

**Antimicrobially active microorganisms associated with  
marine bryozoans**

Wirkstoffproduzierende Mikroorganismen assoziiert mit marinen Bryozoen

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In Erinnerung an meine Oma

*“I love deadlines. I like the whooshing sound they make as they fly by.”*  
Douglas Adams (1952 – 2001)

# Table of contents

Summary .....	1
Zusammenfassung .....	2
General introduction.....	4
Bryozoans.....	4
Bryozoan-natural products .....	8
Bryozoan-microbe associations.....	11
The role of natural products in drug discovery - a focus on anti-infectives.....	14
Thesis outline .....	16
<b>Chapter 1</b>	
Phylogenetic diversity and antimicrobial activities of bryozoan-associated bacteria isolated from Mediterranean and Baltic Sea habitats .....	19
Abstract .....	20
Introduction .....	21
Material and methods .....	23
Results .....	26
Discussion .....	36
<b>Chapter 2</b>	
<i>Tenacibaculum adriaticum</i> sp. nov., from a bryozoan in the Adriatic Sea.....	42
Abstract .....	43
Description of <i>Tenacibaculum adriaticum</i> sp. nov. ....	49
<b>Chapter 3</b>	
<i>Membranipora membranacea</i> associated bacteria: impact of media on their isolation and antimicrobial activity.....	56
Abstract .....	57
Introduction .....	58
Materials and methods .....	60
Results .....	64
Discussion .....	78
Synthesis.....	84
Why are bryozoans underrepresented in natural products research? .....	84
Host-specific and opportunistic microorganisms are associated with bryozoans .....	85
Cultivation-based issues on assessing bacterial diversity .....	88
Antimicrobially active bacterial isolates .....	91
Significance and outlook.....	96
References .....	97
Danksagung.....	109
Scientific contribution to multiple-author publications .....	110
Erklärung.....	111

## Summary

Bryozoans are sessile colonial animals that can be found in various aquatic and mainly in marine environments. Due to their sessile nature, bryozoans compete for surfaces they can colonize but, on the other hand, are confronted with microbial colonizers on their surfaces. Interactions of the bryozoan with its associates, as well as within the microbial community, are mediated chemically. Biofilm formation and composition is mainly influenced by the use of chemical compounds. Studies on the bryozoan-associated microbial diversity are scarce, and surveys on the antimicrobial potential of these associated bacteria are missing. The present study focused on isolating bryozoan-associated bacteria, assessing their antimicrobial properties and classifying them phylogenetically.

Various bryozoan specimens were collected in the Baltic (10 specimens) and the Mediterranean Sea (11 specimens). Bacteria were isolated using a variety of nutrient media and tested for their antimicrobial abilities against Gram-positive and Gram-negative indicator strains, as well as against the yeast *Candida glabrata*. 30% of all isolates displayed activity and were phylogenetically classified on the basis of 16S rDNA gene sequences. Whereas all isolates were active against Gram-positive indicators, four isolates exhibited additional anti-*Escherichia coli* activity, the phylogenetic analysis revealed affiliation to Gram-negative phyla (*Flavobacteria*, *Alpha-* and *Gammaproteobacteria*). One isolate belonged to the Gram-positive *Actinobacteria*. Both species- and strain-specific activity patterns were revealed. Furthermore, site-specific distribution patterns of associated bacteria were found.

Of these antibioticly active isolates, the strain B390 was described as type strain of the novel species *Tenacibaculum adriaticum*.

Also, specimens of the bryozoan *Membranipora membranacea* were sampled in the Baltic Sea for the first more detailed analysis on antimicrobially active isolates. Low-nutrient media featuring “artificial” or “natural” ingredients were used for isolation of bacteria. Additionally, the antibiotic test panel was extended to six different production media. The impact of these media on the phylogenetic diversity, as well as on activity patterns was determined. Although bacteria were affiliated with same phyla (*Alpha-* and *Gammaproteobacteria*, *Actinobacteria*, additionally *Bacilli*), the isolates of this sampling were more diverse as far as genus or phylotype affiliation was concerned. Especially within the *Alphaproteobacteria*, several probably novel bacterial species were found. Furthermore, the use of six different media for activity testing resulted in a more than twofold higher hit rate of active isolates in comparison to only one single medium.

## Zusammenfassung

Bryozoen sind sessile, koloniebildende Tiere, die in vielen aquatischen, und vor allem in marinen Lebensräumen anzutreffen sind. Wegen dieser sessilen Lebensweise konkurrieren Bryozoen um besiedelbare Oberflächen, sind aber andererseits mikrobieller Besiedelung ihrer eigenen Oberflächen ausgesetzt. Interaktionen zwischen Bryozoe und ihrem Bewuchs, als auch innerhalb der mikrobiellen Gemeinschaft werden auf chemischen Weg vermittelt. Biofilmbildung und –zusammensetzung wird hauptsächlich durch die Verwendung chemischer Verbindungen beeinflusst. Studien zur Bryozoen-assoziierten mikrobiellen Diversität sind rar, und Untersuchungen zum antimikrobiellen Potential der assoziierten Bakterien fehlen. Die vorliegende Arbeit richtete sich auf die Isolierung Bryozoen-assoziiierter Bakterien, deren antimikrobiellen Eigenschaften und phylogenetische Klassifizierung.

Verschiedene Bryozoenexemplare wurden in der Ostsee (10 Proben) und im Mittelmeer (11 Proben) gesammelt. Bakterien wurden mit Hilfe unterschiedlicher Nährmedien isoliert und auf antibiotische Aktivität gegen Gram-positive und Gram-negative Indikatorstämme sowie gegen die Hefe *Candida glabrata* getestet. 30% aller Isolate zeigten Aktivität und wurden auf Basis von 16S rDNA Gensequenzen phylogenetisch eingeordnet. Während die Aktivitäten aller Isolate gegen Gram-positive Stämme gerichtet war, vier Isolate waren zusätzlich gegen *Escherichia coli* aktiv, zeigte die phylogenetische Analyse eine Zuordnung zu Gram-negativen Phyla (*Flavobacteria*, *Alpha-* und *Gammaproteobacteria*). Ein Isolat gehörte den Gram-positiven *Actinobacteria* an. Sowohl spezies-, als auch stammspezifische Aktivitätsmuster wurden aufgezeigt. Darüber hinaus wurde eine probennahmestandortspezifische Verteilung der assoziierten Bakterien gefunden.

Eines dieser antibiotisch aktiven Isolate, der Stamm B390, wurde als Typstamm der neuen Spezies *Tenacibaculum adriaticum* beschrieben.

Ferner wurden Proben der Bryozoe *Membranipora membranacea* für die erste detailliertere Analyse antibiotisch aktiver Bakterien aus der Ostsee genommen. Medien mit geringem Nährstoffgehalt, mit „natürlichen“ oder „künstlichen“ Bestandteilen, wurden für die Isolierung verwendet. Weiters wurde die Nährmedienanzahl der antibiotischen Tests auf sechs erweitert. Der Einfluss der Medien auf die phylogenetische Diversität und auf die Aktivitätsmuster wurde bestimmt. Obwohl die Bakterien denselben Phyla zugeordnet wurden (*Alpha-* und *Gammaproteobacteria*, *Actinobacteria*, zusätzlich *Bacilli*), war die Diversität dieser Isolate höher. Insbesondere innerhalb der *Alphaproteobacteria* wurden einige möglicherweise neue Bakterienarten gefunden. Außerdem führte die Verwendung von sechs

verschiedenen Nährmedien für die Aktivitätstests zu einer mehr als doppelt so hohen Ausbeute an antibiotisch aktiven Isolaten im Vergleich zu einem einzigen Medium.

## General introduction

### ***Bryozoans***

The superficial resemblance of bryozoans to corals had led to misclassifications until 1831 when Christian Gottfried Ehrenberg introduced the term “bryozoa” to account for the separate mouth and anus openings, and to distinguish those animals from the “anthozoa”, which lack this complex feature.

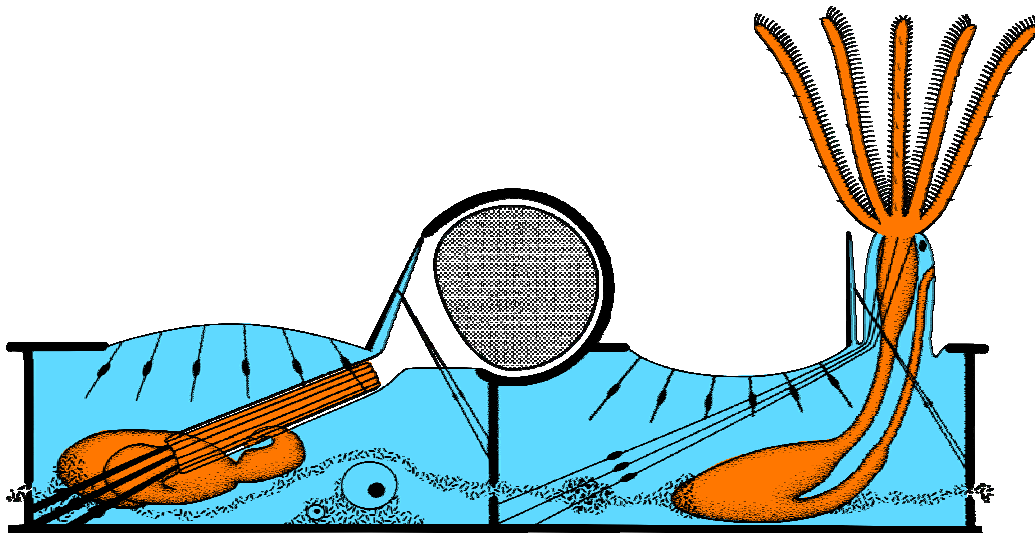
In 1869 the “bryozoa” were separated into two groups, the Ectoprocta and the, at that time recently, discovered Entoprocta. Although both share a similar filter feeding mechanism, they possess a differential internal anatomy. The position of the anus, either outside or inside the ring of tentacles (lophophore), is the main distinguishing feature and name giver of both phyla. Nevertheless, the phylum Ectoprocta is still widely referred to as Bryozoa.

The phylum Bryozoa consists of three classes. The Phylactolaemata contains exclusively freshwater bryozoans and comprises the order Plumatellida. However, it contains less than one hundred recent species in contrast to the several thousands of marine representatives. The Stenolaemata is an exclusively marine class, which has been the predominant bryozoan group of the Paleozoic. Its members have a strongly calcified exoskeleton and form characteristic brood chambers (gonozooids). At the present day it is represented by only one extant order, the Cyclostomata, other orders are only recorded as fossils. Finally, the mainly marine class Gymnolaemata comprises two orders: the Ctenostomata, which lack a calcification of walls and thus appear membranous or gelatinous, and the larger group of Cheilostomata with calcification and presence of a lid (operculum) covering the orifice, a hole in the frontal wall, through which the lophophore extends and retracts. In summary, present day marine species belong either to cyclostome, ctenostome or cheilostome bryozoans. The scientific focus, however, is on extinct representatives, as the number of fossil species is three to four times higher than of extant species.

Bryozoans are colonial animals, except for species of the genus *Monobryozoon*, with single individual modules having a size of about 1 mm and less. As a basic body plan, the polypide (*i.e.* organs and tissues like tentacles, tentacle sheath, alimentary canal, musculature, nerve ganglion) is embedded in a box with calcified or soft walls (cystid). Such a single module is termed a zooid. Many modules are combined to form a colony. Filter-feeding is performed by numerous autozooids, which possess a protrusible lophophore. Other zooids may have a



different morphology (especially within the Cheilostomata) and are specialized to various functions. Avicularia operate a modified operculum (mandible) with strong muscles that can clean off the colony's surface as well as defend the colony by snapping at (small) predators and even catching (small) prey. It decays on the surface setting off nutrients that might be used by the autozooids. Vibracula possess very long and thin mandibles with a wide range of motion and can be used as "legs" by free-living bryozoan colonies. Kenozooids lack a polypide a may act as branching points or spacers. Gonozooids and ovicells are modified as brood chambers for fertilized eggs.

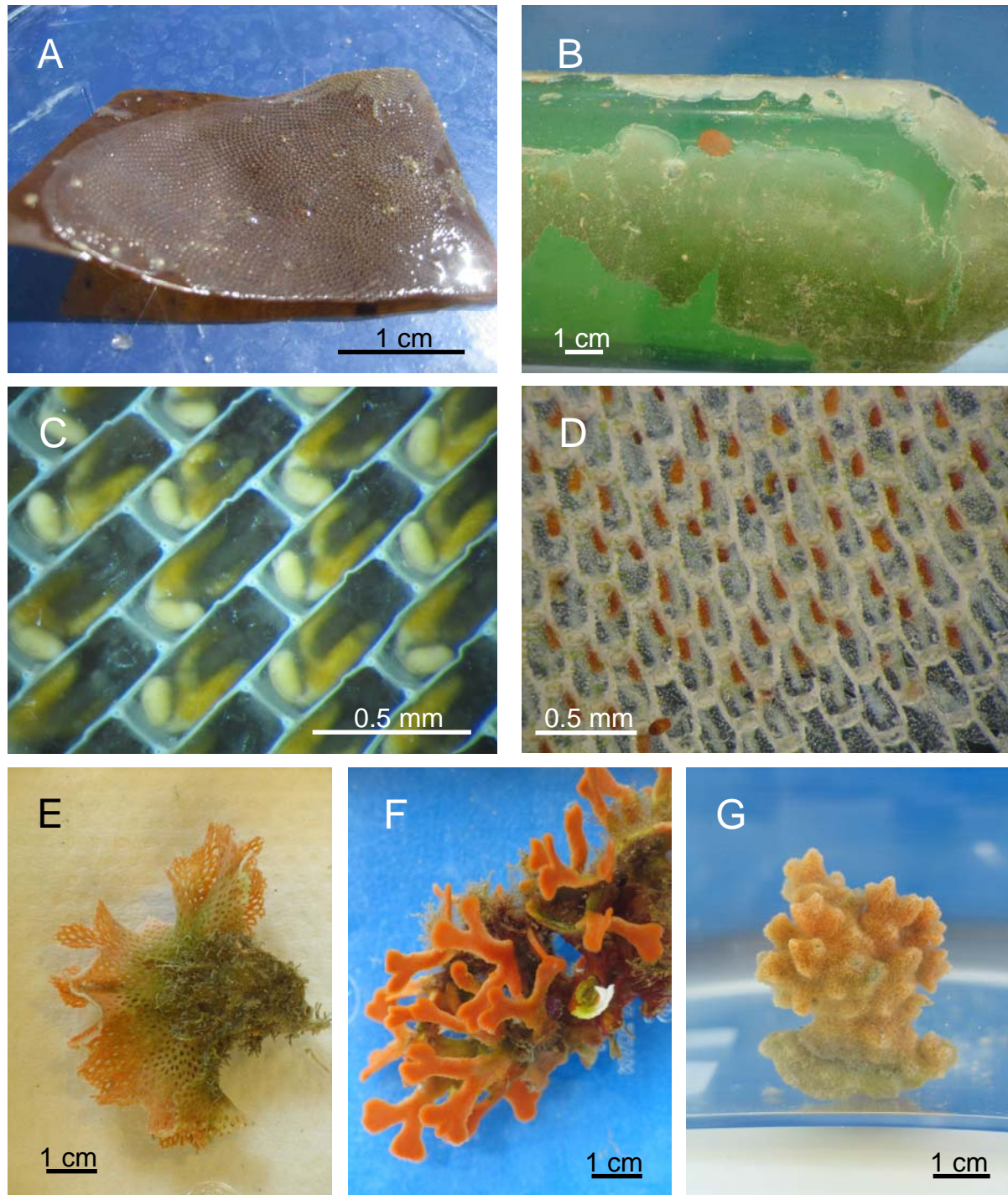


**Figure 1. Schematic image of the anatomy of a generalised bryozoan.** Lophophore (retracted and extended) and digestive tract are shown in orange, skeleton and muscles in black. The funicular system (black dotted strand) connects the zooids. Ovary (bottom) and ovicell (top) are shown in the left zooid, testis (at the funiculus) in the right zooid. © Dr. Claus Nielsen (University of Copenhagen).

Bryozoans reproduce either sexually with a dispersal of larvae or asexually by budding or fission of colony fragments. The pelagic larva seeks for an adequate substrate and enters, after attachment, metamorphosis to form an initial zooid, the ancestrula. A new colony increases in size by budding asexually, *i.e.* all individual zooids are clones of the initial larva. Bryozoan colonies can grow encrusting, erect, free-living or rooted. The pattern and form may be more an adaption to the respective environment than a determined genetic trait (McKinney & Jackson 1991).

Bryozoans colonize various substrates including rocks, coral reefs, seashells or seaweeds, as well as man-made surfaces like glass (waste), ship hulls or piers, which is then referred to as fouling. The vast majority of bryozoans are sessile filter-feeders. When appropriate surfaces for a successful attachment are missing, such as sandy or muddy locations, free-living forms have established. In the case of the cupuladriids, vibracula with long mandibles can lift the circular colony above ground and also allow it to dig back to the sea floor after burial, which is a necessary adaptation to shifting environments (O’Dea 2009).

As sessile animals, bryozoans are exposed to grazers and predators and offer themselves a fine substrate for other colonizers. They rely on mechanical defence strategies and chemical compounds (Gerdes *et al.* 2005a).



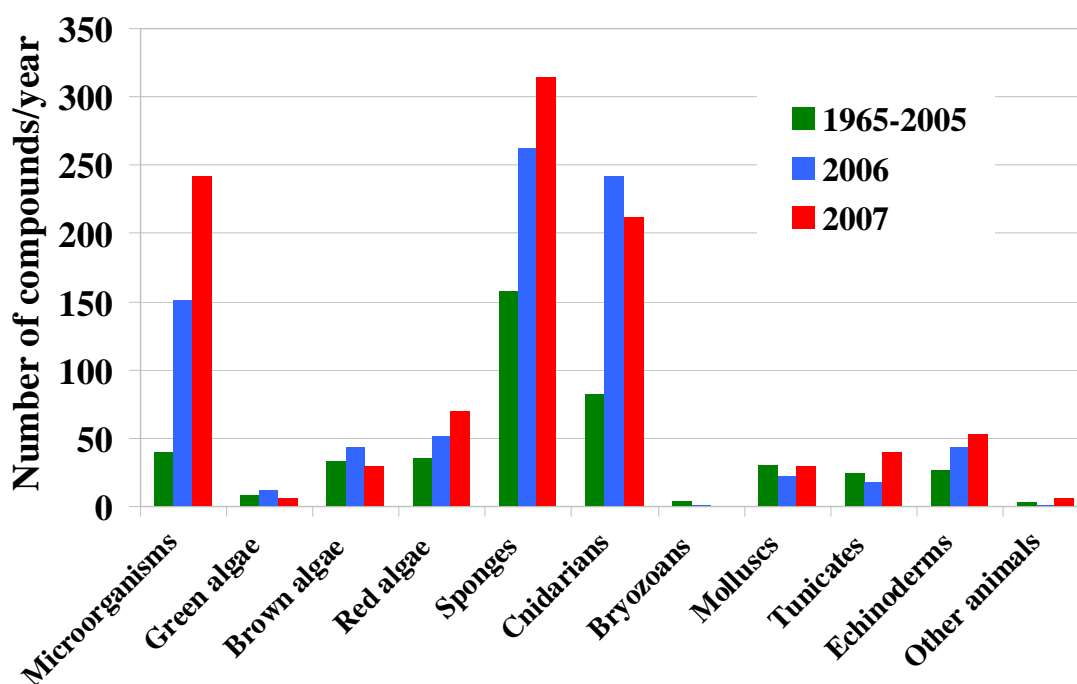
**Figure 2. Photographs of bryozoan samples.** A: cut piece of a *Membranipora membranacea* colony growing on *Saccharina latissima*. (© Dr. Vera Thiel) B: bryozoan colonies on a wine bottle. C: close-up of A, autozooids with clearly visible digestive tract. D: close-up of a colony from B, the funiculus connecting the zooids is visible as a whitish string, the dark orange structure in almost every zooid is the stomach. E: *Reteporella beaniana* (syn. *Sertella beaniana*). F: *Schizotheca serratimargo*. G: *Cellepora pumicosa*.

## **Bryozoan-natural products**

Earlier investigations have focused on whole-bryozoan extracts and revealed antibiotic (Colon-Urban *et al.* 1985), haemolytic (Winston & Bernheimer 1986) or cytotoxic (Newman 1996) activities.

Since 1986, elucidated and published compounds from the marine environment have been annually reviewed in the *Marine Natural Products* (Faulkner 1986). The review features all new chemical entities from marine sources that have been published within a year, as well as recent structural revisions of already known compounds. Usually, a whole year two years before the publication date is covered (*e.g.* the recent 2010 report contains all publications from the year 2008; Blunt *et al.* 2010). Additionally, within the report, all compounds are listed in groups according to their biological origin. One of those groups is the bryozoans, which have been featured in the review since its first issue in 1986.

About 200 new chemical entities from bryozoans have been described so far. Nevertheless, this phylum accounts for only 1% of all marine natural products (Fig. 3).



**Figure 3. Distribution of marine natural products per phylum.** Bars are showing the number of new compounds per year. Data for 1965 to 2005 are averaged. (Blunt *et al.* 2009)

A comprehensive survey of the structural diversity of bryozoan metabolites (*e.g.* terpenes, alkaloids, peptides, macrolides) is given by Sharp *et al.* (2007a), who have covered the years 1980 to 2006. In this review the authors have noted that only 32 species had been studied from a natural products view, whereas the phylum Bryozoa contains more than 8000 species.

Possibly the taxonomic difficulties within the Bryozoa or even the mere recognition of the phylum itself might be a crucial reason for the low diversity of studied species. In fact, the majority of the investigated bryozoan species comprise large, erect and foliose forms, which are easy to recognize and, more important to natural products research, easier to collect in larger quantities, as many secondary metabolites are produced at very low concentrations. The authors have also observed a shift of focus from the isolation and mere description of structural features towards studies on biological activities, but then often in a pharmaceutical context.

### **The discovery of bryostatins**

The most prominent bryozoan-related and elucidated compounds are the bryostatins from *Bugula neritina*. A first collection was sponsored by the national cancer institute (NCI) and took place in 1968 off the west coast of Florida. An aqueous isopropanol extract was produced and tested at the NCI using two leukemic murine *in vivo* models and one *in vitro* cell line. Antitumor activities had been found, but more collections had to be performed in order to isolate and purify the active compounds. After various drawbacks due to storm related damages to the sampling area and variable activities of crude extracts, an activity guided fractionation of new extracts led to the isolation of Bryostatins 1 and 2 in 1981 (Pettit *et al.* 1982). This time *Bugula neritina* was collected in the Gulf of California. In an expanded screen at the NCI against a panel of human tumour cell lines, Bryostatins 1 and 2 displayed a specific inhibition of the leukemia cell lines and in contrast to most other antileukemic agents, stimulating effects on haematopoietic progenitor cells (*i.e.* bone-marrow cells). In 1988, the NCI Decision Network Committee decided to test Bryostatin 1 in clinical studies (Newman 1996). Material for the preclinical and clinical studies was made available by a collection of 13 tons of *Bugula neritina* off the coast of Southern California and subsequent isolation of 18 g Bryostatin 1 following current Good Manufacturing Practices all performed within ten months (Schaufelberger *et al.* 1991). In order to provide a sustainable source for an eventual later market-supply, an aquaculture system was established and tested. A *de novo* synthesis of Bryostatin 1 in the cultures at levels comparable to the “wild” collections was achieved and proved to be cost effective (Mendola 2003). This was also made possible by an improved extraction procedure, which allowed extraction and purification of bryostatins from a reactor within 30 minutes (Castor 1998).

Bryostatin 1 modulates protein kinase C (PKC) activity with stimulating effects on haematopoietic progenitor cell growth and various leukocyte activities (Philip & Zonder

1999). Its ability to sensitize tumour cells to other cytotoxic agents has led to focus on a combination of Bryostatin 1 with other chemotherapeutics (*e.g.* Paclitaxel). Most common side effects are myalgias and flu-like symptoms, which also can cause discontinuation of therapies. Clinical trials are still conducted to test the effectiveness in cancer and more recently, as PKC is involved in various regulative tasks, in Alzheimer's disease.

### ***Bugula neritina* associated microorganisms – the true producers**

In the case of some *Bugula* species, including the bryostatins-harboursing *B. neritina*, rod-shaped bacteria have been found in a particular part of their larvae, the pallial sinus, by thin-section transmission electron microscopy. Interestingly, species that carry these bacteria belong to the most frequently encountered fouling bryozoans (Woolacott 1981). The symbiont of *B. neritina* larvae has been classified as a gammaproteobacterium and named "*Candidatus Endobugula sertula*" (Haygood & Davidson 1997).

Different populations of *B. neritina* contain a different array of bryostatins: whereas the chemotype O (with octadienoate moiety) includes the clinically relevant Bryostatin 1, the chemotype M (minor) lacks these derivatives. These chemotypes could be linked both to a genetic variation within *B. neritina* and to distinct strains of "*Candidatus E. sertula*" existing in the respective populations. According to their water depth occurrence in the Pacific off California, the clades have been termed D, for deep (chemotype O), and S, for shallow (chemotype M) (Davidson & Haygood 1999). A comparable study, but at the east coast of the U.S., could genetically distinguish a northern and southern population of *B. neritina*. Whereas the southern bryozoans contain "*Candidatus E. sertula*" as a symbiont and are highly related to the Pacific shallow clade, the northern specimens lack this bacterium. The authors have suggested that the geographical distribution of populations is a result of different predatory pressure in the respective areas (McGovern & Hellberg 2003), as predators avoid larvae containing bryostatins (and symbionts) (Lopanik *et al.* 2004a). The bacterial symbiont has been identified as the true producer (Davidson *et al.* 2001). Bryostatins are macrocyclic lactones and are biosynthesized by a modular type I polyketide synthase (PKS-I) gene cluster termed *bry* (Sudek *et al.* 2007). The locations of the symbiont "*Candidatus E. sertula*", as well as the bryostatins within the host, have been uncovered during larval settlement, metamorphosis and early development of *B. neritina* (Sharp *et al.* 2007b).

## ***Bryozoan-microbe associations***

The surface of a bryozoan colony offers a habitat for other smaller organisms including diatoms, fungi, bacteria, algae, small invertebrates, sponges and others, depending on the size of the colony. Having such a natural cover and exhibiting similar growth patterns, bryozoans indeed resemble terrestrial mosses (bryophytes), hence the common name moss animals. In contrast to a mere overgrowth of surfaces, symbiotic interrelationships between the habitat-providing and settling organisms may exist, which leads to a mutually influenced formation of the whole structure. Analogous to the prominent macroscopic coral reefs, those zonally organised structures are referred to as (bryozoan-) microreefs (Scholz & Hillmer 1995). But for taxonomic classification the microbial cover is usually removed, and the whole bryozoan bleached in order to expose the informative skeletal features. As a consequence of this, the impact of microorganisms on the bryozoans' growth behaviour has been overlooked for a long time. Indeed, microbial communities influence growth morphologies of bryozoans (Kaselowsky *et al.* 2005, Scholz & Krumbein 1996), but the moss animals, in turn, are able to interfere with bacterial communication as well (Peters *et al.* 2003).

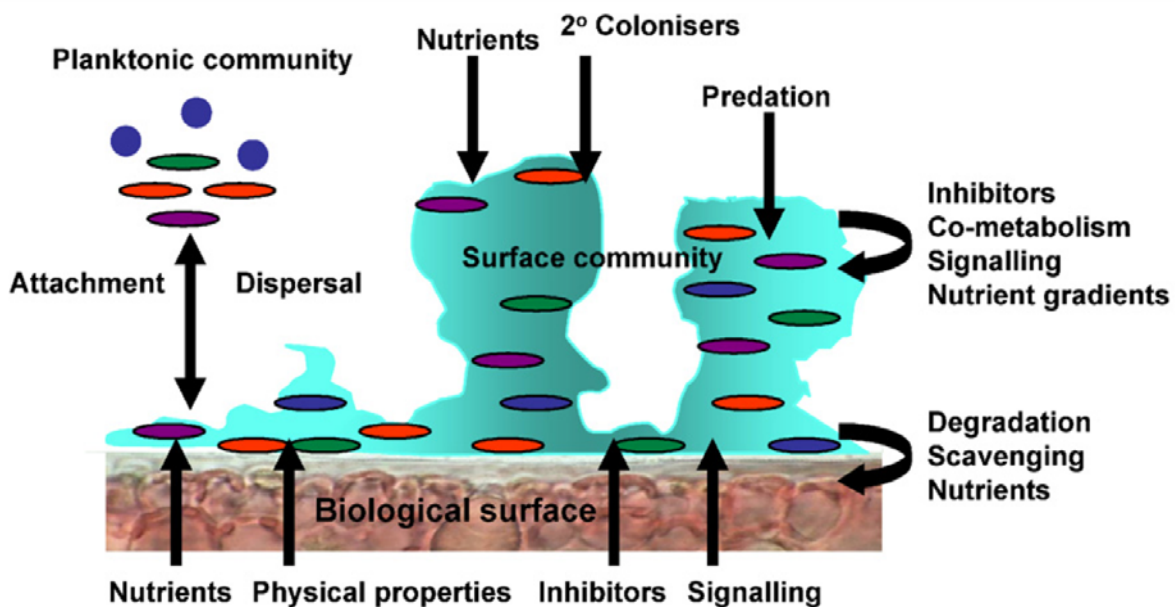
Nevertheless, only a few studies have dealt with bryozoan-associated microorganisms. The most common bryozoan species in the North Sea, *Flustra foliacea*, has been investigated with a cultivation based approach. The bacterial isolates have been phylogenetically classified and comprised mostly common marine representatives. Additionally, the three bryozoan samples have revealed an inconsistent bacterial composition (Pukall *et al.* 2001). With molecular methods, and thus cultivation independent, four different bryozoan species in the North Sea (*Aspidelectra melolontha*, *Conopeum reticulum*, *Electra monostachys* and *Electra pilosa*) have been studied for their associated bacteria. Three of them have displayed host-specific communities, whereas the accompanying bacteria of *C. reticulum* have been site-dependent (Kittelmann & Harder 2005). The degree of microbial fouling on *Pentapora fascialis*, from the Welsh coast, has been analysed and quantified with the use of scanning electron microscopy (Sharp *et al.* 2008). In a combination of microscopic, molecular and cultivation-based methods, the amount and diversity of fouling on various shallow water bryozoans from Japan and New Zealand have been examined. No bryozoan-specific biofilms could be detected but a decrease in biofilm covering towards warmer habitats has been noted (Gerdes *et al.* 2005a). As far as fungi are concerned, similar methods have been applied to identify fungal isolates of several bryozoan species from the coast of Helgoland (*E. pilosa*, *F. foliacea*,



*Tubulipora aperta*, *Membranipora membranacea*, *Bugula plumosa* and *Alcyonidium gelatinosum*), as well as detecting them *in situ* on the bryozoans' surfaces (Hain 2003).

