Physiological characterization and molecular ecological investigation of diverse organisms of the *Roseobacter* clade isolated from the North Sea

Physiologische Charakterisierung und molekularökologische Untersuchung verschiedener Isolate der *Roseobacter* Gruppe aus der Nordsee

Von der Fakultät für Mathematik und Naturwissenschaften
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When we try to pick out anything by itself, we find it hitched to everything else in the universe.

John Muir (1911)

Content

List of publications

This thesis includes four manuscripts that are submitted for publication (chapters II.1 and II.3) or are in preparation to be submitted to scientific journals within the next months (chapter II.4 and II.5); the chapters II.2 and II.6 are already published. My contributions to the manuscripts are listed below:

Hahnke, S., Brock, N.L., Zell, C., Simon, M., Dickschat, J.S., and Brinkhoff, T. (2011) Taxonomic and physiological diversity of roseobacters co-occurring during a phytoplankton bloom. To be submitted to *Systematic and Applied Microbiology*.

Sampling: **S.H.** and T.B.; Isolation of bacterial strains and substrate tests: **S.H.**; Phylogenetic analysis: **S.H.** and T.B.; Diversity analysis, screening of *pufML* genes and biogeopraphy studies: **S.H.**; Chemical analyses: N.B. and C.Z.; First draft of manuscript: **S.H.**, Revision: T.B., J.S.D., M.S.

Hahnke, S., Tindall, B.J., Schumann, P., Sperling, M., Brinkhoff, T., and Simon, M. (2011) *Planktotalea frisia*, gen. nov., sp. nov., isolated from the southern North Sea. *International Journal of Systematic and Evolutionary Microbiology*. doi: 10.1099/ijs.0.033563-0.

Physiological experiments: **S.H.** assisted by M.S.; TEM picture: **S.H.** and Heike Oetting; Phylogenetic analysis: **S.H.**; Fatty acid and lipid analyses: B.T.; Determination of G+C content: P.S.; First draft of manuscript: **S.H.**, Revision: B.T., T.B., M.S.

Hahnke, S., Tindall, B.J., Schumann, P., Simon, M., and Brinkhoff, T. (2011) *Pelagimonas elegans* gen. nov., sp. nov., isolated from the southern North Sea. Submitted to *International Journal of Systematic and Evolutionary Microbiology*.

Physiological experiments: **S.H.** assisted by Martin Sperling; TEM picture: **S.H.** and Heike Oetting; Phylogenetic analysis: **S.H.**; Fatty acid and lipid analyses: B.T.; Determination of G+C content: P.S.; First draft of manuscript: **S.H.**, Revision: B.T., T.B., M.S.

Hahnke, S., Tindall, B.J., Schumann, P., Simon, M., and Brinkhoff, T. (2011) *Marianus varius*, gen. nov., sp. nov., isolated from the southern North Sea. To be submitted to *International Journal of Systematic and Evolutionary Microbiology*.

Physiological experiments: **S.H.** assisted by Martin Sperling; TEM picture: **S.H.** and Heike Oetting; Phylogenetic analysis: **S.H.**; Fatty acid and lipid analyses: B.T.; Determination of G+C content: P.S.; First draft of manuscript: **S.H.**, Revision: B.T., T.B., M.S.

Hahnke, S., Sperling, M., Langer, T., Wichels, A., Gerdts, G., Beardsley, C., Brinkhoff, T., and Simon, M. (2011). Distinct seasonal growth patterns of the bacterium *Planktotalea frisia* in the North Sea and specific interaction with phytoplankton algae. To be submitted to *Environmental Microbiology*.

Development of qualitative PCR: M.S., **S.H.**, T.B.; Screening of North Sea transect samples: M.S.; *Roseobacter*-DGGE analysis of samples of Helgoland 2005: M.S.; Development of qPCR approach and CARD-FISH probes and protocol: **S.H.**; Quantitative screening: **S.H.**; Quantification using CARD-FISH and bacterial cell counts of NHS samples: T.L.; Sampling Neuharlingersiel and sample preparation: T.L., **S.H.**; Preparation of DNA samples and bacterial cell counts of Helgoland samples: G.G. and A.W.; Algal experiments: **S.H.**; Measurement of enzyme activities: C.B.; First draft of manuscript: **S.H.**; Revision: M.S.

Wagner-Döbler, I., Ballhausen, B., Berger, M., Brinkhoff, T., Buchholz, I., Bunk, B., Cypionka, H., Daniel, R., Drepper, T., Gerdts, G., **Hahnke, S.**, *et al.* (2009) The complete genome sequence of the algal symbiont *Dinoroseobacter shibae*: a hitchhiher's guide to life in the sea. *ISME Journal* 4: 61 - 77.

Because of the high number of authors, I only list my own contribution for this publication.

Primer and probe design: **S.H.** and T.B.; Development and application of the CARD-FISH protocol: **S.H.**; Phylogenetic analysis: **S.H.** and T.B.

Presentations at national and international symposia:

Hahnke, S., Brinkhoff, T., and Simon, M. (2008) Investigation of the *Roseobacter* clade in the North Sea by culture-dependent and culture-independent approaches. Royal Netherlands Academy of Arts and Sciences, Acadamy Colloqium; Amsterdam, The Netherlands. **Poster presentation**

Hahnke, S., Sperling, M., Simon, M., and Brinkhoff, T. (2009) Seasonal occurrence of an abundant member of the *Roseobacter* lineage in the North Sea. FEMS; Gothenburg, Sweden**. Poster presentation**

Simon, M., **Hahnke, S.**, Giebel, H.-A., Osterholz, H., Sperling, M., and Brinkhoff, T. (2010) Physiology and biogeography of phytoplankton-associated roseobacters. Workshop: The microbial view of marine biogeochemical cycles; Banyuls, France. **Oral presentation**

Hahnke, S., Giebel, H.-A., Sperling, M., Simon, M., and Brinkhoff, T. (2010) Physiology and biogeography of phytoplankton-associated roseobacters. Kick-off Symposium of the Transregional Collaborative Research Center; Delmenhorst, Germany. **Oral presentation**

Langer, T., **Hahnke, S.**, Brinkhoff, T., and Simon, M. (2011) Response of the *Roseobacter* clade to an experimentally-induced *Phaeocystis* bloom. VAAM; Karlsruhe, Germany. **Poster presentation**

Simon, M., **Hahnke, S.**, Giebel, H.-A., Osterholz, H., Simon, H., Sperling, M., and Brinkhoff, T. (2011) Physiology and interactions of phytoplankton-associated roseobacters. SAME; Rostock/Warnemünde, Germany. **Oral presentation**

List of abbreviations

CARD-FISH	Catalyzed Reporter Deposition Fluorescence *In Situ* Hybridization
BAC	Bacterial Artificial Chromosome
bchl *a*	Bacteriochlorophyll *a*
bp	Base Pairs
Chl *a*	Chlorophyll *a*
DAPI	4',6'-Diamino-2-phenylindol
DGGE	Denaturing Gradient Gel Electrophoresis
DMS	Dimethylsulfide
DMSP	Dimethylsulfoniopropionate
dNTP	Didesoxy ribonucleic acid
DSM, DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DOC	Dissolved Organic Carbon
DOM	Dissolved Organic Matter
et al.	et alii (lat. and others)
FISH	Fluorescence *In Situ* Hybridization
FL	Free-living
gen. nov.	genus novum
HPLC	High Performance Liquid Chromatography
ITS	Intergenic Transcribed Spacer
MPN	Most Probable Number
NCBI	National Center for Biotechnology Information
OD_{600}	Optical density at 600 nm
PA	Particle-associated
POC	Particulate Organic Carbon
PSU	Practical Salinity Unit
qPCR	Quantitative Polymerase Chain Reaction
RCA	*Roseobacter* Clade Affiliated
sp. nov.	species nova
TEM	Transmission Electron Micrograph
μ_{max}	Maximum Growth Rate

Summary

One of the major groups of the marine heterotrophic bacterioplankton is the *Roseobacter* clade (order *Rhodobacterales*) within the *Alphaproteobacteria*, which is widespread in marine ecosystems. Roseobacters exhibit a remarkably variable physiology and are able to use a multitude of organic compounds. They were reported to be among the most active bacterial groups regarding glucose and leucine uptake during a *Phaeocystis* bloom in the southern North Sea and during a coccolithophore bloom in the northern North Sea regarding the consumption of the algal osmolyte dimethylsulfoniopropionate (DMSP). Their metabolic activities significantly contribute to processes in the world's biogeochemical cycles and to the microbial loop of marine food webs, i.e. the utilization of dissolved organic material (DOM) fixed by phytoplankton. As many roseobacters can easily be cultivated, they became one of the most intensively studied groups of marine bacteria.

In this study the *Roseobacter* community of a water sample from the open southern North Sea was analyzed, which was collected during a bloom of phytoflagellates. Overall, 12 different *Roseobacter* clade organisms were detected, seven isolates and five phylotypes obtained by denaturing gradient gel electrophoresis (DGGE) and sequencing of excised bands (one phylotype was obtained by both methods). Three of the phylotypes are affiliated with three of the four major clusters of the *Roseobacter* clade, consisting predominantly of uncultured organisms, i.e. the *Roseobacter* clade-affiliated (RCA) cluster, the NAC11-7 and the CHAB-I-5 cluster, respectively. Physiological characterization of the isolates exhibited different substrate spectra of all strains and feeding experiments with [$^2H_6$]DMSP showed that all isolates converted DMSP into a variety of volatile compounds, mainly via the demethylation pathway. Furthermore, biogeography studies revealed that most of the new *Roseobacter* strains and phylotypes have close relatives previously detected in the Yellow Sea (part of the East China Sea), a habitat with remarkable similarities to the North Sea.

Three of the new isolates were characterized as new species of new genera, *Planktotalea frisia*, *Pelagimonas elegans*, and *Marianus varius*. For *P. frisia* strain SH6-1^T specific primers for qualitative and quantitative (q)PCR were developed to investigate the occurrence and abundance of this phylotype in the North Sea. The results revealed very pronounced seasonal patterns of *P. frisia* in the German Bight and a possible correlation with phytoplankton. In laboratory experiments with the phytoplankton algae *Phaeocystis*

globosa and *Leptocylindrus danicus* the bacterial utilization of algal-derived dissolved organic carbon (DOC) was shown.

Furthermore, in a collaboration work with 37 researchers the genome of *Dinoroseobacter shibae* DFL12^T was analyzed. The genome-based hypothesis that *D. shibae* and its host algal *Prorocentrum lima* act symbiotically was confirmed by experimental studies. *D. shibae* provides the growth-limiting vitamins B1 and B12 to its dinoflagellate host and in turn organic substrates excreted by the algal cell support growth of the bacterium. Using strain-specific CARD-FISH probes it was shown that *D. shibae* attaches to the dinoflagellate. This close association can permit the symbiotic interrelation of these organisms in the water column of the natural environment.

Zusammenfassung

Eine der bedeutendsten Gruppen des marinen heterotrophen Bakterioplanktons ist die *Roseobacter* Gruppe der Ordnung *Rhodobacterales* innerhalb der *Alphaproteobakterien*, die in marinen Ökosystemen sehr weit verbreitet ist. *Roseobacter* weisen eine bemerkenswerte Stoffwechselvielfalt auf und können eine Vielzahl an organischen Verbindungen verwerten. Aus einer früheren Studie ist bekannt, dass *Roseobacter* während einer Phaeocystisblüte in der südlichen Nordsee eine der aktivsten Bakteriengruppen bezüglich der Glucose- und Leucinaufnahme waren sowie während einer Coccolithophoridenblüte in der nördlichen Nordsee bezüglich der Verwertung des Algenosmolyts Dimethylsulfoniopropionat (DMSP). Durch ihre Stoffwechselaktivitäten wirken sie erheblich an den Prozessen der globalen biogeochemischen Kreisläufe mit sowie im *microbial loop* der marinen Nahrungsnetze, d. h. der Verwertung von durch Phytoplankton fixiertem gelösten organischen Material (DOM). Da viele *Roseobacter* leicht kultiviert werden können, sind sie mittlerweile eine der am intensivsten untersuchten Gruppen mariner Bakterien.

In der vorliegenden Studie wurde die *Roseobacter* Diversität in einer Wasserprobe aus der offenen südlichen Nordsee zum Zeitpunkt einer Phytoflagellatenblüte untersucht. Insgesamt wurden 12 verschiedene *Roseobacter* detektiert, sieben Isolate und fünf Phylotypen, die mittels Denaturierender Gradienten-Gelelektrophorese (DGGE) und anschließender Bandensequenzierung identifiziert wurden (ein Phylotyp wurde mittels beider Methoden gefunden). Drei der Phylotypen gehören zu drei der vier größten Cluster innerhalb der *Roseobacter* Gruppe, dem *Roseobacter* Clade Affiliated (RCA) Cluster, dem NAC11-7 und dem CHAB-I-5 Cluster, die alle überwiegend nicht kultivierte Vertreter enthalten. Die Isolate wiesen unterschiedliche Substratspektren auf und Fütterungsexperimente mit [$^2H_6$]DMSP zeigten, dass alle Isolate DMSP in viele verschiedene flüchtige Verbindungen hauptsächlich über den Demethylierungsweg umwandeln können. Biogeographiestudien ergaben, dass die meisten der neuen *Roseobacter* Stämme und Phylotypen nah verwandte Organismen im Gelben Meer (einem Teil des Chinesischen Meeres) aufweisen, einem Habitat, das mit der Nordsee auffallende Ähnlichkeiten hat.

Drei der neuen Isolate wurden als neue Arten neuer Gattungen charakterisiert: *Planktotalea frisia*, *Pelagimonas elegans* und *Marianus varius*. Für *P. frisia* Stamm SH6-1^T wurden spezifische Primer für eine qualitative sowie quantitative (q)PCR

entwickelt, um das Vorkommen und die Abundanzen dieses Phylotypen in der Nordsee zu untersuchen. Die Ergebnisse zeigten ein ausgeprägtes saisonales Vorkommen von *P. frisia* in der Deutschen Bucht sowie eine mögliche Korrelation mit Phytoplankton. In Experimenten mit den Phytoplanktonalgen *Phaeocystis globosa* und *Leptocylindrus danicus* konnte die bakterielle Verwertung von durch Algen produzierten gelösten organischen Kohlenstoff (DOC) gezeigt werden.

Des Weiteren wurde in einer Zusammenarbeit von 37 Wissenschaftlern das Genom von *Dinoroseobacter shibae* DFL12^T analysiert. Die genombasierte Hypothese, dass *D. shibae* und seine Algenwirtszelle *Prorocentrum lima* symbiotisch interagieren können, wurde mittels experimenteller Studien bestätigt. *D. shibae* liefert dem Wirtsdinoflagellaten die wachstumslimitierenden Vitamine B1 und B12 und der Dinoflagellat scheidet organische Substrate aus, die das bakterielle Wachstum fördert. Mittels stammspezifischer CARD-FISH Sonden wurde die Anheftung des Bakteriums an den Dinoflagellaten gezeigt. Diese räumliche Nähe ermöglicht das symbiotische Zusammenspiel beider Organismen in ihrem natürlichen Lebensraum.

Chapter I

Introduction

I.1 Importance of heterotrophic prokaryotes in the marine environment

The importance of heterotrophic bacteria in the oceans has long been unrecognized. In the marine food webs their role was restricted to remineralizers which metabolize organic matter and make inorganic nutrients available for primary producers (Kirchman & Williams, 2000). However, in 1845 Charles Darwin's consideration has already been pointing to the significance of microbes in the marine food webs. He presumed "that the numerous lower pelagic animals persist on the infusoria [microscopic organisms], which are known to abound in the open ocean: but on what, in the clear blue water, do these infusoria subsist?" As pointed out by Sherr & Sherr (2000), until the 1970s the only microbial component of pelagic food webs given serious attention was the "net" phytoplankton (i.e. planktonic algal cells that could be captured using the finest mesh plankton nets, consisting mainly of centric diatoms and dinoflagellates). The food web was considered to base on the "net" phytoplankton primary production consumed by copepods, which in turn were eaten by larger consumers (Steele, 1974). The concept of marine food webs was greatly influenced when it was discovered that the respiration rate in the fraction <366 µm of a seawater sample was about 10 times higher than that of larger plankton. Thus, it was proposed that the main primary producers in the sea were nanoplankton <60 µm, that microbes <366 µm were responsible for the bulk metabolism in seawater, and that the bulk of non-living organic matter is primarily consumed by heterotrophic microbes (Pomeroy, 1974). It was theorized that active bacteria are the primary consumers of phytoplankton-derived dissolved organic matter (DOM) and that these microbial processes play a crucial role in the marine food web (Pomeroy, 1974; Williams, 1981).

Based on the ideas presented by Pomeroy, the concept of the "microbial loop" as component of the marine food web was developed (Azam *et al*., 1983). In the microbial loop, heterotrophic bacteria efficiently utilize DOM for growth (10 - 50% of the carbon fixed by phytoplankton) and are consumed by protozoa (Darwin's infusoria), which in turn are preyed by other microzooplankton and those by copepods and other mesozooplankton and so forth. Thus, a substantial fraction of carbon and energy is channeled into higher trophic levels by bacterial activity (Fig. 1). The enormous overall biomass contribution of bacteria to the food webs of the world's oceans becomes obvious regarding the bacterial activity (Pomeroy *et al*., 2007) and numbers (a global total of about 10^{29} cells, Whitman *et al*., 1998). Covering over 70% of the earth's surface with maximum depth exceeding 11.000 meters, the 365 million square kilometers of the ocean area is the largest habitat on

earth (Ramaiah, 2004). The total biomass of bacteria in the ocean is higher than the mass of zooplankton and fishes combined (Pomeroy *et al*., 2007).

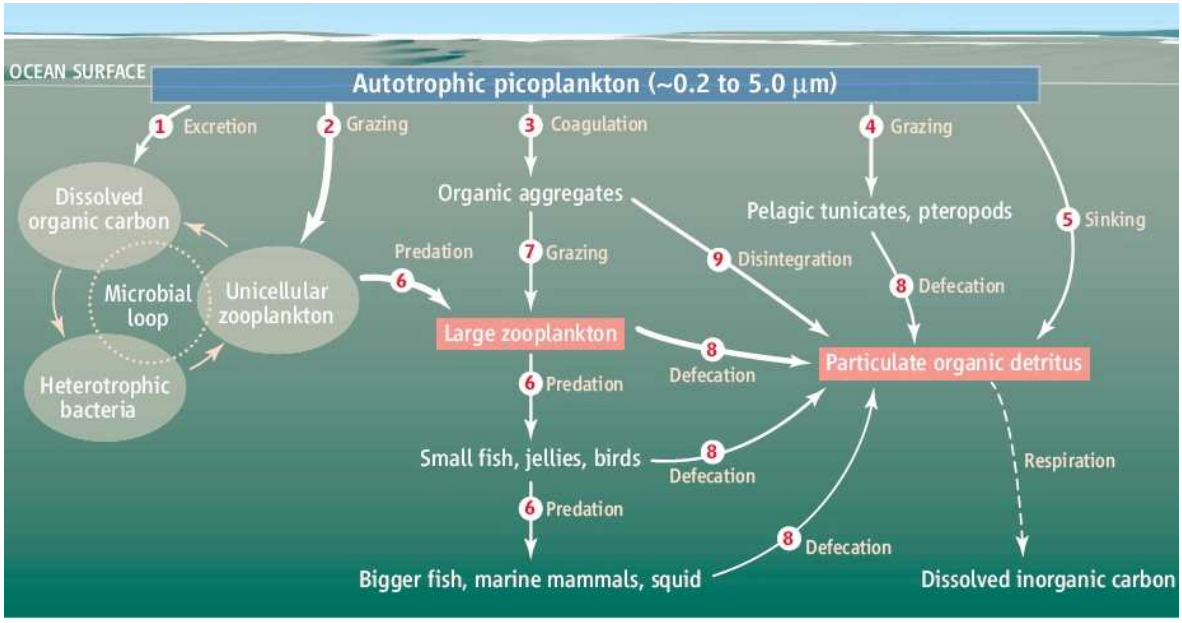

Fig. 1: This oceanic food web based on autotrophic picoplankton shows the paths of organic carbon flux and the microbial loop (on the left) as an important part of the food web. Red boxes: carbon pools that receive substantial export of autotrophic picoplankton carbon. Image: Barber (2007).

Bacteria not only constitute an important component of the marine food webs but also play key roles in biogeochemical cycles, i.e. the movement of chemical elements between biotic and abiotic systems (Maier, 2009). For example, in the sulfur cycle microbial release of dimethylsulfide (DMS) to the atmosphere causes a backflow of sulfur from the sea to the continents (chapter II.1), which was transported to the oceans via rivers. In the atmosphere DMS is rapidly oxidized to methanesulfonic acid and SO_4^{2-}, which rains down to earth to close the cycle (Lovelock *et al*., 1972). The four main elements carbon, nitrogen, phosphorus, and sulfur are necessary for life on earth and critical to comprehensive processes such as climate change and evolution.

I.2 Investigation of prokaryotic communities and functions

I.2.1 Exploring prokaryotic diversity

Until the 1980s the component of heterotrophic bacteria was viewed as a *black box*, a functional unit comprising many unidentified phylotypes, disregarding the phylogenetic and functional variety. Thus, the major players within different habitats and changes in the prokaryotic communities under different conditions have been unrecognized. The small scale as well as the low morphological differentiation of bacteria (microscopically and their appearance on agar plates) hampered the investigation of these communities. Furthermore, to study prokaryotic communities comprehensively the application of cultivation-based methods is insufficient as different cultivation media select for different bacterial groups or strains (e.g. depending on used substrates qualitatively and quantitatively), some fast growing strains might overgrow slow growing strains and many bacteria fail to grow under laboratory conditions at all. The "great plate count anomaly" (a phrase coined by Staley & Konopka, 1985), in which the number of colonies on a plate is orders of magnitude lower than the cell counts in the environmental sample, was first described by Razumov (1932). However, the cultivation efficiency of bacteria in different habitats can vary (e.g. Jannasch & Jones, 1959; Kogure *et al*., 1980; Wagner *et al*., 1993).

Applying molecular techniques, which were introduced in microbial ecology in the late 1980s, prokaryotic diversity and abundances of certain groups *in situ* can be studied more intensively. The denaturing gradient gel electrophoresis (DGGE) bases on the PCR amplification of 16S rRNA genes using group specific primer pairs and is a useful tool to separate different phylotypes of one sample displaying the phylogenetic diversity (Muyzer *et al*., 1993). DGGE gel bands (containing 16S rRNA gene fragments of one phylotype) can be excised, reamplified and sequenced. The 16S rRNA gene sequence information can be used to analyze the phylogenetic affiliation (Woese & Fox, 1977). As PCR causes amplification biases (some sequences are more efficiently amplified than others) and phylotypes present to <1% will not be detected at all, it should be considered that DGGE banding patterns do not display the prokaryotic diversity comprehensively. The construction of large clone libraries can also provide insights into the phylogenetic diversity of prokaryotes present in the environment (e.g. Pommier *et al*., 2007). However, using PCR-based approaches mostly abundant taxa are retrieved. The "rare biosphere", which is thought to account for a considerable fraction of the biodiversity, remains largely unexplored. To improve the situation, group-specific primers, nested PCR (Benlloch *et al*.,

2002) or shotgun cloning, in which PCR is not used (e.g. Venter *et al*., 2004) can be applied. Furthermore, cultivation-dependent techniques could be more suitable to access rare taxa (Pedrós-Alió, 2006).

To quantify different phylogenetic groups fluorescent *in situ* hybridization (FISH) can be used, first described by DeLong *et al*. (1989) and Amann *et al*. (1990). This approach bases on fluorescent-labeled oligonucleotide probes targeting the 16S rRNA. Depending on the physiological state of a cell, which influences the number of 16S rRNA copies, differently intense fluorescent signals are exhibited. In order to be more independent of the physiological state of the cell a modified signaling amplification approach (CARD-FISH, also known as TSA-FISH) was introduced into aquatic microbiology (Pernthaler *et al*., 2002). Furthermore, to quantify prokaryotes of different phylogenetic groups quantitative PCRs are used as a very sensitive method (e.g. Syvanen *et al*., 1988; Suzuki *et al*., 2000; Smith & Osborn, 2009). The accuracy of quantification using methods based on the 16S rRNA gene sequences stands and falls with the probe specificities. Due to the constant increase of 16S rRNA gene sequence databases we now know, that many currently used probes are not as group specific as initially assumed (Amann & Fuchs, 2008).

As frequently reported there is a strong discrepancy regarding phylotypes from one sample found by cultivation-dependent and -independent methods (e.g. Suzuki *et al*., 1997; Kisand & Wikner, 2003; Hirayama *et al*., 2007; Engelen & Cypionka, 2008; chapter II.1). Thus, to analyze prokaryotic communities, it is recommended to use a polyphasic approach based on cultivation and on molecular biological methods. Even though every approach shows certain deficiencies by applying a combination of these methods the *in situ* bacterial diversity and abundances can be assessed.

I.2.2 Dominant bacterial groups in different ecosystems

Prokaryotes of marine waters and soils (as an example for a terrestrial ecosystem) mostly comprise members of the domain *Bacteria* (Table 1).

Table 1: Major bacterial groups of the marine bacterioplankton and soils. Abundances determined by culture-independent approaches. *Giovannoni & Rappé (2000); †Galand *et al*. (2010); ‡Varela *et al*. (2008); ¶Hugenholtz *et al*. (1998) and Frierer *et al*. (2007)

Marine bacterioplankton*	**Soil bacteria¶**
Alphaproteobacteria (class)	*Alphaproteobacteria* (class)
SAR11 clade	*Acidobacteria* (phylum)
Roseobacter clade	*Betaproteobacteria* (class)
SAR116 clade	*Gammaproteobacteria* (class)
Gammaproteobacteria (class)	*Actinobacteria* (phylum)
SAR86 clade	*Verrucomicrobia* (phylum)
Picophytoplankton	
Cytophaga-Flavobacter-Bacteroidetes (phylum)	
Deltaproteobacteria (class)	
SAR324†	
SAR202‡ (phylum *Chloroflexi*)	

The SAR11 clade, first described by Giovannoni *et al*. (1990), comprise the most abundant and ubiquitous phylotypes of the marine bacterioplankton (Giovannoni & Rappé, 2000). As the SAR11 clade, the clades SAR86 and SAR116 are also ubiquitous in seawater and predominantly uncultured. In all three groups genes for the light-driven proton pump proteorhodopsin was detected: in the SAR11 member '*Candidatus* Pelagibacter ubique' (Giovannoni *et al*., 2005), the uncultured SAR86 clade of the *Gammaproteobacteria* (Sabehi *et al*., 2004), and in the SAR116 isolate '*Candidatus* Puniceispirillum marinum' (Oh *et al*., 2010). The clades SAR202 and SAR324, which were found to be abundant in meso- and bathypelagic waters lack cultured representatives (Varela *et al*., 2008; Galand *et al*., 2010). As a result for all of these groups the physiological characteristics are unknown. The *Roseobacter* clade (described in more detail below) of the class *Alphaproteobacteria* is often most abundant associated with marine algae (Buchan *et al*., 2005). The picophytoplankton contributes to the primary production and is often dominated by the cyanobacteria *Synechococcus* and *Prochlorococcus*. The *Cytophaga-Flavobacter-Bacteroidetes* (CFB) often occurr particle-associated and are known to degrade different polymers (Kirchman, 2002).

In soils *Alphaproteobacteria* (containing many nitrogen-fixing bacteria) and *Acidobacteria* are the most abundant groups (Dunbar *et al*., 1999; Frierer *et al*., 2007; Zhang & Xu, 2008). The *Acidobacteria* contain only a few isolates (Sait *et al*., 2002; Joseph *et al*., 2003) and little is known about the metabolic capabilities of this group. *Betaproteobacteria* (containing members known to mediate nitrification) are also abundant as well as *Gammaproteobacteria* (Hugenholtz *et al*., 1998), which contain many phylotypes such as pseudomonads, which are well known for their ability to metabolize a diverse array of carbon compounds (Zhang & Xu, 2008). The commonly, but usually to a minor extend detected *Verrucomicrobia* are rarely cultivated, some are associated with eukaryotic hosts, but in general little is known about their function (Hugenholtz *et al*., 1998; Zhang & Xu, 2008). The high G+C content gram positive *Actinobacteria* are well represented in culture collections, they play an important role in the decomposition of polymeric organic matter and they are well known as secondary metabolite producers.

Results of cultivation-independent studies show, that the microbial communities of different ecosystems are dominated by different phylogenetic groups with different metabolic characteristics. However, it has to be regarded that the abundant groups in an environmental sample do not necessarily contain the active organisms.

I.2.3 Exploring prokaryotic activity and physiology

The phylogenetic affiliation of an organism can give first hints about its physiological properties. However, to confirm and to study these physiological traits it is most suitable to investigate isolates in laboratory experiments. A powerful tool promoting the investigation of isolates is the whole genome sequencing. Once the gene inventory of an organism is known, physiological traits and hence their (possible) function in the environment can be deduced. To achieve these investigations one has to overcome the difficulty of cultivating the particular organism. Furthermore, it is known that some metabolic capabilities are not expressed under laboratory conditions, e.g. in some aerobic anoxygenic phototrophs (containing genes indicating the capability of photosynthesis) bacteriochlorophyll *a* is not expressed or only under certain conditions (Biebl & Wagner-Döbler, 2006). Thus, some metabolic processes, that might be relevant under natural conditions, cannot be studied *in vitro*, showing that the investigation of isolates is limited to some degree.

The application of cultivation-independent methods can shed more light into the function and metabolic potential of cultured and yet uncultured prokaryotes inhabiting different ecosystems. Using metagenome libraries, the gene inventory of an environmental

sample can be studied (i.e. 16S rRNA genes as well as other functional genes present in the sample; e.g. Schmidt *et al*., 1991; Stein *et al*., 1996, Béjà *et al*., 2000 & 2002; Venter *et al*. 2004). To construct such a clone library the environmental DNA molecules are digested by restriction enzymes and ligated into vectors, which are brought into host bacteria (there are different types of vectors which can carry different insert sizes). The insert sequences of these clones can then be analyzed and allocated to different phylotypes or gene functions. Using BAC libraries Béjà *et al*. (2000) uncovered the presence of proteorhodopsin genes and the diversity of aerobic anoxygenic phototrophs (2002) in a water sample from the Monterey Bay. Analog to the metagenomic approach, metatranscriptomic libraries can be constructed by the extraction of RNA from an environmental sample and cloning and sequencing of cDNA fragments. Metatranscriptome data reveal gene expression patterns of bacterial assemblages pointing to microbial processes and activities *in situ* (e.g. Poretsky *et al*., 2005; McCarren *et al*., 2010; Vila-Costa *et al*., 2010). Furthermore, the MAR(or Micro)-FISH technique, a combination of fluorescent *in situ* hybridization and microautoradiography, is used to study the contribution of different phylogenetic groups to biomass production. Therefore, a prokaryotic community is incubated with substrates labeled with a radioactive isotope. By means of microautoradiography the isotopes, i.e. the bacteria which incorporated the labeled compounds, can be localized and by means of FISH the bacteria can be allocated to phylogenetic groups (e.g. Lee *et al*., 1999; Cottrell & Kirchman, 2000; Okabe *et al*., 2004; Malmstrom *et al*., 2007).

Regarding the investigation of the activity and function of prokaryotes, a polyphasic approach is also promising. Cultivation-independent methods are important tools to study prokaryotic *in situ* processes and functions and they provide valuable insights into the physiology of the vast majority of yet uncultured prokaryotes. Using isolates physiological properties can be studied in more detail, such as interactions with other organisms, substrate utilization properties or the response to changing conditions.

I.3 Roseobacters – a prominent and well studied component of the marine heterotrophic bacterioplankton

As mentioned in the previous section, members of the SAR11 clade within the class *Alphaproteobacteria* build the most abundant and ubiquitous group of the bacterioplankton in the oceans (e.g. Giovannoni & Rappé, 2000; Morris *et al*., 2002). Thus, studying the metabolic capabilities of these bacteria and their interaction with other organisms would be most interestingly to assess their ecological role. However, bacteria of the SAR11 group yet are hardly culturable (Rappe *et al*., 2002) making an intense exploration of this clade very difficult. Members of the *Roseobacter* clade are also widespread in the marine environment, they occur in a variety of marine habitats (Buchan *et al*., 2005) and they constitute large fractions of 16S rRNA genes of pelagic marine bacterioplankton communities in surface waters (e.g. Giovannoni & Rappé, 2000; González *et al*., 2000). As they can easily be cultivated, they became one of the most intensively studied groups of marine bacteria. Phylogenetically the cluster belongs to the family *Rhodobacteraceae* within the class *Alphaproteobacteria* and has a within sequence similarity of >88% of the 16S rRNA gene sequences (Binkhoff *et al*., 2008). The group was named after the first isolates, *Roseobacter litoralis* and *Roseobacter denitrificans* (Shiba, 1991), which are pink pigmented due to the carotenoid spheroidenone. Roseobacters are non-obligately phototrophic heterotrophs and they exhibit an enormous diversity of physiological traits (Buchan *et al*., 2005; Wagner-Döbler & Biebl, 2006).

I.3.1 Aspects that are studied

There is a variety of aspects that are currently being studied: for example the degradation and assimilation of the algal osmolyte dimethylsulfoniopropionate (DMSP) (e.g. Ledyard & Dacey, 1994; González *et al*., 1999; Moran *et al*., 2003; Dickschat *et al*., 2010), production of secondary metabolites enabling for quorum sensing and antibacterial effects (e.g. Lafay *et al*., 1995; Ruiz-Ponte *et al*., 1999; Gram *et al*., 2002; Brinkhoff *et al*., 2004; Martens *et al*., 2007; Geng *et al*., 2008), oxidation of the greenhouse gas carbon monoxide (e.g. King, 2003; Moran *et al*., 2004; Tolli, 2006; Cunliffe, 2011), the photosynthesis apparatus of bacteriochlorophyll *a*-containing members and their response to light (e.g. Yurkov & Beatty, 1998; Schwarze *et al*., 2000; Candela *et al*., 2001; Schaefer *et al*., 2007; Holert *et al*., 2010) as well as the distribution and ecological relevance of these aerobic anoxygenic phototrophs (e.g. Shiba, 1995; Allgaier *et al*., 2003; Rathgeber *et al*., 2004), chemotactic properties (Miller *et al*., 2004), proteomics (e.g. Zech *et al*., 2009; Tang *et al*.,

2010a), metabolic fluxes (Fürch *et al*., 2009), interactions with phytoplankton (e.g. Brinkmeyer *et al*., 2000; Geng & Belas, 2010), the distribution of roseobacters (e.g. Selje *et al*., 2004; Buchan *et al*., 2009; Giebel *et al*., 2009 & 2011) and *in situ* activities (Zubkov *et al*., 2001 ; Malmstrom *et al*., 2004; Alonso & Pernthaler, 2006). Whole genomes are used for phylogenomic analyses and to study the metabolic potential of roseobacters (e.g. Moran *et al*., 2004; Swingley *et al*., 2007; Moran *et al*., 2007; Pradella *et al*., 2010; Newton *et al*., 2010; Tang *et al*., 2010b; chapter II.6), for example genome analyses revealed mixotrophy in some organisms (Moran *et al*., 2004; Swingley *et al*., 2007). Furthermore, new *Roseobacter* species and genera are frequently characterized. Currently there are at least 121 validly described species available belonging to at least 55 genera (strains of chapters II.2 - II.4 not included). The characterization of different strains revealed that roseobacters are able to use a multitude of organic compounds and some members showed methylotrophy (Holmes *et al*., 1997). Roseobacters also effectively metabolize phytoplankton dissolved organic carbon (e.g. Riemann *et al*., 2000; Schäfer *et al*., 2001; Grossart *et al*., 2005; chapters II.1 and II.5) and in fact, they are often most abundant associated with marine algae (Buchan *et al*., 2005).

As the characteristics of roseobacters emphasize their ecological significance as an important component of the food webs as well as in the biogeochemical cycles, there is an increasing interest in the investigation of this group, which is reflected by the increasing number of publications within the last years. Based on the ISI Web of Knowledge citation index, within the five years from 1996 - 2000 there were 44 publications found in the database searching for “Roseobacter”, whereas in the successive five years from 2001 - 2005 there were 107 publications, and from 2006 - 2010 with 253 results the number of publications again were more than twice as high, showing an exponential-like increase over the last 15 years.

I.3.2 Roseobacters in the North Sea

In the North Sea roseobacters were reported to be prominent members of the bacterioplankton constituting up to 24% of total bacterial numbers (Eilers *et al*., 2000 & 2001; Zubkov *et al*., 2001; Selje *et al*., 2004; Alderkamp *et al*., 2006). There exist several surveys about roseobacter diversities and their response to changes in the phytoplankton community (e.g. Rink *et al*., 2007 & 2008; Stevens *et al*., 2005; Grossart *et al*., 2005). The presence and diversity of aerobic anoxygenic roseobacters in different North Sea habitats was studied by Allgaier *et al*. (2003). One of the largest cluster within the *Roseobacter*

lineage, the *Roseobacter* clade affiliated (RCA) cluster, was first detected in the North Sea (Selje *et al*., 2004). Giebel *et al*. (2011) studied its distribution and abundances along a North Sea transect and found that members of the RCA cluster might have an important role during decaying phytoplankton blooms. Roseobacters were reported to be among the most active bacterial groups regarding glucose and leucine uptake during a *Phaeocystis* bloom in the southern North Sea (Alderkamp *et al*., 2006; Alonso & Pernthaler, 2006) and during a coccolithophore bloom in the northern North Sea regarding DMSP consumption (Zubkov *et al*., 2001 & 2002). Furthermore, there are roseobacters isolated from North Sea areas that were validly characterized (Wagner-Döbler *et al*., 2003 & 2004; Biebl *et al*., 2005; Martens *et al*., 2006; Sass *et al*., 2010; chapter II.2).

I.4 Taxonomic classification of bacteria

Taxonomic classification of bacteria has emerged as a challenging task and criteria applied to compare bacterial strains and to differentiate them from each other have continuously evolved. Traditionally bacterial classification relied on phenotypic characteristics (e.g. the morphology, pigmentation, Gram reaction, motility, physiological properties or pathogenicity). As these characteristics provide not sufficient information to their evolutionary roots, phenotypic analyses are supplemented by genetic investigations (such as 16S rRNA gene sequence analysis, DNA-DNA hybridization or sequence comparisons of protein-encoding genes).

The comparison of 16S rRNA gene sequences, as pioneered by Woese (1977), is widely used to rapidly allocate bacteria phylogenetically and provides a powerful tool as large 16S rRNA gene databases are available and grow continuously. It has been proposed that a strain, whose 16S rRNA sequence differs by more than 3% (over the almost full length sequence) from that of all other organisms, can be considered a new species (Gevers *et al*., 2005). However, the 16S rRNA gene sequence comparison alone is not sufficient for bacterial classification, but just indicates the affiliation to a certain taxon. When 16S rRNA gene sequence similarity values are higher than 97% other methods such as DNA-DNA hybridization must be used as the resolving power of single 16S rRNA sequences is limited (Rosselló-Mora & Amann 2001; Tindall *et al*., 2010). DNA-DNA hybridization measures the overall genetic similarity among isolates, thus providing a greater resolution. A hybridization value ≥70% has been recommended to define members of a species (e.g. Brenner, 1973; Johnson, 1984; Wayne *et al*., 1987), but should not be used as a strict species boundary (Ursing *et al*., 1995). Usually two bacteria sharing less than 97% 16S rRNA sequence similarity hybridize to less than 70%, but organisms that have very similar rRNA sequences can also have genomes that are quite unrelated (e.g. Fox *et al*., 1992; Martínez-Murcia *et al*., 1992; Rosselló-Mora & Amann 2001; Fig. 2).

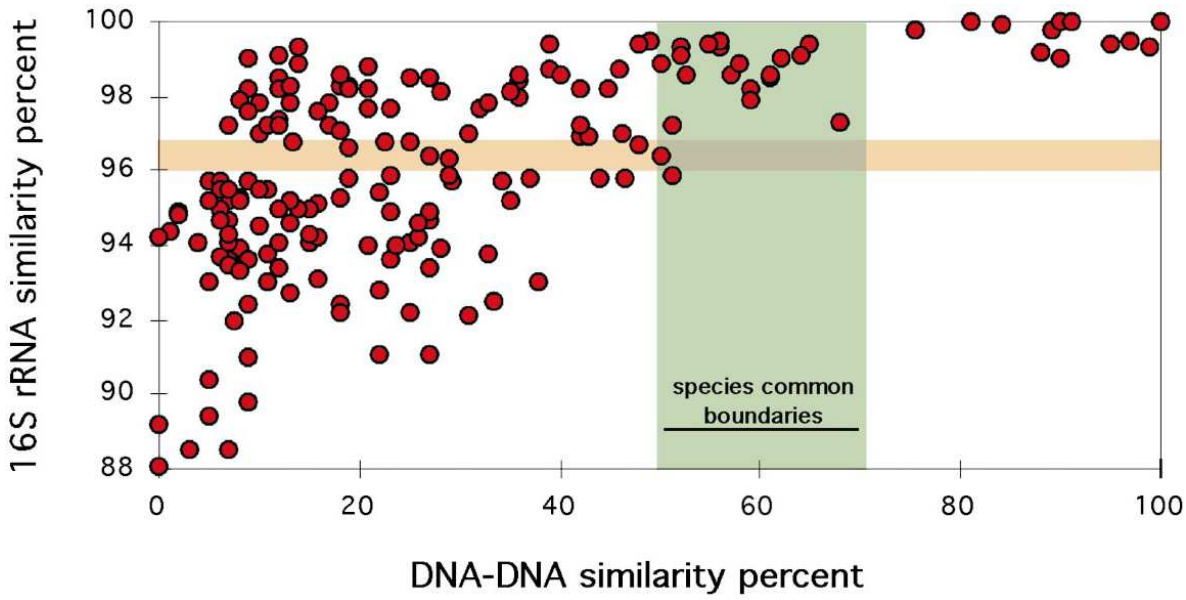

Fig. 2: Comparison of DNA-DNA and 16S rRNA similarities. The dataset is based on 180 values from 27 independent articles of the International Journal of Systematic Bacteriology vol. 49 (1999). Figure: Rosselló-Mora & Amann (2001)

Furthermore, the genomic G+C content can also function as a delineating feature. A 10 mol% difference in DNA G+C content has been proposed as a clear differentiating criterion for species at the genus level (Stackebrandt & Liesack, 1993). However, two organisms can have identical G+C ratios and yet be quite unrelated. As chemotaxonomic characteristics such as the types and proportions of fatty acids and polar lipids of the cell membrane reflect evolutionary groups, these data are helpful to delineate species on the genus level and should be included in strain characterization (Martens *et al*., 2006; Tindall *et al*., 1994 & 2010). In general, for bacterial classification it is crucial to compare all relevant type strains and in case of members of different genera the type species have to be considered as important references (Tindall *et al*., 2010).

In the course of complete genome sequencing, additional data have become available and also used for phylogenetic analyses. The so called phylogenomics include sequence-based methods and analyses that are based on whole-genome features. Whole genome sequences permit the identification of orthologous genes which are assembled for phylogenetic analysis providing higher resolution than sequence comparisons of single genes. Whole-genome features such as the gene repertoire (e.g. the proportion of shared orthologous genes between genomes) or gene order and 'DNA strings' (the distribution of oligonucleotides in genomes) are also used to compare organisms and reconstruct

phylogenetic inference applying tree-building methods (Delsuc *et al*., 2005). As the number of currently available genome sequences is relatively small (about 1000 complete or nearly complete genome sequences of microbes containing also not described strains compared to about 13.700 validly described strains) yet the use of this approach is restricted.

In Conclusion, each and every analysis that describes bacterial characteristics or its phylogenetic affiliation is inadequate as taken as the sole method for identification. Thus, to characterize bacterial strains a polyphasic approach that includes phenotypic and genotypic properties has to be applied. In general, bacterial taxonomy is being influenced by advances in microbial genetics, ecology and genomics (Gevers *et al*., 2005) and can lead to reclassification of some at that time “state of the art classified” bacteria.

I.5 Aims and outline of this thesis

The aim of this thesis was to assess the diversity of the *Roseobacter* clade in the open southern North Sea during a phytoplankton bloom on the basis of cultivation-dependent and -independent approaches. Therefore, a water sample was collected during a ship cruise and new *Rosoeobacter* strains were isolated and used to investigate physiological properties and interrelations with phytoplankton. As many roseobacter studies from coastal North Sea areas exist, this study should base on an open North Sea water sample. In addition, the whole genome of *Dinoroseobacter shibae*, a member of the *Roseobacter* clade also isolated from the North Sea by Biebl *et al*. (2005), was studied within a cooperation project. The aim of this study was to uncover the metabolic capabilities of this strain and to analyze interactions between *D. shibae* and its host algal cell *Prorocentrum lima*.

- Chapter II.1 of this thesis deals with the enrichment and isolation of roseobacters from the open North Sea water sample. The isolates were investigated regarding the utilization of different organic substrates and their capability to degrade the algal osmolyte DMSP. Furthermore, the roseobacter diversity was studied also applying cultivation-independent methods and the sequences and obtained isolates were phylogenetically analyzed. Three new *Roseobacter* isolates were characterized as species of new genera: *Planktotalea frisia*, *Pelagimonas elegans*, and *Marianus varius* (chapter II.2 - II.4).

- Chapter II.5 contains a study about the distribution and seasonal occurrence of *Planktotalea frisia* in the North Sea and its utilization of *Phaeocystis*-derived DOC, a common phytoplankton genus of the North Sea reaching high abundances in spring.

- The genome analysis of *D. shibae* uncovered new physiological traits of this bacterium (chapter II.6), such as the ability to grow anaerobically using the alternative electron acceptors nitrate and dimethylsulfoxide. Furthermore, the genome-based hypothesis that *D. shibae* and *Prorocentrum lima* can act symbiotically was confirmed by laboratory experiments. *D. shibae* is able to synthesize the vitamins B1 and B12, which enables the auxotrophic dinoflagellate to grow and in turn organic substrates released by the algal cell support growth of the bacterium. Using strain-specific CARD-FISH probes, it was

shown that *D. shibae* attaches to the dinoflagellate. This close association can permit the symbiotic interrelation of these organisms in the water column of the natural environment.

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Chapter II

Publications

II.1 Taxonomic and physiological diversity of roseobacters co-occurring during a phytoplankton bloom

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Taxonomic and physiological diversity of roseobacters co-occurring during a phytoplankton bloom

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Running title Roseobacters co-occurring during a phytoplankton bloom

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Abstract

The diversity of roseobacters co-occurring during a phytoplankton bloom in the southern North Sea was investigated. Catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) revealed that these organisms constituted 16% of DAPI and 68% of *Alphaproteobacteria* cells, respectively. Overall, 12 different *Roseobacter* clade organisms were detected in the water column, seven isolates and five phylotypes by denaturing gradient gel electrophoresis (DGGE) and sequencing of excised bands. The isolates were obtained using serial dilution cultures with low-nutrient media. Physiological characterization exhibited different substrate spectra of all strains, in particular with respect to amino acid and monosaccharide utilization. Feeding experiments with [$^2H_6$]DMSP (dimethylsulfoniopropionate) revealed that all isolates converted DMSP into a variety of volatile compounds, identified using GC-MS. The analyses proved that six strains mainly decomposed DMSP via the demethylation pathway, but four strains were also capable to cleave DMSP to DMS and acrylate. Three of the phylotypes are affiliated with three of the four major clusters of the *Roseobacter* clade, consisting predominantly of uncultured organisms, i.e. the *Roseobacter* clade-affiliated (RCA), the NAC11-7 and the CHAB-I-5 cluster, respectively. Most new strains and phylotypes have close relatives previously detected in the Yellow Sea, a habitat with remarkable similarities to the North Sea.

Introduction

One of the most prominent lineages in the marine pelagic mixed layer are *Alphaproteobacteria* of which the SAR11 and *Roseobacter* clades are two major subgroups. The SAR11 clade appears to be restricted to the pelagic zone whereas organisms of the *Roseobacter* clade have been found in greatly differing marine ecosystems and habitats, including pelagic, sediment and surface-associated life styles, often in close association with other organisms such as phytoplankton algae (Buchan *et al.*, 2005). The adaptation of the various organisms of the *Roseobacter* clade to greatly varying environmental conditions is reflected by a high genetic and metabolic diversity, including the ability to perform aerobic anoxygenic photosynthesis, to degrade dimethylsulfoniopropionate (DMSP) and to metabolize a broad range of organic compounds (Buchan *et al.*, 2005; Newton *et al.*, 2010). Culture-independent phylogenetic and metagenomic studies, but more importantly, studies with isolated strains and genomic analyses have contributed to our knowledge of the *Roseobacter* clade, as representatives of almost all major phylogenetic subclusters of this clade have been cultured, physiologically characterized and genome sequenced (Buchan *et al.*, 2005; Brinkhoff *et al.*, 2008; Newton *et al.*, 2010).

Despite these comprehensive analyses compiled from many different habitats and ecosystems, still little information is available on physiological traits of roseobacters occurring simultaneously in one ecosystem. Investigations of the diversity of the *Roseobacter* clade, but also of other bacterial lineages, have been mainly based on cultivation-independent approaches, targeting the 16S rRNA gene and thus making it impossible to deduce detailed physiological traits. To date no systematic analysis of the co-occurrence and physiological traits of different roseobacters within a given ecosystem is available. In order to obtain insight into these traits, investigations on the taxonomic diversity of the *Roseobacter* clade need to be complemented with cultivation-based approaches to obtain isolates for subsequent physiological experiments. As a large number of phylotypes and isolates of the *Roseobacter* clade has been retrieved from coastal seas and phytoplankton (Buchan *et al.*, 2005), the plankton community in neritic seas such as the North Sea is a primary target for studies examining physiological traits of co-occurring roseobacters.

