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DISSERTATION

Titel der Dissertation

Community structure and distribution of functional microbial groups within two complex environments: microorganisms associated with marine sponges and potential sulfur-compounds-reducing microorganisms in peatlands.

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Doktorin der Naturwissenschaften (Dr. rer.nat.)

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Contents

Outline

Part I of this thesis describes the analysis of *dsrAB*-carrying microorganisms in terrestrial wetlands. Newly developed tools such as a functional gene microarray, quantitative PCR assays and *dsrB* based denaturing gradient gel electrophoresis as well as clone libraries were applied to unravel the widespread occurrence of deep-branching *dsrAB* lineages, which outnumbered characterized *dsrAB* lineages in the Schlöppnerbrunnen fen system.

Part II validates the signal variability in DNA microarray hybridizations. A simplified microarray format and numerical simulation tests were applied to investigate systematic variations in detected signal intensities and to explore underlying mechanisms of observed spatial gradients.

Part III gives a comprehensive overview on the community structure of sponge associated microorganisms. A meta-analysis of all public available sponge derived 16S rRNA gene sequences as well as of new sequences retrieved from so far unstudied marine sponges was conducted. Moreover, the mode of transmission of *Archaea* probably involved in ammonia oxidation was analyzed by 16S rRNA and ammonia monooxygenase gene based clone libraries. The presence of detected putative ammonia-oxidizing archaea (AOA) in adult sponges and corresponding sponge larvae was confirmed by fluorescence in situ hybridization.

Part IV includes a summary of the findings obtained in this thesis

The **Appendix** includes a list of the author’s publications, oral and poster presentations, the acknowledgment, and the curriculum vitae.

Part I

Sulfate reducers in peatlands

Introduction

1. Peatlands and their role in the global carbon cycle

Wetlands are a common part of the global landscape, with mainly three distinguishing characteristics to other landform units: (i) the permanent or periodic presence of a water table at, near or above the land surface, which determines (ii) a unique soil development that is frequently characterized by low oxygen content and (iii) specialized plant communities that are adapted to these environments (Cowardin et al. 1995). Peatlands, also called organic wetlands, build up a substantial fraction of these systems and are probably the most widespread type of wetlands by occupying globally an area of about $4{*}10^6$ km^2 (Joosten et al. 2002). They represent ecosystems where net primary production exceeded the rate of decomposition and as a result predominately organic, carbon rich matter, so-called peat, accumulated over time. Their persistence depends on a constant long-term water input, and the origin of this water influences the form and function of peatlands. Based on their main water source, peatlands can be divided in predominantly groundwater-fed fens (minerotrophic peatlands) and in rainwater-fed bogs (ombrotrophic peatlands) (Bridgham et al. 1996). The source of water considerably influences the geochemistry of the peatland. Minerotrophic fens receive nutrient-input from incoming mineralized groundwater and thus higher concentrations of cations and anions are present compared to exclusively precipitation-fed bogs. Bogs are oligotrophic, as isolation from surrounding mineral soil results in low nutrient and mineral content, which is often associated with low pH values (< 5) (Bridgham et al. 1996). Dominating vegetation forms are *Sphagnum* mosses, sedges and ericaceous shrubs, which are adapted to these conditions (Vanbreemen 1995).

Peatlands store about 15–30% of the world's soil carbon (Turunen et al. 2002; Davidson et al. 2006). Thus, they are massive deposits of carbon, acting as a net carbon sink and contributing to global cooling in the past 8,000 to 11,000 years (Frolking et al. 2007). Undisturbed peatlands are likely to continue functioning as net carbon sinks at the decadal scale, despite the large interannual variability of individual peatlands (Moore et al. 1998). However, environmental and anthropogenic factors, such as climate change and land-use, affect the carbon balance of these ecosystems and can lead to substantial carbon loss (Dise 2009). Primary carbon inputs in peatlands derive from plant-assimilated carbon dioxide, whereby energy-rich organic compounds are deposited as litter or released below-ground by plant roots. Carbon loss is dominated by the emission of the gases carbon dioxide and methane (Keller et al. 2007).

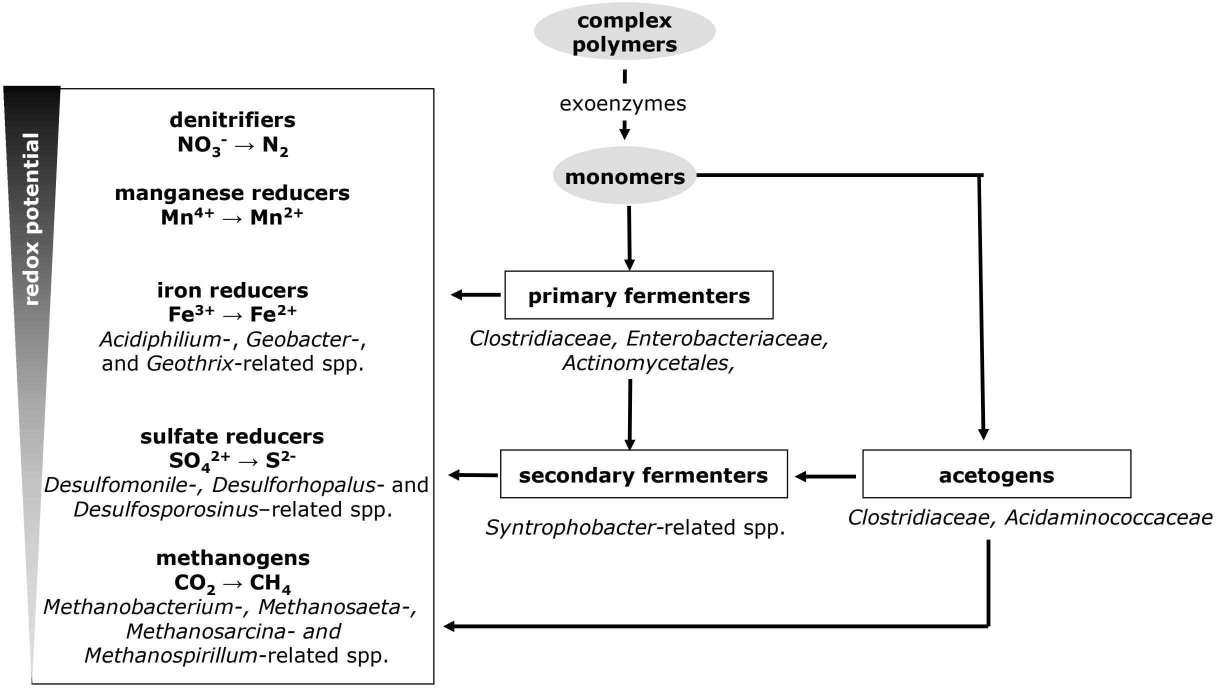

Figure 1: Carbon mineralization processes in peatlands. Extracellular enzymes hydrolyze complex organic matter to monomers. Organic substrates, methanol or carbon dioxide are oxidized to acetate by acetogens or degraded by primary fermenters to organic acids, alcohols and amino acids. Resulting products are available either for secondary fermentation (syntrophic microorganisms) or for the microbial mediated sequential reduction of nitrate, manganese, iron (III), sulfate and finally carbon dioxide. Microorganisms identified in the Schlöppnerbrunnen fen are displayed.

Soil microorganisms that use either oxygen or other electron acceptors as energy sources to decompose organic matter to carbon dioxide, methane and dissolved organic carbon play a major role in carbon effluxes from peatlands. As oxygen supply in peatlands is commonly restricted to small volumes, mainly anaerobic microbial mineralization using alternative electron acceptors takes place (Figure 1). Crucial factors influencing these decomposition processes are on one hand the availability of electron acceptors and on the other hand nutrient content and degradability of organic matter. In this context, the activity and potential environment-mediated inhibition of extracellular enzymes play a key role for substrate supply and microbial growth, as they hydrolyze complex organic matter to low molecular weight compounds (Makoi et al. 2008). Subsequently, acetogens are capable to oxidize a range of organic substrates (e.g. glucose), methanol or carbon dioxide with hydrogen as electron donor to produce acetate. Furthermore, primary fermenters degrade sugars to fermentation products, such as formate, lactate, butyrate and propionate. Resulting end products of these transformations are then available either for secondary fermentation catalyzed by syntrophic microorganisms or for several microbially mediated anaerobic reduction processes. Generally, a sequential reduction of nitrate, manganese, iron (III), sulfate and finally carbon dioxide is assumed, whereby respective respiration processes are regulated by differences in energy yields of the reactions (Achtnich et al. 1995). However, several studies have shown that respective processes can occur spatially and temporally at the same time in these heterogeneous and highly structured systems (Wieder et al. 1990; Yavitt et al. 1990; Koretsky et al. 2007). Processes using inorganic electron acceptors often do not fully explain total anaerobic mineralized carbon amounts in peatlands, and thus the presence of other organic electron acceptors, such as humic acids, and fermentation processes coupled to methanogenesis are supposed to contribute substantially to the decomposition of organic compounds (Lovley et al. 1996; Vile et al. 2003; Keller et al. 2007; Wüst et al. 2009).

2. Biogeochemical cycling of sulfur compounds in peatlands

Microbial mediated transformations of sulfur compounds are closely linked to the carbon cycle as reducing processes are associated with organic matter utilization in anoxic habitats such as peatlands. Peatlands have water saturated anoxic and overlying oxic zones with fluctuating water tables, and thus dynamic sulfur cycling is expected (Figure 2).

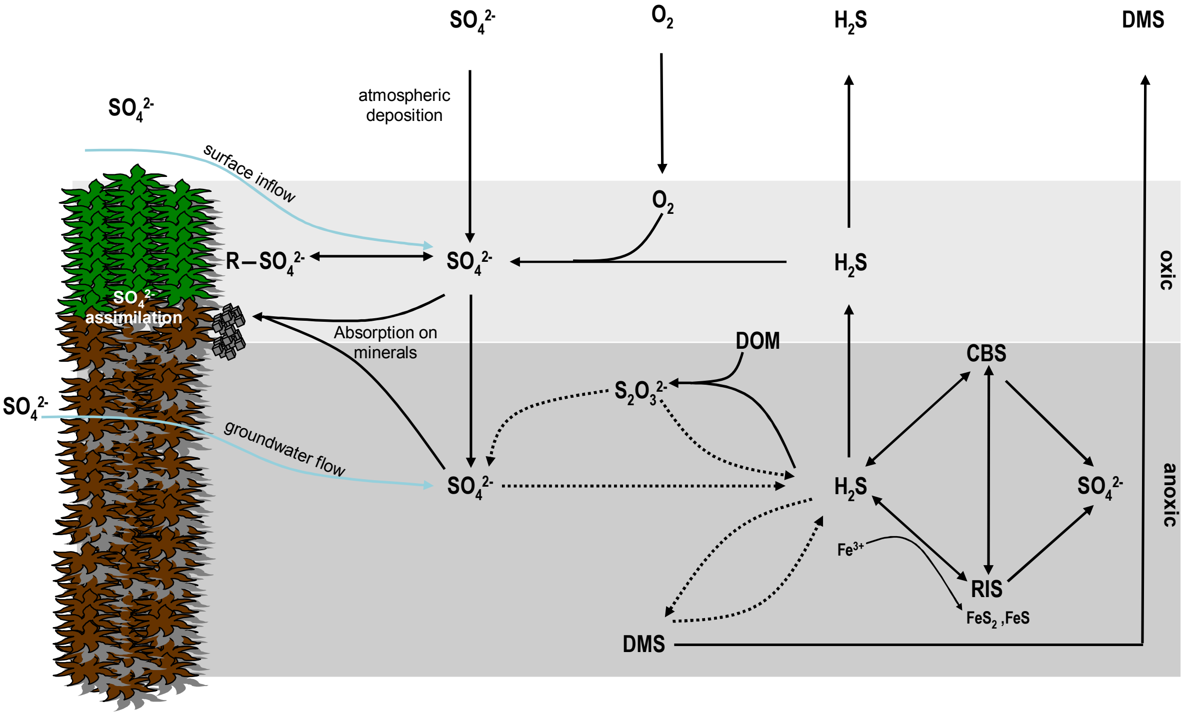

Figure 2: Biogeochemical sulfur cycle in peatlands. Sulfate (SO_4^{2-}) can be assimilated, adsorbed on minerals or forms organic sulfate esters ($R-SO_4^{2-}$). Microbial dissimilatory SO_4^{2-} reduction results in hydrogen sulfide (H_2S) production. In oxic zones, H_2S gets reoxidized chemically to sulfate. H_2S can also react to organic or carbon bonded sulfur (CBS) or inorganic reduced sulfur (RIS). RIS and CBS pools can be re-oxidized either aerobically or anaerobically. Chemical oxidation of H_2S by dissolved organic matter (DOM) to thiosulfate, which subsequently can be utilized by microorganisms for thiosulfate reduction or disproportionation into H_2S and SO_4^{2-} are proposed. Microbial mediated dimethyl sulfide (DMS) production in peatlands is suggested, which would be available for microbial reduction. Exclusively microbial driven transformations are indicated with dotted lines.

In bogs, sulfur is mainly introduced as sulfate (SO_4^{2-}) by atmospheric deposition, while in fens surface-water and groundwater inflows can contribute considerably to the sulfate pools. Sulfate can be assimilated by plants and microorganisms, adsorbed on soil minerals or forms organic sulfate esters ($R-SO_4^{2-}$), which may hydrolyze under sulfate-poor conditions (Mandernack et al. 2000; Clark et al. 2005). In the anoxic zone, sulfate-reducing microorganisms (SRM) use sulfate to gain energy via dissimilatory sulfate reduction resulting in the production of sulfide. Sulfide reacts with hydrogen to hydrogen sulfide (H_2S), which diffuses into the atmosphere or gets re-oxidized chemically to sulfate in oxic zones (Clark et al. 2005). H_2S can also react with organic matter to form organic or carbon bonded sulfur (CBS) (Wieder et al. 1988) or with inorganic compounds, such as iron, contributing to the inorganic reduced sulfur (RIS) pool (Chapman 2001). RIS and CBS pools tend to be unstable in peatlands and can be re-oxidized either aerobically with oxygen if the water table drops (Mandernack et al. 2000), or anaerobically, using iron(III) or other compounds as anaerobic electron acceptor (Wieder et al. 1988; Blodau et al. 2007). An alternative pathway could be chemical oxidation of H_2S by dissolved organic matter to thiosulfate (Heitmann et al. 2006; Heitmann et al. 2007), for example by quinone moieties of humic acids (Blodau *et al.* 2007). Subsequently, thiosulfate can be utilized by microorganisms for thiosulfate reduction to H_2S or oxidation to sulfate, respectively, or might be disproportionated into H_2S and sulfate and may thus maintain the activity of present SRM (Jorgensen 1990). Finally, microbial mediated dimethyl sulfide production in peatlands is suggested (Kiene et al. 1995). Nevertheless, emissions of dimethyl sulfide from peatlands may be insignificant as it seems to be degraded rapidly by SRM and methanogens in freshwater sediments (Lomans et al. 1999).

3. The guild of sulfate-reducing microorganisms and their diversity in wetland systems

SRM represent a phylogenetically and metabolically diverse functional guild of microorganisms. Based on phylogenetic analysis of their 16S rRNA genes, at present, recognized SRM are affiliated to seven different phylogenetic phyla, two within the *Archaea* (i.e. the euryarchaeotal genus *Archaeoglobus* (Stetter 1988) and the crenarchaeotal genera *Caldivirga* (Itoh et al. 1999) and *Thermocladium* (Itoh et al. 1998)), and five within the *Bacteria*. The major fraction of sulfate-reducing bacteria belongs to the class *Deltaproteobacteria*, followed by the *Firmicutes* harboring the sulfate-reducing genera *Desulfotomaculum* (Skerman et al. 1980), *Desulfosporosinus* (Stackebrandt et al. 1997), and *Desulfosporomusa* (Sass et al. 2004). Additionally, thermophilic SRM affiliated to *Nitrospira* (*Thermodesulfovibrio* spp. (Henry et al. 1994)), *Thermodesulfobacteria* (i.e. the genera *Thermodesulfobacterium* (Zeikus et al. 1983) and *Thermodesulfatator* (Moussard et al. 2004)) and the family *Thermodesulfobiaceae* (*Thermodesulfobium narugense* (Mori et al. 2003)) were isolated from various environments.

SRM are characterized by their capability to gain energy for growth and metabolism by coupling the oxidation of organic compounds or hydrogen to the reduction of sulfate to sulfide in a process termed dissimilatory sulfate reduction (Rabus et al. 2000). The multi-step process of dissimilatory sulfate reduction takes place in the cytoplasm or in association with the inner side of the cytoplasmic membrane and involves the transfer of eight electrons. Before being reduced, sulfate has to be activated with adenosine-5'-phosphate (ATP) by an ATP sulfurylase resulting in adenosine-5'-phosphosulfate (APS) and pyrophosphate. Subsequently, APS is converted to (bi-) sulfite by an APS reductase, thereby two electrons are transferred to the sulfur ion and adenosine-5'-monophosphate is released. The reduction of sulfite to sulfide involves the transfer of 6 electrons and is catalyzed by a dissimilatory (bi)sulfite reductase (DSR). The electron transport reactions lead to the formation of a proton motive force that drives ATP synthesis by an ATPase (Rabus et al. 2000). Many SRM are capable to grow also by using numerous other electron acceptors, such as sulfite, thiosulfate, dimethylsulfoxide, nitrate or elemental sulfur (Dalsgaard et al. 1994; Moura et al. 1997; Sass et al. 2009), which are reduced to sulfide as well. Based on their capability to oxidize low-molecular mass organic compounds either completely or incompletely, two main physiological groups are roughly distinguished: SRM involved in incomplete oxidation of organic compounds resulting in acetate as an end product, or those capable of complete oxidation resulting in carbon dioxide (Muyzer et al. 2008). Thereby, dissimilatory sulfate reduction leads to a relatively low energy gain, as for example the free energy change (ΔG) of complete oxidation of acetate or lactate is -48 or -128 kJ/mol, respectively (Rabus et al. 2000). In the absence of sulfate, several SRM can also switch to the fermentation of organic substrates, or shift to a syntrophic lifestyle, such as *Syntrophobacter* species (de Bok et al. 2002).

Several studies used 16S rRNA gene based approaches to investigate SRM diversity and distribution in complex systems. For instance, denaturing gradient gel electrophoresis targeting the 16S rRNA genes were used to detect selected groups of SRM in marine water columns and cyanobacterial mats (Teske et al. 1996; Teske et al. 1998), in industrial bioreactors (Dar et al. 2005) or in industrial paper mill (Maukonen et al. 2006). Furthermore, several 16S rRNA gene targeting probes for fluorescence *in situ* hybridization for the specific detection of different phylogenetic groups of sulfate-reducing bacteria are available (Lücker et al. 2007). As high throughput method, a DNA microarray targeting all 16S rRNA genes of recognized sulfate reducers was used to characterize the diversity of sulfate-reducing communities in tooth pockets and cyanobacterial mats, as well as in peatlands (Loy et al. 2002; Loy et al. 2004). To date, the application of this so-called SRM phylochip was the only study used to investigate specifically the 16S rRNA gene based composition of SRM in wetland systems.

The polyphyletic origin of SRM restricts diversity studies by comparative analysis of their 16S rRNA genes as no single pair of oligonucleotides allows specific detection of all SRM using the

16S rRNA genes as targets. As alternative, phylogenetic marker targeting functional genes encoding for enzymes involved in the metabolic pathway of sulfate reduction can be used to identify SRM. For instance, the genes encoding for the APS reductase (*apsA*) as well as for the DSR (*dsrAB*) were used in several studies to unravel SRM diversity (Hipp et al. 1997; Friedrich 2002; Wagner et al. 2005). The presence of both enzymes is not restricted exclusively to SRM, but they share these enzymes with members of the guild of sulfur oxidizing microorganisms (SOM). In contrast to the APS reductase, where *apsA* of SRM and SOM exhibit a patchy affiliation in comparative phylogenetic analyses (Meyer et al. 2007), *dsrAB* of SRM and SOM are clearly separated in two distinct lineages, allowing a direct assignment of new environmental gene sequences to one of these guilds (Loy et al. 2009). *DsrAB* genes are present in all known SRM, however, some other DSR carrying microorganisms like *Sporotomaculum* (Qiu et al. 2003) and *Pelotomaculum* (Imachi et al. 2007), whose *dsrAB* genes are phylogenetically not clearly separated from those of SRM, are not able to reduce sulfate but live only in syntrophic communities. Beside sulfate, *dsrAB* carrying microorganisms can use several alternative inorganic and organic sulfur electron acceptors for energy conservation (Rabus et al. 2000). In the absence of a suitable electron acceptor, they are also able to use fermentation to gain energy. Accordingly, *dsrAB* based surveys identify not exclusively SRM, but include syntrophic microorganisms as well as sulfite, thiosulfate and/or organosulfonates reducers.

Numerous *dsrAB* based surveys were applied to explore SRM community composition and distribution. For instance, they are well studied in marine ecosystems (Dhillon et al. 2003; Nakagawa et al. 2006; Leloup et al. 2007; Leloup et al. 2009), where high sulfate concentrations of up to 28 mM favor their growth and as a result dissimilatory sulfate reduction was shown to be one of the main mineralization processes in marine sediments (Jørgensen 1982). Their widespread occurrence was also demonstrated in brackish systems such as estuarine sediments (Joulian et al. 2001; Jiang et al. 2009), in freshwater habitats such as lake sediments (Karr et al. 2005; Vladar et al. 2008) and in wastewater treatment plants (Schramm et al. 1999; Dar et al. 2007). In terrestrial ecosystems, research focuses mainly on specialized waterlogged soils like rice paddies (Liu et al. 2009), whereas only few studies investigated the SRM community composition in natural freshwater wetlands such as peatlands. For instance, sulfate-reducing assemblages in the Everglades were investigated using *dsrAB* based clone libraries and terminal restriction fragment length polymorphism (T-RFLP) analysis targeting the DSR subunit A (*dsrA*) (Castro et al. 2002; Castro et al. 2005). Sulfate reduction rates revealed higher activities of SRM in eutrophic zones, which were influenced by sulfate input from agricultural runoff, than in more pristine soils (Castro et al. 2002). There, a highly diverse *dsrAB* composition was detected with the majority of DSR sequences affiliated to different gram-positive *Desulfotomaculum* spp. and to sequences of yet uncultured microorganisms. In the eutrophic regions, most of the *Desulfotomaculum* associated sequences were related to microorganisms that are able to completely oxidize their organic substrates, whereas in the pristine regions all *Desulfotomaculum*-like sequences were exclusively related to incomplete oxidizers (Castro et al. 2002). Additionally, indications were given that sulfate concentration is not the key driver for differences between SRM assemblages, but a selection by the type and amount of electron donor was suggested (Castro et al. 2005). In a more recent approach, spatial relationships between *dsrAB* diversity and concentrations of metals and sulfur were studied in peatland soils in western New York uncovering a close dependency of *dsrAB* based community composition and redox stratification. *DsrAB* genes were mainly detected in deeper soil layers, where sulfur accumulation was evident. In the top peat layers, where manganese accumulation indicated oxic conditions, no *dsrAB* genes were detected (Martinez et al. 2007).

4. The Schlöppnerbrunnen fen as a model system

The main study site of the first part of this thesis, the minerotrophic fen Schlöppnerbrunnen II, lies in the north-eastern part of Bavaria within the forested Lehstenbach catchment (Fichtelgebirge, Germany). The total sulfur concentration in the fen soil as well as in the surrounding forest and mineral soils is in the upper range of the values reported for forest soils in Europe (~3 – 6.5 g*kg^{-1}) (Prietzel et al. 2009). Most likely these high concentrations are caused from intensive deposition of atmospheric sulfur originating from combustion of soft coal in Eastern Europe. Although there has been a substantial reduction in air sulfur emissions since the late 1980s (Klemm et al. 1999), desorbed sulfate is still transported via groundwater flow from upland soils into the groundwater-fed wetland. Most of the introduced sulfate is retained by accumulation of inorganic and organic reduced sulfur species (RIS and CBS) in the soil (Prietzel et al. 2007), whereby estimates of RIS amounts range from <10% (Paul et al. 2006) to ~35% of total sulfur (Prietzel et al. 2009). Intermediate forms, such as sulfones, are abundant and represent 32% - 41% of the sulfur pool and oxidized sulfur species account for 5% - 23% of the total sulfur pool, respectively (Prietzel et al. 2009). Additionally, sulfate concentrations of soil solutions demonstrated unexpected stable concentrations over time, which points to a well-buffered system regarding sulfate dynamics (Alewell et al. 2006).

Biochemical analysis of several studies led to the conclusion that Schlöppnerbrunnen II is dominated by alternating oxidation–reduction cycles (Alewell et al. 2006; Paul et al. 2006; Prietzel et al. 2009), possibly caused by oxygen diffusion from roots of vascular plants, which are common in the fen (Paul et al. 2006). It has been shown that the habitat is characterized by the co-existence of redox processes, such as nitrate, sulfate, iron, manganese reduction and methanogenesis at a small spatial scale of few cm (Alewell et al. 2006; Paul et al. 2006; Alewell et al. 2008). The presence of high dissolved organic carbon concentrations could favor the simultaneous presence of different reduction processes, as electron donors are possibly not reaction limiting (Alewell et al. 2008). Additionally, in the presence of oxygen, organic and inorganic sulfur species will be re-oxidized (Paul et al. 2006) resulting in mobile sulfate formation. Sulfate can to some extent be adsorpted to iron oxide, which is present in high concentrations in the system (Abdelouas et al. 2000; Alewell et al. 2006; Alewell et al. 2008) or is used as electron acceptor by SRM (Loy et al. 2003; Loy et al. 2004; Schmalenberger et al. 2007). Part of the sulfate is most likely lost from the fen with the drainage water. Consequently, although it was shown that the Schlöppnerbrunnen II fen can act temporarily as a hot spot for dissimilatory sulfate reduction (Alewell et al. 2001), long-term maintenance of sulfur species within the system is not expected (Paul et al. 2006; Prietzel et al. 2009).

The microbial diversity in the Schlöppnerbrunnen fen systems was examined in several studies. The application of the so-called SRM phylochip was the only study used to investigate specifically the 16S rRNA gene based composition of SRM in the Schlöppnerbrunnen fen system, whereby only *Syntrophobacter*- and *Desulfomonile*- like bacteria could be identified. Three further studies investigated the Schlöppnerbrunnen fen system using general 16S rRNA gene targeting oligonucleotides. A two-dimensional single strand conformation polymorphism (SSCP) approach revealed significant differences between the microbial communities of oxic and anoxic zones revealing sequences mainly affiliated to *Proteobacteria* and *Acidobacteria* as dominant bacterial groups (Schmalenberger et al. 2008). The application of 16S rRNA based stable isotope probing identified, in addition to the aforementioned groups, sequences related to *Actinobacteria* and *Clostridia* as active anaerobic consumers of monosaccharides like xylose and glucose (Hamberger et al. 2008). Furthermore, uncultured *Archaea* related to *Methanosarcinaceae*, *Methanobacteriaceae* and *Crenarchaeota* were identified in the heavy fraction of the anoxic slurries. Additional anoxic microcosm studies retrieved further formate-consuming and obligate acetoclastic methanogens, related to the families *Methanomicrobiaceae* and *Methanosaetaceae*, as potential trophic partners for moderately acid tolerant fermenters (Wüst et al. 2009). Furthermore, *dsrAB* based clone libraries and T-RFLP analyses were engaged to investigate diversity patterns of SRM in the Schlöppnerbrunnen fen. *DsrAB* based analyses revealed a highly diverse community structure with many novel *dsrAB* sequences unrelated to known SRM (Loy et al. 2004; Schmalenberger et al. 2007). T-RFLP analysis distinguished a high diverse community pattern over a depth gradient of 0 cm to 50 cm clustering in three subgroups, namely the upper 10 cm, 15 – 25 cm and 30 – 50 cm depth zones. The environment in the upper fen soil is controlled by the rhizosphere, which permits cation exchange, O_2 leakage and release of organic acids. Additionally, due to fluctuations in the water table, the upper layers are exposed to oxygenation and drying, which enhance chemical or microbial renewal of reducible electron acceptors. Thus, detected SRM have to be O_2 tolerant and – as sulfate concentrations were very low – a syntrophic lifestyle of the resident SRM was suggested (Schmalenberger et al. 2007).

It was the aim of the first part of this thesis to contribute to a better understanding of the community structure and distribution of *dsrAB* containing microorganisms, which are possibly involved in the biochemical sulfur cycling in terrestrial wetlands. The description of microbial diversity is the first essential step for the exploration of the composition, interactions, and dynamics of microbial communities within their natural environment. Potent and high-throughput techniques are needed to compile habitat specific microbial inventories and thus, novel tools for rapid community screening, such as a *dsrB* based DGGE assay and a newly developed functional gene array, were applied.

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Microorganisms with Novel Dissimilatory (Bi)Sulfite Reductase Genes are Widespread in Geographically Distant, Low-Sulfate Peatlands

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Abstract

Schlöppnerbrunnen peatlands of the Lehstenbach catchment (Germany) house so far unidentified microorganisms with phylogenetically novel variants of the dissimilatory (bi)sulfite reductase genes *dsrAB*. These genes are generally characteristic for microorganisms that reduce sulfate, sulfite or some organosulfonates for energy conservation, but can also be present in anaerobic syntrophs. The abundance, community dynamics, and biogeography of *dsrAB*-carrying microorganisms in peatlands were unknown. Soils from different depths were sampled at three time points over a six-year period to analyze the diversity and distribution of *dsrAB*-containing microorganisms at the Schlöppnerbrunnen II fen site by newly developed functional gene microarray and quantitative PCR assays. Members of novel, uncultivated *dsrAB* lineages (approximately representing species-level groups) (i) dominated a temporally stable but spatially diverse *dsrAB* community and (ii) represented 'core' members (relative abundances of 1-2%; *dsrA* copy number versus the total number of bacterial and archaeal 16S rRNA genes) of the autochthonous microbial community in this fen. A bacterial and archaeal 16S rRNA gene inventory of a fen soil sample with relatively high abundance of two novel *dsrAB* lineages was dominated by clone sequences of the phylum *Acidobacteria*. Members of acidobacterial subdivisions 1, 2, and 3 were present in the Schlöppnerbrunnen fen at relatively high 16S rRNA gene abundances of 5.6-26.3%, 6-15.5%, and 3.6-13.1%, respectively, with low depth-dependent variation, suggesting an important biogeochemical function in oxic and suboxic peat layers. Correlation analysis of quantitative PCR data provided no indications that members of the three acidobacterial subdivisions were carriers of the two novel *dsrAB* variants. Finally, denaturing gradient gel electrophoresis (DGGE)- and clone library-based comparison of the *dsrAB* diversity in soils from a wet meadow, three bogs, and five fens of various geographic locations (distance ~1-400 km), identified one *Syntrophobacter*-related and nine uncultivated *dsrAB* lineages to be widespread in different peatlands. Signatures of biogeography in *dsrB*-DGGE data were not correlated with geographic distance, but could largely be explained by soil pH and wetland type; implying that distribution of *dsrAB*-carrying microorganisms in wetlands on the scale of a few hundred kilometers is not limited by dispersal but determined by contemporary environmental conditions.

Introduction

Peatlands contain 15% to 30% of global soil carbon (24, 114) and represent a net carbon sink that contributed to global cooling in the past 8,000 to 11,000 years (35). While peatlands are generally rather resilient to external perturbation, long-term global changes such as warming, decreased precipitation, and increased atmospheric deposition of reactive nitrogen and sulfur compounds might induce transformation of peatlands to new ecosystem types, accompanied by unforeseeable changes in the carbon balance (30). Carbon loss from peatlands is mainly mediated through anaerobic microbial decomposition of organic matter to the greenhouse gases carbon dioxide and methane (55). Ten to twenty percent of globally emitted methane derives from peatlands (15), (119, 121). Primary and secondary fermentation and methanogenesis are considered to be the main carbon degradation processes because of the absence or limited availability of alternative electron acceptors. However, other microbial processes such as denitrification and dissimilatory iron and sulfate reduction can occur together with methanogenesis in the same peat soil fraction and contribute considerably to anaerobic carbon mineralization (63). Fluctuations in environmental conditions on short- and long-term scales govern trophic interdependencies among the involved microorganisms. Transitions between synergistic (e.g. syntrophic interspecies transfer of hydrogen/formate) and antagonstic (e.g. competition for the same substrates) microbial interactions determine the extent of carbon flow diversion away from methanogenesis. A prime example is the suppression of microorganisms catalyzing methanogenic carbon degradation by sulfate-reducing microorganisms (SRM) that are energetically-favored in the competition for substrates such as acetate, alcohols, and hydrogen (36, 115, 116). While sulfate concentrations are generally low in peatlands (10 to 300 µM), ongoing sulfate reduction at rates (2.5 to 180 nmol cm^{-3} d^{-1}) that are comparable to rates in sulfate-rich environments is fuelled by an anoxic recycling of sulfur compounds via the 'thiosulfate shunt' (10). Alternative replenishment of the sulfate pool by reoxidation of reduced sulfur species in the presence of oxygen is dependent on vegetation type and alternating periods with precipitation and drought (31, 92, 124). In addition, increasing global atmospheric sulfur pollution and acid precipitation contributes to terrestrial sulfate pools and is predicted to repress methane emissions from peatlands by up to 15% within the thirst third of this century (37).

Given the significance of dissimilatory sulfate reduction, it is surprising that most information about the identity of microorganisms that catalyze this process in peatlands derives from studies of a single model fen system (Schlöppnerbrunnen) located in the forested Lehstenbach catchment (Bavaria, Germany). Different redox-processes such as fermentation (43), methanogenesis (44), Fe(III) reduction (100), and sulfate reduction (1, 75) are ongoing on the study site (3). Atmospheric deposition of sulfur originating from combustion of soft coal in Eastern Europe until the 1990s led to the accumulation of sulfur species in the soils of this catchment. Although air pollution decreased in recent years (61), previously stored soil sulfate can desorb and is transported from upland soil into the groundwater-fed fen where it drives dissimilatory sulfate reduction (Alewell 2000). DNA stable isotope probing using in situ concentrations of typical ^{13}C-labeled degradation intermediates (mixture of lactate, acetate, formate, and propionate) has shown that a low abundance *Desulfosporosinus* species, representing on average only 0.006% of the total bacterial and archaeal 16S rRNA genes, could be responsible for a major part of sulfate reduction in the studied fen (Pester *et al.* submitted). In addition, other microorganisms that are potentially involved in sulfate reduction were previously detected in this fen using 16S rRNA gene- and *dsrAB*-based diversity analyses. Few *dsrAB* sequences were affiliated with the SRM genera *Desulfomonile* and *Syntrophobacter*, while most other sequences could derive from new taxa, as these novel *dsrAB* lineages have no close cultivated relative (75, 106) Pester *et al.* submitted). Microorganisms that respire sulfite or sulfate anaerobically depend on the *dsrAB*-encoded key enzyme dissimilatory (bi)sulfite reductase for energy conservation and thus these genes have been widely used as markers for PCR-based molecular diversity studies of this guild (29, 58, 67, 95, 117). However, also some organisms that are phylogenetically related to SRM, but have seemingly lost the ability for sulfite/sulfate reduction, can harbor *dsrAB*. The *dsrAB* sequences of these organosulfonate-reducers (8, 19, 20) or syntrophs (48, 98) can be amplified by the commonly used DSR1F-DSR4R PCR primer mix (74). Consistent with the notion that *dsrAB* can derive from non-SRM, DNA stable isotope probing of *dsrAB* in the model peatland did not indicate that the novel lineages are linked to lactate-, acetate-, formate- or propionate-dependent sulfate reduction (Pester *et al.* submitted). Besides their unknown ecophysiological function in the fen, additional questions regarding the biology of these enigmatic *dsrAB*-containing microorganisms remain unanswered: what is their taxonomic affiliation and are they endemic only to this particular fen site or more widely distributed in different types of wetlands?

Based on the repeated detection of the same or similar operational taxonomic units (OTUs) of *dsrAB* (75, 106) we hypothesize that some of these yet unidentified *dsrAB*-containing microorganisms are part of the autochthonous microbial community in the Schlöppnerbrunnen model peatland. Using newly developed functional gene array (FGA) and quantitative real-time PCR (qPCR) assays, we analyzed the distribution and abundance of individual *dsrAB* lineages in different depths of the peatland soil in samples collected at three time points over a period of six years. For linking of *dsrAB* and phylogenetic sequence information obtained from clone library analyses of bacterial and archaeal 16S rRNA genes, we tested if abundances of selected *dsrAB*-OTUs correlated with the abundances of the dominant 16S rRNA gene sequence clusters in the fen. We further investigated the diversity of *dsrAB* in nine geographically separated wetlands, representing three different wetland types, for patterns in biogeography and endemism by using *dsrB*-denaturing gradient gel electrophoresis (DGGE) and *dsrAB* clone library analyses.

Materials and Methods

Description of sites and sampling of soil.

The geographic distribution and major characteristics of the nine wetland sites analyzed in this study are summarized in Table S1 and Figure S1. The main sampling site, the acidic lowland fen Schlöppnerbrunnen II, is located in the Lehstenbach catchment in the Fichtelgebirge mountains (Bavaria, Germany) (see (2, 64, 75) for a detailed site description). Samples collected in 2001 (July 24th), were retrieved from a single soil core, which was divided in four depth sections (0-7.5, 7.5-15, 15-22.5, and 22.5-30 cm) (75). In 2004 (September 21st) and in 2007 (May 16th), soil cores (diameter ~8 cm) were collected from three random locations within the site (approximate distance between locations was 15 m) and were subsequently divided in four depth sections (0-5, 5-10, 10-15, and 15-20 cm). Two additional depth sections (20-30 and 30-40 cm) were collected from two locations in 2007. Besides Schlöppnerbrunnen II, triplicate soil cores (two depth sections ~0-20 and ~20-40 cm) were taken from random locations at eight additional wetland sites in Italy and Austria (Table S1). Immediately at the sampling site, samples for molecular analyses were homogenized, frozen on dry ice for transportation, and stored at -80 °C upon arrival in the laboratory.

DNA extraction.

For microarray, DGGE and qPCR analysis, DNA was extracted from approximately 250 mg (wet weight) of each soil sample using the Power Soil^{TM} DNA Kit (MoBio Laboratories, Solana Beach, CA). For the preparation of a 16S rRNA gene library from Schlöppnerbrunnen II fen soil (sampled in 2004, 10-15 cm soil section), genomic DNA was additionally extracted from triplicate soil samples using the protocol of Zhou *et al.* (127) and a slightly modified version of the protocol of Griffiths *et al.* (40). In contrast to the original protocol, precipitation of nucleic acids was performed with 0.1 volume of sodium acetate (pH 5.2) and 0.6 volume of isopropanol for 2 h at room temperature.

Fluorescence labeling of target genes for microarray hybridization.

An approximately 1.9 kb fragment of *dsrAB* was PCR amplified from 5 ng reference clone DNA or 50 ng environmental DNA using the degenerate primers DSR1Fmix (equimolar mixture of 10 µM of each DSR1F variant) and DSR4Rmix (equimolar mixture of 10 µM of each DSR4R variant) (Table S2). 16S rRNA and nucleotide transport protein (*ntt*) reference genes, targeted by control probes, were amplified by primer pairs listed in Table S3 (50 µM of each primer). All forward primers contained a T3 promoter site sequence (5'-AATTAACCCTCACTAAAGGG-3') at the 5′ end to enable T3 RNA polymerase-based reverse transcription labeling of the PCR products. PCR mixtures (50 µl) were prepared by using 2 mM of each deoxynucleoside triphosphate, 10x Taq buffer, 2 mM $MgCl_2$ and 2 U of Taq DNA polymerase (Fermentas Inc., Hanover, MD, USA). For amplification of environmental samples, 1 µl of bovine serum albumine (20 µg/µl, New England BioLabs Inc., Beverly, MA, USA) was additionally added to each reaction to enhance PCR efficiency. Reference and control genes were amplified using an initial denaturation step at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 48°C (*dsrAB*), 52°C (16S rRNA gene) or 55°C (*ntt*) for 30 s, and elongation at 72°C for 1 min 10 sec. The cycling was completed by a final elongation step at 72°C for 3 min. PCR amplification of *dsrAB* from environmental DNA extracts was carried out by two successive "hot start" PCRs to minimize unspecific amplification products (23). The first PCR was performed in touch-down mode by using an initial denaturation step at 95°C for 3 min, followed by 10 cycles of denaturation at 95°C for 30 s, annealing at 58-48°C for 30 s (after each cycle the temperature was reduced by 1°C), and elongation at 72°C for 1 min 10 s. Additional 10 PCR cycles at a constant annealing temperature of 48°C were performed prior to the final elongation step at 72°C for 3 min. PCR products were examined by 1%-agarose gel electrophoresis for presence and sizes of amplicons. PCR products were purified from the gel using the Montage™ DNA Gel Extraction kit (Millipore, Bedford, MA, USA) according to the manufacturer's instructions. One microliter of purified PCR product was reamplified in a second PCR applying an initial denaturation step at 95°C for 3 min, followed by 20 cycles of denaturation at 95°C for 30 s, annealing at 48°C for 30 s, and elongation at 72°C for 1 min 10 sec. The cycling was completed by a final elongation step at 72°C for 3 min. PCR products were purified using the QIAquick PCR purification kit (QIAgen, Hilden, Germany).

Target labeling was achieved by *in vitro* transcription according to the following protocol. 500 ng of purified PCR product, 4 µl 5x T3 RNA polymerase buffer (Fermentas), 2 µl dithiothreitol (100 mM), 0.5 µl RNAsin (40 U/µl) (Promega GmbH, Mannheim, Germany), 1 µl each of ATP, CTP, and GTP (each 10 mM), 0.5 µl UTP (10 mM), 2 µl T3 RNA polymerase (20 U/µl) (Fermentas) and 0.75 µl Cy3-UTP (5 mM) were adjusted with RNase-free water to a total volume of 20 µl and incubated at 37°C for 4 hours. The DNA template was subsequently degraded by adding 2 U DNase I (Fermentas) and incubation at 37°C for 15 min. Enzymatic digestion was stopped with 2 µl of EDTA (25 mM, Fermentas). Following adjustment of the volume to 100 µl with TE buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA), RNA was precipitated with 10 µl of 5M NaCl and 300 µl of ethanol. RNA was washed with 500 µl of ice-cold 70% ethanol and resuspended in 50 µl TE buffer. RNA concentration and the amount of incorporated dye were measured with a ND-1000 spectrophotometer (Nanodrop, Inc.). Labeled RNA was fragmented by incubating with 10 mM $ZnCl_2$ and 20 mM Tris/HCl (pH 7.4) at 60°C for 30 min. Fragmentation was stopped by the addition of 10 mM EDTA pH 8.0. Labeled RNA was divided in several aliquots that were stored at -20°C.

Probe design and manufacturing of microarrays.

Probes targeting *dsrA* or *dsrB* were designed using the probe tools of the ARB software package (78) and a *dsrAB* ARB database containing approximately 500 sequences (>1500 nucleotides) from pure cultures (74, 129) and environmental studies. Based on a distance matrix tree of all previously published *dsrAB* clone sequences retrieved from Schlöppnerbrunnen I+II system (75, 106), 146 probes with an average length of 30 bases were designed targeting different clone groups from the study site (Figure S2). Thermodynamic properties of the probes were calculated using the two-state hybridization server of DINAMelt (settings: linear DNA, 37°C, 1 M Na^+, 0 M Mg^{2+}, 0.1 mM strand concentration) (80, 81). Probes lengths were adjusted to obtain similar theoretical free energy (deltaG) values. Final probes had a length of 27 to 34 nucleotides with an average deltaG of -41.0 ± 0.7 kcal/mol. The in silico specificity and number of weighted mismatches to non-target *dsrAB* sequences of each probe were determined using probeCheck (73). Oligonucleotides for microarray spotting were synthesized by Microsynth (Balgach, Switzerland). The 5’- end of each oligonucleotide probe carried a T-spacer consisting of 30 dTTP molecules and the 5'-terminal dTTP was aminated to allow covalent coupling of the oligonucleotides to aldehyde group-coated VSS-25 glass slides (CEL Associates, Houston, Tex.). Each probe was adjusted to a concentration of 50 pmol/µl in 50% dimethyl sulfoxide and printed on the slide by using a BioRobotics MicroGrid spotter (Genomics Solutions, Ann Arbor, MI, USA) under constant temperature (20°C) and humidity (min. 50%) conditions. Each microarray contained three and six replicate spots of each *dsrA*/*dsrB*-targeted and control probe, respectively. Freshly spotted DNA microarrays were incubated overnight at room temperature in a humid chamber. Slides were further treated with sodium borohydride as described previously (76).

Microarray hybridization.

For clones 80 ng and for environmental samples 500 ng of labeled *dsrAB* RNA fragments and defined amounts of labeled RNA targeting the control probes (Table S3) were mixed with 120 µl of hybridization buffer [0.1% SDS, 0.1% 100x Denhardt's reagent (Invitrogen), 6x SSC and 15% formamide] and incubated at 65°C for 5-15 min. The ThermoTWISTER incubator (QUANTIFOIL Instruments GmbH, Jena, Germany) and the HybriWell Sealing System HBW2222-FL (Grace BioLabs) were used for hybridization. Microarrays were hybridized for 17h at 55°C under continuous shaking at 400 rpm. Following hybridization, slides were washed by shaking at room temperature for 5 min in 2x SSC/0.1% SDS, for 5 min in 0.1x SSC, and finally for 20 s in ice-cold double-distilled water. Slides were dried by centrifugation (3 min, 300 g), stored at room temperature in the dark, and scanned the same day.

Scanning and data analysis.

Fluorescence images were recorded by scanning the slides at three lines to average and at 10-µm resolution with a GenePix Personal 4100A array scanner (Axon Instruments, Molecular Devices Corporation, Sunnyvale, CA, USA). Gain of the photo multiplier tube was adjusted to record images with signal intensities just below the saturation level. Scanned images were saved as multilayer tiff images and analyzed with the GenePix Pro 6.0 software (Axon Instruments). Low quality hybridizations were repeated.

Microarray hybridization results were first corrected for differences in the local background according to:

$$SBR_{Pi} = S_{Pi} \times B_{Pi}^{-1}$$

where SBR_{Pi} is the signal-to-noise ratio of the probe spot Pi, S_{Pi} is the median pixel intensity of the specific probe spot and B_{Pi} is the median pixel intensity of the local background area around probe Pi. To account for variations between different hybridizations, the mean signal-to-noise ratio $\bar{x}$ SBR_{Ci} of ten internal control oligonucleotides (dsrCONT1 to 4, dsrCONT7 to 12) and differences in labeling efficiency were used to normalize the SBR_{Pi} using the following formula:

$$nSBR_{Pi} = \left(SBR_{Pi} \times \bar{x}\, SBR_{Ci}^{-1}\right) \times [dye]^{-1} \times [template] \times 100$$

where $nSBR_{Pi}$ is the normalized signal-to-noise ratio of the probe spot Pi, [dye] is the concentration of incorporated Cy3 dye molecules in pmol/µl and [template] is the concentration of labeled RNA in ng/µl. Heatmaps of the microarray results were generated using the software visualization tool JColorGrid (53). Non-metric multidimensional scaling ordinations based on Bray-Curtis similarities between microarray results (based on the mean nSNRs of all probes in the respective probe-target groups, Figure 1A) were performed using the software PRIMER 5 (http://www.primer-e.com/) (18).

Quantitative real-time PCR.

QPCR assays were performed using an iCycler IQ Thermocycler (Biorad Laboratories GmbH, München) and the Platinum® SYBR Green I qPCR Super Mix-UDG (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. 16S rRNA gene- and *dsrA*-targeted primers were designed using the "Probe_Design" and "Probe_Match" tools of the ARB program package. Potential formation of primer dimers and theoretical melting temperatures were determined using the open source program Primer3 (http://primer3.sourceforge.net) (103) and the Oligonucleotide Properties Calculator OligoCalc (http://www.basic.northwestern.edu/biotools/oligocalc.html) (56), respectively. Primers, assay performances, and cycling conditions are given in Table 1. Thermal cycling was initiated by a denaturation step at 94°C for 3 min, followed by 40-45 cycles of denaturation at 94°C for 40 s, annealing at the specific temperature for 40 s, and elongation at 72°C for 40-45 s. PCR products were used as standards for assay calibration and were amplified from the cloned sequences given in Table 1.

Table 1. Primers used for quantitative real-time PCR.

Name	Sequence (5' - 3')	Primer length	(%) GC content	Target gene	Target group	Annealing temp (°C)	Primer conc. [nM]	Approx. length of PCR product (bp)	Reference	Templates for standard synthesis (accession n° or clone names)	qPCR performance[a]		
											Efficiency (%)	Linearity R^2	Dynamic range (target genes/reaction)
DsrA243Fb	CAC GAC CAC CGA TCA GCT	18	61	*dsrA*	OTU 2	64	250	330	This study	AY167467, AY167479, AY167468	83.2 ± 3.0	0.98	$10^2 - 10^6$
DsrA561Rb	GTA CTT GGT CAS TTC GGC C	19	58										
DsrA243Fa	CAC CAC CAC CGA TGA ACT	18	56	*dsrA*	OTU 1	64	250	330	This study	AY167473, AY167466, AY167480	89.8 ± 0.4	0.99	$10^2 - 10^5$
DsrA561Ra	ATA GGC CTT BAC YGC GGC C	19	58 - 68										
DsrA216F	CUC AAC GGG AGA CAU CGU U	19	53	*dsrA*	OTU 6	64	250	360	This study	AY167478, AY167465	73.6 ± 1.4	0.98	$10^2 - 10^6$
DsrA561Re	ATA GGC TTT GAC CGC TGC C	19	58										
SYBAC836F	GGG TAC TCA TTC CTG CTG TG	20	55	16S rRNA	*Syntrophobacter* spp. and related bacteria	64	250	160	This study	X70905, X70906	77.5 ± 0.1	0.99	$10^2 - 10^6$
SYBAC986R	CCG GGG ATG TCA AGC CCA	18	67										
1389F	TG TAC ACA CCG CCC GT	16	63	16S rRNA	*Bacteria* and *Archaea*	52	750	120	*Pester* et al., submitted	X70905, X70906	90.0 ± 2.8	1.00	$10^2 - 10^6$
1492R	GGY TAC CTT GTT ACG ACT T	16	44-50				1000						
Acid303Fa	GCG CAC GGM CAC ACT GGA	18	67-72	16S rRNA	*Acidobacteria* group 1	68.5	1000	360	This study	P4K3f, P5K18f, P5K16f, P1K30f	81.5 ± 0.6	0.99	$10^2 - 10^7$
Acid303Fb	GCG CGC GGC CAC ACT GGA	18	78										
Acid657R	ATT CCA CKC ACC TCT CCC AY	19	50-60										
Acid702Fa	AGA TAT CTG CAG GAA CAY CC	20	45-50	16S rRNA	*Acidobacteria* group 2	62.5	1000	100	This study	P7K24f P4K4f P4K23f P5K23f	86.1 ± 2.3	0.99	$10^2 - 10^6$
Acid702Fb	AGA TAT CCG CAG GAA CAT CC	20	50										
Acid805R	CTG ATS GTT TAG GGC TAG	18	50										
Acid306F	CAC GGC CAC ACT GGC AC	18	71	16S rRNA	*Acidobacteria* group 3	69	500	190	This study	P4K21f, P2K11f	83.1 ± 0.6	0.99	$10^2 - 10^7$
Acid493R	AGT TAG CCG CAG CTK CTT CT	20	50-55										

The specific annealing temperature for each primer pair was determined by gradient PCR using perfectly-matching target genes and a selection of non-target clones with mismatches in the primer target site as templates. For each PCR product, a single band of the expected size was observed by agarose gel electrophoresis. Five nanogram of soil DNA was used as template per PCR. The specificity of each qPCR assay was confirmed by melting-curve analyses of the sample-derived and respective reference clone-derived PCR products. Additionally, environmental PCR products were cloned and the identities of a few selected clones were verified by sequencing. Absence of PCR-inhibitory substances was confirmed by qPCR analyses (using primers 1389F and 1492R) of dilution series of five selected soil DNA extracts, showing similar efficiencies and correlation coefficients as the standards.

Denaturing gradient gel electrophoresis.

Triplicate soil cores from nine different wetlands (including Schlöppnerbrunnen fen samples taken in 2004) were analyzed using newly developed forward primers for *dsrB*-based DGGE. DNA extracts obtained from the different depth fractions of one soil core were pooled prior to PCR. Preparation of GC-clamp tagged *dsrB* amplicons was carried out using a nested PCR approach. First, *dsrAB* fragments were amplified from 20 ng soil DNA in technical duplicates using the degenerate primers DSR1Fmix and DSR4Rmix (Table S2) for hot-start, touchdown PCR as described above, except that only 20 cycles (10 cycles with annealing at 58-48°C, followed by 10 cycles at 48°C) were performed. The *dsrAB* amplicons of duplicate PCRs were mixed, purified by gel electrophoresis using the Montage™ DNA Gel Extraction kit (Millipore), and used as template for the *dsrB*-targeted PCR. Five different PCR reactions per template were performed using the degenerate primers DSR4Rmix (10 µM each variant) and the GC-clamp carrying DSR1728Fmixes A to E (Table S5). Reaction mixes (total volume 100 µl) were prepared with 2 mM of each deoxynucleoside triphosphate, 10x *Taq* buffer, 2 mM $MgCl_2$ and 2 U of *Taq* DNA polymerase (Fermentas), 2 µl of bovine serum albumine (20 mg/ml, New England BioLabs) and 2 µl of template. *A*mplification was started by an initial denaturation step at 95°C for 3 min, followed by 20 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 20 sec. Cycling was completed by a final elongation step at 72°C for 3 min. Successfully amplified PCR products of each sample were pooled and concentrated to ~ 50 µl using a vacuum centrifuge. Additionally, three *dsrAB* plasmids were subjected to the described procedure and used as an internal standard to allow comparison between DGGE gels. DGGE was performed as described earlier (86). In brief, denaturing gradient of 30 to 80% denaturants (100% denaturants mixture consist of 7 M urea and 40% formamide) was used in an 8% (w/v) polyacrylamide gel. DGGE was performed in 1x Tris-acetate-EDTA buffer at 60°C and at a voltage of 150 V for 6 h. Following electrophoresis, the gels were incubated for 60 min in a SYBR® Green I solution. For identification, individual bands were excised, reamplified, purified using the QIAquick PCR purification kit (Qiagen), and sequenced directly.

Principal component analysis (PCA) (Canoco for Windows 4.5 (112)) was performed using a presence/absence matrix of DGGE bands and environmental variables, i.e. pH, sulfate and nitrate). Schlöppnerbrunnen II was excluded from PCA as corresponding environmental parameters were not determined for these samples. In addition, the DGGE band presence/absence matrix, environmental parameters and geographical data were used for calculation of separate Bray-Curtis similarity matrices. The similarity matrices were tested pairwise for similar patterns between wetlands using the RELATE routine of the Primer 5 program (18) (999 permutation, Spearman's rho).

Clone library construction and comparative sequence analysis.

Bacterial and archaeal 16S rRNA gene clone libraries were constructed from Schlöppnerbrunnen II fen soil (sampled in 2004, 10-15 cm soil section) DNA extracts using the primer pairs 616V-1492R (77) and Arch21F-1492R (27, 76), respectively. Prior to PCR, DNA extracts obtained from the three soil cores by using three different isolation methods (n=9) were mixed in equal parts. Cycling started with an initial denaturation step at 95°C for 5 min, followed by 25 cycles (*Bacteria*) or 30 cycles (*Archaea*) of denaturation at 95°C for 40 s, annealing at 52°C (*Bacteria*) or 56°C (*Archaea*) for 40 s, and elongation at 72°C for 1 min 30 sec. PCR was completed by a final elongation step at 72°C for 10 min. After TOPO TA cloning (Invitrogen) and sequencing, sequences were checked for chimeras with the software tools Bellerophon (46) and Pintail (http://www.bioinformatics-toolkit.org/Web-Pintail/) (5) and by comparing the phylogenetic placement of different parts of each 16S rRNA gene sequence (i.e. base positions 26-747 and 747-1471, numbering according to *E. coli*).

A *dsrAB* clone library was construction from pooled DNA extracts of all Rasner Möser fen soil subsamples using the degenerate primers DSR1Fmix and DSR4Rmix (Table S2). Touchdown PCR was performed as described above, with the exception that 25 cycles (10 cycles with annealing at 58-48°C, followed by 15 cycles at 48°C). The cycling was completed by a final elongation step at 72°C for 10 min. The presence and sizes of the amplification products were determined by agarose (1%) gel electrophoresis. Prior to cloning using the TOPO XL cloning kit (Invitrogen), PCR products were purified from the gel using the Montage™ DNA Gel Extraction kit (Millipore).

Operational taxonomic units definitions and Chao1 (17) estimates of richness were carried out using the software package DOTUR (105). Coverage was calculated according to the method described by Good (39). Phylogenetic analyses were performed by using distance matrix, maximum-parsimony and maximum-likelihood methods implemented in the ARB program package (78). 16S rRNA sequences were analyzed using the SILVA 16S rRNA database SSU Ref version 98 (97) and the SILVA Web Aligner SINA was applied to identify the two next related sequences of each fen soil clone. The bacterial 16S rRNA tree was inferred using PHYML and a 50%-conservation filter for *Bacteria* (1,248 valid alignment positions). The iTOL tool was used for tree visualization (69). The archaeal 16S rRNA tree was calculated using TreePuzzle (111) and a 50%-conservation filter for *Archaea* (1,229 valid alignment positions).

Deduced DsrAB and DsrB amino acid sequences were grouped in OTUs based on an identity threshold of 90% (approximately defining species-level groups, (58, 75). Phylogenetic analyses were performed using an indel filter (543 valid alignment positions after exclusion of variable regions with insertions and deletions). A consensus tree was drawn based on PHYLIP ProML (JTT), protein parsimony, and distance-matrix (FITCH, JTT, global rearrangements, and randomized input order of sequences) analyses of sequences >500 amino acids. Sequences <500 amino acids were individually added to the trees without changing the overall topology by using of the ARB "parsimony interactive" method.

Sequence accession numbers.

The sequences determined in this study have been deposited in the GenBank database under accession numbers GU127834 to GU127875 (archaeal 16S rRNA gene clones), GU127668 to GU127833 (bacterial 16S rRNA gene clones), GU127936 to GU127971 (*dsrAB* clones), GU127876 to GU127935 (*dsrB* clones).

Results

Temporal and spatial dynamics of *dsrAB*-containing microorganisms in the Schlöppnerbrunnen II fen.

A habitat-specific FGA, consisting of 146 oligonucleotide probes, was developed and optimized for diversity analysis of *dsrAB*-containing microorganisms in the Schlöppnerbrunnen fen system (Table S4, Figure S2) (75, 106). In a first step, specific hybridization conditions were established by performing a series of hybridizations with clone dsrSBI-3 (AY167467) at increasing formamide concentrations (0 to 60%, at 5% increments) in the hybridization buffer. A formamide concentration of 15% was selected for all subsequent hybridizations as the best compromise between sensitivity (i.e. high signal intensity of perfectly matched probes) and specificity (i.e. high signal intensity ratios between perfectly matched and mismatched probes). In the second step, the specificity of the *dsrAB*-FGA was further evaluated by individual hybridization with 36 *dsrAB* reference clones. This set of reference clones contained at least one perfectly-matched target for 120 probes. 26 probes could not be fully tested, because perfectly-matching reference clones were not available. 98 of the 120 tested probes gave a positive signal only with the perfectly-matching target clone. False positive hybridizations only occurred between probes and non-target sequences with less than two weighted mismatches. Two probes (dsrB40 and dsrA127) were false negative and were thus excluded from further analysis. In summary, only 0.57% and 0.04% of the 5256 individual probe-target hybridizations were false positive and false negative, respectively (Table S6). The nSNR values of perfectly matched probe-target duplexes of the final probe set ranged from 0.04 to 2.2 (factor 55), showing that the signal intensities of individual probes varied considerably. In the third step, the sensitivity of the *dsrAB*-FGA was assessed by a series of hybridizations containing different concentrations of the clone dsrSBI-36 (AY167469). One copy, 10, 100, 1000, 10000, and 100000 copies of the plasmid were added to 60 ng (corresponding to approximately 1×10^7 microbial genomes; assuming an average genome size of 5 Mbp) aliquots of fen soil DNA (sampled in 2007, depth 15-20 cm) prior to PCR amplification and further target preparation. A minimum of 1000 plasmids was required for positive detection of clone dsrSBI-36 by *dsrAB*-FGA hybridization. Given that most *dsrAB*-containing microorganisms only have a single copy of *dsrAB* in their genome, this corresponds to a detection limit of 0.01% of the total microbial community for this target organism (nSBR values of probes targeting clone dsrSBI-36 ranged from 0.17-0.41 in the specificity test). Additionally, the spot-to-spot variability (from triplicate spots of the same slide) and the slide-to-slide variability (from replicate spots of different slides) were consulted to evaluate the robustness of the microarray analysis. The spot-to-spot variability of five randomly chosen arrays, given as mean coefficient of variation of SBR values, was 3.8 ± 0.7% between triplicate spots on a given slide and 7.4 ± 3.7% between spots on three separate slides hybridized with the same fluorescently labeled target RNA. Technical variability of environmental microarray analyses, as determined by hybridizations with labeled RNA prepared from the same environmental sample (year 2001, depth 0-10 cm) but from separate DNA extractions and labeling reactions, showed a mean coefficient of variation of 15.3% based on nSBR values. This variability includes the composite methodological biases introduced by DNA extraction, PCR, labeling, and hybridization.

The newly developed Schlöppnerbrunnen fen-specific *dsrAB*-FGA was applied to analyze the spatial and temporal distribution of *dsrAB* at the site Schlöppnerbrunnen II. Microarray analyses of soil samples obtained from different depths (0 to 40 cm) were performed in technical triplicates (i.e. analysis of three separate DNA extracts obtained from the same soil core) for the year 2001 and in biological triplicates (i. e. analysis of three soil cores) for the years 2004 and 2007. Microarray procedures for target preparation, hybridization, and data analysis were kept identical for all soil samples to allow comparison between different hybridizations. Soil depth profiles of *dsrAB*-FGA hybridization patterns that were obtained for the three years were generally similar (Figure 1A) average Bray-Curtis similarity of 59±18% among all samples). However, in deeper soil layers the *dsrAB* diversity was considerably greater than in the top soil layer, where only a few probe-target groups were detected by the FGA. In accordance with this observation, multidimensional scaling (MDS) analysis of Bray-Curtis similarities between microarray hybridization patterns clearly separated the top soil layers from all other samples (Figure 1B). Members of the novel *dsrAB*-OTUs 1 and 4 (75), which are only distantly related to *Desulfobacca acetoxidans* (DsrAB identities of 69-77%), were present in most deeper soil fractions, as indicated by positive probe signals for probe-target groups 1 and 2-9, respectively.

