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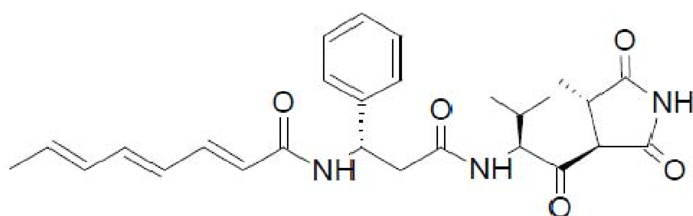
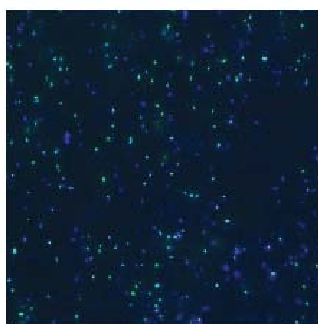
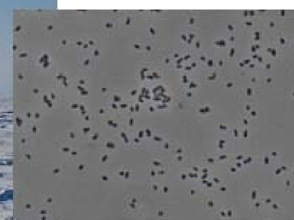
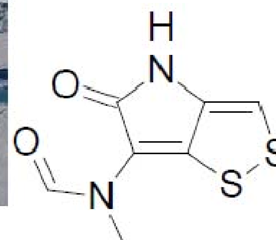
Global patterns of marine bacterioplankton diversity and characterization of bioactive *Vibrionaceae* isolates









DTU

PhD thesis

Matthias Wietz

2011

DTU Food
National Food Institute

Preface

The present PhD study has been conducted at the National Food Institute (previously National Institute for Aquatic Resources) at the Technical University of Denmark from February 2008 until January 2011 under the supervision of Prof. Lone Gram.

The PhD was part of the collaborative research project "Discovery of novel bioactive bacteria and natural products and their use to improve human health and safety" funded by Det Strategiske Forskningsråds Programkomite for Sundhed, Fødevarer og Velfærd (Strategic Research Council for Food, Health and Welfare; FøSu).

The work resulted in the preparation of four papers, which are included in this thesis.

In addition, part of the work was implemented in collaborative research efforts, resulting in one co-authorship.

Matthias Wietz
January 2011

Summary

The purpose of the present study was to analyze the composition of marine bacterial communities around the world, and to investigate bacterial isolates regarding the production of antibiotics. This included molecular analyses of marine bacterioplankton, as well as culture-based studies of marine bacterial isolates with antagonistic activity. The work was based on samples collected during the Galathea 3 and LOMROG-II marine research expeditions that have explored many different oceanic regions worldwide.

A molecular survey of marine bacterioplankton at 24 worldwide stations investigated the abundance of major bacterial groups, potential biogeographical patterns, and their relation to environmental parameters. The original aim was to determine whether the composition of the total microbiota correlates with the occurrence of culturable bioactive bacteria. No such correlation was found. Quantitative community analyses showed latitudinal patterns in bacterial distribution, revealing significantly different relative abundances of *Bacteroidetes*, unclassified *Bacteria* and *Vibrio* between warmer and colder oceans. Absolute cell numbers of most bacterial groups were positively correlated with nutrient concentrations in warmer oceans, and negatively with oxygen saturation in colder oceans. The finding of differing communities in warmer and colder oceans underlined the presence of biogeographical patterns among marine bacteria and the influence of environmental parameters on bacterial distribution.

Studies of antagonistic isolates focused on six bioactive *Vibrionaceae* isolated during Galathea 3. The six strains were identified as *Vibrio coralliilyticus* (two strains), *V. neptunius* (two strains), *V. nigripulchritudo* (one strain), and *Photobacterium halotolerans* (one strain) by sequencing of housekeeping genes. Chemical metabolite profiling underlined genetic relationships by showing highly similar production of secondary metabolites for each species. Two known antibiotics were purified; andrimid from *V. coralliilyticus* and holomycin from *P. halotolerans*. In addition, two novel cyclic peptides from *P. halotolerans* and a novel siderophore-like compound from *V. nigripulchritudo* were isolated. All three compounds interfere with quorum sensing in *S. aureus*.

During LOMROG-II further seventeen strains with antagonistic activity were isolated, affiliating with the *Actinobacteria* (8 strains), *Pseudoalteromonas* (4 strains), the *Vibrionaceae* (3 strains), and *Psychrobacter* (2 strains). Seven of the eight bioactive *Actinobacteria*, being isolated from different sources throughout the Arctic Ocean, were related to *Arthrobacter davidanieli*. Its

broad antibiotic spectrum was likely based on production of the known arthrobacilin antibiotics. The eighth actinomycete, tentatively identified as *Brevibacterium* sp., produces a potentially novel antimicrobial compound.

Most studies of antagonistic marine bacteria have been conducted with the aim of isolating novel antimicrobials with potential clinical applications. However, little is known about production and role of these compounds in the natural environment. This thesis took one step in this direction and demonstrated that *V. coralliilyticus* S2052 produced its antibiotic andrimid when grown with chitin as the sole carbon source. Whilst the strain produced an array of secondary metabolites in laboratory media, it focused on andrimid production with chitin. This indicates that the antibiotic is likely produced in the natural habitat and may serve an ecophysiological function. The finding that two related strains from public culture collections do not produce andrimid and have different biosynthetic temperature optima suggested that *V. coralliilyticus* may comprise different subspecies with different niches.

In summary, the present study shows biogeographical patterns of marine bacterioplankton on a global scale. In addition, the thesis work has demonstrated that marine *Vibrionaceae* and polar *Actinobacteria* are a resource of antibacterial compounds and may have potential for future natural product discovery.

Resumé

Formålet med dette ph.d.-studie var at analysere sammensætningen af marine bakterier fra en række globalt indsamlede prøver og at undersøge produktion af antibiotika fra dyrkbare marine bakterier. Dette omfattede en molekylær undersøgelse af marin bacterioplankton, samt studier af marine bakterier med antagonistisk aktivitet. Arbejdet var baseret på prøver indsamlet under Galathea 3 og LOMROG-II ekspeditioner, der har undersøgt flere forskellige oceaniske regioner.

En molekylær undersøgelse af marine bacterioplankton på 24 stationer havde til formål at kortlægge bakteriesammensætningen, finde mulige biogeografiske mønstre, og deres relation til miljømæssige parametre. Det oprindelige mål var at afgøre, om sammensætningen af den samlede mikrobiota kunne korreleres til forekomsten af dyrkbare bioaktive bakterier. Der blev ikke fundet sådanne korrelationer. Kvantitative samfunds-analyser viste breddegrad-relaterede mønstre i bakteriel distribution, idet der var markant forskellige relative mængder af *Bacteroidetes*, uklassificerede *Bakterier* og *Vibrio* i varmere hhv. koldere oceaner. Det absolutte antal af de fleste bakterie-grupper var positivt korreleret med koncentrationer af næringsstoffer i varmere oceaner, og negativt med iltmætning i koldere have.

Studier af antagonistiske dyrkbare isolater fokuserede på seks bioaktive *Vibrionaceae* isoleret under Galathea 3. De seks stammer blev identificeret som *Vibrio coralliilyticus* (to stammer), *V. neptunius* (to stammer), *V. nigripulchritudo* (en stamme), og *Photobacterium halotolerans* (en stamme) ved sekventering af husholdnings-gener. Kemisk metabolit-profilering understregede de genetiske relationer, idet hver art havde en unik sekundær metabolit-profil. To kendte antibiotika blev isoleret, andrimid fra *V. coralliilyticus* og holomycin fra *P. halotolerans*. Desuden blev to nye cykliske peptider isoleret fra *P. halotolerans* og et nyt siderophore-lignende stof fra *V. nigripulchritudo*. Alle tre stoffer viste sig at interferere med quorum-sensing hos *S. aureus*.

Under LOMROG-II blev yderligere sytten stammer med antagonistisk aktivitet isoleret. De blev identificeret som *Actinobacteria* (8 stammer), *Pseudoalteromonas* (4 stammer), *Vibrionaceae* (3 stammer), og *Psychrobacter* (2 stammer). Syv af de otte bioaktive *Actinobacteria*, isoleret fra forskellige kilder i hele det Arktiske havområde, var relateret til *Arthrobacter davidanieli*. Disse stammer have bred antibiotisk aktivitet, hvilket sandsynligvis skyldes produktion af de kendte antibiotika, arthrobacilin. Den sidste actinomycet, tentativt identificeret som *Brevibacterium* sp., producerede et potentielt nyt antimikrobielt stof.

De fleste undersøgelser af antagonistiske marine bakterier er gennemført med det formål at isolere nye antimikrobielle stoffer med kliniske anvendelsesmuligheder. Vi ved dog uhyre lidt om produktion og betydning af disse stoffer i det naturlige miljø. Denne afhandling tog et skridt i denne retning, og viste, at *V. coralliilyticus* S2052 kunne producere andrimid når den blev dyrket med kitin som eneste kulstofkilde. Mens stammen i laboratorie-substrater producerede en række sekundære metabolitter udover andrimid, så sås andrimid som stort set den eneste sekundære metabolit, når bakterien blev dyrket på kitin. Dette indikerer, at bakterien sandsynligvis producerer sit antibiotikum i det naturlige habitat og at det formodentlig tjener en økofysiologiske funktion. *V. corallilyticus*-stammen blev sammenlignet med to andre ikke-Galathea stammer. Disse producerede ikke andrimid og havde forskellig optimale biosyntetiske temperaturer, hvilket antydet, at *V. coralliilyticus* kan omfatte forskellige underarter med forskellige nicher.

Sammenfattet tilføjer denne undersøgelse til forståelsen af marine bacterioplankton biogeografi jorden rund, et af de store emner i marine mikrobiel økologi. Hertil kommer, har den tese arbejde vist, at marine *Vibrionaceae* og polære *Actinobacteria* er en ressource af antibakterielle stoffer og kan have potentiale for fremtidig opdagelse af naturstoffer.

Table of contents

Preface I
Summary II
Resumé IV
Figure legend VII
List of articles VIII

1. Background and scope of the PhD project 1

2. Marine bacterioplankton communities 4

2.1 Diversity, structure and function of marine bacterioplankton 4
Global patterns of marine bacterioplankton community structure 5
Bacterial biogeography and the influence of environmental parameters 8
2.2 Polar microbiology 13
Molecular characterization of polar marine microbiota 14
Culturable bacteria in polar oceans 15
2.3 Conclusions from chapter 2 17

3. Bacterial interactions and antagonistic marine bacteria 18

3.1 Physical and chemical interactions in the environment 18
3.2 Bioactive microbial secondary metabolites 21
Polyketides and nonribosomal peptides 22
Strategies in natural product discovery 23
Cosmopolitan antibiotics 25
Marine natural products from eukaryotes 28
3.3 Bioactive bacteria from marine environments 29
Antibiotic compounds from marine bacteria 29
Marine bacterial groups harbouring antagonistic strains 32
Bioactive bacteria from polar habitats 35
3.4 Conclusions from chapter 3 37

4. The *Vibrionaceae* 38

4.1 Diversity and phylogeny 39
4.2 Distribution and abundance 41
4.3 Pathogenicity 44
Human pathogens 45
Animal pathogens and role in aquaculture 45
4.4 Physiological traits 47
4.5 Biosynthetic capacities 50
4.6 Non-pathogenic interactions and antibiotic activity 52
Antibiotics from vibrios 53
V. coralliilyticus, *V. neptunius* and *P. halotolerans* 55
Andrimid 57
Holomycin 58
Pathogenic traits 59
Interference with quorum sensing 61
4.7 Conclusions from chapter 4 63

5. Conclusions and Perspectives 65
6. Acknowledgments 68
7. References 69

Figure legend

Fig. 1 The routes of the Galathea 3 and LOMROG-II research expeditions. 1
Fig. 2 Phylogenetic tree of marine antagonistic bacteria collected during Galathea 3 3
Fig. 3 Overview of the major bacterioplankton groups.. 6
Fig. 4 Fluorescence microscopy images of marine bacterioplankton. 7
Fig. 5 Biogeographical patterns among marine bacterioplankton. 11
Fig. 6 Primary production in the oceans by marine phytoplankton 12
Fig. 7 Culturable bacterial counts in different Arctic marine environments 15
Fig. 8 Atomic-force microscopy images of associated marine bacteria 19
Fig. 9 Bacterial interactions in the environment 20
Fig. 10 Overview of the HPLC-MS approach 24
Fig. 11 Rise in resistant pathogenic strains and decrease in approved antibiotics 25
Fig. 12 Cosmopolitan antibiotics 27
Fig. 13 Antibiotic compounds from marine bacteria 31
Fig. 14 SEM micrograph of *V. cholerae* and *Vibrio* colonies on agar. 38
Fig. 15 Phylogenetic tree of the *Vibrionaceae* family 40
Fig. 16 Chitinolytic activity of *Vibrionaceae*. 48
Fig. 17 Increased production of antibiotic with chitin 50
Fig. 18 Cell morphologies and antibiotics from *V. coralliilyticus* and *P. halotolerans* 57
Fig. 19 Nigribactin from *V. nigripulchritudo* S2604. 62
Fig. 20 Solonamides A and B from *P. halotolerans* S2753 63

List of articles

1 Wietz M, Gram L, Jørgensen B, Schramm A (2010) Latitudinal patterns in the abundance of major marine bacterioplankton groups. Aquat. Microb. Ecol. 61:179–189.

2 Wietz M, Månsson M, Ng Y, Gram L (2011) Bioactive bacteria from Arctic marine environments. Manuscript in preparation.

3 Wietz M, Månsson M, Gotfredsen CH, Larsen TO, Gram L (2010) Antibacterial compounds from marine *Vibrionaceae* isolated on a global expedition. Mar. Drugs 8:2946–2960.

4 Wietz M, Månsson M, Gram L (2011) Chitin stimulates producion of the antibiotic andrimid in a *V. coralliilyticus* strain. Manuscript in preparation.

Collaborative article not included in this thesis:

Månsson M, Nielsen A, Kjærulff L, Gotfredsen CH, Wietz M, Ingmer H, Gram L, Larsen TO (2011) Inhibition of virulence gene expression in *Staphylococcus aureus* by a novel depsipeptide from a marine *Photobacterium*. Appl. Environ. Microbiol. (to be submitted)

Global patterns of marine bacterioplankton diversity and characterization of bioactive *Vibrionaceae* isolates

1. BACKGROUND AND SCOPE OF THE PHD PROJECT

The present PhD project included a molecular investigation of marine bacterioplankton community structure on a global scale, as well as culture-based studies of marine bacterial isolates from the *Vibrionaceae* that exhibit antagonistic activity towards other bacteria. In addition, bacteria from high Arctic habitats, in particular strains belonging to the *Actinobacteria*, were investigated for their bioactivity. The work was based on samples collected on two different marine research expeditions, the Danish Galathea 3 and the Danish-Swedish LOMROG-II cruises. The Galathea 3 expedition on board the retrofitted Danish Navy vessel *Vædderen* circumnavigated the globe on a nine-month voyage from August 2006 until May 2007, covering a distance of 55,000 nautical miles and sampling most major oceanic regions, only excluding the high Arctic Ocean. The LOMROG-II expedition explored the pack ice-infested waters of the North Pole region around the central Lomonosov Ridge on board the Swedish icebreaker *Oden* in summer 2009 (Fig. 1). The two expeditions therefore covered a wide geographic range and allowed the investigation of many different oceanic ecosystems, sampling the majority of oceanic surface waters including extreme polar environments.

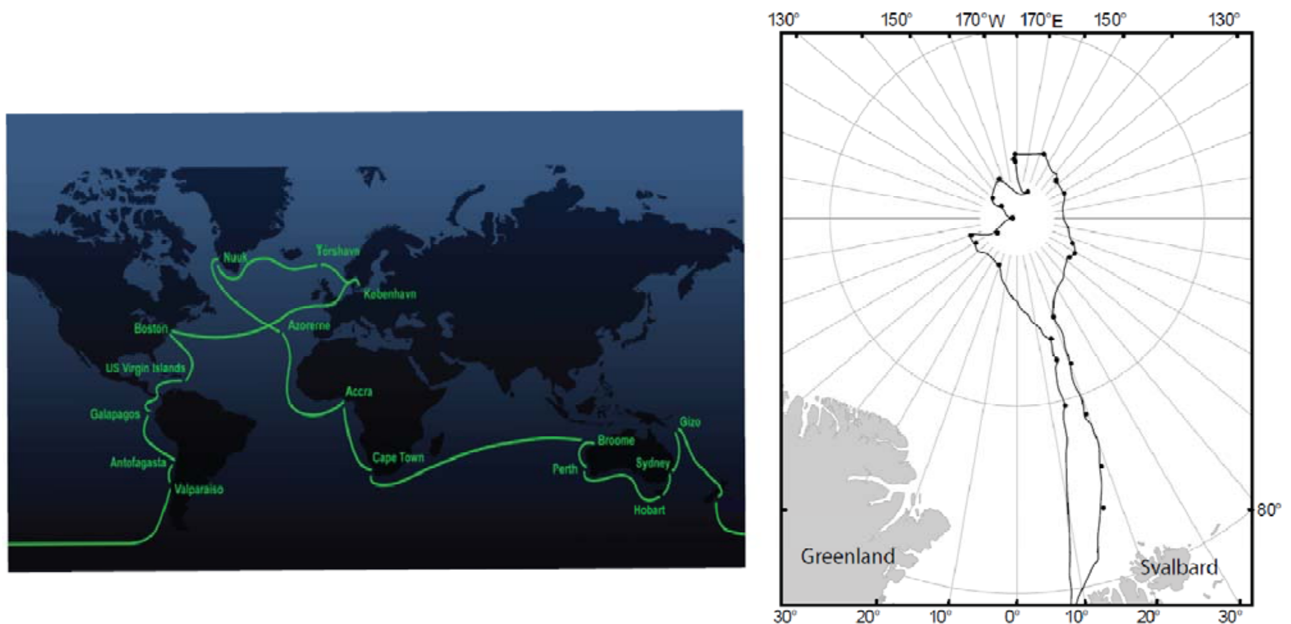


Fig. 1 The routes of the Galathea 3 and LOMROG-II research expeditions.

Marine bacterioplankton communities and biogeography on a global scale

During the global Galathea 3 expedition (http://www.galathea3.dk/uk), one project addressed a molecular survey of marine bacterioplankton community structure around the world. This aimed to study the global distribution of major bacterial groups, analyze potential biogeographical patterns, and relate those to environmental parameters. The original purpose was to characterize the total microbiota in waters that had been sampled for culturable antagonistic bacteria (see below) in order to determine whether particular community patterns or environmental parameters correlate with the occurrence of culturable bioactive strains. Knowledge about the distribution of marine antagonistic bacteria and their preferred “environmental settings” could direct future bioprospecting efforts to “hotspots of bioactivity” predicted to harbour a larger fraction of target organisms. Although a link between the molecular and culture-based analyses could not be established, the present study added to the knowledge of the global distribution and biogeography of marine bacteria. This was achieved by application of catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH), a technique to detect and quantify environmental cells without cultivation (Amann et al. 1995), to bacterioplankton samples from 24 worldwide stations. Data was set in context to various environmental parameters, such as oceanographic variables (temperature, salinity) as well as the concentrations of inorganic nutrients and chlorophyll. All data were evaluated by principal component analysis, a statistical tool to identify patterns and underlying interrelations in a complex dataset (Martens & Martens 2001).

Marine bioactive bacteria

The major microbiological work within the Galathea 3 project at the National Food Institute addressed culturable marine bacteria with inibitory activity towards bacterial pathogens. The present PhD work was based on bacterial isolates from various geographic regions and sample types, including pelagic and coastal waters as well as different biological and abiotic surfaces (Gram et al. 2010). To screen the culturable microbiota for strains with antagonistic activity, marine isolates were replica-plated onto agar plates incorporating the marine fish pathogen *Vibrio anguillarum*, and isolates causing an inhibition zone of the pathogen subsequently pure-cultured. The majority of bioactive bacteria were retrieved from warmer oceanic regions, and especially from surface swabs. Sequencing of 16S rRNA gene fragments revealed that over 90% of the antagonistic

isolates belonged to one of three bacterial genera: *Pseudoalteromonas*, *Vibrio* and the *Roseobacter* clade (Gram et al. 2010) (Fig. 2).

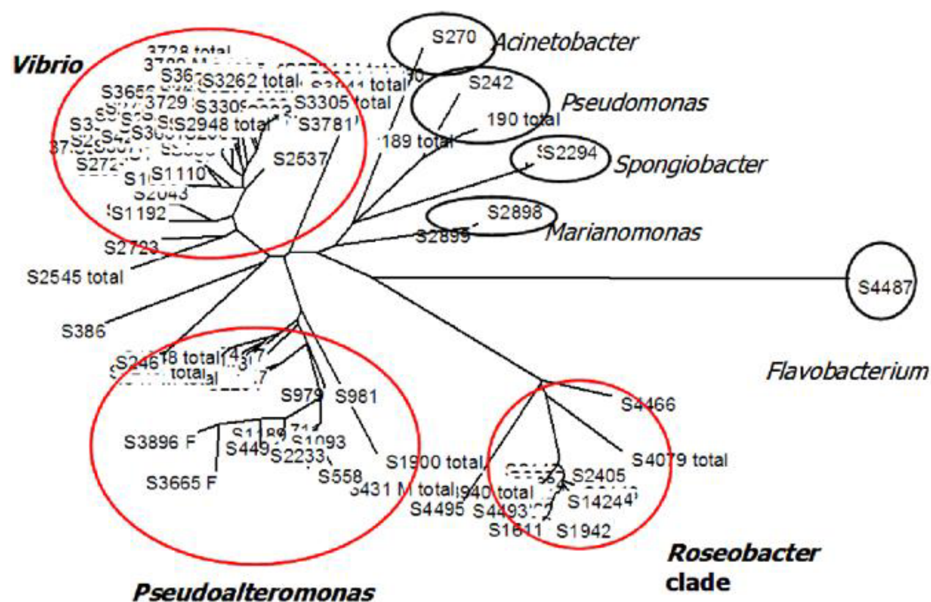


Fig. 2 Phylogenetic tree of marine antagonistic bacteria collected during Galathea 3 (figure by J.B. Bruhn, unpublished).

519 bioactive bacterial strains, among them 301 *Vibrionaceae* investigated in the present PhD, were selected for further study. These strains represent the core of the multidisciplinary collaborative project "Discovery of novel bioactive bacteria and natural products and their use to improve human health and safety" from the Technical University of Denmark (DTU) and the University of Copenhagen (KU). Research is conducted at the National Food Institute (bacterial cultivation, testing for antagonistic activity), DTU Systems Biology (chemical metabolite analyses), KU Life (interference with *S. aureus* quorum sensing; influence on the human immune response), and KU Health (interference with *P. aeruginosa* quorum sensing). Furthermore, the project is followed by the filming company nature&science (Copenhagen), presenting researchers and results in television and internet documentaries.

The strain collection of marine bioactive bacteria has been complemented by isolates from the Arctic LOMROG-II expedition (http://tinyurl.com/lomrogII). Since Galathea 3 did not sample high Arctic waters, LOMROG-II represented a unique opportunity to obtain an almost global collection of marine bioactive strains. A similar experimental approach was applied to ensure that the results can be set in context with those from Galathea 3.

2. MARINE BACTERIOPLANKTON COMMUNITIES

2.1 Diversity, structure and function of marine bacterioplankton

Oceans constitute 95 percent of the world's biosphere and represent the largest ecosystem on Earth. The oceans have been studied from the earliest days of seafaring, and modern marine research continues to explore the biological diversity, biogeochemical processes and physical characteristics of this vast and complex environment.

Marine microorganisms represent the main form of biomass in the oceans, comprising both prokaryotes (bacteria and archaea) and eukaryotes (algae, protists and fungi). Bacterial cell numbers in the upper water layer are typically approx. 10^5 cells per mL, with estimated growth rates of 0.15 divisions per day (Ducklow 2000). The total number of bacteria in oceanic waters has been estimated to 10^{29} cells (Whitman et al. 1998), which by far exceeds the number of stars in the universe (10^{22}). These sheer numbers highlight why the activity, diversity, and ecological importance of marine bacteria have become key research subjects over the previous years. Studies have addressed the structure (Venter et al. 2004; Pommier et al. 2007) and function (Montoya et al. 2004; Niemann et al. 2006) of marine microbial communities on various geographical scales, the physiological capacities of selected bacterial groups (Béjà et al. 2000; Kuypers et al. 2003), as well as the isolation of dominant species (Rappé et al. 2002). This has greatly expanded the understanding of the biological processes driving microbial diversity and activity, including their role in oceanic food webs, nutrient cycling, and the global climate. Marine microbes are also researched regarding their biotechnological potential, including the production of metabolites with pharmacological (e.g. antimicrobials) or industrial applications (enzymes). However, there is still little knowledge in many fields, such as distributional patterns of marine bacteria on a global scale and the role of environmental conditions in these processes. The study of bacterial biogeography has become a major focus in marine microbiology, addressing microbial communities in context with the environment they are living in. The present study adds to the understanding of marine bacterioplankton by molecular analyses of bacterioplankton community structure, the relation of bacterial biogeography to environmental parameters, and physiological studies of culturable bacteria producing antibiotic compounds.

Global patterns of marine bacterioplankton community structure

A major focus in marine microbiology addresses the structure of bacterial communities in oceanic waters. This aims at analyzing the composition of bacterial groups, genera and species as well as their absolute and relative abundances. In general, marine bacteria employ two major lifestyles, free-living (planktonic) or surface-associated (in biofilms). Surface-associated communities, which often sustain significant numbers of microbes, include epibiobic assemblages on higher eukaryotes (e.g., seaweed, zooplankton) or organic particles (marine snow). To date, most studies on marine microbiota have addressed planktonic bacteria, which are also termed the bacterioplankton. Also the present PhD study focuses on this fraction of the marine microbiota.

Most analyses of bacterioplankton community structure have been conducted in the upper water layer or photic zone, whereas habitats such as the deep sea are still largely uncharacterized. In surface water, communities of planktonic marine bacteria largely consist of a few major groups found in all oceanic waters (Fig. 3). These major groups include the *Alphaproteobacteria*, *Gammaproteobacteria*, and *Bacteroidetes* (Giovannoni & Stingl 2005), of which the *Alphaproteobacteria* are generally the most abundant. This dominance is largely attributed to two bacterial clades, SAR11 and *Roseobacter*. SAR11, foremost represented by the species *Pelagibacter ubique*, dominates bacterioplankton communities worldwide (Morris et al. 2002) and can constitute almost 50% of all prokaryotes in some locations (Wietz et al. 2010a). Also the *Roseobacter* clade is often abundant, particularly in areas with phytoplankton blooms, where they can represent over 20% of the bacterioplankton (Wagner-Döbler & Biebl 2006). The present study did not find such high abundances, potentially related to the fact that no samplings were performed during phytoplankton blooms. Roseobacters are generally thought to prevail in temperate and colder waters (Selje et al. 2004). The present study confirmed this for absolute cell numbers (peaking at temperate and polar stations), while highest relative abundances (up to 8%) were measured in tropical waters of the Caribbean Sea (Wietz et al. 2010a). Larger fractions of *Gammaproteobacteria* and *Bacteroidetes* are often found in colder, nutrient-rich waters, with abundances ranging from 2 to 20% of bacteria. The prevalence of *Bacteroidetes* in colder waters was consistent with the present study, showing that *Bacteroidetes* were significantly more abundant in higher latitudes and negatively correlated with water temperature (Wietz et al. 2010a).

Cosmopolitan distribution in marine waters is also seen for rarer microbial groups, including marine *Planctomycetes* (Fuerst 1995) and *Actinobacteria* (Wietz et al. 2010a). However, these two

groups are generally more abundant in surface-associated communities. *Actinobacteria* are often found in sediments and attached to marine snow (Bull & Stach 2007), while *Planctomycetes* have recently been described as common epibionts on the surfaces of macroalgae (Bengtsson & Ovreas 2010). Despite their overall low abundance in marine waters, *Planctomycetes* are important in elemental cycling processes such as anammox, which plays a major role in global nitrogen cycling (Kuenen 2008). In this context, it should be noted that abundance does not necessarily reflect metabolic activity and/or ecological relevance. The so-called rare microbial biosphere, a tail of low-abundance taxa, accounts for most of the marine microbial diversity. While their ecological role is still poorly understood, rare marine microorganisms possibly represent a source of phylotypes that become abundant when environmental conditions change (Galand et al. 2009).

Archaea, the third major branch in the tree of life besides the eukaryotic and bacterial kingdoms, are often absent in surface waters or only present in minute amounts. While it has been suggested that the euryarchaeotal subgroup may be globally present (Fig. 3), the present study detected archaeal cells at only 11 from 24 global sampling sites despite including an *Euryarchaeota*-specific probe (Wietz et al. 2010a).

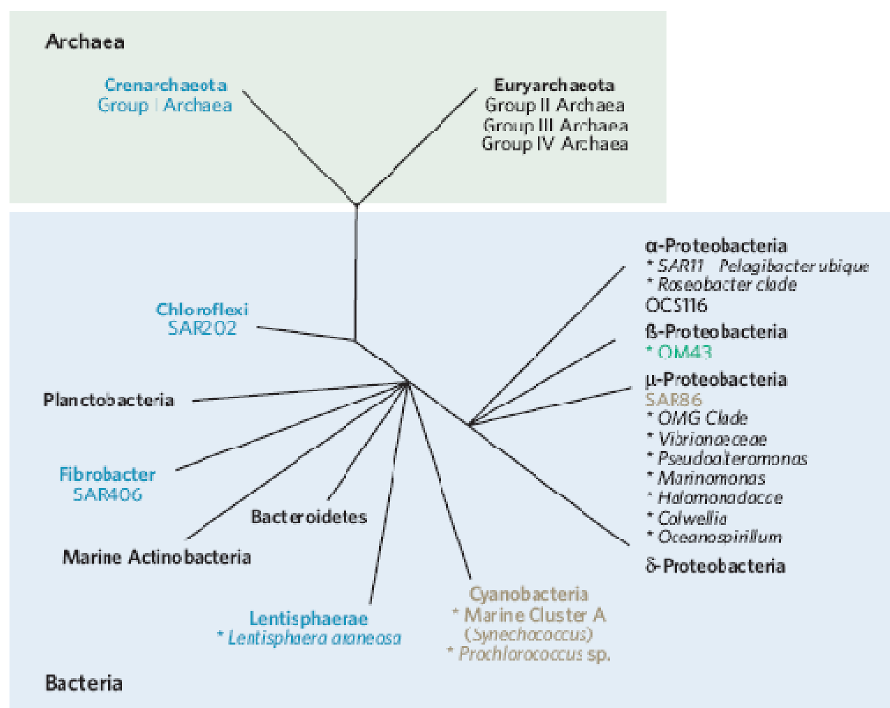


Fig. 3 Overview of the major bacterioplankton groups. Black: potentially ubiquitous in seawater; gold: found in the photic zone; blue: confined to the mesopelagic and surface waters during polar winters; green: associated with coastal ecosystems (Giovannoni & Stingl 2005).

Since only a small fraction of marine bacteria can be cultured (Staley & Konopka 1985) community analyses generally rely on molecular methods. While early, culture-based studies of marine bacteria indicated that the oceans are dominated by genera such as *Vibrio* and *Pseudoalteromonas*, the advent of molecular techniques provided a new conception of marine microbial diversity. The 16S rRNA gene has become the standard in the investigation of microbial community structure. However, 16S rRNA-based fingerprinting methods (DGGE, T-RFLP, clone libraries, qPCR, and microrrays) are based on PCR and therefore affected by potential bias. PCR-independent approaches such as fluorescence *in situ* hybridisation (FISH) are able to circumvent this shortcoming, but have their own limitations (insufficient probe coverages and mismatches in non-target groups). The advantage of hybridization techniques is that they enable *in situ* visualization (Fig. 4) as well as quantitative analyses of bacterioplankton (Amann et al. 1995). A modified protocol (CARD-FISH) provides greater detectability of slow-growing bacteria with low ribosomal content, as typically found in nutrient-limited marine pelagic waters (Pernthaler et al. 2002).

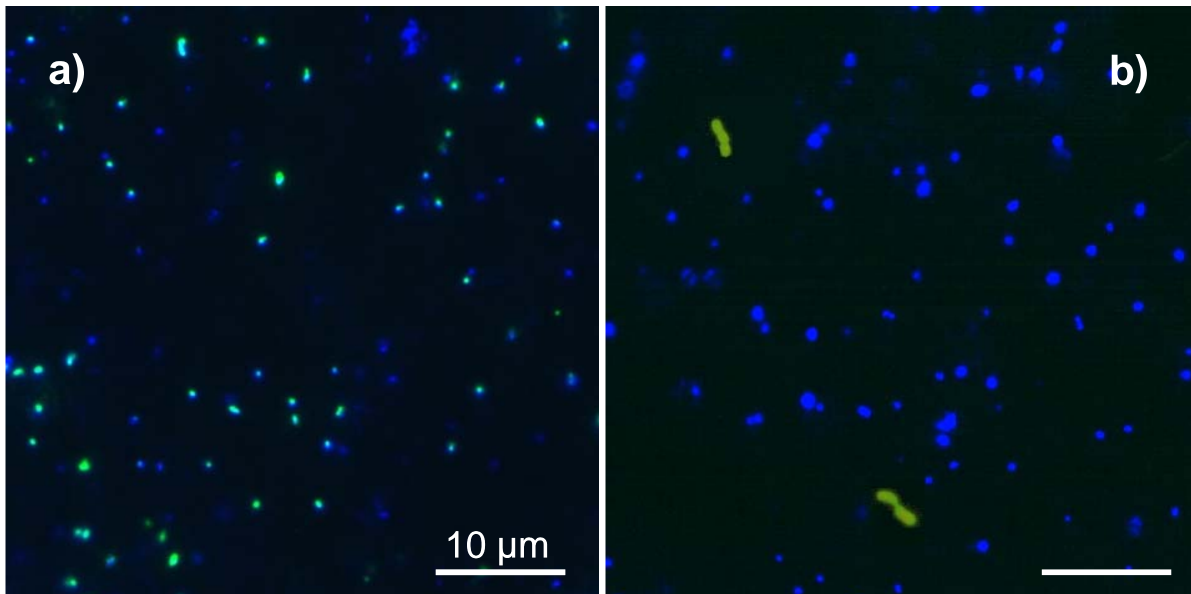


Fig. 4 Fluorescence microscopy images of DAPI-stained marine bacterioplankton (blue) and cells hybridized with CARD-FISH probes (green), showing abundant SAR11 clade bacteria (a) and short chains of SAR86 cells (b) (Wietz et al., unpublished data).

FISH is limited by the fact that not all diversity in a habitat can be analyzed, since each probe only targets a specific bacterial group or genus. Thus, separate probes for each group of

interest must be applied, often allowing only a selection of probes to be applied per sample. Acquiring in-depth information on bacterial diversity is therefore dependent on methods that can analyse the majority of bacterial genomic information in a habitat. This can be achieved by sequencing of genomic community DNA (environmental metagenomics). At present, this provides the best resolution of bacterial community composition and can identify almost all species in a given ecosystem (Venter et al. 2004). This approach also forms the basis of the Global Ocean Survey on board the Sorcerer II expedition (Nealson & Venter 2007). However, a thorough bioinformatic analysis of the resulting enormous datasets is needed to draw any viable conclusions, and only part of the acquired data have been analyzed in greater detail to date (Biers et al. 2009). The search for genes involved in important metabolic pathways, for instance anaerobic ammonium oxidation or aerobic anoxygenic photosynthesis, is an example how metagenomic data can be put into an ecological context to make it more than just a description of "who is out there". In this context, community fingerprinting data should be and is increasingly seen from a broader perspective, which also addresses a potential influence of external factors on microbial community structure. Comparable to the macrobial world, potential patterns in microbial diversity and community structure are likely influenced by environmental parameters. This emphasizes the need for biogeographical analyses to understand the biological, physical and chemical processes behind the distribution of marine microorganisms. The present study added to this field by analyzing biogeographical patterns and their relation to environmental parameters among bacterioplankton communities worldwide (Wietz et al. 2010a).

Bacterial biogeography and the influence of environmental parameters

Studying the distribution of bacteria on smaller and larger geographical scales is important to compare bacterial abundances between different environments, aiming to reveal potential biogeographical patterns and their relation to environmental parameters (Martiny et al. 2006). Almost eighty years ago, Baas-Becking postulated about the distribution of microorganisms that "everything is everywhere, but the environment selects" (Baas-Becking 1934). This implied that all microbial groups and species are present ubiquitously, and only their response to external factors decides about their abundance at a certain location. The discussion about the relation of environmental factors to spatial and temporal patterns among bacteria is still central in microbial

ecology. Some freshwater protists have been indeed found on a global scale (Fenchel & Finlay 2004), and also large bacterial classes such as the *Alpha*- and *Gammaproteobacteria* are present in all aquatic environments (Fig. 3). Some other groups, however, are predominantly found in selected habitats. As mentioned above, *Archaea* are often absent or only marginally present in surface waters. Instead, they often show a vertical stratification in abundance, becoming increasingly dominant in deeper stata of the water column (DeLong et al. 2006). This could be related to the fact that *Archaea* are generally thought to be more extremophilic, although this conception may be outdated since they are not restricted to extreme habitats (Robertson et al. 2005). *Betaproteobacteria* frequently dominate freshwater bacterioplankton but are virtually absent from pelagic assemblages, only being found in coastal and estuarine waters (Giovannoni & Stingl 2005). Also *Actinobacteria* generally reach higher numbers in freshwater communities (Glöckner et al. 2000). In this context, there can be significant differences between coastal and open ocean microbial populations (Rappe et al. 2000), as well as shifts from freshwater-resembling to typically marine community structures along a estuarine gradient (Kirchman et al. 2005). Strong variability in prokaryotic assemblage structure was also demonstrated along coast-ocean transitions (Baltar et al. 2007) and ocean fronts (Pinhassi et al. 2003). These differences are presumably due to differing environmental and biological parameters. Coastal and estuarine waters can have lower salinity levels through fluvial freshwater input; in addition, aeolian deposition and sediment inflow can result in elevated concentrations of nutrients and humic substances. Microbial assemblage structure can also be affected by a larger fraction of phytoplankton at the continental shelves (see below).

The presence of distributional patterns on smaller geographical scales indicates that similar scenarios may be observed at a greater spatial dimension. Large-scale biogeographical patterns likely reflect different physicochemical conditions between oceanic regions, which relates to the fact that the World Ocean can be categorized into major regions according to distinct oceanographic parameters. Seasonal cycles in illumination and nutrient availability form the basis of the classification into the four oceanic biomes, which are designated the Polar, Coastal, Westerlies, and Trades (Longhurst 1998). The Coastal biome includes all coastal locations in warm and temperate waters, but not those in the Arctic and Antarctic which are combined in the Polar biome. The Westerlies and Trades biomes largely correspond to the subtropical and tropical climate zones, respectively. Further classifications of these broad categorizations consider local variation such as coastal upwelling, as found in the Namibian (Benguela) and Peruvian (Humboldt) upwelling systems. In these zones, colder, nutrient-rich waters are transported from deeper strata to the photic

zone, resulting in the formation of highly productive ecosystems that substantially differ from oligotrophic pelagic waters. Also minor seas, such as the Mediterranean or Caribbean, can possess unique oceanographic characteristics. These led to further categorizations into, e.g., 42 provinces (Longhurst 1998) or the 64 Large Marine Ecosystems that were defined by the National Oceanic and Atmospheric Administration (NOAA; see http://www.lme.noaa.gov).

Global studies of bacterioplankton diversity and community structure that address potential biogeographical patterns so far exclusively focused on surface waters. Molecular approaches such as environmental metagenomics (Biers et al. 2009), PCR-based techniques (Baldwin et al. 2005; Pommier et al. 2007; Taniguchi & Hamasaki 2008; Giebel et al. 2009), or CARD-FISH (Schattenhofer et al. 2009) indicated that the composition of surface water bacterioplankton differs between oceanic regions. A global analysis of bacterioplankton phylotypes showed considerable variation on the 16S rRNA sequence level, with a high degree of endemism and few cosmopolitan sequences (Pommier et al. 2007). T-RFLP demonstrated distinct microbial clusters relating to Arctic, Antarctic, temperate and tropical regions, with warm-water communities being similar in composition while being different from cold-water communities (Baldwin et al. 2005). Across an Atlantic Ocean transect, SAR11 was more abundant in the Northern than in the Southern Atlantic, the biomass of *Prochlorococcus* peaked in the tropical regions, and *Bacteroidetes* and *Gammaproteobacteria* bloomed in nutrient-rich temperate waters (Schattenhofer et al. 2009). Also, ribotypes from all major bacterioplankton taxa were shown to be restricted to either higher or lower latitudes (Pommier et al. 2005). A latitudinal gradient in species richness, comparable to the observation in the animal and plant kingdoms, has been shown in this context (Pommier et al. 2007; Fuhrman et al. 2008). Biogeographic variation is also present among the rare microbial biosphere, which was shown to feature only a low amount of cosmopolitan sequences (Galand et al. 2009). The metagenomics-based Global Ocean Survey may be able to provide the most comprehensive picture of marine microbial diversity and biogeography so far (Nealson & Venter 2007), however, further analyses need to be performed before conclusions can be drawn. The present project contributed to the knowledge of bacterioplankton biogeography by showing latitudinal patterns in the abundance of marine bacteria (Wietz et al. 2010a) (Fig. 5). This was mainly related to distinct latitudinal variation in the abundances of a few groups. This included *Bacteroidetes* (peaking at higher latitudes) but also unclassified *Bacteria* and *Vibrio* (both peaking at lower latitudes). On the biome scale, relative abundances of *Alphaproteobacteria* (peaking at subtropical) and

Gammaproteobacteria (polar stations) varied. The latter was consistent with the earlier mentioned increased abundance of this class in colder waters.