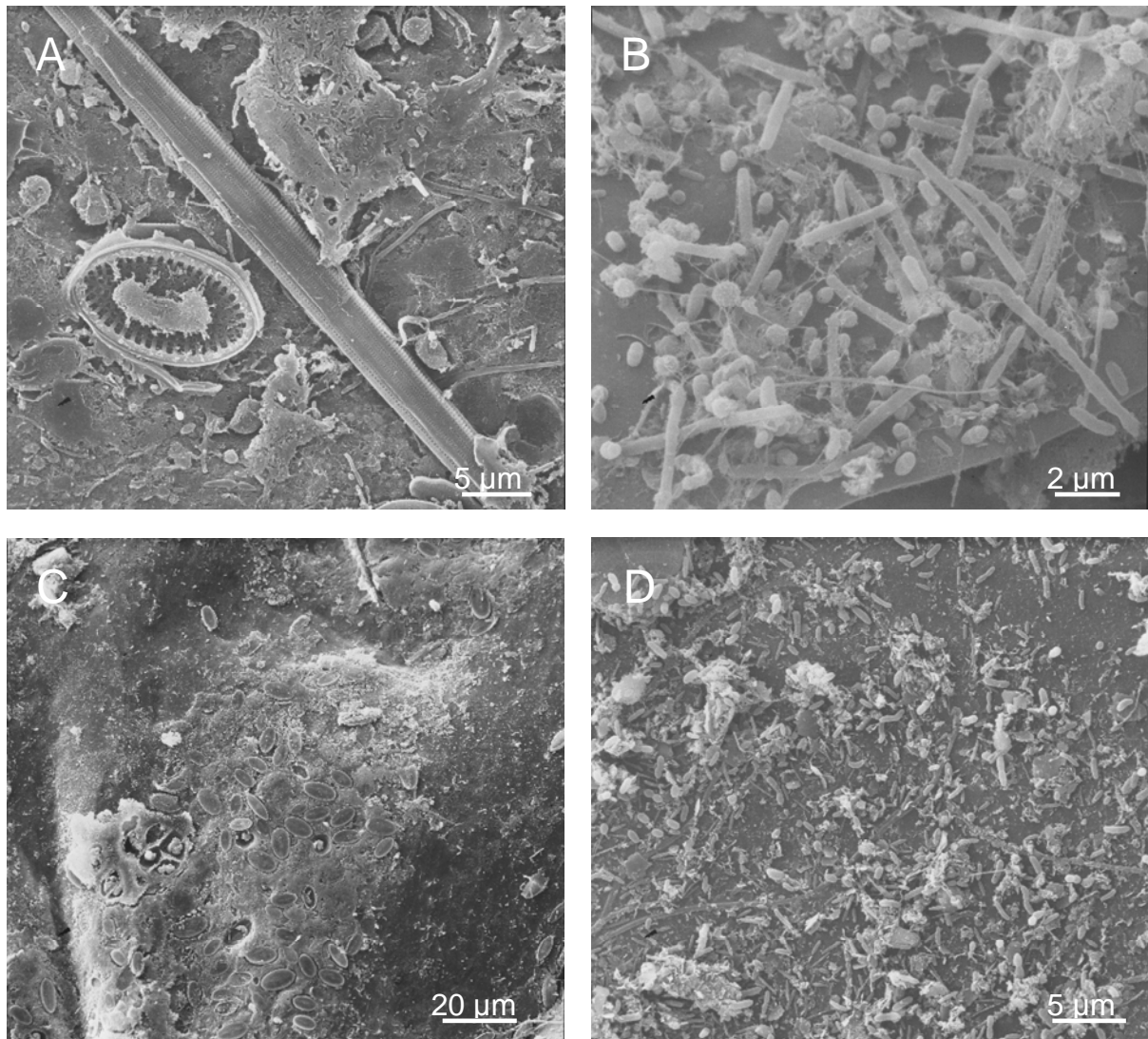
The influence of bryozoans' secondary metabolites on associated microorganisms has been investigated, too. Eleven compounds of *F. foliacea* from the North Sea have been isolated and identified as brominated alkaloids and one diterpene. The effect of these metabolites has been tested in agar diffusion assays on several bacterial isolates obtained from this bryozoan and on some terrestrial strains. Antimicrobial activities have been significant against the marine bacteria, but only weak against the terrestrial ones. Furthermore, two compounds have had antagonistic effects on bacterial communication, in this case *N*-acyl-homoserine lactone dependent quorum sensing (Peters *et al.* 2003).

In general, marine surface attached microorganisms, particularly those living in mixed biofilm communities and in association with eukaryotic hosts, are exposed to space and nutrient limitations, as well as (mostly) antagonistic interactions both with other microorganisms and their hosts (Figs. 4 & 5). This communication is chemically mediated and has a notable impact on the composition and development of the microbial community (Egan *et al.* 2008).



**Figure 4. Interactions of microbial communities and their associated hosts.** The sum of all factors (chemical, physical, biological) shapes a distinct surface community over time. (Egan *et al.* 2008)





**Figure 5. Scanning electron micrographs of bryozoans' surfaces.** A: thick biofilm with diatoms, filamentous and rod-shaped bacteria. B: various bacteria are attached to the lid of an operculum. C: diatoms are embedded in a biofilm. D: aggregates of bacteria with different morphologies

Thus, it's not astonishing that several natural products, which were originally isolated from marine macroorganisms, could rather be of microbial origin, for they strongly resemble metabolites from cultivated bacterial strains (Anthoni *et al.* 1990, König & Wright 1996, König *et al.* 2006).

## ***The role of natural products in drug discovery - a focus on anti-infectives***

The biological activities of natural products have always been utilized by humans to treat various diseases, even though the knowledge of molecules and their interaction with therapeutically relevant targets is a result of modern chemistry and medicine. Traditionally, plants have been used for treatment of diseases, but natural products from microorganisms have had a notable impact on drug discovery since the second half of the last century, especially within the anti-infectives (Newman *et al.* 2000).

The sources of approved drugs for the treatment of human diseases have been assessed from 1981 to 2006 (Newman & Cragg 2007): only new chemical entities have been covered, *i.e.* new combinations, delivery systems or indications of old drugs have been omitted, and grouped into six major categories according to their source of discovery, regardless of their actual large scale production/synthesis process: biologic (B; peptides and proteins), unmodified natural product (N), natural product derived (ND; usually produced semisynthetic), totally synthetic (S), totally synthetic but with natural product derived pharmacophore (S\*), and vaccine (V). In total, 40% are of synthetic origin (S). When only small molecules are concerned, thus excluding the categories B and V, the portion raises to 49%. Within the anti-infectives, 34% of the approved small molecule drugs fit in the S category. They belong only to two classes of active agents: the azol-based antifungals and the quinolone-based antibacterials. On the other hand, only 7% are unmodified natural products (N). Nevertheless, natural products play a crucial role in the development of anti-infectives, because they provide a greater diversity of active lead structures, which can be modified to achieve a better tolerance (leading to the categories ND, 41% and S\*, 18%).

But the extensive and inappropriate use of antimicrobial drugs has caused a loss of their effectiveness within the last decade. Multiple resistances have evolved especially within Gram-positive strains with methicillin-resistant *Staphylococcus aureus* (MRSA) or vancomycin-resistant *Enterococcus* (VRE) as the most prominent. Recently, infections with some strains belonging to the Gram-negative *Enterobacteriaceae*, bearing the resistance gene for New Delhi metallo-beta-lactamase (NDM-1) (Yong *et al.* 2009), have been reported in the media.

Hence, new anti-infectives, in particular with a novel structure and mode of action, are of particular interest. A possible way could be the application of innovative target systems. A genetically modified *S. aureus* strain, which has been rendered hypersensitive to FabF/FabH

inhibition, has been used to identify natural products with activities on fatty acid biosynthesis (Young *et al.* 2006). This has led to the discovery of the selective FabF inhibitor platensimycin (Singh *et al.* 2006) and the dual FabF/FabH inhibitor platencin (Wang *et al.* 2007) from two different *Streptomyces platensis* strains. Both compounds have shown a potent inhibition of various multi-resistant pathogens. In another approach, available genetic information of microorganisms is used to identify genes or gene clusters that are possibly involved in the biosynthesis of secondary metabolites. In the case of the strictly anaerobe *Clostridium cellulolyticum*, such genes have been found, but they remain silent when standard laboratory conditions are applied. By mimicking its natural habitat, plant based compost, the sulphur-containing compound closthioamide has been isolated (Lincke *et al.* 2010). This has been the first reported antibiotic from an anaerobe.

## Thesis outline

The aim of this thesis was to find novel microorganisms with antimicrobial properties. This could be possibly achieved by looking in greater detail into yet unexplored environments that feature a high competition for space and nutrients, *i.e.* struggle for survival. As far as bryozoans were concerned, some reports on the biological activities of whole-bryozoan extracts, as well as a few studies on associated bacteria had been published, but no investigation assessed the antimicrobial traits of bryozoan associated microorganisms. So bryozoans seemed to offer a favourable source.

### Chapter 1

“Phylogenetic diversity and antimicrobial activities of bryozoan-associated bacteria isolated from Mediterranean and Baltic Sea habitats”

In this study, ten bryozoan specimens in the Baltic Sea and eleven in the Adriatic Sea were sampled. Antimicrobial active bacterial isolates (90 out of 340) were phylogenetically classified by 16S rRNA gene sequences. They were affiliated to ten genera: *Tenacibaculum*, *Pseudovibrio*, *Sphingomonas*, *Alteromonas*, *Marinomonas*, *Pseudoalteromonas*, *Pseudomonas*, *Shewanella*, *Vibrio* and the Gram-positive *Cellulosimicrobium*. The distribution of the 27 phlotypes, based on sequence similarities  $\geq 99.5\%$ , turned out to be site-dependent. Antibiotic activities were predominately directed against Gram-positive indicator organisms. Indications for new species within the genera *Pseudoalteromonas*, *Shewanella* and *Tenacibaculum* could be found. Furthermore, biofilm coverage on the Mediterranean samples was investigated by SEM.

This was the first investigation on antimicrobially active bacteria associated with bryozoans.

## Chapter 2

“*Tenacibaculum adriaticum* sp. nov., from a bryozoan in the Adriatic Sea”

The *Flavobacteriaceae* isolate B390, obtained during the study reported in chapter 1, was described as a new species applying a polyphasic approach. Morphological, genetical and physiological characteristics were determined and compared with other type strains of the genus *Tenacibaculum*.

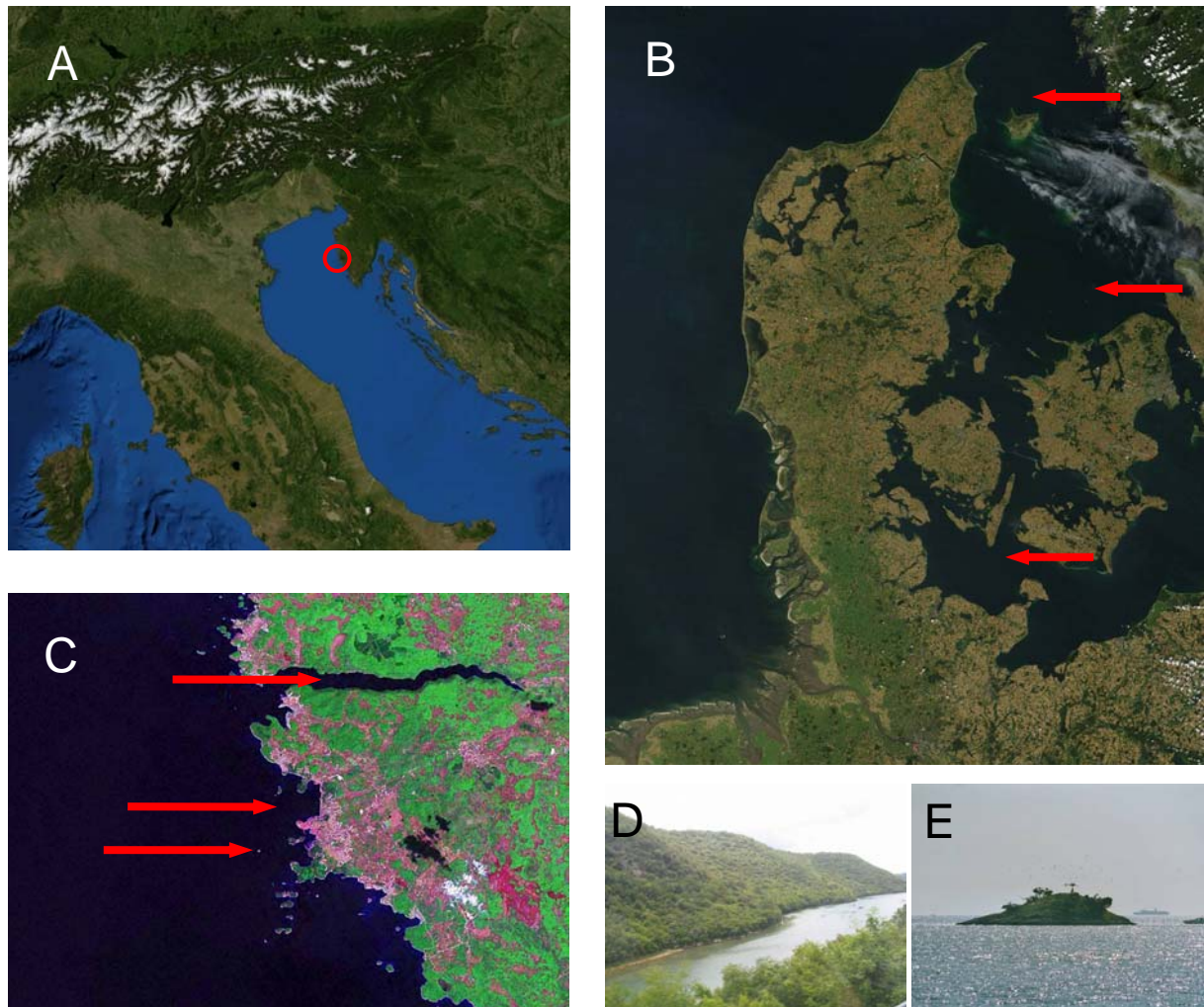
Strain B390 was the first taxonomically described *Tenacibaculum* species from the Mediterranean Sea and the third type strain that was isolated from a bryozoan sample.

## Chapter 3

“*Membranipora membranacea* associated bacteria: impact of media on their isolation and antimicrobial activity”

Whereas the study described in chapter 1 featured various bryozoan samples from different habitats, this survey focused on a single bryozoan species from one location: *Membranipora membranacea* taken in the Kattegat near Læsø. Different isolation media were applied at low nutrient concentrations and their influence on the obtainable bacterial diversity were investigated. “Natural” media featured ingredients from the sampling site (algal extract, bryozoan extract and local sea water). On the other hand, “artificial” media were composed of purchasable components. 96 bacterial isolates could be obtained and sequenced irrespective of their biological activities. They were grouped into 49 phylotypes ( $\geq 99.5\%$  sequence similarity) and belonged to 28 different genera of both Gram-negatives and Gram-positives. Some of them, especially isolates within the *Alphaproteobacteria*, represent very likely new species or even genera. Six different media were applied for antimicrobial testing and resulted in 47 active isolates.

This was the first comprehensive study on *M. membranacea* associated bacterial isolates.



**Figure 6. Sampling sites.** A: Adriatic Sea with Rovinj. B: Kattegat sites (from North to South), Læsø, Lysegrund, Langeland. C: Rovinj sites (from North to South), Limski channel, Rovinj bay, Banjol. D: Limski channel. E: Banjol. Pictures A-C: © NASA.

## **Chapter 1**

# **Phylogenetic diversity and antimicrobial activities of bryozoan-associated bacteria isolated from Mediterranean and Baltic Sea habitats**

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## **Abstract**

To date, only a small number of investigations covering microbe–bryozoa associations have been carried out. Most of them have focused on a few bryozoan species and none have covered the antibacterial activities of associated bacteria. In the current study, the proportion and phylogenetic classification of Bryozoan-associated bacteria with antimicrobial properties were investigated. Twenty-one specimens of 14 different bryozoan species were collected from several sites in the Baltic and the Mediterranean Sea. A total of 340 associated bacteria were isolated, and 101 displayed antibiotic activities. While antibiosis was predominantly directed against Gram-positive test strains, 16S rRNA gene sequencing revealed affiliation of the isolates to Gram-negative classes (*Flavobacteria*, *Alpha*- and *Gammaproteobacteria*). One isolate was related to the Gram-positive *Actinobacteria*. The sequences were grouped into 27 phylotypes on the basis of similarity values  $\geq 99.5\%$ . A host-specific affiliation was not revealed as members of the same phylotype were derived from different bryozoan species. Site-specific patterns, however, were demonstrated. Strains of the genera *Sphingomonas* and *Alteromonas* were exclusively isolated from Mediterranean sites, whereas *Shewanella*, *Marinomonas* and *Vibrio*-related isolates were only from Baltic sites. Although *Pseudoalteromonas* affiliated strains were found in both habitats, they were separated into respective phylotypes. Isolates with 16S rDNA similarity values  $\geq 99.5\%$ , which could possibly represent new species, belonged to the genera *Shewanella*, *Pseudoalteromonas* and *Tenacibaculum*.

**Keywords:** bryozoa; antimicrobial activity; Baltic Sea; Mediterranean Sea; host-bacteria association; 16S rRNA gene analysis



## **Introduction**

Bryozoans mainly populate marine habitats worldwide and they settle on surfaces of different macroorganisms, such as algae and mussels, as well as on non-living hard substrates. Although the bryozoans described so far mostly appear as important index fossils, present-day species still contribute to the discovery of new natural products. However, from more than 5000 estimated recent species (Massard & Geimer 2008, Ryland 2005) only 32 have been studied from the point of view as producers of natural substances, but they have yielded approximately 200 compounds (Sharp *et al.* 2007a). As sessile organisms, which lack an immune system, bryozoans are exposed to grazing and overgrowing pressure and therefore rely on mechanical and chemical defence strategies (Gerdes *et al.* 2005a). With regard to the supposed ecological function of secondary metabolites as antagonistic compounds, many studies have been carried out to assess their possible impact on human health. Thus, screenings have focused on cytotoxic (Winston & Bernheimer 1986) and antibiotic (Colon-Urban *et al.* 1985) extracts obtained from bryozoans, with the bryostatins being shown as the most prominent elucidated compounds (Lopanik *et al.* 2004b, Pettit *et al.* 1982). Bryostatin 1 is a potent protein kinase C modulator and is still undergoing clinical trials to consider its effectiveness in cancer and Alzheimer's disease treatments (see <http://www.clinicaltrials.gov/ct2/results?term=Bryostatin> for details).

Metabolites of bryozoans are able to influence their microbial population as well. In the case of *Flustra foliacea*, eleven compounds (ten brominated alkaloids and one diterpene) were isolated and tested for antibacterial activities and AHL-dependent quorum sensing (QS) antagonism (Peters *et al.* 2003). Five compounds displayed significant antibiotic properties which, in addition, targeted marine-derived strains isolated from *F. foliacea* (*Roseobacter* sp., *Psychroserpens* sp., *Sulfitobacter* sp., and *Paenibacillus pabuli*) more favourably than terrestrial strains (*E. coli* and *Bacillus megaterium*). Two compounds exhibited QS inhibitory effects without inhibiting growth.

Microbial associations with bryozoans on their surfaces or in inner tissues were examined by microscopic methods (Scholz & Krumbein 1996, Woollacott 1981). Nevertheless, studies on the bacterial diversity found on bryozoans are scarce. Gerdes *et al.* (2005b) investigated microbial fouling on 92 bryozoan colonies (e.g. *Disporella* sp., *Callopora* sp., *Celleporella hyalina*, *Watersipora subtorquata*, *Bugula neritina*) sampled from Japan and New Zealand, at four out of six sites in both spring and autumn. Some samples were selected for further microbial and molecular analysis apart from SEM and thin section studies. The biofilms were

quite heterogeneous and thus any specificity to a bryozoan species or sampling site could not be determined. Another cultivation-independent approach was pursued by Kittelmann & Harder (2005). Four bryozoan species (*Aspidelectra melolontha*, *Conopeum reticulum*, *Electra monostachys*, and *Electra pilosa*) were collected at three sites in the Jade area (Southern North Sea), together with empty mussel shells as references. In a comparative DGGE profile analysis, three species (*A. melolontha*, *E. monostachys*, and *E. pilosa*) demonstrated host-specific microbial communities, whereas the fourth (*C. reticulum*) revealed site-specific influences on its epibionts. By cultivation experiments, Pukall *et al.* (2001) classified bacterial isolates associated with *Flustra foliacea* at two sampling sites in the North Sea. Taxonomic affiliation of these 220 bacterial strains to commonly isolated bacteria from marine environments led to the conclusion that *F. foliacea* may accept colonization of the surface.

The attempt to cultivate bacterial strains isolated from bryozoans seems to be more and more crucial, since it is argued that these microorganisms are the real producers of compounds formerly attributed to their hosts (Anthoni *et al.* 1990). In the case of the amathamide alkaloids from the bryozoan *Amathia wilsoni*, strong indications for a bacterial producer were found (Walls *et al.* 1995). Regarding bryostatins from *Bugula neritina*, the microbial symbiont “*Candidatus Endobugula sertula*” was determined as the true producer (Davidson *et al.* 2001), although it could not be cultured.

In this study, twenty-one specimens of 14 different bryozoan species were collected from the Baltic Sea and the Mediterranean Sea. More than 300 associated bacteria were cultivated and their antibiotic activity was assessed. Isolates showing antimicrobial traits were phylogenetically classified by 16S rRNA gene sequencing.

## **Material and methods**

### **Sampling of bryozoans**

Three sites each in the Baltic Sea and the Adriatic Sea were chosen for sampling (Table 1-1). Samples collected by scuba diving were put in sterile plastic containers with screw caps and all samples were stored in local seawater at 4 °C in a cool box until further treatment. Encrusting specimens were cut from their substrata with sterile razorblades and, if necessary, dissected with sterile tweezers. Then, all samples were washed thoroughly with sterile seawater. All bryozoans from the Baltic Sea sites were encrusting species, while some of the Mediterranean species were also arborescent colonies (sample IDs 11-16). Each sample was photographed for later taxonomic classification. Ten samples were collected from the Baltic Sea and they comprised five different bryozoan species. Eleven samples from the Mediterranean Sea covered nine different species.

### **Scanning electron microscopy (SEM) of bryozoan surfaces**

A piece of each Mediterranean bryozoan was fixed in sterile 2% glutardialdehyde in 25‰ saline. After dehydration in a gradient ethanol series (30, 50, 70, 90, and 100%; v/v) the samples were critical point dried with carbon dioxide (Balzers CPD030) and sputter coated with gold-palladium (Balzers Union SCD004). The specimens were examined with a scanning electron microscope (Zeiss DSM960). Pictures were taken with a Contax SLR camera.

### **Isolation and cultivation of bacteria**

Each sample was crushed with a sterile micropestle, and a dilution series with sterile seawater was made ( $10^{-1}$ - $10^{-5}$ ). The serial dilutions (100 µl each), as well as pieces of the bryozoan samples, were spread on agar plates containing different media.

TSB3S25 medium (contained 0.3% (w/v) tryptic soy broth (Difco) and 2.5% (w/v) NaCl) and natural sea water media using Baltic or Mediterranean seawater depending on the sample source were used. 1.5% (w/v) agar was added to the media for solidification. The plates were incubated at 25 °C for at least one week. Colonies were picked and sub-cultured on TSB3S25 agar plates until pure cultures could be obtained.

### **16S rRNA gene sequence-based identification of active bacteria**

Isolates exhibiting antimicrobial activities were chosen for 16S rRNA gene sequence analysis provided they were both from different bryozoans and of diverse colonial morphology (the latter considered within all isolates from one sample). Crude DNA of pure cultures suspended in DNA-free water was extracted either by freezing and boiling (-20 °C overnight, 90 °C for 3 minutes) or by using a Precellys 24 lysis and homogenization device (Bertin Technologies) with a 0961VK05 Precellys grinding kit (Bertin Technologies). These DNA-extracts were used directly for PCR. The primer pairs 27f (5'-GAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') or 27f and 1387r (5'-CGGGCGGTGTGTACAAGG-3') were used for amplification of the 16S rDNA sequences (Lane 1991). PCR reactions were performed as follows: initial denaturation at 93 °C for 2 min, 30 cycles of amplification (each cycle consisting of annealing at 55 °C for 30 s, elongation at 72 °C for 30 s, and denaturation at 93 °C for 30 s), followed by final annealing (42 °C for 1 min) and an elongation step (72 °C for 5 min). The amplified PCR products were verified by agarose gel electrophoresis. PCR product purification, as well as sequencing, was undertaken at the Institute for Clinical Molecular Biology of the University Hospital Schleswig-Holstein, Kiel. For each purification reaction, a mix of 1.5 units Exonuclease I (GE Healthcare) and 0.3 units Shrimp Alkaline Phosphatase (SAP, Roche) was added to the PCR product and incubated for 15 min at 37 °C. The enzymes were inactivated at 72 °C for 15 min. A sequencing reaction was carried out with the BigDye<sup>®</sup> Terminator v1.1 Sequencing Kit (Applied Biosystems) and it was analyzed in a 3730xl DNA-Analyzer (Applied Biosystems), as specified by the manufacturer. Primers used for sequencing were 27f, 534r (5'-ATTACCGCGGCTGCTGG-3'), and 342f (5'-TACGGGAGGCAGCAG-3'), as well as 790f (5'-GATACCCTGGTAGTCC-3'), 1387r, and 1492r. Results were compared with other sequences in the EMBL prokaryotes database using BLAST (Altschul *et al.* 1990) available online at the European Bioinformatics Institute homepage, and they were classified with the tools on the RDP-II Project homepage (Cole *et al.* 2009).

The 16S rRNA gene sequences were submitted to the EMBL database with the accession numbers FN295743 to FN295831 and AM412314.

### **Phylogenetic analysis**

Type strain relatives of all isolates were determined by comparison of 16S rRNA genes in the EMBL prokaryotes database using BLAST and the online database LPSN (<http://www.bacterio.net>) (Euzéby 1997). Isolates were grouped into phylotypes by sequence

similarities  $\geq 99.5\%$ . One representative isolate of each phylotype was selected for phylogenetic calculation. All subsequent relative type strains, as well as selected non-type strains, were included in the phylogenetic analysis. Sequences were aligned using the FastAlign function of the alignment editor implemented in the ARB software package (<http://www.arb-home.de>) (Ludwig *et al.* 2004) and manually refined employing secondary structure information. For phylogenetic calculations, the PhyML program version 2.4.4 (Guindon *et al.* 2005) was used. The evolutionary model GTR+I+G used was determined using the program ModelGenerator, version 0.85 (Keane *et al.* 2006). A total of 100 nonparametric bootstraps were performed.

### **Antibacterial testing**

Pure cultures were inoculated as small dots (app. 1 cm<sup>2</sup>) on TSB3S25 agar plates in quadruplicate. After growing and attaching to the surface (dependent on the respective growth rate this was usually 3 to 7 days) the plates were covered with TSB3S10 soft agar (0.3% (w/v) tryptic soy broth, 1% (w/v) NaCl and 0.8% (w/v) agar) containing one of the indicator strains. The following strains were used for antibiotic testing: *Escherichia coli* DSM 498, *Bacillus subtilis* subsp. *spizizenii* DSM 347, *Staphylococcus lentus* DSM 6672, and the yeast *Candida glabrata* DSM 6425. The presence of inhibition zones was examined the following day as well as three times within a week in order to consider slow emerging activities.

## **Results**

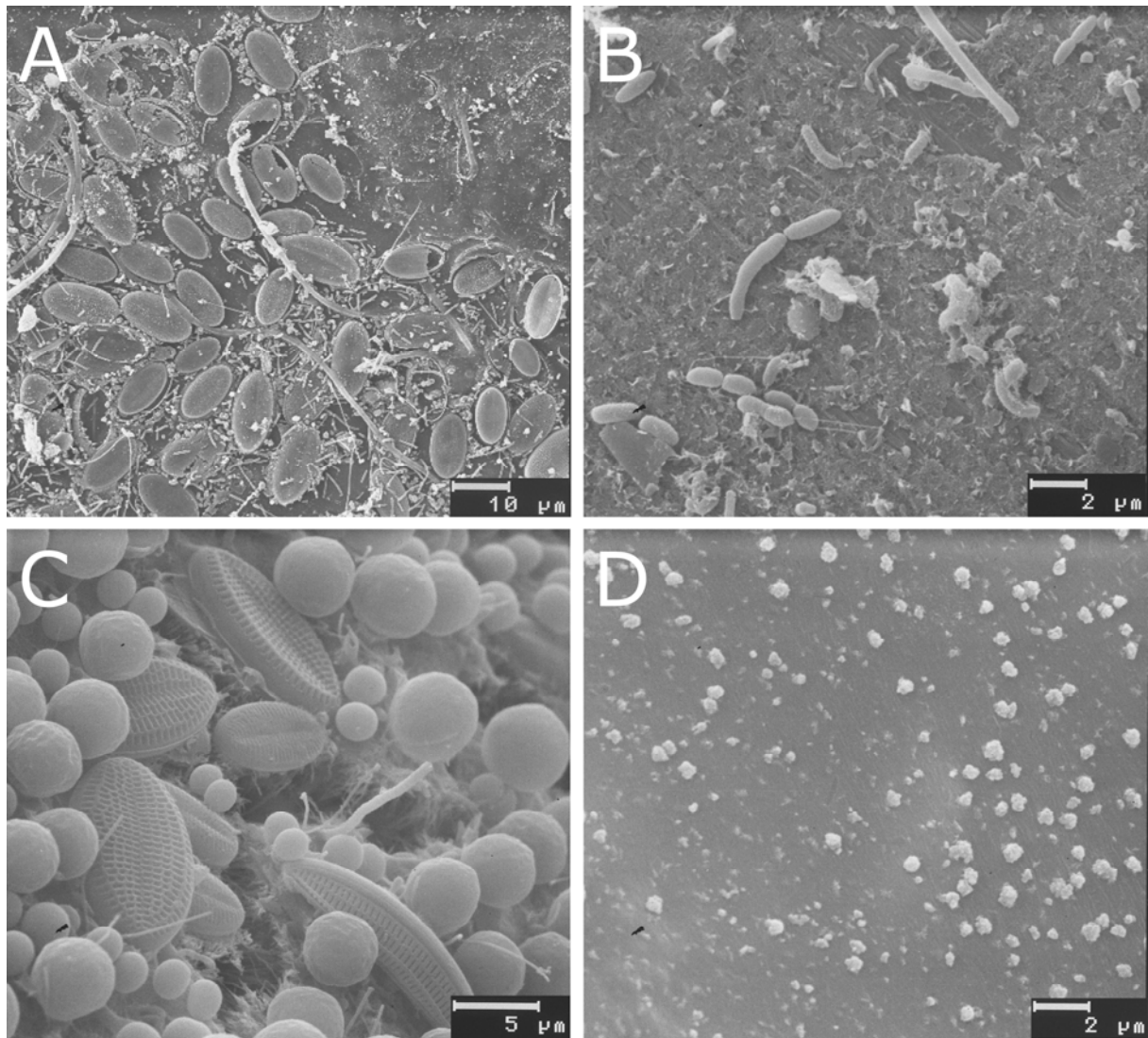
### **Identification of bryozoans**

Bryozoan samples obtained from the Baltic Sea were identified morphologically as *Callopora aurita*, *Electra pilosa*, *Membranipora membranacea*, *Membranipora* sp. and *Tubulipora* sp. Mediterranean samples comprised *Adeonella calveti*, *Carbasea papyrea*, *Cellaria salicornioides*, *Cellepora pumicosa*, *Cryptosula pallasiana*, *Pentapora fascialis*, *Schizobrachiella sanguinea*, *Schizotheca serratimargo*, and *Sertella beaniana*. Details on sampling locations and bryozoan species are given in Table 1-1.