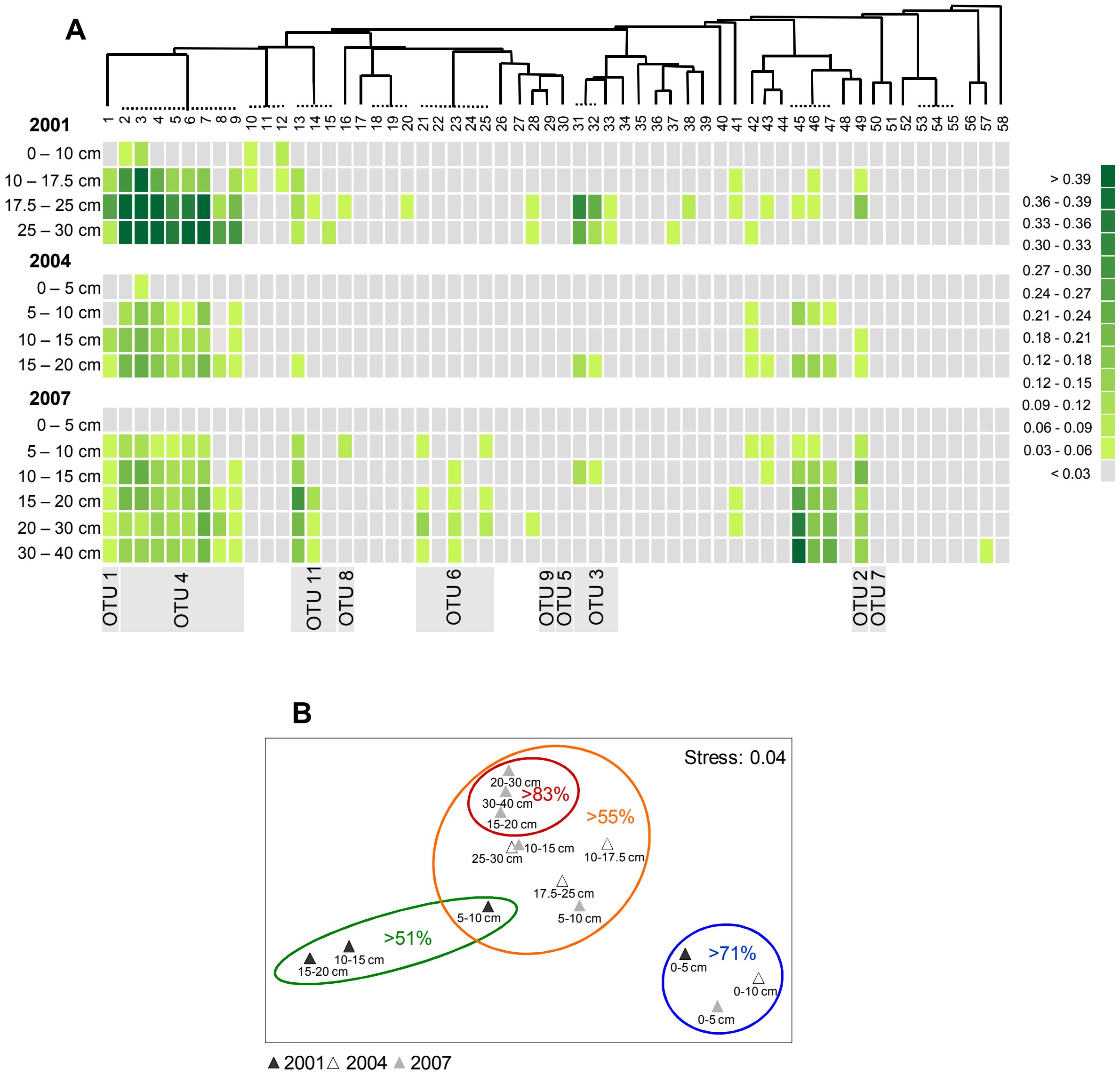
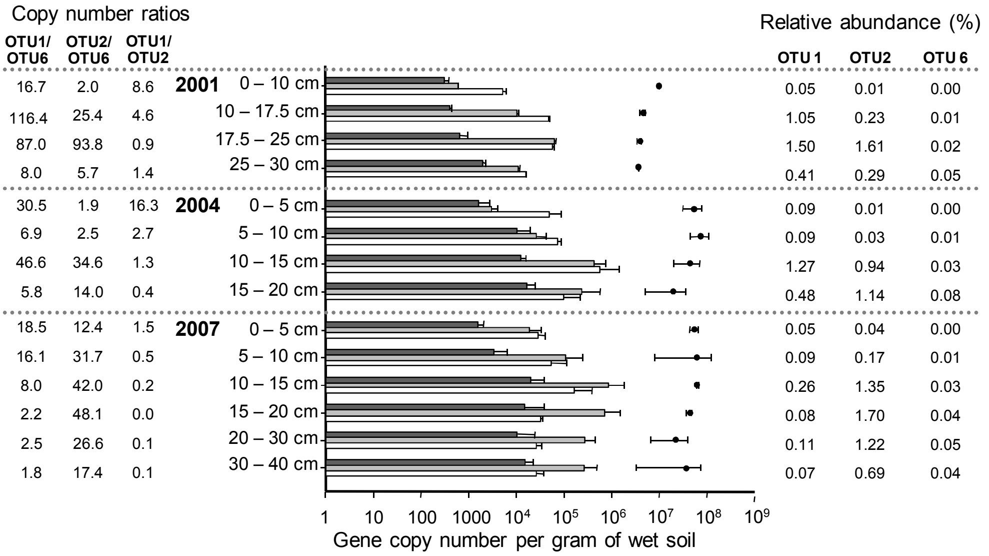


Figure 1. Microarray-based *dsrAB* diversity analysis of Schlöppnerbrunnen II fen soils sampled from different depths in the years 2001, 2004, and 2007. (A) Results of microarray hybridizations are displayed as mean nSBR of all probes within a probe-target group and are averaged between triplicate hybridizations (results of individual replicate analyses are presented in Figure S3). The color code translates into different mean nSBR values. Probe-target groups are arranged phylogenetically according to their position in schematic *dsrAB* neighbor-joining tree. Affiliation of probe-target groups to previously determined *dsrAB*-OTUs (75) is indicated. (B) Multidimensional scaling plot based on Bray-Curtis similarities between microarray hybridization patterns shown in panel A. Minimal Bray-Curtis similarities of all samples in a given group of samples is indicated in color.

The following *dsrAB* groups with no close, cultivated relatives were also detected frequently in some of the deeper soil layers: Members of OTU 2 (probe-target group 49) and the related probe-target groups 45, 46, and 47, probe-target groups 41, 42, and 43, members of OTU 3 (probe-target groups 31-33) and OTU 11 (probe-target groups 13-15). Microorganisms related to the genera *Desulfomonile* (probe-target group 28) and *Syntrophobacter* (OTU 6; probe-target groups 21, 23, 25) were only occasionally detected by *dsrAB*-FGA analysis.

For confirmation of microarray results, *dsrA*-targeted qPCR assays for OTU 1 (probe-target group 1), OTU 2 (probe-target group 49), and OTU 6 (probe-target groups 21, 23 and 25) were developed and applied to determine the abundance of these OTUs in the Schlöppnerbrunnen II soil samples. The average total number of bacterial and archaeal 16S rRNA genes for all samples from different soil depths and years was 3.6±1.6 x 10^7 copies per gram of wet soil, with total numbers being slightly higher in upper soil layers (Figure 2).



OTU1/ OTU6	OTU2/ OTU6	OTU1/ OTU2			OTU 1	OTU2	OTU 6
16.7	2.0	8.6	**2001**	0 – 10 cm	0.05	0.01	0.00
116.4	25.4	4.6		10 – 17.5 cm	1.05	0.23	0.01
87.0	93.8	0.9		17.5 – 25 cm	1.50	1.61	0.02
8.0	5.7	1.4		25 – 30 cm	0.41	0.29	0.05
30.5	1.9	16.3	**2004**	0 – 5 cm	0.09	0.01	0.00
6.9	2.5	2.7		5 – 10 cm	0.09	0.03	0.01
46.6	34.6	1.3		10 – 15 cm	1.27	0.94	0.03
5.8	14.0	0.4		15 – 20 cm	0.48	1.14	0.08
18.5	12.4	1.5	**2007**	0 – 5 cm	0.05	0.04	0.00
16.1	31.7	0.5		5 – 10 cm	0.09	0.17	0.01
8.0	42.0	0.2		10 – 15 cm	0.26	1.35	0.03
2.2	48.1	0.0		15 – 20 cm	0.08	1.70	0.04
2.5	26.6	0.1		20 – 30 cm	0.11	1.22	0.05
1.8	17.4	0.1		30 – 40 cm	0.07	0.69	0.04

Figure 2. Absolute and relative abundances of three *dsrAB*-OTUs in different depths of the Schlöppnerbrunnen II fen site in the years 2001, 2004, and 2007, as determined by qPCR. Error bars are the standard deviations of the mean for the three replicates. Black circles indicate total number of bacterial and archaeal 16S rRNA genes. Copy numbers of *dsrA* of OTUs 1, 2, and 6 are displayed as white, light grey, and dark grey bars, respectively. Additionally, the relative abundance of each *dsrAB*-OTU (given in % of the total number of bacterial and archaeal 16S rRNA genes) and copy number ratios between individual *dsrAB*-OTUs are shown for all years and depths.

In contrast, *dsrA* copy numbers of all three target OTUs were lowest in the top soil layers and generally increased with soil depth, although OTU 1 and OTU 2 were represented by slightly lower *dsrA* copy numbers in the deepest soil layers. In all years and soil depths, *dsrA* of the novel OTUs 1 and 2 outnumbered *dsrA* of the *Syntrophobacter*-related OTU 6; with ratios ranging from 1.8 to 116.4 for OTU 1 and from 1.9 to 93.8 for OTU 2 (Figure 2). The mean *dsrA* copy number per g fen soil was $8.9 \pm 0.6 \times 10^4$, for OTU 1, $2.1 \pm 0.07 \times 10^5$ for OTU 2, and $7.9 \pm 0.6 \times 10^3$, for the OTU 6. The mean relative abundances (percent ratio of *dsrA* copy numbers to total bacterial and archaeal 16S rRNA gene copy numbers averaged over soil depths) of OTUs 1, 2, and 6 were 0.75%, 0.53%, and 0.02% in the year 2001, 0.48%, 0.53%, and 0.03% in the year 2004, and 0.11%, 0.86%, and 0.03% in 2007, respectively.
For *Syntrophobacter*-related bacteria (represented by OTU 6), an additional 16S rRNA gene-targeted qPCR assay was developed based on previously published 16S rRNA gene clone sequences from the Schlöppnerbrunnen fen sites (75). As expected, 16S rRNA gene- and *dsrA*-based qPCR data of the *Syntrophobacter*-related target group showed a high correlation (Pearson correlation coefficient of 0.854, $p<0.01$), confirming that *dsrAB* of OTU 6 indeed derived from *Syntrophobacter*-related bacteria in the Schlöppnerbrunnen fen soil samples.

Comparisons of *dsrA* copy numbers to the nSBR of the corresponding probe-target groups of the *dsrAB*-FGA demonstrated significant correlation ($p<0.01$) between qPCR and microarray data; with Pearson coefficients of 0.811 for OTU 1 (with probe-target group 1), 0.756 for OTU 2 (with probe-target group 49), and 0.734 (with probe-target group 21) and 0.863 (with probe-target group 23) for OTU 6.

Bacterial and archaeal 16S rRNA gene inventories of the Schlöppnerbrunnen II fen.

Soil sampled in the year 2004 from 10 to 15 cm depth contained members of the novel OTUs 1 and 2 at a relatively high abundance and thus was chosen as a representative sample for phylogenetic identification of the abundant Schlöppnerbrunnen II fen soil microorganism. A bacterial and an archaeal 16S rRNA gene clone library were constructed and phylogenetically analyzed. After removing chimeras from the bacterial (n=9) and the archaeal (n=1) dataset, 16S rRNA gene sequences with identities ≥99% (approximately defining species level phylotypes; (109)) were grouped in OTUs/phylotypes.

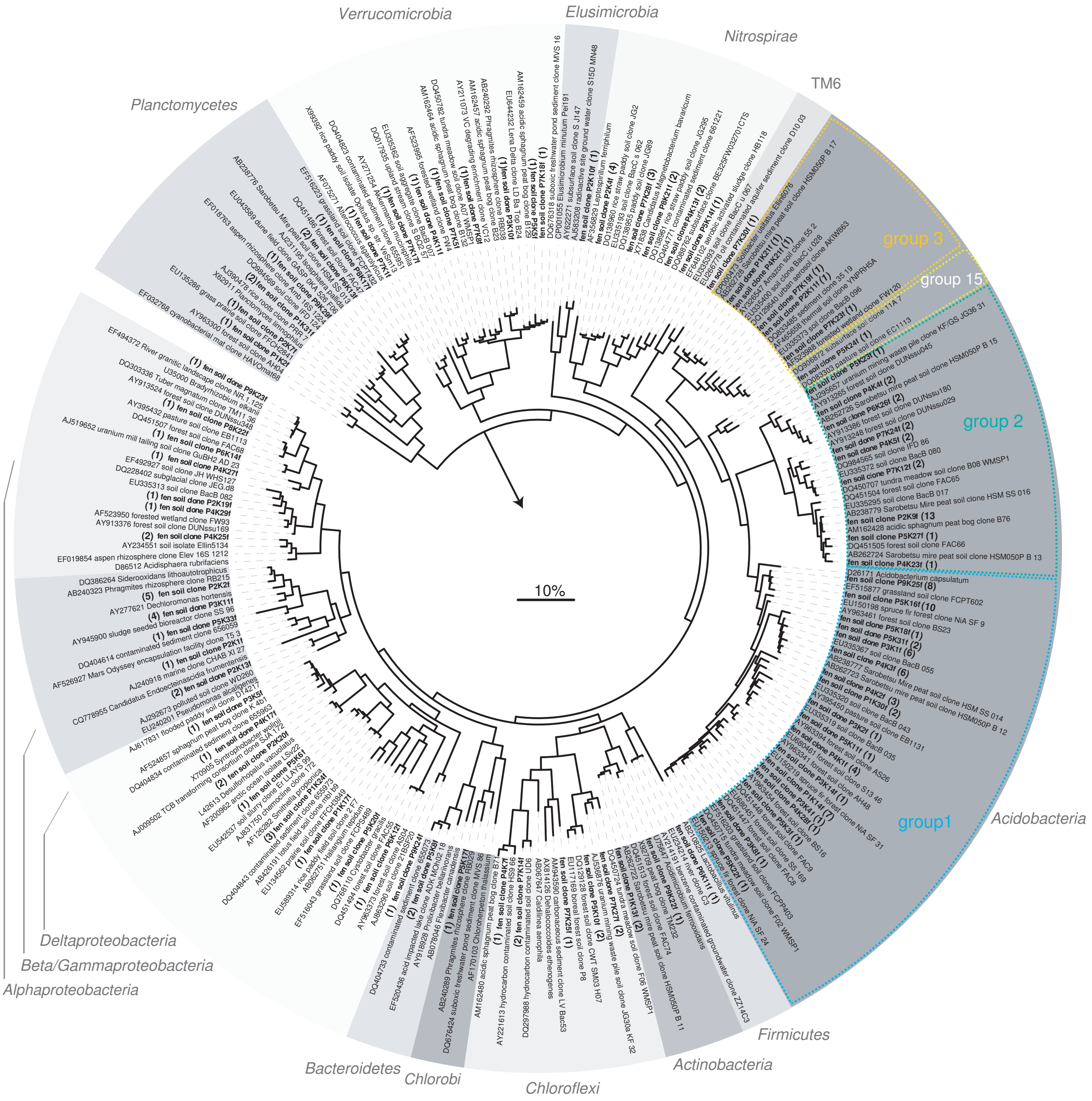


Figure 3. 16S rRNA gene-based maximum likelihood (PhyML) tree showing bacterial sequences retrieved from Schlöppnerbrunnen II fen soil. The tree includes for each OTU a representative clone (shown in bold), sequences from the next closest relatives, and representative cultivated microorganisms in the ARB-SILVA database. For each OTU the number of retrieved clones is depicted in brackets. For the phylum *Acidobacteria*, the grouping of subdivisions 1, 2, 3, and 15 (nomenclature according (7)) is shown. Scale bar indicates 10% estimated sequence divergence.

The 166 bacterial and 42 archaeal clones formed 85 and 18 OTUs and covered 69% and 76% of the expected diversity, respectively. Chao1 richness analyses estimated a minimal number of 160 bacterial and 25 archaeal OTUs. The bacterial OTUs affiliated with members of the eleven described phyla *Actinobacteria*, *Acidobacteria*, *Bacteroidetes, Chloroflexi*, *Chlorobi*, *Elusimicrobia*, *Firmicutes*, *Nitrospirae*, *Planctomycetes*, *Proteobacteria* (including alpha-, beta-, gamma- and delta-classes), and *Verrucomicrobia,* and the uncultured candidate phylum TM6 (Figure 3). Most sequences (53.6%), corresponding to 33 OTUs, derived from the phylum *Acidobacteria*. Most OTUs (n=77) showed low similarity (<90%) to sequences of cultured members of the respective phyla. Only 8 OTUs, represented by the following clones, had 16S rRNA sequence identities of >95% (approximately defining genus level phylotypes): P2K20F (99% to *Desulforhopalus vacuolatus*, L42613, *Deltaproteobacteria*), P9K23f (97.5% to *Bradyrhizobium elkanii*, U35000, *Alphaproteobacteria*), P5K6f (96.1% *to Smithella propionica*, AF126282, *Deltaproteobacteria*), P2K2f (95.78% *to Siderooxidans lithoautotrophicus*, DQ386264, *Betaproteobacteria*), P6K11f (95.4% to *Desulfosporosinus orientis*, Y11570, *Firmicutes*), P1K21f (95.2% to *Solibacter usitatus* Ellin6076, AY673303, *Acidobacteria*), P4K25f (95.1% to isolate Ellin5134, AY234551, *Alphaproteobacteria*), and P3K5f (95% to *Syntrophobacter wolinii*, X90605/06, *Deltaproteobacteria*).

The next relatives to the recovered 16S rRNA sequences of this study originated from a broad range of habitats. However, the majority of sequences (68%, n=87, corresponding to 64 OTUs) were closest related to sequences from soil, with several clones (n=29, corresponding to 20 OTUs) being related to sequences obtained from wetlands or water-saturated soil habitats e.g. forested wetland impacted by reject coal (14), rice paddy field (89), peat bogs (26, 102), alpine tundra and wet meadows (21). Furthermore, 46 sequences (28%) had 16S rRNA sequence identities of >95% to sequences retrieved from heavy DNA fractions after stable isotope probing of Schlöppnerbrunnen II fen soil mesocosms that were incubated with and without sulfate (ZITAT MICHA). Most sequences were in common to sequences obtained from both incubations with and without sulfate and affiliated to *Acidobacteria* (n=36, 95.1% to 99.7%), *Planctomycetes* (n=1, 96.4%), *Actinobacteria* (n=1, 95.1 to 95.5%) and *Alphaproteobacteria* (95.4%). However, similar *Nitrospirae* sequences (n=6, 97 to 99.4%) and *Firmicutes* sequences (n=1, 96.6 to 97.6) were detected only in incubations with sulfate.

With the exception of acidobacterial clones P5K31f (98.2% to AM270582) and P1K30f (99% to AM773947) and the *Chlorobi* clone P5K17f (96% to AM773938), most sequences from our study represent genus level phylotypes that have not been detected by three other 16S rRNA-based studies of the Schlöppnerbrunnen fen system (43, 75, 125).

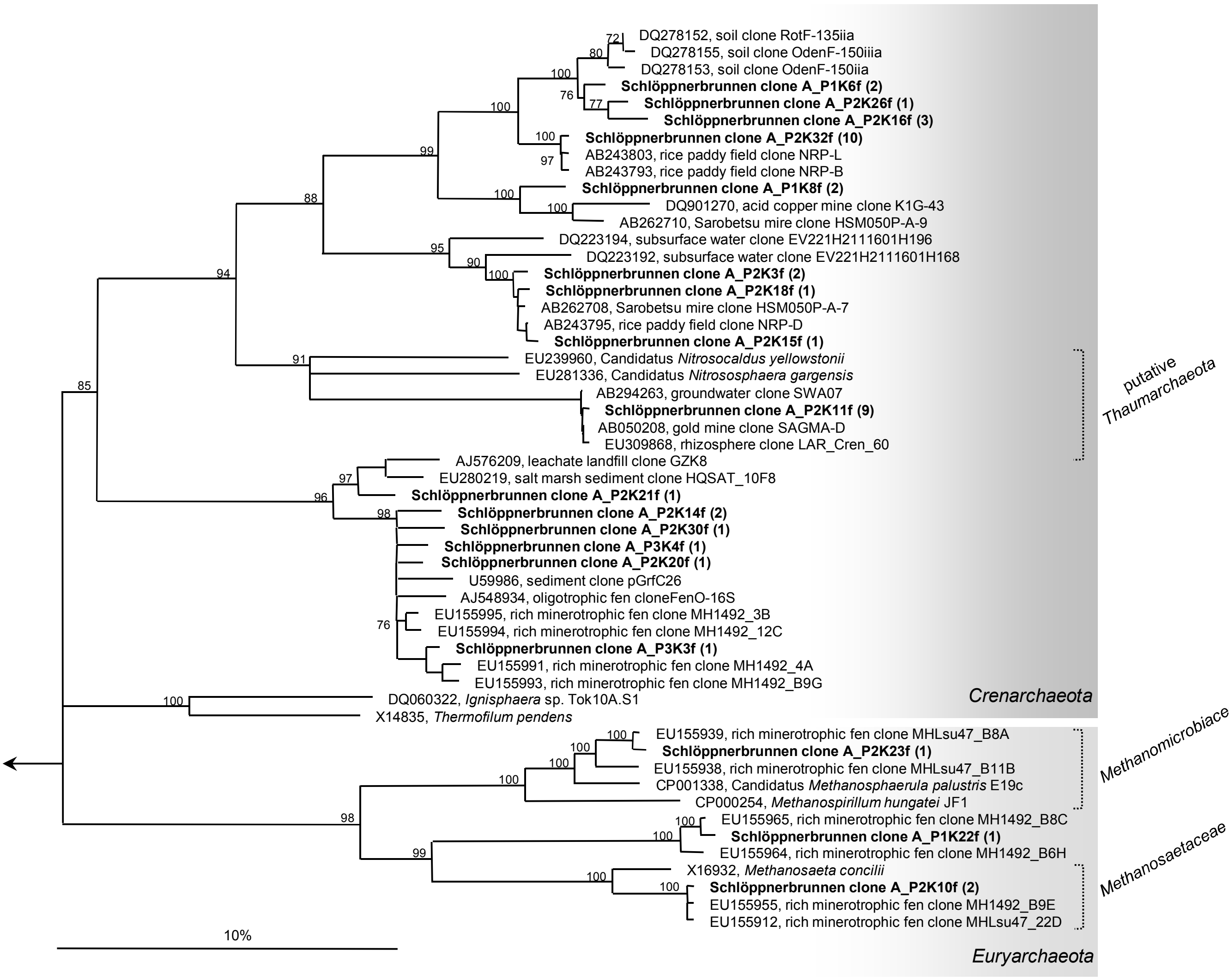


Figure 4. 16S rRNA gene-based maximum likelihood (TreePuzzle) tree showing archaeal sequences retrieved from Schlöppnerbrunnen II fen soil. The tree includes for each OTU a representative clone (shown in bold), sequences from the next closest relatives, and representative cultivated microorganisms in the ARB-SILVA database. For each OTU the number of retrieved clones is depicted in brackets. TreePuzzle support values over 70 are given at the respective nodes. Scale bar indicates 10% estimated sequence divergence.

Most archaeal OTUs (n=15) were affiliated with the phylum *Crenarchaeota*, while only 3 OTUs branched within the *Euryarchaeota* (Figure 4). While none of the crenarchaeal OTUs from the Schlöppnerbrunnen II fen was closely related to a cultivated microorganism (16S rRNA sequence identities <80%), most OTUs grouped with other soil- and wetland-derived sequences. Clone A_P2K11f, representing an OTU consisting of nine sequences, was affiliated with *Nitrososphaera gargensis*, an archaeum recently proposed to belong to a novel archaeal phylum, the *Thaumarchaeota* (13) (Spang *et al.* submitted). The euryarchaeal OTUs were closely related (sequence identities of 98-99%) to clones from a rich minerotrophic, pH neutral fen located in central New York State (16). The closest described relatives of clones A_P2K23f and A_P2K10f were *Candidatus* Methanosphaerula palustris and *Methanosaeta concilii* (each with a sequence similarity of 94%), respectively. In contrast to the bacterial sequences, a considerable proportion of archaeal sequences (43%) had highest similarities to sequences from a previously published 16S rRNA gene inventory of the Schlöppnerbrunnen fen system (125).

Abundance of *Acidobacteria* in the Schlöppnerbrunnen II fen.

Sequences of the phylum *Acidobacteria* predominated in the bacterial 16S rRNA gene library. Development and application of specific 16S rRNA gene-targeted qPCR assays for the three main acidobacterial subdivisions/groups 1, 2, and 3 (Fig. 3, Table 1) confirmed that each of the three groups constituted a major part of the Schlöppnerbrunnen II fen soil microbial community in all soil depths and years (Figure 5).

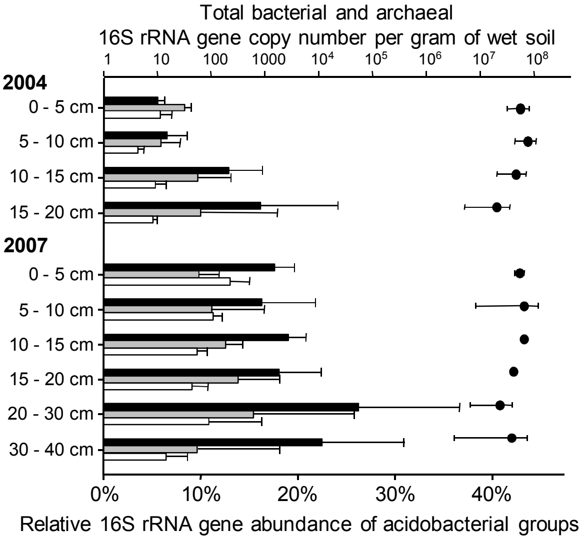


Figure 5. Relative abundance of acidobacterial subdivisions/groups in different depths of the Schlöppnerbrunnen II fen site in the years 2004 and 2007, as determined by of 16S rRNA gene-targeted qPCR. Black circles indicate total numbers of bacterial and archaeal 16S rRNA genes. Relative abundances (given in % of the total number of bacterial and archaeal 16S rRNA genes) of acidobacterial groups 1, 2, and 3 are indicated in black, grey, and white bars, respectively. Error bars are the standard deviations of the mean for the three biological replicates.

Members of group 1 were most abundant, followed by members of group 2 and 3: Mean copy numbers per gram wet soil were 4.3 ± 1.3 x 10^6, 3.9 ± 3.6 x 10^6, and 2.4 ± 0.4 x 10^6 in the year 2004 and 9 ± 2.2 x 10^6, 5.6 ± 5 x 10^6, and 4.9 ± 0.9 x 10^6 in 2007 for group 1, group 2, and group 3, respectively. The relative abundance was 10.3% ± 5.1, 8.5% ± 1.9, and 5.0% ± 1 in the year 2004 and 20.0% ± 3.5, 12.1% ± 6.1, and 3.5% ± 1.7 in 2007 for the groups 1, 2, and 3, respectively. In contrast to *Syntrophobacter*-related microorganisms (see above), 16S rRNA gene abundance of any of the three acidobacterial groups was not correlated with the *dsrA* abundance of any of the novel OTUs 1 and 2 (p=0.123-0.875, Pearson coefficients 0.058-0.521).

Diversity and biogeography of *dsrAB* in geographically separated wetlands.

In order to evaluate if the *dsrAB*-containing microorganisms in the Schlöppnerbrunnen fens are endemic or show a wider geographic distribution, a modified DGGE assay was applied for fingerprinting of *dsrB* in nine wetlands located in Austria, Italy and Germany (Table S1, Figure S1). In comparison to the original DGGE forward primer DSRp2060F (38), which covers only 29% of all sequences with the primer binding site in the *dsrAB* database, the newly developed DSR1728F primer mix has a significantly improved in silico coverage of 95% (Table S5). This modified *dsrB*-DGGE analysis yielded 11 to 40 bands for the different wetland soil samples. The lowest and highest numbers of DGGE bands were detected in the Berndorf wet meadow soil and the Roßbrand II fen soil, respectively. With exception of the Roßbrand peats I and II, located in close distance (~1 km) to each other, the *dsrB*-DGGE profiles between replicate soil cores were more similar to each other than *dsrB*-DGGE profiles between different wetland sites (Figure 6). Furthermore, all precipitation-fed bogs showed greater similarity in *dsrB*-DGGE banding pattern to each other than to other wetland sites. PCA resulted in four principal components (PC), which explained 74.1% of the DGGE-based community composition data, whereby the first two ordination axes (Figure 6) explained 48.8% of the variation. Correlation analysis between principal components and environmental factors revealed that PC1 was strongly negatively correlated with pH (correlation coefficient of -0.9963), whereas PC2 was moderately negatively correlated with sulfate (correlation coefficient of -0.7286) and nitrate (correlation coefficient of -0.6834). Furthermore, the different peatland sites showed no major relationship based on their geographical position. In contrast, the measured environmental factors (pH, concentrations of sulfate and nitrate) explained significantly the differences in the *dsrB*-DGGE profiles of the investigated wetlands (rho=0.568, p<0.001).

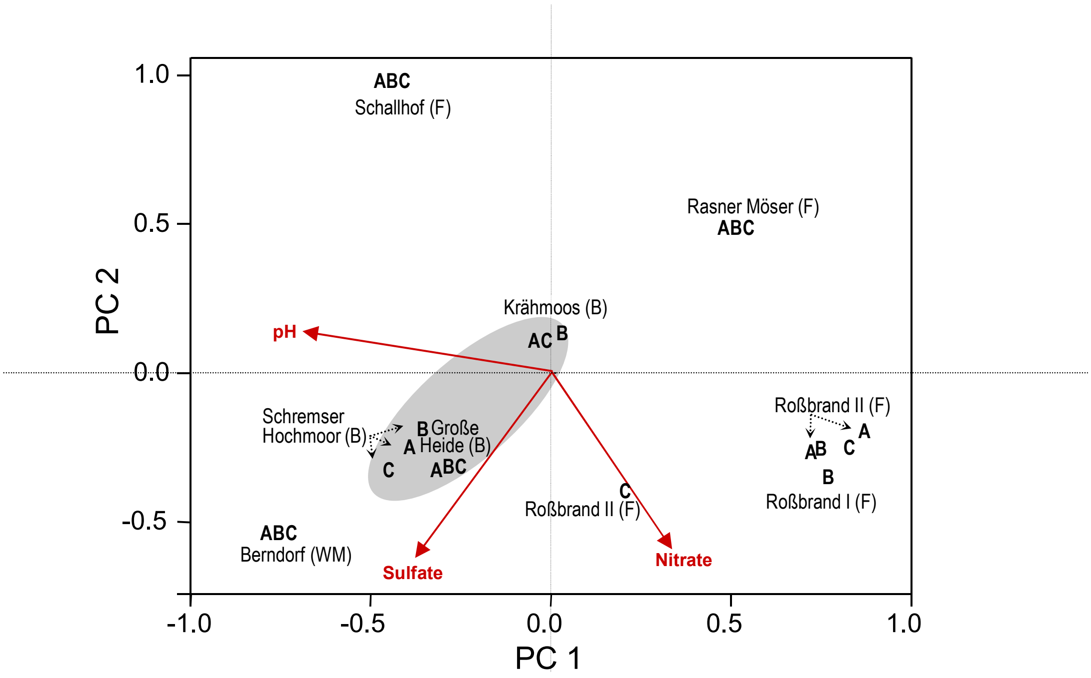


Figure 6. Relationships between *dsrB*-DGGE-based community structure data and environmental factors (pH, sulfate and nitrate concentrations) in eight, geographically separated wetlands were determined by principal component analysis. For each wetland the positions of the three biological replicates (sampling cores A, B, and C) in the ordination biplot is depicted. Wetland type is indicated: bog (B), fen (F), wet meadow (WM). Grey shading additionally highlights grouping of bogs.

For identification, 160 individual DGGE bands were extracted from the gel, reamplified and sequenced. Unambiguous *dsrB* sequences were obtained from 59 DGGE bands and grouped into 22 DsrB-OTUs (Figure 7, Table S7). Only few OTUs were closely affiliated with representatives of characterized SRM. Ten OTUs, including six out of eleven previously defined Schlöppnerbrunnen DsrAB-OTUs (75), were present in at least four out of eight peatlands. Nine OTUs of these widely distributed OTUs were present at bog and fen sites, while *dsrB*-DGGE OTU 6 was only detected in fens.

PCA of individual *dsrB*-DGGE bands closely related to Schlöppnerbrunnen *dsrAB*-OTUs (Table S7) indicate broad distribution of members of Schlöppnerbrunnen OTU 1 along the pH gradient and a preferential association with higher sulfate concentrations. In contrast, DGGE bands corresponding to Schlöppnerbrunnen OTUs 2, 3, and 6 were positioned at low pH and low sulfate concentrations in the PCA plot (data not shown).

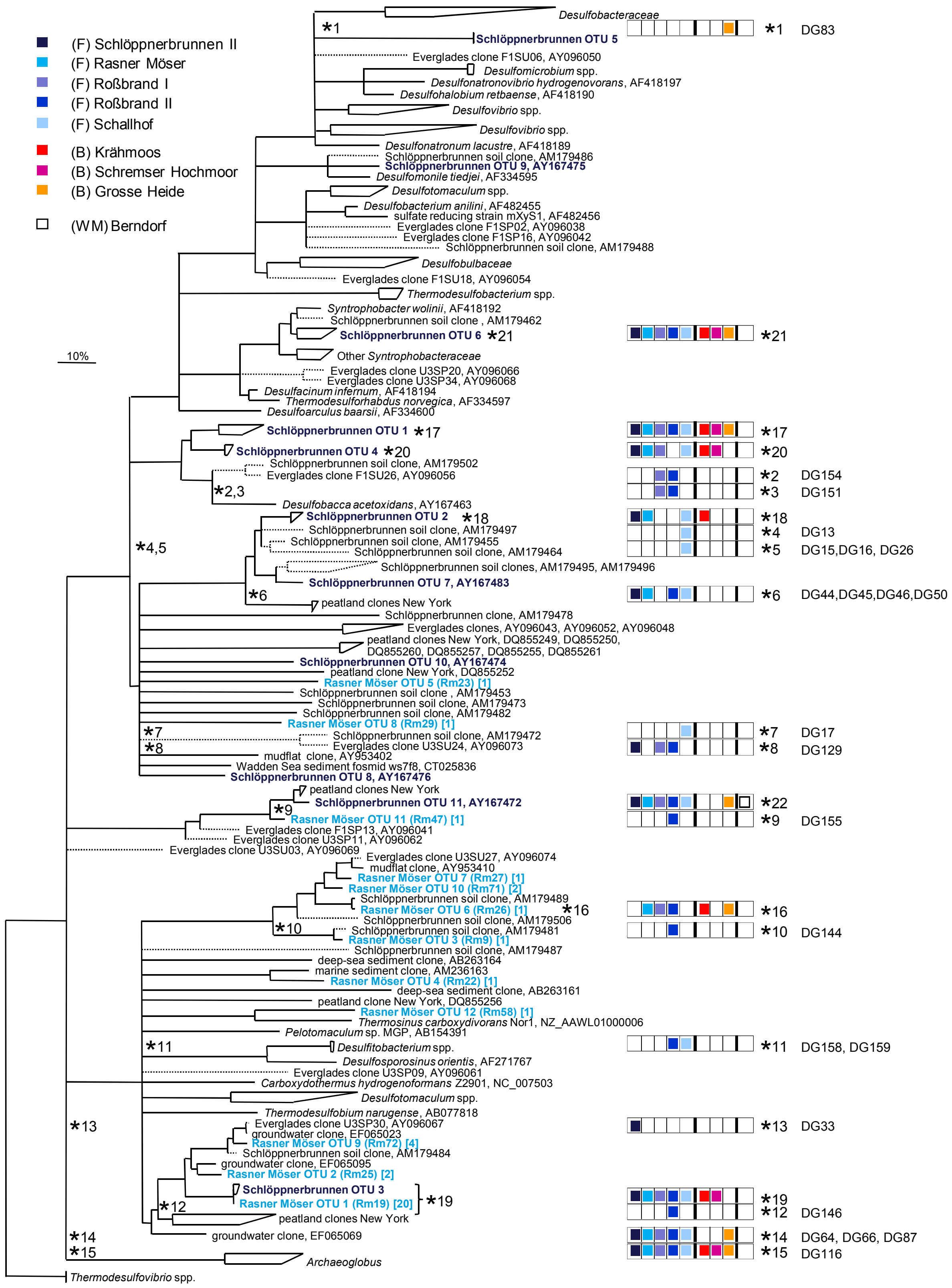

(F) Schlöppnerbrunnen II
(F) Rasner Möser
(F) Roßbrand I
(F) Roßbrand II
(F) Schallhof
(B) Krähmoos
(B) Schremser Hochmoor
(B) Grosse Heide
(WM) Berndorf
10%
Desulfobacteraceae
*1
Schlöppnerbrunnen OTU 5
Everglades clone F1SU06, AY096050
Desulfomicrobium spp.
Desulfonatronovibrio hydrogenovorans, AF418197
Desulfohalobium retbaense, AF418190
Desulfovibrio spp.
Desulfovibrio spp.
Desulfonatronum lacustre, AF418189
Schlöppnerbrunnen soil clone, AM179486
Schlöppnerbrunnen OTU 9, AY167475
Desulfomonile tiedjei, AF334595
Desulfotomaculum spp.
Desulfobacterium anilini, AF482455
sulfate reducing strain mXyS1, AF482456
Everglades clone F1SP02, AY096038
Everglades clone F1SP16, AY096042
Schlöppnerbrunnen soil clone, AM179488
Desulfobulbaceae
Everglades clone F1SU18, AY096054
Thermodesulfobacterium spp.
Syntrophobacter wolinii, AF418192
Schlöppnerbrunnen soil clone , AM179462
Schlöppnerbrunnen OTU 6 *21
Other Syntrophobacteraceae
Everglades clone U3SP20, AY096066
Everglades clone U3SP34, AY096068
Desulfacinum infernum, AF418194
Thermodesulforhabdus norvegica, AF334597
Desulfoarculus baarsii, AF334600
Schlöppnerbrunnen OTU 1 *17
Schlöppnerbrunnen OTU 4 *20
Schlöppnerbrunnen soil clone, AM179502
Everglades clone F1SU26, AY096056
*2,3
Desulfobacca acetoxidans, AY167463
Schlöppnerbrunnen OTU 2 *18
Schlöppnerbrunnen soil clone, AM179497
Schlöppnerbrunnen soil clone, AM179455
Schlöppnerbrunnen soil clone, AM179464
*4,5
Schlöppnerbrunnen soil clones, AM179495, AM179496
Schlöppnerbrunnen OTU 7, AY167483
*6
peatland clones New York
Schlöppnerbrunnen clone, AM179478
Everglades clones, AY096043, AY096052, AY096048
peatland clones New York, DQ855249, DQ855250,
DQ855260, DQ855257, DQ855255, DQ855261
Schlöppnerbrunnen OTU 10, AY167474
peatland clone New York, DQ855252
Rasner Möser OTU 5 (Rm23) [1]
Schlöppnerbrunnen soil clone , AM179453
Schlöppnerbrunnen soil clone, AM179473
Schlöppnerbrunnen soil clone, AM179482
Rasner Möser OTU 8 (Rm29) [1]
*7
Schlöppnerbrunnen soil clone, AM179472
*8
Everglades clone U3SU24, AY096073
mudflat clone, AY953402
Wadden Sea sediment fosmid ws7f8, CT025836
Schlöppnerbrunnen OTU 8, AY167476
peatland clones New York
Schlöppnerbrunnen OTU 11, AY167472
*9
Rasner Möser OTU 11 (Rm47) [1]
Everglades clone F1SP13, AY096041
Everglades clone U3SP11, AY096062
Everglades clone U3SU03, AY096069
Everglades clone U3SU27, AY096074
mudflat clone, AY953410
Rasner Möser OTU 7 (Rm27) [1]
Rasner Möser OTU 10 (Rm71) [2]
Schlöppnerbrunnen soil clone, AM179489
Rasner Möser OTU 6 (Rm26) [1] *16
Schlöppnerbrunnen soil clone, AM179506
*10
Schlöppnerbrunnen soil clone, AM179481
Rasner Möser OTU 3 (Rm9) [1]
Schlöppnerbrunnen soil clone, AM179487
deep-sea sediment clone, AB263164
marine sediment clone, AM236163
Rasner Möser OTU 4 (Rm22) [1]
deep-sea sediment clone, AB263161
peatland clone New York, DQ855256
Rasner Möser OTU 12 (Rm58) [1]
Thermosinus carboxydivorans Nor1, NZ_AAWL01000006
Pelotomaculum sp. MGP, AB154391
*11
Desulfitobacterium spp.
Desulfosporosinus orientis, AF271767
Everglades clone U3SP09, AY096061
Carboxydothermus hydrogenoformans Z2901, NC_007503
Desulfotomaculum spp.
Thermodesulfobium narugense, AB077818
*13
Everglades clone U3SP30, AY096067
groundwater clone, EF065023
Rasner Möser OTU 9 (Rm72) [4]
Schlöppnerbrunnen soil clone, AM179484
groundwater clone, EF065095
Rasner Möser OTU 2 (Rm25) [2]
Schlöppnerbrunnen OTU 3
*19
*12
Rasner Möser OTU 1 (Rm19) [20]
peatland clones New York
*14
*15
groundwater clone, EF065069
Archaeoglobus
Thermodesulfovibrio spp.
*1 DG83
*21
*17
*20
*2 DG154
*3 DG151
*18
*4 DG13
*5 DG15,DG16, DG26
*6 DG44,DG45,DG46,DG50
*7 DG17
*8 DG129
*22
*9 DG155
*16
*10 DG144
*11 DG158, DG159
*13 DG33
*19
*12 DG146
*14 DG64, DG66, DG87
*15 DG116

Figure 7. DsrAB consensus tree showing the affiliation of *dsrAB* clone and *dsrB* DGGE sequences recovered nine different wetlands; five fens (F), three bogs (B), and a wet meadow (WM). OTUs from the Schlöppnerbrunnen (75) and Rasner Möser fens were based on DsrAB sequences longer than 500 amino acids and are depicted in bold and colored type. For each Rasner Möser OTU, a representative clone and the number of sequenced clones are given in round and square brackets, respectively. Sequences shorter than 500 amino acids are indicated by dashed branches and asterisks, the latter depict the phylogenetic positions of 22 *dsrB*-OTUs, which were identified by sequencing of *dsrB*-DGGE bands. In addition, the presence/absence of each *dsrB*-OTU in the nine wetlands was inferred from comparison of DGGE banding pattern and is indicated by presence/absence of the respective colored square. Scale bar indicates 10% estimated sequence divergence.

Soil samples from the groundwater-fed fen site Rasner Möser were additionally subjected to *dsrAB* clone library analysis. A total of 39 fully sequenced *dsrAB* clones (~1900 bp) formed 12 OTUs; resulting in a Good's coverage of 78%. Only OTU 12 was moderately related to *Thermosinus carboxydovorans*, while the remaining OTUs had no close cultivated relative (Figure 7). Rasner Möser OTUs 1 (represented by more than half of the *dsrAB* sequences, n=20) and 11 were most closely related to the deep-branching Schlöppnerbrunnen OTUs 3 (88.2-97.3% amino acid identities) and 11 (90% amino acid identity), respectively. Only these two Rasner Möser OTUs and OTU 6 were also identified by *dsrB*-DGGE analysis (Table S7).

Discussion

Novel lineages are 'core' organisms and dominate a temporally stable but spatially heterogeneous *dsrAB*-containing population in the Schlöppnerbrunnen II fen.

Environmental diversity surveys of the dissimilatory (bi)sulfite reductase genes *dsrAB* have repeatedly demonstrated that clone libraries from diverse ecosystems can be largely composed of sequences forming new branches with no cultivated representative in the DsrAB tree (66, 75). These novel *dsrAB* sequences can be indicative of sulfite/sulfate reducers (or organosulfonate reducers) that belong to either previously undescribed phylogenetic lineages or described lineages not yet known to contain these functional guilds of microorganisms.

Additionally, anaerobes that live in syntrophic association with other microorganisms might be carriers of these novel *dsrAB*, which are presumably genomic remains of an ancestral sulfate-reducing metabolism (50, 120). The fact that evolutionary distribution of *dsrAB* among SRM was influenced by some lateral gene transfer events (59, 129) complicates phylogenetic interpretation of environmentally retrieved *dsrAB* sequences. Hence, these novel *dsrAB* sequences might also derive from microorganisms which are phylogenetically related to known cultivated microorganisms but have received their *dsrAB* via lateral gene transfer from a yet unknown donor. The true identity and also the environmental relevance of microorganisms carrying these novel *dsrAB* types are currently unknown.

Here, we have developed and extensively tested the diagnostic performance of a habitat-specific *dsrAB*-based microarray and three OTU-specific qPCR assays for revealing the dynamics of the *dsrAB*-containing microbial community in the Schlöppnerbrunnen II fen, a model peatland and long-term study site that was previously shown to harbor several novel *dsrAB* lineages (75, 106). As expected, qPCR assays were more sensitive than FGA analysis (Figure 1A, Figure 2). However, qPCR results confirmed the experimentally inferred detection limit of the FGA (0.01% of the total microbial community) as *dsrAB*-OTUs with abundances of ≥0.05% (relative to the total number of bacterial and archaeal 16S rRNA genes) were also consistently detected in fen soil samples by microarray analysis. FGA hybridization pattern of soils sampled over a period of six years indicated little long-term changes in the number and types of *dsrAB*-containing microorganisms, confirming that members of several *dsrAB*-lineages are part of the autochthonous community in the Schlöppnerbrunnen II fen. In contrast, clear spatial differences were evident in FGA hybridization pattern, with only very few probe-target groups being detected in the most upper fen layer (Figure 1). Consistent with this observation, copy numbers of *dsrA* of OTUs 1, 2, and 6 analyzed by qPCR were always considerably lower in the most upper than in deeper soil layers. Interestingly, the overall dynamics of the *dsrAB*-harboring community are mirrored by spatially highly variable but temporally relatively stable sulfate concentrations at the study site (2). In contrast, highly variable δ^{34}S values were indicative of past changes in the activity of fen soil SRM in space and time. Comparably stable but low bulk concentrations of sulfate over time thus rather reflect an active buffering/recharging system for sulfate than balanced consumption and production of sulfate at the Schlöppnerbrunnen II fen site (2). Factors other than sulfate availability must thus be responsible for the reduced *dsrAB* diversity in the most upper soil layer. While generally regarded as anaerobic microorganisms, it is well known that some SRM (i) can be highly active in oxic-anoxic transition zones and even oxic environments (84, 85, 113) and (ii) tolerate or respire oxygen in pure culture (22, 33, 68, 108). However, to date growth under oxic conditions was shown only for one sulfate reducing bacterium, *Desulfovibrio desulfuricans* ATCC 27774 (72). Recurring events of drought and precipitation are major determinants of short- and long-term changes in the water table and thus the redox status of peatlands (28, 99). Oxygen exposure in upper peat zones is more substantial than in deeper zones due to increased impact of drought events (28, 99) and release of oxygen from aerenchymatic plant roots (123). It is thus conceivable that growth inhibition due to prolonged exposure to oxygen restricts the abundance of anaerobic, *dsrAB*-containing microorganisms in the upper fen soil layer (approximately 0-10 cm) on a long-term scale.

Interestingly, FGA and qPCR analyses both indicated that members of novel *dsrAB* lineages (e.g. OTUs 1, 2, 3, 4, and 11) outnumbered members of lineages that contain cultivated microorganisms. In particular, all soil fractions contained a higher copy number of *dsrA* from the novel OTUs 1 and 2 than from the *Syntrophobacter wolinii*-related OTU 6. In some soil samples from depths of 10-30 cm, members of OTUs 1 and 2 even accounted for a significant proportion of the total microbial community (1-2% *dsrA* copy number relative to the total number of bacterial and archaeal 16S rRNA genes) (Figure 2). These relatively high abundances of ≥0.1-1% imply that unknown OTU 1 and OTU 2 members are so-called ‘core’ microorganisms (93) and actively contribute, at least occasionally, to the prevalent biogeochemical processes in the fen.

Identity and putative functional properties of dominant Schlöppnerbrunnen II fen microorganisms.

Classical 16S rRNA gene clone library analysis showed that common community members in the model fen belong to twelve bacterial and two archaeal phyla. The list of 85 bacterial and 18 archaeal species-level 16S rRNA-OTUs detected in the fen represents a first general, albeit incomplete inventory of microorganisms that might harbor the novel *dsrAB* gene types. Clones from *Acidobacteria* groups/subdivisions 1, 2, and 3 (7, 47, 128) accounted in total for over 50% of the bacterial 16S rRNA gene sequences. Although acidobacterial sequences tend to be overrepresented in general clone libraries [e.g. (26, 60)], qPCR-based quantification of the three main acidobacterial groups supported their high frequency of occurrence in the fen soil (5.6-26.3%, 6-15.5%, and 3.6-13.1%, for group 1, 2, and 3, respectively) and comparably stable distribution across soil layers (Figure 5). High abundance of these microorganisms in deeper, mainly anoxic peat layers implies an anaerobic or facultative aerobic lifestyle of at least some group members. No correlation was found between abundances of individual acidobacterial groups and *dsrAB*-OTUs 1 or 2. This could have two reasons: First, members of these *Acidobacteria* subdivisions in the peat are not carriers of these novel *dsrAB* types. Second, because qPCR assays target microorganisms at highly different phylogenetic levels (subdivision level for *Acidobacteria* versus approximately species level for *dsrAB*-OTUs), it is possible that some acidobacterial group members harbor these *dsrAB* but correlations are obscured by other group members lacking these genes.

Independent from being *dsrAB* carriers or not, *Acidobacteria* are commonly detected in other acidic peatlands and akin environments (6, 26, 52) and thus presumably have an important ecological role in such soil ecosystems. It is likely that the phylogenetically different acidobacterial OTUs (n=33) in the Schlöppnerbrunnen II fen also have different physiological features. Assigning general metabolic capabilities across all detected members of the entire phylum is difficult, if not impossible. Nevertheless, the following physiological traits have been attributed to *Acidobacteria* and provide some hints why they are successful inhabitants of acidic peatlands. First, many isolated representatives are moderately acidophilic or acid tolerant and favor environments with pH 3.5 to 6.5 (57, 65, 90, 91). This is especially true for members of subdivision 1 (104), the most abundant group detected in our study (Figure 4). Second, peat soil *Acidobacteria* are able to utilize a variety of carbon substrates, in particular complex, plant-derived compounds (90, 91, 122). 16S rRNA-based stable isotope probing of the Schlöppnerbrunnen fen has indicated that some members of group 1 (clone AM773995) are involved in fermentation of the monosaccharide xylose, an important lignin-derived intermediate in the carbon degradation network in wetlands (43). Furthermore, aerobic strains of the species *Bryobacter aggregatus* (subdivision 3), isolated from acidic *Sphagnum* peatlands, consumed galacturonic and glucuronic acids, which are typically released from the cell walls of decaying *Spagnum* mosses (65). Third, genomic and physiological data postulate that ecological functions of some *Acidobacteria* are nitrate/nitrite reduction and iron oxidation/reduction (91, 122);(90), redox processes that are also taking place in the Schlöppnerbrunnen fen model system (2, 64, 101).

Most of the detected bacterial OTUs represent novel family/genus-level phylotypes within known phyla, while only eight OTUs could be assigned to described genera. Interestingly, members of four of those genera, namely *Smithella*, *Syntrophobacter*, *Desulforhopalus,* and *Desulfosporosinus*, are endowed with metabolic features for sulfate reduction and/or syntrophic growth. The propionate-oxidizing syntrophs *Smithella propionica* and *Syntrophobacter wolinii* were both initially cultivated in co-culture with hydrogen/formate-utilizing methanogens (71). Potential methanogenic partners for a thermodynamically favorable syntrophic lifestyle have been detected in the fen and belong to the obligate acetate-consuming family *Methanosaetaceae* and the hydrogen/formate-consuming family *Methanomicrobiaceae* (Figure 4) (44, 125). *Smithella*- and *Syntrophobacter*-related bacteria were actively involved in syntrophic oxidation of propionate in a flooded rice field soil and it is tempting to speculate that the Schlöppnerbrunnen II microorganisms have the same function. Although propionate is an important carbon degradation intermediate, which is rapidly formed and consumed after anoxic incubation of fen soil, DNA stable isotope probing of Schlöppnerbrunnen II soil that was incubated for two months with a ^{13}C-labelled mixture of acetate, propionate, formate, and lactate showed no indications for ^{13}C-labeling of *Smithella*- or *Syntrophobacter*-DNA (Pester *et al.*, submitted). However, growth of these bacteria under thermodynamically limited, syntrophic conditions was probably too low to sustain sufficient labeling of their DNA for separation by density gradient centrifugation (79).

One fen OTU has species-level 16S rRNA sequence identity to *Desulforhopalus vacuolatus*, a deltaproteobacterial SRM that can use sulfate, sulfite or thiosulfate as electron acceptor for incomplete oxidation of propionate, lactate, pyruvate, propanol or ethanol (51). Additionally, disproportionation of sulfite allows *D. vacuolatus* to grow on acetate as sole carbon source. In the absence of an electron acceptor, *D. vacuolatus* is able to switch to fermentation of lactate or pyruvate.

Although *Desulforhopalus* and *Syntrophobacter* species are capable of dissimilatory sulfate reduction, only *Desulfosporosinus* species were shown to be catalyzing this process in the Schlöppnerbrunnen II fen (Pester *et al.*, submitted). Albeit *Desulfosporosinus* species are members of the ‘rare biosphere’ (<0.01% of the total archaeal and bacterial community) in the fen, they can sustain such a high metabolic activity to account for a major part of the bulk sulfate reduction rates measured in situ. However, besides sulfate also thiosulfate or sulfite, present in high amounts in the fen system (200.3 mg/kg S) (96), might be employed by the detected SRM as alternative electron acceptors for anaerobic growth.

It is unlikely that some of the novel *dsrAB* types derive from the genera *Syntrophobacter*, *Desulforhopalus*, and *Desulfosporosinus*, because *dsrAB* sequences related to sequences from cultivated members of these genera have already been recovered from the Schlöppnerbrunnen fens (9, 75) (Pester *et al.*, submitted). However, archaeal sulfite reducers of the genus *Pyrobaculum* contain two or more very distantly related copies of *dsrAB* in their genomes (55-91% DsrAB sequence identity) (4, 34). Although the genome of *Syntrophobacter fumaroxidans*

MPOB has only one copy of *dsrAB* (http://genome.jgi-psf.org/synfu/synfu.home.html), it is at least theoretically conceivable that some of the *Syntrophobacter*, *Desulforhopalus* or *Desulfosporosinus* fen species in the fens contain in addition to their orthologous *dsrAB* a xenologous *dsrAB* copy that was acquired from a yet unknown donor via lateral gene transfer. Although members of the genus *Smithella* are not capable of dissimilatory sulfite/sulfate reduction, they could still harbor *dsrAB*, analogous to the situation in other syntrophs such as some *Pelotomaculum* species (48, 49), but this remains to be shown.

Novel *dsrAB*-carrying microorganisms are widespread in wetlands.

DGGE analyses of nine wetlands with geographical distances ranging from 1 km to 400 km showed that many of the *dsrAB*-containing microorganisms (six OTUs) found in the Schlöppnerbrunnen fens are not endemic but also inhabit other peatlands. Previous molecular fingerprinting studies of anthropogenically impacted sediments (94) and river floodplains (83) observed signatures of biogeography in *dsrAB* data, and the overall *dsrB*-DGGE banding pattern also indicated that the spatial distribution of *dsrAB*-containing microorganisms between the nine wetland sites is non-random. On condition that *dsrB*-DGGE analysis with degenerated primers is prone to biases [e.g. (11, 87, 88)] and restricted by its detection limit, we sought to identify environmental and/or geographic factors that govern the biogeography of *dsrAB*-containing microorganisms in wetlands. Spatial variations in microbial community composition can generally depend on contemporary environmental factors, on past events (such as dispersal limitation and past environmental conditions) that lead to genetic differentiation and possibly speciation, or on both (82). If community structure is only influenced by the currently prevailing environmental parameters, known as Baas-Becking hypothesis “everything is everywhere, but the environment selects” (25), we would expect no geographical distance dependent differences. Indeed, geographical distance between sites was found to be a poor predictor of *dsrB*-DGGE banding pattern distribution, suggesting that the current biogeography of *dsrAB*-containing microorganisms in wetlands is not a legacy of historical events (82). Although only few environmental parameters (Table S1) were measured, the observed biogeography pattern significantly reflected the influence of environmental variations. In particular, soil pH was the dominant factor that determined the community structure. This finding is consistent with a general study of bacterial biogeography in soils, where bacterial richness, diversity, and overall community composition were found to be mainly influenced by soil pH (32). In our study, the *dsrAB*-containing communities in the wetlands seemed to be additionally impacted by the source of water, as bogs, which are fed by precipitation, clustered together in principal component analysis (Figure 6). The type of water source can be regarded as a proxy for the nutrient content of peatlands (12, 62). Therefore, microorganisms inhabiting precipitation-dependent bogs generally have to cope with a lower availability of minerals and nutrients compared to microorganisms that live in fens, which receive additional substrates by the groundwater flow. Phylogenetic analyses of *dsrB*-DGGE bands and a *dsrAB* clone library from the Rasner Möser fen revealed ten OTUs that were broadly distributed among different bogs and fens; these OTUs include the *Syntrophobacter wolinii*-related OTU and nine OTUs with no close cultivated relatives. However, only microorganisms belonging to one of these more widely distributed OTUs (*dsrB*-DGGE OTU 6) appeared to preferentially colonize fens (Figure 7) and thus could be potential bioindicators for this type of wetland.

Conclusions

We show that microorganisms with phylogenetically novel types of the dissimilatory (bi)sulfite reductase genes *dsrAB* are persistent and numerically significant inhabitants of a long-term experimental peatland field site. Comparative analysis of *dsrAB*-carrying microorganisms in different peatlands has identified soil pH as one of the most influential community structure-shaping environmental factors. In contrast, dispersal limitation of *dsrAB*-carrying microorganisms does not seem to contribute largely to endemism, because members of various novel *dsrAB* lineages display a rather cosmopolitan distribution among peatlands that are located up to several hundred kilometers apart.

Numerical abundance and broad distribution of these novel *dsrAB*-carrying microorganisms clearly suggest that they have a considerable impact on peatland ecosystem functioning. Revealing the identity and actual physiological features of these mysterious microbes thus far proved to be a challenge (Pester *et al.*, submitted). However, the relatively high abundance of these yet unidentified microorganisms at a given time and location holds much promise for future research, because it makes them amenable to techniques that allow sorting and genomic or physiological analysis of individual microbial cells (45, 70, 110, 126).

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Supplementary material

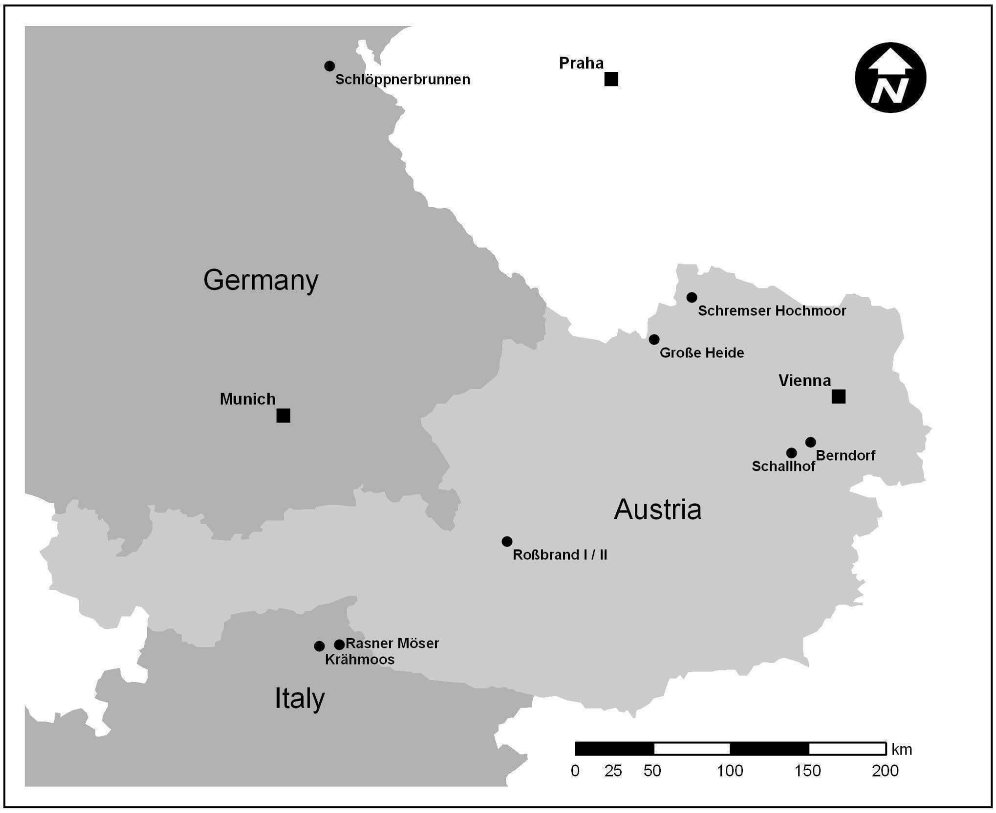


Figure S1. Geographic location of the investigated wetlands.