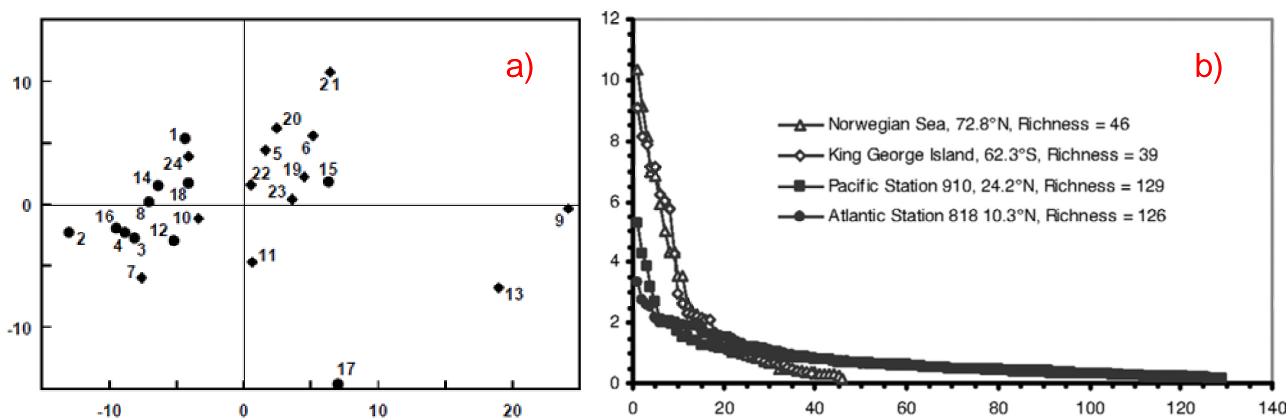


Fig. 5 Biogeographical patterns among marine bacterioplankton. a) Clustering of sampling stations according to higher and lower latitudes after comparison of bacterial abundances worldwide (Wietz et al. 2010a). b) Rank-abundance curves of bacterial samples taken along an equator-to-pole transect, illustrating decreasing species richness with latitude (Fuhrman et al. 2008).

Distributional patterns among marine microbiota likely relate to environmental factors, such as the water temperature (Baldwin et al. 2005; Fuhrman et al. 2008) or nutrient availability (Abell & Bowman 2005). Known linkages can even be used to predict community composition from oceanographic conditions (Fuhrman et al. 2006). In Mediterranean waters (Teira et al. 2008), a multitude of correlations between environmental parameters and bacterial groups have been shown. For instance, *Gammaproteobacteria* were positively correlated with nitrite and nitrate, while *Bacteroidetes* were negatively correlated (Teira et al. 2008). On a global scale, such interrelations appear to be more complex (Wietz et al., 2010a).

Biogeographical patterns among marine bacterioplankton can also be linked to the abundance of non-bacterial microorganisms, most notably photosynthesizing phytoplankton. Primary production through photosynthesis is not equal throughout oceanic regions, but concentrated to continental margins and nutrient-rich upwelling zones (Fig. 6), zones that also typically harbor distinct bacterioplankton communities. Certain bacterial groups, such as the *Roseobacter* clade (Wagner-Döbler & Biebl 2006), *Bacteroidetes* (Fandino et al. 2005) and SAR86 (Gonzalez et al. 2000), are known to respond to phytoplankton blooms and therefore dominant in these habitats. In the present study, the fraction of *Bacteroidetes* in the Benguela upwelling system

was shown to constitute 20% of all bacteria, being almost twice the global average (Wietz et al. 2010a). Furthermore, both *Bacteroidetes* and the *Roseobacter* clade correlated with the concentration of chlorophyll (Wietz et al. 2010a), an indicator of phytoplankton biomass.

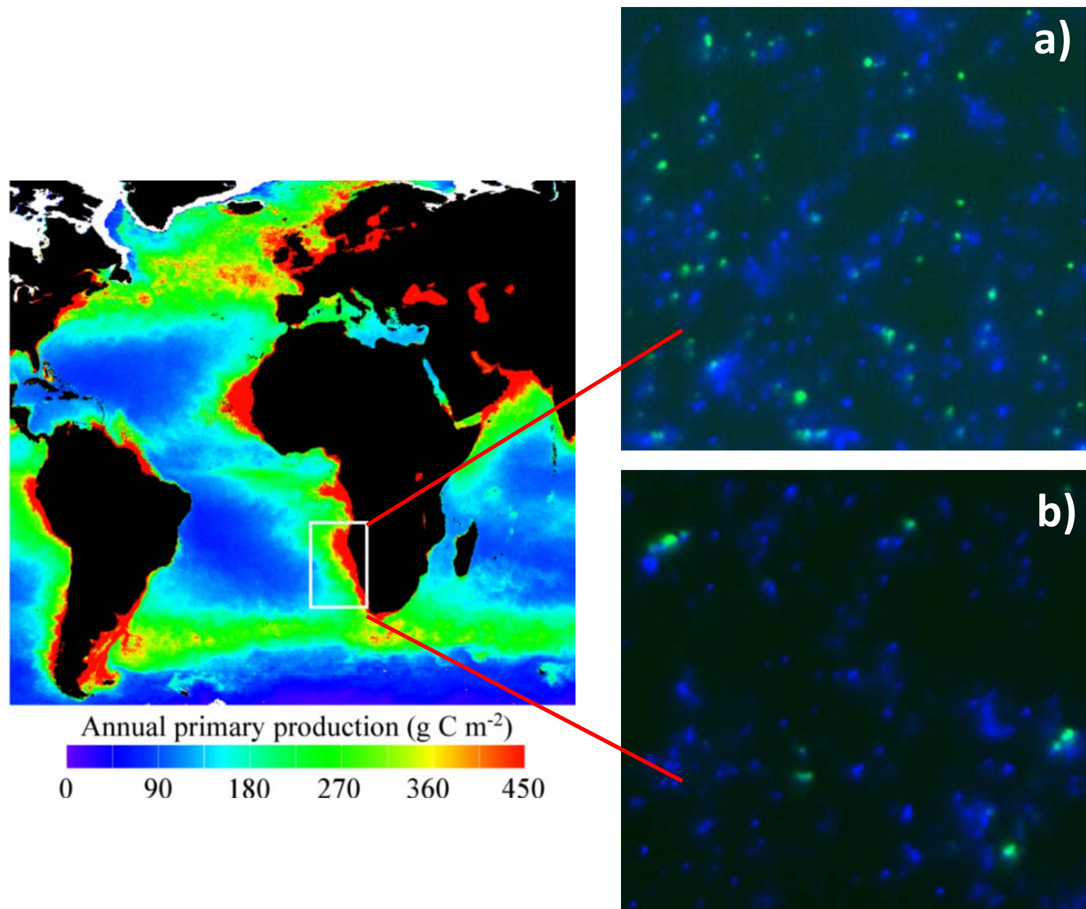


Fig. 6 Primary production in the oceans by marine phytoplankton (Kuypers et al. 2005). The white box marks the Benguela upwelling, a zone of higher phytoplankton abundance and increased production. Algal blooms stimulate the growth of certain bacterial groups, including *Bacteroidetes* (a) and the *Roseobacter* clade (b).

Biogeographical patterns also possibly reflect variability in the genetic and enzymatic repertoire of bacteria from different regions. Molecular approaches (Zehr et al. 2003; Mou et al. 2008) and mesocosm experiments (Carlson et al. 2002; Zubkov et al. 2003) have demonstrated that bacterioplankton community structure and physiological capacities can be linked. Consequently, metabolic specialization may have contributed to niche speciation and the emergence of

biogeographical patterns. Different members of the *Roseobacter* clade are capable of sulfite reduction (Park et al. 2007), aerobic anoxygenic photosynthesis (Béjà et al. 2002), and utilization of dimethylsulfoniopropionate (DMSP). It is possible that such differences contribute to the restriction of certain species clusters to defined oceanic regions (Selje et al. 2004) or the existence of endemic ecotypes, genetically closely related yet physiologically distinct microbes occupying different niches (Cohan 2002). For instance, *Prochlorococcus* ecotypes show a distinct partitioning among the four major oceanic biomes (Zwirglmaier et al. 2008). Metabolic variation as the basis of biogeographical variation is also seen for certain *Cytophaga*-related bacteria (Rosselló-Mora et al. 2008) and the actinobacterial genus *Salinispora* (Jensen & Mafnas 2006).

The linkage of bacterioplankton community structure, physiological capacities of marine bacteria and environmental parameters are illustrated by the multitude of correlations between bacterial abundances and abiotic factors (Wietz et al. 2010a). Future studies are likely to reveal more biogeographical variation on global and local scales, and correlations of these patterns with environmental parameters. However, there is apparently no link between particular community patterns and the occurrence of culturable bioactive bacteria (Wietz et al., unpublished data). This is an important reminder of the "great plate count anomaly" (Staley & Konopka 1985) and the often selective enrichment of certain culturable bacteria from marine samples (Eilers et al. 2000), making it difficult to link molecular and culture-based approaches.

2.2 Polar microbiology

During the course of the PhD study, our group was offered to take part in the LOMROG-II expedition to the high Arctic. In many aspects, the polar environments are different from temperate and warm waters, and are therefore introduced separately in the following section.

Polar seas are affected by greater seasonal variation, including the characteristic annual cycle of sea ice formation and melting. In the Arctic Ocean, the effective absence of wind mixing results in a very stable, highly stratified water column consisting of three major water masses (Aagaard et al. 1981). The upper water layer is found in the first 60 m and consists of low-salinity water, with a temperature of around 0 °C. The intermediate layer (between 75 and 150 m) contains high-salinity and cold (–2 to –4 °C) water. Below, moderately warm (2 to 4 °C) water originating from the Atlantic Ocean prevails. The Southern Ocean is characterized by different oceanographic

conditions. In contrast to the land-locked Arctic Ocean that receives terrestrial inflow from Russia, North America and Greenland, the Southern Ocean surrounds a continent (Antarctica) with narrow, deep continental shelves. It receives virtually no freshwater inflow and no terrigenous organic matter (Kumar et al. 1995).

Molecular characterization of polar marine microbiota

The isolation of cold polar oceans and their unique oceanographic conditions suggest that polar assemblages of marine prokaryotes may have evolved independently, which implies the presence of unique communities. For instance, the cyanobacterial genus *Prochlorococcus* is abundant in temperate and tropical but virtually absent above 45° latitude (Zwirglmaier et al. 2008). In contrast, gas vacuolate bacteria are found in sea ice-associated, but not in warm-water microbiota (Walsby 1994). Such scenarios are thought to rely on the unique signature to the water mass structure by annual cooling and the resulting formation of sea ice.

Although there is still comparatively little data available, it has become apparent that waters of the Arctic Ocean harbour an increased abundance of *Gammaproteobacteria* and *Bacteroidetes* (Brinkmeyer et al. 2003; Malmstrom et al. 2007), and that *Archaea* are present in higher numbers (Galand et al. 2006) compared to temperate and warm oceans. Overall, the species richness in Arctic waters is lower, resembling observations in the macrobial world and resulting in latitudinal patterns (Fuhrman et al. 2008). Interestingly, archaeal communities showed local biogeographical variation, with communities from inflowing rivers being different from those in adjacent coastal waters (Galand et al. 2006). In deep Arctic waters, bacterial biogeography was related to hydrographic variations between the previously mentioned three water masses. More specifically, the abundance of specific phylotypes within the SAR11 (*Alphaproteobacteria*), SAR406, SAR202 (*Chloroflexi*), and SAR324 (*Deltaproteobacteria*) clades significantly varied with depth. This was thought to reflect that water masses act as physical barriers limiting bacterial dispersal (Galand et al. 2010). Biogeographical patterns were also seen for members of the rare microbial biosphere, showing that rare Arctic phylotypes did not have a ubiquitous distribution but rather followed patterns similar to those of the most abundant community members (Galand et al. 2009).

Specialized polar microbiota are represented by sea-ice microbial communities (SIMCO), which often involve the co-occurrence of bacterial and algal cells. Arctic sea ice bacteria comprise

common (*Proteobacteria*, *Bacteroidetes*, *Actinobacteria*) but also less common marine groups such as the orders *Verrucomicrobiales* and *Chlamydiales* (Brown & Bowman 2001). Although some microbes can be present in both sea ice and the underlying seawater, many species appeared to be unique to the sea-ice habitat (Bowman et al. 1997). This is probably related to the fact that most microbial activity takes place in sea ice cavities, high-saline microenvironments requiring a pronounced salt tolerance that potentially selects for highly adapted species groups. Molecular studies (FISH and clone libraries) of Arctic and Antarctic pack ice demonstrated that the *Alpha*- and *Gammaproteobacteria* as well as *Bacteriodetes* were the dominant taxonomic bacterial groups (Brinkmeyer et al. 2003). 16S rRNA sequence analyses revealed a high incidence of closely overlapping sequences, indicating that communities from both poles may be similar in composition. FISH revealed a distinct abundance of specialized genera including *Octadecabacter*, *Glaciecola*, *Psychrobacter*, *Marinobacter*, *Shewanella*, and *Polaribacter*. The finding of limnic phylotypes in Arctic samples suggested that the bacterial community was influenced by terrestrial input (Brinkmeyer et al. 2003).

Culturable bacteria in polar oceans

Studies on culturable bacteria in polar environments have largely focused on sea-ice associated strains. Sea ice microbiota are characterized by higher cell densities than found in other polar habitats, such as the adjacent water column. The culturability is often substantially higher than commonly found in marine systems, reaching up to 25% of all bacteria (Brinkmeyer et al. 2003). The present study confirmed this observation, showing that sea ice samples yielded viable counts of up to twice as high than water and zooplankton samples (Wietz et al. 2011b) (Fig. 7).

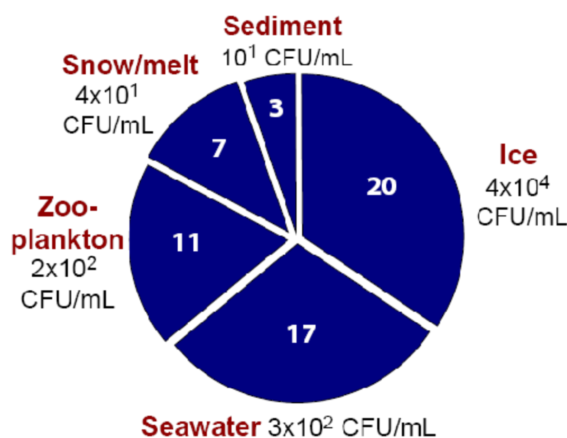


Fig. 7 Culturable bacterial counts in fifty-eight samples from different Arctic marine environments (Wietz et al. 2011b)

The culturable microbiota of sea ice contains comparatively limited species diversity. From the *Alphaproteobacteria* the genus *Octadecabacter* (belonging to the *Roseobacter* clade) was found to be especially abundant, while communities of *Gammaproteobacteria* (mainly *Marinobacter*, *Colwellia* and *Glaciecola* spp.) and *Bacteroidetes* (mainly *Salegentibacter* and *Psychroserpens* spp.) were more diverse (Junge et al. 2002; Brinkmeyer et al. 2003). In polar seawater, the culturable microbiota is dominated by the *Roseobacter* clade, various *Gammaproteobacteria* and Gram-positive *Actinobacteria* (Mergaert et al. 2001). Little is known about epibiotic communities associated with higher eukaryotes. The epibiotic flora of polar zooplankton includes *Vibrionaceae*-like strains in association with amphipods (Atlas et al. 1982) and different invertebrates (Jøstensen & Landfald 1997).

The environmental extremes, foremost the year-round low temperatures, require a high potential of physiological adaptation. Cold is a physical stress that considerably changes the physical and chemical parameters of a living cell, including membrane fluidity, enzyme kinetics, and the performance of macromolecules (Rodrigues & Tiedje 2008). Low temperatures necessitate bacteria to be psychrophilic or –tolerant, while the number of psychrotolerant species (growing both at low and higher temperatures) in polar sea ice appears to be higher than that of true psychrophiles (only growing below 15 °C) (Rodrigues & Tiedje 2008). Also, high salinities in brine pockets of sea ice and seasonal variation in illumination have to be endured. Cell membranes are often enriched in unsaturated and shorter-length fatty acids to maintain membrane integrity. Cold-acclimation proteins include approx. 20 macromolecules that are permanently synthesized at low, but not at milder temperatures (Hebraud et al. 1994). Specialized cold-active chaperones and nucleases ensure replication, translation and the processing of nutrients (Feller & Gerday 2003). The high activity of psychrophilic enzymes at low temperatures could provide economic benefits, and are therefore widely researched in biotechnology. While psychrophilic enzymes could provide energy savings in large-scale industriual processes, the most commonly named example lies in the domestic market. Cold-active lipases and glycosidases being implemented in washing detergent could help to hydrolyse macromolecular stains at low temperatures, thereby reducing energy consumption and protecting the colours of fabrics (Feller & Gerday 2003).

2.3 Conclusions from chapter 2

The molecular investigation of marine bacterioplankton communities, including their biogeographical patterns, represents one major focus in contemporary marine microbiology. There is increasing evidence of distributional patterns among marine bacteria on global and local scales, which are likely related to environmental parameters. Recent studies have revealed that latitudinal patterns not only exist in the macrobial world, but may likewise be present among marine microbes. This has been underlined by the present study, showing that warmer and colder oceans harbour distinct bacterioplankton communities. While the original aim of linking the composition of the overall microbiota to the occurrence of bioactive culturable genera could not be accomplished, the results are valuable for the understanding of marine bacterioplankton distribution and biogeography. Future work will likely reveal more comparable patterns, and increasingly address how abundance patterns are linked with metabolic activity.

A detailed molecular investigation of samples collected throughout the Arctic during LOMROG-II would be valuable to obtain further potential evidence that polar microbiota are different from those in warmer waters.

3. BACTERIAL INTERACTIONS AND ANTAGONISTIC MARINE BACTERIA

The second part of the PhD study addressed the antagonistic activity and biosynthetic potential of marine bacterial isolates. Being a culture-based approach, this part therefore clearly separates from the molecular investigation of marine microbiota as introduced in chapter 2. Nevertheless, bacterial antagonism probably plays a role in environmental community dynamics, indicating that physiological traits are likely interlinked with bacterioplankton community structure. Nevertheless, a direct link between the overall composition of the microbiota (Wietz et al. 2010a) and the occurrence of culturabl antagonistic bacteria could not be shown (see above).

3.1 Physical and chemical interactions in the environment

Microbial interactions are a keystone in bacterial ecophysiology. When microbes occur in higher densities, for example in biofilms, the proximity of individual cells results in direct physical contact that is likely accompanied by chemical interactions. The ecological function of microbes is often dependent on symbiotic interactions (Davey & O'̈Toole 2000), and co-existing species can show commensal interactions depending on their physical positioning (Christensen et al. 2002). This can even lead to genomic mutations to adapt to neighbouring microbes, thereby forming intimate and specialized associations (Hansen et al. 2007).

In the marine environment, physical and chemical interactions are potentially more widespread than previously thought. On a micro- and nanometer scale, many presumably "free-living" bacteria were in fact closely associated with another (Fig. 8) (Malfatti & Azam 2009). This is contrasting the common perception of marine bacterioplankton communities and how to categorize their members. The standard approach – and also the basis for the present study (Wietz et al. 2010a) – is a dichotomy of particle-associated versus planktonic bacteria. Water samples are therefore commonly filtrated through different pore sizes (5 µm and 0.2 µm) to obtain two fractions with either particle-associated or free-living cells. The shown close interactions between presumably single planktonic bacteria indicate that this perception may be outdated.

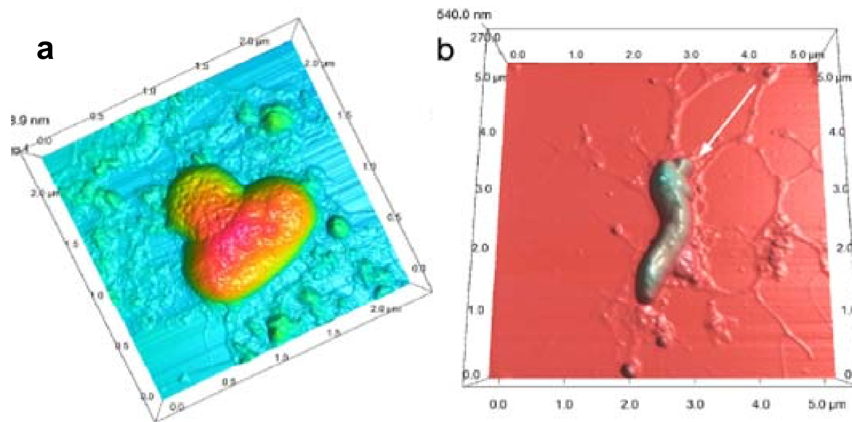


Fig. 8 Atomic-force microscopy images of associated marine bacteria (Malfatti & Azam 2009). a) Conjoint cocci and rod-shaped cell; b) pair of heterotrophic bacteria, showing a large S-shaped cell in association with a much smaller bacterium (arrow).

Recent work has shown that many aquatic bacteria potentially possess a complex lifestyle and alternate between a free-living and a surface-associated stage (Grossart 2010). This is believed to be facilitated by the existence of various microbial networks that are connected via microhabitats, for instance organic aggregates, fecal pellets, and higher organisms. Within these networks, bacterial cells can travel long distances and interact with other bacteria, for instance exchange genetic information (Grossart 2010).

Fig. 9 summarizes three possible ways bacteria can interact and communicate. While electrical interactions are still poorly understood, chemical interactions are more widely researched. A prominent mode of interaction bases on secreted signalling molecules which are sensed by neighbouring cells and trigger an intracellular response. Quorum sensing (QS), a density-dependent modulation of gene expression, is a major example for this kind of interactions. Classic QS molecules are *N*-acyl homoserine lactones of varying length, but also other types of signalling compounds exist, for instance the furanone autoinducer AI-2 or the pseudomonas-specific quinolone signal (Camilli & Bassler 2006). Quorum sensing effects usually base on two-component pathways, e.g. via membrane-bound kinases that mediate a signalling pathway in the receptor cell. This results in a regulation of DNA transcription and gene expression according to environmental stimuli. Such mechanisms, for instance, affect the virulence in *Pseudomonas aeruginosa* (Pesci et al. 1999), *Vibrio cholerae* (Zhu et al. 2002), and fish pathogenic bacteria (Bruhn et al. 2005a).

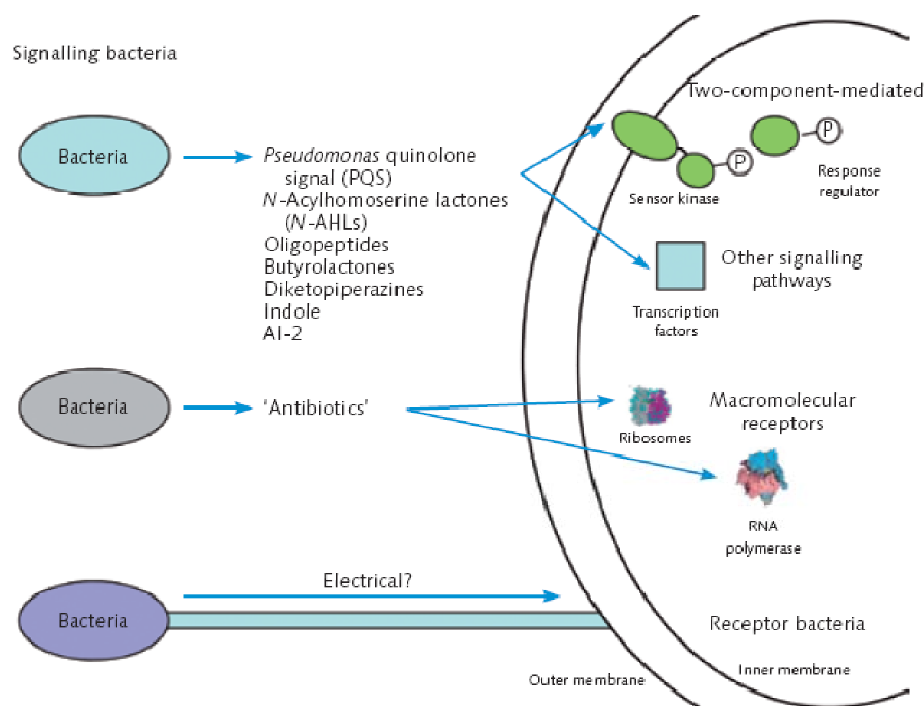


Fig. 9 Bacterial interactions in the environment (Davies 2009).

Another important mode of interaction is mediated by production of antagonistic compounds, such as antibiotics. High quantities of such molecules, for example when microorganisms are grown to high cell densities under laboratory conditions, can be potent inhibitors of bacterial growth. In the natural habitat, however, cell densities are much lower, and antibiotic compounds are consequently only present in dilute concentrations. In environmental microbiota, antagonistic compounds may therefore have additional functions than growth inhibition of competing microbes. At sublethal concentrations, the primary effect of antagonistic metabolites probably lies in cell signalling, such as the modulation of metabolic function, gene expression, and global transcription processes (Davies et al. 2006). The feature of possessing contrasting effects at high and low concentrations has been referred to as hormesis (Yim et al. 2007). Such processes can have an influence on different phenotypic characteristics, including virulence (Kastbjerg et al. 2010) and exoprotein production (Tanaka et al. 2005). The conception of antibiotic compounds as a tool of chemical interaction and intercellular communication (Hibbing et al. 2010) probably also applies for aquatic environments, where secreted microbial metabolites are immediately diluted in the aqueous surrounding.

3.2 Bioactive microbial secondary metabolites

Bacterial metabolites can generally be distinguished into primary and secondary metabolites. While the former (comprising nucleotides, amino acids, and vitamins) are synthesized by all organisms and involved in vital cellular functions, secondary metabolites form part of an additional metabolic repertoire. In many cases, these so-called natural products possess an ecological role and confer a beneficial effect. As a result, natural products are commonly termed “bioactive”.

In the microbial realm, especially soil actinomycetes and fungi have been characterized regarding their secondary metabolome (Baltz 2008). Most microbial natural products are small organic molecules with a molecular weight below 3000 Dalton, and despite an immense structural diversity they can be categorized into a few major compound classes. These are polyketides and fatty acids, terpenoids and steroids, alkaloids, phenylpropanoids, as well as specialized amino acids, peptides, and carbohydrates. Monomeric secondary metabolites are often arranged in polymers, the so-called extracellular polymeric substances (EPS). EPS have a major biological function in biofilm formation as surface adhesins, nutrient reserves, protection from predation and desiccation, and dispersants to release bacteria from nutrient-depleted surfaces (Weiner 1997). Another example of bacterial secondary metabolite polymers are polyhydroxyalkanoates, storage polyesters accumulated under excess carbon supply (Reddy et al. 2003). Among marine bacteria, hydroxybutarate polymers (PHB) were found in *Vibrio* (Chien et al. 2007) and *Brachyomonas* (Halet et al. 2007). The present study confirmed the production of PHB in marine *Vibrionaceae*, including *Vibrio coralliilyticus* and *Photobacterium halotolerans* (Wietz et al. 2010b).

Secondary metabolites also include pigments, which can provide ecological advantage by preventing ultraviolet light damage, interfering with proteolytic and hydrolytic enzymes, and acting as nutrient sink in biofilms (Weiner 1997). For bacteria in surface waters, increased pigmentation can be used to counteract higher UV radiation (Hermansson et al. 1987). Pigments can also serve as cryoprotectants, and carotenoid-containing Antarctic bacteria were more resistant to freeze-thaw cycles thane non-pigmented strains (Dieser et al. 2010). In addition, many pigments have antibiotic properties, and a link between pigmentation and antibiosis has been demonstrated in *Streptomyces coelicolor* (Rudd & Hopwood 1980), *Chromobacterium violaceum* (Durán & Menck 2001), and *Pseudoalteromonas* spp. (Bowman 2007).

The most widely researched class of bioactive secondary metabolites are pharmacologically active compounds. Over the past decades, the majority of new drugs have been generated from

bioactive microbial natural products or synthetic derivatives. Biotechnological and medicinal applications are diverse, ranging from antibiotic, antimalarial, immunosuppressant, to anticancer and many other treatments (Li & Vederas 2009). The present PhD focuses on antibiotic compounds that inhibit the growth of bacterial pathogens. Antibiotic natural products often belong to two structural compound classes, the polyketides and nonribosomal peptides.

Polyketides and nonribosomal peptides

Polyketides harbour an enormous structural diversity. Their biosynthesis bases on condensation of malonyl-CoA derived monomeric units, a process similar to the synthesis of fatty acids (Robinson 1991). This process is catalyzed by polyketide synthetases (PKS), large modular enzymes (up to 2000 kDa) that elongate the polyketide in an assembly-line manner. In most bacteria, modular synthetases are organized into functional units with several catalytic domains, each performing one elongation step before transferring the nascent polyketide chain to the next unit. While polyketides can undergo a complex series of modifications, they can be broadly divided into three classes. Type I polyketides are often macrolides containing a lactone ring with attached deoxy sugars, while Type II and III polyketides are molecules with aromatic rings (Robinson 1991). Phylogenetic prediction, a genetic screening of environmental isolates for presence of synthetase genes, can assist in the identification of strains with the potential of polyketide synthesis and natural product discovery (Kim et al. 2006). Such knowledge is of interest given the diverse pharmacological properties of polyketides, including antibiotic (Sujatha et al. 2005) and anticancer (Davidson et al. 2001) activities.

Also the nonribosomal peptides are a diverse family of natural products, consisting of short amino acid chains (usually less than 20 amino acids) that are condensed by transamination. This process is catalyzed by mRNA-independent, large multi-domain nonribosomal peptide synthetases (NRPS) (Sattely et al. 2008) with architectural and organizational similarities to polyketide synthetases. Nonribosomal peptides encompass a great structural diversity, such as cyclized or branched dimers of identical sequences. Peptides can contain non-proteinogenic and D-amino acids, and can be modified by a series of chemical alterations including glycosylation, acylation, halogenation, or hydroxylation. Also, methyl and formyl groups are frequently added.

Nonribosomal peptides show a broad range of biological and pharmacological properties including antibiotic activity (Schwarzer et al. 2003).

Given the similarities in polyketide and nonribosomal peptide synthetases, hybrid enzymatic megacomplexes exist. The *pks* gene cluster in *Bacillus subtilis* is present in many copies throughout the genome, each encoding subunits which ultimately organize into a single organelle-like complex of tens to hundreds of megadaltons (Straight et al. 2007). In the present study, the hybrid nonribosomal-polyketide antibiotic andrimid, a compound whose biosynthesis involves both PKS and NRPS (Jin et al. 2006), has been isolated from a marine *Vibrio* (Wietz et al. 2010b).

Strategies in natural product discovery

The investigation of microbial natural products typically combines microbiological and chemical analyses. In the early stages of the discovery process, crude extracts of microbial cultures are analyzed on a small scale in order to assess the chemical novelty within a collection of strains (metabolite profiling). These initial analyses are based on a separation of the extract components by high-performance liquid chromatography (HPLC) typically coupled to an ultraviolet diode-array detector (UV/DAD) and a mass spectrometer (MS). The resulting chromatogram can be analyzed by extracting UV and MS spectra for each individual compound, providing structural information relating to the presence of conjugated double bonds (the chromophore) and molecular masses (Fig. 10). This data can then be used to search available databases containing reported structures (Laatsch 2010) to recognize and eliminate already studied compounds (dereplication).

This initial analysis to visualize the array of molecules produced by an organism is usually followed by bioassay-guided fractionation and iterations of dereplication. In combination with techniques allowing the further chemical characterization of molecules in the crude extracts, such as explorative solid-phase extraction that can assign characteristics such as polarity, charge, and size (Månsson et al. 2010), this can be used to determine the most suitable purification strategy of a desired compound. Subsequently, mass cultivation is necessary to obtain sufficient biomass for structural elucidation, which is acquired by nuclear magnetic resonance (NMR) spectroscopy of purified compounds.

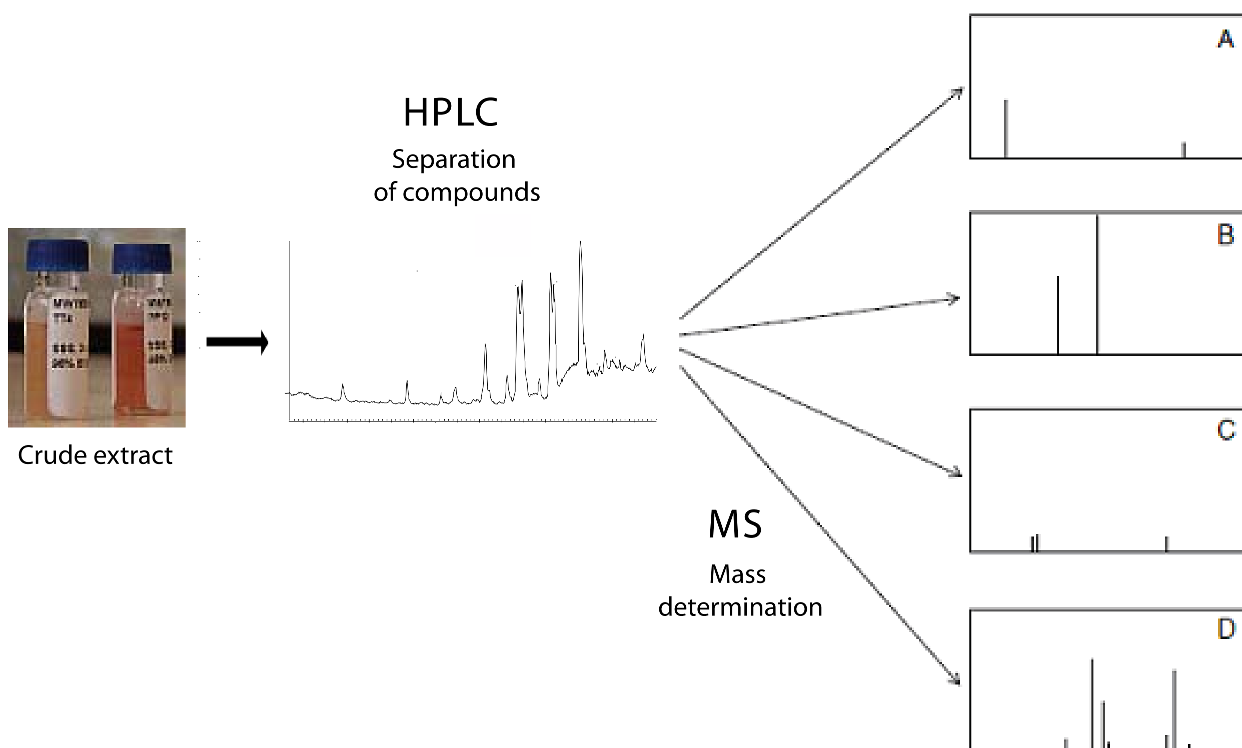


Fig. 10 Overview of the HPLC-MS approach to separate and analyse compounds from a crude culture extract (modified from Back to Basics 2002).

Natural product research yielded the discovery of potent antibiotics including penicillin, streptomycin, tetracycline, and many others. Terrestrial microbes, foremost fungi and filamentous actinomycetes (Berdy 2005), have proven a rich source of antibiotic natural products. The "golden era" of antibiotic discovery in the middle of the 20th century was, however, followed by steadily decreasing numbers of newly discovered and approved antibiotics. The concurrent increase of resistances of pathogenic microorganisms against known drugs underlines the need for novel antimicrobial substances for pharmaceutical, food and aquaculture industries. In human medicine, rapidly increasing resistances of clinically relevant pathogens are a major problem. These include multiresistant *Staphylococcus aureus* (MRSA) causing a large fraction of hospital-based infections, as well as vancomycin-resistant *Enterococcus* (VRE) and fluoroquinoline-resistant *Pseudomonas aeruginosa* (FQRP) (Fig. 11).

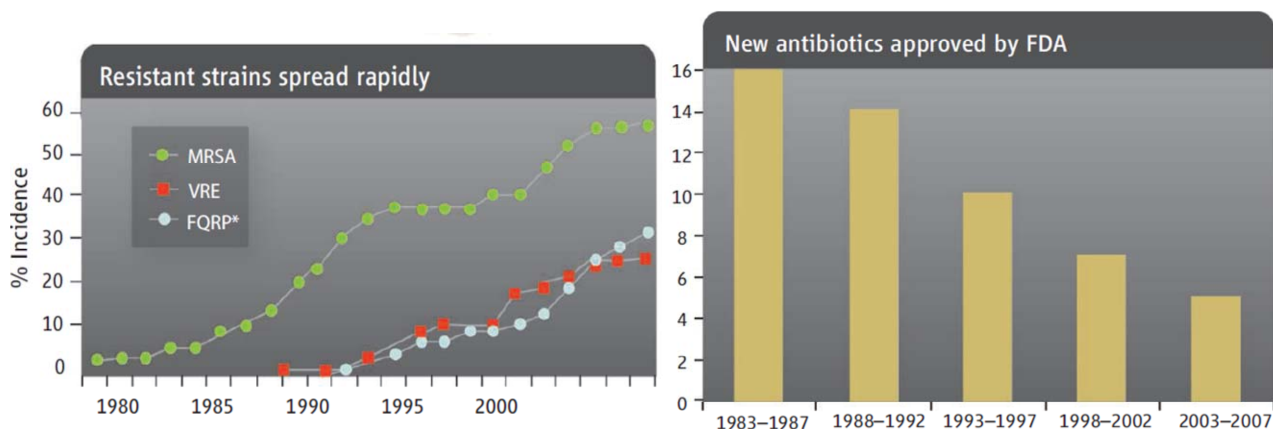


Fig. 11 Rise in the number of resistant pathogenic strains and concurrent decrease in the number of new antibiotics approved in the United States (Taubes 2008).

Also many industrial settings are challenged by increasing occurrence of unwanted, persistent and/or resistant microbes. The food industry has to cope with bacterial contaminants, such as *Listeria monocytogenes*, which can lead to severe cases of food poisoning (Mead et al. 1999). Aquaculture industries are hampered by outbreaks of pathogenic microbes leading to mass mortalities of cultured organisms (Karunasagar et al. 1994). This demands the identification of novel antibacterial compounds, and the discovery of new products is thought be dependent on extensive screening efforts, targeted discovery strategies, and the exploration of largely untapped biological resources.

Cosmopolitan antibiotics

Sequences of microbial genomes revealed that only about 1% of the natural product diversity is known, and that only approx. 10% of the metabolites of known producer species have been analyzed (Fischbach 2009). While this should permit a steady discovery rate, only few novel antimicrobial compounds have been identified in recent years, and research into natural products has gradually declined (Li & Vederas 2009). Rediscovery of already known compounds in evolutionary distant organisms (Fischbach 2009) is a major obstacle in natural product research, since even unrelated microorganisms share metabolic pathways and biosynthetic capacities. As seen in Fig. 12 and 13 identical antibiotics are produced by species from different phylogenetic groups that inhabit different habitats. For instance, violacein is produced by both *Chromobacterium*

(*Betaproteobacteria*) and *Pseudoalteromonas* (*Gammaproteobacteria*) that belong to entirely different bacterial classes with different niches. As described in chapter 2.1, *Betaproteobacteria* including *Chromobacterium* are mostly abundant in freshwater and other terrestrial ecosystems, while *Pseudoalteromonas* is a strictly marine genus. As shown by the collaborative Galathea 3 project, marine *Pseudoalteromonas* species also produce the antibiotic indolmycin (Vynne et al. 2011b), which has so far been only found in terrestrial streptomycetes. Other metabolites are shared between even more distantly related groups including Gram-negative *Proteobacteria*, Gram-positive *Actinobacteria*, and *Firmicutes*. The cosmopolitanism of antibiotics not only includes bacterial classes, but can even extend across the distinct border between pro- and eukaryotes (Fig. 13). Cephaolosporin as well as some antibiotics from the beta-lactam family (Liras & Martín 2006) occur in both bacteria and eukaryotic fungi. The shared biosynthetic capacities probably originate from an ancestral bacterial gene cluster, which was transmitted from producer to non-producer microorganisms by horizontal gene transfer (Fischbach 2009).

The widespread occurrence of some antibiotics severely influences natural product discovery strategies, since “unexpected” discoveries in non-investigated microbes can occur. Extensive screening, purification and isolation efforts can therefore eventually result in the rediscovery of a known compound that was previously found in another microorganism. Despite the existence of natural product databases containing information about known compounds (molecular mass, absorbance spectra, bioactivities) that can be used for comparison, it is not guaranteed that a rediscovery remains undetected until final structural elucidation by NMR. Many compounds in natural product databases such as AntiBase (Laatsch 2010) have similar masses, so even the combination of UV spectra, accurate mass data, and explorative fractionation of crude extracts (Månsson et al. 2010) is not always discriminatory enough. The cosmopolitanism of identical antibiotics has major implications for natural product discovery strategies and stresses the need for careful dereplication in the initial stages of screening. To avoid isolation of redundant chemistry, high-resolution chemical analyses have to be performed. Small mass differences between database-deposited and the own determined can already yield that a probable rediscovery is overlooked.