### **SEM examination of Mediterranean bryozoans**

In case of encrusting species (sample IDs 17 to 21), only the side that faced the water column was examined. Due to the sample preparation, the membrane that covers the skeletal structures of the bryozoans broke open.

Although bacteria could be found on the surface of all samples, the degree of microbial cover was highly variable (Fig. 1-1). A distinct biofilm with diatoms and bacteria embedded in a thick extracellular matrix could be found on both encrusting *C. pallasiana* species (sample IDs 17 and 19). The arborescent bryozoan *S. serratimargo* (ID 16) showed a comparable cover but diatoms were lacking. Biofilms also consisting of diatoms and bacteria grew to a lesser degree on both encrusting *S. sanguinea* species (IDs 18 and 20). Hence, all specimens taken from the Limski Channel (IDs 16-19) displayed a distinct biofilm. With regard to the arborescent samples *C. salicornioides* (ID 12) and *P. fascialis* (ID 15) showed comparable cover. The surface of *A. calveti* (ID 11) was covered with spherical particles, which could not be identified. Diatoms were not found on samples *C. papyrea*, *S. beaniana*, *S. serratimargo*, and *C. pumicosa* (IDs 13, 14, 16, and 21). The surface of *C. pumicosa* (ID 21) was uncolonised except for a few areas showing bacteria.



**Figure 1-1. SEM pictures of bryozoan surfaces.** A: sample ID 19 (*Cryptosula pallasiana*), thick biofilm with diatoms and microorganisms embedded in an extracellular matrix. B: sample ID 13 (*Carbasea papyrea*), bacteria attached on a surface. C: sample ID 11 (*Adeonella calveti*), diatoms and unidentified cocci. D: sample ID 21 (*Cellepora pumicosa*), unpopulated surface area.

**Bacterial isolates from bryozoans and 16S rDNA sequence-based phylogenetic analysis of bioactive bacteria**

For isolation of pure cultures, 340 strains were selected from the agar spread plates according to colony morphology and colour from each medium and bryozoan host (Table 1-1). A total of 90 strains with antibiotic activity were classified phylogenetically (Table 1-2, Fig. 1-2).

The great majority of the isolates belonged to the *Proteobacteria* phylum. Members of the *Gammaproteobacteria* class (77 isolates) were affiliated to the genera *Alteromonas* (15), *Marinomonas* (2), *Pseudoalteromonas* (37), *Pseudomonas* (1), *Shewanella* (13), and *Vibrio* (9). Whereas *Gammaproteobacteria* could be isolated from all 14 bryozoan species, representatives of the *Alphaproteobacteria* class (11 isolates) were exclusively found on eight out of nine different bryozoan species obtained from the Mediterranean Sea. A single isolate of the genus *Pseudovibrio* (B411) was derived from *C. pumicosa* and ten isolates from seven different bryozoan species were related to the genus *Sphingomonas*. In addition, one isolate from *E. pilosa* was classified as a member of the *Actinobacteria* (B150) and one strain from *S. sanguinea* was identified as a representative of the *Flavobacteria* (B390).

The sequences were grouped into following 27 phlotypes, according to a sequence similarity value  $\geq 99.5\%$ : “B130b” (containing B22, B28, B30, B36, B44, B49, B60, B62, B94, B125a, B130b, B137, B149, B158, B162b, B164, B178, B328), “B35” (B35, B131b, B129, B172, B179, B193), “B296” (B281, B296, B303, B304), “B199b” (B145, B169, B180, B199b), “B201” (B160, B166, B170.2, B201), “B327” (B327, B330, B331, B344, B345, B348, B349, B359, B362, B377, B408), “B326” (B275, B278, B326, B358), “B69” (B69, B109, B131a), “B231” (B80, B231), “B105” (B105, B234), “B147” (B147, B153, B159, B165, B194), “B163” (B163, B171), “B200” (B200, B206), “B205” (B167, B205), “B273” (B273, B285, B291, B309, B350, B364), “B382” (B269, B340, B382, B394) and the single sequence phlotypes “B173.2”, “B130a”, “B142”, “B157”, “B225”, “B237a”, “B246”, “B151”, “B411”, “B390” and “B150” (Fig. 1-2, Table 1-2).

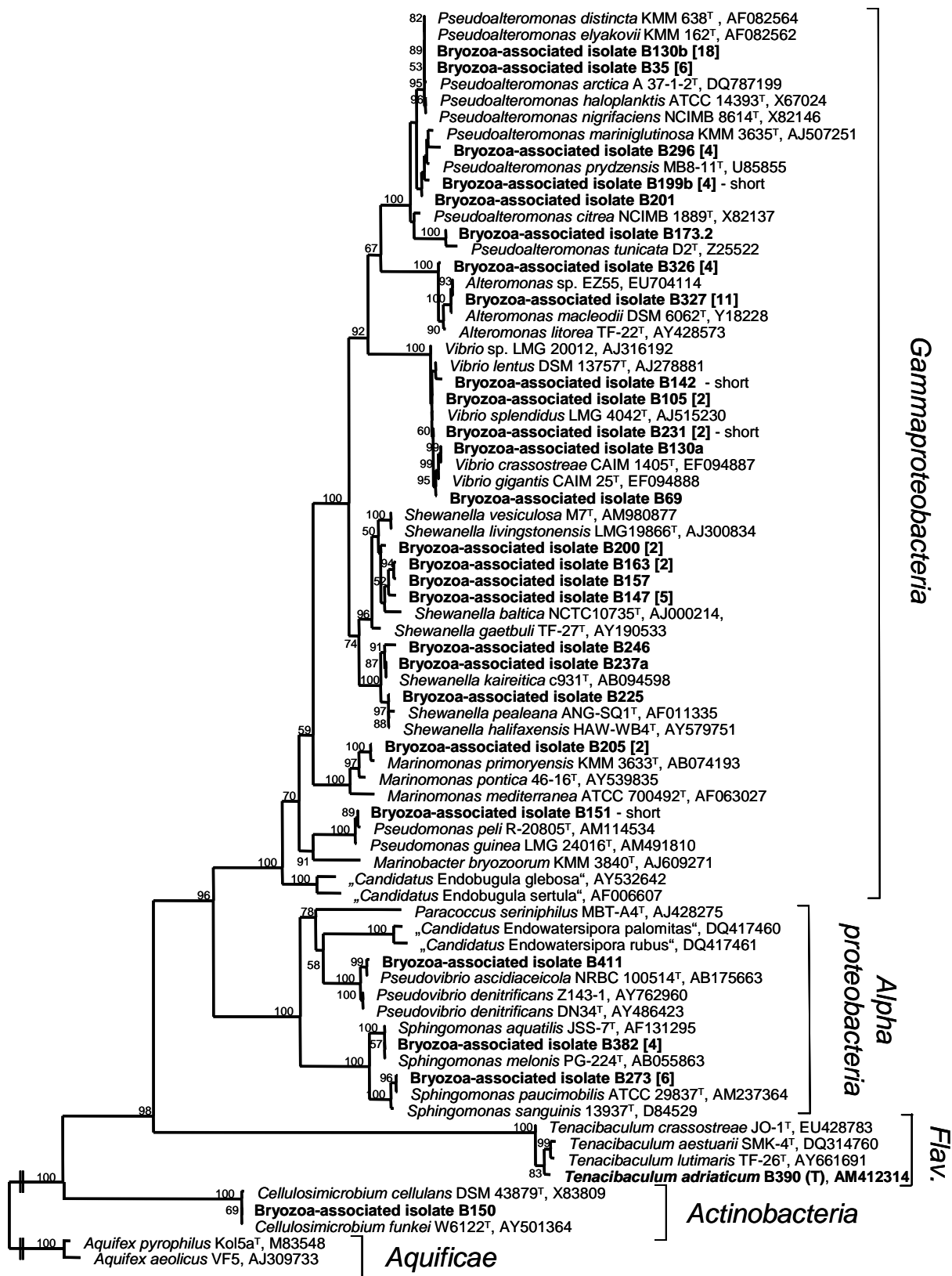
All strains affiliated to phlotypes “B199b” (*Pseudoalteromonas*, 4 isolates), “B201” (*Pseudoalteromonas*, 3 isolates) and “B147” (*Shewanella*, 5 isolates) were found in all bryozoan samples from Langeland (sample IDs 6-8, all *E. pilosa*) but not elsewhere.

Also, strains affiliated to phlotypes “B296” (*Pseudoalteromonas*, 4 isolates), “B69” (*Vibrio*, 3 isolates) or “B205” (*Marinomonas*, 2 isolates) could only be found at Rovinj dredge 1 (IDs 12-14), Læsø (IDs 3-5) or Langeland (IDs 7 and 8), respectively. The other phlotypes exhibited a non site-specific dispersal but were divided into Baltic and Mediterranean-only groups with the exception of “B130b” (*Pseudoalteromonas*, 18 isolates) that contained



representatives from both seas. Neither *Alteromonas* nor *Sphingomonas* representatives were found in bryozoans sampled from the Baltic Sea. In contrast, *Vibrio*, *Shewanella* and *Marinomonas*-related strains were obtained only from the Baltic Sea bryozoans. All but one *Shewanella*-related isolates were obtained from *Electra pilosa*.

Some isolates, which showed similarity values of less than 98% to validly described species, could possibly represent members of new species (Stackebrandt & Ebers 2006). New *Shewanella* species might be represented by the strains of phylotypes “B147”, “B163” (both <97% to *S. gaetbuli* TF-27<sup>T</sup>), “B157” (<98% to *S. baltica* NCTC10735<sup>T</sup>) and “B200” (<97% to *S. vesiculosa* M7<sup>T</sup>), as well as by the *Pseudoalteromonas*-related strain B173.2 (<98% to *P. tunicata* D2<sup>T</sup>). Another new species, the *Flavobacteriaceae* related strain B390, a *S. sanguinea*-associated isolate, was described as the type strain of *Tenacibaculum adriaticum* elsewhere (Heindl *et al.* 2008).



**Figure 1-2. Maximum-likelihood tree** constructed from 16S rRNA gene sequences showing the phylogenetic relationships of strains from this study to closely related species and some other selected strains. Non-parametric bootstrapping analysis (100 datasets) was conducted. Values equal to or greater than 50 are shown. The scale bar indicates the number of substitutions per nucleotide position. The total number of represented sequences is given in square brackets. “short” indicates sequences <1000 bp. *Flav.*, *Flavobacteria*.

**Antimicrobial activity**

Approximately 30% of the isolated 340 bacteria from the bryozoan samples showed antibacterial activities against at least one indicator strain (Table 1-1). *B. subtilis* was inhibited by 84%, *S. lentus* by 67% and *E. coli* by 4% of all active isolates. A total of 50% were active against both Gram-positive indicator bacteria. No inhibitory activity against *C. glabrata* was observed. Almost the same percentage of active isolates could be found referring to the bryozoans sampled from the Baltic Sea and from the Mediterranean Sea (30% and 29%, respectively) but bacteria from the Baltic Sea sites inhibited growth of both *B. subtilis* and *S. lentus* more often (67% of Baltic Sea active bacteria versus 20% of Mediterranean active bacteria).

Samples from *E. pilosa* taken near Langeland (IDs 6, 7 and 8) yielded the highest counts of active bacteria, since 37 out of 53 isolates (70%) displayed antibiotic activities. Among these active strains, 27 (73%) showed inhibition of both *B. subtilis* and *S. lentus*. On the other hand, *E. pilosa* samples from the Lysegrund site (IDs 3 and 4) provided the lowest numbers of active isolates (9%).

The bulk of *Alteromonas*-related isolates (14 out of 15), and *T. adriaticum* B390<sup>T</sup> exclusively inhibited the growth of *B. subtilis* (Table 1-2). On the other hand, most of *Sphingomonas*-related isolates (8 out of 10) and the *Cellulosimicrobium* isolate B150 inhibited the growth of *S. lentus* only. The majority of *Pseudoalteromonas* (31 out of 37), as well as *Shewanella*-related (11 out of 13) strains, suppressed growth of both *B. subtilis* and *S. lentus*. Inhibition of *E. coli* occurred in addition to antibiotic activity against both Gram-positive tests strains and was demonstrated by four isolates, including members of *Pseudoalteromonas* (B131b and B62) as well as *Pseudovibrio* (B411) and *Vibrio* (B131a).

Interestingly activity tests of ten *Alteromonas* isolates (B275, B278, B326, B344, B348, B349, B358, B359, B362 and B377) indicated enhanced growth of *S. lentus*, *E. coli*, and *C. glabrata* besides their inhibition of *B. subtilis* (data not shown).

## Chapter 1

**Table 1-1. Classification of bryozoan specimens, sampling sites and antibiotic activities.**

Sampling date	Sampling site	Location & Method	Sample ID	Bryozoan species	Number of bacteria	Isolated	Active	Activity in detail <sup>a</sup>			
								Sl	Bs	Ec	Sl&Bs
					total	218	66	50	60	3	44
2003-11-20	Kattegatt (56°16,10'N 11°47,41'E) at depth 8.4 -9.0 m	station Lysegrund dredged	1	<i>Tubulipora</i> sp.	29	3	3	3	0	3	
			2	<i>Callopora aurita</i>	30	6	6	5	1	5	
			3	<i>Electra pilosa</i> (on <i>Polysiphonia</i> sp.)	32	3	2	2	0	1	
			4	<i>E. pilosa</i> (on <i>Fucus</i> sp.)	22	2	0	2	0	0	
			5	<i>Membranipora</i> sp.	26	8	5	8	2	5	
						139	22	16	20	3	14
2004-05-17	Baltic Sea (54°40,09'N 10°45,25E) at depth 24.8 m	Langeland dredged	6	<i>E. pilosa</i> (on <i>Laminaria saccharina</i> )	19	13	10	12	0	9	
			7	<i>E. pilosa</i> (on <i>L. saccharina</i> )	20	7	7	6	0	6	
			8	<i>E. pilosa</i> (on <i>Delesseria</i> sp.)	14	7	7	6	0	6	
						53	37	31	33	0	27
2004-05-26	Kattegatt (57°27,98'N 11°9,41'E) at depth 23.3 m	Læsø dredged	9	<i>E. pilosa</i> (on <i>L. saccharina</i> )	13	5	2	5	0	2	
			10	<i>Membranipora membranacea</i> (on <i>L. saccharina</i> )	13	2	1	2	0	1	
						26	7	3	7	0	3
					total	122	35	18	25	1	7
2004-06-08	Adriatic Sea at depth 6 m	Rovinj: Banjol scuba diving	11	<i>Adeonella calveti</i>	13	4	3	1	0	0	
2004-06-09	Adriatic Sea at depth 25 - 30 m	Rovinj dredge 1 dredged	12	<i>Cellaria salicornioides</i>	8	4	2	3	0	1	
			13	<i>Carbacea papyrea</i>	10	3	3	2	0	2	
			14	<i>Sertella beaniana</i>	12	4	5	2	0	2	
			15	<i>Pentapora fascialis</i>	9	3	1	2	0	0	
						39	14	11	9	0	5
2004-06-11	Adriatic Sea at depth 5 m	Rovinj: Limski channel scuba diving	16	<i>Schizotheca serratimargo</i>	10	5	2	3	0	0	
			17	<i>Cryptosula pallasiana</i> (on a stone)	11	5	1	4	0	0	
			18	<i>Schizobrachiella sanguinea</i> (on a stone)	9	4	1	3	0	0	
			19	<i>C. pallasiana</i> (on a wine bottle)	13	2	1	2	0	1	
						43	16	5	12	0	1
2004-06-13	Adriatic Sea at depth 25 - 30 m	Rovinj dredge 2 dredged	20	<i>S. sanguinea</i> (on a bivalve)	16	2	0	2	0	0	
			21	<i>Cellepora pumicosa</i> (on a stone)	11	3	2	2	1	1	
						27	5	2	4	1	1
					all isolates	340	101	68	85	4	51

<sup>a</sup>Sl, *Staphylococcus lentus*; Bs, *Bacillus subtilis*; Ec, *Escherichia coli*.

**Table 1-2. 16S rDNA based classification (BLAST) and antibiotic activity of the isolates**

Isolate	Base-pairs	Nearest phylogenetic neighbour	Accession No.	Similarity (%)	Sample ID <sup>b</sup>	Antibiotic activity <sup>c</sup>		
						Sl	Bs	Ec
<b>Actinobacteria</b>								
<i>Cellulosimicrobium</i>								
B150	1120	<i>Cellulomonas</i> sp. BCHID458428	AY545638	99	6	+	-	-
<b>Flavobacteria</b>								
<i>Tenacibaculum</i>								
B390 <sup>a</sup>	1441	<i>Tenacibaculum adriaticum</i> B390 <sup>T</sup>	AM412314	100	20	-	+	-
<b>Alphaproteobacteria</b>								
<i>Pseudovibrio</i>								
B411	1406	<i>Pseudovibrio ascidiaceicola</i> F423	AB175663	99	21	+	+	+
<i>Sphingomonas</i>								
B382	1065	<i>Sphingomonas melonis</i> strain MPU95	AB334774	100	19	+	+	-
B269	767	<i>Sphingomonas</i> sp. Camargue	DQ218322	98	11	+	-	-
B340	781	<i>Sphingomonas</i> sp. Camargue	DQ218322	100	16	+	-	-
B394	779	<i>Sphingomonas</i> sp. Camargue	DQ218322	100	20	-	+	-
B273	1072	<i>Sphingomonas</i> sp. SKJH-30	AY749436	100	11	+	-	-
B285	753	<i>Sphingomonas paucimobilis</i> ATCC29837	U37337	100	12	+	-	-
B291	775	<i>Sphingomonas paucimobilis</i> ATCC29837	U37337	99	13	+	-	-
B309	768	<i>Sphingomonas paucimobilis</i> ATCC29837	U37337	99	14	+	-	-
B350	752	<i>Sphingomonas paucimobilis</i> ATCC29837	U37337	100	17	+	-	-
B364	778	<i>Sphingomonas paucimobilis</i> ATCC29837	U37337	100	18	+	-	-
<b>Gammaproteobacteria</b>								
<i>Alteromonas</i>								
B326	1467	<i>Alteromonas macleodii</i> 'Deep ecotype'	CP001103	99	15	-	+	-
B275	1106	<i>Alteromonas macleodii</i> 'Deep ecotype'	CP001103	99	11	-	+	-
B278	1140	<i>Alteromonas</i> sp. strain KE10	AB015135	100	12	-	+	-
B358	742	<i>Alteromonas</i> sp. SCSWC25	FJ461449	99	18	-	+	-
B327	1455	<i>Alteromonas</i> sp. CF14-2	FJ170032	99	15	-	+	-
B331	744	<i>Alteromonas macleodii</i> DSM 6062	Y18228	100	16	-	+	-
B330	757	<i>Alteromonas macleodii</i> DSM 6062	Y18228	100	16	-	+	-
B344	675	<i>Alteromonas macleodii</i> DSM 6062	Y18228	100	17	-	+	-
B345	709	<i>Alteromonas macleodii</i> DSM 6062	Y18228	99	17	-	+	-
B348	1392	<i>Alteromonas</i> sp. CF14-2	FJ170032	99	17	-	+	-
B349	775	<i>Alteromonas macleodii</i> DSM 6062	Y18228	99	17	-	+	-
B359	1137	<i>Alteromonas</i> sp. S1613	FJ457262	100	18	-	+	-
B362	1455	<i>Alteromonas</i> sp. CF14-2	FJ170032	99	18	-	+	-
B377	773	<i>Alteromonas macleodii</i> DSM 6062	Y18228	98	19	-	+	-
B408	1371	<i>Alteromonas</i> sp. CF14-2	FJ170032	99	21	+	-	-
<i>Marinomonas</i>								
B205	1464	<i>Marinomonas</i> sp. M12-1	FN377704	99	8	+	-	-
B167	775	<i>Marinomonas</i> sp. NJ522	DQ191961	99	7	-	+	-
<i>Pseudoalteromonas</i>								
B35	1458	<i>Pseudoalteromonas</i> sp. M12-3	FN377726	99	2	+	+	-
B129	1155	<i>Pseudoalteromonas</i> sp. L203	EU828455	99	5	+	+	-
B131b	1458	<i>Pseudoalteromonas</i> sp. SM495	FJ436981	100	5	+	+	+
B172	1100	<i>Pseudoalteromonas</i> sp. IC006	U85856	99	7	-	+	-
B179	789	<i>Pseudoalteromonas</i> sp. L203	EU828455	100	7	+	+	-
B193	1448	<i>Pseudoalteromonas arctica</i> strain A 37-1-2	DQ787199	100	8	+	+	-

Continued on next page

Table 1-2. continued

Isolate	Base-pairs	Nearest phylogenetic neighbour	Accession No	Similarity (%)	Sample ID <sup>b</sup>	Antibiotic activity <sup>c</sup>		
						SI	Bs	Ec
B130b	1458	<i>Pseudoalteromonas</i> sp. M12-3	FN377726	99	5	-	+	-
B22	1458	<i>Pseudoalteromonas</i> sp. M12-3	FN377726	100	1	+	+	-
B28	1076	<i>Pseudoalteromonas</i> sp. IC013	U85859	99	1	+	+	-
B30	1458	<i>Pseudoalteromonas</i> sp. M12-3	FN377726	100	1	+	+	-
B36	985	<i>Pseudoalteromonas</i> sp. IC013	U85859	100	2	+	+	-
B44	1458	<i>Pseudoalteromonas</i> sp. M12-3	FN377726	100	2	+	-	-
B49	859	<i>Pseudoalteromonas</i> sp. IC013	U85859	100	2	+	+	-
B60	757	<i>Pseudoalteromonas</i> sp. IC013	U85859	100	2	+	+	-
B62	1163	<i>Pseudoalteromonas</i> sp. BSw20441	EF639368	99	2	+	+	+
B94	1364	<i>Pseudoalteromonas</i> sp. M12-3	FN377726	100	3	+	+	-
B125a	762	<i>Pseudoalteromonas</i> sp. IC013	U85859	99	5	+	+	-
B137	1458	<i>Pseudoalteromonas</i> sp. M12-3	FN377726	100	5	+	+	-
B149	774	<i>Pseudoalteromonas</i> sp. IC013	U85859	100	6	-	+	-
B158	789	<i>Pseudoalteromonas</i> sp. IC013	U85859	100	6	-	+	-
B162b	782	<i>Pseudoalteromonas</i> sp. IC013	U85859	100	7	+	+	-
B164	772	<i>Pseudoalteromonas</i> sp. IC013	U85859	100	7	-	+	-
B178	782	<i>Pseudoalteromonas</i> sp. IC013	U85859	100	7	+	+	-
B328	750	<i>Pseudoalteromonas</i> sp. IC013	U85859	100	16	-	+	-
B199b	776	<i>Pseudoalteromonas porphyrae</i> S2-65	AY771715	100	8	+	+	-
B145	761	<i>Pseudoalteromonas porphyrae</i> S2-65	AY771715	100	6	+	+	-
B169	766	<i>Pseudoalteromonas porphyrae</i> S2-65	AY771715	99	7	+	+	-
B180	774	<i>Pseudoalteromonas porphyrae</i> S2-65	AY771715	100	7	+	+	-
B296	1463	<i>Pseudoalteromonas</i> sp. CF14-5	FJ170035	99	13	+	+	-
B281	774	<i>Pseudoalteromonas</i> sp. S4491	FJ457244	100	12	+	+	-
B303	752	<i>Pseudoalteromonas</i> sp. S1650	FJ457155	99	14	+	+	-
B304	772	<i>Pseudoalteromonas</i> sp. S1650	FJ457155	99	14	+	+	-
B201	1464	<i>Pseudoalteromonas</i> sp. BSw20083	EU365608	100	8	+	+	-
B160	788	<i>Pseudoalteromonas</i> sp. RS-61B	FN377725	100	6	+	+	-
B166	775	<i>Pseudoalteromonas</i> sp. RS-61B	FN377725	100	7	+	+	-
B170.2	788	<i>Pseudoalteromonas</i> sp. RS-61B	FN377725	100	7	+	+	-
B173.2	1200	<i>Pseudoalteromonas</i> sp. L145	AM913917	100	7	+	-	-
<i>Pseudomonas</i>								
B151	780	Arctic seawater bacterium Bsw20350	DQ064611	99	6	-	+	-
<i>Shewanella</i>								
B200	1467	<i>Alteromonadaceae</i> bacterium E1	AF539787	99	8	+	+	-
B206	1467	<i>Alteromonadaceae</i> bacterium E1	AF539787	99	8	+	+	-
B237a	1380	<i>Shewanella kaireiae</i> c931	AB094598	100	9	-	+	-
B225	1465	<i>Shewanella halifaxensis</i> HAW-EB4	CP000931	99	9	-	+	-
B163	1467	<i>Shewanella</i> sp. CR07-A1	EU979479	99	7	+	+	-
B171	1467	<i>Shewanella</i> sp. CR07-A1	EU979479	99	7	+	+	-
B147	1467	<i>Shewanella</i> sp. IE9-1	EF105397	100	6	+	+	-
B153	1467	<i>Shewanella</i> sp. IE9-1	EF105397	99	6	+	+	-
B159	1467	<i>Shewanella</i> sp. IE9-1	EF105397	100	6	+	+	-
B165	1467	<i>Shewanella</i> sp. IE9-1	EF105397	100	7	+	+	-
B194	1467	<i>Shewanella</i> sp. IE9-1	EF105397	100	8	+	+	-
B157	1467	<i>Shewanella</i> sp. CR07-A1	EU979479	99	6	+	+	-
B246	1379	<i>Shewanella piezotolerans</i> WP3	CP000472	98	10	+	+	-

Continued on next page

**Table 1-2.** continued

Isolate	Base-pairs	Nearest phylogenetic neighbour	Accession No	Similarity (%)	Sample ID <sup>b</sup>	Antibiotic activity <sup>c</sup>		
						Sl	Bs	Ec
<i>Vibrio</i>								
B69	1477	<i>Vibrio splendidus</i> isolate PB1-10rrnA	EU091325	99	3	+	-	-
B109	1125	<i>Vibrio</i> sp. S4738	FJ457608	100	4	-	+	-
B131a	755	<i>Vibrio</i> sp. S4738	FJ457608	99	5	+	+	+
B130a	1371	<i>Vibrio</i> sp. S3897	FJ457573	99	5	-	+	-
B231	740	<i>Vibrio</i> sp. S648	FJ457326	99	9	+	+	-
B80	709	<i>Vibrio splendidus</i> LGP32	FM954972	99	3	-	+	-
B105	1164	<i>Vibrio splendidus</i> LGP32	FM954972	100	4	-	+	-
B234	1125	<i>Vibrio</i> sp. S4734	FJ457604	99	9	+	+	-
B142	770	<i>Vibrio splendidus</i> LGP32	FM954972	100	5	-	+	-

<sup>a</sup> type strain of the species *Tenacibaculum adriaticum*.

<sup>b</sup> Bryozoan species: ID1, *Tubulipora* sp.; ID2, *Callopora aurita*; IDs3, 4, 6, 7, 8, 9, *Electra pilosa*; ID5 *Membranipora* sp.; ID10 *Membranipora membranacea*; ID11 *Adeonella calveti*; ID12, *Cellaria salicornioides*; ID13, *Carbasea papyrea*; ID14, *Sertella beaniana*; ID15, *Pentapora fascialis*; ID16, *Schizotheca serratimargo*; IDs17, 19, *Cryptosula pallasiana*; IDs18, 20, *Schizobrachiella sanguinea*; ID21, *Cellepora pumicosa*.

<sup>c</sup> Sl, *Staphylococcus lentus*; Bs, *Bacillus subtilis*; Ec, *Escherichia coli*

## **Discussion**

### **Microbial cover on bryozoan samples from the Mediterranean Sea**

As far as we know, among the bryozoans collected in this study, only *Electra* sp., *Tubulipora* sp., *Membranipora* sp., and *Callopora* sp. had been examined previously for a bacterial biofilm (Gerdes *et al.* 2005b, Kittelmann & Harder 2005). Therefore, scanning electron microscopic studies were carried out with all nine bryozoan species sampled from the Mediterranean Sea. *S. serratimargo*, *C. pallasiana* and *S. sanguinea*, which were collected in the Limski Channel, displayed a thick microbial coverage. There is a constant water exchange between this channel and the Adriatic Sea. Therefore, since fresh water from land does not mix and drains from the surface into the sea, the bottom water and its fauna are of marine origin. Stable thermoclines can establish in summer and these are a possible cause of oxygen depletion to a minimum of about 70%, whereas prevailing homoeothermic conditions in winter allow for full oxygenation (Daniels 1970, Uffenorde 1970). The reduced oxygen availability in the Limski Channel during the summer could impair the bryozoans' fitness and impede the disposal of epibionts, therefore favouring colonization on their surfaces.

Since the *C. pallasiana* and *S. sanguinea* specimens, collected either in the Limski Channel or via dredging (Rovinj dredge 2), showed the same extent of microbial cover, it might also be possible that acceptance of microbial growth is a characteristic trait of these species. This hypothesis is supported by the finding that, in contrast to *S. sanguinea*, the surface of *C. pumicosa* (also collected by Rovinj dredge 2) was almost free from microorganisms. This contrast in microbial colonization might also be the result of a different defensive strategy exhibited by *C. pumicosa*. The *P. denitrificans*-related isolate B411, which was isolated only from this bryozoan, demonstrated antibiotic activities against both Gram-positive and Gram-negative strains. Thus, this strain might also play a role in the defence of its host by producing biologically active substances.