Investigations of the bacterioplankton composition of the North Sea frequently demonstrated the presence of a substantial fraction of organisms of the *Roseobacter* clade

(Eilers *et al.*, 2001; Zubkov *et al*., 2001; Alderkamp *et al*., 2006; Rink *et al.*, 2007). In the southern North Sea strong seasonal variations of the proportion of roseobacters were reported with highest abundances of up to 20% of total bacterioplankton cells at the decline of a phytoplankton bloom (Eilers *et al.*, 2001). During a coccolithophore bloom in the northern North Sea the *Roseobacter* clade constituted up to 24% of the bacterioplankton (Zubkov *et al.* 2001). In the Wadden Sea, the shallowest near coastal region of the southern North Sea, the community composition of roseobacters was shown to vary significantly with changes in the phytoplankton composition (Rink *et al.*, 2007).

One possible reason why members of the *Roseobacter* clade are abundant components of the bacterioplankton during phytoplankton blooms may be their ability to degrade the osmolyte DMSP which is released from various phytoplankton algae (Yoch, 2002). It has been shown that the occurrence of roseobacters is linked to phytoplankton blooms releasing DMSP (González *et al.*, 2000; Zubkov *et al.*, 2001) and that pelagic roseobacters are major consumers of DMSP in the sea (Malmstrom *et al.*, 2004). Two pathways to break down DMSP exist in the *Roseobacter* clade. DMSP can either be cleaved to acrylate and the volatile and climate-relevant dimethyl sulfide (DMS), or demethylated to 3-methylmercaptopropionate, which is incorporated into bacterial biomass and serves as a carbon and sulfur source (Moran *et al.*, 2003). The demethylation pathway appears to be more widely distributed in the *Roseobacter* clade than the cleavage pathway (Newton *et al.*, 2010). Even though the sulfur metabolism of several species of the *Roseobacter* clade has been examined (Holmes *et al.*, 1997; González *et al*., 1999; Dickschat *et al.*, 2010), there is still rather little information available on the metabolism and production of organic sulfur compounds, in particular of roseobacters occurring during phytoplankton blooms.

The aim of this study was to investigate the taxonomic diversity as well as physiological traits of roseobacters occurring during a phytoplankton bloom. Therefore, a sample from the southern North Sea was collected in May 2007 and investigated for the presence of organisms affiliated with the *Roseobacter* clade by cultivation-dependent and -independent approaches. The bacterioplankton community was analyzed with CARD-FISH (catalyzed reporter deposition fluorescence *in situ* hybridization) and DGGE (denaturing gradient gel electrophoresis) based on PCR with primers specific for the *Roseobacter* clade and *Alphaproteobacteria*. Physiological studies including the degradation of DMSP were carried out with seven new *Roseobacter* isolates to obtain a more detailed insight into their traits and niche separation to explain their coexistence

during the phytoplankton bloom and their growth requirements in the pelagic zone in general.

Materials and methods

Sampling site and isolation of bacteria

A water sample was collected on 12 May 2007 in the southern North Sea (54°42' N, 06°48' E; depth: 36 m) from 2 m depth with 5 l-Niskin bottles attached to a conductivity-temperature-depth profiler (CTD, Sea-Bird SBE 19plus). In order to enrich bacteria of different abundance, dilution cultures in most probable number (MPN) series (10^{-1} to 10^{-8}) were applied using various media (composition see below). In order to obtain isolates from free-living and phytoplankton-associated bacteria, media were inoculated with unfiltered and 1.2 µm prefiltered seawater and with phytoplankton concentrated on a 10 µm screen. One set of MPN dilution series was incubated in triplicates in the dark and one set without replicates at a 12:12 h dark-light cycle at 15°C for four weeks. Subsequently, 100 µl of each dilution culture were transferred to agar plates with the same medium as used for enrichment, with artificial saltwater medium (medium M6), and additionally dilutions of filtered algae were spread on medium M7 (compositions see below). The former incubation conditions were retained. Morphologically different as well as randomly selected colonies were picked and restreaked several times for purification. Isolates affiliated to the *Roseobacter* clade were identified with a PCR specific for the 16S rRNA genes of organisms of this clade (Rink *et al.*, 2007) and their 16S rRNA genes were subsequently sequenced (see below).

Media and culture conditions

Seven different media were applied (Table 1), four on the basis of sea water (M1, M2, M3, M5), two with artificial salt water [M6 and M7 based on Schut *et al.* (1993); for exact salt water composition see Table S1] and one with Marine Broth (M4, composition as in Difco 2216). Yeast extract (Roth, Karlsruhe, Germany) and Bacto-peptone (Difco/BD, Heidelberg, Germany) were used for four media at different concentrations. As cyclic adenosine monophosphate (cAMP) was previously described to increase the cultivation efficiency of heterotrophic bacteria (Bruns *et al.*, 2002) cAMP was added to M2 from a 10 mM stock solution. M4, M6 and M7 were adjusted to pH 8 after autoclaving. For M5 an

autoclaved cell extract of the diatom *Thalassiosira weissflogii* was added to unfiltered seawater. The multi vitamin solution (Balch *et al.*, 1979) was added to M5, M6 and M7 from a 5-fold concentrated stock solution. To M6 15 ml of 1 M $NaHCO_3$ (autoclaved separately) was added as a supplement after autoclaving.

Table 1. Composition of the various media used in this study. Seawater was collected in April 2007 in the southern North Sea near Neuharlingersiel (53°42' N, 07°42' E). Supplements were added after autoclaving. Trace element and vitamin solutions were filtered sterile (0.2 µm) prior to addition.

Medium	Basic medium	Yeast extract (mg l^{-1})	Peptone (mg l^{-1})	Other supplements
M1	Sea water [1]	5	10	-
M2	Sea water [1]	50	150	cAMP (10 µM)
M3	Sea water [1]	-	-	DMSP (10 µM)
M4	Marine Broth	100	500	-
M5	Sea water	-	-	*Thalassiosira weissflogii* extract (100 mg l^{-1}, ww) Trace element solution (1 ml l^{-1}) [4] Vitamin solution (2 ml l^{-1}) [5]
M6	Artificial salt water [2]	30	60	Trace element solution SL10 (1 ml l^{-1}) [6] Vitamin solution (1 ml l^{-1}) [5]
M7	Artificial salt water [3]	-	-	100 mg glucose l^{-1} Trace element solution SL10 (1 ml l^{-1}) [6] Vitamin solution (1 ml l^{-1}) [5]

[1] 10 µm prefiltered
[2] for the composition see Table S1.
[3] for the composition see Table S1.
[4] Zech *et al.* (2009)
[5] Balch *et al.* (1979)
[6] Tschech & Pfennig (1984)

MPN series were screened with a *Roseobacter* clade specific PCR (see below). Based on these results we determined the number of culturable roseobacters in our sample using the MPN Index (Trolldenier 1993). The upper and lower 95% confidence limits were calculated after Cornish & Fischer (1937).

To isolate different *Roseobacter* strains, agar plates of the different media were prepared with 20 g purified agar l^{-1} (washed five times in distilled water). Further cultivation of the isolated *Roseobacter* strains was performed using medium M6 with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone.

Substrate utilization

To determine the substrate spectra of the isolates, growth on single substrates was tested as described by Martens *et al.* (2006) using artificial seawater medium according to Zech *et al.* (2009) with addition of vitamins as described above. For a full list of tested substrates see Table 4. Amino acids were added to a final concentration of 5 mM (1 mM tyrosine was used because of precipitation at 5 mM), DMSP was added to a final concentration of 100 µM, other organic substrates such as sugars were tested with 1 g l^{-1}. Since on single substrates no or only very weak growth was observed for strains SH6-1 and SH36, assays were repeated and supplemented with 0.1 g l^{-1} yeast extract.

Screening for bacteriochlorophyll *a*

Screening for bacteriochlorophyll *a* was carried out by spectrophotometry. A dense suspension of cells grown in the dark was examined for light absorbance with a Beckmann DU 520 General Purpose UV/VIS Spectrophotometer at 400 - 900 nm. *Dinoroseobacter shibae* DFL12 (DSM 16493) was used as positive control.

Conversion of DMSP into volatile compounds

The seven *Roseobacter* clade strains isolated during this study were precultured as described by Dickschat *et al.* (2010) with the following modifications. Feeding experiments were carried out not only on agar plates but also in liquid medium and only with 1 mM [$^2H_6$]DMSP·HCl. The feeding experiments on agar plates were done as described previously (Dickschat *et al.*, 2010). For the feeding experiments in 50% MB liquid medium (Roth, Karlsruhe, Germany) 1 mM [$^2H_6$]DMSP·HCl was added, the cultures (50 ml) were inoculated with 1 ml of the preculture, incubated for 1 day at 28°C, and directly subjected to headspace analysis. The volatiles emitted by the bacterial cultures were collected by use of a closed loop stripping apparatus (CLSA) and detected by GC-MS by comparison of mass spectra to data base spectra of the Wiley 7 library. For further details see Dickschat *et al.* (2010).

Microbial and biogeochemical parameters

Water samples for bacterial cell counts were fixed with 1% glutardialdehyde (GDA) and stored at –20°C until further analysis. Total bacterial numbers were determined on black 0.2 µm Nuclepore filters by epifluorescence microscopy after staining with SybrGreen I (Lunau *et al.*, 2005). For phytoplankton fixation, 1 ml acid Lugol's solution was added to

99 ml of water sample and stored in brown glass bottles. Phytoplankton cells were counted and identified by Aqua Ecology (Oldenburg, Germany) according to Utermöhl (1958).

For chlorophyll analysis, 1500 ml of seawater were filtered onto a Whatman GF/F filter in two replicates and kept frozen at –20°C in the dark until further processing. Filters were extracted at 70°C in 90% ethanol, and concentrations of chlorophyll *a* and phaeopigments, after acidification, were determined spectrophotometrically (Nusch, 1999).

For determination of dry weight (DW) of SPM, 1500 ml seawater were filtered on pre-weighted glass fiber filters in duplicates (Whatman GF/F). DW was determined after drying at 60°C for 12 h. For particulate organic carbon analysis (POC), 350 ml of seawater were filtered onto pre-combusted (500°C, 2 h) glass fiber filters in duplicates (Whatman GF/F) and kept frozen at –20°C in the dark until further processing. Analyses were performed using a FlashEA 1112 CN Filters Analyzer (Thermo Fisher Scientific).

Bacterioplankton composition analyzed by CARD-FISH

Six ml of seawater were filtered onto white 0.2 µm Nuclepore polycarbonate filters and covered with paraformaldehyde (2% w/v) for 1 h. After fixation, filters were subsequently rinsed with 1 ml of phosphate-buffered saline (PBS 1x) and ultraclean water. Filters were stored at –20°C until further processing. CARD-FISH was performed according to Sekar *et al.* (2003). Hybridization conditions were 2 h at 35°C, 30 min washing at 37°C and 30 min signal amplification at 37°C using tyramine-HCl labeled with fluorescein-5-isothiocyanate (FITC). To avoid unspecific accumulation of dye in the cells, the last washing step in PBS (1x) amended with TritonX-100 (0.05%) was extended to 30 min. Counterstaining was performed with Vectashield-mounting medium with DAPI (1.5 mg ml^{-1}; Vector Laboratories, Peterborough, England). Filter sections were analyzed with an epifluorescence microscope (Axioskop, Zeiss, Germany) equipped with filter sets 02 (LP 420) and 09 (LP 515), for viewing DAPI and fluorescein, respectively. For each probe 10 randomly selected viewfields (>1000 DAPI cells) from one filter section were analyzed. The following oligonucleotide probes labeled with horseradish peroxidase (HRP) were used: EUB338 (Amann *et al.*, 1990), ALF968 (Neef, 1997), GAM42a (Manz *et al.*, 1992), CF319a *Cytophaga-Flavobacter-Bacteroidetes* (Manz *et al.*, 1996), ROS536 (Brinkmeyer *et al.*, 2000) and NON338 (Wallner *et al.*, 1993).

Nucleic acid extraction

Particle-associated bacteria from the untreated water sample and from phytoplankton concentrated on a 10 µm gaze were collected on 5 µm Nuclepore filters. Free-living bacteria from the 5 µm and 1.2 µm filtrate were filtered onto 0.2 µm Nuclepore filters. DNA was extracted by a standard protocol with phenol-chloroform-isoamylalcohol, sodium-dodecyl-sulfate (SDS) and 500 mg per 1.5 ml circonia beads (Selje & Simon, 2003). Precipitation was done at –80°C for 1 h using isopropanol (Rink *et al.*, 2007). The DNA was resuspended in molecular grade water (Eppendorf) and stored at –20°C until further processing.

PCR amplification of 16S rRNA genes and DGGE

Amplification and purification of almost complete 16S rRNA genes was carried out as described by Brinkhoff & Muyzer (1997). *Alphaproteobacteria*- and *Roseobacter*-specific PCR and DGGE were performed using the primer sets GC-341F and ALF968r and GC-ROSEO536Rf and GRb735r (Rink *et al.* 2007). DGGE bands of *Alphaproteobacteria* were excised from DGGE gels using a sterile scalpel and reamplified using the primer set 341F and ALF968r and the following cycling parameters: 95°C for 3 min, followed by 30 cycles of 95°C for 1 min, 55°C for 45 s, and 72°C for 3 min. PCR products were purified with the peqGOLD MicroSpin Cycle-Pure Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany). Sequencing was performed by GATC Biotech AG (Konstanz, Germany). The 16S rRNA gene sequences of the isolates and sequenced DGGE bands obtained in this study were deposited in GenBank under accession numbers FJ882052 to FJ882058 and GU815092 to GU815097, respectively.

Phylogenetic analysis

All 16S rRNA gene sequences obtained in this study were compared with those in GenBank at the NCBI database using the BLAST tool (http://www.ncbi.nlm.nih.gov/). To compare the results obtained with sequences of the excised DGGE bands with those of the isolated strains, the almost complete 16S rRNA gene sequences of the seven isolates as well as fragments of their 16S rRNA genes as usually obtained from DGGE gels (sequence from *E. coli* position 341 to 907) were used for BLAST analyses. Sequences with ≥99% similarity were considered as closely related. Phylogenetic trees were calculated with the ARB software package [http://www.arb-home.de (Ludwig *et al.*, 2004)].

Screening for *pufLM* genes

Detection of genes coding for subunits of the photosynthetic reaction centre complex (*pufL* and *pufM*) was performed as described by Beja *et al.* (2002). Reactions were cycled in an Eppendorf thermocycler with the following parameters: 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, with a final extension step at 72°C for 10 min. The PCR products were purified by using the peqGOLD MicroSpin Cycle-Pure Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany). Sequencing was performed by GATC Biotech AG (Konstanz, Germany).

Results

Sampling site characteristics

Satellite images of the southern North Sea taken between May 7 and 9 2007 (http://koflux1.gkss.de) showed a phytoplankton bloom at the study site. The bloom with a concentration of 2.55 µg chlorophyll *a* l^{-1} at the sampling site was dominated by small (<10 µm) flagellates and raphidophytes (Fig. S1). Environmental parameters of the sampling site are summarized in Table 2. CARD-FISH analysis revealed that *Bacteria* constituted 84% of the DAPI cell counts in the unfiltered and 90% in the 1.2 µm prefiltered sample. Respective numbers of *Alpha-* and *Gammaproteobacteria* in both fractions were 23% and 21%, and 40% and 26%, respectively. Prefiltration caused a relative loss of bacteria from the *Cytophaga-Flavobacter-Bacteroidetes* (CFB) phylum from 38% to 9%, but a relative enrichment of cells of the *Roseobacter* clade from 16% to 30% of total DAPI cell counts, equivalent to 68% and 74% of *Alphaproteobacteria*, respectively (Fig. 1).

Table 2. Sampling site characteristics.

Parameter	
Depth (m)	2.0
Water temperature (°C)	11.0
Salinity	34.17
Chlorophyll *a* (µg l^{-1})	2.55
Phaeopigments (µg l^{-1})	0.53
Dry weight (mg l^{-1})	20.6
POC (mg l^{-1})	0.2
Bacterial cell counts:	
Unfiltered sample (10^5 ml^{-1})	8.0
1.2 µm prefiltered sample (10^5 ml^{-1})	1.9

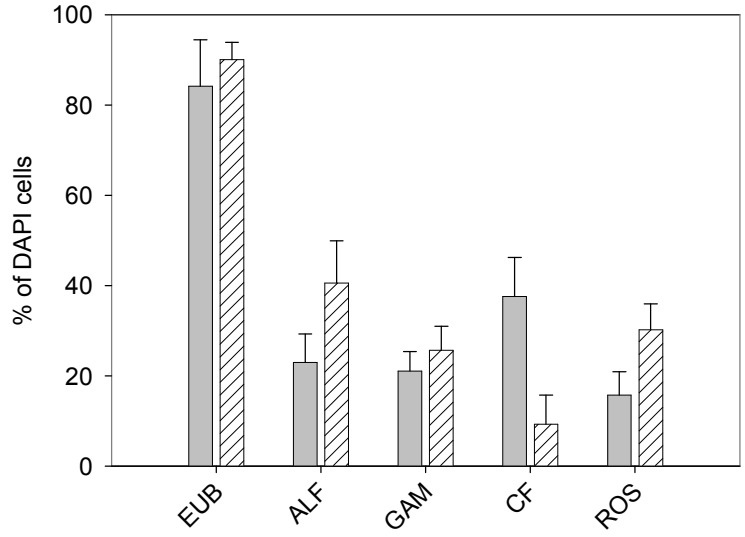


Fig. 1. CARD-FISH analysis of the composition of the bacterial community of the sample collected in the North Sea in May 2007. Proportions of the phylogenetic groups as % of DAPI cell counts (± standard deviation calculated from ten counts of each filter section). Filled bars: untreated sample, hatched bars: sample filtered through 1.2 µm. EUB: *Bacteria*, ALF: *Alphaproteobacteria*, GAM: *Gammaproteobacteria*, CF: *Cytophaga-Flavobacter-Bacteroidetes*, ROS: *Roseobacter* clade.

Enrichment and isolation of strains from the *Roseobacter* clade

The most suitable media for the enrichment of roseobacters were media with peptone and yeast extract (media M4 and M6; Table 1) and a medium containing autoclaved cells of the diatom *Thalassiosira weissflogii* (medium M5). Applying the most probable number (MPN) technique with these media we determined abundances ranging from 1.4 x 10^4 to 2.6 x 10^5 (medium M4), 2.8 x 10^4 to 4.6 x 10^5 (medium M5), and 1.4 x 10^4 to 4.8 x 10^5 (medium M6) roseobacter cells ml^{-1} for the 1.2 µm-prefiltered sample, corresponding to 7 to 137%, 15 to 242%, and 7 to 253% of total cell counts, respectively.

Seven novel *Roseobacter* strains (SH4-1, SH6-1, SH22-1, SH22-2a, SH24-1b, SH36, and SH40) were isolated with different media, from different dilution steps, and from different inocula (Table 3). Identical phylotypes, on the basis of 16S rRNA gene sequences, to strains SH4-1, SH6-1, and SH24-1b were repeatedly isolated also applying different conditions (Table S2). No roseobacters were isolated with media containing peptone, yeast extract and DMSP or autoclaved cells of the diatom *Thalassiosira weissflogii* (medium M3 and M5). It is remarkable that although medium M5 was suitable for the enrichment of roseobacters, no strains were isolated from agar plates with this medium.

Table 3. Isolation conditions used for the seven *Roseobacter* strains obtained in this study. For the composition of the media see Table 1.

Strain	Medium for enrichment	Inoculated with	Obtained from dilution 10^{-x}	Incubation conditions	Isolated on medium
SH4-1	- *	Prefiltered seawater (1.2µm)	-	Dark-Light cycle, 15°C	M6
SH6-1	M1	Prefiltered seawater (1.2µm)	6	Dark, 15°C	M6
SH22-1	M4	Prefiltered seawater (1.2µm)	1	Dark-Light cycle, 15°C	M6
SH22-2a	M4	Prefiltered seawater (1.2µm)	1	Dark-Light cycle, 15°C	M6
SH24-1b	- *	Filtered algae (≥10 µm)	-	Dark-Light cycle, 15°C	M6
SH36	M2	Prefiltered seawater (1.2µm)	4	Dark, 15°C	M2
SH40	M1	Filtered algae (≥10 µm)	7	Dark, 15°C	M1

* Obtained by direct plating

Diversity and phylogeny

Analyses with the *Roseobacter* clade-specific DGGE revealed clear differences in the particle-associated (>10 µm and >5 µm) and the free-living fractions (<5 µm or <1.2 µm; Fig. 2). While the patterns of the free-living fractions showed 10 bands, the particle-

associated fractions exhibited at least 15 bands. Furthermore, the patterns of both particle-associated fractions exhibit some differences whereas those of the two free-living fractions are almost identical. The strains RCA23 (Giebel *et al*., 2011), SH6-1 and SH36 could be allocated to three dominant bands of the free-living fraction of the DGGE banding patterns.

Phylogenetic analysis of the 16S rRNA gene sequences of the seven new isolates as well as of the sequences of six bands obtained with an *Alphaproteobacteria*-specific DGGE approach demonstrated their affiliation to 12 different clusters within the *Roseobacter* clade (Fig. 3, for *Alphaproteobacteria*-specific DGGE pattern see Fig. S2). As the sequence of DGGE band 5.2 is 99% identical to that of strain SH6-1, we consider both sequences as the same phylotype. Ten of the 12 clusters contain strains or clones with sequence identities ≥99% found previously in the North Sea including the Weser estuary and the Skagerrak (Fig. 3, Table S3).

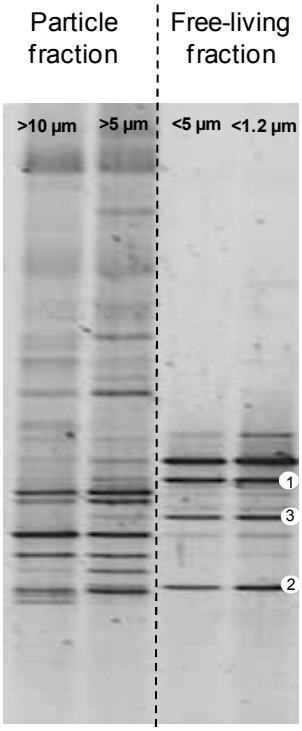
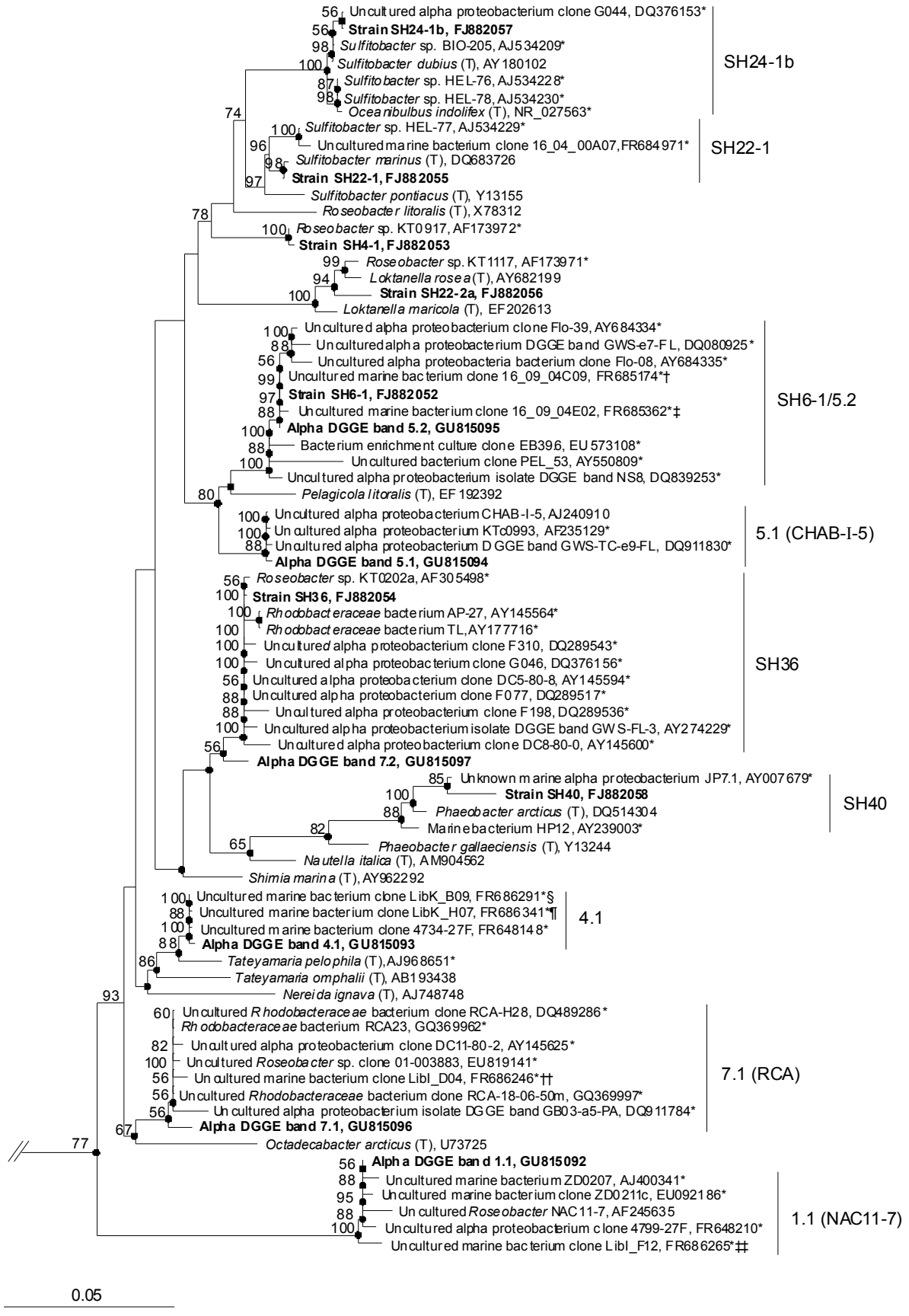


Fig. 2. *Roseobacter*-specific DGGE analysis of the particle-associated (>10µm, >5 µm) and free-living (<5 µm, <1.2 µm) bacterial communities of the sample collected in May 2007 in the North Sea. Bands with identical migration behavior were observed for 1) strain RCA23 (Giebel *et al*., 2011), 2), strain SH36, and 3) strain SH6-1.

Fig. 3. Neighbor-joining tree based on 16S rRNA gene sequence similarity showing the phylogenetic positions of sequences of isolates and DGGE bands obtained in this study (written in bold face) within the *Roseobacter* clade. The tree was generated with selected type species (T) and the nonredundant sequences of North Sea strains and clones that are closely related to the roseobacters detected in this study. Numbers at the nodes are bootstrap values (only >50% are shown) from 1000 replicates. Sequences <1300 nt were added after calculation of the backbone tree. Filled circles indicate nodes also recovered reproducibly with maximum-likelihood calculation. Selected members of the *Gammaproteobacteria* were used as outgroup (not shown). The scale bar indicates 5% sequence divergence. *: Strains or clones obtained from the North Sea during previous studies. For some phylotypes BLAST analysis resulted in many sequence with the same identity from the same study. †: obtained eight times; ‡: obtained 13 times; §: obtained 59 times; ¶: obtained 50 times; ††: obtained 436 times; ‡‡: obtained 163 times.


Uncultured alpha proteobacterium clone G044, DQ376153*
Strain SH24-1b, FJ882057
Sulfitobacter sp. BIO-205, AJ534209*
Sulfitobacter dubius (T), AY180102
Sulfitobacter sp. HEL-76, AJ534228*
Sulfitobacter sp. HEL-78, AJ534230*
Oceanibulbus indolifex (T), NR_027563*
SH24-1b
Sulfitobacter sp. HEL-77, AJ534229*
Uncultured marine bacterium clone 16_04_00A07, FR684971*
Sulfitobacter marinus (T), DQ683726
Strain SH22-1, FJ882055
SH22-1
Sulfitobacter pontiacus (T), Y13155
Roseobacter litoralis (T), X78312
Roseobacter sp. KT0917, AF173972*
Strain SH4-1, FJ882053
Roseobacter sp. KT1117, AF173971*
Loktanella rosea (T), AY682199
Strain SH22-2a, FJ882056
Loktanella maricola (T), EF202613
Uncultured alpha proteobacterium clone Flo-39, AY684334*
Uncultured alpha proteobacterium DGGE band GWS-e7-FL, DQ080925*
Uncultured alpha proteobacteria bacterium clone Flo-08, AY684335*
Uncultured marine bacterium clone 16_09_04C09, FR685174*†
Strain SH6-1, FJ882052
Uncultured marine bacterium clone 16_09_04E02, FR685362*‡
Alpha DGGE band 5.2, GU815095
SH6-1/5.2
Bacterium enrichment culture clone EB396, EU573108*
Uncultured bacterium clone PEL_53, AY550809*
Uncultured alpha proteobacterium isolate DGGE band NS8, DQ839253*
Pelagicola litoralis (T), EF192392
Uncultured alpha proteobacterium CHAB-I-5, AJ240910
Uncultured alpha proteobacterium KTc0993, AF235129*
Uncultured alpha proteobacterium DGGE band GWS-TC-e9-FL, DQ911830*
Alpha DGGE band 5.1, GU815094
5.1 (CHAB-I-5)
Roseobacter sp. KT0202a, AF305498*
Strain SH36, FJ882054
Rhodobacteraceae bacterium AP-27, AY145564*
Rhodobacteraceae bacterium TL, AY177716*
Uncultured alpha proteobacterium clone F310, DQ289543*
Uncultured alpha proteobacterium clone G046, DQ376156*
Uncultured alpha proteobacterium clone DC5-80-8, AY145594*
Uncultured alpha proteobacterium clone F077, DQ289517*
Uncultured alpha proteobacterium clone F198, DQ289536*
Uncultured alpha proteobacterium isolate DGGE band GWS-FL-3, AY274229*
Uncultured alpha proteobacterium clone DC8-80-0, AY145600*
SH36
Alpha DGGE band 7.2, GU815097
Unknown marine alpha proteobacterium JP7.1, AY007679*
Strain SH40, FJ882058
Phaeobacter arcticus (T), DQ514304
Marinebacterium HP12, AY239003*
Phaeobacter gallaeciensis (T), Y13244
Nautella italica (T), AM904562
Shimia marina (T), AY962292
SH40
Uncultured marine bacterium clone LibK_B09, FR686291*§
Uncultured marine bacterium clone LibK_H07, FR686341*¶
Uncultured marine bacterium clone 4734-27F, FR648148*
Alpha DGGE band 4.1, GU815093
4.1
Tateyamaria pelophila (T), AJ968651*
Tateyamaria omphalii (T), AB193438
Nereida ignava (T), AJ748748
Uncultured Rhodobacteraceae bacterium clone RCA-H28, DQ489286*
Rhodobacteraceae bacterium RCA23, GQ369962*
Uncultured alpha proteobacterium clone DC11-80-2, AY145625*
Uncultured Roseobacter sp. clone 01-003883, EU819141*
Uncultured marine bacterium clone LibI_D04, FR686246*††
Uncultured Rhodobacteraceae bacterium clone RCA-18-06-50m, GQ369997*
Uncultured alpha proteobacterium isolate DGGE band GB03-a5-PA, DQ911784*
Alpha DGGE band 7.1, GU815096
7.1 (RCA)
Octadecabacter arcticus (T), U73725
Alpha DGGE band 1.1, GU815092
Uncultured marine bacterium ZD0207, AJ400341*
Uncultured marine bacterium clone ZD0211c, EU092186*
Uncultured Roseobacter NAC11-7, AF245635
Uncultured alpha proteobacterium clone 4799-27F, FR648210*
Uncultured marine bacterium clone LibI_F12, FR686265*‡‡
1.1 (NAC11-7)
0.05

The number of closely related sequences differed when either the whole 16S rRNA gene sequence or the DGGE fragment (ca. 560 bp) was used for BLAST analysis (Table S3). Also almost complete 16S rRNA gene sequences were identified as closest relatives when the DGGE fragments were used for BLAST analysis, but not vice versa. Thus the results presented are based on BLAST analyses of the DGGE fragments.

No North Sea sequences with an identity of ≥99% to sequences of SH22-2a and 7.2 were found. For strain SH22-2a the closest relative detected in the North Sea, *Roseobacter* sp. KT1117 (Eilers *et al*., 2001), showed 98.4% similarity. For 9 of the 12 clusters, except those with isolate SH40 and DGGE bands 4.1 and 7.2, phylotypes with sequence identities ≥99% were also detected in the Yellow Sea (Tables S3 and S4). In other marine regions not more than four of the 12 North Sea roseobacter phylotypes were detected.

The sequences of DGGE bands 1.1, 5.1, and 7.1 are affiliated with three of the four major clusters of the *Roseobacter* clade consisting mainly or exclusively of uncultured phylotypes, i.e. the NAC11-7 cluster (González *et al*., 2000), the CHAB-I-5 cluster (Schäfer *et al*., 2000), and the *Roseobacter* clade-affiliated (RCA) cluster (Selje *et al*., 2004), respectively. The CHAB-I-5 cluster is exclusively composed of uncultured phylotypes mainly derived from coastal seawater (Buchan *et al*., 2005). The RCA cluster is the largest cluster within the *Roseobacter* clade and is restricted from temperate to polar seas (Selje *et al*., 2004; Buchan *et al*., 2005). DGGE band 7.2 showed affiliation to the SH36 cluster (Fig. 3) which contains the isolates and clones AP-27, DC5-80-8, and DC8-80-0 (Selje *et al*., 2005), and TL (Brinkhoff *et al*., 2004). For these organisms high abundance was deduced by Stevens *et al.* (2005b) and can also be assumed for strain SH36 as it was obtained from a high dilution step (10^{-4}).

For strains SH22-1 and SH24-1b BLAST results with the DGGE fragments and phylogenetic analysis with the almost complete 16S rRNA gene sequences showed some discrepancies. Sequences with identities ≥99% obtained by BLAST analysis with the DGGE fragment of strain SH24-1b form a cluster with strain SH22-1 and vice versa. BLAST results obtained with the almost complete 16S rRNA gene sequences of both strains, however, reflected the phylogenetic positions in the tree. Furthermore, BLAST analysis of the DGGE fragment of strain SH22-1 resulted in two sequences, which fell between the clusters of SH22-1 and SH24-1b, showing more than 3% difference to both isolates based on the almost full 16S rRNA gene sequence (not shown).

Substrate utilization and presence of *pufML* genes

Strains SH6-1 and SH36 showed no or only very weak growth on single substrates. Growth of both strains was considerably improved by the addition of 0.1 g l^{-1} yeast extract. Strains SH4-1, SH22-1, SH24-1b, SH36, and SH40 grew on most amino acids, strain SH22-2a on 10 and strain SH6-1 only weakly on six amino acids. None of the strains grew on lysine and valine (Table 4). Most of the tested sugars were used by isolates SH4-1, SH6-1, and SH22-2a, whereas strains SH24-1b and SH40 (both isolated from the phytoplankton fraction) as well as strain SH22-1 grew only on some of them. In particular, growth of strain SH24-1b on sugars was generally weak. Strain SH36 did not grow on any of the tested sugars. Arabinose, rhamnose, and fucose were only used by strain SH4-1. None of the isolates showed growth on glucosamine and on any of the polysaccharides (Table 4). Almost all strains grew well on the tested citric acid cycle intermediates as well as on glycolysis end products and on propionate, glycerol, and tween 80. DMSP was not used as sole carbon and energy source, however, all strains were able to metabolize DMSP (see below).

The analysis of the substrate spectra demonstrated that roseobacters from the same habitat show remarkable differences in the utilization of organic compounds. Most striking are the differences between the strains SH36 and SH6-1 (see above). Three of the other five strains (SH22-1, SH24-1b, and SH40) predominantly grew on amino acids. Strain SH22-2a showed a weak preference for sugars, while strain SH4-1 was very versatile growing on most amino acids and sugars (Fig. 4).

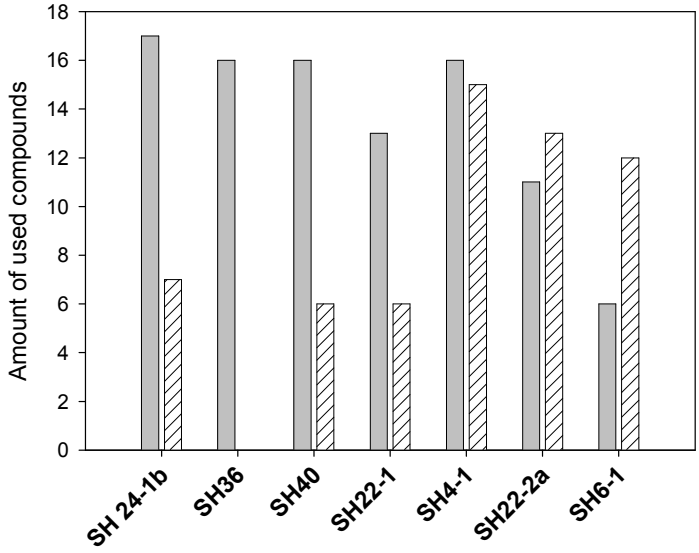


Fig. 4. Number of sugars and amino acids used by the strains isolated in this study. Filled bars: amino acids (20 amino acids were tested), hatched bars: sugars (21 sugar compounds were tested).

Table 4. Substrate spectra of the *Roseobacter* isolates obtained in this study. Yeast extract (0.1 g l^{-1}) was added to cultures of strains SH6-1 and SH36 (+ Y) because no or only very weak growth was observed without addition of yeast extract. Cell cultures with 0.1 g l^{-1} yeast extract were used as negative control. +: growth, -: no growth, w: weak growth (OD_{600} ≤0.2).

Substrate \ Strain	SH4-1	SH6-1 (+ Y)	SH22-1	SH22-2a	SH24-1b	SH36 (+ Y)	SH40
Amino acids:							
L-Alanine	+	w	+	+	+	w	+
L-Arginine	+	w	+	w	+	+	w
L-Asparagine	-	-	+	+	+	w	+
L-Aspartic acid	+	w	+	+	w	+	+
L-Cysteine	-	-	+	w	+	w	+
L-Glutamine	+	-	+	+	+	+	+
L-Glutamic acid	+	-	+	-	+	+	+
Glycine	w	-	-	w	w	w	+
L-Histidine	+	-	-	-	w	+	-
L-Isoleucine	+	-	-	-	-	w	w
L-Leucine	+	-	+	w	+	w	+
L-Lysine	-	-	-	-	-	-	-
L-Methionine	w	-	-	-	+	-	-
L-Phenylalanine	+	-	+	-	w	+	+
L-Proline	+	w	+	w	w	+	+
L-Serine	+	w	+	w	+	+	+
L-Threonine	+	-	-	-	w	+	+
L-Tryptophan	+	w	+	-	w	-	+
L-Tyrosine	+	-	+	-	w	w	+
L-Valine	-	-	-	-	-	-	-
Sugars:							
(+)-D-Xylose	+	w	+	w	w	-	+
(+)-D-Glucose	+	w	+	+	w	-	+
(+)-D-Mannose	+	w	w	+	w	-	+
(+)-D-Galactose	+	w	-	+	-	-	-
(-)-D-Fructose	+	w	+	+	w	-	+
(+)-L-Arabinose	+	-	-	-	-	-	-
(-)-D-Ribose	-	w	-	-	+	-	w
(+)-L-Rhamnose	+	-	-	-	-	-	-
(-)-L-Fucose	+	-	-	-	-	-	-
(-)-D-Mannitol	+	w	+	+	w	-	+
(-)-D-Sorbitol	+	-	+	+	+	-	-
(+)-D-Glucosamine	-	-	-	-	-	-	-
Lactose	+	w	-	+	-	-	-
Sucrose	+	+	-	+	-	-	-
Maltose	+	w	-	+	-	-	-
Cellobiose	+	+	-	+	-	-	-
Trehalose	+	w	-	+	-	-	-
Laminarin	-	-	-	-	-	-	-
Starch	-	-	-	-	-	-	-
Inulin	-	-	-	-	-	-	-
Xylan	-	-	-	-	-	-	-
Other organic compounds:							
Sodium formiate	-	-	-	-	-	-	-
Sodium acetate	+	w	+	+	+	+	+
Sodium pyruvate	+	+	+	+	+	+	+
Sodium malate	+	w	+	+	w	+	+
Citric acid	+	w	+	+	w	+	+
Disodium succinate	+	-	+	-	w	+	+
Sodium lactate	+	+	+	+	w	w	+
Sodium propionate	+	-	+	+	w	w	+
Glycerol	+	w	+	+	+	-	+
Tween 80	+	w	+	-	w	-	w
DMSP	-	-	-	-	-	-	-

None of the strains produced any pigments or bacteriochlorophyll *a*, however, analysis by a specific PCR approach and subsequent sequencing of the PCR products revealed that strains SH6-1 and SH22-2a contain *pufML* genes, coding for subunits of the bacterial photosynthetic reaction centre complex. This indicates that these organisms might be capable to perform aerobic anoxygenic photosynthesis.

Degradation of DMSP

Headspace analyses of the new isolates grown in liquid cultures and on agar plates spiked with [$^2H_6$]DMSP were performed to investigate the catabolism to sulfur volatiles. The isotopic labelling of DMSP was required to unambiguously distinguish between DMSP and other sulfur sources such as sulfate as precursors for sulfur volatiles. Volatiles were collected by use of a closed-loop stripping apparatus (CLSA) and analyzed via GC-MS (for details of all compounds detected see Table S5 and Fig. S3).

All strains produced large amounts of dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), and *S*-methyl methanethiosulfonate (Fig. 5), while the higher homolog dimethyl tetrasulfide was only found as trace compound in some extracts. Methyl methylthiomethyl disulfide was produced by all strains growing on agar plates and also occasionally by liquid cultures. The principal components DMDS, DMTS, and *S*-methyl methanethiosulfonate are derived from MeSH by oxidations (Chin & Lindsay, 1994; Bashkova *et al.*, 2002), and methyl methylthiomethyl disulfide arises by photolysis of DMDS (Buttery & Seifert, 1977). The deuterium incorporation into these compounds with rates of 30 - 96% by all strains, but <5% by strain SH6-1, proves catabolism of [$^2H_6$]DMSP via demethylation and lysis to [$^2H_3$]MeSH, whereas the additional formation of [$^2H_6$]dimethyl sulfoxide by strains SH4-1, SH24-1b, SH36, and SH40 demonstrates their potential for cleavage of [$^2H_6$]DMSP to [$^2H_6$]DMS. Several further sulfur volatiles originating from DMSP were identified in strain-specific patterns that, apart from methyl 3-(methylsulfanyl)propionate, have been previously found in other bacteria from the *Roseobacter* clade (Dickschat *et al*., 2010; Dickschat *et al*., 2005a, 2005b; for a brief discussion cf. Supporting Information). Strain SH22-1 additionally released methyl 3-(methyldisulfanyl)propionate that has not been described as natural product before. Its structure has been verified by synthesis (see Supporting Information).

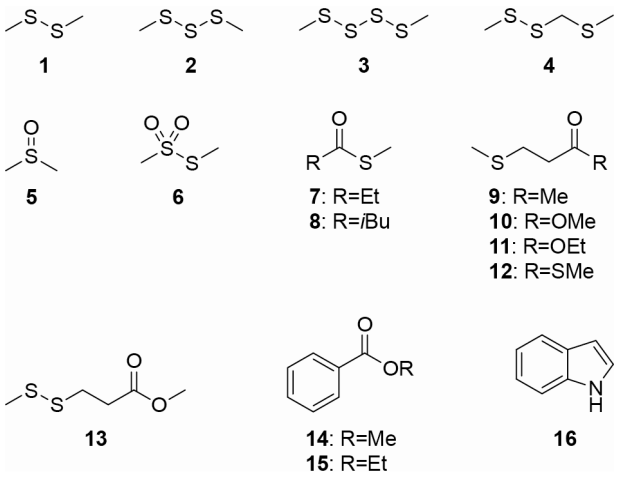


Fig. 5. Volatiles released by the seven strains from the *Roseobacter* clade isolated in this study. Compound **1**: dimethyl disulfide (DMDS), **2**: dimethyl trisulfide (DMTS), **3**: dimethyl tetrasulfide, **4**: methyl methylthiomethyl disulfide, **5**: dimethyl sulfoxide (DMSO), **6**: *S*-methyl methanethiosulfonate, **7**: *S*-methyl propanethioate, **8**: *S*-methyl 3-methylbutanethioate, **9**: 4-(methylsulfanyl)butan-2-one, **10**: methyl 3-(methylsulfanyl)propionate, **11**: ethyl 3-(methylsulfanyl)propionate, **12**: *S*-methyl 3-(methylsulfanyl)propanethioate, **13**: methyl 3-(methyldisulfanyl)propionate, **14**: methyl benzoate, **15**: ethyl benzoate, **16**: indole.

Discussion

Our results show that roseobacters constitute the majority of *Alphaproteobacteria* in the North Sea during a phytoplankton bloom and represent one of the most abundant groups of the bacterioplankton community. The proportions of bacteria of the *Roseobacter* clade and other phylogenetic groups we found are within ranges reported previously for the North Sea (Eilers *et al*., 2001; Zubkov *et al*., 2001; Sekar *et al*., 2004). Members of the phylum *Bacteroidetes* are known to have a preference for growth on high molecular weight material and in particular on organic particles such as phytoplankton detritus (Kirchman 2002; Grossart *et al.*, 2005; Rink *et al.*, 2007), thus explaining the loss of most of these organisms after sample filtration through 1.2 µm. By use of a combination of cultivation-dependent and -independent methods we identified 12 different co-occurring roseobacters. These organisms are probably common North Sea inhabitants since for most of the phylotypes closely related strains or clones were previously detected in the North Sea at different times and locations (e.g. Eilers *et al*., 2001; Zubkov *et al*., 2001; Allgaier *et al*., 2003; Brinkhoff *et al*., 2004; Selje *et al*., 2005; Stevens *et al*., 2005a; Rink *et al*., 2007). In

none of these studies, however, such a broad diversity of roseobacters has been demonstrated.

As reported previously (e.g. Suzuki *et al*., 1997; Kisand & Wikner, 2003), we also found a strong discrepancy between culturable and non-culturable organisms. Only one phylotype, represented by strain SH6-1, was obtained by isolation as well as by DGGE. While use of dilution cultures with different media allowed the isolation of a variety of strains, three of the roseobacters detected only by DGGE belong to three of the four major clusters of the *Roseobacter* clade consisting exclusively or mainly of uncultured phylotypes (i.e. RCA, NAC11-7 and CHAB-I-5, Buchan *et al*., 2005). Thus, for detecting a broad diversity of co-occurring roseobacters, the combination of culture-dependent and -independent approaches is necessary.

Isolation and physiology of new *Roseobacter* strains

The use of a variety of media and enrichment conditions was suitable to obtain seven different *Roseobacter* strains from one sample. Random selection of colonies, and screening with the specific PCR approach, combined with DGGE analyses, was a successful and timesaving strategy to detect different roseobacters. We did not observe an increased cultivation efficiency of heterotrophic bacteria by addition of cAMP to the medium as described by Bruns *et al.* (2002). Strain SH36, however, was exclusively isolated using the medium containing cAMP even though it also grows on some of the other media. To enrich roseobacters the most suitable media were artificial saltwater media with peptone and yeast extract, and seawater with autoclaved cells of the diatom *Thalassiosira weissflogii*. With these media the cultivation efficiencies for roseobacters were in the same order of magnitude as the proportion detected with CARD-FISH. Stevens *et al.* (2009) also yielded high cultivation efficiencies with MPN cultures using Marine Broth medium and dried and pestled material of the macroalga *Fucus vesiculosus* as substrate. Since roseobacters could be efficiently enriched, also in high dilutions, but not isolated with the media containing DMSP or *T. weissflogii* cell extracts, it is possible that these strains simply cannot be isolated on solid media as recently described for a strain affiliated with the RCA cluster (Giebel *et al*., 2011).

The addition of phytoplankton-derived dissolved organic matter (DOM) to bacterial communities mimics the situation during phytoplankton blooms and shows by the high cultivation efficiencies that organisms of the *Roseobacter* clade can effectively use this material. Also other studies, based on culture-independent approaches, have found that

members of the *Roseobacter* clade are particularly responsive to phytoplankton-derived DOM (Riemann *et al.*, 2000; Schäfer *et al.*, 2001; Grossart *et al.*, 2005). High abundances of strains of the RCA cluster during blooms of diatoms (Giebel *et al.*, 2011) and dinoflagellates (Mayali *et al.*, 2008) and of strain SH6-1 during a *Phaeocystis* bloom (S. Hahnke *et al.*, unpubl. results) further support these notions. Strains SH6-1, SH36, and RCA23, allocated to three of the dominant DGGE bands of the free-living fraction, needed the addition of complex protein-based substrates to grow reliably (this study; Giebel *et al.*, 2011). This finding also suggests a growth stimulation of these strains during phytoplankton blooms, since excretion by phytoplankton and cell lysis provide a milieu of diverse organic matter.

DMSP degradation

Our results show that all seven newly isolated strains can degrade the algal osmolyte DMSP to MeSH and produce various MeSH-derived volatile compounds, whereas only four strains (SH4-1, SH24-1b, SH36, and SH40) emitted traces of the DMS oxidation product DMSO. These data are in line with previous findings, demonstrating that the demethylation pathway is more widely distributed within the *Roseobacter* clade than the cleavage pathway and that the major portion of marine DMSP is catabolized to MeSH and not to DMS (Kiene *et al.*, 2000; Dickschat *et al.*, 2010; Newton *et al.*, 2010).