New approaches and alternative cultivation techniques, such as dilution-to-extinction, low-nutrient and virus-depleted incubation conditions (Bull & Stach 2007), are needed to amplify the potential for biodiscovery. Also, the exploration of less investigated environments, such as the deep sea or permanently cold habitats, are regarded as a promising future direction. Although 90% of the Earth’s oceanic waters have a temperature of 5°C or less, relatively few scientific studies have

investigated secondary metabolites from polar bacteria. There may be great potential of metabolite discovery, as polar habitats are a potential source of novel compounds with biotechnological potential (Thomas & Dieckmann 2002) and extremophilic bacteria possess antagonistic traits (O'Brien et al. 2004; Lo Giudice et al. 2007; Shekh et al. 2010). The latter has been confirmed by the present study (Wietz et al. 2011b).

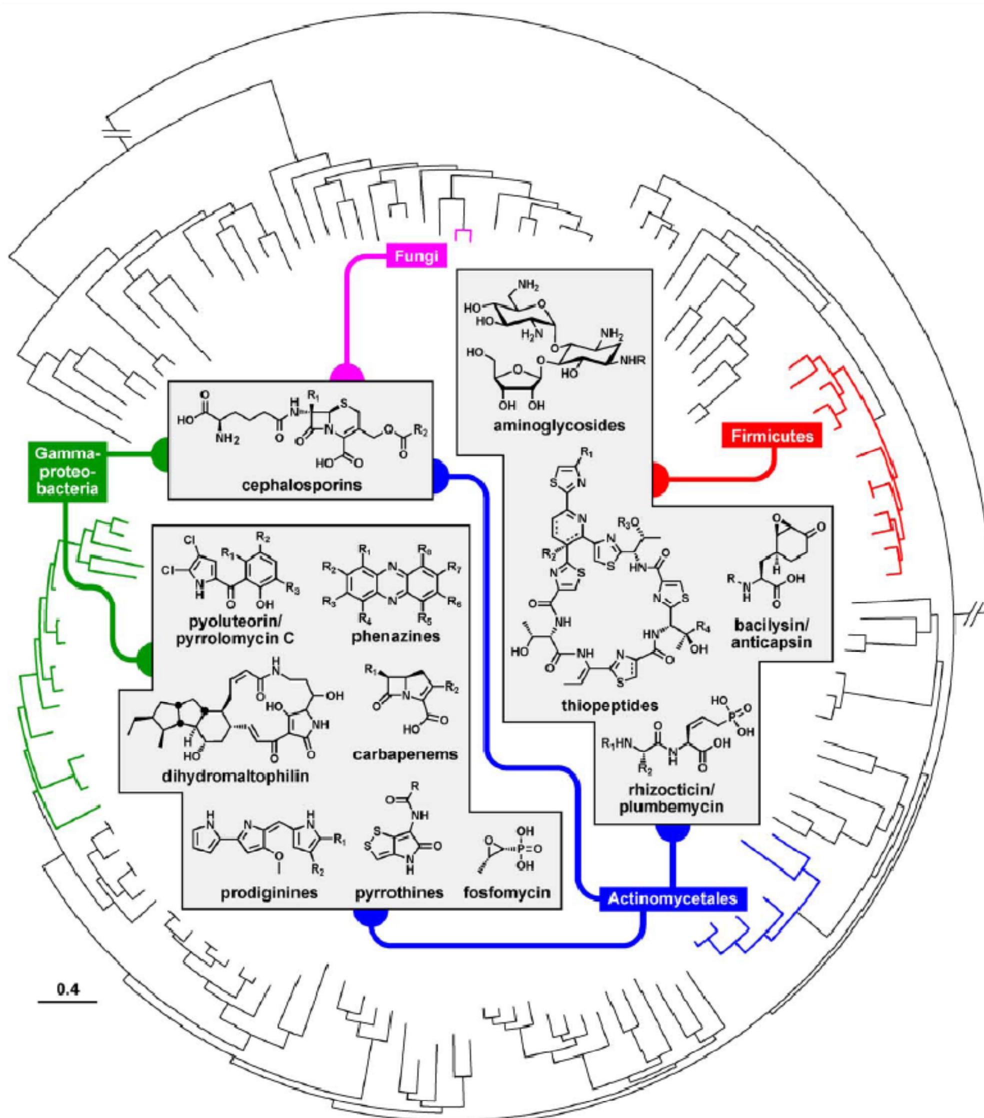


Fig. 12 Cosmopolitan antibiotics (Fischbach 2009).

Marine natural products from eukaryotes

During the past decades, the marine environment has been increasingly regarded as a potential source of novel natural products, motivating marine "blue" biotechnology to explore the vast reservoir of the World Ocean for valuable commodities. Marine natural product research to date largely focused on sessile macroorganisms, since these regularly employ chemical cues as anti-predation mechanisms.

Marine macroalgae and invertebrates are the most widely characterized producers of secondary metabolites to date (Sridhar & Vidyavathi 1991; Taylor et al. 2007). Sponges are one of the richest sources of bioactive compounds and a model organism for natural product research (Taylor et al. 2007). Bioactivities from marine-derived compounds include antibacterial, anticancer, cytotoxic, antithelminthic, immunomodulatory, and many others properties (Mayer et al. 2009). As of October 2010, seventeen marine-derived compounds are in later-phase clinical trials (http://marinepharmacology.midwestern.edu/clinPipeline.htm), but only four of those have been approved to date (Table 1).

Table 1 Approved marine-derived bioactive compounds.

Name	Source	Chemical class	Activity
Cytaribine	Sponge	Nucleoside	Anticancer
Vidarabine	Sponge	Nucleoside	Antiviral
Ziconotide	Cone snail	Peptide	Painkiller
Trabectedin	Tunicate	Alkaloid	Anticancer

Almost all of the above mentioned 17 compounds were originally isolated from sessile or benthic invertebrates. Only 2 of the 17 compounds originated from microorganisms, one eukaryotic (*Aspergillus* fungus) and one prokaryotic (*Salinispora*-affiliated actinomycete). It cannot be excluded, however, that some of the reported compounds were in fact produced by symbiotic bacteria inside the animal tissue. For instance, the anticancer polyketide bryostatin currently in phase 1 of clinical trials likely originates from an associated but uncultured symbiont (Davidson et al. 2001). Also concerning sponges, there is often debate about the true origin of reported bioactive compounds. Sponges harbour a highly diverse microflora that could be responsible for compound

production, and it is not always obvious whether production is related to the host or prokaryotic symbionts (Taylor et al. 2007). This also indicates that bioactive compounds may contribute to commensal or symbiotic interactions between pro- and eukaryotes. In a hypothetical symbiosis, the host would gain microbial chemical defense against predators, and in turn provide a niche for bacterial colonization.

3.3 Bioactive bacteria from marine environments

The potential of marine bacteria to produce antimicrobial compounds has been known for decades (Burkholder et al. 1966; Gauthier & Flatau 1976; Nair & Simidu 1987). Long & Azam (2001) emphasized that marine bacteria represent an underutilized resource of novel antimicrobials, and subsequent studies have shown that the marine environment indeed comprises a multitude of bacterial species producing bioactive metabolites (Jensen & Fenical 2000; Debbab et al. 2010). Screening of environmental isolates using metagenomics (Handelsman 2004) for the presence of genes important for bioactive metabolite synthesis could represent a valuable tool to discover novel compounds. At present, however, most pharmaceutical firms do not undertake such efforts (Li & Vederas 2009). Furthermore, it is a prerequisite that the mechanisms and genetic background of antagonistic activity are known. Despite the only small fraction of microbes that can be cultured, the isolation of bacteria is therefore still a valuable approach (Giovannoni & Stingl 2007) and promising way to isolate bioactive strains with desired physiological traits (Bull 2004). New cultivation approaches, such as 'dilution-to-extinction' techniques, may assist in the isolation of marine isolates that so far refused cultivation. Such approaches were already proven successful to obtain cultures of bacterioplankton groups that dominate surface water communities (Rappé et al. 2002).

Antibiotic compounds from marine bacteria

Most studies on marine antagonistic bacteria have focused on the upper oceanic layer, investigating both biofilm-associated as well as planktonic bacteria. A number of bioactive species have been isolated from surface (Gram et al. 2010) and deep waters (Hohmann et al. 2009), but the

majority originated from biotic surfaces (Penesyan et al. 2010; Gram et al. 2010). Epibiotic communities usually harbour diverse microbiota, and bacterial densities on marine aggregates can be 60-fold higher than in the adjacent water (Schweitzer et al. 2001). High cell densities may select for microorganisms with unique bioactivities (Egan et al. 2008), reflected by a higher percentage of antagonistic strains among surface-attached microbiota compared to their planktonic counterparts (Nair & Simidu 1987; Long & Azam 2001; Gram et al. 2010). Consequently, many bioactive strains have been isolated from eukaryotic surfaces, including zooplankton and macroalgae (Wiese et al. 2009), corals (Rypien et al. 2010), bryozoans (Herndl et al. 2010), and especially sponges (Taylor et al. 2007).

Antibiotic compounds include growth-inhibiting as well as surface-active molecules (biosurfactants) that prevent microbial attachment and colonization in biofilms (Ron & Rosenberg 2001; Das et al. 2008). The latter is especially interesting since biofilms can be a reservoir for pathogenic microorganisms (Hall-Stoodley et al. 2004). Antibiotics are produced by both Gram-negative (*Proteobacteria* and *Bacteroidetes*) and Gram-positive (*Actinobacteria* and *Firmicutes*) marine bacteria. A number of marine-derived antimicrobials have been characterized in greater detail by NMR spectroscopy, including several polyketide or nonribosomal peptide antibiotics. Structurally elucidated molecules include halogenated compounds such as the bromosubstituted pentabromopseudilin (Andersen et al. 1974) or the chlorinated lynamicin (McArthur et al. 2008). Sulphuric antibiotics include tropodithietic acid (Geng et al. 2008) and holomycin (Kenig & Reading 1979; Hou et al. 2008; Wietz et al. 2010b) that each contain two sulfur atoms. Marine bacteria also produce several antimicrobial peptides, including depsi- (Romanenko et al. 2008) and lipopeptides (Das et al. 2008). Also surface-active glycolipids have been reported (Kiran et al. 2010). Among high molecular weight structures, an antibiotic amino acid oxidase (Gomez et al. 2008) and proteins with anticandidal activities (Selvin et al. 2009) have been isolated. An overview about antibiotics from marine bacteria is given in Fig. 13 and several reviews, for instance by Debbab et al. (2010).

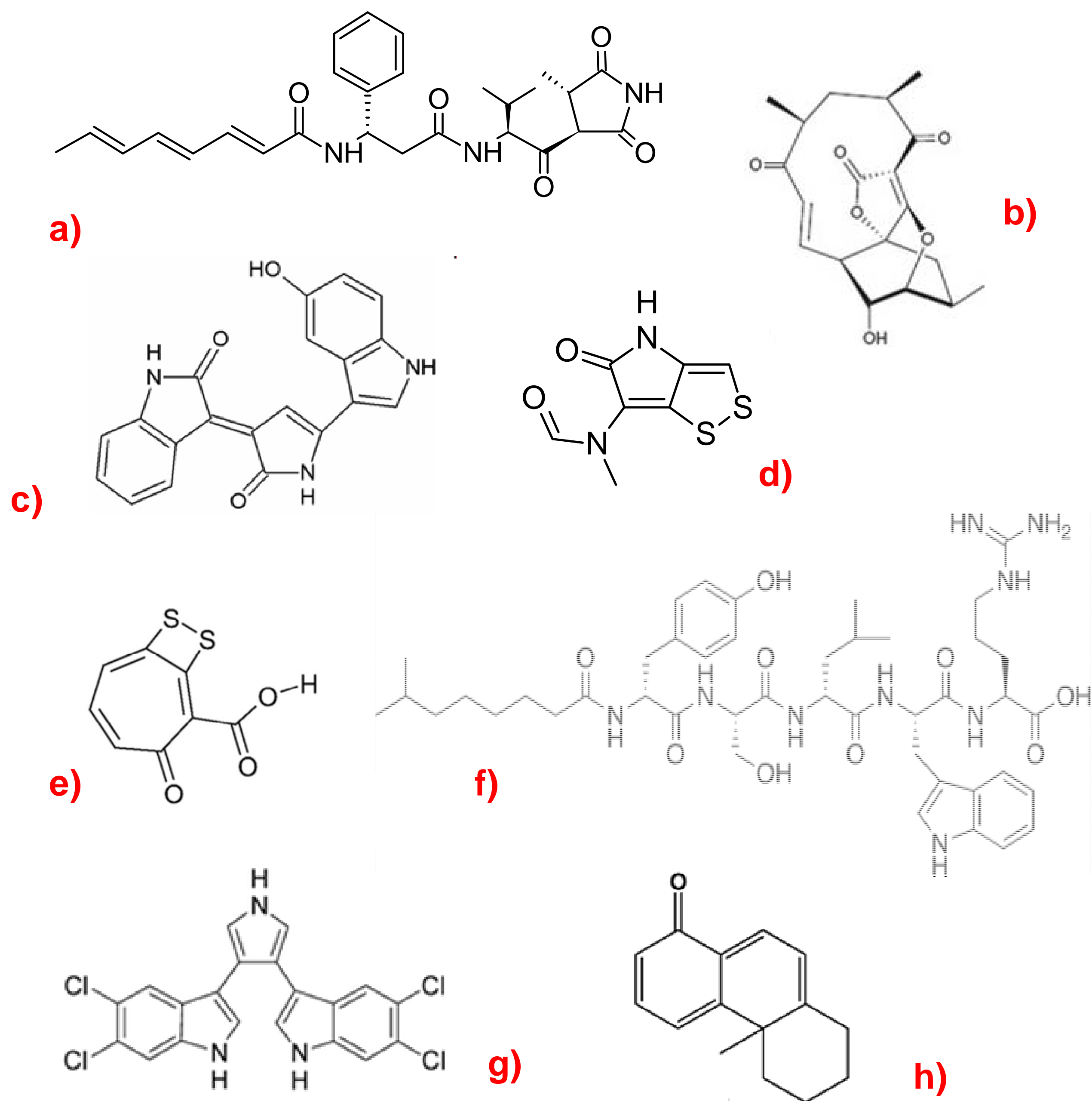


Fig. 13 Antibiotic compounds from marine bacteria. a) Andrimid from *Vibrio* and other *Proteobacteria*; b) abyssomycin from *Verrucosispora* (*Actinobacteria*); c) violacein from *Chromobacterium* (*Betaproteobacteria*) and *Pseudoalteromonas* (*Gammaproteobacteria*); d) holomycin from *Photobacterium* (*Gammaproteobacteria*) and *Streptomyces* (*Actinobacteria*); e) tropodithietic acid from roseobacters (*Alphaproteobacteria*); f) tauramamide from *Brevibacillus* (*Firmicutes*): g) lynamicin from *Marinispora* (*Actinobacteria*); h) zafrin from *Pseudomonas* (*Gammaproteobacteria*).

Marine bacterial groups harbouring antagonistic strains

Some bacterial groups and genera are especially known to harbour antagonistic strains, and three of them – *Actinobacteria*, the *Roseobacter* clade and *Pseudoalteromonas* – are introduced in greater detail. The *Vibrionaceae* family, which represents the focus of the present study, has also been linked with antibacterial activity, while much more is known about their role in human and animal diseases (Thompson et al. 2004). An introduction to the diversity, physiology and ecology of vibrios including antagonistic traits will be given in Chapter 4.

Actinobacteria

Gram-positive *Actinobacteria* are one of the most well known producers of bioactive secondary metabolites. *Actinobacteria* have been mainly investigated in soil ecosystems, where they are often abundant due to their saprophytic lifestyle (Goodfellow & Williams 1983). The genus *Streptomyces* alone accounts for over 70% of all described actinomycetal natural products, possessing an estimated repertoire of approx. 10^5 compounds (Watve et al. 2001).

In aquatic habitats, *Actinobacteria* are common in freshwater ecosystems (Glöckner et al. 2000) but also found in the marine environment. Some lineages are obligate marine, being widespread and persistent in many habitats (Mincer et al. 2002). *Actinobacteria* are a global component of the bacterioplankton (Wietz et al. 2010a), but generally more abundant in sediment as well as on organic surfaces (Bull & Stach 2007). The diverse actinobacterial flora associated with sponges apparently possesses host specificity (Li et al. 2006), indicating the presence of commensal or symbiotic-like relationships with higher eukaryotes.

Although research on marine actinobacterial natural products is still at an early stage compared to what is known about terrestrial relatives, the enormous potential of marine strains as producers of bioactive secondary metabolites has already been proven. The discovery rate among marine strains surpasses that of their terrestrial counterparts (Bull & Stach 2007), likely attributed to the fact that marine assemblages harbour a greater diversity of bioactive representatives (Bredholdt et al. 2007). So far isolated metabolites cover many different compound classes and biological activities, ranging from antibacterial, anticancer, antimalarial, antifungal, to anti-inflammatory (Lam 2006).

Many sponge-associated actinomycetes show antibacterial activity (Li & Liu 2006), and marine *Nocardiopsis* spp. have been widely studied for their production of antibiotic (Schumacher et al. 2001; Engelhardt et al. 2010) and anticancer compounds (Shin et al. 2010). Abyssomicin C (Fig. 13), a polycyclic polyketide antibiotic produced by a marine *Verrucosispora* strain, is a potent inhibitor of para-aminobenzoic acid biosynthesis and possesses potent activity against Gram-positive bacteria, including multiresistant *Staphylococcus aureus* (Bister et al. 2004). During the present study, eight bioactive actinobacterial strains were isolated from high Arctic marine environments (Wietz et al. 2011b). While one strain (tentatively identified as *Brevibacterium* sp.) produces a potentially novel antibiotic compound, activity of seven *Arthrobacter* isolates was likely related to production of the known arthrobacilin antibiotics (see below).

The anticancer agent salinisporamide A is currently in preclinical trials (Fenical et al. 2009) and may become the first commercially available pharmaceutical product from marine actinomycetes. The producer genus, *Salinispora*, has been isolated from sediment at worldwide locations and shows biogeographical variation related to its bioactivity. Although all three known species co-occur at six distant locations (Jensen & Mafnas 2006), the compound was only produced by *S. tropica* from the Caribbean (Jensen et al. 2005). This underlines that biogeographical variation is not necessarily only related to abundance variation between locations, but also to physiological differences between species. Species-specific secondary metabolism provides a new perspective on the ecological and evolutionary significance of antagonistic traits. The occurrence of different natural product signatures between globally distributed populations indicates that bioactive secondary metabolites have ecological function, for example in niche speciation.

Roseobacter clade

Besides playing a role in global biogeochemical cycles, such as aerobic anoxic photosynthesis and turnover of DMSP (Wagner-Döbler & Biebl 2006), several members of the *Roseobacter* clade produce bioactive compounds (Bruhn et al. 2005b; Martens et al. 2007). As mentioned earlier, roseobacters are often associated with phytoplankton blooms. While this prevalence is likely facilitated by their ability to degrade algal-derived compounds (Wagner-Döbler & Biebl 2006) the production of antimicrobials could play a role as well. Approx. 20 strains of *Ruegeria mobilis* collected around the world (Gram et al. 2011) all produced the same antibiotic, tropodithietic acid (Geng et al. 2008). This indicated that the compound may serve an ecological

function, e.g. by assisting surface colonization, antagonizing competing bacteria, or providing a mean of chemical interaction. The potential ecological role of tropodithietic acid (Fig. 13) in natural community dynamics was substantiated by showing inhibition of indigenous bacterial and interestingly also algal isolates by a wadden sea roseobacter (Brinkhoff et al. 2004). A *Phaeobacter* isolate from an aquaculture farm has been characterized in greater detail regarding the physiological background of bioactivity, showing that culture conditions influenced antibiotic production. *Phaeobacter* bioactivity was apparently linked to the formation of biofilms and cell rosettes at the air-liquid interface (Bruhn et al. 2007). Surface-associated growth therefore facilitates antibiotic production in some roseobacters, and can yield significant reduction or complete killing of pathogens in co-culture experiments (D'Alvise et al. 2010). Biofilm-specific production of antimicrobials has also been described for other bacterial genera (Yan et al. 2003). In contrast, marine *Ruegeria* species isolated during Galathea 3 and other closely related roseobacters strains (Porsby et al. 2008) are able to form the compound under both aerated and stagnant culture conditions.

Pseudoalteromonas spp.

Many species within the marine bacterial genus *Pseudoalteromona*s, which are often found in association with eukaryotes (Holmström & Kjelleberg 1999), produce antibacterial compounds (Bowman 2007; Gram et al. 2010). This supports the notion that surface-associated growth can be linked with the production of antimicrobials (Long & Azam 2001; Bruhn et al. 2007). Pigmented pseudoalteromonads produce a variety of bioactive compounds with antimicrobial, antifouling, and algicidal activities, whereas non-pigmented strains often lack these traits (Bowman 2007). This feature has been underlined by a global collection of *Pseudoalteromonas* strains isolated during Galathea 3 (Vynne et al. 2011b). The secondary metabolome of pseudoalteromonads includes polyanionic exopolymers, alkaloids, cyclic peptides, and different brominesubstituted compounds (Bowman 2007). Small bioactive molecules are, for example, violacein and pentabromopseudilin from *P. luteoviolacea* (Andersen et al. 1974). Tambjamine, a yellow compound with antifungal activity, has been isolated from various marine invertebrates but is believed to originate from associated *P. tunicata* (Burke et al. 2007). *P. phenolica* produces a bactericidal compound active against methicilin-resistant *S. aureus* (MRSA). This compound, a brominated phenyldiol, was found in methanol extracts of whole cells and not in the surrounding agar, and was therefore

suggested to be cell membrane-bound. The mechanism of action seems to be based on permeabilization of the target cell membrane (Isnansetyo & Kamei 2003).

Surface-active molecules, such as antifouling agents or antibacterial biosurfactants (Das et al. 2008), can prevent biofilm formation. Antifouling compounds from *P. tunicata* inhibit the settlement of bacteria and higher organisms (Dobretsov et al. 2006). Pharmacological application of such molecules could be used to avert microbial colonization of medical devices, a major cause of serious infection (Hall-Stoodley et al. 2004). Surface-active molecules may also prove useful as disinfectants in food processing plants or as antifouling component in paint (Burgess et al. 2003). The latter may help to prevent macrofouling of immersed surfaces – the colonization of ship hulls and other marine equipment by barnacles and other organisms – which often results in major economic losses. Macrofouling is thought to be a follow-up of initial microbial colonization, and could thus be prevented by antifouling agents.

Bioactive bacteria from polar habitats

Since most studies on antagonistic bacteria have been conducted in warmer oceanic waters, relatively little is known about such traits among polar bacteria. As mentioned above, the polar oceans harbor a microbial flora that is different from tropical and temperate waters, so it is likely that also the diversity of antagonistic strains differs.

Antagonistic traits in polar bacteria have so far been mainly investigated in Antarctic strains, reporting antimicrobial activities with *Actinobacteria* (O'Brien et al. 2004; Lo Giudice et al. 2007), bacilli and enterobacteria (Shekh et al. 2010), *Bacteriodetes* (Al-Zereini et al. 2007), as well as *Gammaproteobacteria* (O'Brien et al. 2004). These originated from a variety of environmental sources, including microbial mats, soils, lake sediments, water, as well as faeces and feathers from penguins. Some of the bacteria displayed antibiotic activity against food-borne pathogens at low temperatures, indicating a potential use of the responsible compound(s) as food preserving agents in cold storage of food (O'Brien et al. 2004). Chemical analyses indicated the presence of novel antimicrobials (Biondi et al. 2008), including alkaloids from *Pseudomonas* (Jayatilake et al. 1996), aromatic nitro compounds from *Salegentibacter* (Al-Zereini et al. 2007), a naphthalene compound from the cyanobacterium *Nostoc* (Asthana et al. 2009), and a novel angucyclinone antibiotic, designated frigocyclinone, from *Streptomyces* (Bruntner et al. 2005).

The present PhD study provides the first description of bioactive strains from high Arctic environments (Wietz et al. 2011b), describing seventeen bioactive strains affiliating with the *Actinobacteria* (8 strains), *Pseudoalteromonas* (4 strains), the *Vibrionaceae* (3 strains), and *Psychrobacter* (2 strains) (Table 2).

Table 2 Antagonistic bacteria from the Arctic isolated during the LOMROG-II expedition.

	Strain	Source	Closest relative (based on 16S rRNA gene sequences)
Actinobacteria	PP12	Sea ice	*Arthrobacter davidanieli*
	SS14	Copepods	
	TT4	Meltwater	
	ZZ3	Sea ice	
	LM7	Surface water	
	WX11	Deep water	
	MB182	Sea ice	
	SS12	Copepods	*Brevibacterium* sp.[#]
Gammaproteobacteria	RR12	Amphipods	*Vibrio* spp.
	EF14	Deep water	
	RS9	Sea ice	
	XX5	Sea ice	*Psychrobacter nivimaris*
	ST4	Sea ice	
	MB33	Copepods	*Pseudoalteromonas* B201
	MB205	Surface water	
	MB220	Sea ice	
	MB240	Sea ice	

[#] Based on short (400 bp) 16S rRNA sequence – resequencing necessary for verification

Arthrobacter davidanieli, being isolated from different sources throughout the Arctic Ocean, inhibited both Gram-positive and –negative pathogens, while the eighth isolated actinomycete (tentatively identified as *Brevibacterium* sp.) only inhibited Gram-negative strains. *Arthrobacter* spp. are known antibiotic producers both in terrestrial (Carnio et al. 1999) and aquatic habitats, including polar environments (Lo Giudice et al. 2007; Rojas et al. 2009). Vaccination with live *A. davidanieli* cells was effective against bacterial disease in Atlantic as well as coho salmon, with above 80% survival in experimental challenge trials. The *Arthrobacter* strain is the first live

organism to be licensed as a vaccine for use in aquaculture (Salonius et al. 2005). The mechanism of action, however, has not been described, but could rely on bioactive compounds that are similar to the metabolite(s) responsible for antibacterial activity in the Arctic *A. davidanieli* isolates. Extracts from one representative strain, WX11, inhibited a broad range of both Gram-positive and Gram-negative bacterial pathogens, including *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus* (Gram-positives), *Salmonella* Enteritidis, *Aeromonas salmonicida*, *Vibrio vulnificus*, *V. parahaemolyticus*, *V. harveyi*, *Yersinia enterolitica*, and *Y. ruckeri* (Gram-negatives). The broad antibiotic spectrum against clinical, food-borne and aquaculture-related pathogens indicated production of a potent antibiotic compound. Initial chemical analyses by LC-UV/MS indicated that it is identical to or a structural analogue of the arthrobacilins A-C (Ohtsuka et al. 1992), but careful dereplication and compound purification need to be done for final evaluation.

Also *Brevibacterium* spp. is a known antibiotic producer, but this trait has to date only been described in terrestrial representatives (Kato et al. 1991; Valdes-Stauber & Scherer 1994; Maisnier-Patin & Richard 1995). *Brevibacterium* has been found in Arctic ecosystems, but the present study is the first showing antagonistic traits in a polar strain. *Brevibacterium* sp. SS12 produces several metabolites that do not match any previously reported compounds (Laatsch 2010). It is therefore possible that a novel antimicrobial can be isolated from this strain. Final compound purification and structural elucidation by NMR spectroscopy is underway to address this question.

3.4 Conclusions from chapter 3

Bacterial secondary metabolites are believed to represent an important tool in chemical interaction and communication. Many bioactive secondary metabolites also have biotechnological potential, and bacteria from the marine environment represent a promising source of so-far unknown chemical diversity. Metabolite profiling will likely yield the isolation of novel antimicrobials from the sea, potentially opening new avenues in microbial control and antibiotic therapy. The chance of obtaining novel compounds can potentially be increased by investigated less explored habitats, such as polar environments. Also the investigation of less studied bacterial groups represents a promising approach. As described in the next chapter, marine *Vibrionaceae* – to date mainly researched regarding their pathogenic potential – may represent a bacterial group with a high potential of natural product discovery.

4. The *Vibrionaceae*

During the global Galathea 3 expedition, approx. 300 strains from the bacterial *Vibrionaceae* family were isolated from the marine environment due to their ability to inhibit pathogenic bacteria (Gram et al. 2010). A part of this thesis was devoted to the identification and characterization of the strains with strongest antagonistic activity. This included phylogenetic studies based on housekeeping gene sequences, activity testing in relation to culture conditions, and the identification of antibiotic compounds. The work was done in close collaboration with the Centre for Microbial Biotechnology (DTU Systems Biology).

The *Vibrionaceae* family, commonly abbreviated vibrios, belongs to the Gamma subclass of *Proteobacteria*. Vibrios are Gram-negative, mostly oxidase positive, chemoorganotrophic, and have a facultative fermentative metabolism. They are usually motile rods by means of one or several polar flagella, and often have curved cell morphology (Fig. 14a). Vibrios are readily cultivated on Marine Agar or the selective medium thiosulfate-citrate-bile salt-sucrose agar (TCBS), where they form green or yellow colonies (Fig. 14b). The *Vibrionaceae* are a diverse group of heterotrophic bacteria able to utilize a wide range of carbon and nitrogen sources (Farmer & Hickman-Brenner 2006), and include many facultative symbiotic and pathogenic strains. Most vibrios contain two chromosomes (Okada et al. 2005), which has been suggested as important for rapid DNA replication and fast doubling times (Yamaichi et al. 1999). Quorum sensing, a density-dependent modulation of gene expression relying on the production of signalling molecules, is present in many species (Thompson et al. 2004).

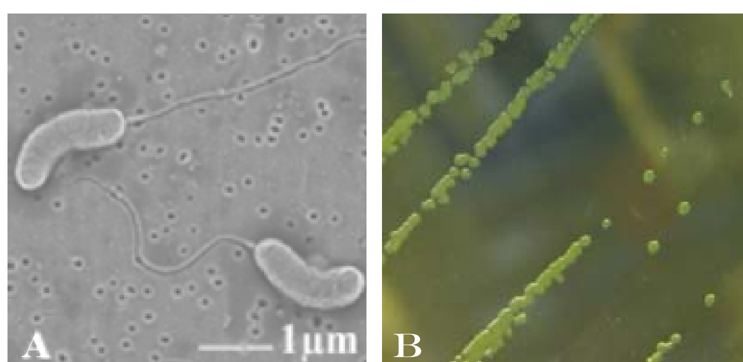


Fig. 14 (a) SEM micrograph of *V. cholerae*, illustrating the typical curved cell morphology with a polar flagellum (Chaiyanan et al. 2007). (b) Colonies of *V. coralliilyticus* S2052 on TCBS agar.

As per January 2011, nineteen genomes of *Vibrionaceae* strains have been fully sequenced and annotated, with about half from representatives of the human pathogen *V. cholerae*. The sequenced strains provided novel insights into the architecture of the two chromosomes, showing that the larger chromosome 1 typically contains housekeeping genes that encode proteins for translation, replication, and motility. Most likely, it is therefore replicated independently (Dryselius et al. 2008) from the smaller chromosome 2 which only contains accessory genes involved in pathogenicity, antimicrobial resistance, and host avoidance mechanisms (Makino et al. 2003). *V. parahaemolyticus* is an atypical example, with several vital genes being located on chromosome 2 (Makino et al. 2003). The high genomic diversity among vibrios is indicative of their adaptive ability (Chen et al. 2003) and may also play a role in niche speciation and biogeographic separation (Pollock et al. 2010).

4.1 Diversity and phylogeny

Every year new *Vibrionaceae* species are being isolated from the marine environment, more than doubling the number of isolates between 1981 and 2002 (Thompson et al. 2004). At present, 119 species are recognized within the family (Fig. 15). However, species identification and determination of phylogenetic relationships are often difficult. While the *Vibrionaceae* are currently perceived as a single family containing eight different genera (*Vibrio*, *Aliivibrio*, *Enterovibrio, Salinivibrio*, *Listonella, Photobacterium*, *Catenococcus*, and *Grimontia*) other phylogenetic studies indicated that the taxonomy is more diversified and vibrios are organized in four different families (*Vibrionaceae, Photobacteriaceae*, *Salinivibrionaceae*, and *Enterovibrionaceae*) (Thompson et al. 2004). One major obstacle is the genetic homogeneity among vibrios, especially among the 16S rRNA gene which has become the standard for bacterial taxonomy. While the threshold for species distinction is 97% 16S rRNA gene similarity, the interspecies relatedness among vibrios can be as high as 99%. Phylogeny and taxonomy is further complicated by the fact that some species can contain multiple copies of the 16S rRNA gene that differ in their respective sequence (Jensen et al. 2009). Consequently, *Vibrio* phylogeny is increasingly based on analyses of housekeeping genes that encode constitutively expressed proteins and possess greater sequence variation. Genes suitable for species discrimination include *recA*, *rpoA* (Thompson et al. 2005a), *atpA* (Thompson et al. 2007), *dnaJ* (Nhung et al. 2007), *pyrH*, and *toxR* (Pascual et al. 2009). Sequence variation,

exemplified with the *rpoA* and *recA* genes, can be at least 84 and 73%, respectively. Species identification can also be done by DNA-DNA hybridization, amplified fragment length polymorphism (Vos et al. 1995), or repetitive extragenic palindromic PCR (Versalovic et al. 1991). An alternative approach to gene-based phylogeny is chemical metabolite profiling of strains (also termed chemotyping), which is able to identify vibrios (Dieckmann et al. 2010) and complement gene-based phylogenies (Wietz et al. 2010b).

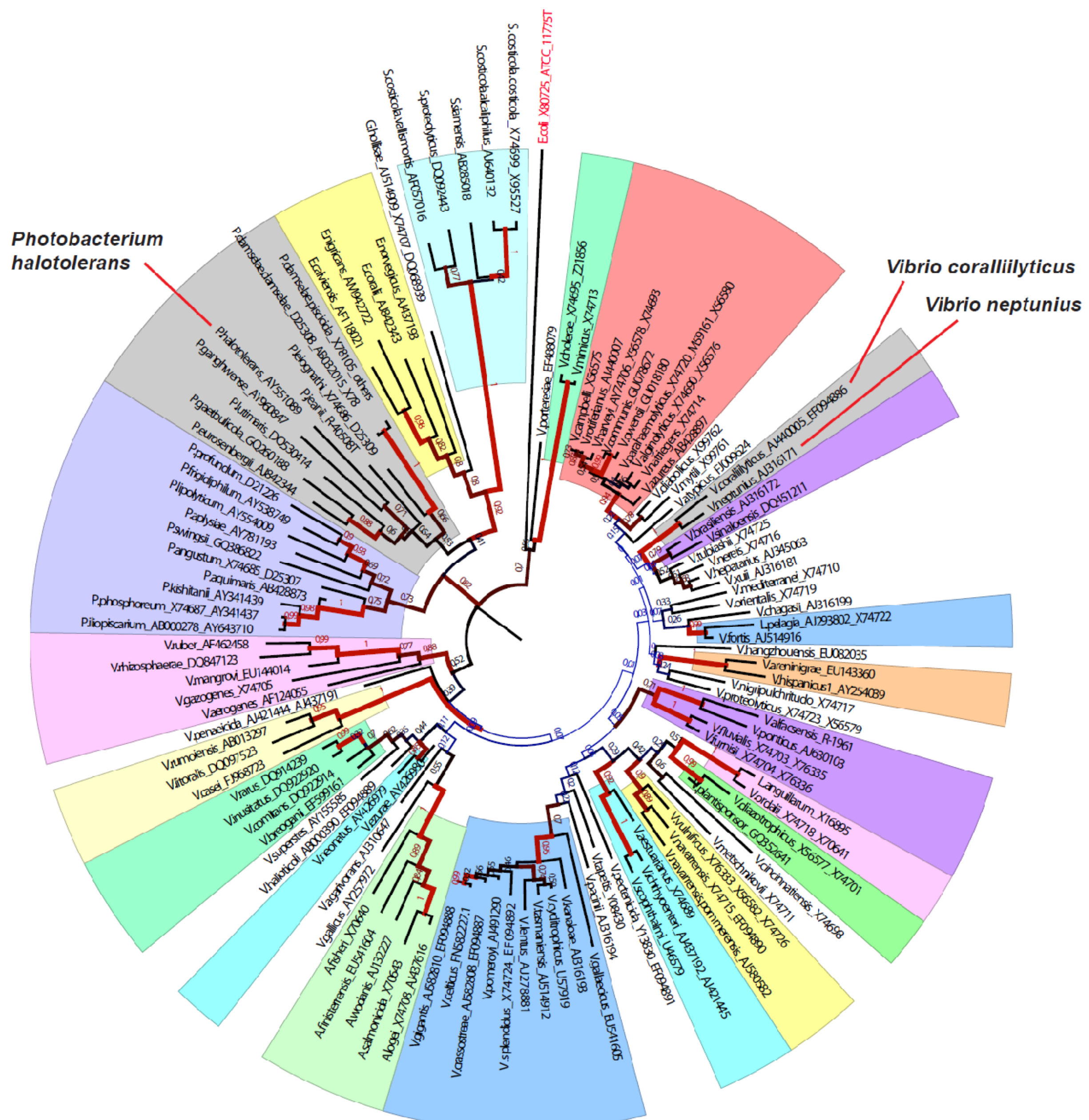


Fig. 15 Phylogenetic tree of the *Vibrionaceae* family, illustrating the taxonomic positioning of the strains investigated here (modified from http://www2.ioc.fiocruz.br/vibrio/AVib/Dendro.html)

The majority of species within the *Vibrionaceae* belong to the genera *Vibrio* (81) and *Photobacterium* (20 species). Also the present study focused on the investigation of strains from these two genera, addressing five strains from the genus *Vibrio* and one from the genus *Photobacterium*. Only one strain, *V. nigripulchritudo* S2604, could be assigned to the species level by 16S rRNA gene similarity and phenotypic characteristics. For the other 5 strains, only the sequencing of *recA* and *rpoA* genes was able to provide this information (Wietz et al. 2010b). This showed that each two vibrios were affiliated with *V. coralliilyticus*, and another two with *V. neptunius*. These two form a species cluster and are highly similar on the genetic level (Fig. 15), which was underlined by highly similar secondary metabolite profiles (Wietz et al. 2010b). The *Photobacterium* isolate was closely related to *P. halotolerans* (Fig. 15), and its evolutionary distance was reflected in a unique metabolite profile (Wietz et al. 2010b).

4.2 Distribution and abundance

Vibrionaceae are found in almost all marine and brackish environments worldwide, including estuaries, coastal and pelagic waters, sediments, and aquaculture systems (Thompson et al. 2004). While vibrios tend to be more abundant in subtropical and tropical regions, the present study underlined that they also occur in polar and extreme environments including sea ice and the deep sea (Wietz et al. 2011b). The family shows considerable seasonal variation in population diversity and density (Heidelberg et al. 2002b; Turner et al. 2009), as well as variability in both the minimal sodium concentrations and temperature required for optimal growth (Thompson et al. 2006). *Vibrio* growth and persistence is linked with several environmental parameters, including the availability of dissolved organic matter (Mourino-Perez et al. 2003), water temperature and salinity (Kaspar & Tamplin 1993), as well as physical associations with higher organisms or solid substrates (Huq et al. 2005). Also viral infection by bacteriophages (Fuhrman & Schwalbach 2003) and predation through protozoan grazing (Langenheder & Jürgens 2001) impact the structure and diversity of environmental *Vibrionaceae* assemblages.

In pelagic bacterioplankton where oligotrophic conditions prevail, the abundance of vibrios rarely exceeds 1% of all bacterial cells. While their biodiversity and ecological role in pelagic ecosystems is still poorly understood (Thompson et al. 2004), the present study provided additional data on the *in situ* abundance and distribution of vibrios on a global scale (Wietz et al. 2010a).

While this confirmed that vibrios are overall only a minor component of marine microbiota (global average abundance of 1.5%), some warmer oceanic regions, for instance equatorial African waters, were shown to harbour abundances of up to 3% (Table 3). The present study also revealed that the abundance of vibrios showed a latitudinal pattern, with relative abundances in lower (0-35°; Tropical and Subtropical climate zones) being significantly higher than in higher latitudes (>35°; temperate and Polar climate zones). In contrast, absolute abundances did not differ between warmer and colder regions (Table 3) (Wietz et al. 2010a).

Table 3 Relative (% of bacteria) and absolute (cells mL^{-1}) abundances of *Vibrionaceae* at 24 stations around the world (Wietz et al. 2010a).

	Location	**% of bacteria**	**Cells mL^{-1}**
Higher latitudes (>35°)	North Atlantic	1.0	1.6×10^{4}
	Greenland (2 stations)	0.7	1.7×10^{4}
	Azores	0.3	7.9×10^{2}
	Indian Ocean	2.0	4.9×10^{4}
	South Australia	0.8	1.1×10^{4}
	New Zealand	1.6	1.2×10^{4}
	Southern Ocean (3 stations)	1.3	3.6×10^{3}
	Humboldt upwelling	1.2	1.9×10^{4}
	Mean	**1.1**	$\mathbf{1.3\times10^{4}}$
Lower latitudes (0-35°)	West Atlantic	1.3	6.1×10^{3}
	Equatorial Africa	3.2	4.5×10^{3}
	Benguela upwelling	0.6	8.2×10^{3}
	NW Australia (2 stations)	2.1	2×10^{4}
	South Australia	1.1	6.2×10^{3}
	Solomon Islands	2.4	1.9×10^{4}
	Humboldt upwelling	2.7	5.3×10^{4}
	Pacific/Costa Rica	1.9	1.4×10^{4}
	Sargasso Sea (3 stations)	1.9	5.2×10^{3}
	Mean	**1.8**	$\mathbf{1.3\times10^{4}}$
	Global mean	**1.5**	$\mathbf{1.3\times10^{4}}$

In contrast to the overall low planktonic abundance of vibrios, several cultivation-dependent and -independent studies have shown a frequent occurrence of *Vibrio* spp. on immersed surfaces. Biofilm-mediated attachment may be essential for the survival of vibrios in the natural habitat, and their planktonic existence only be mean of dispersal (Stoodley et al. 2002). Growth in biofilms provides multiple ecological advantages, including increased resistance against acid stress (Zhu & Mekalanos 2003) and protozoan grazing (Matz et al. 2002). Investigating biofilm formation under laboratory conditions has shown that both *V. parahaemolyticus* and *V. vulnificus* have greater rates of attachment in minimal medium compared to LB broth (Wong et al. 2002), and that addition of glucose and other carbohydrates inhibited attachment (McDougald & Kjelleberg 2006). In contrast, the influence of temperature and salinity on biofilm formation remains unclear (McDougald & Kjelleberg 2006).