Kaselowsky *et al.* (2005) and Gerdes *et al.* (2005b) investigated the growth behaviour in several different bryozoan species in response to microbial fouling. They stated a decrease of biofilm coverage towards warm water habitats and no specific biofilm type selected by the investigated bryozoans. In contrast, our findings favour a rather bryozoan-species dependent microbial coverage in the case of *S. sanguinea*.



### **Classification of antibiotically active bacteria**

Regarding specific bacterial associations with the hosts it has to be considered that sampling and sample preparation methods have to be viewed critically, especially during the dredging procedure, since contamination with alien bacteria could not be completely prevented.

However, in this study, members of the genera *Cellulosimicrobium*, *Tenacibaculum*, *Pseudovibrio*, *Sphingomonas*, *Alteromonas*, *Marinomonas*, *Pseudoalteromonas*, *Pseudomonas*, *Shewanella* and *Vibrio* were found to be associated with bryozoans.

The major part of related validly described species originates from marine environments. In fact, all type strains of the genera *Tenacibaculum*, *Marinomonas*, *Pseudovibrio* and *Alteromonas* have been isolated from marine habitats. In the case of *Pseudoalteromonas* and *Shewanella*, only *P. nigrifaciens* NCIMB 8614<sup>T</sup> (isolated from dairy butter) and *S. baltica* NCTC10735<sup>T</sup> (isolated from oil brines in Japan) were not of marine origin. No marine representatives have been found among the next related type strains of the genera *Cellulosimicrobium*, *Sphingomonas* and *Pseudomonas*.

Previous studies on bacteria associated with the bryozoan *Flustra foliacea* showed contrasting results (Pukall *et al.* 2001). In one sample taken from the North Sea, west of Helgoland, members of the *Gammaproteobacteria* (genera *Shewanella*, *Pseudoalteromonas*, *Psychrobacter* and *Pseudomonas*), as well as several *Bacteroidetes* isolates, were predominantly obtained, which was similar to our results. However, bacterial isolates from two other *Flustra foliacea* samples from Steingrund, northeast of Helgoland, were dominated by Gram-positive representatives (with *Bacillus* as the most abundant genus). The authors concluded that these bryozoans may tolerate colonization by bacteria that are common in the marine environment.

Our data suggest a site-specific rather than a bryozoan-specific distribution for most of the isolates because their phylogenetic affiliation was identical regardless of the bryozoan host used for isolation. Differences in the occurrence of bacteria belonging to different genera could be observed between bryozoans from the Baltic Sea and the Mediterranean Sea with *Shewanella* and *Vibrio* representatives only found in the former, members of *Sphingomonas* and *Alteromonas* in the latter and *Pseudoalteromonas* strains in both.

The cultivation-based method used in this work could generally favour microorganisms that easily adapt to different environments and therefore grow under various conditions, including those of the laboratory. Nevertheless, a specific bacterial community would be strongly dependent on its host's attributes, which are difficult to simulate *in vitro*.

In a culture-independent approach, biofilms on various bryozoan species sampled in Japan and New Zealand were examined by Gerdes *et al.* (2005b). Selected bryozoan specimens (e.g. *Cellepora* sp., *Bugula neritina*, *Callopora decidua*) sampled in shallow water of Japan and New Zealand were investigated by DGGE analysis of 16S rDNA fragments. The heterogeneous DNA-banding pattern, using general bacterial primers, indicated neither host nor site-specific associations. However, when using *Bacillaceae* specific primers some DNA-profile conformities were revealed. The bryozoans used for this DGGE analysis lived adjacent on kelp roots and were not further identified.

Kittelmann & Harder (2005) also performed DGGE-based analyses on four bryozoan species from the North Sea and compared them to empty mussel shells as references. *Conopeum reticulum* revealed site-specific influences on the bacterial population. In contrast, *Aspidelectra melolontha*, *Electra monostachys*, and *E. pilosa* showed consistencies in their related bacterial populations throughout three different sampling locations. The authors considered these bacterial communities as bryozoan-specific but details on the phylogenetic classification were not given.

In the case of several *Bugula* bryozoans, species-specific endosymbionts (*Candidatus* Endobugula) could be found with molecular techniques and they were assigned to the *Gammaproteobacteria* (Lim-Fong *et al.* 2008), whereas those of the bryozoan genus *Watersipora* belonged to the *Alphaproteobacteria* (*Candidatus* Endowatersipora) (Anderson & Haygood 2007). The symbiont of *Bugula neritina*, *Candidatus* Endobugula sertula, was shown to be the true originator of the clinically interesting bryostatins, which were also thought to play a biological role in the protection of bryozoan larvae from predation (Sharp *et al.* 2007b).

Apart from the *Flavobacteriaceae* bacterium *Tenacibaculum adriaticum* B390<sup>T</sup> (Heindl *et al.* 2008), two more bryozoan-associated isolates have been validly described as new species: the alphaproteobacterium *Paracoccus seriniphilus* MBT-A4<sup>T</sup> from a *Bugula plumosa* sample taken at Helgoland (North Sea) (Pukall *et al.* 2003) and the gammaproteobacterium *Marinobacter bryozorum* KMM 3840<sup>T</sup> from an unidentified bryozoan occurring in the Bering Sea (Pacific Ocean) (Romanenko *et al.* 2005). Nevertheless, whether or not these isolates were specifically associated with their hosts was not examined further.

**Antibiosis**

The antibiosis of practically all active isolates (96%) was directed exclusively against Gram-positive indicator strains. Only four isolates displayed an additional inhibitory effect on *E. coli*. Since all but one antibiotically active isolates were Gram-negative they could hinder the growth of Gram-positive bacteria on bryozoan surfaces *in vivo*. Antagonistic interactions between marine bacteria are an important aspect in shaping microbial communities, which are associated with non-living surfaces or with macroorganisms, such as sponges (Grossart *et al.* 2004, Long & Azam 2001, Mangano *et al.* 2009).

Concerning the specificity of antibiotic activities, two different observations were made. While consistent antibiosis profiles were found within the phylotypes “B273” (*Sphingomonas*), “B326” (*Alteromonas*), “B199b”, “B296”, “B201” (*Pseudoalteromonas*), “B200”, “B163” and “B147” (*Shewanella*), isolates affiliated to “B382” (*Sphingomonas*), “B327” (*Alteromonas*), “B205” (*Marinomonas*), “B35”, “B130b” (*Pseudoalteromonas*), “B69”, “B231” and “B105” (*Vibrio*) inhibited target organisms in a strain-specific manner. (Table 1-2)

Shnit-Orland & Kushmaro (2009) isolated similar bacteria, (*inter alia* members of the genera *Vibrio*, *Pseudoalteromonas*, and *Shewanella* comprising 67.4% of all isolates) from coral-mucus, and tested them against different indicator organisms (*B. cereus*, *E. coli*, *Staphylococcus aureus*, and others). In this case, antibiosis was also favourably aimed at Gram-positive indicator bacteria and was found to be rather strain-specific. Comparable studies of psychrotrophic bacteria from Antarctica also showed strain-specific antibiotic patterns, but those isolates were predominantly affiliated to *Actinobacteria* (Lo Giudice *et al.* 2007a, Lo Giudice *et al.* 2007b).

Among the producers of natural products from marine sources, the marine microorganisms play a major role (Blunt *et al.* 2009). The bacterial *Streptomyces* species (> 240 new chemical entities) followed by *Alteromonas* (47), *Bacillus* (37), *Vibrio* (29), *Pseudomonas* (28), *Actinomyces* (25), and *Pseudoalteromonas* (25) were the most prominent ones (Laatsch 2006). With the exception of the actinomycetes and *Bacillus*, these genera also represent the greater number of isolates obtained in this study.

However, only a few substances from bacterial genera related to those found in this study were reported as antibiotically active. Such products have been reported for marine *Alteromonas* (Shiozawa *et al.* 1993, Stierle & Stierle 1992), *Pseudomonas* (Bultel-Poncé *et al.* 1999, Jayatilake *et al.* 1996), *Vibrio* (Kobayashi *et al.* 1994), *Pseudoalteromonas* (Hayashida-Soiza *et al.* 2008, Yoshikawa *et al.* 1997) and *Marinomonas* species (Lucas-Elio *et al.* 2005).

*Pseudovibrio denitrificans*-related strains with antibacterial properties were the dominant cultured isolates of several sponges sampled near Rovinj (Muscholl-Silberhorn *et al.* 2008). A close relative of isolate B411, the *P. denitrificans* Z143-1 strain, was reported to produce the red pigment heptylprodigiosin, a substance with activity against *S. aureus* (Sertan-de Guzman *et al.* 2007). Although *Shewanella* strains were reported as antibiotically active isolates (De La Rosa-García *et al.* 2007, Mangano *et al.* 2009, Shnit-Orland & Kushmaro 2009), similar substances with antimicrobial properties have not been published as far as we know.

Since compounds with antibacterial properties had been published for members of six related genera from this study, the phylogenetic affiliation of active bacteria found may not be surprising. Nevertheless, these reports, as cited above, were scarce, and the production of secondary metabolites could differ at the species or strain level. Studies regarding the identification of antibiotically active compounds produced by strains of the remaining genera from this study (*Cellulosimicrobium*, *Shewanella*, *Sphingomonas* and *Tenacibaculum*) are either not known or are very scarce.

Apart from growth inhibiting activities, growth enhancing capabilities were also observed in this study with some *Alteromonas*-related isolates. In an effort to obtain axenic cultures of the cyanobacterium *Prochlorococcus* strain MIT 9215, a heterotrophic contaminant, *Alteromonas* sp. EZ55, has been used as a growth supporter (Morris *et al.* 2008). Other marine bacteria with growth stimulating effects on other bacteria comprised members of the genera *Pseudoalteromonas* and *Vibrio*, as well as the family *Flavobacteriaceae*. In this case, the growth supporting effect could be linked to a reduction of oxidative stress. In another survey, “helper strains”, members of *Pseudoalteromonas*, *Psychrobacter* and others, were used to grow hitherto “uncultivable” bacteria *in vitro*. A 5-amino-acid peptide was identified as a strong growth inducer of the otherwise “uncultivable” strain MSC33 (Nichols *et al.* 2008).

## **Conclusion**

Our study has demonstrated that bryozoa provide a rich source of bacteria with antimicrobial activities. Among those isolates the identification of new bacterial species is likely (in the case of *Shewanella*) or proven (*T. adriaticum* B390<sup>T</sup>). Phylogenetic identification of the antibiotically active isolates suggests a more site-specific distribution than a bryozoan-specific association for most strains. Nevertheless, some isolates might interact with their host in a specific manner, and this has to be studied by further experiments.

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## Chapter 2

# ***Tenacibaculum adriaticum* sp. nov., from a bryozoan in the Adriatic Sea**

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The supplemental material (a neighbour-joining tree and Biolog utilization results) has been added to this chapter.

**Abstract**

A rod-shaped, translucent yellow-pigmented, Gram-negative bacterium, strain B390<sup>T</sup>, was isolated from the bryozoan *Schizobrachiella sanguinea* collected in the Adriatic Sea, near Rovinj, Croatia. 16S rRNA gene sequence analysis indicated affiliation to the genus *Tenacibaculum*, with sequence similarity levels of 94.8 - 97.3% to type strains of species with validly published names. It grew at 5-34 °C, with optimal growth at 18-26 °C, and only in the presence of NaCl or sea salts. In contrast to other type strains of the genus, strain B390<sup>T</sup> was able to hydrolyse aesculin. The predominant menaquinone was MK 6 and major fatty acids were iso-C<sub>15:0</sub>, iso-C<sub>15:0</sub>3-OH, and iso-C<sub>15:1</sub>. The DNA G+C content was 31.6 mol%. DNA-DNA hybridization and comparative physiological tests were performed with type strains *Tenacibaculum aestuarii* JCM 13491<sup>T</sup> and *Tenacibaculum lutimaris* DSM 16505<sup>T</sup>, since they exhibit 16S rRNA gene sequence similarities above 97%. These data, as well as phylogenetic analyses, suggest that strain B390<sup>T</sup> (= DSM 18961<sup>T</sup> = JCM 14633<sup>T</sup>) should be classified as the type strain of a novel species within the genus *Tenacibaculum*, for which the name *Tenacibaculum adriaticum* sp. nov. is proposed.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain B390<sup>T</sup> is AM412314.

The genus *Tenacibaculum* was first proposed by Suzuki *et al.* (2001) as a consequence of the reclassification of the species [*Flexibacter*] *maritimus* (Wakabayashi *et al.* 1986) and [*Flexibacter*] *ovolyticus* (Hansen *et al.* 1992) as *Tenacibaculum maritimum* and *Tenacibaculum ovolyticum*, respectively, and the description of *Tenacibaculum mesophilum* and *Tenacibaculum amylolyticum*. At present, the genus includes five further species: *Tenacibaculum skagerrakense* (Frette *et al.* 2004), *T. lutimaris* (Yoon *et al.* 2005), *T. litoreum* (Choi *et al.* 2006), *T. aestuarii* (Jung *et al.* 2006), and *T. litopenaei* (Sheu *et al.* 2007). All of them were isolated from marine environments. Strain B390<sup>T</sup>, reported in this study, represents the first taxonomically described *Tenacibaculum* species from the Mediterranean Sea.

Bryozoan specimens were collected in the Adriatic Sea near Rovinj, Croatia, and used as sources for isolation and characterization of associated micro-organisms. Strain B390<sup>T</sup> was obtained from a specimen of *Schizobrachiella sanguinea* by the dilution plating technique on tryptic soy broth (TSB; Difco) supplemented with 2.5% (w/v) NaCl and 1.5% (w/v) agar. Subcultivation was done on TSB and half-strength marine broth 2216 (MB; Difco) plates at room temperature (approx. 22 °C). Unless otherwise noted, half-strength MB medium was used in the studies. Agar (1.5% w/v) was added for solidification if needed. Strain B390<sup>T</sup> was maintained in MB supplemented with 10% (v/v) DMSO at -80 °C.

Phenotypic and phylogenetic characterization was done according to the minimal standards for describing new taxa of the family *Flavobacteriaceae* proposed by Bernardet *et al.* (2002). Colony morphology was determined with 5-day-cultures on agar plates with a binocular microscope (Wild Heerbrugg). Gram staining was performed with standard stains (carbol-gentian violet solution, Lugol's iodine, 96% (v/v) ethanol, and carbol-fuchsin solution) and by cell lysis using 6% (w/v) KOH as described by Gregersen (1978). Cell morphology and flagellation were examined by phase-contrast microscopy (Axiophot; Zeiss). The ability to glide was checked with cultures grown under optimal and harsh conditions (regarding temperature, pH and salinity) as described by Bowman (2000) and Bernardet *et al.* (2002). Scanning electron micrographs were taken from cultures grown overnight or for 4 days at room temperature with a Zeiss DSM 940. The presence of flexirubin-type pigments was tested with 20% KOH (Reichenbach 1992); Congo red adsorption was determined as described by Bernardet *et al.* (2002).



Growth at various temperatures from 2 to 50 °C was measured with a custom-made temperature-gradient bar over 7 days. Salinity requirement was determined with saltless MB-medium (per litre distilled water: 5.0 g Bacto peptone, 1 g yeast extract and 0.1 g ferric citrate) and saltless TSB-medium (per litre distilled water: 1.7 g Bacto tryptone, 0.3 g papain-digested soy bean meal, 0.25 g glucose and 0.25 g K<sub>2</sub>HPO<sub>4</sub>). The media were supplemented with NaCl (0-9% (w/v) in 1% increments) or artificial sea salts (Tropic Marin; 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, and 80 g l<sup>-1</sup>) and incubated for 7 days at room temperature. Growth at different pH values (2.3, 3.5, 5.0, 6.0, 8.5, 9.0, and 9.5) was also investigated.

Type strains of *T. aestuarii* (JCM 13491<sup>T</sup>) and *T. lutimaris* (DSM 16505<sup>T</sup>), the most closely related species based on 16S rRNA gene sequences, were tested as references in the phenotypic characterization. The API 20E Kit (bioMérieux) for identification of Gram-negative rods was performed in duplicates according to the manufacturer's instructions with API NaCl 0.85% medium and artificial sea water (ASW; Bruns *et al.* 2001) as suspension fluids. The utilization of different carbon sources was checked with the GN2 MicroPlate (Biolog) following the manufacturer's instructions. Duplicate tests were carried out with bacteria suspended in 1% (w/v) NaCl solution, triplicate tests in ASW as suspension medium. The hydrolysis of aesculin was studied as described by Cowan & Steel (1965) and alternatively with half-strength MB plates supplemented with 0.1% (w/v) aesculin. The tested strains grew considerably better on the MB plates than on the standard plates, and addition of ferric salts for complexation of aesculetin was not necessary since MB contains ferric citrate. L-Tyrosine and starch hydrolysis tests were carried out as described by Cowan & Steel (1965) with the difference that half-strength MB was used as the nutrient source. Casein hydrolysis was determined with plates made as follows: 2.5 g casein was suspended in 1000 ml distilled water, followed by 0.1 g CaCl<sub>2</sub> and a few drops of 10 M NaOH. The suspension was left to macerate for 30 min. After addition of 1.5% (w/v) agar and 18.7 g marine broth 2216, the suspension was brought carefully to the boil, filtrated and autoclaved (15 min 121 °C). In order to test chitin hydrolysis, a bottom-agar plate was covered with a top agar layer containing chitin. The bottom agar consisted of 15 g agar and 20 g artificial sea salts per litre distilled water. The top agar comprised two solutions: 0.8 g chitin was suspended in 100 ml distilled water (solution 1), and 6 g agar was suspended in 300 ml half-strength MB (solution 2). After autoclaving, the two solutions were combined and poured on the bottom-agar plates.

Chemotaxonomic analyses (DNA G+C content, DNA-DNA hybridization, menaquinones, fatty acid methyl esters) were carried out by the Identification Service at the DSMZ (Braunschweig, Germany). DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified as described by Cashion *et al.* (1977). The DNA G+C content of strain B390<sup>T</sup> was determined according to Mesbah *et al.* (1989). DNA-DNA hybridization was carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huß *et al.* (1983). Fatty acid analysis was carried out using the MIDI system (MIDI Inc.).

Genomic DNA for phylogenetic analyses was isolated by consecutive freezing and boiling of picked colonies in DNA-free water (Fluka). The 16S rRNA gene amplification was done by PCR with puReTaq Ready-To-Go PCR Beads (Amersham Biosciences) and primers 27f and 1492r (Lane 1991). PCR product purification and sequencing were done at the Institute for Clinical Molecular Biology of the University Hospital Schleswig-Holstein in Kiel. For each purification reaction, a mixture of 1.5 U exonuclease I (GE Healthcare) and 0.3 U shrimp alkaline phosphatase (SAP, Roche) was added to the PCR product and incubated for 15 min at 37 °C. The enzymes were inactivated at 72 °C for 15 min. A sequencing reaction was done with the BigDye Terminator v1.1 sequencing Kit (Applied Biosystems) and analysed in a 3730xl DNA-Analyser (Applied Biosystems) as specified by the manufacturer. The 16S rRNA gene sequence obtained was a continuous stretch of 1441 bp. The sequence was compared in the EMBL nucleotide database using FASTA (Pearson 1990), available online at the European Bioinformatics Institute homepage. Related sequences from cultivated bacteria were downloaded from the RDP-II Project homepage (Cole *et al.* 2007) and aligned with the CLUSTAL W program (Thompson *et al.* 1994). The alignment was checked and, if necessary, corrected manually. Phylogenetic analyses were conducted using MEGA version 3.1 (Kumar *et al.* 2004) and the PhyML online web server (Guindon *et al.* 2005). A distance matrix-based tree using the neighbour-joining method (Kimura's two-parameter model) was calculated on the basis of 1000 bootstrap replicates. A maximum-likelihood tree was generated using the general time reversible (GTR) substitution model with estimated proportion of invariable sites and bootstrap values of 500.

Cells of strain B390<sup>T</sup> were rod-shaped, 0.3 µm wide and 1.5-3.5 µm long and Gram-negative. Considerably longer filaments could sometimes be observed, and spherical cells occurred regularly under suboptimal growth conditions such as adverse temperature, salinity or pH.

Characteristic morphological, physiological, and chemotaxonomic properties are summarized in Table 2-1 and are included in the species description. The DNA G+C content of strain B390<sup>T</sup> was 31.6 mol%, which falls in the range reported for other *Tenacibaculum* species (30-35.2 mol%). Consistent with other *Tenacibaculum* species, as well as with members of the *Flavobacteriaceae* family in general, the major isoprenoid quinone was MK-6 (98.5%); others were MK-5 (1%) and MK-7 (0.5%). The major components of the fatty acid profile were iso-C<sub>15:0</sub>, iso-C<sub>15:0</sub>3-OH, iso-C<sub>15:1</sub>, summed feature 3 (C<sub>16:1</sub>ω7c, and/or iso-C<sub>15:0</sub>2-OH), iso-C<sub>17:0</sub>3-OH and C<sub>15:1</sub>ω6c, which strengthens the affiliation of strain B390<sup>T</sup> to the genus *Tenacibaculum*. Several other fatty acids were detected, but made up less than 5% of the total mass. A comprehensive list of fatty acids in comparison with other *Tenacibaculum* species is given in Table 2-2.

A detailed Biolog respiration profile (including profiles of *T. aestuarii* JCM 13491<sup>T</sup> and *T. lutimaris* DSM 16505<sup>T</sup> and in comparison to published profiles of *T. skagerrakense* D28 and D30<sup>T</sup> and *T. litopenaei* B-I<sup>T</sup>) is given in Table 2-3. Important differences were that strain B390<sup>T</sup> could utilize α-D-glucose and several D-glucose-derived compounds (such as α-cyclodextrin, dextrin, glycogen, cellobiose, gentiobiose, maltose, and D-glucose 6-phosphate) as well as succinic acid, succinamic acid, and succinic acid monomethyl ester, whereas *T. aestuarii* JCM 13491<sup>T</sup> and *T. lutimaris* DSM 16505<sup>T</sup> could not. *T. litopenaei* B-I<sup>T</sup>, *T. skagerrakense* D28 and D30<sup>T</sup> were able to utilize α-D-glucose, the latter two strains were also positive for utilization of α-cyclodextrin, dextrin, glycogen, cellobiose and maltose, but not succinic acid, succinamic acid, or succinic acid monomethyl ester. All strains were respiration-positive for acetic acid, L-glutamic acid and L-proline.

The API 20E test revealed positive responses for gelatinase and cytochrome oxidase. Flexirubin-type pigment was absent and strain B390<sup>T</sup> did not adsorb Congo red, indicating the absence of extracellular glycans.

Phylogenetic analysis based on 16S rRNA gene sequences showed that strain B390<sup>T</sup> clustered within other *Tenacibaculum* sequences, but formed a distinct branch that was separated from type strains of all hitherto described *Tenacibaculum* species. This branching was demonstrated by the maximum-likelihood tree (Fig. 2-1) as well as by the neighbour-joining tree (Fig. 2-2).

Sequence similarities below 97% were found with the type strains of all other species of *Tenacibaculum* with validly published names except for *T. aestuarii* SMK-4<sup>T</sup> and *T. lutimaris* TF26<sup>T</sup>, which showed similarities of 97.2 and 97.3% using FASTA. DNA-DNA hybridization tests were performed. Results of a replicate hybridization test are given in parentheses. The DNA relatedness of strain B390<sup>T</sup> was 27.4 (32.9) % to *T. aestuarii* JCM13491<sup>T</sup> and 27.1 (20.5) % to *T. lutimaris* DSM 16505<sup>T</sup>. According to the recommendation of Wayne *et al.* (1987), who defined a threshold value over 70% for DNA-DNA relatedness as a definition of bacterial species, strain B390<sup>T</sup> is not a member of *T. aestuarii* or *T. lutimaris*.

In conclusion, the phenotypic and phylogenetic data presented indicate that strain B390<sup>T</sup> should be classified within a novel species, for which the name *Tenacibaculum adriaticum* sp. nov. is proposed.

**Description of *Tenacibaculum adriaticum* sp. nov.**

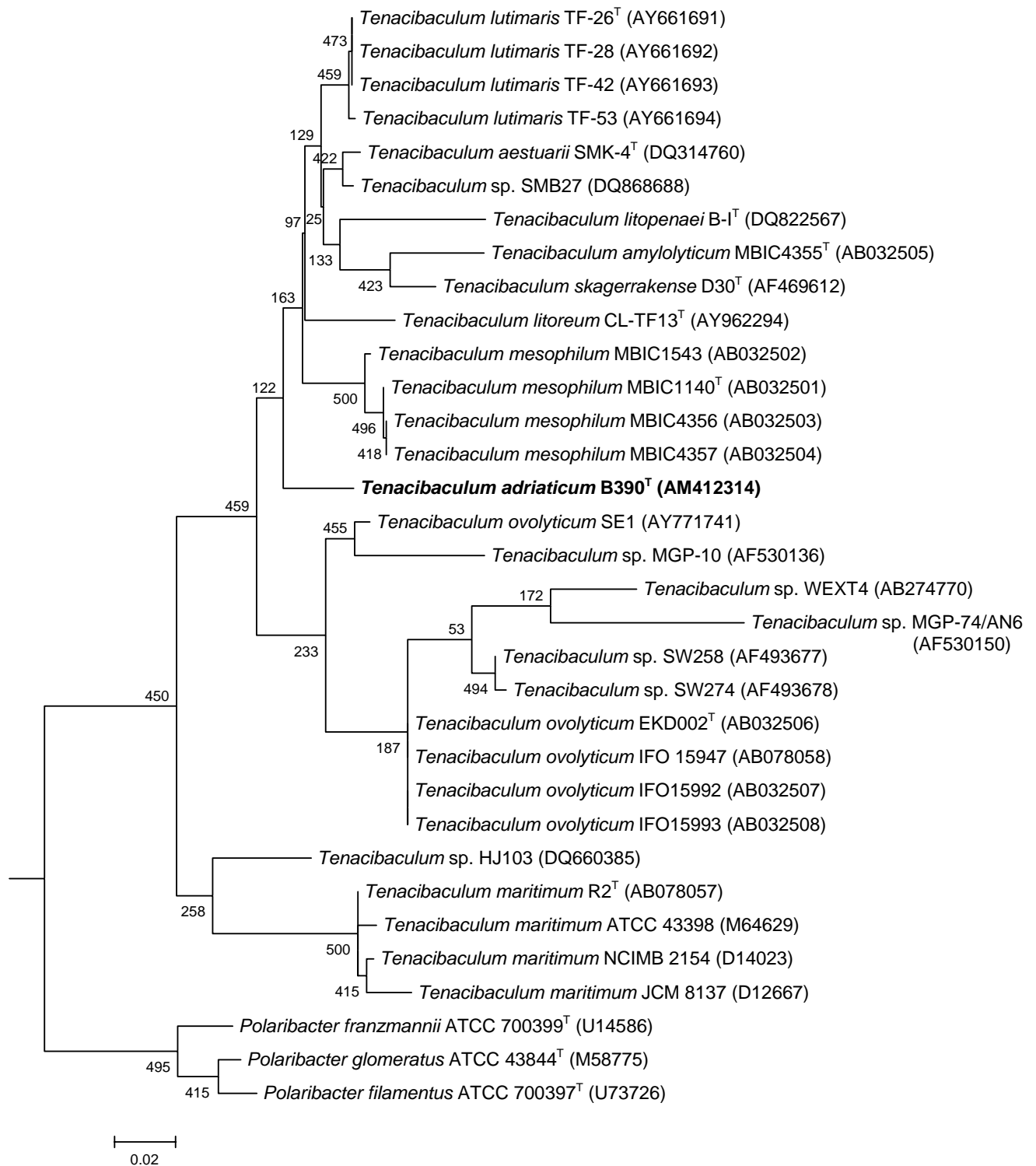
*Tenacibaculum adriaticum* (ad.ri.a'ti.cum. L. neut. adj. *adriaticum* of the Adriatic Sea).

Cells are Gram-negative, rod-shaped, 0.3  $\mu\text{m}$  wide and 1.5-3.5  $\mu\text{m}$  long, non-flagellated, but motile by means of gliding. Spherical cells (0.5-0.8  $\mu\text{m}$  in diameter) occur in the stationary phase and under suboptimal growth conditions. Longer rods (up to 35  $\mu\text{m}$  in length) are observed regularly. Colonies are circular with a regular edge and translucent yellow; flexirubin-type pigment is absent. Requires NaCl for growth (optimum 1-2%) and grows in media containing artificial sea salts up to 70  $\text{g l}^{-1}$ . Temperature range for growth is 5-34  $^{\circ}\text{C}$  with an optimum at 18-26  $^{\circ}\text{C}$ . Grows at pH 5-9 with an optimum at pH 7. Aesculin, casein, tyrosine, starch and gelatine are hydrolysed. Oxidase-positive. According to Biolog tests,  $\alpha$ -cyclodextrin, dextrin, glycogen, cellobiose, gentiobiose, maltose, D-glucose 6-phosphate, succinic acid, succinamic acid, and succinic acid monomethyl ester are utilized, as well as Tween 40, maltose, acetic acid, D- and L-lactic acid, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-ornithine and L-proline as carbon sources. The G+C content of the DNA is 31.6 mol%. The major isoprenoid quinone is MK-6 (98.5%). Major fatty acids are iso-C<sub>15:0</sub> (25.3%), iso-C<sub>15:0</sub>3-OH (13.7%), and iso-C<sub>15:1</sub> (13.1%).

