further related sponge-derived sequences present within these phylogenetic clusters.

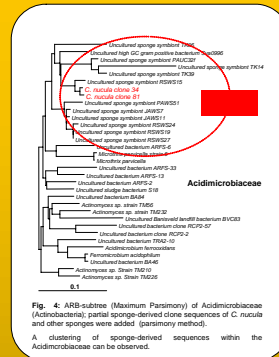


Fig. 4: ARB-subtree (Maximum Parsimony) of Acidimicrobiaceae (Actinobacteria); partial sponge-derived clone sequences of *C. nucula* and other sponges were added (parsimony method).
A clustering of sponge-derived sequences within the Acidimicrobiaceae can be observed.

Materials & Methods

Sponge collection. Specimens of the Mediterranean sponge *Chondrilla nucula* were collected by scuba diving. The sample was obtained from the Adriatic Sea and was collected in April 2003 in the Limsky Canal in Croatia in a depth of ~15m. The sponge was collected into sterile plastic bags, cooled on ice and transported immediately to the laboratory. There the sponge was washed in sterile filtered seawater (0.2 µm) and cut. Part of the sponges was homogenized in sterile seawater cooled on ice, shock frozen and transported to Germany.

DNA extraction and amplification. Genomic DNA was extracted and purified using the DNA Mini Spin Column DNA Purification Kit tissue protocol and genomic DNA purification protocol (Qiagen, Germany). Amplification of 16S rDNA was performed with the subcloning primers 27F and 1492R. DNA was purified using the High Pure PCR Product Purification Kit (Roche). Cloning was performed using the TOPO TA® Cloning Kit (Invitrogen, Germany), inserts were amplified using the M13MT1/Sev primer set. Correct insert size was verified by using agarose gel electrophoresis.

Sequencing and phylogenetic analysis. Sequencing was performed using the ABI PRISM® BigDye™ Terminator Ready Reaction Kit (Applied Biosystems) and a ABI PRISM® 310 Genetic Analyzer (Perkin Elmer Applied Biosystems, Foster City, USA). Sequence data were edited with Chromas, version 1.45 (www.technetysystem.com) and checked for possible chimera origins (CHECK_CHIMERA software of the Ribosomal Database Project). Phylogenetic analyses were performed with the ARB software package [3]. Partial clone sequences of 670-890 nucleotides length were added subsequently to the respective tree (sequences >1,400bp) without changing tree topology using the ARB maximum parsimony method.

Denaturing Gradient Gel Electrophoresis (DGGE). Extracted DNA from whole-sponge tissue was PCR-amplified by analysis with denaturing gradient-gel electrophoresis (DGGE) with bacterial 16S rDNA DGGE primers [4]. PCR products were loaded onto denaturing gradient-gels with 50-80% denaturant and 6-8% Acrylamide [5]. Bands were visualized with UV transillumination.

Acknowledgements

We gratefully acknowledge help of R. Batel and the personnel at the Ruder Boskovic Institut (Rovinj, Croatia) during sampling of the sponges. We thank F. Brümmer and W. Zucht for identification of the sponges. This work was supported by the bmb+g as part of the Kompetenz-Zentrum BIOTECmarin (grant no. 03F0345B)

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Sponge specific bacterial clusters in *Chondrilla nucula* from two different locations in the Mediterranean Sea



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Abstract

Sponges are known to contain large numbers of (extracellular) bacteria generally located within the mesohyl. It has been suggested that symbiotic interactions between sponges and microorganisms exist. Sponge-specific phylogenetic clusters have been found, but a sponge species specific association has not been demonstrated, yet. We examined the bacterial diversity associated with the Mediterranean sponge *Chondrilla nucula* (Fig. 1) from different sampling sites (Adriatic Sea and Ligurian Sea) (Fig. 2). Further, we compared the species and sampling site specific bacterial phylogeny by 16S rDNA cloning experiments.

C. nucula possesses three distinct specific monophyletic clusters of 16S rDNA sequences (Fig. 3-5). In two of them the *C. nucula* specific clone-sequences from the Adriatic and the Ligurian Sea showed similarities to each other of more than 99% and less than 87% to other known sequences from the database.

The high similarities of up to more than 99% between sequences obtained from *Chondrilla nucula* specimens from different sampling sites suggest that specific *C. nucula* associated bacterial groups exist.



Fig. 1: Map of the Mediterranean Sea showing the different sampling sites in the Ligurian and Adriatic Sea.