Vibrios are found on the exterior surfaces of many marine eukaryotes, in particular zooplankton (Heidelberg et al. 2002a). The high nutrient concentrations in organic-rich microenvironments selectively enrich vibrios and other heterotrophic bacteria (Thompson et al. 2004). *Vibrionaceae* are also commonly found on the exterior of corals (Ben-Haim et al. 2003a), fish (Grisez et al. 1997), eel (Marco-Noales et al. 2001), molluscs (Sawabe et al. 2003), shrimp (Gomez-Gil et al. 1998), squid (Visick & Ruby 2008), phytoplankton (Kumazawa et al. 1991), and macroalgae (Wiese et al. 2009). Vibrios also constitute a major part of the intestinal flora of many marine fish species, including cod (Fjellheim et al. 2007), salmon (Hovda et al. 2007), and flounder (Eddy & Jones 2002). Surface attachment can be accompanied by production of EPS or capsules that exist in either smooth/rugose (EPS) and translucent/opaque (capsule) forms, respectively. Some strains can switch between the two different types, potentially related to niche speciation in the natural habitat. For instance, translucent strains of *V. vulnificus* seem better adapted to attach to algal surfaces, while opaque strains preferably attach to oysters (Joseph & Wright 2004). Many *Vibrio* associations with higher organisms, however, coincide with disease conditions and opportunistic infections by pathogenic strains. Thus, as described below, vibrios are widely researched regarding their pathogenic potential towards aquatic organisms or man (Thompson et al. 2004). Biofilms can thereby constitute a reservoir of pathogenic strains, representing a significant contributor to pathological conditions.

The understanding of *Vibrio* ecology and activity in the natural habitat is still hampered by missing knowledge of what genes are functional *in situ*. While many studies addressed laboratory phenotypes of vibrios, still little is known about their ecology in major natural habitats (Grimes 1991). This understanding substantially improved over the past decades through the increased availability of genomic-based information, including microarrays and proteomics (Sharkey et al. 2004). Metatranscriptomics, the analysis of gene expression patterns of whole environmental populations (Frias-Lopez et al. 2008), unveiled immediate responses to external change and expanded the understanding of *Vibrio in situ* ecology (Smith & Oliver 2006; Jones et al. 2008).

4.3 Pathogenicity

Vibrios are widely researched due to their pathogenic potential towards man or aquatic animals (Thompson et al. 2004). However, most infections by *Vibrionaceae* (often combined under the term vibriosis) are probably opportunistic (Saulnier et al. 2000) according to the 'damage-response' framework (Casadevall & Pirofski 2003) that microbial pathogenesis and pathological manifestation are attributable to neither the microorganism nor the host alone. Infections therefore often rely on and are acerbated by an immunocompromised health status and/or external stress of the host. *Vibrio* spp. with pathogenic potential can be found on both healthy and diseased populations of fish (Thune et al. 1993), prawns (Karunasagar et al. 1994), and corals (Bourne & Munn 2005), emphasizing that vibrios are normally part of the commensal flora and only cause disease when the host is compromised. Also, not all strains within a potentially pathogenic species are indeed causing disease. For instance, only two of over 200 serotypes of *V. cholerae* are capable of generating cholera epidemics, while others with only partial combinations of virulence genes only cause attenuated disease symptoms (Singh et al. 2001).

In general, the mode of infection consists of three basic steps. Chemotactic motility of the bacterium enables the initial penetration of the host tissue. Once inside the host, the bacterium deploys iron-sequestering systems, typically siderophores, to obtain iron from the host's metabolism. Eventually, the secretion of virulence factors, including enterotoxins, cytotoxins, haemolysins, proteases, lipases, phospholipases, and haemagglutinins, leads to tissue damage (Thompson et al. 2006). Quorum sensing (see below) is regularly employed to regulate the expression of virulence factors (McDougald & Kjelleberg 2006).

Human pathogens

V. cholerae is the most serious human pathogen within the *Vibrionaceae*, with serotypes O1 and O139 being able to generate cholera epidemics. Serotype O1 is responsible for the majority of outbreaks, and pathogenic strains often show regional specificity. Serotype O139 is confined to South-East Asia, but recently, new variants potentially able to cause more severe cases with higher fatality rates have been detected in Asia and Africa (WHO 2010).

Transmission of *V. cholerae* primarily occurs through contaminated drinking water; in addition, colonized plankton can act as a vehicle (Huq et al. 1983). Interestingly, removal of larger zooplankton by filtration through local Sari cloth is an effective prevention in rural tropical areas (Huq et al. 1996). Also lethal wound infections have been linked to *V. cholerae* (Lukinmaa et al. 2006). Upon infection, the bacterium proliferates in the gastrointestinal tract and adheres to the epithelium. Production of the cholera enterotoxin causes severe diarrhoea, vomiting, electrolyte imbalance and rapid dehydration that need to be treated by oral or intravenous rehydration. Interestingly, cholera toxin appears to have no function in the bacterium's natural habitat (Reidl & Klose 2002). Other major human pathogens are *V. vulnificus* and *V. parahaemolyticus* that are involved in fatal seafood poisoning (Lipp & Rose 1997). However, also vibrios that are mainly pathogenic towards aquatic life can cause disease in humans, mainly if these are immunocompromised. Water- and seafood-related gastrointestinal infections have been implicated in *V. mimicus* (Campos et al. 1996), *V. alginolyticus*, and *V. hollisae* (Oliver & Kaper 1997). Also *V. fluvialis*, *V. furnisii*, and *V. harveyi* (Thompson et al. 2004) were sporadically reported as pathogenic towards humans. Since vibrios tend to be more abundant in warmer waters, the pathogenic potential and risk of infection in humans (Sechi et al. 2000) and marine animals (Rosenberg & Ben-Haim 2002) may be coupled with global warming and the subsequent rise of seawater temperatures.

Animal pathogens and role in aquaculture

Vibrios are capable of causing disease in many aquatic animals. The infection route in marine eukaryotes has been investigated with *V. proteolyticus*, revealing that *Vibrio* cells that had been seeded into the rearing water penetrated the gut epithelium of *Artemia* nauplii after one day,

subsequently causing extensive tissue damage spreading toward the host's body cavity (Verschuere et al. 2000).

Pathogenicity of *Vibrio* species is often linked to a certain eukaryotic host. For instance, *V. harveyi* is a predominant pathogen of shrimps, while *V. splendidus* is often associated with diseased molluscs. Several vibrios possess notable pathogenic potential towards corals, and the already severely affected status of coral reefs worldwide is often amplified by opportunistic *Vibrio* infections (Thompson et al. 2004). *V. shilonii* is responsible for bleaching of the Mediterranean coral *Oculina patagonica*, reaching up to 10^9 CFU cm^{-3} coral tissue within 5 days of infection. The life cycle of *V. shilonii* encompasses overwintering in an invertebrate host, the fireworm *Hermodice carunculata*, which then serves as a vector of infection during summer (Sussman et al. 2003). Another important coral pathogen is *V. coralliilyticus*, which will be introduced further below since strains of this species harbour antagonistic traits (Gram et al. 2010). Common to microbial disease of corals is a generally increasing virulence of coral pathogens at higher temperatures. Since the continuous warming of tropical waters is predicted to further increase, *Vibrio*-based bleaching events will occur even more frequently (Hoegh-Guldberg 1999). Temperature-dependent pathogenicity of vibrios is however not restricted to corals, but also affects benthic invertebrates (Vezzulli et al. 2010).

Besides their role as opportunistic pathogens in the wild, vibrios play a fundamental role in marine aquaculture. While vibrios can be abundant with healthy aquacultured organisms and even contribute to their well-being (Sawabe et al. 2003), their proliferation in aquaculture systems is often linked with disease conditions. It is believed that infections normally relate to sudden proliferation of a single hypervirulent strain upon favourable external conditions, commonly during early larval stages when the immune system is not yet fully operational. In cultures of adult animals, high stocking densities and artificial culture conditions in combination with high inputs of antibiotics and feed further support the proliferation of vibrios. The resulting stress among culture animals even multiplies the risk of opportunistic infections (Thompson et al. 2004).

V. anguillarum, *V. salmonicida*, and *V. vulnificus* are major bacterial pathogens of aquacultured fish (Austin & Austin 2007). Shrimp pathogens include *V. harveyi*, *V. penaeicida* and *V. nigripulchritudo* (Karunasagar et al. 1994; Goarant et al. 2006). *V. harveyi* is also the causative agent of disease in adult and larval lobsters (Webster et al. 2006; Abraham et al. 2009). Traditionally, vibriosis in aquaculture settings has been treated with antibiotics, subsequently leading to resistances among aquaculture-inhabiting strains. About 70% of *Vibrio* spp. from

aquaculture systems can be multidrug-resistant (Roque et al. 2001). The potential spread of resistant strains to natural environments is a major concern, since aquaculture systems could serve as reservoirs for pathogenic vibrios that could be transmitted to adjacent coastal waters where contacts with wild animals or humans can occur (Ben-Haim et al. 2003a). This advises the need for alternative antimicrobial treatments, such as vaccination with killed cells (Buchmann et al. 2001) or the administration of live probionts (FAO/WHO 2001). Regarding the latter, the presence of certain vibrios in shrimp rearing systems seemed to enhance the survival and growth of culture animals (Moss et al. 2000). Furthermore, a probiotic strain of *V. alginolyticus* reduced mortalities within cultures of the Atlantic salmon by inhibiting the growth of several fish pathogens (Austin et al. 1995).

4.4 Physiological traits

Vibrios possess a typical 'starvation-survival' response, a transient growth state characterized by decreased cell size and low, but constant metabolic rate (Kjelleberg et al. 1987). Given the nutrient-deprived conditions throughout most oceanic regions, most marine bacterial cells including vibrios will be present in such a dormant state. Upon favourable conditions, vibrios and other heterotrophs can exploit suddenly increasing nutrient availability and proliferate rapidly, which is however counterbalanced by a higher grazing mortality (Beardsley et al. 2003). The rapid response of vibrios to changing environmental conditions is attributed to their opportunistic growth characteristics reflecting a “feast-and-famine” life strategy (Flardh et al. 1992), which also explains their selective enrichment from marine samples (Eilers et al. 2000). In contrast, many other microbes cannot tolerate such sudden changes in environmental conditions. This is reflected in the low culturability of most marine bacteria when transferred from the oligotrophic natural habitat to rich isolation agar, being summarized in the theory of ‘viable but nonculturable cells’ (Oliver 2005).

Due to their metabolic versatility (Farmer & Hickman-Brenner 2006) vibrios probably play a role in the cycling of nutrients and organic matter (Thompson et al. 2006), thereby transferring dissolved organic carbon to higher trophic levels of the marine food web (Grossart et al. 2005). Some strains are able to fix atmospheric nitrogen, which contributes to the nitrogen inventory by regenerating losses through degradative processes such as anammox. Diazotrophic strains are found

among *V. parahaemolyticus* (Criminger et al. 2007), *V. diazotrophicus*, *V. natriegens*, and the opportunistic human pathogen *V. cincinnatiensis* (Urdaci et al. 1988).

The frequent occurrence of vibrios in association with eukaryotic organisms is probably in part related to their ability to utilize host-derived substances. One of the most prominent physiological traits is the utilization of chitin $(C_8H_{13}O_5N)_n$, a long-chain polymer of the glucose derivative *N*-acetylglucosamine, as carbon and nitrogen source. Being the oceanic counterpart to cellulose, chitin is the most abundant biopolymer in the marine environment, particularly found in the exoskeletons of crustaceans and the cell walls of fungi (Gooday 1990). The global yearly production and steady-state amount of chitin in the oceans has been estimated to 10^{10}-10^{11} tons (Gooday 1990). Chitinolytic activity through production of chitinases is a core function of the *Vibrionaceae* (Hunt et al. 2008), and chitinolytism a potential explanation of their ubiquitous occurrence (Riemann & Azam 2002) and frequent association with zooplankton (Thompson et al. 2004). The interaction of *V. cholerae* and chitin is one of the most widely studied examples, being linked at multiple hierarchical levels including chemotaxis, biofilm formation, natural competence, nutrient cycling, pathogenicity, as well as commensal and symbiotic relationships with higher organisms (Pruzzo et al. 2008). All *Vibrionaceae* investigated in the present study were able to utilize chitin (Fig. 16).

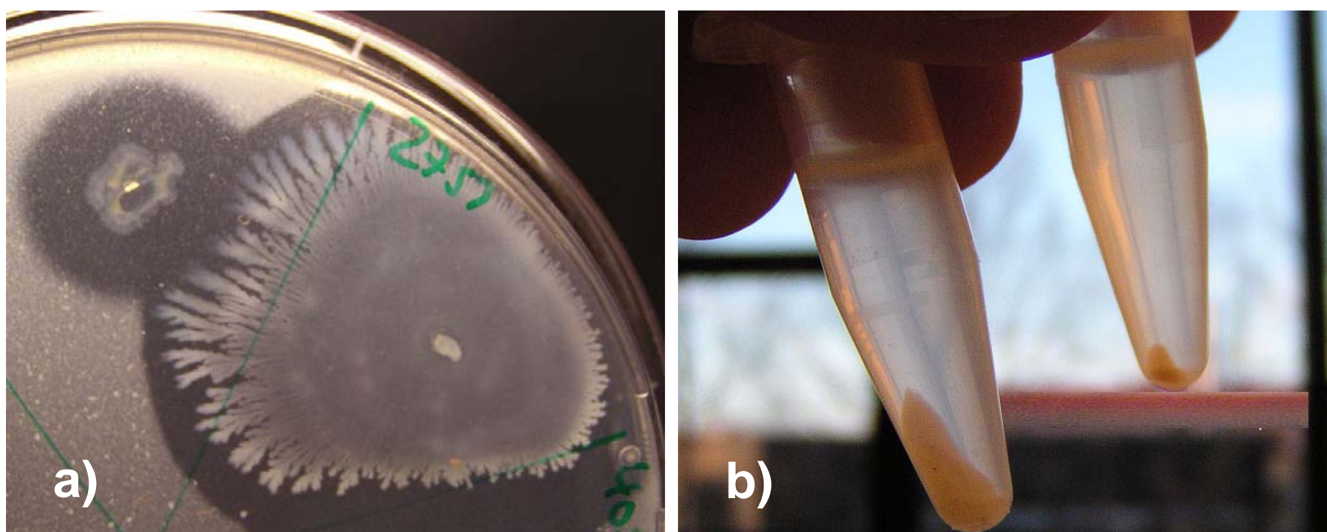


Fig. 16 Chitinolytic activity of *Vibrionaceae*, exemplified with *P. halotolerans* S2753. a) Clearing of the chitin precipitate and swarming on chitin agar; b) degradation of chitin in broth, illustrated by comparing pelleted chitin from sterile chitin broth (left tube) to pelleted cells and remaining chitin after 3 days of incubation (right tube).

The degradative abilities of vibrios also include the utilization of algal substrates, e.g. cyanobacterial-derived organic matter (Eiler et al. 2007) or structural polysaccharides that are contained in the cell walls of macroalgae. The occurrence of vibrios in epiphytic communities of, for instance, the brown seaweed *Laminaria* (Laycock 1974) is therefore potentially linked to the degradation of algal-derived poly- and monosaccharides. Vibrios produce agarase, alginase, mannanase, cellulase, and pectinase enzymes (Goecke et al. 2010). Also laminarin and fucoidan, the major polymeric components of the brown macroalgae *Laminaria* and *Fucus* that are widespread throughout temperate oceans, can be utilized (Furukawa et al. 1992; Alderkamp et al. 2007). Laminarase and alginase activities in *Vibrio* spp. can be induced even after nutrient starvation of up to 8 months, and starved cells previously grown in the presence of inducer substrates maintained measurable degradation for several weeks (Davis 1992). Degradation of algal substrates was also observed for vibrios collected during Galathea 3, but distinct growth was mainly restricted to cultures containing crude algal extracts as nutrient source. With pure algal carbohydrates as sole nutrient source, only *V. coralliilyticus* (with laminarin), *V. neptunius* S2394 (with alginate), and *P. halotolerans* S2753 (with laminarin) showed considerable growth (Wietz et al., unpublished data).

The physiology and growth kinetics of vibrios can be influenced by the nutrient source. In this context, it is important to remember that most studies of bacterial physiology are conducted using laboratory substrates that do not reflect conditions in the natural habitat. However, laboratory growth conditions can shift the phenotype towards a metabolic state that is potentially unlike the environmental ecology of a microorganism (Palkova, 2004). The source of carbon (Sanchez et al., 2010) and nutrient availability (Demain et al., 1983) can have a substantial influence on bacterial metabolism. An excess of nutrients – typically not encountered in the oligotrophic marine environment – can increase the synthesis of storage compounds (Chien et al., 2007) but suppress production of antibiotic compounds (Doull and Vining, 1990).

The present study showed that growth conditions mimicking natural habitats of vibrios can significantly change the phenotype, demonstrating that *V. coralliilyticus* S2052 produced a variety of compounds in rich medium whereas metabolite synthesis was focused on a specific antibacterial compound when grown with chitin (Fig. 17) (Wietz et al. 2011a). The use of culture conditions more closely resembling those encountered in the natural habitat may give a more realistic view about phenotypic traits that are expressed in the environment. While such knowledge is interesting from an ecological perspective, the finding of culture conditions that stimulate the production of a desired compound may also be valuable for potential large-scale applications in biotechnology.

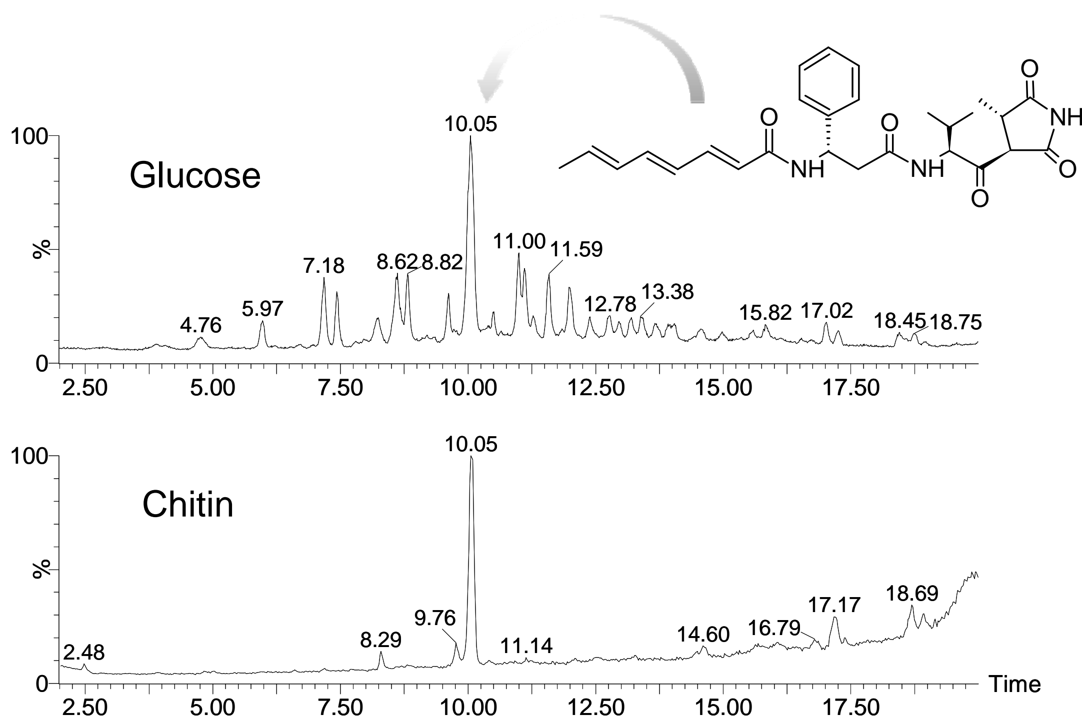


Fig. 17 Growth with chitin results in increased production of antibiotic (exemplified with andrimid from *V. coralliilyticus* S2052) while the biosynthesis of other metabolites that are produced in glucose medium is largely abolished.

4.5 Biosynthetic capacities

The biosynthetic capacities of vibrios have been mainly researched regarding the production of virulence factors, typically large proteinaceous toxins or extracellular enzymes (Thompson et al. 2004). In contrast, less is known about their potential to produce small organic molecules with biological functions. Being Gram-negative bacteria, vibrios were suggested to be less prolific in their production of secondary metabolites than, for instance, actinomycetes (Fenical 1993). While their true chemical diversity is poorly understood, several studies have however indicated that vibrios may possess greater biosynthetic potential than previously thought. Several vibrios associated with estuarine grasses produce the phytohormone indole-3-acetate, suggesting that those strains play a role in shaping estuarine landscapes and the cycling of carbon (Gutierrez et al. 2009). Vibrios dominate the intestinal microbiota of pufferfish and produce the "pufferfish poison" tetrodotoxin (Noguchi et al. 1987), therefore being potentially responsible for toxification after

ingestion of pufferfish (Lee et al. 2000). Another interesting metabolite from *Vibrionaceae* is kahalalide F (Hill et al. 2004) that is cytotoxic towards various cancer cell lines and currently undergoing Phase II cliical trials.. Some vibrios are also able to produce antibiotics, which will be introduced further below. Throroughly studied small molecules from vibrios are quorum sensing signalling molecules and iron-chelating siderophores.

Quorum sensing autoinducers

As mentioned in chapter 3.1, bacterial communication in part relies on quorum sensing (QS), the secretion and uptake of small signalling molecules (autoinducers) that influence gene expression and phenotypic characteristics in a density-dependent manner.

The classical example of QS in vibrios is the production of bioluminescence. At high cell densities, symbiotic *Vibrio fischeri* within the light organ of the Hawaiian bobtail squid (Visick & Ruby 2008) emit visible light. This process bases on the positive influence of *N*-acyl homoserine lactones on the *lux* operon (Devine et al. 1989), which triggers a signalling cascade and biosynthesis of luciferase, resulting in the production of blue/green light. Interestingly, *V. fischeri* employs a second QS system which negatively modulates *lux* expression and therefore diminishes luminescence (McDougald & Kjelleberg 2006). Bioluminescence also occurs in *V. harveyi*, with similarities to QS signalling pathways in *V. cholerae* (Higgins et al. 2007). Besides controlling bioluminescence QS is involved in the regulation of virulence, biofilm formation (McDougald & Kjelleberg 2006), pigment production (Croxatto et al. 2002), resistance to oxidative stress (McDougald et al. 2002), starvation adaptation (McDougald et al. 2001), and colony opacity (McCarter 1998). Many coral-associated vibrios produce QS molecules, which could play a role in surface colonization but potentially also in pathogenicity towards the coral host. The same study also showed a distinct influence of temperature on autoinducer production (Tait et al. 2010).

In many bacterial species, the production of antibiotics is quorum-regulated (Eberl 1999). Therefore, we investigated production of *N*-acyl homoserine lactones during antibiotic production in the antagonistic strain *V. coralliilyticus* S2052. Despite production of the antibiotic andrimid was detected from a certain cell density only, no QS molecules could be detected using two microbiological monitors (Wietz et al. 2011a). Given the existence of multiple QS systems in vibrios and a variety of signalling molecules from *V. coralliilyticus* (Tait et al. 2010) potentially

untraceable in our approach, it remains possible that andrimid synthesis is density-regulated but relies on unknown QS autoinducers.

Siderophores

Iron-sequestering siderophores are essential to scavenge free iron from the aqueous surrounding, which is important in the typically iron-limited marine waters. Despite possessing a large structural diversity, the majority of siderophores are synthesized by enzymes of the NRPS family (Crosa & Walsh 2002). These enzymatic complexes, as explained in chapter 3.2, are also involved in the biosynthesis of other bioactive secondary metabolites (Sattely et al. 2008).

The siderophore anguibactin from *V. anguillarum* can be involved in pathogenicity (Di Lorenzo et al. 2004) but also possesses beneficial activities, such as cytotoxicity against murine cancer cell lines (Sandy et al. 2010) and growth inhibition of bacterial pathogens (Pybus et al. 1994). Nigribactin from *V. nigripulchritudo* S2604 collected during Galathea 3 interferes with *S. aureus* quorum sensing (see below). Bisucaberin from *V. salmonicida* (Winkelmann et al. 2002) is, unlike most other *Vibrio* siderophores, synthesized through an NRPS-independent route (Kadi et al. 2008). This siderophore was found to be useful in combinatorial anticancer therapy (Kameyama et al. 1987).

4.6 Non-pathogenic interactions and antibiotic activity

The association of vibrios with healthy marine animals in the wild and in aquaculture systems underline that many interactions with higher organisms are non-pathogenic, and include commensal or even symbiotic relationships. The above-mentioned symbiosis of *Vibrio fischeri* (recently reclassified as *Allivibrio fischeri*) with the Hawaiian bobtail squid (Visick & Ruby 2008), where high numbers of vibrios are found in the light organs of the eukaryotic host, is the best studied non-pathogenic interaction. The squid benefits from bacterial bioluminescence by using it to attract prey and counteract ultraviolet radiation. *A. fischeri* benefits by obtaining an exclusive and secure habitat, where it can sustain much higher population densities than in the surrounding water (Visick & Ruby 2008).

Commensal colonization of zooplankton constitute a survival strategy during environmental stresses (Wai et al. 1999), and associations with plant surfaces may be facilitated by molecular communication with the host (Gutierrez et al. 2009). In these interactions, the host potentially benefits from bacterial traits such as antagonism that could help controlliing the colonization of undesired microoganisms. While the producion of antagonistic compounds could contribute to the common occurrenece of *Vibrionaceae* on organic surfaces, the knowledge of antagonistic activity among vibrios and their interactions with other bacteria is relatively limited. While *Vibrionaceae* have been shown to produce antimicrobial substances (Long & Azam 2001), it is unknown whether antagonistic activity is widespread among the family. The knowledge of antibiotic-producing vibrios has been recently expanded by isolation of over 300 antagonistic marine vibrios during Galathea 3 (Gram et al. 2010). The finding of antagonistic activity in strains collected on a global expedition could indicate that the production of antagonistic compounds has an ecological role, and may contribute to the worldwide occurrence of vibrios (Thompson et al. 2004). Inhibitory compounds from certain vibrios are able to reduce the number of other community members, which has been suggested to influence microscale variations in competing bacterial populations. The antagonistic microflora of Atlantic cod is dominated by vibrios (Fjellheim et al. 2007), and antibacterial activities have been described with *V. parahaemolyticus* (Radjasa et al. 2007), *V. anguillarum* (Hjelm et al. 2004), *V. alginolyticus* (Austin et al. 1995), and several unidentified *Vibrio* spp. (Long & Azam 2001; Castro et al. 2002). However, the antibacterial activity has in many cases not been related to chemical compound classes, and only few *Vibrio* antibiotics have been structure elucidated to date.

Antibiotics from vibrios

Compared to other marine bacteria, such as *Pseudoalteromonas* or actinomycetes that are widely studied regarding their antibiotic secondary metabolites, comparatively few antibiotic *Vibrio* compounds have been structurally elucidated by NMR spectroscopy. An overview about published compounds as per January 2011 is given in Table 4. From 24 compounds, 17 were produced by strains only identified to the genus level, and no further species or strain identification was carried out. The present study is one of the few linking structure-elucidated antimicrobial compounds to specific species that had been identified by sequencing of housekeeping genes. This revealed

production of andrimid and holomycin by *V. coralliilyticus* and *P. halotolerans*, respectively (Wietz et al. 2010b).

Table 4. Antibiotic compounds from *Vibrionaceae* (reviewed by Månsson et al. 2011).

Antibiotic	Compound class	Source	Reference
Andrimid	Pyrrolidinedione	*Vibrio sp.*	Oclarit 1994
		Vibrio sp.	Long 2004
		V. coralliilyticus	Wietz 2010
Aqabamycin (A-G)	Maleimide	*Vibrio sp.*	Yao 2010
Cycloprodigiosin	Prodiginine	*V. gazogenes*	Gerber 1983
3,5-Dibromo-2-(3',5'-dibromo-2'-methoxyphenoxy)-phenol	Diphenyl ether	*Vibrio sp.*	Elyakov 1991
Vibrindole	Indole	*V. parahaemolyticus*	Bell 1994
Griseoluteic acid	Phenazine	*Vibrio sp.*	Sato 1995
Holomycin	Pyrrothine	*P. halotolerans*	Wietz 2010
Indazole-3-carbaldehyde	Indazole	*Vibrio sp.*	Yao 2010
Magnesidin A	Tetramic acid	*V. gazogenes*	Imamura 1994
Ngercheumicin I and II	Depsipetide	*Photobacterium sp.*	Adachi 2007
Pelagiomicin C	Phenazine	*Vibrio sp.*	Sato 1995
Prodigiosin	Prodiginine	*V. psychroerythrus*	D'Aoust 1974
		V. gazogenes	Harwood 1978
		V. ruber	Shieh 2003
Turbomycin	Indole	*Vibrio sp.*	Veluri 2003
Unnarmicin A and C	Depsipeptide	*Photobacterium sp.*	Oku 2008
Vibrindole A	Indole	*V. parahaemolyticus*	Bell 1994

The red pigment and antibiotic prodigiosin is another example of cosmopolitan antibiotics. Besides *V. psychroerythrus*, *V. gazogenes* and *V. ruber* (Table 4), it is produced by diverse other Gram-negatives (*Pseudoalteromonas rubra*, *Serratia marinorubra*, *Hahella chejuensis*) as well as Gram-positive *Streptomyces* spp. (Månsson 2011). The prodigiosin biosynthetic gene cluster contains genetic signatures related to PKS and NRPS enzymes (Harris et al. 2004). Besides antibiotic activity, prodigiosin and analogues thereof have antimalarial, immunosuppressive, and anticancer activities (Månsson 2011). A synthetic derivative is currently in Phase I and II clinical trials, while native prodigiosin is in preclinical trials (Pandey et al. 2009). Recently, prodiginine production in an estuarine *Vibrio* was demonstrated to inferre competitiveness against an indigenous *Bacillus* strain, suggesting that the compound may provide a competitive advantage in the environment (Staric et al. 2010). Interestingly, the prodigiosin-producing *V. gazogenes* was

found to produce another antibiotic, the unique magnesium-containing compound magnesidin A (Imamura et al. 1994).

The ngercheumicins, cyclic depsipeptides with a peptide (type I) or 3-hydroxyhexanoic fatty acid tail (type II) that were isolated from *Photobacterium* sp., also possess antibacterial activity (Adachi et al. 2007). From *Photobacterium halotolerans* S2753 collected during Galathea 3, five different type I ngecheumicins were isolated, but they did not display antibiotic properties. This could have been related to the fact that Adachi et al. (2002) tested for activity against an environmental strain of *Pseudovibrio denitrificans*. While they claim that ngecheumicins can treat infections by this bacterium, *Pseudovibrio* has never been reported as being pathogenic. Nonetheless, missing activity in our assay could reflect that *Pseudovibrio* as a potential non-pathogenic strain may be more susceptible to the ngecheumicins as compared to the pathogens used in the present analysis.

V. coralliilyticus, V. neptunius and P. halotolerans

The present study focused on the investigation of five antagonistic *Vibrionaceae* strains that were collected during Galathea 3. On the basis of *recA* and *rpoA* housekeeping gene similarities, the five strains were identified as *V. coralliilyticus* (two strains), *V. neptunius* (two strains), and *P. halotolerans* (one strain) (Wietz et al. 2010b). Each pair of "strain siblings" shared high genetic and metabolic similarities despite being isolated from distant oceanic regions and different sample types (Wietz et al. 2010b). Inhibition was seen against a wide range of human and aquatic bacterial pathogens, as well as bioactive marine bacteria from the *Pseudoalteromonas* and *Roseobacter* groups (Table 5). The latter included the antibacterial isolates *Phaeobacter gallaciensis* 27-4 from an aquaculture farm (Bruhn et al. 2005b), as well as *Ruegeria* sp. S1926 and *Pseudoalteromonas* sp. S2471 collected during Galathea 3 (Gram et al. 2010).

Table 5 Inhibition of various Gram-negative and Gram-positive bacteria by marine *V. coralliilyticus*, *V. neptunius* and *P. halotolerans*.

	Target strain	Source/characteristics	Inhibition by		
			S2052/4053	**S2394/4051**	**S2753**
Gram–	*V. anguillarum*	Fish pathogen	+	+	+
	V. harveyi	Shrimp pathogen	+	+	+
	V. vulnificus	Human pathogen	+	–	+
	V. parahaemolyticus	Human pathogen	–	–	+
	Phaeobacter 27-4	Aquaculture system	–	nd	+
	Ruegeria S1926	Seawater	–	nd	+
	Ps.alteromonas S2471	Seawater	–	nd	–
	E. coli AAS-EC-009	Multiresistant	–	–	+
	S. Enteritidis	Human pathogen	+	–	+
	K. pneumoniae	Human pathogen	+	–	+
	P. aeruginosa	Human pathogen	–	–	–
	S. marcescens	Human pathogen	–	–	–
	Y. enterolitica	Human pathogen	+	+	+
Gram+	*L. monocytogenes*	Human pathogen	–	–	+
	B. cereus	Human pathogen	+	–	+
	S. aureus	Human pathogen	+	–	+

The observed activities in *V. coralliilyticus* and *P. halotolerans* were due to the production of andrimid and holomycin, respectively (Fig. 18) (Wietz et al. 2010b), already known antibiotics. Since also the other investigated bacterial groups collected during Galathea 3 (*Pseudoalteromonas* and the *Roseobacter* clade) did not yield any novel compound (Gram et al. 2011; Vynne et al. 2011b), the discovery potential of novel antibiotics from Galathea 3 samples was therefore minimized. Nevertheless, the present study still underlined that marine *Vibrionaceae* are a resource of antibacterial compounds and may have potential for future natural product discovery. There is still a chance of finding chemical novelty among the isolates, including antimicrobial compounds. Neither andrimid nor holomycin were produced by *V. neptunius* S2394 and S4051, and more detailed chemical analyses including LC-UV/MS, dereplication, fractionation and purification are needed to identify the compound(s) responsible for their antibacterial activity. Furthermore, several

compounds from Galathea 3 isolates are indeed novel, and have interesting bioactivities besides direct antibiosis (see below).

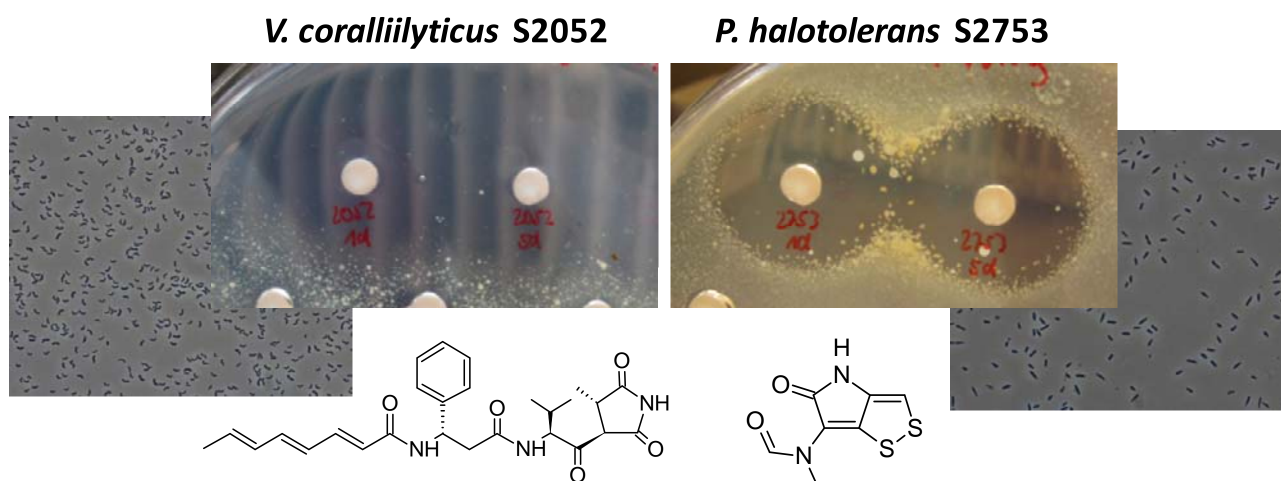


Fig. 18 Cell morphologies, inhibition of *V. anguillarum* (left) and *S. aureus* (right), and antibiotics from *V. coralliilyticus* S2052 and *P. halotolerans* S2753 responsible for the antibacterial activity (Wietz et al. 2010b).

Andrimid

Andrimid is a potent peptide-polyketide antibiotic effective against a wide range of Gram-negative and –positive bacteria, including methicilin-resistant *Staphylococcus aureus* (Singh et al. 1997). Its mode of action is different from many other antibiotics, since it acts as acetyl-CoA carboxylase inhibitor and targets fatty acid synthesis (Freiberg et al. 2004). Andrimid biosynthesis was investigated by cloning the biosynthetic gene cluster from a natural producer species into *E. coli*, yielding a cosmid that conferred robust production. The gene cluster was found to encode a hybrid nonribosomal peptide/polyketide synthase with several unusual features, including enzymes that insert β-amino acids, transglutaminase-like enzymes potentially functioning as condensation catalysts, and four densely hybrid elongation domains involved in precursor formation. The architecture of the andrimid synthase is furthermore unlike conventional, large multidomain type I nonribosomal peptide/polyketide synthases (Jin et al. 2006). Andrimid biosynthesis likely involves numerous interactions between proteins along the assembly line and consists of four basic steps; (i)

formation of the polyunsaturated fatty acid, (ii) formation and insertion of β-phenylalanine, (iii) construction of an succinimide precursor, and (iv) host resistance and enzyme priming (Jin et al. 2006). Interestingly, andrimid-producing strains were demonstrated to encode an acetyl-CoA carboxyltransferase subunit that renders them resistant to the antibiotic (Liu et al. 2008).

Andrimid was first described from an endosymbiont of a planthopper (Fredenhagen et al. 1987) and later found in other microbial species, including the terrestrial enterobacterium *Pantoea agglomerans* (Jin et al. 2006) and marine *Pseudomonas fluorescens* (Needham et al. 1994). Also marine vibrios have been shown to produce andrimid, but none of the species has been identified to the species level (Oclarit et al. 1994; Long et al. 2005). The present PhD study is thus the first linking production of andrimid to a specific *Vibrio* species. However, similar phenotypic traits (decreasing andrimid production above 30 °C) was seen with both *V. coralliilyticus* S2052 and *Vibrio* sp. SWAT3 (Long et al. 2005), suggesting that the latter was also a *V. coralliilyticus* isolate. The existence of almost identical andrimid gene clusters and a transposase pseudogene in two producer species suggested horizontal gene transfer as the most likely explanation behind the widespread occurrence of the antibiotic (Fischbach 2009). While convergent evolution is a theoretical option, it can be assumed that occurrence of andrimid in *V. coralliilyticus* is also related to horizontal gene transfer. The recently annotated full genome sequence of the *V. coralliilyticus* type strain (LMG20984) may be able to answer this question. However, opposed to the Galathea isolate S2052, the type strain as well as another close relative (LMG10953) do not produce andrimid (Wietz et al. 2011a). A BLAST search of the deposited sequence of the andrimid biosynthetic gene cluster (Jin et al. 2006) confirmed that LMG20984^T does not contain any related sequence. To test whether *V. coralliilyticus* S2052 contains a similar gene cluster, the application of primers targeting a sequence within the cluster could determine whether andrimid biosynthesis in S2052 is mediated via a comparable enzymatic pathway.

Holomycin

Holomycin belongs to the pyrrothine class of antibiotics that comprise a characteristic pyrrolinonodithiole nucleus with two sulphur atoms (Celmer & Solomons 1955). Although its mode of action could not be fully elucidated to date, holomycin likely interferes with RNA synthesis. However, effects on macromolecular synthesis were not becoming apparent in all performed experiments, indicating that holomycin may be a prodrug requiring intracellular conversion to an

active RNA polymerase inhibitor (Oliva et al. 2001). Minimum inhibitory concentrations (MIC) of holomycin to *E. coli* resulted in partial suppression, while 2-8 MIC equivalents caused complete cessation of growth. This exemplifies a bacteriostatic effect (Oliva et al. 2001).