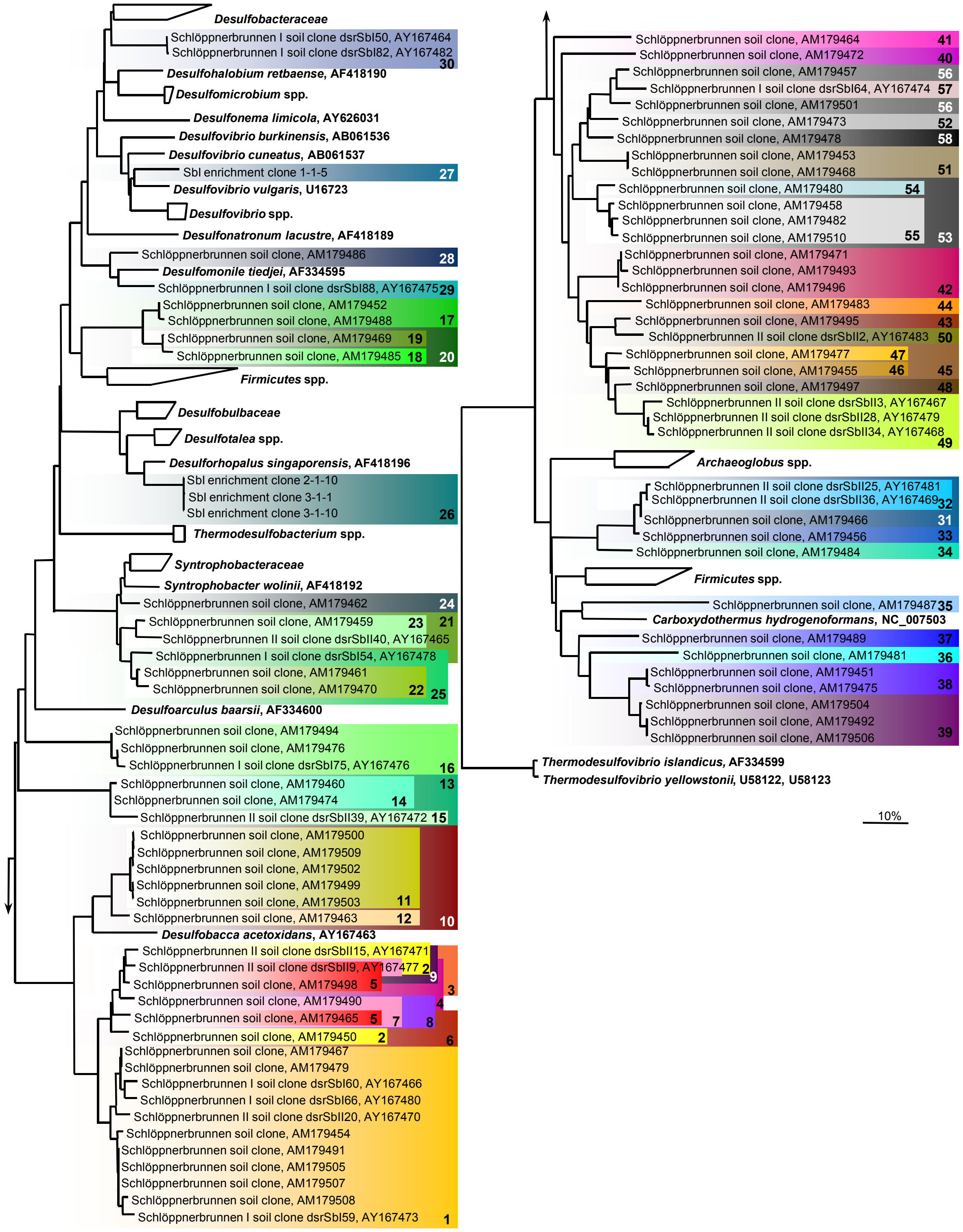


Figure S2. Target groups of *dsrAB*-based oligonucleotide microarray probes. *DsrAB* phylogeny of Schlöppnerbrunnen *dsrAB* sequences (6, 7) was inferred by distance matrix analysis. Colored boxes indicate perfectly-matched target sequences of probe-target groups 1 to 58, as defined in Table S4.

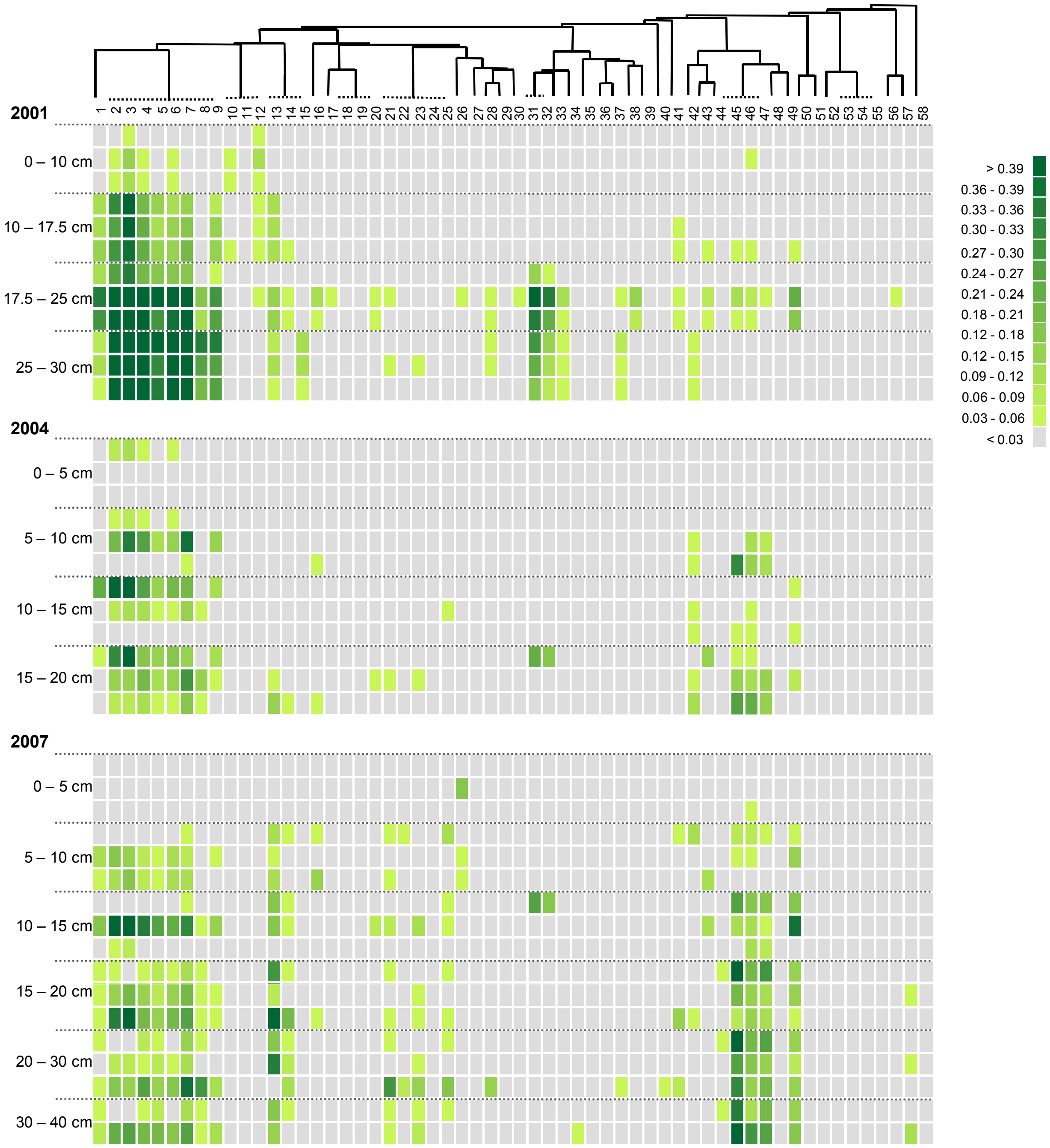


Figure S3. Microarray-based *dsrAB* diversity analysis of Schlöppnerbrunnen II fen soils sampled from different depths in the years 2001, 2004, and 2007. Results of microarray hybridizations are displayed as mean nSBR of all probes within a probe-target group and are shown for each replicate. The color code translates into different mean nSBR values. Probe-target groups are arranged phylogenetically according to their position in a *dsrAB* neighbor-joining tree.

TableS1. Description and location of sampling sites.

Wetland name	Geographical position	Wetland type[a]	pH	Sulfate [µM][c]	Nitrate [µM][c]
Schlöppnerbrunnen	50° 09' 21" N 11° 52' 22" O Germany	fen	4.0-6.1[b]	22-240 [b]	<5-120[b]
Krähmoos	46° 47' 60" N 11° 54' 18" O Italy	bog	4.0	10.2	1.1
Rasner Möser	46° 48' 27" N 12° 04' 25" O Italy	fen	4.3	9.1	0.2
Roßbrand I	47° 24' 27" N 13° 28' 27" O Austria	fen	4.1	18.3	2.1
Roßbrand II	47° 24' 39" N 13° 28' 56" O Austria	fen	4.9 [d]	57.5 [d]	2.3 [d]
Große Heide	48° 34' 06" N 14° 45' 26" O Austria	bog	5.3	41.1	12.3
Schremser Hochmoor	48° 48' 17" N 15° 06' 04" O Austria	bog	4.7	30.5	1.8
Schallhof	47° 53' 23" N 15° 56' 43" O Austria	fen	7.3	2.1	0.2
Berndorf	47° 56' 41" N 16° 06' 46" O Austria	wet meadow	7.6	116.8	0.5

[a] Wetland classification: bog, ombrotrophic (rainwater-fed); fen, minerotrophic (mostly groundwater-fed)
[b] Data from (7)
[c] Sulfate and nitrate was determined in the soil water by ion chromatography
[d] Soil core C was measured separately: pH = 5.8, nitrate: 3.3 µM, sulfate: 95.6 µM

Table S2. *dsrAB*-targeted primers.

Primer	Sequence (5'-3')	G+C (%)	Theoretical melting temperature [a]	Reference
DSR1F	ACSCACTGGAAGCACG	63	42-51	(9)
DSR1Fa	ACCCAYTGGAAACACG	50-56	43-45	(6)
DSR1Fb	GGCCACTGGAAGCACG	69	51	(6)
DSR1Fc	ACCCATTGGAAACATG	44	40	(10)
DSR1Fd	ACTCACTGGAAGCACG	56	47	(10)
DSR1Fe	GTTCACTGGAAACACG	50	42	Pester *et al.* submitted
DSR1Ff	AGCCACTGGAAACACG	56	47	Pester *et al.* submitted
DSR1Fg	GGCCACTGGAAACATG	56	44	Pester *et al.* submitted
DSR1Fh	GGCTATTGGAAGCACG	56	46	Pester *et al.* submitted
DSR4R	GTGTAGCAGTTACCGCA	53	48	(9)
DSR4Ra	GTGTAACAGTTTCCACA	41	41	(6)
DSR4Rb	GTGTAACAGTTACCGCA	47	45	(6)
DSR4Rc	GTGTAGCAGTTKCCGCA	53-59	49	(6)
DSR4Rd	GTGTAGCAGTTACCACA	47	43	(10)
DSR4Re	GTGTAACAGTTACCACA	41	40	(10)
DSR4Rf	GTATAGCARTTGCCGCA	47-53	47-48	Pester *et al.* submitted
DSR4Rg	GTGAAGCAGTTGCCGCA	59	52	Pester *et al.* submitted

[a] Melting temperatures were estimated using oligoCalc (5)

Table S3. Primers and targets for PCR- and in vitro transcription-based preparation of a defined mixture of fluorescently-labelled control RNA for microarray hybridization.

Target	Primer	Primer sequence (5'-3')	Reference	PCR annealing temp. (°C)	Amount of labeled target RNA per hybridization
16S rRNA gene of *Desulfonema limicola* DSM 2076	BACT8F BACT1529R	AGAGTTTGATYMTGGCTC CAKAAAGGAGGTGATCC	(4)	52	100 ng
Nucleotide transporter NTT1 gene of *Protochlamydia amoebophila* UWE25	NTT1F NTT1R	ATGTCGCAAGATGCGAAA TTAGCTAGTAGCTATTTC	(8)	55	75 ng
16S rRNA gene of *Desulfohalobium retbaense* DSM 5692	BACT8F BACT1529R	AGAGTTTGATYMTGGCTC CAKAAAGGAGGTGATCC	(4)	52	50 ng
Nucleotide transporter NTT5 gene of *Protochlamydia amoebophila* UWE25	NTT5F NTT5R	ATGAAAAATCAACAAAAT TTATCCATGGGAAGCTTC	(3)	55	25 ng
Nucleotide transporter NTT4 gene of *Protochlamydia amoebophila* UWE25	NTT4F NTT4R	ATGAGTAAAACAAACCAG TTATTTTTTTATAAAAGC	(2)	55	10 ng

Table S4. Oligonucleotide probes for microarray hybridization.

Probe name	Perfectly-matching target organisms/sequences	Target gene	Probe-target group	*dsrAB*-OTU [d]	Sequence (5'-3')	deltaG [kcal/mol]	Probe length
dsrCONT1	*Desulfohalobium retbaense* DSM 5692 T	16S rRNA	-	-	CGCAGAGTTATCCCATACCTCGAGGTAGATTATC	-41.2	34
dsrCONT2		16S rRNA	-	-	GACCCGTATTGGGAATCACCCATTTCTTCCCT	-40.8	32
dsrCONT3	*Desulfonema limicola* DSM 2076	16S rRNA	-	-	GCTCCCCGAAGGGCACTATCTCCTTTCAAAG	-40.7	31
dsrCONT4		16S rRNA	-	-	TAGAGGCCATCTTTCATCATTAATACCGGGGTA	-40.7	33
dsrCONT7	*Protochlamydia amoebophila* UWE25	*ntt1*	-	-	GGATAGCTGCAGGGACAACACCAAAAGACTTC	-41	32
dsrCONT8		*ntt1*	-	-	GCCATGCATCAACATTTGCAGGTAAATGCTTG	-40.6	32
dsrCONT9	*Protochlamydia amoebophila* UWE25	*ntt4*	-	-	GCGTGATTATAGGTCCCATTACAGCGCCTAAA	-40.5	32
dsrCONT10		*ntt4*	-	-	ATCCATATTGGCTGCAAAACGCGTTTGTGCAA	-42.2	32
dsrCONT11	*Protochlamydia amoebophila* UWE25	*ntt5*	-	-	GCCATATTTCCAACGAACATACAGAGGGGGTA	-40.5	32
dsrCONT12		*ntt5*	-	-	TGTCAGTCCAAACCCCACTACCAATACAGCTA	-40.7	32
dsrA1	AY167466, AY167480, AM179479, AM179467, AY167470, AY167473, AM179507, AM179505, AM179491, AM179508, AM179454	*dsrA*	1	OTU 1	TCAGTTCGGCGAAGGTGGGTTCCAGTTCAT	-40.8	30
dsrA2		*dsrA*			AAGGTGGGTTCCAGTTCATCGGTGGTGGTG	-40.7	30
dsrA3		*dsrA*			TTCATCGGTGGTGGTGCCCAGGAAGATCAT	-40.1	30
dsrB4		*dsrB*			GTTGTCTTTGATGATGGGCGGCAAGAACTTGT	-41.4	32
dsrA5		*dsrA*			CCAGGATGGGGGCGTGCGATCCCAGGAG	-41.5	28
dsrB6		*dsrB*			GGGGGGCCAATATCAGTTATACGGTCAGCC	-39.6	30
dsrA7	AY167467, AY167479, AY167468	*dsrA*	49	OTU 2	ACGGGTTATCTCTGGGTGCCGAGACCATTT	-40,0	30
dsrA8		*dsrA*			GCACGCGCGATGGAAGCGACGCAATCG	-41.6	27
dsrB9		*dsrB*			CTCGCAAACTCGGGGAAGAATGTCGTGCTT	-40.7	30
dsrB10		*dsrB*			ACGCCGGGCGCCAGGTTCTCGTGATACTT	-42.4	29
dsrA11		*dsrA*			GGGACAGCCCGCGCACTTGATCTTGAACTT	-41.7	30
dsrB12		*dsrB*			AGTTCTTCTTCACAATCGGAGGCAGGAACTTGT	-41.8	33
dsrA13[a]	AM179497	*dsrA*	48	-	CAATCATTGGGACAACCGGCACACTTGATTTT	-40.5	32
dsrA14[a]		*dsrA*			GCTGGTATAAAACCATCCCGCCGGATGGTT	-40,0	30
dsrA15[a]		*dsrA*			GTGCATGTTGGTCAATCCGGATCCGTGCTT	-40.8	30
dsrA16	AM179495	*dsrA*	43	-	TGGAAAGCCGGCTCAAGTTCCGGTGTGGTT	-42.1	30
dsrA17		*dsrA*			GCCCAAATCGAATCCCGCCCTCGCAAGTT	-41.1	29
dsrA18		*dsrA*			GCGCCATACCGACGCAACACGAAGGCGT	-42.2	28
dsrA19	AY167483	*dsrA*	50	OTU 7	TCGTAGGGCAGAGATCGCAAACGTGCTTTT	-40.5	30
dsrB20		*dsrB*			TACTCGCCGGATCTGGTCCTTTTGGACCTT	-40.5	30
dsrA21		*dsrA*			CACGCAGTTCCGGTTGTCAATCGCGAGTTT	-41.5	30
dsrA22	AM179496, AM179471, AM179493	*dsrA*	42	-	CTCGAGTAGAACCACGCCGCCGGTTGGTT	-41.8	29
dsrA23		*dsrA*			CATCGGGAAATTGCTCGCCTACGTCACTGT	-40.4	30
dsrA24		*dsrA*			TGAAAGGTGGGCTCCAACTGGTCCGTGGTT	-41.5	30
dsrA25	AM179468, AM179453	*dsrA*	51	-	CGGCGTATTCGGCGACCTTCTCATCGTTTT	-41,0	30
dsrA26		*dsrA*			CACGGATGGTGTGGAAGCGCGCCACGTT	-42,0	28
dsrA27		*dsrA*			GTGGAAGCGCGCCACGTTGGGGAATTCTT	-41.4	29
dsrA28	AM179483	*dsrA*	44	-	CGCCTTGGCAAACTCCTGAAACGCGGGTT	-41.5	29
dsrA29		*dsrA*			GTGCACGTTGGTCACGCCCGAACCGTTG	-41.5	28
dsrA30		*dsrA*			CGCAGATGGTGCGAATGGCGTCACTGGTG	-41.9	29
dsrA31	AM179510, AM179482, AM179458, AM179480	*dsrA*	53	-	CTCCTTGAAGGTGGGCTCGAGCTCATCGG	-40.2	29
dsrA32	AM179510, AM179482, AM179458	*dsrA*	55		TCGGGCATCCACTGCACTTGAACTTGAACTT	-41,0	31
dsrA33		*dsrA*		-	TTGACACGCAGCGTATGGAAGTGCGCGAC	-41.8	29

dsrA34		*dsrA*			CTCATCCTGGTAGGTCCGCGTCAAGTCGTC	-41.1	30
dsrA35	AM179480	*dsrA*	54	-	GAGGTCGAATCCCGCGGCGGTCAGCTCC	-42.3	28
dsrA36		*dsrA*			CTCATCCTGGTAGCTGCGCGTCAAGTCGTC	-41.9	30
dsrA37	AM179501, AM179457	*dsrA*	56	-	GCGGTCGCACACGTCTTTCTTGATGTCGAG	-41.6	30
dsrA38		*dsrA*			GACAGCGGTCGCACACGTCTTTCTTGATGT	-40.9	30
dsrA39	AY167474	*dsrA*	57	OTU10	ATACTCATCCCAGAATTCCCACACGCGTTTC	-39.8	31
dsrB40[b]		*dsrB*			CCGCAGCTCCTGCTTGAGCGGTTCGATCT	-41.8	29
dsrA41		*dsrA*			GCGTGGGGCAGCGATCGCACACATCCTT	-41.6	28
dsrA42	AM179478	*dsrA*	58	-	CTTGAAGGCAGGCTCCAGCTCAGAGGTCTT	-40.3	30
dsrA43		*dsrA*			GTAGGCGCGGGTGAGGTCATCGCACAGTT	-41.8	29
dsrA44		*dsrA*			ACGCGCATGGTATGAAAGTGCGCGACCTT	-41.3	29
dsrA45	AM179473	*dsrA*	52	-	GCATGTTCGTCAAGCCGGATCCGTGACGTT	-42.2	30
dsrA46		*dsrA*			CGTGATGCGATCGCACATCTCGAGCGTGT	-41.6	29
dsrA47		*dsrA*			ACTTGTAAGGAAAAGGCGGACGGTGCAGTT	-40,0	30
dsrA48[a]	AM179472	*dsrA*	40	-	GTCATTGGGGCACCCCGCGGCCTTGAATT	-42.1	29
dsrA49[a]		*dsrA*			GAGAAGGTGGGCTCCAGTTGATCCGTTCGC	-41.5	30
dsrA50[a]		*dsrA*			TGGTGTGAAAGTGGGCGACCTCGGGGAATT	-41.6	30
dsrA51[a]	AM179464	*dsrA*	41	-	GTACTGCATCGTCAGATCGTAGGTCAGGTTGA	-41.2	32
dsrA52[a]		*dsrA*			GGAGGTGTAGAACCAGGCGCTGGGCTGGT	-41.9	29
dsrA53[a]		*dsrA*			CTTCACCTGGGCCTGGTCGATCTGGATGTT	-40.4	30
dsrA54	AY167478, AM179461, AM179470	*dsrA*	25	OTU 6	AGTCGGAACGCGCGATCGATGCGACGC	-41.6	27
dsrA55		*dsrA*			CTGGTATTCCTGCGTCAGGTCGTAAATGATGT	-40.4	32
dsrA56		*dsrA*			TCGCAACGGGACATTCCCAGGCAGCCCT	-41.4	28
dsrA57	AY167465, AM179459	*dsrA*	23	OTU 6	AGAAAACAGGCTCGAGCTGGTCGGTAGTCG	-40.9	30
dsrA58		*dsrA*			GGTCCTCAAGTTGGATCCCGAGCCGCCGA	-42.4	29
dsrA59		*dsrA*			GCGCAAGACTTTCGTGTCGTAGTACTTGCT	-39.7	30
dsrA60	AM179462	*dsrA*	24	-	CCACAGATCGCACAGACCGCGGATGACTTT	-41.6	30
dsrA61		*dsrA*			TCCCAGGCAACTTTCCGGAGTCCTGAGGTT	-41,0	30
dsrA62		*dsrA*			GCGTCAGATCATAGATGATGTCCTGCGTGTT	-39.9	31
dsrA63	AY167469, AM179466, AY167481	*dsrA*	31	OTU 3	TTCTCCGGGCAGTCCGAGTACCGGCCGA	-41.9	28
dsrA64		*dsrA*			CAGTCATTGGGACAGGCCGCGACCTTGAT	-40.4	29
dsrB65	AY167469, AY167481	*dsrB*	32	OTU 3	CGCGCACCGTATAGAGCGCTTCTCCCGT	-41.1	28
dsrB66		*dsrB*			GATTTCACAGAGATTCGGGATGCGCGTGTT	-39.8	30
dsrB67		*dsrB*			GATATCGCATATCTCCCGGATGTGGTCCGTT	-40.2	31
dsrA68	AM179456	*dsrA*	33	OTU 3	AAGCGGCGTAGGCCGCCACTTCCTTATGG	-41.7	29
dsrA69		*dsrA*			GATGCAGGCCCACTCACATCGTCCCGGT	-40.4	28
dsrA70		*dsrA*			ATAGACCATGCACCTCGTCGGGCAGAGGTT	-41.2	30
dsrA71	AY167471, AY167477, AM179450	*dsrA*	2	OTU 4	CTTGTACGGGAAAGACGGCCGGTGCATATC	-40.7	30
dsrA72	AY167471, AY167477, AM179498	*dsrA*	3	OTU 4	ATGGTGTCGTAGCAGGCGTATTCGCAGCG	-41.2	29
dsrA73	AY167477, AM179498, AM179490	*dsrA*	4	OTU 4	CTTCCAGACGCCGATGACGCTCATATCGGC	-42,0	30
dsrA74	AY167477, AM179498, AM179465	*dsrA*	5	OTU 4	TCCGGCAGGTCGGAGTAACGGCCGATGAT	-42,0	29
dsrA75	AY167477, AM179498, AM179490	*dsrA*	4	OTU 4	CCCGCAGGGCCTTGGTGGTGTAGAACCAG	-41.4	29
dsrA76	AM179465, AM179450	*dsrA*	6	OTU 4	GAAGCAGGGCTCCAACTGATCGGTGGTGGT	-41.9	30
dsrA77		*dsrA*			GACGGCCGGTGCATATCGAACTGGAATTCC	-40.9	30
dsrA78	AY167477, AM179490, AM179465	*dsrA*	7	OTU 4	TGGAGCCGTGCTCGTCCCAGATGGCGC	-41.3	27

dsrA79	AM179490, AM179465	*dsrA*	8	OTU 4	CCGGTGCATATCGAACTGGAATTCCTGGGT	-39.7	30
dsrB80	AY167477, AY167471	*dsrB*	9	OTU 4	CGAGCTTTTTGCATTCGGCCTTCACGCCG	-41.6	29
dsrB81		*dsrB*			TCCACTTGCCGTAGTTCTCTTTGATCACCGG	-40.8	31
dsrA82	AM179502, AM179509, AM179500, AM179503, AM179499, AM179463	*dsrA*	10	-	GCGGTGTAATACCAGCCGGAGGGCTGGTT	-41.2	29
dsrA83		*dsrA*			CATGGTGTGGAAATGTTCCAGACCGGGGAA	-39.7	30
dsrA84	AM179502, AM179509, AM179500, AM179503, AM179499	*dsrA*	11	-	GTGGAAATGTTCCAGACCGGGGAACTCATCC	-40.8	31
dsrA85[a]	AM179463	*dsrA*	12	-	GTGGAAATGTTCCAGACCGGGGAAATCATCC	-39.9	31
dsrA86	AY167472, AM179474, AM179460	*dsrA*	13	OTU11	GGTGCCGATGATGGACATGTCGGCGCGG	-42,0	28
dsrA87[a]	AM179474, AM179460	*dsrA*	14	OTU11	CCTTTGCCGTTGTTTCCGCAAAGATGGCTT	-40.4	30
dsrA88[a]		*dsrA*			GTTGGCGCATCCTGAAACCTTGATCTTGAAC	-39.9	31
dsrA89	AY167472	*dsrA*	15	OTU11	CCTTTGCAGTTGTTTCCGCGAAGATGGCCT	-41,0	30
dsrA90		*dsrA*			GTTGGCGCAACCGGCAACCTTGATCTTGAA	-41.3	30
dsrA91	AM179506, AM179492, AM179504	*dsrA*	39	-	TGCCTCGGTCAGTGCATCGAAGCACGGC	-41.5	28
dsrA92		*dsrA*			TGACGCGCACGGTGTGGAAGGCGGTGA	-41.6	27
dsrA93		*dsrA*			CTTGTACGGCCAGCGGGGTCGATGCATCT	-41.6	29
dsrA94[a]	AM179475, AM179451	*dsrA*	38	-	ATGACATCGCCGGTCGCGCCGTGAAAGTT	-42.4	29
dsrA95[a]		*dsrA*			TCATCCTGATACGTCATCGTAAGGTCGTGTATC	-40.8	33
dsrA96[a]		*dsrA*			GATGCGCATGGTGTGAAATGCGGTCACGTT	-41.8	30
dsrA97[a]		*dsrA*			GATACGTCATCGTAAGGTCGTGTATCAGCGC	-40.5	31
dsrA98	AM179506, AM179492, AM179504	*dsrA*	39	-	GATAGGTCATGGTAAGGTCGTGTATCAGCGC	-39.8	31
dsrA99[a]	AM179481	*dsrA*	36	-	TTAGCTCGTCGAAGCGGGGTTGCAGGCTTT	-42.4	30
dsrA100[a]		*dsrA*			TGGTATGGAACATCTCAACGGCGGGGAATTTA	-41.6	32
dsrA101[a]		*dsrA*			GTGCCGGGCCAACACAGCAACTGATGGTT	-41.2	29
dsrA102	AM179489	*dsrA*	37	-	TTCCCAGACATCACAGAGCTTTCTCAAGGTTTT	-40.6	33
dsrA103		*dsrA*			AAGATTGGCGGTGTTCGTGCCCAAAAGAATG	-41,0	31
dsrA104		*dsrA*			ACCTCCGAGATCGAACCCTATCTCTGTCAGTG	-41.2	32
dsrA105[a]	AM179487	*dsrA*	35	-	CCAGATATCGCAGAGTCGTCGAAGGTATTTTGT	-41.2	33
dsrA106[a]		*dsrA*			TCATCGAAGCATGGTTGCAGCTCGTTGGTT	-40.8	30
dsrA107[a]		*dsrA*			CACCTGAACCGCCTAAGTCGTAGCCTGCTT	-41,0	30
dsrA108	AM179488, AM179452	*dsrA*	17	-	CCGCATCCTTATTGATACGGATGTCATCTTTCC	-40.6	33
dsrA109		*dsrA*			CAATATCACAGAAACCTCTCAGCCAATCAGTTGT	-40.9	34
dsrA110		*dsrA*			CAGGTCCATGGTATCGTAACATGCGAACTCG	-40.3	31
dsrA111[a]	AM179485	*dsrA*	18	-	CACATGCCCATGGTATCGTAACATGCGAACTC	-41.6	32
dsrA112	AM179469	*dsrA*	19	-	AACACATGTCCATGGTATCGTAACATACGAACTC	-41,0	34
dsrA113	AM179485, AM179469	*dsrA*	20	-	TACCAATACAATCAGAAGGTGTACGCAGGTTTC	-40.7	33
dsrA114		*dsrA*			TGAACGTGCGATACTTGCAGTACAGCCATTC	-40.5	31
dsrA115	AY167464, AY167482	*dsrA*	30	OTU 5	CCCTTCTCTCCCACAAGTCACAAATCTTTTCCA	-40.9	33
dsrA116		*dsrA*			TATCGTGGGTCAGTTCCCAAAAAACCTCTTCC	-40.7	32
dsrB117		*dsrB*			GGCTGCTTGCGGCTCAGGAGATCCTGTTTT	-41.5	30
dsrB118		*dsrB*			AATGACCTCGGCGTCGATCATGGGCGGTTT	-42.4	30
dsrA119	AY167475	*dsrA*	29	OTU 9	CCCCAAACAGCACTCGGGAGTTCTAAGGTTA	-40.6	31
dsrB120		*dsrB*			GTTTGATCCGCCCGGGAACTTACGGCTTTT	-40.7	30
dsrB121		*dsrB*			GCAACCACCTACGCGCACGGAGAAAACCTT	-42.1	30
dsrA122	AM179486	*dsrA*	28		CAGGTTCGACCCTGAACCGCCCAAGTCCTG	-42.4	30
dsrA123		*dsrA*		-	TGGTCCGTATTAGTCCCCAGGAAGACGATGTC	-41.5	32

dsrA124		*dsrA*			TTGAGGCTGGTCGCAATACCTGCCGATGGT	-41.9	30
dsrA125	AM179484	*dsrA*	34	-	CATCAAAGCAGGGCTGAAGATTCTCCGTCTTG	-41.1	32
dsrA126		*dsrA*			CCCCTAAATCAAAACCCGCATCCGAAAGGTC	-40.4	31
dsrA127[b]		*dsrA*			AGCAGATATCCAGGCTGTGGACGCAGGCC	-41.1	29
dsrA128	Schlöppnerbrunnen fen soil enrichment clones 2-1-10, 3-1-10, 3-1-1[c]	*dsrA*	26	-	GCCAGAAGACATAGGGATTGGAACGAGGTTCT	-40.8	32
dsrA129		*dsrA*			CCAGAGATTCATCAGGTCGCGAAGATTCTTTG	-40,0	32
dsrB130		*dsrB*			TTTCCATCAATTTCCACCTTGGCGGGCTTTAC	-41.2	32
dsrB131[a]	Schlöppnerbrunnen fen soil enrichment clone 1-1-5RV[c]	*dsrB*	27	-	TCGTTGTCCCCCATGAATTCAACGTTGTTTCT	-40.9	32
dsrA132[a]		*dsrA*			GCTTCTGGAAAGAGAGGCGCTTCATGGTTTC	-40.6	31
dsrA133[a]		*dsrA*			CCAGTCCCAGATCTTGCCGACAACGTCTTT	-40,0	30
dsrA134	AY167476, AM179476, AM179494	*dsrA*	16	OTU 8	AGAGGTCGGTGAAGATGTCCTCAAGGTGTTCG	-42.1	32
dsrA135		*dsrA*			GTCGCAGAGATAACGGAGAGAAGACGTGTTGT	-41.5	32
dsrA136		*dsrA*			GGCATATCGCCATTGTATCCATGCAGGCGT	-40.7	30
dsrA137	AM179477, AM179455	*dsrA*	45	-	ACCTGAATGTCGTCTTTCCAGGTGCCGAT	-38.5	29
dsrA138	AM179455	*dsrA*	46	-	TCAGGTCGTAGCAGAGCTTCATGGTGTCGTA	-41.7	31
dsrA139		*dsrA*			CATTCGCGATTCTCGATGGCCAGCTTCTTG	-40.3	30
dsrA140		*dsrA*			CCAGATCGAAACCGGCGTGTGCGAGTTCC	-41.6	29
dsrA141		*dsrA*			GCATATTGGTCAGGCCAGACCCGTGCTTCT	-41	30
dsrA142	AM179477	*dsrA*	47	-	TCGCGGTTCTCAATGGCGAGCTTCTTGCC	-41.5	29
dsrA143		*dsrA*			ACTTGTAGGCAAACGCGGGACGATGCAGTT	-41.6	30
dsrA144		*dsrA*			CACAGAAGGTGCGCAGGGCATCGCTGGT	-41.3	28
dsrA145	AY167465, AM179459, AY167478	*dsrA*	21	OTU 6	CGCCAGGTCCCGATAATCGAGCAGTCGGA	-41.4	29
dsrA146	AM179461, AM179470	*dsrA*	22	-	CGCCAGGTCCCGATAATCGAACAGTCGGA	-40.3	29

[a] Perfectly-matched reference target was not available for testing.

[b] Probe was false-negative during testing and was excluded from further analysis.

[c] unpublished *dsrAB* clones from a Schlöppnerbrunnen I fen soil enrichment (1)

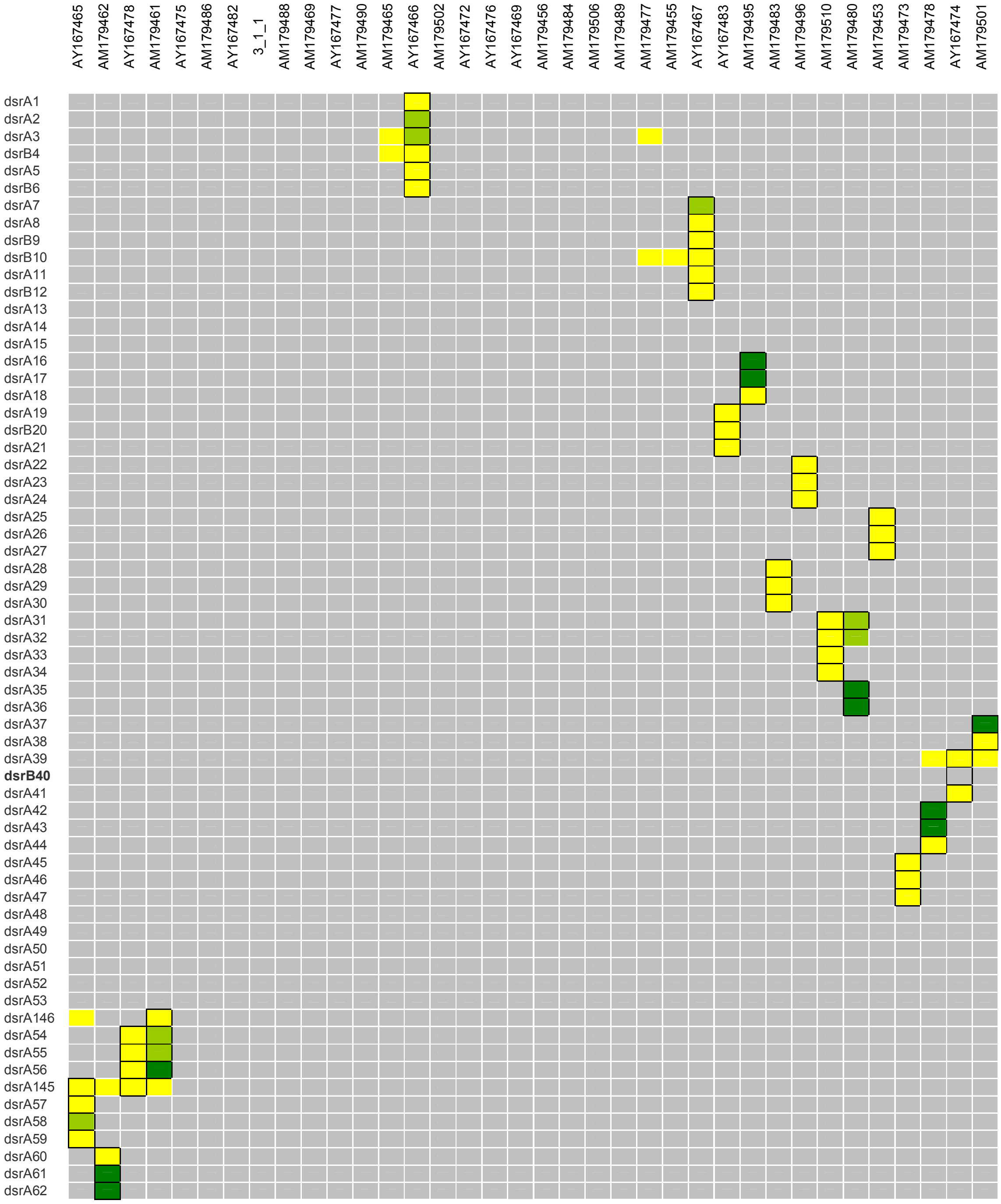
[d] Affiliation of probe-target group to previously defined Schlöppnerbrunnen *dsrAB*-OTU (6)

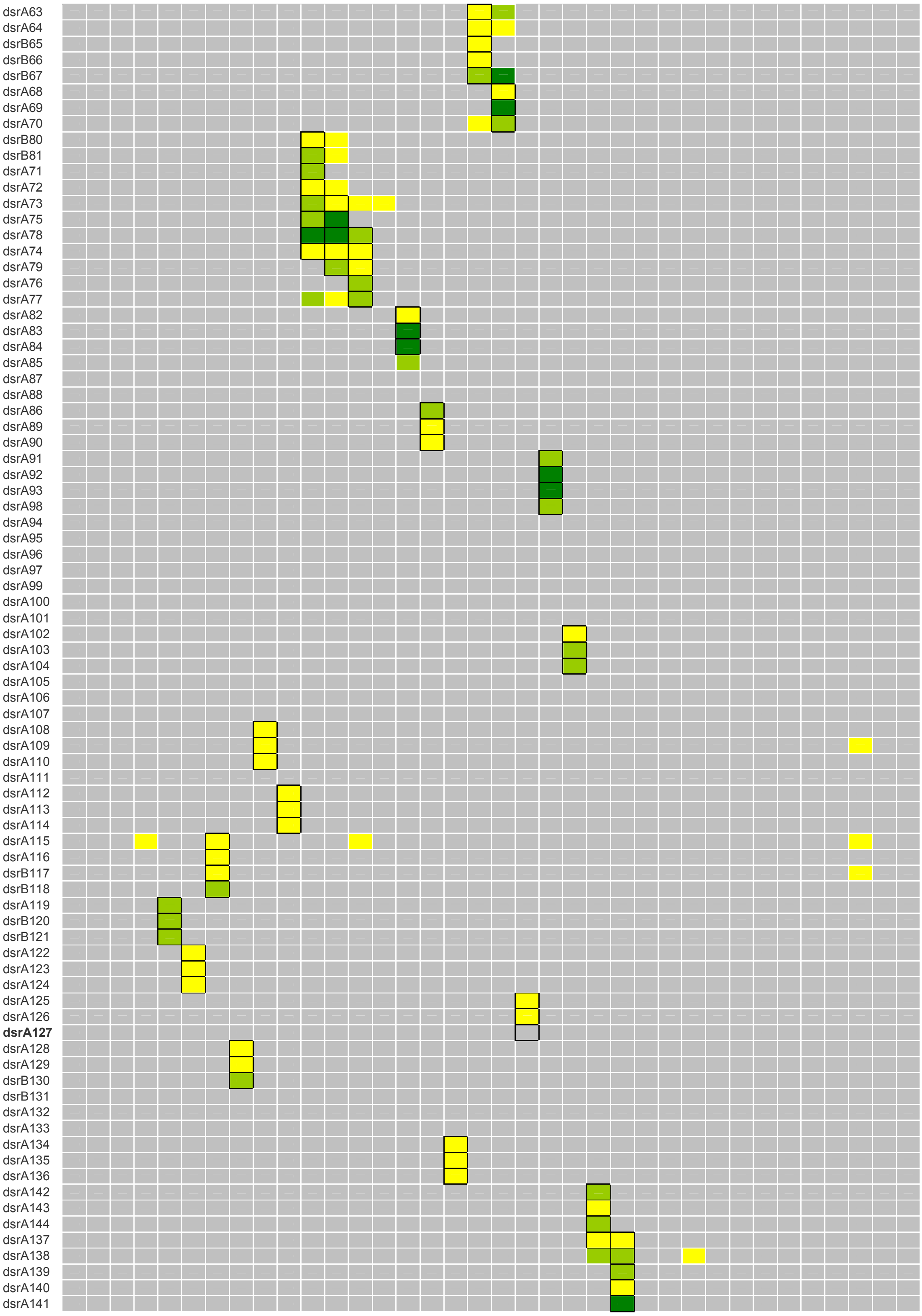
Table S5. *dsrB*-targeted primers used for DGGE analysis.

Primer	Sequence (5'-3')	G+C (%)	In silico coverage (%)[a]	Number of non-degenerated primer variants	Primer-Mix	In silico coverage (%)[a]
DSR1728Fa	cay acc cag ggy tgg	60-73	67.0	4	DSRp1728FmixA	67,0
DSR1728Fb	cay acc cag ggr tgg	60-73		4		
DSR1728FcI	cac acb caa ggd tgg	53-67	6.0	9	DSRp1728FmixB	6,0
DSR1728FcII	cay acb caa ggc tgg	53-67		6		
DSR1728Fd	cat acd cag ggh tgg	53-67	4.4	9	DSRp1728FmixC	4,4
DSR1728Ff	cac acd cag ggr tgg	60-73	11.4	6	DSRp1728FmixD	11,4
DSR1728Fe	cac acd cag ggy tgg	60-73		6		
DSR1728Fg	cat acc cag ggn tay	47-60	6.3	8	DSRp1728FmixE	5,3
DSR1728Fh	cat acw cag ggh tat	40-47		6		1,0

[a] Coverage was determined using a customized *dsrAB* database including 1641 *dsrB* sequences with sequence information at the primer binding site.

Table S6. Specificity of *dsrAB*-FGA probes as tested by individual hybridization with 36 reference clones. Grey colored boxes indicated hybridization events with normalized signal-to-background ratios (nSBR) below 0.04 and were considered negative. Yellow boxes indicate hybridization signals between 0.04 and 0.39. Light green boxes specify hybridizations with nSBR between 0.4 and 0.79. Dark green depicts hybridizations with nSBR ≥0.8. Hybridizations between reference clones and perfectly-matching probes are framed in black. False positive hybridizations are thus indicated by colored boxes without black frames, false negative hybridizations by grey boxes with black frames (n=2, probes are additionally indicated in bold).




dsrA63
dsrA64
dsrB65
dsrB66
dsrB67
dsrA68
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dsrA140
dsrA141

Table S7. Classification of *dsrB*-DGGE sequences in OTUs (based on an amino acid identity threshold of 90%) and corresponding *dsrAB*-OTUs from Schlöppnerbrunnen (6) and Rasner Möser wetland sites.

OTU	*dsrB* DGGE sequences	Corresponding Schlöppnerbrunnen (Sb) and Rasner Möser (Rm) OTUs	
1	DG83	-	-
2	DG154	-	-
3	DG151	-	-
4	DG13	-	-
5	DG15, DG16, DG26	-	-
6	DG44, DG45, DG46, DG50	-	-
7	DG17	-	-
8	DG129	-	-
9	DG155	-	-
10	DG144	-	-
11	DG158, DG159	-	-
12	DG33	-	-
13	DG146	-	-
14	DG64, DG65, DG66, DG87	-	-
15	DG116	-	-
16	DG67	-	Rm OTU 6
17	DG112, DG113, DG152, DG153, DG156, DG74, DG79, DG80, DG81	Sb OTU 1	-
18	DG58	Sb OTU 2	-
19	DG55, DG101	Sb OTU 3	Rm OTU 1
20	DG91, DG92, DG131	Sb OTU 4	-
21	DG35, DG37, DG40, DG51, DG52, DG117, DG123, DG141, DG140	Sb OTU 6	-
22	DG18, DG19, DG20, DG21, DG22, DG27, DG31, DG124, DG135, DG82	Sb OTU 11	Rm OTU 11

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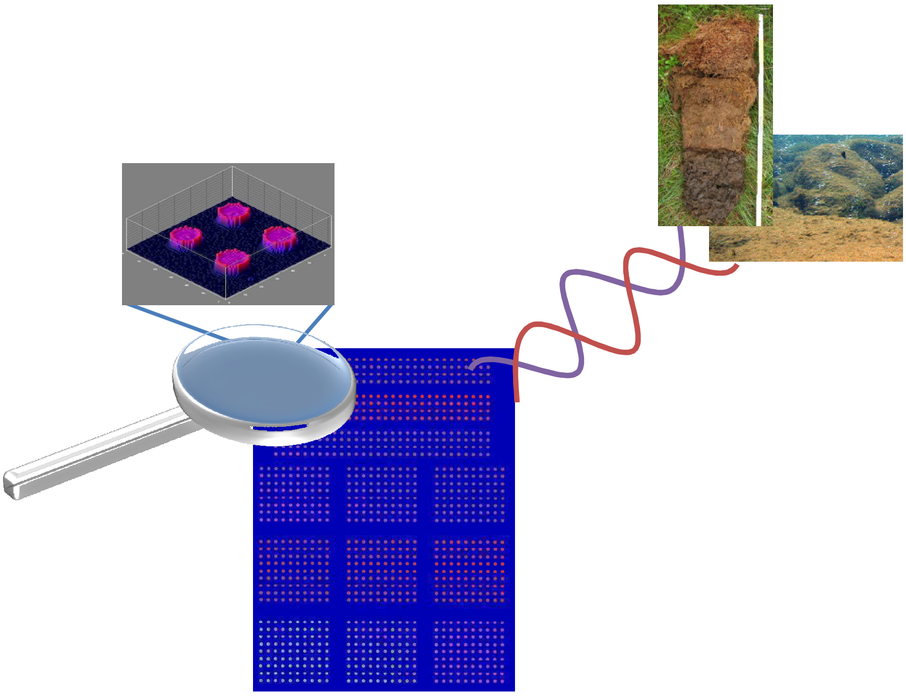
Part II

Hybridization regimes in DNA microarray experiments



Introduction

1. DNA Microarrays as high throughput method for community structure analysis

With the ascent of 16S rRNA gene-based diagnostic DNA microarrays a powerful method was available to rapidly screen numerous samples. Initially, this tool was used to analyze gene expression patterns (Schena *et al.* 1995). With the progress in development of the techniques several other applications came up and thus, DNA microarrays are now widely used to profile microbial community structures (Loy *et al.* 2002; Peplies *et al.* 2004; Lehner *et al.* 2005; Loy *et al.* 2005; Huyghe *et al.* 2008), to detect pathogens (Wang *et al.* 2007; Wiesinger-Mayr *et al.* 2007), or to study bacterial community dynamics and seasonal variations (Brodie *et al.* 2006; Garrido *et al.* 2008). In addition, microarrays opened the door to highly parallel structure-function analyses of microbial communities with the development of the isotope-array approach (Adamczyk *et al.* 2003; Wagner *et al.* 2006). DNA microarrays are based on the parallel detection of tens to thousands different sequence types via multiple hybridization reactions of target sequences with specific probes, which are immobilized on a solid substrate. The DNA microarray technique involves different steps including probe design, microarray fabrication and processing, target preparation, hybridization, and data analysis. Two types of phylochips can be distinguished based on the total number of probes. The high capacity of the microarray format is best exploited with high-density phylochips that carry several thousands of oligonucleotide probes (DeSantis *et al.* 2007). In contrast, only up to a few hundred oligonucleotides are immobilized on low-density phylochips. These custom-made microarrays usually target microorganisms that are defined by their phylogeny or taxonomy (Loy et al. 2002; Lehner et al. 2005), the environment they live in (Neufeld *et al.* 2006) or their physiological capabilities. The latter one include also the so-called functional gene arrays (FGAs), which consist of DNA probes targeting genes encoding key enzymes of specific functional or metabolic pathways of microorganisms (Wu *et al.* 2001) (Bodrossy *et al.* 2003; Rhee *et al.* 2004; He *et al.* 2007; Jaing *et al.* 2008; Nyyssonen *et al.* 2009; Waldron *et al.* 2009).

2. Challenges in DNA microarray experiments

Although large-scale comparison of DNA microarray data confirmed an overall high reproducible and reliable data generation by this tool (Shi *et al.* 2006), substantial technical issues and complex physicochemical processes are associated with the use of this technology, which can hamper the interpretation of microarray data. On one hand, signal variations can originate in the binding behavior and sequence dependence of molecular probe-target hybridizations (Zhang et al. 2003; Wu et al. 2005), the probe length (Chou et al. 2004) or the influence of target folding on heteroduplex formation (Mir et al. 1999). On the other hand, variations such as dye biases caused by dye-dye interactions or dye-nucleotide interactions (Dobbin et al. 2005; Jeon et al. 2007), spatial artifacts (Yuan et al. 2006) and several other technical variations deriving from microarray manufacturing, sample processing and hybridization (Novak et al. 2002) are known. Systematic variations of retrieved microarray data seem to be one major limitation for correct data analysis, especially if quantitative conclusions are made (Brody *et al.* 2002; Tan *et al.* 2003). Underlying mechanisms and their resolution was addressed in several studies, where thermodynamic and kinetic mechanisms such as diffusion limitations or the effect of reaction rates on hybridization were analyzed in several studies (Held *et al.* 2003; Pappaert *et al.* 2003; Gadgil *et al.* 2004; Dandy *et al.* 2007; Gong *et al.* 2008; Ono *et al.* 2008; Singh *et al.* 2009).

Basically, microarray hybridizations are solid-phase hybridizations which are subjected to thermodynamic and kinetic restrictions. The kinetics of hybridizations is mainly determined by mass transport (diffusion) and reaction limited processes (Levicky *et al.* 2005). Diffusion describes the movement of particles, in the case of microarrays of nucleic acids, with a certain molecular velocity, which depends on the size of the molecule and on the temperature, along a free path. These properties, which are characteristic for different particles in a defined environment, are summarized as diffusion coefficient. Mainly Fick's laws are used to describe diffusion, which add a concentration gradient as driving force to the diffusion coefficient. Microarray experiments are usually dependent on the diffusional transport of labeled nucleic acid targets to their perfect matching complementary probes and hence, diffusion is a key mechanism governing microarray hybridization processes (Dandy *et al.* 2007). Besides mass transport effects, microarray kinetics also involves hybridization reaction rate considerations, i.e. how quickly the equilibrium of these reactions is approached. Initially, the target DNA strand diffuses towards the immobilized probe strand. During the subsequent collision, a so-called nucleation site, involving the formation of the first short stretch of base pairing concerning at least 3 contiguous bases, is formed. In the following phase, either a stable base pair is formed during a rapid ‘zippering’ reaction, or, in the case of a mismatch, the nucleation site loses its stability, and the probe strand detaches and prepares for another attempt, either with the same, or with a different probe strand (Southern *et al.* 1999; Pappaert *et al.* 2003). In the majority of such reaction rate analyses, the Langmuir isotherm is used as basis for theoretical model development (Halperin *et al.* 2006). The Langmuir isotherm, originally developed to express the dependence of adsorbed gas molecules on the pressure of the gas above the surface, describes the equilibrium between molecules in solution and corresponding adsorbed molecules bound to the surface of a solid support.

To date, mainly the principal nature of hybridization events in microarray experiments was investigated considering single probe–target or single probe spot–target hybridization behavior. Thus, comprehensive analyses of effectors influencing the hybridization mechanisms in relation to the dimensions of such an assay are still missing.

It was the aim of the second part of this thesis to unravel restrictions of DNA microarray experiments by applying a simplified assay format, which allows the analysis of spatial trends and their effectors in DNA microarray hybridizations.

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Spatial gradients of target nucleic acids govern DNA microarray hybridization assays.

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Draft not corrected by co-authors

Concept by D.S. and A.L. *P. amoebophila* array conducted by S.H., D.S. performed all other experiments. R.S. performed diffusion modeling. D.S. wrote the manuscript.

Abstract

Nucleic acid surface hybridization assays are routinely applied in science and clinical diagnosis for high-throughput multiplex analyte detection and quantification. This study reports major and systematically arranged spatial gradients of detected target signal intensities within DNA microarrays. An experimental approach shows the general nature of these variations, which halves the amount of detected target in the array center and thus has a major impact on data interpretation and reliability. Additionally, simulation tests unravel mass transfer limitations as causing mechanism.

Introduction

DNA microarrays, developed in the mid-nineties (Schena et al. 1995), represent a widely used tool in clinical diagnostics (Yoo et al. 2009), gene expression profiling (Lazazzera 2005; Shyamsundar et al. 2005), genotyping (Sachse et al. 2008; Dufva et al. 2009), microbial ecology (Wagner et al. 2007) or in the detection of alternative splicing (Kechris et al. 2008). They consist of either oligonucleotide or cDNA based probes immobilized on a solid surface and are applied as high-throughput hybridization formats for the identification and quantification of up to several thousand different nucleic acids targets in parallel. It has been shown that DNA microarrays generate overall robust and reliable data with generally high consistencies in identified genes using different microarray platforms (Shi et al. 2006) and thus nowadays array based diagnostic tests are widely spread onto the medical market. However, several studies unraveled systematic variations in microarray data (Brody et al. 2002; Tan et al. 2003) affecting their reproducibility or quantitative power. Possible explanations of such observations can be found in the highly complex physicochemical processes driving these surface capture assays. DNA microarrays are solution-solid-phase hybridizations and thus, underlying dynamics are more complex than in solution-solution hybridizations. Probes are commonly immobilized on a planar substrate, such as glass slides or nylon membranes, which can interfere with target access (Levicky et al. 2005). Thereby, thermodynamic and kinetic mechanisms regulate nucleic acid hybridizations (Wetmur 1991; Levicky et al. 2005). In DNA microarray experiments two facets of kinetic processes are believed to play a major role: On one hand mass transfer limitations seem to dominate surface hybridizations and on the other hand influence of reaction rates on hybridization has been reported (Held et al. 2003; Pappaert et al. 2003; Gadgil et al. 2004; Dandy et al. 2007; Gong et al. 2008; Ono et al. 2008; Singh et al. 2009). In detail, reaction and/or diffusion modeling of nucleic acid surface hybridization has unraveled diverse chemico-physical phenomena of hybridization events. Pappaert *et al*. have shown that after an initial reaction-limited time period, target depletion renders the hybridization process diffusion-limited (Pappaert et al. 2003). In another study hybridization of short targets was diffusion limited, but long targets were kinetically limited (Singh et al. 2009). Gong *et al.* reported recently that at low ionic strengths electrostatic forces are dominant, while at high ionic strength more complex hybridization regimes take place (Gong et al. 2008). Studies analyzing probe-target interactions contribute to a better understanding of processes and effectors in hybridization experiments and thus support technical progress in the field and a more accurate interpretation of retrieved data. Thereby, most work focuses on the principal nature of reaction and does not consider spatial scales of such hybridization assays with the exception of one study investigating the influence of microarray spot sizes. In the respective work the authors demonstrated that hybridization efficiencies depend inversely on feature size and increase from spot center to spot edge (Dandy et al. 2007).

The present work bases upon the observation of strong signal intensity gradients across DNA microarrays, which were positioned non-randomly with significantly lower signal intensities in the array centers. These findings could be confirmed both experimentally by a simplified microarray approach and by diffusion based modeling unveiling strong positional effects in microarray experiments rendering quantitative array data interpretation problematic.

Materials and Methods

Image analyses of *Protochlamydia amoebophila* array hybridizations and public DNA microarray data.

For the evaluation of systematic variations in microarray experiments, a data obtained by hybridization of a whole genome array for *Protochlamydia amoebophila* (data unpublished) as well as publically accessible microarray data were analyzed.

The *P. amoebomophila* array consisted of 5´-amino modified oligonucleotide probes with a length of 45 to 55 bases. The 5’- end of each oligonucleotide probe was tailed with an additional 20 dATP spacer. Oligonucleotides had a concentration of 100 µM in 50% dimethyl sulfoxide (DMSO) and were printed onto silylated amine-aldehyde glass slides (VSS-25C, CEL Associates, Houston, Tex.) using a BioRobotics MicroGrid spotter (Genomics Solutions, Ann Arbor, MI, USA) and 16 MicroSpot 2500 split pins (Zinsser Analytic GmbH). The average spot diameter was about 150 µm and spot centers were 300 µm apart. The array consists of 48 blocks, with each block comprising 12 spots per row and column (144 spots/block). The distance between the different blocks was about 1050 µm. Analyzed *Protochlamydia amoebophila* DNA microarrays were hybridized with 1 - 2 µg fluorescently labeled genomic DNA. Briefly, fragmented genomic DNA was random primed and labeled with Cy3 or Cy5 fluorophores by using the DecaLabel DNA Labeling Kit (Fermentas Inc., Hanover, MD, USA). Unincorporated nucleotides were removed using the QIAquick Nucleotide Removal Kit (Qiagen). Purified labeled genomic DNA was vacuum-dried in a Speed Vac (Eppendorf concentrator 5301). Before hybridization, slides were pre-hybridized by addition of blocking reagent in 5x SSC, 0.1% SDS and 1% bovine serum albumin for at least 2 hours at 42°C. Pre-hybridized slides were washed in double-distilled water and in isopropanol, and dried by centrifugation. After denaturation at 95°C for 10 min, labeled targets were hybridized in 400 µl of hybridization buffer (35% formamide, 5x SSC, 0.1% SDS, 0.1% *n*-lauryl sarcosine, 0.1% blocking reagent, 50 µg ml^{-1} salmon sperm DNA) using sealed coverslips (HybriWell Sealing System; GRACE Bio Labs) under constant shaking at 400 rpm (ThermoTWISTER Comfort, QUANTIFOIL Instruments GmbH). Following 16 hours of incubation at 42°C, the slides were washed at 42°C applying three serial washes with decreasing stringency and images were recorded scanning the slides with a GenePix Personal 4100A array scanner (Axon Instruments, Molecular Devices Corporation, Sunnyvale, CA, USA). Raw hybridization images were used for analysis.

The Stanford Microarray database (SMD) was used to evaluate exemplarily public accessible microarray data as it provides a comprehensive and easy-to-use database including raw images. Analyzed microarrays can be identified by experiment ID (ExptID) given in the figure legends (Demeter et al. 2007). Signal intensity surface plots of microarray hybridization images were generated using the open-source software ImageJ with the Interactive three-dimensional Surface Plot plug-in (Abramoff et al. 2004). Microarray data were analyzed using the normalized intensities of the average red signal ("R", Cy5) and the average green signal ("G", Cy3) of all open reading frames. Positive and negative controls, as well as additional control oligonucleotides were excluded from analysis.

Microarray system and hybridization experiments

Microarray manufacture and processing. The oligonucleotide, namely DVHO831 (S-*-Dv.h.o-0831-a-A-18, GAA CCC AAC GGC CCG ACA), for microarray spotting was published previously (Loy et al. 2002) and synthesized from MWG Biotech (Ebersberg, Germany). The 5’-end of each oligonucleotide probe was tailed with 12 dTTP molecules (T-spacer) and the terminal dTTP was aminated to allow covalent coupling of the oligonucleotides to aldehyde group-coated VSS-25 glass slides (CEL Associates). The concentration of probes before printing was adjusted to 50 pmol μl^{-1} in 50% DMSO. Oligonucleotides were printed at constant temperature (20°C) and humidity (at least 50%) using a BioRobotics MicroGrid spotter (Genomics Solutions). The DVHO831 probe was printed 900 times per array with 30 features per row and column, respectively. The detailed dimensions of the array are shown in the supplementary Figure 1. Spotted DNA microarrays were incubated overnight at room temperature in a wet chamber. Slides were processed with sodium borohydride as described previously (Loy et al. 2002).

Target preparation. For target preparation, 16S rRNA gene fragments of *Desulfovibrio halophilus* (DSM5663) were first PCR amplified by the primer pair 616V and 630R (Juretschko et al. 1998) using a concentration of 50 µM of each primer. A T3 promoter site tag (5'-AAT TAA CCC TCA CTA AAG GG-3') on the 5′ end of the forward primers was added to allow a T3 RNA polymerase based reverse transcription labeling of the PCR products. Reaction mixtures were prepared using 2 mM of each deoxynucleoside triphosphate, 10x *Taq* buffer, 2 mM $MgCl_2$ and 2 U of Taq DNA polymerase (Fermentas). Cycling was accomplished by an initial denaturation step at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, and elongation at 72°C for 1 min. PCR was completed by a final elongation step at 72°C for 3 min. Prior labeling, PCR products were purified using the QIAquick PCR purification kit (QIAgen, Hilden, Germany). Target labeling was achieved by *in vitro* transcription as follows: 500 ng PCR product, 4 µl 5x T3 RNA polymerase buffer (Fermentas), 2 µl DTT (100 mM), 0.5 µl RNAsin (40 U/µl) (Promega), 1 µl each of ATP, CTP, GTP (10 mM), 0.5 µl UTP (10 mM), 2 µl T3 RNA polymerase (20 U/µl) (Fermentas) and 0.75 µl Cy3-UTP or Cy5-UTPs (5 mM) in a total volume of 20 µl were incubated at 37 °C for 4 hours. DNA was degraded with 2 U DNase I (Fermentas). Enzymatic digestion was stopped with 2 µl of 25 mM EDTA (Fermentas). Following adjustment of reaction volume to 100 µl with TE buffer, RNA was precipitated with 10 µl of 5M NaCl and 300 µl of $\text{ethanol}_{(abs)}$. RNA was washed with 500 µl of ice cold 70% ethanol and resuspended in 50 µl H_2O_{bidest}. RNA concentration and dye incorporation rates were measured spectrophotometrically. RNA was fragmented by incubating with 10 mM $ZnCl_2$ and 20 mM Tris/HCl (pH 7.4) at 60 °C for 30 min. Fragmentation was stopped by the addition of 10 mM EDTA pH 8.0. Labeled RNA was aliquoted and stored in the dark at -20°C.

Mircoarray hybridization and washing. Prior to microarray hybridization, fluoresecently-labeled 16S rRNA of *D. halophilus* was added to 0.1% SDS, 0.1% 100x Denhardt's reagent, 6x SSC and 15% formamide and incubated at 65 °C for 5-15 min. For hybridization, the

HybriWell^{TM} Hybridization Sealing System (HBW2222-FL, Grace BioLabs) with 0.25 mm deep wells was used. Microarray slides were inserted into a custom-made hybridization chamber and were hybridized in a waterbath without shaking over night for 18h at 42°C, with the exception of the time series. Subsequently, microarrays were washed by shaking at room temperature for 5 min in 2x SSC, 0.1% (w/v) SDS; for 5 min in 0.1x SSC and finally for 20 s in ice-cold MQ. Slides were dried by centrifugation (3 min, 300 g) and stored at room temperature in the dark and scanned the same day.