Fig. 2: *Chondrilla nucula* (Adriatic Sea specimen) (A) in situ (M. Sidri & W. Zucht) (B) electron microscopic (REM) exposure (Dr. R. Schmaljohann)

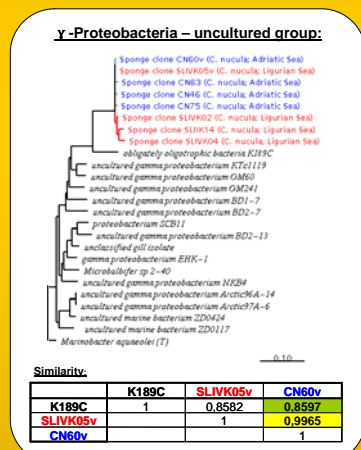


Fig. 3: phylogenetic tree (ARB/Maximum Parsimony) and similarity matrix (Neighbor Joining) of the *C. nucula* specific cluster I

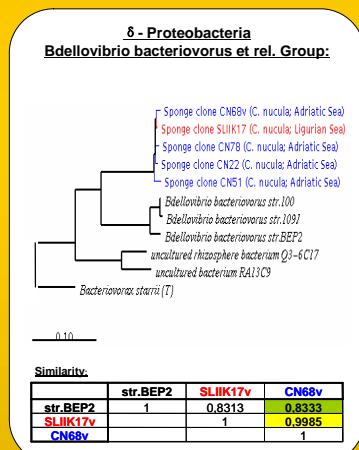


Fig. 4: phylogenetic tree (ARB/Maximum Parsimony) and similarity matrix (Neighbor Joining) of the *C. nucula* specific cluster II

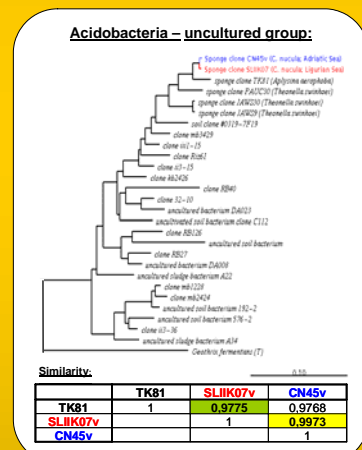


Fig. 5: phylogenetic tree (ARB/Maximum Parsimony) and similarity matrix (Neighbor Joining) of the *C. nucula* specific cluster III

Results

We observed a high bacterial diversity at both sampling sites. Sequences show that specimens from both habitats harboured Gamma- and Delta- Proteobacteria, Actinobacteria, Chloroflexi and a division of uncultured bacteria with uncertain affiliation. *C. nucula* contained three distinct specific monophyletic clusters of 16S rDNA sequences (Fig. 3-5): one each within the Gamma- (Fig. 3) and Delta-Proteobacteria (Fig. 4) and within the Acidobacteria (Fig. 5). In two of these clusters the *C. nucula* specific clone-sequences from the Adriatic and the Ligurian Sea show similarities to each other of more than 99% and less than 87% to other known sequences from the database. Further, we found a third monophyletic cluster within a sponge specific cluster of the Acidobacteria (Fig. 5). This is represented by sequences from *C. nucula* and sequences previously found in *Theonella swinhoei* and *Aplysina aerophoba*. Within the Actinobacteria, sequences from *C. nucula* from both sampling sites clustered together with sequences also found in other sponges. These did not show the high similarities like the former 3 (data not shown).

Sequences from the Alpha-Proteobacteria and representatives of the Nitrospina-OP8-division were specific to samples from the Ligurian Sea, while sequences belonging to the Bacteroidetes, the TM7-division and two divisions of uncultured, partly symbiotic bacteria were particular to the specimen from the Adriatic Sea.




Acknowledgements

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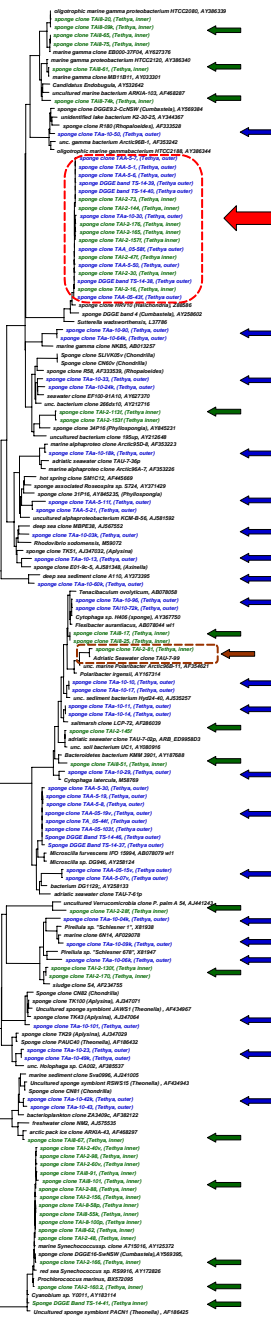
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IFM-GEOMAR

Spatial distribution of sponge associated bacteria within *Tethya aurantium*

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Abstract:
Stability and composition of the sponge associated bacterial community of the Mediterranean sponge *Tethya aurantia* was studied. A different bacterial community was observed in different parts of the sponges within one individual, both differing from that of the surrounding seawater, while inner and outer parts of the sponges are highly similar between different individuals and sampling time as shown by DGGE bandpatterns.