Even though growth of bacteria on DMSP as the sole carbon and energy source has been observed (Ledyard & Decay, 1994; Yoch, 2002), our strains did not grow on DMSP as the sole carbon source and only metabolized added DMSP when grown on complex media. There is ample information that roseobacters show chemotactic behaviour towards DMSP, are capable of metabolizing and consuming DMSP under *in situ* conditions and that DMSP serves as the major sulfur source in marine bacterioplankton (Kiene *et al.*, 1999; Zubkov *et al.*, 2001; Moran *et al.*, 2003; Malmstrom *et al.*, 2004; Miller *et al.*, 2004; Dickschat *et al.*, 2010; Newton *et al.*, 2010). Our study adds to this information by experimentally showing how DMSP is metabolized in different ways in *Roseobacter* strains of different phylogenetic subclusters co-occurring during a phytoplankton bloom. As we applied DMSP concentrations in the mM range as compared to nM concentrations occurring *in situ* during phytoplankton blooms (Zubkov *et al.*, 2001; Malmstrom *et al.*, 2004) we cannot directly infer that similar DMSP metabolites as we found also occur at ambient conditions. However, our study demonstrates that the *Roseobacter* strains we tested have the potential to produce these metabolites. Future studies need to show whether

these compounds are also produced under *in situ* conditions, and if so, whether they exhibit specific functions.

Niche separation of different *Roseobacter* isolates

Since microenvironments cannot be separated during sampling, different isolation techniques and subsequent characterization of the organisms are needed to learn about the possible eco-niche of a specific bacterium (Simu *et al*., 2005). This is also true for members of the *Roseobacter* clade and directed our sampling and isolation strategy, because single organisms of this clade have been detected and isolated in rather specific environments, including various phytoplankton species, organic aggregates and the free-living phase (Allgaier *et al.*, 2003; Buchan *et al*., 2005; Grossart *et al*., 2005). Many roseobacters have been characterized so far and it was shown that they are able to use a large variety of organic substances (Brinkhoff *et al*., 2008; Newton *et al.*, 2010). However, in one single strain only a subset of these physiological properties is present, making the strains unique in the composition of their physiological abilities (Newton *et al.*, 2010). This is also reflected by the different substrate utilization spectra of the seven isolated roseobacters obtained in this study and probably reflects specific adaptations of the individual organisms. Most noticeable in that respect is the substrate spectrum of strain SH36, which did not grow on any of the tested sugars. On the other hand almost every tested substrate was used by at least one of the strains. Furthermore, in strains SH6-1 and SH22-2a *pufML* genes were found, indicating that they are capable of aerobic anoxygenic photosynthesis and exploring light as an additional energy source (Wagner-Döbler & Biebl, 2006). The ability to use different substrates and energy sources illustrates that the *Roseobacter* strains occupy different niches and partially explains their ability to coexist. A spatial segregation, which effectively reduces competition between species according to classical theories (e.g. Wilson, 1990; Lyons, 2010), was found for roseobacters by the specific DGGE approach, demonstrating clear differences between the particle-associated and the free-living communities. The strains SH24-1b and SH40, obtained from the particle-associated fraction, show very similar substrate utilization spectra and use mainly amino acids. Proteins and amino acids are highly enriched and solubilized in organic aggregates (Smith *et al*., 1992), thus making these microstructures well suitable for the life style of both roseobacters.

Continuous variation in environmental conditions is another factor permitting high biodiversity (e.g. Konopka *et al*., 2007; Spencer *et al*., 2007). Even in seemingly

homogeneous habitats, such as the surface mixed layer of the ocean due to variations in phytoplankton composition and growth, grazing pressure and phage infection, the bacterial community never reaches a state of equilibrium (Scheffer *et al*., 2003). During the course of a phytoplankton bloom bacteria undergo temporal changes in the availability of organic resources. Variations in DOM composition are caused by the differential release of individual organic compounds by the complex phytoplankton in various growth stages and release from other detrital material and processing by heterotrophic organisms. The composition of DOM released by algae is species specific and the nutrient status also profoundly affects the amount and composition of exudates (Myklestad, 1974; van Rijssel *et al*., 2000; Biersmith & Benner, 1998). Fluctuation in nutrient and substrate availability might provide niches for bacteria responding differently to different substrate concentrations (Konopka, 1999).

It is striking that nine of the 12 different phylotypes detected in this study were also found in the Yellow Sea. The Yellow Sea shows remarkable similarities to the North Sea. Both are semi-enclosed shallow seas of similar size and low mean depths (458.000 km^2 and 44 m, compared to 575.300 km^2 and 74 m) (Lü *et al*., 2007; Otto *et al*., 1990) and both possess extensive stretches of tidal flats. Tidal dynamics lead to an intense exchange of organic and inorganic nutrients with the open sea. Furthermore, these ecosystems also receive a remarkable nutrient and sediment input from rivers. As a result, both systems are characterized by high productivity (Poremba *et al*., 1999; Tian *et al*., 2005). Cho *et al.* (1994) reported that the ranges of bacterial and phytoplankton variables, water temperature, nutrient concentrations, and well-mixed conditions along a transect in the mouth of the Yellow Sea were similar to those found in the North Sea during a spring bloom. Thus, the North Sea and the Yellow Sea might provide conditions, which promote growth of similar heterotrophic bacterial communities.

This study demonstrates a high diversity of roseobacters present in the North Sea and shows that co-occurring *Roseobacter* isolates exhibit remarkable physiological differences. The latter explains to a certain extent their simultaneous presence and niche separation in a phytoplankton bloom in the southern North Sea. Our results also confirm that organisms of the *Roseobacter* clade represent one of the most abundant groups of the bacterial community in surface waters of the North Sea, playing an important role in organic matter cycling in this ecosystem, especially during phytoplankton blooms. The mechanisms leading to and explaining the coexistence of different roseobacters are, however, only partially understood and need to be analyzed in more depth in future studies.

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Supporting Information

Synthesis of dimethyl 3,3'-disulfanediyldipropanoate (**25**)

A solution of methyl 3-mercaptopropanoate (**24**, 12.031 g, 100.26 mmol, 1.0 eq.) and Et_3N (10.240 g, 101.39 mmol, 1.0 eq.) in DMF (100 ml) was treated for 24 h in an ultrasonic bath at 40°C. The reaction was quenched with water, extracted with ethyl acetate, washed with water, dried with $MgSO_4$, and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (hexane / ethyl acetate = 5:1) to give **25** (6.591 g, 27.69 mmol, 55%) as a yellowish oil. TLC (hexane / ethyl acetate = 5:1): R_f = 0.19. GC: I = 1746. MS (EI, 70 eV): m/z (%) = 238 (M^+, 100), 207 (26), 175 (17), 151 (6), 118 (87), 109 (6), 92 (4), 87 (59), 77 (13), 64 (9), 59 (97), 55 (60), 45 (28), 43 (5). ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 3.71 (s, 6 H, 2 x CH_3), 2.93 (t, 4 H, $^3J_{H,H}$ = 7.4 Hz, 2 x CH_2), 2.75 (t, 4 H, $^3J_{H,H}$ = 7.2 Hz, 2 x CH_2). ^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 172.1 (C_q), 51.8 (CH_3), 33.8 (CH_2), 33.0 (CH_2). IR (diamond-ATR): ν□ (cm^{-1}) = 2953 (w), 1731 (s), 1436 (m), 1354 (m), 1239 (m), 1215 (m), 1195 (m), 1171 (s), 1138 (m), 1017 (w), 979 (w), 911 (m), 823 (w), 729 (s), 648 (w).

Synthesis of methyl 3-(methyldisulfanyl)propanoate (**13**) (Fig. S5)

To a solution of dimethyl 3,3'-disulfanediyldipropanoate (**25**, 6.591 g, 27.69 mmol, 1.0 eq.) and dimethyl disulfide (**1**, 2.624 g, 27.91 mmol, 1.0 eq.) in dry CH_2Cl_2 (100 ml) and CH_3NO_2 (100 ml) was added $BF_3 \cdot Et_2O$ (0.393 g, 2.77 mmol, 0.1 eq.) at 0°C. After stirring at 0°C for 3 h and at room temperature overnight, the reaction was quenched with water, extracted with ethyl acetate, dried with $MgSO_4$, and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (hexane / ethyl acetate = 5:1) to give **13** (3.975 g, 23.95 mmol, 43%) and recovered **25** (3.393 g, 14.26 mmol, 51%) both as yellowish oils. TLC (hexane / ethyl acetate = 5:1): R_f = 0.45. GC: I = 1260. MS (EI, 70 eV): m/z (%) = 166 (M^+, 100), 135 (22), 118 (63), 107 (25), 93 (18), 87 (39), 79 (49), 64 (12), 59 (74), 55 (18), 45 (38), 41 (4). ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 3.71 (s, 3 H, CH_3), 2.95 (t, 2 H, $^3J_{H,H}$ = 7.2 Hz, CH_2), 2.77 (t, 2 H, $^3J_{H,H}$ = 7.2 Hz, CH_2), 2.41 (s, 3 H, CH_3). ^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 172.1 (C_q), 51.8 (CH_3), 33.9 (CH_2), 32.3 (CH_2), 23.1 (CH_3). IR (diamond-ATR): ν□ (cm^{-1}) = 2994 (w), 2951 (w), 2917 (w), 2843 (w), 1733 (s), 1434 (m), 1354 (m), 1239 (m), 1213 (m), 1170 (s), 1138 (m), 1017 (w), 979 (w), 953 (w), 822 (w), 667 (w).

Volatiles produced from fatty acids or the amino acid pool

Other volatiles originating either from fatty acids or the amino acid pool were found in strain-specific patterns and were most efficiently produced by agar plate cultures. The fatty acyl-CoA-derived thioesters **7** and **8** arise by enzymatic transesterification with MeSH (Helinck *et al.*, 2000). Transamination of L-methionine, oxidative decarboxylation to **20**, and formal transesterification with EtOH or MeSH, respectively, yields the ester **11** or the thioester **12** (Dickschat *et al.*, 2010; Fig. S6). Acid liberation from **20** and methylation with *S*-adenosyl methionine could account for the methyl ester **10**, while its chain elongation in analogy to fatty acid biosynthesis followed by spontaneous decarboxylation of the 3-oxo acid **23** furnishes the ketone **9** (Fig. S6). The deuterium incorporation into **9** – **12** points to the uptake of [$^2H_3$]MeSH into the amino acid pool by reaction with *O*-acetyl homoserine (**17**) to [$^2H_3$]-L-methionine.

A few compounds were specifically emitted by single strains such as the major component of strain SH22-1, indole (**16**). An unknown sulfur volatile from this strain was proposed to be methyl 3-(methyldisulfanyl)propanoate (**13**), based on its mass spectrum (Fig. S4). A synthesis via ultrasonic dimerisation of methyl 3-mercaptopropionate (**24**) followed by disulfide interchange with dimethyl disulfide (**1**) established its identity (Fig. S5). Further, the aromates methyl benzoate (**14**) and ethyl benzoate (**15**) were produced only by strain SH40.

Table S1. Composition of media M6 and M7.

Component	M6	M7
NaCl (mM)	416.0	513.0
$MgCl_2$ x $6H_2O$ (mM)	49.2	4.9
$CaCl_2$ x $2H_2O$ (mM)	10.0	1.0
KCl (mM)	8.9	9.4
Na_2SO_4 (mM)	28.2	28.2
HEPES (mM)	10.0	10.0
KBr (µM)	840.0	840.0
H_3BO_3 (µM)	400.0	400.0
$SrCl_2$ (µM)	150.0	150.0
NH_4Cl (µM)	400.0	400.0
KH_2PO_4 (µM)	40.0	40.0
NaF (µM)	70.0	70.0

Table S2. Enrichment and isolation conditions of the seven *Roseobacter* phylotypes. The first column of each phylotype indicates the origin of the strain used for further analyses, the following columns specify the origins of identical phylotypes obtained in this study (based on 16S rRNA gene sequence comparison).

Strain / Conditions	SH4-1				SH6-1				SH22-1	SH22-2a	SH24-1b					SH36	SH40
Inoculum:																	
Prefiltered seawater (1.2 µm)	x	x	x		x	x	x		x	x					x	x	
Filtered algea (≥10 µm)				x				x			x	x	x	x			x
Enrichment medium:																	
M1, seawater (10 µm) + yeast extract + peptone				x	x			x									x
M2, seawater (10 µm) + yeast extract + peptone + cAMP																x	
M4, substrate reduced Marine Broth									x	x							
M6, artificial saltwater medium						x	x										
Dilution step (10^{-x})	-*	-*	-*	8	6	4	7	5	1	1	-*	-*	-*	-*	-*	4	7
Isolation medium:																	
M1, seawater (10 µm) + yeast extract + peptone																	x
M2, seawater (10 µm) + yeast extract + peptone + cAMP																x	
M4, substrate reduced Marine Broth								x									
M6, artificial saltwater medium	x	x	x		x	x	x		x	x	x	x	x	x	x		
M7, + glucose				x													

* Obtained by direct plating

Table S3. Number of BLAST hits of the almost full length 16S rRNA gene sequences and the corresponding DGGE fragments (including the hits from the almost full length sequence), numbers of closely related (≥99% identity) non-redundant sequences occurring in the North Sea, and occurrence in the Yellow Sea. +: present; -: absent.

	Number of BLAST hits ≥99%		Sequence occurrence	
Strain/DGGE band	Full sequence	DGGE fragment	North Sea	Yellow Sea
SH4-1	3	22	1	+
SH6-1	7	60	8	+
SH22-1	42	106	6	+
SH22-2a	8	36	0	+
SH24-1b	37	114	4	+
SH36	41	76	10	+
SH40	1	17	2	-
1.1 (NAC11-7)	-	490	4	+
4.1	-	118	3	-
5.1 (CHAB-I-5)	-	79	2	+
5.2 (SH6-1)	-	30	8	+
7.1 (RCA)	-	763	7	+
7.2	-	0	0	-

Table S4. Accession numbers of 16S rRNA gene sequences obtained from isolates and clones from the Yellow Sea showing identities ≥99% to the roseobacter phylotypes obtained from the North Sea within this study.

North Sea phylotype (Acc. no.)	Acc. no. of sequences of the Yellow Sea
DGGE band 1.1 (GU815092)	FJ825803, FJ825835, FJ825840, FJ825897, FJ825902, FJ825907, FJ825910, FJ825913, FJ825918, FJ825921, FJ825922, FJ825924, FJ825933, FJ825944, FJ825951, FJ825956, FJ825957, FJ825958, FJ825970, FJ825987, FJ825995, FJ826083, FJ826087, FJ826094, FJ826101, FJ826103, FJ826110, FJ826113, FJ826118, FJ826135, FJ826150, FJ826178, FJ826387, FJ826508, GU061023, HM057657, HM057668, HM057687
DGGE band 5.1 (GU815094)	FJ545507, FJ545513, FJ545522, FJ825934, FJ826232, FJ826260, FJ826325, FJ826400, FJ826501, HM057611, HM057744
DGGE band 5.2 (GU815095)	EU005298, EU005307, EU005290
DGGE band 7.1 (GU815096)	GU061061, GU061179, GU061182, GU061290, GQ468662, GQ468663, GQ468664, HM057656, HM057659, HM057689, HM057693
Strain SH4-1 (FJ882053)	EU005317
Strain SH6-1 (FJ882052)	EF215753, EF215778, EF215824, EF491378, EU005307, EU005290, EU005298, GU061021, GU061063, GU061132, GU061135, GU061151, GU061161
Strain SH22-1 (FJ882055)	EF491425, EU617818, EU939703, GU061005, GU061007, GU061009, GU061017, GU061020, GU061022, GU061025, GU061028, GU061032, GU061046, GU061050, GU061065, GU061060, GU061062, GU061079, GU061085, GU061111, GU061118, GU061126, GU061128, GU061130, GU061133, GU061147, GU061165, GU061168, GU061169, GU061198, GU061226, GU061283, HM032815
Strain SH22-2a (FJ882056)	EF491281, EU919202, EU939711, GU061018, GU061083, GU061152, GU061240, GU061270
Strain SH24-1b (FJ882057)	AY456219, EU617818, GU061005, GU061007, GU061009, GU061017, GU061020, GU061022, GU061025, GU061028, GU061029, GU061032, GU061050, GU061060, GU061062, GU061085, GU061111, GU061118, GU061126, GU061128, GU061129, GU061130, GU061133, GU061147, GU061150, GU061165, GU061168, GU061169, GU061198, GU061226, GU061283, HM032815, HM544041
Strain SH36 (FJ882054)	EF215736, EF491274, EF491293, EF491323, EF491328, EF491389, EF491416, FJ154967, FJ436731, FJ436732, GU061006, GU061090, HM057661, HM057670

Table S5. Volatiles released by the isolated strains from the *Roseobacter* clade obtained from MB liquid cultures (LC) or agar plate cultures (AP) amended with 1 mM [$^2H_6$]DMSP.

Compound[a]	*I*[b]	Ident.[c]	SH4-1[d]		SH6-1		SH22-1		SH22-2a		SH24-1b		SH36		SH40	
			LC	AP	LC	AP	LC	AP	LC	AP	LC	AP	LC	AP	LC	AP
[$^2H_6$]Dimethyl disulfide		ms	++	+++		+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
[$^2H_3$]Dimethyl disulfide		ms	+++	++		+	++	+++	++	++	+++	++	+++	+++	+++	+++
Dimethyl disulfide (**1**)		ms, syn	+++	+	+++	+++	++	+++	+	+	+++	+	+	+++	+	+++
[$^2H_3$]-*S*-Methyl propanethioate	800	ms		+				+						+		
S-Methyl propanethioate (**7**)	802	ms, syn						+			+			+		
[$^2H_6$]Dimethyl sulfoxide (**5**)	831	ms, syn		+							+			+		+
[$^2H_3$]-*S*-Methyl 3-methylbutanethioate	939	ms		++				++								
S-Methyl 3-methylbutanethioate (**8**)	941	ms, syn		+				++								
[$^2H_6$]Dimethyl trisulfide	962	ms	+	+		+	+	++	++	+	+	+	+	+	+	+
[$^2H_3$]Dimethyl trisulfide	964	ms	+	+		+	+	++	+	+	++	+	+	+	+	++
Dimethyl trisulfide (**2**)	966	ms, syn	+	+	+++	+++	+	+	+	+	++	+	+	+	+	++
[$^2H_3$]-4-(Methylsulfanyl)butan-2-one	989	ms								+		+		+		
4-(Methylsulfanyl)butan-2-one (**9**)	991	ms, syn								+		+		+		
[$^2H_3$]Methyl 3-(methylsulfanyl)propionate	1025	ms		+				+								
Methyl 3-(methylsulfanyl)propionate (**10**)	1028	ms, syn		+				+								
[$^2H_6$]-*S*-Methyl methanethiosulfonate	1061	ms	+	+		+	+	+++	++	+	+	+	++	+	+	+
[$^2H_3$]-*S*-Methyl methanethiosulfonate	1063	ms	++	+		+	+	++	+	+	+	+	+	+	+	++
S-Methyl methanethiosulfonate (**6**)	1065	ms, syn	++	+	++	++	+	+	+	+	+	+	+	+	+	++
Methyl benzoate (**14**)	1095	ms, syn														+
[$^2H_3$]Ethyl 3-(methylsulfanyl)propionate	1098	ms						+								
Ethyl 3-(methylsulfanyl)propionate (**11**)	1100	ms						+								
[$2H_8$]Methyl methylthiomethyl disulfide	1118	ms		+				+		+		+		+	+	+
[$2H_6$]Methyl methylthiomethyl disulfide	1119	ms		+				+		+		+		+	+	+
[$2H_5$]Methyl methylthiomethyl disulfide	1121	ms		+				+		+		+		+	+	+

[$^2H_3$]Methyl methylthiomethyl disulfide	1123	ms	+				+		+		+		+	+	+
[$^2H_2$]Methyl methylthiomethyl disulfide	1125	ms	+				+				+		+	+	+
Methyl methylthiomethyl disulfide (**4**)	1128	ms	+	+		+	+				+		+	+	+
Ethyl benzoate (**15**)	1170	ms, syn													+
[$^2H_6$]-*S*-Methyl 3-(methylsulfanyl)propanethioate	1193	ms	+				+		+						
[$^2H_3$]-*S*-Methyl 3-(methylsulfanyl)propanethioate	1195	ms	+				+		+						
S-Methyl 3-(methylsulfanyl)propanethioate (**12**)	1197	ms, syn					+								
[$^2H_6$]Dimethyl tetrasulfide	1208	ms			+					+					+
[$^2H_3$]Dimethyl tetrasulfide	1211	ms			+					+					+
Dimethyl tetrasulfide (**3**)	1213	ms		+	+					+					+
[$^2H_3$]Methyl 3-(methyldisulfanyl)propionate	1239	ms				+									
Methyl 3-(methyldisulfanyl)propionate (**13**)	1241	ms, syn				++									
Indole (**16**)	1293	ms, syn				+++									

[a] Unidentified compounds, artifacts, and compounds arising from the medium are not mentioned. Compound numbers refer to numbers in Fig. 5 (main text). [b] Retention indices determined from a homologous series of *n*-alkanes. [c] Identification of compounds based on their mass spectra by comparison to data base spectra (ms) and by comparison to synthetic references (syn). [d] Strains were grown in 50% MB medium as liquid cultures (LC) or on agar plates (AP). The relative amounts of the compounds in the headspace extracts are noted by +: trace compound (0–2%), ++: minor compound (2–8%), and +++: main compound (>8% of total area in GC).

The compounds *S*-methyl propanethioate (**7**), *S*-methyl 3-methylbutanethioate (**8**), 4-(methylsulfanyl)butan-2-one (**9**), ethyl 3-(methylsulfanyl)propionate (**11**), and *S*-methyl 3-(methylsulfanyl)propanethioate (**12**) have been found in other bacteria from the *Roseobacter* clade (**7**: *Phaeobacter gallaeciensis*, *Oceanibulbus indolifex*; **8**: *P. gallaeciensis*, *O. indolifex*; **9**: *Loktanella* sp., *Octadecabacter* sp.; **11**: *O. indolifex*; **12**: *O. indolifex*), whereas methyl 3-(methylsulfanyl)propionate (**10**) has not been described from the *Roseobacter* clade and methyl 3-(methyldisulfanyl)propionate (**13**) is a new natural product.

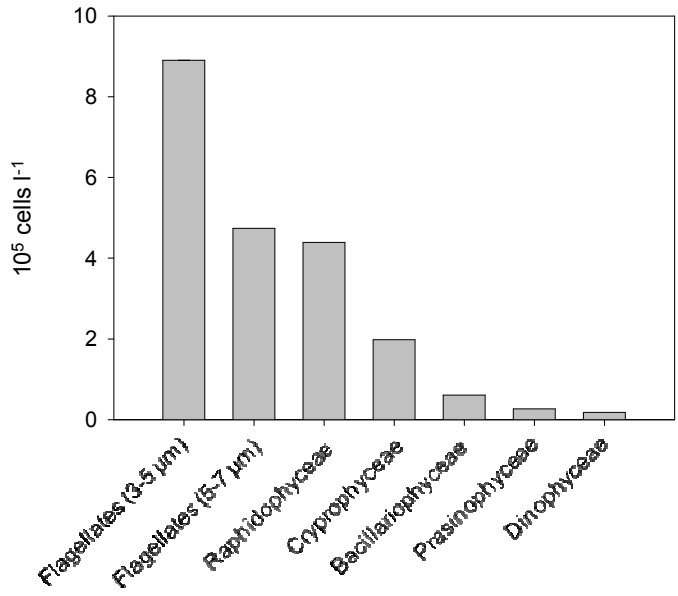

Fig. S1. Phytoplankton composition on the taxonomic level of families. The not further described flagellates contain bacteriovorous and autotrophic cells. A mean value of duplicates is shown.

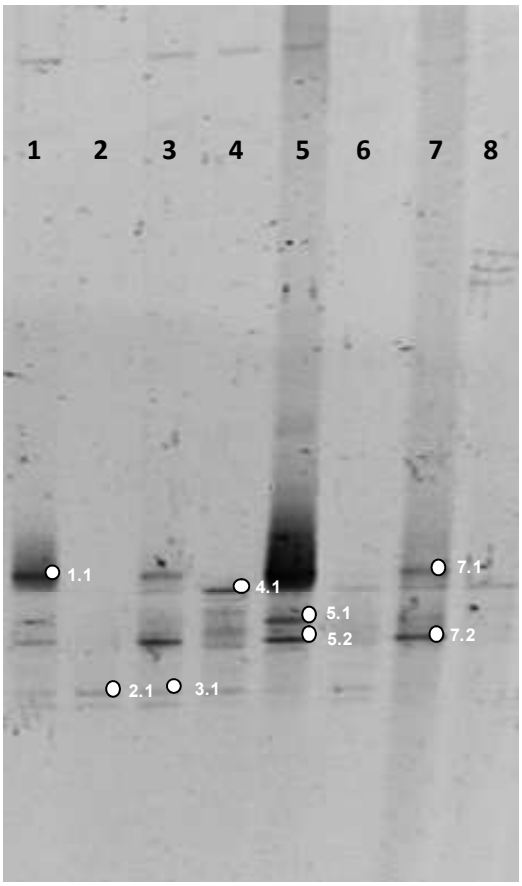

Fig. S2. DGGE with *Alphaproteobacteria* specific primers. Marked bands were excised, reamplified and sequenced. Lanes 1 and 5: Free-living fractions on 0.2 µm (prefiltered through 1.2 µm); lanes 2 and 6: Particle-associated fractions (>5 µm); lanes 3 and 7: Free-living fractions on 0.2 µm (prefiltered through 5 µm); lanes 4 and 8: Filtrated algae (>10 µm). The bands 1.1 (lane.band), 4.1, 5.1, 5.2, 7.1, and 7.2 were identified as roseobacters. Bands 2.1 and 3.1 were identified as *Methylobacterium* (*Rhizobiales*).

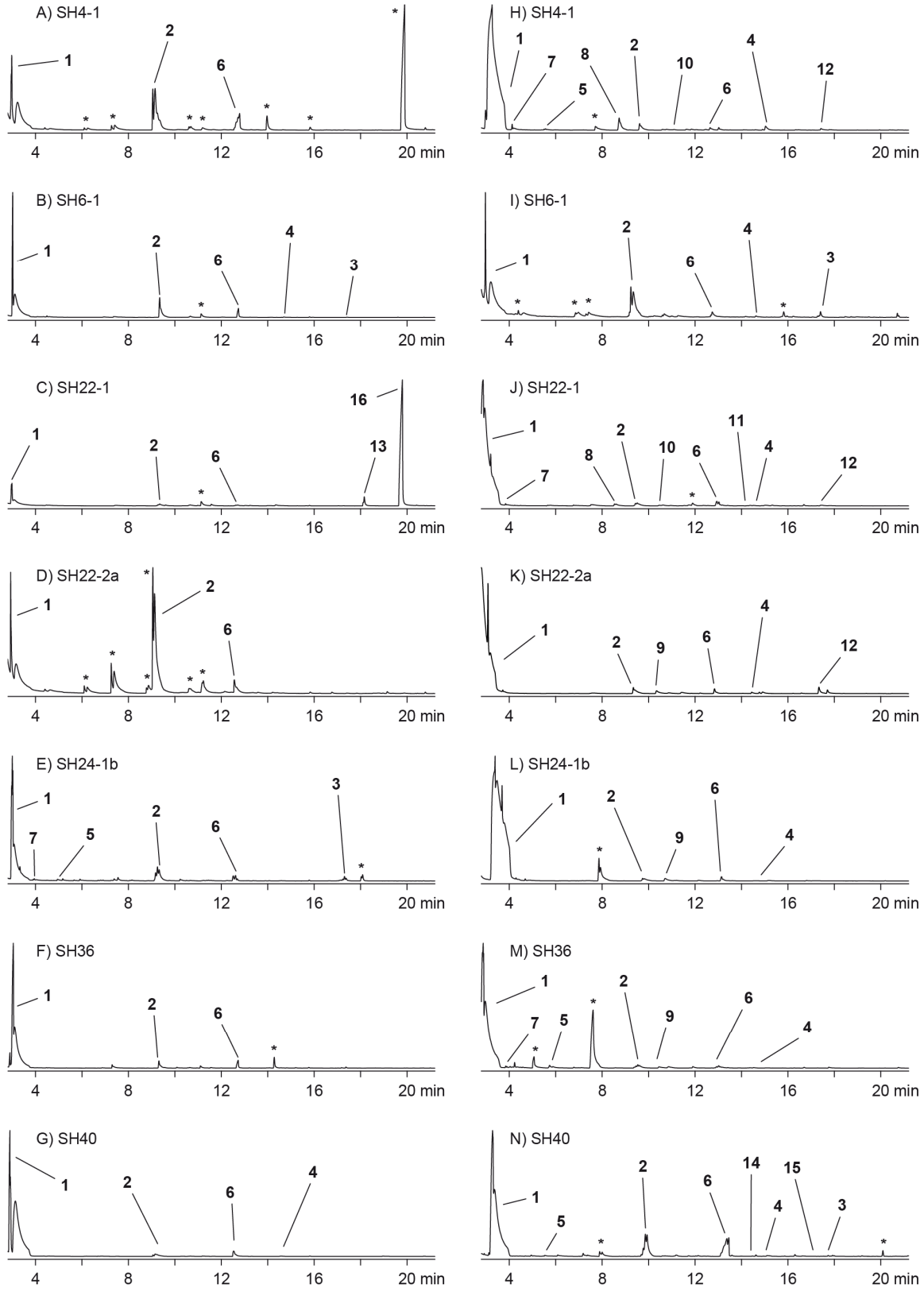

Fig. S3. Total ion chromatograms of the CLSA extracts of the seven isolated strains obtained from liquid cultures (A–G) or agar plates (H–N) both amended with 1 mM [$^2H_6$]DMSP. The peaks are marked with the compound numbers as shown in Fig. 5. Signals arising from the medium, artifacts, and unidentified compounds are labeled with asterisks.

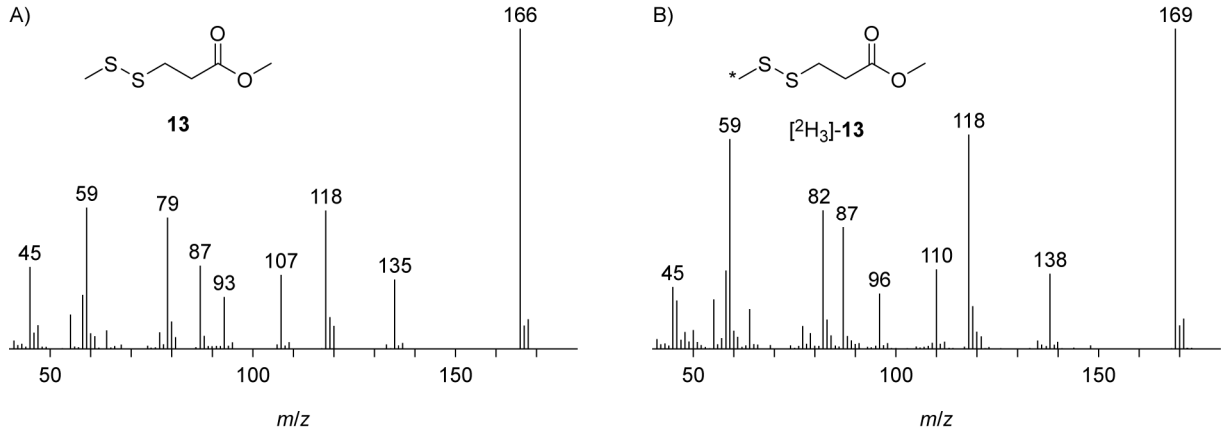

Fig. S4. Mass spectra of methyl 3-(methyldisulfanyl)propionate (**13**, A) and its deuterated analogon [$^2H_3$]-**13** obtained after feeding of [$^2H_6$]DMSP (B).

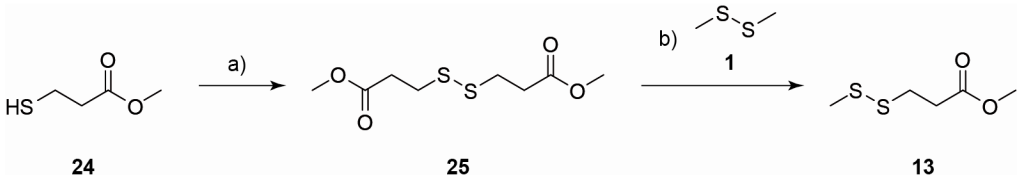

Fig. S5. Synthesis of methyl 3-(methyldisulfanyl)propanoate (**13**). Reagents and conditions: a) Et_3N, DMF; 40°C (55%). b) $BF_3{\cdot}Et_2O$, CH_2Cl_2, CH_3NO_2, 0°C; then **1** (43%).

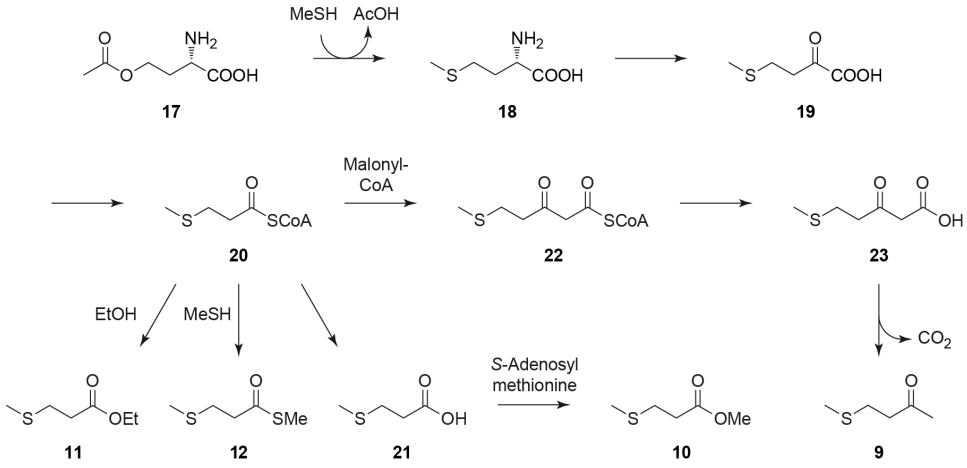

Fig. S6. Uptake of MeSH into the amino acid pool and biosynthesis of the sulfur volatiles **9–12**. Compound **9**: 3-(methylsulfanyl)butan-2-one, **10**: methyl 3-(methylsulfanyl)propionate, **11**: ethyl 3-(methylsulfanyl)propionate, **12**: S-methyl 3-(methylsulfanyl)propanethioate, **17**: *O*-acetylhomoserine, **18**: methionine, **19**: 4-(methylsulfanyl)-2-oxobutanoate, **20**: 3-(methylsulfanyl)propionyl-SCoA, **21**: 3-(methylsulfanyl)propionic acid, **22**: 5-(methylsulfanyl)-3-oxopentanoyl-SCoA, **23**: 5-(methylsulfanyl)-3-oxopentanoic acid.

II.2 *Planktotalea frisia,* gen. nov., sp. nov., isolated from the southern North Sea

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Planktotalea frisia, gen. nov., sp. nov., isolated from the southern North Sea

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Running title: *Planktotalea frisia*

Subject category: New taxa, subsection *Proteobacteria*

The GenBank accession number for the 16S rRNA gene sequence of *Planktotalea frisia* SH6-1^T is FJ882052.

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SUMMARY

A heterotrophic, aerobic bacterium, designated strain SH6-1^T, was obtained from a seawater sample collected from the open North Sea during a phytoplankton bloom. Strain SH6-1^T was isolated from a 10^{-6} dilution culture, indicating high abundance of this organism in the environmental sample. The 16S rRNA gene sequence comparison revealed that the strain SH6-1^T belongs to the marine *Roseobacter* clade (order *Rhodobacterales*) within the class *Alphaproteobacteria*. *Pelagicola litoralis* was the closest described species with 96.4% similarity based on 16S rRNA gene sequence comparison. Cells of strain SH6-1^T were small to elongated irregular rods. Optimal growth occurred between 20 to 25°C and at a pH between 7.5 and 9.0 with peptone and yeast extract. On marine agar it formed non-pigmented, small, circular, convex colonies. Cells required the vitamins pantothenic acid and nicotinic acid amide as well as sodium ions for growth. The DNA G+C content was 53.8 mol%. The fatty acids (>1%) comprised 10:0 3-OH, 16:0, 12:1, 12:1 3-OH, 16:0, 18:0, 18:1ω7c, 18:2, and 11-methyl 18:1ω7c. The polar lipid pattern indicated the presence of phosphatidylcholine, phosphatidylglycerol, an unidentified aminolipid, and one unidentified phospholipid. The major respiratory lipoquinone was ubiquinone 10 (Q-10). Strain SH6-1^T contained the *pufLM* genes, coding for the bacterial photosynthesis reaction center, however, no bacteriochlorophyll *a* (Bchl *a*) could be detected. Physiological, geno- and phenotypic differences to *Pelagicola litoralis* support the description of a new genus and species for which we suggest the name *Planktotalea frisia* gen. nov., sp. nov., with strain SH6-1^T (= DSM 23709 = LMG 25294) as the type strain.

Bacteria of the *Roseobacter* clade within the *Alphaproteobacteria* have been detected in a large variety of marine habitats, often in high abundance. Currently, more than 40 described genera are considered to be members of the *Roseobacter* clade (Brinkhoff *et al.*, 2008). They gain energy from a multitude of organic compounds and some members are capable of aerobic anoxygenic photosynthesis, however, they are all unable to grow autotrophically (for reviews see Buchan *et al.* & 2005; Wagner-Döbler & Biebl, 2006). In pelagic habitats of the North Sea roseobacters were found to constitute 5 to 24% of DAPI cell counts (Eilers *et al.*, 2001; Alderkamp *et al.*, 2006). A new member of the *Roseobacter* clade, strain SH6-1^T, was isolated from the German North Sea showing 96.4% 16S rRNA sequence similarity to its closest described relative, *Pelagicola litoralis* (Kim *et al*., 2008). Based on our results we describe strain SH6-1^T as a new species of a new genus, *Planktotalea frisia*.

Strain SH6-1^T was isolated from a water sample collected at 2 m depth on 12 May 2007 in the southern North Sea (54°42' N, 06°48' E; 36 m depth; salinity: 34.17) during a phytoplankton bloom. In order to enrich the abundant bacteria present in the water sample, serial dilution cultures were used. Prior to inoculation of the serial dilution, the seawater sample was filtered through a 1.2 µm filter to obtain only single planktonic cells. Strain SH6-1^T was isolated from a 10^{-6} dilution in low-nutrient seawater medium (North Sea water filtered through 10 µm and amended with 5 mg l^{-1} yeast extract and 10 mg l^{-1} peptone, pH 8.0, sterilized by autoclaving). After incubation in the dark at 15°C for four weeks, 100 µl of each dilution culture were spread on agar plates with saltwater medium (composition see below). As substrate 30 mg l^{-1} yeast extract and 60 mg l^{-1} peptone were used. For purification, agar-agar was washed five times in distilled water. The composition of saltwater medium (SWM) was: 416 mM NaCl, 49.2 mM $MgCl_2.6H_2O$, 10 mM $CaCl_2.2H_2O$, 8.9 mM KCl, 28.2 mM Na_2SO_4, 10 mM HEPES, 840 µM KBr, 400 µM H_3BO_3, 150 µM $SrCl_2$, 400 µM NH_4Cl, 40 µM KH_2PO_4, 70 µM NaF, pH was adjusted to 8.0. Supplements after autoclaving: 1 ml l^{-1} sterile filtered trace element solution SL 10 (Tschech & Pfennig, 1984), 1 ml l^{-1} of a sterile filtered 5-fold concentrated multi vitamin solution (Balch *et al.*, 1979), and 15 ml of 1 M $NaHCO_3$ (autoclaved separately). Agar plates were prepared with 20 g agar l^{-1} and incubated at 15°C in the dark. Single colonies were picked and re-streaked five times for purification. Further cultivation of strain SH6-1^T was performed using saltwater medium with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone.

Production of Bchl *a* was determined by spectrophotometric analysis. Cells grown in the dark were harvested by centrifugation at 10.000 rpm for 10 min, and pigments were extracted with acetone:methanol 7:2 v/v as described by Clayton (1966). The extract was examined for light absorbancy (350 - 900 nm) with a Beckmann® DU 520 General Purpose UV/VIS Spectrophotometer. *Dinoroseobacter shibae* DFL12^T (DSM 16493^T) was used as positive control.

For determination of temperature and pH optima and range, cells were grown in SWM with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone (composition see above). Temperature range was determined between 4 and 45°C (at 4, 10, 17, 20, 25, 28, 30, 32, 35, 37, 42 and 45°C). The pH range (5 - 11) was tested in increments of 0.5, adjusted with sterile NaOH and HCl solutions based on Wagner-Döbler *et al*., (2004). It is known that pH values above 9.0 does not maintain stable due to CO_2 which dissolve in the medium, either from the air or from the metabolic activity of the strains causing the pH to drop. Therefore, only the growth behaviour during the first 48 h was observed. Clear differences could be detected and growth that occurred after 48 h was scored as negative. Further growth experiments were performed in artificial seawater medium (ASW) according to Zech *et al*. (2009) with addition of 15 ml 1 M $NaHCO_3$ solution (autoclaved separately). After autoclaving 1 ml l^{-1} sterile filtered 5-fold concentrated vitamin solution (Balch *et al.* 1979) was added.

Salinity range and optimum was determined in test tubes. To prepare salt solutions of 0, 12.5, 25, 37.5, 50, 65, 80 and 100 g salts l^{-1}, a concentrated stock solution was used: (l^{-1}) 80 g NaCl, 16 g Na_2SO_4, 1 g NH_4Cl, 12 g $MgCl_2.6H_2O$, 2 g KCl, 0.6 g $CaCl_2.2H_2O$, 0.76 g $NaHCO_3$ (autoclaved separately). From another solution (l^{-1}) 0.2 g KH_2PO_4, 0.25 g NH_4Cl, 0.05 g yeast extract and 0.05 g peptone were added as well as 1 ml 5-fold concentrated vitamin solution (Balch *et al.* 1979) and 1 ml trace element solution (Zech *et al*., 2009). Before inoculation, cells from a preculture grown in SWM with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone, were washed once with the lowest salt solution. The osmotolerance was studied with NaCl only. Therefore a concentrated NaCl solution was added to a minimal medium consisting of (l^{-1}) 1 g Mg-acetate, 40 mg yeast extract, 7 g $MgSO_4.7H_2O$, 0.2 g KH_2PO_4, 0.25 g NH_4Cl, 3 g $MgCl_2.6H_2O$, 0.5 g KCl, 0.15 g $CaCl_2.2H_2O$, 0.3 g $KHCO_3$ (autoclaved separately), vitamins and trace elements as mentioned above to final concentrations of 0.01, 0.05, 0.1, 0.25, 0.37, 0.5, 0.7, 1.0, 1.5 and 2.0 M NaCl.

To determine the maximal doubling time, growth in SWM with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone under optimal conditions (25°C, pH 7.5, 3.75% salinity) was followed in triplicates. Every 1-2 h the test tubes were vigorously shaken and growth was monitored by measuring the OD600. Growth rates (μ) and doubling time (td = ln2/μ) were determined by linear regression of semi-logarithmic plots of cell density against time.

Requirement for single vitamins was tested in ASW supplemented with all 20 amino acids as carbon source (each 1 mM). All amino acids were added, since strain SH6-1^T did not grow in minimal medium with single amino acids. The vitamins nicotinic acid amide, thiamine, pantothenic acid, pyridoxal hydrochloride, cyanocobalamine, riboflavin and biotin (all at 0.05 mg l^{-1}) were tested in mixtures from which one was omitted. Cells were precultured in SWM with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone. Prior to inoculation cells were washed with ASW without substrate.

Tests of growth on single substrates (1 g l^{-1}) were performed in ASW as described by Martens *et al.* (2006). Additionally the algal osmolyte dimethylsulfoniopropionate (DMSP) was tested (at 100 μM). Utilization of amino acids was tested using a final concentration of 5 mM except for tyrosine, which was added to 1 mM, because of precipitation at 5 mM. The pH was adjusted to 7.5. All substrates are listed below (see description of *Planktotalea frisia*). Cells were precultured in SWM with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone. Exponentially grown cells were washed twice with ASW without substrate in 2 ml reaction vessels. For each substrate two replicates of 5 ml medium were inoculated with 10 μl washed cells. Since no or only very weak growth was observed on single substrates, assays were supplemented with 0.1 g l^{-1} yeast extract similar to Wagner-Döbler *et al.* (2004). Cell cultures with 0.1 g l^{-1} yeast extract were used as negative control. Growth was determined after incubation for at least one week at 20°C by an increase in OD_{600} as measured with a spectrophotometer (Spectronic 70). Growth was scored as negative when equal to or less than that in the negative control.

Gram staining was performed using the Hucker staining method as described by Murray *et al.* (1994). Cytochrome oxidase test was carried out by streaking a single colony on a cellulose strip saturated with test reagent (1 g l^{-1} ascorbic acid, 10 g l^{-1} tetramethyl-p-phenylendiamine-HCl). Blue coloration shows oxidase activity (positive control *Micrococcus luteus*). To assay catalase reaction, a drop of 5% H_2O_2 was added to a dense

cell culture. *E. coli* was used as positive control. Exoenzyme activities (hydrolysis of gelatin, starch and Tween 80) were analysed with saltwater medium solidified with 9% (w/v) gelatin or 1.5% (w/v) agarose and supplemented with 0.2% (w/v) starch and 1% (v/v) Tween 80, respectively (Smibert & Krieg, 1994). Reduction of nitrate was tested in anoxic ASW containing resazurin (0.5 g l^{-1}). After autoclaving, the medium was reduced by addition of ~ 1 mg of sterile sodium dithionite (causing discoloration of resazurin). Nitrate was added as 1 g l^{-1} sodium nitrate. Medium was filled into test tubes, containing a small inverted glass tube. The headspace was flushed with N_2/CO_2 (80/20, v/v) and tubes were sealed airproof. Substrates (0.2 g l^{-1} glucose and 0.1 g l^{-1} yeast extract, respectively) and sodium nitrate were injected from anoxic stock solutions. Exponentially grown cells were preincubated under N_2 gassing before inoculation. Cells were precultured in SWM with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone. Accumulation of bubbles in the inverted glass tube indicates N_2 formation by nitrate reduction. *Roseobacter denitrificans* DSM 7001^T was used as positive control.

Antibiotic susceptibility was tested with agar diffusion tests using penicillin G, streptomycin sulfate, chloramphenicol and kanamycin sulfate as described in Brinkhoff *et al.* (2004). *Phaeobacter gallaeciensis* BS107 and *Rhodobacter sphaeroides* 2.4.1 were used as control organisms.

For transmission electron microscopy (TEM), cells from the exponential phase, grown in SWM with peptone and yeast extract, were once washed in destilled water and studied unstained as described previously (Cháves *et al.*, 2004). After air drying, copper grids (200 mesh; Plano) were examined by TEM (EM 902A; Zeiss).

The amplification and purification of the 16S rRNA gene was carried out as described by Brinkhoff & Muyzer (1997) using the primer pair 27F (GM3F) and 1492R (GM4R). Sequencing was performed by GATC Biotech AG (Konstanz, Germany). Phylogenetic analysis was performed with the ARB software package [http://www.arb-home.de (Ludwig et al., 2004)]. Detection of genes coding for the subunits of the photosynthetic reaction centre complex (*pufL* and *pufM*) was performed by PCR using the primers pufL, forward (5'-CTKTTCGACTTCTGGGTSGG-3') and pufM, reverse (5'-CCATSGTCCAGCGCCAGAA-3') (Beja *et al.*, 2002). Reactions were cycled in an Eppendorf thermocycler at the following parameters: 94°C for 3 min, followed by 30

cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, with a final extension step at 72°C for 10 min. The PCR product was purified by using the peqGOLD MicroSpin Cycle-Pure Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany). Sequencing was performed by GATC Biotech AG (Konstanz, Germany).

For the following analyses cell biomass of strain SH6-1^T and *P. litoralis* CL-ES2^T was obtained from exponentially growing cells cultivated in SWM with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone at 20°C. Respiratory lipoquinones and polar lipids were extracted from 100 mg freeze-dried cells using the method described by Tindall (1990a, b). Respiratory lipoquinones were separated into their structural classes (menaquinones, ubiquinones etc.) by TLC. Bands were eluted and further separated and identified by HPLC, using an RP_{18} column (Tindall, 1996). Polar lipids were separated by two-dimensional chromatography and identified on the basis of their R_F values in combination with their reaction with specific staining reagents (Tindall, 1990a, b). Fatty acid methyl esters were released from 10 mg freeze-dried cells using methodologies which release only ester-linked fatty acids (M1) or ester- and amide-linked fatty acids (M2) (see Labrenz *et al.*, 1998; Strömpl *et al.*, 1999). The samples were initially run using the MIDI Sherlock system (version 6) and the fatty acids identified using the TSBA40 peak naming tables. Peaks that were not identified by the MIDI Sherlock system or were not unambiguously identified were further characterized by GC-MS using a Varian CP-3800 gas chromatograph coupled to a Varian 320-MS single quadrapole mass spectrometer operated in electron impact mode (EI).

The DNA G+C content of cells of strain SH6-1^T was determined by extracting genomic DNA according to Cashion *et al.* (1977) and subsequent determination of deoxyribonucleosides by HPLC (Mesbah *et al.*, 1989; Tamaoka & Komagata, 1984).

After 7 days on agar plates with SWM at 20°C, colonies were circular, convex, whitish with a shiny surface and up to 0.5 mm in diameter. Single cells grown in liquid medium were short rods to irregular elongated rods with a length of 0.5 µm - 4 µm and a width of 0.5 µm - 1 µm (Fig. S1). Cells stained Gram-negatively. Flagella were not detected by microscopy and cells did not show clear motility, however, cells exhibited slight wobbling.