Scanning and data analysis. Fluorescence images were recorded by scanning the slides at three lines to average and at 10-µm resolution with a GenePix Personal 4100A array scanner (Axon Instruments, Molecular Devices Corporation, Sunnyvale, CA, USA). Photomultiplier tube gain was first adjusted to record spot signal intensities just below the saturation level. For each experimental series, identical scanning settings were used to enable the comparison of absolute signal intensities between different hybridizations. Scanned images were saved as multilayer tiff images and analyzed with the GenePix Pro 6.0 software (Axon Instruments). Low quality hybridizations were repeated and single bad quality spots were excluded from analysis. Signal intensity surface plots of the microarray results were generated using the tool ImageJ with the Interactive three-dimensional Surface Plot plug-in (Abramoff et al. 2004). Signal intensities were analyzed according to the defined spot positions as illustrated in supplementary Figure 1. For data analysis, median pixel intensities of probe spots were used. Mean relative signal intensities for each spot position were calculated as ratio of the spot position with highest or lowest absolute signal intensity, respectively. One-way analysis of variance (ANOVA) was used to compare the mean differences of signal intensities of different spot positions.

Results and Discussion

Spatial gradients in microarray experiments.

Image analysis of hybridization experiments with fluorescently labeled genomic DNA on a whole genome array of *Protochlamydia amoebophila* pointed to periodic intra-array variations in intensities of fluorescent signals (data unpublished). An obvious increase in detected fluorescence intensities from the array center to the array edge was observed via interpretation of absolute signal intensity as height in a three dimensional surface plot (Figure 1). These systematic differences in signal intensities could be observed both, over the whole width of the microarray (Figure 1A) and within the single sub-blocks (Figure 1B), respectively.

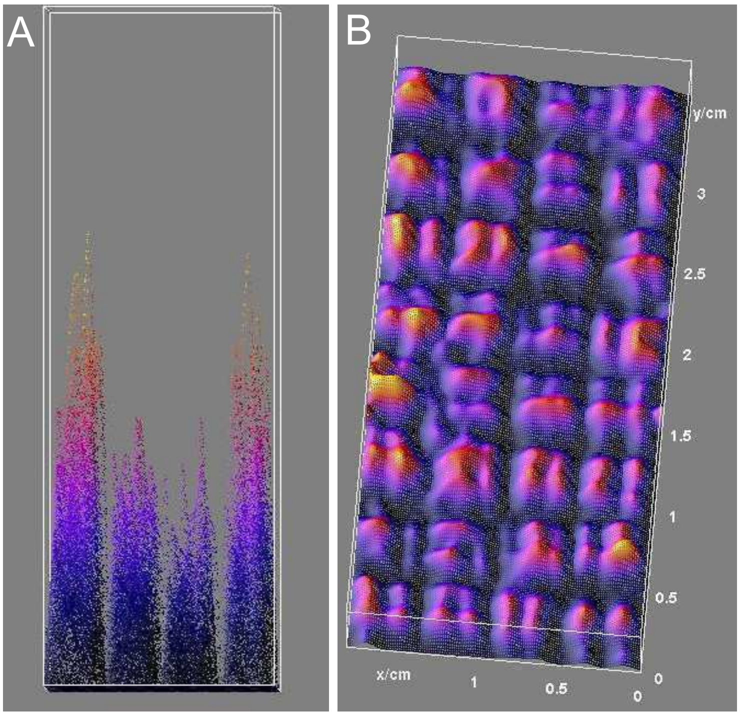


Figure 1: Image analysis of a hybridizations with fluorescently labeled genomic DNA on a genome array of *Parachlamydia pneumophila.* Signal intensities are shown as height in a three dimensional surface plot using ImageJ and displayed as **(A)** lateral view and **(B)** top view.

Additionally, publically accessible microarray experiments, which were hybridized with fluorescently labeled genomic DNA, exhibited comparable variations in signal intensities with considerably higher signal intensities at the border of the array or the array sub-blocks than in the array or block centers (supplementary Figure 2) (Demeter et al. 2007). These findings indicate that observed phenomenon concerns not only the *P. amoebophila* array but could be of common relevance in microarray hybridizations.

In order to verify and characterize the nature of the observed hybridization behavior, a simplified array layout was chosen, which allows a reduction in complexity of putative influencing factors. Therefore, the same oligonucleotide probe was immobilized in 900 spots (30 spots per row and column) to exclude different reaction rates of dissimilar probes as driving force for variability in signal intensities. The microarray was hybridized with varying concentrations of target RNA for different time periods (18, 65 and 140 hours) without agitation. Figure 2 shows hybridization of Cy3 labeled RNA under target-limiting conditions (300 ng) for 18 hours, a typical hybridization setup in DNA microarray experiments (Ulger et al. 2003; Bodrossy et al. 2006; Han et al. 2006; Manak et al. 2006).

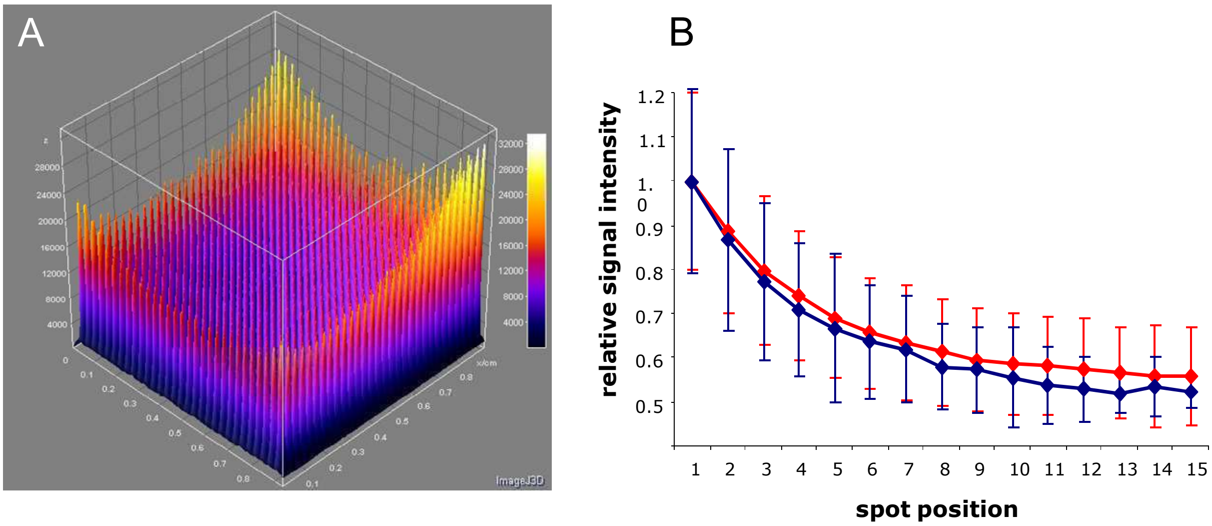


Figure 2: Analysis of target hybridizations on a simplified assay under target limiting conditions for 18 hours. Fluorescently labeled RNA was hybridized on an array with 900 spotted features consisting of 18-mer oligonucleotides complementary to the target. **(A)** Overlay of 3 replicate hybridizations using Cy3 labeled RNA Mean signal intensities are shown as height in a three dimensional surface plot using ImageJ **(B)** Microarray hybridization of Cy3 (red) and Cy5 (blue) labeled target RNA. Mean relative signal intensities are given as fraction of the spot position with highest absolute signal intensity and are displayed as function of the respective position. Error bars represent relative standard deviations of the mean signal intensities per spot position (including values of 3 independent experiments).

Comparison of the different spot positions as defined in supplementary Figure 1 revealed a significant decline of detected amount of hybridized target ($p < 0.001$) from the array edge (spot position 1) to the array center (spot position 15) (Figure 2B). If measured signal intensities were expressed as fraction of the strongest signal, mean signal intensities in the array center almost halved in relation to those at the array edge (Figure 2A). To exclude dye specific effects, equivalent hybridizations were carried out using Cy5 instead of Cy3 labeled RNA and obtained results corresponded to those received in the former experiment (Figure 2B) (Pearson correlation coefficient between Cy3 and Cy5 signals of the different spot positions = 0.99, $p < 0.01$). Moreover, after comparison of apparent differences in signal intensities within the distinct spot positions, highest values in the array corners were detected (Figure 3). By considering the signal intensities of the diagonal and center line transect separately, significant higher amounts of hybridized target could be observed at the outer spots of the diagonal transect compared to the corresponding features of the center line transect ($p < 0.01$).

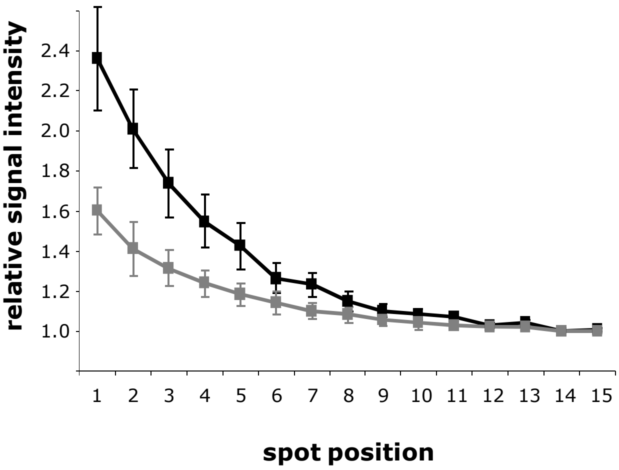


Figure 3: Comparison of relative signal intensities of diagonal (black) and center line transects (grey) (see supplementary Figure 1) of a hybridization with Cy3 labeled RNA under target limiting conditions for 18 hours. Mean relative signal intensities are given as fraction of the spot position with lowest absolute signal intensity and are displayed as function of the respective position. Error bars represent relative standard deviations of the mean signal intensities per spot position (including values of 3 independent experiments).

It can be assumed, that spots at the array corners have a positional advantage to access the provided target. As they have fewer spots in their direct surroundings, they have less competition for RNA strands in solution. Moreover, physical interference by other adjacent probe spots hindering the free diffusion of the target is reduced. Our results strongly point to mass transfer limitations as key driver in such surface hybridizations as the same probe was spotted at all position and thus different probe-target reaction kinetics can be excluded. In a study of Livshits *et al.*, brighter signals in DNA hybridization assays with oligonucleotides immobilized in a polyacrylamide gel layer have been observed at the edges. Retarded diffusion was shown to direct the kinetics of hybridization on the investigated assay (Livshits et al. 1996). Essentially, the movement of DNA into the center of the assay was shown to be driven by diffusion, whereby repeated duplex formation between probes and free analyte molecules is assumed (Livshits et al. 1996). As a result their diffusion is slowing. In a more recent work, spatial biases in DNA microarray experiments were described as a major source of inaccuracies in such studies (Koren et al. 2007). Although this study did not provide any explanation for the observed variations, they could show that the described biases might be responsible for more than 15% false data per experiment independent of microarray platforms and experimental procedures.

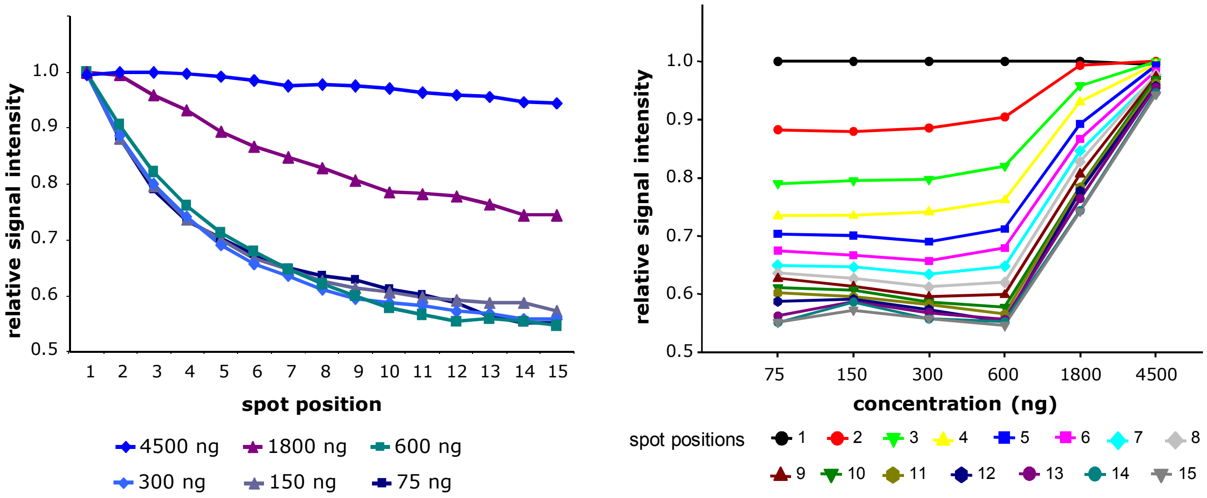


Figure 4: (A) Hybridization series with different concentrations of Cy3 labeled target RNA (75 – 4,500 ng) for 18 hours. Mean relative signal intensities are given as fraction of the spot position with highest absolute signal intensity and are displayed as function of the respective position. Error bars are omitted for clarity. Relative standard deviations of the mean signal intensities per spot position (including values of three independent experiments) ranged between 0.22–0.08 for 75 ng, 0.21–0.13 for 150 ng, 0.20–0.10 for 300 ng, 0.20–0.11for 600 ng, 0.12–0.7 for 1,800 ng and 0.12–0.7 for 4,500 ng, respectively. **(B)** Comparison of relative signal intensities for the different spot positions using different target amounts. Mean relative signal intensities (including 3 independent experiments) are given as fraction of the spot position with highest absolute signal intensity and are displayed as concentration.

To further investigate probe–target hybridizations on a surface, a series of experiments using several target concentrations were conducted. A remarkable decrease of spatial gradients at 1800 ng and their almost complete disappearance using 4,500 ng of target rRNA was observed (Figure 4A). Comparison of the different target amounts hybridized to the distinct spot positions mirror their dissimilar hybridization behavior at respective spot locations (Figure 4B). This finding is not surprising, as mass transfer and hybridization rates, both key processes in hybridization experiments, are strongly dependent on the concentrations of analyzed targets (Bhanot et al. 2003; Dandy et al. 2007).

Under target limiting conditions, a similar picture is presented by the time series with a decrease in variation of the signal intensities for the different spot positions over time (Figure 5A). A closer look at the single spot positions over different time confirms a heterogeneous hybridization behavior of target RNA to the immobilized probe spots (Figure 5B).

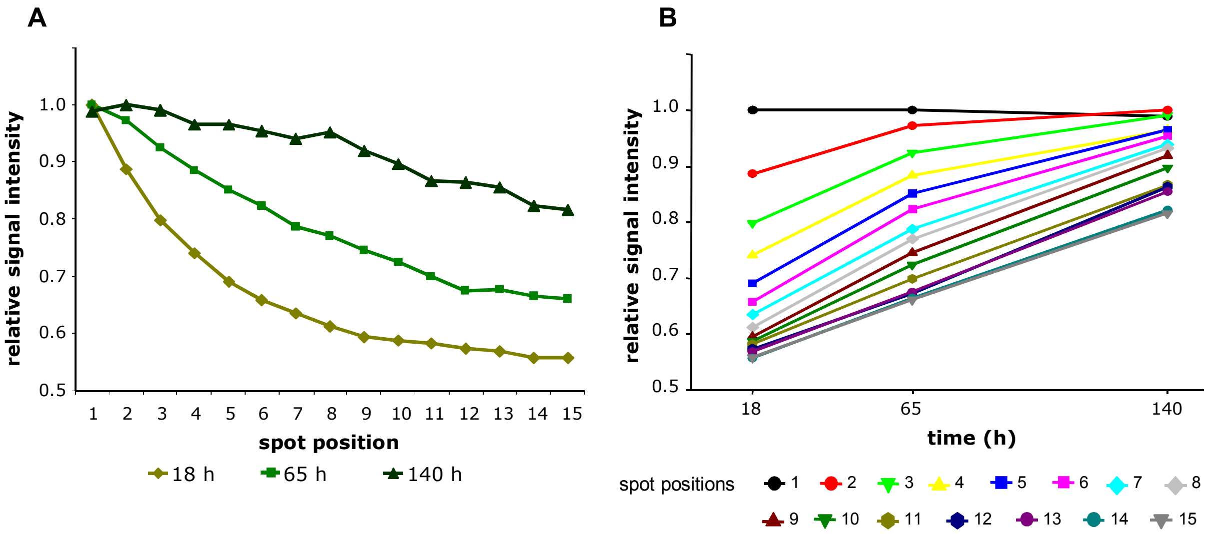
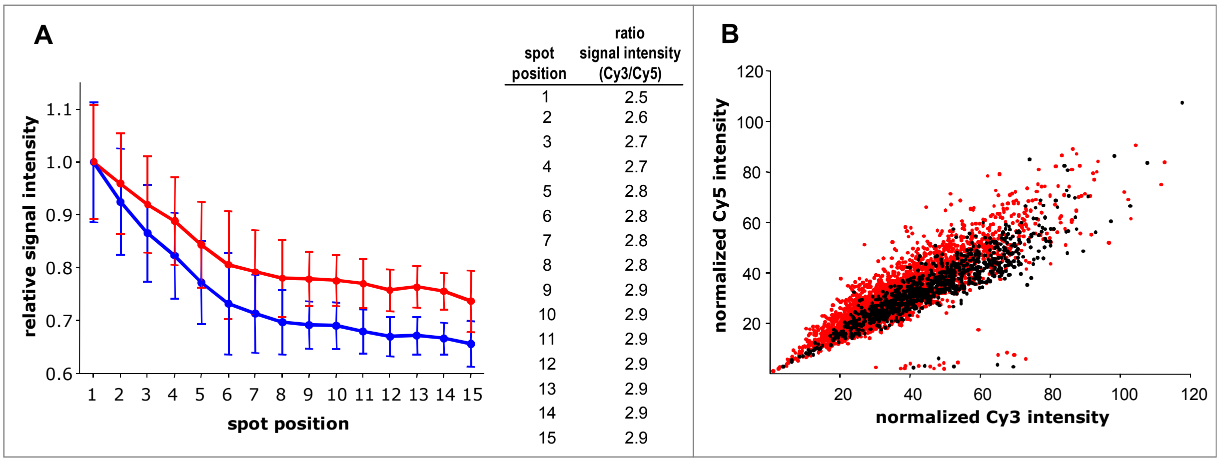


Figure 5: (A) Hybridization series of Cy3 labeled target RNA under target limiting conditions for 18, 65 and 140 hours. Mean relative signal intensities are given as fraction of the spot position with highest absolute signal intensity and are displayed as function of the respective position. Error bars are omitted for clarity. Relative standard deviations of the mean signal intensities per spot position (including values of three independent experiments) ranged between 0.20–0.10 for 18 h, 0.12–0.04 for 65 h and 0.21–0.11 for 140h, respectively. **(B)** Comparison of relative signal intensities for the different spot positions over time under target limiting conditions (Cy3 labeled RNA). Mean relative signal intensities (including 3 independent experiments) are given as fraction of the spot position with highest absolute signal intensity and are displayed as function of time.

While the inner spot positions 11 to 15 display a constant increase in signal intensities, the slope diminishes for the positions 2 to 10. Finally, the outer spot position 1 demonstrates no obvious differences in the amount of hybridized target over time with a tendency to decreasing signal intensities after 65 hours. These results underpin the assumption of mainly diffusion limiting transport. If the overall hybridization performance on the array would be mostly reaction rate limited, the same probe-target hybridization behavior would be expected at all spot positions. Thus, simultaneous presence of different reaction states within one array is likely. In the case of spot position 1, the amount of hybridized targets does not change significantly over time. Accordingly, reaction rate decreases suggesting depletion of available free analyte strands in the surroundings of these probes or saturation of the spots at position 1, respectively. In contrast, spot hybridization reactions in the array center seem to be distant from steady state, likely caused by target limitation and continuous new influx of free targets by diffusion.



spot position	ratio signal intensity (Cy3/Cy5)
1	2.5
2	2.6
3	2.7
4	2.7
5	2.8
6	2.8
7	2.8
8	2.8
9	2.9
10	2.9
11	2.9
12	2.9
13	2.9
14	2.9
15	2.9

Figure 6: Analysis of simultaneously hybridized Cy3- and Cy5 labeled targets on DNA microarrays. **(A)** Double hybridization of 900 ng Cy3 (red) and 300 ng Cy5 (blue) labeled target RNA on the simplified assay (see supplementary Figure 1) for 18 hours. Mean relative signal intensities are given as fraction of the spot position with highest absolute signal intensity and are displayed as function of the respective position. Error bars represent relative standard deviations of the mean signal intensities per spot position. Additionally, ratios of Cy3 and Cy5 median pixel intensities per probe spot positions are shown. **(B)** Linear scatter plot of a double genomic DNA hybridization (ExpID 68809). Each ORF is represented by a point with coordinates consisting of the normalized intensities of the average Cy5 signals and Cy3 signals. The array consisted of 12 sub-blocks with eight blocks located on the borders of the assay and four in the assay center (compare with supplementary Figure 2A). ORFs located in the outer blocks are displayed in red (n=2882), ORFs of the array center in black (n=959).

Finally, double hybridization of varying Cy3- (900 ng) and Cy5- (300 ng) labeled RNA concentrations also reflects a differentiated hybridization behavior. The continuous decrease of hybridized target amount to complementary probes of the spot positions 1 to 15 was lower for the applied three-fold higher concentration of Cy3-labeled target strands than for the Cy5-labeled targets (Figure 6A). This heterogeneous hybridization behavior is a crucial factor in microarray data interpretation, as gene expression array experiments usually include different concentrations of targets, ranging from abundant to rare target strands. Highly concentrated targets are closer to steady state than targets that have a low concentration level. Consequently, an asymmetric precision in the identification of up- and down-regulated genes was anticipated (Bhanot et al. 2003). In detail, lower target concentrations correspond usually to down-regulated genes and according to their extended equilibrium times, longer hybridizations would be needed to obtain the same accuracy as for genes that are up-regulated (Sartor et al. 2004). Hybridization times needed to reach equilibrium depends on target concentration and thus, underestimation of fold change of differentially expressed genes and miss of low abundant genes is suggested when hybridization reactions are not driven to completion (Wei et al. 2005).

Furthermore, comparison of the actual applied target ratio of 3:1 with the experimentally measured Cy3-to-Cy5 ratios, fitted best in the center of the array (2.9:1, spot position 9 to 15), where target-limiting and non-equilibrium conditions are assumed (Figure 6A). It has been shown that linearity between target concentrations and measured amounts of hybridized targets is sustained only within a narrow concentration range. Thereby very low abundant targets are affected by detection limit issues and high abundant targets are influenced by saturation penalties (Chudin et al. 2002). Most studies emphasize the importance of equilibrium conditions for adequate quantification of target molecules, especially in regard to multiplex hybridization as cross-hybridizations are enhanced under non-equilibrium condition (Bhanot et al. 2003). Nevertheless, our results suggest best agreement of actually present and hybridized target concentrations under non-equilibrium conditions. Accordingly, theoretical modeling by Gadgil *et al.* supports this finding in diffusion driven reactions, since intensities of hybridized targets corresponded best to their actual concentrations, when the reaction was far from equilibrium (Gadgil et al. 2004).

In order to verify these findings, public accessible data of a genome microarray hybridized simultaneously with Cy3 (1µg) and Cy5 (4 µg) labeled genomic DNA of different *Vibrio cholerae* strains were analyzed exemplarily using the normalized intensities of the average Cy5 and Cy3 signals of all open reading frames. By comparison of the Cy5 and Cy3 signal intensities of the sub-blocks positioned on the boundaries of the array against those located in the array center, an uneven distribution of analyzed signal intensities was observed. In detail, spots positioned in the array center possess a narrow range of Cy3-to-Cy5-ratios, whereas the outer sub-blocks exhibited a broader distribution with an apparent overrepresentation of Cy5 labeled strands hybridized on the respective spots (Figure 6B). This analysis underlines the importance of the observed effect in real microarray experiments, such as gene expression analyses, as multiple targets with different specificities and concentrations appear to be affected by the same hybridization mechanisms than our simplified array format. Although the effect can be influenced by the sequence composition and thus by unequal hybridization behavior of the two hybridized strains, a preferential hybridization driven by higher affinities of the Cy5 labeled *V. cholerae* strain only to spots of the outer blocks seems to be highly unlikely.

Diffusion drives the hybridization process.

The confirmation of the obtained experimental data was accomplished by simulation of the passive mass transport using a diffusion model. It was presumed that target transport is only driven by Fickian diffusion kinetics. Thereby, the diffusion equation was solved for the simplified array format used for the experiments with the dimensions as illustrated in supplementary Figure 1, with the exception that the chamber height assumed in the numerical simulation was 150 µm and 750 µm, respectively. In detail, a hybridization chamber of a half-width L=10.8 cm and a height H=150 µm, containing a square block of 30 x 30 spots (0.85 x 0.85 cm), each spot having 150 µm in diameters and a distance of 150 µm between them, is assumed. A time-dependent simulation with a diffusion coefficient of $D=10^{-9}$ m^2/s and a diffusion time of 86400 s was applied. The flux of target strands to each spot at each instant in time was calculated and was subsequently integrated up for the total time (24 h) for each spot. Thereby, three different cases were considered. Case A is most similar to the above described situation on the applied simplified array with a large and shallow hybridization chamber (L=10.8 mm, H=150 µm), with the exception that the actual chamber height was 250 µm. Case B assumes a large and deep hybridization chamber (L=10.8 mm, H=750 µm) and is used to study the effect of the chamber's height. Finally, case C tests the effect of the amount of surface area not covered with spots by assuming a small and shallow chamber (L=4.5 mm, H=150 µm).

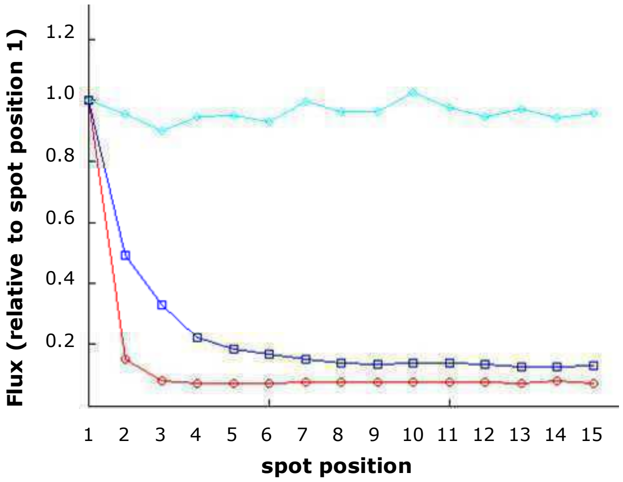


Figure 7: Numerical simulations of target diffusion relative to the spot positions as defined in supplementary Figure 1. The flux of target strands to each spot position was estimated for a hybridization time of 24 h. Three different cases were considered: Case A (red) corresponds to an array with a large and shallow hybridization chamber (L=10.8 mm, H=150 µm). Case B (dark blue) assumes a large and deep hybridization chamber (L=10.8 mm, H=750 µm) and case C (light blue) assumes a small and shallow chamber (L=4.5 mm, H=150 µm).

Resulting profiles displayed as relative flux of target RNA to the respective spot positions (Figure 7) revealed a significant decrease in the amount of RNA transported to the spots at the array center considering case A and B, and thus confirmed the results obtained in the hybridization experiments. Thereby, chamber height influences the amount of target molecules reaching the array center. In detail, the shallower the hybridization chamber is, the more pronounced the spatial variability of hybridized target will be. In fact, when the hybridization chamber is very shallow, most target molecules will hybridize to the outer spots before they can penetrate the block, whereas in deeper ones changes of RNA getting "captured" by the outer spots is lower. Actually, diffusion-reaction modeling of DNA hybridizations on microarray showed a linear increase of hybridized targets with chamber height. Furthermore it was implied that the time range for the change from reaction to diffusion limited hybridization is a function of chamber height (Pappaert et al. 2003). Thereby, targets within the first 100 nm of the fluid layer above the probe spots were fully hybridized in 10 ms, whereas the depletion of target strands within 100 µm took 1000 s, as the process switched to a diffusion controlled regime. Furthermore, in an experimental study, free targets appeared to be quickly depleted perpendicular to the array. By increasing the chamber height, greater advantage of vertical diffusion is taken, and thus the shift to a lateral diffusion limited hybridization is delayed (Borden et al. 2005).

Finally, comparison of case A and C uncovered the presence of a free surface area not covered with probe spots within the hybridization chamber as crucial factor in determining the spatial variability (Figure 7). If the chamber included no spot-free surface, no spatial variability was estimated (case C). Accordingly, each spot depletes rapidly all free targets in its neighborhood (over a time scale of approximately H^2/D, i.e. 9.3 minutes for a height of 750 µm). Thereby, all spots have equal access conditions to free target strands perpendicular to their positions. In contrast, when an uncovered surface area is included in the array, which in our case was 6.38 cm, target strands will diffuse for the most part laterally towards the spotted area, and will therefore hybridize preferentially to the outer spots in the block. The differences of size of the applied hybridization chamber with a half-width within cm- and height within µm-scale, respectively, governs observed hybridization regimes.

Conclusions

Spatial gradients present in nucleic acid surface hybridizations were investigated systematically by hybridization experiments and numerical simulations. A simplified microarray approach confirmed the preferential hybridization of analytes at the array edges resulting in 50% lower signal intensities in the array center at the given array dimensions. Observed gradients appeared also in two-color analyses, usually applied in gene expression analysis. As a consequence, quantitative data interpretation is heavily biased by systematic spatial variations. Theoretical simulations emphasized these results and identified diffusion processes as key driver for the heterogeneous hybridization behavior at different spot positions.

This study reports and elucidates a drastic and systematic spatial effect tampering certainly most nucleic acid surface hybridization experiments. Reduction of the influence of described variations could be approached by the adaptation of already existing normalization methods such as integration of embedded and adequately distributed technical replicates for bias correction (Yuan et al. 2006).

Furthermore, reduction of diffusion driven reactions is approached by the application of non-static hybridization devices. Most commercial DNA microarray formats, including Affymetrix Gene Chips, utilize only passive mixing by rotation of the entire microarray, and thus still suffer from diffusion-limited hybridization rates. However, possibilities to overcome diffusion limitations are provided by actively mixing hybridization systems. One promising attempt is the use of pressure driven flow-through systems. The porous substrate was shown to reduce dramatically diffusion limitations on the hybridization rate (Mocanu et al. 2009); nevertheless, further effort will be needed to unravel the complex physicochemical interactions in these relatively novel assays.

Finally, a straightforward improvement was highlighted by the simulation experiments. The extension of the height and contemporary reduction of free surface areas within the array would be an easy and effective way to allow faster and equal analyte transfer to all spots. However, larger hybridization solution volumes would be required and therefore the detection of low abundant target might be lowered.

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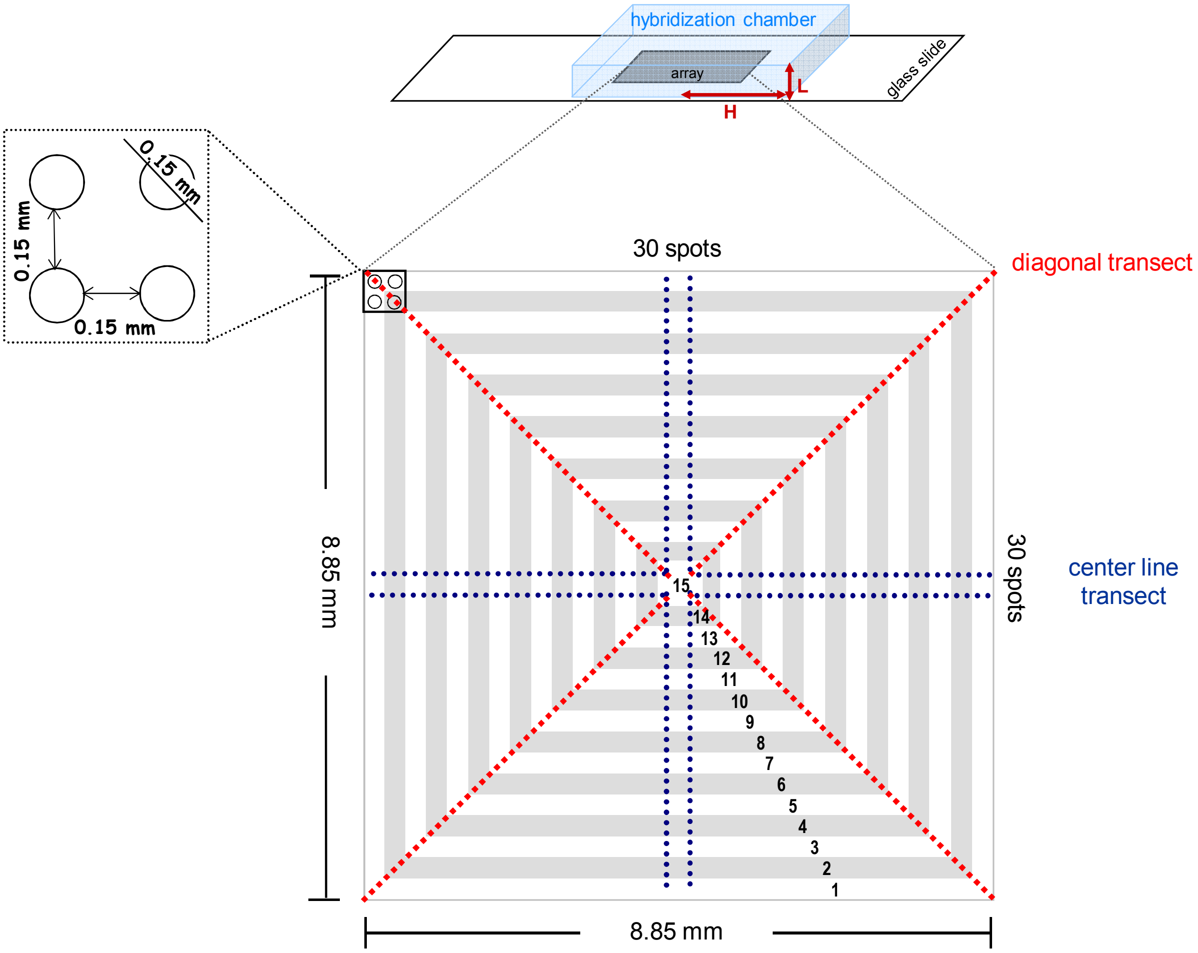
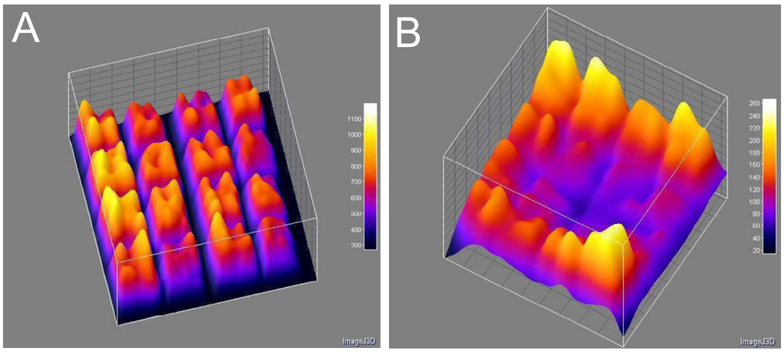
Supplementary Figures



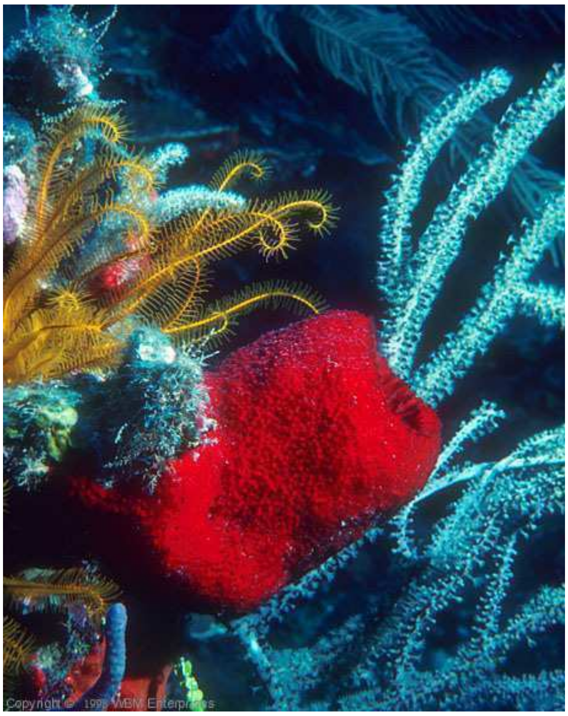
Supplementary Figure 1: Assay layout and dimensions. Hybridization chambers have a half-width of L=10.8 cm and a height of H=0.150 cm. Spot positions (1–15) are labeled with white and grey surrounds. Features within one defined position are treated as replicate spots with decreasing numbers of replicates from spot position 1 (n=116) to spot position 15 (n=4). Definitions of diagonal transects (n=4) and center line transects (n=8) are shown as dotted lines. Spots have a diameter of 150 µm and have a distance of 150 µm to each other.



Supplementary Figure 2: Image analysis of public accessible microarray images. Analyzed microarrays can be identified by the experiment ID (ExptID) in the Stanford Microarray database. Genomic DNA hybridizations on different target genome arrays were chosen for analysis. Signal intensities are shown as height in a three dimensional surface plot using ImageJ. **(A)** ExpID 68809 **(B)** ExpID 75165.

Part III

Microorganisms in marine sponges



Introduction

Sponges (Phylum *Porifera*) are the most primitive of the multi-cellular animals (*Metazoa*). They form one of the deepest branches of the metazoans, whose evolutionary roots date back to precambrian times over 600 million years ago (Müller *et al.* 2009). There exist about 15000 known and 8000 validly described species with the vast majority (85%) of the formally described species belonging to the class *Demospongiae* (Hooper *et al.* 2002). These sessile, filter-feeding organisms inhabit a wide variety of marine and freshwater systems. Sponges frequently colonize tropical reefs but are also found in temperate and polar regions as well as in freshwater streams and lakes. They represent important members of benthic communities, as they contribute substantially to the biomass present in the benthic habitat such as reefs (Diaz *et al.* 2001). In this connection, marine sponges play an important functional role in benthic or pelagic processes, as they offer a habitat for various reef species and they are heavily involved in benthic food webs (Gili *et al.* 1998; Diaz *et al.* 2001). Two key factors direct research interest towards marine sponges: on one hand marine sponges are recognized for their production of secondary metabolites with a wide range of biological and pharmaceutical activities (Braekman *et al.* 2004; Laport *et al.* 2009) and on the other hand many species harbor dense and highly diverse microbial communities (Hentschel *et al.* 2006; Webster *et al.* 2009) which have been postulated to be important for the fitness of the animal and which might contribute to the production of secondary metabolites (Piel 2004; Wang 2006; Muscholl-Silberhorn et al. 2008; Hertiani et al. 2010).

1. Microorganisms associated with marine sponges

To date, several types of marine sponge-microbe associations are known. Microorganisms are used as food source by these filter-feeding organisms, but they comprise also disease-causing parasites or mutualistic symbionts (Wilkinson et al. 1984; Webster et al. 2002; Müller et al. 2004; Thacker 2005). This variety in the nature of interactions is also reflected by the phylogenetic diversity of microorganisms that occur within sponges: all three domains of life, namely *Bacteria*, *Archaea* and *Eukarya*, are now known to inhabit marine sponges.
Microorganisms occur both intracellularly in specialized sponge host cells, the bacteriocytes, and extracellularly in the sponge mesohyl (Friedrich *et al.* 1999). Thereby, intracellular sponge symbionts are less frequently observed then the often dense microbial communities

inhabiting the sponge mesohyl, an extracellular matrix which separates the internal acquiferous systems from the outer sponge cells. Twenty-three bacterial phyla, both major archaeal lineages, and various microbial eukaryotes have been described so far in marine sponges (Webster *et al.* 2001; Taylor *et al.* 2007; Granados *et al.* 2008; Webster *et al.* 2009). Thus, marine sponges contain highly diverse microbial communities with often immense amounts of microorganisms detected within one sponge (up to 10^9 microbial cells per g) (Hentschel *et al.* 2006). Since microbes detectable in sponges can either be symbionts or a food source, it is challenging to reliably assign them to one of these two groups.

One approach to distinguish between the different types of sponge associated microorganisms employs the phylogenetic characterization of these associations. For instance, comparative taxonomic analysis of selected marine sponges and their bacterial associates suggested that some sponge symbionts have co-evolved with their hosts, implying a permanent and maybe beneficial role of these bacterial groups (Erpenbeck *et al.* 2002). Furthermore, numerous sponge associated microorganisms were affiliated to sponge-specific clusters, based on their 16S rRNA gene sequences (Hentschel *et al.* 2002), suggesting selective occurrence of certain bacterial and archaeal groups within sponge hosts. Per definition, these clusters have to fulfill the following conditions: they have to comprise at least three microbial sequences that (i) have been retrieved from different sponges and/or from distinct geographic locations, (ii) are more closely related to each other than to any other sequence from non-sponge sources, and finally (iii) cluster together independent of the treeing method used (Hentschel *et al.* 2002). Out of a total number of 190 analyzed 16S rRNA gene sequences, 133 sponge-derived sequences (70%) were originally assigned to 14 monophyletic sponge-specific clusters in 2002. This basis was extended comprehensively in the course of this thesis in 2007 by phylogenetic analyses of about 1700 microbial sponge-derived 16S rRNA gene sequences with 546 16S rRNA gene sequences affiliating to sponge-specific clusters (32%). Recent bacterial 16S rRNA gene tag pyrosequencing of three different marine sponge species and surrounding seawater samples confirmed the validity of 48% out of 33 detected clusters (Webster *et al.* 2009). Some of the remaining sponge-specific clusters were also found at very low numbers in the surrounding seawater. Consequently, the rare seawater biosphere possibly provides microbes as seed organisms to sponges resulting in selective enrichment of specific microbes from the marine environment. Thus, described host-microbe associations might have evolved much more recently than previously thought (Hentschel *et al.* 2002; Webster *et al.* 2009).

Beside the possibility of environmental acquisition of microorganisms by the host sponge, evidence of vertical transmitted bacteria via sponge larvae was obtained in several studies, which supports stable associations of microorganisms in at least some sponges. A first insight into vertical transmission in marine sponges was provided by ultrastructural studies. The presence of cyanobacteria located throughout the mesohyl of adult *Chondrilla australiensis* sponges, was additionally shown inside developing eggs and nurse cells in 25% of female hosts using transmission electron microscopy (Usher *et al.* 2001). Furthermore, yeast cells were maternally transmitted in *Chondrilla* species through the oocytes to the fertilized eggs (Maldonado *et al.* 2005). Following molecular studies revealed that multiple and phylogenetically diverse groups of microorganisms are transferred between sponge generations through their reproductive stages (Enticknap et al. 2006; Schmitt et al. 2007; Sharp et al. 2007; Schmitt et al. 2008; Lee et al. 2009). Combined application of 16S rRNA gene analyses, fluorescence *in situ* hybridization and electron microscopy resulted in a model for symbiont transmission in marine sponges with high microbial abundances (Schmitt *et al.* 2008). In this model sponge reproductive stages take up microbes from the adult mesohyl. Nurse cells might be involved essentially in microbial incorporation by promoting selection of these microbial communities. Developing sponge larvae are non-filter feeding stages and might be regarded as a closed system, where no exchange with the environment occurs. Vertical transmission leads consequently to separation of microbial types in different sponges which would result in genetic differentiation and finally in co-speciation. After the metamorphosis of sponge larvae into juveniles, the filter-feeding life phase begins, which induces uptake of microorganisms from the surrounding seawater (Schmitt *et al.* 2008). Thereby, horizontal transfer of microbes between sponge individuals or environmental acquisition as discussed above appears to be possible.

2. Ecological functions of sponge associated microorganisms

There are a variety of functions that have been attributed to microorganisms living in marine sponges. Host sponges may benefit from microbial metabolite production, sponge skeleton stabilization, nutrient acquisition and metabolic waste removal (Wilkinson 1978; Wilkinson 1992; Unson *et al.* 1994; Hentschel *et al.* 2001). For instance, symbiont-produced bioactive metabolites may serve to protect the sponge from pathogens, predators and fouling organisms (Hentschel *et al.* 2001). Furthermore, facultatively anaerobic bacteria are commonly found in sponges (Wilkinson 1978; Santavy *et al.* 1990) and through their ability to metabolize a wide range of compounds, these organisms may play a role in removing waste products from the sponge during periods of low pumping activity (Wilkinson 1978).

A well studied example of sponge-microbe interactions is presented in the beneficial contribution of cyanobacterial symbionts to their host. Many tropical sponges contain substantial populations of these oxygenic photoautotrophs and it was demonstrated that photosynthetic produced glycerol and organic phosphate are provided from cyanobacterial symbionts to their hosts (Wilkinson 1979). In this case, the host sponge may receive up to 50% of its energy requirements and 80% of its carbon budget (Wilkinson 1992; Cheshire *et al.* 1997). A further advantage for the host may include the provision of nitrogen, a limiting nutrient in many marine systems, by cyanobacteria which are capable of fixing atmospheric nitrogen (Wilkinson *et al.* 1979).

Further microorganisms involved in the nitrogen cycle have been identified in marine sponges. The nitrogen cycle in sponges comprises four main microbial mediated transformation processes, namely nitrogen fixation, nitrification, anaerobic ammonia oxidation and denitrification. During nitrogen fixation, carried out by specialized microorganisms like the above mentioned *Cyanobacteria*, nitrogen is converted to ammonia. Under aerobic conditions, ammonia is transformed to nitrite and subsequently to nitrate by ammonia- and nitrite-oxidizing microorganisms (nitrification). Under anaerobic conditions however, members of the genus *Planctomycetales* are able to convert nitrite and ammonium directly into N_2 in a process called anaerobic ammonia oxidation (Jetten *et al.* 2001). Finally, nitrate and nitrite can be reduced anaerobically to N_2 (or NO and N_2O) by diverse heterotrophic bacteria (denitrification). It has been shown, that marine sponges assimilate and release dissolved inorganic nitrogen like ammonia, nitrite and nitrate, as well as dissolved and particulate organic nitrogen (Bayer *et al.* 2008). In addition, microbial mediated denitrification and anaerobic ammonium oxidation in sponges was quantified by application of incubation experiments with stable isotopes (Hoffmann *et al.* 2009). Also molecular methods were employed using 16S rRNA genes and functional genes as phylogenetic markers, to uncover the microorganisms involved in these transformations. Environmental genome sequencing of the sponge associated crenarchaeon *Cenarchaeum symbiosum* reported the presence of an ammonia monooxgenase gene (amoA), a key enzyme in the aerobic ammonia oxidation, in this uncultivable sponge symbiont (Hallam *et al.* 2006). Additionally, 16S rRNA gene and/or *amoA* genes of *Nitrosospira*, *Nitrospira* and *Crenarchaeota*, all groups involved in aerobic nitrification, were detected in several marine sponges (Hentschel *et al.* 2002; Bayer *et al.* 2008; Hoffmann *et al.* 2009; Mohamed *et al.* 2009). Recently, 16S rRNA gene based analyses revealed furthermore first molecular evidence for the presence of anaerobic ammonia oxidizing bacteria in sponges (Hoffmann *et* *al.* 2009; Mohamed *et al.* 2009). Finally, investigation of functional genes catalyzing the anaerobic reduction of nitrite to nitrogen confirmed the occurrence of denitrifiers belonging to the *Betaproteobacteria* in sponges (Hoffmann *et al.* 2009).

3. Archaea associated with marine sponges

Once thought to inhabit only extreme environments, *Archaea* are now known to occur in a wide range of habitats (Stein *et al.* 1996). In the last years several studies emerged, which describe the detection of *Archaea* in marine sponges. The first reported archaeon in a sponge was *C. symbiosum*, a psychrophilic microorganisms affiliated to the crenarchaeal group I.1A, which builds a specific association with the sponge *Axinella mexicana* (Preston *et al.* 1996). Whereas *C. symbiosum* was the only crenarchaeal phylotype inhabiting this sponge, broader association of *Crenarchaeota* with marine sponges has been demonstrated in other host species (Margot *et al.* 2002; Lee *et al.* 2003; Holmes *et al.* 2007). It has been shown, that members of the group I.1A *Crenarchaeota* are widespread in marine environments (Karner *et al.* 2001) and nearly all sponge-derived archaeal sequences are affiliated with this group. The marine isolate *Candidatus* “Nitrosopumilus maritimus” (Könneke *et al.* 2005) that grows chemolithoautotrophically by oxidizing ammonia to nitrite, is the only cultivated member of this group. Aerobic ammonia oxidation as energy metabolism for autotrophic growth is also proposed for *C. symbiosum*. Analysis of the genome sequence of *C. symbiosum* predicted genes encoding ammonia monooxygenase subunits, ammonia permease, urease, and urea transporters, which implies the use of reduced nitrogen compounds as energy sources for autotrophic growth by ammonia oxidation (Hallam *et al.* 2006). Furthermore, an autotrophic carbon assimilation cycle with utilization of carbon dioxide as carbon source, as well as an oxidative tricarboxylic acid cycle indicative for additional consumption of organic carbon was supported by genomic analysis.

Although most archaeal sequences retrieved from the host affiliate to the crenarchaeotal lineage, *Archaea* belonging to the Phylum *Euryarchaeota* were also reported from marine sponges (Webster *et al.* 2001; Holmes *et al.* 2007). Additionally, lipid biomarker analysis as well as fluorescence *in situ* hybridizations suggested the presence of both *Crenarchaeota* and *Euryarchaeota* in the mesohyl of an arctic deep-water sponge (Pape *et al.* 2006). Whereas for at least some sponge associated *Crenarchaeota* strong evidence for a functional role in the nitrogen cycle is given, the function of *Euryarchaeota* remains unclear, although members of this group possess metabolic capabilities of potential benefit to host sponges (e.g. sulfur metabolism).

It was the aim of the third part of this thesis to contribute to a better understanding of these complex sponge-microbe associations by (i) performing a comprehensive meta-analysis of all public available sponge derived 16S rRNA gene sequences supplemented by sequences retrieved from three additional sponges as well as by (ii) addressing the mode of transmission of sponge associated *Archaea* with a putative role in the nitrogen cycle.

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PAPER I

Sponge-Associated Microorganisms: Evolution, Ecology, and Biotechnological Potential

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Sponge-Associated Microorganisms: Evolution, Ecology, and Biotechnological Potential†

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INTRODUCTION

Marine sponges represent a significant component of benthic communities throughout the world, in terms of both biomass and their potential to influence benthic or pelagic processes (73, 74, 124, 220). Sponges (phylum Porifera) are among the oldest of the multicellular animals (Metazoa) and possess relatively little in the way of differentiation and coordination of tissues (26, 371). They are sessile, filter-feeding organisms which, despite a simple body plan, are remarkably efficient at obtaining food from the surrounding water (290, 308, 443). The more than 6,000 described species of sponges inhabit a wide variety of marine and freshwater (somewhat more restricted) systems and are found throughout tropical, temperate, and polar regions (167). Sponges have been the focus of much recent interest (Fig. 1) due to the following two main (and often interrelated) factors: (i) they form close associations with a wide variety of microorganisms and (ii) they are a rich source of biologically active secondary metabolites. This increasing research interest has greatly improved our knowledge of sponge-microbe interactions, and yet, as apparent throughout this article, many gaps remain in our knowledge of these enigmatic associations. For example, we still lack a clear picture of microbial diversity—and the factors which influence it—in these hosts. Similarly, the physiology of most sponge-associated microorganisms remains unclear, as do many fundamental aspects of sponge symbiont ecology. (Throughout this article, the terms "symbiont" and "symbiosis" are used in their loosest possible definitions, to refer simply to two [or more] different organisms that live together over a long period

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† Supplemental material for this article may be found at http://mmbr.asm.org/.

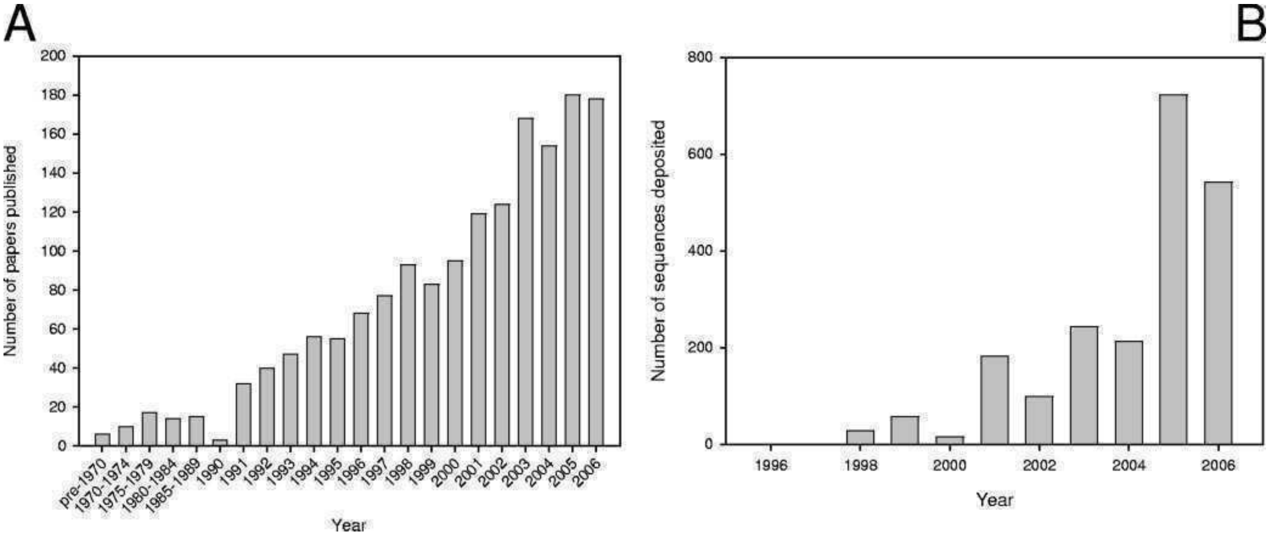

FIG. 1. Increasing research interest in marine sponge-microorganism associations. (A) Number of publications retrieved from the ISI Web of Science database by using the following search string: (sponge* or porifera* or demospong* or sclerospong* or hexactinellid*) and (bacteri* or prokaryot* or microbe* or microbial or microorganism* or cyanobacteri* or archaeon or archaea* or crenarchaeo* or fung* or diatom* or dinoflagellate* or zooxanthella*) not (surgery or surgical). (B) Number of sponge-derived 16S rRNA gene sequences deposited in GenBank per year. The 2006 value includes the 184 sequences submitted to GenBank from this article. The search string used to recover sequences was as follows: (sponge* or porifera*) and (16S* or ssu* or rRNA*) not (18S* or lsu* or large subunit or mitochondri* or 23S* or 5S* or 5.8S* or 28S* or crab* or alga* or mussel* or bivalv* or crustacea*).

of time, similar to the original de Bary definition. No judgment is made regarding benefit to either partner.) Here we aim to provide a comprehensive review of the current knowledge of the evolution, ecology, and biotechnological potential of sponge-microbe associations.

We begin with an introduction to the host organism. The phylum Porifera is a paraphyletic grouping consisting of three major sublineages (classes), namely, the Hexactinellida (glass sponges), Calcarea (calcareous sponges), and Demospongiae (demosponges), with the last group containing the majority of extant species (38, 167). Sponge architecture is unlike that for any other taxon, and sponge morphology greatly affects many aspects of sponge biology, including interactions with microorganisms. The basic body plan comprises several different cell layers (Fig. 2) (371). The outer surface, or pinacoderm, is formed by epithelial cells known as pinacocytes. Through pores

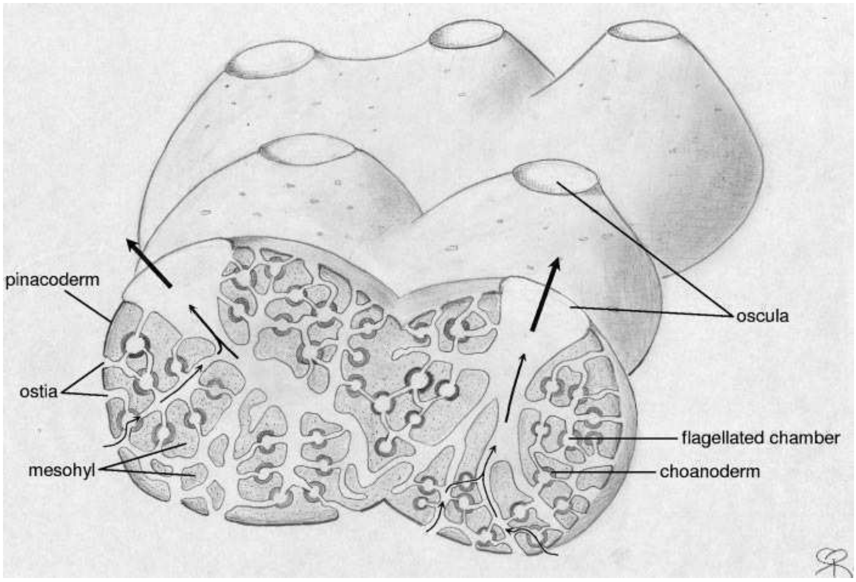

FIG. 2. Schematic representation of a sponge. Arrows indicate the direction of water flow through the sponge. (Adapted from reference 328 with permission of Brooks/Cole, a division of Thomson Learning.)

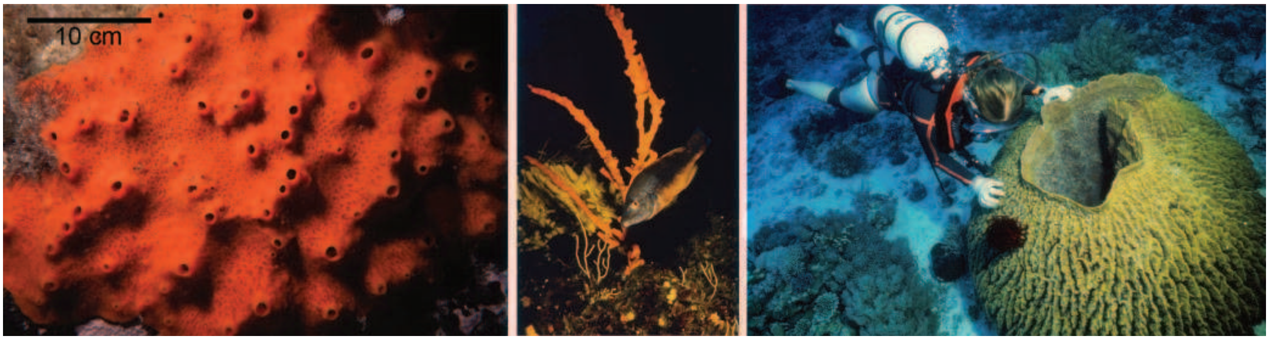

FIG. 3. Sponges of diverse size, shape, and color. The encrusting sponge *Tedania digitata* (left), the branching sponge *Axinella cannabina* (center), and the giant barrel sponge *Xestospongia testudinaria* (right) are shown. The last two images were kindly provided by Armin Svoboda (Ruhr-Universität, Bochum, Germany).

(ostia) on the sponge surface, these cells also extend along the interior canals which permeate the sponge. Inside the sponge, specialized flagellated cells (choanocytes) form a series of chambers where feeding takes place. In these chambers, collectively called the choanoderm, the flagellated choanocytes beat to pump water in through the ostia and along the often elaborate aquiferous systems within the sponge. Choanocytes also filter out food particles (including bacteria and microalgae) from the water, and these are transferred to the mesohyl, an extensive layer of connective tissue (Fig. 2). In the mesohyl, food particles are digested via phagocytosis by another group of sponge cells, the archaeocytes. These totipotent cells are capable of differentiating into any of the other sponge cell types. Also present in the mesohyl of many sponges are dense communities of microorganisms (106, 430, 471–473). The existence of these putative symbionts alongside bacterium-digesting archaeocytes is somewhat paradoxical and implies either recognition of different microbial types by the sponge cells or shielding of symbiont cells to prevent consumption (482). Once filtered in the choanocyte chambers, water is eventually expelled from the sponge via the exhalant opening, or osculum. It has been estimated that up to 24,000 liters of water can be pumped through a 1-kg sponge in a single day (443).

Beyond the basic body plan described above, sponge morphology is highly diverse. Inspection of any marine "sponge garden" will reveal a colorful array of encrusting, branching, cup-shaped, and massive (amorphous) types (Fig. 3), with individuals ranging in size from a few millimeters to more than a meter in diameter (328). Sponge morphology can also reflect ecological function, as seen in the many cyanobacterium-containing species whose flattened shapes allow optimal light reception for their photosynthetic symbionts (337, 474, 477). Structural integrity is conferred upon most sponges by siliceous or calcareous spicules (371), and these skeletal components are the basis for much of sponge biology and taxonomy. A wide range of spicule types are secreted, many of which are characteristic of particular taxa (167). Collagenous tissues, such as spongin, also play a role in providing structural support and, together with spicules, allow the development of very large individuals, such as those found among many tropical species.

Sessile organisms such as sponges and other marine invertebrates (including corals and ascidians) rely heavily on the production of chemicals as a form of defense against natural enemies, such as predators and competitors. Marine sponges have attracted particularly intense scrutiny in this regard, with a wide variety of sponge natural products characterized to date (see reference 32 and its preceding versions). More novel bioactive metabolites are obtained from sponges each year than from any other marine taxon, and a range of pharmacological properties have been demonstrated (32, 250). Various ecological roles have also been proposed for these compounds, including defense against predators (20, 55, 275), competitors (94, 395, 411), fouling organisms (363, 487), and microbes (19, 254, 398). Interestingly, in at least some cases, the compounds appear to be produced by associated microorganisms rather than by the sponge (27, 285, 351). Continued investigations of sponge-derived compounds and their biotechnological and ecological implications should guarantee vigorous interest in sponge-microbe associations for some time to come.

Interactions between sponges and microorganisms occur in many forms. To a sponge, different microbes can represent food sources (290, 307, 308), pathogens/parasites (16, 171, 199, 455), or mutualistic symbionts (474, 477). Microbial associates can comprise as much as 40% of sponge tissue volume (427), with densities in excess of 10^9 microbial cells per ml of sponge tissue (159, 453), several orders of magnitude higher than those typical for seawater. The diversity in types of interaction is matched by the phylogenetic diversity of microbes that occur within host sponges. It was already evident from early microscopy and culturing studies of sponge-associated microbes that high levels of morphological and metabolic diversity were present (62, 218, 336, 430, 471–473). The application of molecular tools over the past decade has greatly extended the known diversity of microorganisms within these hosts (100, 106, 146, 214, 294, 390, 458). Each of the three domains of life, i.e., *Bacteria*, *Archaea*, and *Eukarya*, are now known to reside within sponges. We now consider in detail this enormous diversity together with the evolutionary mechanisms driving its existence.