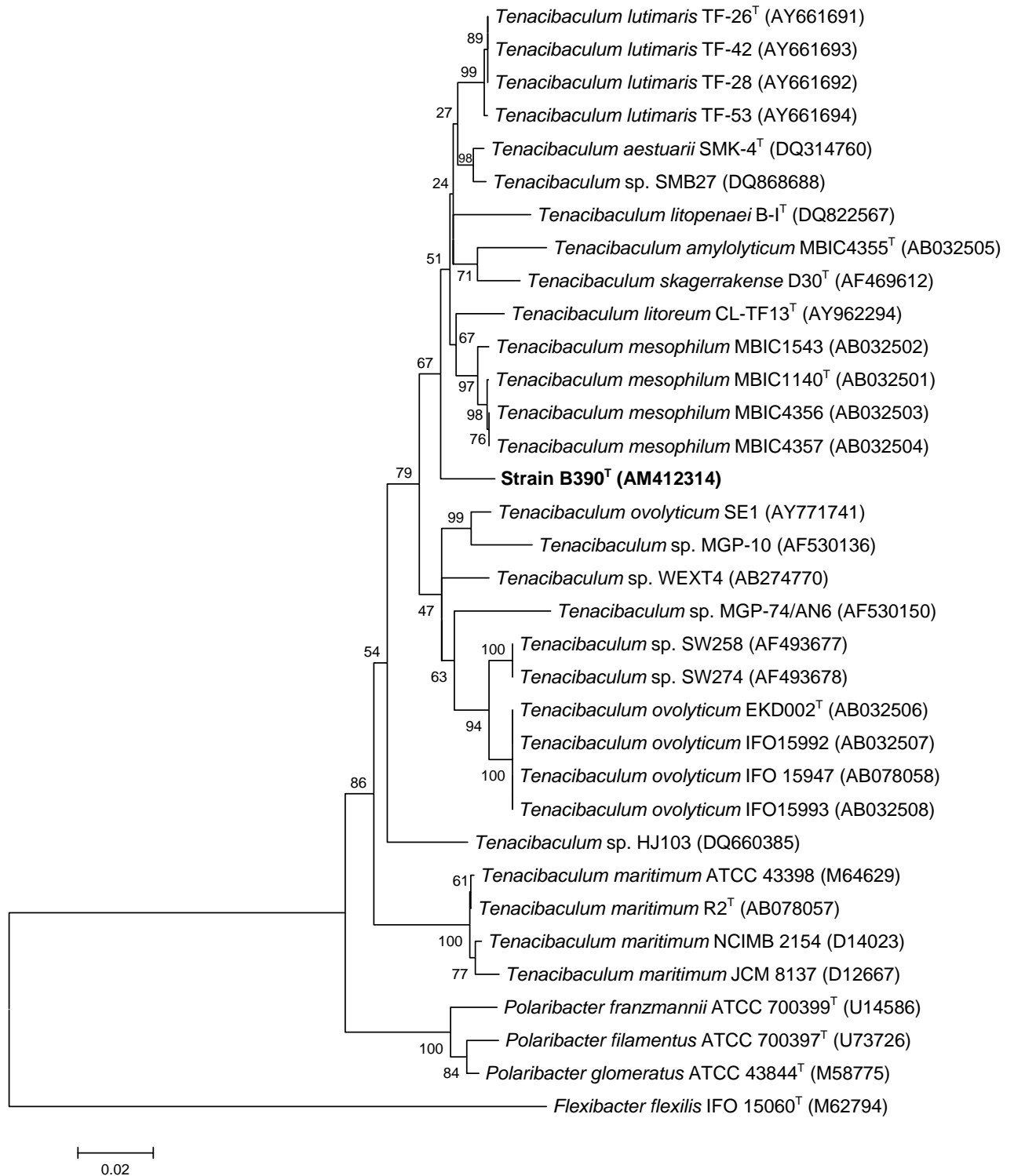
The type strain is B390<sup>T</sup> (=DSM18961<sup>T</sup> =JCM 14633<sup>T</sup>), isolated from the bryozoan *Schizobrachiella sanguinea* from the Adriatic Sea.

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**Figure 2-1. Maximum-likelihood tree** based on 16S rRNA gene sequences available from the databases (accession numbers in parentheses). Bootstrap values based on 500 replications are given at branching points. The tree was calculated using *Flexibacter flexilis* ATCC 23079<sup>T</sup> (GenBank accession no. M62794) as an outgroup (not shown). Bar, 0.02 substitution per nucleotide position.



**Fig. 2-2. Neighbour-joining tree** based on 16S rRNA gene sequences available from the databases (accession numbers are given in parentheses). Bootstrap values based on 1000 replications are given in percentages at the branching points. The tree was calculated using *Flexibacter flexilis* ATCC 23079<sup>T</sup> (GenBank/EMBL/DDBJ accession no. M62794) as an outgroup.

**Table 2-1. Morphological and physiological characteristics** of strain B390<sup>T</sup> and the most closely related *Tenacibaculum* species

Taxa: 1, strain B390<sup>T</sup>; 2, *T. aestuarii* (data from Jung *et al.*, 2006); 3, *T. litoreum* (Choi *et al.*, 2006); 4, *T. lutimaris* (Yoon *et al.*, 2005); 5, *T. skagerrakense* (Frette *et al.*, 2004); 6, *T. mesophilum* (Suzuki *et al.*, 2001); +, Positive; -, negative; (+), weakly positive; ND, no data available; NG, no growth.

Characteristic	1	2	3	4	5	6
Origin of type strain	Bryozoan, Croatia	Tidal flat, Korea	Tidal flat, Korea	Tidal flat, Korea	Seawater, Skagerrak (Denmark)	Sponge, Japan
Cell size (µm)	0.3 x 1.5-3.5	0.3 x 2.0-3.5	0.3-0.5 x 2-35	0.5 x 2-10	0.5 x 2-15	0.5 x 1.5-10
Filaments (µm)	11-35	ND	ND	ND	ND	ND
Colony morphology						
Shape	Circular, regular edge	Irregular, spreading edge	Irregular, spreading edge	Irregular, spreading edge	Circular, spreading edge	Circular or irregular, spreading edge
Diameter at 5 days (mm)	<5	5-10	5-10	10-20	5-20	30-60
Colour	Trans-lucent yellow	Pale yellow	Pale yellow	Pale yellow, glistening	Bright yellow	Bright yellow
pH Range	5-9	5.5-8.5	6-10	5-8	6-8	5.3-9.0
Temperature for growth (°C)						
Range	5-33.5	9-41	5-40	10-39	10-40	15-40
Optimum	18-26	30-37	35-40	30-37	25-37	28-35
Salinity range						
NaCl (% (w/v))	1-5	0.5-7	3-5	<8 (2-3)	NG	1-7
Sea water (% (v/v))	12.5-175*	ND	25-250*	25-175*	25-150*	10-100
Nitrate reduction	-	-	+	-	+	-
Utilization of:						
DL-Aspartate	+	+† <sup>a</sup>	-	+† <sup>b</sup>	+	+
L-Proline	+	+† <sup>a</sup>	+	+† <sup>b</sup>	+	+
L-Glutamate	+	+† <sup>a</sup>	-	+† <sup>b</sup>	+	+
Citrate	-	-†	-	-†	+	-
L-Leucine	(+)	(+)† <sup>a</sup>	-	(+)† <sup>b</sup>	+	-
D-Glucose	+	-	-	-	+	ND
D-Mannose	+	-	-	-	+	ND
Cellobiose	+	-	ND	-	+	ND
Hydrolysis of:						
Starch	+	-	+	-	+	-
Aesculin	+	-	-	-	ND	-
Chitin	-	-†	ND	-†	-	-
DNA G+C content (mol%)	31.6	33.6	30.0	32.2-32.8	35.2	31.6-32.0

\*Percentage calculated using a relation of 100% sea water = 40 g artificial sea salts l<sup>-1</sup> (Frette *et al.*, 2004).

†Data from this study. Where indicated, a negative result was reported by: *a*, Jung *et al.* (2006); *b*, Yoon *et al.* (2005).



**Table 2-2. Cellular fatty acid contents (%) of strain B390<sup>T</sup> and other *Tenacibaculum* species**

Taxa: 1, strain B390<sup>T</sup>; 2, *T. aestuarii* (data from Jung *et al.*, 2006); 3, *T. litoreum* (Choi *et al.*, 2006); 4, *T. lutimaris*; 5, *T. skagerrakense*; 6, *T. mesophilum*; 7, *T. maritimum* (data in columns 4-7 from Yoon *et al.*, 2005); 8, *T. litopenaei* (Sheu *et al.*, 2007); TBSA, Tuberculostearic acid (10-methyl C<sub>18:0</sub>); ECL, equivalent chain length; tr, trace (<1 %).

Fatty acid	1	2	3	4	5	6	7	8
Straight-chain								
C <sub>15:0</sub>	2.7	6.1	2.7	8.9	4.9	3.6	2.9	
C <sub>16:0</sub>		0.4	0.9	0.6	0.6	0.7	0.3	1.8
C <sub>18:0</sub>			0.3				1.4	
Branched								
iso-C <sub>13:0</sub>	1.6	1.3	1.4	0.7	0.2	0.8	1.8	tr
iso-C <sub>14:0</sub>	0.4	2.2	0.7	1.7	0.9	0.8	0.8	tr
iso-C <sub>15:0</sub>	25.3	18.9	18.8	17.2	9.5	13.2	16.8	22.0
iso-C <sub>15:1</sub>	13.1	8.7	8.2	5.3	8.2	7.1	7.6	8.7
iso-C <sub>16:0</sub>	0.2	2.0	2.3	3.8	1.3	1.7	0.3	1.8
iso-C <sub>16:1</sub>	0.8	2.3	1.3	1.7	1.7	0.8		1.6
iso-C <sub>17:1</sub> ω <sup>9</sup> c	2.0	0.8	1.6	0.4		0.6		1.6
anteiso-C <sub>15:0</sub>	0.5	1.3	1.8	0.7		1.1	0.8	tr
TBSA 10	0.4							
Unsaturated								
C <sub>15:1</sub> ω <sup>6</sup> c	5.6	3.0	1.7	4.2		1.6	2.2	1.6
C <sub>17:1</sub> ω <sup>6</sup> c	0.7	1.6	0.9	1.5	1.2	0.9	0.3	1.9
C <sub>18:1</sub> ω <sup>5</sup> c	1.2							
C <sub>18:3</sub> ω <sup>6</sup> c			1.5					
Hydroxylated								
C <sub>10:0</sub> 3-OH		0.7						
C <sub>15:0</sub> 2-OH	0.4	0.8	0.7	1.2	2.5	1.1	1.1	tr
C <sub>15:0</sub> 3-OH	3.2	4.2		3.4	8.6	2.9	3.8	2.7
C <sub>16:0</sub> 3-OH	0.2	1.0	1.6	1.3	2.1	3.2	1.5	5.4
C <sub>17:0</sub> 2-OH		0.4	0.9	0.2		0.8		tr
C <sub>17:0</sub> 3-OH		0.6	0.3	0.9	2.5	0.7	0.6	1.0
iso-C <sub>14:0</sub> 3-OH	0.3	0.5						
iso-C <sub>15:0</sub> 3-OH	13.7	6.1	6.6	4.6	7.8	8.0	19.8	4.6
iso-C <sub>16:0</sub> 3-OH	2.8	12.3	6.8	12.8	12.2	9.0	5.0	3.4
iso-C <sub>17:0</sub> 3-OH	10.8	9.6	13.6	8.4	11.7	14.9	13.7	12.7
Unknown								
(ECL 13.565)	0.2		1.3					1.9
(ECL 16.582)	0.8	1.0	1.3	0.7	0.7	1.0	1.0	tr
Summed features*								
1	1.2							
3	11.8	11.9	19.6	18.1	22.5	24.4	17.9	21.3
4			1.3					

\*Summed features contained one or more of the following fatty acids. Summed feature 1, iso-C<sub>15:1</sub> H, iso-C<sub>15:1</sub> I and C<sub>13:0</sub>3-OH; summed feature 3, C<sub>16:1</sub>ω<sup>7</sup>c and iso-C<sub>15:0</sub>2-OH; summed feature 4, iso-C<sub>17:1</sub> I and anteiso-C<sub>17:1</sub> B.

**Table 2-3. Biolog test results.**

Strains: 1, B390<sup>T</sup>; 2, *T. aestuarii* JCM 13491<sup>T</sup>; 3, *T. lutimaris* DSM 18961<sup>T</sup>; 4, *T. skagerrakense* D30<sup>T</sup>, data from Frette *et al.* (2004); 5, *T. skagerrakense* D28, data from Frette *et al.* (2004); 6, *T. litopenaei* B-I<sup>T</sup>, data from Sheu *et al.* (2007); +, positive; -, negative; (+), weakly positive; ND, not determined.

*Continued on next page*

Utilization of	1	2	3	4	5	6
$\alpha$ -Cyclodextrin	+	-	-	+	+	-
Dextrin	+	-	-	+	+	-
Glycogen	+	-	-	+	+	-
Tween 40	+	+	(+)	+	+	-
Tween 80	-	-	-	-	-	-
N-Acetyl-D-galactosamine	-	-	-	-	-	-
N-Acetyl-D-glucosamine	-	-	-	-	-	ND
Adonitol	-	-	-	-	-	-
L-Arabinose	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-
D-Cellobiose	+	-	-	+	+	-
i-Erythritol	-	-	-	-	-	-
D-Fructose	-	-	-	ND	ND	-
L-Fucose	-	-	-	ND	ND	-
D-Galactose	-	-	-	-	-	-
Gentiobiose	+	-	-	-	-	-
$\alpha$ -D-glucose	+	-	-	+	+	+
m-Inositol	-	-	-	-	-	-
$\alpha$ -D-lactose	-	-	-	-	-	-
Lactulose	-	-	-	-	-	-
Maltose	+	-	-	+	+	-
D-Mannitol	-	-	-	-	-	-
D-Mannose	+	-	-	+	+	-
D-Melibiose	-	-	-	ND	ND	-
$\beta$ -Methyl-D-glucoside	-	-	-	ND	ND	-
D- Psicose	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-
L-Rhamnose	-	-	-	-	-	-
D-Sorbitol	-	-	-	ND	ND	-
Sucrose	-	-	-	+	+	-
D-Trehalose	-	-	-	-	-	-
Turanose	-	-	-	+	+	-
Xylitol	-	-	-	-	-	-
Pyruvic acid methyl-ester	-	-	-	ND	ND	-
Succinic acid mono-methyl-ester	+	-	-	ND	ND	-
Acetic acid	+	+	+	+	+	+
Cis-Aconitic acid	-	-	-	+	+	-
Citric acid	-	-	-	+	+	-
Formic acid	-	-	-	-	-	-
D-Galactonic acid lactone	-	-	-	-	-	-
D-Galacturonic acid	-	-	-	-	-	-
D-Gluconic acid	-	-	-	-	-	-
D-Glucosaminic acid	-	-	-	+	-	-
D-Glucuronic acid	-	-	-	ND	ND	-
$\alpha$ -Hydroxybutyric acid	-	-	-	-	-	-
$\beta$ -Hydroxybutyric acid	-	-	-	-	-	-
$\gamma$ -Hydroxybutyric acid	-	+	-	+	-	-
p-Hydroxy phenylacetic acid	-	-	-	-	-	-

**Table 2-3.** continued.

Utilization of	1	2	3	4	5	6
Itaconic acid	-	-	-	-	-	-
$\alpha$ -Keto butyric acid	(+)	(+)	+	+	+	+
$\alpha$ -Keto glutaric acid	-	-	-	ND	ND	+
$\alpha$ -Keto valeric acid	(+)	-	-	+	+	+
D,L-Lactic acid	+	+	(+)	+	+	-
Malonic acid	-	-	-	+	-	-
Propionic acid	(+)	+	+	+	+	-
Quinic acid	-	-	-	+	-	-
D-Saccharic acid	-	-	-	-	-	-
Sebacic acid	-	-	-	ND	ND	-
Succinic acid	+	-	-	-	-	-
Bromosuccinic acid	-	-	-	-	-	-
Succinamic acid	+	-	-	-	-	-
Glucuronamide	-	-	-	-	-	-
L-Alaninamide	-	-	-	ND	ND	-
D-Alanine	-	-	-	-	-	-
L-Alanine	(+)	(+)	-	+	+	-
L-Alanylglycine	(+)	(+)	(+)	+	+	-
L-Asparagine	(+)	+	+	+	+	-
L-Aspartic acid	+	+	+	+	+	-
L-Glutamic acid	+	+	+	+	+	+
Glycyl-L-aspartic acid	+	+	+	+	+	-
Glycyl-L-glutamic acid	+	+	+	+	+	-
L-Histidine	-	(+)	-	-	-	-
Hydroxy-L-proline	-	(+)	+	+	+	+
L-Leucine	(+)	(+)	(+)	+	+	-
L-Ornithine	+	(+)	(+)	+	+	-
L-Phenylalanine	-	-	-	-	-	-
L-Proline	+	+	+	+	+	+
L-Pyroglutamic acid	-	-	-	ND	ND	-
D-Serine	-	-	-	-	-	-
L-Serine	-	-	(+)	+	+	+
L-Threonine	(+)	-	(+)	+	+	+
D,L-Carnitine	-	-	-	-	-	-
$\gamma$ -Amino butyric acid	-	-	-	+	+	-
Urocanic acid	-	(+)	-	-	-	-
Inosine	-	-	-	+	+	-
Uridine	-	-	-	+	+	+
Thymidine	-	-	(+)	-	-	-
Phenylethylamine	-	-	-	-	-	-
Putrescine	-	-	-	-	-	-
2-Aminoethanol	-	-	-	-	-	-
2,3-Butanediol	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-
D,L- $\alpha$ -Glycerol phosphate	-	-	-	-	-	-
$\alpha$ -D-Glucose-1-phosphate	-	-	-	+	+	-
D-Glucose-6-phosphate	+	-	-	ND	ND	-

## Chapter 3

# ***Membranipora membranacea* associated bacteria: impact of media on their isolation and antimicrobial activity**

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Submitted

## **Abstract**

Specimens of the bryozoan *Membranipora membranacea* were collected in the Baltic Sea for the first extensive culture-dependent study on the associated bacteria and their antimicrobial activities. Low-nutrient media were used for the isolation and, according to the habitat, were composed of “bryozoan extract” and “algal extract”, or “artificial” nutrient components. All isolates were classified by 16S rRNA gene sequence analysis and tested for antimicrobial properties using a panel of five indicator strains and six different media.

The 96 bacterial isolates were affiliated to the *Alpha*- and *Gammaproteobacteria*, as well as to the *Bacilli* and *Actinobacteria*. They belonged to 28 genera and were assigned to 49 phlotypes on the basis of sequence similarities  $\geq 99.5\%$ . Members of 37 phlotypes were exclusively isolated from one of the media, strains of the remaining phlotypes were found on two or three different media, but none on all four. 22 isolates, especially within the *Alphaproteobacteria*, very likely represent new species.

47 strains displayed antibiotic activities, which were predominately directed against Gram-positive indicator strains. 63.8% of the active strains exhibited antibiotic traits only on one or two of the media, whereas only 12.8% inhibited growth on five or all six media. Applying six different media for antimicrobial testing resulted in twice the number of positive hits as compared with only one medium. The combination of different isolation and test media presented in this work, as well as probing microbiologically untapped sources like *M. membranacea*, demonstrates a possible way to discover novel bacteria that might produce new antibiotics.

## **Introduction**

Surfaces in the marine environment, whether biotic or abiotic, are exposed to colonization by other organisms. Accordingly, the encrusting bryozoan *Membranipora membranacea* and related species populate kelps in temperate waters all over the world. *Membranipora* is a potent colonizer and disperser which had started its global distribution most probably in the North Pacific several million years ago (Schwaninger 2008). In the Baltic Sea, a preferred substrate is provided by phylloids of *Saccharina latissima* (newer synonym of *Laminaria saccharina* (Lane *et al.* 2006)). However, the bryozoan surfaces are subjected to colonizers and grazers, too. Like other sessile and colony-forming organisms in the marine environment, bryozoans rely on mechanical and chemical defence strategies (Gerdes *et al.* 2005a).

In order to investigate their potential therapeutic use, several bryozoans have been collected, extracted and screened for biological activities, leading to the bryostatins as the most prominent compounds (Pettit *et al.* 1982). Nevertheless, bryozoan metabolites account only for about 1% of natural products as annually reviewed by Blunt *et al.* (2010). Although the phylum *Bryozoa* contains several thousands of recent species, studies on natural products have been focused only on a few species with a bias towards those of the Cheilostome infraorder Flustrina (Sharp *et al.* 2007a). It is to note that the genus *Bugula*, which is known for the production of the bryostatins, is located within this infraorder. To our knowledge, there is no report on natural products produced by *M. membranacea*.

The resemblance of many natural products from marine macroorganisms to those identified from bacteria and the knowledge of microbial associations with higher organisms in the marine environment led to the conclusion that these compounds could be indeed of microbial origin (König & Wright 1996, König *et al.* 2006). This has also been demonstrated for the bryostatins which are produced by the bacterial symbiont “*Candidatus Endobugula sertula*” (Davidson *et al.* 2001). The bacteria are vertically transmitted (Sharp *et al.* 2007b) and are considered to protect the bryozoan larvae from predation (Lopanik *et al.* 2004a). The identification of the true producer offers the possibility to produce the bryostatins by fermentation processes rather than non-sustainable collections or costly aquacultures of the host. Although the bacterial symbiont is not in culture so far, heterologous expression of the relevant bryostatin PKS gene clusters would provide access to larger quantities of the desired compounds (Sudek *et al.* 2007).

Host-associated bacteria, in particular those colonizing surfaces and living in biofilm communities, encounter complex interactions both with other microorganisms and the host.

Communication is mediated chemically, and the sum of all factors shapes the composition of microbial covers, which can differ considerably from the surrounding environment (Egan *et al.* 2008). Therefore, regarding the discovery of bioactive compounds, marine surface-associated microorganisms represent a suitable source (de Carvalho & Fernandes 2010, Penesyan *et al.* 2010). Bryozoan-associated microorganisms have been studied using microscopic (Palinska *et al.* 1999, Woolacott 1981), genetic (Kittelmann & Harder 2005), and cultivation-based methods (Heindl *et al.* 2010, Pukall *et al.* 2001), as well as combinations thereof (Gerdes *et al.* 2005b).

In this study, bacteria associated with the bryozoan *Membranipora membranacea* and their antibiotic properties were investigated. Bacteria were isolated from three specimens of *M. membranacea* from the Baltic Sea using four different media with low nutrient levels. All isolates were classified by 16S rRNA gene sequence analysis. The impact of six different media on their antimicrobial activities against five indicator strains was determined.

## **Materials and methods**

### **Sampling site and sample preparation**

Bryozoan samples were collected by dredging in the Baltic Sea north of Læsø (Kattegat, coordinates N57°28.3' – E11°10.4' at depth 20 m) and identified as *Membranipora membranacea* growing on phylloids of *Saccharina latissima*. Three separate bryozoan colonies were cut out, washed with sterile filtered surrounding sea water and transferred aseptically into sterile tubes containing 50% (v/v) glycerol and 3% (w/v) sodium chloride. The tubes were immediately frozen and stored at -18 °C until further treatment. Excess bryozoan samples growing on algae were put aside in a closeable beaker, containing local sea water (total volume about one liter) and stored at 4 °C until used for media preparation. Moreover, two liters of seawater from the sampling site were collected before dredging, filtered through a 0.2 µm cellulose acetate filter and added to selected agar media.

### **Culture media**

For isolation of microorganisms four media were prepared: “bryozoan extract medium” (BM), “algal extract medium” (AM), diluted “Reasoner’s 2A medium” (R2Ad), and diluted “Difco all culture medium” (ACd). For antibiotic activity testing six media were prepared: “Väätänen nine salt solution medium” (VNSS), “*Pseudoalteromonas* specific medium” (PSA), “Reasoner’s 2A medium” (R2A), “Difco all culture medium” (AC), “marine broth medium” (MB), and “tryptic soy broth medium” (TSB).

Isolation media were prepared as follows: the excess samples from the beaker were used for the media which resembled the natural habitat (BM and AM). Bryozoans were cut out from the algae and minced. An equivalent weight of 3% (w/v) saline was added. This material was thoroughly blended with an Ultraturrax-homogenizer (IKA Werke, Germany), frozen at -100 °C and lyophilized to obtain a “bryozoan extract”. The remaining algae were recombined with the seawater in a beaker, homogenized, frozen and lyophilized to yield an “algal extract”. Both extracts were dissolved in sea water collected from the sampling site at concentrations of 0.06% (w/v) yielding BM and AM media. Additionally, R2Ad medium (contained each 0.01% (w/v) Bacto yeast extract, Difco proteose peptone, Difco casamino acids, glucose, soluble starch, 0.006% (w/v) sodium pyruvate, 0.006% (w/v) K<sub>2</sub>HPO<sub>4</sub>, and 0.00048% (w/v) MgSO<sub>4</sub>), and ACd medium (0.06% (w/v), both with 3% (w/v) sea salt (Tropic Marin) were prepared.



The six media for the activity tests were prepared as follows: VNSS medium: (0.1% (w/v) peptone from soymeal (Merck), 0.05% (w/v) yeast extract, 0.05% (w/v) glucose, 0.5% (w/v) soluble starch, 0.001% (w/v)  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.001% (w/v)  $\text{Na}_3\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 1.76% (w/v) sodium chloride, 0.147% (w/v)  $\text{Na}_2\text{SO}_4$ , 0.008% (w/v)  $\text{NaHCO}_3$ , 0.025% (w/v)  $\text{KCl}$ , 0.004% (w/v)  $\text{KBr}$ , 0.187% (w/v)  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.041% (w/v)  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.001% (w/v)  $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.001% (w/v)  $\text{H}_3\text{BO}_3$ ) (according to Mården *et al.* (1985)); PSA medium: (0.2% (w/v) peptone from soymeal, 0.2% (w/v) yeast extract, 0.1% (w/v) glucose, 0.02% (w/v)  $\text{KH}_2\text{PO}_4$ , 0.005% (w/v)  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1% (w/v)  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.01% (w/v)  $\text{KBr}$ , and 1.8% (w/v) sea salt) (according to Kalinovskaya *et al.* (2004)); R2A medium and AC medium (fivefold concentrated compared to isolation media and with 3% (w/v) sea salt); MB medium (0.5% (w/v) peptone, 0.1% (w/v) yeast extract, and 3.14% (w/v) sea salt) and TSB medium (0.3% (w/v) tryptic soy broth (Difco) and 2.5% (w/v) sodium chloride). 1.5% (w/v) agar was added to all media for solidification.

### **Isolation and cultivation of bacteria**

For comparison, two different methods of sample preparation were applied. The first two bryozoan samples were crushed with a sterile micropestle, the third was processed with a Precellys 24 lysis & homogenization device with a hard tissue grinding MK28 kit (Bertin Technologies) at 6300 rpm for 20 s. Dilution series with sterile seawater were made ( $10^{-1}$  to  $10^{-5}$ ). The dilution series (100  $\mu\text{l}$  each) as well as pieces of the bryozoan samples were spread on agar plates containing four different media. The plates were incubated at 25 °C in the dark. All visible colonies were picked and sub-cultured on MB agar plates. For preservation, pure cultures were suspended in liquid MB medium containing 5% (v/v) DMSO and stored at -100 °C.

### **Screening for inhibitory activities against indicator organisms**

Bacterial isolates were grown on MB agar plates directly from the DMSO stock. Colonies were picked and suspended in 1 ml sterile 3% (w/v) saline. 15  $\mu\text{l}$  each were pipetted on agar plates with six different media. After growing for 3 to 4 days at room temperature (approx. 22 °C), the bacterial colonies were checked for the presence of clearance zones to anticipate false positive results. The plates were then covered with 5 ml TSB soft agar (with 1% (w/v) sodium chloride and 0.8% (w/v) agar) containing one of the following indicator strains: *Escherichia coli* DSM 498, *Bacillus subtilis* subsp. *spizizenii* DSM 347, *Staphylococcus lentus* DSM 6672, *Pseudomonas fluorescens* NCIMB 10586, and the yeast *Candida glabrata*

DSM 6425. The presence of inhibition zones was examined the next day as well as on day 3, 7, and 14.

### **Amplification, sequencing and classification of the isolates**

Amplification, sequencing and phylogenetic analysis of the 16S rRNA gene sequences from the bacterial isolates were carried out as previously described (Heindl *et al.* 2010). Isolates were grouped into phylotypes by sequence similarities  $\geq 99.5\%$ . Genus affiliation was determined using the RDP classifier (Wang *et al.* (2007b). If resulting confidence values were  $< 60\%$  for the classified genus, the affiliation was specified by constructing phylogenetic trees and comparing BLAST results. This was the case for phylotypes 1 (*Erwinia*), 16 (*Roseobacter*), and 19 (*Ruegeria*).

In the case of strain BB77, a 16S rRNA gene clone library was constructed because direct sequencing of the PCR product was not successful. The PCR product was purified after gel electrophoresis with a MinElute Gel Extraction Kit (Qiagen, Hilden, Germany) and excision of the band. The purified 16S rRNA gene was ligated into the pCR<sup>®</sup>2.1-TOPO<sup>®</sup> vector and transformed into One Shot<sup>®</sup> TOP10 chemically competent *E. coli* cells, using the TOPO TA Cloning<sup>®</sup> Kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Correct insertion was checked by PCR with vector binding primers included in the kit. 14 clones were chosen for sequencing and classification of the inserted 16S rRNA gene as described above. The 16S rRNA gene sequences were deposited with the EMBL Nucleotide Sequence Database under the accession numbers FR693269 to FR693364.

### **Cluster analysis**

The distribution patterns of phylotypes and antibiotic activities of isolates were compared by cluster analysis using the Bray-Curtis similarity index, and dendrograms were generated with the program PAST applying the paired group algorithm (Hammer *et al.* 2001).

## Results

### Isolation of *M. membranacea* associated bacteria

All colonies on the four isolation media were picked from the agar plates and purified on MB agar. The majority of the 96 isolates (60.4% of all isolates) derived from media inoculated with a piece of the bryozoan, 30.2% from dilution step  $10^{-1}$ , 8.3% from step  $10^{-2}$ , and 1.0% from step  $10^{-4}$ . Most isolates (43.8% of all isolates) were obtained from ACd medium, fewer isolates resulted from R2Ad medium (34.4%), BM medium (15.6%), and AM medium (6.2%). Nearly equal portions of isolates were obtained from the three bryozoan samples (38.5%, 32.3%, and 29.2% from samples 1, 2, and 3, respectively).

### Phylogenetic affiliation

All isolates were classified phylogenetically based on 16S rRNA gene sequences and grouped into phylotypes according to sequence similarity values of  $\geq 99.5\%$ . The resulting 49 phylotypes were affiliated to 28 different genera (see Table 3-2). A cluster analysis regarding the presence and absence of phylotypes within the three bryozoan samples revealed low similarity values at  $\leq 0.3$ , with samples 1 and 2 as the most related (see Fig. 3-1A). This indicated that clearly distinct phylotypes were obtained from each sample, especially from the third bryozoan specimen, which had been prepared differently as described above. The four media had a significant influence on the isolated bacteria and resulted in dissimilar phylotype patterns. 37 phylotypes (75.5%) were unique to one of the media, *i.e.* representatives of these phylotypes were not found elsewhere. Most “unique” phylotypes derived from R2Ad medium (17 of the 23 phylotypes of this medium) followed by ACd medium (11 of 23), BM medium (6 of 12), and AM medium (3 of 6). No representatives of Gram-positive bacteria were isolated from AM medium. Accordingly, similarity values calculated for the presence and absence of phylotypes in the different media were low and ranged from 0.1 to 0.3 (see Fig. 3-1B).

The bacterial isolates were affiliated to four classes: to the Gram-negative *Gammaproteobacteria* (40 isolates) and *Alphaproteobacteria* (21) as well as to the Gram-positive *Bacilli* (12) and *Actinobacteria* (23). Representatives of these classes were isolated from each bryozoan sample, with the exception of the *Alphaproteobacteria*, which were not obtained from sample 3 (Fig. 3-2).