Strain SH6-1^T grew between 4°C and 32°C with a maximum between 20°C and 25°C. The pH range tolerated for growth was 6.0 to 9.5 with an optimum between 7.5 and 9.0. Growth was observed at salt concentrations of 1.25% to 8% with an optimum at 3.75%. Strain SH6-1^T has an absolute requirement of Na^+, its osmotolerance ranges from 0.25 to 0.7 M NaCl. Under optimum conditions in saltwater medium, grown with 0.02 g l^{-1} peptone and 0.01 g l^{-1} yeast extract, the doubling time was 3.7 h. Strain SH6-1^T showed no or only very weak growth on minimal medium, however, with the addition of 0.1 g l^{-1} of yeast extract growth was stimulated. The following carbon sources supported cell growth: L-alanine, L-arginine, L-aspartic acid, L-proline, L-serine, L-tryptophan, L-tyrosine, (+)-D-xylose, (+)-D-glucose, (+)-D-mannose, (+)-D-galactose, (-)-D-fructose, (-)-D-ribose, (-)-D-mannitol, sucrose, maltose, cellobiose, trehalose, lactose, sodium acetate, sodium pyruvate, sodium malate, citric acid, disodium succinate, sodium lactate, glycerol and Tween 80. No growth was observed when no vitamins were added. Strain SH6-1^T was dependent on pantothenic acid and nicotinic acid amide. Cells of strain SH6-1^T are susceptible to penicillin G, streptomycin sulfate and chloramphenicol but not to kanamycin sulfate. Strain SH6-1^T exhibited oxidase but no catalase activity.

Analysis of the 16S rRNA gene sequence indicated that strain SH6-1^T shared 96.4% sequence similarity with the sequence of its nearest described relative *Pelagicola litoralis* CL-ES2^T (Fig. 1). The G+C base content of strain SH6-1^T is 53.8 mol%. Cells contain *pufLM* genes coding for the bacterial photosynthesis reaction center. However, in laboratory cultures no Bchl *a* or any other pigments could be detected.

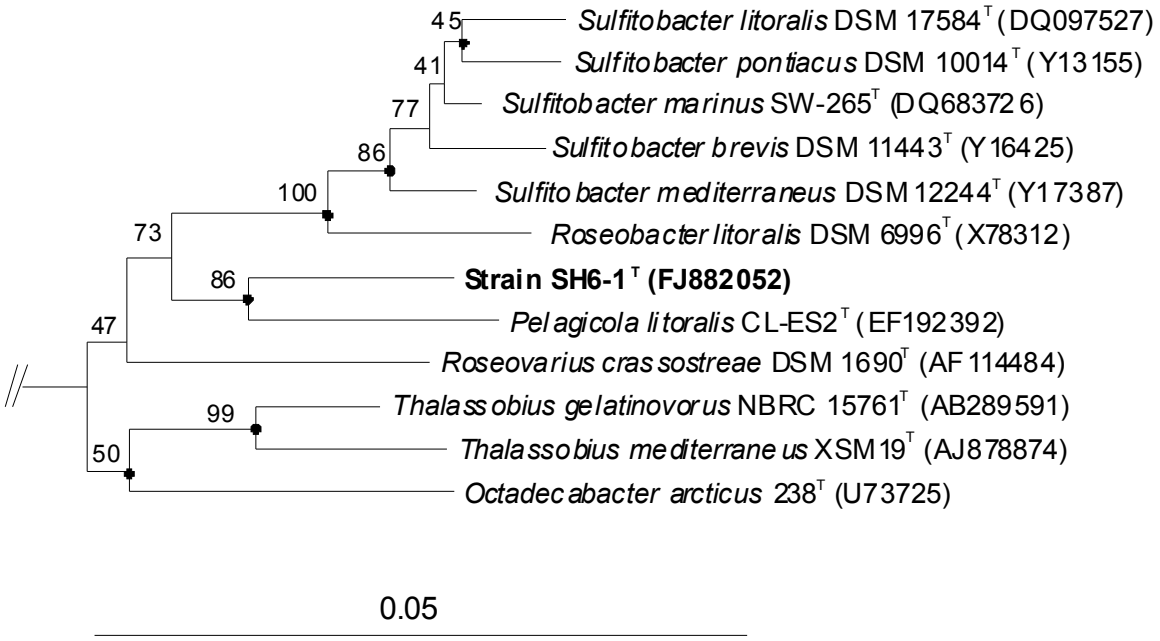

Fig. 1. Neighbour-joining tree showing the phylogenetic relationships of strain SH6-1^T and representatives of the family *Rhodobacteraceae* within the class *Alphaproteobacteria* based on 16S rRNA gene sequence similarity. Bootstrap values were derived from 1000 replicates. Filled circles indicate nodes also recovered reproducibly with maximum-likelihood. Selected members of the class *Gammaproteobacteria* were used as an outgroup. The scale bar indicates 5% sequence divergence.

The predominant respiratory lipoquinone of strain SH6-1^T was ubiquinone 10 (Q-10). The fatty acid composition was dominated by 18:1ω7c; other fatty acids (>1%) were 10:0 3-OH, 16:0, 12:1, 12:1 3-OH, 16:0, 18:0, 18:2, and 11-methyl 18:1ω7c. The polar lipids found in strain SH6-1^T were phosphatidylcholine, phosphatidylglycerol, one unidentified aminolipid, and one unidentified phospholipid.

Comparison of the polar lipid patterns of strain SH6-1^T and *Pelagicola litoralis* CL-ES2^T revealed clear differences (Fig. 2). The fatty acid compositions of these strains showed the main differences in fatty acids that were present in low concentrations. *P. litoralis* contained the fatty acid 16:0 2-OH, which was not present in strain SH6-1^T and much larger amounts of 14:1 3-OH than strain SH6-1^T. Strain SH6-1^T, however, contained 12:1 3-OH not present in *P. litoralis* (Table 1). Further differential characteristics of strain SH6-1^T and *P. litoralis* are summarized in Table 2.

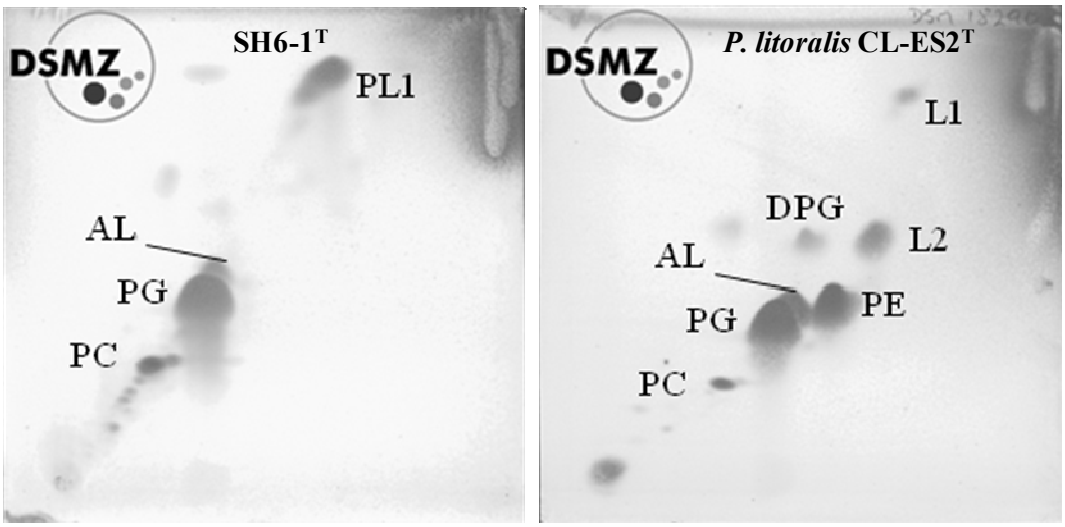

Fig. 2. Polar lipid composition of strain SH6-1^T and *Pelagicola litoralis* CL-ES2^T. AL, unidentified aminolipid; DPG, Diphosphatidylglycerol; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PG, Phosphatidylglycerol; PL1, Phospholipid; L1 - L2, Lipid

Table 1. Fatty acid composition of cells of strain SH6-1^T and *Pelagicola litoralis* CL-ES2^T using methodologies which release only ester-linked (M1) or ester- and amide-linked fatty acids (M2). Strains were grown under identical cultivation contiditons. *, these fatty acids are not listed in the MIDI Sherlock TSBA40 peak naming table and their identity has been confirmed by GC-MS, †, Summed feature 7 contains C19:1ω6c and/or unknown ECL 18.846.

Fatty acid	**SH6-1^T**		***P. litoralis* CL-ES2^T**	
	M1	**M2**	**M1**	**M2**
C16:0	2.44	6.44	2.91	3.26
C18:0	2.57	1.75	0.75	0.70
C12:1*	1.68	2.56	2.24	2.46
C18:1ω7*c*	75.36	70.97	75.91	71.31
C18:2*	10.34	11.45	10.09	9.54
C10:0 3-OH	1.54	1.36	4.01	3.37
C12:1 3-OH	2.53	1.82	-	-
C14:1 3-OH*	-	0.18	-	**2.67**
C16:0 2-OH	-	-	1.13	1.11
11-methyl C18:1ω7*c*	2.29	2.74	2.96	2.73
Summed feature 7†	1.18	0.34	-	0.14

Table 2. Differential characteristics of strain SH6-1^T and *Pelagicola litoralis* CL-ES2^T (Kim *et al.*, 2008). +, positive result or growth; -, negative result or no growth; w, weak (OD_{600} ≤0.2); ND, no data available.

Characteristic	**SH6-1^T**	***P. litoralis* CL-ES2^T**
Colony morphology	circular, convex, whitish	circular, convex, creamy
Cell morphology	irregular rods	club-shaped rods
Cell size (µm)	0.5-4 x 0.5-1.0	1.1-7.0 x 0.5-1.4
Motility	(+)	-
pufLM genes	+	ND
Catalase	-	+
Growth at 4°C	+	-
G+C content (mol%)	53.8	47
Salinity range	1.25-8%	2-6%
Salt optimum	3.75%	3-4%
Osmotolerance	0.25-0.7 M NaCl	ND
Temperature range (°C)	4-32	20-30
Temperature optimum (°C)	20-25	25
pH range	6.0-9.5	6.0-8.0
pH optimum	7.5-9.0	7.0
Substrates used:		
Proline	w	+
Serine	w	+
Citrate	w	-
Sucrose	w	-
Trehalose	w	-
Succinate	w	-
Pyruvate	w	-
Glycerol	w	-
Hydrolysis of:		
Starch	-	+
Tween 80	w	+

Based on all the distinctive features, we propose that strain SH6-1^T be assigned to a novel genus and species, for which the name *Planktotalea frisia* gen. nov., sp. nov. is proposed.

Description of *Planktotalea* gen. nov.

Planktotalea (Plank.to.ta´le.a. Gr. adj. planktos roaming, wandering; L. fem. n. talea a slender staff, a rod; N. L. fem. n. Planktotalea a planktonic/roaming rod).

Gram-negative, aerobic, oxidase-positive and catalase-negative. Cells are small to irregular rods, approximately 0.5 µm - 4 µm in length and 0.4 µm - 1 µm in width. Cells contain *pufLM* genes coding for subunits of the bacterial photosynthetic reaction centre complex. Growth in minimal medium on single substrates is poor. Cells require vitamins as well as sodium ions. The major respiratory lipoquinone is Q-10. The polar lipids comprise phosphatidylcholine, phosphatidylglycerol, an unidentified aminolipid, and one

unidentified phospholipid. The fatty acids comprise 10:0 3-OH, 12:1, 12:1 3-OH, 16:0, 18:0, 18:1ω7c, 18:2, 11-methyl 18:1ω7c, and traces of 14:1 3-OH. Whereas most of the fatty acids were ester-linked, the small amount of 14:1 3-OH present was amide-linked. The genus is a member of the class *Alphaproteobacteria*, order *Rhodobacterales*, and so far contains only one species, *Planktotalea frisia*, which is the type species.

Description of *Planktotalea frisia* sp. nov.

Planktotalea frisia (fri'si.a. L. fem. adj. frisia, Frisian, pertaining to Frisia, a coastal region along the southeastern corner of the North Sea, i.e. the German Bight, from where the organism was obtained).

In addition to the characteristics that define the genus, the type species has the following characteristics. On marine agar colonies are small, circular, convex and whitish with a shiny surface up to 1 mm in diameter. Cells grow at temperatures ranging from 4 to 32°C, with an optimum between 20 and 25°C, and pH ranging from 6.0 to 9.5 (optimum pH 7.5 to 9.0). Cells grow in the presence of salt concentrations of 1.25% and 8%, optimal salinity is at 3.75%. Strain SH6-1^T has an absolute requirement of Na^+, its osmotolerance ranged from 0.25 to 0.7 M NaCl. No growth is observed without the vitamins pantothenic acid and nicotinic acid amide. Strain SH6-1^T does not reduce nitrate to N_2. Amylase and gelatinase negative, Tweenase positive. Substrate tests in artificial seawater medium with different carbon sources additionally containing 0.1 g/l yeast extract showed utilization of L-alanine, L-arginine, L-aspartic acid, L-proline, L-serine, L-tryptophan, L-tyrosine, (+)-D-xylose, (+)-D-glucose, (+)-D-mannose, (+)-D-galactose, (-)-D-fructose, (-)-D-ribose, (-)-D-mannitol, sucrose, maltose, cellobiose, trehalose, lactose, sodium acetate, sodium pyruvate, sodium malate, citric acid, disodium succinate, sodium lactate, glycerol and Tween 80. No growth was observed on L-asparagine, L-cysteine, L-glutamine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-threonine, L-valine, (+)-L-arabinose, (+)-L-rhamnose, (-)-L-fucose, (-)-D-sorbitol. (+)-D-glucosamine, laminarine, starch, inulin, xylan, sodium formate, sodium propionate, and dimethylsulfoniopropionate (DMSP). Strain SH6-1^T is susceptible to penicillin G, streptomycin sulfate and chloramphenicol but resistant to kanamycin sulfate. In laboratory cultures cells of strain SH6-1^T did not express Bchl *a* or any other pigments. The G+C content is 53.8 mol%.

The type strain SH6-1^T (= DSM 23709 = LMG 25294), was isolated from a North Sea water sample from 2 m depth. The GenBank accession number for the 16S rRNA gene sequence of *Planktotalea frisia* SH6-1^T is FJ882052.

ACKNOWLEDGEMENTS

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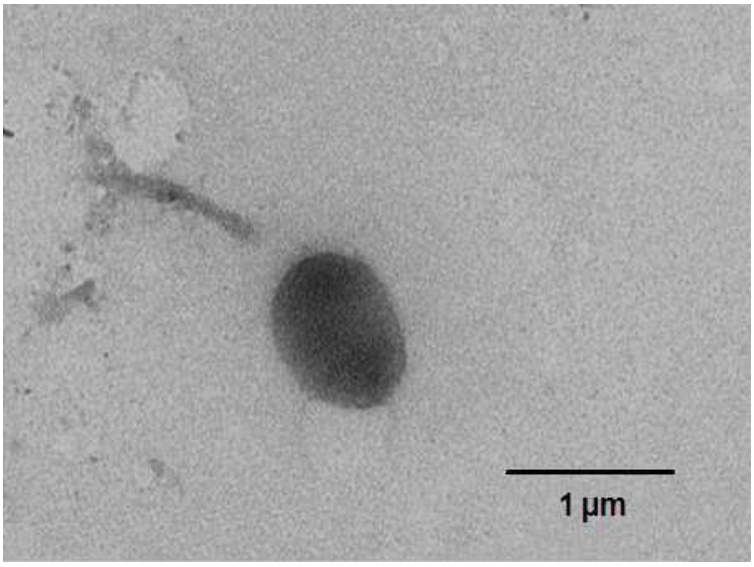
Supplementary material

Fig. S1. Electron micrographs of strain SH6-1^T showing the rod-shaped cell morphology.



II.3 *Pelagimonas elegans*, gen. nov., sp. nov., isolated from the southern North Sea

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Pelagimonas elegans gen. nov., sp. nov., isolated from the southern North Sea

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Running title: *Pelagimonas elegans* gen. nov, sp. nov.

Subject category: *Proteobacteria*

The GenBank accession number for the 16S rRNA gene sequence of *Pelagimonas elegans* SH36^T is FJ882054.

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SUMMARY

A heterotrophic, aerobic bacterium, designated strain SH36^T, was obtained from a seawater sample collected from the open North Sea during a phytoplankton bloom. Isolation was performed applying dilution series and subsequent transfer to agar plates using artificial saltwater medium with peptone and yeast extract. Comparison of the 16S rRNA gene sequence revealed affiliation to the *Roseobacter* clade (*Alphaproteobacteria*) with *Nautella italica* and *Shimia marina* as the closest related species, both showing 96% sequence similarity. Calculation of phylogenetic trees indicated, however, that strain SH36^T clusters with *Tateyamaria pelophila* (95.5 %), *Tateyamaria omphalii* (95.7 %), and *Nereida ignava* (95.3 %). Cells of strain SH36^T are coccoid to elongated rods. Optimal growth occurs between 30 to 32 °C and at a pH between 7.0 and 9.0. On saltwater medium strain SH36^T builds small, white, circular, convex and shiny colonies. Growth was not observed with any of the tested sugars but on almost all amino acids. For growth cells require the vitamins pantothenic acid and nicotinic acid amide as well as sodium ions. The DNA G+C content is 56.6 mol%. The fatty acids (>1 %) comprised 10:0 3-OH, 12:1, 14:1 3-OH, 16:0, 18:0, 18:2, 18:1ω7c, and 11-methyl 18:1ω7c. The lipid pattern indicated the presence of phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, an unidentified aminolipid, one unidentified phospholipid, and one unidentified lipid. Physiological, geno- and phenotypic differences to described species support the description of strain SH36^T as a new species within a new genus for which we propose the name *Pelagimonas elegans* gen. nov., sp. nov. (= DSM 23330^T = LMG 25292^T).

Organisms of the *Roseobacter* clade within the family *Rhodobacteraceae* were detected in a large variety of marine habitats, from coastal areas to open oceans, and constitute up to 25 % of the total bacterial community (e.g. Buchan *et al*., 2005). Members of the *Roseobacter* clade share >88% identity of the 16S rRNA gene sequence (Brinkhoff *et al*., 2008), which may also be indicative that this group should constitute a separate higher taxonomic rank. Very diverse physiological traits have been reported for this group, e.g. turnover of the algal osmolyte dimethylsulfoniopropionate (DMSP) (Ledyard *et al.*, 1993; Ledyard & Dacey, 1994; González *et al.*, 1999), oxidation of the greenhouse gas carbon monoxide (King, 2003; Tolli, 2003; Moran *et al.*, 2004), production of secondary metabolites (Lafay *et al.*, 1995; Brinkhoff *et al.*, 2004), and methylotrophy (Holmes *et al.*, 1997). Members of the *Roseobacter* clade use a multitude of organic compounds and some members are capable of aerobic anoxygenic photosynthesis (Yurkov & Beatty, 1998). Here we describe a new member of the *Roseobacter* clade, strain SH36^T, isolated from the southern North Sea during a phytoplankton bloom. The 16S rRNA gene sequence of strain SH36^T is closely affiliated with sequences of different strains and clones previously obtained from different North Sea areas. Based on our results we describe strain SH36^T as a new species of a new genus, *Pelagimonas elegans*.

Strain SH36^T was isolated from a water sample collected at 2 m depth on 12 May 2007 in the southern North Sea (54°42' N, 06°48' E; 36 m depth; salinity: 34.17) during a phytoplankton bloom. In order to enrich the abundant roseobacters of the water sample, serial dilution cultures were used. Prior to inoculation of the serial dilution, the seawater sample was filtered through a 1.2 µm filter to obtain only single pelagic cells. Strain SH36^T was isolated from a 10^{-4} dilution in seawater medium (North Sea water filtered through 10 µm amended with 50 mg l^{-1} yeast extract and 150 mg l^{-1} peptone, pH 8.0, sterilized by autoclaving and supplemented with 10 µM cAMP). After incubation in the dark at 15 °C for four weeks, 100 µl of each dilution culture were spread on agar plates with the respective medium. Agar plates were prepared with 20 g agar l^{-1} and incubated at 15 °C in the dark for about three weeks. Single colonies were picked and re-streaked five times for purification. Further cultivation of strain SH36^T was performed using saltwater medium with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone. The composition of saltwater medium (SWM) was: 416 mM NaCl, 49.2 mM $MgCl_2.6H_2O$, 10 mM $CaCl_2.2H_2O$, 8.9 mM KCl, 28.2 mM Na_2SO_4, 10 mM HEPES, 840 µM KBr, 400 µM H_3BO_3, 150 µM $SrCl_2$, 400 µM NH_4Cl, 40 µM KH_2PO_4, 70 µM NaF, pH was adjusted to 8.0. Supplements after

autoclaving: 1 ml l^{-1} sterile filtered trace element solution SL 10 (Tschech & Pfennig, 1984), 1 ml l^{-1} of a sterile filtered 5-fold concentrated multi vitamin solution (Balch *et al.*, 1979), and 15 ml 1 M $NaHCO_3$ (autoclaved separately).

Production of Bchl *a* was determined by spectrophotometric analysis. Cells grown in the dark were harvested by centrifugation at 10.000 rpm for 10 min, and pigments were extracted with acetone:methanol 7:2 v/v as described by Clayton (1966). The absorption spectrum of the extract was examined in the range 350 – 900 nm using a Beckmann® DU 520 General Purpose UV/VIS Spectrophotometer. *Dinoroseobacter shibae* DFL12^T (DSM 16493^T) was used as positive control.

For determination of temperature and pH optima and range, cells were grown in SWM (composition see above) with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone. Temperature range was determined between 4 and 45 °C (at 4, 10, 17, 20, 25, 28, 30, 32, 35, 37, 42 and 45 °C). The pH range (5 - 11) was tested in increments of 0.5, adjusted with sterile NaOH and HCl solutions based on the method described by Wagner-Döbler *et al*. (2004). Further growth experiments were performed in artificial seawater medium (ASW): 342 mM NaCl, 28.2 mM Na_2SO_4, 1.47 mM KH_2PO_4, 4.7 mM NH_4Cl, 14.8 mM $MgCl_2.6H_2O$, 6.7 mM KCl, 1 mM $CaCl_2.2H_2O$. After autoclaving 1 ml l^{-1} sterile filtered 5-fold concentrated vitamin solution (Balch *et al.* 1979), 1 ml l^{-1} sterile filtered complex trace element solution (Zech *et al.*, 2009), and 15 ml 1 M $NaHCO_3$ solution (autoclaved separately) were added.

Salinity range and optimum were determined in test tubes. To prepare salt solutions of 0, 12.5, 25, 37.5, 50, 65, 80 and 100 g salts l^{-1}, a concentrated stock solution was used: (l^{-1}) 80 g NaCl, 16 g Na_2SO_4, 1 g NH_4Cl, 12 g $MgCl_2.6H_2O$, 2 g KCl, 0.6 g $CaCl_2.2H_2O$, 0.76 g $NaHCO_3$ (autoclaved separately). From another solution (l^{-1}) 0.2 g KH_2PO_4, 0.25 g NH_4Cl, 0.05 g yeast extract and 0.05 g peptone were added as well as 1 ml 5-fold concentrated vitamin solution (Balch *et al.* 1979) and 1 ml trace element solution (Zech *et al*., 2009). Before inoculation, cells from a pre-culture, grown in SWM with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone, were washed once with the lowest salt solution. The osmotolerance was studied with NaCl only. A concentrated NaCl solution was added to a minimal medium consisting of (l^{-1}) 1 g Mg-acetate, 40 mg yeast extract, 7 g $MgSO_4.7H_2O$, 0.2 g KH_2PO_4, 0.25 g NH_4Cl, 3 g $MgCl_2.6H_2O$, 0.5 g KCl, 0.15 g $CaCl_2.2H_2O$, 0.3 g $KHCO_3$, 5-fold

concentrated vitamin solution and complex trace element solution (see above) to final concentrations of 0.01, 0.05, 0.1, 0.25, 0.37, 0.5, 0.7, 1.0, 1.5 and 2.0 M NaCl.

To determine the maximal doubling time, growth in SWM with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone and under optimal conditions (30 °C, pH 7.5, 3.75 % salinity) was followed in triplicates. Every 1-2 h the test tubes were vigorously shaken and growth was monitored by measuring the OD_{600}. Growth rates (μ) and doubling time ($t_d = \ln 2/\mu$) were determined by linear regression of semi-logarithmic plots of cell density against time.

Requirement for single vitamins was tested in ASW supplemented with all 20 amino acids as carbon source (each 1 mM). All amino acids were added, since strain $SH36^T$ did not grow sufficiently in minimal medium with single amino acids. The vitamins nicotinic acid amide, thiamine, pantothenic acid, pyridoxal hydrochloride, cyanocobalamine, riboflavin and biotin (all at 0.05 mg l^{-1}) were tested in mixtures from which one was omitted. Cells were pre-cultured in SWM with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone. Prior to inoculation cells were washed with ASW without vitamins. When growth was detected, cells from the stationary phase were transferred to fresh medium to test whether growth was only possible as the result of residual vitamins associated with the cells or due to self-sufficient growth.

Tests of growth on single substrates (1 g l^{-1}) were performed in ASW as described by Martens *et al.* (2006). Additionally the algal osmolyte dimethylsulfoniopropionate (DMSP) was tested (at 100 µM). Utilization of amino acids was tested using a final concentration of 5 mM except for tyrosine, which was added to 1 mM, because of precipitation at 5 mM. The pH was adjusted to 7.5. All substrates are listed below (see description of *Pelagimonas elegans*). Cells were pre-cultured in SWM with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone. Exponentially grown cells were washed twice with ASW without substrate in 2 ml reaction vessels. Two replicates of 5 ml medium were inoculated with 10 µl washed cells for each substrate. Due to the fact that no or only very weak growth was observed on single substrates, cultures were supplemented with 0.1 g l^{-1} yeast extract, similar to the procedure described by Wagner-Döbler *et al.* (2004) for the characterization of *Oceanibulbus indolifex*. Cell cultures with 0.1 g l^{-1} yeast extract and no further substrate were used as a negative control. Growth was determined after incubation for at least one week at 20 °C by measuring an increase in OD_{600} with a spectrophotometer (Spectronic 70). Growth was

scored as negative when the optical density was equal to or less than that in the negative control.

Gram staining was performed using the Hucker staining method described by Murray *et al.* (1994). Cytochrome oxidase test was carried out by streaking a single colony on a cellulose strip saturated with test reagent (1 g l^{-1} ascorbic acid, 10 g l^{-1} tetramethyl-p-phenylendiamin-HCl). Blue coloration shows oxidase activity (*Micrococcus luteus* was used as positive control). Catalase reaction was tested with a drop of 5 % H_2O_2 added to a dense cell culture (*Escherichia coli* was used as positive control). Exoenzyme activities (hydrolysis of gelatin, starch and Tween 80) were analysed with saltwater medium solidified with 9 % (w/v) gelatin or 1.5 % (w/v) agarose and supplemented with 0.2 % (w/v) starch and 1 % (v/v) Tween 80, respectively, based on the methods given in Smibert & Krieg (1994). Reduction of nitrate was tested in anoxic ASW containing resazurin (0.5 g l^{-1}). After autoclaving, the medium was reduced by addition of ~1 mg sterile sodium dithionite (causing discoloration of resazurin). Nitrate was added as 1 g l^{-1} sodium nitrate. Medium was filled into test tubes, containing a small inverted glass tube. Headspace was flushed with N_2/CO_2 (80/20, v/v) and tubes were sealed airproof. Substrates (0.2 g l^{-1} glucose, 0.2 g l^{-1} glucose + 0.2 g l^{-1} sodium acetate, 1 g l^{-1} pyruvate, and 0.1 g l^{-1} yeast extract, respectively) and sodium nitrate were injected from anoxic stock solutions. Exponentially grown cells were preincubated under N_2 gassing before inoculation. Cells were pre-cultured in SWM with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone. Accumulation of bubbles in the inverted glass tube indicates N_2 formation by nitrate reduction. *Roseobacter denitrificans* DSM7001^T was used as a positive control.

Antibiotic susceptibility was tested with agar diffusion tests using penicillin G, streptomycin sulfate, chloramphenicol and kanamycin sulfate as previously described (Brinkhoff *et al.*, 2004). *Phaeobacter gallaeciensis* DSM 17395^T and *Rhodobacter sphaeroides* 2.4.1^T were used as control organisms.

For transmission electron microscopy (TEM), cells from the exponential phase, grown in ASW with all 20 amino acids (each 1 mM) were once washed in distilled water and studied unstained as described previously (Cháves *et al.*, 2004). After air drying, copper grids (200 mesh; Plano) were examined by TEM (EM 902A; Zeiss).

The amplification and purification of the 16S rRNA gene was carried out as described by Brinkhoff & Muyzer (1997). Sequencing was performed by GATC Biotech AG (Konstanz, Germany). Phylogenetic analysis was performed with the ARB software package [http://www.arb-home.de (Ludwig *et al.*, 2004)]. Detection of genes coding for the subunits of the photosynthetic reaction centre complex (*pufL* and *pufM*) was performed by PCR using the primers pufL and pufM (Beja *et al.*, 2002). Reactions were cycled in an Eppendorf thermocycler at the following parameters: 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, with a final extension step at 72 °C for 10 min. The PCR product was purified by using the peqGOLD MicroSpin Cycle-Pure Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany). Sequencing was performed by GATC Biotech AG (Konstanz, Germany).

For the following analyses cell biomass was obtained from exponentially growing cells cultivated in SWM with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone at 20 °C. Respiratory lipoquinones and polar lipids were extracted from 100 mg freeze-dried cells using the method described by Tindall (1990a, b). Respiratory lipoquinones were separated into their structural classes (menaquinones, ubiquinones etc.) by TLC. Bands were eluted and further separated and identified by HPLC, using an RP_{18} column (Tindall, 1996). Polar lipids were separated by two-dimensional chromatography and identified on the basis of their R_F values in combination with their reaction with specific staining reagents (Tindall, 1990a, b). Fatty acid methyl esters were released from 20 mg freeze-dried cells using methodologies that release only ester-linked fatty acids (M1) or ester- and amide-linked fatty acids (M2) (see Labrenz *et al.*, 1998; Strömpl *et al.*, 1999). Initial identification relied on the use of the MIDI Sherlock system (version 6.1), with fatty acids being initially identified against the TSBA4 database. Where peaks were not identified by the MIDI Sherlock system peaks were identified using GC-MS.

The DNA G+C content of cells of strain $SH36^T$ was determined by extracting genomic DNA according to Cashion *et al.* (1977) and subsequent determination of deoxyribonucleosides by HPLC (Mesbah *et al.*, 1989; Tamaoka & Komagata, 1984).

After 7 days incubation on agar plates with SWM supplemented with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone at 20 °C, colonies were white circular, and convex with a shiny surface and up to 1 mm in diameter. Single cells grown for seven days in liquid saltwater

medium were coccoid to elongated rods with a length of 0.6 µm - 1.5 µm and a width of 0.6 µm - 0.7 µm (Fig. 1). Cells were not motile and Gram-negative.

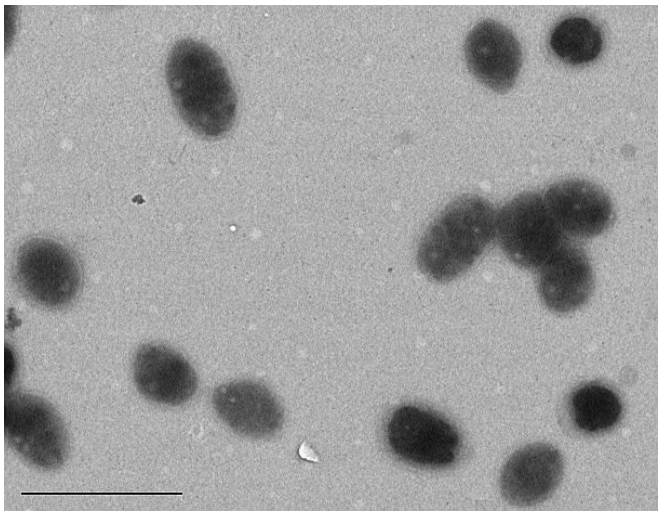

Fig. 1. Transmission electron microscopic image of cells of strain SH36^T from the exponential phase, grown on ASW medium with all 20 amino acids. Bar, 2 µm.

Strain SH36^T showed growth between 4 °C and 37 °C with a maximum between 30 °C and 32 °C. The pH range tolerated for growth was 6.0 to 9.5 with an optimum between 7.0 and 9.0. Growth was observed at salt concentrations of 1.25 to 8 % with an optimum between 1.25 and 3.75 %. Strain SH36^T has an absolute requirement of Na^+, its osmotolerance ranged from 0.05 to 1.5 M NaCl. Under optimum conditions in saltwater medium, grown with 0.02 g l^{-1} peptone and 0.01 g l^{-1} yeast extract, the doubling time was 2.6 h. Strain SH36^T showed no or only very weak growth on minimal medium with single carbon sources, however, with the addition of 0.1 g l^{-1} of yeast extract growth was stimulated. Growth on sugars was not observed. The following carbon sources supported cell growth: L-arginine, L-aspartic acid, L-glutamine, L-glutamic acid, L-histidine, L-phenylalanine, L-proline, L-serine, L-threonine, sodium acetate, sodium pyruvate, sodium malate, citric acid, and disodium succinate. L-alanine, L-asparagine, L-cystein, glycine, L-leucine, L-isoleucine, L-tyrosin, sodium lactate, and sodium propionate supported growth only weakly. No growth was observed when no vitamins were added. Strain SH36^T was dependent on pantothenic acid and nicotinic acid amide. Cells of strain SH36^T were susceptible to penicillin G, streptomycin sulfate and chloramphenicol, but not to kanamycin sulfate. Strain SH36^T exhibited oxidase, but no catalase activity.

Analysis of the 16S rRNA gene sequence showed that strain SH36^T shared 96 % sequence similarity with both, *Nautella italica* and *Shimia marina* (95.5 % with *Shimia isoporae*). In the phylogenetic tree strain SH36^T, however, does not form a cluster with these strains but with *Tateyamaria pelophila*, *Tateyamaria omphalii*, and *Nereida ignava* (95.5 %, 95.7 %, and 95.3 % identity, respectively) (Fig. 2). Cluster formation of *Nereida ignava* and phylotypes closely related to strain SH36^T was also found by Pujalte *et al*. (2005) and Kurahashi & Yokota (2007). Furthermore, strain SH36^T is closely affiliated (>99.4 % sequence identity) with eleven sequences obtained previously from several locations of the North Sea (the marine section of the Weser estuary, the German Wadden Sea, and from offshore samples close to the island Helgoland). Four of these strains and clones were also detected in high dilution steps, i.e. clones AP-27, DC5-80-8, and DC8-80-0 (Selje *et al*., 2005), and strain TL (Brinkhoff *et al*., 2004), pointing to a high abundance of these organisms (Fig. 2). Six of these sequences (including that of strain SH36^T) were obtained from samples taken during phytoplankton blooms or from phytoplankton cultures, respectively (Sapp *et al*., 2007a, b).

pufLM genes encoding for the pufL and pufM subunits of the bacterial photosynthetic reaction centre complex were not detected in strain SH36^T and in laboratory cultures cells did not express bacteriochlorophyll *a* or any other pigments. The G+C base content of strain SH36^T is 56.6 mol%.

The predominant respiratory lipoquinone of strain SH36^T was Q-10, a feature typical of the vast majority of the class *Alphaproteobacteria*. The fatty acid composition was dominated by 18:1ω7c; other fatty acids (>1 %) were 10:0 3-OH, 12:1, 14:1 3-OH, 16:0, 18:0, 18:2, and 11-methyl 18:1ω7c. It should be noted that the common features of all taxa listed in Table 1 were 18:1ω7c; 10:0 3-OH, 16:0, and 11-methyl 18:1ω7c, while differentiating features of strain SH36^T center on the presence/absence of the amide-linked fatty acids 14:1 3-OH/ 12:0 3-OH, and the ester-linked 18:2, 12:1 and 16:0 2-OH. The polar lipids found in strain SH36^T were phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, an unidentified aminolipid, one unidentified phospholipid, and one other unidentified lipid (Fig. 3). In the case of the polar lipids the common features of all taxa examined in figure 3 was the presence of phosphatidylcholine, phosphatidylglycerol, an unidentified aminolipid (AL) and one other unidentified lipid (L2). The differentiating features of the taxa were the presence/absence of

phosphatidylethanolamine, diphosphatidylglycerol, the unidentified phospholipid PL1, and the unidentified lipids L3, L4 and L5. In all instances the identity of the unidentified polar lipids should be linked to the R_F values in the solvent systems used and their staining behaviour.

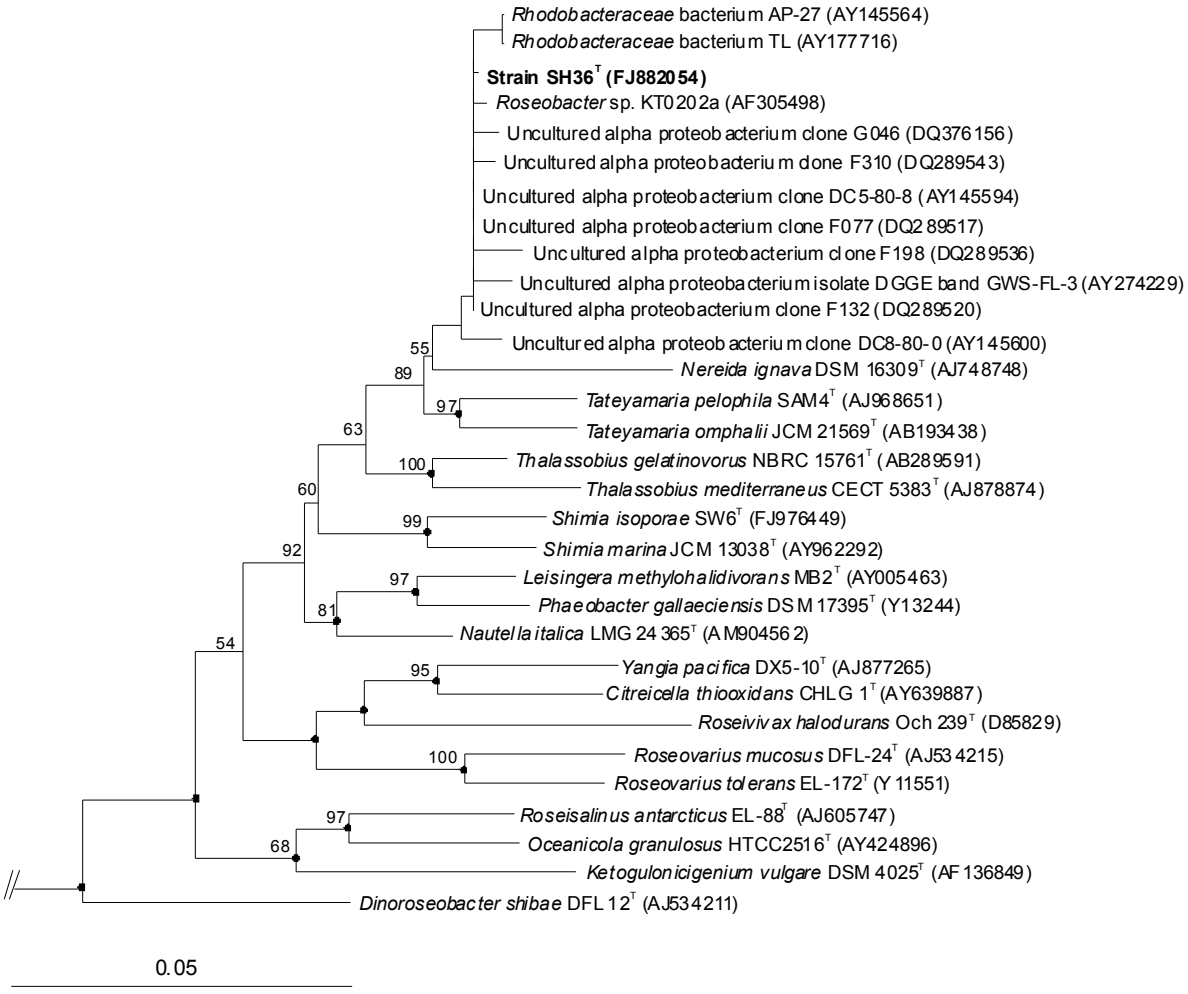

Fig. 2. Neighbor-joining tree based on 16S rRNA gene sequence similarity showing the phylogenetic relationships of strain SH36^T, closely affiliated strains and clones from the North Sea, and representatives of the *Rhodobacteraceae* within the *Alphaproteobacteria*. Bootstrap values were derived from 1000 replicates. Filled circles indicate nodes also recovered reproducibly with maximum-likelihood. Selected members of the *Gammaproteobacteria* were used as an outgroup (not shown). The scale bar indicates 5 % sequence divergence.

Table 1. Fatty acid composition of strain SH36^T and the type species of *Nereida*, *Shimia, Nautella*, and *Tateyamaria* using methodologies which release only ester-linked (M1) or ester- and amide-linked fatty acids (M2). tr, traces.

Fatty acid	SH36^T		*N. ignava* DSM 16309		*S. marina* JCM 13038		*N. italica* LMG 24365		*T. omphalii* JCM 21569	
	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2
16:0	6.31	7.53	1.90	1.94	2.42	2.93	5.35	6.00	3.31	3.74
18:0	1.65	1.53	3.46	3.47	tr	tr	1.16	1.14	tr	tr
12:1	2.03	2.80	tr	tr	1.12	1.44	1.87	1.72	1.06	1.40
18:1ω7c	69.78	59.44	80.22	79.08	88.73	83.26	71.51	69.18	79.67	76.35
11-methyl 18:1ω7c	2.69	6.85	6.82	6.77	1.45	1.38	8.77	8.49	8.75	8.24
18:2	11.33	15.50	1.69	1.68			5.66	5.46		
10:0 3-OH	3.58	2.41	2.55	2.13	2.99	4.71	1.94	1.71	2.30	2.33
12:0 3-OH						tr	tr	1.22		tr
14:1 3-OH		1.89	tr	2.15	tr	2.03		tr	tr	1.96
16:0 2-OH					tr		1.12	1.25	tr	tr
20:1ω7c			tr	1.04	tr	tr				tr
Summed feature 7*	1.37	tr	tr							

* Summed feature 7 contains C19:1ω6c and/or unknown ECL 18.846 (due to the same amounts present no attempt was made to resolve the identity of the fatty acids)

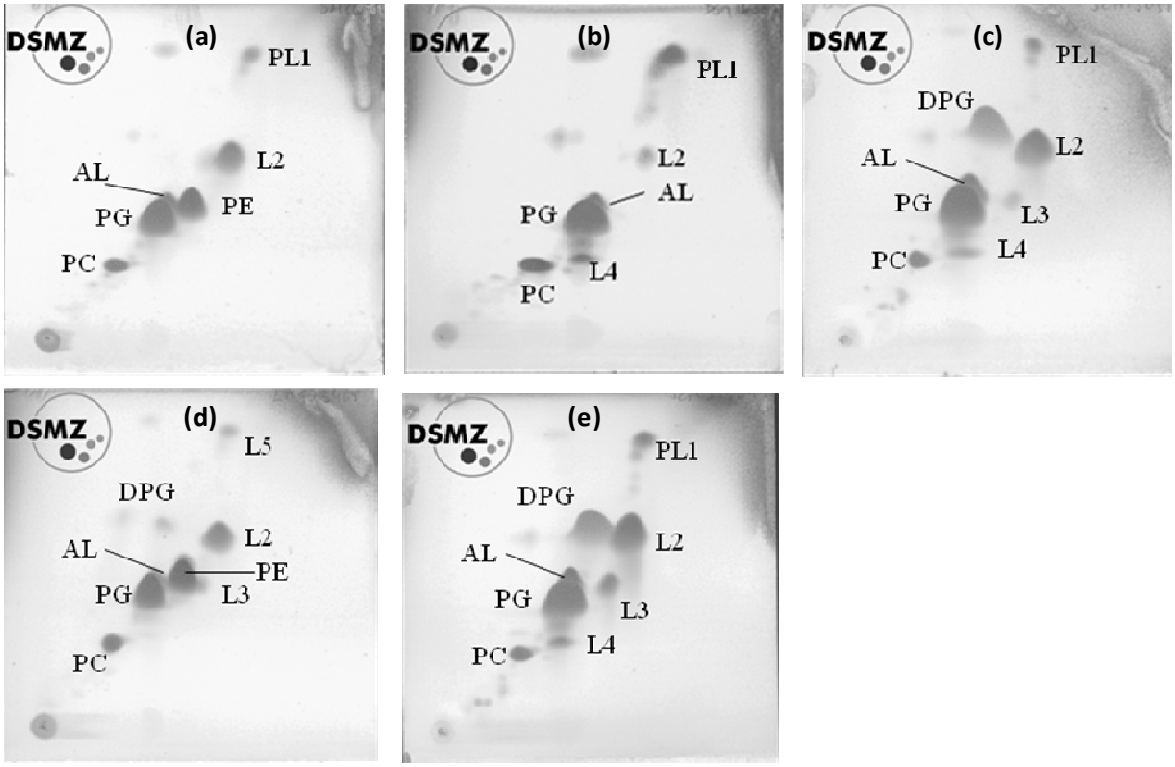

Fig. 3. Polar lipid composition of (a) strain SH36^T; (b) *Nereida ignava* DSM 16309^T; (c) *Shimia marina* JCM 13038^T; (d) *Nautella italica* LMG 24365^T; (e) *Tateyamaria omphalii* JCM 21569^T. AL, unidentified aminolipid; DPG, Diphosphatidylglycerol; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PG, Phosphatidylglycerol; PL1, Phospholipid; L2 - L5, Lipid.

Comparison of the polar lipid patterns of strain SH36^T and the type species of the closest related described genera revealed clear differences (Fig. 3). The fatty acid compositions of these strains showed the main differences in fatty acids that were present in low concentrations. All reference genera showed low amounts of both, certain ester- and amide-linked fatty acids, which were not present in strain SH36^T. Furthermore, all strains except of strain SH36^T contained small amounts of the fatty acid 12:0 (Table 1). Further differential characteristics are summarized in Table 2.

Table 2. Differential phenotypic characteristics of strain SH36^T compared to its closest phylogenetic relatives (based on 16S rRNA sequence analysis) of the genera *Nereida*, *Nautella*, *Shimia*, and *Tateyamaria.*

Taxa: 1, Strain SH36^T (data from this study); 2, *Nereida ignava* DSM 16309^T (Pujalte *et al*., 2005); 3, *Shimia marina* JCM 13038^T (Choi & Cho, 2006); 4, *Shimia isoporae* SW6^T (Chen *et al*., 2010); 5, *Nautella italica* LMG 24365^T (Vandecandelaere *et al*., 2009); 6, *Tateyamaria omphalii* JCM 21569^T (Kurahashi & Yokota, 2007); 7, *Tateyamaria pelophila* SAM4^T (Sass *et al*., 2010). +, positive result or growth; -, negative result or no growth; w, weak growth (OD_{600} ≤0.2); ND, no data available. *Data from Chen *et al*., 2010.

Characteristics	**1**	**2**	**3**	**4**	**5**	**6**	**7**
16S rRNA gene sequence similarity to SH36^T(%)	100	95.3	96	95.5	96	95.7	95.5
Colony color and morphology	white, circular, convex, shiny	unpigmented opaque	colourless or beige, circular, convex, opaque	beige, circular, convex	beige, round, smooth surface, convex	beige, circular, smooth, convex	beige/pinkish
Cell shape	coccoid to elongated rods	elongated rods, tear shaped	rods	rods	rods	coccoid to shortrods	rods
Cell size (µm)	0.6-0.5x0.6-0.7	0.2-0.3x1-3	0.3-0.6x0.8-3.6	0.9-1.0x2.1-2.3	0.7-0.1 1x1.5-2.1	0.8-1.0x1.0-1.5	1.5-3x0.7-1.4
Motility	-	-	+	+	+	-	+
BChl*a*	-	-	+	ND	ND	-	+
Catalase	-	+	ND	+	+	+	+
Growth at 4 °C	+	-	ND	ND	+	-	+
Growth at 37 °C	-	-	ND	ND	+	-	+
Nitrate reduction	-	-	+		-	+	ND
G+C content (mol%)	56.6	56	57.2	54.9	61	61.6	56.4
Salinity range (%)	1.25-8	1.36-8	3-7	ND	1-7	0.2-6	0.3-10.2
Salt optimum (%)	1.25-3.75	ND	3-5	ND	2-3	ND	ND
Temp. range (°C)	4-35	13-28	15-35	20-30	ND	ND	ND
Temp. optimum (°C)	30-32	ND	30-35	25-30	20-28	25-30	25
pH range	6.0-9.5	ND	6.0-10	7-11	5.5-9	5.4-8.9	6.0-9.0
pH optimum	7.0-9.0	ND	ND	7-8	ND	ND	ND
Substrates used:							
L-Alanine	w	-	-*	+	ND	ND	+
L-Arginine	+	-	-	ND	ND	ND	+
L-Asparagine	w	-	-	-	ND	ND	+
L-Aspartic acid	+	-	-	+	ND	ND	+
Glycine	w	-	+	ND	ND	ND	+
L-Histidine	+	-	ND	-	ND	ND	+
L-Leucine	w	-	+	-	ND	ND	+
L-Lysine	-	-	+	ND	ND	ND	+
L-Proline	+	-	-	+	ND	ND	ND
L-Serine	+	w	-*	+	ND	ND	+
L-Glutamic acid	+	-	-/+*	-	ND	ND	+
L-Methionine	-	ND	ND	ND	ND	ND	+
L-Threonine	+	-	-*	+	ND	ND	+
L-Tyrosine	w	-	ND	ND	-	ND	+
L-Tryptophan	-	ND	ND	ND	ND	ND	+
L-Phenylalanine	+	-	ND	-	ND	ND	+
L-Valine	-	ND	ND	ND	ND	ND	+
D-Glucose	-	w	-	ND	-	w	+
D-Fructose	-	-	-	+	ND	ND	+
Maltose	-	+	-*	+	-	+	+
D-Mannitol	-	w	-	-	ND	ND	+
D-Mannose	-	w	-	-	-	+	+
D-Xylose	-	-	-	ND	ND	ND	+
D-Trehalose	-	-	-	-	ND	+	+
D-Sorbitol	-	-	w*	-	ND	ND	+
L-Rhamnose	-	-	-	-	ND	ND	+
D-Ribose	-	ND	-	ND	ND	ND	+
Cellobiose	-	-	-	-	ND	+	+
Sucrose	-	-	w*	-	ND	ND	+
Lactose	-	-	-	+	ND	ND	ND
Acetate	+	w	-	+	ND	+	+
Citrate	+	w	+	+	ND	-	+
Formate	-	-	-	+	ND	ND	+
Lactate	W	+	ND	+	ND	+	+
Propionate	W	-	ND	+	ND	ND	ND
Malate	+	+	ND	ND	-	ND	+
Glycerol	-	-	-	+	ND	ND	+
Xylan	-	ND	ND	ND	ND	ND	+
Laminarin	-	ND	ND	ND	ND	ND	+
Hydrolysis of:							
Starch	-	-	w	+	-	+	+
Tween 80	-	-	+	-	+	-	ND
Gelatine	-	-	+	-	-	-	-

Based on all the distinctive features, we propose that strain SH36^T be assigned to a novel genus and species, for which the name *Pelagimonas elegans* gen. nov., sp. nov. is proposed.