EVOLUTION AND DIVERSITY OF SPONGE-ASSOCIATED MICROORGANISMS

Marine sponges are widely considered the most primitive of the metazoans, arising at least as early as the Precambrian, some 600 million years ago (206). According to molecular clocks, the divergence of sponges from the ancestors of other metazoans may have occurred even earlier, around 1.3 billion years ago (144). During subsequent periods of the Paleozoic era, sponges accounted for much of the biomass on marine reefs (167, 491). Today, they remain important members of both shallow- and deep-water communities, occupying as much as 80% of available surfaces in some areas (74). Such sustained evolutionary and ecological success is probably due, at least in part, to their intimate associations with microbial symbionts. However, unlike many other studied host-microbe associations, in which only a very small number of participants are involved (e.g., squid-*Vibrio fischeri* [258], amoeba-*Chlamydiae* [168], and *Bugula*-"*Endobugula*" symbioses [142, 210]), it is apparent that sponge-associated microbial communities can be highly diverse, with a range of different microorganisms consistently associated with the same host species. In this section, we describe the extent of this diversity, providing in-depth phylogenetic analyses of all known sponge-associated microorganisms. We summarize current evidence for the existence of sponge-specific microorganisms and conclude by considering whether sponge-microbe associations are evolutionarily ancient or are, instead, recently initiated relationships involving microorganisms which are present in the surrounding seawater.

Known Diversity of Microorganisms from Sponges

Prior to this review, the diversity of microorganisms known from sponges was categorized in 14 recognized bacterial phyla (and one candidate phylum), both major archaeal lineages, and assorted microbial eukaryotes (145, 148, 477). Sequences representing the following bacterial phyla have been recovered from 16S rRNA gene libraries and/or excised denaturing gradient gel electrophoresis (DGGE) bands: *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Deinococcus-Thermus*, *Firmicutes*, *Gemmatimonadetes*, *Nitrospira*, *Planctomycetes*, *Proteobacteria* (*Alpha*, *Beta*, *Delta*, and *Gammaproteobacteria*), *Spirochaetes*, and *Verrucomicrobia* (7, 95, 123, 146, 148, 151, 154, 214, 317, 342, 383, 390, 391, 396, 404, 407, 421, 452, 454, 458; S. R. Longford, N. A. Tujula, G. R. Crocetti, A. J. Holmes, C. Holmström, S. Kjelleberg, P. D. Steinberg, and M. W. Taylor, unpublished data). In addition, a seemingly sponge-specific candidate phylum, "*Poribacteria*," has also been reported for several sponges (100). The most frequently recovered sequences in general 16S rRNA gene surveys of sponges include those from the *Acidobacteria*, *Actinobacteria*, and *Chloroflexi* (148). Members of several bacterial phyla, namely, the *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, *Planctomycetes*, *Proteobacteria*, and *Verrucomicrobia*, have also been isolated in pure culture from marine sponges (46, 47, 56, 81, 95, 147, 187, 188, 198, 202, 214, 235, 263, 264, 292, 334, 341, 365, 453, 458). Sequences from the *Chlorobi* (green sulfur bacteria) have not been obtained from sponges, although positive fluorescence in situ hybridization (FISH) signals were obtained from *Rhopaloeides odorabile* with a specific probe for this phylum (458). In contrast to the case for marine sponges, the (limited) available evidence for freshwater species suggests that bacterial diversity and abundance are both much lower. Only sequences from the *Actinobacteria*, *Chloroflexi*, and *Alpha*- and *Betaproteobacteria* were recovered in a recent 16S rRNA gene library constructed from the freshwater sponge *Spongilla lacustris* (123). Moreover, many of these sequences were highly similar to those known previously from freshwater habitats, suggesting that they may not represent true symbionts.

With a few exceptions in the *Euryarchaeota* (164, 456), archaea reported from marine sponges are members of the phylum *Crenarchaeota* (164, 200, 226, 294, 454, 456). Lipid biomarkers also suggested the presence of both crenarchaeotes and euryarchaeotes in a deep-water Arctic sponge, though no phylogenetic information was provided in that study (272). The group I.1A *Crenarchaeota* are extremely prevalent in marine environments (180), and almost all sponge-derived archaeal sequences are affiliated with this group. The best-studied sponge-associated archaeon is the psychrophilic crenarchaeote "*Candidatus* Cenarchaeum symbiosum," which comprises up to 65% of prokaryotic cells within the Californian sponge *Axinella mexicana* (135, 294, 343, 345).

Eukaryotic microbes also occur in sponges. Sponge-inhabiting dinoflagellates (120, 152, 153, 338, 339, 355, 382, 454, 477) and diatoms (16, 47, 51, 53, 65, 113, 305, 390, 409, 454) have been reported, with the latter seemingly most prevalent in polar regions (16, 51, 53, 113, 305, 409, 454). Freshwater sponges often contain endosymbiotic microalgae, primarily zoochlorellae (30, 108, 109, 331, 333, 475, 488). Two previous reports of cryptomonads in sponges were noted by Wilkinson (477), while marine sponge-derived fungi are receiving increasing attention due to their biotechnological potential (44, 163, 191). Interestingly, of 681 fungal strains isolated worldwide from 16 sponge species, most belonged to genera which are ubiquitous in terrestrial habitats (e.g., *Aspergillus* and *Penicillium*) (163). It thus remains unclear in most cases whether such fungi are consistently associated with the source sponge, or even whether they are obligate marine species. Compelling evidence for symbiosis of a yeast with sponges of the genus *Chondrilla* was obtained by extensive microscopy studies of both adult sponge tissue and reproductive structures, with strong indications of vertical transmission of the yeast symbiont (221).

Little is known about viruses in sponges, although virus-like particles were observed in cell nuclei in *Aplysina* (*Verongia*) *cavernicola* (432). It was suggested that these particles could be involved in sponge cell pathology. Infection of an *Ircinia strobilina*-derived alphaproteobacterium by a bacteriophage isolated from seawater has also been demonstrated (211), although the propensity of this siphovirus to infect the bacterium in nature is not known.

In addition to the realization of high microbial diversity per se, we are now beginning to recognize more subtle patterns of host-symbiont distribution. For example, it appears that a given species of sponge contains a mixture of generalist and specialist microorganisms (390) and that the associated microbial communities are fairly stable in both space and time (105, 390, 391, 454). One particularly interesting pattern to emerge is the apparent widespread existence of sponge-specific bacterial clusters, i.e., closely related groups of bacteria which are found only in sponges (146). In the following section, we examine the published evidence for such clusters.

Existing Evidence for Sponge-Specific Microorganisms

The notion that marine sponges might contain a specific microbiota arose some 3 decades ago from the seminal work of Vacelet et al. and Wilkinson et al. (427, 430, 469, 471–473, 483). Based on electron microscopy and bacterial cultivation studies, these pioneers of sponge symbiont research proposed the following three broad types of microbial associates in sponges: (i) abundant populations of sponge-specific microbes in the sponge mesohyl, (ii) small populations of specific bacteria occurring intracellularly, and (iii) populations of nonspecific bacteria resembling those in the surrounding seawater (427, 472). One type of bacterial isolate, regarded as a single species, was recovered from 35 taxonomically diverse sponges from several geographic regions, but never from seawater (469, 483). Immunological experiments in which these same isolates cross-reacted with other "sponge-specific" bacteria but not with seawater isolates were taken as further evidence of sponge specificity (469). Another significant advance came in 2002, when Hentschel and coworkers integrated these concepts into the molecular age (146). They defined sponge-specific clusters as sponge-derived groups of at least three 16S rRNA gene sequences which (i) are more similar to each other than to sequences from other, nonsponge sources; (ii) are found in at least two host sponge species and/or in the same host species but from different geographic locations; and (iii) cluster together irrespective of the phylogeny inference method used (146).

The hypothesis of widespread, sponge-specific microbial communities put forward by Hentschel and colleagues (146) was compelling and was constrained only by the limited data set available at that time. They performed phylogenetic analyses with the 190 publicly available sponge-derived 16S rRNA gene sequences, the majority of which were from *Aplysina aerophoba*, *Rhopaloeides odorabile*, and *Theonella swinhoei*. These three sponges are phylogenetically only distantly related and were collected from the Mediterranean Sea, the Great Barrier Reef, and Micronesia/Japan/Red Sea, respectively, yet they contained largely overlapping microbial communities. Together with the earlier work of Wilkinson and contemporaries (e.g., see reference 483), these remarkable results suggested that even unrelated sponges with nonoverlapping geographic ranges might share a common core of bacterial associates. Indeed, subsequent studies have lent further weight to this notion, with numerous reports of similar (and in some cases sponge-specific) bacteria found in different sponge species (100, 151, 154, 198, 235, 342, 404, 407). Furthermore, both cultivation-based and molecular methods have provided evidence for distinct microbial communities between sponges and the surrounding seawater (151, 265, 334, 391, 472). Taken together, these results appear to indicate that sponge-associated microbial communities are indeed unique and at least partially sponge specific, and the existence of sponge-specific microorganisms has consequently become something of a paradigm in this field.

A total of 14 monophyletic, sponge-specific sequence clusters were identified in the original study of Hentschel et al. (146). These occurred in the *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Nitrospira*, and *Proteobacteria* (*Alpha*, *Delta*, and *Gammaproteobacteria*) and, in most cases, were strongly supported by bootstrap analyses (in all cases, the clusters were found with three different tree construction methods). Three further clusters—each sponge specific, with the exception of a single nonsponge sequence—were also identified in the *Acidobacteria* and in a lineage of uncertain affiliation (later recognized as *Gemmatimonadetes* (146, 499). Overall, 70% of the 190 sponge-derived sequences available at the time fell into one of these monophyletic clusters or the other. Interestingly, within-cluster 16S rRNA sequence similarities ranged down to as low as 77% (146), often considered indicative of phylum-level differences (170). Several subsequent, mostly cultivation-independent studies have also led to the recovery of apparently sponge-specific sequences. Approximately 50% of 16S rRNA gene sequences in a gene library obtained from the unidentified Indonesian sponge 01IND 35 were most closely related to sequences derived from other sponges (154). These included members of the *Acidobacteria*, *Nitrospira*, *Bacteroidetes*, and *Proteobacteria*, as well as several sequences in a group of uncertain affiliation (our analyses indicate that these may be deltaproteobacterial sequences [see Fig. 8]). A similar situation was reported for *Discodermia dissoluta*, whereby three-quarters of 160 retrieved 16S rRNA sequences were most similar to other sponge-derived sequences (342). Conversely, of 21 unique sequences (each representing a unique restriction fragment length polymorphism [RFLP] type) obtained from the Caribbean sponge *Chondrilla nucula*, only 5 retrieved other sponge-derived 16S rRNA sequences during BLAST searches (although with the advantage of our larger data set, we found indications that several more of the *C. nucula* sequences are in fact from members of sponge-specific clusters) (151). Perhaps the most impressive sponge-specific cluster to be reported so far is the candidate phylum "*Poribacteria*" (100). Fieseler and colleagues found members of this lineage, which is moderately related to the *Planctomycetes*, *Verrucomicrobia*, and *Chlamydiae* (446), in several sponges from geographically diverse locations, but never in adjacent seawater or sediment samples (100). It will be especially interesting to see whether "*Poribacteria*" sequences are recovered from other environments in the future.

The sheer number of reports dealing with sponge-specific microorganisms is compelling. However, one must be cautious when proposing a sponge-specific cluster. Of crucial importance is the selection of nonsponge reference organisms for phylogenetic analyses. In principle, any group of sequences can appear sponge specific if the most appropriate reference organisms (i.e., those that are most closely related to the sponge-derived sequences) are not also included. The length of analyzed sequences is also of concern, with the level of phylogenetic information obtainable increasing with sequence length. Every effort should be made to obtain at least one near-full-length sequence per sequence type (or operational taxonomic unit). Decreasing sequence costs render this eminently achievable, and in many cases, it would only involve performing a few additional sequencing reactions. These are not new ideas and we are certainly not the first to advocate the use of full-length sequences (e.g., see reference 216), but during our analyses of sponge-derived 16S rRNA sequences, it became apparent that many of these sequences are rather short and therefore phylogenetically not particularly informative. Indeed, we encountered many problems with inser-

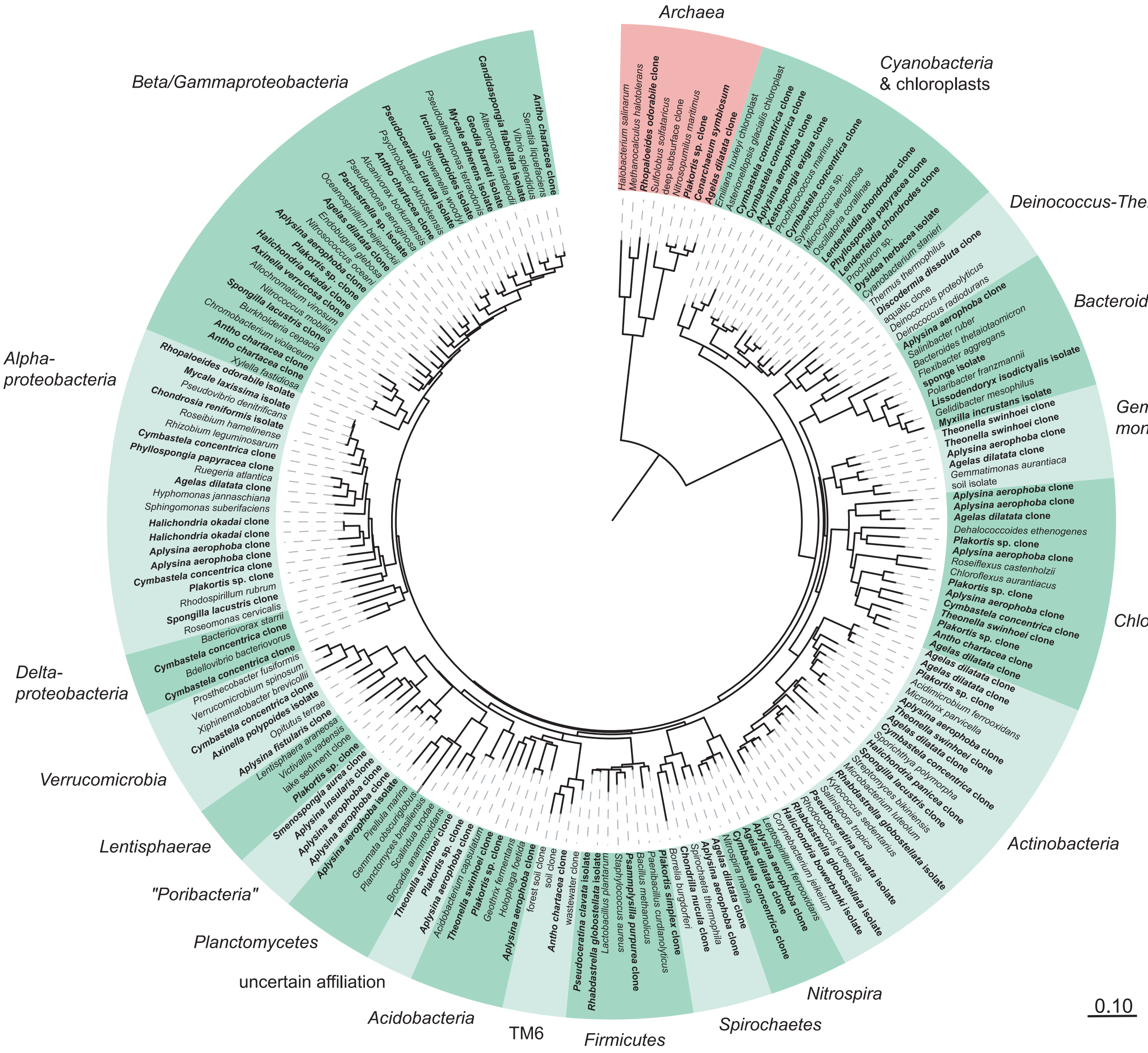

FIG. 4. 16S rRNA-based phylogeny showing representatives of all bacterial and archaeal phyla from which sponge-derived sequences have been obtained. Sponge-derived sequences are shown in bold, with additional reference sequences also included. The displayed tree is based on a maximum likelihood analysis. Bar, 10% sequence divergence.

tion of short sponge-derived sequences into our phylogenetic trees, and in some cases, we were not even certain of their phylum-level affiliation.

Census of Sponge-Associated Microorganisms

Increasing interest in sponge-microbe associations has resulted in a concomitant increase in the amounts of 16S rRNA sequence data obtained from sponges (Fig. 1B). There are currently ~1,500 sponge-derived 16S rRNA gene sequences available in GenBank (http://www.ncbi.nlm.nih.gov/), in contrast to only 190 such sequences available for the 2002 study by Hentschel et al. (146). We carried out an extensive phylogenetic analysis of all currently available sponge-derived 16S rRNA gene sequences, with two main objectives, as follows: (i) to provide an overview of microbial diversity in sponges and (ii) to critically assess the occurrence of monophyletic, sponge-specific sequence clusters. As mentioned above, such clusters are often discussed, yet their existence has not been reevaluated rigorously in light of the rapidly expanding 16S rRNA sequence databases. It is thus unclear whether these clusters are truly sponge specific or merely reflect a greater sampling effort for these communities than for others.

We began, using the ARB program package (217), by establishing an encompassing database that contains all sponge-derived 16S rRNA sequences which were available in GenBank on 28 February 2006. In addition to these 1,499 sequences (plus 11 18S rRNA sequences amplified from eukaryotic microbes in sponges), we contributed a further 184 bacterial and archaeal sequences from three hitherto unstudied sponges,

TABLE 1. Summary of all publicly available sponge-derived 16S rRNA sequence data (as of 28 February 2006) plus 184 bacterial and archaeal sequences contributed from this article

Phylogenetic affiliation	Total no. of sequences	No. of sequences of >1,000 bp	No. of sequences		% of sequences in clusters obtained from:		
			Uncultivated	Obtained from an isolate	Exclusively sponges[a]	Sponges and corals	Sponges and one nonsponge organism
Bacteria	1,630	592					
Acidobacteria	66	9	66	0	5	64	24
Actinobacteria	266	99	190	92	38 (45)	0	1
Bacteroidetes	77	20	46	31	0 (4)	0	10
Chloroflexi et al.	109	48	109	0	62 (75)	0	0
Cyanobacteria	119	68	111	7	79	0	0
Deinococcus-Thermus	2	0	2	0	0	0	0
Firmicutes	96	31	45	51	9	0	0
Gemmatimonadetes	16	5	16	0	25	56	0
Lentisphaerae	1	1	1	0	0	0	0
Nitrospira	14	6	14	0	57	29	0
Planctomycetes	11	4	9	2	0	0	0
"*Poribacteria*"	21	10	21	0	100	0	0
Proteobacteria							
Alpha-	311	125	196	115	14 (22)	0	4
Beta/Gamma-	430	114	298	134	34 (37)	0	0
Delta-	48	25	48	0	15	40	6
Spirochaetes	6	2	6	0	67	0	0
TM6	1	1	1	0	0	0	0
Verrucomicrobia	13	13	12	1	23	0	0
Uncertain affiliation	23	11	23	0	78	0	0
Archaea	44	10					
Crenarchaeota	43	10	43	0	28 (42)	0	0
Euryarchaeota	1	0	1	0	0	0	0
Eukarya[b]	20	6	18	2			
Total	1,694	608	1,259	435	546	74	43

[a] Numbers in parentheses are inclusive of clusters which are supported by only two of three tree construction methods.

[b] Includes both 18S rRNA- and 16S rRNA (plastid)-derived sequences.

namely, *Agelas dilatata*, *Plakortis* sp. (both from the Bahamas; kindly provided by U. Hentschel), and *Antho chartacea* (from southeastern Australia). Preliminary phylogenetic analyses identified members of putative sponge-specific clusters, and for each cluster, the most similar nonsponge sequences were retrieved by BLAST searches (from both regular NCBI and environmental genome databases) and imported into ARB for subsequent alignment (automatic and manual). The resulting ARB database, containing an alignment of all sponge-derived sequences and their nearest relatives (together with annotated information [e.g., host species and collection location] for the sponge sequences), is available upon request. Extensive phylogenetic analyses (see the supplemental material for details) were conducted, taking all (n = 1,694) sponge-derived sequences into account. In order to rigorously test the existence of monophyletic, sponge-specific sequence clusters, we employed multiple tree construction methods (maximum likelihood, neighbor joining, and maximum parsimony), together with the use of various sequence conservation filters and correction parameters.

In total, sequences representing 16 bacterial phyla and both major archaeal lineages (*Crenarchaeota* and *Euryarchaeota*) have been recovered from sponges (Fig. 4). In addition to those known prior to this study (*Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Deinococcus-Thermus*, *Firmicutes*, *Gemmatimonadetes*, *Nitrospira*, *Planctomycetes*, "*Poribacteria*," *Proteobacteria* [*Alpha-*, *Beta-*, *Delta-*, and *Gammaproteobacteria*], *Spirochaetes*, *Verrucomicrobia*, and *Chlorobi* FISH signals), we report for the first time the presence in sponges of 16S rRNA sequences affiliated with the phylum *Lentisphaerae* and the candidate phylum TM6. The number of sequences representing each phylum varied widely, from single sequences for the *Lentisphaerae* and TM6 to more than 250 sequences for each of the *Actinobacteria*, *Alphaproteobacteria*, and *Beta/Gammaproteobacteria* (Table 1). The proportions of sequences derived from cultivated versus noncultivated microorganisms also varied greatly among phyla.

The phylogenetic analyses presented here strongly support the existence of monophyletic, sponge-specific 16S rRNA sequence clusters. These occurred in many of the bacterial and archaeal phyla found in sponges, with approximately one-third (32%) of all sponge-derived sequences falling into such clusters (Table 1; Fig. 5 to 15; also see the supplemental material). If sequences derived from cultured isolates are excluded, this figure rises to 42%. This result was expected since tightly linked symbionts—those presumed to occur in sponge-specific clusters—are likely difficult to cultivate and therefore underrepresented in culture collections. Several additional clusters each contained a single nonsponge sequence, with the extra sequences often, but not always, obtained from marine envi-

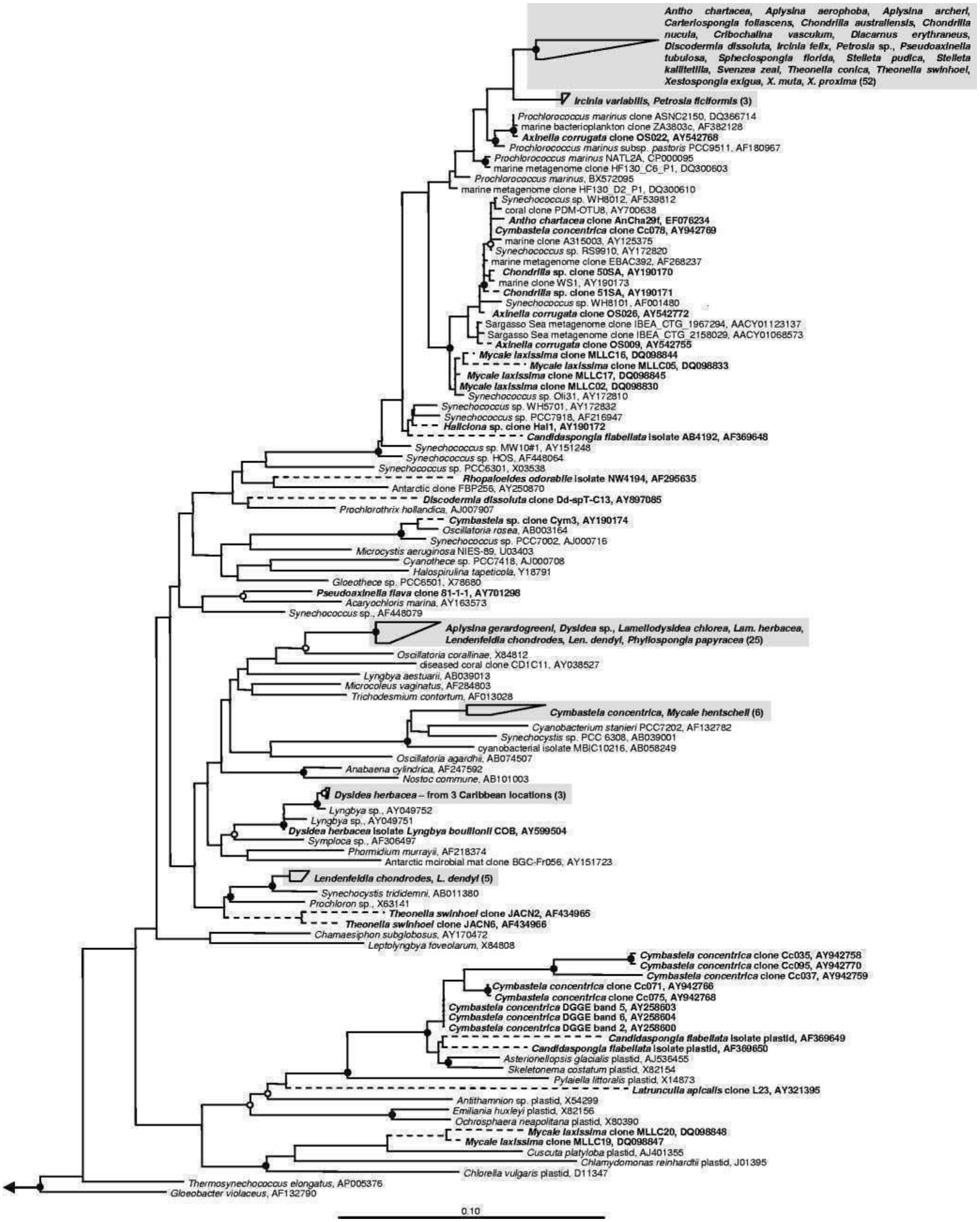

FIG. 5. 16S rRNA-based phylogeny of sponge-associated cyanobacteria and chloroplasts. Sponge-derived sequences are shown in bold. The displayed tree is a maximum likelihood tree constructed based on long (≥1,000 nucleotides) sequences only. Shorter sequences were added using the parsimony interactive tool in ARB and are indicated by dashed lines. Shaded boxes represent sponge-specific monophyletic clusters, as defined

ronments. It is also possible that sponge-specific microbes are more prevalent in those sponges which contain very dense microbial communities (Ute Hentschel, personal communication), i.e., the so-called bacteriosponges or high-microbial-abundance sponges (148, 430). Due to a lack of microbial abundance data for most host sponges, we did not attempt to take this factor into account during our analyses. Overall, while representation of sequences in sponge-specific clusters was quite high, it should be noted that the proportions of sequences falling within such clusters differed greatly among the various phyla.

More than three-quarters of the 119 available sponge-derived *Cyanobacteria* sequences fell into monophyletic, sponge-specific clusters (Table 1; Fig. 5). Most of these were in two clusters, with one comprising 25 sequences from at least 7 sponge species and the other comprising 52 sequences from 21 sponges. The latter cluster represents the recently described candidate species "*Candidatus* Synechococcus spongiarum" (426) and was the sole *Cyanobacteria* cluster in the study of Hentschel et al. (146), while the former corresponds to the filamentous cyanobacterium *Oscillatoria spongeliae* (39, 157). Sequences representing *O. spongeliae* were not available for the 2002 study. Several other, smaller clusters are also evident among the cyanobacteria (Fig. 5). Additionally, in a number of cases, microalgal plastids have also been amplified using 16S rRNA primers.

Another bacterial phylum containing many sponge-specific sequence clusters is the *Chloroflexi* (Table 1; Fig. 6). Of the 109 sponge-derived sequences analyzed, 62% comprised such clusters, while the occurrence of a further 13% of sequences in clusters was weakly supported. In the new analyses, all but one of the members of a sponge-specific cluster described by Hentschel and coworkers (146) remained in a cluster, although these sequences were now dispersed over four different clusters. Such movement of sequences was frequently observed and is not surprising given the much larger data set at our disposal now (i.e., many new related sequences, both sponge- and non-sponge-derived, were included in the phylogenetic analyses described here). None of the sponge-derived sequences were closely related to the few described *Chloroflexi* species, although many were similar to sequences from uncultivated organisms, particularly from marine environments (Fig. 6).

Interestingly, many sponge-derived 16S rRNA sequences formed exclusive monophyletic clusters with sequences obtained from corals (Table 1). This was particularly apparent for the *Acidobacteria* and *Deltaproteobacteria* (Fig. 7 and 8, respectively) but was also evident for the *Gemmatimonadetes* (Fig. 9) and *Nitrospira* (Fig. 10). No coral-derived sequences shared monophyletic clusters with sponge sequences in the original study of Hentschel et al. (146), no doubt reflecting the fact that most of the relevant coral sequences were deposited in GenBank since then. It is too early to speculate whether some sort of marine invertebrate-specific sequence cluster exists, but further sampling of taxa such as ascidians and bryozoans should help to resolve this issue. A study of two marine macroalgae and the cooccurring sponge *Cymbastela concentrica* gave no indication of specific clusters spanning these taxonomically disparate groups (Longford et al., unpublished data).

Sponge-specific sequence clusters were not prevalent for, among others, the *Bacteroidetes* (see Fig. S1 in the supplemental material) and *Firmicutes* (see Fig. S2 in the supplemental material), perhaps reflecting the relatively high proportions of sequences derived from cultivated organisms in these phyla.

We report for the first time the recovery of *Lentisphaerae* (Fig. 11) and candidate phylum TM6 (Fig. 12A) sequences from sponges. Each phylum was represented by a single 16S rRNA sequence, from the marine sponges *Plakortis* sp. and *Antho chartacea*, respectively, and it cannot be ruled out that these represent contaminating sequences from the surrounding environment (although arguably this also applies to many, more commonly recovered sequence types). The *Lentisphaerae* phylum comprises part of the so-called *Planctomycetes-Verrucomicrobia-Chlamydiae* (PVC) superphylum (446), with sponge-derived sequences from the superphylum additionally being found in the *Verrucomicrobia*, *Planctomycetes*, and "*Poribacteria*" (Fig. 11). Members of the superphylum are frequently associated with eukaryotes. There is also a group of uncertain affiliation which falls near the PVC superphylum (but without strong bootstrap support) during phylogenetic analyses. This group includes sequences from many sponges, such as *Agelas dilatata*, *Aplysina aerophoba*, *Discodermia dissoluta*, and *Theonella swinhoei*. Those sequences most closely related to the sponge sequences are also from marine environments.

Several large sponge-specific clusters were found among the *Actinobacteria* sequences, particularly in the family *Acidimicrobiaceae* (Fig. 13). The largest comprised 54 sequences obtained from sponges from the Caribbean (*Agelas dilatata*, *Discodermia dissoluta*, *Plakortis* sp., and *Xestospongia muta*), Indonesia (*Xestospongia testudinaria*), the Red Sea (*Theonella swinhoei*), and the South China Sea (*Dysidea avara*). None of the sequences within this cluster were obtained from cultured bacteria, with the nearest (but still distantly related) cultured actinobacteria being the wastewater bacterium *Microthrix parvicella* and the acidophilic *Acidimicrobium* spp. (Fig. 13).

Although not representing a sponge-specific cluster, the group of sequences affiliated with the marine *Pseudovibrio* spp. within the *Alphaproteobacteria* deserves special mention (Fig. 14). Members of this genus are frequently found in sponge-

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by Hentschel et al. (146), i.e., a group of at least three sponge-derived 16S rRNA gene sequences which (i) are more similar to each other than to sequences from other, nonsponge sources, (ii) are found in at least two host sponge species and/or in the same host species but from different geographic locations, and (iii) cluster together irrespective of the phylogeny inference method used (all clusters shown here also occurred in neighbor-joining and maximum parsimony analyses). Names outside wedges of grouped sequences represent the sponges from which the relevant sequences were derived; the number in parentheses indicates the number of sequences in that wedge. Filled circles indicate bootstrap support (maximum parsimony, with 100 resamplings) of ≥90%, and open circles represent ≥75% support. The outgroup (not shown) consisted of a range of sequences representing several other bacterial phyla. Bar, 10% sequence divergence.

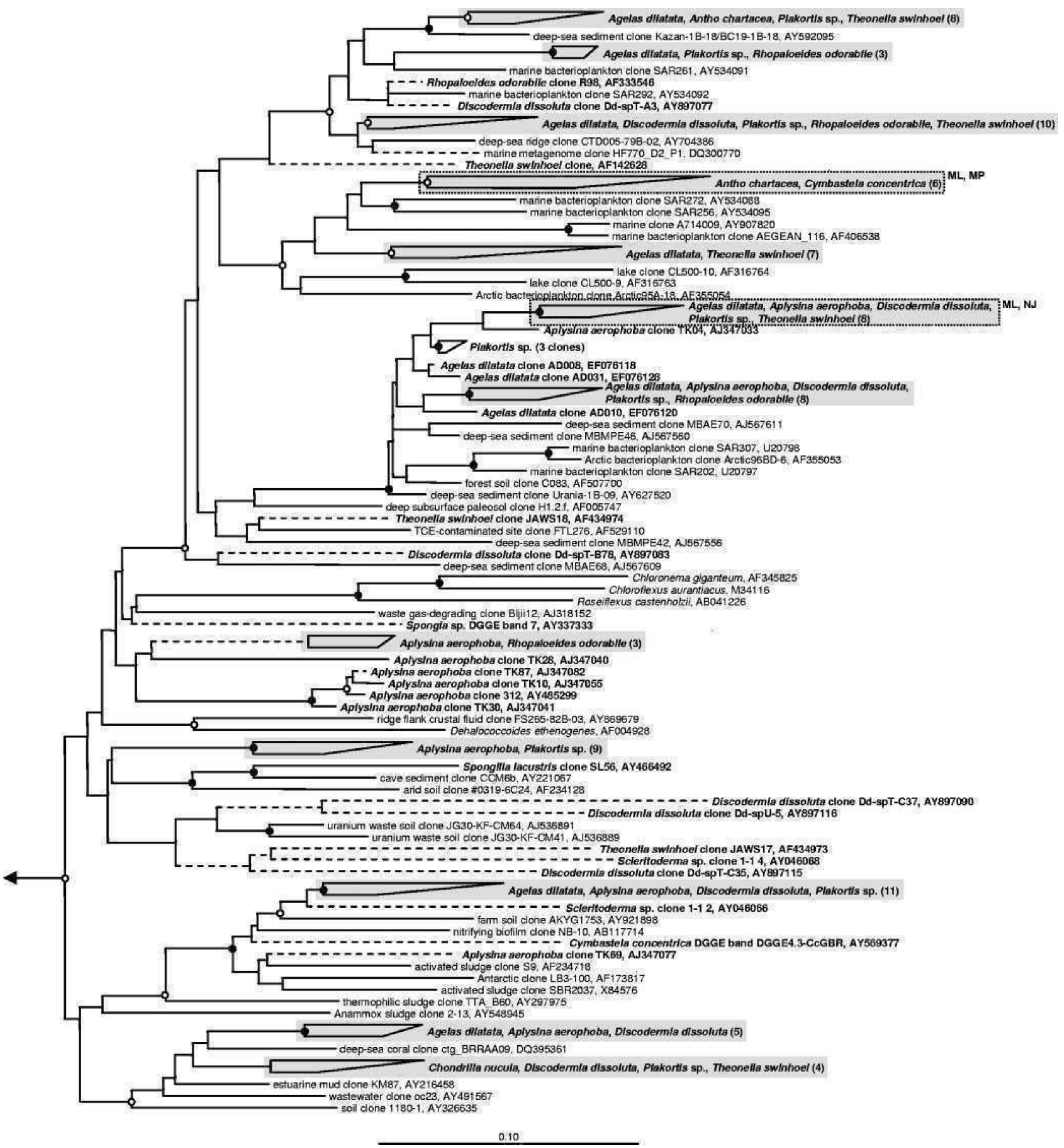

FIG. 6. 16S rRNA-based phylogeny of sponge-associated *Chloroflexi* organisms. Details are the same as those provided for Fig. 5, with the following additions. Shaded boxes contained within dotted lines represent sponge-specific clusters supported by only two tree construction methods (ML, maximum likelihood; MP, maximum parsimony; and NJ, neighbor joining), and new sequences from our laboratory have the prefix "AD" (for the sponge *Agelas dilatata*), "AnCha" (*Antho chartacea*), or "PK" (*Plakortis* sp.).

derived cultivation-based and molecular studies (95, 96, 147, 187, 198, 263, 453), and there is strong evidence for its being a true sponge symbiont (95, 453).

Only 28% of sponge-derived *Archaea* sequences fell into well-supported sponge-specific clusters (Fig. 15), although the fact that almost all of these were within the group I.1A *Crenarchaeota* bears testimony to their high degree of phylogenetic relatedness. The recently isolated ammonia-oxidizing archaeon "*Candidatus* Nitrosopumilus maritimus" (192) is the only cultivated member of this group, with the well-studied

FIG. 7. 16S rRNA-based phylogeny of sponge-associated *Acidobacteria* organisms. Details are the same as those provided for Fig. 5 and 6, with the following two additions. Open boxes represent monophyletic clusters containing sponge-derived sequences and a single, nonsponge origin sequence, and open boxes with asterisks outside them signify clusters containing only sponge- and coral-derived sequences (the number of asterisks corresponds to the number of coral-derived sequences within the cluster).

FIG. 8. 16S rRNA-based phylogeny of sponge-associated *Deltaproteobacteria* organisms. Details are the same as those provided for Fig. 5 to 7.

(but still uncultivated) archaeon "*Ca*. Cenarchaeum symbiosum" being the best known sponge-associated member. A genome project for the latter has recently been completed (134). At the time of sequence collection, 44 archaeal sequences had been recovered from sponges, all of which were marine sponges (Table 1; Fig. 15). All but one of these was from the *Crenarchaeota*, with a single *Euryarchaeota* sequence from the Great Barrier Reef sponge *Rhopaloeides odorabile* (456). An

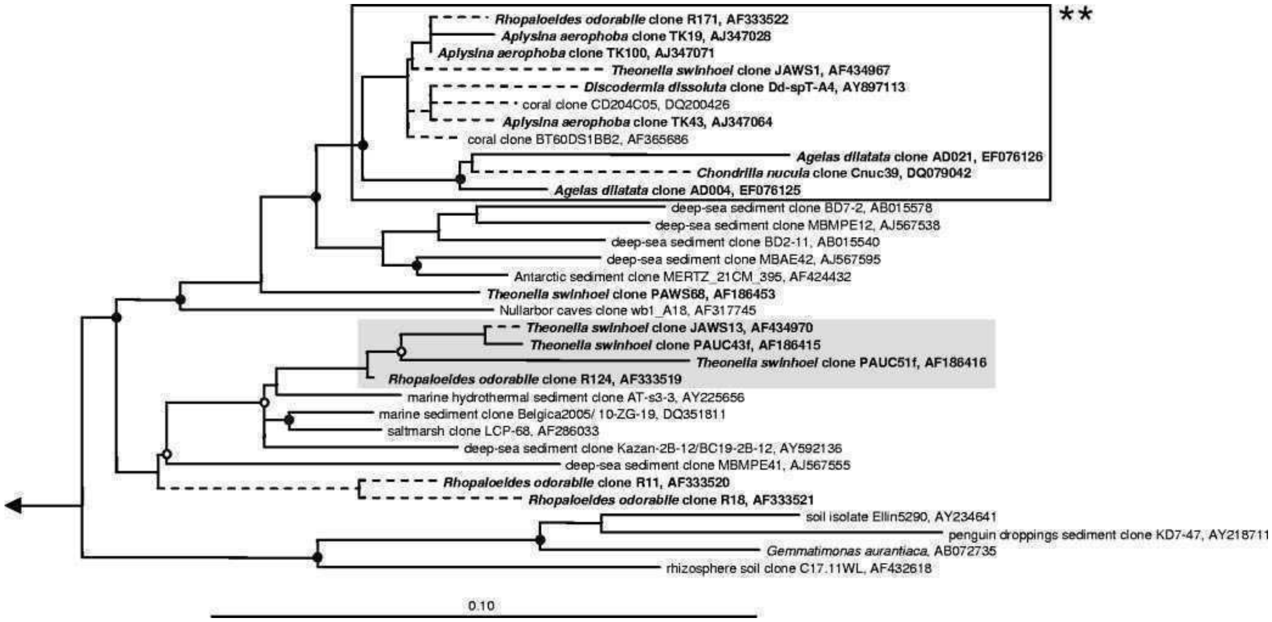

FIG. 9. 16S rRNA-based phylogeny of sponge-associated *Gemmatimonadetes* organisms. Details are the same as those provided for Fig. 5 to 7.

article which appeared in mid-2006 (whose sequences were not available on 28 February 2006 and were therefore not included in our study) reported more euryarchaeotal sequences from various sponges, although the majority of sequences in that study were still affiliated with the *Crenarchaeota* (164).

All sponge-derived 16S rRNA sequences available on 28 February 2006 were analyzed phylogenetically, but for practical reasons the larger trees are available only in the supplemental material. Broadly speaking, the results of our analyses are consistent with the earlier study by Hentschel et al. (146), with, for example, the *Actinobacteria*, *Nitrospira*, and *Acidobacteria* still well represented by sponge-specific microorganisms. As could be expected, some sponge-specific clusters from the 2002 study now form parts of several new clusters, while others do not comprise clusters at all in the new data set. Conversely, the addition of more sequences meant that many formerly single sequences are now in specific clusters with other sponge-derived sequences.

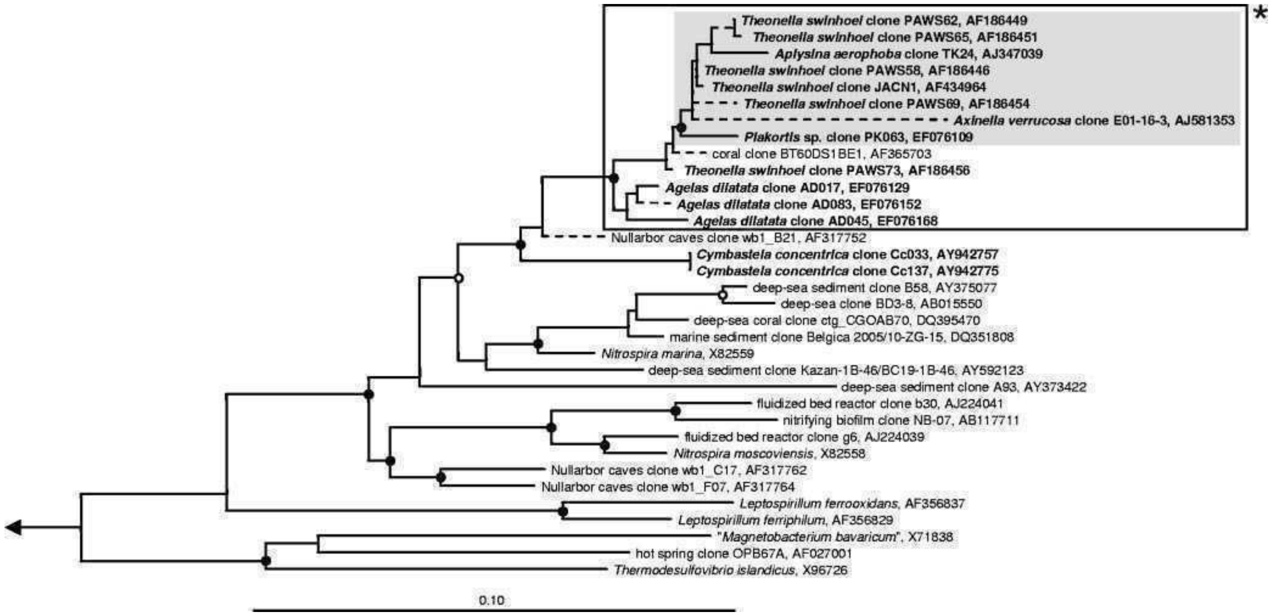

FIG. 10. 16S rRNA-based phylogeny of sponge-associated *Nitrospira* organisms. Details are the same as those provided for Fig. 5 to 7.

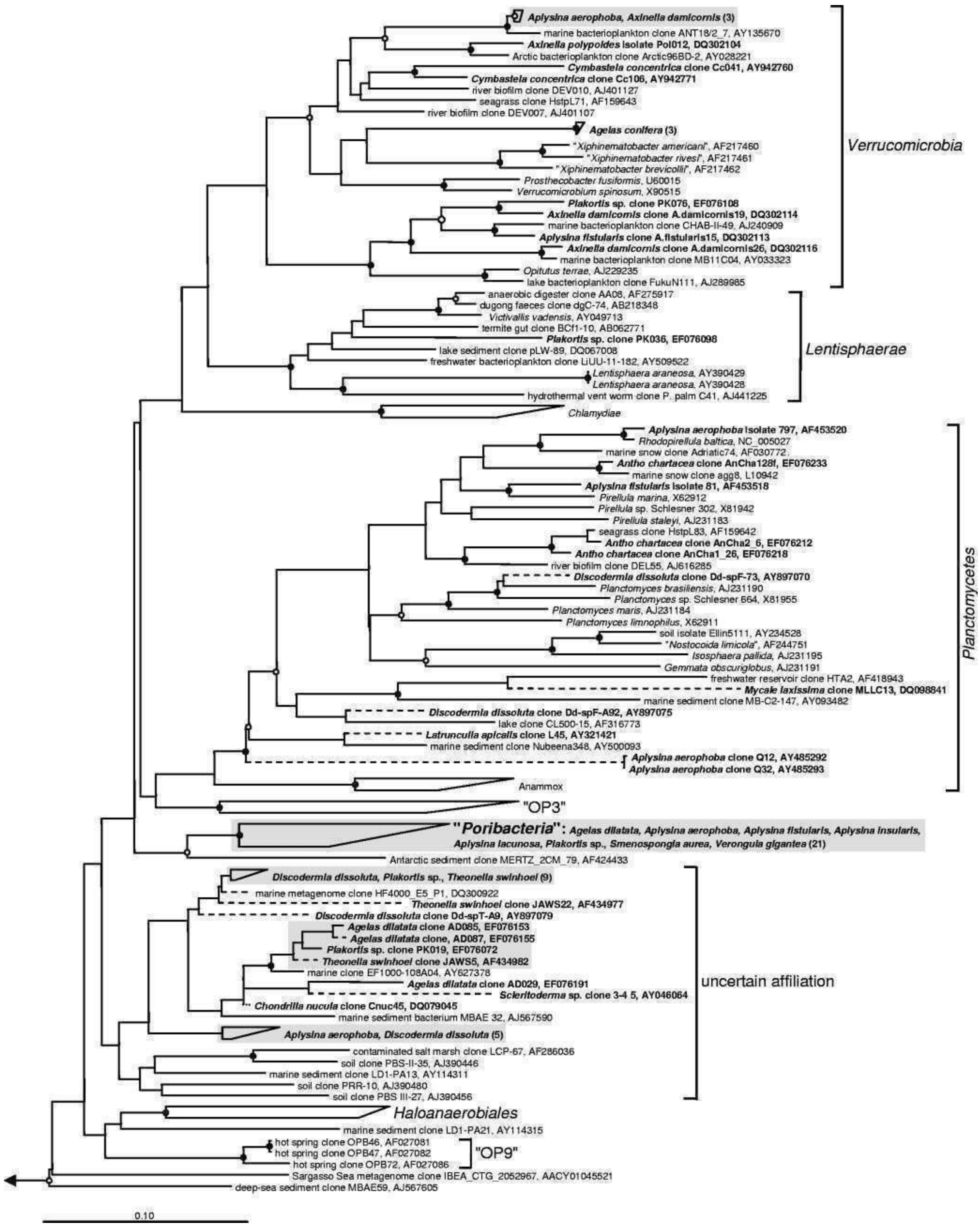

FIG. 11. 16S rRNA-based phylogeny of sponge-associated *Verrucomicrobia*, *Planctomycetes*, *Lentisphaerae*, and “*Poribacteria*” organisms and of a lineage of uncertain affiliation. These and associated lineages comprising the PVC superphylum (446) are shown. Details are the same as those provided for Fig. 5 to 7.

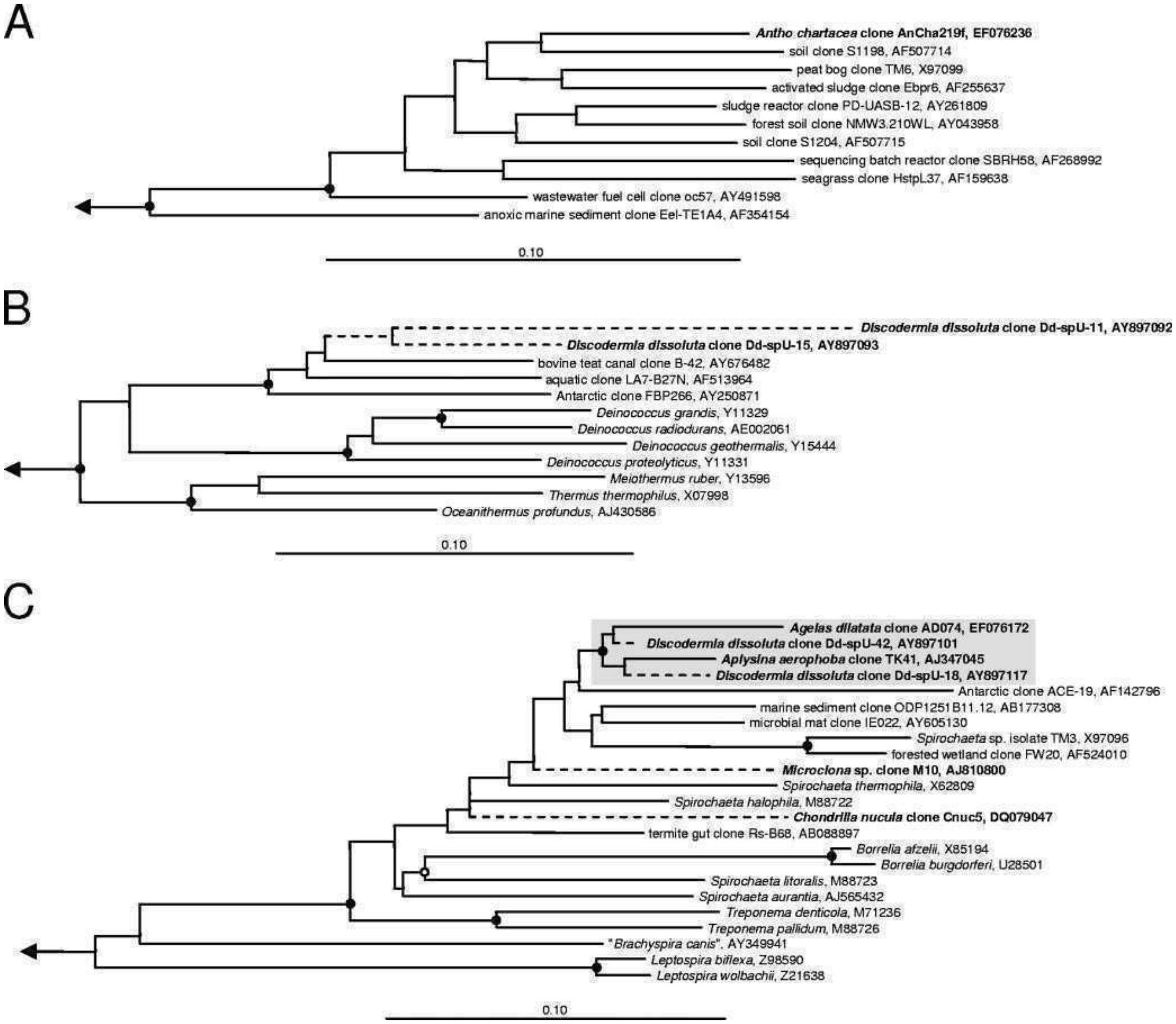

FIG. 12. 16S rRNA-based phylogeny of sponge-associated members of the candidate phylum TM6 (A), *Deinococcus-Thermus* organisms (B), and *Spirochaetes* organisms (C). Details are the same as those provided for Fig. 5 to 7. (B) In our analyses, the position of clone Dd-spU-11 (from the sponge *Discodermia dissoluta*) was not stable, and we are not certain of its phylogenetic affiliation.

Very few sequences were available from sponge-associated eukaryotic microbes at the time of database establishment (since then, some 45 18S rRNA sequences derived from sponge-associated fungi have been deposited in GenBank). Those that are included in our database include 9 16S rRNA sequences derived from diatom chloroplasts (Fig. 5) and 11 18S rRNA sequences obtained from diatoms and dinoflagellates. All but one of the 18S rRNA sequences were obtained from Antarctic sponges (454), with the remaining sequence representing a zooxanthella (*Symbiodinium* sp.) from the Palauan sponge *Haliclona koremella* (49).

We endeavored to be as thorough and as careful as possible throughout our analyses, yet there remain some caveats to our results. Despite extensive BLAST searches using members of all putative sponge-specific clusters, it is not inconceivable that we failed to include some key sequences which would have broken up otherwise specific sponge clusters. Another factor relates to the short lengths of many sponge-derived 16S rRNA sequences. We constructed our trees using only sequences longer than 1,000 bp, but more than two-thirds of all sponge-derived sequences are shorter than this (Table 1), and we added these via the parsimony interactive tool in ARB. In principle, this method allows the insertion of short sequences without changing tree topology (217). However, when many short sequences are added at once, they can influence each other's positioning (and potentially bias the analysis towards the formation of sponge-specific clusters). We attempted to gauge the severity of this problem by (for a selection of sequences) sequentially adding and removing individual short sequences and comparing their placement to the outcome

FIG. 13. 16S rRNA-based phylogeny of sponge-associated *Actinobacteria* organisms belonging to the family *Acidimicrobiaceae*. Other sponge-derived actinobacteria are shown in Fig. S3 in the supplemental material. Details are the same as those provided for Fig. 5 to 7.

when they were all added at once. The results were highly consistent, but it should not be assumed that this will always be the case. The alternative is to perform the entire phylogenetic analysis with short sequences and to truncate longer sequences to leave only the homologous region; this results in the loss of much phylogenetic information and is not recommended under any circumstances (216). Again, we reiterate the importance of obtaining at least one near-full-length sequence for each operational taxonomic unit obtained. This is not possible in some cases (e.g., excised DGGE bands) but is feasible in many others.

It is prudent to consider whether the apparent occurrence of sponge-specific sequence clusters could have a more dubious origin, namely, laboratory contamination. Theoretically, a 16S

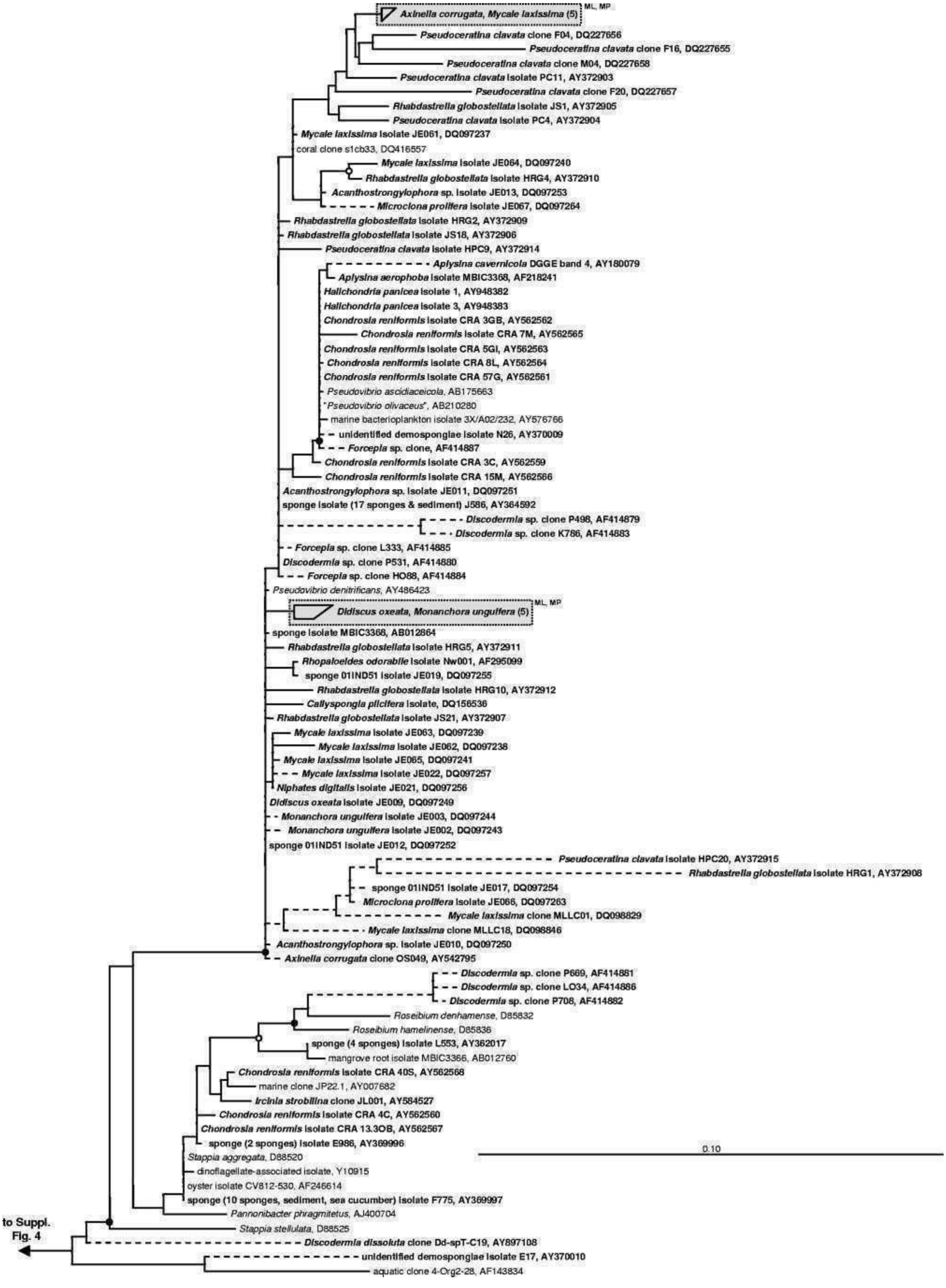

FIG. 14. 16S rRNA-based phylogeny of sponge-associated *Alphaproteobacteria* organisms affiliated with the genus *Pseudovibrio* and its relatives. Other sponge-derived alphaproteobacteria are shown in Fig. S4 and S5 in the supplemental material. Details are the same as those provided for Fig. 5 to 7.

FIG. 15. 16S rRNA-based phylogeny of sponge-associated archaeal organisms. Details are the same as those provided for Fig. 5 to 7.

rRNA gene-containing plasmid or PCR product could, if accidentally spread to DNAs from several sponges in the same laboratory, appear to form its own sponge-specific cluster. However, the available evidence strongly suggests that this is not the case, since many or most clusters contain sequences originating from several independent laboratories.

With almost 1,700 sponge-derived 16S rRNA sequences falling into some 16 or more bacterial and archaeal phyla, we sought to address the following question: how well sampled are marine sponge-associated microbial communities? If current studies are recovering mainly sequences which were previously obtained from sponges (as the presence of sponge-specific clusters might imply), then we may have already uncovered most of the microbial diversity in these hosts, suggesting that our current descriptive phase might be nearing its logical conclusion. Unfortunately, the available data are insufficient to satisfactorily address this issue for sponges. In a recent article in this journal, Schloss and Handelsman (348) used the program DOTUR to estimate richness at different levels of phylogenetic relatedness for each bacterial phylum represented in the Ribosomal Database Project (61). To perform an analogous study with the sponge symbiont data set, we were restricted to sequences which met the following criteria: (i) they were part of attempts at extensive microbial community surveys using general 16S rRNA gene primers for the construction of clone libraries; (ii) they overlapped a sufficient distance to be useful (*Escherichia coli* positions 100 to 500 would have been appropriate for a reasonable portion of the sponge data set); and (iii) they were not obtained from prescreened gene libraries (e.g., by RFLP analysis), as this would heavily bias results—thus, all collected sequences must have been deposited in GenBank. After applying these (in our eyes) minimal criteria, only 317 sequences (of 1,694) were deemed suitable for use with DOTUR or similar programs. For many phyla, only a few sequences were retained (e.g., for *Cyanobacteria*, 8 of 119 sequences were kept, and for *Alphaproteobacteria*, 21 of 311 sequences were kept), precluding meaningful analyses. Furthermore, even if 50 or more sequences were suitable (as in the case of the *Beta*/*Gammaproteobacteria*), these were not necessarily representative of the known (sponge-derived) diversity within that phylum, again preventing the drawing of meaningful conclusions. Although statistically robust analyses are therefore not possible at this stage, data from two recent studies can add greatly to this discussion. In the first, Lopez and colleagues at the Harbor Branch Oceanographic Institution (213) obtained more than 700 sequences from 20 different sponge-derived gene libraries by using general 16S rRNA primers, with the vast majority of these belonging to phyla already obtained from sponges, such as *Chloroflexi*, *Cyanobacteria*, *Nitrospira*, *Planctomycetes*, and *Spirochaetes*. Of the recovered sequences, *Epsilonproteobacteria* was the only major taxonomic group not previously obtained from sponges. In another study, examining the Adriatic sponges *Chondrilla nucula* and *Tethya aurantium*, Thiel and coworkers recovered representatives of only known sponge-associated phyla (*Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Gemmatimonadetes*, *Planctomycetes*, *Proteobacteria* [*Alpha*-, *Beta*-, *Delta*-, and *Gammaproteobacteria*], *Spirochaetes*, and *Verrucomicrobia*) (404; V. Thiel, T. Staufenberger, and J. F. Imhoff, presented at the 11th International Symposium on Microbial Ecology, Vienna, Austria, 20 to 25 August 2006). The lack of new phyla in these data allow one to speculate that the majority of sponge-associated microorganisms may have already been encountered in gene libraries, at least at the phylum level. However, two major caveats exist. Although we may arguably be nearing the point of diminishing returns with respect to using current techniques to recover novel lineages from sponges (i.e., gene libraries constructed using general 16S rRNA primers), it is highly likely that the use of phylum-specific primers and/or metagenomic (i.e., PCR-independent) approaches will reveal phyla previously unknown to exist in these hosts or even unknown to science (e.g., "*Poribacteria*") (100). To our knowledge, there is no example of a sponge for which the results of general versus specific 16S rRNA gene libraries have been compared. A second point is that few gene libraries, including those from sponges, are sequenced to full coverage, and it is possible that the recurring sequences obtained from different sponges are merely those that are most abundant (or those that PCR is most biased toward) in each sponge, with the unsequenced remainder of the library potentially contributing new sequence types. The advent of high-throughput sequencing technologies (e.g., see reference 227) offers the potential to sequence gene libraries to much greater depth, illuminating the rare biosphere within sponges (376).