Production of holomycin in *Photobacterium halotolerans* S2753, as demonstrated in the present study, is the first report of this antibiotic in a Gram-negative heterotrophic bacterium (Wietz et al. 2010b). To date, holomycin had only been described in Gram-positive *Streptomyces* from both terrestrial (Kenig & Reading 1979) and marine (Hou et al. 2008) environments. While parallel evolution of this trait is possible, horizontal gene transfer is the more likely explanation for its occurrence in both *Vibrionaceae* and actinomycetes. The NRPS biosynthetic cluster encoding holomycin was recently identified in *Streptomyces clavuligerus* (Li & Walsh 2010), and genomic analyses of S2753 could reveal whether it contains the same enzymatic repertoire. In combination with the observation of andrimid production in *V. coralliilyticus* and other microorganisms, this is the second example that vibrios potentially share biosynthetic genes for antibiotic synthesis with evolutionary distant microbes. This indicates that the *Vibrionaceae* may have acquired a variety of genes during evolution and species diversification, which may contribute to their ubiquity in marine and brackish environments worldwide.

The type strain of *P. halotolerans* was isolated from a saline lake located in Mallorca (Spain), while *P. halotolerans* S2753 originated from a mussel surface in the tropical Pacific. Interestingly, comparison of the antagonistic potential between the two strains revealed that only S2753 produced holomycin, while the two strains shared a number of other metabolites (Månsson et al. 2011). This highlighted that despite genetic relatedness, the physiology of related strains not necessarily resemble in all metabolic details. Bacterial taxonomy could therefore benefit from complementation by metabolite analyses. The secondary metabolome has already been shown useful to group strains of the marine antibiotic producer species *Salinispora tropica* (Jensen et al. 2007) and *Pseudoalteromonas luteoviolacea* (Vynne et al. 2011a).

Pathogenic traits

V. coralliilyticus is a model organism for the study of coral disease, being responsible for bleaching and tissue lesions in *Pocillipora damicornis* (Ben-Haim et al. 2003b) and the potential aetiological agent of White Syndrome throughout the Indo-Pacific (Sussman et al. 2008). White Syndrome is a general term for diseases of scleractinian coral, characterized by acute tissue lesions

and the exposure of the white calcium carbonate skeleton. White Syndrome often yields total mortality of coral colonies, and is therefore a significant threat to tropical reefs worldwide. For a better understanding on the disease ecology of White Syndrome caused by *V. coralliilyticus*, studies have addressed the physiological and genetic background of its virulence (Sussman et al. 2009; Meron et al. 2009) that includes a characteristic temperature-dependent pathogenicity only seen at seawater temperatures above 25 °C (Ben-Haim et al. 2003a). Raising seawater temperatures due to global warming may therefore facilitate outbreaks of coral disease (Rosenberg & Ben-Haim 2002).

There is substantial genetic variation between *V. coralliilyticus* strains from distant oceanic regions, which indicates the existence of endemic populations around the world (Pollock et al. 2010). Potentially, this could also be reflected in a presence of strains with different niches based on physiological variation, for example antibiotic production (see above). Also other physiological traits differ between *V. coralliilyticus* strains. The andrimid-producing isolate S2052 had a different temperature optimum for secondary metabolism than the related strains LMG20984^T and LMG10953, producing more metabolites at 25 compared to 30 °C (Wietz et al. 2011a). The presence of different phenotypes suggested different subspecies among *V. coralliilyticus*, potentially related to the intraspecies genetic variability (Pollock et al. 2010). Whether this is also related to varying pathogenic potential remains unknown, but the presence of a zinc metalloprotease gene linked to disease signs in corals (Sussman et al. 2008; 2009) in S2052 contradicted this hypothesis. It has to be noted, though, that the presence of the gene does not necessarily imply pathogenicity, since coral disease such as White Syndrome is likely multifactorial (Sussman et al. 2008)

Pathogenic *V. neptunius* have been primarily linked to disease in mussels. In oyster larvae, infection by *V. neptunius* leads to reduced motility, morphological anomalies and swarming of bacteria inside and around dead and moribund larvae (Prado et al. 2005). A *V. neptunius*-like isolate was also responsible for mortalities in larvae of Greenshell™ mussels, New Zealand's largest aquaculture industry that represents 70% of the national aquaculture product and is valued at US$120 million per annum (Kesarcodi-Watson et al. 2009). The fact that some of the antagonistic strains investigated in the present study belong to species that are known to harbour pathogenic strains suggests that some vibrios may possess a dual physiology, being antagonistic against other prokaryotes but pathogenic towards higher organisms.

P. halotolerans has not been linked with pathogenicity to date, but the genus harbours pathogenic species, most notably *P. damselae* (Love et al. 1981). Given the close relationship of *P. halotolerans* to *P. rosenbergii* that is involved in coral bleaching (Thompson et al. 2005b), it cannot

be excluded that pathogenic traits are present as well. However, metabolic differences between *P. halotolerans* and *P. rosenbergii* (Månsson et al. 2011) suggested that their physiological traits may be rather different.

Interference with quorum sensing

Part of the thesis work contributed to collaborative research that investigated other potential bioactivities of marine vibrios. One experimental approach addressed the interference of *Vibrio* compounds with quorum sensing in pathogenic bacteria. Quorum sensing (QS) inhibitors not necessarily kill but rather modulate microbial phenotypes, e.g. attenuate virulence (Cámara et al. 2002). Since virulence factors are unique to bacteria, QS inhibition has a high degree of selectivity and represents a promising alternative for antimicrobial therapy (Alksne & Projan 2000).

Unpublished work by Phipps et al. and Månsson et al. (DTU Systems Biology, Copenhagen University) showed interference of *Vibrio* culture extracts with QS-regulated virulence in *Staphylococcus aureus*. *S. aureus* is one of the main causes of nosocomical infections, and conventional therapeutics are hindered by rapid emergence of methicillin-resistant strains (Grundmann et al. 2006). The expression of staphylococcal virulence factors is coordinated through several key regulators, of which the QS-controlled Agr (accessory gene regulator) is the best described system (Novick 2003). Inhibition of Agr could represent an alternative therapeutic target, but only few natural antagonists of this system have been reported to date (Qazi et al. 2006). During the FøSu project, a *lacZ* reporter assay was used to monitor the effect of *Vibrio* culture extracts on expression of three virulence gene reporter fusions (*spa*, *hla*, and *RNAIII*) and hence potential interference with Agr (Nielsen et al. 2010). This assay revealed QS interference by *V. nigripulchritudo* S2604 and *P. halotolerans* S2753, and these two strains were further characterized to identify the compounds responsible for the observed activity.

Nigribactin from V. nigipulchritudo S2604

V. nigripulchritudo S2604 was isolated from a lavastone in the Solomon Islands, and differs from most other vibrios by production of a dark pigment (brown to blueish depending on culture

age) when streaked on marine agar. In broth, growth and pigment production are influenced by the carbon source. Melibiose yields considerable growth and a greenish-blue pigmentation, while glucose only yields little growth and pigmentation. S2604 is only weakly growth inhibitory, but strongly interferes with *S. aureus* QS. Stagnant compared to aerated incubation yielded higher bioactivity, comparable to observations with marine roseobacters (Bruhn et al. 2007). QS interference was found to be related to a peptide-like catecholate compound, preliminary designated as nigribactin. While being a novel structure, it is analogous to vibriobactin produced by *V. fluvialis* (Yamamoto et al. 1993) only varying by the loss of a single methyl group (Fig. 19). Vibriobactin and other related structures act as iron-sequestering siderophores (Bergeron et al. 1983), and it is likely that also nigribactin has a related function.

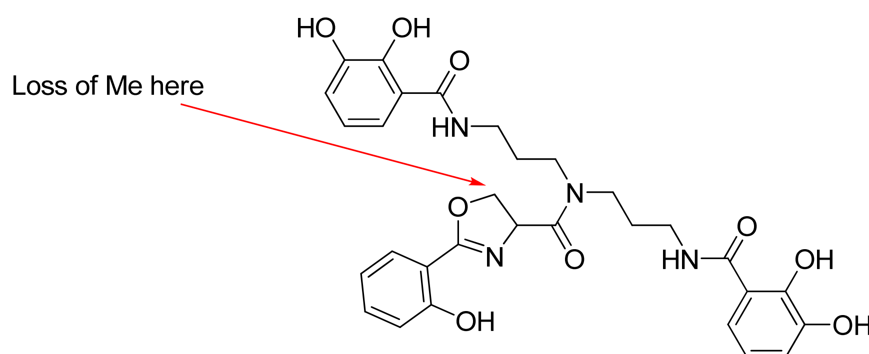


Fig. 19 Nigribactin from *V. nigripulchritudo* S2604 (Phipps et al.; unpublished).

Solonamides from P. halotolerans S2753

During the purification of holomycin, the obtained fractions were tested in a *S.aureus lacZ* reporter assay (Nielsen et al. 2010), revealing that QS interference was caused by another fraction than the one containing holomycin. Thus, interference was independent from the antibiotic. This second bioactive trait of S2753 was demonstrated to relate to two peptides, which were named solonamide A and B due to their site of isolation (Månsson et al. 2011). Both solonamides were isolated as white powder, and NMR showed them to be cyclodepsipeptides consisting of four amino acids and a 3-hydroxy fatty acid. The amino acid composition was elucidated as alanine, phenylalanine, and two leucines for both peptides (Fig. 20). Interestingly, the solonamides contain each two D-amino acids, which are less common than the L configuration. The fatty acid differs

between the two peptides, with solonamide A containing a 3-hydroxyhexanoic acid and B containing a 3-octahexanoic acid. Solonamide A has a molecular mass of 558 Da, while it is 586 Da for solonamide B (Månsson et al. 2011).

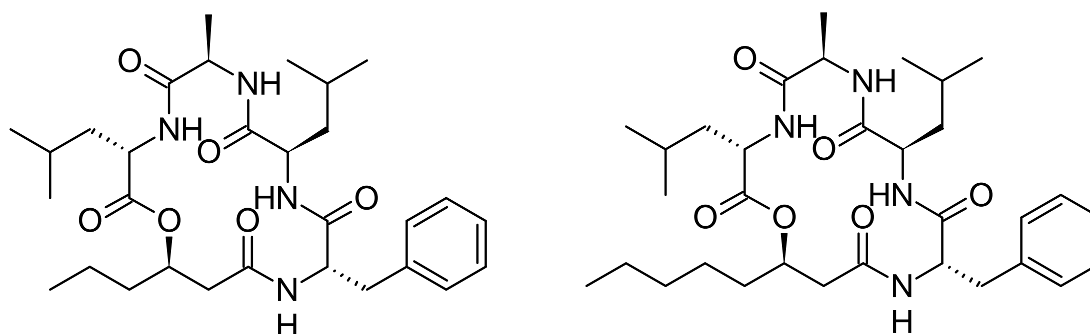


Fig. 20 Solonamides A and B from *P. halotolerans* S2753 (Månsson et al. 2011).

Northern blot analyses confirmed QS interference for solonamide B on the genetic level. Reduction of *hla* and *RNAIII* expression compared to induction of *spa* confirmed a regulatory effect of the solonamides on the Agr system (Månsson et al. 2011). This is one of the first reports of natural antagonists of the Agr system, and could provide new opportunities in anti-infective therapy. Interestingly, solonamides A and B were not produced by related *Photobacterium* strains, despite S2753 and the *P. halotolerans* type strain shared a number of metabolites (Wietz et al. 2010b; Månsson et al. 2011). This potentially reflected the observations among related *V. coralliilyticus* strains with different metabolite profiles despite high genetic similarities (Wietz et al. 2011a).

4.7 Conclusions from chapter 4

The present study expanded the knowledge about marine *Vibrionaceae*, highlighting that vibrios harbour more physiological traits than being opportunistic pathogens. The isolation of antagonistic vibrios over large spatial scales illustrated a greater biosynthetic potential than previously thought. While the rediscovery of known antibiotics suggests that the true potential of discovering chemical novelty among vibrios may be overestimated, microbiological and chemical screening should

continue to maintain the chance of identifying novel compounds. With respect to the finding that natural nutrient sources can stimulate antibiotic production, it can be hypothesized that *Vibrio* antimicrobials play a role in natural community dynamics. This may provide a new perspective on the ubiquity of vibrios and their frequent association with eukaryotes. Antibiotics could be important in bacterial competition, surface colonization, cellular communication, but potentially also pathogenicity or grazing resistance. Antagonistic traits may contribute to the adaptability and niche breadth of strains, while physiological variations between closely related strains suggested that *Vibrionaceae* species can harbour different ecotypes with phenotypical variation. This could contribute to the global occurrence and significance of this bacterial family.

5. Conclusions and Perspectives

The present PhD study included both a culture-independent and culture-dependent investigation of marine bacterioplankton. This addressed a molecular analysis of marine bacterioplankton communities, as well as studies of bioactive culturable bacteria. The work was based on the Galathea 3 and the LOMROG-II marine research expeditions that covered the majority of oceanic surface waters, including extreme polar environments.

Marine bacterioplankton community structure and biogeography

This part addressed the abundance of major marine bacterioplankton groups around the world, investigating potential biogeographical patterns and their relation to environmental conditions. Quantitative analyses of bacterioplankton samples from surface seawater at 24 worldwide stations confirmed previous studies by showing that *Alphaproteobacteria* including SAR11, *Gammaproteobacteria*, and *Bacteroidetes* globally dominate the bacterioplankton. This study was the first to provide a global quantification of the SAR86, *Roseobacter*, *Actinobacteria*, *Pseudoalteromonas* and *Vibrio* groups, expanding the knowledge of their global distribution. Latitudinal patterns in abundance and variation on the scale of oceanic biomes emphasized that marine bacterioplankton shows geographical variation, and that bacterial distribution may follow similar principles as seen for higher organisms. Linkages of environmental parameters with bacterial abundances underlined that external factors have an influence on bacterial distribution, and suggested that warmer and colder oceans harbour distinct populations. In conclusion, this analysis added to one of the widely discussed topics among marine microbiologists by providing further insights into the distributional patterns of marine bacteria on a global scale.

Marine bioactive bacteria

This part addressed antagonistic marine bacteria isolated from various geographic regions and sample types, including seawater, biotic and abiotic surfaces, and polar habitats. The

investigation focused on *Vibrionaceae* from warmer waters and *Actinobacteria* from high Arctic environments.

The analysis of five bioactive *Vibrionaceae* (*Vibrio coralliilyticus*, *V. neptunius*, and *Photobacterium halotolerans*) isolated form distant oceanic regions showed that marine vibrios produce potent antibiotic compounds. The reflection of genetic relationships in the secondary metabolome showed that metabolite analyses can complement *Vibrio* taxonomy and phylogeny. Different antibiotic production and biosynthetic temperature optima showed that closely related strains can have great physiological variation. Production of antibiotics under natural conditions indicated an ecological function of antibiotic compounds that could play a role in bacterial competition and communication, or in interactions with higher organisms. The rediscovery of andrimid and holomycin underlined the presence of cosmopolitan antibiotics shared between many different microorganisms, which may limit the chance of antibiotic discovery. This study nevertheless shows that *Vibrionaceae* have potential for future natural product discovery. Novel bioactive molecules with quorum sensing interference, such as nigribactin or the solonamides, could provide an attractive alternative to conventional antibiotic treatment.

The finding of seventeen strongly active strains from high Arctic environments showed that also polar and extreme habitats are a promising source of bacteria with antagonistic traits. While also bioactive vibrios were isolated from these regions, the most active strains belonged to the *Actinobacteria*, one of the most widely recognized groups of antibiotic producers. The isolation of seven closely related *Arthrobacter* strains throughout the Arctic suggested that its antibiotic compounds, likely related to the arthrobacilins, serve an ecological function and may contribute to its widespread occurrence. Future analyses will reveal the true potential of a novel discovery from a *Brevibacterium* isolate.

Outlook

Within the coming years, research on marine bacterioplankton communities will probably reveal more biogeographical patterns and their relation to environmental conditions. With the increasing availability and cost-effectiveness of large-scale community analyses, the knowledge of fine-scale community composition and species diversity at locations around the world is likely to increase. Linking this to environmental parameters and metabolic activity *in situ* will provide a new

picture on the role of marine bacterioplankton in global biogeochemical events. Also considering the influence of some bacterial groups on the cycling of greenhouse gases, it is imperative to investigate whether a globally changing macrobial world also affects the microbiota. Bacteria represent the basis of the food web, and drastic changes in community composition may impact on higher trophic levels and ultimately affect human society.

Regarding the culturable fraction of marine bacteria and their physiological traits, the chance of discovering novel bioactive compounds will probably depend on more sophisticated isolation, cultivation and screening techniques. However, since even the investigation of largely unexplored bacteria or habitats can yield rediscoveries of known compounds, the scale of cosmopolitanism among antibiotics may be greater than expected and severely limit natural product discovery. It is therefore advised to investigate bioactivities that have received less attention so far, such as interference with quorum sensing. Overall, the limitations of traditional culture-based approaches need to be considered. The use of standard media will likely yield the continuous isolation of similar strains, thereby minimizing the chance of discovering biological and chemical novelty. Targeted isolation techniques and genomic screening of clone libraries for biosynthetic genes should be increasingly used on larger scales. The vast and still largely uncharacterized bacterial diversity in the oceans remains a treasure for future scientific discoveries, having both an academic value but potentially also a benefit for our society.

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Latitudinal patterns in the abundance of major marine bacterioplankton groups

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ABSTRACT: The present study describes the abundance of major marine bacterioplankton groups and 2 bacterial genera (*Pseudoalteromonas* and *Vibrio*) in surface seawater at 24 stations around the world. Catalyzed reporter deposition–fluorescence *in situ* hybridization showed that *Alphaproteobacteria* (average relative abundance 37%, average absolute abundance 3.7×10^5 cells ml^{-1}) including SAR11 [30% / (3×10^5)], *Gammaproteobacteria* [14% / (1.2×10^5)] and *Bacteroidetes* [12% / (1.3×10^5)] globally dominated the bacterioplankton. The SAR86 clade [4.6% / (4.1×10^4)] and *Actinobacteria* [4.5% / (4×10^4)] were detected ubiquitously, whereas *Archaea* were scarce [0.6% / (4.2×10^3)]. The *Roseobacter* clade [averaging 3.8% / (3.5×10^4)], *Pseudoalteromonas* [2.6% / (2.1×10^4)] and *Vibrio* [1.5% / (1.3×10^4)] showed cosmopolitan occurrence. Principal component analysis revealed a latitudinal pattern in bacterial abundances by clustering samples according to lower and higher latitudes. This was related to significantly different relative abundances of *Bacteroidetes* (peaking at higher latitudes) and of unclassified *Bacteria* and *Vibrio* (both peaking at lower latitudes) between warmer and colder oceans. Relative abundances of *Alphaproteobacteria* (peaking at subtropical) and *Gammaproteobacteria* (polar stations) varied between major oceanic biomes, as did absolute abundances of *Roseobacter* (peaking at temperate and polar stations). For almost all groups, absolute abundances were positively correlated with nutrient concentrations in warmer oceans and negatively correlated with oxygen saturation in colder oceans. On a global scale, *Roseobacter* and SAR86 were correlated with chlorophyll *a*. Linkages of environmental parameters with relative abundances were more complex, with e.g. *Bacteroidetes* being associated with chlorophyll *a*. The finding of differing communities in warmer and colder oceans underlined the presence of biogeographical patterns among marine bacteria and the influence of environmental parameters on bacterial distribution.

KEY WORDS: Marine bacterioplankton · Global quantification · Latitudinal pattern · Biogeography · CARD-FISH · PCA

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INTRODUCTION

The diversity, activity and ecology of marine microbes — the main form of biomass in the oceans — have become key research subjects over the previous years, greatly expanding our knowledge of the structure and function of marine microbiota (Giovannoni & Stingl 2005). Given the role of marine bacteria in global nutrient turnover, biogeochemical processes and climate events (Arrigo 2005), the study of microbial distribution and biogeography has received increasing attention (Martiny et al. 2006). Still, there is only a basic understanding of marine bacterial community structure on a global scale, such as to whether 'everything is everywhere, but the environment selects' (Baas-Becking 1934) or if geographically separated regions harbour distinct communities. This discussion also addresses the relation of environmental factors to spatial and temporal patterns among bacteria (Dolan 2005, Martiny et al. 2006, Van der Gucht et al. 2007).

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Characterization of marine bacterial communities on larger geographical scales has been done by shotgun sequencing (Rusch et al. 2007), polymerase chain reaction (PCR)-based techniques (Baldwin et al. 2005, Pommier et al. 2007, Taniguchi & Hamasaki 2008) and catalyzed reporter deposition–fluorescence *in situ* hybridization (CARD-FISH) (Schattenhofer et al. 2009). These studies have indicated that the composition of bacterioplankton differs between oceanic regions, probably related to oceanographic factors such as water temperature (Baldwin et al. 2005, Fuhrman et al. 2008), nutrient availability (Abell & Bowman 2005), or water masses (Teira et al. 2006, Galand et al. 2010). The analysis of a global sample set showed marked variation in bacterial community structure on the 16S rRNA sequence level, with a high degree of endemism and few cosmopolitan sequences (Pommier et al. 2007). A latitudinal gradient in species richness, comparable to observations in the animal and plant kingdoms, has been shown in this context (Pommier et al. 2007, Fuhrman et al. 2008). Terminal–restriction fragment length polymorphism (T-RFLP) demonstrated distinct microbial clusters relating to Arctic, Antarctic, temperate and tropical regions, with mid-latitudinal and equatorial communities being more similar in composition to each other than to cold water communities (Baldwin et al. 2005). Quantitative analyses showed that across an Atlantic Ocean transect, SAR11 was more abundant in the northern Atlantic Ocean than in the southern Atlantic gyre, the biomass of *Prochlorococcus* peaked in the tropical Atlantic Ocean, and *Bacteroidetes* and *Gammaproteobacteria* bloomed in nutrient-rich temperate waters (Schattenhofer et al. 2009). Furthermore, prokaryotic assemblage structure was shown to exhibit strong variability along estuarine gradients (Kirchman et al. 2005), coast–ocean transitions (Baltar et al. 2007) and ocean fronts (Pinhassi et al. 2003).

The present study adds to the understanding of marine bacterial biogeography by analyzing bacterioplankton community structure and environmental parameters along the route of the worldwide Galathea 3 expedition. The Earth's circumnavigation included both a global survey of culturable bacteria with antibacterial activity (Gram et al. 2010) and a cultivation-independent investigation of bacterioplankton by CARD-FISH. This enabled the direct quantification of domains (*Bacteria* and *Archaea*), higher phylogenetic groups (*Alpha-* and *Gammaproteobacteria*, *Bacteroidetes*, *Actinobacteria*), important bacterial clades (SAR11, SAR86, *Roseobacter*) and 2 individual genera (*Pseudoalteromonas* and *Vibrio*). *Roseobacter*, *Pseudoalteromonas* and *Vibrio* were selected, since >90% of the antagonistic strains isolated during the culture-based survey (Gram et al. 2010) were affiliated with one of the 3 groups. The purpose of the present study was to investigate potential patterns in their *in situ* distribution. While species cluster distribution of the *Roseobacter* clade has been studied (Selje et al. 2004), the biogeography of *Pseudoalteromonas* (Skovhus et al. 2007) and *Vibrio* (Thompson et al. 2006) in pelagic marine ecosystems is poorly documented.

We analyzed CARD-FISH and environmental data by principal component analysis (PCA), a statistical tool to identify patterns, associations between factors and underlying causative links within a complex dataset (Martens & Martens 2001). PCA has been used for bacterial community analyses (Sekiguchi et al. 2002, Teira et al. 2008) and served here to investigate global patterns in the bacterioplankton community structure and their relation to environmental parameters. This contributes to the understanding of marine bacterial biogeography and the factors that influence microbial distribution on a global scale.

MATERIALS AND METHODS

Sampling procedures. At 24 stations along the route of Galathea 3 (www.galathea3.dk/uk), surface seawater was sampled using Niskin bottles on an SBE32 rosette (Seabird) connected with a conductivity-temperature-depth (CTD) profiler (MacArtney). Sampling sites covered different oceanic regions, including coastal and pelagic waters (Fig. 1, Table S1 in the supplement at www.int-res.com/articles/suppl/a061p179_supp.pdf). Using a 5 × 100 ml filtration manifold (DHI LAB) 5 samples were prepared per station. Each 50 ml seawater sample was filtered through 5 µm polycarbonate filters (diameter: 25 mm) to remove particle-associated bacteria. Planktonic cells were collected from the 5 µm filtered water on 0.2 µm polycarbonate filters (diameter: 25 mm) and fixed with 2% paraformaldehyde for 1 h in the dark. Filters were washed with 1× phosphate-buffered saline (PBS) and sterile Milli-Q water for 1 min each, air dried and stored at –80°C until further processing. A range of physicochemical environmental parameters were measured by standard methods at each station at which bacterioplankton was sampled. These included (1) oceanographic data (temperature, salinity, density, *in vivo* fluorescence, photosynthetically active radiation); (2) oxygen concentration and saturation; (3) concentrations of inorganic nutrients (total inorganic nitrogen [N] and phosphorus [P], nitrite [NO_2^-], nitrate [NO_3^-], ammonium [NH_4^+], phosphate [PO_4^-], silica [SiO_2]); and (4) the concentration of chlorophyll *a* (Table S1).

Selection of oligonucleotide FISH probes. Bacterioplankton communities were characterized using a range of horseradish peroxidase-labelled FISH probes (Table S2 in the supplement at www.int-res.com/

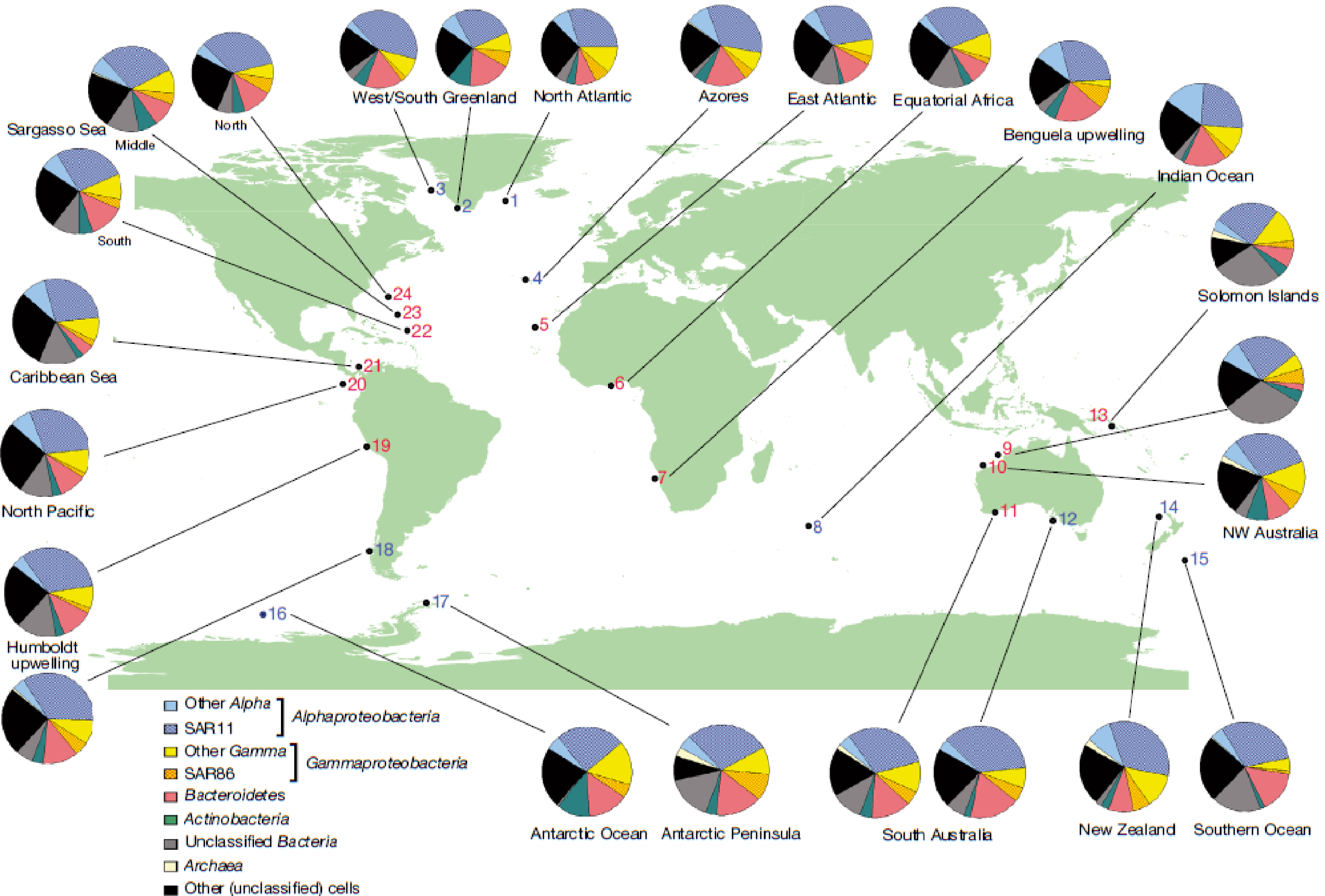

Fig. 1. Relative abundances of higher phylogenetic bacterioplankton groups (in %DAPI) at 24 stations worldwide. Blue numbers designate stations at higher (>35°), red numbers designate stations at lower (0 to 35°) latitudes. Other *Alpha*: cells detected by ALF968, but not by SAR11-441; Other *Gamma*: cells detected by GAM42a, but not by SAR86-1249; Unclassified *Bacteria*: cells detected by EUB338, but not by ALF968, GAM42a, CF319a, or HGC69a; Other (unclassified) cells: DAPI-stained cells not detected by EUB338. All EUB338 fractions refer to the average detectability from lysozyme and combined lysozyme/achromopeptidase permeabilizations

articles/suppl/a061p179_supp.pdf) purchased from biomers. net. All clade- and group-specific probes (SAR11-441, SAR86-1249, ROS536, PSU730, GV) were checked for specificity and coverage of their target groups against the SILVA database, release 96 (Pruesse et al. 2007), using the ARB software package (Ludwig et al. 2004).

CARD-FISH. Cells on filters were permeabilized by different enzyme treatments depending on the subsequent hybridization. For hybridizations targeting *Bacteria*, *Proteobacteria* and *Bacteroidetes*, cells were permeabilized with lysozyme (10 mg ml^{-1} in 1× TE buffer [0.01 M EDTA, 0.1 M Tris-HCl, pH 8.0]) for 60 min at 37°C. For hybridizations targeting *Bacteria* and *Actinobacteria*, cells were permeabilized for 30 min with achromopeptidase (60 U ml^{-1} in 0.01 M NaCl, 0.01 M Tris-HCl, pH 8.0), followed by 30 min with lysozyme (1 mg ml^{-1} in 1× TE buffer) at 37°C (Sekar et al. 2003). For hybridizations targeting *Archaea*, cells were permeabilized with Proteinase K (2 µg ml^{-1} in 1× TE buffer) for 60 min at 37°C (Teira et al. 2004). Filters were stored at –20°C until further processing. CARD-FISH was carried out according to Pernthaler et al. (2004) using FITC-labelled tyramides for signal amplification.

Epifluorescence microscopy. Stained filter sections were inspected on an Axiovert 200M inverse fluorescence microscope (Carl Zeiss) equipped with 63× and 100× objective lenses and Filter Sets 02 (Cat. No. 488002-0000-000) for DAPI and 10 (Cat. No. 488010-0000-000; both Carl Zeiss) for FITC. Per hybridization, from 600 to 1000 DAPI-stained cells were counted in ≥10 microscopic fields that were randomly selected across the filter section. Relative fractions of bacterial groups were corrected by subtraction of negative control counts with probe NON338. To validate reproducibility of hybridizations and accuracy of counts, a selection of samples from widely separated stations was again hybridized with probes targeting higher phylogenetic groups. Deviations in detected relative fractions were ≤3%, which was considered acceptable.

Data analysis. PCA was performed to reduce the complexity of multivariate data (prokaryotic abundances and physicochemical environmental parameters) in order to identify variables accounting for most of the variability in the original data. PCA was performed for each of the following datasets: (1) relative prokaryotic abundances, (2) relative abundances and environmental parameters, (3) absolute abundances and (4) absolute abundances and environmental parameters. Input data were the abundances of *Actinobacteria*; *Bacteroidetes*; SAR11, *Roseobacter*, other *Alphaproteobacteria* (difference between the sum of SAR11 and *Roseobacter* to the total abundance of *Alphaproteobacteria*); SAR86, *Pseudoalteromonas*, *Vibrio*, other *Gammaproteobacteria* (difference between the sum of SAR86, *Pseudoalteromonas* and *Vibrio* to the total abundance of *Gammaproteobacteria*); unclassified *Bacteria* (difference between the sum of *Alphaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes* and *Actinobacteria* to total bacterial abundance as detected by EUB338-I to -III); and unclassified cells (DAPI-stained cells not detected by probes EUB338-I to -III, EURY806, CREN537/CREN554). In addition, 2-tailed *t*-tests comparing relative and absolute abundances between latitudes and biomes were performed. Abundances were sorted by higher (combining stations from the tropical and subtropical climate zones, 0 to 35°) and lower latitudes (temperate and polar zones, >35°), as well as their association with the 4 major oceanic biomes (Longhurst 1998). These are designated the Polar, Coastal, Westerlies and Trades biomes and categorized by oceanographic characteristics, including seasonal cycles of nutrient availability and illumination. Furthermore, Pearson product moment correlation coefficients, *r*, between prokaryotic abundances of bacterial groups, and between prokaryotic abundances and physicochemical parameters, were calculated (Tables S3 to S5 in the supplement at www.int-res.com/articles/suppl/a061p179_supp.pdf). All reported correlations were statistically significant ($p < 0.05$).

RESULTS

Environmental parameters

Highest concentrations of oxygen (>6.5 ml l^{-1}), total inorganic nitrogen (>35 µM l^{-1}) and phosphorus (>2 µM l^{-1}), nitrate (>20 µM l^{-1}), nitrite (>0.3 µM l^{-1}), ammonium (>0.28 µM l^{-1}) and phosphate (>1 µM l^{-1}) were recorded in temperate and cold waters of the Southern Ocean (Table S1). The concentration of silica peaked in Antarctic waters (up to 76 µM l^{-1}). Elevated nutrient levels were also recorded in the northern Atlantic and in the Benguela (Namibian) and Humboldt (Peruvian) upwelling systems. Temperature ranged from –0.1 (Antarctic Peninsula) to 30.1°C (Solomon Islands), and its negative correlation with nutrients, oxygen, as well as chlorophyll *a* and *in vivo* fluorescence (indicators of phytoplankton biomass), confirmed colder waters as being nutrient richer, more aerated and more productive (*r* between –0.42 and –0.86). The chlorophyll *a* concentration peaked in the pelagic Indian Ocean (1.6 µg l^{-1}) and the Benguela upwelling system (1.2 µg l^{-1}). Solar radiation as a mean of photosynthetically active radiation (PAR) and surface PAR (SPAR) was highest in several subtropical and tropical locations (>630 and >2500 µM photons m^{-2} s^{-2}, respectively).

Abundance of major bacterioplankton groups

Relative fractions of *Bacteria* as detected by probe mix EUB338 ranged from 65% (Stn 21; Caribbean Sea) to 88% (Stn 17; Antarctic Peninsula) of DAPI-stained cells upon cell permeabilization with lysozyme, with variations in absolute numbers between 1×10^5 and 3×10^6 cells ml^{-1} (Table 1). For 15 out of the 24 stations, the detectability increased between 3 and 14% (corresponding to 3.3×10^3 and 5.4×10^5 cells ml^{-1}) upon cell permeabilization with both achromopeptidase and lysozyme. *Archaea* were only detected at 11 out of the 24 stations, most of them in the southern hemisphere, with relative fractions >1% occurring at only 5 locations.

Alphaproteobacteria, *Gammaproteobacteria* and *Bacteroidetes* constituted the major fraction of surface bacterioplankton around the globe (Fig. 1, Table 1). Their combined relative abundance accounted for up to 72% (globally averaged 63%) of DAPI-stained cells, and up to 93% (averaged 81%) of cells detected by EUB338 (average detectability of lysozyme and combined lysozyme/achromopeptidase permeabilization). At 2 out of 5 polar stations (South Greenland and Antarctic Ocean) *Actinobacteria* represented a considerable fraction with 10 and 12%, respectively. Their lowest relative abundances (approximately 1.5%) occurred at pelagic stations in the Atlantic, Indian and Southern Oceans, resulting in an average relative fraction of 4.5%.

Alphaproteobacteria were the most abundant group across all samples, with relative abundances between 29% (Stn 13; Solomon Islands) and 44% (Stn 3; West Greenland) and a global average of 37%. Absolute alphaproteobacterial cell numbers varied between 3.9×10^4 and 1.8×10^6 ml^{-1}, with a global average of 3.7×10^5 ml^{-1}. The SAR11 clade, a subclass of *Alphaproteobacteria*, constituted between 24 and 41% (average 30%) and therefore represented the majority of surface water *Alphaproteobacteria*. The relative abundance of *Gammaproteobacteria* was highest (19 to 20%) at Stns 10, 16 and 17 (NW Australia, Antarctica) and lowest (6%) at Stn 15 (Southern Ocean), globally averaging 14%. The SAR86 clade, a subclass of *Gammaproteobacteria*, constituted between 1% (Stn 15; Southern Ocean) and 9% (Stns 7 and 17; Benguela upwelling and Antarctic Peninsula), with a global average of 4.6%. *Bacteroidetes* outnumbered *Gammaproteobacteria* at several stations, but had a lower average relative abundance (12%) due to their more patchy distribution, which varied between 3% (Stn 9; NW Australia) and 20% (Stn 7; Benguela upwelling). The absolute abundance of *Bacteroidetes*, however, was, on a global average, slightly higher (1.3 $\times 10^5$ cells ml^{-1}) than that of *Gammaproteobacteria* (1.2 $\times 10^5$ cells ml^{-1}). This highlighted that absolute cell numbers did not always reflect relative abundances. While, for instance, the relative *Bacteroidetes* abundance at Stns 3 and 4 varied by only 0.7%, actual cell numbers were 16-fold different (6.4×10^5 compared to 3.9×10^4 cells ml^{-1}).

Pseudoalteromonas, *Vibrio* and the *Roseobacter* clade were detected ubiquitously (Table 1). The *Roseobacter* clade (*Alphaproteobacteria*) was most abundant in the Caribbean Sea (Stn 21; 8.2%) and least at equatorial Africa (Stn 6; 1.5%), globally averaging 3.8% corresponding to 3.5×10^4 cells ml^{-1}. *Pseudoalteromonas* (*Gammaproteobacteria*) was most abundant at the Antarctic Peninsula (Stn 17; 6%) and least at South Australia (Stns 11 and 12; 0.5%), globally averaging 2.6% (2.1×10^4 cells ml^{-1}). The abundance of *Vibrio* (*Gammaproteobacteria*) peaked at equatorial Africa (Stn 6; 3.2%), while being lowest in the Azores and Caribbean Sea (Stns 4 and 21; 0.3%), globally averaging 1.5% (1.3×10^4 cells ml^{-1}).

Biogeographical patterns of bacterial distribution

PCA comparing relative bacterial abundances between all 24 stations showed 2 clusters, while Stns 9 (NW Australia), 13 (Solomon Islands) and 17 (Antarctic Peninsula) were positioned separately (Fig. 2). The 2 clusters corresponded to the Earth's colder and warmer climate zones comprising stations from higher (>35°; temperate and polar zone) and lower latitudes (0 to 35°; tropical and subtropical zone), respectively. Four stations (Stns 7, 10, 15 and 24) did not fit the pattern and were positioned in the opposite latitude cluster. Stn 7 is located in the Benguela upwelling system, the colder, nutrient-rich waters of which probably yielded a community structure similar to colder oceans despite the subtropical location. The exclusion of Stn 10 (NW Australia) from the pattern cannot be related to an obvious factor, but was potentially due to unique oceanographic characteristics that also resulted in the entirely separate positioning of its 'sister station' Stn 9. Stns 15 (Southern Ocean) and 24 (northern Sargasso Sea) are located in regions where colder and warmer climates meet, and the opposite positioning probably reflected the fact that those communities were sampled at a transition between colder and warmer waters. PCA with absolute abundances yielded a similar latitudinal grouping of stations, albeit being less distinct as with relative values (data not shown).