*Gammaproteobacteria*

The 40 isolates of the *Gammaproteobacteria* could be grouped into 15 phylotypes and assigned to ten genera: *Erwinia*, *Pseudoalteromonas*, *Vibrio*, *Shewanella*, *Halomonas* (4 phylotypes), *Marinobacter*, *Psychrobacter*, *Microbulbifer* (2), *Alcanivorax*, and *Pseudomonas* (2) (Fig. 3-3A; Table 3-2). The majority of these bacteria (85%, covering 14 phylotypes) were picked from media inoculated with a piece of the bryozoan, and most isolates (47.5%, covering 11 phlotypes) were obtained using ACd medium (Table 3-1).

Bacteria related to *Halomonas* were isolated from all three bryozoan samples and from all four media. In contrast, all eight isolates assigned to *Pseudoalteromonas* originated from sample 3 but were picked from three different isolation media. Representatives of *Psychrobacter*, *Microbulbifer*, and *Pseudomonas* derived each from more than one medium and bryozoan sample. Single isolates were obtained of *Vibrio*, *Shewanella*, *Marinobacter*, *Alcanivorax*, and *Erwinia*. The latter (BB49, phylotype 1) could represent a new species as indicated by 16S similarity values  $\leq 97\%$  to validly described species (Table 3-2). This is below the threshold value of 98.7% for 16S similarity values proposed by Stackebrandt & Ebers (2006), which indicate genomic uniqueness of novel isolates.

*Alphaproteobacteria*

Members of 16 phylotypes (21 isolates) were affiliated with the *Alphaproteobacteria* and assigned to ten genera: *Roseobacter*, *Roseovarius* (2 phylotypes), *Ruegeria* (4), *Jannaschia*, *Paracoccus*, *Anderseniella*, *Amorphus*, *Erythrobacter* (2), *Sphingopyxis* (2), and *Pelagibius*. (Fig. 3-3B; Table 3-2). In contrast to the *Gammaproteobacteria*, these isolates were predominantly picked from R2Ad medium (47.6%, covering 9 phylotypes) and plates from the dilution series (71.4%, covering 12 phylotypes) (Table 3-1).

Except for the isolates affiliated to *Sphingopyxis* (both phylotypes) and *Ruegeria* (phylotype 21), all *Alphaproteobacteria* were single isolates. New species are possibly represented by members of 13 phylotypes: *Roseobacter* (phylotype 16), *Roseovarius* (17), *Ruegeria* (19, 21), *Jannaschia* (23), *Paracoccus* (24), *Anderseniella* (25), *Amorphus* (26), *Erythrobacter* (27, 28), *Sphingopyxis* (29, 30), and *Pelagibius* (31) (Table 3-2).

### *Bacilli*

This class (Ludwig *et al.* 2009) is represented by seven phylotypes (12 isolates) affiliated to the genera *Staphylococcus*, *Bacillus* (5 phylotypes), and *Exiguobacterium* (Fig. 3-3C; Table 3-2). All but one of the isolates were obtained from plates inoculated with bryozoan samples 2 and 3 (41.7% and 50%, covering four and three phylotypes, respectively). ACd and R2Ad media yielded five isolates each. *Bacillus* and *Exiguobacterium* related isolates originated predominantly from agar plates inoculated with a bryozoan piece, while those of *Staphylococcus* derived from the dilution series (Table 3-1).

### *Actinobacteria*

The 11 phylotypes (23 isolates) affiliated to this class (Stackebrandt *et al.* 1997) were assigned to the genera *Mycobacterium* (4 phylotypes), *Streptomyces* (2), *Arthrobacter* (3), *Pseudonocardia*, and *Microbacterium* (Fig. 3-3C; Table 3-2). The majority of the isolates was obtained from ACd medium (52.2%, 6 phylotypes). Isolates affiliated to *Streptomyces* and *Arthrobacter* originated from media inoculated with a piece of the bryozoan specimens, while all others were obtained from dilution series (Table 3-1). Isolates belonging to *Mycobacterium* (phylotype 42) and *Arthrobacter* (47) possibly represent new species (Table 3-2).

### **Antibiotic activity**

Antibiotic activity against at least one indicator strain was shown by 50.5% of all isolates (47 out of 93 tested bacteria). The vast majority of the tested isolates inhibited growth of Gram-positive test strains (45.2% *B. subtilis*, 10.8% *S. lentus*, 7.5% both), while only a minor part (5.4%) was active against Gram-negative indicator bacteria (1.1% *E. coli*, 4.3% *P. fluorescens*) or against the eukaryote *C. glabrata* (1.1%). Three isolates were not analyzed due to insufficient growth on the test media (Table 3-1).

### **Activity profiles on different media**

Antibiotic activities were analyzed on six different media (TSB, MB, VNSS, PSA, R2A, and AC medium). 21 isolates each displayed activity on R2A and MB media, followed by TSB (20 isolates), AC (19), PSA (18), and VNSS (15). The majority (63.8% of active isolates) hindered growth of indicator strains on a single medium or on two media. Only six isolates (12.8% of active isolates) showed antibacterial activities on five or all six media (Table 3-1). The media had a clear influence on the pattern of antibiotic activities. A corresponding cluster analysis revealed similar activity patterns on R2A and AC as well as on MB and VNSS

media, whereas patterns on TSB and PSA media were distantly related to the other media (Fig. 3-1C).

#### **Activity profiles according to phylogenetic affiliation**

The number of active isolates affiliated to the four bacterial classes, as well as their percentage within each class, according to the six media used, is depicted in Fig. 3-4.

While the *Gammaproteobacteria* were mainly active on TSB and MB media, most of the *Alphaproteobacteria* were active on R2A and AC media and most of the *Bacilli* and *Actinobacteria* were active on PSA and TSB plates.

Regarding antibiosis against individual indicator strains, the following observations were made. As nearly all active isolates displayed activity against *B. subtilis* (89.4% of all active isolates), the media preferences were virtually identical to those considering all activities. In contrast, activity against *S. lentus* was basically shown by Gram-positive strains: while *Actinobacteria* were active on all media, *Bacilli* were mainly active on PSA and AC plates, and strains of the *Gammaproteobacteria* displayed activity against *S. lentus* particularly on AC agar plates (Table 3-1).

#### *Gammaproteobacteria*

Almost 50% of the *Gammaproteobacteria* (19 of 39 tested strains) displayed antimicrobial properties. Antibiosis was mostly directed against *B. subtilis* (78.9% of active isolates), followed by *S. lentus* (15.8%) and *P. fluorescens* (10.5%). Antibiotically active isolates were affiliated to *Erwinia* (phylotype 1), *Shewanella* (4), *Marinobacter* (9), *Pseudoalteromonas* (2, 7 out of 8 isolates), *Halomonas* (5, 3 of 4), *Psychrobacter* (10, 1 of 5), *Microbulbifer* (11 and 12, all isolates), and *Pseudomonas* (14, 1 of 4). All *Microbulbifer* affiliated isolates were active on R2A medium, while most *Pseudoalteromonas* isolates displayed activity on MB agar plates. Whereas activity against *B. subtilis* was shown by almost all active strains, bacteria of phylotype 5 (assigned to *Halomonas*) were active against *S. lentus* and *P. fluorescens* instead. Only single isolates, *Microbulbifer* sp. BB44 (phylotype 11) on AC medium and *Pseudoalteromonas* sp BB67 (phylotype 2) on TSB medium, were active against *E. coli* or *C. glabrata*, respectively. No activity was exhibited by phlotypes 3 (*Vibrio*), 6, 7, 8 (all *Halomonas*), and 15 (*Pseudomonas*).

#### *Alphaproteobacteria*

Among the *Alphaproteobacteria* more than 75% (16 out of 21 strains) were antimicrobially active. Activity was directed exclusively against *B. subtilis*. Active isolates were members of

*Roseobacter* (phylotype 16), *Roseovarius* (17 and 18), *Ruegeria* (19 to 22), *Paracoccus* (25), *Erythrobacter* (28, 1 of 2 isolates), *Sphingopyxis* (29, 1 of 3 isolates, and 30), and *Pelagibius* (31). Most of the active strains (68.8%) showed antimicrobial activities on R2A medium, followed by AC (50.0%) medium. Especially isolates that belonged to the *Roseobacter*-clade, which was represented by the phylotypes 16 to 23, displayed activity (90% of *R.*-clade isolates) preferentially on R2A medium (88.9% of active *R.*-clade isolates). The *Jannaschia* related strain BB23 (phylotype 23) was the only non-active member of this clade. In addition, the strains of phylotypes 26 (*Amorphus*) and 27 (*Erythrobacter*) displayed no activity, as did two strains of phylotype 29 (*Sphingopyxis*).

### *Bacilli*

Antibiotic activity of isolates related to *Bacilli* was directed against *B. subtilis* (7 strains) or both *B. subtilis* and *S. lentus* (3 strains, all *Bacillus*, phylotypes 34, 35, and 36). One isolate (BB42, phylotype 35) showed additional anti-*P. fluorescens* activity. This *Bacillus* affiliated isolate was also capable of expressing antimicrobial activities on all media. All *Exiguobacterium* affiliated strains (phylotype 38) were active against *B. subtilis*. One representative of phylotype 32 (*Staphylococcus*) and 34 (*Bacillus*) as well as phylotype 33 (*Bacillus*) did not impede growth of any indicator strain.

### *Actinobacteria*

Only five out of 23 *Actinobacteria* related isolates inhibited growth of indicator strains. Four strains each were active against *B. subtilis* and *S. lentus*. Three of them (phylotypes 44 and 45, both *Streptomyces*) displayed activity against both Gram-positive test strains on all six media. Isolate BB84 (phylotype 44) was additionally active against *P. fluorescens*. The *Mycobacterium* related strain of phylotype 41 showed anti-*S. lentus* activity. Phylotypes with no active representatives were: 39, 40, and 42 (all *Mycobacterium*), 43 (*Pseudonocardia*), 46 to 48 (all *Arthrobacter*), and 49 (*Microbacterium*).



**Table 3-1. Origin, affiliation and antimicrobial activity of *M. membranacea* associated bacteria**

Phylo type	Isolate	Affiliation	Isolation <sup>a</sup>			Activity <sup>b</sup>				
			Specimen	Medium	Source	Bs	Sl	Ec	Pf	Cg
1	BB49	<i>Erwinia</i>	2	BM	p	T				
2	BB66a	<i>Pseudoalteromonas</i>	3	BM	p					
2	BB67	<i>Pseudoalteromonas</i>	3	R2Ad	p					T
2	BB69	<i>Pseudoalteromonas</i>	3	R2Ad	p	M				
2	BB71	<i>Pseudoalteromonas</i>	3	ACd	p	M				
2	BB72b	<i>Pseudoalteromonas</i>	3	ACd	p	TM				
2	BB73	<i>Pseudoalteromonas</i>	3	ACd	p	TM				
2	BB74	<i>Pseudoalteromonas</i>	3	ACd	p	TM				
2	BB68	<i>Pseudoalteromonas</i>	3	R2Ad	p	M				
3	BB8	<i>Vibrio</i>	1	ACd	p					
4	BB86	<i>Shewanella</i>	1	ACd	p	TMV	A			
5	BB12b	<i>Halomonas</i>	1	ACd	p					
5	BB66b	<i>Halomonas</i>	3	BM	p			A		T
5	BB85	<i>Halomonas</i>	2	ACd	p			PRA		
5	BB7	<i>Halomonas</i>	1	AM	p					P
6	BB10	<i>Halomonas</i>	1	ACd	p					
6	BB3	<i>Halomonas</i>	1	ACd	1					
6	BB6	<i>Halomonas</i>	1	AM	p					
6	BB65	<i>Halomonas</i>	3	BM	p					
7	BB9	<i>Halomonas</i>	1	ACd	p					
8	BB11b	<i>Halomonas</i>	1	ACd	p					
8	BB81	<i>Halomonas</i>	1	R2Ad	p					
8	BB82b	<i>Halomonas</i>	1	R2Ad	p					
9	BB15	<i>Marinobacter</i>	1	ACd	p	T	A			
10	BB13	<i>Psychrobacter</i>	1	R2Ad	p					
10	BB14	<i>Psychrobacter</i>	1	R2Ad	p					
10	BB20	<i>Psychrobacter</i>	1	ACd	p					
10	BB62	<i>Psychrobacter</i>	3	BM	1	T	V			
10	BB83	<i>Psychrobacter</i>	1	ACd	p					
11	BB44	<i>Microbulbifer</i>	2	R2Ad	p	MVPRA		A	A	
12	BB27	<i>Microbulbifer</i>	1	AM	2	R				
12	BB34	<i>Microbulbifer</i>	2	ACd	1	PR				
12	BB48	<i>Microbulbifer</i>	2	ACd	p	R				
13	BB31	<i>Alcanivorax</i>	2	R2Ad	1	n.t.	n.t.	n.t.	n.t.	n.t.
14	BB5	<i>Pseudomonas</i>	1	R2Ad	1					
14	BB82a	<i>Pseudomonas</i>	1	R2Ad	p					
14	BB79	<i>Pseudomonas</i>	1	R2Ad	p					
14	BB80	<i>Pseudomonas</i>	1	R2Ad	p	T	V	A		
15	BB75a	<i>Pseudomonas</i>	3	ACd	p					
15	BB78	<i>Pseudomonas</i>	3	ACd	p					
16	BB43	<i>Roseobacter</i>	2	R2Ad	p	R				
17	BB22	<i>Roseovarius</i>	1	R2Ad	1	R				
18	BB19	<i>Roseovarius</i>	1	AM	2	RA				
19	BB50b	<i>Ruegeria</i>	2	BM	p	V	RA			
20	BB40	<i>Ruegeria</i>	2	R2Ad	p	MVPRA				
21	BB2	<i>Ruegeria</i>	1	ACd	1	MV	A			
21	BB29	<i>Ruegeria</i>	2	R2Ad	1	RA				
21	BB45	<i>Ruegeria</i>	2	R2Ad	p	MV	R			
22	BB33	<i>Ruegeria</i>	2	ACd	1	MV	RA			
23	BB23	<i>Jannaschia</i>	1	R2Ad	1					
24	BB51b	<i>Paracoccus</i>	2	BM	p	T	RA			
25	BB54	<i>Andersenella</i>	2	AM	2	R				
26	BB18	<i>Amorphus</i>	1	AM	2					
27	BB32	<i>Erythrobacter</i>	2	R2Ad	1					
28	BB17	<i>Erythrobacter</i>	1	R2Ad	4	MV				
29	BB1	<i>Sphingopyxis</i>	1	ACd	2	T				
29	BB4	<i>Sphingopyxis</i>	1	ACd	1					
29	BB46	<i>Sphingopyxis</i>	2	ACd	p					
30	BB24	<i>Sphingopyxis</i>	1	R2Ad	1	VP				
30	BB28	<i>Sphingopyxis</i>	2	ACd	2	M				
31	BB21	<i>Pelagibius</i>	1	R2Ad	1	M	PRA			

Continued on next page

Table 3-1. continued

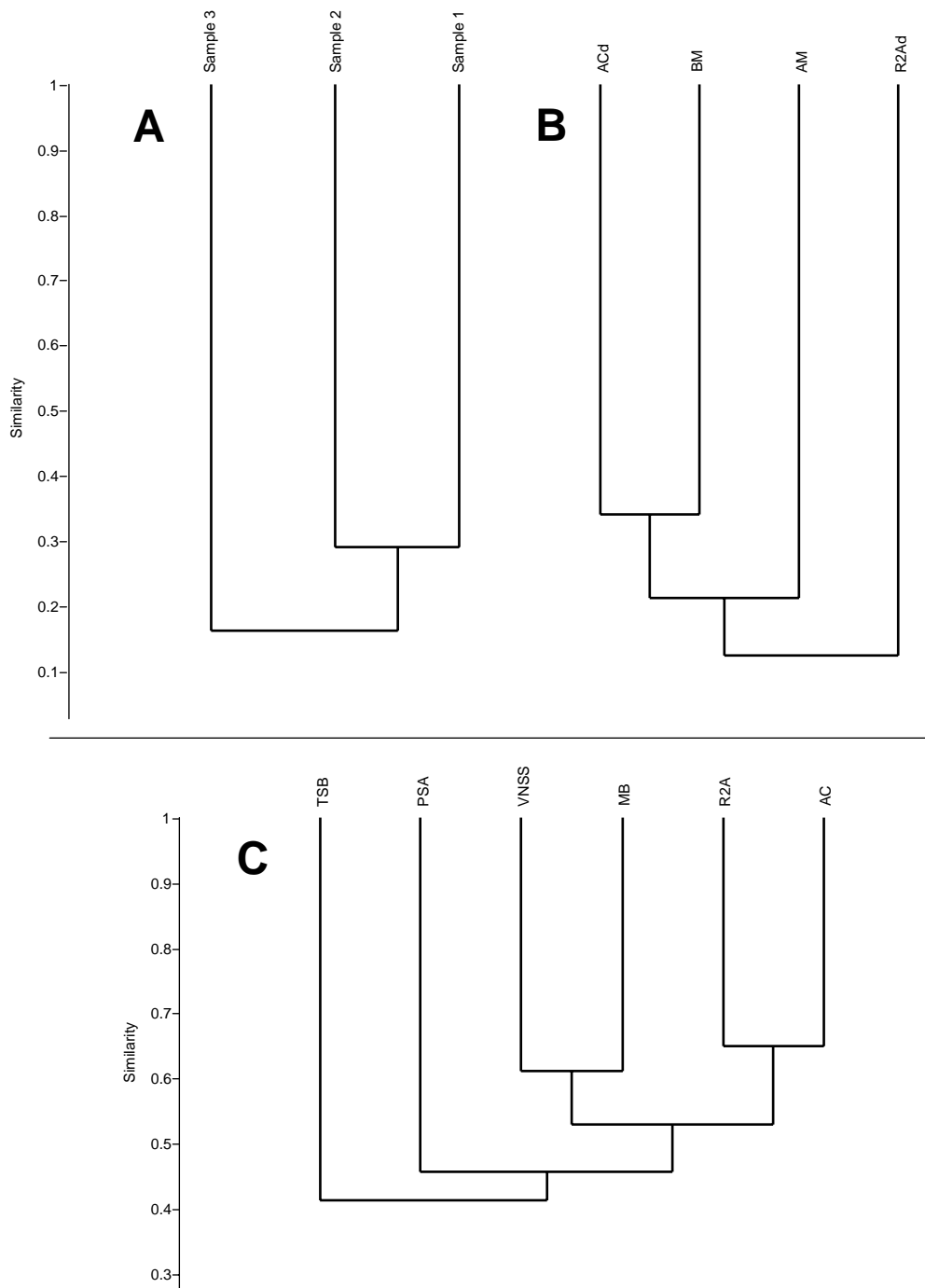
Phylo type	Isolate	Affiliation (Genus)	Isolation <sup>a</sup>			Activity <sup>b</sup>				
			Specimen	Medium	Source	Bs	Sl	Ec	Pf	Cg
32	BB58a	<i>Staphylococcus</i>	3	ACd	1	<i>n.t.</i>	<i>n.t.</i>	<i>n.t.</i>	<i>n.t.</i>	<i>n.t.</i>
32	BB58b	<i>Staphylococcus</i>	3	ACd	1	<i>n.t.</i>	<i>n.t.</i>	<i>n.t.</i>	<i>n.t.</i>	<i>n.t.</i>
32	BB60	<i>Staphylococcus</i>	3	R2Ad	1	P				
32	BB26	<i>Staphylococcus</i>	1	ACd	1					
33	BB41	<i>Bacillus</i>	2	R2Ad	p					
34	BB50c	<i>Bacillus</i>	2	BM	p	P	P			
34	BB51c	<i>Bacillus</i>	2	BM	p					
35	BB42	<i>Bacillus</i>	2	R2Ad	p	T VPRA	TMVPRA		VR	
36	BB52	<i>Bacillus</i>	2	R2Ad	p	T	P A			
37	BB61	<i>Bacillus</i>	3	R2Ad	1	T P				
38	BB75b	<i>Exiguobacterium</i>	3	ACd	p	TM PR				
38	BB76	<i>Exiguobacterium</i>	3	ACd	p	M				
39	BB36	<i>Mycobacterium</i>	2	ACd	1					
39	BB55	<i>Mycobacterium</i>	3	ACd	1					
39	BB56	<i>Mycobacterium</i>	3	ACd	1					
39	BB57	<i>Mycobacterium</i>	3	ACd	1					
39	BB64	<i>Mycobacterium</i>	3	BM	2					
40	BB35	<i>Mycobacterium</i>	2	ACd	1					
41	BB37	<i>Mycobacterium</i>	2	ACd	1		T P			
42	BB38	<i>Mycobacterium</i>	2	BM	1					
43	BB63	<i>Pseudonocardia</i>	3	R2Ad	2					
44	BB16	<i>Streptomyces</i>	1	BM	p	P				
44	BB47a	<i>Streptomyces</i>	2	ACd	p					
44	BB47b	<i>Streptomyces</i>	2	ACd	p					
44	BB50a	<i>Streptomyces</i>	2	BM	p					
44	BB51a	<i>Streptomyces</i>	2	BM	p					
44	BB11a	<i>Streptomyces</i>	1	ACd	p	TMVPRA	TMVPRA			
44	BB84	<i>Streptomyces</i>	1	ACd	p	TMVPRA	TMVPRA		T	
45	BB12a	<i>Streptomyces</i>	1	ACd	p	TMVPRA	TMVPRA			
46	BB72a	<i>Arthrobacter</i>	3	ACd	p					
47	BB59	<i>Arthrobacter</i>	3	R2Ad	1					
47	BB70	<i>Arthrobacter</i>	3	R2Ad	p					
48	BB77	<i>Arthrobacter</i>	3	BM	p					
49	BB25	<i>Microbacterium</i>	1	R2Ad	1					
49	BB30	<i>Microbacterium</i>	2	R2Ad	1					

<sup>a</sup> p, piece; 1-4, dilution series 1-4.

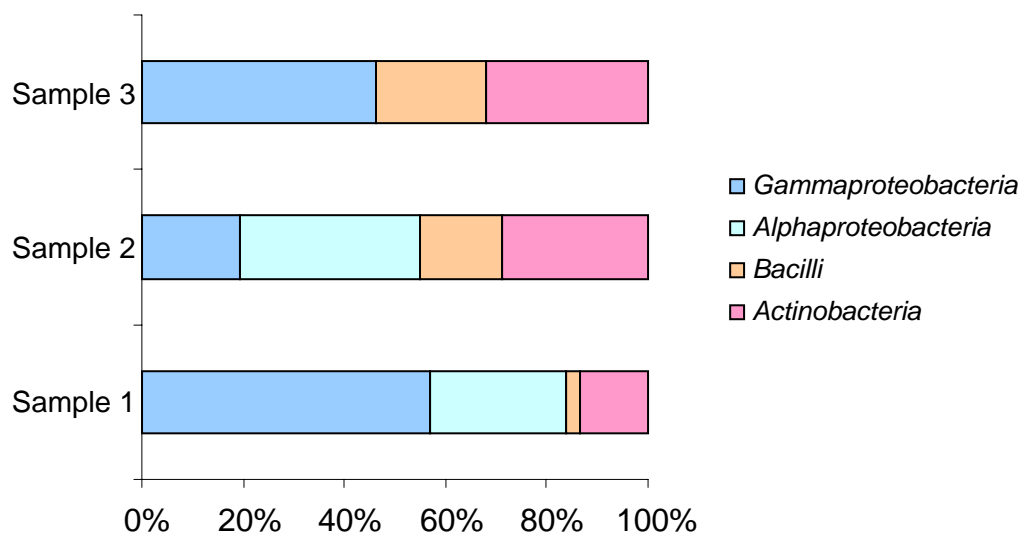
<sup>b</sup> Bs, *B. subtilis*; Sl, *S. lentus*; Ec, *E. coli*; Pf, *P. fluorescens*; Cg, *C. glabrata*; T, TSB; M, MB; V, VNSS; P, PSA; R, R2A; A, AC, *n.t.*, not tested.

**Table 3-2. Phylogenetic affiliation (RDP) and nearest type strains (BLAST)**

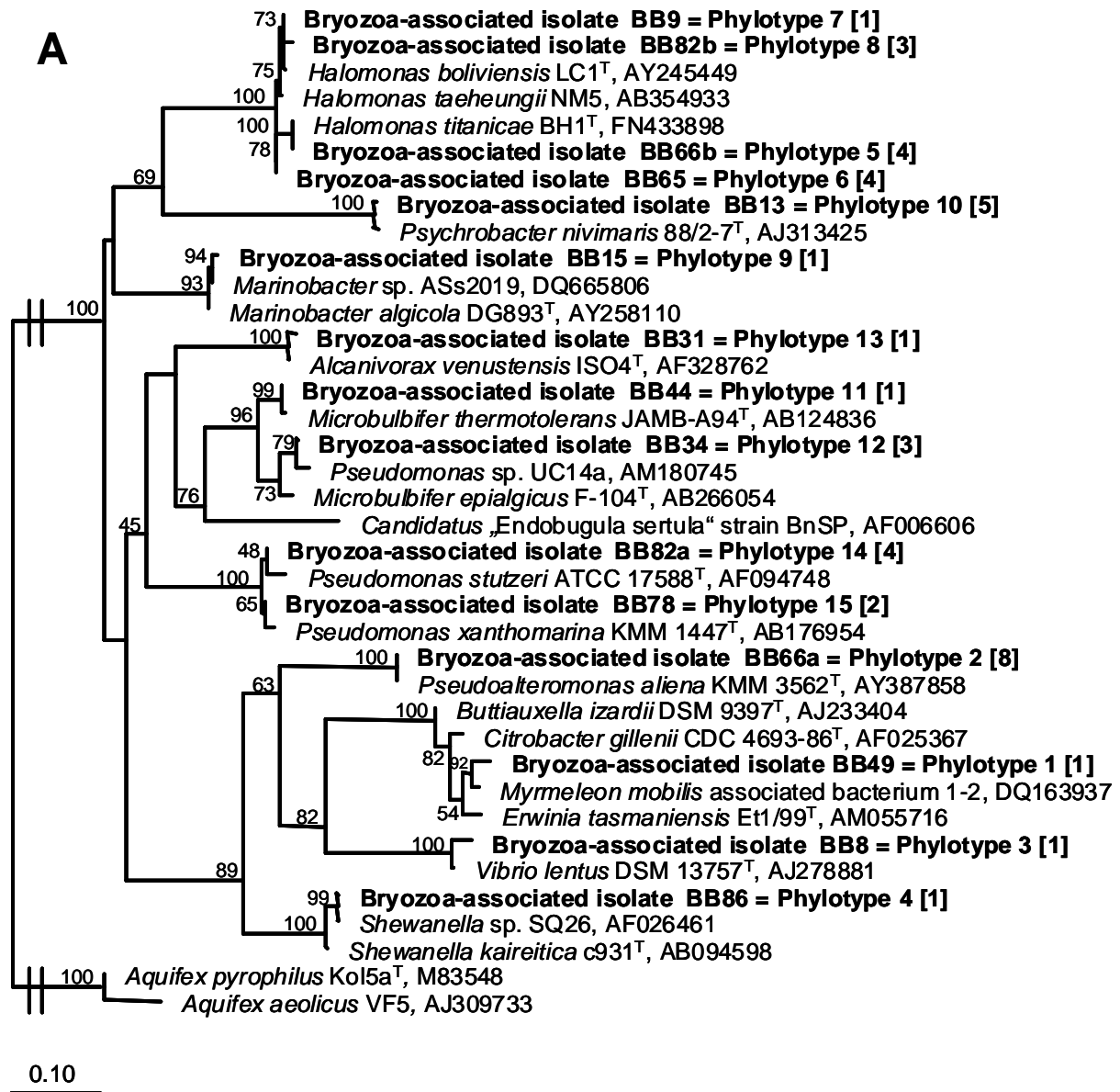
Phylo-type	Re-presentative	No. of isolates	Affiliation	Nearest type strain	Similarity (%)	Accession no.
1	BB49	1	<i>Erwinia</i>	<i>Erwinia tasmaniensis</i>	97	AM055716
				<i>Citrobacter gilleni</i>	97	AF025367
2	BB66a	8	<i>Pseudoalteromonas</i>	<i>Pseudoalteromonas aliena</i>	99	AY387858
3	BB8	1	<i>Vibrio</i>	<i>Vibrio tasmaniensis</i>	98	AJ316192
4	BB86	1	<i>Shewanella</i>	<i>Shewanella kaireitica</i>	98	AB094598
5	BB66b	4	<i>Halomonas</i>	<i>Halomonas titanicae</i>	99	FN433898
6	BB65	4	<i>Halomonas</i>	<i>Halomonas boliviensis</i>	99	AY245449
7	BB9	1	<i>Halomonas</i>	<i>Halomonas boliviensis</i>	99	AY245449
8	BB82b	3	<i>Halomonas</i>	<i>Halomonas boliviensis</i>	99	AY245449
9	BB15	1	<i>Marinobacter</i>	<i>Marinobacter algicola</i>	99	AY258110
10	BB13	5	<i>Psychrobacter</i>	<i>Psychrobacter piscatorii</i>	99	AB453700
11	BB44	1	<i>Microbulbifer</i>	<i>Microbulbifer thermotolerans</i>	99	AB124836
12	BB34	3	<i>Microbulbifer</i>	<i>Microbulbifer epialgicus</i>	98	AB266054
13	BB31	1	<i>Alcanivorax</i>	<i>Alcanivorax venustensis</i>	99	AF328762
14	BB82a	4	<i>Pseudomonas</i>	<i>Pseudomonas perfectomarina</i>	100	U65012
15	BB78	2	<i>Pseudomonas</i>	<i>Pseudomonas chloritidismutans</i>	99	AY017341
16	BB43	1	<i>Roseobacter</i>	<i>Leisingera nanhaiensis</i>	97	FJ232451
				<i>Seohicola saemankumensis</i>	96	EU221274
17	BB22	1	<i>Roseovarius</i>	<i>Roseovarius aestuarii</i>	97	EU156066
18	BB19	1	<i>Roseovarius</i>	<i>Roseovarius aestuarii</i>	99	EU156066
19	BB50b	1	<i>Ruegeria</i>	<i>Ruegeria scottomollicae</i>	96	AM905330
20	BB40	1	<i>Ruegeria</i>	<i>Ruegeria scottomollicae</i>	98	AM905330
21	BB2	3	<i>Ruegeria</i>	<i>Ruegeria atlantica</i>	96	D88526
22	BB33	1	<i>Ruegeria</i>	<i>Ruegeria atlantica</i>	99	D88526
23	BB23	1	<i>Jannaschia</i>	<i>Jannaschia pohangensis</i>	97	DQ643999
24	BB51b	1	<i>Paracoccus</i>	<i>Paracoccus homiensis</i>	97	DQ342239
25	BB54	1	<i>Andersenella</i>	<i>Andersenella baltica</i>	97	AM712634
26	BB18	1	<i>Amorphus</i>	<i>Amorphus coralli</i>	95	DQ097300
27	BB32	1	<i>Erythrobacter</i>	<i>Erythrobacter longus</i>	97	AF465835
28	BB17	1	<i>Erythrobacter</i>	<i>Erythrobacter aquimaris</i>	98	AY461441
29	BB1	3	<i>Sphingopyxis</i>	<i>Sphingopyxis litoris</i>	98	DQ781321
30	BB24	2	<i>Sphingopyxis</i>	<i>Sphingopyxis litoris</i>	98	DQ781321
31	BB21	1	<i>Pelagibius</i>	<i>Pelagibius litoralis</i>	92	DQ401091
32	BB58b	4	<i>Staphylococcus</i>	<i>Staphylococcus epidermidis</i>	99	D83363
33	BB41	1	<i>Bacillus</i>	<i>Bacillus hwajinpoensis</i>	98	AF541966
34	BB50c	2	<i>Bacillus</i>	<i>Bacillus hwajinpoensis</i>	99	AF541966
35	BB42	1	<i>Bacillus</i>	<i>Bacillus stratosphericus</i>	99	AJ831841
36	BB52	1	<i>Bacillus</i>	<i>Bacillus licheniformis</i>	99	CP000002
37	BB61	1	<i>Bacillus</i>	<i>Bacillus cereus</i>	99	AE016877
38	BB76	2	<i>Exiguobacterium</i>	<i>Exiguobacterium oxidotolerans</i>	99	AB105164
39	BB64	5	<i>Mycobacterium</i>	<i>Mycobacterium frederiksbergense</i>	99	AJ276274
40	BB35	1	<i>Mycobacterium</i>	<i>Mycobacterium aurum</i>	99	X55595
41	BB37	1	<i>Mycobacterium</i>	<i>Mycobacterium aurum</i>	98	X55595
42	BB38	1	<i>Mycobacterium</i>	<i>Mycobacterium komossense</i>	97	X55591
43	BB63	1	<i>Pseudonocardia</i>	<i>Pseudonocardia carboxydivorans</i>	99	EF114314
44	BB51a	7	<i>Streptomyces</i>	<i>Streptomyces griseorubens</i>	99	AB184139
45	BB12a	1	<i>Streptomyces</i>	<i>Streptomyces praecox</i>	99	AB184293
46	BB72a	1	<i>Arthrobacter</i>	<i>Arthrobacter parietis</i>	99	AJ639830
47	BB70	2	<i>Arthrobacter</i>	<i>Arthrobacter tumbae</i>	97	AJ315069
48	BB77	1	<i>Arthrobacter</i>	<i>Arthrobacter agilis</i>	98	X80748
49	BB25	2	<i>Microbacterium</i>	<i>Microbacterium schleiferi</i>	99	Y17237



**Figure 3-1. Similarity dendrograms** of shared phylotypes within the three *M. membranacea* samples (A), the four isolation media (B), as well as of antimicrobial activities expressed by the isolates on different media (C).