Statistical comparisons of microbial community compositions allow for the inclusion of more sequences (relative to species richness estimates via DOTUR) due to less stringent selection criteria. We thus used the so-called parsimony test, implemented in the program TreeClimber (347), to compare our three new gene libraries (from the sponges *Agelas dilatata*, *Antho chartacea*, and *Plakortis* sp.) with selected sponge-derived libraries from the literature and those deposited in GenBank. The parsimony test compares phylogenetic trees rather than sequence data per se (228, 347), and various tree construction algorithms can be employed. Our criteria for sequence inclusion were that (i) general 16S rRNA gene primers were used and (ii) at least 25 sequences were available from each library. The main caveats are that prescreening of clones (e.g., by RFLP analysis) with subsequent representation of each operational taxonomic unit by a single sequence prevents strict application of the parsimony test (347), while low sequencing coverage of some libraries may obscure true similarities or differences among libraries by missing overlapping or distinct sequences, respectively. With these considerations in mind, we compared the three libraries obtained from this study with those from the marine sponges *Theonella swinhoei* (146), *Aplysina aerophoba* (146), *Rhopaloeides odorabile* (458), *Cymbastela concentrica* (Longford et al., unpublished data), *Discodermia dissoluta* (342), and *Chondrilla nucula* (151) and the freshwater sponge *Spongilla lacustris* (123). An initial analysis comprising all 10 libraries yielded a highly significant ($P < 0.001$) result (i.e., the differences in sequence composition among the various libraries were not due to chance). Likewise, comparisons of the marine versus freshwater (*S. lacustris*) libraries, as well as comparisons among the marine libraries and among broad geographic locations, were all highly significant. The usefulness of such analyses should increase as more 16S rRNA gene libraries are sequenced from sponges (and with greater sequencing coverage), including multiple species from the same location and/or from the same genus or family.

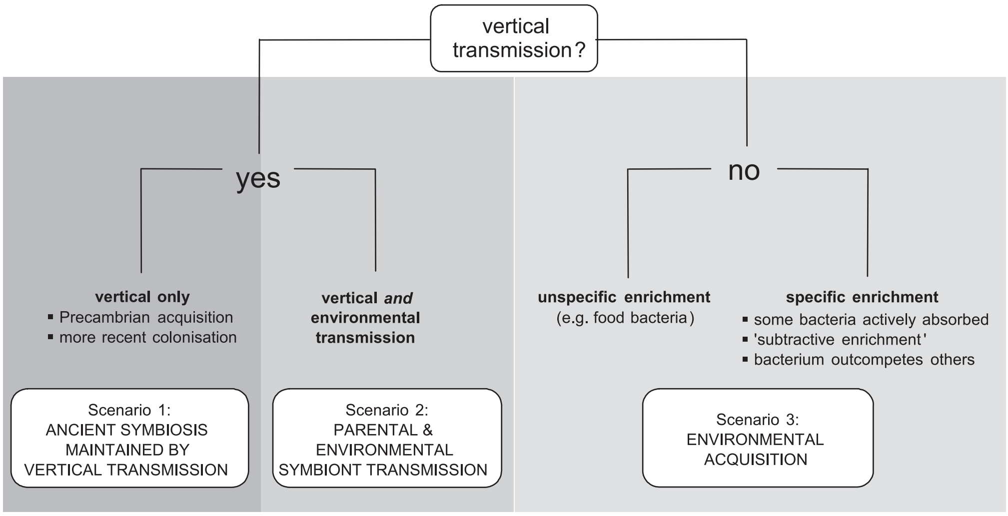

FIG. 16. Summary of various evolutionary scenarios for sponge-microorganism associations.

Sponge-Associated Microorganisms: Ancient Partners or Recent Visitors That Have Come To Stay?

Based largely upon arguments centering on immunological evidence dating back to the 1980s (469), it is often stated that sponge-bacterium symbioses have existed for as long as 600 million years. This would date such associations back to the Precambrian, prior to the bulk of taxonomic radiation in sponges. Moreover, given the likely basal position of sponges in the metazoan phylogenetic tree (38, 133), this would presumably make sponges and microorganisms the most ancient of all metazoan-microorganism associations. So what is the evidence for this oft-cited ancient symbiosis? In his 1984 study, on which the majority of these arguments rest, Wilkinson used a collection of 296 sponge isolates which, on the basis of morphological and physiological characteristics, comprised one bacterial species (469). In addition, 128 seawater and nonspecific sponge isolates were included as control strains. It is important to note that the sponge-specific isolates were obtained from phylogenetically distant sponges in widely separated geographical regions. From seven of the specific isolates and five of the others, Wilkinson prepared antisera and performed agglutination tests. Many of the "sponge-specific" strains reacted positively in these tests to one or more of the antisera derived from sponge-specific bacteria, but none of them reacted with sera derived from non-sponge-specific strains, nor did cross-reactions occur between the 128 non-sponge-specific strains and the sera prepared from sponge-specific bacteria. The implication of these results was that the studied, widespread, sponge-specific bacterium did indeed form a single species group distinct from isolates found in the surrounding seawater (469). According to Wilkinson, the most logical explanation for the occurrence of this specific bacterial type in such diverse hosts and locations was that these bacteria became associated with an ancestral sponge prior to the evolution of current sponge classes (i.e., during the Precambrian). One should bear in mind, however, that the enormous complexity of microbial communities in seawater could have led to this bacterium being missed in Wilkinson's culture libraries.

In the 22 years since the Wilkinson study, a wealth of molecular data has become available for sponge-associated microorganisms, ranging from sequences of single genes to an entire genome (for the archaeon "*Candidatus* Cenarchaeum symbiosum") (134). Here we ponder whether such data can be exploited to address the issue of the ancientness (or otherwise) of sponge-microbe associations. First, we consider some of the many possible evolutionary scenarios (summarized in Fig. 16), as follows.

Scenario 1: Ancient symbioses maintained by vertical transmission. A given sponge-specific cluster in the phylogenetic tree of life may contain 16S rRNA gene sequences derived from distantly related, geographically disparate sponge species. If the microorganisms represented by these sequences do not occur outside sponges today, then the ancestral strain (the future symbiont) may have first inhabited a sponge during one or several colonization events prior to sponge speciation (~600 million years ago) (the Precambrian acquisition hypothesis of Wilkinson [469]). Such a symbiosis could have been maintained in the intervening years via vertical transmission (see "Establishment and Maintenance of Sponge-Microbe Associations"), and the microbes evolved to become sponge (or even species) specific. A related but subtly different hypothesis is that an association could still be ancient but not predate the bulk of sponge speciation. In this scenario, it is conceivable that one sponge could have been colonized very early on, resulting in the evolution of a sponge-adapted microorganism. Millions (or hundreds of millions) of years later, this microbe could have spread across the oceans and, upon encountering other sponges, colonized them. Perhaps it is no longer present in seawater, or perhaps it is still there but in very small numbers. Yet another scenario is that today's sponge-specific microbes were once a generalist marine species, thriving in all marine ecosystems, including sponges. Those strains that inhabited sponges have since evolved to become genetically distinct from their free-living counterparts. Support for these scenarios comes from another quarter, with various fatty acids of likely microbial origin occurring in a wide range of sponges irrespective of host phylogeny or geographic location (401, 403). The apparent absence of some of these biomarkers from marine sediments and seawater led to the suggestion that the compounds and their microbial producers have been present in the sponges since ancient times (403).

It is likely that any ancient sponge-microbe symbiosis would be obligate for one or both partners, potentially involving a reduction in microbial genome size if the symbiont has developed a nutritional dependence on its host. This has been demonstrated for many obligate insect endosymbionts (e.g., see references 252, 437, and 501), but it is unknown whether such tight host-symbiont coupling occurs in sponges. Integration of host and symbiont genomes was discussed in the sponge context by Sara and colleagues (337), while a recent paper offers evidence for lateral gene transfer from a fungus to the mitochondrion of its host sponge (327). Such gene transfer would not be without precedent among marine invertebrates, as it is believed that the ascidian *Ciona intestinalis* laterally acquired a cellulose synthase gene from a bacterium (253). Future genome sequencing of sponges and their microbial associates should offer valuable insights into the nature of these symbioses.

As noted earlier, not all sponge species harbor abundant microbial communities, and it is worthwhile to take a moment to consider these organisms. Freshwater sponges, for example, typically contain a paucity of microbial associates, and it has been suggested that this is due to an obligate requirement for sodium ions by the symbiotic bacteria (469). When freshwater sponges colonized their new habitat from the sea some 20 to 50 million years ago, it is presumed that existing symbionts were lost. Many marine sponges also harbor only relatively small numbers of microorganisms. These so-called low-microbial-abundance sponges (148) often cooccur with the high-microbial-abundance bacteriosponges, so habitat variation cannot be invoked as an explanation for these differences. Whether these sponges once contained, but later lost, large communities of microbial symbionts is unknown. It is also unknown whether the (comparatively few) microorganisms in low-microbial-abundance sponges are phylogenetically similar to those in their high-microbial-abundance counterparts.

Based on sequence information already at hand, the nearest we can come to addressing these and the following hypotheses is to consider estimated rates of 16S rRNA evolution for members of given sponge-specific clusters and to attempt to infer when the last common ancestor of sponge-specific microbes from different sponges might have occurred. If one assumes equal mutation rates in different bacterial lineages and asserts that a 1 to 2% 16S rRNA sequence difference corresponds to approximately 50 million years of evolution (259), then sequence differences of at least 10% would be required to place a common ancestor of these organisms back in the late Precambrian (~600 million years ago). Here we consider two examples, the cluster representing the cyanobacterium "*Candidatus* Synechococcus spongiarum" (426) and the "*Poribacteria*" (100). The "*Ca*. Synechococcus spongiarum" cluster is one of the largest of all sponge-specific sequence clusters, is well supported by all tree construction methods, and contains 52 sequences from 21 sponges located around the world (Fig. 5). We chose three of these sequences as an example, derived from the sponges *Theonella conica* (sampled from east Africa; GenBank accession number AY701309), *Aplysina aerophoba* (from the Mediterranean Sea; GenBank accession number AJ347056), and *Antho chartacea* (from southeastern Australia; GenBank accession number EF076240). The minimum pairwise 16S rRNA similarity among these sequences (after correcting for different sequence lengths) is 97.9%. This is a very minor difference when one considers the phylogenetically disparate hosts (the last two sponges are in different orders, while *T. conica* is in a different subclass) and their geographically distinct locations. Even if one assumes that cyanobacteria evolve very slowly, we argue that greater sequence divergence would be expected if these bacteria had indeed been living (separately) within their host sponges for 600 million years. This should be especially true for endosymbiotic microorganisms, which are believed to evolve more rapidly due to increased fixation of mutations within their small populations (259). These members of the "*Ca*. Synechococcus spongiarum" cluster may therefore have a much more recent common origin, reflecting a role of horizontal (i.e., environmental) transmission consistent with scenario 2 or 3 in Fig. 16. However, consideration of other sequences within the same cluster can yield a quite different result. The two least similar sequences within the "*Ca*. Synechococcus spongiarum" cluster are only ~93% similar, suggesting a much older separation of these particular strains. A comparable degree of similarity is seen in comparing sequences from the "*Ca*. Synechococcus spongiarum" cluster with those from free-living relatives. We suggest that a combination of vertical and horizontal symbiont transmission (scenario 2) could explain the observed data. Possible vectors responsible for horizontal symbiont transmission could include sponge-feeding animals, such as fishes and turtles (205, 274), analogous to the coral-feeding fireworm *Hermodice carunculata*, which acts as a vector for the coral pathogen *Vibrio shilonii* (386).

In our second example, we consider the "*Poribacteria*." At first glance, there appears to be a strong case for an evolutionarily ancient relationship between these bacteria and their sponge hosts. The members of this monophyletic, exclusively sponge-specific bacterial lineage differ in their 16S rRNA sequences by up to 15% and are some 20% dissimilar to their nearest nonsponge relative (derived from Antarctic sediment) (Fig. 11). Such high divergence within the cluster, together with the low similarity to the next most similar known organism, is suggestive of an ancient symbiosis with sponges. However, the two least similar "*Poribacteria*" sequences were taken from closely related (same family) sponges collected at the same Bahamas location, perhaps indicating horizontal symbiont transfer between these hosts. If the associations were ancient and involved strict coevolution of host and symbiont, then their respective phylogenies would be more congruent, with the least similar microbes being hosted by the least similar sponges. Furthermore, the long naked branch leading to the "*Poribacteria*" in the 16S rRNA tree could potentially be explained by faster rates of evolution in these bacteria. "*Poribacteria*" are a sister phylum to the *Planctomycetes* (446), which are sometimes believed to exhibit higher rates of evolution than other lineages (392). Like the case for "*Ca.* Synechococcus spongiarum," a combination of vertical and horizontal symbiont transmission is thus the most likely scenario here, although the acquisition of these bacteria exclusively from the environment also cannot be ruled out.

Perhaps the most convincing evidence for a long-standing, symbiotic relationship between sponges and at least some microorganisms comes from demonstrations of coevolution. Despite difficulties in addressing this issue due to the phylogenetic complexity of sponge-associated microbial communities, several authors have now shown coevolution between sponges and microbes. In the first study, the mitochondrial cytochrome oxidase subunit 1 (*CO1*) gene and its bacterial homolog were amplified from several halichondrid sponges and their associated bacteria (98). A *CO1*-based phylogenetic tree of six putatively alphaproteobacterial symbionts was largely congruent with a tree containing sequences from the corresponding host sponges, suggesting that cospeciation had occurred (although there also appeared to have been a host switch event at one point). Subsequent studies of the filamentous cyanobacterium *Oscillatoria spongeliae* indicated a high degree of host specificity for various dictyoceratid sponges, with evidence of cospeciation as well as indications of some host switching (316, 396). Ongoing studies of this system by Thacker and coworkers (R. W. Thacker, personal communication) should further elucidate the complex evolutionary relationships among these tropical sponges and their cyanobacterial associates. Coevolution requires that the host and symbiont maintain close associations over evolutionary time, and as mentioned above, the mechanism by which this presumably occurs in sponges is vertical transmission of microorganisms in host eggs or larvae. An additional point to consider at this stage is that the phylogeny of sponges themselves is not fully resolved (40). Molecular data are often incongruent with traditional sponge taxonomy, which is based largely on morphological properties, such as growth form and spicule characteristics (8, 37, 190). Accordingly, our understanding of symbiont evolution in sponges will continue to develop only in parallel with improvements in our knowledge of host phylogeny. A recently initiated *CO1* sequencing project for taxonomically diverse sponges (www.spongebarcoding.org) is a step in the right direction for achieving the latter goal.

The final type of evidence for ancient, close associations between sponges and microorganisms comes from the fossil record (43, 261, 377). Reef mounds constructed by siliceous sponges and cyanobacterial mats, with the latter represented in part by stromatolites still found today, flourished in (sub)tropical marine waters as far back as the early Cambrian (43). The fact that sponges and microbes closely coexisted hundreds of millions of years ago is thus clear, but the nature of that interaction (e.g., whether microbes lived within sponge tissues) remains less certain.

Scenario 2: Parental and environmental symbiont transmission. Demonstrated vertical transmission is generally considered a strong indicator of symbiosis, yet this does not rule out the possibility of horizontal (e.g., environmental) transmission of the same microbe as an additional mechanism. Indeed, this phenomenon has already been shown for insect-bacterium symbioses (reviewed in reference 67), and here we borrow from the well-developed literature on this topic. In aphids, the primary (obligate) bacterial symbiont *Buchnera aphidicola* is vertically transmitted, whereas secondary (facultative) symbionts can be transferred either vertically or horizontally (329). Given that facultative symbionts can confer fitness advantages upon their hosts, maintenance of these populations—by whatever mechanism—is of clear benefit. Interestingly, it was recently shown that secondary symbionts in aphids can also be transmitted via the sperm, yielding a different infection pattern from that which would be expected based on strictly maternal transmission (237). As discussed in a subsequent section of this article, both maternal and paternal transmissions of cyanobacterial symbionts have been documented for a marine sponge (424). Another finding from the insect world which is relevant to our discussion is that certain bacteria can invade novel host species and form stable associations, perhaps using similar mechanisms for invasion to those found in pathogenic bacteria (67). Provided that host chemical and immune defenses can be evaded, it therefore seems entirely plausible that marine microbes could invade, and establish themselves within, sponges from which they were previously absent.

While phylogenetic trees of primary insect symbionts are congruent with those of their hosts, episodes of horizontal transfer in secondary symbionts obscure the coevolutionary signal for these microorganisms. As mentioned in the preceding section, in which the cases of the "*Poribacteria*" and "*Candidatus* Synechococcus spongiarum" were highlighted, the available molecular evidence for sponge-associated microorganisms supports a combination of vertical and horizontal transmission (not just overall, but for specific individual lineages). Another salient example is the alphaproteobacterial sponge associate represented in Fig. 14; this bacterium occurs widely in sponges (95, 453) and appears to be vertically transmitted (95) yet is closely related to bacteria isolated from seawater. Issues of 16S rRNA sequence resolution notwithstanding (i.e., minor rRNA differences may hide major ecological or even genomic differences) (178, 323), this example underscores the complexities involved with considerations of sponge-microbe evolution.

Scenario 3: Environmental acquisition. In the third scenario, putatively sponge-specific microorganisms are, in fact, also present in the surrounding seawater, but at such low abundance that standard methods fail to detect them. Several mechanisms exist by which the same microbes may then be detected upon contact with a sponge. Firstly, it is possible that sponges absorb specific microbial types from seawater, a process which would imply some degree of recognition of particular microorganisms (e.g., by the sponge's innate immune system) (244). Recognition of symbionts versus food bacteria has already been proven experimentally (see the following section), and if a given microbe encounters favorable conditions (e.g., high nutrients), it may multiply to the extent to which it can then be detected by the applied methods. Alternatively, a type of subtractive enrichment may occur, whereby those microbes which cannot resist phagocytosis by sponge cells are consumed and hardier bacteria (e.g., those with protective capsules) (482) survive and are physically enriched near the choanocyte chambers due to the sponge's filtering activities. If such resistant bacteria are capable of out-competing other potential colonizers, they may establish themselves within the sponge tissue. These possibilities can be placed under a banner of specific enrichment. Unspecific enrichment is another, at least theoretical, alternative; in this case, microorganisms would simply be concentrated by sponges during filtering to the extent to which they can then be detected by the applied methods. Although it is not easy to prove any of these hypotheses correct, it is, in principle, even more difficult to prove them wrong. Finding a sponge-specific microbe actively living in the ocean—independent from a host sponge—would lend support to the enrichment hypothesis. The converse is less convincing: if such cells are not detected outside sponges, it may simply reflect insufficient sampling.

Methodological considerations are of paramount importance in discussing the environmental acquisition scenario. Even after many studies of marine microbiology, it still cannot be discounted that many or most of the so-called sponge-specific microbes are in fact also present in seawater, but only at a low abundance which is not detected by standard methods. But how likely is it that sponge-specific microbes are actually present in other environments and that, due to methodological limitations, we simply fail to detect them? Given recent findings regarding the high level of diversity of uncommon microorganisms in the so-called rare biosphere (276, 376), the enrichment scenarios seem entirely plausible. Deep sequencing of seawater-derived 16S rRNA amplicons should provide further insights into the diversity of marine microorganisms, perhaps also yielding sequence types which are presently considered sponge specific. An interesting aside is that remarkably few of the >1,000 16S rRNA sequences obtained from the Sargasso Sea metagenome (440) are closely related to those sequences in sponge-specific clusters. That study employed a direct cloning approach and was thus free of PCR biases which might otherwise have resulted in the missing of certain sequence types. In this context, it will be very interesting to examine the upcoming results of the Sorcerer II expedition (www.venterinstitute.org), during which seawater samples are being collected from around the world. Irrespective of what such studies find, if the sponge specificity of any microbe is to be disproven, then it will be necessary to demonstrate activity of the said microbe outside a sponge host, as merely demonstrating its presence is not sufficient.

Highly relevant to this discussion is an interesting point made recently by R. Hill (154) regarding the abundance of microorganisms in seawater and the potential consequences for sponge microbiology. Central to this argument is the immense filtering capacity of sponges, i.e., up to 24,000 liters of seawater per day for a 1-kg sponge (443). Given that the typical cell density of bacteria in seawater is about 10^6 cells/ml, then such a sponge could take in a staggering 2.4×10^{13} bacterial cells per day. Thus, even if a specific bacterium is present in seawater at only 1 cell/ml (i.e., one-millionth of all cells present), then during a single day a sponge could still filter some 24 million cells of this organism from the water column (154). The implications are obvious: an organism which is perennially extremely rare in seawater (and therefore never detected by applied molecular methods) could conceivably be concentrated within a pumping sponge (e.g., in the choanocyte chambers, prior to phagocytotic ingestion) to an extent which is readily detectable by PCR or even hybridization-based methods. If it occurs widely (but is undetected) in seawater, then one can imagine a situation in which it is easily detected from different sponges at different locations and erroneously considered a sponge symbiont. Hunting for sponge-specific microbes in other prolific filter feeders, such as ascidians (rather understudied thus far, from a microbial perspective, but see references 239 and 413), may lead to the identification of these organisms from other, nonsponge sources. These arguments might seem to paint a grim picture for proponents of the sponge-specific microbe concept, but the following must also be considered. If it were really the case that such nonspecific enrichment of rare seawater microbes occurs in sponges, then surely sponge-derived 16S rRNA gene libraries would be dominated by other microbes which are known to be abundant in seawater. This does not happen. To illustrate the point, we use again the example of the cyanobacterium "*Candidatus* Synechococcus spongiarum": if "*Ca*. Synechococcus spongiarum"-type organisms are very rare in the ocean but are concentrated to sufficient levels to be detected in gene libraries from sponges, then the exceedingly abundant (and closely related) planktonic *Prochlorococcus* and *Synechococcus* strains should be much better represented. However, a cursory look at Fig. 5 reveals only a few such sequences from sponges. We thus feel there is a strong case for rejection of the unspecific enrichment hypothesis.

Regrettably, a lack of sufficient (appropriate) data prevents us from ending this section with firm conclusions regarding the origin of sponge-microbe associations. Even with almost 2,000 sponge-derived 16S rRNA sequences at our disposal, it is sobering how little can actually be inferred about the evolution of sponge-microbe associations. For almost every argument in favor of an ancient symbiosis, there exists a rational counterargument which invokes (recent) environmental acquisition as the probable driver of the association. For example, a sponge-specific cluster at the end of a long naked branch in a phylogenetic tree could reflect early divergence of this group from its relatives, or it may simply reflect insufficient sampling of closely related lineages and/or accelerated evolutionary rates of the sponge-specific organisms. Additionally, while low levels of sequence divergence within a sponge-specific cluster argue against an ancient association, the corollary does not necessarily hold: extensive intracluster sequence divergence could equally indicate a long, separate evolutionary history (e.g., among different hosts) or selective enrichment of diverse (but still monophyletic) organisms from seawater. So where do we stand at the moment? At this stage, there is no clear indication for scenario 1 (ancient symbioses), though this will be addressed more satisfactorily in the future once additional information on sponge phylogeny is available. The existing data point more towards scenario 2, whereby particular microbes may be passed vertically between generations, but with some horizontal exchange also occurring. Further consideration of potential vectors for the latter process would be worthwhile. Although we consider this scenario most likely, the supporting data are also consistent with scenario 3, i.e., environmental acquisition. This highlights a major current limitation from a methodological perspective, namely, our inability to distinguish between facultative sponge symbionts and specifically enriched microorganisms. Presumably, the former prefer to inhabit sponges but can tolerate conditions in seawater, while enriched microbes may simply tolerate sponges long enough to be detected by applied methods. Such apparently complex patterns of microbial transmission are not without precedent in the animal kingdom, with insect-bacterium symbioses providing a valuable framework for future considerations of sponge-microbe symbioses. A well-studied marine system that has contributed greatly to our understanding of symbiont transmission—and, indeed, of host-microbe associations in general—is that of the squid *Euprymna scolopes* and its bioluminescent symbiont, *Vibrio fischeri* (195, 230, 258, 442). *V. fischeri* is acquired from the surrounding seawater by the juvenile squid, which, via a remarkable stepwise process coined "winnowing," prevents all other bacteria from being established, culminating in a monoculture of *V. fischeri* in its light organ (258). The phylogenetically less complex microbial communities encountered in hosts such as insects and the squid light organ (typically, in insects, there are one or two primary endosymbionts and one or two secondary endosymbionts) facilitate a depth of understanding of these processes thus far not achievable for the highly diverse microbial communities in sponges.

ECOLOGICAL ASPECTS: FROM SINGLE CELLS TO THE GLOBAL SCALE

Establishment and Maintenance of Sponge-Microbe Associations

The mechanisms by which associations between sponges and microorganisms are established are not well understood. As discussed at length in the previous section, the fundamental question of symbiont origin (i.e., whether symbionts were passed down from an ancestral sponge or obtained contemporaneously from seawater) remains unresolved, as do many of the mechanisms of sponge-microbe interactions and the regulation of microbial communities in these hosts. Extensive studies by Müller and colleagues have attempted to address the underlying bases of sponge-microbe interactions at the molecular level (36, 241, 243, 249, 399, 400, 444, 465). Sponges are often regarded as primitive animals, yet their morphological simplicity belies the possession of a surprisingly complex immune system (244). Indeed, the refinement of such a system has undoubtedly contributed to the success of sponges throughout their long evolutionary history, especially when one considers the immense numbers of (potentially pathogenic) microorganisms to which they are exposed due to their filter-feeding activities. Detailed studies of the Adriatic sponge *Suberites domuncula* have revealed immune responses against both gram-negative (36, 243, 464) and gram-positive (400) bacteria, illuminating one means by which sponges may select for and against certain microbes from the surrounding environment. In the case of the former, exposure of *S. domuncula* to the bacterial endotoxin lipopolysaccharide (LPS)—derived from gram-negative cell walls—elicited an increase in synthesis of two compounds with pronounced antibacterial activity (243). Confirmation that the compounds were indeed synthesized by the sponge was obtained by cloning of the gene encoding a key enzyme in the relevant biosynthetic pathway. A receptor for LPS on the sponge cell surface was later identified, as was a signal transduction pathway which is upregulated upon exposure to increased LPS levels (464). The immune response of *S. domuncula* to gram-positive bacteria takes a quite different form: upon exposure to peptidoglycan in the bacterial outer cell wall, the sponge responds with a rapid activation of endocytosis, followed by the release of lysozyme (400). Receptors for fungi (277) and viruses (466) also occur in sponges. For more detailed discussions of the various immune responses and signal transduction pathways in sponges, the reader is referred to recent reviews dealing with this topic (e.g., see references 244 and 246).

In another recent study from the Müller group, using *S. domuncula* and its alphaproteobacterial symbiont SB2 as a model system, the importance of oxygenation of sponge tissue in mediating the relationship was demonstrated (241). Specifically, it was shown that strain SB2 grew preferentially on minimal media with the aromatic compound protocatechuate, rather than glucose, as the carbon source. The bacterium can obtain protocatechuate in situ from the sponge, which produces this and other diphenols via the activities of the enzyme tyrosinase. Interestingly, tyrosinase activity and expression of the tyrosinase-encoding gene in *S. domuncula*, as well as the number of *pcaDC* genes in strain SB2 (responsible for bacterial utilization of protocatechuate and used here as a proxy for SB2 abundance on the surface [exopinacoderm] of the sponge), were all maximal under aerated conditions (241). Coupled with the observed loss of SB2 cells from the sponge surface under low-oxygen conditions, it was asserted that the oxygen level is responsible for regulating the bacterial fauna in sponges. Whether this type of mechanism is important in other sponge-microbe systems remains to be determined.

The coexistence of microbial symbionts with bacterium-digesting archaeocytes in the sponge mesohyl has long interested sponge biologists. In a series of landmark experiments, Wilkinson and coworkers fed tritium-labeled sponge- and seawater-derived bacteria back to host sponges and found that sponge symbionts passed through uneaten, whereas seawater bacteria were largely consumed (482). Two different mechanisms were proposed to account for this: either (i) symbionts are specifically recognized by the sponge and deliberately not ingested or (ii) bacteria use extracellular masking capsules to avoid detection by sponge cells (482, 483). While neither theory has been tested explicitly (but see the preceding discussion on sponge immune responses), the latter explanation is in favor today, with several studies reporting the existence of slime layers and sheaths on symbiotic bacteria (106, 427, 473). The results of recent experiments by the Hentschel group were consistent with earlier findings: seawater-derived bacteria were consumed by *Aplysina aerophoba* some 2 orders of magnitude faster than was a consortium of sponge-derived bacteria (459). In addition, when a green fluorescent protein-labeled food bacterium was fed to the sponge, it was rapidly digested within the sponge tissues. All of these findings carry interesting implications for the evolution of sponge-microbe associations (also see the previous section). If presumed symbionts are not taken from the seawater (i.e., either as colonizers or as a food source), then this suggests vertical transmission as the mechanism by which these associations are maintained.

It is now established beyond doubt that many sponges (at least among those in marine environments) harbor diverse and abundant microbial communities. What is far from established is how, if at all, the composition and density of these communities are regulated. The potential role of phages and protozoa in regulating microbial communities within sponges is of interest, but virtually nothing is known about this to date (but see reference 211). Predatory bacteria, perhaps related to the deltaproteobacterial genus *Bdellovibrio*, could also be involved in structuring microbial communities in sponges (468). At least for cyanobacteria, it has been suggested that their abundance is directly proportional to the number of sponge cells, implying some degree of influence by the sponge over symbiont growth and reproduction (477). The high photosynthetic rates of cyanobacteria in sponges (see also the following section) should, with all things being equal, result in cyanobacterial growth to the extent that host tissues would be overwhelmed. It is thus likely that the sponge exerts some control over its symbiont populations, with several mechanisms being proposed, including the following: sponges consume excess symbionts, sponges eject symbionts when stressed, the host sponge steals photosynthate from the cyanobacteria, and sponges starve the symbionts (477). With some debate over the extent of sponge consumption of symbionts and no evidence for expulsion of excess symbionts, Wilkinson argued that the last two scenarios (steal and starve) are most likely. There is strong evidence for stealing of photosynthate from symbionts in other systems (e.g., coral-zooxanthella [128] and freshwater *Hydra-Chlorella* [232] symbioses), and it seems plausible that sponges may also produce some sort of host release factor to induce the release of large quantities of fixed carbon from the cyanobacteria. Along these lines, a host release factor was recently described for the symbiosis between the sponge *Haliclona cymaeformis* and its macroalgal symbiont *Ceratodictyon spongiosum* (129). In contrast, the starve hypothesis holds that if a sponge can somehow restrict the symbiont's access to essential nutrients, such as nitrogen and phosphorus, then symbiont protein synthesis and cell division would be restricted. Consequently, an excess supply of carbon-rich photosynthate would be excreted from the symbiont (156, 477). To the best of our knowledge, neither scenario has been proven unequivocally or disproven for any sponge-microorganism association. More generally, remarkably little is known about communication, or chemical cross talk, between sponges and their microbial associates. Marine sponges produce a wide variety of secondary metabolites, some of which could potentially enable them to select for or against particular types of microorganisms (185) (although the nonspecific nature of many antimicrobial compounds suggests that such selection may generally be limited to broader classes of microbes, e.g., gram-positive versus gram-negative bacteria). Conserved bacterial signaling systems, as exemplified by the acyl homoserine lactone (AHL) regulatory systems of many gram-negative bacteria (111, 369), often mediate colonization-related traits (e.g., biofilm formation, swarming, and virulence) and offer one means by which sponges could interact or interfere with bacteria. Bacteria capable of AHL production have already been reported from marine sponges (361, 389), as have other putative signaling molecules, such as diketopiperazines (1, 162, 182). It is highly likely that sponges produce metabolites which allow them to disrupt AHL-regulated phenotypes, as shown for the macroalga *Delisea pulchra* (126, 127, 223, 224) and various terrestrial plants (302, 393). Indeed, inhibition of bacterial swarming by chemical extracts from sponges has recently been shown, though it has yet to be clarified whether this is an AHL-specific effect (184).

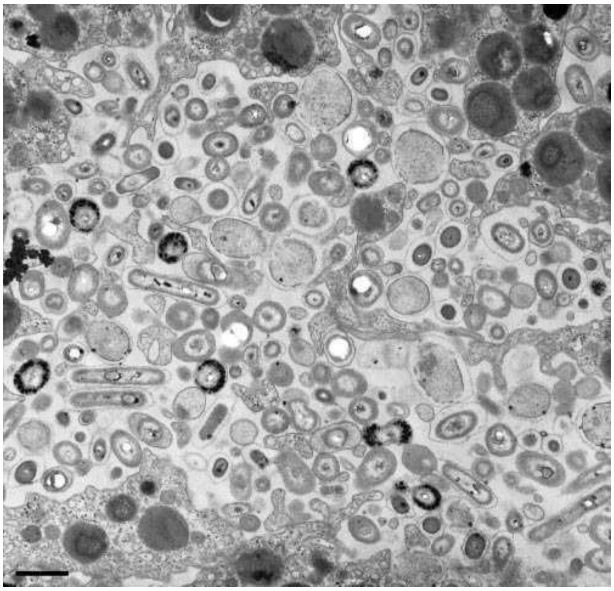

FIG. 17. Vertical transmission of microbial symbionts by a marine sponge. A transmission electron micrograph of a *Chondrilla australiensis* larva is shown, indicating a diverse range of bacterial morphotypes. Bar = 1 μm. (Modified from reference 420 with permission of the publisher.)

Associations between sponges and microorganisms can be maintained over different generations in either of the following two ways: (i) microbes can be recruited from the surrounding water by filter feeding (i.e., horizontal or environmental transmission) or (ii) microbial symbionts can be passed on from the parent sponge via reproductive stages (i.e., vertical transmission) (Fig. 17). Although horizontal transmission of symbionts has been demonstrated convincingly for several marine symbioses (e.g., squid-*Vibrio fischeri* [258] and hydrothermal vent tubeworm-chemoautotrophic bacterium [257] symbioses), it is the latter mechanism which has received most attention for sponges. Bacteria have now been found in embryos or larvae from all three classes of Porifera (see reference 97 and references therein), including species with highly varied reproductive strategies. Sexual reproduction in sponges involves either vivipary (where larvae are brooded within the animal) or ovipary (whereby eggs, generally fertilized externally, develop outside the sponge). Evidence for vertical transmission of bacteria has been reported for both types (97, 116, 118, 181, 362, 419, 422, 431), while asexual reproduction, i.e., budding, could also contribute to symbiont transfer in some species (146). Indeed, gemmules, the asexual buds of freshwater sponges, contain symbiotic zoochlorellae in at least some species (372), while a bud protruding from the surface of the marine sponge *Tethya orphei* contained a symbiotic cyanobacterium (117).

The vast majority of reports dealing with vertical transmission in sponges have been based on transmission electron microscopy (TEM) observations. Such studies have contributed greatly to our understanding of this phenomenon, with the identification of several mechanisms by which symbiotic microbes can be incorporated from maternal mesohyl tissue into eggs or embryos (reviewed in reference 97). These include phagocytosis of microbial cells by the oocyte directly from the adult mesohyl as well as transfer of microbes from parent sponge to embryo along an "umbilical cord." Intriguingly, in the Australian sponge *Chondrilla australiensis*, eggs containing a cyanobacterial symbiont (of the "*Candidatus* Synechococcus spongiarum" type [426]) were distributed throughout the sponge mesohyl, whereas cyanobacteria are normally confined to the better-illuminated periphery, or cortex, of the sponge (422). Nurse cells, probably derived from choanocytes (425), have been invoked as a possible mechanism by which cyanobacteria are transported to the eggs (422, 424). These cells, which fuse with eggs and release their contents (including cyanobacteria) into the egg cytoplasm prior to spawning, presumably phagocytose the symbionts in the cortex before moving to the developing eggs deeper within the sponge. Remarkably, Usher and coworkers were also able to demonstrate the presence of cyanobacteria in sperm cells, indicating that both parents are capable of transferring symbionts to offspring (424). Sponges of the genus *Chondrilla* were also the subject of another recent TEM study (which additionally employed immunocytochemical techniques), in which vertical transmission of an endosymbiotic yeast was shown (221).

The drawback of the TEM approach is that, with some exceptions (e.g., cyanobacteria [423, 424]), even phylum-level identification of the relevant microorganisms is not possible due to an insufficient number of distinguishing morphological characters. Multiple bacterial morphotypes have been observed in sponge larvae (suggesting transmittance of a complex assemblage) (e.g., see reference 424), yet little or nothing is known of their phylogenetic affiliations. The recent application of molecular techniques in this area (95, 266) thus offers the potential for exciting new insights into the phylogenetic (and, in principle, metabolic) complexity of transmitted microbial assemblages. A 16S rRNA gene library constructed using cyanobacterium-specific PCR primers confirmed the presence (as indicated by TEM) of a single cyanobacterial type in both larvae and adults of the Red Sea sponge *Diacarnus erythraenus* (266). The transmitted cyanobacterium is highly similar to the aforementioned "*Ca.* Synechococcus spongiarum"-type symbiont of *Chondrilla australiensis*. A range of molecular techniques were used to examine the bacterial community in larvae of the Caribbean sponge *Mycale laxissima*, revealing a much more diverse population than that recovered by cultivation efforts (95). A single alphaproteobacterium, related to *Pseudovibrio denitrificans* and previously reported from many sponges (Fig. 14), was the only bacterium from the larvae that could be grown on a standard marine medium. In contrast, sequences representing a diverse assemblage comprising *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Planctomycetes*, and *Proteobacteria* (including the isolated alphaproteobacterium) were recovered from a 16S rRNA gene library based on DNAs isolated from the larvae (95). Similarly diverse microbial communities were found in larvae of the sponges *Corticium* sp. (366) and *Ircinia felix* (352), using 16S rRNA-based approaches, such as gene libraries, FISH, and DGGE. Broad congruence between larva- and adult-associated microbial communities in these studies indicated that a significant subset of the resident microbes is transferred in this way. Future molecular studies could provide information on the metabolic properties of the transferred symbionts (e.g., via analysis of functional genes or FISH-microautoradiography), which should improve our understanding of the mechanistic bases of these symbioses.

Whatever the underlying mechanisms for the establishment and maintenance of sponge-microbe associations, it is apparent that in many cases such associations are highly stable and resistant to external disturbance (reviewed in reference 148). In at least some other instances (e.g., see reference 457), this is not the case. Briefly, neither starvation, exposure to antibiotics, nor transplantation to different depths could elicit major changes in bacterial community composition in *Aplysina aerophoba* (105) and *Aplysina cavernicola* (407). Similarly, even translocation of *Aplysina fistularis* from its natural depth of 4 m to a new depth of 100 m was not enough to significantly affect cyanobacterial abundance in this sponge (although the sponge did die at depths of >100 m) (222). In contrast, this change resulted in a loss of cyanobacterial symbionts from the cooccurring sponge *Ircinia felix*. The importance of cyanobacterial symbionts—at least to some sponges—was recently demonstrated by Thacker in a series of elegant field experiments (394). To test the hypothesis that a greater benefit to the host is derived from more specialized symbionts, shading experiments were conducted with the tropical sponges *Lamellodysidea chlorea* (containing the host-specific cyanobacterium *Oscillatoria spongeliae*) and *Xestospongia exigua* (which contains the generalist symbiont "*Ca*. Synechococcus spongiarum"). Shaded individuals of the encrusting sponge *L. chlorea* lost >40% of their initial area, but the chlorophyll *a* concentration in the remaining sponge tissue (a proxy of cyanobacterial abundance) did not change, implying a close, putatively mutualistic relationship between the sponge and *O. spongeliae*. In contrast, *X. exigua* lost relatively little mass but did lose many of its "*Ca*. Synechococcus spongiarum" symbionts, suggesting that this relationship is less tight-knit than the other (394). While the mechanism by which symbionts are lost from *X. exigua* is unclear, the existing data do suggest that the specialist *O. spongeliae* provides a greater benefit for its host sponge than does the generalist "*Ca*. Synechococcus spongiarum" for its host. Although the necessary data are currently lacking, one could speculate that the degree of host specificity (of individual symbionts or even entire communities) may explain some of the different results obtained from previous perturbation experiments (148). More generally, the extent of host specificity among marine eukaryote-associated microbes may have substantial implications for microbial diversity on a wider scale (390). If most symbionts are highly host specific, then their overall diversity will be much higher than if the same hosts harbor mostly generalist species. Finally, Roberts and coworkers recently reported the results of experimental manipulations with the photosynthetic symbiont-containing Australian sponge *Cymbastela concentrica* (322). Shade and, to a lesser extent, silt treatments (both designed to mimic the physical effects of sewage effluent discharge) led to lowered chlorophyll *a* concentrations within the sponge, while increased salinity and nutrient loads had negligible effects.

Physiology of Sponge-Associated Microorganisms

A lack of pure cultures for most sponge-associated microorganisms has contributed to a paucity of knowledge about their physiological characteristics. What we do know has arisen from a combination of the existing pure-culture studies, process data, and inferred metabolic properties from analysis of 16S rRNA, functional genes, and metagenome data. Collectively, microbes in sponges are capable of, among other processes, photosynthesis, methane oxidation, nitrification, nitrogen fixation, sulfate reduction, and dehalogenation. Here we summarize the current knowledge by first examining, in turn, the major nutrient cycles within sponges.

Carbon. Heterotrophy is a common form of carbon metabolism in sponges, either via consumption of microbes from seawater or via microbial uptake of dissolved organic carbon (495). However, for many sponges, particularly those in tropical regions, carbon metabolism centers around the activities of photosynthetic microorganisms, such as cyanobacteria (11, 58, 59, 381, 470, 474, 477, 486). Many tropical sponges contain substantial populations of these oxygenic autotrophs, and nowhere is the contribution of microbial symbionts to the host sponge more evident than in this case (see also the next section). Translocation of photosynthates (mostly as glycerol) from cyanobacteria to the host has been shown for marine sponges (476), while glucose produced by a chlorella-like green alga was passed to its freshwater sponge host, *Ephydatia fluviatilis* (475). Phototrophic sponges—those whose carbon nutrition depends heavily on cyanobacterial symbionts—receive $>50\%$ of their energy requirements from cyanobacteria (474), allowing these species to thrive in the low-nutrient, high-light areas commonly found on tropical reefs. On the Great Barrier Reef, phototrophic sponges comprise approximately half of the total sponge biomass on outer reefs, where the water is cleaner, but are much less common inshore, where terrestrial runoff and turbidity are greater (470, 485). Similarly, phototrophic sponges are largely absent from Caribbean reefs, where only small numbers of sponge-associated cyanobacteria are present (470). Phototrophy has also been demonstrated for at least one temperate sponge (57), while numerous others are known to contain photosynthetic symbionts (321, 336, 390, 430). The sponge *Cymbastela* sp. from temperate South Australia was capable of compensating photosynthetically (i.e., the rate of photosynthesis equals its rate of respiration) at a 4- to 5-m depth in winter, while it was a net producer at the same depth in summer (57). In contrast, Great Barrier Reef sponges may derive much of their nutrition from photosynthetic symbionts as deep as 15 to 30 m, due to the clearer water and, therefore, decreased light attenuation (58). Some sponges are apparently obligate phototrophs, with their lower depth limits determined by the availability of light for photosynthesis (58). Others function as mixotrophs, combining symbiont-derived nutrition with filter feeding, while still others contain no photoautotrophic symbionts and derive all of their carbon nutrition from filter feeding (477).

The extent to which other photosynthetic associates (e.g., diatoms, dinoflagellates, and phototrophic sulfur bacteria) contribute to carbon cycling within sponges is less clear. The Mediterranean sponges *Cliona viridis* and *Cliona nigricans* both contain symbiotic dinoflagellates (zooxanthellae), and for *C. viridis*, at least, it appears that sponge metabolism depends on the photosynthetic activity of these symbionts (353). Indeed, the growth of *C. viridis* was greater in individuals maintained under natural light conditions than in those maintained in constant darkness, reflecting the contribution of photosynthetic symbionts to host metabolism (324). Conversely, in at least one case, it appears that diatoms in Antarctica may parasitize the sponge host, using its metabolic products as an energy source (16). The sponge *Cymbastela concentrica* in southeastern Australia contains high densities of diatom-like cells in the illuminated periphery (M. W. Taylor, unpublished data), but further work is required to elucidate whether this association is phototrophic in nature. The occurrence in this sponge of at least some cyanobacteria in addition to the diatoms (Longford et al., unpublished data) will complicate efforts in this direction. Some freshwater sponges (e.g., *Spongilla lacustris*) contain zoochlorellae, and although the symbiosis is not obligate (aposymbiotic individuals occur in areas of deep shade), it appears that algal photosynthesis can contribute to host metabolism and growth (109, 333, 475).

An unusual form of nutritional symbiosis is that between methanotrophic bacteria and deep-sea cladorhizid sponges (428, 429, 431). These remarkable sponges, which possess no aquiferous system but instead prey on tiny swimming organisms, are believed to obtain a significant portion of their nutrition from the consumption of methanotrophs. Methane serves as a carbon source and substrate for energy production in methanotrophs, and in this particular system it is derived from a deep-sea mud volcano (429). In other sponges, the presence of methanogenic archaea may lead to methane production within anoxic zones. A 16S rRNA gene sequence affiliated with the methanogens of the phylum *Euryarchaeota* (456) is the sole piece of evidence for this at present, but the documented existence of anoxic microhabitats within sponges (159) suggests that these associations could be more widespread. Other chemoautotrophic microbial processes that have been observed in sponges and may also contribute to sponge nutrition are nitrification and sulfur oxidation. These are dealt with in the following sections.

Nitrogen. After carbon, nitrogen is the most important nutrient for life, as it is required for the synthesis of amino acids and, subsequently, proteins. In oligotrophic waters where nitrogen levels are low (e.g., coral reefs), symbiotic microorganisms may contribute to the nitrogen budget of sponges via fixation of atmospheric nitrogen, N_2 (479, 484). The first evidence for this came from measurements of nitrogenase activity in three Red Sea sponges (479). The activity of this enzyme, the catalyst for microbial N fixation, was estimated using an acetylene reduction test (for caveats, see reference 484) and could be measured only in *Siphonochalina tabernacula* and *Theonella swinhoei*, both of which contained cyanobacteria. In contrast, *Inodes erecta*, which contained only noncyanobacterial microorganisms, showed no evidence of N fixation. Additionally, nitrogenase activity was higher in illuminated tissue than in that maintained in the dark and did not correlate with the abundance of the heterotrophic bacterial communities in *S. tabernacula* and *T. swinhoei*. Taken together, these data suggested that nitrogenase activity was due mainly to the presence of cyanobacteria, many of which are capable of N fixation (479). A subsequent study provided more concrete proof of N fixation in sponges by demonstrating incorporation of the stable isotope $^{15}N_2$ into various amino acids in *Callyspongia muricina* (484). Whether microbial N fixation is of major ecological significance for sponges remains uncertain, but it does appear that its occurrence in sponges is not limited to cyanobacteria.

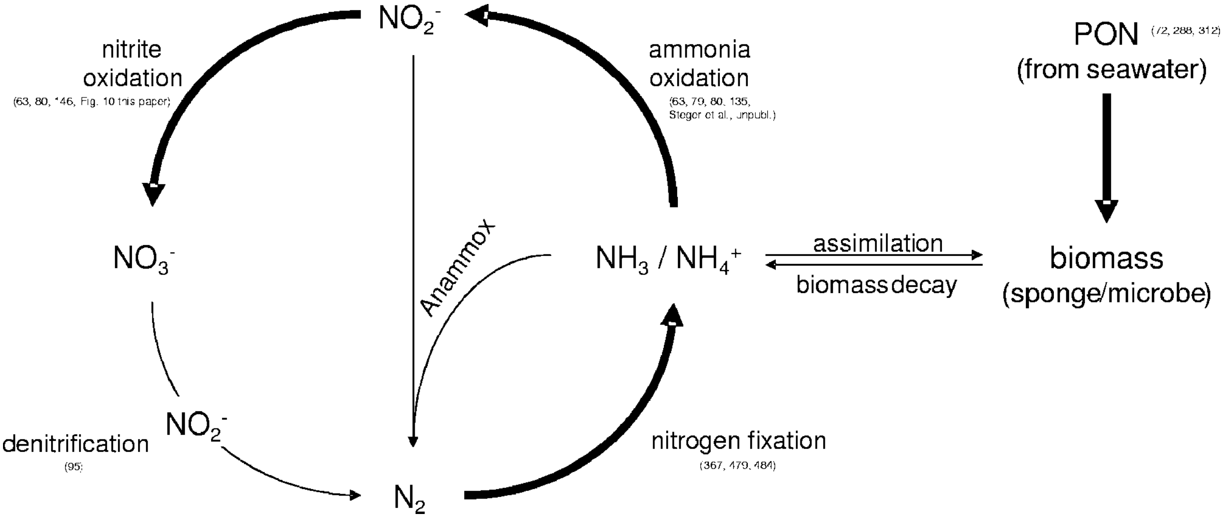

FIG. 18. Current state of knowledge about the nitrogen cycle in sponges. Thick arrows signify those processes which have been demonstrated in sponges; references (given in parentheses) pertain to either the process or the implicated microorganisms. PON, particulate organic nitrogen.

Heterotrophic nitrogen-fixing bacteria were reported from a *Halichondria* sp. (367), and the *nifH* gene (encoding a subunit of the nitrogenase reductase enzyme) has been amplified from both alpha- and gammaproteobacteria inhabiting several Caribbean sponges (N. M. Mohamed, Y. Tal, and R. T. Hill, presented at the 11th International Symposium on Microbial Ecology, Vienna, Austria, 20 to 25 August 2006).

Nitrification in sponges has also received attention. The two steps of nitrification, i.e., the biological conversion of ammonia to nitrite and then to nitrate, are catalyzed, in turn, by ammonia-oxidizing and nitrite-oxidizing microorganisms (33, 194). Ammonia, which can be toxic to eukaryotes, is a metabolic waste product and could accumulate within sponge tissues, particularly during periods of low pumping activity. The release of nitrate (and in some cases nitrite) from incubated sponges provided the first indication of nitrification within these organisms, with estimated rates often far exceeding those for other benthic substrata (63, 80). These results suggested the presence of nitrifying microorganisms, and indeed, 16S rRNA sequences from both ammonia-oxidizing betaproteobacteria (79; Taylor et al., unpublished data) and nitrite-oxidizing bacteria of the genus *Nitrospira* (Fig. 10) (146; Longford et al., unpublished data) have been recovered in molecular surveys of sponges. The widespread presence of *Nitrospira* in sponges may indicate low nitrite availability in these hosts, as members of the *Nitrospira* typically favor low-nitrite habitats (356, 448). Nitrifying microorganisms are among the few for which metabolic capabilities can generally be inferred from 16S rRNA data, and sequences representing several types of ammonia-oxidizing bacteria–in the genera *Nitrosospira* and *Nitrosomonas*–-were identified from the Australian sponge *Cymbastela concentrica* (Taylor et al., unpublished data). The finding of only *Nitrosomonas eutropha/europaea*-affiliated ammonia oxidizers in a previous study of six tropical sponges (79) may have been due to the use of overly specific PCR primers (both of the primers used have mismatches to all *Nitrosospira*-affiliated ammonia oxidizers and also to many *Nitrosomonas* spp. [193, 300]). Nitrite oxidizers belonging to the *Nitrospira* genus are frequently recovered in 16S rRNA gene surveys of sponges, yet in at least one case (80), extensive release of nitrite indicated that oxidation of ammonia and nitrite could be uncoupled. Interestingly, ammonia-oxidizing archaea, whose existence was just proven in 2005 (192), also exist in sponges. Metagenomic reconstruction of "*Candidatus* Cenarchaeum symbiosum," the abundant and yet uncultivated crenarchaeote in the Californian sponge *Axinella mexicana* (294), revealed the existence of ammonia monooxygenase (*amoA*) genes (required for ammonia oxidation) in this organism (135), while PCR-based surveys for *amoA* indicated that archaeal ammonia oxidizers are widespread in marine sponges (D. Steger et al., unpublished data). Whether archaea or bacteria are the key ammonia oxidizers in sponges remains to be determined, but in at least one system (soils), ammonia-oxidizing archaea appear to greatly outnumber their bacterial counterparts (204).

Numerous gaps remain in our knowledge of nitrogen cycling in sponges (Fig. 18). For example, the existence of anoxic zones in at least some sponges (159) suggests the potential for both denitrification and anaerobic ammonium oxidation (anammox). Neither process has been reported for sponges thus far, and our own efforts to amplify 16S rRNA genes from known anammox bacteria from several sponges have yielded no results. Denitrification is catalyzed by a phylogenetically diverse range of microorganisms, and it is risky to infer the ability to denitrify from 16S rRNA sequence data alone. Nonetheless, it is worth noting that a common alphaproteobacterial associate of marine sponges (95, 147, 453) (Fig. 14) is very closely related to the marine denitrifier *Pseudovibrio denitrificans* (368), and at least some of the sponge-derived strains have also tested positive for denitrification (95). The role of sponge filter feeding in providing particulate organic nitrogen is also of interest (312), with evidence that uptake of ultraplankton can yield sufficient nitrogen to sustain both the tropical sponge *Haliclona cymaeformis* and its macroalgal symbiont (72, 288).

Sulfur. Several lines of evidence point to the widespread occurrence of sulfur-metabolizing microorganisms in sponges. For starters, two of the key microbial players in the sulfur cycle, namely, sulfate reducers and sulfur oxidizers, have been found in multiple sponges. Sulfur-oxidizing bacteria from the families *Rhodospirillaceae* and *Chromatiaceae* (*Alpha*- and *Gammaproteobacteria*, respectively) were isolated from *Ircinia* sp. and *Euspongia officinalis* in the 1970s (173). In that paper, the bacteria were referred to as phototrophic sulfur bacteria, and additional, earlier isolations of green sulfur bacteria (phylum *Chlorobi*) were also discussed (see reference 173 and the Eimhjellen [1967] citation within). FISH signals for *Chlorobi* were later found in the Great Barrier Reef sponge *Rhopaloeides odorabile* (458). The above-mentioned sulfur bacteria oxidize reduced sulfur compounds such as hydrogen sulfide. This substrate is presumably derived from the activities of sulfate-reducing bacteria, which have also been obtained from sponges (159, 160, 173, 225, 358). An endosymbiotic sulfur cycle comprising sulfate-reducing and sulfide-oxidizing bacteria has already been demonstrated for a marine oligochaete (83), and the above data suggest that a similar process takes place in at least some sponges.

The most extensive work on sulfur metabolism within sponges has been conducted by Hoffmann, Reitner, and colleagues (159, 160, 225, 310, 358). They detected sulfate-reducing bacteria by FISH in the Mediterranean sponges *Chondrosia reniformis* and *Petrosia ficiformis* (225, 358), as well as in the cold-water sponge *Geodia barretti* (159, 160). In *G. barretti*, FISH detection of sulfate reducers belonging to members of the genera *Desulfoarculus*, *Desulfomonile*, and/or *Syntrophus* (estimated abundance, 1.8% of the total bacterial community) was complemented by isotopic measurements of sulfate reduction rates and analysis of oxygen profiles within the sponge (159). Sulfate reduction is an anaerobic process, and through the use of microelectrodes (354), these authors were able to demonstrate the presence of anoxic zones within the sponge, particularly during periods of pumping inactivity (159). The estimated sulfate reduction rates in *G. barretti*, of up to 1,200 nmol SO_4^{2-} cm^3 sponge day^{-1}, are among the highest recorded in natural systems. Intriguingly, analysis of lipid biomarkers suggested that bacterially derived carboxylic acids (perhaps from sulfate reducers) may be transferred to the host for subsequent synthesis into other compounds (159). The accumulation of toxic hydrogen sulfide was also addressed by Hoffmann and coworkers, who calculated that the activities of sulfide-oxidizing bacteria could, together with chemical reoxidation processes and the use of oxidized iron from seawater as an electron acceptor, be sufficient to balance microbial sulfide production. Although it is pure speculation at this stage, it is also possible that sulfur-oxidizing symbionts enable sponges to occupy sulfide-rich environments. The base of the Micronesian sponge *Oceanapia* sp., for example, can be buried up to 20 cm deep in the sediment (360), where anoxic conditions with high sulfide concentrations may prevail.

Interestingly, 16S rRNA gene sequences which are highly similar to those from known sulfate reducers (e.g., *Desulfobacterium* or *Desulfomicrobium* spp.) (Fig. 8) have only rarely been recovered from sponges. Ahn and colleagues did find *Desulfovibrio*-related organisms in enrichment cultures grown on *Aplysina aerophoba*-derived brominated phenolic compounds (3), but these sequences do not appear to have been deposited in GenBank and were therefore not included in our analyses. Analysis of functional genes [e.g., *dsrAB*, encoding the dissimilatory (bi)sulfite reductases] (447, 502) would be one way to gain further insights into the composition of sulfur-metabolizing microbial guilds within sponges.

A final aspect of sulfur metabolism that has received much attention in marine systems is that of dimethylsulfoniopropionate (DMSP) and its cleavage product, dimethylsulfide (DMS). These compounds are thought to play a role in global climate regulation (497), while DMSP may also protect marine algae and invertebrates from herbivores/predators and oxidative damage (384, 436). In a recent survey of DMSP content in a variety of coral reef invertebrates, high levels in corals were attributed to symbiotic zooxanthellae, while the much lower DMSP concentrations typical of sponges were presumed to be diet derived (435). It is currently unknown whether the levels in sponges are sufficient to play a role in predator deterrence or whether those sponges with symbiotic zooxanthellae have higher DMSP contents.

Other aspects of microbial metabolism in sponges. In contrast to the case for several major chemical elements (namely, carbon, nitrogen, and sulfur), to our knowledge virtually nothing is known about phosphorus cycling within marine sponges. We assume that sufficient phosphorus is obtained from the sponge's diet of microorganisms.

The degradation of halogenated chemicals within the Mediterranean sponge *Aplysina aerophoba* was the subject of an interesting recent study (3). This and other sponges are rich sources of brominated compounds such as bromophenols and bromoindoles, and it was predicted that microorganisms within such sponges may be capable of dehalogenation. Indeed, by establishing enrichment cultures from sponge tissue, in the presence or absence of various electron acceptors, the authors of that study were able to demonstrate reductive debromination under methanogenic and sulfidogenic, but not denitrifying, conditions (3). Antibiotic inhibition of dehalogenation activity indicated that it was the microbes, not the sponge, which were responsible.

The production of a wide range of secondary metabolites by sponge-associated microorganisms is well known. We provide examples of some pharmacologically relevant metabolites in a later section (see "Biologically Active Chemicals from Marine Sponge-Microbe Consortia and Their Commercial-Scale Supply") and, immediately below, outline the potential benefit(s) of these metabolites to the sponge-microbe association.

The Varied Nature of Sponge-Microbe Interactions

Sponges and the microorganisms living within and around them display the full gamut of interactions, from microbial pathogenesis and parasitism (sometimes resulting in sponge death) to microbes as the major food source for heterotrophic sponges and to mutualistic (or at the very least commensalistic) associations in which both partners appear to benefit. We first consider the putative benefits of symbiotic microorganisms to the host sponge.

Mutualism/commensalism. It is clear that sponges benefit greatly from the diverse metabolic properties of their associated microorganisms (see the preceding section). The provision of photosynthates and (perhaps to a lesser extent) fixed nitrogen from cyanobacteria (11, 474, 476, 477, 479, 486) is presumably a key factor in the ecological success of many sponges on nutrient-poor tropical reefs. Cyanobacterial symbionts may be equally important to juvenile and adult sponges. Sponge larvae are generally thought to be lecithotrophic (i.e., nourished from finite stored nutrients) (219), with no capacity for filter feeding (though some may assimilate dissolved organic matter from seawater [175]), so the energy gained from photosynthetic cyanobacteria should contribute to (i) gamete and larval longevity in the water column (424) and (ii) (once sponges are settled) the rapid growth required to outcompete algae and other photosynthetic organisms for substratum in illuminated areas (477, 478). Larval mortality may consequently be lower for those harboring cyanobacterial symbionts. The importance of photosynthetic symbionts to their hosts is evident in the typically flattened morphologies of phototrophic sponges, with the thinner species containing dense accumulations of cyanobacteria throughout the tissue. In contrast, mixotrophic sponges—those which utilize both filter feeding and photosynthetic symbionts for nutrition—may reduce their reliance on symbionts with age. Juveniles possess a high proportion of symbiont-containing tissue, which reduces as the sponge grows thicker and increases the amount of filter-feeding tissue (474, 478). Interestingly, endosymbiont photosynthesis can also bring with it certain costs for the sponge, such as the following: (i) morphological adaptation for improved light capture may occur at the expense of filter-feeding capacity (477), and (ii) oxidative stress may result from the presence of high levels of photosynthetically produced molecular oxygen, necessitating an enhancement of antioxidant defenses compared with those of asymbiotic specimens (303, 304). Another role for cyanobacteria and their pigments has also been proposed, namely, protection of sponges from excessive illumination (336). Although this role has not been proven experimentally, one expects that this could be particularly important for intertidal species, where radiation (UV and photosynthetically active radiation) is especially high (381). The documented occurrence of UV-absorbing mycosporine-like amino acids in sponges harboring cyanobacteria (e.g., *Dysidea herbacea* [13]) also supports the hypothesis of shading by the symbionts.

Microbial metabolism may benefit the host sponge in other ways. As mentioned earlier, Hoffmann and colleagues (159) described the likely transfer of carboxylic acids from anaerobic bacteria to the sponge *Geodia barretti*. Methanotrophic bacteria may supplement the nutrition of non-filter-feeding, carnivorous sponges in methane-rich deep-sea habitats (429, 431), while symbiotic zooxanthellae (dinoflagellates) enhance boring and growth rates in clionid sponges (153). Elimination of toxic metabolic by-products is another possible role played by sponge-associated microbes. The sulfur-oxidizing bacteria mentioned above oxidize reduced sulfur compounds, such as highly toxic hydrogen sulfide, to less harmful forms. Sulfide may accumulate in anoxic zones due to the activities of sulfate reducers, particularly during periods of low pumping activity (159). Similarly, ammonia and nitrite, which can be toxic to eukaryotes, are products of sponge and microbial metabolism but may be oxidized to harmless forms via the activities of nitrifying microorganisms. While negative effects of nitrite on the development of some juvenile freshwater sponges have been demonstrated in laboratory experiments (179), it is less clear whether ammonia or nitrite ever accumulates to sponge-harming levels in nature. Microelectrode studies to address such questions (354) would be of great interest and should further our understanding of the role of nitrifying microorganisms in sponges. Also of interest will be the data derived in the future from sponge genome projects. These should help to identify possible absent metabolic pathways in the host, whose functions may instead be filled by symbiont-derived factors.

Further putative benefits for sponges from their microbial partners include increased structural rigidity (due to mucous production by bacteria) (472), direct incorporation of dissolved organic matter from seawater (480, 495), digestion and recycling of insoluble sponge collagen (481), and microbial production of secondary metabolites that are of use to the host. In several cases, production of bioactive metabolites has tentatively been ascribed to bacterial symbionts, and these may serve to protect the sponge from pathogens, predators, and foulers (e.g., see references 147, 351, and 417). In some other cases, molecules produced by microbial symbionts could potentially be used as precursors for the biosynthesis of defense metabolites by sponges. Whatever the exact mechanism, it is likely that the chemical defenses of many sponges include both host- and symbiont-produced metabolites (see also “Harming the host: pathogenesis, parasitism, and fouling” and Biotechnology of Sponge-Microbe Associations: Potential and Limitations).