Description of *Pelagimonas* gen. nov.

Pelagimonas (Pe.la.gi.mo´nas. L. n. *pelagus*, the sea; L. fem. n. *monas*, a unit, monad; N.L. fem. n. *Pelagimonas*, a sea monad).

Gram-negative, aerobic, oxidase-positive and catalase-negative cells, coccoid to irregular rod shaped. Chemoorganoheterotrophic growth on various compounds. Cells do not express any pigments or bacteriochlorophyll *a*, *pufLM* genes coding for subunits of the bacterial photosynthetic reaction centre complex are not present. Cells require vitamins as well as sodium ions. The predominant respiratory lipoquinone is Q-10. The fatty acid composition is dominated by 18:1ω7c (feature common to many members of the class *Alphaproteobacteria*), 16:0, and 18:2; other fatty acids (>1 %) are 10:0 3-OH, 12:1, 14:1 3-OH, 18:0, and 11-methyl 18:1ω7c. The polar lipids are phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, an unidentified aminolipid (AL), one unidentified phospholipid (PL1), and one other unidentified lipid (L2). On the basis of the 16S rRNA gene sequence analysis the genus represents a separate branch within the class *Alphaproteobacteria*, order *Rhodobacterales*, and so far contains only one species, *Pelagimonas elegans*, which is the type species.

Description of *Pelagimonas elegans* sp. nov.

Pelagimonas elegans (e´le.gans. L. adj. *elegans* fastidious – with respect to utilization of substrates).

In addition to the characteristics that define the genus, the type strain of the type species has the following characteristics. On SWM medium colonies are white circular, and convex with a shiny surface and up to 1 mm in diameter. Cells grow at temperatures ranging from 4 to 37 °C, with an optimum between 30 and 32 °C, and pH ranging from 6.0 to 9.5 (optimum pH 7.0 to 9.0). Cells grow in the presence of salt concentrations of 1.25 to 8 %, optimal salinity is at 1.25 to 3.75 %. Strain SH36^T has an absolute requirement of Na^+, its osmotolerance ranges from 0.05 to 1.5 M NaCl. No growth was observed without the vitamins pantothenic acid and nicotinic acid amide. Does not reduce nitrate to N_2. Oxidase-positive but catalase-negative and amylase, gelatinase and tweenase negative. Growth in minimal medium on single substrates is poor, however, with the addition of

0.1 g l^{-1} of yeast extract growth was stimulated. Substrate tests in artificial seawater medium with different carbon sources additionally containing 0.1 g l^{-1} yeast extract showed utilization of L-arginine, L-aspartic acid, L-glutamine, L-glutamic acid, L-histidine, L-phenylalanine, L-proline, L-serine, L-threonine, sodium acetate, sodium pyruvate, sodium malate, citric acid, and disodium succinate. L-alanine, L-asparagine, L-cystein, glycine, L-leucine, L-isoleucine, L-tyrosin, sodium lactate, and sodium propionate supported growth only weakly. No growth was observed on L-lysine, L-methionine, L-tryptophan, L-valine, all tested sugars (i.e. (+)-D-xylose, (+)-D-glucose, (+)-D-mannose, (+)-D-galactose, (-)-D-fructose, (+)-L-arabinose, (-)-D-ribose, (+)-L-rhamnose, (-)-L-fucose, (-)-D-mannitol, (-)-D-sorbitol, (+)-D-glucosamine, sucrose, maltose, cellobiose, trehalose, lactose, laminarin, starch, inulin, and xylan), sodium formate, glycerol, tween 80, and dimethylsulfoniopropionate (DMSP). Strain SH36^T is susceptible to penicillin G, streptomycin sulfate, and chloramphenicol but resistant to kanamycin sulfate. The G+C content is 56.6 mol%.

The type strain SH36^T (= DSM 23330^T = LMG 25292^T) was isolated from a North Sea water sample collected in 2 m depth during a phytoplankton bloom. The GenBank accession number for the 16S rRNA gene sequence of *Pelagimonas elegans* SH36^T is FJ882054.

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II.4 *Marianus varius,* gen. nov., sp. nov., isolated from the southern North Sea

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General comment on chapter II.4 - *Marianus varius*, gen. nov., sp. nov., isolated from the southern North Sea

The following draft classifying strain SH4-1 as a new species of a new genus bases on currently available data found in the literature. For phenotypic comparison, however, cultivation of all relevant strains should be carried out under identical or at least comparable conditions rather than using the data of previously published work (Tindall *et al*., 2010). Fatty acid and polar lipid patterns of the cell membrane are useful characteristics in bacterial classification as the chemical composition generally does not fluctuate significantly within a taxonomic group (Tindall, 1994). Such chemotaxonomic analyses are particularly helpful to distinguish between taxa when the phylogenetic position based on the 16S rRNA sequence is uncertain, which is the case for strain SH4-1. The chemical composition of membranes is affected by environmental parameters and can in turn differ due to the cultivation conditions. Thus, prior to submission of this manuscript analyses have to be carried out using cells cultivated under identical conditions to compare all reference strains properly. As these analyses of fatty acid and polar lipid patterns are not yet available, which will be generated by Brian Tindall from the German Collection of Microorganisms and Cell Cultures (DSMZ), it could not be included in this manuscript. Such comparable data might reveal slightly different distinguishing features than outlined in this chapter and classification of strain SH4-1 maybe has to be revised.

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Marianus varius, gen. nov., sp. nov., isolated from the southern North Sea

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Running title: *Marianus varius* gen. nov, sp. nov.

Subject category: *Proteobacteria*

The GenBank accession number for the 16S rRNA gene sequence of *Marianus varius* SH4-1^T is FJ882053.

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SUMMARY

A heterotrophic, Gram-negative, aerobic bacterium, designated strain SH4-1^T, was obtained from a seawater sample collected from the southern North Sea during a phytoplankton bloom. Isolation was performed using artificial saltwater medium with peptone and yeast extract. The 16S rRNA gene sequence comparison revealed affiliation to the *Roseobacter* clade (class *Alphaproteobacteria*) with *Sulfitobacter marinus* as the closest characterized species, showing 97.2% sequence similarity. Calculation of phylogenetic trees indicated, however, that members of the genus *Roseobacter* (95 and 96% sequence similarity, respectively) fall between strain SH4-1^T and the *Sulfitobacter* cluster including *Oceanibulbus indolifex* (≥95.4% identities). Cells of strain SH4-1^T are irregular rods with at least one flagellum. Optimal growth occurred between 28 and 32°C and at a pH between 7.0 and 8.5. On saltwater medium cells build small, white, circular, convex colonies. For growth cells require the vitamin nicotinic acid amide as well as sodium ions. The DNA base composition is 55.1 mol%. The fatty acids (>1%) comprised 10:0 3-OH, 12:1, 14:1 3-OH, 16:0, 18:0, 18:2, 18:1ω7c, and 11-methyl 18:1ω7c. The polar lipid pattern indicated the presence of phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylmonomethyl-ethanolamine, an unidentified aminolipid (AL), one unidentified phospholipid (PL1), and one other unidentified lipid (L2). Physiological, geno- and phenotypic differences to members of the genus *Sulfitobacter*, *Oceanibulbus* and *Roseobacter* support the description of a new species within a new genus for which we suggest the name *Marianus varius* gen. nov., sp. nov., with strain SH4-1^T (= DSM 23678 = LMG 26343) as the type strain.

The genus *Sulfitobacter* was first described by Sorokin (1995) and currently comprises nine species. In phylogenetic trees based on the 16S rRNA sequence, *Oceanibulbus indolifex* also falls within the *Sulfitobacter* cluster showing between 94.9 and 97.2% sequence similarities to the *Sulfitobacter* species. Phylogenetically, the genera belong to the *Roseobacter* clade, which falls within the family *Rhodobacteraceae* and has a within-cluster sequence similarity of >88% of the 16S rRNA gene (Brinkhoff *et al*., 2008). The first described species of this clade were *Roseobacter litoralis* and *Roseobacter denitrificans* (Shiba, 1991). Very diverse physiological traits have been reported for this group, e.g. turnover of the algal osmolyte dimethylsulfoniopropionate (DMSP) (Ledyard *et al.*, 1993; Ledyard & Dacey, 1994; González *et al.*, 1999), oxidation of the greenhouse gas carbon monoxide (King, 2003; Tolli, 2003; Moran *et al.*, 2004), production of secondary metabolites (Lafay *et al.*, 1995; Brinkhoff *et al.*, 2004), and methylotrophy (Holmes *et al.*, 1997). For the first characterized *Sulfitobacter* strain, *S. pontiacus*, Sorokin (1995) reported the capability of sulfite oxidation, however, this is not a feature that all *Sulfitobacter* strains have in common. Members of the *Roseobacter* clade use a multitude of organic compounds and some members are capable of aerobic anoxygenic photosynthesis (Yurkov & Beatty, 1998). In pelagic habitats of the North Sea roseobacters were found to constitute 5 to 24% of DAPI cell counts (Eilers *et al.*, 2001; Alderkamp *et al.*, 2006). Here we describe a new member of to the *Roseobacter* clade, strain SH4-1^T, isolated from the southern North Sea during a phytoplankton bloom. The 16S rRNA gene sequence of strain SH4-1^T is closely affiliated with the sequence of *Sulfitobacter marinus* (97.2% similarity), however, in phylogenetic trees members of the genus *Roseobacter* fall between strain SH4-1^T and the *Sulfitobacter* cluster. Based on our results we describe strain SH4-1^T as a new species of a new genus, *Marianus varius*.

Strain SH4-1^T was isolated from a water sample collected at 2 m depth on 12 May 2007 in the southern North Sea (54°42' N, 06°48' E; 36 m depth; salinity: 34.17) during a phytoplankton bloom. In order to obtain only pelagic cells, prior to inoculation the seawater sample was filtered through 1.2 µm. For isolation 100 µl of the prefiltered seawater were spread on agar plates with saltwater medium (composition see below) containing 30 mg l^{-1} yeast extract and 60 mg l^{-1} peptone. The composition of saltwater medium (SWM) was: 416 mM NaCl, 49.2 mM $MgCl_2.6H_2O$, 10 mM $CaCl_2.2H_2O$, 8.9 mM KCl, 28.2 mM Na_2SO_4, 10 mM HEPES, 840 µM KBr, 400 µM H_3BO_3, 150 µM $SrCl_2$, 400 µM NH_4Cl, 40 µM KH_2PO_4, 70 µM NaF, pH was adjusted to 8.0. Supplements after

autoclaving: 1 ml l^{-1} sterile filtered trace element solution SL 10 (Tschech & Pfennig *et al.*, 1984), 1 ml l^{-1} of a sterile filtered 5-fold concentrated multi vitamin solution (Balch *et al.*, 1979), and 15 ml 1 M $NaHCO_3$ (autoclaved separately). Agar plates were prepared with 20 g agar l^{-1} and incubated at 15°C. Single colonies were picked and restreaked five times for purification. Further cultivation of strain SH4-1^T was performed using saltwater medium with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone.

Production of Bchl *a* was determined by spectrophotometric analysis. Cells grown in the dark were harvested by centrifugation at 10.000 rpm for 10 min, and pigments were extracted with acetone:methanol 7:2 v/v as described by Clayton (1966). The extract was examined for light absorbancy (350 - 900 nm) with a Beckmann® DU 520 General Purpose UV/VIS Spectrophotometer. *Dinoroseobacter shibae* DFL12^T (DSM 16493^T) was used as positive control.

For determination of temperature and pH optima and range, cells were grown in SWM (composition see above) with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone. Temperature range was determined between 4 and 45°C (at 4, 10, 17, 20, 25, 28, 30, 32, 35, 37, 42 and 45°C). The pH range (5 - 11) was tested in increments of 0.5, adjusted with sterile NaOH and HCl solutions based on Wagner-Döbler *et al*. (2004). Further growth experiments were performed in artificial seawater medium (ASW): 342 mM NaCl, 28.2 mM Na_2SO_4, 1.47 mM KH_2PO_4, 4.7 mM NH_4Cl, 14.8 mM $MgCl_2.6H_2O$, 6.7 mM KCl, 1 mM $CaCl_2.2H_2O$. After autoclaving 1 ml l^{-1} sterile filtered 5-fold concentrated vitamin solution (Balch *et al.* 1979), 1 ml l^{-1} sterile filtered complex trace element solution (Zech *et al.*, 2009), and 15 ml 1 M $NaHCO_3$ solution (autoclaved separately) were added.

Salinity range and optimum were determined in test tubes. To prepare salt solutions of 0, 12.5, 25, 37.5, 50, 65, 80 and 100 g salts l^{-1}, a concentrated stock solution was used: (l^{-1}) 80 g NaCl, 16 g Na_2SO_4, 1 g NH_4Cl, 12 g $MgCl_2.6H_2O$, 2 g KCl, 0.6 g $CaCl_2.2H_2O$, and 0.76 g $NaHCO_3$ (autoclaved separately). From another solution (l^{-1}) 0.2 g KH_2PO_4, 0.25 g NH_4Cl, 0.05 g yeast extract and 0.05 g peptone were added as well as 1 ml 5-fold concentrated vitamin solution (Balch *et al.* 1979) and 1 ml trace element solution (Zech *et al*., 2009). Before inoculation, cells from a preculture, grown in SWM with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone, were washed once with the lowest salt solution. The osmotolerance was studied with NaCl only. Therefore a concentrated NaCl solution was

added to a minimal medium consisting of: (l^{-1}) 0.1 g Mg-Acetate, 7 g $MgSO_4.7H_2O$, 0.2 g KH_2PO_4, 0.25 g NH_4Cl, 3 g $MgCl_2.6H_2O$, 0.5 g KCl, 0.15 g $CaCl_2.2H_2O$, 0.3 g $KHCO_3$, 5-fold concentrated vitamin solution and complex trace element solution (see above) to final concentrations of 0.01, 0.05, 0.1, 0.25, 0.37, 0.5, 0.7, 1.0, 1.5 and 2.0 M NaCl.

To determine the maximal doubling time, growth in SWM with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone and under optimal conditions (30°C, pH 7.5, 3.75% salinity) was followed in triplicates. Every 1-2 h the test tubes were vigorously shaken and growth was monitored by measuring the OD_{600}. Growth rates (μ) and doubling time ($t_d = \ln 2/\mu$) were determined by linear regression of semi-logarithmic plots of cell density against time.

Requirement for single vitamins were tested in ASW supplemented with all 20 amino acids as carbon source (each 1 mM). The vitamins nicotinic acid amide, thiamine, pantothenic acid, pyridoxal hydrochloride, cyanocobalamine, riboflavin and biotin (all at 0.05 mg l^{-1}) were tested in mixtures from which one was omitted. Cells were precultured in SWM with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone. Prior to inoculation cells were washed with ASW without vitamins. When growth was detected, cells from the stationary phase were transferred to fresh medium to test whether growth was only possible as the result of residual vitamins associated with the cells or due to self-sufficient growth.

Tests of growth on single substrates (1 g l^{-1}) were performed in ASW as described by Martens *et al.* (2006). Additionally the algal osmolyte dimethylsulfoniopropionate (DMSP) was tested (at 100 µM). Utilization of amino acids was tested using a final concentration of 5 mM except for tyrosine, which was added to 1 mM, because of precipitation at 5 mM. The pH was adjusted to 7.5. All substrates are listed below (see description of *Marianus varius*). Cells were precultured in SWM with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone. Exponentially grown cells were washed twice with ASW without any substrate in 2 ml reaction vessels. Two replicates of 5 ml medium were inoculated with 10 µl of washed cells for each substrate. Cell cultures in ASW without any substrate were used as a negative control. Growth was determined after incubation for at least four weeks at 20°C by measuring an increase in OD_{600} with a spectrophotometer (Spectronic 70).

Gram staining was performed using the Hucker staining method as described by Murray *et al.* (1994). Cytochrome oxidase test was carried out by streaking a single colony on a

cellulose strip saturated with test reagent (1 g l^{-1} ascorbic acid, 10 g l^{-1} tetramethyl-p-phenylendiamin-HCl). Blue coloration shows oxidase activity (*Micrococcus luteus* was used as positive control). Catalase reaction was tested with a drop of 5% H_2O_2 added to a dense cell culture (*E. coli* was used as positive control). Exoenzyme activities (hydrolysis of gelatin, starch and Tween 80) were analysed with saltwater medium solidified with 9% (w/v) gelatin or 1.5% (w/v) agarose and supplemented with 0.2% (w/v) starch and 1% (v/v) Tween 80, respectively, based on the methods given in Smibert & Krieg (1994). Reduction of nitrate was tested in anoxic ASW containing resazurin (0.5 g l^{-1}). After autoclaving, the medium was reduced by addition of ~1 mg sterile sterile sodium dithionite (causing discoloration of resazurin). Nitrate was added as 1 g l^{-1} sodium nitrate. Medium was filled into test tubes, containing a small inverted glass tube. Headspace was flushed with N_2/CO_2 (80/20, v/v) and tubes were sealed airproof. Substrates (0.2 g l^{-1} glucose and 0.1 g l^{-1} yeast extract, respectively) and sodium nitrate were injected from anoxic stock solutions. Exponentially grown cells were preincubated under N_2 gassing before inoculation. Cells were precultured in SWM with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone. Accumulation of bubbles in the inverted glass tube indicates N_2 formation by nitrate reduction. *Roseobacter denitrificans* DSM7001^T was used as a positive control.

Antibiotic susceptibility was tested with agar diffusion tests using penicillin G, streptomycin sulfate, chloramphenicol and kanamycin sulfate as previously described (Brinkhoff *et al.*, 2004). *Phaeobacter gallaeciensis* DSM17395^T and *Rhodobacter sphaeroides* 2.4.1^T were used as control organisms.

For transmission electron microscopy (TEM), cells from the exponential phase, grown in ASW with all 20 amino acids (each 1 mM) were once washed in distilled water and studied unstained as described previously (Cháves *et al.*, 2004). After air drying, copper grids (200 mesh; Plano) were examined by TEM (EM 902A; Zeiss).

The amplification and purification of the 16S rRNA gene was carried out as described by Brinkhoff & Muyzer (1997). Sequencing was performed by GATC Biotech AG (Konstanz, Germany). Phylogenetic analysis was performed with the ARB software package [http://www.arb-home.de (Ludwig *et al.*, 2004)]. Detection of genes coding for the subunits of the photosynthetic reaction centre complex (*pufL* and *pufM*) was performed by PCR using the primers pufL and pufM (Beja *et al.*, 2002). Reactions were cycled in an

Eppendorf thermocycler at the following parameters: 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, with a final extension step at 72°C for 10 min. The PCR product was purified by using the peqGOLD MicroSpin Cycle-Pure Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany). Sequencing was performed by GATC Biotech AG (Konstanz, Germany).

For the following analyses cell biomass of strain SH4-1^T was obtained from exponentially growing cells cultivated in SWM with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone at 20°C. Respiratory lipoquinones and polar lipids were extracted from 100 mg freeze-dried cells using the method described by Tindall (1990a, b). Respiratory lipoquinones were separated into their structural classes (menaquinones, ubiquinones etc.) by TLC. Bands were eluted and further separated and identified by HPLC, using an RP18 column (Tindall, 1996). Polar lipids were separated by two-dimensional chromatography and identified on the basis of their RF values in combination with their reaction with specific staining reagents (Tindall, 1990a, b). Fatty acid methyl esters were released from 20 mg freeze-dried cells using methodologies that release only ester-linked fatty acids (M1) or ester- and amide-linked fatty acids (M2) (see Labrenz *et al.*, 1998; Strömpl *et al.*, 1999). Initial identification relied on the use of the MIDI Sherlock system (version 6.1), with fatty acids being initially identified against the TSBA4 database. Where peaks were not identified by the MIDI Sherlock system peaks were identified using GC-MS.

The DNA G+C content of cells of strain SH4-1^T was determined by extracting genomic DNA according to Cashion *et al.* (1977) and subsequent determination of deoxyribonucleosides by HPLC (Mesbah *et al.*, 1989; Tamaoka & Komagata, 1984).

After 7 days incubation on agar plates with SWM supplemented with 0.1 g l^{-1} yeast extract and 0.2 g l^{-1} peptone at 20°C, colonies were white, circular, convex, shiny and up to 1.5 mm in diameter. Single cells grown for four days in liquid saltwater medium were irregular rods with a length of 0.75 - 3.3 µm and a width of 1.0 - 1.4 µm (Fig. 1). By microscopy cells showed at least one flagellum, however, they did not show clear motility. Cells stained Gram-negatively.

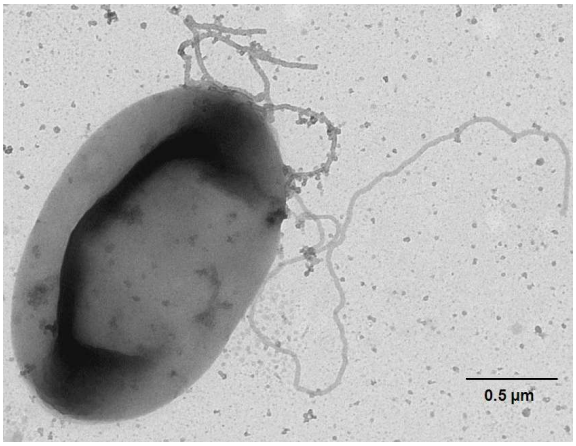

Fig. 1. Transmission electron microscopic image of cells of strain SH4-1^T from the exponential phase, grown on ASW medium with all 20 amino acids.

Strain SH4-1^T grew between 4°C and 37°C with a maximum between 28°C and 32°C. The pH range tolerated for growth was 6.0 to 9.5 with an optimum between 7.0 and 8.5. Growth was observed at salt concentrations of 1.25% to 8% with an optimum between 1.25 and 5%. Strain SH4-1^T has an absolute requirement of Na^+, its osmotolerance ranged from 0.1 to 0.7 M NaCl. Under optimum conditions in saltwater medium, grown with 0.02 g l^{-1} peptone and 0.01 g l^{-1} yeast extract, the doubling time was 2.9 h. The following carbon sources supported cell growth: L-alanine, L-arginine, L-aspartic acid, L-glutamine, L-glutamic acid, L-histidine, L- isoleucine, L-leucine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, (+)-D-xylose, (+)-D-glucose, (+)-D-mannose, (+)-D-galactose, (-)-D-fructose, (+)-L-arabinose, (+)-L-rhamnose, (-)-L-fucose, (-)-D-mannitol, (-)-D-sorbitol, lactose, sucrose, maltose, cellobiose, trehalose, sodium acetate, sodium pyruvate, sodium malate, citric acid, disodium succinate, sodium lactate, sodium propionate, glycerol, and tween 80. Glycine and L-methionine supported growth only weakly. No growth was observed without the addition of the vitamin nicotinic acid amide. Cells of strain SH4-1^T were susceptible to penicillin G, streptomycin sulfate and chloramphenicol but not to kanamycin sulfate. Strain SH4-1^T exhibited no oxidase and only weak catalase activity.

Analysis of the 16S rRNA gene sequence showed that strain SH4-1^T shared 97.2% sequence similarity with *Sulfitobacter marinus* SW-265 as the closest characterized species. Calculation of phylogenetic trees indicated, however, that members of the genus *Roseobacter* (with 95 and 96% sequence similarity, respectively) fall between strain SH4-1^T and the *Sulfitobacter* cluster including *Oceanibulbus indolifex* (≥95.4% sequence

identities; Fig. 2). The G+C base content of strain SH4-1^T is 55.1 mol%. *pufLM* genes coding for the pufL and pufM subunits of the bacterial photosynthetic reaction centre complex were not detected in strain SH4-1^T and in laboratory cultures cells did not express bacteriochlorophyll *a* or any other pigments.

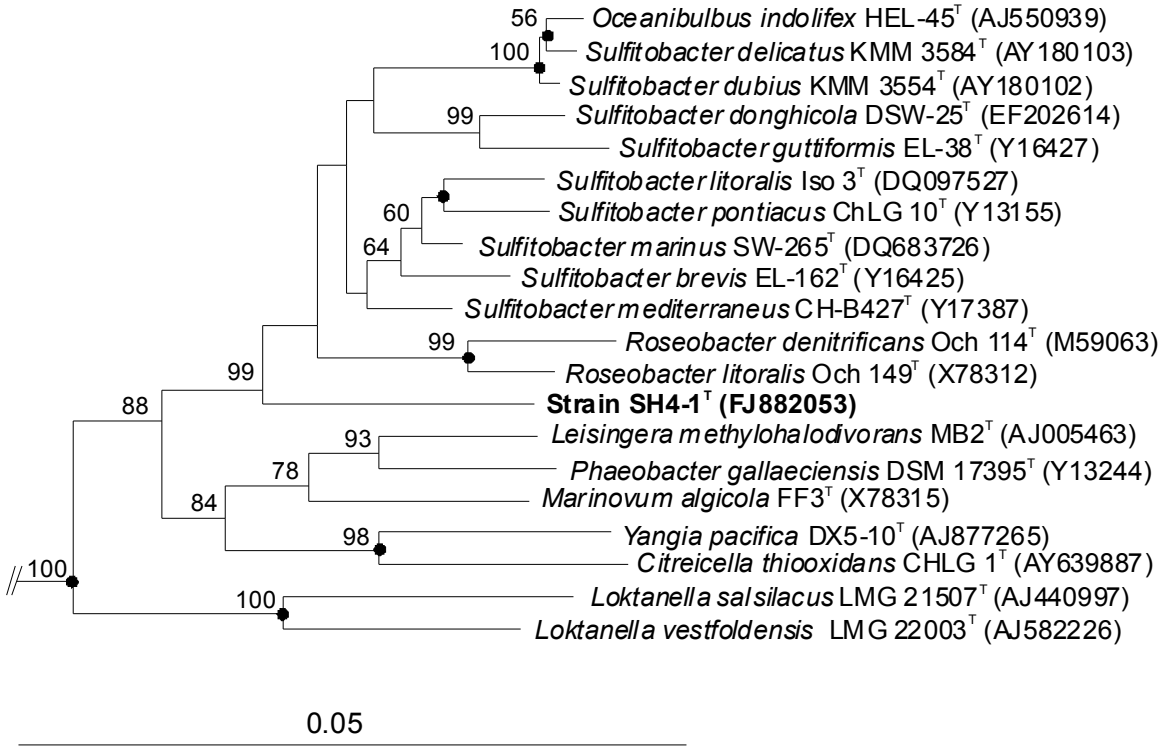

Fig. 2. Neighbour-joining tree showing the phylogenetic relationships of strain SH4-1^T and representatives of the family *Rhodobacteraceae* within the class *Alphaproteobacteria* based on 16S rRNA gene sequence similarity. Bootstrap values were derived from 1000 replicates. Filled circles indicate nodes also recovered reproducibly with maximum-likelihood. Selected members of the class *Gammaproteobacteria* were used as an outgroup. The scale bar indicates 5% sequence divergence.

The predominant respiratory lipoquinone of strain SH4-1^T was Q-10, a feature typical of the vast majority of the class *Alphaproteobacteria*. The fatty acid composition was dominated by 18:1ω7c, which is also common to many members of the class *Alphaproteobacteria*; other fatty acids (>1%) were 10:0 3-OH, 12:1, 14:1 3-OH, 16:0, 18:0, 11-methyl 18:1ω7c, and 18:2. The polar lipids found in strain SH4-1^T were phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylmonomethylethanolamine, an unidentified aminolipid (AL), one unidentified phospholipid (PL1), and one unidentified lipid (L2).

To compare strain SH4-1^T with the genera *Roseobacter*, *Sulfitobacter* and *Oceanibulbus*, the fatty acid and polar lipid compositions of representative strains were analyzed, i.e. the type strains of the two characterized *Roseobacter* species, *S. pontiacus* as the type species of the genus *Sulfitobacter*, the type strain of *S. marinus* as the closest described relative of strain SH4-1^T based on 16S rRNA comparison, and *O. indolifex* as the type species of the genus *Oceanibulbus*. Strain SH4-1^T contained the fatty acids 12:1, 11-methyl 18:1ω7c, and 18:2 which were not or only in small amounts present in the *Roseobacter* species and it showed clear differences in the polar lipid composition compared to *R. litoralis* and *R. denitrificans* (Table 1). *O. indolifex* did not contain the fatty acids 12:1, 14:1 3-OH, 18:0, 11-methyl 18:1ω7c, and 18:2, which were present in strain SH4-1^T, but 16:1w7c was present in higher amounts than in strain SH4-1^T. Furthermore, the two strains showed some differences in the polar lipid composition. For the two *Sulfitobacter* species the fatty acids 12:1 and 18:2 were not reported, strain SH4-1^T contained higher amounts of 14:1 3-OH and also the composition of polar lipids differed. Thus strain SH4-1^T can be distinguished from the reference genera *Roseobacter*, *Sulfitobacter* and *Oceanibulbus*, however, *S. pontiacus* and *S. marinus* showed only a few differences. Further differential characteristics are summarized in Table 2.

Table 1. Fatty acid and polar lipid composition of 1, strain SH4-1^T; 2, *Sulfitobacter pontiacus* ChLG 10 (Wagner-Döbler *et al*., 2004); 3, *Sulfitobacter marinus* SW-265 (Yoon *et al*., 2007); 4, *Oceanibulbus indolifex* HEL45 (Wagner-Döbler *et al*., 2004); 5, *Roseobacter litoralis* Och 149 and 6, *Roseobacter denitrificans* Och 114 (Martens *et al*., 2006) using methodologies which release only ester-linked (M1) or ester- and amide-linked fatty acids (M2). tr, traces; ND, no data available.

	1		**2**		**3**	**4**		**5**		**6**	
Fatty acid	**M1**	**M2**	**M1**	**M2**	**M1**	**M1**	**M2**	**M1**	**M2**	**M1**	**M2**
16:0	7.03	4.34	9.97	10.10	8.3	4.66	5.24	1.51	1.11	1.58	1.56
18:0	1.50	2.21	1.32	1.16	tr	-	-	1.59	1.39	2.71	2.75
12:1	2.59	2.21	-	-	-	-	-	-	-	-	-
16:1ω7c	tr	tr	-	tr	1.0	1.87	2.02	-	tr	-	tr
18:1ω7c	59.48	64.69	78.86	77.37	77.1	88.38	85.19	91.84	86.44	92.74	87.22
11 methyl 18:1ω7c	7.03	2.51	5.0*	-	6.9	-	-	-	tr	-	-
18:2	15.55	9.32	-	-	-	tr	tr	1.4†	-	-	-
10:0 3-OH	3.63	3.22	1.79	2.85	3.6	1.61	2.70	3.25	2.78	2.12	2.14
12:1 3-OH	-	tr	-	-	-	-	1.21	-	-	-	-
14:1 3-OH	tr	5.48	-	1.04	-	-	-	-	3.58	-	3.78
Polar lipid											
Phosphatidylglycerol	+		+		ND	+		+		+	
Diphosphatidylglycerol	+		+		ND	+		+‡		+‡	
Phosphatidylethanolamine	+		+		ND	+		-		-	
Phosphatidylchonline	+		+		ND	+		-		+	
Phosphatidylmonomethyl-ethanolamine	+		-		ND	-		-		-	
Unidentified aminolipid (AL)	+		+		ND	+		+		+	
Unidentified phospholipid (PL1)	+		-		ND	-		+		+	
Unidentified phospholipid (PL2)	-		-		ND	-		+		+	
Unidentified lipid (L)	+		-		ND	-		-		-	

*Park *et al*., 2007
†Biebl *et al*., 2005
‡Shiba, 1991

Table 2. Differential characteristics of 1, strain SH4-1^T; 2, *Sulfitobacter pontiacus* ChLG 10 (Sorokin, 1995); 3, *Sulfitobacter marinus* SW-265 (Yoon *et al*., 2007); 4, *Oceanibulbus indolifex* HEL45 (Wagner-Döbler *et al*., 2004); 5, *Roseobacter litoralis* Och 149 and 6, *Roseobacter denitrificans* Och 114 (Shiba, 1991).

+, positive result or growth; -, negative result or no growth; W, weak (OD_{600} ≤0.2); ND, no data available; V, variable.

Characteristics	**1**	**2**	**3**	**4**	**5**	**6**
16S rRNA sequence similarity to SH4-1^T (%)	100	96.2	97.2	95.5	96	95
Colony morphology	White, circular, convex	Colourless, flat	Cream-coloured	whitish, shiny surface	Pink	Pink
Cell morphology	Irregular rods	Rod	Rod or oval	Irregular rods	Rod or ovoid rod	Rod or ovoid rod
Cell size (µm)	1.0-1.4x0.75-3.3	0.45-1.3x2-5	0.2-0.5x0.6-1.5	1.8-2.5x3-5	0.6-0.9x1.2-2.0	0.6-0.9x1.0-2.0
Rosettes formed	+	+	ND	ND	-	-
Motility	(+)	+	-	-	+	+
BChl *a*	-	-	ND	-	+	+
pufML genes	-	ND	ND		+†	+†
Oxidase	-	+	+	W	+	+
Catalase	W	+	+	+	ND	ND
Growth at 37°C	W	-	-	-	-	-
Nitrate reduction	-	+‖	-	-	-	+
G+C content (mol%)	55.1	62-62.5	57.8	60.1	57.2	59.6
Temperature range (°C)	4-37	4-35	4-35	8-30	2-30	2-30
Temperature optimum (°C)	28-32	22-25	30	25-30	20-30	20-30
pH range	6·0-9·5	6.5-8.5	5·0-ND	7.0-9.0	7.5-9.5	7.5-9.5
pH optimum	7·0-8·5	7.3-7.5	7·0-8·0	7.0-8.0	ND	ND
Substrates used:						
L-Alanine	+	+	-	+	ND	ND
L-Arginine	+	+	ND	-	ND	ND
L-Aspartic acid	+	+	-	+	ND	ND
L-Glutamic acid	+	+	-	+	+	+
L-Tryptophan	+	ND	ND	-	ND	ND
D-Glucose	+	W*	-	+	ND	ND
D-Mannose	+	ND	-	-	ND	ND
Glucosamine	-	ND	-	-	+‡	+‡
D-Galactose	+	ND	-	-	ND	ND
Maltose	+	ND	-	-	-	-
D-Fructose	+	ND	-	-	ND	+
L-Fucose	+	ND	-	-	+‡	+‡
L-Rhamnose	+	ND	-	-	+‡	+‡
L-Arabinose	+	ND	-	-	V‡	+‡
D-Xylose	+	ND	-	-	ND	ND
Acetate	+	+	-	-	+	+
Citrate	+	w*	-	+	+	+
Propionate	+	ND	ND	-	ND	ND
Cellobiose	+	ND	-	-	-‡	-§
α-Lactose	+	ND	-	-	ND	ND
Sucrose	+	ND	-	-	-	-
Ribose	W	ND	-	-	ND	ND
Trehalose	+	ND	-	-	-§	-§
Glycerol	+	+	ND	ND	-	-‡
Starch	-	+*	ND	-	ND	ND
Hydrolysis of:						
Amylase	-	+	-*	-	-	-
Gelatine	+	-*	-	-	+	+
Tween 80	-	+*	+	-	+§	+§

*Ivanova *et al*., 2004
†Newton *et al*., 2010
‡Labrenz *et al*., 1999
§Lafay *et al*., 1995
‖Yoon *et al*., 2007

Based on all the distinctive features, we propose that strain SH4-1^T be assigned to a novel species of a novel genus, for which the name *Marianus varius* gen. nov., sp. nov. is proposed.

Description of *Marianus gen. nov.*

Marianus (Ma.ri.a´nus. N.L. masc. marianis, belonging to the sea).

Gram-negative, aerobic, oxidase-negative and catalase-positive cells, irregular rod shaped. Chemoorganoheterotrophic growth on various compounds. Cells do not express any pigments or bacteriochlorophyll *a*, *pufLM* genes coding for subunits of the bacterial photosynthetic reaction centre complex are not present. Cells require vitamins as well as sodium ions. The major respiratory lipoquinone is Q-10. The fatty acid composition is dominated by 18:1ω7c (feature common to many members of the class *Alphaproteobacteria*) and 18:2; other fatty acids (>1%) are 10:0 3-OH, 12:1, 14:1 3-OH, 16:0, 18:0, and 11-methyl 18:1ω7c. The polar lipids are phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylmonomethylethanolamine, an unidentified aminolipid (AL), one unidentified phospholipid (PL1), and one other unidentified lipid (L2). On the basis of the 16S rRNA gene sequence analysis the genus represents a separate branch within the class *Alphaproteobacteria*, order *Rhodobacterales*, and so far contains only one species, *Marianus varius*, which is the type species.

Description of *Marianus varius* sp. nov.

Marianus varius (va´ri.us. L. masc. adj. *varius*, various, diverse, manifold - with respect to substrate utilization).

In addition to the characteristics that define the genus, the type strain of the type species has the following characteristics. Cells are variable in size (0.75 - 3.3 x 1.0 - 1.4 µm) with at least one flagellum. On SWM medium colonies are white, circular, convex and shiny up to 1.5 mm in diameter. Cells grow at temperatures ranging from 4 to 37°C, with an optimum between 28 and 32°C, and pH ranging from 6.0 to 9.5 (optimum pH 7.0 to 8.5). Cells grow in the presence of salt concentrations of 1.25% and 8%, optimal salinity is between 1.25% and 5%. Strain SH4-1^T has an absolute requirement of Na^+, its osmotolerance ranges from 0.05 to 1.5 M NaCl. No growth was observed without the vitamin nicotinic acid amide. Does not reduce nitrate to N_2. Oxidase-negative but weakly catalase-positive, amylase and tweenase negative and gelatinase positive. Substrate tests in

artificial seawater medium with different carbon sources show utilization of L-alanine, L-arginine, L-aspartic acid, L-glutamine, L-glutamic acid, L-histidine, L-isoleucine, L-leucine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, (+)-D-xylose, (+)-D-glucose, (+)-D-mannose, (+)-D-galactose, (-)-D-fructose, (+)-L-arabinose, (+)-L-rhamnose, (-)-L-fucose, (-)-D-mannitol, (-)-D-sorbitol, lactose, sucrose, maltose, cellobiose, trehalose, sodium acetate, sodium pyruvate, sodium malate, citric acid, disodium succinate, sodium lactate, sodium propionate, glycerol, and tween 80; glycine and L-methionine supported growth only weakly. No growth was observed on L-asparagine, L-cysteine, L-lysine, L-valine, (+)-D-glucosamine, (-)-D-ribose, sodium formiate, starch, inulin, xylan, laminarin, and dimethylsulfoniopropionate (DMSP). Cells of strain SH4-1^T were susceptible to penicillin G, streptomycin sulfate and chloramphenicol but resistant to kanamycin sulfate. The G+C content is 55.1 mol%.

The type strain SH4-1^T (= DSM 23678 = LMG 26343), was isolated from a North Sea water sample from 2 m depth. The GenBank accession number for the 16S rRNA gene sequence of *Marianus varius* SH4-1^T is FJ882053.

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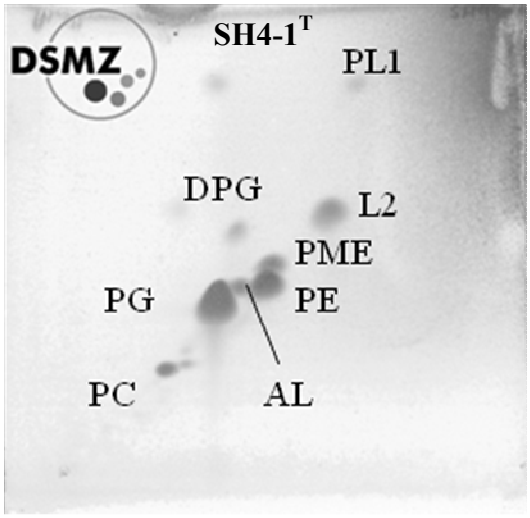
Supplementary material

Fig 1S. Polar lipid composition of strain SH4-1^T. AL, unidentified aminolipid; DPG, Diphosphatidylglycerol; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PG, Phosphatidylglycerol; PME, Phosphatidylmonomethylethanolamine; PL1, Phospholipid; L2, Lipid.



II.5 Distinct Seasonal Growth Patterns of the Bacterium *Planktotalea frisia* in the North Sea and Specific Interaction with Phytoplankton Algae

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Distinct Seasonal Growth Patterns of the Bacterium *Planktotalea frisia* in the North Sea and Specific Interaction with Phytoplankton Algae

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Running title: Seasonal growth patterns of *P. frisia* SH6-1 in the North Sea

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Summary

We investigated the spatio-temporal occurrence of the newly described member of the *Roseobacter* clade *Planktotalea frisia* strain SH6-1^T in the North Sea and interactions with phytoplankton algae with a special emphasis on the carbohydrate metabolisms. This bacterium was present in the first half of the year throughout the North Sea. Detailed studies in the German Bight in several years showed that *P. frisia* exhibited distinct peaks during and after phytoplankton blooms. Highest abundances, as detected by a highly specific quantitative PCR assay and CARD-FISH, were 0.5 to 0.9% of total bacterial abundance. In the second half of the year, *P. frisia* was not detected in any samples throughout the North Sea. Experimental studies in which *P. frisia* was grown in the presence of axenic cultures of *Phaeocystis globosa*, *Leptocylindrus danicus* and *Thalassiosira rotula* exhibited distinctly different responses with the best growth together with *P. globosa* and *T. rotula* and very low growth together with *L. danicus*. The results show that the algae greatly differed in the composition of their exuded carbohydrates and that *P. frisia* was rather selective in their consumption, suggesting that the distinct carbohydrate metabolisms is a key feature to explain its seasonal occurrence in the North Sea.

Introduction

Molecular, culture-independent techniques revealed during the recent past that the ocean harbors an unknown wealth of prokaryotes. Most of these organisms are rare and only a minority of them constitutes the majority of bacterioplankton cells in the water column, even though some changes and variations occur in space and time (Pedrós-Alió, 2006, Sogin *et al*., 2006). Temporal changes in the abundance of phylogenetic groups in a given pelagic system are the result of responses to primary production, substrate availability and predation by grazing or viral infection (Brown *et al*., 2005; Alderkamp *et al*., 2006; Sapp *et al*., 2007a; Teira *et al*., 2009; Alonso-Gutiérrez *et al*., 2009). Even though we know that also the abundance of individual bacterioplankton taxa changes over time and space (Eilers *et al*., 2000; Giebel *et al*., 2011; Carlson *et al*., 2009; Mayali *et al*., 2011) there is still little detailed understanding of the controlling mechanisms of their growth. It has been shown that phytoplankton species exhibit distinct positive and negative interactions with heterotrophic bacteria (Grossart, 1999; Grossart *et al*., 2005; Rooney-Varga *et al*., 2005; Grossart and Simon, 2007; Sher *et al*., 2011) and that protozoan grazing and resistance to phage infection can select for distinct taxa (Beardsley *et al*., 2003; Holmfeldt *et al*., 2007). Such information is important to better understand and dissect changes in the bacterioplankton community composition but, so far, little such information is available for abundant bacterioplankton taxa.

In order to enlarge our knowledge on the significance of individual members of the bacterioplankton it is important to obtain more isolates of abundant bacterioplankton taxa. Refined culturing techniques such as dilution cultures can greatly help to achieve this goal (Giovannoni and Stingl, 2007). Obtaining isolates of relevant bacterioplankton taxa is also important in the context of metagenomic, metatranscriptomic and metaproteomic studies because information on individual taxa can only be extracted when they have been isolated and their genomes sequenced, such as for *Prochlorococcus* and Cand. *Pelagibacter ubique* (Giovannoni *et al*., 2005; Coleman *et al*., 2006; Sowell *et al*., 2008). Examining the physiological properties of relevant isolates can further help to understand their significance in organic matter cycling and is an important complement for postgenomic studies.

The *Roseobacter* clade within *Alphaproteobacteria* is one of the most prominent phylogenetic lineages in the marine bacterioplankton and in particular in temperate coastal seas such as the North Sea. Various studies have shown that this clade constitutes up to ~24% of the total bacterioplankton in the North Sea and even higher fractions of its active

subcommunity (Zubkov *et al*., 2001; Eilers *et al.*, 2001; Pernthaler *et al.*, 2002; Sekar *et al.*, 2004; Alonso and Pernthaler, 2006; Alderkamp *et al*., 2006). Much less information exists on the abundance and significance of individual taxa of the *Roseobacter* clade in the North Sea and in general in pelagic marine systems. Zubkov *et al*. (2001) showed that one taxon of the *Roseobacter* clade dominated the consumption of dimethylsulfoniopropionate (DMSP) in a coccolithophore bloom in the North Sea. The largest cluster of this clade, the *Roseobacter* Clade Affiliated (RCA) cluster with the isolate Cand. *Planktomarina temperata*, constitutes 5 to 20% of total bacteria in the North Sea and its occurrence is significantly correlated with phaeopigments (Giebel *et al*., 2011).

The aim of this study was to examine the occurrence and abundance of a *Roseobacter* clade strain, *Planktotalea frisia* SH6-1^T, in the North Sea and some physiological traits with respect to its sugar metabolism with potential relevance to its occurrence. *P. frisia* was isolated from the southern North Sea from a 10^{-6} dilution culture in a phytoplankton bloom dominated by small flagellates (<10 µm) and raphidophytes (Hahnke *et al*., 2011). The retrival of this organism from a high dilution step indicated a high abundance in the sample and suggested that it is, at least temporally, an important component of the *Roseobacter* clade in the North Sea.

Results

Occurrence of P. frisia SH6-1 in the North Sea

Planktotalea frisia SH6-1 was detected at all except one station of a south-north transect in the North Sea from the German Bight to southern Norway visited during a cruise in May 2006 (Fig. S1A). This strain was detected in 27 of 31 samples collected between the surface and 90 m depth and predominantly in the free-living (FL; 0.2 - 5 µm) bacterial fraction (Fig. S1C). During a cruise on the same transect in September 2005 this strain was not detected at all (Fig. S1B).

Quantitative analyses of samples collected in the German Bight at Helgoland Roads and in the Wadden Sea over annual cycles of various years showed that *P. frisia* SH6-1 was present during most of the growing season with highest abundances in spring and summer (Fig. 1). Highest proportions reached 0.42% of total bacterial 16S rRNA genes at Helgoland Roads and 0.47% in the Wadden Sea. At Helgoland Roads, *P. frisia* SH6-1 peaked in the beginning of May and at the end of July 1998, coinciding with a peak of total bacterial cells, and in the middle of February and May 1999 (Fig. 1A). The increase of

P. frisia SH6-1 in April and May 1999 was paralleled by two phytoplankton blooms, a first one consisting of diatoms, and a second larger bloom consisting of diatoms and small phytoflagellates. After the complete collapse of this bloom *P. frisia* SH6-1 reached its annual maximum (Fig. 1B). *P. frisia* SH6-1 did not respond to further blooms of phytoflagellates in June and of diatoms in July. Unfortunately, information on the detailed composition of the phytoplankton during this time is not available.

The abundance of *P. frisia* SH6-1 was further assessed at Helgoland Roads from February to August 2005 showing its presence from May to July with a pronounced peak in mid-June after a *Phaeocystis* bloom but without any obvious covariation with total phytoplankton cell numbers (Fig. 1C). *P. frisia* SH6-1 was mainly found in the FL bacterial fraction but was also detected particle-associated (PA; >3 µm) after the break down of the *Phaeocystis* bloom. A *Roseobacter*-specific DGGE analysis confirmed the presence of *P. frisia* SH6-1 in the FL and the PA bacterial fraction (Fig. S2).