**Figure 3-2. Relative abundance** of isolates from three *M. membranacea* samples affiliated with the four bacterial classes observed in this study.



**Figure 3-3. Maximum likelihood tree** constructed from 16S rRNA gene sequences showing the phylogenetic relationships of isolates from this study to closely related species and some other selected representatives of the *Gammaproteobacteria* (A), the *Alphaproteobacteria* (B) and Gram-positive bacteria (C). Non-parametric bootstrapping analysis (100 datasets) was conducted. Values equal to or greater than 50 are shown. The scale bar indicates the number of substitutions per nucleotide position. The total number of represented sequences is given in square brackets.

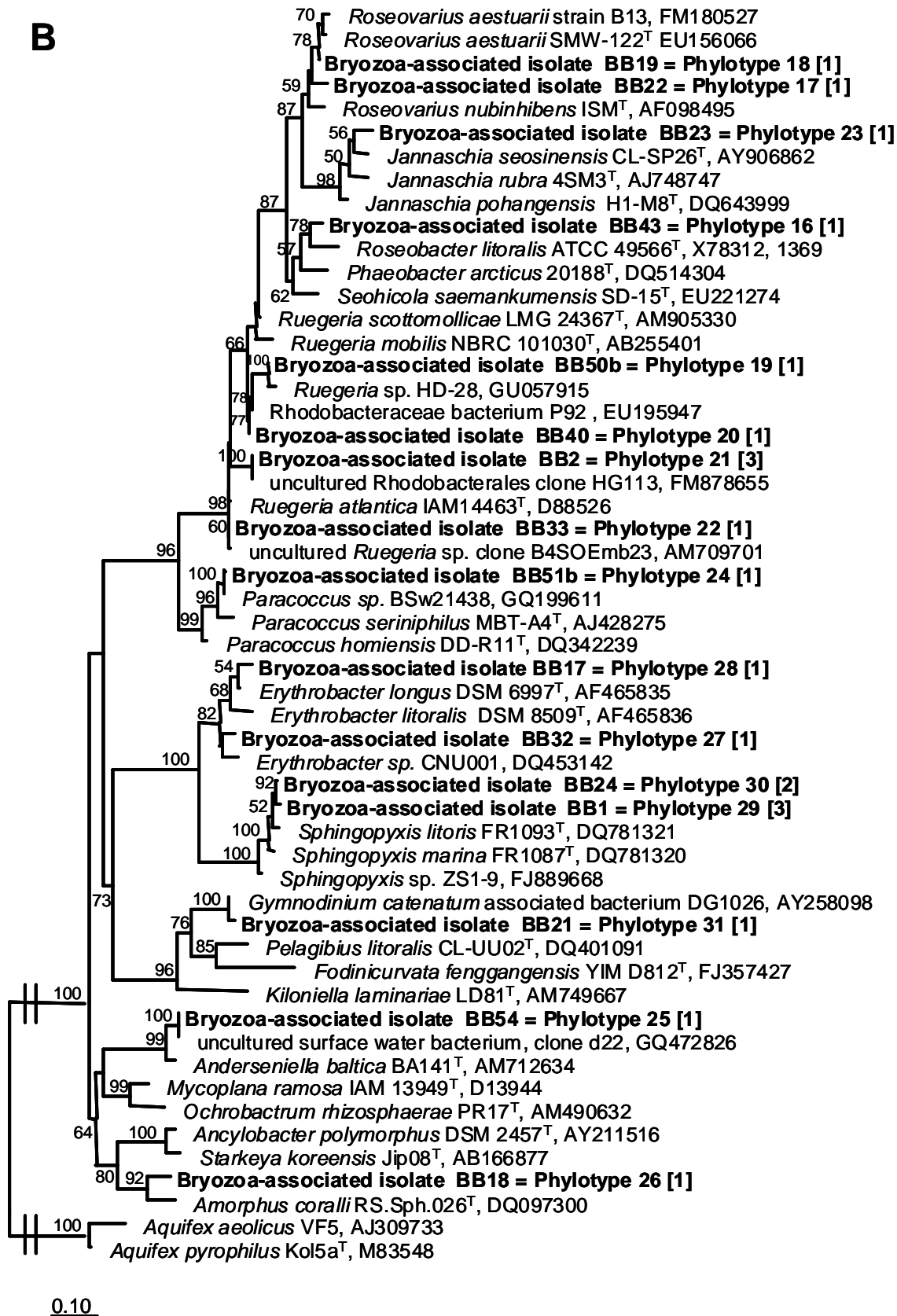
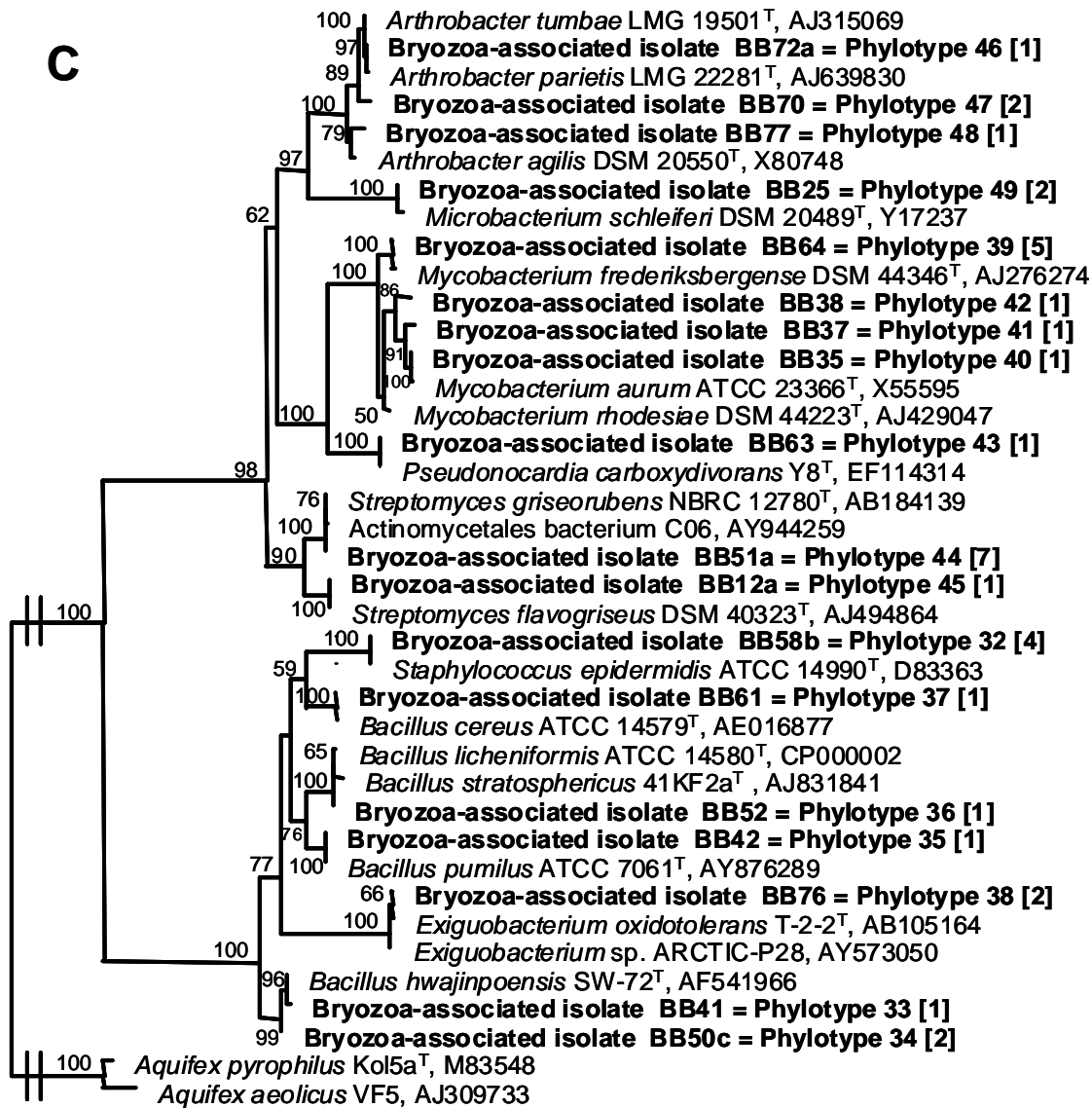


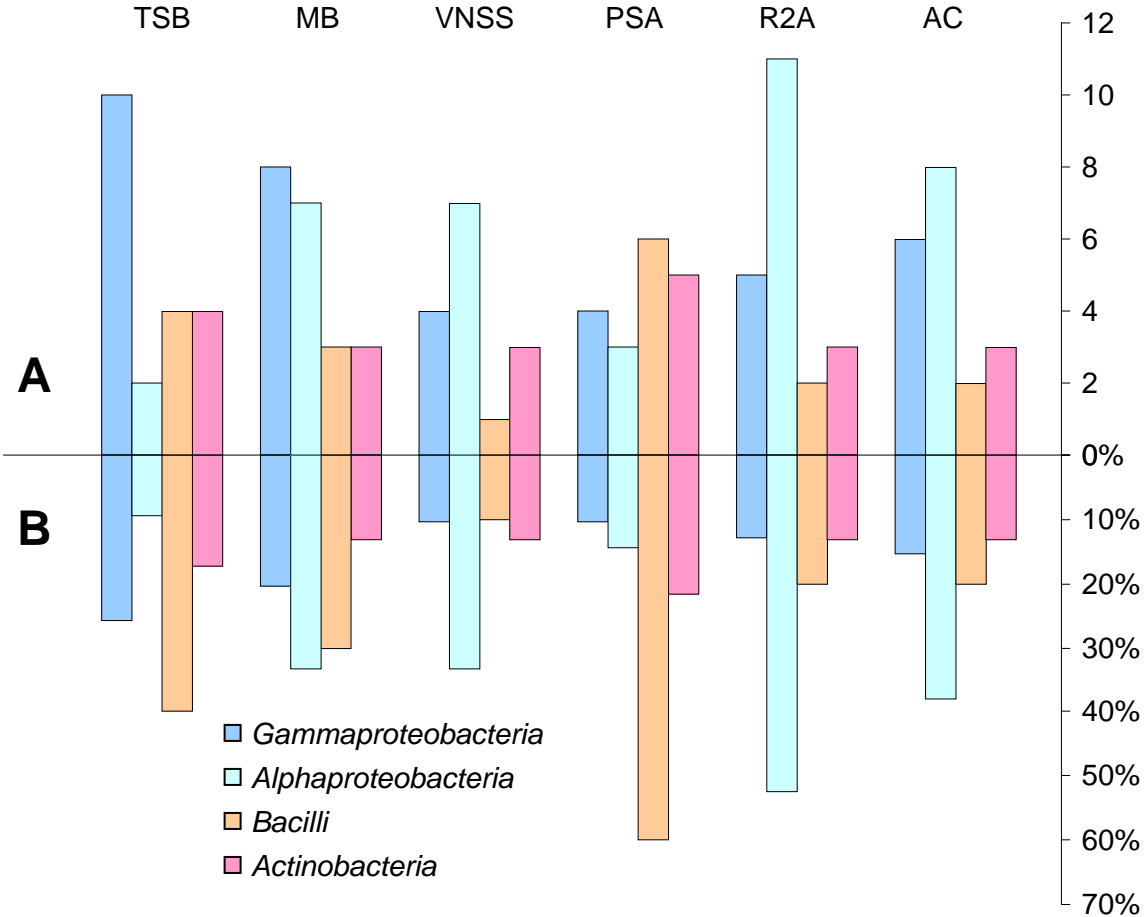
Figure 3-3. continued



0.10

Figure 3-3. continued





**Figure 3-4. Absolute counts** of active isolates (A). Percentage of active isolates within each bacterial class (B).

## **Discussion**

### **Isolated bacteria**

#### *Impact of media*

In this study, BM and AM media resembled the natural habitat, whereas the other two media (R2Ad and ACd) were composed of “artificial” ingredients. Interestingly, the “natural” media yielded a third of the isolates compared to the amount that grew on the “artificial” plates. Nevertheless, each medium featured a rather unique set of phylotypes. This resulted in a phylogenetically diverse collection of isolates, which is discussed in more detail below. Nutrients in the “artificial” media, especially glucose, were possibly easier accessible and favoured growth of microorganisms. On the contrary, potentially inhibitory compounds that originate from bryozoans or algae hindered the growth, since such sessile macroorganisms rely on chemical defence mechanisms (Gerdes *et al.* 2005a, Peters *et al.* 2003). The fewest isolates were obtained from “algal extract medium” (AM). As marine algae are producers of a variety of active metabolites to prevent biofouling of their own surfaces (Bhadury & Wright 2004), some of these natural products may have been stable enough to express growth inhibiting properties even after autoclaving. Unsaturated fatty acids have been identified as antibacterially active agents from brown algae which also maintained their properties even if stored at room temperature for several years (Rosell & Srivastava 1987). The activities were especially directed against Gram-positive bacteria. This correlates with the finding that no Gram-positive bacteria were obtained from AM plates in this study.

The detrimental effects of inhibitory compounds and inaccessibility of nutrients in the “natural” isolation media seemed to have outbalanced the usually beneficial impact of habitat water as shown in previous studies (Eguchi 1999, Muscholl-Silberhorn *et al.* 2008).

#### *Distribution of phylotypes*

The use of four different isolation media was advantageous in order to obtain a diverse collection of bacteria. The high number of phylotypes that were exclusively found on one medium (75.5%) and the resulting low similarity values (Fig.3-1B) indicated a quite “unique” set of isolates obtained from each medium. This observation correlates with the known fact, that phylogenetically different bacteria express different needs on nutrients, growth factors, salt composition, trace elements, *et cetera*, which could not be covered by a single medium. Nonetheless, clear media preferences could not be narrowed down to a specific group of phylotypes or genera, as all media yielded phylogenetically diverse isolates. However, a

certain bias of ACd medium towards the isolation of *Gammaproteobacteria* and *Actinobacteria*, as well as of R2Ad medium towards *Alphaproteobacteria* and *Bacilli* was noted.

Interestingly, the three bryozoan specimens yielded a similar amount of exclusive phylotypes: 38 phylotypes (77.6%) originated from single samples exclusively, which resulted also in low similarity values between the samples (Fig.3-1A). Analogously to the influence of the isolation media, this reflected quite “unique” collections of bacteria obtained from each specimen. A previous cultivation based study on the microbial diversity with samples of the North Sea bryozoan *Flustra foliacea* showed similar results (Pukall *et al.* 2001): although a great array of different isolation media was used and the same procedures were applied to all specimens, the distribution of bacterial taxons was very divergent. Indeed, no single genus could be found on all three samples. Another culture independent study on bacterial communities of bryozoans in the North Sea has demonstrated species-specific associations for three of the four bryozoan species (*Aspidelectra melolontha*, *Electra monostachys*, and *E. pilosa*). In contrast, a site-dependent influence was observed in *Conopeum reticulum* specimens (Kittelmann & Harder 2005).

The lower similarities between the bryozoan specimens in this study are likely due to the cultivation based approach. Nevertheless, specific but yet not cultivated bacterial associates may exist in the case of *M. membranacea*. Culture-independent methods cover a greater variety of bacterial species, which would increase the chance to detect any specific communities and hence raise the similarity between samples. Furthermore, host-specific bacteria will be strongly dependant on their host’s attributes and would be outcompeted *in vitro* by other associated microorganisms that can more easily adapt to varying conditions.

In this study, sample preparation procedures also had an impact on the subsequent phylogenetic composition of the isolated bacteria. *M. membranacea* sample 3 showed a striking dissimilarity in associated bacteria compared to the other samples, especially due to its lack of *Alphaproteobacteria*. The different preparation procedure prior to plating generated shear stress and a higher temperature on this sample, which probably had “inactivated” or killed sensitive bacteria.

*Phylogenetic affiliation of isolates*

Members belonging to 29 different genera were associated with *M. membranacea* growing on *S. latissima*. Three of these genera, *Shewanella*, *Pseudoalteromonas*, and *Pseudomonas*, have also been reported in three comparable, cultivation based studies on bryozoans from the North and Baltic Sea, as well as from Baltic *S. latissima* samples (Heindl *et al.* 2010, Pukall *et al.* 2001, Wiese *et al.* 2009). Other genera from this study which were also listed in these surveys (but not in all three of them) included *Bacillus*, *Arthrobacter*, *Vibrio*, as well as *Psychrobacter*, *Ruegeria*, *Staphylococcus*, and *Streptomyces*. Consequently, members of only one-third of the genera found in this study have been isolated from comparable sources before. This corroborates the bryozoan *M. membranacea*'s suitability as source of novel bacterial isolates.

A prominent group of heterotrophic bacteria, the 'Bacteroidetes', was also reported in all of these studies (Heindl *et al.* 2010, Pukall *et al.* 2001, Wiese *et al.* 2009) but corresponding isolates were lacking in this work. It belongs together with the *Proteobacteria* to the two most abundant groups in aquatic ecosystems as determined by FISH (Kirchman 2002). However, it is also known for its local and seasonal variability (Alonso *et al.* 2007). This could explain why members of this group had not been obtained in this study.

Within the *Alphaproteobacteria* half of the phylotypes (16 to 23) and isolates of this study were affiliated to members of the *Roseobacter* lineage. Organisms of this clade can account for up to 27% of quantitative 16S rRNA gene clone libraries in seawater and are also abundant in various marine bacterial communities associated with algal blooms, biofilms, cephalopods, *et cetera*. A characteristic trait is the requirement for sodium ions, and validly described species of this clade originate almost entirely from marine habitats (Brinkhoff *et al.* 2008). In contrast to the *Roseobacter* clade, where several cultivated strains are also closely related to retrieved environmental sequences (Buchan *et al.* 2005), readily culturable members of other phylogenetic groups (*Gammaproteobacteria*: *Pseudoalteromonas*, *Alteromonas*, *Vibrio*, *Halomonas* and others) are underrepresented in the marine environment when cultivation-free techniques are applied (Eilers *et al.* 2000). Hence, the 'Roseobacter clade'-isolates from this work could actually represent *in vivo* abundant bacteria on *M. membranacea*.

In this study, members of the *Alphaproteobacteria* also accounted by far for the highest number of possibly novel species as far as 16S rRNA gene sequence similarities are concerned. The low nutrient media used for isolation have probably favored growth of otherwise outcompeted bacterial strains. Additionally, the milder grinding of the first two

bryozoan specimens proved advantageous in order to isolate *Alphaproteobacteria*, because none were obtained from the third sample which had been prepared more vigorously. At last cultivation attempts from bryozoans are still very scarce compared to other marine sources and thus provide higher chances of finding novel bacteria. Validly described type strains to date, which have been originally isolated from bryozoans, are *Tenacibaculum adriaticum* (*Flavobacteria*), *Marinobacter bryozorum* (*Gammaproteobacteria*), and *Paracoccus seriniphilus* (*Alphaproteobacteria*) (Heindl *et al.* 2008, Pukall *et al.* 2003, Romanenko *et al.* 2005). Representatives of the latter two genera were also isolated in this study, albeit with single isolates each.

### **Antimicrobial activity**

Bacterial isolates from this study displayed predominately antibiosis against Gram-positive test strains. This result is similar to those obtained in our previous study on activities of bryozoan-associated bacteria where the same indicator organisms for antibacterial testing were used (Heindl *et al.* 2010). However, isolates from the present work were affiliated with different genera (except for *Vibrio*, *Shewanella*, *Pseudoalteromonas*, and *Pseudomonas*) and consisted also of numerous Gram-positive representatives. Activity against *B. subtilis* and *S. lentus* could be advantageous on surfaces *in vivo* as members of both genera were also isolated from the bryozoan specimens.

Both phylotype/genus and strain-specific activities could be observed. All isolates of the genera *Microbulbifer*, *Roseovarius*, *Ruegeria*, and *Exiguobacterium* showed consistent profiles (genus-specific activity). Moreover, this antibiosis was expressed on the same media each with single exceptions of *Ruegeria* affiliated strains (Table 3-1). In contrast, only some isolates related to *Pseudoalteromonas*, *Psychrobacter*, *Pseudomonas*, *Sphingopyxis*, *Bacillus*, *Staphylococcus*, or *Streptomyces* inhibited target organisms (strain-specific activity). It is to note that strain-specific activities seem to be more probable when larger subsets of analogous isolates, as determined by 16S rDNA sequencing, were tested (*e.g.* *Pseudoalteromonas* or *Streptomyces* related isolates from this study) (LoGiudice *et al.* 2007a, LoGiudice *et al.* 2007b).

In addition to these phylogenetic traits, “suitable” testing conditions might reveal bioactivity of otherwise non-active strains. Each of the *Sphingopyxis* related strains from this work expressed activities on different media. The activation of secondary metabolite pathways which remain silent under standard laboratory conditions is a feasible way to access new natural products in microorganisms (Brakhage & Schroeckh 2010, Perić-Concha & Long

2003). In this study, the influence of test media on antibiotic traits reflected this dependency of the bacterial isolates on a “suitable” environment. Correspondingly, most activities were expressed on one or two media only (63.8%), whereas merely a minor fraction of the isolates was able to hinder growth on all or five media (12.8%). Likewise the use of six different test media resulted in a twofold higher discovery of active bacteria than it would using only a single medium.

Microorganisms belong to the prominent producers of natural products in the marine environment. The best studied genera in terms of published metabolites are *Streptomyces*, *Alteromonas*, *Bacillus*, *Vibrio*, *Pseudomonas*, *Actinomyces*, and *Pseudoalteromonas*, which were all isolated in this work too. Other genera of this study like *Microbacterium*, *Marinobacter*, *Halomonas*, *Ruegeria*, and *Erythrobacter* also contribute to published marine natural products but to a lesser extent (Laatsch 2006). However, only some of these substances were reported as antimicrobial active. For example, in the case of *Pseudoalteromonas* and *Pseudomonas* some secondary metabolites displayed antibiotic properties (Bowman 2007, Isnansetyo & Kamei 2009). Members of the *Roseobacter* clade were reported to produce the antimicrobial metabolite thiotropocin and its precursor tropodithietic acid (Brinkhoff *et al.* 2004, Bruhn *et al.* 2005, Bruhn *et al.* 2007). Sponge and ascidian-associated *Microbulbifer* strains produced variations of parabens, which preservative properties were widely applied in pharmaceuticals, cosmetics or food (Peng *et al.* 2006, Quévrain *et al.* 2009). At last, other well-documented producers of antimicrobially active compounds like *Streptomyces* strains can still be a source of novel compounds: in a recent study, the production of the antiproliferative and antibacterial compound mayamycin by a marine *Streptomyces* related strain could be induced by variation of culture conditions (Schneemann *et al.* 2010). Several members of those genera known for the production of antimicrobial substances were also found within this study. Except for *Streptomyces* or *Bacillus*, however, these reports are rare, and especially novel species, in particular the *Alphaproteobacteria* related isolates of this work, would likely produce novel antimicrobial compounds.

The association of bacteria with marine surfaces, in this study with the bryozoan *M. membranacea*, which in turn grew on the alga *S. latissima*, likely reflects the need for appropriate conditions *in vitro* to stimulate the production of antibiotic substances. Microbes on marine surfaces are exposed to competition, and associations are based on chemical/metabolic interactions. While the host-organisms could take advantage of antifouling activities made available by colonizing bacteria and let establish a stable

association, inanimate surfaces lack this ability and microbial settling would be more variable over time. This makes “living surfaces” a potential target for finding bioactive microorganisms. Moreover, the active compounds should feature high potencies to compensate for their dilution in the water (Egan *et al.* 2008, Penesyán *et al.* 2010).

### **Conclusion**

Our results have demonstrated that living substrates like the bryozoan *M. membranacea* are a promising source of bacteria with antimicrobial activities. Many isolates belonged to genera which are known for their occurrence in marine environments (*e.g.* *Pseudoalteromonas*, *Shewanella*, *Marinobacter*) or antibiotic properties (*e.g.* *Streptomyces*, *Bacillus*). Nevertheless, several isolates, especially those related to the *Alphaproteobacteria*, could very likely represent new bacterial species with antimicrobial traits. The use of low nutrient media as well as both “artificial” and “natural” ingredients has contributed to the high phylogenetic diversity of bacterial isolates. Additionally, surveys on bryozoan-associated bacteria are still scarce, which possibly had an advantageous effect on obtaining new bacterial species. As far as detecting antimicrobial activities is concerned, an array of six different “production” media has led to a twofold higher positive hit rate than it would with only a single medium.

### **Acknowledgements**

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## Synthesis

### ***Why are bryozoans underrepresented in natural products research?***

Bryozoans populate worldwide various marine and freshwater habitats. They are potent colonizers of submerged surfaces, but, as sessile animals, have to deal with overgrowth themselves, preferentially with the synthesis and release of chemical compounds. Nevertheless, research on natural products rather focused on other sessile filter feeders, namely sponges and corals, as well as algae (Blunt *et al.* 2009). A possible reason could be that bryozoans are mainly recognized as fouling organisms which cause economical damage. So, efforts are primarily made to control or prevent their settlement using anti-fouling agents. Other reasons might include their inconspicuous appearance compared to the often colourful sponges or corals and difficulties with their taxonomy, especially of the encrusting species (Sharp *et al.* 2007a). Additionally, their extensive fossil record attracts preferably scientists with geological background, who are of course more interested in addressing paleo-geological questions than in exploring the bryozoans' biotechnological potential. However, fouling/colonization on bryozoans' surfaces and interactions between the hosts and their microbial cover have been investigated, but these studies, as cited in the introduction of this thesis, are scarce, and biological activities or phylogenetic affiliation of their associated microorganisms have never or seldom been assessed.

Consequently, bryozoans seem to provide a mainly untapped source as far as natural products and biological activities are concerned. Furthermore, as bryozoan-associated microorganisms have been hardly characterized so far, a focus on their microbial fauna would be advantageous, especially when previously host-attributed compounds are most likely produced by their accompanying microorganisms (Anthoni *et al.* 1990, König & Wright 1996, König *et al.* 2006, Walls *et al.* 1995).

A prominent example is the origin of tetrodotoxin (TTX), a highly neurotoxic compound of several *Takifugu* species, the Japanese pufferfish. At first it seemed that TTX was exclusive to some species of the family *Tetraodontidae*, but later this poison could be found in other marine animals, like starfish, gastropods, cephalopods (blue-ringed octopus) or crabs. Also pufferfish become non-toxic when being fed artificially and regain their toxicity after eating TTX containing food. So a dietary uptake is more likely than an endogenous production. In



fact, TTX producing bacteria, affiliated to the genera *Vibrio*, *Alteromonas* and *Shewanella*, have been isolated from these animals (Noguchi & Arakawa 2008).

In the similar case of the bryozoan *Bugula neritina* and its cytotoxic bryostatins, the fluctuating activities of whole-animal extracts in the NCI screenings could later be explained by the presence or absence of a specific bacterial symbiont, the true producer. On the other hand, if the scientists had focused on isolating associated bacteria, the bryostatins would have probably not been discovered, as the symbiotic producer can still not be cultured in the laboratory.