Fig. 1: *Tethya aurantia* (V.Thiel)

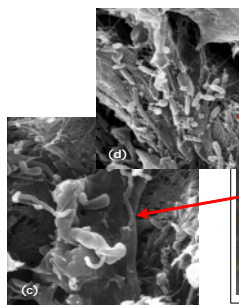


Fig. 3: *Tethya aurantia* cut in diameter (a) (S. Neuling), with REM pictures of the outer (b) and inner (c)(d) parts (R. Schmaljohann)

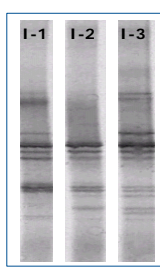


Fig. 2a: DGGE patterns of inner tissue samples (I) of different *Tethya aurantia* individuals (1-3) from 2004 (1) and 2005 (2,3)

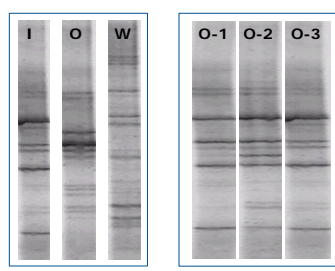


Fig. 2b: DGGE patterns of outer tissue samples (O) of *Tethya aurantia* and surrounding seawater (W)


Fig. 2c: DGGE patterns of outer tissue samples (O) of different individuals (1-3) *Tethya aurantia* from 2004 (1) and 2005 (2,3)

Fig. 4: Phylogenetic tree of 16S rDNA sequences resembling the different clustering of clone and DGGE sequences obtained from *Tethya aurantia* inner (green arrows) and outer parts (blue arrows). A shared cluster is indicated by a red arrow. One example for sequences found in the surrounding water as well as in the sponge is pointed out by a brown arrow. The tree was calculated with only nearly full length sequences using the parsimony method. Partial sequences were added without changing the topology of the tree using the ARB parsimony method

Approach and Results:
Bacterial community composition has been studied using the molecular, culture independent methods of double gradient gel electrophoresis (DGGE) and 16S rDNA cloning experiments. Sponge samples were obtained by SCUBA diving from the Limsky Canal, a fjord like bay at the Adriatic coast north off Rovinj, Croatia. Sponge individuals from different positions and different sampling dates over a period of 3 years were studied. DGGE patterns of inner and outer tissue samples differed constantly from surrounding seawater and each other (Fig. 2b), while inner (Fig. 2a) and outer (Fig. 2c) tissue samples shared a similar pattern between different individuals at different sampling times. 16S rDNA clones obtained from two different individuals from 2003 and 2004 show a high dissimilarity (Fig. 4 indicated by green and blue arrows) in the bacterial community composition, sharing only one phylogenetic cluster (Fig. 4 indicated by red arrow). Some bacterial sequences from surrounding seawater were also found in sponge samples (Fig. 4 example indicated by brown arrow) reflecting the sponge taking up bacteria by filter feeding.

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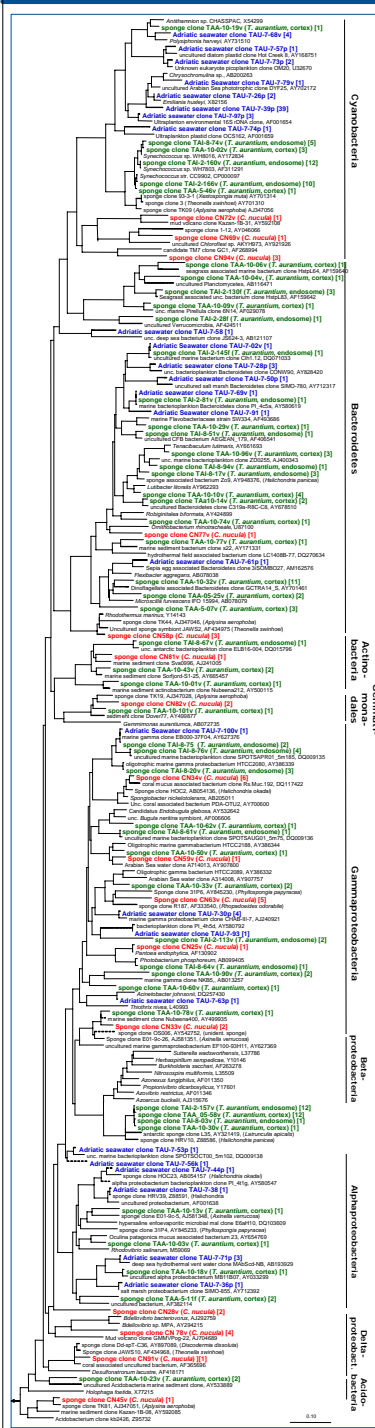
Leibniz Gemeinschaft