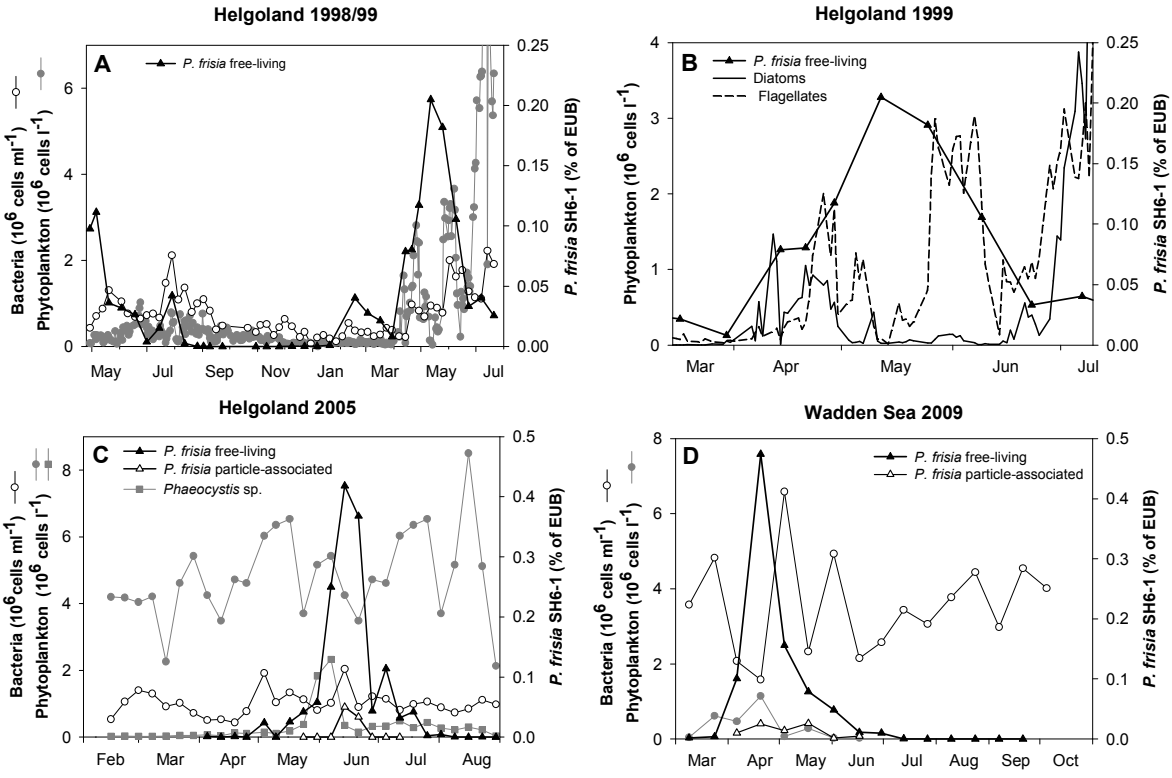

Figure 1: Abundance of *P. frisia* SH6-1 and total abundance of bacteria and phytoplankton in the North Sea at Helgoland Roads from May 1998 to July 1999 (A), from March to July 1999 (B), from February to August 2005 (C) and in the Wadden Sea from March to October 2009 (D).

In the Wadden Sea data set of 2009 *P. frisia* SH6-1 occurred from April to June, predominantly in the FL bacterial fraction, with highest abundance in the end of April (Fig. 1D). The increase of FL *P. frisia* SH6-1 cells was preceded by a small phytoplankton peak, mostly composed of *Chaetoceros* spp. and unidentified centric diatoms. The main peak of *P. frisia* SH6-1 coincided with the main peak of total phytoplankton, constituted mostly of *Phaeocystis* sp. and unidentified small (3 - 5 μm) flagellates (Figs. 1D, S3). Dynamics of total bacterial cells neither covaried with phytoplankton cell numbers nor with abundance of *P. frisia* SH6-1. From the beginning of April to mid-June a small proportion of *P. frisia* SH6-1 was particle-associated. Enumeration by CARD-FISH confirmed the proportions of *P. frisia* SH6-1 assessed by qPCR, yielding 0.89% ± 0.25 of total *Bacteria* in the sample of 20 April (including FL and PA cells).

Utilization of algal-derived organic matter and carbohydrates

In order to better understand the distinct seasonal patterns of *P. frisia* SH6-1 we carried out growth tests with axenic phytoplankton species with which this organism appeared to exhibit positive or negative interactions, *Phaeocystis globosa* and *Leptocylindrus danicus*, and with specific model substrates, i.e. mono- and polysaccharides. From previous studies we knew that *P. frisia* SH6-1 shows a high preference for sugars against amino acids as it grows on 12 mono- and disaccharides but only on 7 amino acids as a sole carbon source (Hahnke *et al*., 2011).

After addition of *P. frisia* SH6-1 to axenic cultures of stationary phase *Phaeocystis* cells, the bacterial cells immediately started growing and reached the stationary phase at day 9 (Fig. 2A). During the same period, algal cell numbers decreased but reached a second peak on day 16 before they reached baseline values on day 26. In contrast, numbers of axenic *Phaeocystis* remained significantly higher than those in the treatment with *P. frisia* SH6-1 from day 9 until day 21 (P=0.023; t-test), the stationary phase of bacterial growth. At the start of the experiment concentrations of dissolved organic carbon (DOC) in the *Phaeocystis* cultures were ~12 mM and ~2 mM higher than in the controls with only the medium in which *Phaeocystis* was grown and which contains natural seawater and submillimolar concentrations of TRIS, EDTA and glycerophosphate (Fig. 2B). In the axenic algal cultures, DOC concentrations steadily increased until the late phase of the experiment. In contrast, in the treatment with added *P. frisia* SH6-1, DOC concentrations remained nearly constant until day 16 in the stationary phase and were significantly lower

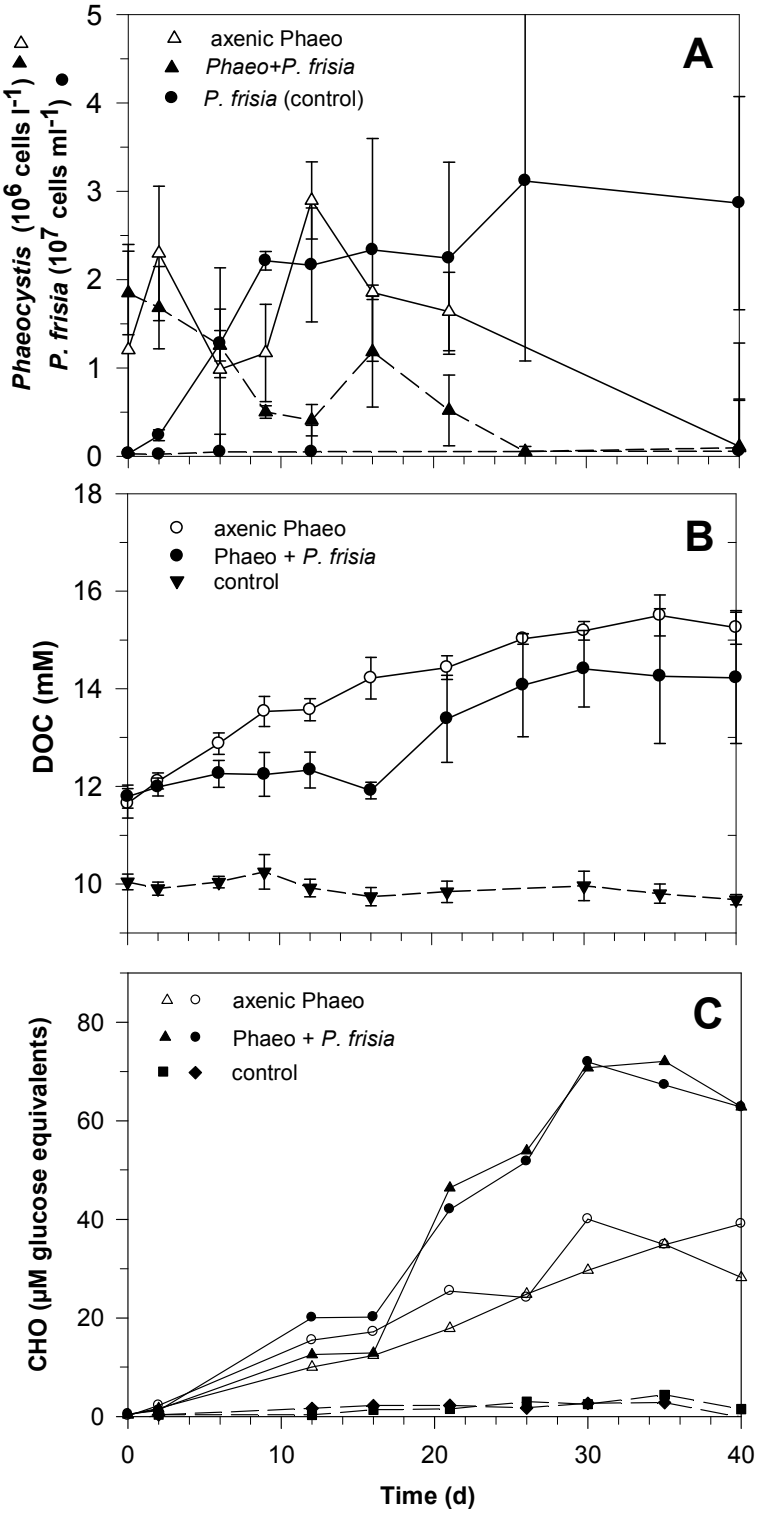

Figure 2: Growth experiment of *P. frisia* SH6-1 with *Phaeocystis globosa* in treatments of axenic *Phaeocystis*, cultures of *Phaeocystis* and *P. frisia* and a control of *P. frisia* in the algal medium. At the start *Phaeocystis* was in the stationary growth phase. A: Abundance of *P. frisia* SH6-1 and *P. globosa* over time; B: DOC concentrations; C: dissolved free reducing sugars. Data in A and B are mean values of triplicates ± standard deviation. Reducing sugars are only available for duplicates because the third replicate was used to measure the concentrations of total dissolved neutral monosaccharides (Fig. 3).

(*P*=0.016; t-test) than in the axenic cultures from day 2 until day 16. DOC concentrations substantially increased from day 16 to 21, simultaneously with the break down of the second peak of the *Phaeocystis* numbers. In the control assays without algal cells but addition of bacteria, no significant changes in DOC concentrations and cell numbers were detected (Fig. 2A, B), indicating that cells of *P. frisia* SH6-1 were not able to consume the DOC of the natural seawater, EDTA, TRIS or glycerophosphate, constituting the organic components of the *Phaeocystis* medium.

Free reducing sugars were not detected in any of the assays at the start of the experiment but only a few days later and increased constantly in both, the axenic and the cultures with addition of *P. frisia* SH6-1. In the late stationary phase, the concentrations of free reducing sugars increased much stronger in the non-axenic cultures as compared to the axenic ones (Fig. 2C), suggesting hydrolysis of polysaccharides by the added bacteria. In the control with the algal medium and bacteria the concentration of reducing sugars was much lower than in the assays with algae (Fig. 2C). During the course of the experiment, however, it increased slightly, indicating that hydrolysis of polysaccharides occurred, presumably originating from the seawater used to prepare the medium. Glucose and galactose, and to a lesser extent rhamnose, were the main dissolved free neutral monosaccharides (DFNCHO) released by *Phaeocystis* cells, primarily during senescence (Fig. 3A). Net release in the non-axenic assays was lower than in the axenic ones, indicating that during the break down of *Phaeocystis* these monosaccharides were consumed by bacteria, like rhamnose which was below the detection limit. The occurrence of arabinose and mannose suggested that some hydrolysis of polysaccharides also occurred, as reflected by the occurrence of these monosaccharides in the axenic culture (Fig. 3A, B). Concentrations of total dissolved neutral monosaccharides (TDNCHO) were one to two orders of magnitude higher than those of DFNCHO.

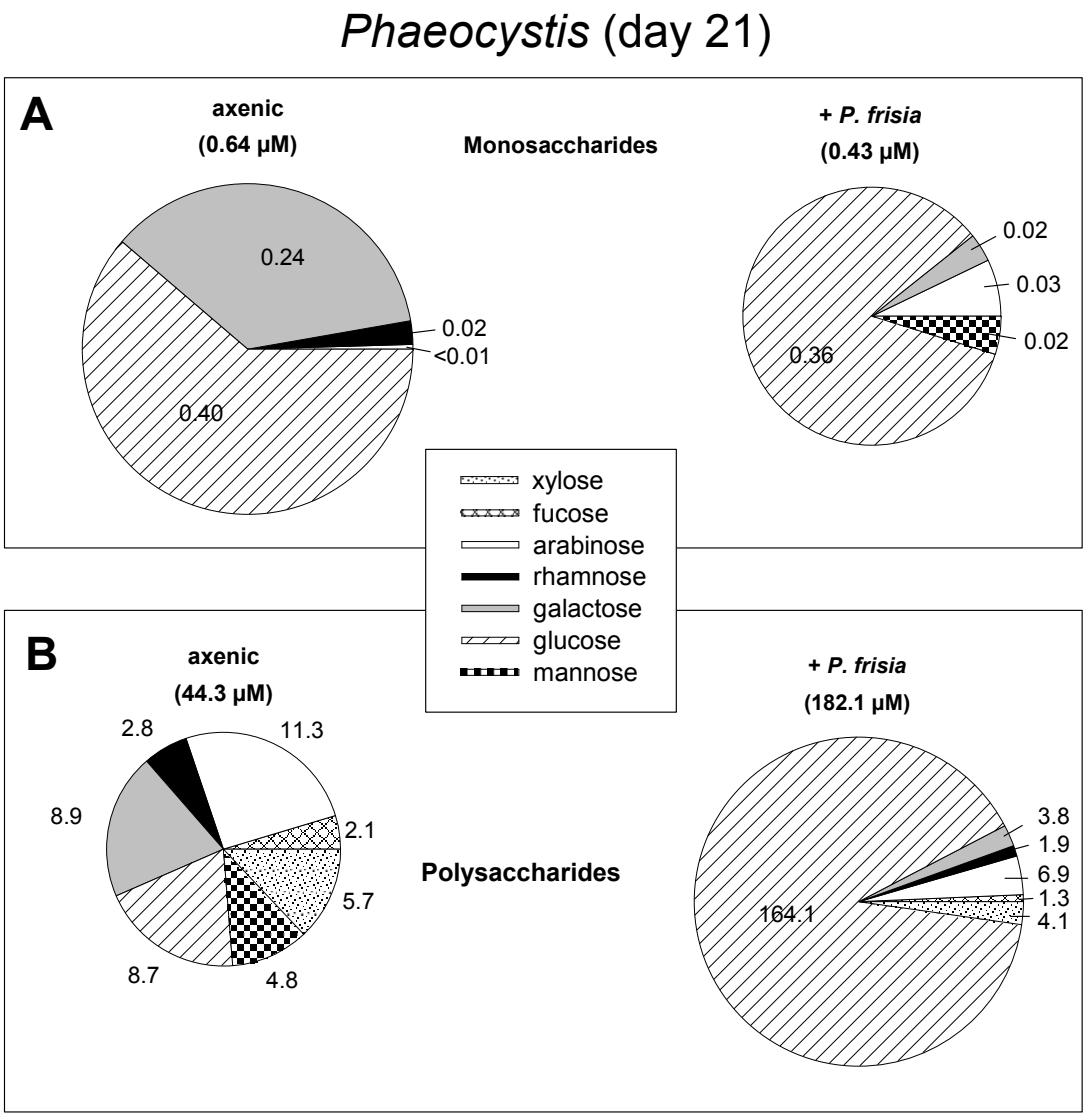

Figure 3: A: Dissolved free neutral monosaccharides (DFNCHO) and B: total dissolved neutral monosaccharides (TDNCHO) at day 21 of the experiment in the *Phaeocystis* cultures, axenic and with addition of *P. frisia*. DFNCHO are given as means of duplicates and TDNCHO as single values of the third replicate.

In the axenic algal cultures, arabinose, galactose and glucose were the main constituents, followed by xylose, mannose, rhamnose, and fucose (Fig. 3B). Concentrations reached 44 µM on day 21 and further increased to 112 µM until day 40 without a change in composition. In the cultures with the addition of *P. frisia* SH6-1, TDNCHO exhibited much higher concentrations than in the axenic cultures and glucose greatly dominated. The concentrations of the other sugars were reduced as compared to the axenic cultures (Fig. 3B), indicating that the bacteria metabolized them, presumably by transforming them into glucose which was released in a polymeric form. In addition, the bacteria obviously

hydrolyzed the particulate polysaccharides of the *Phaeocystis* cells as shown by epifluorescence microscopical observations that the single *Phaeocystis* cells were colonized by *P. frisia* SH6-1 (data not shown). Hence, a polysaccharide, composed mainly of glucose, was secreted by *P. frisia* SH6-1, presumably because the bacteria were strongly nitrogen limited. In the control containing bacteria and no algae, only very low concentrations of glucose (~2 µM) and arabinose (~0.5 µM) were detected (not shown).

In the experiment with *Leptocylindrus danicus* growth patterns of axenic and cultures with added *P. frisia* cells differed much less than in the *Phaeocystis* experiment. In the later phase on days 19 and 37 algal cell numbers of both treatments were not significantly different (Fig. 4A). Cell numbers of *P. frisia* continuously increased from the beginning, but numbers remained almost one order of magnitude lower as compared to the *Phaeocystis* experiment. As growth in the replicates differed substantially the standard deviation was large. No growth of *P. frisia* occurred in the control with the *Leptocylindrus* medium only. DOC concentrations were more than one order of magnitude lower than in the *Phaeocystis* experiment as the *Leptocylindrus* medium did not contain any organic ingredients except natural seawater. Differences between the axenic cultures and the treatments with the added *P. frisia* did not exceed 0.5 mM, and were not significantly different (Fig. 4B). In the control without algal cells DOC concentrations remained constant. Concentrations of free reducing sugars increased over the course of the experiment but were not significantly different between both treatments (Fig. 4C). The composition of DFNCHO released by *Leptocylindrus* was completely different compared to DFNCHO in the *Phaeocystis* cultures. The main constituents were xylose and fucose (Fig. 5), which were not detected in the *Phaeocystis* cultures. Glucose, galactose, rhamnose and arabinose were only present in concentrations <0.2 µM. Furthermore, the proportions of individual DFNCHO in the axenic treatments and in those with added *P. frisia* SH6-1 showed only small differences, while they greatly differed in the respective *Phaeocystis* treatments. Total concentrations of DFNCHO in the non-axenic cultures were lower than in the axenic ones, indicating that most of these sugars and mainly xylose were utilized by the bacteria. Unfortunately, concentrations of TDNCHO are not available for this experiment.

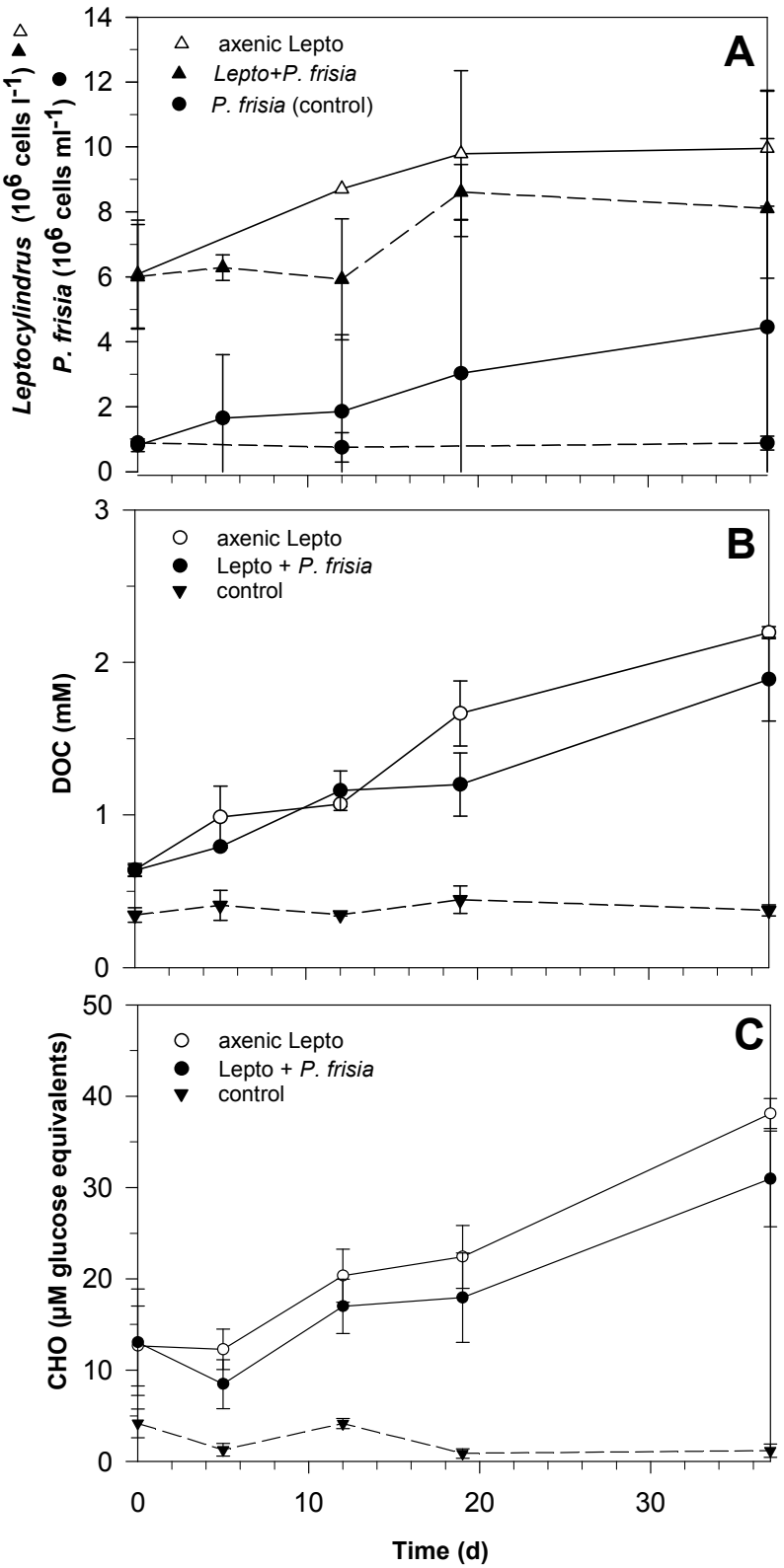

Figure 4: Growth experiment of *P. frisia* SH6-1 with *Leptocylindrus danicus* in treatments of axenic *Leptocylindrus*, cultures of *Leptocylindrus* and *P. frisia* and a control of *P. frisia* in the algal medium. At the start *Leptocylindrus* was in the late exponential growth phase. A: Abundance of *P. frisia* SH6-1 and *L. danicus* over time; B: DOC concentrations; C: dissolved free reducing sugars. Given are mean values of triplicates ± standard deviation.

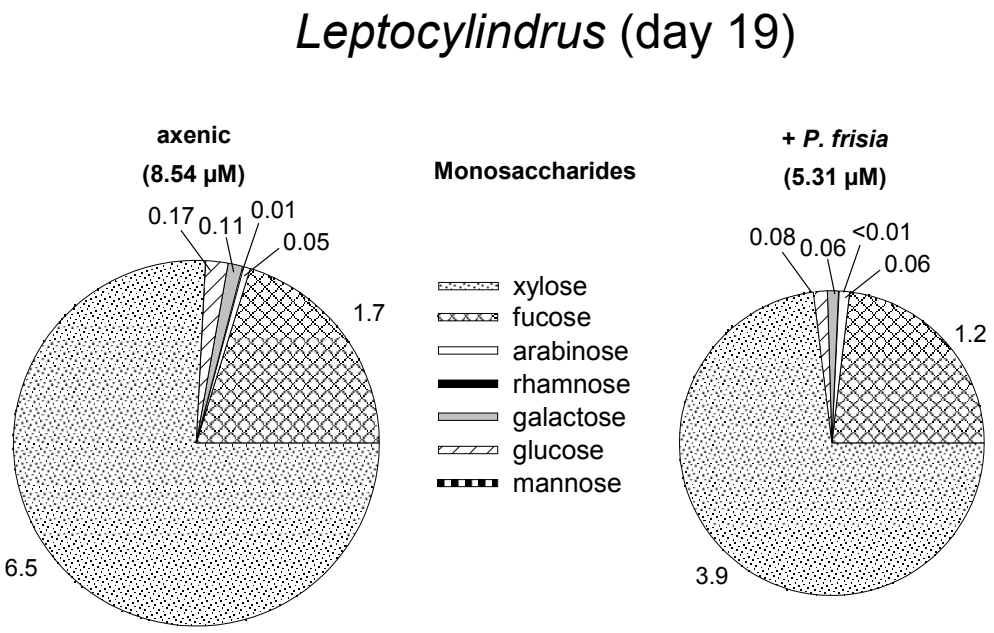

Figure 5: Dissolved free neutral monosaccharides (DFNCHO) at day 19 of the experiment in the *Leptocylindrus* cultures, axenic and with addition of *P. frisia*. Given are mean values of triplicates ± standard deviation.

Utilization of polysaccharides

We tested whether *P. frisia* SH6-1 was able to grow directly on the main storage polysaccharide of *Phaeocystis*, laminarin and to hydrolyse chitin and cellulose, structural polysaccharides of *Phaeocystis* and other microalgae (Alderkamp *et al*., 2007). The results showed that this strain does not grow on laminarin (Fig. 6), thus confirming previous observations (Hahnke *et al*., 2011). However, when laminarinase was added, *P. frisia* did grow on laminarin, obviously on the expense of the cleaved monomer, glucose, as high concentrations of glucose were detected in this assay. In the control assays containing *P. frisia* and laminarin or laminarinase only, no growth was detected. In the assays with laminarinase only OD increased slightly which was presumably due to a slight change in color as the enzyme solution showed a brown color that became more intense over time.

When *P. frisia* SH6-1 grew in pure culture in artificial seawater medium (ASW) and yeast extract it did not exhibit any activity of N-acetyl-glucosaminidase or beta-glucosidase. However, when this strain grew in the presence of exudates of the diatom *Thalassiosira rotula*, beta-glucosidase and N-acetyl-glucosaminidase activities were detected after five and eight days, respectively (data not shown). Growth of *P. frisia* SH6-1 was enhanced in the presence of these exudates relative to the control without exudates.

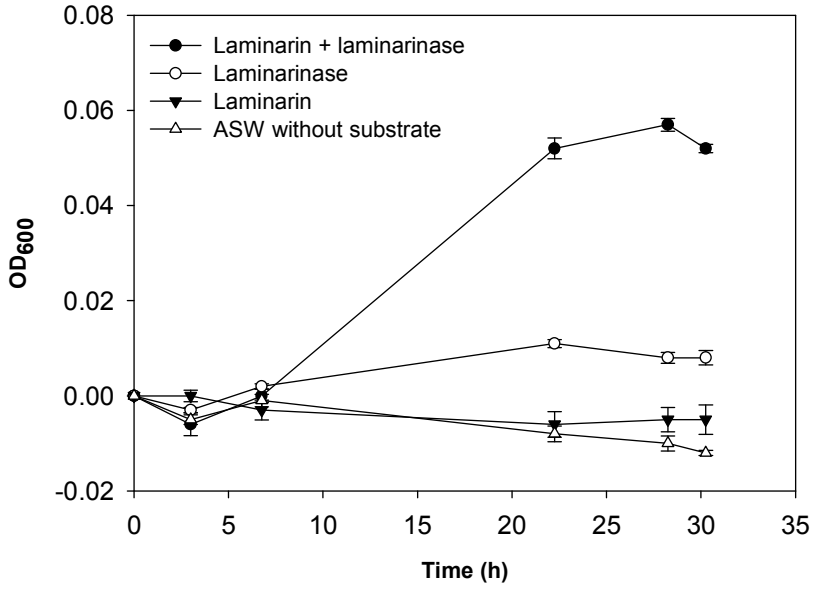

Figure 6: Growth of *P. frisia* SH6-1 in ASW medium and the addition of laminarin and laminarin+laminarinase and controls with laminarinase only and no laminarin addition. Growth of *P. frisia* was measured by optical density (OD) at 600 nm. The ASW medium did not contain any other organic carbon source. Mean ± standard deviation of five replicates are given.

Discussion

Our results show that *P. frisia* SH6-1 exhibits very pronounced seasonal patterns with distinct short term maxima during phytoplankton blooms and their break down phases in the first half of the year in the southern North Sea. A survey in May and September revealed that these seasonal patterns are obviously not restricted to the German Bight but occur also throughout the North Sea up to the Norwegian coast. In the German Bight, these recurrent patterns were found in different years and different locations. Highest abundances of *P. frisia* SH6-1 at the maxima were 0.2 to 0.5% of total bacterial 16S rRNA genes as assessed by qPCR and 0.9% as assessed by CARD-FISH. These numbers may appear low as compared to quantitative data on the occurrence of other phylogenetic lineages in the bacterioplankton (Brown *et al*., 2005; Sapp *et al*., 2007a; Giebel *et al*. 2011; Mayali *et al*., 2011; Carlson *et al*., 2009; Teira *et al*., 2009; Gomez-Pereira *et al*., 2010). One has to keep in mind that in most cases, in particular when applying FISH or CARD-FISH, the target lineages are wider than a narrow population of a type strain and most closely related ecotypes, like in the case of *P. frisia* SH6-1. Hence, our very specific quantitative data on the occurrence of this type strain, based on qPCR and targeting the genomic intergenic spacer (ITS) region, are not that low and add new information at higher

resolution on individual bacterioplankton populations. Our results indicate that the population of this type strain of the newly described species *P. frisia* (Hahnke *et al*., 2011) is a regularly occurring member of the bacterioplankton in the North Sea. It has a specific, but still unknown function during phytoplankton blooms and their break down in the first half of the growing season in the southern North Sea.

Several studies exist which showed that distinct bacterial phylotypes or phylogenetic clusters exhibit pronounced temporal changes which are related to biotic and/or abiotic parameters such as phytoplankton abundance, composition, nutrients or flagellate abundance (Brown *et al*., 2005; Sapp *et al*., 2007a; Mayali *et al*., 2011; Steele *et al*., 2011). Even though such studies provide detailed insight into the co-occurrence of distinct bacterial taxa and co-variations with other parameters, they do not allow explaining these correlations on a mechanistic or even deterministic level. For such explanations, experimental approaches with the bacterial community or with individual members are necessary, looking for the physiological traits, interactions with other bacteria, phytoplankton species and other parameters controlling their growth. Previous studies based on such approaches showed that individual phytoplankton species do exhibit distinct positive or negative interactions with bacterial species and harbor distinct bacterial communities (Grossart 1999; Grossart *et al*., 2005; Rooney-Varga *et al*., 2005; Sapp *et al*., 2007b; Grossart and Simon, 2007; Mayali *et al.*, 2008). However, so far, most of these studies were carried out with model bacteria and selected phytoplankton species, not considering their relevance *in situ*. The main reason is the fact that so far only few bacteria which are abundant members of the heterotrophic bacterioplankton are available as isolates, i.e. Cand. *Pelagibacter ubique* (Rappé *et al*., 2002), *Congregibacter litoralis* strain KT71 (Fuchs *et al*., 2007) and Cand. *Planktomarina temperata* (Giebel *et al*., 2011). We were able to isolate a regular member of the North Sea bacterioplankton, *P. frisia* SH6-1, affiliated to the *Roseobacter* clade, from a 10^{-6} dilution culture of a sample from the German Bight. This notion indicates that the isolate was present at a rather high abundance which was later substantiated by our qPCR and FISH assays. Hence, a model organism was available for detailed studies to examine potential controls of its growth and seasonal occurrence *in situ*.

A previous study showed that only 7 of 20 amino acids, but 7 of 12 monosaccharides and all 5 disaccharides tested supported growth of *P. frisia* SH6-1 (Hahnke *et al*., 2011). Fucose did not support growth and xylose only weakly. The polysaccharides xylan, laminarin, starch and inulin were not consumed either. Hence, these

tests showed that *P. frisia* SH6-1 has very distinct substrate requirements and in general has a broader potential for using carbohydrates than amino acids. The results of the present study complement these findings by showing that the presence of exudates of *Thalassiosira rotula* induces N-acetyl glucosaminidase and beta-glucosidase activity in *P. frisia* SH6-1, implying that chitin and cellulose can be hydrolyzed and used as substrate by this bacterium. These traits are in contrast to those of other characterized members of the *Roseobacter* clade isolated from the North Sea which have a much higher preference for amino acids than for carbohydrates (Wagner-Döbler *et al*., 2004; S. Hahnke *et al*., unpubl. results).

These distinct traits may explain the different responses of *P. frisia* SH6-1 when grown in the presence of *Phaeocystis globosa* and *Leptocylindrus danicus*. The growth yield of *P. frisia* SH6-1 was an order of magnitude higher in the presence of *Phaeocystis* as compared to *Leptocylindrus*. *Planktotalea frisia* SH6-1 was able to break down the *Phaeocystis* cells, as shown by the reduced cell numbers and the lower DOC concentrations relative to the axenic *Phaeocystis* culture and by bacterial cells attached to *Phaeocystis* cells, Growth of *Leptocylindrus*, however, was unaffected by the presence of *P. frisia* SH6-1. Obviously, this bacterium was unable to degrade *Leptocylindrus* over a period of 40 days.

One explanation for these different responses may be the composition of the dissolved carbohydrates of both algal cultures which were strikingly different. DFNCHO of *Phaeocystis* were dominated by glucose and galactose but those of *Leptocylindrus* by xylose and fucose. TDNCHO of *Phaeocystis* were composed of seven different monosaccharides with arabinose, galactose and glucose as the dominant components. N-acetyl glucosamine, the monomeric building block of chitin, is not detected by the HPLC analysis and pulsed amperometric detection (PAD) detection and completely removed by the desalting pretreatment of the samples by ion exchange chromatography we applied. It is known, however, that *Phaeocystis* contains chitin in its flagellated cells (Alderkamp *et al*., 2007). The reduction of the concentration of all monosaccharides detected, except glucose, in the TDNCHO and DFNCHO pools by *P. frisia* SH6-1 in the *Phaeocystis* cultures after 21 days indicates that this bacterium was able to hydrolyze the polysaccharides and to consume the cleaved monosaccharides. Possibly, another mechanism for polysaccharide hydrolysis of the *Phaeocystis* cells were autolytic enzymes of the decaying algal cells. These patterns of hydrolysis and uptake of polysaccharides and DFNCHO, except for arabinose, are in line with utilization patterns of monosaccharides by

P. frisia SH6-1 reported in a previous study (Hahnke *et al*., 2011) and with our observation of the induction of beta-glucosidase and N-acetyl glucosaminidase activities by algal exudates. The findings of hydrolytic enzyme induction are in line with observations by Grossart (1999) that the presence of single algal species greatly enhanced beta-glucosidase and aminopeptidase activities of individual bacterial strains. Arabinose did not support growth of *P. frisia* SH6-1 as a single carbon source in the study by Hahnke *et al*. (2011), but our study showed that its concentration was reduced in the polysaccharide pool released by *Phaeocystis* when *P. frisia* SH6-1 was present. Possibly, arabinose can not serve as a single carbon source for this bacterium but is co-metabolized with another monosaccharide. A striking result of the growth experiments with *P. frisia* SH6-1 and *Phaeocystis* was that the concentration of TDNCHO increased by >4-fold and the concentration of free reducing sugars twofold, relative to the axenic algal cultures, and that glucose dominated this pool by 90%. Obviously, *P. frisia* SH6-1 secreted large amounts of polymeric glucose, presumably because of N-limitation. Most of this glucose presumably originated in the glucose- and N-acetyl glucosamine-dominated pool of particulate polysaccharides. As shown by the reduced concentrations of other monosaccharides in the TDNCHO and DFNCHO pools after 21 days in the culture with added bacteria relative to the axenic *Phaeocystis* culture, they must have been hydrolyzed from the pool of dissolved and particulate polysaccharides, converted to glucose after uptake, polymerized and secreted. Even though nitrogen limitation for growth of *P. frisia* SH6-1 and other bacteria may also occur at ambient conditions, we assume that such high net exudation of polymeric glucose, i.e. mucopolysaccharides, by *P. frisia* SH6-1 is untypical and a rare event at ambient conditions.

Our results show that *P. frisia* SH6-1 can greatly accelerate the break down of a *Phaeocystis* culture in the stationary phase. As in our experiment *Phaeocystis* grew as single cells and not as colonies we do not know whether *P. frisia* SH6-1 is also able to degrade the mucus material of *Phaeocystis* colonies which consists of glucan and heteropolysaccharides with beta-glycosidic bonds (Alderkamp *et al*., 2007). The fact that the *Thalassiosira* exudates induced beta-glucosidase and N-acetyl glucosaminidase activities is a strong indication that *P. frisia* SH6-1 is able to at least partly degrade this mucus material. Thus, growth of this bacterium is favored by *Phaeocystis* and *T. rotula* and presumably by other bacteria which exude DOM of a similar quality.

In contrast to *Phaeocystis*, *Leptocylindrus* was little affected by the presence of *P. frisia* SH6-1 and growth of this bacterium was an order of magnitude lower. Xylose, the

dominant DFNCHO released by *Leptocylindrus*, was consumed by *P. frisia* SH6-1 to a certain extent, but fucose, dissolved free reducing sugars and total DOC only marginally. Unfortunately, the composition of TDNCHO of *Leptocylindrus* and data on their decomposition by *P. frisia* SH6-1 are not available. However, the other data suggest that polysaccharides were only little decomposed by this bacterium. We assume that the different structure and composition of the dissolved and particulate polysaccharides of *Leptocylindrus* as compared to *Phaeocystis* prevented hydrolysis by *P. frisia* SH6-1. Our observations indicate that *Leptocylindrus* does not provide favorable growth conditions to *P. frisia* SH6-1.

The findings of the different utilization patterns of DFNCHO and TDNCHO and DOC of *P. frisia* SH6-1 when grown in the presence of these algal cultures and the information on substrate utilization patterns of this bacterium by Hahnke *et al*. (2011) provide some clues to better understand its seasonal occurrence in the North Sea. *P. frisia* SH6-1 obviously takes advantage of situations when large amounts of sugars such as galactose, but to a lesser extent glucose, are available. In addition, chitin and polysaccharides with beta-glycosidic bonds are also hydrolyzed and consumed. These situations occur in the first half of the year and typical indicators appear to be *Phaeocystis* and *T. rotula*. The rapid rise of *P. frisia* SH6-1 populations indicates that such situations occur within short periods of time. Our data on the direct colonization of *Phaeocystis* cells by *P. frisia* SH6-1 suggest that this bacterium actively contributes to decomposing blooms of this alga such as in June 2005 at Helgoland Roads (Fig. 1C). The increasing abundance of *P. frisia* SH6-1 in April and May 1999 at Helgoland Roads during and after the break down of the diatom-dominated spring bloom (Fig. 1B) is another indication of the functional response of this bacterium to favorable ecological conditions. The rapid decline of the *P. frisia* populations may indicate a specific phage infection as has been observed for certain *Flavobacteria* (Holmfeldt *et al*., 2007). The observation of different types of beta-glucosidases over the course of a *Phaeocystis* bloom in the North Sea is a good indication that physiologically different bacteria occur in the course of the bloom (Arrieta and Herndl, 2002) and supports the view of the dynamic occurrence of individual bacterial populations such as *P. frisia* SH6-1.

In the second half of the year, favorable growth conditions for *P. frisia* SH6-1 apparently do not prevail, presumably because digestable carbohydrates are not available any more. Obviously, the phytoplankton produces carbohydrates of a different composition which can not be digested by *P. frisia* SH6-1, as exemplified by *Leptocylindrus danicus*

and the strikingly different composition of the exuded DFNCHO. *P. frisia* SH6-1 obviously can not or only little consume these monosaccharides and hydrolyze such polysaccharides. It may depend on their hydrolysis by other bacteria such as *Flavobacteria* and *Sphingobacteria* (Cottrell and Kirchman, 1999). As *P. frisia* has a rather limited potential to utilize amino acids (Hahnke *et al*., 2011) switching to explore this class of substrates may be of limited use. Because of the low growth rates of *P. frisia* SH6-1 under these conditions this bacterium presumably is outcompeted by other bacteria which are better adapted to these conditions. During the second half of the year, *P. frisia* SH6-1 has to be considered as a member of the rare biosphere whereas in the first half it often qualifies as a member of the abundant biosphere (Pedrós-Alió, 2006; Sogin *et al*., 2006). Hence, these data provide further evidence that a bacterium is not *per se* a member of either biosphere but has a dynamic occurrence which depends on the actual situation of its ecological niche.

Other studies already have shown specific positive or negative interactions of individual bacteria with phytoplankton algae (Grossart, 1999; Grossart *et al*., 2005; Grossart and Simon, 2007, Mayali *et al*., 2008; Sher *et al.*, 2011). Mayali and coworkers showed that an isolate of the *Roseobacter* Clade Affiliated (RCA) cluster can cause the break down of a bloom of the dinoflagellate *Lingulodinium polyedrum*, presumably by ectohydrolytic activities of bacterial cells attached to the alga. This parasitic behavior appears to occur also *in situ* (Mayali *et al.*, 2011). Our results add to the findings of these studies by showing that growth of a single bacterium is largely dependent on the exudation of suitable carbohydrates by a given alga or phytoplankton community, thus shedding some light on the conditions of the occurrence of this bacterium in the North Sea.

The occurrence of *P. frisia* is not restricted to the North Sea. 16S rRNA gene sequences with a similarity of ≥99% to that of *P. frisia* SH6-1 were also found in other seas such as the Adriatic Sea and the North East Pacific (Table S1). Whether substrate preferences and growth properties of these members of the species *P. frisia* is similar to the type strain *P. frisia* SH6-1 remains to be tested.

Experimental Procedures

Study areas and sampling

The seasonal occurrence of *P. frisia* SH6-1 was studied at two distinctly different locations in the German Bight, a pelagic off shore station at Helgoland Roads (54°18'N, 07°90'E) and a near shore station in the German Wadden Sea at Neuharlingersiel (53°42'N, 07°42'E). At Helgoland Roads, samples were collected weekly from 1 m depth between May 1998 and July 1999 and from February to August 2005. At Neuharlingersiel, samples were collected at the surface biweekly between March and October 2009. PA bacteria at Helgoland Roads were collected from 1 l samples on 3 µm polycarbonate filters (47 mm diameter) and at Neuharlingersiel from 100 ml samples on 5 µm polycarbonate filters. FL bacteria at both sites were collected on 0.2 µm polycarbonate filters. The 5 µm filters for collecting PA bacteria in the Neuharlingersiel samples were used to avoid clogging because of the high load of suspended particulate matter (SPM). Filters were stored at -80°C until further processing.

The occurrence of *P. frisia* in the south- and northeastern North Sea between the Weser estuary and the Norwegian coast was assessed during two cruises with RV Heincke at 13 and 15 stations in September 2005 and May 2006, respectively. For the exact location of stations see Fig. S1 and Giebel *et al*. (2011). Samples were taken by Niskin bottles mounted on a CTD rosette (Hydrobios) between 1 and 90 m depth. Bacterioplankton was concentrated by filtering 0.5 - 1.0 l of seawater onto a 0.2 µm polycarbonate filters (47 mm diameter). At coastal stations with high concentrations of SPM and chlorophyll *a*, samples were prefiltered through 5 µm polycarbonate filters to also collect PA bacteria. Filters with the bacterial biomass were transferred into sterile Eppendorf tubes and stored at -80°C until further processing.

Nucleic acid extraction

Samples of Helgoland Roads: DNA of PA and FL bacteria was extracted from filter pieces as described by Sapp *et al*. (2007a). Briefly cell lysis was performed by adding lysozyme (1 mg ml^{-1}) and sodium dodecyl sulfate (1%). DNA extraction was carried out using phenol/chloroform/isoamyl (PCI) alcohol (25:24:1). After precipitation of the DNA with isopropanol, all DNA extracts were eluted in sterile water and stored at -20°C until further analyses.

DNA of the samples collected at Neuharlingersiel and during the two cruises was extracted with PCI, sodium-dodecyl-sulfate (SDS) and 500 mg per 1.5 ml circonium beads according to Selje and Simon (2003). Precipitation was done at -80°C for 1 h using isopropanol (Rink *et al.*, 2007). The DNA was resuspended in molecular grade water (Eppendorf) and stored at -20°C until further processing.

Phytoplankton composition

Samples from Neuharlingersiel were preserved with 1% Lugols' solution. Twent five ml samples were prepared for enumeration by inverted microscopy according to Utermöhl (1958). Phytoplankton data from Helgoland Roads were provided by K. Wiltshire (see Wiltshire *et al.*, 2008).

Design of P. frisia SH6-1^T specific oligonucleotides for qualitative PCR and FISH

Specific primers and probes were designed for *P. frisia* SH6-1^T (FJ882052) and the *Rhodobacteraceae* bacterium DG1250 (DQ486490), which shows 100% sequence similarity to strain SH6-1^T, using the ProbeDesign function of the ARB software package (http://www.arb-home.de). Specificity of the oligonucleotide sequences was checked with the NCBI and RDP databases (http://www.ncbi.nlm.nih.gov/, http://rdp.cme.msu.edu/html/). Sequences of specific primers are SH6-1-67f (5' - CGC TTA CCT TCG GGT AGG - 3') and SH6-1-1019r (5' - CCA GCC TAG CTG AAA GCT C - 3'). PCR was performed at the following conditions: 95°C for 3 min, followed by 34 cycles of 95°C for 1 min, 63°C for 1 min, and 72°C for 3 min, with a final extension step at 72°C for 10 min. For FISH analyses probes SH6-1-67 (5' - CCT ACC CGA AGG TAA GCG - 3') and SH6-1-578 (5' - GAC TAA TCC GCC TAC GTG - 3') were used in combination as there was no single probe with sufficient specificity.

Quantification of P. frisia SH6-1^T using quantitative PCR

A specific quantitative (q)PCR approach for *P. frisia* SH6-1^T was developed targeting the intergenic spacer (ITS) region as there were no suitable primers found on the 16S rRNA gene sequence. For primer design the ITS sequence was aligned to the ITS sequence of strain HTCC2083 (the nearest relative of *P. frisia* SH6-1^T, showing 99.1% identity based on the 16S rRNA sequence). Specificity of potential primer sequences was checked with the NCBI and RDP databases (http://www.ncbi.nlm.nih.gov/, http://rdp.cme.msu.edu/html/). The primers SH6-1-ITSf (5' - GGT CAG ACT AGC TTG

CTA G - 3') and SH6-1-ITSr (5' - GGT TAG CTC GAC GCA TGT T - 3') did not result in any other bacterial hits.

qPCR was performed as follows: 10 µl template (dilution of the environmental DNA extract) were added to 15 µl of the PCR mastermix containing 10 pmol of each primer and 12.5 µl of a qPCR kit (DyNamo™ HS SYBR® Green qPCR Kit, Finnzyme). Amplification was performed with a Rotorgene 3000 thermocycler (Corbett Research) at the following parameters: 95°C for 15 min, followed by 50 cycles of 94°C for 10 sec, 62°C for 15 sec, 72°C for 15 sec, 79°C for 20 sec, and 80°C for 20 sec (fluorescence signals for the final analysis were measured at 80°C). A subsequent extension step was carried out at 50°C for 2 min. In each run every sample including standards were set up in triplicates. Data were analyzed using the Rotorgene software package 6.0 supplied by Corbett Research. As standards a PCR fragment from the 16S rRNA to the beginning of the 23S rRNA gene of strain SH6-1 was amplified using the universal primer set 341f (Muyzer *et al.*, 1995) and 23S-189r (Hunt *et al*., 2006). PCR was performed at the following conditions: 95°C for 3 min, followed by 30 cycles of 95°C for 1 min, 46°C for 1 min, and 72°C for 3 min, with a final extension step at 72°C for 10 min. The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen) and the DNA concentration was quantified fluorometrically with PicoGreen (Martens-Habbena *et al*., 2006). For each qPCR run a separate standard curve was generated with 10-fold dilutions of standard DNA within the range of the sample concentrations. Quantification of *Bacteria*-specific 16S rRNA genes was performed according to Beckmann *et al*., (2011) using the primer set 519f (Lane, 1991) and 907r (Muyzer *et al.*, 1995). The abundance of strain SH6-1 was determined as the ratio of strain specific ITS genes over total bacterial 16S rRNA genes.

Quantification of P. frisia SH6-1^{T} *by CARD-FISH*

Samples were fixed for 1 h with formaldehyde (2% final concentration) and filtered onto white 0.2 µm polycarbonate filters (47 mm). Filters were once rinsed with 1 ml of phosphate-buffered saline (PBS 1x) and with 10 - 20 ml distilled water and stored until further processing at -20°C. CARD-FISH was performed according to Sekar *et al.* (2003). Since one single probe was found to be not specific enough, CARD-FISH was carried out by performing a double hybridization using the probes SH6-1-67 (5' - CCT ACC CGA AGG TAA GCG - 3') and SH6-1-578 (5' - GAC TAA TCC GCC TAC GTG - 3'). Hybridization was done for 2 h at 35°C and subsequent washing for 30 min at 37°C. First, hybridization with probe SH6-1-67 was carried out using 50% formamide and 28 mM

NaCl for washing. Per 400 µl of hybridization buffer, 15 µl of the horseradish peroxidase (HRP) probe (50 ng μl^{-1}) was used. Signal amplification was performed at 37°C using tyramine-HCl labeled with cyanine dye (Cy3; Pernthaler *et al.,* 2002). To avoid unspecific accumulation of dye in the cells, the last washing step in PBS (1x) amended with TritonX-100 (0.05%) was extended to 30 min. To inactivate peroxidase, filters were incubated at 55°C in 10 mM HCl for 20 min and subsequently washed twice in distilled water. The second hybridization with probe SH6-1-578 was performed using 70% formamide and 7 mM NaCl for washing and further conditions were as described for probe SH6-1-67. Signal amplification was carried out with fluorescein-5-isothiocyanate (FITC) labeled tyramine. Counterstaining was performed with Vectashield-mounting medium with DAPI (1.5 mg ml^{-1}; Vector Laboratories). Filter sections were analyzed with an epifluorescence microscope (Axioskop, Zeiss) equipped with filter sets LP 420, BP 505 - 530, and BP 575 - 640 for viewing DAPI, FITC, and Cy3, respectively. DAPI stained cells with both, positive FITC and positive Cy3 signals, were accounted for target organisms. For each probe 20 view fields (each with ca. 100 DAPI cells) from three filter sections of three filters were counted.