The observant reader may have noticed that the preceding discussion deals almost exclusively with putative benefits for the host, with little mention of advantages for the symbiont(s). Even when the benefits to the host sponge are obvious (e.g., in phototrophic sponges), it is not necessarily clear what benefit the microbial partner derives from the association. It is thus difficult, and often impossible, to confidently assign a mutualistic rather than just a commensalistic label to a sponge-microbe association. Presumed benefits for microbial symbionts of sponges include a generous supply of nutrients, as well as shelter—from predators or high light levels—in the sponge tissue (336).

Microorganisms as a food source for sponges. With the primary exception of phototrophic sponges (described above), most sponges are thought to obtain the bulk of their carbon nutrition via the consumption of microorganisms from the water column (though uptake of dissolved organic carbon may also be significant [e.g., see reference 495]). Bacteria (including both cyanobacteria and presumed heterotrophs) as well as eukaryotic microalgae can satisfy the entire food requirements of sponges (307), with the potential for dense sponge communities to significantly deplete the surrounding water of microbial cells (289, 309). Early studies of particle feeding in sponges indicated that as much as 96% of bacterial cells were removed from the inhalant seawater by the filtering activities of the sponge (308). These results were supported by the later application of more sophisticated techniques, in particular flow cytometry (23, 289, 290). Pile and colleagues reported grazing of the Atlantic sponge *Mycale lingua* on various types of plankton (<10 μm in size), with retention efficiencies ranging from 93% for *Prochlorococcus*-type cyanobacteria down to 72% for the smallest photosynthetic eukaryotes (290). Similar methodologies applied to the encrusting New Zealand sponge *Polymastia*

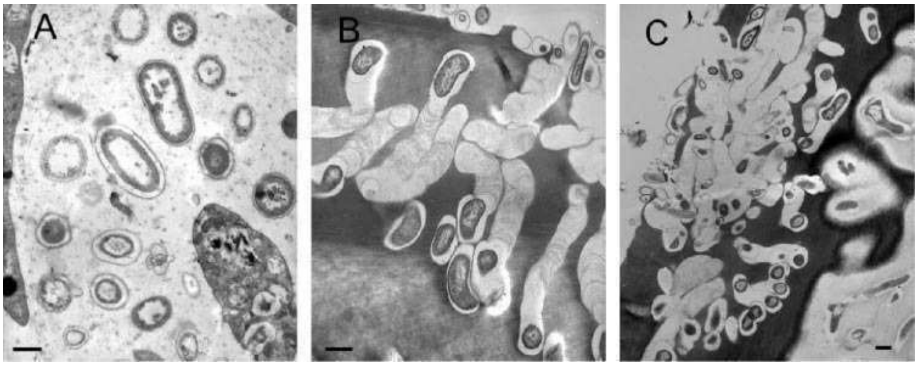

FIG. 19. Effect of a bacterial pathogen on a marine sponge. Transmission electron micrographs of *Rhopaloeides odorabile* tissue are shown, displaying (A) the diversity of bacterial morphotypes in healthy tissue, (B) a sponge experimentally infected with the alphaproteobacterial pathogen strain NW4327, and (C) consequent necrosis of the sponge tissue. Bar = 500 nm. (Reprinted from reference 455 with permission of the publisher.)

croceus showed the best retention of *Synechococcus*-type cyanobacteria (94%) and picoeukaryotes (88%), with somewhat poorer retention of *Prochlorococcus*-type cyanobacteria and other (noncyano-) bacteria (74 and 46%, respectively) (23). The lower retention of some cell types suggested that *P. croceus* was selective in its feeding. Laboratory experiments involving the feeding of symbiotic versus seawater bacteria to other sponges lend strong support to the notion of selective feeding (459, 482).

The generally highly efficient removal of particles from seawater is due largely to the extraordinarily large number of choanocyte chambers ($\sim 1 \times 10^7$ per cm^3) in sponge tissues (306). With each chamber containing as many as 150 choanocytes (371), coupled with the ability of pinacocytes (epithelial cells) to capture larger particles (308, 412), any food particle passing through the intricate aquiferous system of a sponge is subjected to intense grazing pressure. Interestingly, it now appears that even viruses can be retained by sponges, with some 23% of viral particles being removed from seawater by the Red Sea sponge *Negombata magnifica* (131). Considering the enormous abundance of viruses in seawater (1 million to 100 million per ml) (387), this could represent a significant flux of nutrients in ecosystems containing large sponge populations. While most studies of sponge feeding have been conducted with demosponges (e.g., see references 89, 196, 307, 313, and 379), microbial retention efficiencies of 90% or more have also been reported for hexactinellids (494, 496). The deep-sea hexactinellid *Sericolophus hawaiicus* was somewhat less efficient, with microbial retention efficiencies ranging from 47% for bacteria to 54% for photosynthetic eukaryotes of <3 μm (291).

Consumption of symbiotic microorganisms has also been raised as a possible food source for sponges (336, 337). The first report of apparent widespread disintegration, both intra- and extracellularly, of cyanobacterial symbionts was from Sara in the early 1970s (336). His TEM observations suggested that the Mediterranean sponge *Ircinia variabilis* actively degraded *Aphanocapsa*-type cyanobacteria (now considered *Synechococcus* spp.) (421) both in the sponge mesohyl and within certain sponge cells, providing an important source of photosynthetically fixed carbon to the host sponge (336). These results have since been questioned, with the suggestion that the observed lysis of symbionts was in fact an artifact of the histology procedure (477). Wilkinson argued that while some cyanobacterial cells may be digested intracellularly (e.g., see reference 473), this is the exception rather than the rule. However, several other reports of bacterial (including cyanobacterial) consumption by sponges have since emerged (172, 222, 266, 422), lending weight to the notion that certain sponges may "farm" bacteria as a food source (172). Indeed, phagocytosis and subsequent intracellular digestion of bacteria are the presumed mechanisms of nutrient transfer between a carnivorous *Cladorhiza* sponge and its methanotrophic symbionts (429, 431).

Harming the host: pathogenesis, parasitism, and fouling. Deleterious effects of microbes on sponges may be direct (i.e., pathogenesis or parasitism) or indirect (e.g., microbial films promoting surface fouling). The various reported instances of sponge disease have generally been attributed to bacteria or fungi (199), yet in most cases the responsible microbe(s) has not been identified unequivocally. A notable exception is a 2002 study by Webster and colleagues (455) in which they isolated a pathogenic alphaproteobacterium (designated strain NW4327) from an infected individual of the Great Barrier Reef sponge *Rhopaloeides odorabile*. Strain NW4327, which is related to the tumor-forming symbionts of *Prionitis* sp. macroalgae (12) and to the causative agent of juvenile oyster disease (34, 35), was shown to infect and kill healthy sponge tissue (455). The mechanism by which this occurred was via degradation of the collagenous spongin fibers, with almost the entire sponge surface subject to tissue necrosis following experimental inoculation with strain NW4327 (Fig. 19). Similarly infected tissue, with documented bacterial attack of spongin fibers, was evident during a devastating outbreak of disease in commercially important Mediterranean sponges during the late 1980s (433). Mass mortalities of commercially important sponges (e.g., *Spongia* spp.) have occurred several times in both the Mediterranean (199, 298) and Caribbean (119, 199, 375), virtually eliminating commercial sponge fisheries in some areas. Not only sponges, but also corals and other epibenthic organisms, experienced extensive mortality during a 1999 episode in the northwestern Mediterranean (52). This outbreak coincided with a sudden increase in seawater temperature, with subsequent laboratory studies suggesting the additional involvement of both protozoans and fungi. Increased microbial virulence and/or compromised host resistance linked to global warming has already been postulated as a cause of many mass mortalities of marine organisms (139, 140), and it will be of great interest (and concern) to see how marine sponges are affected by predicted rises in seawater temperature in the future. Other reports of diseases in sponges include the so-called *Aplysina* red band syndrome, afflicting aplysinid sponges on Bahaman reefs (262), cyanobacterial overgrowth of *Geodia papyracea* (330), and repeated observations of diseased sponges on a Panamanian coral reef over a 14-year period (492). Bleaching of *Xestospongia muta* and other Caribbean sponges has also been reported (64, 91, 251, 441), but it remains to be established whether, as is the case for some corals (325, 326) and an Australian macroalga (R. J. Case, A. Low, W. C. Chen, S. Longford, G. R. Crocetti, N. A. Tujula, P. Steinberg, and S. Kjelleberg, presented at the 11th International Symposium on Microbial Ecology, Vienna, Austria, 20 to 25 August 2006), bacterial pathogens are to blame. Bacteria of two genera (*Bacillus* and *Pseudomonas*) were identified as possible agents of disease in the Papua New Guinean sponge *Ianthella basta* (54). This fan-shaped sponge has undergone significant mortality at a number of inshore sites, leading to speculation that the putative pathogen(s) may be of terrestrial origin. A simple model was recently developed to describe the role of sponge morphology in recovery from disease, with branching sponges being the most likely to recover (493). To our knowledge, nothing is known about diseases of freshwater sponges.

Parasitism of sponges by diatoms has been reported for several Antarctic species (16, 51). Bavestrello and coworkers found a negative correlation between chlorophyll *a* (used as a proxy for diatom abundance) and sponge carbohydrate levels (16), while in a parallel study of the hexactinellid sponge *Scolymastra joubini*, they described a degradation of sponge internal tissue in areas of dense diatom aggregations (51). The diatoms in *S. joubini* were of the genus *Melosira* and appeared to enter the host either through the ostia (inhalant openings) (Fig. 2) or via active incorporation by the sponge pinacoderm (dermal membrane). Why sponges should actively incorporate potentially harmful diatoms is not clear, although consumption of diatoms as a food source is one possibility (113, 114). Alternatively, the silica-encased diatoms may "trick" the sponge cells into taking them up (51), a plausible explanation given the tendency of some sponges to incorporate siliceous particles from the surrounding environment (15, 17, 18, 107). Currently, nothing is known about the nature of the interactions between sponges and diatoms in tropical and temperate systems (65, 390).

Microbes may also harm sponges in a less direct manner, for example, by promoting the fouling of sponge surfaces. Any surface in the marine environment, biotic or abiotic, is subject to intense fouling pressure. During the colonization process, a new surface will first develop a biochemical conditioning film, followed by microbial fouling (e.g., colonization by bacteria and diatoms). This biofilm then acts as a precursor to attachment by macrofouling organisms, such as invertebrates and macroalgae (71, 450), which in the worst cases can negatively affect sponge nutrition by blocking feeding channels or can increase hydrodynamic drag, resulting in sponge dislodgment from the substratum.

It is important that sponges not be considered mere helpless targets for potentially harmful microorganisms. Compounds with antibacterial, antifungal, or antifouling properties are produced by many sponges (19, 32, 112), and those chemicals with more specific effects may allow the host sponge to select for harmless or even beneficial microbes while deterring deleterious types. Interestingly, the resident microbial community may also participate in host defense, and there are numerous examples of the antimicrobial potential of apparently indigenous microbes (56, 147, 203, 397, 402). In addition, at least one sponge, *Halichondria panicea*, prevents fouling and sedimentary clogging of its ostia by sloughing off its outer tissue layer every few weeks (14), while the innate immune systems of sponges (see "Establishment and Maintenance of Sponge-Microbe Associations") are also believed to play a role in the prevention of microbial invasion.

The Big Picture: Temporal and Biogeographic Variability in Microbial Communities of Sponges

Variability in sponge-associated microbial communities has been examined at a number of levels, such as over time (days to months) (105, 201, 390, 462), within and among individuals of the same sponge species (mm to thousands of km) (7, 146, 201, 365, 390, 391, 404, 454, 462), and among different host species (146, 151, 208, 390). Other studies have investigated the spatial and/or temporal distributions of specific microbial taxa within sponges (100, 226, 294, 421, 453). If an emergent theme is to be identified from these studies, it is that, with some exceptions, sponge-associated microbial communities appear to be relatively stable, with little variation in time and space (148). The main caveat to this statement is that the methods employed may not always detect the variability which is present. We review the main published findings on these topics and, where appropriate, use our own phylogenetic analyses (see Evolution and Diversity of Sponge-Associated Microorganisms) to aid our discussion of symbiont biogeography.

Considering first those studies in which the whole microbial community was targeted, only a few papers deal with temporal variability. The first examined such variation in aquarium-maintained *Aplysina aerophoba* (105). The authors used several methods to characterize the resident microbial community, including cell counts (both 4′,6′-diamidino-2-phenylindole [DAPI]- and cultivation-based), TEM, FISH, and DGGE. What they revealed was an extremely abundant bacterial community (6.4×10^8 cells per g sponge tissue) which varied little during an 11-day incubation period, even under starvation conditions or upon exposure to antibiotics. Although DGGE banding patterns changed slightly during the antibiotic treatment, relative levels of abundance of the major bacterial groups, as assessed by FISH, stayed fairly constant irrespective of treatment (105). The lack of observed temporal variability as well as the apparent resistance of the community to disturbance suggests that *A. aerophoba* harbors a highly stable microbial community. Similarly, cultivation for up to 8 months did not seem to greatly alter the bacterial community in *Geodia barretti*, at least at the broad phylogenetic levels targeted by the applied FISH probes (160). Temporal variability has also been examined among sponges in the field. A 16S rRNA gene-DGGE study found bacterial communities in the temperate Australian sponges *Callyspongia* sp., *Stylinos* sp., and *Cymbas-*

tela concentrica to be highly stable over the course of a year, while additional sampling of the last species revealed a similar lack of variation on a shorter (days to weeks) time scale (390). Another DNA fingerprinting method, terminal RFLP analysis, also identified only relatively minor temporal changes in bacterial community composition on the surface of the sponge *Mycale adhaerens* from Hong Kong (201). In contrast to these studies, the bacterial community profile of the North Sea sponge *Halichondria panicea*, as assessed by 16S rRNA gene-DGGE, varied considerably over a 10-month period (462). The archaeal community, also assessed by DGGE, varied little. Another study by the same group, on the North Sea sponge *Pachymatisma johnstonia*, demonstrated stable bacterial communities in specimens sampled at different times (2 years apart) from two Orkney Isles collection sites (A. Wichels, S. Kuppardt, and G. Gerdts, presented at the 10th International Symposium on Microbial Ecology, Cancun, Mexico, 2004).

Spatial variability in sponge-associated microbial communities has been studied from the millimeter to the interocean scale. Taylor and coworkers examined spatial variability within and among individuals of three cooccurring Australian sponges (390). In all cases, the variation in 16S rRNA gene-DGGE banding patterns (and inferred community compositions) was minor, with even the least similar samples for a species sharing >70% of bands. For the 30% or less of bands which did vary, most of the variation could be ascribed to differences among rather than within individual sponges. Considerable differences were seen among different host species, with one sponge in particular harboring a distinct bacterial community compared to those in the other two species (390). In another study, Wichels et al. found differences between mesohyl-inhabiting microorganisms and transient microbes present in the sponge aquiferous system (462). The latter fraction of microbes was targeted by gently compressing *Halichondria panicea* tissue within a syringe and collecting the outflowing water. Although incomplete separation of aquiferous system and tissue fractions may have sometimes disguised differences (462), in general there did seem to be distinct communities between the two sample types. Marked differences were also evident between outer (cortex) and inner (endosome) tissues in the Mediterranean sponge *Tethya aurantium* (404). Cell separation techniques used in natural product research on sponges have also identified patterns of microbial distribution within sponge tissues. For example, cyanobacterial symbionts in the ectosome (outer tissues) of *Theonella swinhoei* were readily separated from a filamentous bacterium (later identified as the deltaproteobacterium "*Candidatus* Entotheonella palauensis" [351]) which occurs exclusively in the inner endosome (27, 28). It is typical for phototrophic symbionts, such as cyanobacteria, to be prevalent in the outer, well-illuminated surfaces of host sponges, while other microorganisms may dominate the inner core (148).

Moving up to the next spatial scale, we consider geographic patterns of variability. A 16S rRNA gene-DGGE study of the Antarctic sponges *Homaxinella balfourensis*, *Kirkpatrickia varialosa*, *Latrunculia apicalis*, *Mycale acerata*, and *Sphaerotylus antarcticus* revealed that associated bacterial communities were highly consistent, both among individual sponges at the same sampling site and also among three different sampling sites separated by some 10 km (454). The first molecular study of large-scale biogeographic variability in sponges was the 2002 study by Hentschel and colleagues (146), which we discussed at length in previous sections. The sponges *Rhopaloeides odorabile* (458), *Aplysina aerophoba*, and *Theonella swinhoei* contained substantially overlapping microbial communities whose sequences often fell in monophyletic, sponge-specific clusters, despite wide (host) phylogenetic and geographic separation (146). A 2005 study employed 16S rRNA gene-DGGE to investigate the bacterial community in the sponge *Cymbastela concentrica* along the eastern Australian coast (391). At eight sampling sites spanning 500 km of coastline within the temperate range of the sponge, bacterial community composition varied little. However, *C. concentrica* sponges from a tropical location >1,000 km away had a seemingly very different resident bacterial community. Seawater collected during sponge sampling varied comparatively little between the tropical and temperate locations. Allopatric speciation (resulting from adaptation to geographically separated hosts) is one possible explanation for the different communities, although latitudinal changes in environmental factors (e.g., temperature and light) could also be responsible. A more prosaic explanation is that the *C. concentrica* individuals from the two locations could simply be distinct (sub)species, although molecular taxonomy studies would be needed to confirm this (391). Cultivation efforts by Sfanos and colleagues in which 17,000 bacterial isolates were obtained and more than 2,000 were screened by 16S rRNA-based RFLP fingerprinting and/or sequencing often yielded the same bacterium from many sponge hosts from multiple locations (365). The most extreme case was that of an alphaproteobacterium (GenBank accession no. AY362009), which was recovered from 18 sponge species (plus several nonsponge sources, such as a coral and a sea cucumber) spread among various Caribbean and eastern Atlantic locations (365).

Several papers have examined the temporal and spatial variability of particular sponge-associated microorganisms, often providing valuable clues to the nature of the sponge-microbe association (i.e., true symbionts would be expected to maintain a long and consistent relationship). For example, "*Candidatus* Cenarchaeum symbiosum," the sole archaeon present in the marine sponge *Axinella mexicana*, was recorded from all 23 individuals of this sponge collected from the Californian coast over a 9-month period (294). Furthermore, archaeal rRNA levels stayed relatively constant (and high) for more than a year in aquarium-maintained *A. mexicana*, indicating a highly stable relationship between the sponge and its archaeal inhabitant. A subsequent study demonstrated temporally and spatially stable associations between three Mediterranean axinellid sponges (*Axinella damicornis*, *Axinella verrucosa*, and *Axinella* sp.) and their single, host-specific crenarchaeal associates (which, in all cases, were related to "*Ca.* Cenarchaeum symbiosum") (226). The cultivable fraction of the microbial community in *Rhopaloeides odorabile* was always dominated, irrespective of sampling time or location, by a specific alphaproteobacterium (453). This bacterium, which is closely related to *Pseudovibrio denitrificans* (Fig. 14), comprised >80% of the total heterotrophic bacterial colony count in samples collected over a 460-km portion of the Great Barrier Reef as well as in those collected during four consecutive seasons at one reef. Another alphaproteobacterium, affiliated with the genus *Rhodobacter*, was found in all samples of the sponge *Halichon-*

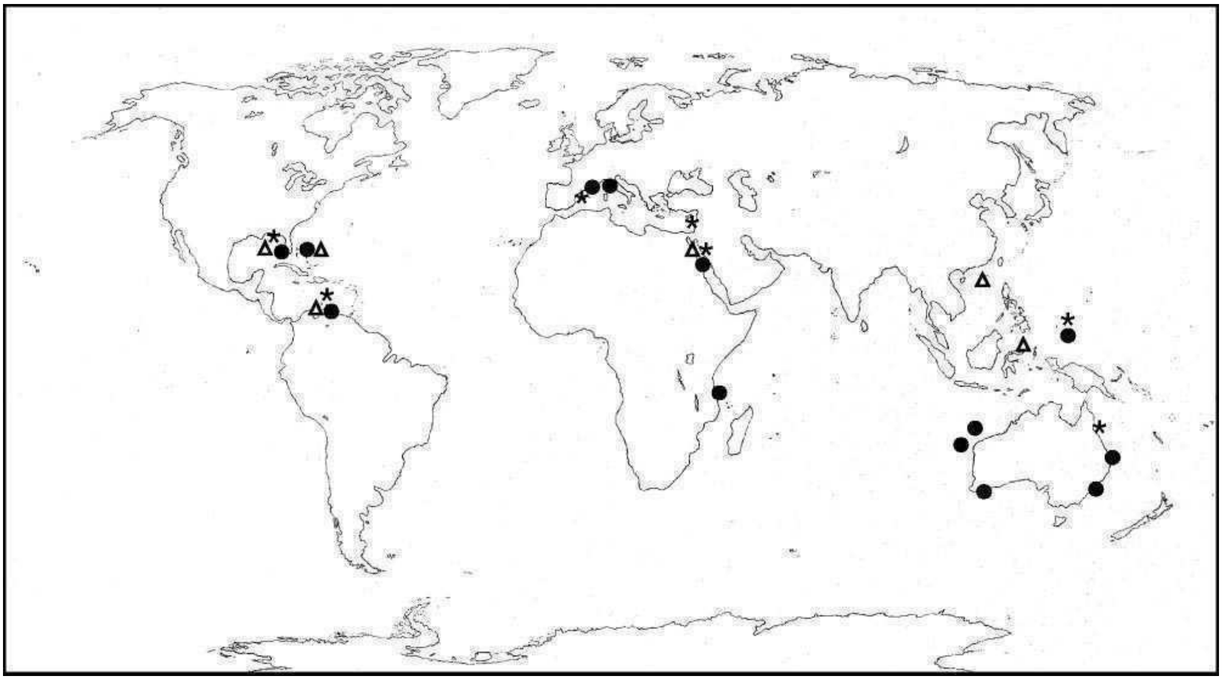

FIG. 20. Global distributions of selected monophyletic, sponge-specific clusters. Symbols refer to collection locations for representatives of the "*Ca.* Synechococcus spongiarum" (*Cyanobacteria*) (circles), *Actinobacteria* (triangles), and *Acidobacteria* (stars) clusters. In the last cluster, coral-derived sequences from the Mediterranean are also present.

dria panicea from the Adriatic, Baltic, and North seas (7). In a much earlier study, Wilkinson et al. isolated a particular bacterium (or at least a highly similar one, as molecular data were not feasible at that time) from several sponges in the Mediterranean and the Great Barrier Reef (483).

The biogeography of sponge-associated cyanobacteria has recently come under close scrutiny. Usher and colleagues performed an extensive survey of cyanobacterial symbionts, sampling nine sponge species (from six genera) in the Mediterranean Sea and the Pacific, Southern, and Indian Oceans (421). In addition, one of these sponges, *Chondrilla australiensis*, was sampled from eight Australian locations spanning several thousand kilometers and a wide temperature range. 16S rRNA gene sequences representing at least four closely related lineages of *Synechococcus* spp. were recovered from the various host sponges and included, most notably, "*Candidatus* Synechococcus spongiarum" (426), which was present in four of the sampled sponges, including all sampled individuals of *C. australiensis* (421). Interestingly, the "*Ca.* Synechococcus spongiarum" sequences from the Usher et al. study comprise, together with other sequences obtained independently from the Caribbean (78, 151, 342, 383), Red Sea (383), east Africa (383), Micronesia (146, 394), Mediterranean (146), and southeastern Australia (this study), one of the largest documented monophyletic, sponge-specific clusters (Fig. 5). In total, "*Ca.* Synechococcus spongiarum"-like sequences have been recovered from 21 sponge species from around the world, making this organism similarly widely distributed as its free-living *Synechococcus*/*Prochlorococcus* counterparts (273, 340).

Many of the sponge-specific clusters from other phyla are also widely distributed. The "*Poribacteria*," for example, have so far been identified from sponges in the Mediterranean, Caribbean, and eastern Pacific (100; this study), while a large sponge-specific cluster within the *Actinobacteria* (Fig. 13) contains sequences from the Red Sea (146), South China Sea (data not shown), Indonesia (235), and various Caribbean locations (235, 342; this study). Numerous other such examples exist, indicating an apparently global distribution of many sponge-specific microbes (Fig. 20). Hill and colleagues (151) attempted to relate the occurrence of major (sponge-associated) bacterial taxa to the geographic location where the host sponge was collected. They suggested that some patterns could be discerned whereby specific taxa might be better represented in, e.g., tropical but not cold-temperate sponges. However, we argue that such statements are premature and that there are insufficient data to draw firm conclusions at present. For example, many (or most) of the existing 16S rRNA gene libraries from sponges have not been sampled exhaustively, and missing taxa may well be represented in a library of clones but not yet identified due to low sequence coverage. Furthermore, different geographic areas (and different habitat types within an area) have not been studied equally well, so there could be biases towards apparently more diversity in some areas which have received more attention. The construction of 16S rRNA gene libraries from sponges in underrepresented locations (e.g., Africa, South America, and western North America) would go a long way towards improving our understanding of sponge symbiont biogeography.

Throughout this review, we have attempted to carefully evaluate published data in the context of the methods and ap-

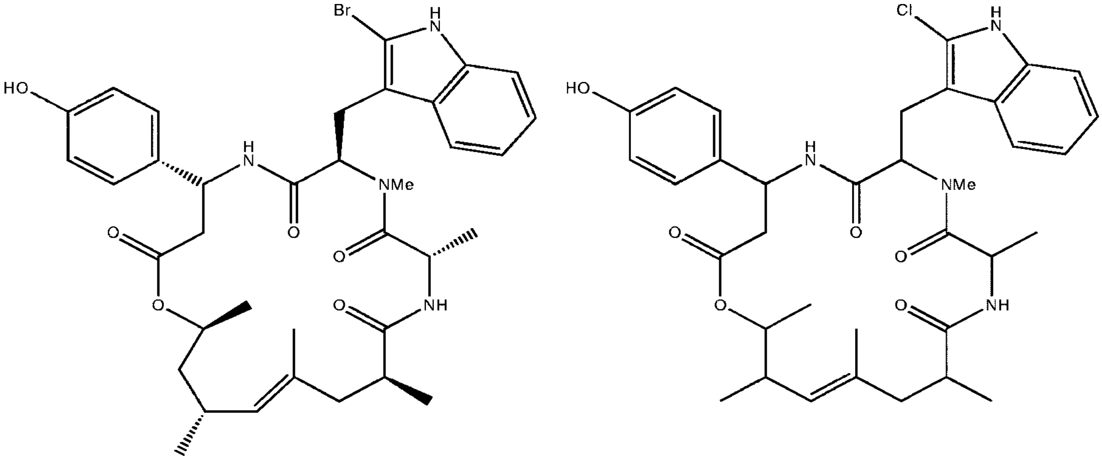

FIG. 21. Chemical structures of jaspamide (left), from *Jaspis* sp. sponges, and chondramide D (right), from the deltaproteobacterium *Chondromyces crocatus*. Note the remarkable structural similarities between the compounds.

proaches used for a particular study. This is never more critical than when considering variability in complex microbial communities. Appropriate sampling designs are important for investigations of any ecological system, yet in the past this has often been overlooked in microbial ecology circles (238, 390). The fact that one should analyze sufficient replicate samples to encompass biological variability is beyond question, yet many of the available methods for characterizing microbial communities are seemingly incompatible with this goal. DNA fingerprinting techniques such as DGGE, terminal RFLP analysis, and automated ribosomal intergenic spacer analysis offer high-throughput analyses of large numbers of samples, but together they suffer from one major drawback: without considerable efforts (e.g., sequencing of excised DGGE bands), the bands or peaks representing particular microorganisms have no identity. If banding patterns alone are compared as proxies of community composition, one must acknowledge that observed changes in a community could equally likely be due to changes among two strains of the same microbial species or to a much more significant shift, such as from a member of one bacterial phylum to another. Conversely, FISH and 16S rRNA gene library analyses can provide detailed quantitative and phylogenetic information, respectively, yet neither approach is well suited to analyzing large numbers of samples. Based on our own experience as well as reports in the literature (e.g., see reference 458), autofluorescence in sponges can also create difficulties for FISH analyses. A need therefore exists for a phylogenetically informative yet rapid means of assessing microbial community structure. Microarrays offer particular promise in this regard, with a range of 16S rRNA- and functional gene-based microarrays already available (reviewed in references 122 and 215). The highly parallel nature of microarrays provides the potential, for example, to survey the presence of multiple sponge-specific clusters in a single assay, something which is not possible with other existing techniques. Importantly, symbiont function could also be addressed via the so-called isotope array approach (2).

BIOTECHNOLOGY OF SPONGE-MICROBE ASSOCIATIONS: POTENTIAL AND LIMITATIONS

Biologically Active Chemicals from Marine Sponge-Microbe Consortia and Their Commercial-Scale Supply

An enormous number of biologically active compounds have been isolated from marine sponges and their associated microorganisms. Indeed, sponges are the most prolific marine producers of novel compounds, with more than 200 new metabolites reported each year (see reference 32 and preceding reviews in that series). Furthermore, more sponge-derived compounds are in clinical and preclinical trials (e.g., as anticancer or anti-inflammatory agents) than compounds from any other marine phylum (31). The occurrence in unrelated sponges of structurally similar compounds, particularly those which were otherwise known exclusively from microorganisms, led to speculation that such compounds (including some already in drug trials) were of microbial origin (27, 143, 280, 427) (Fig. 21). Since chemical synthesis of natural products can be problematic and expensive due to their structural complexity (4, 48, 373), the realization that at least some compounds may be produced by microbes raised hopes of obtaining a sustainable, essentially unlimited supply of compounds for testing and subsequent drug production (e.g., via cultivation of the relevant bacteria) (280, 297). Today, the true origin of most sponge compounds has still not been proven unambiguously and remains a key issue among marine natural product chemists. The possibility of convergent evolution of biosynthetic pathways among different sponges has also been raised (332). It is not our intention to comprehensively review sponge-derived natural products; such reviews are the subject of chemistry rather than microbiology per se, and many excellent reviews dedicated to this topic already exist (31, 32, 191, 236, 279, 280). Rather, we focus our attention on selected important examples and highlight some of the difficulties involved with obtaining a consumer-ready end product.

Sponge (or microbe)-derived compounds span a wide range of chemical classes (e.g., terpenoids, alkaloids, peptides, and

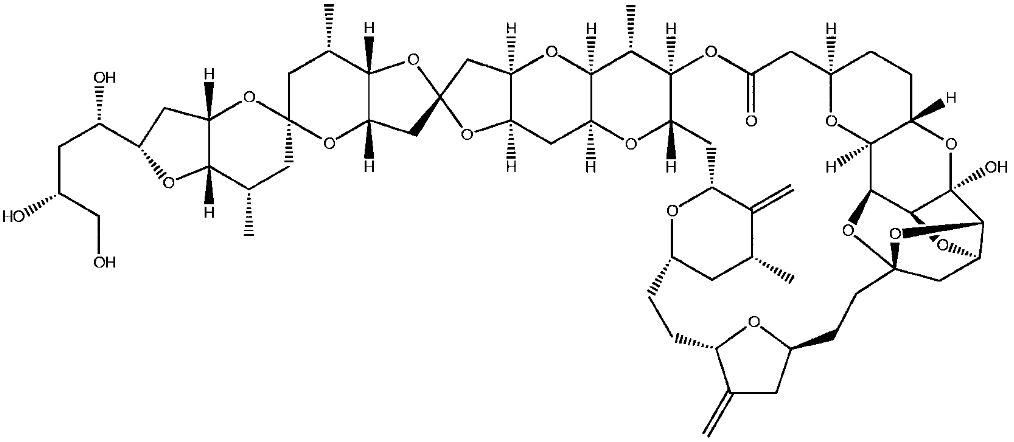

FIG. 22. Chemical structure of halichondrin B.

polyketides) with an equally wide range of biotechnologically relevant properties (e.g., anticancer, antibacterial, antifungal, antiviral, anti-inflammatory, and antifouling) (31, 32, 112, 186, 229, 236, 279, 280). The attention of natural product chemists and pharmaceutical companies, at present, is focused firmly on anticancer drugs, with several promising sponge-derived compounds in clinical and preclinical cancer trials (31, 255, 370). The large number of novel, active metabolites being reported from sponges every year begs the question of why such chemicals have not yet made it to pharmacy shelves. To date, and to the best of our knowledge, not a single compound obtained from a sponge has been approved as a drug, with a major brake on progress being the so-called supply problem (138, 297, 408). (The nucleoside analogs Ara-A and Ara-C, commercialized as antiviral and anticancer agents, respectively, could arguably be considered the sole exceptions. They were not isolated directly from sponges but are synthetic derivatives based on compounds from the Caribbean sponge *Cryptotethia crypta* [24, 25].) Biologically active natural products are often produced in relatively small amounts, and often by rare animals whose natural populations cannot sustain the extensive collections required for clinical trials. Alternative means for producing large amounts of metabolites are therefore required. We illustrate this issue by using two examples, the anticancer compounds halichondrin B and peloruside A.

The halichondrins are a group of polyether macrolides that exhibit potent antitumor activities (158, 415). First isolated from the Japanese sponge *Halichondria okadai* in the mid-1980s (158), they were subsequently found in several other sponges from diverse geographic locations, including *Axinella* spp., *Phakiella carteri*, *Raspailia agminata*, and *Lissodendoryx* sp. (138). Halichondrin B (Fig. 22) was particularly sought after due to its high cytotoxicity, and its total synthesis was reported as early as 1992 (4). However, due to the structural complexity of the compound, many steps were required for its synthesis, rendering total synthesis impractical for industrial-scale production. While the occurrence of halichondrins in many unrelated sponges suggested a microbial origin, little was known about the microbiology of the relevant sponges, and thus alternative avenues were investigated (to our knowledge, the precise [i.e., sponge versus microbial] origin of the halichondrins has never been determined unambiguously). *Lissodendoryx* sp., collected from the coast of southern New Zealand, yielded the largest amounts of halichondrins and therefore became a focus of drug supply efforts (138, 250). Based on the potency of halichondrin B and its projected demand if approved for human use, the requirement for clinical trials was estimated to be ~10 g, with annual requirements as a commercial drug of 1 to 5 kg (138). Given that 1 metric ton of *Lissodendoryx* sp. sponges yielded only 300 mg of halichondrin B and that the entire natural biomass of the sponge was estimated to be only 289 metric tons, collection from the wild was quickly ruled out. Aquaculture of *Lissodendoryx* sp. was then investigated, with promising initial results (250). However, scale-up of the operation to the levels necessary for commercial production was not achieved due to a lack of funding (M. J. Page, personal communication), and the small amounts of compound present in the sponge tissue may render the aquaculture option economically untenable in this case anyway (373). Nevertheless, halichondrin B may yet prove to be a success story, with a synthetic analog, E7389, in phase I clinical trials as an anticancer compound (370). This simplified version of halichondrin B is more amenable to chemical synthesis but retains the biological activities of the original compound (60).

Our second example concerns the macrocyclic lactone peloruside A (460) (Fig. 23). Isolated from the New Zealand demosponge *Mycale hentscheli*, peloruside A shows promising

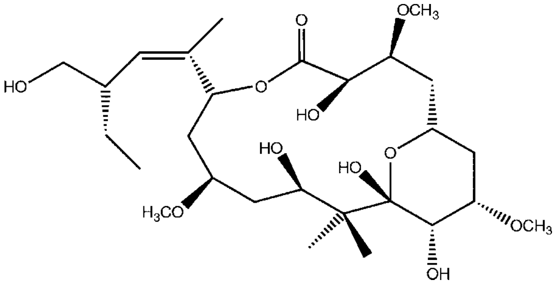

FIG. 23. Chemical structure of peloruside A.

anticancer properties, acting in a similar manner and potency to the widely used cancer drug paclitaxel (Taxol) (166). With the compound currently in preclinical trials, two avenues are being pursued in parallel to ensure a sufficient supply of the compound for subsequent clinical trials. Chemical synthesis is one approach, with several groups recently reporting partial or total synthesis (e.g., see reference 177). A New Zealand consortium, working together with a U.S. pharmaceutical company, is currently investigating whether cost-effective, industrial-scale synthesis is achievable (137). An alternative supply option for peloruside A is being explored by the same group, with aquaculture of *M. hentscheli* looking highly encouraging (137, 271). With 200 kg of sponge yielding a mere 2 g of pure peloruside A, scaling-up is a priority, with the goal of growing >500 kg of sponge over the coming year (137). Other compounds of pharmaceutical interest are also produced by *M. hentscheli*, namely, the cytotoxic polyketide mycalamide A and the macrolide pateamine (165, 256, 270, 278). Concentrations of these metabolites in natural sponge populations vary significantly in time and/or space (270), suggesting that complex ecological and physical factors may be involved in their production. An improved understanding of the ecological roles of these and other compounds could greatly benefit metabolite harvesting programs, and indeed, ecological observations are often used to guide the initial stages of drug discovery in marine environments (295, 359). Ongoing microbiological investigations with *M. hentscheli* (452; S. A. Anderson, unpublished data) should also benefit future drug development efforts with this sponge.

Supply issues notwithstanding, the pharmacological potential of marine sponges and other sessile invertebrates (e.g., corals, bryozoans, and ascidians) is enormous. Although progress toward the commercial product stage has been slow, it is highly likely that at least one of the several compounds now in clinical trials (or a synthetic analog) will be commercialized within the next few years. A combination of improved chemical synthesis methods with the various approaches outlined in the following section should ensure a bright future for this field, with sponge-derived natural products being utilized either in their natural form or as inspiration for new, laboratory-generated compounds (e.g., via chemical proteomics) (287). As a footnote to this discussion, the freshwater sponges should also be mentioned. Their chemistry has received much less attention than that of their marine counterparts, and while various lipids and a compound with antipredator activity have been reported (77, 311), it is unclear whether these sponges produce many, if any, compounds of pharmaceutical interest.

Methods for Accessing the Hidden Chemistry of Marine Sponges

A number of (nonsynthesis) approaches are available for accessing biologically active natural products from sponges and the microorganisms within them (Fig. 24). For convenience, we split these into the following three main themes: cultivation of metabolite-producing microbes, sponge culture, and molecular biological methods, such as metagenomics. In addition, we highlight the importance of metabolite localization studies for improving our knowledge of which partner (sponge or microorganism) is responsible for metabolite production.

Cultivation of metabolite-producing microorganisms. Cultivation of sponge-associated microorganisms that produce bioactive compounds is the most direct method for large-scale production of these chemicals (154), and cultivation approaches are widely practiced among those targeting bioactive compounds (46, 47, 81, 130, 147, 154, 163, 176, 189, 235, 364, 365, 378, 453). The potential payoffs from the cultivation approach are obvious and substantial: if metabolite producers can be isolated on artificial media and grown to significant cell numbers (while continuing to produce the relevant metabolite), then this obviates the need for large-scale harvesting of natural sponge populations, with its environmentally and financially negative implications.

Two broad strategies for isolating microbial producers of bioactive compounds were outlined by Hill in a recent review (154). The first is to use a wide range of media in an effort to grow as many different sponge-associated microbes as possible. Since growth under different culture conditions may influence which metabolites are produced, the use of many different media and conditions should help to maximize the chemical diversity from a given microorganism (154). Bacteria associated with deep-sea benthic invertebrates have been the subject of extensive cultivation efforts by the Harbor Branch Oceanographic Institution, with a range of nutrient-poor to nutrient-rich media being utilized (130, 264, 365). Approximately 17,000 isolated microbes, most from deep water and mostly from sponges, are present in the Harbor Branch Oceanographic Marine Microbial Culture Collection (365). These include representatives of the *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* and are the subject of natural product screening. An alternative, more targeted approach is to go after specific microbial groups with proven track records in the production of bioactive compounds. Many such groups, including cyanobacteria, fungi, and actinomycetes, are well known from sponges (148, 163), with actinomycetes being the subject of a particularly interesting success story. Sponge-derived actinomycetes of the genus *Micromonospora* produce manzamines, alkaloids with, among other things, potent antimalarial properties (10, 93, 155, 301). The first hint that manzamines were of microbial origin came from the finding of these compounds in many distantly related, geographically disparate sponge species. Subsequent cultivation-dependent and -independent characterization of the microbial communities in two Indonesian manzamine-producing sponges, 01IND 35 and 01IND 52, revealed highly diverse assemblages, with the recovery of actinomycetes provoking intensive culturing efforts in their direction (154). Growth of the sponge-derived *Micromonospora* sp. has since been achieved on a large scale in 20-liter fermentations, with maintenance of manzamine production (R. T. Hill, personal communication). Improvements in this process should greatly facilitate passage of these compounds through the various stages of the drug-testing process. Actinomycete-selective media were also used successfully with the sponges *Pseudoceratina clavata* (188), *Xestospongia* spp. (235), *Hymeniacidon perlevis* (498), and *Craniella australiensis* (209). In the last study, many of the cultivated actinomycetes displayed broad-spectrum antimicrobial activities.

There are numerous other examples of the production of biologically active compounds by sponge-derived microbial isolates. An antibacterial peptide was isolated from both the

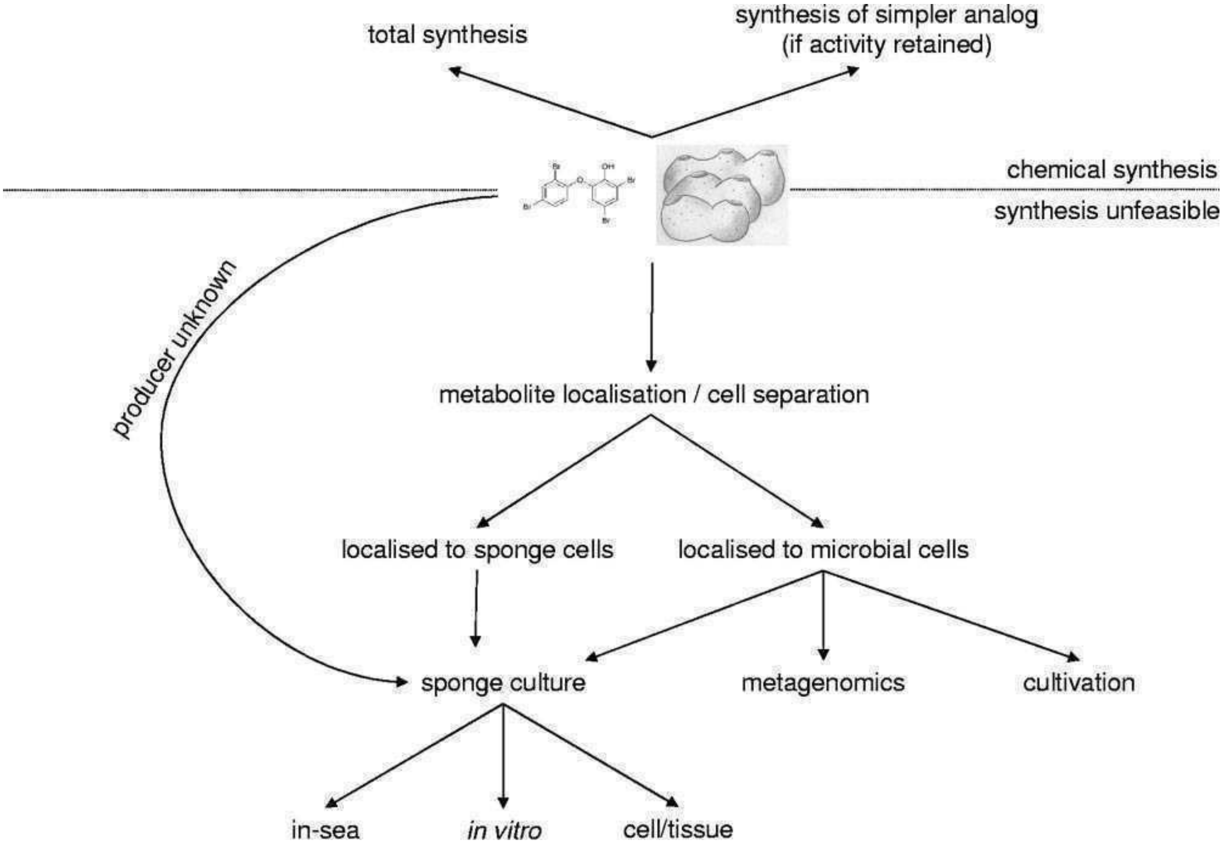

FIG. 24. Approaches for obtaining bioactive metabolites from marine sponges.

sponge *Hyatella* sp. and an associated *Vibrio* sp. (260), while a glycoglycerolipid produced by a *Halichondria panicea*-derived *Microbacterium* sp. had antitumor properties (463). In another study, several quinolones, including one with both antimicrobial and cytotoxic activities, were isolated from a pseudomonad from the Pacific sponge *Homophymia* sp. (45). Although the mechanistic basis was not identified, 27 bacteria isolated from the Mediterranean sponges *Aplysina aerophoba* and *A. cavernicola* exhibited antimicrobial activities in a series of assays (147). Given the activity of some of these isolates against clinically important multiresistant *Staphylococcus aureus* and *Staphylococcus epidermidis* strains, assay-guided fractionation and subsequent chemical characterization of any active components could prove particularly profitable. Terrestrial fungi have a long-standing reputation as prolific producers of bioactive natural products (44), and it is hardly surprising that sponge-associated fungi also show promise in this regard. Many examples exist of sponge-derived fungi that produce bioactive compounds (29, 42, 44, 163, 174, 176, 295, 296, 451). Although many of the isolated fungi are of suspected terrestrial origin (i.e., they are closely related to typical terrestrial species), to some extent this does not matter for drug discovery purposes; even if sponges act only as mere accumulators of contaminant fungi, these microorganisms can still be targeted, and once they are isolated, there may be no need to attempt reisolation from the original sponge hosts. Interestingly, it appears that unlike bacteria, fungi are not the source of any natural products previously ascribed to marine sponges (191).

The success of efforts to isolate sponge-associated microorganisms that produce bioactive compounds is dependent upon a number of factors. Most significantly, the majority of environmental microorganisms, including those in sponges, have proven resistant to cultivation by standard techniques (105, 380). Although various authors have reported improved culturability of sponge-associated bacteria via, for example, supplementing the media with sponge tissue extracts (458) or catalase and sodium pyruvate (264), the proportion of total sponge bacteria that can be isolated has remained low. Only 0.06, 0.1, 0.15, and 0.7% of total bacteria could be cultured from the sponges *Candidaspongia flabellata* (47), *Rhopaloeides odorabile* (47, 453), *Aplysina aerophoba* (105), and 01IND 35/01IND 52 (154), respectively. Santavy and colleagues were able to achieve somewhat better recovery (3 to 11%) from the Caribbean sclerosponge *Ceratoporella nicholsoni*, although obviously, even in this case, some 90% of the resident bacteria were not captured by cultivation attempts (335). While cultivation difficulties are hardly confined to sponges, it is nevertheless likely that many of the sponge associates are obligate symbionts which may have evolved with the sponge over hundreds of millions of years (see Evolution and Diversity of

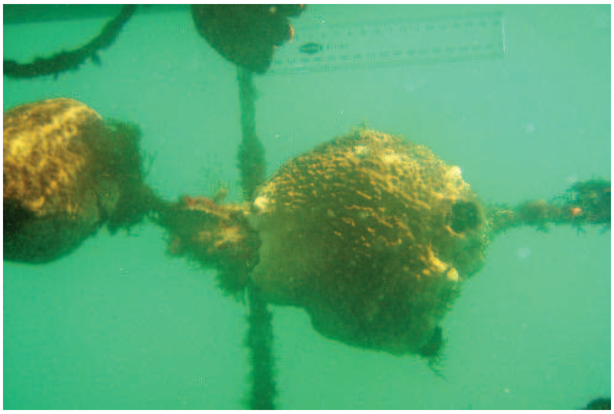

FIG. 25. In-sea aquaculture of the Great Barrier Reef sponge *Rhopaloeides odorabile*. (Image courtesy of Rocky de Nys [James Cook University, Australia], reproduced with permission.)

Sponge-Associated Microorganisms) and will, due to nutritional or other dependencies, be extremely difficult to obtain in pure culture. Furthermore, those that can be isolated may not necessarily produce the compound anymore, as they may require some as yet unknown cue or metabolic intermediates from the host sponge. Additionally, and for unknown reasons, some bacteria simply stop producing the compound of interest after a certain time on artificial media (145). This could be due to any of a number of genetic reasons relating to a lack of selective pressure in pure culture, e.g., point mutation in a key gene or loss of a mobile genetic element carrying the biosynthesis genes.

Sponge culture. Culturing sponges is another way to address the supply problem, irrespective of whether compound production is due to the sponge or the symbiont. Methods employed for the cultivation of sponges for drug production vary widely in scale and sophistication, from sea-based aquaculture to in vitro cultivation (in closed or semiclosed systems) to cell and tissue cultures (22). The technical and economic potential of each of these was reviewed recently (373).

In-sea sponge aquaculture has received considerable attention for its potential to cost-effectively address the metabolite supply issue (Fig. 25) (84–88, 132, 137, 231, 248, 271, 299, 439). Of all approaches, it has the advantage of most closely simulating the conditions encountered by sponges in nature, and practitioners have been able to draw upon more than a century of experience in farming bath sponges. On the negative side, the inherent unpredictability of marine environments can create problems (e.g., due to atypical climatic conditions or storm damage) (439) which are easily avoided in controlled in vitro systems. The outcomes of sponge aquaculture trials have varied widely, with success dependent upon a number of factors, including the type of farming structure (84, 85, 132), sponge growth form (86), farming location (271), and season of transplantation (86, 87). If sponge survival can be ensured, then growth increases of up to 5000% per year (relative to the starting size) are achievable, depending on the sponge species examined (271). Crucially, bioactive metabolites are typically retained in farmed sponges (84, 87, 132, 248, 250, 271).

The consequences of environmental variability can be sidestepped by cultivating sponges under semienclosed or even fully closed conditions (22, 90, 141, 161, 267–269). Although this is generally more expensive than sea-based aquaculture, an obvious advantage is the ability to control environmental parameters, such as growth temperature, water movement, and food supply, as well as to eliminate biomass loss due to storms or disease outbreaks (see "The Varied Nature of Sponge-Microbe Interactions"). Potential problems in recirculating systems include a buildup of toxic secondary metabolites and metabolic wastes, such as ammonia (22). Considerable growth (>200% in 1 to 2 months) of *Pseudosuberites andrewsi* explants was achieved in a bioreactor (268), with even more growth (>1,000% in 45 days) observed for the sponge *Crambe crambe* in a closed system (21). Like the case for sea-grown sponges, metabolite production has been observed for sponges subjected to in vitro cultivation (e.g., see references 75 and 90). However, one must remember that despite its promise, this technology remains in its infancy, and to our knowledge, there are no examples of industrial-scale in vitro cultivation of sponges.

Sponge cell culture for the production of biologically active metabolites represents the other extreme of the scale continuum from sea-based aquaculture. Although sponge cells can be dissociated readily and even induced to divide in suspension for several cycles, it has so far proven impossible to establish continuous cell lines (reviewed in references 293, 319, and 320). Primmorphs, which are three-dimensional aggregates comprising proliferating and differentiating sponge cells, have therefore generated much recent interest since they can be maintained for long periods (66, 240, 242, 247, 293). Particularly exciting was the finding by Müller and coworkers that primmorphs from *Dysidea avara* grown in a bioreactor produced the secondary metabolite avarol, which is both characteristic of this sponge and of great pharmacological interest due to its strong biological activities (e.g., antitumor, antibacterial, and antiviral activities) (242). In contrast, single *D. avara* cells did not produce avarol. Another interesting aspect of primmorphs, especially within the context of this review, is that symbiotic microorganisms can be retained within them (247, 398), potentially allowing for primmorph production of both sponge- and microbe-derived compounds. Since their initial demonstration in *Suberites domuncula* (66, 247) and then *D. avara*, primmorphs have been generated from a wide range of sponges, including *Axinella polypoides*, *Cliona celata*, *Halichondria panicea*, *Petrosia ficiformis*, and *Stylotella agminata* (374, 434, 500). In the coming years, it should become clear whether primmorphs can be scaled up sufficiently to overcome the supply problem for many promising drug leads.

Surprisingly little information exists on the microbiology of cultured sponges (in any system), yet this could be of vital importance if metabolites are produced by microbial associates. For example, if sponges are cultured away from their natural environment, then metabolite-producing symbionts may conceivably be lost, or if metabolites are diet derived (as suspected for okadaic acid) (297), then a change in diet would presumably result in a loss of compounds. Moreover, even if the desired metabolite is produced by the sponge itself, microbial symbionts may still be of direct (e.g., by providing metabolic precursors) or indirect (e.g., by affecting general sponge health) significance (154).

Metagenomics. One of the most exciting developments in molecular biology from a drug discovery perspective has been the advent of environmental genomics, or metagenomics (92, 104, 136, 207, 346, 438). Metagenomics refers to the analysis of genome fragments from a complex microbial community and offers the potential for large-scale, sustainable production of bioactive metabolites, including those produced by uncultivated microorganisms. If biosynthesis genes can successfully be cloned and expressed in another (cultivated) microorganism, such as *E. coli*, then this could ensure an unlimited supply of a specific metabolite (48, 143). Different approaches for the cloning and heterologous expression of biosynthesis genes from marine invertebrate symbionts were recently the subject of a comprehensive review by Hildebrand and colleagues (149). The successful application of many of these methods is exemplified by studies by Haygood and coworkers on the symbiosis between the bryozoan *Bugula neritina* and its bryostatin-producing gammaproteobacterial symbiont, "*Candidatus* Endobugula seritina" (69, 70, 142, 143, 149, 150). Here we focus our attention on the results of recent metagenomic studies involving sponges.

Two main types of analysis have been used to extract biotechnologically relevant information from metagenome libraries: one is based on function, whereby libraries are screened for the expression of specific traits, and the other is based on screening for sequences themselves (346). While screening for functional traits (e.g., antibiotic production or quorum-sensing inhibitors) has been successful (to various degrees) in other environments (68, 318, 489), studies of sponge metagenomics, to our knowledge, have been exclusively sequence based. To date, the major foci of such studies have been the polyketide synthase (PKS) and nonribosomal peptide synthetase genes (187, 284, 285, 342). The PKSs are responsible for the synthesis of bacterial polyketides, a diverse group of pharmacologically important natural products which include the antibiotics erythromycin and tetracycline as well as antitumor, immunosuppressive, and cholesterol-lowering agents (197). Type I PKSs are organized in a modular fashion, lending themselves to the combinatorial biosynthesis of novel polyketides with potentially useful pharmaceutical properties (197). A number of marine drug candidates, including the bryostatins, discodermolide, and the aforementioned peloruside A, belong to this class of compounds (104, 150). The modular nature of the polyketides suggests that environmentally retrieved PKS fragments, which may not produce intact bioactive compounds, could still be useful by providing modules for combinatorial polyketide synthesis (187, 197). A large part of the sponge-PKS story comes from work by Piel and colleagues (279–286). Their study of the polyketide onnamide in sponges was greatly facilitated by prior metagenomic investigations into the production of a structurally highly similar antitumor compound, pederin, in beetles of the genus *Paederus* (281, 283, 286). The microbial community within these beetles is much less complex than that of sponges, allowing easier access to the genome of the bacterial pederin producer (349). Ultimately, pederin production was linked to a beetle symbiont closely related to *Pseudomonas aeruginosa*, although evidence for lateral gene transfer of the pederin-type genes suggests that compound production may not necessarily correlate with rRNA-based bacterial phylogeny (283, 285). Armed with a sound understanding of the genetic bases of pederin biosynthesis, Piel et al. investigated the production of onnamide in the sponge *Theonella swinhoei* from Japan (285). Ketosynthase (KS) fragments were PCR amplified directly from the sponge metagenome, revealing a diverse range of sequence types. More importantly, PCR-based screening of a 60,000-clone cosmid library with the same primers yielded a single KS-positive clone, which was fully sequenced. Strong indications existed for a bacterial origin of this genome fragment (e.g., a lack of introns and small intergenic distances), which should correspond to almost the entire region of the polyketide structure needed to obtain an antitumor compound (285). In addition to the obvious biotechnological importance of this study, as heterologous expression of such gene clusters could lead to an inexhaustible supply of target metabolites for clinical trials, interesting ecological and evolutionary questions were also raised. For example, how did such similar biosynthetic pathways come to be present in symbionts of such dissimilar hosts, i.e., marine sponges and terrestrial beetles?

PKSs have also been studied in other sponges by using metagenomic approaches. In an attempt to isolate genes encoding the promising antitumor compound discodermolide from the Caribbean sponge *Discodermia dissoluta*, Schirmer and colleagues (342) employed a two-step approach. First, degenerate KS primers were used to amplify 256 sequences (85 different KS sequences), including several from *trans*-AT-type PKS domains of the pederin and onnamide types. A selection of the derived sequences was then used to create a probe pool for the screening of 155,000 macroarrayed fosmid and cosmid clones; given the proportion of bacterial inserts in the studied libraries (>90%) and the average insert size (35 kb), >4 Gb (some 1,000 bacterial genome equivalents) were screened (342). In total, 1,025 PKS-positive clones (0.7% of all analyzed clones) were identified. Interestingly, sequencing of selected fosmid and cosmid clones revealed suprisingly little overlap between these KS domains and those derived from the same sample by the direct PCR approach. A PKS consistent with the biosynthesis of discodermolide was also not found (342). Direct amplification of KS domains was also combined with the construction of fosmid libraries to study PKSs in another sponge, the Great Barrier Reef species *Pseudoceratina clavata* (187, 188). Each approach led to the retrieval of five KS domains, all of which fell into an apparently sponge-specific KS cluster (together with sequences obtained from *Discodermia dissoluta*, *Theonella swinhoei*, and an unidentified sponge) following phylogenetic analysis (187). Cultivated bacteria from *P. clavata* were also screened by PCR for KS domains, with 10 such domains detected in representatives of the *Actinobacteria*, *Alphaproteobacteria*, and *Firmicutes*. None of the KS domains from isolates clustered with the metagenome-derived sequences, highlighting the importance of a polyphasic approach to encompass as much of the PKS diversity as possible (187).

Although not part of a drug isolation strategy per se, no discussion of sponge metagenomics would be complete without considering the seminal work of DeLong and colleagues (134, 135, 294, 343–345). Over the past decade, the symbiosis between the Californian sponge *Axinella mexicana* and the psychrophilic crenarchaeote "*Candidatus* Cenarchaeum symbiosum" (294) has been a model system for environmental genomics. "*Ca.* Cenarchaeum symbiosum" was the first known symbiotic crenarchaeote, and despite its as yet uncultivated status, several factors have made it a suitable target for genomic studies: it is the only archaeon in the sponge and dominates the microbiota, consistently comprising some 65% of all prokaryotic cells; it is always associated with the sponge; and the symbiosis is maintained for a long time in aquaria (294). Relatively large amounts of biomass and DNA have therefore been available, with physical enrichment for the archaeal cells greatly facilitating the construction of large-insert genomic libraries for this organism. The first published results from the genomic analyses outlined the characterization of a DNA polymerase which was homologous to those of cultivated archaeal hyperthermophiles and yet, as revealed by heterologous expression in *E. coli*, was inactivated at temperatures above 40°C, reflecting the symbiont's low-temperature lifestyle (345). Initial studies based on the 16S rRNA gene indicated the presence of a single archaeal phylotype in *A. mexicana* (294), so subsequent genome-derived indications of the presence of two closely related variants were unexpected (343). Although the two variants differed <0.8% in their 16S and 23S rRNA genes and had an identical gene order for a studied 28-kb region, variations in DNA identity of up to 20% were observed for protein coding regions, with up to 30% variation for intergenic regions (343). These findings thus highlighted the difficulties created by genomic microheterogeneity in assembling environmentally retrieved genome fragments, and these difficulties remain today (76, 414, 440). Despite such obstacles, the DeLong group was able to assemble a closed genome for "*Ca.* Cenarchaeum symbiosum" (134), which has already yielded important insights into the metabolism of the sponge symbiont in particular and of marine *Crenarchaeota* in general (135). For example, genome reconstruction revealed the potential of "*Ca.* Cenarchaeum symbiosum" to function either autotrophically (as an ammonia oxidizer) or mixotrophically. In an extension of the environmental genomics approach, homologues of genes involved in carbon and nitrogen metabolism were also found in metagenome libraries from ocean waters worldwide, demonstrating the ubiquity of these metabolic pathways among marine crenarchaeotes (135). Conversely, certain genes encoding cell surface, regulatory, or defense mechanisms were not recovered from the free-living relatives of "*Ca.* Cenarchaerum symbiosum," suggesting that these could be involved specifically with the establishment and maintenance of the symbiosis (134).

The organism-oriented approach taken for "*Ca.* Cenarchaeum symbiosum" was also employed in a recent study of the sponge-specific candidate phylum "*Poribacteria*" (100, 101). Virtually nothing is known about the physiology and genetic makeup of these bacteria, and since there are no cultured representatives, metagenomics offered a promising approach. The sole entry point into the "*Poribacteria*" genome was the 16S rRNA gene sequence, and a single fosmid clone among 29,000 clones (corresponding to 1.1 Gb of DNA in total) was positive in an initial 16S rRNA PCR-based screening (101). Analysis of this 39-kb insert revealed 27 open reading frames, including one encoding a new kind of molybdenum-containing oxidoreductase and several encoding unusual transmembrane proteins (101).

The potential of metagenomics and other cloning approaches to revolutionize natural product research with sponges is undeniable, yet there remain considerable technical challenges. For example, if the microbial communities under study are highly complex, then the genomes of target organisms (e.g., "*Poribacteria*") may remain largely hidden against a background of genomes from other symbionts (but see Conclusions and Future Directions for possible means of enriching specific genomes). Other potential problems include the use of inappropriate host organisms for expression studies (some hosts may not express the genes of interest) (104, 136) and the large sizes of many gene clusters, which can prohibit their successful cloning (although there exists the theoretical possibility of reconstructing biosynthetic pathways via the assembly of many smaller, overlapping sequence reads). In still other cases, nonclustering of biosynthesis genes may be a problem, with the genes of interest spread across different parts of the symbiont's genome (281). On the bright side, the relevant technology is developing quickly. Massive sequencing efforts, such as the recent Sargasso Sea study (440), may provide one means of accessing rare genomes and the biotechnologically relevant information within them. Indeed, an ambitious small- and large-insert sequencing study along these lines was recently initiated for a temperate Australian sponge (S. Kjelleberg, P. Steinberg, and T. Thomas, personal communication). Similarly, the new pyrosequencing technology from 454 Life Sciences (227) has already been applied successfully to complex environmental samples (204) and may prove valuable in future sponge metagenomics projects. Furthermore, high-throughput screening techniques have developed to the extent that a single worker can now screen, from start to finish, a library of 400,000 clones for a particular gene in a mere 4 days (169).