PCA demonstrated that the relative abundances of *Bacteroidetes* and unclassified *Bacteria* (cells detected by probe mix EUB338, but not by ALF968, GAM42a, CF319a, or HGC69a) accounted most for the latitudinal pattern. This was consistent with significantly different relative fractions of *Bacteroidetes* (peaking at higher lat-

Table 1. Total bacterial numbers and absolute abundances (cells ml^{-1}) of bacterioplankton groups at 24 stations worldwide from the 4 oceanic biomes (P: Polar; C: Coastal; W: Westerlies; T: Trades) sorted according to higher (>35°) and lower (0 to 35°) latitudes. EUB: *Bacteria* (L: lysozyme permeabilization, L + A: combined lysozyme/achromopeptidase permeabilization); ARCH: *Archaea*; ALF: *Alphaproteobacteria*; ROS: *Roseobacter* clade; GAM: *Gammaproteobacteria*; PSA: *Pseudoalteromonas*; VIB: *Vibrio*; BAC: *Bacteroidetes*; ACT: *Actinobacteria*; Unclassified EUB: cells detected by EUB338 (average detectability from L and L + A permeabilizations), but not by ALF968, GAM42a, CF319a, or HGC69a; Other: unclassified cells, not detected by EUB338 (average detectability from L and L + A permeabilizations). na: not applicable (sum of ALF968, GAM42a, CF319a and HGC69a counts was higher than the EUB338 count); nd: not detected

Stn	Location	Biome	Total count	EUB L	EUB L + A	ARCH	ALF	SAR11	ROS	GAM	SAR86	PSA	VIB	BAC	ACT	Unclassified EUB	Other
Higher latitudes (>35°)																	
1	North Atlantic	P	1.6×10^6	1.1×10^6	1.2×10^6	nd	6×10^5	4.7×10^5	5.7×10^4	2.8×10^5	1×10^5	5.8×10^4	1.6×10^4	1.5×10^5	5.8×10^4	7.8×10^4	4.3×10^5
2	S Greenland	P	7.2×10^5	5×10^5	6×10^5	nd	2.5×10^5	1.9×10^5	1.9×10^4	1.1×10^5	4.7×10^4	1.7×10^4	4.3×10^3	1.3×10^5	7.3×10^4	na	1.7×10^5
3	W Greenland	P	4×10^6	3×10^6	3.5×10^6	nd	1.8×10^6	1.6×10^6	7.7×10^4	4.3×10^5	8.3×10^4	6.1×10^4	3.1×10^4	6.4×10^5	2.2×10^4	1.6×10^5	7.8×10^5
4	Azores	W	2.3×10^5	1.8×10^5	1.8×10^5	1.3×10^3	9.7×10^4	7.7×10^4	4.7×10^3	2.7×10^4	9×10^3	9.3×10^3	7.9×10^2	3.9×10^4	1.1×10^4	5.4×10^3	4.9×10^4
8	Indian Ocean	W	2.5×10^6	1.8×10^6	2.1×10^6	nd	1×10^6	6.3×10^5	1.5×10^5	3.4×10^5	8.8×10^4	3.9×10^4	4.9×10^4	4.2×10^5	4×10^4	8.3×10^4	5.8×10^5
12	South Australia	C	1.5×10^6	1.2×10^6	1.2×10^6	nd	6×10^5	5.4×10^5	3.9×10^4	1.9×10^5	7.6×10^4	6.9×10^3	1.1×10^4	2.7×10^5	3.2×10^4	1.1×10^5	3.1×10^5
14	New Zealand	W	7.3×10^5	5×10^5	6×10^5	1.8×10^4	3.1×10^5	2.4×10^5	5.1×10^4	1.3×10^5	4.3×10^4	2.4×10^4	1.2×10^4	6.8×10^4	1.9×10^4	1.7×10^4	1.6×10^5
15	Southern Ocean	C	7.1×10^5	5.4×10^5	5.7×10^5	2.1×10^3	2.5×10^5	2.1×10^5	2.7×10^4	4.3×10^4	8.7×10^3	6.8×10^3	3.4×10^3	1.1×10^5	1.3×10^4	1.3×10^5	1.7×10^5
16	Antarctic Ocean	P	1.3×10^5	1×10^5	1.1×10^5	nd	3.9×10^4	3.2×10^4	3.2×10^3	2.7×10^4	6.1×10^3	1.7×10^3	1.3×10^3	1.9×10^4	1.6×10^4	1×10^3	2.8×10^4
17	Ant. Peninsula	P	2.5×10^5	2.2×10^5	2.2×10^5	7.9×10^3	8.4×10^4	7.1×10^4	1.2×10^4	4.8×10^4	2.4×10^4	1.5×10^4	6.1×10^3	4×10^4	9.6×10^3	4×10^4	2.2×10^4
18	Humboldt	C	1.6×10^6	1.1×10^6	1.3×10^6	1.3×10^4	6.3×10^5	5.5×10^5	7.2×10^4	2.2×10^5	8.1×10^4	4.6×10^4	1.9×10^4	2×10^5	6.3×10^4	9.9×10^4	3.8×10^5
Lower latitudes (0 to 35°)																	
5	East Atlantic	T	4.8×10^5	3.3×10^5	5.9×10^4	nd	1.7×10^5	1.4×10^5	9.4×10^3	5.1×10^4	1.7×10^4	9.3×10^3	6.1×10^3	6×10^4	7.6×10^3	5.9×10^4	1.3×10^5
6	Equat. Africa	C	1.4×10^5	1×10^5	2.1×10^4	nd	4.6×10^4	4.1×10^4	2.1×10^3	1.7×10^4	2.9×10^3	4.5×10^3	4.5×10^3	1.4×10^4	5×10^3	2.1×10^4	3.8×10^4
7	Benguela	C	1.5×10^6	1.1×10^6	6×10^4	3.1×10^3	5.9×10^5	4.3×10^5	5.3×10^4	1.7×10^5	1.3×10^5	1.4×10^4	8.2×10^3	3×10^5	7.4×10^4	6×10^4	3×10^5
9	NW Australia	C	8.6×10^5	7×10^5	7×10^5	nd	2.8×10^5	2×10^5	2.1×10^4	1×10^5	5.1×10^4	3×10^4	1.6×10^4	2.2×10^4	3.7×10^4	2.7×10^5	1.6×10^5
10	NW Australia	C	9×10^5	6.4×10^5	7.5×10^5	2.1×10^4	3.3×10^5	2.6×10^5	5×10^4	1.7×10^5	6.3×10^4	4.2×10^4	2.1×10^4	8.2×10^4	7.3×10^4	4.2×10^4	1.9×10^5
11	South Australia	C	5.8×10^5	4×10^5	4.7×10^5	9.5×10^3	2×10^5	1.8×10^5	1.6×10^4	9.3×10^4	2.9×10^4	3.3×10^3	6.2×10^3	8.2×10^4	2.6×10^4	6.5×10^4	1×10^5
13	Solomon Islands	T	7.9×10^5	6.7×10^5	6.7×10^5	2.2×10^4	2.3×10^5	2×10^5	3.1×10^4	1.3×10^5	2.6×10^4	2×10^4	1.9×10^4	6.1×10^4	3.7×10^4	2.1×10^5	9.9×10^4
19	Humboldt	C	2×10^6	1.5×10^6	1.6×10^6	nd	7.4×10^5	6.5×10^5	4.4×10^4	2×10^5	3.7×10^4	4.9×10^4	5.3×10^4	2.4×10^5	5.9×10^4	3×10^5	4.7×10^5
20	North Pacific	T	7.2×10^5	4.8×10^5	5.7×10^5	nd	2.7×10^5	2.1×10^5	2.1×10^4	7.4×10^4	1.1×10^4	1.5×10^4	1.4×10^4	7.3×10^4	2.2×10^4	8.9×10^4	2×10^5
21	Caribbean Sea	T	4.2×10^5	2.7×10^5	3.2×10^5	nd	1.6×10^5	1.2×10^5	3.4×10^4	4.6×10^4	8.5×10^3	6.8×10^3	1.1×10^3	2×10^4	1.1×10^4	6.2×10^4	1.3×10^5
22	S Sargasso Sea	T	4.1×10^5	3.2×10^5	3×10^5	nd	1.4×10^5	1.1×10^5	2.4×10^4	5.4×10^4	1.5×10^4	1.8×10^4	5.1×10^3	5.5×10^4	2×10^4	4.2×10^4	9.9×10^4
23	Mid Sargasso Sea	W	2.6×10^5	2.1×10^5	1.9×10^5	2.3×10^3	9.2×10^4	7.6×10^4	1.3×10^4	3.5×10^4	1.1×10^4	8.8×10^3	6.4×10^3	2.4×10^4	1.9×10^4	3.2×10^4	5.6×10^4
24	N Sargasso Sea	W	2×10^5	1.4×10^5	1.6×10^5	2.3×10^2	7.7×10^4	6.7×10^4	9.1×10^3	2.4×10^4	1.3×10^4	5.6×10^3	4.1×10^3	2.3×10^4	9.9×10^3	1.2×10^4	5.3×10^4

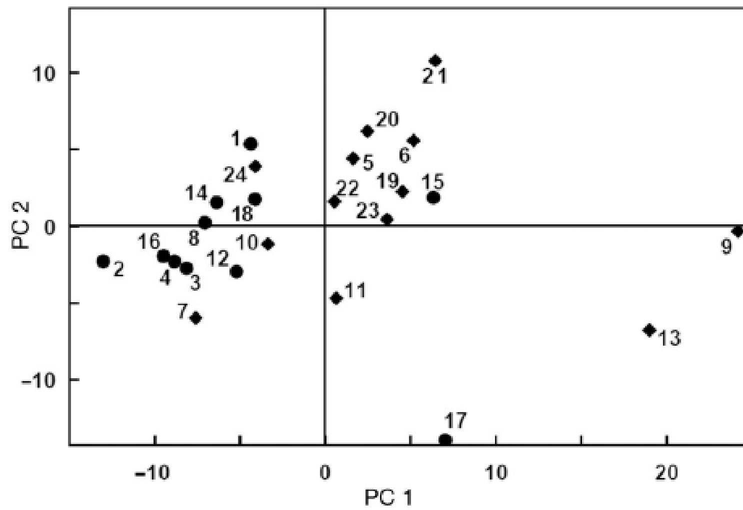

Fig. 2. Principal Component (PC) 2 versus 1 scores comparing relative bacterial abundances between 24 stations worldwide showing 2 clusters comprising samples from higher (●) and lower (◆) latitudes, respectively. The principal components explain 51 and 17 %, respectively, of the total variance between stations

Table 2. Pearson product moment correlation coefficients (*r*) between environmental parameters and absolute abundances of bacterioplankton groups. ALF: *Alphaproteobacteria*; ROS: *Roseobacter* clade; GAM: *Gammaproteobacteria*; BAC: *Bacteroidetes*; ACT: *Actinobacteria*; chl-a: chlorophyll *a*; O_2sat: oxygen saturation. Black: global correlations, blue: correlations at higher (>35°) latitudes, red: correlations at lower (0 to 35°) latitudes

	ALF	SAR11	ROS	GAM	SAR86	BAC	ACT
NO_2	0.78	0.71	0.62	0.62	0.82	0.94	0.66
NO_3	0.58				0.79	0.8	0.54
PO_4	0.9	0.84	0.7	0.78	0.83	0.96	0.78
SiO_2	0.61		0.74	0.66	0.97	0.72	0.81
chl-a			0.47		0.38 0.81	0.67	
O_2sat	−0.65 −0.65	−0.68 −0.69		−0.64 −0.65	−0.5 −0.61	−0.62 −0.63	−0.74 −0.76

Table 3. Pearson product moment correlation coefficients (*r*) between environmental parameters and relative abundances of bacterioplankton groups. ALF: *Alphaproteobacteria*; BAC: *Bacteroidetes*; Temp: temperature; Total N: total inorganic nitrogen; chl-a: chlorophyll *a*; O_2c: oxygen concentration. Black: global correlations, blue: correlations at higher (>35°) latitudes, red: correlations at lower (0 to 35°) latitudes

	ALF	SAR86	BAC
Temp	0.72 −0.61	−0.53	−0.64 −0.75
Total N	−0.74	0.49	0.39
NO_2	−0.84 0.55		0.45 0.68
NO_3	−0.85 0.59	0.38	0.39 0.66
PO_4	−0.81	0.4	0.56
chl-a	0.54	0.65	0.47
O_2c	−0.85 0.53	0.6	0.59 0.79

itudes; p = 0.015) and unclassified *Bacteria* (peaking at lower latitudes; p = 0.034) between colder and warmer oceans. Also *Vibrio* showed latitudinal variation (peaking at lower latitudes; p = 0.039). Comparison between biomes revealed relative abundances of *Alphaproteobacteria* peaking in the Westerlies biome (p = 0.023) and of *Gammaproteobacteria* in the Polar biome (p = 0.028). For *Roseobacter* relative abundances were highest in subtropical and tropical locations (p = 0.027), while absolute abundances peaked in temperate and polar waters (p = 0.041).

Relation of community structure to environmental parameters

Linkages of bacterial distribution with environmental parameters were analyzed by PCA and Pearson product moment correlations. PCA, being a multivariate analysis comparing all parameters, provides insights into more complex correlations and interdependencies. In contrast, Pearson correlations point out direct pairwise associations between abundances and physicochemical parameters (Tables 2 & 3, Tables S4 & S5) and/or between bacterial groups with a comparable response to environmental conditions (Table S3). Both approaches revealed relations of community structure to environmental parameters, with distinct variations depending on whether the analysis was done using relative or absolute bacterial abundances. Absolute abundances of most bacterial groups showed a latitude-dependent pattern, being correlated with nutrient concentrations in warmer, but with oxygen saturation in colder oceans (Tables 2 & S4). PCA highlighted these associations by positioning oxygen saturation and bacterial abundances opposite each other, and nutrients and bacterial abundances at similar positions on the abscissa (Fig. 3a). Absolute cell numbers of *Roseobacter* and SAR86 were globally correlated with chlorophyll *a* (Table 2). The influence of environmental parameters on relative abundances was more complex. While PCA clustered SAR86 with nitrogenous and phosphorous nutrients, *Actinobacteria* with chlorophyll *a*, ammonium and nitrite, and *Roseobacter* with solar radiation, salinity and oxygen saturation (Fig. 3b), only the first was reflected in Pearson correlations. In

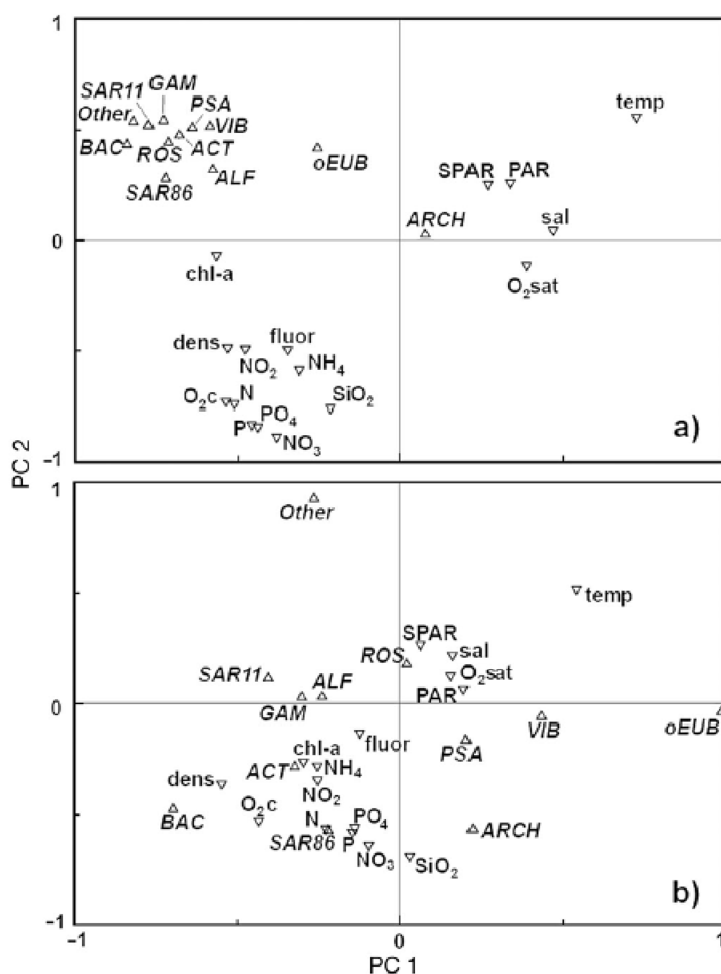

Fig. 3. Principal Component (PC) 2 versus 1 correlation loadings comparing environmental parameters (∇) with (a) absolute and (b) relative bacterial abundances (Δ) between 24 stations worldwide. The principal components explain (a) 30 and 28% and (b) 46 and 17% of the total variance between samples. *ARCH*: *Archaea*; *ALF*: *Alphaproteobacteria*; *ROS*: *Roseobacter* clade; *GAM*: *Gammaproteobacteria*; *PSA*: *Pseudoalteromonas*; *VIB*: *Vibrio*; *BAC*: *Bacteroidetes*; *ACT*: *Actinobacteria*; *oEUB*: unclassified *Bacteria* detected by EUB338, but not by ALF968, GAM42a, CF319a, or HGC69a; *Other*: unclassified cells not detected by EUB338. All EUB338 fractions refer to the average detectability from lysozyme and combined lysozyme/achromopeptidase permeabilizations. temp: temperature; sal: salinity; dens: density; N: total inorganic nitrogen; P: total inorganic phosphorus; chl-a: chlorophyll *a*; fluor: *in vivo* fluorescence; O_2c: oxygen concentration; O_2sat: oxygen saturation; PAR: photosynthetically active radiation; SPAR: surface PAR

contrast, the global associations of *Bacteroidetes* with chlorophyll *a* and different inorganic nutrients were only seen in Pearson correlations (Tables 3 & S5). Several correlations were restricted to either higher or lower latitudes. Furthermore, 4 out of 10 found associations of *Alphaproteobacteria* with environmental parameters differed between colder and warmer waters, having a positive correlation at higher, but a negative correlation at lower latitudes and vice versa (Table 3).

DISCUSSION

The present study adds to recent large-scale surveys of marine bacterial community structure in surface seawater (Baldwin et al. 2005, Pommier et al. 2007, Fuhrman et al. 2008, Biers et al. 2009) by presenting a quantitative *in situ* dimension of marine bacterioplankton around the world. We confirmed the ubiquitous dominance of *Alphaproteobacteria* and the SAR11 clade (Morris et al. 2002, Pommier et al. 2007), medium to high abundance of *Gammaproteobacteria* and *Bacteroidetes* (Giovannoni & Stingl 2005) and low numbers of *Archaea* in oceanic surface waters (DeLong et al. 2006). In addition, this is the first quantitative report of *Actinobacteria*, the SAR86 and *Roseobacter* clades, and the *Pseudoalteromonas* and *Vibrio* genera on a global scale.

In comparison to other CARD-FISH surveys, we reached similar conclusions regarding the relative abundance of higher phylogenetic groups in coastal tropical Atlantic (Baltar et al. 2007) and Antarctic waters (Topping et al. 2006), but found differing fractions of SAR11, *Gammaproteobacteria* and *Bacteroidetes* in the Benguela upwelling system (Schattenhofer et al. 2009). This probably reflected the fact that the samplings were conducted in different seasons (boreal autumn versus spring) and at different coordinates (24° S versus 30° S). The Benguela province harbours several, oceanographically distinct upwelling centres. While the present sampling was done in the most intense upwelling zone, which is additionally influenced by the input of aerosol particulates (representing an environmental barrier), Schattenhofer et al. (2009) sampled in a centre and period of minimal upwelling (Longhurst 1998). Seasonal variation plays an important role in bacterial community dynamics, but cannot be addressed here since samplings were only possible at one time point at each station.

The present study provides further evidence for spatial variation in marine bacterioplankton community structure on a global scale. Finding latitude- and biome-related variations in bacterial distribution highlighted the presence of latitudinal patterns among

marine microbiota (Pommier et al. 2007, Fuhrman et al. 2008) and emphasized that warm water communities are more similar in composition to each other than to cold water communities (Baldwin et al. 2005). Environmental parameters were correlated with both relative and absolute bacterial abundances, indicating a complex interplay of abiotic factors behind the structuring of bacterioplankton communities. The strong influence of nutrient concentrations on bacterial population sizes at lower latitudes probably reflected the characteristic nutrient scarcity in warmer waters. In the richer waters of colder oceans, however, nutrients were likely not limiting; instead, a negative correlation of population sizes with oxygen saturation was found, possibly reflecting the increased oxygen consumption of the larger active bacterial populations.

For *Alphaproteobacteria*, several correlations with environmental parameters differed between latitudes. We assume that these variations reflected the existence of variable dominant populations, or ecotypes (genetically closely related but physiologically distinct populations with unique niches; Cohan 2002) between colder and warmer waters. Widespread bacterial groups commonly share a high diversity of distinct low-level taxa (Kirchman et al. 2005), and ribotypes from all major bacterioplankton groups were shown to be restricted to either higher or lower latitudes (Pommier et al. 2005). Spatially diverging communities were also observed for *Roseobacter* (Selje et al. 2004) and *Prochlorococcus* (Bouman et al. 2006) ecotypes.

The present study highlighted the prevalence of *Gammaproteobacteria* and *Bacteroidetes* in colder waters (Malmstrom et al. 2007, Schattenhofer et al. 2009). Furthermore, the *Bacteroidetes*–chlorophyll *a* correlation substantiated the group as being responsive to phytoplankton blooms (Fandino et al. 2005, Pommier et al. 2007). It should be considered, however, that any apparent correlation can be a side-effect of uncharacterized, superior dependencies (Levin et al. 2001). Furthermore, bacterial biogeography is likely influenced by more parameters than analyzed here, such as hydrography (Teira et al. 2006, Galand et al. 2010) or carbon-flux related variables (Teira et al. 2008).

The *Roseobacter* clade, *Pseudoalteromonas* and *Vibrio* were quantified to complement results from a culture-based survey of antagonistic bacteria performed during the same expedition, and to provide further insight into the marine ecology of these groups. The *Roseobacter* clade is a heterogeneous group involved in global sulphur and greenhouse gas cycling (Moran et al. 2003, Wagner-Döbler & Biebl 2006). While the peak of relative abundances in warmer waters contradicted earlier observations, the finding of significantly higher absolute numbers in colder waters was consistent (Selje et al. 2004). The detection of smaller relative *Roseobacter* fractions than reported elsewhere (Wagner-Döbler & Biebl 2006) potentially reflected the fact that no samplings were performed during phytoplankton blooms, when the clade often dominates the bacterioplankton. Nevertheless, the global correlation of absolute *Roseobacter* cell numbers with chlorophyll *a* underlined the association with algal communities. *Pseudoalteromonas* spp. is often associated with marine eukaryotes (Holmström & Kjelleberg 1999) and produces various antibacterial compounds (Bowman 2007). Despite its prevalence on surfaces, we detected a considerable planktonic population, corresponding to findings by Schattenhofer et al. (2009). *Vibrio* is mainly researched regarding its pathogenicity to humans or aquatic animals (Thompson et al. 2006). The detected fractions of *Vibrio* were consistent with other studies using a hybridization approach (Heidelberg et al. 2002, Schattenhofer et al. 2009). In contrast, the Global Ocean Survey — a metagenomics-based and hence also quantitative analysis — reported an approximately 1% smaller abundance (Biers et al. 2009).

In conclusion, the present study provides a global quantitative survey of the major bacterioplankton groups, important clades and 2 bacterial genera in surface seawater around the world. The latitudinal patterns in community structure, together with the clustering and correlations of bacteria with physicochemical parameters, underlined the existence of biogeographical variation among marine bacteria and the relation of abiotic factors to these patterns. The latitude-dependent variations in both relative and absolute bacterial abundances indicated that bacterial biogeography may follow principles similar to those seen for higher eukaryotes.

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Paper 2

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Bioactive bacteria from Arctic marine environments.

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Bioactive bacteria from Arctic marine environments

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Abstract

During an expedition to the high Arctic Ocean (84° to 89° N) seventeen strains with antagonistic activity towards bacterial pathogens were isolated from 58 different marine samples. The bioactive strains originated from sea ice, zooplankton, sea- and meltwater, and affiliated with the *Actinobacteria* (8 strains), *Pseudoalteromonas* (4 strains), the *Vibrionaceae* (3 strains), and *Psychrobacter* (2 strains). No bioactive strains were isolated from snow or sediment samples. Seven of the eight bioactive *Actinobacteria*, being isolated from different sources throughout the Arctic Ocean, were related to *Arthrobacter davidanieli*. All *A.davidanieli* strains showed a broad antibiotic spectrum against Gram-positive and -negative human and animal pathogens, including *Salmonella*, *Listeria* and *Vibrio* spp. The eighth isolated actinomycete, *Brevibacterium* sp., had a different antibiotic spectrum and only inhibited Gram-negative pathogens, including *K. pneumoniae* and multiresistant *E. coli*. Bioactive *Pseudoalteromonas*, *Vibrionaceae*, and *Psychrobacter* strains inhibited *Staphylococcus aureus* and/or *Vibrio anguillarum*. Chemical metabolite analyses of *A. davidanieli* indicated that arthrobacilins were likely responsible for the antagonistic activity, while the antibacterial(s) from *Brevibacterium* sp. remain to be determined. The finding of bioactive bacteria being widespread in different Arctic marine environments contributes to the knowledge of antagonistic traits in polar environments, and indicates that bioactivity may play an ecological role in these habitats.

Introduction

Bioactive secondary metabolites represent a keystone in microbial interactions (Hibbing et al. 2010), but also have biotechnological potential as antibiotics, biosurfactants, antiviral, or anticancer agents (Demain & Sanchez 2009). Antagonistic bacteria from marine environments (Nair & Simidu 1987, Long & Azam 2001, Gram et al. 2010) are a still comparatively untapped resource of bioactive natural products. The structural diversity and broad activity spectrum of marine bioactive compounds (Debbab et al. 2010) highlight the potential of novel discoveries among marine microorganisms, advising the investigation of largely unexplored marine environments.

Polar habitats as a resource of natural products with biotechnological potential (Thomas & Dieckmann 2002) have so far been mainly researched for bioactive macromolecules, such as cold-

active enzymes and antifreeze proteins from both pro- and eukaryotes (Feller & Gerday 2003). However, relatively few studies have investigated secondary metabolites, small organic molecules with a molecular weight less than 3000 Dalton, from culturable polar bacteria. In Arctic and Antarctic pack ice, the culturable microbiota is dominated by *Alphaproteobacteria* such as *Octadecabacter* spp., but its potential for secondary metabolite biosynthesis seems to be low (Newton et al. 2010). Other common sea-ice associated bacteria belong to the genera *Marinobacter*, *Colwellia*, and *Glaciecola* (*Gammaproteobacteria*) as well as *Salegentibacter* and *Psychroserpens* (*Bacteroidetes*) (Junge et al. 2002, Brinkmeyer et al. 2003), of which *Glaciecola* and *Salegentibacter* are known to produce bioactive secondary metabolites (Al-Zereini et al. 2007, Wiese et al. 2009). In Arctic seawater, culturable bacteria are mainly represented by the *Roseobacter* clade, various *Gammaproteobacteria* and Gram-positive *Actinobacteria* (Mergaert et al. 2001). The epibiotic flora of polar zooplankton includes *Vibrionaceae*-like strains in association with amphipods (Atlas et al. 1982) and different invertebrates (Jøstensen & Landfald 1997). Many of these bacterial groups harbour strains with biosynthetic potential and antagonistic traits (Jensen et al. 2005, Bowman 2007, Martens et al. 2007, Gram et al. 2010, Wietz et al. 2010)

Antagonistic traits in polar bacteria have been mainly investigated in Antarctic strains, describing antimicrobial activities with *Actinobacteria* (O'Brien et al. 2004, Lo Giudice et al. 2007), bacilli and enterobacteria (Shekh et al. 2010), and different *Gammaproteobacteria* (O'Brien et al. 2004). Also, cyanobacteria from benthic mats produced multiple antimicrobial compounds (Biondi et al. 2008). Structure-elucidated antibiotics include phenazines from *Pseudomonas* (Jayatilake et al. 1996), aromatic nitro compounds from *Salegentibacter* (Al-Zereini et al. 2007), and an angucyclinone from *Streptomyces* (Bruntner et al. 2005).

The present study aimed at the isolation of antagonistic bacteria and bioactive secondary metabolites from high Arctic marine environments, a so far virtually unexplored habitat. We report the isolation of seventeen bioactive isolates from sea ice, seawater, and zooplankton samples collected between 84° and 89° N. Our finding of bioactive bacteria widespread throughout the marine Arctic contributes to the understanding of antagonistic traits among polar bacteria, and suggests a potential ecological role of the antibiotic compounds.

Materials and methods

Collection of environmental samples. Environmental samples (Table S1) were collected from a variety of sources throughout the Arctic Ocean during the LOMROG-II expedition (Fig. 1, Table S1). Sea ice cores from first-, second- and multiyear ice were obtained with a Mark II coring system (Kovacs, Lebanon, USA). Each core was cut into 20 cm subsections and scraped with a sterile blade to remove contaminating material. Sections were transported to the lab inside sterile plastic bags while kept on ice, and melted in an equal volume of sterile-filtered artificial seawater (47 PSU) at 4 °C. From 20 selected core section melts, 0.75 mL was frozen in 0.75 mL of sterile cryoprotectant solution at –80°C. Water (18 samples) was sampled from various depths using 7 L Niskin bottles on a SBE32 rosette (Seabird, Bellevue, USA) and collected from meltwater ponds (4 samples). Per sample, 0.75 mL was frozen in 0.75 mL of sterile cryoprotectant solution (60% glycerol in MilliQ water) at –80°C. Live zooplankton (10 samples) was collected using plankton nets and processed depending on size. Larger specimen were swabbed using sterile cotton swabs, which were transferred to 4 ml of sterile-filtered seawater and vortexed vigorously to remove bacteria from the cotton. Smaller zooplankton (copepods and amphipods) were homogenized in 4 mL of sterile-filtered seawater using sterile mortar and pestle. 0.75 mL of each homogenate was frozen in 0.75 mL of sterile cryoprotectant solution at –80°C. In addition, enrichment cultures were prepared from selected sea ice, water, and zooplankton samples by transferring 1 mL of sample to half-strength Marine Broth 2216 (Gram et al. 2010) and incubation at 20 °C for several weeks. Snow (3 samples) was collected with a sterile shovel, and 0.75 mL was frozen in 0.75 mL of sterile cryoprotectant solution at –80°C. Sediment (3 samples) was obtained using a piston sediment corer, and a piece of approx. 3 mL was extracted from selected core sections using a sterile 10 mL syringe. The middle part of the sediment piece was transferred to 0.75 mL of sterile cryoprotectant solution, vortexed vigorously and frozen at –80°C.

Bacterial isolation substrates. Bacteria were isolated from cryopreserved samples using two commercial media (Marine Agar 2216, Difco 212185; Actinomycete Isolation Agar, Sigma 17177) and three custom mixtures in 0.2 µm-filtered polar seawater. Marine Agar 2216 (MA) was prepared in half strength (Gram et al. 2010). Chitin agar (CA) was prepared according to Helmke and Weyland (1995) with a final concentration of 0.2% colloidal chitin purified as follows: 10 g practical grade chitin (Sigma P7170) were hydrolyzed in 400 mL ice-cold 37% HCl for 20 min and

stirred vigorously at 37 °C until clear. The solution was poured into 4 L of dH_2O and placed at 4 °C overnight for settlement of chitin. The supernatant was aspirated and chitin resuspended in 2 L of dH_2O. Chitin was collected by centrifugation (4000 *g* for 12 min), resuspended in 1 L dH_2O, and adjusted to pH 7 using KOH pellets. The solution was homogenized for 5 min using an Ultra-Turrax (IKA, Staufen, Germany) and autoclaved. The final concentration of colloidal chitin was determined from a dried (70 °C overnight) subsample. Low-nutrient medium (LN) was prepared according to Cho and Giovannoni (2004) in polar seawater amended with 1% agar, 1 µM NH_4Cl, 1 µM KH_2PO_4 and sterile-filtered vitamin solution at a 10^{-4} dilution of stock (thiamine hydrochloride 0.2 mg, biotin 1 µg, vitamin B_{12} 1 µg, folic acid 2 µg, PABA 10 µg, nicotinic acid 0.1 mg, inositol 1 mg, calcium panthotenate 0.2 mg, pyridoxine hydrochloride 0.1 mg; all amounts per liter) (Davis & Guillard 1958). Low-nutrient medium for *Actinobacteria* (LA) was prepared in polar seawater amended with 1.8% agar, sodium propionate (0.2 g L^{-1}), cycloheximide (50 mg L^{-1}), and thiamine hydrochloride (4 mg L^{-1}).

Isolation of bacteria from environmental samples. Samples were thawed at 4 °C, serially diluted in sterile 3% Instant Ocean (Aquarium Systems, Sarrebourg, France), and spread-plated on MA, CA and LN (incubated at both 20 °C and 5 °C), AA (20 °C), and LA (15 °C). During the incubation period over 500 colonies were randomly picked and pure-cultured to obtain a variety of colony morphotypes. This was done by selecting 2 to 15 colonies per sample, depending on the total number of colonies as well as the number of distinguishable colony morphologies. Culturable counts on MA, CA, and AA plates incubated at 20 °C were determined after 1 week, and on LN and LA plates after 3 weeks. Culturable counts on MA, CA and LN plates incubated at 5 °C were determined after 1 week, and again after four weeks to see whether additional slow-growing colonies had appeared. Culturable counts were compared between the agar types as well as the incubation temperatures using a Student's *t*-test ($p < 0.05$). After 3 and 6 months, Marine Broth enrichments were subcultured on MA for 5 days at 20 °C.

Screening for bioactive bacteria. Randomly picked and pure-cultured strains were tested for antibacterial activity by spotting colony mass onto agar plates seeded with a pathogenic *Vibrio anguillarum* strain 90-11-287. In addition, 254 plates were replica-plated onto pathogen-seeded agar (Gram et al. 2010). Pure cultures of strains causing a clearing of the pathogen were frozen at −80 °C in cryoprotectant solution. To identify the strains retaining activity upon freeze-storage,

strains were retested twice against *V. anguillarum* and additionally *Staphylococcus aureus* strain 8325.

Phylogenetic analysis. Seventeen bioactive strains with consistent antibacterial activity in spottests were identified by 16S rRNA gene sequencing. DNA was extracted from liquid overnight cultures using the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. For extractions with low DNA yield (strains PP12 and SS12) culture pellets were subjected to bead-beating in 500 µL lysis buffer (40 mM Na_2EDTA, 0.75 M sucrose, 50 mM Tris-HCl) followed by treatment with lyzoyme (1 mg mL^{-1}; 30 min at 37 °C), SDS (1%), and Proteinase K (2 mg mL^{-1}; overnight at 56 °C). DNA was purified using phenol:chloroform:isoamylalcohol (25:24:1) and chloroform:isoamylalcohol (24:1) extraction, and precipitated with 0.6 volumes isopropanol and 0.1 volumes 3 M sodium acetate for 1 h at −20 °C. DNA was pelleted by centrifugation (20 min, 20000 *g* at 4 °C), washed with 70% ethanol, and resuspended in sterile MilliQ water. 16S rRNA gene sequences were amplified using primers 27F and 1492R (Kennedy et al. 2009) and PCR products checked by agarose gel electrophoresis. Products were sequenced using primers 27F and 1492R, and aligned to their closest sequence relatives (Altschul et al. 1997).

Bioactivity testing of culture extracts. To test whether antibacterial compound(s) were extractable in organic solvent, the seventeen strains were grown aerated (200 rpm) in 30 mL sea salt solution (Sigma S9883; 40 g L^{-1}) containing 0.4% glucose and 0.3% casamino acids for 3 days at 25 °C. Whole cultures were frozen at −20 °C overnight, followed by freezing with liquid nitrogen before freeze-drying (Heto Drywinner; Heto-Holten, Denmark). Lyophilized cells were extracted with 15 mL 96% ethanol by vortexing for partial desalting. Cells and undissolved media components were removed by centrifugation (10 min at 3000 *g*). The supernatant was collected and evaporated under nitrogen. The residue was redissolved in 1 mL 50% acetonitrile in MilliQ water before testing for activity against *V. anguillarum* strain 90-11-287 and *S. aureus* strain 8325 using a well diffusion agar assay (Hjelm et al. 2004). Active extracts (strains WX11 and SS12) were analyzed by LC-UV/MS and tested against a larger panel of pathogenic bacteria.

LC-UV/MS of active culture extracts. Extracts from two actinobacterial strains, WX11 and SS12, were analyzed by liquid chromatography-diode array/mass spectrometry (LC-UV/MS). Extracts

were evaporated under nitrogen, redissolved in methanol and analyzed on an Agilent 1100 liquid chromatograph with a diode array detector (Agilent, Waldbronn, Germany) coupled to an LCT TOF mass spectrometer (Micromass, Manchester, UK) using a Z-spray ESI source. Separation was obtained on a Luna II C_{18} column (50 × 2 mm, 3 µm; Phenomenex, Torrance, CA) fitted with a security guard system using a linear gradient starting from 15% acetonitrile (MeCN) in water (both buffered with 20 mM formic acid) increasing to 100% MeCN over 20 minutes at a flow rate of 0.3 mL min^{-1}. In each chromatographic run, the first minute eluate from the column was by-passed the MS detector to prevent salting of the cone.

Results

Bacterial isolates and culturable counts

Fifty-eight samples, originating from sea ice (20 samples), seawater (18), zooplankton (10), meltwater/snow (7), and sediment (3), were collected at 26 locations throughout the high Arctic Ocean (Fig. 1, Table S1).

Culturable counts as determined by the number of colony-forming units (CFU) distinctly varied between the sample types. Highest culturable counts were obtained from sea ice samples (average of 3.8×10^4 mL^{-1}), followed by zooplankton (3.3×10^2 mL^{-1} homogenate) and seawater (2.4×10^2 CFU mL^{-1}). Samples from sediment and meltwater/snow yielded counts below 10^2 CFU mL^{-1}. There was no significant difference in culturable counts between rich and low-nutrient agars, or between high and low incubation temperatures. Therefore, we tested the abilities of randomly selected strains that had been isolated on low-nutrient media and/or at 5 °C for their ability to grow at high nutrient concentrations and temperature, respectively. From 30 strains isolated on low-nutrient media, 27 (90%) were able to grow on rich medium (MA). From 48 strains isolated at 5 °C, 38 (80%) were able to grow at 20 °C.

Chitinolytic bacteria, as identified by the formation of clearing zones on chitin agar, were found in 13 samples, mainly from zooplankton (6) and water (5). Four strongly chitinolytic isolates were identified by 16S rRNA gene sequencing and assigned to *Paenibacillus*, *Shewanella*, *Vibrio*, and *Pseudoalteromonas* spp.

Isolation and identification of antagonistic bacteria

From 511 picked colonies, 50 strains (10%) were identified as being antagonistic towards *V. anguillarum* strain 90-11-287. Replica-plating of 254 plates (containing 32,385 colonies) resulted in the isolation of 61 antagonistic strains (0.2%). The screening of randomly selected, single colonies was therefore much more effective in the identification of bioactive strains, although only a smaller number of strains could be tested.

From the total of 111 bioactive strains, 17 retained considerable antibacterial activity upon repeated testing. These originated from sea ice (8 strains), zooplankton (4), seawater (4), and a meltwater pond (1), and were identified as *Actinobacteria* (8 strains) and *Gammaproteobacteria* (9 strains) (Table 2). Seven isolates inhibitory towards both *V. anguillarum* and *S. aureus* were almost identical in the 16S rRNA gene and closely related to *Arthrobacter davidanieli*. These strains were isolated from different sources, ranging from the deep sea to low-salinity meltwater, at distant geographical sites (Fig. 1, Table 2). Inhibition of both *V. anguillarum* and *S. aureus* was also found with the eighth actinobacterial isolate, tentatively identified as *Brevibacterium* sp. Gammaproteobacterial antagonists comprised three *Pseudoalteromonas s*pp. obtained from different enrichment cultures, having almost identical 16S rRNA gene sequences. In addition, three bioactive *Vibrionaceae* and two strains related to *Psychrobacter nivimaris* were isolated. Two of the three vibrios inhibited both *V. anguillarum* and *S. aureus*, whereas *Pseudoalteromonas* and *Psychrobacter* only inhibited *V. anguillarum* (Table 2).

Metabolite analyses of selected bioactive strains

Seventeen strains with pronounced, reproducible bioactivity were extracted with ethanol from freeze-dried cultures to test whether antibacterial compounds were extractable in organic solvent. All *Arthrobacter* strains and *Brevibacterium* sp. SS12 provided active extracts. The LC-UV/MS profiles of all *Arthrobacter* strains were identical and dominated by a series of large compounds (*m/z* 1000-1100; retention times Rt = 14.92, 16.24, 17.23, 17.75/17.91, 19.22/19.36, 20.55 min) with no characteristic UV absorptions (Fig. 2). Careful dereplication by high-resolution MS and comparison of the data to compounds reported in AntiBase (Laatsch 2010) led to the tentative identification of arthrobacilins A-C, each present as two conformers (Rt = 17.75/17.91 min $C_{54}H_{96}O_{21}$, 19.22/19.36 min $C_{56}H_{100}O_{21}$, and 20.55/20.62 min $C_{58}H_{104}O_{21}$) (Fig. 3) (Ohtsuka et al. 1992), as well as some potential novel analogues (Rt = 14.85/14.92 min $C_{50}H_{90}O_{21}$ and 16.24/16.33 min $C_{52}H_{94}O_{21}$). *Brevibacterium* sp. SS12 showed a unique metabolite profile and had only few

compounds detectable in the extract (Rt = 3.33, 3.92, 9.82, and 18.15 min) (Fig. 3). These peaks matched no previously reported compounds (Laatsch 2010).

Inhibitory spectrum of *Arthrobacter davidanieli* WX11 and *Brevibacterium* sp. SS12

The ethanolic extracts of *Arthrobacter davidanieli* WX11 and *Brevibacterium* sp. SS12 were tested against a larger panel of bacterial strains, comprising human and animal pathogens as well as a pathogenic yeast (Table S2). Extracts from WX11 inhibited a broad range of both Gram-positive and Gram-negative bacterial pathogens, including *B. cereus*, *L. monocytogenes*, *S. aureus* (Gram-positives), *S.* Enteritidis, *A. salmonicida*, *V. vulnificus*, *V. parahaemolyticus*, *V. harveyi*, *Y. enterolitica*, and *Y. ruckeri* (Gram-negatives). In contrast, extracts from SS12 only inhibited Gram-negative bacteria, despite whole colonies were inhibitory towards *S. aureus* when spotted on pathogen-seeded agar. SS12 inhibited *A. salmonicida*, *V. vulnificus*, *V. parahaemolyticus*, *V. harveyi*, *Y. enterolitica*, and *Y. ruckeri*. In addition, SS12 antagonized strains not inhibited by WX11, including *K. pneumonia* and multiresitant *E. coli*.