Phytoplankton strains and cultivation conditions

Cultivation of axenic *Phaeocystis globosa* strain Ph-91 mf (obtained from the Royal Netherlands Institute for Sea Research (NIOZ)) was carried out at 15°C in a light-dark cycle of 16:8 h in a 1:1 mixture of f/2 medium (Guillard, 1975; without silicate) and enriched artificial seawater medium ESAW (Harrison *et al*., 1980) modified by the addition of 5 mM Tris-HCl and 10 nM Na_2SeO_3 (Cottrell and Suttle 1991). This strain was isolated by Louis Peperzak from the Oosterschelde (a North Sea estuary in Zeeland, the Netherlands). *P. globosa* grew as single cells and did not form colonies. Cultivation of axenic *Leptocylindrus danicus* CCMP 470 and axenic *Thalassiosira rotula* CCMP 1647, obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP, USA), was carried out in f/2 medium at 17°C in a light-dark cycle of 12:12 h.

Algal growth experiments

To study the capability of *P. frisia* to grow by utilizing phytoplankton exudates, cells of *P. frisia* were added to axenic cultures of stationary phase cultures of *P. globosa* and *L. danicus* and of *T. rotula* kept physically separated in dialysis bags with a molecular cut off of 25 KDa. As control assays of *P. globosa* and *L. danicus* axenic algal cultures as well

as the respective algal medium without algae but with the addition of bacterial cells were used. Each assay was carried out in triplicates of 215 ml. Incubation conditions were 17°C in a 12:12 h light-dark cycle for *P. globosa* and *L. danicus* and 15°C for *T. rotula*. Precultivation of the bacterial strain was done in saltwater medium (SWM) with 0.5 g l^{-1} yeast extract and 0.5 g l^{-1} peptone (for composition of SWM see Hahnke *et al.*, 2011). Prior to inoculation of algal cultures, bacterial cells were washed twice in the respective algal medium in a 2 ml reaction vessel by centrifugation for 5 min at 7000 g and subsequent resuspension. After inoculation of the algal cultures with *P. frisia* SH6-1^T (~1 x 10^6 cells ml^{-1}) the following parameters were determined: a) bacterial cell counts, b) algal cell counts, c) concentrations of dissolved organic carbon (DOC), d) concentrations of dissolved free reducing sugars, and e) concentrations of dissolved free and total neutral monosaccharides. To exclude contamination during the course of the experiment the composition of the bacterial community was analyzed in selected samples using DGGE.

Bacterial and algal cell counts

Samples for bacterial and phytoplankton cell counts were fixed with 1% glutardialdehyde (GDA) and stored at -20°C until further use. Total cell numbers were determined on black 0.2 µm polycarbonate filters by epifluorescence microscopy after staining with SYBR® Green I (Invitrogen). For further details see Lunau *et al.* (2005).

Dissolved organic carbon (DOC)

For DOC analyses, algal cultures were filtered through precombusted (2 h, 500°C) GF/F filters (Whatman). The filtrate was acidified with 6 M HCl (20 µl per 5 ml) and stored in sealed glass ampuls at 4°C. Analyses were performed by a Shimadzu TOC-V CSH total organic carbon analyzer.

Free reducing sugars and dissolved free and total neutral monosaccharides

Algal cultures were filtered as described for DOC measurements (see above). Samples were stored in precombusted glass vials at -20°C. Concentrations of free reducing sugars were determined spectrophotometrically using the method by Myklestad *et al.* (1997). Concentrations of dissolved free neutral monosaccharides (DFNCHO) were analyzed by high-performance liquid chromatography (HPLC) with a Carbopac PA 10 column (Dionex) and PAD according to Mopper *et al.* (1992). NaOH (20 mM) was used as eluent. Prior to analysis, samples were desalted by ion-exchange chromatography and the retention

efficiency for neutral monosaccharides of the ion exchange resins was determined in each analytical batch by a standard containing arabinose, fucose, galactose, glucose, mannose, rhamnose, xylose (Borch and Kirchman, 1997). Total dissolved neutral monosaccharides (TDNCHO) were measured after acid hydrolysis. Therefore, the sample (6 ml) was acidified with 429 µl of concentrated H_2SO_4, yielding a concentration of 7.9 M H_2SO_4, purged with N_2 for 30 sec and hydrolyzed at 100°C for 3 h in sealed glass ampuls. The chilled hydrolysate was neutralized by adding 1.05 g precombusted $CaCO_3$ and the precipitate removed by centrifugation at 3000 g for 5 min. The hydrolyzed monosaccharides in the supernatant were analyzed by HPLC as described above.

PCR amplification of 16S rRNA genes and DGGE

For DGGE analysis 1 ml of each assay was centrifuged for 10 min at 10.000 g. Cells were resuspended in 50 µl of PCR water and cracked by consecutive freezing and thawing for three times. *Bacteria*-specific PCR was performed using the primer set GC-341F and 907RM (Muyzer *et al*., 1998). The diversity of the *Roseobacter* clade in samples from Helgoland Roads 2005 was analyzed using the *Roseobacter*-specific primer set GC-ROSEO536Rf and GRb735r as described by Rink *et al*. (2007). DGGE was performed with the INGENY phorU System (INGENY International BV) according to Rink *et al*. (2007). Gels were stained with SYBR® Gold (Invitrogen) after electrophoresis and documented digitally using a BioDoc Analyze Transilluminator (Biometra).

Utilization of laminarin degradation products by P. frisia

In artificial seawater medium (ASW) 0.25 g l^{-1} sterile filtered laminarin (from *Laminaria digitata*; Sigma-Aldrich) was degraded by addition of 0.001 U ml^{-1} laminarinase (from *Trichoderma* sp.; Sigma-Aldrich) at 30°C for ~65 h. ASW medium was prepared according to Zech *et al*. (2009) with addition of 15 ml of 1 M $NaHCO_3$ solution (autoclaved separately). After autoclaving, 1 ml l^{-1} sterile filtered 5-fold concentrated vitamin solution (Balch *et al*., 1979) was added. Laminarinase was sterile filtered using low protein binding 0.22 µm PVDF membrane filters. The following treatments were incubated under the same conditions: a) ASW with addition of laminarin, b) ASW with addition of enzyme, and c) ASW without additions. Five replicates of 5 ml medium of each treatment were inoculated with 10 µl of washed cells of *P. frisia*. Precultivation was done in saltwater medium (SWM) and cells were washed twice in ASW as described for the algal growth experiment

(see above). The assays were incubated at 20°C and growth was monitored by an increase in OD600 spectrophotometrically (Spectronic 70).

Hydrolytic enzyme activities

In the cultures of *P. frisia* growing in the presence of *T. rotula* exudates hydrolytic activities of β-glucosidase and N-acetyl glucosaminidase were measured by using the fluorogenic substrate analogs 4-methyl-umbelliferyl-β-D glucoside and 4-methyl-umbelliferyl-β-D-glucosaminide, respectively, according to Hoppe (1993) and modified by using microtiter plates with bacterial concentrates.

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Supplementary material

Table S1.

Phylotypes with a sequence similarity of ≥99% to *P. frisia* SH6-1. For this analysis the almost complete (>1300 bp) 16S rRNA gene sequence was compared with those in GenBank at the NCBI database using the BLAST tool (http://www.ncbi.nlm.nih.gov/).

Phylotype (Acc. no.)	Origin
Rhodobacteraceae bacterium DG1250 (DQ486490)	*Lingulodinium polyedrum* CCAP 1121/2 laboratory culture
Uncultured marine bacterium clone 16_09_04C09 (FR685174)	Coastal water, Raunefjord (Norway)
Uncultured marine bacterium clone 16_09_04E02 (FR685362)	Coastal water, Raunefjord (Norway)
Uncultured marine bacterium clone 16_02_00C11 (FR683713)	Coastal water, Raunefjord (Norway)
Uncultured marine bacterium clone 16_06_03C12 (FR683500)	Coastal water, Raunefjord (Norway)
Uncultured marine bacterium clone 16_07_05E02 (FR684002)	Coastal water, Raunefjord (Norway)
Uncultured *Rhodobacteraceae* bacterium clone M7CS04_10C11 (HQ242519)	Coastal water, North East Pacific
Uncultured bacterium clone MF-Jan-21 (HQ225108)	Not indicated
Uncultured bacterium clone SW-Jan-21 (HQ203846)	Not indicated
Rhodobacteraceae bacterium MOLA 361 (AM945591)	Adriatic Sea (Italy)

Figure S1:

Concentrations of chlorophyll a (mg/m^3) and occurrence of *P. frisia* SH6-1 on a North Sea transect in May 2006 (A) and September 2005 (B). Δ: *P. frisia* SH6-1 present; o: *P. frisia* SH6-1 absent. Satellite image is courtesy of HZG (http://www.hzg.de/institute/coastal_research/structure/operational_systems/KOF/index.html). C: Occurrence of *P. frisia* SH6-1 in the water column (0-90 m) on the transect in May 2006.

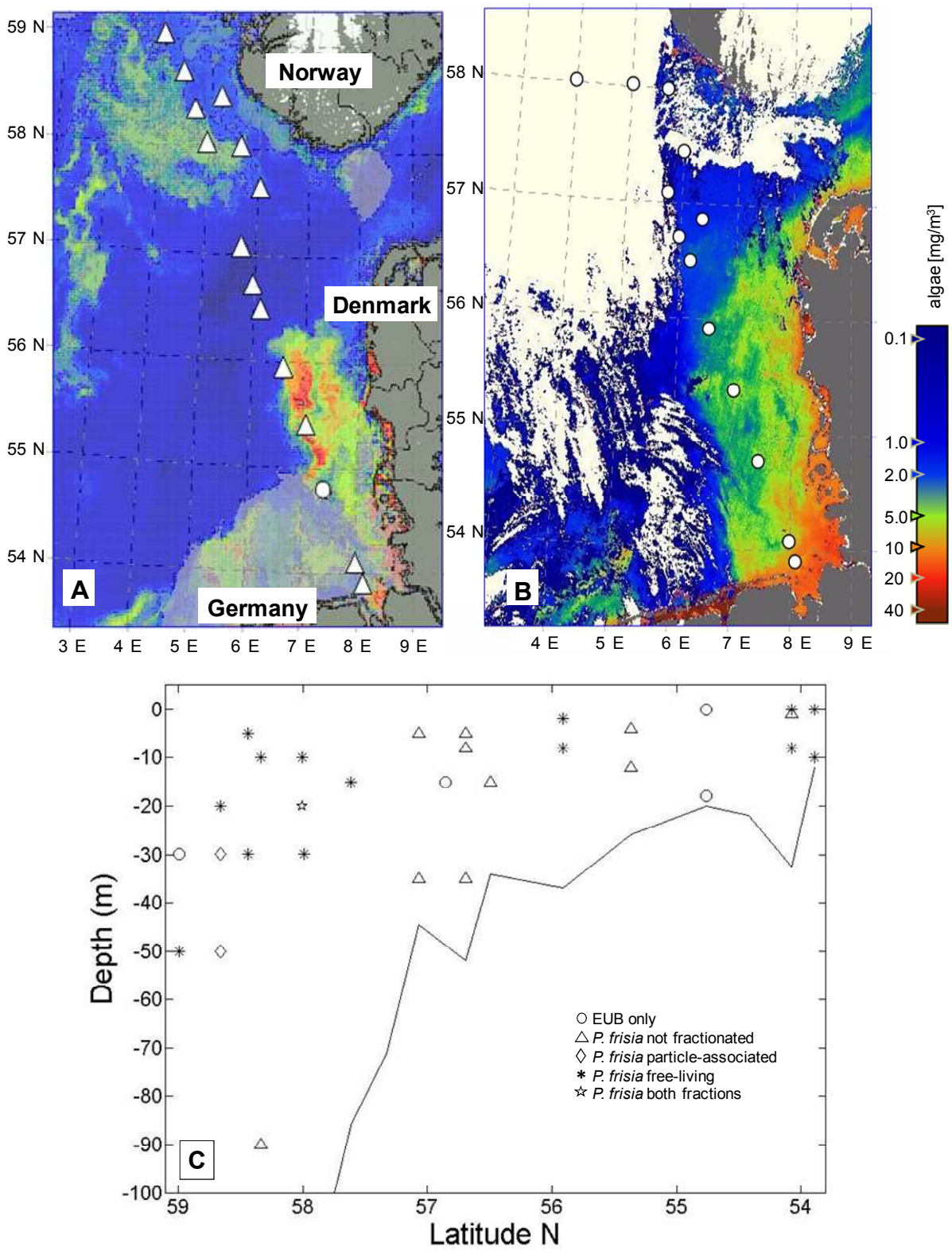


Figure S2:

Roseobacter-specific DGGE analysis of the bacterial communities of samples collected in the North Sea at Helgoland Roads from late April to August 2005. Bands associated to *P. frisia* SH6-1 are marked by the ellipse. PA: Particle-associated fraction (>5 µm); FL: Free-living fraction (0.2 - 5 µm); M: Marker.

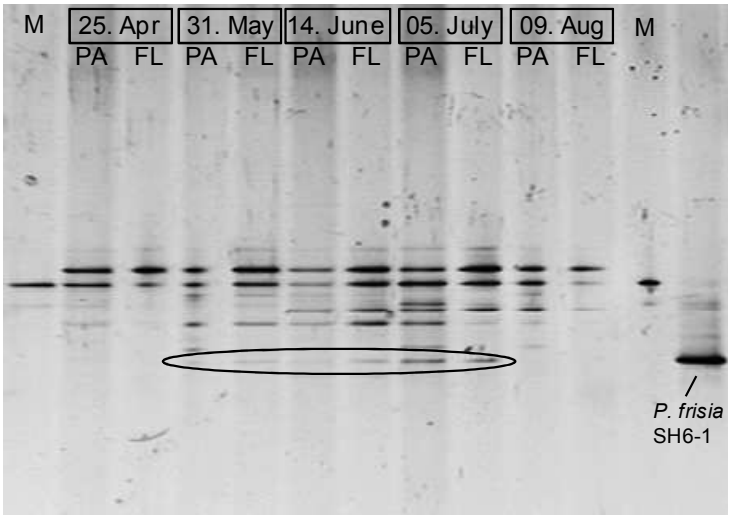
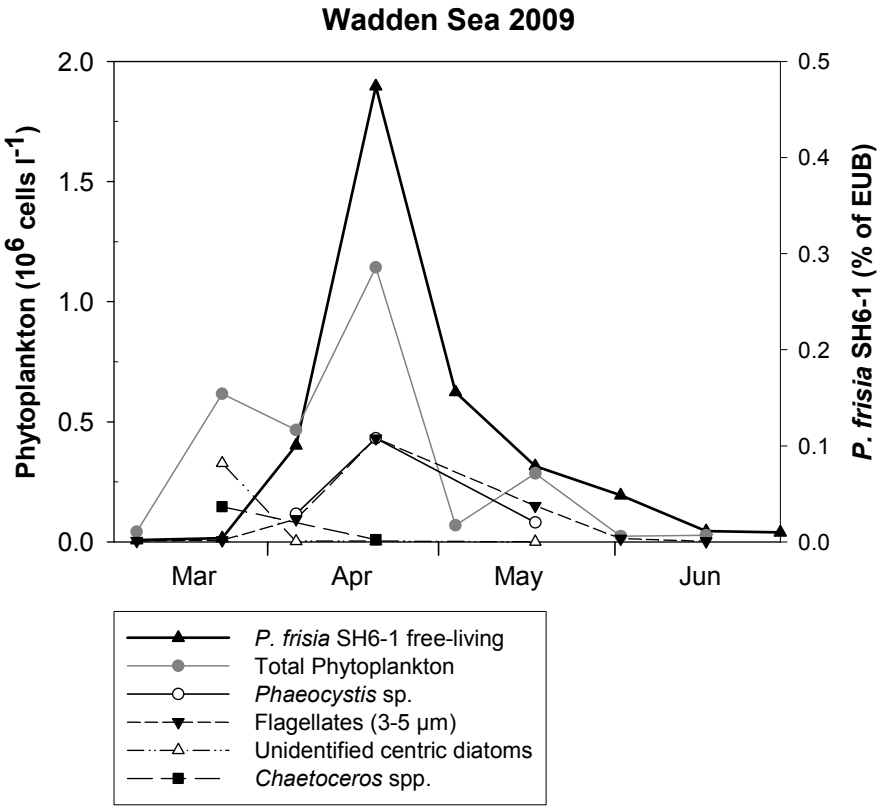


Figure S3:

Abundance of *P. frisia* SH6-1 and abundance of total phytoplankton and their major taxonomic groups in the Wadden Sea from March to June 2009.



II.6 The complete genome sequence of the algal symbiont *Dinoroseobacter shibae*: a hitchhiker's guide to life in the sea

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ORIGINAL ARTICLE

The complete genome sequence of the algal symbiont *Dinoroseobacter shibae*: a hitchhiker's guide to life in the sea

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***Dinoroseobacter shibae* DFL12^T, a member of the globally important marine *Roseobacter* clade, comprises symbionts of cosmopolitan marine microalgae, including toxic dinoflagellates. Its annotated 4 417 868 bp genome sequence revealed a possible advantage of this symbiosis for the algal host. *D. shibae* DFL12^T is able to synthesize the vitamins B_1 and B_{12} for which its host is auxotrophic. Two pathways for the *de novo* synthesis of vitamin B_{12} are present, one requiring oxygen and the other an oxygen-independent pathway. The *de novo* synthesis of vitamin B_{12} was confirmed to be functional, and *D. shibae* DFL12^T was shown to provide the growth-limiting vitamins B_1 and B_{12} to its dinoflagellate host. The *Roseobacter* clade has been considered to comprise obligate aerobic bacteria. However, *D. shibae* DFL12^T is able to grow anaerobically using the alternative electron acceptors nitrate and dimethylsulfoxide; it has the arginine deiminase survival fermentation pathway and a complex oxygen-dependent Fnr (fumarate and nitrate reduction) regulon. Many of these traits are shared with other members of the *Roseobacter* clade. *D. shibae* DFL12^T has five plasmids, showing examples for vertical recruitment of chromosomal genes (*thiC*) and horizontal gene transfer (*cox* genes, gene cluster of 47 kb) possibly by conjugation (*vir* gene cluster). The long-range (80%) synteny between two sister plasmids provides insights into the emergence of novel plasmids. *D. shibae* DFL12^T shows the most complex viral defense system of all *Rhodobacterales* sequenced to date.**

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Keywords: symbiosis; vitamin; cobalamin; thiamine; *Roseobacter*; dinoflagellate

Introduction

One of the most abundant and metabolically versatile groups of bacteria in the world's oceans is the *Roseobacter* clade. Members of this alphaproteobacterial lineage are prominently involved in the global marine carbon and sulfur cycles (Moran *et al.*, 2004; Selje *et al.*, 2004; Buchan *et al.*, 2005; Wagner-Dobler and Biebl, 2006; Howard *et al.*, 2006; Moran and Miller, 2007; Brinkhoff *et al.*, 2008). Many *Roseobacter* species live as epibionts on marine algae where they reach high abundance during phytoplankton blooms (reviewed by Buchan *et al.* (2005)). Some phytoplankton blooms, the so-called

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red tides, are caused by toxic dinoflagellates, and *Roseobacter* species were frequently isolated from their laboratory cultures (Lafay *et al.*, 1995; Prokic *et al.*, 1998; Miller and Belas, 2004), and also detected on dinoflagellates in nature (Hasegawa *et al.*, 2007). A direct interaction between these algae and their associated bacteria is suggested by the fact that *Roseobacter* cells have been found attached to the surface of toxic *Pfiesteria* species using fluorescent *in situ* hybridization (FISH) (Alavi *et al.*, 2001).

The *Roseobacter* isolates from dinoflagellate cultures must be able to grow on metabolites excreted by the algae because they are heterotrophs (Moran and Miller, 2007) and there are no carbon sources present in the mineral media used for cultivating phototrophic algae. Many *Roseobacter* bacteria can degrade dimethylsulfoniopropionate (Moran *et al.*, 2007), an osmoprotectant released in large quantities during algal blooms, and they show positive chemotaxis toward this compound (Yoch 2002; Miller *et al.*, 2004; Miller and Belas, 2006). A strain of the abundant RCA (*Roseobacter* clade-affiliated) cluster could be cultivated in coculture with an axenic dinoflagellate (Mayali *et al.*, 2008). These studies show that *Roseobacter* bacteria can thrive on algal metabolites. However, it is presently not known whether this relationship provides an advantage to the dinoflagellate host.

In this study, we report the complete genome sequence of *Dinoroseobacter shibae* DFL12^T, the type strain of a species from the *Roseobacter* clade. It was isolated from *Prorocentrum lima*, a benthic dinoflagellate, by picking a single dinoflagellate cell from a culture, washing it several times and placing it on an agar surface (Allgaier *et al.*, 2003; Biebl *et al.*, 2005). *P. lima* produces okadaic acid, which can cause diarrhetic shellfish poisoning during red tides (Pan *et al.*, 1999). *D. shibae* DFL12^T was chosen for sequencing because of its symbiosis with dinoflagellates, its ability to perform light-driven ATP synthesis using bacteriochlorophyll *a* in the presence of oxygen (Allgaier *et al.*, 2003; Biebl and Wagner-Dobler, 2006; Wagner-Dobler and Biebl, 2006), the large number of extrachromosomal replicons (Pradella *et al.*, 2004) and its novel acylated homoserine lactone (AHL) compounds (Wagner-Dobler *et al.*, 2005). The complete genome sequence of *D. shibae* DFL12^T revealed traits that presumably are highly adaptive in the habitat of bloom-forming algae and that may be characteristic for the whole *Roseobacter* clade. Moreover, we discovered an essential gift provided to the dinoflagellate by the bacteria.

Materials and methods

Sequencing, assembly and finishing

The genome of *D. shibae* DFL12^T was sequenced at the Joint Genome Institute (JGI) Production Genomics Facility using a combination of 3 and 8 kb (plasmid), and 40 kb (fosmid) DNA libraries. All general aspects of library construction and sequencing performed at the JGI can be found at http://www.jgi.doe.gov. The Phred/Phrap/Consed software package (http://www.phrap.com) was used for sequence assembly and quality assessment (Ewing and Green, 1998; Ewing *et al.*, 1998; Gordon *et al.*, 1998). After the shotgun stage, reads were assembled with parallel phrap (High Performance Software, LLC). Mis-assemblies were corrected with Dupfinisher (Han and Chain, 2006) or transposon bombing of bridging clones (Epicentre Biotechnologies, Madison, WI, USA). Gaps between contigs were closed by editing in Consed, custom primer walk or PCR amplification. A total of 742 additional reactions were necessary to close the gaps and to raise the quality of the finished sequence. The completed genome sequences of *D. shibae* DFL12^T contain 67 596 reads, achieving an average of a 11-fold sequence coverage per base with an error rate less than 1 in 100 000. The sequences of *D. shibae* DFL12^T, comprising a chromosome and five plasmids, can be accessed using the GenBank accession numbers NC_009952, NC_009955, NC_009956, NC_009957, NC_009958 and NC_009959. Manual curation and reannotation of the genome was carried out using The Integrated Microbial Genomes Expert Review System (img/er http://imgweb.jgi-psf.org) (Markowitz *et al.*, 2008) and the Artemis software package (http://www.sanger.ac.uk/Software/Artemis/v9).

Phylogenetic analysis

Phylogenetic analyses of 16S rRNA gene sequences were performed with the ARB software package (http://www.arb-home.de) (Ludwig *et al.*, 2004). A phylogenetic tree was constructed using neighbor-joining and maximum-likelihood analyses. Only sequences with more than 1200 bp were considered in these calculations. Sequences <1200 bp were added afterward using parsimony.

Design of D. shibae*-specific oligonucleotides for PCR and FISH*

Specific primers and probes were developed using the ProbeDesign function of the ARB software package (http://www.arb-home.de). Sequences of specific primers are DSH176f (5′-CGTATGTGGCC TTCGGGC-3′) and DSH1007r (5′-GCTTCCGTCTCC GGAAGCC-3′). Specificity of primer sequences was checked with the NCBI and RDP databases (http://www.ncbi.nlm.nih.gov and http://rdp.cme.msu.edu/) and resulted in at least one mismatch to 16S rRNA gene sequences of non-target organisms. Probe DSH176-HRP (5′-GCCCGAAGGCCACATACG-3′) was used for FISH, in combination with helper probes DSH176-H1 (5′-GTATTACTCCCAGTTTCC CA-3′) and DSH176-H2 (5′-ATCCTTTGGCGATAAA TCTTTC-3′).

Sample preparation for CARD-FISH
In total, 5 ml of the *Prorocentrum* culture were fixed with 5 ml paraformaldehyde (4% w/v) for 1 h and filtered onto a 0.2-µm filter (Nucleopore Track Etch PC MB 25 mm, Whatman Nr. 1110656, Whatman plc, Maidstone, Kent, UK) or a 10-µm filter (Polycarbonate Membrane Filters, TCTP04700, Millipore Corporation, Billerica, MA, USA). Cells were washed eight times with distilled water. Reference strains *D. shibae* DFL12^T (0 mismatches to probe DSH176) and *Sulfitobacter guttiformis* DSM11458^T (two mismatches to probe DSH176) were filtered onto 0.2-µm polycarbonate filters and fixed with paraformaldehyde (2% w/v) for 1 h, rinsed with 1 ml 1 × phosphate buffer solution and subsequently with 1 ml distilled water. Filters were air dried and stored at −20 °C until further processing.

CARD-FISH
CARD-FISH (Catalyzed reporter deposition FISH) was performed according to Sekar *et al.* (2003). Hybridization conditions were as follows: 2 h of hybridization at 35 °C (60% formamide), 30 min washing at 37 °C (14 mM NaCl) and 30 min amplification at 37 °C. Per 400 µl hybridization buffer, 15 µl of the HRP (horseradish peroxidase) probe DSH176-HRP and 15 µl of each unlabeled helper oligonucleotide (DSH176-H1 and DSH176-H2) were used (probe solutions 50 ng µl^{-1}): Tyramine-HCl was labeled with fluorescein-5-isothiocyanate as described by Pernthaler *et al.* (2002). To avoid unspecific accumulation of dye in the cells, the last washing step in phosphate buffer solution (1 ×) amended with TritonX-100 (0.05%) was extended to 30 min. Counterstaining was performed with Vectashield-mounting medium with 4′,6-Diamidino-2-phenylindole (1.5 µg ml^{-1}; Vector Laboratories, Peterborough, England).

Comparative genomics
The Integrated Microbial Genomes Expert Review System (img/er http://imgweb.jgi-psf.org (Markowitz *et al.*, 2008) was used for comparative analysis on the basis of the nine presently available completely sequenced genomes of the *Rhodobacterales*: *Jannaschia sp.* CCS1 (Moran *et al.*, 2007), *Roseobacter denitrificans* (Swingley *et al.*, 2007), *Silicibacter pomeroyi* (Moran *et al.*, 2004), *Silicibacter sp.* TM1040 (Moran *et al.*, 2007), three strains of *Rh. sphaeroides* (Choudhary *et al.*, 2007) as well as *Paracoccus denitrificans* (JGI Institute, unpublished).

CRISPR
Homologs of *cas* genes were detected using tools of the BLAST package (Altschul *et al.*, 1990). CRISPR arrays were detected by the CRISPR recognition tool (Bland *et al.*, 2007). Comparative analysis of repeat sequences was done both manually and by CRISPRdb (Grissa *et al.*, 2007). The origin of spacer sequences was searched for in the CAMERA databases (Seshadri *et al.*, 2007) (http://camera.calit2.net).

Regarding the methods for dinoflagellate cultivation and sampling, isolation of genomic DNA, PCR, cloning and sequencing, determination of vitamin B_{12} and coculture experiments, see Supplementary material 10.

Results and discussion

Ecological niche and phylogeny
D. shibae DFL12^T was isolated from *P. lima* (Biebl *et al.*, 2005). This phototrophic dinoflagellate occurs in sand and sediment and to some extent also in the water column, but reaches its highest densities in biofilms on marine macroalgae, macrophytes, corals, mussels and oysters (Levasseur *et al.*, 2003; Vershinin *et al.*, 2005; Okolodkov *et al.*, 2007; Parsons and Preskitt, 2007).

Several closely related strains were isolated from the dinoflagellate *Alexandrium ostenfeldii* (Allgaier *et al.*, 2003). Using PCR with *D. shibae*-specific primers for the 16S rRNA gene and sequencing of the obtained bands for confirmation, *D. shibae* was also detected in cultures of *Protoceratium reticulatum,* a dinoflagellate isolated from the North Sea. All three host species are cosmopolitan, toxic algae causing diarrhetic shellfish poisoning (Aasen *et al.*, 2005). Finally, *D. shibae* was isolated from *Isochrysis galbana*, a member of the class *Haptophyceae* (Genbank accession EF512132). This nontoxic alga is cultivated as food for bivalves in aquaculture. Thus, *D. shibae* is not restricted to toxic dinoflagellates, but is associated with a variety of cosmopolitan marine microalgae. Figure 1 shows the phylogenetic position of all currently known *D. shibae* strains within the *Roseobacter* clade and indicates from which algae they were isolated. The similarity of these sequences to *D. shibae* DFL12^T is between 97.2% and 99.8%, indicating that they probably belong to the same species, but additional data are needed (Rossello-Mora and Amann, 2001; Stackebrandt *et al.*, 2002; Gevers *et al.*, 2005).

A physical association between host algae and bacteria is suggested by the fact that *D. shibae* DFL12^T was isolated from single washed cells of the dinoflagellate (Biebl *et al.*, 2005). Moreover, it can be seen attached to *P. lima* in coculture (Figure 2). In this study, dinoflagellates were separated from suspended bacteria using 10-µm polycarbonate filters, washed eight times and stained using CARD-FISH with probes specific for *D. shibae* DFL12^T. Many bacteria can be seen adhering to the surface of the dinoflagellate, and some are freely suspended in the medium. It remains to be seen if a genetic program coordinates the switch between planktonic growth and growth as an epibiont in *D. shibae*.

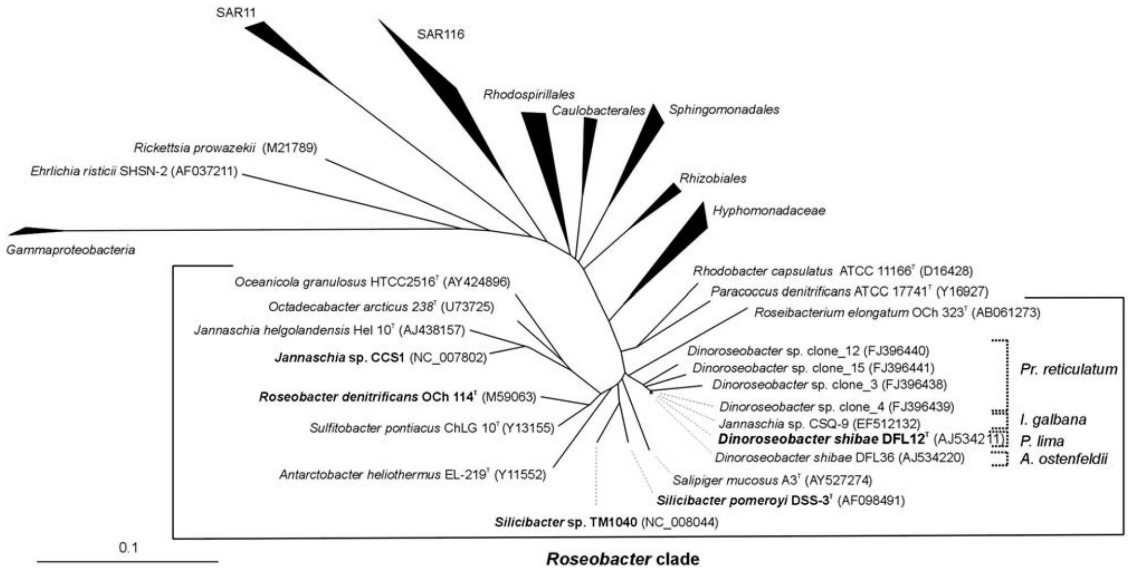


Figure 1 Phylogenetic position of *Dinoroseobacter shibae* DFL12^T and closely related strains and phylotypes within the *Rhodobacteraceae* (*Alphaproteobacteria*) based on 16S rRNA gene comparisons. All names written in boldface indicate organisms whose complete genome sequences have already been determined. The phylogenetic tree was generated using the maximum-likelihood method. Selected members of the *Gammaproteobacteria* were used as an outgroup (not shown). The eukaryotic host algae from which the strains were isolated are indicated next to the dotted brackets. *Pr., Protoceratium* (Dinophyceae); *I., Isochrysis* (Haptophyceae); *P., Prorocentrum* (Dinophyceae); *A., Alexandrium* (Dinophyceae). Bar = 0.1 substitutions per nucleotide position.

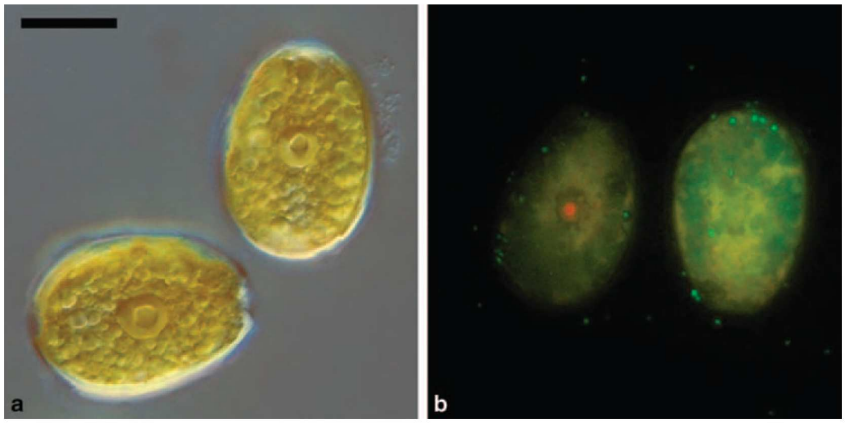


Figure 2 Specific attachment of *D. shibae* DFL12^T to its dinoflagellate host *Prorocentrum lima*. (**a**) Differential interference contrast (DIC) image of dinoflagellates from a non-axenic culture to which cells of strain DFL12^T had been added 2 months before; (**b**) Catalyzed reporter deposition fluorescent *in situ* hybridization (CARD-FISH) with *Dinoroseobacter*-specific 16S rRNA probes showing most cells of *D. shibae* DFL12^T (green dots) closely attached to the dinoflagellate. Combined images generated by means of PICOLAY (http://www.picolay.de) from a series of six pictures taken at sequential focus levels. Bar = 20 µm. The central round structure in the cell center seen in (**a**) is the pyrenoid, a specialized area of the plastid that contains high levels of Rubisco. It is typically surrounded by a starch sheath that can also be seen. The crystal-like ultrastructure of the pyrenoid (Kowalik, 1969) may be the reason for its orange appearance in the left cell in (**b**).

General characteristics of the genome

The *D. shibae* type strain DFL12^T (= DSM 16493^T = NCIMB 14021^T) has a genome size of 4417 kbp, similar to the other fully sequenced *Roseobacter* genomes (Supplementary material S1). The genome contains 4198 protein-encoding genes. About 28% of the derived gene products have no predicted function. The finished genome sequence indicates a circular conformation of the chromosome and the five plasmids (Figure 3). Previously the presence of plasmids with linear conformation was hypothesized for two *D. shibae* strains and

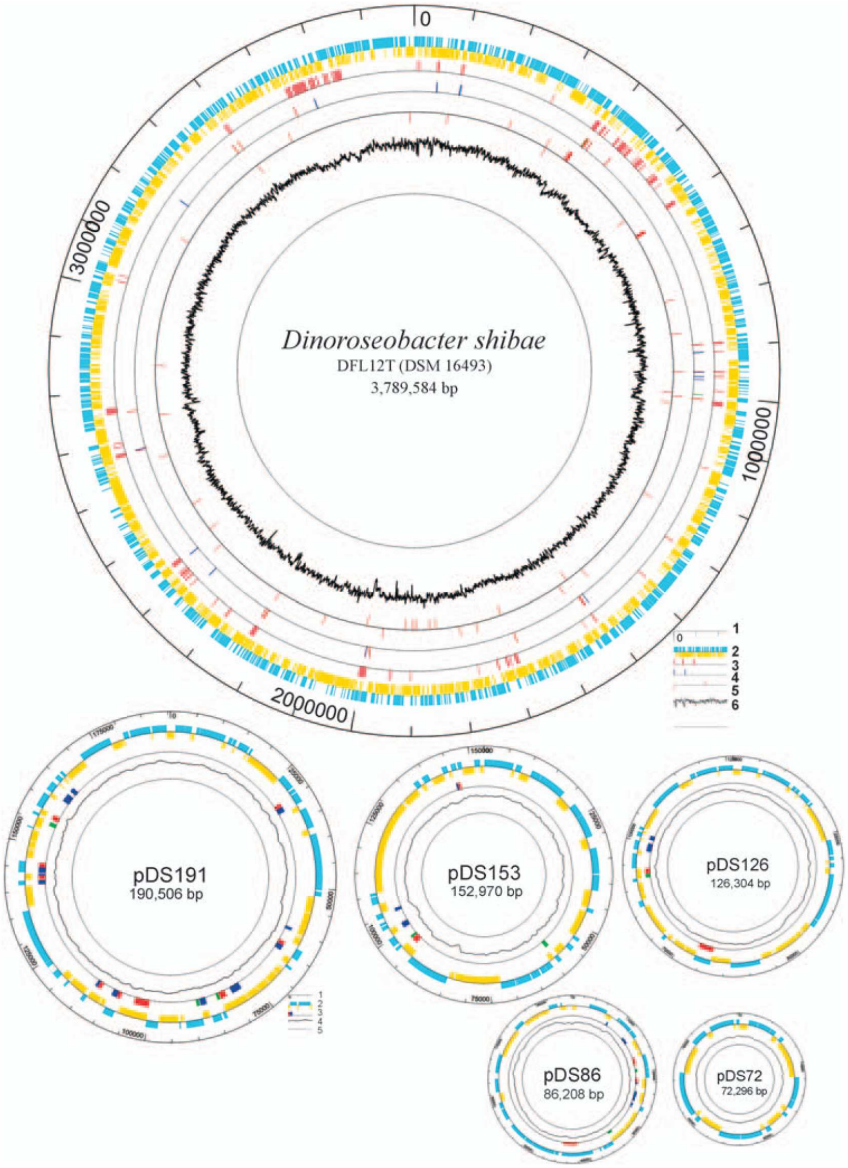


Figure 3 Circular representation of the chromosome and plasmids of *D. shibae* DFL12^T. The ring of the chromosome depicts (starting with the outer ring) (1) the scale in basepairs; (2) all protein-encoding genes, clockwise-transcribed genes in blue and counterclockwise-transcribed genes in yellow; (3) alien genes, that is, genes that are likely acquired by horizontal gene transfer; (4) genes related to DNA flexibility: transposases (blue), integrases (red) and recombinases (green); (5) rRNA and tRNA genes; (6) the GC content. The rings of the plasmids depict (1) the scale in base pairs; (2) all protein-encoding genes; (3) genes related to DNA flexibility; (4) rRNA and tRNA genes and (5) the GC content. Alien genes are not shown in the plasmids, because these contigs are too small for their calculation.

several related species (Pradella *et al.*, 2004). We investigated the plasmid structure in *D. shibae* DFL12 by a restriction assay combined with length separation through pulsed-field gel electrophoresis and could confirm the circular conformation (Supplementary material S2). This is in agreement with the absence of genes typically associated with linear plasmids, for example, the telomere resolvase (Meinhardt *et al.*, 1997; Kobryn and Chaconas, 2001).

Comparative genomics

D. shibae DFL12^T harbors all biogeochemically important traits reported previously from fully

sequenced *Roseobacter* isolates, namely anaerobic anoxygenic photosynthesis (*pufLM* genes), carbon monoxide oxidation (*cox* genes), aromatic compound degradation (*pcaGH* and *boxC* genes), sulfur oxidation (*soxB*), denitrification (*nirS/K*), nitrate assimilation (*nasA*), phosphonate use (*phn*), type IV secretion (*vir* genes) and dimethylsulfoniopropionate degradation (*dmdA*) (Supplementary material S3). Several families of transposases/integrases (61 and 43 copies, respectively) and site-specific recombinases/resolvases (11 copies) belong not only to the largest gene families, but also they are more frequent than in other members of the *Roseobacter* clade, indicating a large potential for DNA exchange in *D. shibae* DFL12^T. Proteins involved in information processing usually represent the role categories showing the least variation between closely related organisms. Their comparative analysis is described in Supplementary material S1.

The central energy metabolism and its regulation

Central carbon metabolism

The central carbon metabolism is reconstructed in Supplementary material S4. Glucose breakdown is potentially possible through glycolysis (Embden-Meyerhoff-Parnas pathway) and the Entner-Doudoroff pathway, the latter being closely connected to the oxidative pentose phosphate pathway. Interestingly, a fluxom analysis using ^{13}C labeling techniques showed that glucose breakdown is carried out exclusively using the Entner-Doudoroff pathway; thus, the alternative routes are used only for anabolic purposes (Fürch *et al.*, 2009). The Calvin cycle is lacking, indicated by the absence of the key enzyme Rubisco, as in all *Roseobacter* strains (Swingley *et al.*, 2007). Carbon dioxide might be fixed by anapleurotic enzymes either through (1) a two-step reaction involving pyruvate-orthophosphate dikinase and phosphoenolpyruvate carboxylase or through (2) pyruvate carboxylase. Genes for both carboxylases are present in all fully sequenced *Roseobacter* strains. Pyruvate carboxylase requires biotin as a cofactor, and hence the need to supply *D. shibae* DFL12^T with this vitamin when it is grown on minimal medium (Biebl *et al.*, 2005). A fluxom analysis revealed that, indeed, this enzyme displays the major CO_2-assimilating route in *D. shibae* (Fürch *et al.*, 2009). *D. shibae* DFL12^T is the only fully sequenced *Roseobacter* strain harboring the arginine deiminase pathway, which allows one to carry out a survival fermentation process known from the *Bacillus* and *Pseudomonas* species (Eschbach *et al.*, 2004; Williams *et al.*, 2007).

Electron transport chains

Complex electron transport systems were found (Supplementary material S5), including seven electron-donating primary dehydrogenases, such as for L- and D-lactate, glycerol-3-phosphate, NADH and succinate, and respiratory dehydrogenases for glucose and gluconate. The presence of the aerobically performed anoxygenic photosynthesis further complicates the electron transport machinery. Just as in the mitochondria, which are derived from *Alphaproteobacteria*, a cytochrome bc_1 complex is available. Electron flux can occur from ubiquinol, the reduced form of ubichinone, through the bc_1 complex and different cytochrome c molecules to two types of cytochrome c oxidases to reduce molecular oxygen to water. Consequently, a close coordination between light-dependent cyclic electron transport (photosynthesis) and respiration is required, the mechanism of which remains to be elucidated.

A complete denitrification pathway and a dimethylsulfoxide (DMSO) reductase are available for the reduction of the alternative electron acceptors DMSO and nitrate. Interestingly, the organism uses the Nap (periplasmic) type nitrate reductase, which is oriented toward the periplasmic space and is active both under aerobic and anaerobic conditions, instead of the Nar (respiratory) type facing the cytoplasm, and is induced only under anaerobic/microaerophilic conditions. Anaerobic growth using nitrate as an electron acceptor was confirmed experimentally in a defined mineral medium (data not shown).

Oxygen-dependent regulation of the central energy metabolism

We deduced the regulon for the global oxygen-dependent transcriptional regulator for fumarate and nitrate reduction, Fnr (Supplementary material S3). Six Fnr/Crp (cAMP receptor protein) type regulators are encoded by the genome of *D. shibae* DFL12^T. However, only one of the deduced proteins carries the cysteine residues required for iron sulfur cluster formation. Two others resemble Fnr-type regulators such as Dnr and DnrD (transcription factor for denitrification gene expression). Interestingly, Dnr is located directly upstream of the *nos* operon encoding a nitrous oxide reductase, the enzyme catalyzing the transformation of N_2O to N_2, the last step of denitrification. Such a gene arrangement has not been found before in denitrifying bacteria, for example, in *R. denitrificans*.

Using a position weight matrix approach (Munch *et al.*, 2005), the genome was searched for potential Fnr-binding sites. In agreement with findings for other Gram-negative bacteria, the promoter region of genes for high-affinity oxygen-dependent cytochrome c oxidases (cbb_3-type), alternative anaerobic systems including the NADH dehydrogenase, the quinone oxidoreductase and the corresponding cofactor biosynthesis genes (*hemN1*, *hemN2*, *hemA* and *moaC*) are all carrying Fnr-binding sites. No obvious Fnr boxes were found upstream of the various operons involved in denitrification and DMSO reduction. Interestingly, three universal

stress protein-like genes and eight potential regulator genes are also carrying Fnr boxes. Two of these regulators are suppressor proteins of the heat shock chaperone DnaK (DksA), and the function of the other six regulators is unknown. The Fnr box is also present in promoters of bacteriochlorophyll biosynthesis genes, but it is not possible to predict whether Fnr induces or represses the expression of the corresponding genes.

The Fnr regulator is not a unique feature of *D. shibae* DFL12^T. Searching the complete genome sequences of the *Roseobacter* clade with the *D. shibae* Fnr by reciprocal best hit BLAST showed that one copy is present in all of them. Thus, the ability to fine-tune their metabolism to anoxic conditions is a general trait of the *Roseobacter* clade.

Nutrients provided by the algal host and their uptake

The pathways for the known growth substrates of *D. shibae* DFL12^T (Biebl *et al.*, 2005) could be reconstructed from the genome sequence. Interestingly, most of them are Krebs cycle intermediates (fumarate, succinate, pyruvate and citrate). They may be released from the algal host after cell death or during photosynthesis. Photosynthate release from phytoplankton can be up to 40% of primary production (Wang and Douglas, 1997) and forms the basis of the microbial loop (Azam *et al.*, 1983). *D. shibae* DFL12^T also uses acetate and glucose, important components of the marine dissolved organic carbon (Aluwihare *et al.*, 2002; Biersmith and Benner, 1998). Growth also occurs on fructose and glyceraldehyde-3-phosphate, products of the photosynthetic Calvin–Benson cycle. The dimethylsulfoniopropionate breakdown product DMSO can be used as an alternative electron acceptor by *D. shibae* DFL12^T.

For the uptake of nutrients from the environment, tripartite ATP-independent periplasmic transporters (TRAP) seem to be preferentially used by *D. shibae* DFL12^T rather than ABC-type transporters, which are only about half as frequent as in other members of the *Roseobacter* clade. TRAP transporters are composed of a periplasmic solute receptor (DctP or TAXI) and a secondary transporter (DctM(Q)) (Forward *et al.*, 1997). Transport is driven by an electrochemical ion gradient rather than by ATP hydrolysis, and this may be the reason why TRAP systems have their highest prevalence among marine bacteria (Rabus *et al.*, 1999; Mulligan *et al.*, 2007). Their design allows the coupling of high-affinity solute reception (formerly attributed only to primary transporters) with energy-efficient ion gradient-driven permeases. The substrate range of TRAP systems also comprises, besides C4-dicarboxylates, pyruvate and two other oxoacids, glutamate, sialic acid, ectoine and 2,3-diketogulonate. With 27 complete TRAP systems encoded in its genome, *D. shibae* DFL12^T is second only to *S. pomeroyi* (28 complete systems). Efficient uptake of organic nutrients available only in the sub-micromolar concentration range could provide members of the *Roseobacter* clade with a competitive advantage.

Plasmid biology

Characteristic features of the plasmids

The most striking observation regarding the extrachromosomal elements is the long-range synteny between the 191 and the 126 kb plasmid (Figure 4). About 80% of the sequence of the smaller replicon, including a gene cluster of 47 kb, a *vir* operon and a short inverted region (shown in blue), are highly conserved. This pattern provides strong evidence for a common origin, thus justifying their designation as sister plasmids. The differences among the non-conserved regions result from genomic rearrangements likely assisted by transposition events, an explanation that is supported by the frequent occurrence of transposases and integrases (Supplementary material S6A). Moreover, comparative sequence analyses indicate that a common ancestor of the two sister plasmids may have been recruited through conjugational gene transfer. The *D. shibae* DFL12^T chromosome has a GC content of 66%, which is in the upper range of *Roseobacters*, and the respective values for the plasmids vary between 60% and 69% (Supplementary material S6A). The two sister plasmids show the lowest GC content and the analysis with the Artemis software revealed a poor so-called 'GC Frame Plot' (Supplementary material 6B–D). This finding probably reflects their relatively recent recruitment in an evolutionary time scale and an early stage of sequence adaptation within the new host bacterium. This scenario is supported by the observation that in *D. shibae*, DFL12^T *vir* operons are exclusively present on the sister plasmids (Figure 4). The *vir* gene cluster encoding the type IV secretion system is the structural prerequisite for the formation of sex pili required for conjugation. It is structurally highly conserved among *Roseobacter* strains and also present in *Silicibacter* sp. TM1040 (Moran *et al.*, 2007).