Fig. B-4: Poster presented at the annual meeting of the ‘Vereinigung für Allgemeine und Angewandte Mikrobiologie, VAAM’, March 2006 in Jena, Germany

Sponge-associated bacterial diversity differs highly between sponge species at the same location in the Adriatic Sea

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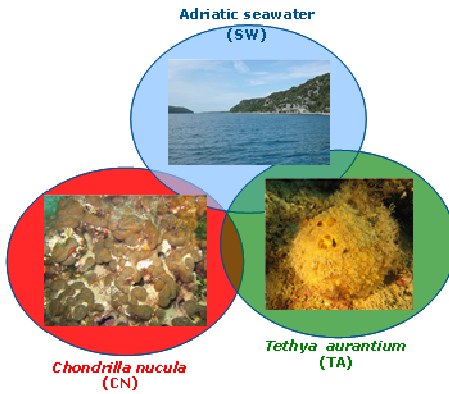
IFM-GEOMAR



Abstract:
Sponge-associated bacterial communities of two distinct sponge species (*Chondrilla nucula*, Fig. 1 and *Tethya aurantium*, Fig. 3), living in close proximity in an Adriatic Sea habitat, were studied. Despite sharing one habitat and being exposed to the same bacterial seawater population, the sponge-associated bacterial communities differ significantly between the two sponge species. The impact of ambient seawater on the composition of the SAB-population thus seems to be low.



Fig. 1: *Chondrilla nucula*. In-situ photograph at the sampling site, in the Limski kanal, a fjord like bay at the Adriatic coast north off Rovinj, Croatia.



Results 1:
Distinct denaturing gradient gel electrophoresis (DGGE) band patterns show differences in the bacterial community structures (Fig. 2). The sponge-associated bacterial communities differ between the two sponges ('TA' and 'CN') as well as between endosome ('TA-E') and cortex ('TA-C') within one sponge specimen (*T. aurantium*). Both sponges clearly differ in their associated bacterial community from the surrounding seawater (Fig. 2).

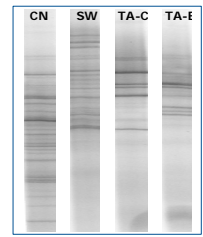


Fig. 2: DGGE band patterns of 16S rDNA derived from sponges and seawater. (CN *C. nucula*; SW ambient seawater; TA *T. aurantium*; TA-E endosome, TA-C cortex)

Results 2:
Analysis of sponge- and seawater-derived 16S rDNA clone libraries, revealed distinct bacterial communities in each of the samples (Fig. 4). Bacterial communities of the sponges did not overlap with each other. Few phylotypes were found in both, sponge and seawater.

A high phylogenetic diversity was observed for both sponges and seawater. *T. aurantium*-derived clone libraries (132 seq.) revealed 45 phylotypes of which the majority was affiliated with *Proteobacteria* (40%), *Bacteroidetes* (26%) and *Cyanobacteria* (23%). The *C. nucula*-derived clone library (34 seq.) revealed 16 phylotypes distributed among 6 different bacterial divisions with the majority (62%) belonging to the *Proteobacteria* (Fig. 4). Bacterial sequences obtained from *C. nucula* were in large parts (62%) affiliated to other sponge-derived sequences available from databases, while a minor fraction (22%) of *T. aurantium*-associated phylotypes shared similarity with other sponge-derived sequences. From the seawater 41 clone sequences were retrieved, representing 27 phylotypes of 5 bacterial divisions.

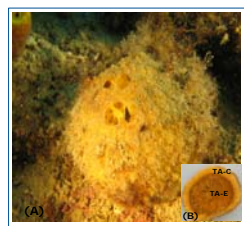


Fig. 3: *Tethya aurantium*. (A) In-situ photograph. (B) cut in diameter; the cortex (TA-C) surrounding the endosome (TA-E)

Sequences identical to those obtained from ambient seawater were found in *T. aurantium* endosome only. Microscopic studies revealed that *T. aurantium* compared to *C. nucula* contains less associated bacteria in its mesohyl (not shown). The impact of seawater-derived bacteria on the sequences obtained in sponge-derived 16S rDNA clone libraries thus seems to increase with a decreasing amount of associated bacteria present in the sponge mesohyl.

Acknowledgements
We gratefully acknowledge help of R. Batel and the personnel at the Ruder Boskovic Institut (Rovinj, Croatia) during sampling of the sponges. We thank F. Brümmer and W. Zucht for identification of the sponges. This work was supported by the tmh-f as part of the Kompetenz-Zentrum BIOTECmarin (grant no. 03F345B)

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