Discussion

Although 90% of the Earth's oceanic waters are cold (Rodrigues & Tiedje 2008), there is only limited knowledge about bioactive secondary metabolites from polar marine bacteria. In combination with the high culturability of bacteria from sea ice (Brinkmeyer et al. 2003) and the often higher number of psychrotolerant compared to psychrophilic species (Helmke & Weyland 1995), polar habitats therefore represent a promising source of strains with biological and chemical novelty.

The present study adds to the understanding of antagonistic traits in polar bacteria by the investigation of high Arctic marine environments. High culturable counts from sea ice were consistent with previous observations (Brinkmeyer et al. 2003). Interestingly, culturable counts did not significantly vary between rich and low-nutrient media. Our use of rich and low-nutrient media was based on the fact that many bacteria refuse cultivation under high nutrient regimes but become culturable under nutrient limitation (Cho & Giovannoni 2004). Here, most isolates however tolerated both high and low nutrients, and the culturable fraction apparently comprised a large fraction of generalist species. Sampling from polar waters, we also chose a low incubation

temperature but only obtained a marginal fraction of true psychrophilic strains, which was consistent with previous findings (Rodrigues & Tiedje 2008).

Since our experimental approach did not yield the isolation of genera commonly found in Arctic marine environments, these are likely not capable of antagonism, at least not under the conditions applied in this study. The isolation of a bioactive *Arthrobacter* species from diverse, distant habitats indicated a ubiquitous distribution in the marine Arctic. While *Arthrobacter* has been found in polar marine environments (Bowman et al. 1997, Junge et al. 1998), this is the first study describing isolation over large spatial scales. The metabolic versatility and resistance of *Arthrobacter* spp. against environmental stresses (Mongodin et al. 2006) likely contributed to the widespread occurrence, ranging from high pressure (deep sea), as well as low (meltwater) and high (sea ice) salinities. The bioactivity of the species may further facilitate the colonization of different niches.

Terrestrial *Arthrobacter* spp. are known to produce bioactive compounds (Carnio et al. 1999), including quinolone antibiotics (Kamigiri et al. 1996). The siderophore arthrobactin was shown to transport antimicrobials into foreign cells when attached to synthetic beta-lactam conjugates (Ghosh & Miller 1993), but it is possible that a related process could enable environmental *Arthrobacter* spp. to introduce self-produced antimicrobials into competing cells. Bioactive *Arthrobacter* have also been isolated from aquatic habitats, including polar environments (Lo Giudice et al. 2007, Rojas et al. 2009), sponges (Hentschel et al. 2001), macroalgae (Wiese et al. 2009) and aquaculture facilities (Li et al. 2006). However, there is no detailed knowledge about the bioactive compound(s). Here, we tentatively identified three cyclic glycolipids, arthrobacilin A-C, from *Arthrobacter davidanieli* based on accurate mass as well as spectroscopic and chromatographic properties. We also observed two potentially novel analogues ($C_{52}H_{94}O_{21}$ and $C_{50}H_{90}O_{21}$) that are interesting candidates for full structural characterization. Arthrobacilins have previously been isolated from soil *Arthrobacter* sp. and reported as having weak cytotoxic effects against human cancer cell lines (Ohtsuka et al. 1992). It is likely that these compounds are also responsible for the antibacterial activities observed in the present study. A report of reduced mortalities from bacterial disease in salmonid fish by vaccination with live *Arthrobacter davidanieli* (Salonius et al. 2005) was also potentially related on arthrobacilins.

Also the other genera isolated in the present study are known to comprise antibiotic producer species. Terrestrial *Brevibacterium* sp. of industrial relevance produce large proteinaceous bacteriocins (Kato et al. 1991, Valdes-Stauber & Scherer 1994, Maisnier-Patin & Richard 1995).

Here, we show that the genus also produces bioactive metabolites with low molecular weight. Future work will concern the purification and structural elucidation of the unknown compounds.

Pseudoalteromonas sp. B201 was previously isolated from a Baltic sea bryozoan (Herndl et al. 2010), and our results show a geographically wider distribution of this bioactive strain. A number of bioactive compounds have been described in *Psychrobacter* (Li et al. 2008), while this is the first report linking *Psychrobacter* bioactivity to a specific species. Also marine *Vibrionaceae* comprise antagonistic traits (Long & Azam 2001), but were so far mainly reported in strains from warmer waters (Gram et al. 2010, Wietz et al. 2010). Interestingly, antagonistic *Vibrio* spp. from the Arctic were closely related to warm-water bioactive strains (Gram et al. 2010, Herndl et al. 2010), indicating that bioactive potential among vibrios may be limited to a few related species that are widespread in oceanic environments.

In summary, the present study showed the presence of bioactive bacteria in different Arctic marine environments. The ubiquity of bioactive species throughout the marine Arctic suggested that antagonistic traits may be of ecological importance and could provide a selective advantage in niche colonization. Secondary metabolite analyses indicated the presence of known and novel bioactive compounds that will be further investigated for biomedical potential. This adds to the understanding of marine and extreme environments as a potential source of biotechnologically relevant microorganisms.

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Table 1. Culturable counts (CFU mL^{-1}) of 58 Arctic samples using a series of different cultivation conditions.

Sample	No	Marine agar		Chitin agar		Low-nutrient agar		Actino-bacteria	Low-nutrient Actinobacteria	Mean/sample type
		20 °C	5 °C	20 °C	5 °C	20 °C	5 °C	20 °C	15 °C	
Ice	20	3.7×10^{4}	4.4×10^{4}	3.8×10^{4}	4.1×10^{4}	2.9×10^{4}	4.4×10^{4}	3.1×10^{4}	4.3×10^{4}	3.8×10^{4}
Water	18	4.3×10^{2}	3×10^{2}	2.6×10^{2}	1.7×10^{2}	3×10^{2}	8×10^{1}	9.5×10^{1}	2.9×10^{2}	2.4×10^{2}
Zooplankton	10	3.8×10^{2}	4×10^{2}	7.4×10^{2}	2.4×10^{2}	2.1×10^{2}	1.3×10^{2}	2.6×10^{2}	2.5×10^{2}	3.3×10^{2}
Meltwater/snow	7	2.3×10^{1}	1.8×10^{1}	6.4×10^{1}	1.5×10^{1}	1.9×10^{1}	n.a.	1.7×10^{1}	n.a.	3.6×10^{1}
Sediment	3	9.1×10^{1}	1.8×10^{1}	3×10^{1}	n.a.	n.a.	n.a.	n.a.	n.a.	1.7×10^{1}
Mean/agar type		2.2×10^{3}	1.5×10^{4}	1.3×10^{4}	1.6×10^{3}	1.6×10^{4}	1.6×10^{4}	1×10^{4}	1.6×10^{4}	

n.a. – not applicable (no growth)

Table 2 Site of isolation, source, species affiliation and activity spectrum of antagonistic Arctic bacterial isolates when spotted on pathogen-seeded agar.

	Strain	Site	Source	Species	Inhibition of	
					V. anguillarum	*S. aureus*
Actinobacteria	PP12	16	Sea ice	*Arthrobacter davidanieli*	+	+
	SS14	17	Copepods		+	+
	TT4	18	Meltwater		+	+
	ZZ3	18	Sea ice		+	+
	LM7	22	Surface water		+	+
	WX11	26	Deep water		+	+
	MB182	16	Sea ice		+	+
	SS12	17	Copepods	*Brevibacterium* sp.[#]	+	+
Gammaproteobacteria	RR12	17	Amphipods	*Vibrio* spp.	+	−
	EF14	21	Deep water		+	+
	RS9	23	Sea ice		+	+
	XX5	18	Sea ice	*Psychrobacter nivimaris*	+	−
	ST4	24	Sea ice		+	−
	MB33	5	Copepods	*Pseudoalteromonas* B201	+	−
	MB205	19	Surface water		+	−
	MB220	18	Sea ice		+	−
	MB240	20	Sea ice		+	−

[#] Based on short (400 bp) 16S rRNA sequence – resequencing necessary for verification

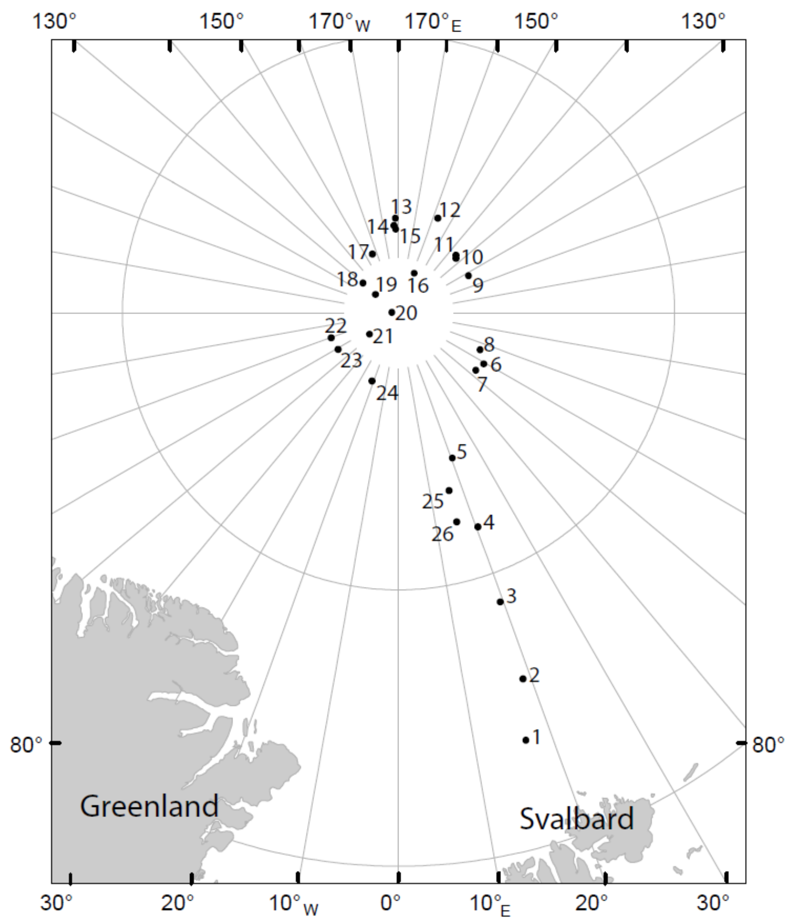


Fig. 1 Sampling sites for isolation of bioactive bacteria.

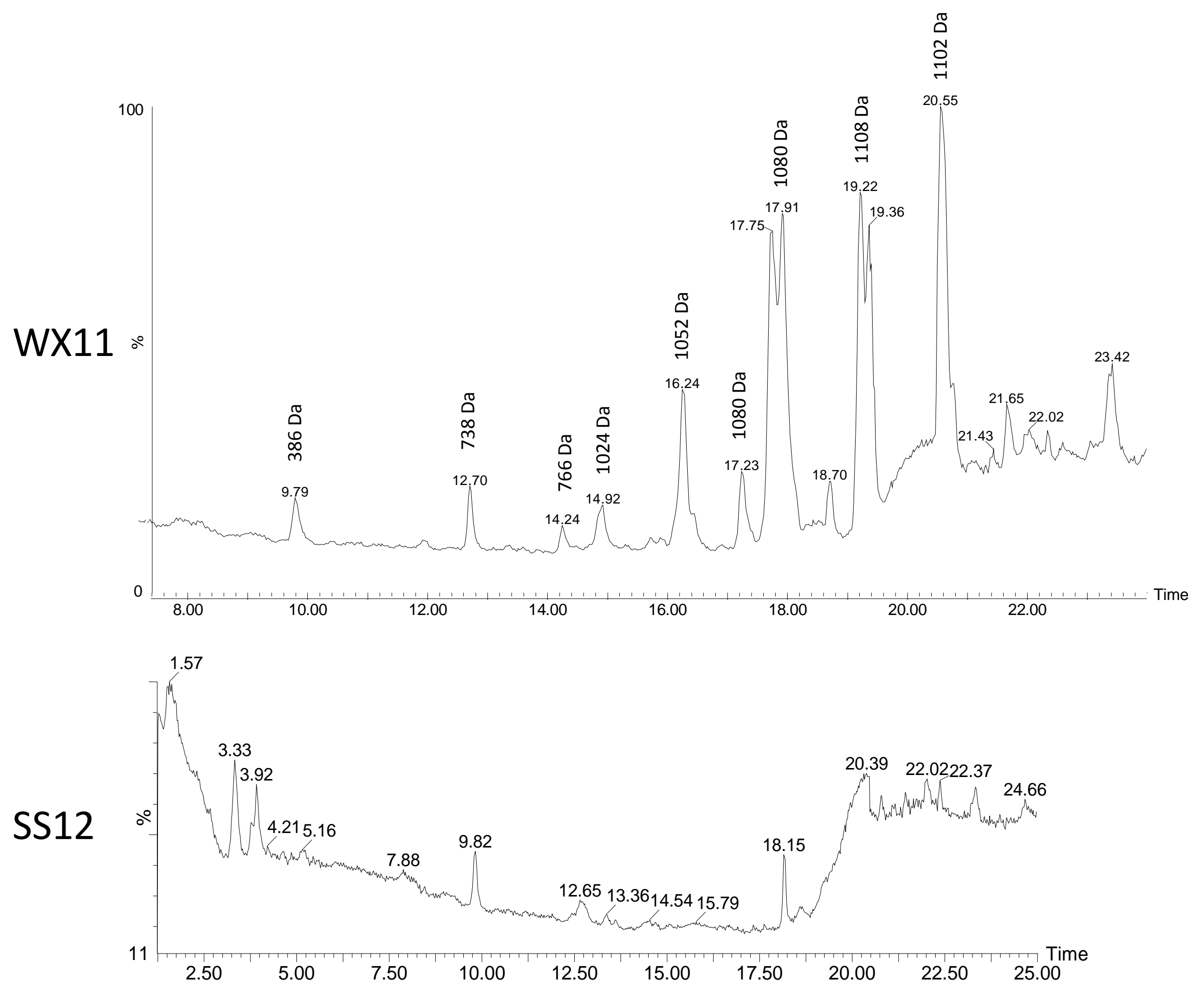
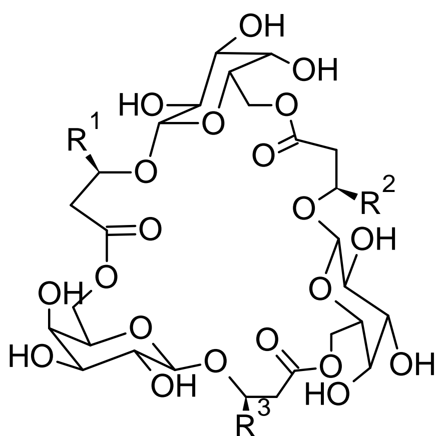


Fig. 2 LC-MS total ion chromatograms (ESI$^+$) of *Arthrobacter davidanieli* (strain WX11 as representative of *A. davidanieli* isolates) and *Brevibacterium* sp. SS12. Peaks corresponding to retention times 17.91 (*m/z* 1080), 19.22 (*m/z* 1108), and 20.55 (*m/z* 1102) minutes in the profile of WX11 were tentatively identified as arthrobacilins A-C.



A: $R^1 = R^2 = R^3 = (CH_2)_8CH_3$

B: $R^1 = R^2 = (CH_2)_8CH_3$, $R^3 = (CH_2)_{10}CH_3$

C: $R^1 = (CH_2)_8CH_3$, $R^2 = R^3 = (CH_2)_{10}CH_3$

Fig. 3 Structure of arthrobacilins A-C.

Table S1 Coordinates and sample types of 26 sampling sites throughout the high Arctic.

Site	Latitude	Longitude	Sample taken
1	81.94083	16.69166	Water
2	83.01258	18.85367	Sea ice
3	84.46333	19.45667	Sea ice (2 samples)
4	85.87666	20.46566	Zooplankton
5	87.19650	20.52900	Zooplankton (3 samples)
6	88.19916	59.13233	Zooplankton (2 samples), water
7	88.25067	53.62550	Sea ice
8	88.37566	65.7252	Sea ice
9	88.55883	117.87066	Water (3 samples)
10	88.56533	133.26783	Water (3 samples), zooplankton
11	88.53008	134.83075	Sea ice
12	88.14666	157.12316	Water (3 samples)
13	89.22831	157.9785	Sea ice, meltwater, snow
14	88.29400	–178.4500	Sediment (3 samples)
15	88.49328	–178.4085	Sea ice
16	88.42214	–177.2886	Sea ice, meltwater
17	88.84483	–156.27416	Zooplankton (2 samples)
18	89.47536	–128.51161	Sea ice (3 samples), meltwater, snow
19	89.1677	–130.2940	Water (3 samples)
20	89.88600	–95.59417	Sea ice (4 samples), meltwater, snow
21	89.35416	–53.40016	Deep water
22	88.72933	–58.67183	Water (2 samples)
23	88.70621	–69.74980	Sea ice (2 samples)
24	88.68383	–20.95466	Zooplankton
25	86.08367	15.64067	Sea ice, dirty ice
26	86.6630	15.9375	Deep water

Table S2. Strains used for bioactivity testing of Arctic isolates.

Human pathogens	Characteristics
Bacillus cereus	Human pathogen; food-borne
Listeria monocytogenes Scott A	Human pathogen; food-borne
Staphylococcus aureus 8325	Human pathogen; nosocomical
Escherichia coli ATCC 25922^{T}	Control strain for susceptibility testing
E. coli AAS-EC-009	Multiresistant clinical pathogen
Klebsiella pneumoniae ATCC 13883^{T}	Human pathogen
Pseudomonas aeruginosa O1	Human pathogen
Salmonella Enteritidis	Human pathogen; food-borne
Serratia marcescens ATCC 8100	Human pathogen, nosocomical
Vibrio parahaemolyticus ATCC 17802^{T}	Human pathogen; food poisoning
Vibrio vulnificus ATCC 27562^{T}	Human pathogen
Yersinia enterolitica	Human pathogen; food-borne
Candida albicans	Pathogenic yeast
Animal pathogens	
Aeromonas salmonicida	Fish pathogen
Vibrio anguillarum	Fish pathogen
Vibrio harveyi	Shrimp and lobster pathogen
Yersinia ruckeri	Fish pathogen

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Paper 3

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Antibacterial Compounds from Marine *Vibrionaceae* Isolated on a Global Expedition

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Abstract: On a global research expedition, over 500 bacterial strains inhibitory towards pathogenic bacteria were isolated. Three hundred of the antibacterial strains were assigned to the *Vibrionaceae* family. The purpose of the present study was to investigate the phylogeny and bioactivity of five *Vibrionaceae* strains with pronounced antibacterial activity. These were identified as *Vibrio coralliilyticus* (two strains), *V. neptunius* (two strains), and *Photobacterium halotolerans* (one strain) on the basis of housekeeping gene sequences. The two related *V. coralliilyticus* and *V. neptunius* strains were isolated from distant oceanic regions. Chemotyping by LC-UV/MS underlined genetic relationships by showing highly similar metabolite profiles for each of the two *V. coralliilyticus* and *V. neptunius* strains, respectively, but a unique profile for *P. halotolerans*. Bioassay-guided fractionation identified two known antibiotics as being responsible for the antibacterial activity; andrimid (from *V. coralliilyticus*) and holomycin (from *P. halotolerans*). Despite the isolation of already known antibiotics, our findings show that marine *Vibrionaceae* are a resource of antibacterial compounds and may have potential for future natural product discovery.

Keywords: *Vibrio coralliilyticus*; *Vibrio neptunius*; *Photobacterium halotolerans*; chemotyping; andrimid; holomycin

1. Introduction

Bioactive secondary metabolites are believed to play a key role in microbial interactions by mediating antagonistic activity and intercellular communication [1]. In addition, many microbial natural products have biotechnological potential as antibiotics, biosurfactants, antifungal, or anticancer agents [2]. Sequences of microbial genomes revealed that only a small fraction of the natural product diversity is known, highlighting the potential for finding novel bioactive compounds in environmental microorganisms [3]. The need for novel antimicrobials to combat increasing antibiotic resistances in pathogenic bacteria has stimulated the exploration of other than the traditional sources, such as terrestrial actinomycetes or fungi [4].

The marine environment harbors bacteria with antagonistic traits [5,6], and marine microorganisms are a potential source of novel antimicrobials [7]. Antagonistic marine bacteria have been isolated from surface [8] and deep waters [9], but the majority originated from biotic surfaces such as sponges [10], zooplankton and macroalgae [8,11], corals [12], and bryozoans [13]. Bioactive bacterial strains predominantly belong to *Pseudoalteromonas* spp. [14], the *Roseobacter* clade [15], and *Actinobacteria* [16]. A number of marine-derived antimicrobials have been characterized in greater detail, including halogenated [17] and sulfuric [18] compounds, depsipeptides [19] and lipopeptides [20], glycolipids [21], as well as high molecular weight structures such as amino acid oxidases [22].

Also the *Vibrionaceae* family, Gram-negative *Gammaproteobacteria* ubiquitous in marine and brackish environments [23], harbors strains with antagonistic activity [8]. The family comprises eight genera, with *Vibrio* and *Photobacterium* constituting the majority of species. To date, *Vibrionaceae* have primarily been investigated due to their pathogenic potential to humans and aquatic animals, but they also occur in commensal or symbiotic associations with eukaryotic organisms [23]. While the abundance of *Vibrionaceae* in nutrient-rich microenvironments such as chitinous zooplankton is potentially related to a superior nutrient utilization based on their metabolic versatility [24], antagonism of competing bacteria through production of antimicrobial compounds may also contribute to a selective advantage. Antimicrobials from *Vibrio* spp. can reduce the number of other microbial community members and influence microscale variations in competing bacterial populations [6]. Antibacterial activities have been described from *V. alginolyticus* [25], *V. parahaemolyticus* [26], *V. anguillarum* [27], and several unidentified *Vibrio* spp. [28,29]. However, the nature and frequency of antagonism among vibrios is still largely unknown, and only a few antibiotic *Vibrio* compounds have been structure elucidated to date [30,31].

The present study describes the analysis of bioactive *Vibrionaceae* strains collected during a global marine expedition [8]. The purpose was to (i) provide phylogenetic and chemical analyses of the strains with strongest antibacterial activity; (ii) characterize their bioactivity depending on culture conditions; and (iii) isolate and elucidate the structure of bioactive metabolites. We report the

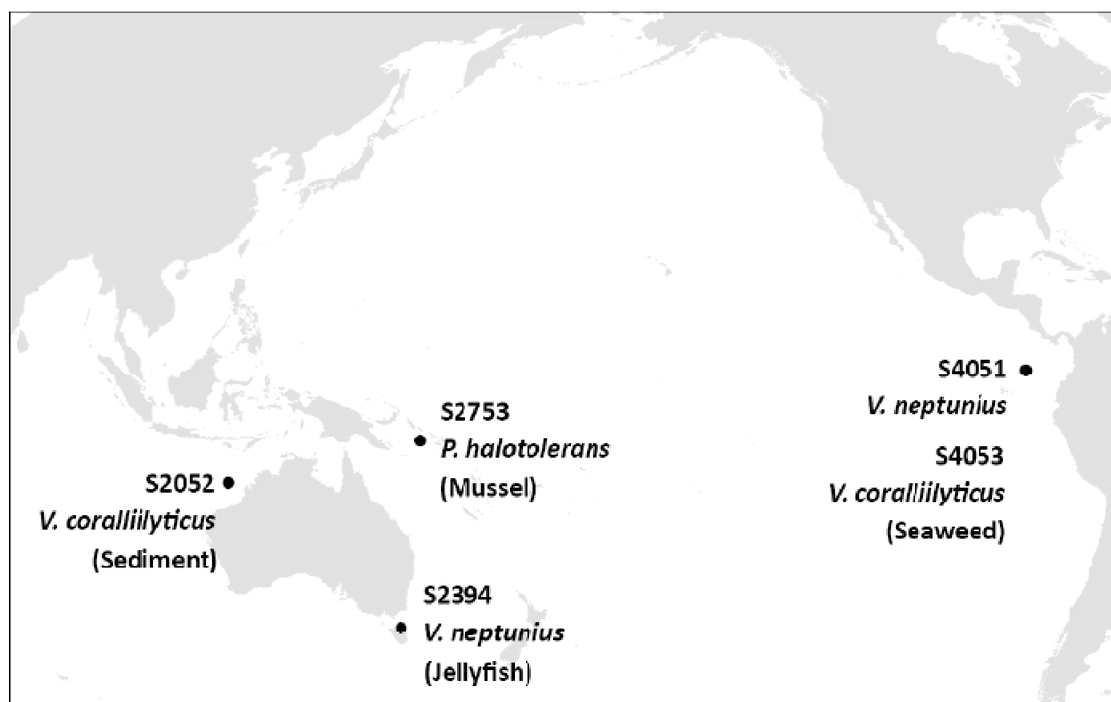
identification of five *Vibrionaceae* strains with pronounced antibacterial activity, the use of chemotyping to support genetic identification, and the structures of two antibacterial compounds.

2. Results and Discussion

2.1. Selection of Strains with Pronounced Antibacterial Activity

Three hundred and one *Vibrionaceae* strains were isolated during a global marine expedition (http://www.galathea3.dk/uk) based on their ability to antagonize the fish pathogen *Vibrio anguillarum* strain 90-11-287 [8]. After being stored at −80 °C for between six and 12 months, all strains were retested for antibacterial activity against *V. anguillarum* strain 90-11-287 and the human pathogen *Staphylococcus aureus* strain 8325 by spotting colony mass on pathogen-seeded agar [8]. Activity was assessed by the formation of clearing zones around spotted colony mass. From 301 strains, only 138 retained antibacterial activity, being a small fraction compared to other antagonistic marine bacteria [32,33]. One hundred strains causing pronounced inhibition (diameter of clearing zones larger than 10 mm) were retested using the same set-up, resulting in a subselection of 39 strains with reproducible strong antibacterial activity when spotted on pathogen-seeded agar. This subselection was inoculated in liquid cultures and extracted with ethyl acetate to determine if antibacterial compounds were extractable with organic solvent. Activity was seen in ethyl acetate extracts from five strains, which were selected for further analyses. The five bioactive strains originated from different surface samples collected in distant oceanic regions (Figure 1).

Figure 1. Site of isolation, source, and species identification of five bioactive marine *Vibrionaceae*. Strains were identified to the species level by sequence analysis of several housekeeping genes (see below).



2.2. Phylogenetic Identification and Chemotyping of Strains

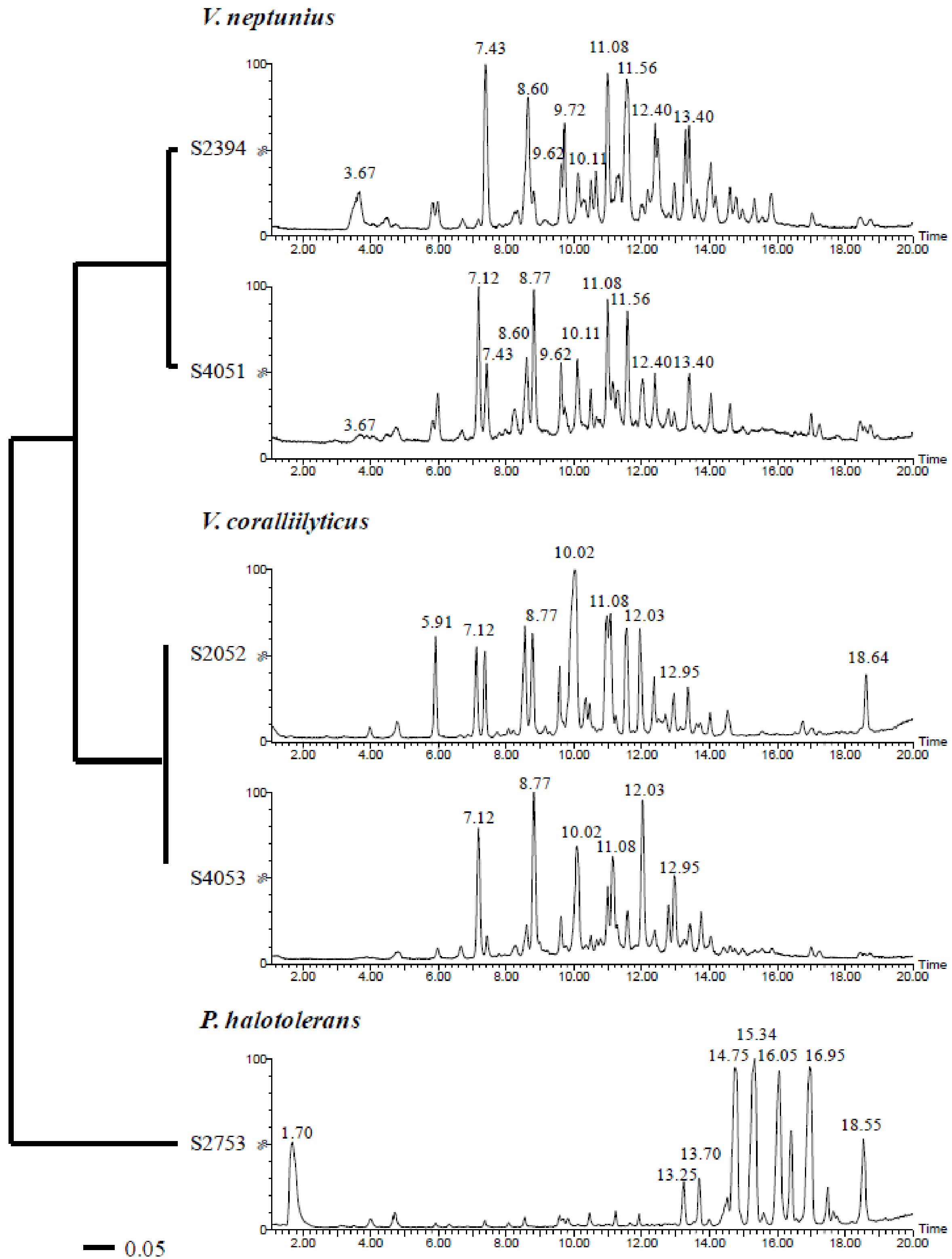
All strains investigated in the present study had previously been assigned to the *Vibrionaceae* family based on 16S rRNA gene similarities [8]. However, the 16S rRNA gene is highly conserved among the *Vibrionaceae* and is not well suited for identification to the species level [34]. Therefore, additional sequence analyses of three housekeeping genes (*recA*, *rpoA*, and *toxR*) were performed. These genes encode constitutively expressed proteins and are suitable for phylogenetic studies of *Vibrionaceae* [34,35]. On the basis of *recA* and *rpoA* sequence similarities, strains S2052 and S4053 were identified as *Vibrio coralliilyticus*, S2394 and S4051 as *Vibrio neptunius*, and S2753 as *Photobacterium halotolerans* (Figure 1). The *toxR* gene was less suited for general species identification due to its high variability even in closely related vibrios, as well as comparatively few *toxR* sequence data available in public gene libraries [36]. However, multiple alignments and neighbor-joining analyses of *toxR* sequences provided the best phylogenetic resolution for determining the relationship between the five strains (Figure 2). The usefulness of *toxR* for species discrimination was consistent with previous reports [35]. LC-UV/MS metabolite profiles underlined the close relationship between *V. coralliilyticus* S2052/4053 and *V. neptunius* S2394/4051, respectively. The evolutionary distance of *P. halotolerans* S2753 to the other strains was reflected by a unique metabolite profile (Figure 2). All five strains were consistent in their metabolite production in separate cultivations over a one-year interval.

Several metabolites were produced by all *V. coralliilyticus* and *V. neptunius* strains, for instance those related to the peaks at retention times Rt = 11.08 and 12.03 min (Figure 2). Although they are different species, *V. coralliilyticus* and *V. neptunius* are closely related vibrios with only 2–3% sequence variation in the *recA* and *rpoA* genes (data not shown), signifying why biosynthetic pathways are shared between the species. Based on their molecular formulas, UV, and MS characteristics [37], most of the metabolites produced by both species were assigned as smaller peptides (*m/z* 300–500), a class of molecules commonly produced by marine culturable bacteria [38,39]. Despite the presence of shared metabolites between *V. coralliilyticus* and *V. neptunius*, clearly distinguishable peaks were seen as well. For instance, the major peak at retention time Rt = 10.02 min (MW 479 Da) was only seen in the two *V. coralliilyticus*, and the peak at Rt = 10.11 min (MW 493 Da) only in the two *V. neptunius* strains.

The metabolites produced by *P. halotolerans* S2753 comprised a series of larger peptides (*m/z* 500–900) [40]. The large peak at Rt = 1.70 min (MW 213 Da) displayed a unique UV spectrum characteristic of that of a highly conjugated system. However, this peak could not be ascribed to any known compound or compound class based on LC-UV/MS data alone.

Several metabolites (Rt = 4.70, 7.41, 8.60, 9.60, and 10.50 min) were found in all five strains and assigned as poly-β-hydroxybutyric acid polymers (PHB) of varying lengths (repeating unit n = 86 Da). This was verified by NMR for some of the compounds (data not shown). PHB are common bacterial storage compounds accumulated when growing on an excess carbon source [41].

Figure 2. Phylogenetic and chemical relationship between five bioactive *Vibrionaceae* based on neighbor-joining analyses of aligned *toxR* gene sequences and LC-MS Total Ion Chromatograms (TIC). The scale bar relates to the number of base substitutions in *toxR* gene sequences (as displayed by branch lengths in the phylogenetic tree).



Chemotyping of prokaryotes has mostly been restricted to analyses of fatty acids and sugars [42], but we show that also the profiling of small molecules can be used for species discrimination. This highlights the usefulness of metabolomics for bacterial classification, adding to recent work of whole-cell laser desorption MALDI-TOF mass spectrometry for characterization of vibrios [43] and secondary metabolite profiling to assess the biosynthetic potential of marine *Pseudoalteromonas* [32]. While our study is limited to the analysis of only three species from the *Vibrionaceae* family, the isolation of two genetically and chemically closely related "strain siblings" from distant oceanic regions indicated that production of certain secondary metabolites is a preserved trait. Similar secondary metabolite profiles were also shown for marine actinomycetal *Salinispora* spp. [44] from distant habitats. Also, all *Ruegeria mobilis* strains from worldwide locations produced the same antibiotic, tropodithietic acid [33].

2.3. Bioassay-Guided Identification of Antibacterial Compounds

V. coralliilyticus (strains S2052 and S4053) and *P. halotolerans* (S2753) inhibited both *V. anguillarum* and *S. aureus*, whereas *V. neptunius* (strains S2394 and S4051) only inhibited *V. anguillarum* (Table 1). Antibacterial activity was highest in aerated cultures and detected after one, three, and five days of incubation. No significant difference in activity was seen between the tested culture media.

Table 1. Inhibition of *V. anguillarum* strain 90-11-287 and *S. aureus* strain 8325 by ethyl acetate extracts from five marine *Vibrionaceae*. Antibacterial activity is displayed by the diameter of clearing zones (−: no activity; +: between 0 and 15 mm; ++: between 15 and 30 mm; +++: over 30 mm).

		Inhibition of	
Strain	**Species**	***V. anguillarum***	***S. aureus***
S2052	*V. coralliilyticus*	+++	++
S2394	*V. neptunius*	++	−
S2753	*P. halotolerans*	+++	++
S4051	*V. neptunius*	++	−
S4053	*V. coralliilyticus*	++	+

The finding of bioactivity among marine *Vibrionaceae* underlined marine microorganisms being a source of antimicrobials. To our knowledge, none of the species investigated here have previously been studied with respect to their secondary metabolome including antibacterial compounds.

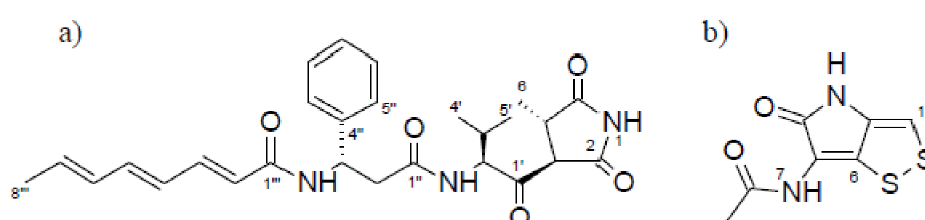
To identify the compounds responsible for the observed activity, large-scale cultivations and fractionations were undertaken for *V. coralliilyticus* S2052 and *P. halotolerans* S2753, representing two distant *Vibrionaceae* species with different metabolite profiles. All fractionation steps were guided by activity testing against *V. anguillarum* strain 90-11-287.

Initial dereplication of S2052 by LC-UV/MS [37] and explorative solid-phase extraction (E-SPE) [45] indicated that andrimid (Rt = 10.02 min; Figure 2) could be responsible for the antibacterial activity. This compound (Figure 3a), a hybrid nonribosomal peptide-polyketide antibiotic, was first described from an insect endosymbiont [46] and later found in other microbial species [47,48] including marine

vibrios [29,31]. Pure andrimid was isolated for NMR analysis, and our data was in accordance with literature data [47]. Andrimid acts as an acetyl-CoA carboxylase inhibitor [49], and we extended its broad antibiotic spectrum [50] by showing inhibition of the bacterial pathogens *Salmonella* Enteritidis, *Bacillus cereus*, *Yersinia enterolitica*, *Yersinia ruckeri*, *Vibrio harveyi*, and *Vibrio vulnificus* (data not shown). Production of andrimid was also confirmed for the other isolated *V. coralliilyticus* strain, S4053. We furthermore speculate whether a recent report of antagonism in the *V. coralliilyticus* type strain [12] was also attributed to this compound. Previous studies have revealed almost identical andrimid gene clusters and a transposase pseudogene in two producer species, suggesting horizontal gene transfer as the most likely explanation behind the cosmopolitanism of the antibiotic [51]. We hypothesize that such transfer is also the reason for its presence in *V. coralliilyticus* S2052 and S4053. Our study is the first linking andrimid production to a specific *Vibrio* species, with production occurring in two strains isolated from very different geographical regions and sources.

The antibacterial compound of *P. halotolerans* S2753 was identified as holomycin (Rt = 1.70 min; Figure 2), a compound belonging to the pyrrothine class of antibiotics acting by interference with RNA synthesis [52]. Our NMR data (Figure 3b) was consistent with previous reports [53]. Holomycin has until now only been found in Gram-positive *Streptomyces* [54,55], and the present study is the first demonstrating production of this antibiotic in a Gram-negative heterotrophic bacterium. While parallel evolution of this trait is possible, horizontal gene transfer is the more likely explanation for its occurrence in both *Vibrionaceae* and actinomycetes. We extended the broad-spectrum activity of holomycin [52] by showing inhibition of the bacterial pathogens *Listeria monocytogenes*, *Serratia marcescens*, *S.* Enteritidis, *B. cereus*, *Y. enterolitica*, *Y. ruckeri*, *V. harveyi*, *V. vulnificus* and *V. parahaemolyticus*, as well as of several marine strains from the *Roseobacter* and *Pseudoalteromonas* groups (data not shown).

Figure 3. Structures of andrimid (**a**) and holomycin (**b**) isolated from marine *Vibrionaceae*.



Neither andrimid nor holomycin were produced by *V. neptunius* S2394 and S4051, and further fractionation and purification is needed to identify the compound(s) responsible for their antibacterial activity. Interestingly, *V. neptunius* S4051 and *V. coralliilyticus* S4053 were isolated from the same seaweed sample, showing that two antagonistic *Vibrio* species whose antibacterial activity is based on different compounds co-occur in the same microenvironment. Moreover, the same sample also contained an antibiotic-producing *Pseudoalteromonas* strain [8].

Two of the antagonistic *Vibrionaceae* species harbor pathogenic strains, with *V. coralliilyticus* being pathogenic to corals [56] and *V. neptunius* being pathogenic to oysters [57]. While we do not know whether *V. coralliilyticus* S2052 has pathogenic potential, the *V. coralliilyticus* type strain has both antagonistic and pathogenic traits [12]. Hence, our results suggest that some vibrios possess a

dual physiology, being antagonistic against other prokaryotes but pathogenic towards higher organisms. Moreover, the production of antibiotics in several species suggests that these compounds may be of ecological importance [1].

This study highlights one of the challenges in natural product discovery. Despite major screening efforts for novel antimicrobials to be used in pharmaceutical, food, and aquaculture industries, only a limited amount of compounds have been discovered in recent years [58]. While the isolation of culturable bacteria remains a promising approach [42] and the secondary metabolome of marine vibrios has not been extensively studied, we only isolated known compounds despite careful dereplication prior to any compound purification. Dereplication is apparently troubled by the high degree of gene transfer between distantly related bacteria such as Gram-positive actinomycetes and Gram-negative *Proteobacteria* [3]. Many compounds in natural product databases such as AntiBase [59] have similar masses (<5 ppm difference), so even the combination of UV/VIS spectra, accurate mass data (<5 ppm), and E-SPE [45] is not sufficiently discriminatory for these organisms. To avoid isolation of redundant chemistry, dereplication by NMR [60] or ultra high-resolution mass spectrometry (<1 ppm) with high isotope accuracy ratios for correct elementary composition determination [61] is imperative to exclude previously isolated compounds.

3. Experimental Section

3.1. Isolation of Bioactive Marine Vibrionaceae

During a global research expedition (http://www.galathea3.dk/uk), marine bacterial strains were isolated from environmental samples and screened for antagonistic activity against a pathogenic *Vibrio anguillarum*, strain 90-11-287. Three hundred and one bioactive strains were identified as *Vibrionaceae* based on 16S rRNA gene similarities [8]. Pure cultures of strains were stored in cryoprotectant solution at −80 °C until being analyzed in the present study.