Plasmid replication and maintenance

We identified the core functions for plasmid replication initiation, partitioning and stability representing the 'functional heart' of a plasmid (Supplementary material 7A-C). They provide the structural basis for compatibility versus incompatibility (Petersen *et al.*, 2009). We found three different types for replication initiation including the *repABC* module that is characteristic for the sister plasmids (Figure 4). Our phylogenetic analyses document that their crucial replicases (*repC*) are only distantly related, indicating that at least one of them has been exchanged by a gene transfer event. The respective *repABC* modules hence belong to

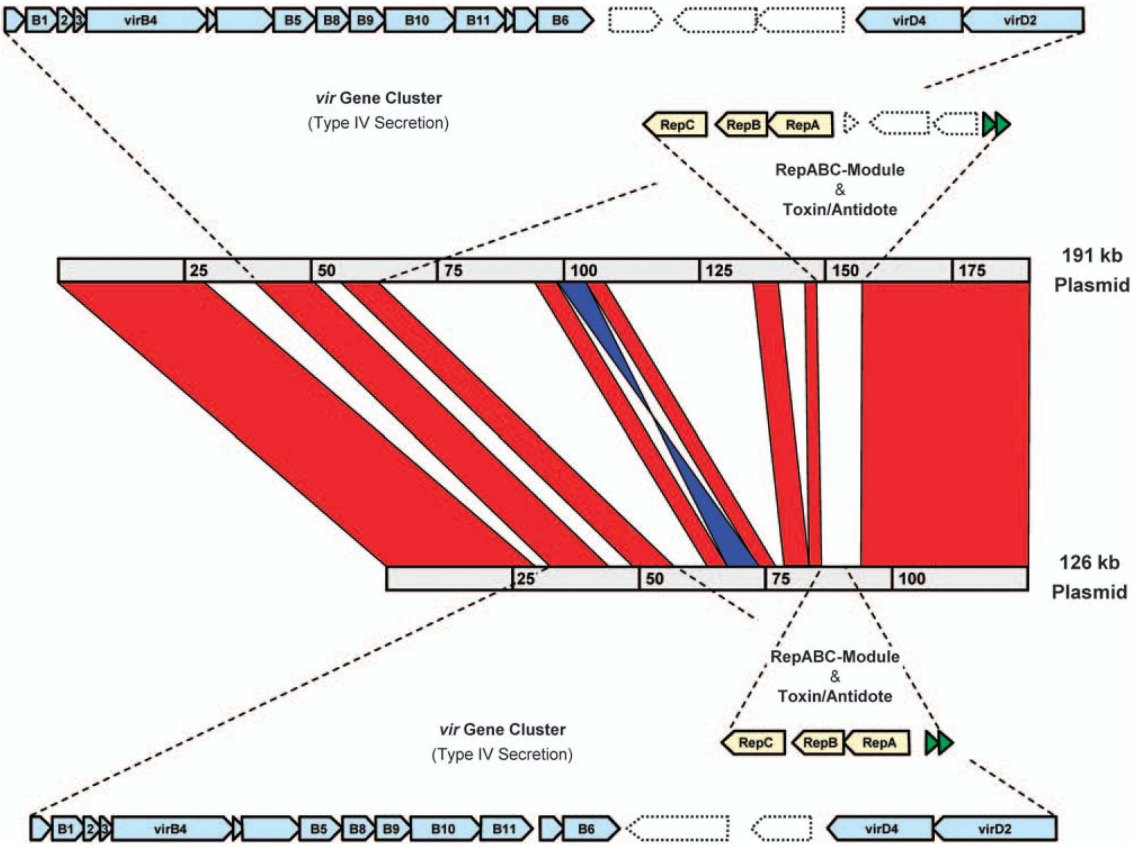


Figure 4 Synteny plot of the 191 and 126 kb plasmids of *D. shibae* DFL12^T. Long-range homologies are shown with red bars and an inverted region is printed in blue. The left and right borders of the plasmids are completely conserved. Homologous *vir* clusters and the distantly related *repABC*-type replicons as well as the adjacent toxin/antidote addiction systems for plasmid stability are shown in detail. Adjacent genes that are not involved in plasmid core functions are indicated with dotted lines. The analysis was calculated with the Artemis Comparison Tool (WebACT, http://www.webact.org/WebACT/generate).

different compatibility groups (Petersen *et al.*, 2009), the prerequisite for a stable coexistence of the syntenous plasmids. Finally, at least four of the five replicons contain putative addiction systems for plasmid maintenance (Zielenkiewicz and Ceglowski, 2001). They are represented by operons encoding a stable toxin as well as a less stable antidote (Figure 4). These selfish units indirectly ensure the maintenance of the low copy replicons in the offspring, as plasmid loss would be lethal for the host cell.

Metabolic capabilities and localization of genes

The extrachromosomal replicons of *D. shibae* DFL12^T can roughly be classified according to their genetic composition (Supplementary material S7D). The two sister plasmids are characterized by the *vir* gene cluster (see above), whereas the other plasmids harbor gene clusters for the degradation of aromatic compounds (153 kb), *cox* operons that presumably contribute to energy production (72 kb) and a wealth of genes for sugar metabolism (86 kb). The latter replicon represents an aged and possibly dying plasmid owing to its conspicuous amount of pseudogenes and transposases. Transposition may dominate intracellular genetic rearrangements as documented for *thiC*, an essential gene for thiamine (vitamin B_1) biosynthesis (Vander Horn *et al.*, 1993). In contrast to all other genes of this pathway that are localized on the *D. shibae* DFL12^T chromosome, altogether three copies of *thiC* are located on different plasmids (191 kb, 153 kb and 86 kb). The genomic neighborhood of an integrase, resolvase and transposase strongly supports an underlying dispersal mechanism based on DNA transposition.

Interaction with other organisms

Quorum sensing

D. shibae DFL12^T produces three different quorum-sensing signals from the group of AHLs, namely a

C_8-homoserine lactone (C8-HSL), and two AHLs with a side chain of 18 carbon atoms, C_{18}-en-HSL and C_{18}-dien-HSL (Wagner-Dobler *et al.*, 2005). This is the maximum side chain length for AHLs and it was, for the first time, detected in *Sinorhizobium meliloti,* a root nodule-forming symbiont (Marketon *et al.*, 2002), where this signal downregulates motility at high cell density (Hoang *et al.*, 2008). The unsaturations found in the C_{18} side chain of DFL12^T are novel and may be critical for signal specificity. The genome analysis predicted three autoinducer synthases (*luxI* type) and five *luxR* type genes for AHL-controlled transcriptional regulators (Supplementary material S3). $luxI_1$ and $luxI_2$ of *D. shibae* DFL12^T are located on the chromosome adjacent to a *luxR* gene with an interspacer region of appr. 100 kb, whereas $luxI_3$ is on the 86-kb plasmid without an adjacent *luxR* gene. *Roseobacter* strains usually have one or two autoinducer synthases, whereas up to four have been found in root nodule-forming *Rhizobia* (Case *et al.*, 2008). The orphan *luxR* genes may allow eavesdropping on competing bacterial species in the environment (Case *et al.*, 2008).

Production of secondary metabolites

Polyketide synthases (PKSs) catalyze the synthesis of polyketides, a large class of secondary metabolites, from acetyl-CoA precursors. *D. shibae* DFL12^T harbors a 12-gene cluster containing two adjacent modular type I PKS genes among hypothetical and putative proteins (Supplementary material S3). The first PKS gene is composed of only one module containing the domains essential for chain elongation (β-ketoacylsynthase domain, acyltransferase domain, and two ACP (acyl carrier protein) domains), a 2-nitropropane dioxygenase domain and a β-ketoacyl-ACP reductase domain. No reductive domain required for the modification of the keto-group could be identified. The second PKS gene contains two modules. In the N-terminal module, a β-ketoacyl-synthase domain and an acyltransferase domain were identified; the C-terminal module shows similarity to a phosphopantetheinyltransferase domain (PKS loading enzyme). The other genes in this cluster, such as the two putative short-chain dehydrogenases or the two putative NAD-dependent epimerases/dehydratases, may be involved in chain modification reactions to give the final active product.

Close homologs of these genes are not found in the *Roseobacter* clade, but a similar gene cluster is harbored by *Solibacter usitatus* (*Acidobacteria*) and *Gloeobacter violaceus* (*Cyanobacteria*), suggesting that these genes may have been acquired by horizontal gene transfer. Thus, *D. shibae* DFL12^T encodes a multiprotein complex that may catalyze the synthesis of a novel polyketide that could have a role in interactions with competing microbes. The dinoflagellate surface is colonized by a range of other bacteria, including *Vibrios* and *Flavobacteria*. Tropodithietic acid, an antibiotic produced by at least two *Roseobacter* isolates, is especially active against *Vibrios* (Brinkhoff *et al.*, 2004; Bruhn *et al.*, 2005) and is encoded by a different pathway (Geng *et al.*, 2008).

Resistance against viral attack

Viruses are the most abundant biological entity on the planet (Williamson *et al.*, 2008). By lysing a significant fraction of bacterial communities, bacteriophages influence the cycling of organic matter in the sea (Fuhrman, 1999; Weinbauer, 2004; Brussaard *et al.*, 2008; Danovaro *et al.*, 2008) and the genetic diversity of their host populations (Muhling *et al.*, 2005). Continuous viral attack accompanies red tides (Tomaru and Nagasaki, 2004) and contributes to their sudden collapse (Nagasaki *et al.*, 2005; Rhodes *et al.*, 2008). Thus, resistance against phage attack must be highly adaptive in the habitat of *D. shibae* DFL12^T, which has evolved the most complex viral defense system of all *Roseobacter* strains sequenced to date.

CRISPRs (**c**lustered **r**egularly **i**nterspaced **s**mall **p**alindromic **r**epeats) are small, transcribed DNA spacers separated by short palindromic repeats that are located next to a cluster of *cas* (**C**RISPR **as**sociated) genes. Many spacer sequences have been shown to originate from bacteriophages, leading to the current hypothesis that CRISPRs are involved in an RNAi-like mechanism resulting in antiviral resistance (Haft *et al.*, 2005; Makarova *et al.*, 2006; Barrangou *et al.*, 2007). Two CRISPRs were detected in the genome of *D. shibae* DFL12^T, whereas no other completed genome from the *Rhodobacterales* contains more than one. Five organisms of this group revealed a CRISPR (Supplementary material S8). Remarkably, four of them lack the characteristical CRISPR marker gene *cas1*, which is reportedly present in all CRISPR-containing organisms, with *Pyrococcus abyssii*, as formerly, a single exception. Therefore, *cas1* is apparently no reliable marker for CRISPRs in the *Rhodobacterales*.

Figure 5 shows that the two CRISPR/*cas* gene clusters of *D. shibae* DFL12^T have totally different structures. Comparative sequence analyses indicated that they are complete and independent units derived from different microbial lineages. It is most likely that their acquisition occurred through horizontal gene transfer, as suggested earlier (Godde and Bickerton, 2006). To discover the origin of the CRISPR spacer sequences in *D. shibae* DFL12^T, these sequences were compared with the marine metagenomics database, CAMERA (Seshadri *et al.*, 2007), but no homologs were found, most likely because the habitats of *D. shibae* DFL12^T have not yet been searched for phages. Diversification of the CRISPR region is inferred to be a population-level response to the rapidly changing selective pressure of phage predation (Tyson and Banfield, 2008), and hence phage predation must represent a significant selective force in the habitat of *D. shibae* DFL12^T.

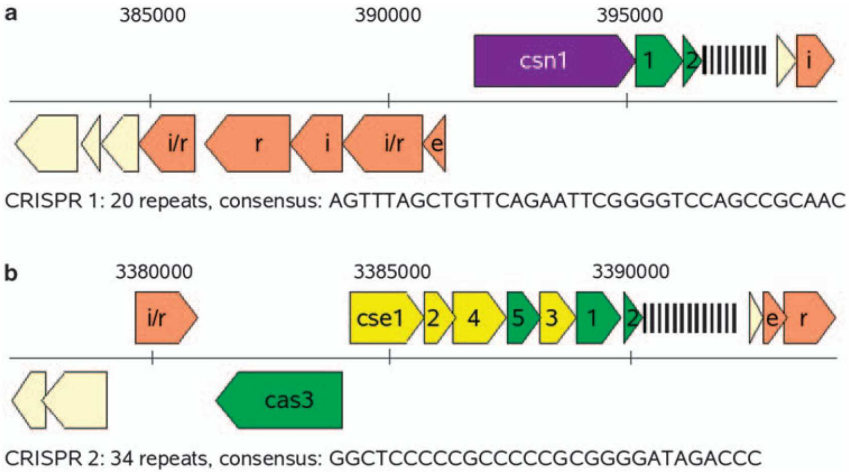


Figure 5 Genomic context of the two CRISPRs of *D. shibae* DFL12^T. The numbers indicate the base position in the genome. Barcode-like series of vertical black bars symbolize CRISPR arrays. The colored gene arrows show the orientation, length and assignment to functional gene groups: Green—*cas* core genes; 1 = *cas1*; 2 = *cas2*; 5 = *cas5*. Purple—*csn1* gene, specific for subtype *Neisseria meningitidis*. Yellow—*cas* genes specific for subtype *Escherichia coli*; 2 = *cse2*; 3 = *cse3*; 4 = *cse4*. Orange—genes for polynucleotide processing; i = integrase; r = recombinase; e = excisionase.

Synthesis and utilization of vitamin B_{12}

Vitamin B_{12}-dependent enzymatic reactions

Most Bacteria and Archaea including *D. shibae* DFL12^T have enzymes that require B_{12} as a cofactor. The genome revealed a gene encoding a B_{12}-dependent methionine synthase, catalyzing the final step in methionine biosynthesis. A gene encoding an enzyme that catalyzes the same reaction in a B_{12}-independent manner was also found. Genes encoding a B_{12}-dependent ribonucleotide reductase and a methylmalonyl-CoA mutase were also found, but genes for other B_{12}-dependent enzymes (glutamate mutase, ethanolamine ammonia lyase, diol dehydratase and glycerol dehydratase) were not present.

Cobalamin biosynthesis

Vitamin B_{12}, which is only synthesized *de novo* by some Bacteria and Archaea, is derived from uroporphyrinogen III, a precursor in the synthesis of heme, siroheme, cobamides, chlorophylls and the methanogenic F430 (Scott and Roessner, 2002; Warren *et al.*, 2002; Rodionov *et al.*, 2003). At least 25 enzymes are uniquely involved (Roth *et al.*, 1996). The genome of *D. shibae* DFL12^T harbors all the genes required for *de novo* B_{12} synthesis (Supplementary material S9), similar to other fully sequenced members of the *Roseobacter* clade, for example, *R. denitrificans* and *S. pomeroyi*. However, it has an unusual route for vitamin B_{12} synthesis. Two different biosynthetic routes for cobalamin are known in Bacteria: (i) an oxygen-dependent (aerobic) pathway and (ii) an oxygen-independent (anaerobic) pathway (Martens *et al.*, 2002; Scott and Roessner, 2002; Warren *et al.*, 2002). They differ in the first part, the insertion of cobalt into the corrin ring, whereas the second part is common for both the routes. In the aerobic route, cobalt insertion is performed by an ATP-dependent cobalt chelatase, which is encoded in *P. denitrificans* by *cobN*, *cobS* and *cobT*. In the anaerobic pathway, single subunit ATP-independent cobalt chelatases such as CbiX of *B. megaterium* are performing the reaction. *D. shibae* DFL12^T contains putative genes for both types of chelatases and in this respect it is unique among the fully sequenced *Rhodobacterales*. Other pathway-specific genes were also present, but *cobG*, regarded as a signature gene for the aerobic route (Scott and Roessner, 2002), was absent. The gene for an alternative enzyme found in *Rhodobacter capsulatus*, CobZ, was also not present (McGoldrick *et al.*, 2005). Thus, *D. shibae* DFL12^T might have the property to synthesize B_{12} by either the aerobic or the anaerobic pathway depending on the availability of oxygen. Alternatively, it might synthesize B_{12} by an unusual combination of both pathways.

We experimentally confirmed that the *de novo* pathway for vitamin B_{12} synthesis is functional in *D. shibae* DFL12^T. The bacteria can grow in a defined mineral medium, but to exclude that traces of vitamin B_{12} might be present even in such media, we compared the B_{12} content of cells grown in the presence and absence of methionine. *D. shibae* DFL12^T has two methionine synthases, one requiring B_{12} and another that is B_{12} independent. In the absence of methionine, the content of B_{12} in the cells should increase, as the B_{12}-dependent methionine synthase requires B_{12} as a cofactor. The vitamin B_{12} content of the cells was 340 ± 36 ng mg^{-1} protein in the presence of methionine and increased to 450 ± 26 ng mg^{-1} protein in the absence of methio-

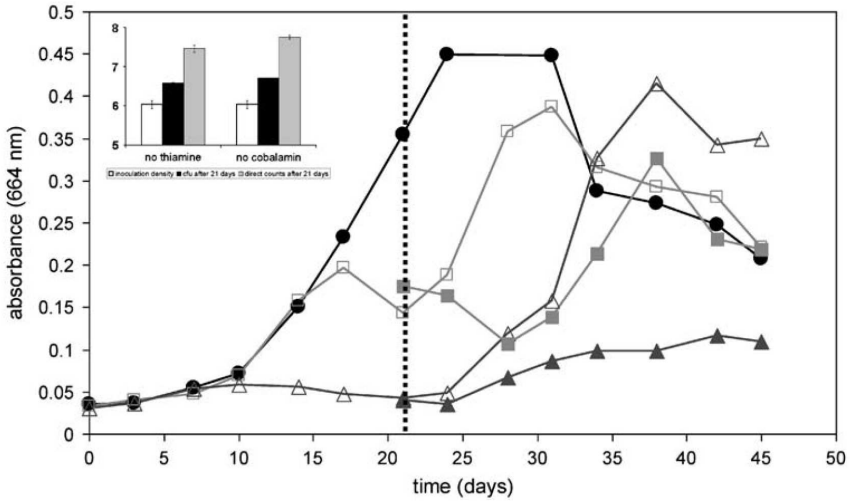


Figure 6 The bacterium *D. shibae* DFL12^T provides thiamine (B_1) and cobalamin (B_{12}) to the dinoflagellate *Prorocentrum minimum* in coculture. Growth of the algae is shown as the amount of chlorophyll *a* (absorbance at 664 nm). Symbols show cultures on L1 mineral salts medium Δ lacking thiamine; □ lacking cobalamin and ● control (complete L1 medium). On day 22 (dotted line), 1% of the cultures lacking thiamine or cobalamin were transferred to fresh medium of the same composition. One half of the culture was provided with the lacking vitamin (open symbols), and the other half was inoculated with *D. shibae* DFL12^T (filled symbols). The inset shows log cell densities of *D. shibae* DFL12^T at the time of inoculation and at the end of the experiment (cfu and direct microscopic counts). A full colour version of this figure is available at *The ISME Journal* online.

nine, showing that a complete *de novo* pathway for vitamin B_{12} synthesis is functional.

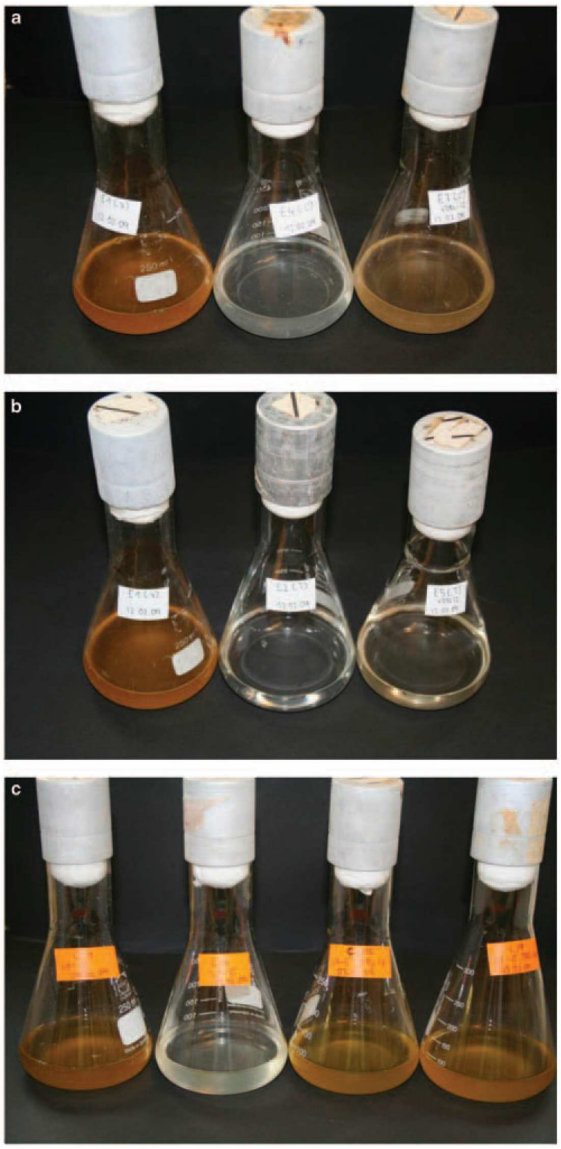
Symbiosis with dinoflagellates

Besides supplying its own B_{12}-dependent enzymes with the cofactor, the versatility in B_{12} production might also contribute to a successful symbiosis with the dinoflagellate. Axenic (bacteria-free) cultures of dinoflagellates can be obtained on defined mineral media, but supplementation with three vitamins is necessary, namely thiamine (B_1), cobalamin (B_{12}) and biotin (B_7 or vitamin H) (Guillard and Hargraves, 1993). *D. shibae* DFL12^T is able to synthesize two of these, B_{12} and B_1. It is auxotrophic for biotin, similar to many *Roseobacter* species (Shiba, 1991), and thus this vitamin must be provided by other sources in nature to both the dinoflagellate and the *Roseobacter* species.

To experimentally determine whether *D. shibae* DFL12^T can provide B_1 and B_{12} to its dinoflagellate host, we performed coculture experiments. Axenic cultures for *P. lima* are not available in culture collections, but as the symbiosis of *D. shibae* with algae is not strictly host specific, we reasoned that proof of principle might be obtained using the closely related dinoflagellate *P. minimum* CCMP1329.

First, auxotrophy of *P. minimum* for B_1 and B_{12} was investigated, as it had not been tested earlier (Croft *et al.*, 2005). Supplementary material S10 shows that *P. minimum* was not able to grow on a synthetic mineral medium from which either B_1 or B_{12} had been omitted. Next, either B_1 or B_{12} were replaced by 1.1×10^6 cells per ml of *D. shibae* DFL12^T. In the experiment shown in Figure 6, the dinoflagellate was first cultivated for 22 days without bacteria; there was no growth without thiamine, and cobalamin became growth limiting after about 17 days. On day 22, the cultures were divided. One half was provided with the lacking vitamin, and the other half was inoculated with *D. shibae* DFL12^T. Figure 6 shows that *D. shibae* DFL12^T stimulated the growth of the alga in the absence of B_1 or B_{12}, and that the alga allowed the bacterium to grow too (inlay in Figure 6). From an initial density of 1.1×10^6 cfu ml^{-1}, they increased to 5.1×10^6 cfu ml^{-1}. Bacterial densities determined by direct microscopic counts were even higher by more than one order of magnitude. The density of the dinoflagellate at the end of the experiment was appr. 2.5×10^6 cells ml^{-1}, as determined by microscopic counts. Supplementation with pure vitamins resulted in faster recovery of the dinoflagellate culture in the case of B_{12}, and in much stronger recovery in the case of B_1 compared with inoculation with *D. shibae* DFL12^T. The bacteria were entirely dependent on carbon sources excreted by the algae, and thus may have been initially starved in the non-growing algal culture. The concentration of thiamine present in the cultivation medium is 200-fold higher than that of B_{12}, and therefore the amount synthesized by the relatively low density of bacteria might not have been sufficient.

Experiments with the haptophyte *I. galbana* CCMP1323, from which *D. shibae* DFL12^T has also been isolated and which is known to be auxotrophic for B_{12} (Croft *et al.*, 2005), showed similar results for cobalamin, but this alga did not require thiamine for



***Prorocentrum minimum* CCMP1329.**
Media are (from left to right) L1 medium, L1 medium lacking cobalamin, and L1 medium lacking cobalamin inoculated with *D. shibae* DFL12^T.

***Prorocentrum minimum* CCMP1329.**
Media are (from left to right) L1 medium, L1 medium lacking thiamine, and L1 medium lacking thiamine inoculated with *D. shibae* DFL12^T.

***Isochrysis galbana* CCMP1323.**
Media are (from left to right) L1 medium, L1 medium lacking cobalamin, L1 medium lacking cobalamin inoculated with 10 µl *D. shibae* DFL12^T and L1 medium lacking cobalamin inoculated with 100 µl *D. shibae* DFL12^T

Figure 7 Cultures of the axenic dinoflagellate *Prorocentrum minimum* CCMP1329 (**a** and **b**) and the axenic haptophyte *Isochrysis galbana* CCMP1323 (**c**) inoculated with *D. shibae* DFL12^T in the absence of vitamin B_{12} and (**a** and **c**) or vitamin B_1 (**b**).

growth. Figure 7 shows the effects of cocultivation with *D. shibae* DFL12^T on cultures of *P. minimum* and *I. galbana* in media lacking cobalamin or thiamine.

Discussion

Adaptation to periodic anoxia

D. shibae DFL12^T is clearly optimized for respiratory modes of energy conservation. Its electron transport systems are fueled both by organic carbon compounds and light, with ubiquinone as a central intermediate pool. However, the arginine deiminase survival fermentation pathway and the ability to use alternative electron acceptors (nitrate, DSMO) allow *D. shibae* DFL12^T to sustain an active energy metabolism in the complete absence of oxygen. Several of the key genes involved are also found in other *Roseobacter* species. Therefore, the current paradigm that the *Roseobacter* clade comprises only obligate aerobes needs to be revised. Periodic anoxia is regularly encountered in photosynthetic biofilms at night as a result of intense respiration, as shown,

for example, for microbial mats (Steunou *et al.*, 2008). Moreover, steep gradients of oxygen and nitrate are present in marine sediments (Lorenzen *et al.*, 1998), and as microorganisms are easily transported by bioturbation to deeper layers (Pischedda *et al.*, 2008), the ability of *D. shibae* DFL12^T to switch between alternative electron acceptors is crucial.

D. shibae *DFL12^T is able to provide the dinoflagellate host with vitamins*

The genome data suggest that *D. shibae* DFL12^T is able to synthesize two nutrients that are essential and potentially growth limiting for their hosts, vitamin B_{12} (cobalamin) and vitamin B_1 (thiamine). The pathways for both of them have unique features: Altogether, three copies of the thiamine synthetase, *thiC*, are present on different plasmids (191 kb, 153 kb and 86 kb), whereas all the other thiamine synthesis genes are located on the chromosome. Synthesis of vitamin B_{12} should be possible by an oxygen-dependent as well as an oxygen-independent pathway in *D. shibae* DFL12^T, whereas organisms studied to date use only one of these pathways, pointing to both the importance of anaerobic conditions in the life of *D. shibae* DFL12^T and to the importance of B_{12}.

We experimentally confirmed that a pathway for *de novo* synthesis of B_{12} is functional in *D. shibae* DFL12^T and that the bacteria can provide both B_1 and B_{12} to the dinoflagellate *P. minimum* in a defined coculture. Thus, a symbiotic relationship between *D. shibae* DFL12^T and its dinoflagellate host may exist, which is based on an exchange of micronutrients synthesized at extremely low concentrations but high metabolic costs by the bacteria against photosynthate leaking from the algae during photosynthesis. As *D. shibae* is associated with several phylogenetically diverse species of algae, this symbiosis is not host specific. The dinoflagellate may replace *D. shibae* with other bacteria, provided they carry out the same essential functions, for example, synthesize B_{12}. Conversely, *D. shibae* may live with another algae if it excretes photosynthate, dimethylsulfoniopropionate and so on A variety of symbioses ranging from loose mutualistic interactions to obligate endosymbiotic relationships are increasingly discovered on the basis of genomic data and novel experimental methods (Moran, 2006). However, *D. shibae* DFL12^T could also be a scavenger of dead algae during the collapse of algal blooms. The metabolic flexibility as shown in the genome suggests that it might even switch from a symbiotic to a parasitic mode of life.

Growth limitation of phytoplankton by vitamins in the sea

Although 50% of all dinoflagellate species require B_{12}, they may not be dependent on a symbiosis with bacteria to obtain it (Croft *et al.*, 2005), as the requirements are so low that ambient concentrations in seawater may suffice (Droop, 2007). New methods have been used to determine the pmolar concentrations of B_1 and B_{12} *in situ* (Okbamichael and Sanudo-Wilhelmy, 2005). B_{12} concentrations correlate with bacterial densities at certain times and in some locations and decreased 90% during dinoflagellate blooms, relative to pre-bloom values (Gobler *et al.*, 2007). B_{12} colimitation with iron was, for the first time, shown in coastal waters of the Antarctic peninsula (Panzeca *et al.*, 2006) and later on in the Ross Sea, one of the most productive areas in the Southern Ocean (Bertrand *et al.*, 2007). In this study, the bacterial densities are low; cyanobacteria, which are able to synthesize B_{12}, are absent, and UV irradiance is high, all accounting for low ambient levels of B_{12}. These phytoplankton communities were iron limited, but adding B_{12} together with iron resulted in an additional increase in biomass. One of the dominant dinoflagellates present at the time was *P. minimum*, which was shown here to be auxotrophic for B_{12}, supporting the results of Bertrand *et al.* Thus, B_{12} can clearly be growth limiting in the sea under certain conditions. The composition of the bacterial flora may therefore have consequences for the succession of phytoplankton species (Panzeca *et al.*, 2008). Field investigations of bacterial communities associated with algae at such a level of detail, deciphering not only phylogenetic composition but also metabolic function, have not yet been carried out, but the breathtaking progress of sequencing whole microbial communities (Nealson and Venter, 2007) and, in the future, even single cells will eventually change this.

Horizontal and vertical gene transfer and the adaptive Roseobacter *gene pool*

The members of the *Roseobacter* clade are characterized by a significant fraction of extrachromosomal elements in their genomes (Pradella *et al.*, 2004), with *D. shibae* DFL12^T currently housing the largest number of plasmids among the fully sequenced strains. Conjugation obviously has a crucial role in the horizontal spread of these genetic elements, even if the experimental proof is still lacking. A conspicuous example is the presence of a 47-kb gene cluster on the two sister plasmids of *D. shibae* DFL12^T and on several other extrachromosomal elements of the *Roseobacter* clade, which also harbor the *vir* gene cluster required for conjugation. Genes that have been found on *Roseobacter* plasmids include not only typical plasmid-encoded traits such as degradation of aromatic compounds, but also key genes for biogeochemical cycles, for example, carbon monoxide oxidation in *D. shibae* DFL12^T and even the complete photosynthesis gene cluster in *R. litoralis* ((Pradella *et al.*, 2004) and integrated microbial genomes). The horizontal transfer of these traits within the *Roseobacter* clade is

consistent with their patchwork distribution in the phylogenetic tree (Buchan *et al.*, 2005). Our data show that the boundary between the chromosome and the plasmid is dynamic, as outsourcing of chromosomal genes to plasmids can be observed and increases the shared adaptive gene pool in the lineage.

Besides plasmids, phages also may mediate horizontal gene transfer. In total, 5% of the genome of *S.* TM1040 consists of prophages (Chen *et al.*, 2006; Moran *et al.*, 2007). A phage-like structure, the so-called GTA (gene transfer agent), was discovered many years ago in *Rh. capsulatus.* GTA particles contain only a few kilobases of DNA, are not inducible by mitomycin and do not form plaques. They contain small random pieces of host DNA, rather than a phage genome, suggesting that their main activity is the lateral transfer of host DNA. A search for the 15 GTA genes from *Rh. capsulatus* in complete and draft bacterial genomes showed that they are exclusively present in *Alphaproteobacteria*, and that all but one of the genome sequences in the *Roseobacter* clade have a GTA-like gene cluster, including *D. shibae* DFL12^T (Biers *et al.*, 2008). Thus, the genetic adaptability of the *Roseobacter* gene pool appears to be because of the richness of extrachromosomal elements, some of which are conjugative, the mobilization of genes from the chromosome to the plasmid, and a phage-like gene transfer mechanism unique in *Alphaproteobacteria*.

Outlook

Roseobacter bacteria are often associated with marine algae and reach high abundances during algal blooms. Our findings shed new light on this association, suggesting that it might be a symbiotic relationship in which a bacterium is required by the alga for the supply of essential nutrients, which can be growth limiting under certain conditions. Conversely, the bacteria may scavenge on dead algae and possibly switch between both types of interactions.

Studying the specificity and molecular mechanisms of this relationship in detail, both in the laboratory and in the sea, remains a fascinating field for future analyses.

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Chapter III

Discussion & Outlook

III.1 Discussion

This thesis focused on the isolation and investigation of new *Roseobacter* strains from the open southern North Sea. Applying dilution cultures with different media and low substrate concentrations new *Roseobacter* strains were successfully isolated (chapter II.1). This work provided the basis for studying certain physiological and ecological aspects of these roseobacters of which three strains were characterized as new species of new genera (chapter II.2 - II.4). In one study, the occurrence and abundance of *Planktotalea frisia* strain SH6-1^{T} in the North Sea was investigated using cultivation-independent approaches. Resulting hypotheses with potential relevance to its seasonal occurrence, i.e. specific interactions with phytoplankton algae, were examined in laboratory experiments (chapter II.5).

In general, studies on isolates provide valuable information addressing one of the fundamental questions in community ecology, i.e. what is the relationship between biodiversity and ecological functions in ecosystems? However, because the majority of prokaryotes can not be cultured yet, instead of laboratory experiments with isolates the application of cultivation-independent approaches increased within the last two decades (Fig. 1). In fact, several bacterial groups found to be ubiquitous and dominant in marine environments lack cultured representatives or at best achieve low cell densities in laboratory cultures (i.e. the clades SAR11, SAR86, SAR116, SAR202, SAR324, and RCA; cf. introduction). As a result, little is known about the physiology of members of these groups. To improve growth and to obtain isolates of yet uncultured taxa it is necessary to develop refined cultivation strategies, which is a challenging and time-consuming task (Giovannoni & Stingl, 2007; Stingl *et al*., 2007). Consequently, one could question if this is worth the effort or can cultivation-dependent studies easily be replaced?

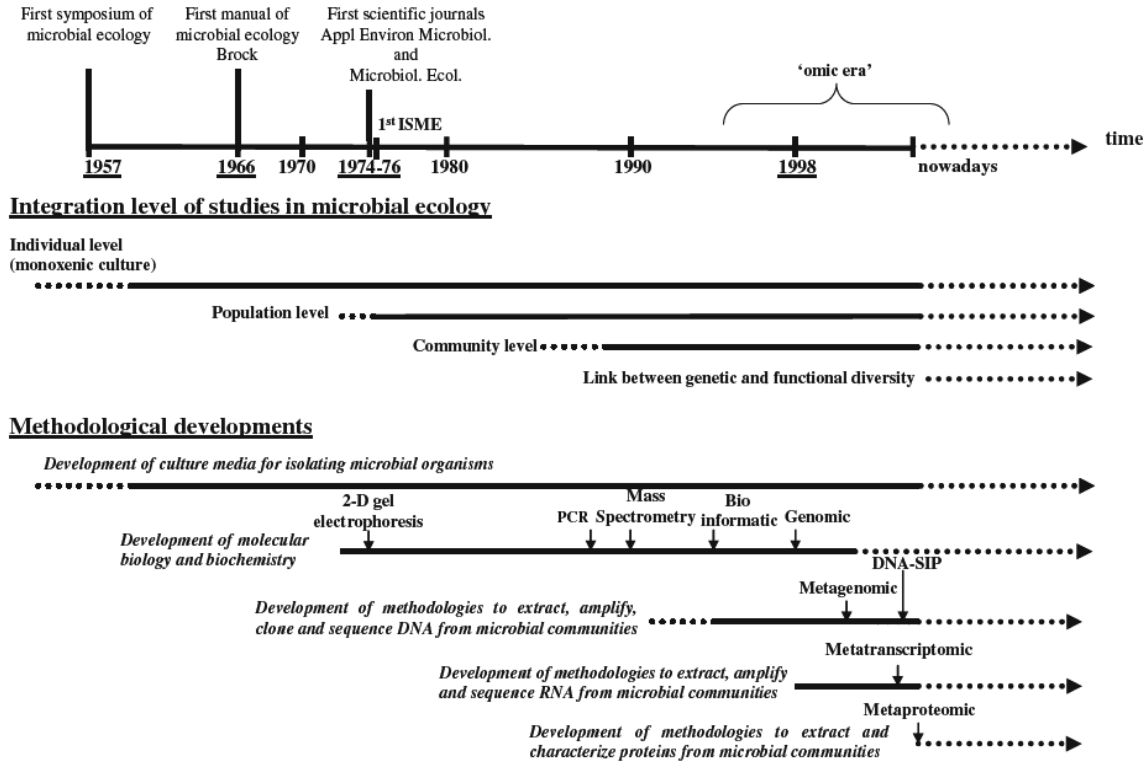

Fig. 1: Historical progressions in microbial ecology. Image: Maron *et al*., 2007

III.1.1 'Omics' versus the investigation of isolates

The cultivation-independent 'omic' techniques metagenomics, -transcriptomics, and -proteomics revolutionized the abilities to investigate aspects in marine microbiology and ecology as these methods provide data of total microbial communities including yet unknown organism and they represent *in situ* conditions. 'Omic' approaches entail several advantages but they also have certain limitations (reviewed by Morales & Holben (2010) for the nucleic acid-based techniques). Metagenomic analyses were first applied by Schmidt *et al*. (1991) to analyze the phylogenetic diversity of marine picoplankton. To construct metagenome libraries, environmental DNA has to be carefully extracted and either large fragments, which contain several genes arranged in precise order can be cloned in appropriate vectors such as fosmids or BAC (bacterial artificial chromosome) constructs (e.g. Béjà *et al*., 2000), or small fragments can be shotgun cloned in conventional vectors (e.g. Venter *et al*., 2004). One benefit of both ways is the bypass of PCR reducing the introduction of bias by amplification. Besides containing phylogenetic information in terms of 16S rRNA genes, metagenomic data provide access to genes encoding for proteins of biochemical pathways giving insights into the physiological potential of microbial assemblages. Due to the vast diversity of microorganisms in many environments, however,

the complete coverage of a metagenome or the complete assembly of individual genomes within a community is difficult. Exceptions are extremely simple communities such as acid mine drainage environments where the metagenome led to the reconstruction of almost complete bacterial genomes (Tyson *et al*., 2004). Metagenome libraries are particularly useful to investigate abundant taxa, species of lower abundance may be represented by only a few sequences.

To focus on the active population and *in situ* processes in environmental samples, the metagenomic approach was refined to metatranscriptomics targeting RNAs (Poretsky *et al*., 2005; Vila-Costa *et al*., 2010). Both nucleic acid-based techniques led to novel findings, the discovery of bacterial phototrophy mediated by proteorhodopsin in samples from the Sargasso Sea (Béjà *et al*., 2000) and the uncovering of new gene products (Rondon *et al*., 2000; Daniel, 2004; Steele *et al*., 2009) and unique small RNAs (Shi *et al*., 2009). As metatranscriptomics, metaproteome libraries also provide insights into *in situ* activities and processes and contain peptide sequences based on proteins extracted from an environmental sample (e.g. Kan *et al*., 2005; Maron *et al*., 2007; Sowell *et al*., 2009). In contrast to static genomes, transcriptomes and proteomes are dynamic, thus such data can be used to study responses to environmental conditions.

'Omic' techniques can facilitate the exploration of the uncultured majority of microorganisms, their metabolic capabilities and functional roles, however, physiological properties of complex microbial assamblages can only be inferred from fragmentary sequence data. In contrast, the investigation of isolates permits a comprehensive physiological characterization of whole organisms. Another shortcoming of the massive data generated by 'omic' techniques is that most nucleic acid and protein sequences can only be assigned to putative functions. Characterization of genes and proteins mostly rely on cultured organisms but as uncultured organisms represent the vast majority of microbial diversity (Rappé & Giovannoni, 2003; Floyd *et al*., 2005; Janssen, 2006; Jones *et al*., 2009; Spain *et al*., 2009) reference databases grossly underrepresent the functional potential of microorganisms. Furthermore, short sequences, which in particular are generated in shotgun libraries, lack information about the gene surrounding, which often provides the context to discern whether putative gene sequences are capable of producing a viable protein to ascertain an ecological function (Sorek & Cossart, 2009). To interpret sequence data properly, isolates are pivotal, which provide whole genomes. Using isolates physiological pathways and involved genes can be elucidated as well as conditions controlling its induction. An increase in assigning sequences to certain functions is being

accomplished as a result of the numerous genome-sequencing efforts such as the DOE-supported Genomic Science Program (http://genomicscience.energy.gov/), the NIH-sponsored Human Microbiome Project (http://nihroadmap.nih.gov/hmp/) and the Genomic Encyclopedia of Bacteria and Archaea Project (http://www.jgi.doe.gov/programs/GEBA/). The latter is a collaboration of the Joint Genome Institute (JGI, Walnut Creek, CA, USA) and the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), which aims to sequence the genome of at least one representative of every validly characterized bacterial and archaeal type species. This systematic genomic exploration is important to cover prokaryotic functional potentials present in diverse phylogenetic groups and will improve the interpretation of 'omic' data.

As bacteria in pure laboratory cultures do not necessarily exhibit metabolic activities which are carried out in their natural habitat, laboratory experiments also provide only limited information about a strain's physiology and its function *in situ*. Furthermore, nucleic acid-based 'omic' analyses eliminate the selective pressure imposed by laboratory cultivation, which can produce phenotypes that differ compared to those present in the environment (Elena & Lenski, 2003). Nonetheless, using isolates, different physiological properties can be demonstrated and responses to changing conditions can be studied in detail. In contrast, 'omic' data represent only a snapshot of a particular situation.

III.1.2 'Omics' and isolate studies – complementary tools in microbial ecology

As outlined above, cultivation-indepenent approaches can overcome shortcomings of cultivation-dependent techniques and vice versa. Using only cultivation-dependent methods, nothing would be known about some ubiquitous and abundant bacterial groups in marine environments, not even about their existence. 'Omic' data give valuable information about these yet uncultured organisms but in turn isolates are necessary to interpret such data properly. Although 'omics' information has not been a major factor in the isolation of new organisms, it might be valuable to design media to culture members of yet uncultured groups of marine prokaryotes. For the host-associated bacterium *Tropheryma whipleii* genomic information was successfully used to develop an isolation medium (Renesto *et al*., 2003). As mentioned above, genome assembly from metagenomic data of total complex microbial communities is very difficult, but when complexity is reduced by cell sorting, metagenomic data could lead to almost complete genomes (Morales & Holben, 2010) and be used to develop cultivation strategies. Laboratory studies

with isolates are also useful to confirm hypotheses that emerge from genomic data (cf. chapter II.6).

If we will be able to isolate organisms of many relevant groups of marine microorganisms, still the question remains, if we really will be able to mimic the complex marine environments in laboratory experiments to deduce bacterial functions in their natural habitats. Or do we also need approaches that probe cell activities *in situ*? In fact, we need to do both (Pedrós-Alió, 2006; Giovannoni and Stingl, 2007). 'Omic' analyses are at this time insufficient, but they provide valuable information about *in situ* processes and physiological potentials. Well-directed 'omic' studies such as comparative matatranscriptome or -proteome analyses of two different settings at almost the same location (e.g. at the edge of an algal bloom and outside of the bloom) could illustrate which processes are initiated by these changing conditions and how it affects the abundances of active taxa. Subsequently, resulting hypotheses can be studied in detail on relevant isolates in laboratory experiments.

III.2 Outlook

Further aspects regarding some of the new *Roseobacter* isolates obtained within this thesis seem to be interesting. As for *Pelagimonas elegans* strain SH36^T and closely related strains high abundances are indicated in the North Sea and the German Wadden Sea, respectively, these phylotypes might constitute an important component of bacterial communities in North Sea habitats. To study distribution and abundance patterns, the design of specific primers for qualitative and quantitative real-time PCR would be necessary (indeed, the former is currently being done at the ICBM, University of Oldenburg). Potential physiological characteristics and interactions with environmental factors deduced from such studies could be investigated in laboratory experiments.

For *Planktotalea frisia* strain SH6-1^T further investigations could be studies of expression patterns of its *puf* gene in the North Sea, which is involved in phototrophic energy generation. In general little is known about the ecological relevance of this physiological capability, which was found in several bacterioplankton members and different habitats (e.g. Béjà *et al*., 2002; Allgaier *et al*., 2003; Cottrell *et al*. 2006; Rathgeber *et al*., 2008). As *P. frisia* does not build bacteriochlorophyll *a* (bchl *a*) in laboratory cultures, an interesting question is, if this bacterium is able of photosynthetic energy conservation in its natural habitat at all or under which conditions, respectively. Gene expression patterns in coastal and offshore habitats in relation to the annual occurrence could indicate the function of this physiological trait or confirm the hypothesis of phototrophic energy generation as a way of maintaining the cells energy status under starvation conditions (Holert *et al*., 2011). Strain-specific PCR primers targeting the *puf* gene were already developed and tested during this thesis (however, so far only for qualitative analyses; see appendix). Additionally the expression of bchl *a* in cells of *P. frisia* could be investigated in environmental samples using a combination of microscopic infrared detection of chlorophyll and strain-specific fluorescent *in situ* hybridization.

In the near future, the environmental study of chapter II.1 will be extended by metagenomic and -proteomic data from biomass of the same sampling site collected in the course of this thesis. These analyses are currently being done at the University of Göttingen and the TU Braunschweig, respectively. Metagenomic data particularly give information about abundant taxa and their potential physiological properties, leading to hypotheses about capabilities which might support growth of bacterioplankton during the

algal bloom event. Additionally metaproteomic data provide information about active phylogenetic groups and about important processes, such as the degradation of specific phytoplankton products, carried out in this particular environmental setting. Such insights might lead to the screening for specific genes in the isolates of this study (as they are not genome sequenced) and to further investigations in laboratory experiments.

The exploration of marine microbial communities is one of the most challenging tasks in marine ecology as such communities are very complex and not easy accessible. Due to continuously refinded cultivation-dependent and -independent approaches along with advances in sequencing techniques, light could be shed on the main questions in marine microbial ecology, i.e. which microbial groups are ubiquitous in the ocean and which are abundant in different habitats? What kind of metabolic processes occur and which organisms are involved? How do microbial communities respond to changing conditions? Ongoing efforts in culturing marine prokaryotes and systematic genome sequencing provide an increasing potential to illuminate yet unanswered ecological questions about the unseen majority in the oceans.

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Appendix

Development of PCR primers targeting the *pufM* gene of *Planktotalea frisia* strain SH6-1^T

A fragment of the *pufL* and *pufM* genes of *P. frisia* strain SH6-1^T was amplified according to Béjà *et al*. (2002) and subsequently partly sequenced using the reverse primer pufMR. The sequence was compared to those in GenBank of the NCBI database using the BLAST function (http://www.ncbi.nlm.nih.gov/) and aligned to the *pufM* gene sequences of the uncultured bacterium clone 22-26 (FJ669188) and the uncultured bacterium env20m1 (AY044246) showing 87% and 82% similarity, respectively. Additionally, the *pufL* and *pufM* gene sequences of the closely related and genome sequenced *Rhodobacteraceae* bacterium HTCC2083 (99.1% 16S rRNA gene sequence identity to that of strain SH6-1^T) was used for the alignment. Using the BLAST function of the NCBI database this sequence was not detected, but in fact it showed highest sequence similarities. Based on the alignment possible primer sequences were deduced and specificities were checked with the NCBI databases. Sequences of specific primers were pufF(1b) (5' - AAG ACG CCA TAT GGC ACG - 3') and pufR(6) (5' - TCT AGA GAG CGC TTA AAA GG - 3') covering a 461 bp long gene sequence. Primers were tested under conditions shown in Fig. A1, as annealing temperature 62°C was considered (Fig. A2).

Component	µl
dNTP-Mix (je 2.5 mM)	5
10 x Puffer + 15 mM $MgCl_2$ GENECRAFT	5
BSA (3 mg/ml)	5
$MgCl_2$ (100 mM)	0,5
Primer A (20 pmol/µl)	0,5
Primer B (20 pmol/µl)	0,5
PCR H_2O	32,4
Taq-Polymerase (5U/µl), SupraTherm GENECRAFT	0,1

Mastermix: 49 µl
Cells of strain SH6-1^T: 1 µl

PCR conditions:

Temp (°C)	Time (min)	Cycles (n)
95	3	
95	1	
56 - 66	1	30
72	3	
72	10	
8	Stop	

Fig. A1: Gradient PCR conditions using the primers pufF(1b) and pufR(6).

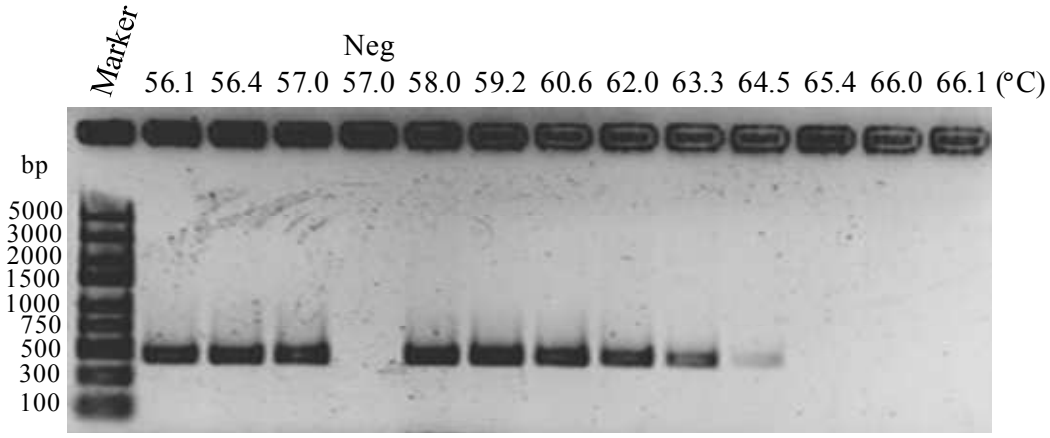

Fig. A2: Gradient PCR to determine the temperature optimum for amplification of the *pufM* gene of strain SH6-1^{T}.

Reference:

Béjà, O., Suzuki, M.T., Heidelberg, J.F., Nelson, W.C., Preston, C.M., Hamada, T., Eisen, J.A., Fraser, C.M., and DeLong, E.F. (2002) Unsuspected diversity among marine aerobic anoxygenic phototrophs. *Nature* **415:** 630-633.

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig verfasst und nur die angegebenen Quellen und Hilfsmittel verwendet habe.

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