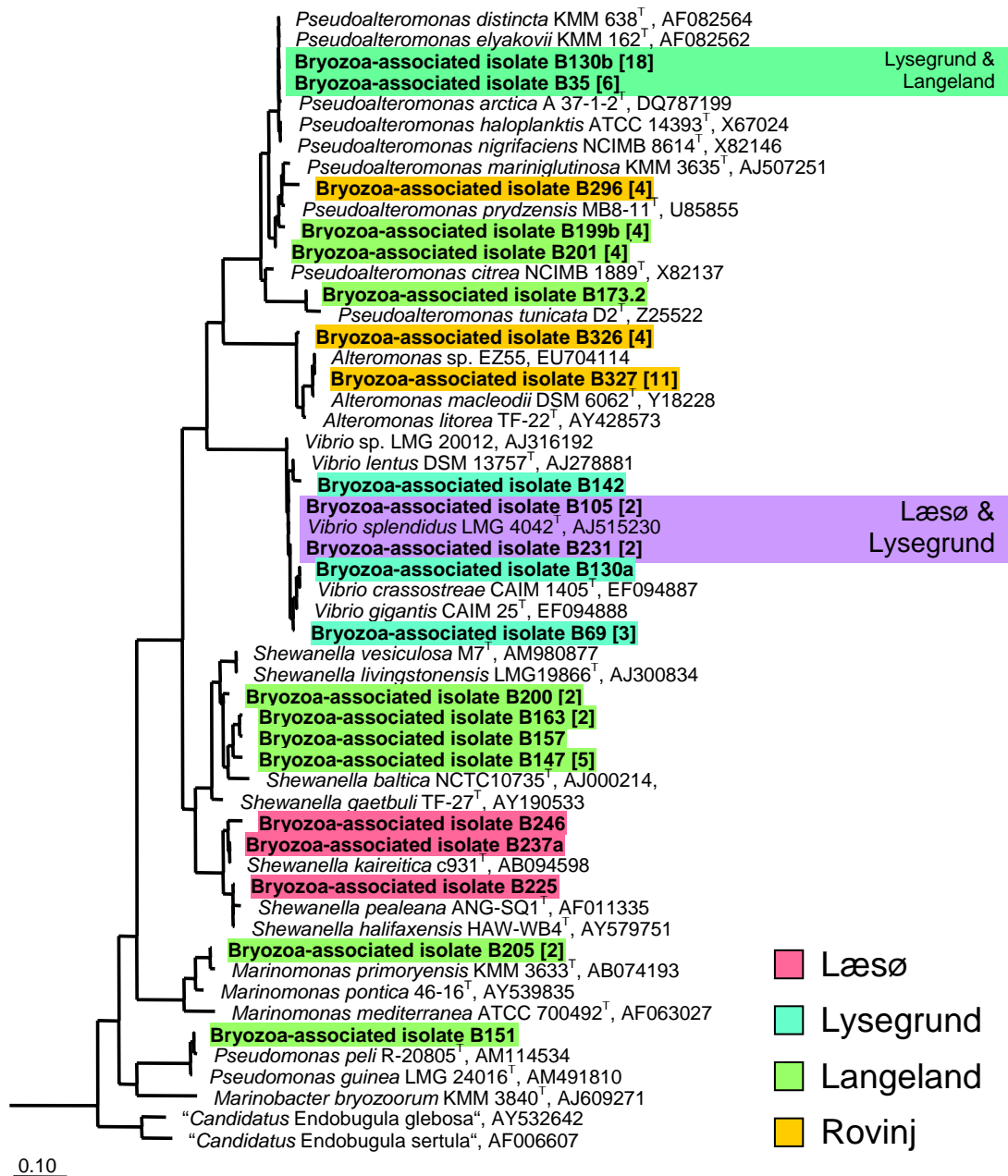
### ***Host-specific and opportunistic microorganisms are associated with bryozoans***

Some of the few studies on bryozoan-associated microorganisms have revealed host-specific bacteria: *Candidatus* Endobugula in four out of seven investigated *Bugula* species (Lim-Fong *et al.* 2008) and *Candidatus* Endowatersipora in two *Watersipora* species (Anderson & Haygood 2007). These symbionts have been originally discovered in the respective bryozoans' larvae, where they can be located at well defined structures (either in the pallial sinus or in a supracoronal groove) in high abundances, very likely as the only occurring bacteria. Furthermore each bryozoan species harbours a distinct bacterial symbiont species. A plausible reason of this symbiosis might be the protection of larvae from predators (Lindquist & Hay 1996, Lopanik *et al.* 2004a). Moreover, the bacteria may play a role in successful settlement and metamorphosis of the larvae, because they are released as a cloud over the substrate (Woollacott 1981). Apart from these symbiotic bacteria, only one further study, as far as I know, has revealed specific associations of bryozoans with microorganisms: host-specific bacterial communities have been found on three out of four investigated encrusting bryozoan species (*Aspidelectra melolontha*, *Electra monostachys* and *Electra pilosa*, not on *Conopeum reticulum*) in the North Sea by means of analysing DNA banding patterns in a comparative DGGE analysis (Kittelman & Harder 2005). However, any phylogenetic classification of these bacteria is not available, as sequence data have not been obtained.

Other studies have demonstrated that rather opportunistic microbial communities exist in association with bryozoans. Various bryozoan species (*E. pilosa*, *Membranipora membranacea*, *Flustra foliacea*, *Tubulipora aperta*, *Bugula plumosa* and *Alcyonidium gelatinosum*) have been sampled in the North Sea near Helgoland and used for isolation as well as *in situ* identification of accompanying fungi. Neither a bryozoan species-specific nor a

persistent community throughout the two years of sampling could be detected (Hain 2003). Regarding the biofilm related community of microorganisms on bryozoans, similar observations have been made. Bryozoan species (*e.g.* *M. membranacea*, *Electra angulata*, *Callopora* sp.) collected in the Pacific Ocean at Japan and New Zealand have shown inconsistent biofilm composition and densities. The authors have concluded that the bryozoans may tolerate their corresponding biofilms (Gerdes *et al.* 2005b). Finally, specimens of *F. foliacea* near Helgoland have also been used for a cultivation based investigation of its bacterial community (Pukall *et al.* 2001). Likewise, the phylogenetic affiliation of the bacterial isolates, as based on 300 bp 16S rDNA sequences and amplified ribosomal RNA restriction analysis (ARDRA), has indicated that the bryozoan simply accepts its colonization with these bacteria.

The phylogenetic data presented in chapters 1 and 3 also suggest a rather opportunistic role of the isolated bacteria. The antimicrobially active isolates (chapter 1) could be clearly subdivided into genera that were found either in the Baltic Sea or in the Mediterranean Sea. Only *Pseudoalteromonas* related bacteria were isolated from both locations. Also, a more detailed phylogenetic analysis showed that the Baltic isolates could be grouped into clusters that were specific to their respective sampling site: the geographically more distant sites Læsø and Langeland featured also distinct phylogenetic clusters of their respective isolates. Active bacteria obtained from Lysegrund, which was located between the other two sites, shared some clusters with either Læsø or Langeland isolates. Active bacteria from the Mediterranean Sea, however, did not cluster in any site-specific groups, which was likely due to the close vicinity of these sampling areas (Fig. 7).



**Figure 7. Sampling site specific phylotypes within the Gammaproteobacteria.** Detail of the phylogenetic tree presented in chapter 1 (Fig. 1-2). Note that phylotype B130b contained also one isolate from Rovinj.

Representatives of the genera *Pseudomonas*, *Pseudoalteromonas*, *Shewanella* and *Vibrio* were also isolated during the second sampling at Læsø as mentioned in chapter 3. However, a greater diversity of genera (29 vs. 10) and phylotypes (49 vs. 27) could be covered. The distribution of the isolates on the three *M. membranacea* specimens was very heterogeneous, which also indicated an opportunistic role of these bacteria. Solely isolates related to one *Halomonas*-phylotype were found on all three samples. Interestingly, only this phylotype contained also antimicrobially active *Halomonas*-isolates. In a comparable study, Pukall *et al.* (2001) have found a similar varying diversity of cultivated bacterial isolates from three *F. foliacea* specimens.

### ***Cultivation-based issues on assessing bacterial diversity***

Results of cultivation-based studies must be interpreted very cautiously when conclusions on a real microbial diversity were drawn. Counts of microorganisms, especially bacteria, were an order of magnitude higher when microscopic methods were applied, than the counts of colony forming units (CFUs) acquired with cultivation media. This discrepancy has been termed “great plate count anomaly” (Staley & Konopka 1985). For example, the percentage of culturable marine bacterioplankton has been determined for the Skagerrak and the central Baltic Sea (Simu *et al.* 2005). CFU numbers on agar plates compared to total DAPI staining counts have reached 0.14% or 0.5%, respectively. However, counts that are determined with staining protocols (including DAPI) can largely overestimate microbial abundances, because non-nucleoid containing bacteria (*i.e.* without DNA) are covered with this method as well. These “ghosts”, which are without doubt inactive and non-viable, can account for up to 98% of the total cell count (Zweifel & Hagström 1995). Consequently, the cultivation success of bacteria compared to the real viable community that exists in the environment should be rated higher. Within the study of Simu *et al.* (2005), 1.33% (Skagerrak) and 17.7% (Baltic Sea) of the viable bacteria have been successfully cultured on agar plates. Interestingly, these isolates are members of the marine *Alphaproteobacteria* (belonging to the *Roseobacter*, *Rhodobacter* or *Sphingomonas* clade).

Although cultivation-based investigations may not reflect the actual microbial diversity, bacterial cultures offer the advantage to investigate complete genome sequences, metabolic pathways, phenotypic characteristics, thus serving as model organisms, and, of course, to detect biological activities (Giovannoni & Stingl 2007, Nichols 2007).

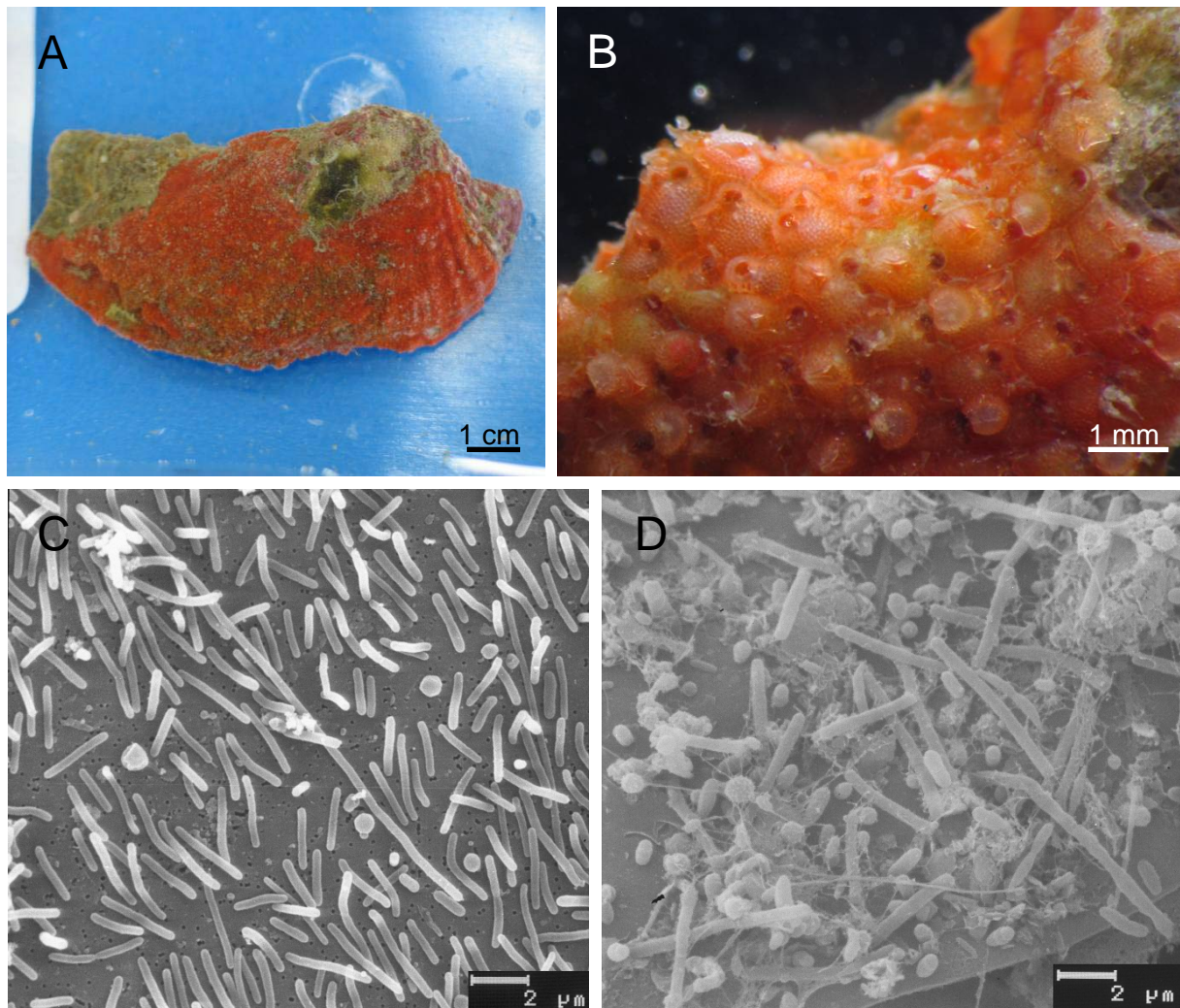
Various cultivation techniques have been elaborated and applied in order to get novel bacterial species into culture. These methods have included for example encapsulation in gel microdroplets (Zengler *et al.* 2002), enrichment in diffusion chambers (Kaeberlein *et al.* 2002), “dilution to extinction” (Connon & Giovannoni 2002, Rappé *et al.* 2002) or host derived extracts as medium ingredients (Olson *et al.* 2000). As a common feature, they have tried to resemble the natural habitat and have used nutrient media at low concentrations.

The bacterial isolates (chapter 1) were obtained using mainly media with nutrient concentrations of 0.3% (w/v). Antimicrobially active bacteria were basically affiliated with five genera of the *Gamma*- and *Alphaproteobacteria* (93.3% belonged to *Pseudoalteromonas*, *Alteromonas*, *Vibrio*, *Shewanella* and *Sphingomonas*). A survey on the abundance of culturable bacteria in Mediterranean sponges has revealed that the largest morphological diversity could be found using oligotrophic media (0.01% and 0% nutrient addition) and water from the habitat (Muscholl-Silberhorn *et al.* 2008). However, bacteria picked from such agar plates have been largely resistant to subsequent cultivation attempts.

In chapter 3 isolation media with 0.06% (w/v) nutrient concentration were used. Furthermore two media resembled the natural habitat (“bryozoan extract” and “algal extract” with habitat water), whereas the other media were composed of “artificial” ingredients. A higher diversity of bacteria could be isolated compared to the results of chapter 1, even if only antimicrobially active isolates were concerned (22 vs. 10 active genera, 31 vs. 27 active phylotypes). Furthermore, bacteria belonging to only four genera were isolated in both studies (chapter 1 and 3: *Pseudoalteromonas*, *Shewanella*, *Vibrio* and *Pseudomonas*). Whereas representatives of those genera accounted for up to 66.7% of all isolates from chapter 1, they made up only 16.7% of chapter 3’s isolates. These findings suggested that the different isolation media approach led to phylogenetically different and more diverse bacterial isolates.

Additionally, adaptation to a surface attached way of life in natural habitats, for example provided by any host-microbe association, may also favour the detection of corresponding, *i.e.* surface-adapted, microorganisms within cultivation-based attempts, especially when agar media were used. The *Flavobacteriaceae* related isolate B390 was described as the type strain of the novel species *Tenacibaculum adriaticum* (chapter 2). It is a member of the *Cytophaga-Flavobacterium* group, which features a special mechanism for movement that does not involve flagella or type IV pili. This is termed gliding motility and is restricted to a movement on surfaces. It allows the cells to move quite rapidly and occasionally reverse the direction. However, in the case of this bacterial group, the exact mechanism is still speculated (McBride 2004). Gliding motility strongly indicates a surface attached way of life because the

mechanism is apparently useless in a pelagic habitat. In fact, scanning micrographs of the bryozoan's surface, from which B390 was isolated, revealed many attached cells with a striking similar morphology to a pure culture of this strain (Fig. 8).

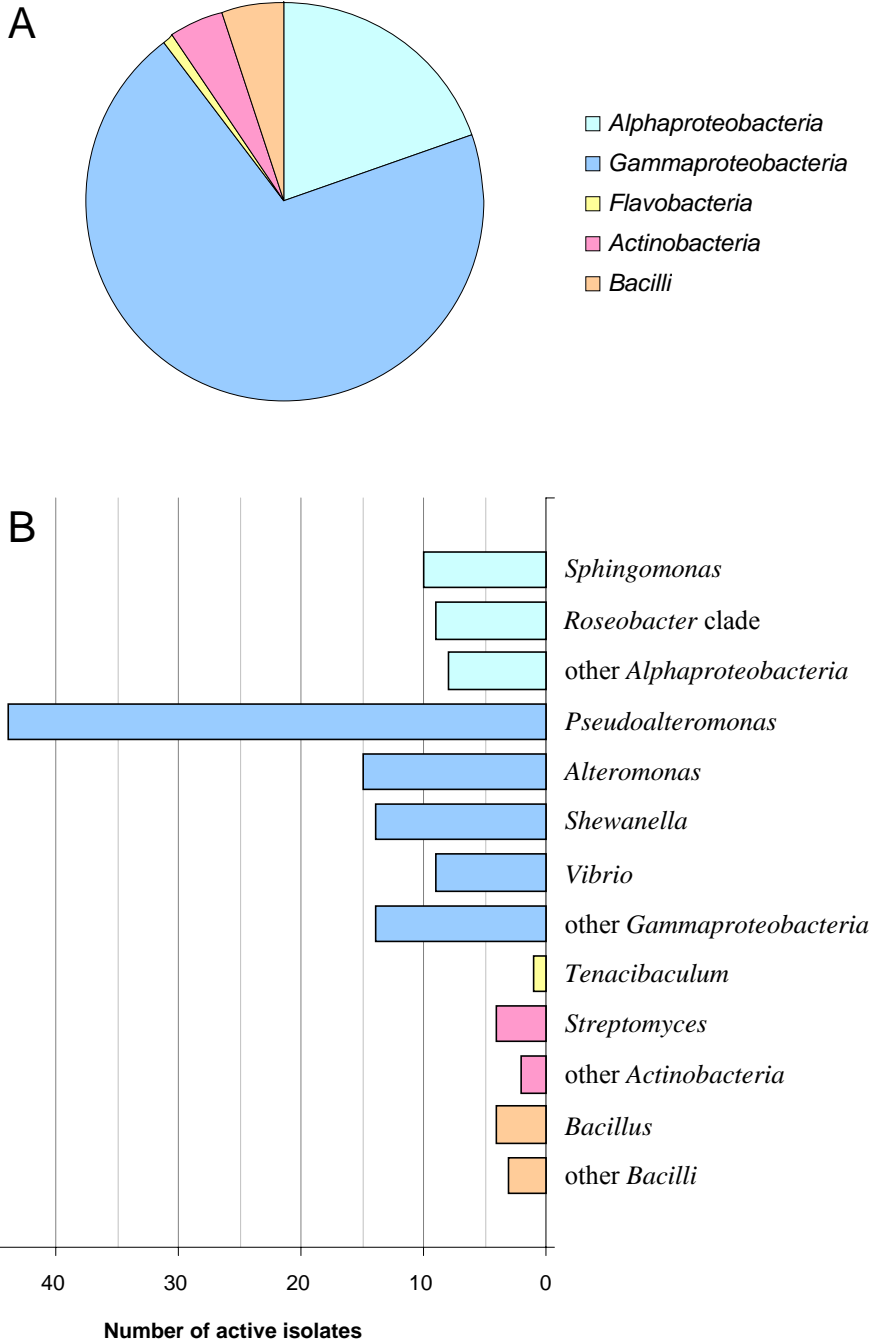


**Figure 8.** *Tenacibaculum adriaticum* B390<sup>T</sup>. A: bryozoan *Schizobrachiella sanguinea*, the source of B390, growing on a bivalve. B: close-up view on the colony. C: scanning electron micrograph of a pure culture of B390. D: scanning electron micrograph of the bryozoan's surface reveals bacteria with a similar morphology.

### ***Antimicrobially active bacterial isolates***

In chapter 3 antibacterial activities were tested with isolates that derived directly from DMSO stocks, *i.e.* these bacteria were only subjected to the sub-cultivation steps necessary to obtain pure cultures. This was done because earlier studies in the lab with bryozoan-associated microorganisms resulted in a loss of activity when isolates were sub-cultured too often. Similar observations have been made with marine bacteria collected on a global research expedition (Gram *et al.* 2010): 78% of all active strains from the first screening have retained activity upon re-testing. The “loss” of activity has been connected with distinct sub-groups or species (*e.g.* non pigmented *Pseudoalteromonas* related strains). Additionally, the authors have noted a higher ratio of active bacteria that derived from surfaces (13% active) than of those from seawater (3%). In another study, a shift of antibacterial activities from Gram-negative towards Gram-positive indicator strains has been noticed after repetitive testing and sub-culturing of bacterial isolates associated with sponges. Basically, former activities against *E. coli* have vanished (Muscholl-Silberhorn *et al.* 2008).

In this thesis, 33.9% of all bacterial isolates displayed antimicrobial activities in the agar diffusion tests. However, as activity testing in chapter 1 was assessed on TSB plates only, this percentage drops to 27.8%, when solely TSB related activities from chapter 3 would be counted: 57.4% of otherwise active isolates did not display activities on TSB plates. As far as the phylogenetic affiliation of active bacteria was concerned, most isolates by far were members of the *Gamma*- (70.1%) and *Alphaproteobacteria* (19.7%) (see Fig. 9A). On the genus level most active bacteria belonged to *Pseudoalteromonas* (32.1%), *Alteromonas* (10.9%), *Shewanella* (10.2%), *Sphingomonas* (7.3%), *Vibrio* (6.6%) or to the *Roseobacter*-clade (6.6%) (see Fig. 9B).



**Figure 9. Active bacterial isolates.** A: relative abundance of isolates affiliated to five phyla found within this work. B: number of active isolates affiliated to the most abundant genera.



Other investigations on marine bacteria with antimicrobial activities have also featured *Gamma*- and *Alphaproteobacteria*, but not necessarily in the same extent. Nevertheless, active *Pseudoalteromonas* related isolates, as well as *Vibrio*, *Shewanella* or *Roseobacter* clade affiliated bacteria, have been found in association with corals (Shnit-Orland & Kushmaro 2009), sponges (Mangano *et al.* 2009, Muscholl-Silberhorn *et al.* 2008), algae (Wiese *et al.* 2009) or in seawater (LoGiudice *et al.* 2007a). On a global sampling of marine bacteria, a higher portion of active isolates has been obtained from biotic or abiotic surface-swabs (12.7% of surface derived isolates) than from seawater samples (3.2% of seawater derived isolates). Active bacteria have been identified as members of *Vibrio* or *Photobacterium* (both *Vibrionaceae*, 59.5% of all active bacteria) and *Pseudoalteromonas* (24.7%), which have been predominantly isolated from marine surfaces, and as members of the *Roseobacter*-clade (mostly *Ruegeria*, 5.6%), which, in contrast, have mainly originated from seawater samples (Gram *et al.* 2010).

So members of the *Gamma*- and *Alphaproteobacteria* belong to the common antimicrobially active isolates from the marine environment. Nonetheless, several bacterial isolates (chapters 1 and 3) represented possibly novel species, as far as 16S rDNA sequence similarities were concerned. Values <98.7% very likely imply DNA-DNA reassociation values below 70% and thus genomic uniqueness of the respective bacterial strain, which is necessary to establish a separate bacterial species, (Stackebrand & Ebers 2006). The corresponding active proteobacterial isolates were affiliated with *Shewanella* (phlotypes “B147”, “B163”, “B157” and “B200), *Pseudoalteromonas* (“B173.2”), *Erwinia* (phlotype 1), *Roseobacter* (16), *Roseovarius* (17), *Ruegeria* (21), *Paracoccus* (24), *Andersenella* (25), *Erythrobacter* (28), *Sphingopyxis* (29 and 30) and *Pelagibius* (31).

The genus *Pseudoalteromonas* has been introduced in 1995, when the genus *Alteromonas* has been divided into these two genera (Gauthier *et al.* 1995). Members of these genera are often reported in association with marine hosts and exhibit various biological activities (*e.g.* algicidal, antimicrobial or agarolytic). They are also known for antifouling capabilities which could possibly be used by their host for biofilm control (Holmström & Kjelleberg 1999). They can be divided into non-pigmented and pigmented clades, whereas pigmentation correlates with a preference for secondary metabolite synthesis (Bowman 2007). Members of the genus *Shewanella* are known for their metabolic flexibility and are widely distributed in aquatic environments. Although they are mentioned as antibiotically active, studies have rather focused on their potential in the bioremediation of toxic waste or on their use in microbial fuel cells (Fredrickson *et al.* 2008, Hau & Gralnick 2007). *Sphingomonas* related

bacteria feature a comparable metabolic versatility but are not as prominent in aquatic ecosystems (Balkwill *et al.* 2006). Antimicrobially active compounds have not been published for these two genera as far as I know. An abundant genus in aquatic environments is *Vibrio*, which can be found in and on several marine organisms. Strains appear as coral and human pathogens (*e.g.* the recent cholera epidemic on Haiti), but are also known as symbionts. They serve as model organisms for bacterial communication, termed quorum sensing (QS) (Thompson *et al.* 2004). And in the end, members of the *Roseobacter*-clade represented the sixth most common group of active bacterial isolates within this work. These bacteria are commonly found in the marine environment and are the only lineage that features cultivated bacteria which are closely related to environmental clones (Wagner-Döbler & Biebl 2006).

Whereas the majority of active isolates belonged to Gram-negative taxa, 9.5% were related to Gram-positive groups with the genera *Bacillus* and *Streptomyces* as the most frequent (2.9% each). Both genera, as well as *Alteromonas/Pseudoalteromonas*, *Vibrio* and *Pseudomonas*, belong to the bacterial taxa with the most published secondary metabolites in a marine context (Laatsch 2006).

Antimicrobial activities of this work's isolates were almost entirely directed against Gram-positive indicator strains (98.5% of all active isolates). A possible cause for this bias could be a greater susceptibility of these indicators to antibioticly active metabolites than their Gram-negative counterparts. Also, the majority of active isolates (90.5%) belonged to Gram-negative taxa. They could have probably hindered the growth of any Gram-positive bacteria during the isolation procedures on the agar plates.

Interestingly, *Bacillus* related isolates also exhibited anti-*B. subtilis* activity. "Cannibalism" is known among *B. subtilis* populations to delay spore morphogenesis (González-Pastor *et al.* 2003). Although they consist of genetically identical cells the genes are expressed heterogeneously by random switching mechanisms. This bistability leads to distinct subpopulations that are presumably better adapted to present or pending conditions (*e.g.* nutrient limitation, antibiotics) (Dubnau & Losick 2006). More indicator organisms, especially *Bacillus* related strains from this work, would be advantageous to determine more specific antibacterial activities including "cannibalism".

Finally, surface attached or host associated bacteria are confronted with chemically mediated interactions within the biofilm community and the host. Furthermore, they encounter physical and chemical conditions which are specific for the given surface they attempt to colonize (Egan *et al.* 2008). It seems very likely that all these "colonizing" bacteria are capable of

preventing growth or colonization of other microorganisms, in other words they are probably all able to produce antimicrobially active substances. Finding the proper conditions *in vitro* to induce biosynthesis of these compounds is a challenge in natural products research.

Some studies have revealed various inducible influences on secondary metabolite production. For example, marine animal associated *Pseudoalteromonas* and *Bacillus* isolates have exhibited higher antimicrobial activities when cultivated on hydrophilic surfaces, but the number of attached cells has been greater on hydrophobic material (Ivanova *et al.* 1998). A marine *Bacillus licheniformis* strain has produced antimicrobial compounds when it has grown attached to a surface, in contrast to shake flask cultures. Furthermore, spent medium, which has been obtained from surface attached cultivation of this strain and other *Bacillus* species, could induce the production of those antimicrobials even in shake flask cultures. The authors have suggested that biofilm-dependent signalling compounds may exist which can induce planktonically grown cells to produce antimicrobials as if they have been grown as a biofilm (Yan *et al.* 2003). Also, some members of the *Roseobacter* clade have produced antibacterial compounds when being cultivated under static conditions but not when shaken. (Bruhn *et al.* 2007). Variation of cultivation media might also result in altered secondary metabolite synthesis. In chapter 3 of this work, the impact of six different media on the detection of antimicrobial activities was determined. This resulted in 50.5% positive hits, whereas only 16.1 to 22.6% of all tested strains would have shown antibiotic activities, if just one of the media had been used. Accordingly, the media favoured antibiotic production of differing bacterial isolates, but with a preference for certain groups.

## Significance and outlook

Up to now, only a few studies have dealt with biological activities of bryozoans or surveyed on their microbial colonizers. The present work provides the first insight into the phylogeny and the antimicrobial activities of bryozoan-associated bacteria. Most notably it was discovered that several novel bacterial species can be recovered from bryozoans, and that these isolates provide decent antibacterial properties, too.

Future research on bryozoan-bacteria associations could address two main topics: firstly, a comprehensive phylogeny of the bacterial associates and secondly, their possible role within these associations, especially regarding the occurrence and impact of natural products. Whereas questions like “Do stable associations of microorganisms with bryozoans exist?” or “Are there any bryozoan species-specific associations?” might be answered conclusively, the answer to their speculated roles will probably never be satisfactorily given.

Nevertheless, as microbes-host interactions are likely mediated chemically, the elucidation of occurring natural products and the assessment of their biological activities certainly will give good hints for their function in the natural environment.

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## Scientific contribution to multiple-author publications

### Chapter 1

“Phylogenetic diversity and antimicrobial activities of bryozoan-associated bacteria isolated from Mediterranean and Baltic Sea habitats”

Sampling of bryozoans at the Lysegrund site, as well as isolation of their bacteria was done by Jutta Wiese. Sampling at the remaining sites and isolation of the respective bacteria was done by Herwig Heindl. Experimental work (16S rDNA library, antimicrobial activity testing) was done by Herwig Heindl under supervision of Johannes F. Imhoff. Phylogenetic calculations were done by Vera Thiel. Evaluation of data and preparation of the manuscript was done by Herwig Heindl. The co-authors contributed to the manuscript by critical revision.

### Chapter 2

“*Tenacibaculum adriaticum* sp. nov., from a bryozoan in the Adriatic Sea”

Experimental work, phylogenetic analysis and preparation of the manuscript was done by Herwig Heindl under supervision of Jutta Wiese. Johannes F. Imhoff contributed to the manuscript by critical revision.

### Chapter 3

“*Membranipora membranacea* associated bacteria: impact of media on their isolation and antimicrobial activity”

Sampling of *M. membranacea* was done by Vera Thiel. Isolation of bacteria and experimental work was done by Herwig Heindl under supervision of Jutta Wiese. Phylogenetic trees were calculated by Vera Thiel. Evaluation of data and preparation of the manuscript was done by Herwig Heindl under critical revision by Johannes F. Imhoff and Jutta Wiese.

## **Erklärung**

Hiermit erkläre ich, dass ich die vorliegende Dissertation unter Einhaltung der Regeln guter wissenschaftlicher Praxis verfasst habe, und dass sie nach Form und Inhalt meine eigene Arbeit ist. Sie wurde keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt. Das mein einziges und bisher erstes Promotionsverfahren.

Teile dieser Arbeit wurden bereits veröffentlicht oder zur Publikation eingereicht.

Kiel, den

Herwig Heindl