3.2. Selection of Strains with Pronounced Antibacterial Activity

All 301 strains were retested for antibacterial activity by spotting colony mass on agar seeded with either *V. anguillarum* strain 90-11-287 or *S. aureus* strain 8325. Activity was assessed by the formation of clearing zones around spotted colony mass. Selected active strains were grown both stagnant and aerated (200 rpm) in 30 mL Marine Broth 2216 (Difco 279110) for 3 days at 25 °C in 250 mL glass bottles. Cultures were extracted with an equal volume of HPLC-grade ethyl acetate (EtOAc) for 30 min. The organic phase was transferred to fresh sample vials and evaporated under nitrogen until dryness. Extracts were redissolved in 1 mL of EtOAc and stored at −20 °C until further analysis. EtOAc extracts were tested in a well diffusion agar assay [62] for activity against *V. anguillarum* strain 90-11-287 and *S. aureus* strain 8325.

3.3. Phylogenetic Analysis

Genomic DNA was extracted from 1-day cultures using the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. PCR for *recA* and *toxR* gene fragments was performed according to [35], and PCR for *rpoA* gene fragments according to [34]. PCR products

were checked by agarose gel electrophoresis and purified using the Wizard PCR Preps DNA Direct Purification System (Promega, Madison, USA) according to the manufacturer's instructions. Obtained nucleotide sequences were edited using Chromas Lite (Technelysium, Australia) and aligned to its closest sequence relative [36]. The phylogenetic relationship between the five isolates was determined by neighbor-joining analyses (1000 bootstrap replicates) of nucleotide and amino acid alignments (translated using EMBOSS Transeq, http://www.ebi.ac.uk/Tools/emboss/transeq/) done in ClustalX. Gene sequences have been deposited at GenBank under the accession numbers HQ452614–452618 (*toxR*), HQ452619–452623 (*recA*), and HQ452624–452628 (*rpoA*).

3.4. Influence of Culture Conditions on Bioactivity

The five strains with strongest antibacterial activity (S2052, S2394, S2753, S4051, and S4053) were grown both stagnant and aerated (200 rpm) at 25 °C in either Marine Broth (MB) or Marine Minimal Medium [63] containing 0.4% glucose and 0.3% casamino acids (MMM). Per strain and culture condition, three bottles were inoculated with 30 mL of medium each, of which each one was sampled after 1, 3, and 5 days of incubation. In addition, strains were grown in 30 mL sea salt solution (Sigma S9883; 40 g L^{-1}) with 0.4% glucose and 0.3% casamino acids for 3 days (200 rpm) at 25 °C. EtOAc extracts were prepared as described above, and tested in a well diffusion agar assay [62] for activity against *V. anguillarum* strain 90-11-287 and *S. aureus* strain 8325.

3.5. Chemotyping

Liquid chromatography-diode array/mass spectrometry (LC-UV/MS) analyses were performed on dried EtOAc extracts redissolved in methanol (MeOH) from all tested culture conditions to visualize the array of produced molecules. In addition, 3-day MMM cultures were extracted and analyzed in biological triplicate. LC-UV/MS was performed on an Agilent 1100 liquid chromatograph with a diode array detector (Agilent, Waldbronn, Germany) coupled to an LCT TOF mass spectrometer (Micromass, Manchester, UK) using a Z-spray ESI source. The separation was done on a Luna II C_{18} column (50 mm × 2 mm, 3 μm) (Phenomenex, Torrance, CA) fitted with a security guard system using a linear gradient starting from 15% acetonitrile (MeCN) in water (H_2O) to 100% MeCN over 20 min at a flow rate of 300 μL min^{-1}. Both MeCN (HPLC grade) and H_2O were buffered with 20 mM HPLC-grade formic acid (FA).

3.6. Isolation and Structural Elucidation of Antibacterial Compounds

Strains S2052 and S2753 were grown in 20 L sea salt solution (Sigma S9883; 40 g L^{-1}) with 0.4% glucose and 0.3% casamino acids for 3 days (100 rpm) at 25 °C. On day 3, sterile Dianion HP20SS resin (Sigma-Aldrich, St. Louis, MO) was added to the broth (12 g of resin L^{-1}). After 24 h, the resin was filtered off and washed with H_2O (2 × 1 L), followed by extraction with MeCN/H_2O (80/20 v/v; 2 × 1500 mL).

For S2052, all organic extracts were pooled, absorbed onto 90 g Sepra ZT C18 (Phenomenex), and dried before packing into a 100 g SNAP column (Biotage, Uppsala, Sweden) with pure resin (10 g) in the base. Using an Isolera flash purification system (Biotage), the extract was subjected to a crude

fractionation using a $MeCN/H_2O$ gradient (flow rate 30 mL min^{-1}) starting with 10% MeCN (10 min, isocratic), increasing to 100% MeCN (25 min) before washing with 100% MeCN (15 min). Fractions were automatically collected using UV detection (210 and 320 nm). The fraction with antibacterial activity (185 mg) was subjected to further purification on a Luna II C_{18} column (250 × 10 mm, 5 µm) (Phenomenex) using a 45–70% $MeCN/H_2O$ gradient (buffered with 20 mM FA, flow rate 5 mL min^{-1}) over 20 minutes on a Gilson 322 liquid chromatograph with a 215 liquid handler/injector (BioLab, Risskov, Denmark). This yielded 7.6 mg of pure andrimid.

For S2753, the $MeCN/H_2O$ extract from Dianion HP20SS extraction was evaporated until dryness on a rotary evaporator. The extract was redissolved in EtOAc, absorbed onto 5 g Isolute diol (Biotage), and added to a glass column with pure diol (95 g). A total of 12 fractions were collected from the diol column (100 g, 20 × 350 mm) ranging from heptane, dichloromethane, EtOAc to pure MeOH, running under gravity. The fraction with antibacterial activity (172 mg, 100% EtOAc) was further separated on the Isolera flash purification system, on Sepra ZT C18 (10 g SNAP) using a $MeCN/H_2O$ gradient (flow rate 12 mL min^{-1}) starting with 5% MeCN increasing to 30% MeCN (12 min), quickly increasing to 100% MeCN (10 min). Fractions were automatically collected using UV detection (210 and 380 nm). Pure holomycin (4.3 mg) was obtained after final purification on a Luna II C_{18} column (250 × 10 mm, 5 µm) (Phenomenex) using a $MeCN/H_2O$ (buffered with 20 mM FA) gradient from 7–37% MeCN over 17 min.

NMR spectra were recorded on a Bruker Avance 800 MHz spectrometer with a 5 mm TCI Cryoprobe at the Danish Instrument Center for NMR Spectroscopy of Biological Macromolecules, using standard pulse sequences. The NMR data used for the structural assignment of andrimid and holomycin were acquired in DMSO-d_6 (δ_H 2.49 and δ_C 39.5 ppm).

Optical rotation was measured on a Perkin Elmer Model 341 polarimeter (Perkin Elmer, Waltham, MA) (α_D at 589 nm).

Andrimid: orange-yellow amorphous solid; UV ($MeCN/H_2O$) λ_{max} 200 (100%), 280 (40%) nm; $[\alpha]_D^{20}$ −62.9° (*c* 0.24, MeOH); ^1H NMR δ_H ppm: 0.75 (3H, d, 6.7 Hz, H-4’), 0.80 (3H, d, 6.7 Hz, H-5’), 1.07 (d, 7.2 Hz, 1H, H-6), 1.78 (3H, d, 6.7 Hz, H-8’’’), 2.30 (m, 1H, H-3’), 2.65 (1H, dd, 14.6, 6.2 Hz, H-2_a’’), 2.77 (1H, dd, 14.6, 8.2 Hz, H-2_b’’), 2.91 (m, 1H, H-4), 3.92 (d, 5.6 Hz, 1H, H-3), 4.62 (dd, 8.4, 5.4 Hz, 1H, H-2’), 5.28 (1H, m, H-3’’), 5.90 (1H, m, H-7’’’), 6.01 (1H, d, 15.2 Hz, H-2’’’), 6.18 (1H, m, H-6’’’), 6.26 (1H, dd, 14.5, 11.4 Hz, H-4’’’), 6.53 (1H, dd, 14.5, 10.0 Hz, H-5’’’), 7.00 (1H, dd, 15.2, 11.4 Hz, H-3’’’), 7.20 (1H, m, H-7’’), 7.29-7.31 (4H, m, H-5’’/H-6’’), 8.11 (1H, d, 8.4 Hz, NH-2’), 8.42 (1H, d, 8.5 Hz, NH-3’’), 11.36 (s, 1H, NH-1); ^{13}C NMR δ_C ppm: 14.5 (C-6), 17.2 (C-4’), 18.3 (C-8’’’), 19.4 (C-5’), 28.1 (C-3’), 39.0 (C-4), 41.9 (C-2’’), 57.8 (C-3), 63.1 (C-2’), 124.2 (C-2’’’), 126.4 (C-5’’), 126.9 (C-7’’), 128.1 (C-4’’’), 128.2 (C-6’’), 131.5 (C-6’’’), 133.4 (C-7’’’), 139.0 (C-5’’’), 139.4 (C-3’’’), 142.9 (C-4’’), 164.3 (C-1’’’), 169.9 (C-1’’), 173.8 (C-2), 180.0 (C-5), 203.9 (C-1’); HRESIMS *m/z* 479.2435 (calcd for $C_{27}H_{33}N_3O_5$, 479.2420).

Holomycin: orange-yellow prisms; UV ($MeCN/H_2O$) λ_{max} 200 (100%), 280 (40%) nm; ^1H NMR δ_H ppm: 2.01 (s, 1H, H-9), 7.04 (s, 1H, H-1), 9.86 (s, 1H, NH-7), 10.69 (s, 1H, NH-3); ^{13}C NMR δ_C ppm: 22.4 (C9), 110.6 (C-1), 115.4 (C-5), 133.7 (C-2), 133.9 (C-6), 167.9 (C-4), 168.8 (C-8); HRESIMS *m/z* 213.9860 (calcd for $C_7H_6N_2O_2S_2$, 213.9871).

4. Conclusions

The present study adds to the knowledge of *Vibrionaceae* bioactivity and physiology by showing a worldwide occurrence of marine strains producing antibacterial compounds. In addition, we underlined that chemotyping can support gene-based species identification and help resolving phylogenetic relationships within a genetically homogenous family such as the *Vibrionaceae*. The discovery of known antibiotics that are also produced by evolutionary distant microbes suggests an involvement of horizontal gene transfer, and indicates that these compounds are fundamental to compete and communicate in the natural habitat. The cosmopolitanism of identical antibiotics has major implications for natural product discovery strategies and stresses the need for careful dereplication in the initial stages of screening. An alternative approach could be the screening for largely untested bioactivities, for instance, interference with quorum sensing or modulation of gene expression.

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Paper 4

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Chitin stimulates production of the antibiotic andrimid in a *V. coralliilyticus* strain.

Manuscript in preparation

Chitin stimulates production of the antibiotic andrimid in a *Vibrio coralliilyticus* strain

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Running title – Andrimid production in *V. coralliilyticus*

Keywords: *Vibrio coralliilyticus*, andrimid, chitin, seaweed, biosynthetic temperature optima, secondary metabolism

Abstract

Vibrio coralliilyticus is a putative coral pathogen in tropical oceans, but also possesses antagonistic traits. We previously reported antibacterial activity in *Vibrio coralliilyticus* strain S2052 based upon the antibiotic andrimid. The purpose of the present study was to determine whether the antibiotic is produced under conditions mimicking natural habitats of vibrios. *V. coralliilyticus* S2052 synthesized andrimid with both chitin and seaweed extracts as sole nutrient source. In laboratory medium, S2052 produced a range of secondary metabolites, including andrimid. With chitin, the biosynthesis of metabolites other than andrimid was largely abolished, and the yield of the antibiotic was two-fold higher. The metabolic focus on andrimid production with chitin indicates that the antibiotic serves an ecophysiological function. Comparison of S2052 with two related *V. coralliilyticus* strains (LMG20984^T and LMG10953) revealed physiological differences between these close relatives. Despite overall similar secondary metabolomes, LMG20984^T and LMG10953 did not produce andrimid, and their optimum biosynthetic temperature was 30 as compared to 25 °C for S2052. Although the true pathogenic potential of S2052 is unknown, we showed that it contains a zinc metalloprotease gene linked to coral disease. Different physiologies of S2052 and closely related strains indicated that *V. coralliilyticus* subspecies may be adapted to different niches.

Introduction

The *Vibrionaceae* are a diverse family of Gram-negative heterotrophic bacteria commonly found in marine and brackish environments. Vibrios contain several commensal and symbiotic species, but are primarily researched due to their pathogenic potential towards man and marine eukaryotes (Thompson et al., 2004). *Vibrio coralliilyticus* is a model organism for the study of coral disease, being responsible for bleaching and tissue lesions in *Pocillipora damicornis* (Ben-Haim et al., 2003b) and the potential aetiological agent of White Syndrome throughout the Indo-Pacific (Sussman et al., 2008). The physiological and genetic background of its virulence (Sussman et al., 2009; Meron et al., 2009) includes a characteristic temperature-dependent pathogenicity which only occurs above 25 °C (Ben-Haim et al., 2003a). Increasing seawater temperatures due to global warming may therefore facilitate outbreaks of coral disease by *V. coralliilyticus* and other

pathogens (Rosenberg and Ben-Haim, 2002). The species is also able to infect crustaceans, rainbow trout (Austin et al., 2005), and bivalve larvae (Ben-Haim et al., 2003a). There is substantial genetic variation between *V. coralliilyticus* strains, indicating the existence of endemic populations in different oceanic regions (Pollock et al., 2010a). Potentially, this could also be reflected in a presence of strains with different niches based on physiological variation, for instance their pathogenicity or metabolic activity.

The *Vibrionaceae* also harbour strains with antibacterial activity (Long and Azam, 2001), and several antagonistic vibrios, including *V. coralliilyticus*, were recently isolated from the marine environment (Gram et al., 2010). *V. coralliilyticus* strain S2052 was isolated from sediment in the tropical Indian Ocean, and antagonized bacterial pathogens by production of the antibiotic andrimid (Wietz et al., 2010). Also the *V. coralliilyticus* type strain has antibacterial activity (Rypien et al., 2010), but it has not been determined whether this was related to the same compound. Andrimid is a hybrid nonribosomal polyketide-peptide antibiotic and effective against a wide range of bacteria (Singh et al., 1997) by interference with fatty acid synthesis (Freiberg et al., 2004). The antibiotic is also produced by other microbes, including an insect endosymbiont (Fredenhagen et al., 1987), an enterobacterium (Jin et al., 2006), as well as marine pseudomonads (Needham et al., 1994) and vibrios (Oclarit et al., 1994; Long et al., 2005). The cosmopolitan occurrence of andrimid is likely related to horizontal gene transfer (Fischbach, 2009).

Many studies of bacterial physiology are conducted using laboratory substrates that do not reflect conditions in the natural habitat. However, laboratory growth conditions can shift the phenotype towards a metabolic state that is potentially unlike the environmental ecology of a microorganism (Palkova, 2004). The source of carbon (Sanchez et al., 2010) and nutrient availability (Demain et al., 1983) can have a substantial influence on secondary metabolism. An excess of nutrients – typically not encountered in the oligotrophic marine environment – can increase the synthesis of storage compounds, for example polyhydroxybutyrates (Chien et al., 2007), but suppress antibiotic production (Doull and Vining, 1990).

Also the presence of a natural growth substrate, such as chitin, can influence bacterial physiology. Chitin is the most abundant biopolymer in the marine environment, particularly found in the exoskeletons of crustaceans and zooplankton (Gooday, 1990), and profoundly linked to the ecology of vibrios. In the human pathogen *V. cholerae*, chitin influences bacterial ecophysiology at multiple hierarchical levels including chemotaxis, biofilm formation, nutrient cycling, pathogenicity, as well as commensal and symbiotic relationships with higher organisms (Pruzzo et

al., 2008). Vibrios are often abundant in chitinous microenvironments (Thompson et al., 2004) due to their ability to utilize chitin as carbon and nitrogen source by secretion of chitinases (Hunt et al., 2008). Vibrios also occur in epiphytic communities of macroalgae, including the brown seaweed *Laminaria* (Laycock, 1974), which is potentially linked to the degradation of algal-derived carbohydrates (Goecke et al., 2010). The utilization of chitinous and algal substrates, possibly combined with the production of antagonistic compounds, could provide vibrios with a selective advantage to colonize biotic surfaces.

The purpose of the present study was to determine whether *V. coralliilyticus* S2052 produces the antibiotic andrimid under conditions mimicking natural habitats of vibrios. Production of andrimid with seaweed extracts and stimulated biosynthesis with chitin suggested an ecological role of the antibiotic. Furthermore, comparison of antibiosis, secondary metabolism and potential pathogenicity traits (Sussman et al., 2008; 2009) in S2052 and two related strains suggested that *V. coralliilyticus* harbours several subspecies with different antagonistic potential and ecophysiological characteristics.

Materials and methods

Preparation of natural growth substrates. Colloidal chitin was prepared from practical grade chitin (Sigma P7170) as follows: 10 g chitin were hydrolyzed in 400 mL ice-cold 37% HCl for 20 min and stirred at 37 °C until clear. The solution was poured into 4 L of dH_2O and placed at 4 °C overnight for settlement of chitin. The supernatant was aspirated and chitin resuspended in 2 L of dH_2O. Chitin was collected by centrifugation (4000 *g* for 12 min) and resuspended in 1 L dH_2O. The pH was adjusted to 7 using KOH pellets. The solution was homogenized for 5 min using an Ultra-Turrax (IKA, Staufen, Germany) and autoclaved. The final concentration of colloidal chitin was determined from a dried (70 °C overnight) subsample. Aqueous seaweed extracts were prepared from the brown macroalgae *Fucus vesiculolus* and *Laminaria saccharina* freshly collected from Danish coastal waters. Algae were cut in strips and homogenized in 8 mL sea salt solution (Sigma S9883, 40 g L^{-1}) per 1 g algae using an Ultra-Turrax (IKA). Extracts were centrifuged (15 min at 2000 *g*) to precipitate larger pieces, filtered through Whatman No 1, and centrifuged again (10 min at 6000 *g*) to remove remaining particles. Extracts were sterilized by filtration (0.2 µm) and stored at 4 °C. The pure algal carbohydrates fucoidan (Sigma F5631; 1% w/v), fucose (Sigma

F8150; 10% w/v), laminarin (Sigma L9634; 2% w/v), and mannitol (Sigma M4125; 10% w/v) were readily dissolved in dH_2O. A 1% alginate solution was prepared by slow addition of alginate (Sigma 05550) and sea salts (40 g L^{-1}) to dH_2O to prevent gelling. All solutions were sterilized by filtration (0.2 µm).

Bacterial strains and growth conditions. *V. coralliilyticus* S2052 (Gram et al., 2010) was inoculated in marine minimal medium (MMM) (Östling et al., 1991) containing (i) 0.2% colloidal chitin, (ii) 10, 25 and 50% of seaweed extracts, and (iii) 0.05, 0.2 and 0.4% of algal carbohydrates. In addition, undiluted (100%) seaweed extracts, as well as stock solutions of fucoidan/alginate (1%) and laminarin (2%) amended with sea salts (40 g L^{-1}), were tested as growth substrate. Furthermore, S2052 was inoculated in MMM containing 0.4% glucose and 0.3% casamino acids, representing laboratory medium. Cultures were grown aerated (200 rpm) at 25 °C for 3 (laboratory medium, chitin, 1% alginate and 50/100% seaweed extract) or 7 days (10/25% seaweed extract). Metabolite production in S2052 was compared to two related strains (LMG20984^T and LMG10953). Cultures were grown aerated (200 rpm) in each 30 mL MMM with 0.4% glucose and 0.3% casamino acids at 15, 20, 25, 30, and 35 °C for 3 days. All strains were furthermore screened by PCR for a *dnaJ* gene fragment conserved in *V. coralliilyticus* (Pollock et al., 2010b) and the presence of a *Vibrio* zinc metalloprotease gene (Sussman et al., 2008).

Andrimid detection. All cultures were extracted with an equal amount of HPLC-grade ethyl acetate for 30 min. The organic phase was transferred to fresh sample vials and evaporated under nitrogen until dryness. The residue was redissolved in 1 mL of ethyl acetate and tested for activity against *V. anguillarum* strain 90-11-287 using a well diffusion agar assay (Hjelm et al., 2004). Blank samples representing all tested culture conditions were analyzed to exclude antibacterial activity by any components of the growth substrates. All active samples were analyzed for the presence of andrimid by liquid chromatography-diode array/mass spectrometry (LC-UV/MS). Extracts were redissolved in methanol and analyzed on an Agilent 1100 liquid chromatograph with a diode array detector (Agilent, Waldbronn, Germany) coupled to an LCT TOF mass spectrometer (Micromass, Manchester, UK) using a Z-spray ESI source. Separation was obtained on a Luna II C_{18} column (50 × 2 mm, 3 µm; Phenomenex, Torrance, CA) fitted with a security guard system using a linear gradient starting from 15% acetonitrile (MeCN) in water (both buffered with 20 mM formic acid) increasing to 100% MeCN over 20 minutes at a flow rate of 0.3 mL min^{-1}. In LC-

UV/MS profiles of seaweed cultures, the bacterial metabolites were camouflaged by a large number of co-extracted algal compounds, but andrimid could be detected by its high-resolution molecular mass using the selective ion-trace (*m/z* 480.25, ESI^+).

Growth kinetics of S2052. S2052 was inoculated at approx. 10^3 cells mL^{-1} in 300 mL MMM with (i) 0.4% glucose and 0.3% casamino acids and (ii) 0.2% colloidal chitin (each in duplicate). Cultures were grown aerated (200 rpm) at 25 °C. Sampling points were set according to growth characteristics in a preliminary test (data not shown). Glucose/casamino acid cultures were sampled after 0, 2, 4, 6, 8, 10, 12, 15, 24, 48 and 72 h, and chitin cultures after 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 48 and 72 h. At each time point, 12 mL of culture were sampled. A serial dilution series was plated on Marine Agar 2216 (Difco 212185) and determination of colony forming units (CFU mL^{-1}) performed after 2 days of incubation at 25 °C.

Andrimid quantification. At each sampling point, the amount of andrimid was quantified directly from sterile-filtered (0.2 µm) culture using ultra-high liquid chromatography-diode array (UPLC-UV) analyses on a Dionex RSLC Ultimate 3000 (Dionex, Sunnyvale, CA) equipped with a diode-array detector. Separation was obtained on a Kinetex C_{18} column (150 × 2.1 mm, 2.6 µm; Phenomenex) maintained at 60 ºC using a linear gradient starting from 15% MeCN in water (both buffered with 50 ppm trifluoroacetic acid) increasing to 100% MeCN over 7 minutes at a flow rate of 0.8 mL min^{-1}. Injection volume was 20 µL. Andrimid (Rt = 4.44 min) was detected from the 302 ± 2 nm chromatogram using quantification by external standard calibration with an NMR validated standard of pure andrimid (Wietz et al., 2010). A standard curve from six different andrimid concentrations (0.06, 0.2, 0.3, 0.45, 0.65, and 2.5 mM) resulted in a linear calibration curve with R^2 = 0.9991. In addition, 5 mL culture from each time point were extracted twice with ethyl acetate (with and without 1% formic acid) to investigate the overall production of secondary metabolites over time. The extracts were pooled and evaporated under nitrogen until dryness. The residue was redissolved in 1 mL methanol and filtered (0.45 µm) before subjected to UPLC-UV analyses as described above using a 3 µL injection volume.

Results

Andrimid production under natural growth conditions

V. coralliilyticus S2052 inhibited pathogenic bacteria by production of the antibiotic andrimid when grown in laboratory medium (Wietz et al., 2010). Here, we investigated growth and andrimid production under natural conditions with chitin, seaweed extracts, or pure algal carbohydrates as sole nutrient source. S2052 grew well in chitin medium, and chitinolytic activity was visually detected as disappearance of the colloidal chitin precipitate. Antibacterial activity and production of andrimid was confirmed by bioassay testing against *V. anguillarum* strain 90-11-287 and LC-UV/MS analyses with comparison to an NMR validated standard. With chitin, S2052 abolished the production of an array of metabolites produced in laboratory medium (Fig. 1) that had been assigned as polyhydroxybutyrates and smaller peptides by NMR spectroscopy (Wietz et al., 2010).

In seaweed media, the type and concentration of seaweed extract influenced growth characteristics and andrimid production. With *Fucus*, only 50 and 100% extract concentration yielded distinct growth. Bioactivity and considerable production of andrimid was detected with 50, but hardly any with 100% extract concentration. With *Laminaria*, high cell densities were reached at 25, 50, and 100%. Bioactivity and production of andrimid was confirmed for all extract concentrations. We tested whether the observed bioactivities reflected an ability to degrade known components from *Fucus* and *Laminaria* likely present in the crude extracts. Therefore, the polysaccharide fucoidan and its main monomer fucose (mainly present in *Fucus*), the polysaccharide laminarin (mainly present in *Laminaria*), as well as the polysaccharide alginate and the monosaccharide mannitol (present in both), were tested as sole carbon source. Distinct growth was only seen with 1% alginate; however, no andrimid was produced as confirmed by bioactivity testing and LC-UV/MS.

Growth and quantification of andrimid in chitin versus laboratory media

In laboratory medium, there was no lag phase and exponential growth started immediately. Andrimid was detected after 10 hours at an approximate culture density of 5×10^{8} cells mL^{-1}. The yield of andrimid (production per cell) peaked after 10 h, reaching approx. 10 pmol per cell. Despite continuing growth between 12 and 24 h, the first decreasing and then stagnant yield illustrated that no additional andrimid was produced (Fig. 2). With chitin, there was a three-hour lag phase before

exponential growth started, and the generation time was longer than in laboratory medium. Andrimid production was detected after 18 h and at a slightly lower culture density (10^7 cells mL^{-1}). The yield peaked after 24 h at over 20 pmol per cell, being two-fold higher than with laboratory medium. Production stopped along with an increase in growth between 24 and 30 h. The slightly decreasing yield towards the end of the incubation period indicated that the antibiotic was partially degraded with time. UPLC-UV analyses of ethyl acetate extracts obtained at every sampling point underlined earlier observations (Fig. 1) in a time-dependent manner, showing that over 72 h very few metabolites other than andrimid were produced when grown with chitin.

Influence of temperature on metabolite production in S2052 and related strains

We compared the production of andrimid and metabolite profiles of S2052 with two related *V. coralliilyticus* strains, LMG20984^T and LMG10953. Since the pathogenic potential (Ben-Haim et al., 2003a) and intercellular chemistry (Boroujerdi et al., 2009) of *V. coralliilyticus* is temperature-regulated, we tested growth at 15, 20, 25, 30, and 35 °C to see whether temperature also influences secondary metabolite and antibiotic production.

All strains grew from 15 to 35 °C, with fast doubling times (estimated 20-30 minutes) above 30 °C. The three strains had almost identical secondary metabolite profiles, but andrimid (peak at 10.01 min) was only produced by S2052. In contrast, another metabolite (peak at 9.87 min) was mainly produced by LMG20984^T and LMG10953 (Fig. 3). S2052 produced andrimid at 15, 20 and 25 °C, but only limited amounts at 30 and not at all at 35 °C. We tested whether the lack of production above 30 °C was related to compound degradation over the 3-day incubation period, yet andrimid was neither detected in cultures incubated for only 12 h. Temperature optima for secondary metabolite production differed between the strains. Higher relative abundances of metabolites revealed that S2052 had highest biosynthetic capacity at 25 °C, while only limited amounts were produced at 30 °C. In contrast, LMG20984^T and LMG10953 had the maximum biosynthetic capacity at 30 °C (Fig. 3). These physiological distinctions questioned the identification of S2052 as *V. coralliilyticus*, however, we verified the species affiliation by successful amplification of a *dnaJ* gene fragment using *V. coralliilyticus* specific primers (Pollock et al., 2010b). The relationship of S2052 to the demonstrated coral pathogens LMG20984^T and LMG10953 was furthermore underlined by amplification of a zinc metalloprotease gene fragment linked to pathogenicity (Sussman et al., 2008; 2009).

Discussion

The *Vibrionaceae* family comprises symbiotic, commensal, pathogenic (Thompson et al., 2004), as well as antagonistic strains (Long and Azam, 2001; Gram et al., 2010). The relatively widespread production of antibiotics in marine vibrios including *V. coralliilyticus* (Wietz et al., 2010) indicated that antagonistic activity may be of ecological importance. To further test this hypothesis, the present study investigated andrimid production in *V. coralliilyticus* S2052 under conditions mimicking natural habitats of vibrios. Antagonism based upon the utilization of natural substrates could facilitate the colonization of biotic surfaces, and contribute to the association of vibrios with zooplankton (Thompson et al., 2004) and macroalgae (Laycock 1974).

Chitinolytic activity is a core function of the *Vibrionaceae* (Hunt et al., 2008), making it a potential reason for their ubiquitous occurrence (Riemann and Azam, 2002). Chitin controls several genetic and physiological characteristics of vibrios (Pruzzo et al., 2008), and the present study showed that it also influences antagonistic activity. The stimulation of antibiotic production by chitin was potentially related to observations in streptomycetes (Rigali et al., 2008), the paradigm of antibiotic-producing bacteria (Hopwood, 1999). The almost complete shut-down of metabolite production except for andrimid indicated that all available carbon was used for its biosynthesis, suggesting that a competitive phenotype was formed that could be of advantage in the natural habitat. We can, however, only speculate about the ecological role of andrimid. The combination of chitinolytism and antibiosis could provide a selective advantage in the colonization of chitinous microenvironments by using andrimid to antagonize competing bacteria. Andrimid could also represent a chemical mean to provide grazing resistance. Such a scenario has been shown for the bioactive bacterial compound violacein, which has both antibacterial and antiprotozoan activity (Matz et al., 2004). The link between chitin and andrimid could also play a role in pathogenicity, comparable to the importance of chitinase activity in *V. cholerae* infections (Kirn et al., 2005). Considering the ubiquity of quorum sensing in vibrios including *V. coralliilyticus* (Tait et al., 2010) we hypothesized that andrimid production and/or chitinolytism would be controlled by such mechanisms. Although quorum sensing can be linked to chitinase activity (Chernin et al., 1998; Defoirdt et al., 2010) we did not detect any signalling molecules using two different microbiological AHL monitors (McClean et al., 1997; Cha et al., 1998) (data not shown).

We hypothesized that variations in growth and andrimid production between *Fucus* and *Laminaria* extracts reflected differing abilities of S2052 to utilize species-specific algal compounds.

Degradation of fucoidan (Furukawa et al., 1992) and laminarin (Alderkamp et al., 2007) has been shown in vibrios, but S2052 was unable to utilize these as sole nutrient source. In contrast, S2052 could utilize alginate, but no andrimid was produced. In combination with missing antibiosis in undiluted compared to diluted *Fucus* extract, this suggested that biosynthesis of the antibiotic is not a constitutive trait, requires a mixture of algal nutrients, and can be influenced by substrate type or concentration.

The almost identical metabolite profiles of S2052, LMG20984^T and LMG10953, together with the presence of a conserved *dnaJ* gene fragment, confirmed that all three strains belong to *V. coralliilyticus*. However, the production of andrimid in S2052 but its absence in LMG20984^T and LMG10953 showed that even such closely related strains can possess considerable physiological variation. Missing production in LMG20984^T furthermore implied that its antagonism against coral-associated bacteria (Rypien et al., 2010) is caused by a compound that is untraceable in our approach. Nevertheless, the increased antibiosis of LMG20984^T at 25 compared to 30°C was potentially related to the observation in S2052. In combination, these results suggest that antagonistic interactions involving vibrios play a greater role at lower temperatures. Stopping andrimid production above 30 °C was consistent with another andrimid-producing *Vibrio* (Long et al., 2005), and potentially related to the fact that the synthesis of polyketides and nonribosomal peptides can be thermoregulated (Rohde et al., 1998).

The increased biosynthetic capacity of the known pathogens LMG20984^T and LMG10953 at higher temperatures was consistent with a previous report using metabolomics (Boroujerdi et al., 2009), and probably reflected that pathogenicity only occurs above 25 °C (Ben-Haim et al., 2003a). The higher metabolite production of S2052 at lower temperatures implied that its pathogenic potential may differ from LMG20984^T and LMG10953. However, the presence of a zinc metalloprotease gene linked to disease signs in corals (Sussman et al., 2008; 2009) contradicted this hypothesis. It has to be noted, though, that the presence of the gene does not necessarily imply pathogenicity, since coral disease such as White Syndrome is likely multifactorial (Sussman et al., 2008). The observation of two different phenotypes – one pathogenic, non-andrimid producing with a temperature optimum of 30 °C (LMG20984^T and LMG10953) and one andrimid-producing with potential pathogenicity and a temperature optimum of 25 °C (S2052) – possibly reflects genetic variability (Pollock et al., 2010a) and differing enzymatic capacities (Ben-Haim et al., 2003a) among *V. coralliilyticus*. Differing physiologies could even relate to geographically separated, endemic populations within the species (Pollock et al., 2010a). Our results suggest that bacterial

taxonomy could benefit from complementation by metabolite analyses (Gevers et al., 2005). The potential of metabolomics for bacterial typing has been exemplified with 12 strains of *E. coli*, showing that all had a different metabolic fingerprint and only shared a core set of compounds (Maharjan and Ferenci, 2005). Also strains from the marine bacterium *Pseudoalteromonas luteoviolacea* could be grouped into subspecies according to their secondary metabolome (Vynne et al., unpublished data).

In conclusion, production of andrimid with seaweed extracts and stimulated biosynthesis with chitin implied that antagonistic traits in *V. coralliilyticus* serve an ecological function. Antagonism based upon the utilization of natural substrates could provide a competitive advantage, facilitate the colonization of biotic surfaces, or play a role in pathogenicity. The presence of different phenotypes among *V. coralliil*yticus indicated the existence of several subspecies with different ecophysiogical characteristics. The variations in antibiosis and biosynthetic temperature optima suggested that closely related *V. coralliilyticus* strains may occupy different niches and respond differently to environmental variation.

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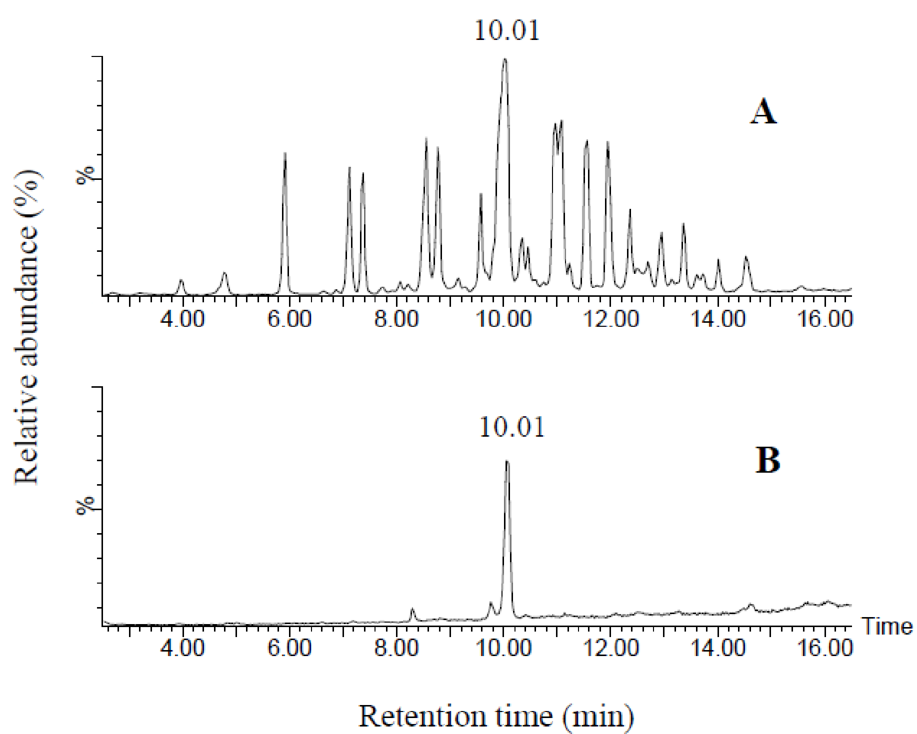

Fig. 1 LC-MS metabolite profiles (total ion chromatogram ESI^{+}) of *V. coralliilyticus* S2052 in laboratory (A) and chitin (B) medium. The peak with retention time Rt = 10.01 relates to andrimid (Wietz et al. 2010b).

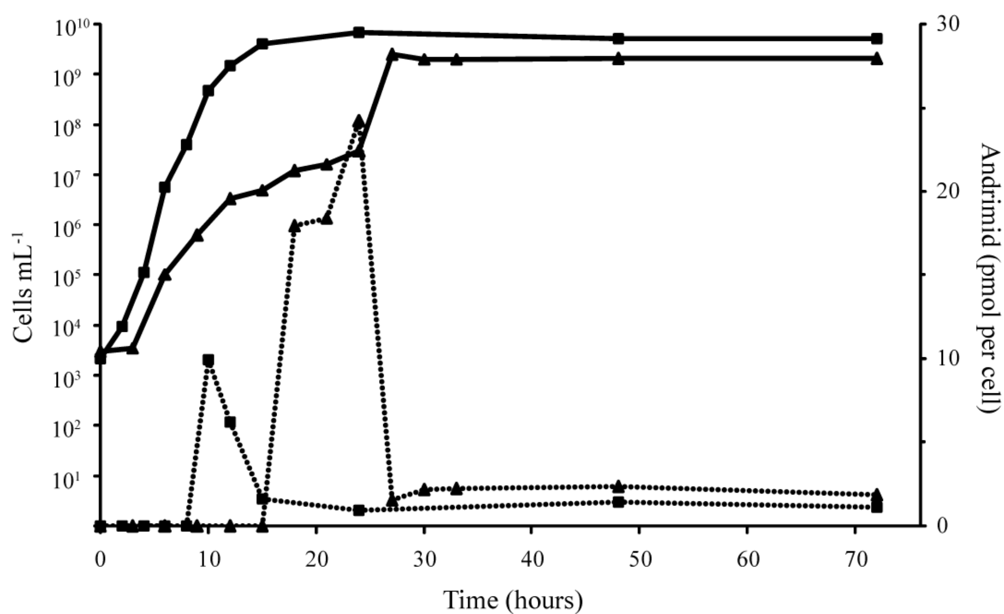

Fig. 2 Growth and production of andrimid in *V. coralliilyticus* S2052 over 72 h in laboratory versus chitin medium. Closed lines: cell density (CFU mL^{-1}), dotted lines: yield of andrimid (pmol per cell) in laboratory (■) and chitin (▲) medium.

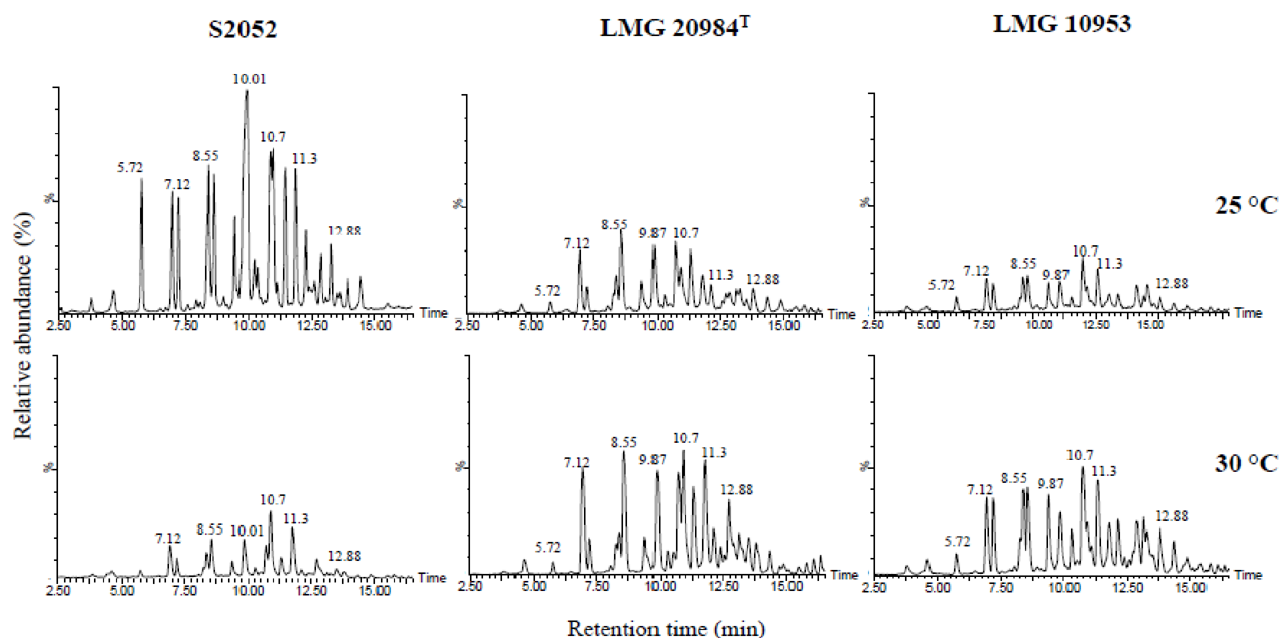

Fig. 3 LC-MS metabolite profiles (total ion chromatogram ESI^{+}) of *V. coralliilyticus* S2052, LMG20984^{T} and LMG10953 grown at 25 and 30 °C.

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