Cell separation and metabolite localization. The aforementioned techniques each offer the potential to generate large quantities of biologically active metabolites from sponge-microbe associations. All three approaches (microbial cultivation, sponge culture, and metagenomics) can, in principle, be undertaken without prior knowledge of which partner (sponge or microorganism) produces a given compound. However, the most rational approach is to first establish which cell type(s) is responsible for metabolite production, as this can determine a logical strategy for future efforts (Fig. 24). Many researchers have employed cell separation techniques for this purpose (27, 28, 99, 103, 120, 314, 315, 332, 416, 417). A major breakthrough in this area came with the realization that chemically fixed sponge and microbial cells retain their natural products in a form amenable to chemical analyses, such as high-performance liquid chromatography and nuclear magnetic resonance spectroscopy (416). Coupled with the relative ease with which one can dissociate sponge tissue, this opened up a number of possibilities for natural product research. In the 1990s, researchers in Faulkner's laboratory thus took advantage of cyanobacterial autofluorescence to separate, via flow cytometry, the filamentous cyanobacterium *Oscillatoria spongeliae* from sponge and other microbial cells in dissociated tissue of *Dysidea herbacea* (416, 417). Of three major types of metabolites known previously from this sponge, two—polychlorinated peptides (416) and polybrominated biphenyl ethers (417)—were found exclusively inside *O. spongeliae* cells. In contrast, sesquiterpenoids were confined to the sponge cells. While autofluorescing cells (e.g., cyanobacteria) have been separated from other cell types (e.g., sponge and noncyanobacterial microbes) by flow cytometry, other characteristics, such as cell size and, provided the natural products are retained, FISH probe-conferred fluorescence, could also be used to differentiate sponge and symbiont cells by this technique.

A more common approach has been to separate different cell types in dissociated sponge tissue via centrifugation (27, 28, 314). Again, the Faulkner group was instrumental in establishing these approaches for sponge natural product study. In the chemically rich lithistid sponge *Theonella swinhoei*, Bewley and colleagues (28) identified four distinct cell types by electron microscopy (sponge cells, unicellular cyanobacteria, unicellular heterotrophic bacteria, and filamentous heterotrophic bacteria [the term "heterotrophic bacteria" is used here in reference to noncyanobacterial microorganisms; these cells could, in principle, be from chemolithoautotrophic bacteria]). By following gross dissection of the sponge (into the endosome [inner tissues] and ectosome [outer tissues]) with dissociation and centrifugation, they were able to obtain relatively clean fractions comprising each cell type. The macrocyclic polyketide cytotoxin swinholide A was found only in the fraction containing the unicellular heterotrophic bacteria, while the potent antifungal glycopeptide theopalauamide (350) was associated with a filamentous bacterium (later identified as a deltaproteobacterium, "*Candidatus* Entotheonella palauensis" [351]) (28). Density gradient centrifugation provides yet another opportunity for separating sponge and microbial cells. In this case, dissociated sponge tissue is placed above a density gradient (consisting of, e.g., Ficoll or Percoll), and following centrifugation, various cell fractions are formed based on differing densities (103, 120, 121, 315). Individual fractions can then be examined chemically and microscopically to correlate the occurrence of compounds with specific cell types. For *Haliclona* sp., Garson and coworkers used Percoll gradients to demonstrate localization of the cytotoxic alkaloids haliclonacyclamine A and B within the sponge cells rather than in associated dinoflagellates (120).

Depending on the properties of and prior knowledge about the compounds under study, localization may be achieved without the need for prior cell separation. For example, brominated compounds, which are often biologically active and can be present in large amounts in sponges, may be visualized in situ by X-ray energy-dispersive microanalysis (406, 410). Specific antibodies for the toxin latrunculin B were used to prove its localization in sponge but not symbiont cells within the Red Sea demosponge *Negombata magnifica* (125). In a novel approach for sponges, catalyzed reporter deposition-FISH was recently used to demonstrate mRNA expression from the *dysB1* genes (responsible for the biosynthesis of polychlorinated peptides) in cells of the cyanobacterium *Oscillatoria spongeliae* in *Dysidea* (*Lamellodysidea*) *herbacea* (102). The biological result was consistent with earlier work by others (103, 416), but more significantly, this study proved the utility of the method for the investigation of target chemicals in sponges. The main limitation of the method is that a certain level of genetic information must first be available for the studied, or a related, biosynthetic pathway.

Although strongly suggestive, even the localization of a metabolite to a particular cell type is not unequivocal proof of its production in that cell or, indeed, even in that organism at all. A compound may diffuse or be exported away from its site of synthesis, especially if it is toxic to the producer (149). The recent demonstration of swinholide A production by free-living marine cyanobacteria (9) is a case in point: while Bewley and coworkers (28) found this compound only in heterotrophic bacteria (outlined above), it now seems plausible that cyanobacterial symbionts in *Theonella swinhoei* may produce the compound but excrete it to be stored elsewhere. Alternatively, both the heterotrophic bacteria and the cyanobacteria may share the capacity for swinholide A biosynthesis, perhaps due to lateral gene transfer (9). Interphylum and even interdomain transfer of natural product pathways has been reported a number of times (e.g., the β-lactam biosynthesis genes, responsible for the production of antibiotics, including penicillins and cephalosporins, are found in both fungi and bacteria [41]). Yet another potential explanation is that a given metabolite could be derived from the sponge's diet. There is evidence suggesting that this may be the case for okadaic acid, which has been isolated from various sponges but is known to be produced by free-living marine dinoflagellates (297, 467). Interestingly, while localization studies have very often implicated the host sponge as the source of bioactive metabolites, in at least some cases it appears likely that intracellular or cell surface-associated bacteria may have been present but overlooked (149). In such cases, microbial production of metabolites cannot be ruled out completely.

It is important to note that while any of the aforementioned localization techniques may give an indication of which is the metabolite-producing organism, none of them directly allows the harvesting of large amounts of the desired compounds (in contrast to cultivation of the relevant microorganism, sponge culture, or heterologous expression of biosynthesis genes). The chief benefit of such studies is therefore to improve our understanding of metabolite production within the sponge-microbe association, identifying the putative producer(s) and thus providing the basis for targeted cultivation and/or molecular approaches.

Other Biotechnologically Relevant Aspects of Sponges

In addition to the by now well-known pharmacological potential of marine sponges and their associated microbiota, several other aspects are also worth highlighting. For example, the farming of certain sponges, particularly those in the genera *Spongia* and *Hippospongia*, for their use as bath sponges has been going on for more than a century. The industry, based mainly in the Caribbean and Mediterranean, has been hit numerous times by mass outbreaks of sponge disease (see "Harming the host: pathogenesis, parasitism, and fouling"). A better understanding of sponge microbiology in general, and disease ecology in particular, should contribute to the future success of these endeavors. The enormous filtering capacity of sponges has led to the suggestion that they be farmed in a bioremediation context, e.g., to reduce the high bacterial loads resulting from sewage discharges and marine fish farms (110, 233, 379). Such an approach could be optimized financially by farming sponges that, in addition to their role as a biofilter, are useful as bath sponges or produce bioactive metabolites which can subsequently be harvested (233).

Another aspect relates to the remarkable structural properties of the silica skeletons of demosponges and hexactinellid (glass) sponges. A series of recent studies examined in detail the siliceous spicules of *Euplectella aspergillum*, a deep-sea hexactinellid from the western Pacific (5, 6, 385). Individual spicules exhibit, in addition to their role in providing structural support, fiber-optic properties similar to those of fibers utilized in the telecommunications industry (5, 385). Importantly from a technical point of view, spicules are formed at ambient temperatures, and the study of this process may allow some of the inherent problems associated with the commercial (high-temperature) manufacture of optical fibers to be overcome (385). Further insights into how organisms synthesize such sophisticated biological materials were gained from electron microscopy observations of spicule organization in the same sponge (6). The spicules of *E. aspergillum* provide great structural stability due to their intricate arrangement at several spatial levels, ranging from nanometers to centimeters, perhaps inspiring the future development of new materials by humans (6). As mentioned earlier, sponges (e.g., the hexactinellid sponge *Scolymastra joubini*) (51) may derive at least some of their silica from diatoms, while in turn, spicules may extend the range of photosynthetic symbionts within sponge tissue via their conduction of light (115). Ongoing work on sponge skeletogenesis (e.g., see references 245, 357, 418, and 490) should provide fascinating insights into its biological and, potentially, commercial implications.

CONCLUSIONS AND FUTURE DIRECTIONS

As indicated at the beginning of this article, even the significant recent advances in our understanding of sponge-microorganism associations have not closed numerous gaping holes in our knowledge of these systems. It is startling how little is known about many fundamental aspects of sponge symbiont biology, particularly in the areas of symbiont metabolism and evolution. On the other hand, the ever-increasing research interest in this topic (Fig. 1) promises a bright future for the field. To close, we offer our thoughts on where some of this research attention could best be directed.

Detailed studies of symbiont transmission and sponge-microbe coevolution will greatly facilitate our understanding of the evolution of these systems. Improved host phylogenies, from species to class levels, will be critical in this regard, and recent efforts in this direction are highly encouraging. “Hunting” for apparently sponge-specific microorganisms in other, nonsponge habitats (e.g., seawater and other filter-feeding invertebrates) will also be important. However, merely proving the presence of a sponge-specific microbe outside a sponge host is not sufficient to disprove its sponge-specific nature. For example, a sponge damaged by a predator or storm may disintegrate and spread its microbial inhabitants into the water column. It is thus necessary to prove the activity of such microorganisms outside the sponge host, using methods which link microbial identity with function (e.g., FISH-microautoradiography or stable isotope probing). This will be no small feat if the organism is extremely rare in the ocean, as will almost certainly be the case.

The function and physiology of sponge-associated microbes are increasingly important research topics, reflecting our current paucity of knowledge about many of the microbial associates of sponges. For many, or even most, symbionts in sponges, all that is known so far is their 16S rRNA gene sequence: their metabolism remains a black box. Despite various caveats, the 16S rRNA data assembled here, together with analyses of so-called functional genes, will provide a solid framework for the application of recently developed molecular tools for ecophysiological analyses of uncultivated microbes (2, 445, 449). Moreover, future combinations of (hypothesis-generating) metagenomic data with such tools should be particularly profitable, especially for enigmatic microbes such as the “*Poribacteria*.” Another area warranting further attention is that of the molecular and biochemical bases of sponge-microbe interactions. Recent work on the innate immune responses of sponges has already provided many important insights, but much remains unknown about host-symbiont signaling and regulation.

Although many aspects of sponge-microbe associations are interesting and important from a basic research perspective, we acknowledge that it is largely biotechnological interest that will sustain this field into the future. It is thus fitting to end our review with this topic. The biotechnological potential of sponge-microbe associations has been widely (and justifiably) lauded, yet the transition from initial compound discovery to large-scale commercial production remains difficult. Chemical synthesis of sponge-derived compounds or their simpler derivatives offers the most reliable option for sustainable, long-term drug supply if the said compounds can be produced cost-effectively. Emerging technologies such as metagenomics and high-throughput microbial cultivation approaches offer exciting potential for accessing those compounds which are produced by microorganisms. Current problems for metagenomics due to the complexity of microbial communities in sponges may be overcome in the future by single-cell genomic approaches (such as multiple displacement amplification) applied to specific cell types which have been separated by, e.g., FISH and flow cytometry (e.g., see reference 183). The use of sponge larvae as starting material for metagenomic studies of drug-producing microbes has also been suggested, as these are most likely to represent the true symbionts and, perhaps, may be simpler communities (279). The demonstrated complexity of vertically transmitted communities (reviewed in this article) suggests, however, that even this will be challenging. Combinatorial biosynthesis, as exemplified by an elegant recent study of *Prochloron* sp. symbionts of ascidians (82), provides further potential for exploiting the chemical novelties present in sponge-microbe associations. Looking to the future, it is clear that even greater integration among microbiologists, chemists, geneticists, zoologists, and aquaculture experts will be crucial in order to wring the most (data) out of sponges and their microbial partners.

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Supplemental Material for Taylor et al., "Sponge-associated microorganisms: evolution, ecology and biotechnological potential"

Supplemental Figures

Suppl. Fig. 1 16S rRNA-based phylogeny of sponge-associated *Bacteroidetes*. Details are as provided for Figs. 5-7 in the article.

Suppl. Fig. 2 16S rRNA-based phylogeny of sponge-associated *Firmicutes*. Details are as provided for Figs. 5-7 in the article.

Suppl. Fig. 3 16S rRNA-based phylogeny of sponge-associated *Actinobacteria* (excluding Family *Acidimicrobiaceae* – shown in Fig. 13). Details are as provided for Figs. 5-7 in the article.

Suppl. Fig. 4 16S rRNA-based phylogeny of sponge-associated *Alphaproteobacteria*. Other members of this class are shown in Fig. 14 and Suppl. Fig. 5. Details are as provided for Figs. 5-7 in the article.

Suppl. Fig. 5 16S rRNA-based phylogeny of sponge-associated *Alphaproteobacteria*. Other members of this class are shown in Fig. 14 and Suppl. Fig. 4. Details are as provided for Figs. 5-7 in the article.

Suppl. Fig. 6 16S rRNA-based phylogeny of sponge-associated *Gammaproteobacteria*. Other members of this class are shown in Suppl. Figs. 7 and 8. Details are as provided for Figs. 5-7 in the article.

Suppl. Fig. 7 16S rRNA-based phylogeny of sponge-associated *Gammaproteobacteria*. Other members of this class are shown in Suppl. Figs. 6 and 8. Details are as provided for Figs. 5-7 in the article.

Suppl. Fig. 8 16S rRNA-based phylogeny of sponge-associated *Gammaproteobacteria*. Other members of this class are shown in Suppl. Figs. 6 and 7. Details are as provided for Figs. 5-7 in the article.

Suppl. Fig. 9 16S rRNA-based phylogeny of sponge-associated *Betaproteobacteria*. Details are as provided for Figs. 5-7 in the article.

DNA extraction, PCR amplification, and cloning of 16S rRNA from three marine sponges

The sponges *Agelas dilatata* and *Plakortis* sp. were collected from Little San Salvador Island, Bahamas, and kindly provided to us by Dr Ute Hentschel (University of Würzburg). *Antho chartacea* was collected from Bare Island, Sydney, Australia by Sharon Longford and Megan Huggett, whose efforts we gratefully acknowledge. Collections were performed under as sterile conditions as possible, and samples were frozen prior to later freeze-drying.

We extracted DNA from 5 mg of freeze-dried tissue, from each of three individuals per sponge species, using the UltraClean Soil DNA Isolation Kit (MoBio), according to the manufacturer's instructions. PCR amplification was performed in a BioRad iCycler, using the following primers:

616V (5'- AGA GTT TGA TYM TGG CTC -3') and 1492R (5'- GGY TAC CTT GTT ACG ACT T -3') for *Bacteria* (616V and 1390R (5'- GAC GGG CGG TGT GTA CAA -3') were used for amplifications with *A. chartacea*)

ARCH21F (5'- TTC CGG TTG ATC CYG CCG GA -3') and 1492Rdeg (5'- GGY YAC CTT GTT ACG ACT T -3') for *Archaea*

POR389F (5'- ACG ATG CGA CGC CGC GTG -3') and POR1130R (5'- GGC TCG TCA CCA GCG GTC -3') for "*Poribacteria*"

Cycling conditions were: 94°C for 3 min; 30 cycles of 94°C for 40 s, 52°C for 40 s, 72°C for 1 min 30 s; 72°C for 10 min.

Correct-sized amplicons were excised from a low-melting point agarose gel, then cloned using the TOPO-TA Cloning Kit (Invitrogen), according to the manufacturer's instructions. Clones containing an insert of the correct length were selected randomly for sequencing. The obtained sequences are available in Genbank under accession numbers EF076059 to EF076242.

Construction of an ARB database comprising all publicly available sponge-derived 16S rRNA sequences

We imported into ARB all sequences which were retrieved from Genbank on 1 March 2006 using the search string: (sponge* or porifera*) and (16S* or ssu* or rRNA*). We then removed all extraneous sequences, including 18S rRNA sequences derived from the sponge genome, and 16S rRNA sequences which contained 'sponge' somewhere in the Genbank record but were in fact e.g. seawater-derived. After removing 26 sequences due to insufficient quality for reliable alignment, and adding a further 184 16S rRNA sequences from the sponges *Agelas dilatata*, *Antho chartacea* and *Plakortis* sp. (see below), we were left with a total of 1683 sponge-derived 16S rRNA sequences and 11 18S rRNA sequences from eukaryotic microbes in sponges. The annotated ARB database and alignment, with these 1694 sponge-derived 16S and 18S rRNA sequences, is available from the authors upon request.

Phylogenetic analyses of sponge-derived 16S rRNA sequences

Preliminary analyses were performed to ascertain which sponge-derived sequences fell into putative monophyletic, sponge-specific clusters. Representatives of every such cluster were then screened against both the regular and environmental NCBI databases using the BLAST function. The several most similar sequences to those in putative sponge-specific clusters were then imported into ARB and aligned as described above. These and other related sequences were used as reference sequences for subsequent phylogenetic analyses.

The majority of phylogenetic analyses were performed in the ARB programme package. Maximum likelihood, maximum parsimony and neighbour-joining trees were constructed on long (≥1000 nt) sequences using the programs AxML, DNAPARS and Neighbor Joining, respectively. In three cases (*Actinobacteria*, *Alphaproteobacteria* and *Beta*/*Gammaproteobacteria*) the number of sequences was too high to perform the maximum likelihood analyses in ARB, so the program RAxML-VI-HPC was used (2). We compared the topology of smaller trees obtained with AxML (implemented in ARB) and RAxML-VI-HPC, and the results were highly consistent. Short sequences (>1000 nt) were subsequently added to trees using the Parsimony Interactive tool in ARB, with no apparent change in tree topology. Bootstrap analyses (100 resamplings) were performed in ARB using the DNAPARS program, except for the *Actinobacteria*, *Alphaproteobacteria* and *Beta*/*Gammaproteobacteria* trees, for which neighbour-joining bootstraps were calculated in PAUP* 4.0b 10 (PPC). Positional conservation filters of 50% were applied for each respective phylum, with outgroups consisting of sequences representing several other bacterial phyla.

Maximum likelihood trees are displayed in the article, with sponge-specific clusters only marked if they occurred consistently with all three treeing methods (some clusters are an exception, occurring only in two methods – these are marked accordingly in the relevant figures). The circular tree displayed in Fig. 4 was calculated in RAxML-VI-HPC, then refined using the tools available at itol.embl.de (1).

References

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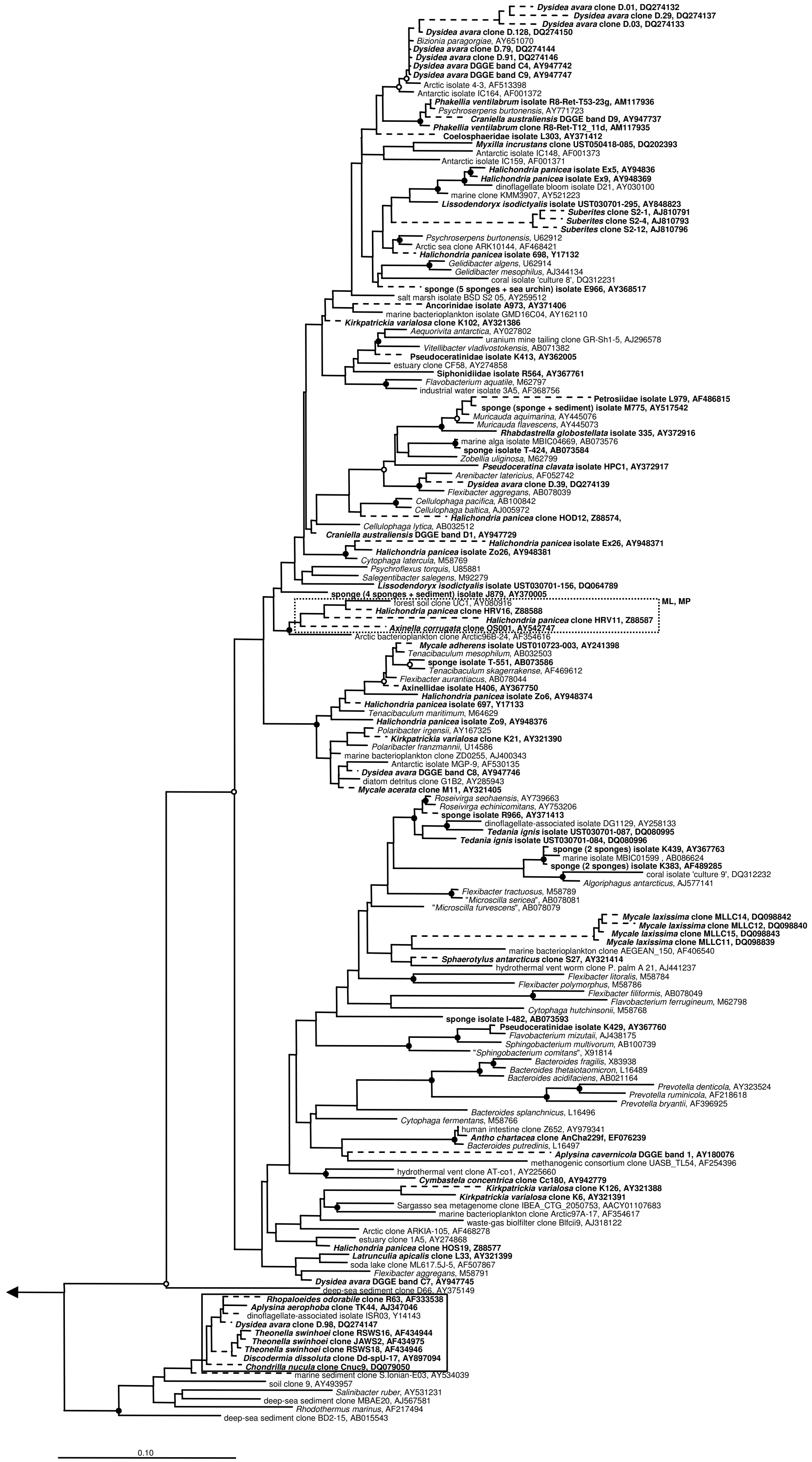
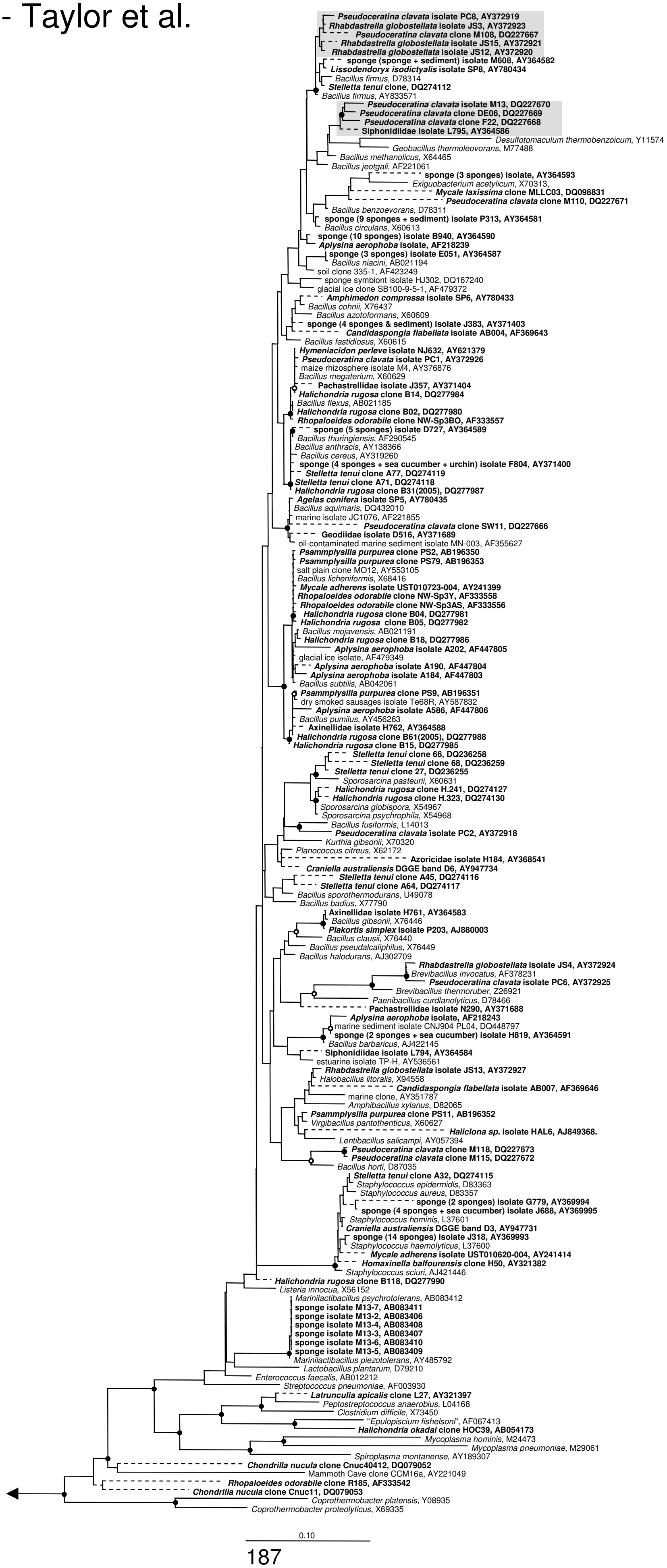
2. **Stamatakis, A.** 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics **22:**2688-2690.

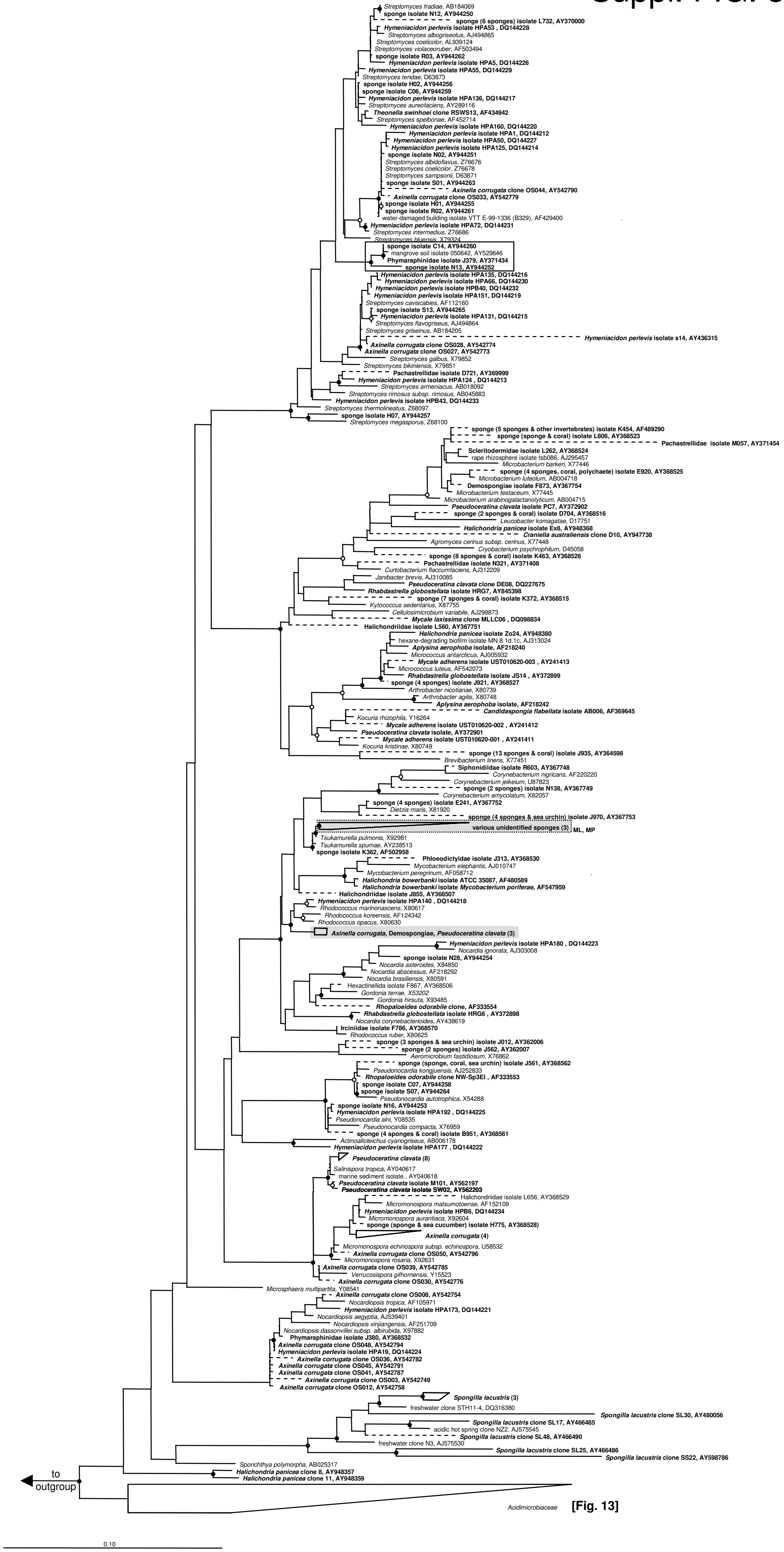
Suppl. FIG. 1 - Taylor et al.



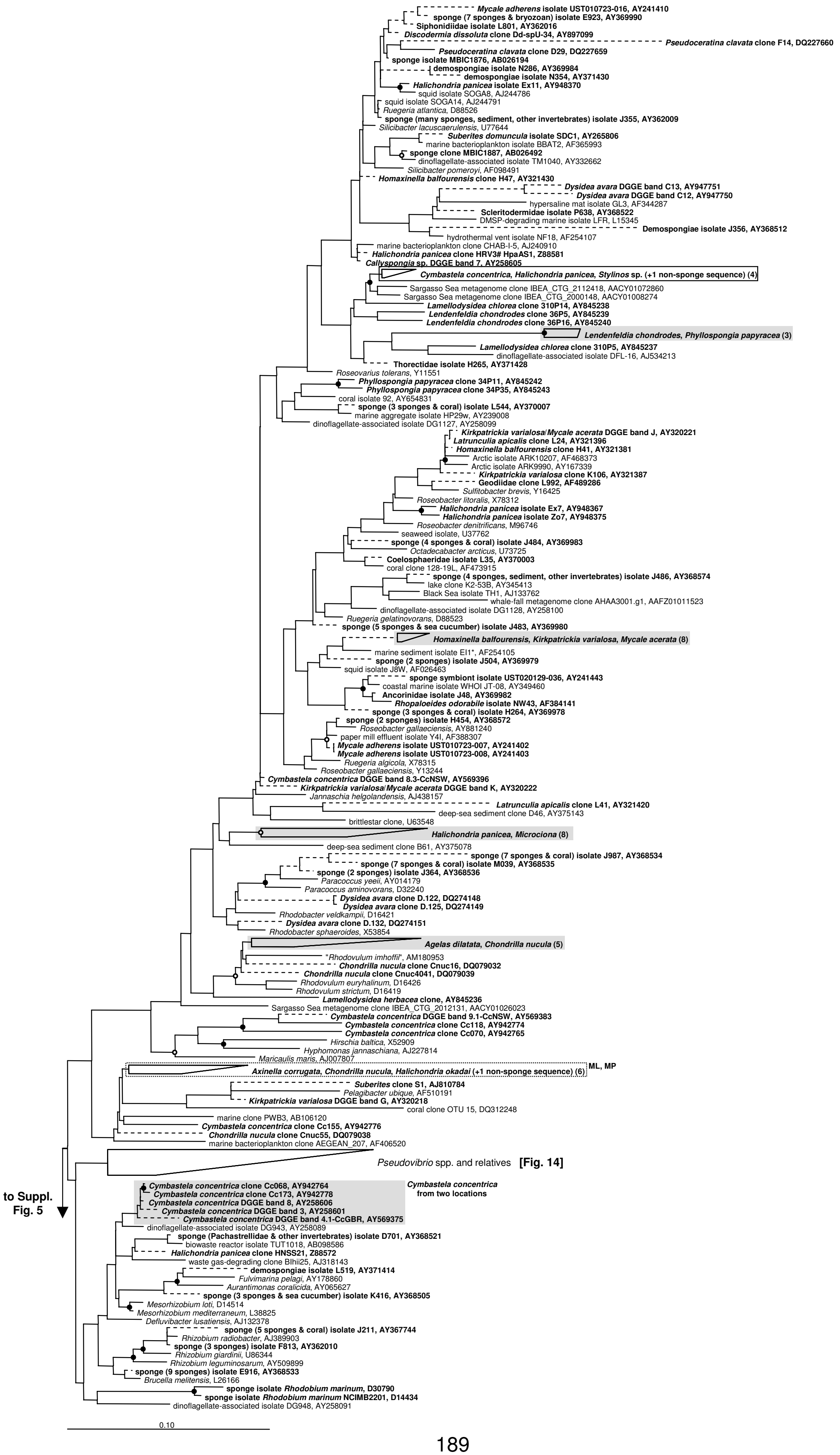
Suppl. FIG. 2 - Taylor et al.



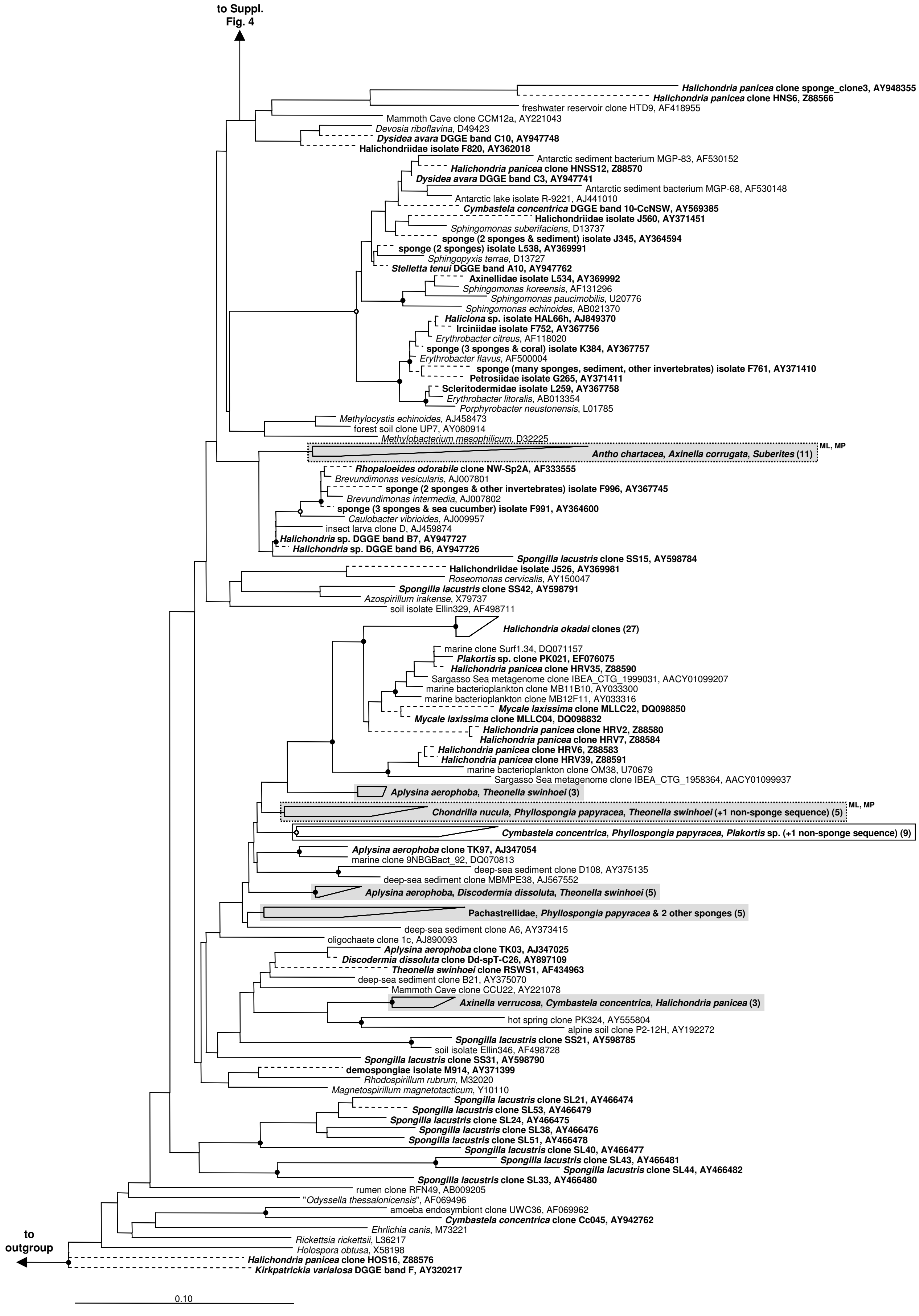
Suppl. FIG. 3 - Taylor et al.



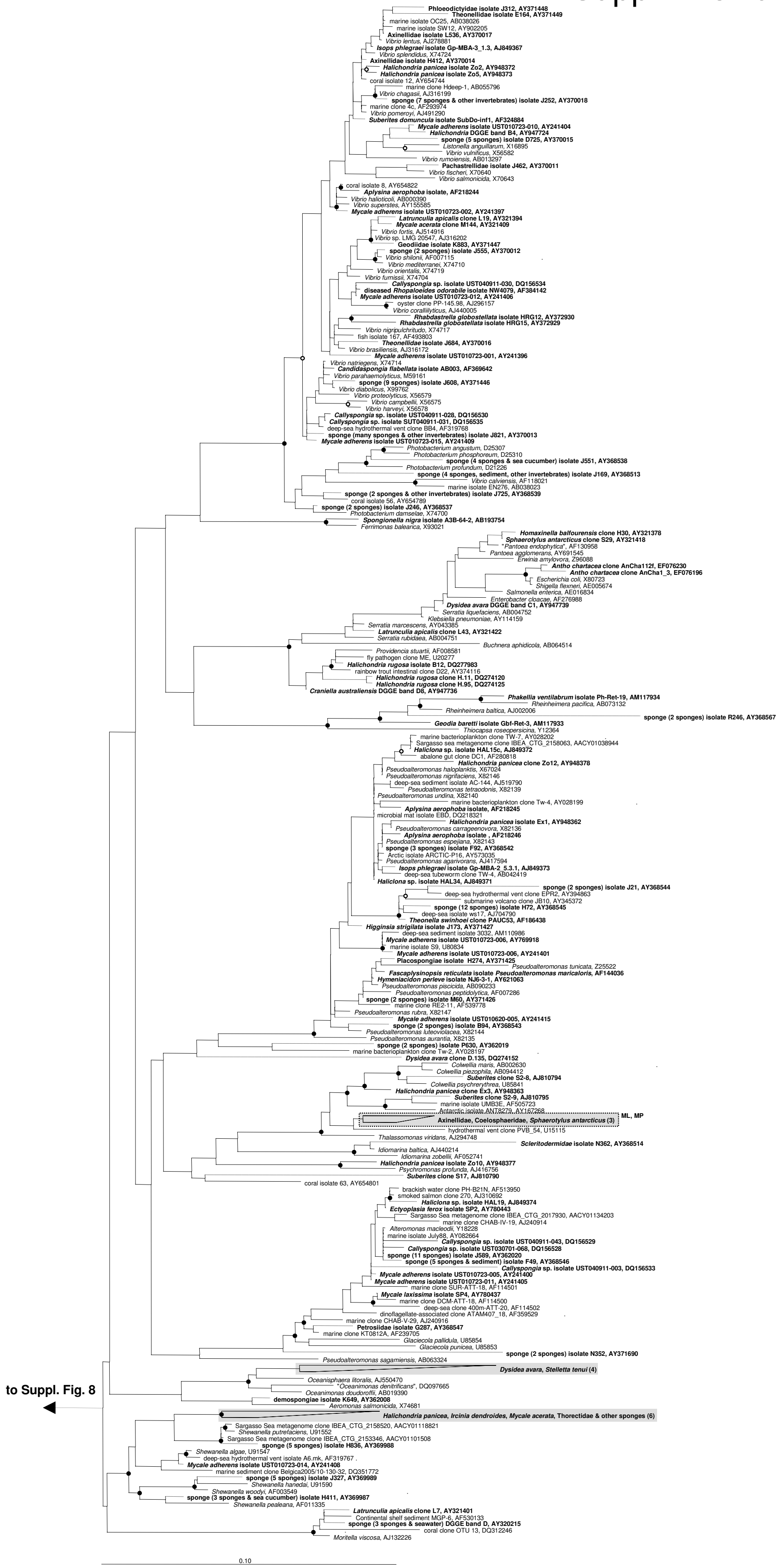
Suppl. FIG. 4 - Taylor et al.



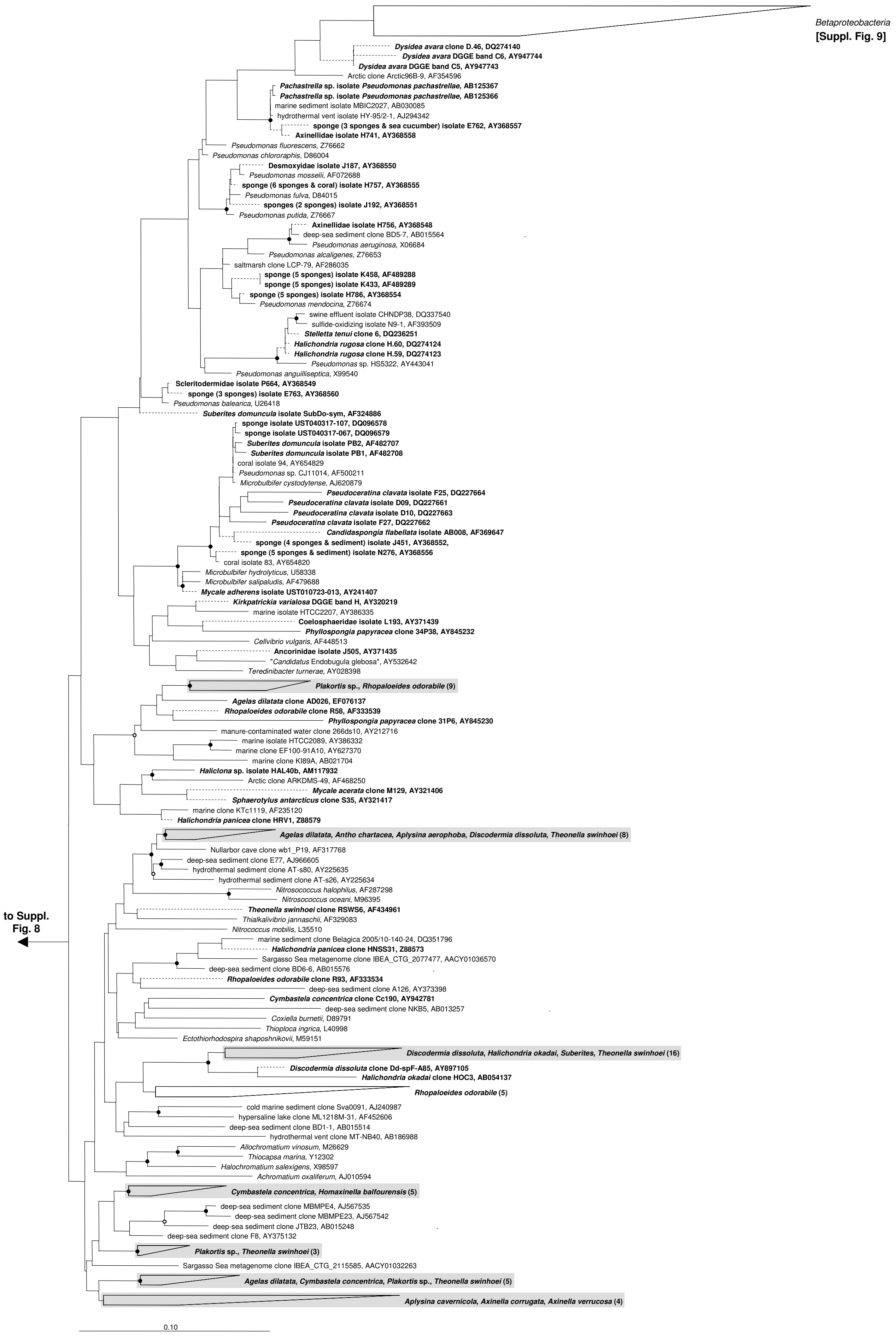
Suppl. FIG. 5 - Taylor et al.



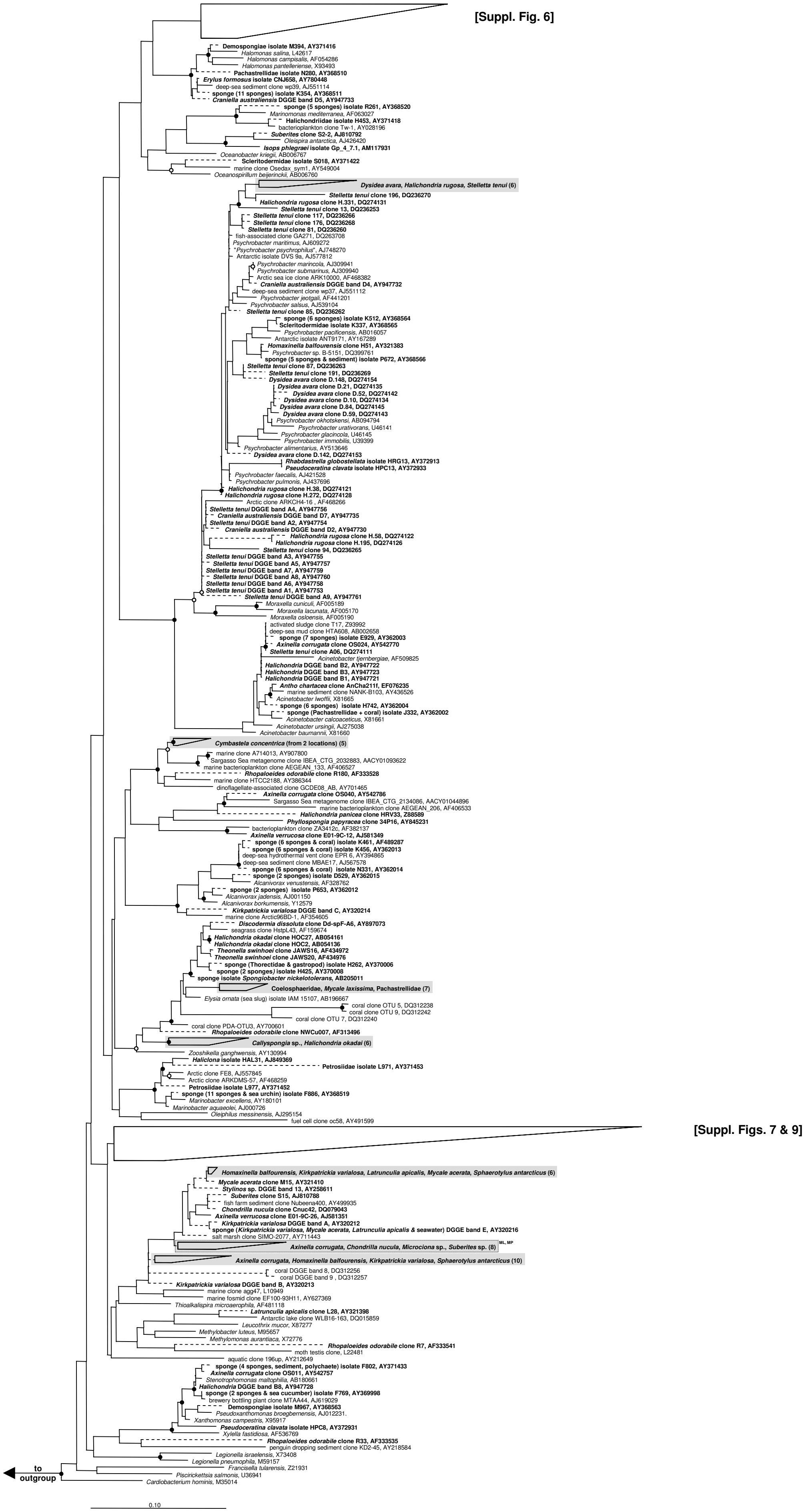
Suppl. FIG. 6 - Taylor et al.



Suppl. FIG. 7 - Taylor et al.



Suppl. FIG. 8 - Taylor et al.



Suupl. FIG. 9 - Taylor et al.

PAPER II

Diversity and mode of transmission of ammonia-oxidizing archaea in marine sponges

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Concept by D.S. and M.W.T. Sampling was done by P.E-E., S.W., U.H. and R.N. D.S. performed all experiments. M.W.T., M.W. and D.S. wrote the manuscript.

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Brief report

Diversity and mode of transmission of ammonia-oxidizing archaea in marine sponges

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Summary

The model marine crenarchaeote '*Cenarchaeum symbiosum*' is until now the only ammonia-oxidizing archaeon known from a marine sponge. Here, phylogenetic analyses based on the 16S rRNA and ammonia monooxygenase subunit A (*amoA*) genes revealed the presence of putative ammonia-oxidizing archaea (AOA) in a diverse range of sponges from the western Pacific, Caribbean and Mediterranean. *amoA* diversity was limited even between different oceans, with many of the obtained sequences (75.9%; $n_{total} = 83$) forming a monophyletic, apparently sponge- (and coral-) specific lineage, analogous to those previously inferred from comparative 16S rRNA gene studies of sponge-associated microbes. The presence of AOA in sponge larvae, as detected by 16S rRNA and *amoA* PCR assays as well as by fluorescence *in situ* hybridization, suggests they are vertically transmitted and thus might be of importance for ammonia detoxification within the sponge.

Introduction

Many marine sponges harbour diverse and abundant microbial communities, including bacteria, archaea and eukaryotic microorganisms (Taylor *et al*., 2007). Sponge-associated archaea were first discovered in 1996, with the notable finding of a psychrophilic crenarchaeote, '*Candidatus* Cenarchaeum symbiosum', within the Californian sponge *Axinella mexicana* (Preston *et al*., 1996). '*C. symbiosum*' is consistently associated with *A. mexicana*, comprising up to 65% of prokaryotic cells within the sponge (Preston *et al*., 1996), and has become an important model organism within the field of environmental genomics (Schleper *et al*., 2005; Hallam *et al*., 2006a,b). Numerous reports of archaea associated with sponges have since emerged (Webster *et al*., 2001a; Margot *et al*., 2002; Lee *et al*., 2003; Holmes and Blanch, 2006; Pape *et al*., 2006), and it is now apparent that archaea form part of the natural microbiota of many demosponges.

With a handful of reported exceptions (Webster *et al*., 2001a; Holmes and Blanch, 2006), archaea within marine sponges are members of the phylum *Crenarchaeota*. The ubiquity and abundance of crenarchaeotes within marine systems is beyond question (DeLong, 1992; Karner *et al*., 2001), yet until recently their likely functional role(s) was unknown. The 2005 isolation in pure culture of an ammonia-oxidizing marine crenarchaeote (Könneke *et al*., 2005) confirmed earlier predictions from environmental genomics studies (Venter *et al*., 2004; Treusch *et al*., 2005) that crenarchaeotes could be capable of chemolithoautotrophic ammonia oxidation. Subsequent recovery of archaeal ammonia monooxygenase (*amoA*) gene sequences from varied seawater and sediment samples, together with compelling abundance and process data, indicates that archaeal ammonia oxidation may be widespread and important in marine systems (Francis *et al*., 2005; Ingalls *et al*., 2006; Wuchter *et al*., 2006; Coolen *et al*., 2007; Lam *et al*., 2007; Mincer *et al*., 2007).

Given the frequent association of *Crenarchaeota* with marine sponges, together with evidence of nitrification within these host organisms (Corredor *et al*., 1988; Diaz and Ward, 1997; Bayer *et al*., 2007), it seems likely that ammonia-oxidizing archaea (AOA) should occur in sponges. Indeed, Hallam and coworkers recently reported the presence of *amo* gene homologues within the sequenced genome of the sponge symbiont

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Table 1. Details of sponge samples used for this study.

Sponge species	Order/Family	Collection location (latitude, longitude)
Axinella polypoides	*Halichondrida/Axinellidae*	Rovinj, Croatia (45°05′N, 13°38′E)
Chondrosia reniformis	*Chondrosida/Chondrillidae*	Rovinj, Croatia (45°05′N, 13°38′E)
Aplysina insularis	*Verongida/Aplysinidae*	Little San Salvador Island, Bahamas (24°35′N, 75°58′W)
Cliona sp.	*Hadromerida/Clionaidae*	Little San Salvador Island, Bahamas (24°35′N, 75°58′W)
Plakortis sp.	*Homosclerophorida/Plakinidae*	Little San Salvador Island, Bahamas (24°35′N, 75°58′W)
Coscinoderma sp.	*Dictyoceratida/Spongiidae*	Orpheus Island, Queensland, Australia (18°36′S, 146°29′E)
Luffariella variabilis	*Dictyoceratida/Thorectidae*	Orpheus Island, Queensland, Australia (18°36′S, 146°29′E)
Rhopaloeides odorabile	*Dictyoceratida/Spongiidae*	Pelorus Island, Queensland, Australia (18°34′S, 146°29′E)
Callyspongia sp.	*Haplosclerida/Callyspongiidae*	Bare Island, Sydney, Australia (33°59′S, 151°14′E)
Siphonochalina sp.	*Haplosclerida/Callyspongiidae*	Bare Island, Sydney, Australia (33°59′S, 151°14′E)
Stylinos sp.	*Halichondrida/Halichondriidae*	Bare Island, Sydney, Australia (33°59′S, 151°14′E)

In all cases, three individuals of each sponge species were collected. All listed sponges are in the class *Demospongiae*.

'*C. symbiosum*' (Hallam *et al.*, 2006a,b). Also present in the '*C. symbiosum*' genome are other genes encoding components associated with the ammonia oxidation pathway, including urease and nitrite reductase. Intriguingly, no homologue was found for hydroxylamine oxidoreductase (*hao*), a key component of ammonia oxidation in bacteria (Hallam *et al.*, 2006a).

Confirmation of the occurrence of AOA within a marine sponge brings with it many questions. For example, are AOA associated with many sponges, or merely limited to the '*C. symbiosum*'–*A. mexicana* symbiosis? What is the magnitude of AOA diversity within sponges, that is do different phylotypes occur or are they all phylogenetically closely affiliated with '*C. symbiosum*'? How are AOA transferred between generations of the host sponge? Addressing such questions is important in order to provide a sound framework for subsequent, detailed studies of nitrification in sponges. The accumulation of metabolic wastes (including ammonia and nitrite) may have adverse effects on sponge health, with evidence for the detrimental impact of nitrite on juvenile sponge development (Kahlert and Neumann, 1997). The ability to metabolize nitrogenous waste products may thus be the basis for a mutualistic relationship between ammonia and nitrite oxidizers and marine sponges, and it is important to improve our understanding of the basic ecological features of the sponge–AOA association.

Results and discussion

To establish whether AOA are frequent associates of marine sponges, we screened sponges sampled from the Caribbean, Mediterranean and western Pacific, for the presence of archaeal *amoA* genes. Although possession of this gene does not provide unequivocal proof that a given organism can oxidize ammonia, it is strongly suggestive and we can label such archaea as putative ammonia oxidizers. *amoA* sequences were obtained from each of three tested individuals of the Caribbean sponges *Aplysina insularis*, *Cliona* sp. and *Plakortis* sp., the Mediterranean sponges *Axinella polypoides* and *Chondrosia reniformis*, the Great Barrier Reef sponges *Coscinoderma* sp., *Luffariella variabilis* and *Rhopaloeides odorabile* and the south-eastern Australian sponges *Callyspongia* sp., *Siphonochalina* sp. and *Stylinos* sp. (Table 1). All of these sponges are abundant at their respective collection locations. There was no clear biogeographic effect, with many of the sponge-derived sequences forming a large monophyletic cluster comprising sequences from the Great Barrier Reef, the Bahamas, the Mediterranean and California (= '*C. symbiosum*') (Fig. 1). Also present in this cluster are several coral-derived crenarchaeotal *amoA* sequences (Beman *et al.*, 2007). Although the relatively limited archaeal *amoA* data set ($n = 1547$ in the database we used) currently available constrains speculation, this putatively 'sponge- and coral-specific' crenarchaeotal cluster is reminiscent of those reported previously for the 16S rRNA gene (Taylor *et al.*, 2007; see also Fig. 2). It will be of interest to see whether the cluster remains intact once more marine *amoA* data are obtained, but it is noteworthy that none of the more than 920 *amoA* sequences from marine sediments and water samples, including 45 *amoA* sequences deposited in the marine metagenomic data sets (Venter *et al.*, 2004; Rusch *et al.*, 2007; Seshadri *et al.*, 2007), are members of this cluster. Other sponge-derived *amoA* sequences obtained in this study were similar to those from other marine environments. Several sponges contained more than one *amoA* sequence type (Fig. 1). Overall, *amoA* variability in sponges was relatively low (lowest similarity value of two retrieved sponge-derived *amoA* sequences was 94.7% amino acid identity), even among sequences obtained from different oceans, although it cannot be ruled out that further sequencing may have uncovered a greater diversity of phylotypes. The recognized *amoA* diversity of sponges is lower than that of corals and marine sediments, while a comparison to the AOA *amoA* diversity retrieved from the marine water column revealed a more

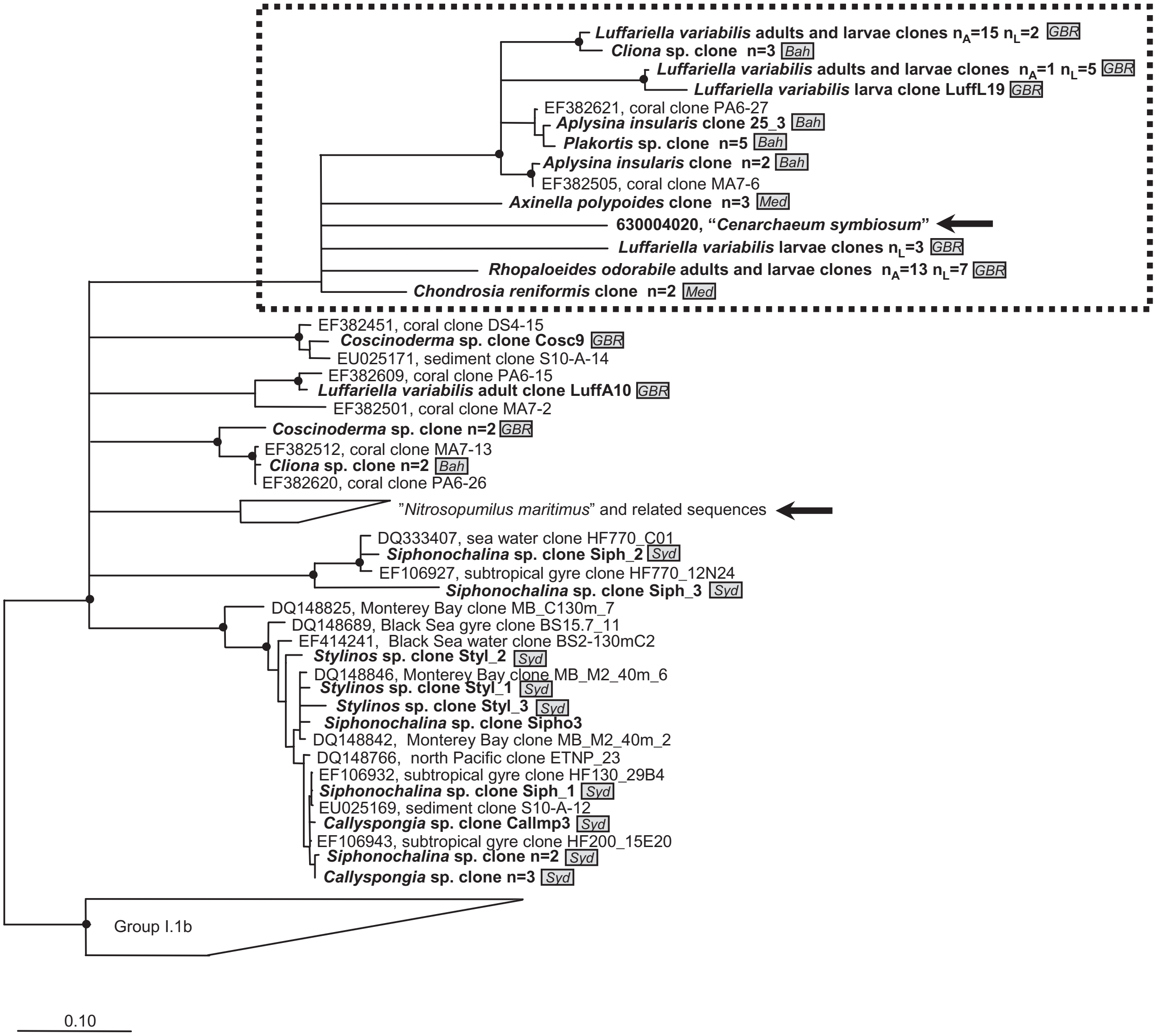

Fig. 1. *amoA*-based phylogenetic consensus tree of AOA affiliated with the marine group I.1a *Crenarchaeota* including sponge-derived sequences (in bold). Polytomies indicate that respective branching order could not be unambiguously resolved by different treeing methods. Codes in shaded boxes indicate collection location: Bah = Bahamas, GBR = Great Barrier Reef, Med = Mediterranean, Syd = Sydney. The large box represents a monophyletic cluster consisting exclusively of sponge- and coral-derived sequences. n_L = number of identical sequences obtained from the respective larvae; n_A = number of identical sequences obtained from adult sponges. Arrows indicate the two known ammonia-oxidizing archaea, '*Candidatus* Cenarchaeum symbiosum' and '*Cand.* Nitrosopumilus maritimus'. Filled circles indicate parsimony bootstrap values (100 resamplings) of 90–100%. Bar, 10% sequence divergence. For sequences obtained during this study, DNA extractions were performed on 5 mg of freeze-dried sponge tissue using the UltraClean Soil DNA Kit (MoBio Laboratories, USA), followed by PCR modified slightly from the protocol of Francis and colleagues (Francis *et al*., 2005). The cycling conditions were: 94°C for 3 min; 35 cycles of 94°C for 40 s, 53°C for 40 s, 72°C for 40 s; 72°C for 20 min. Cloning was carried out using the TopoTA Cloning Kit (Invitrogen, USA). The displayed consensus tree was constructed based on distance (Fitch), maximum parsimony and maximum likelihood phylogenetic analyses conducted in ARB (Ludwig *et al*., 2004), with 585 characters used for tree construction. The tree was calculated with a larger data set than displayed, but to improve clarity those clusters (with the exception of the '*N. maritimus*' cluster) which did not contain sponge-derived sequences were removed after calculation.

complicated picture (Fig. 3). More *amoA* operational taxonomic units (OTUs) are found in sponges than in marine water samples, if a nucleic acid sequence similarity threshold of 80–85% is applied, while at the microdiversity level (sequence similarity threshold for OTU definition > 90%) the OTU number of planktonic AOA by far exceeds the one observed in sponges. For ammonia-oxidizing bacteria 80% sequence similarity of the *amoA* gene can be used as a threshold for species differentiation (Purkhold *et al*., 2000). Under the assumption that this

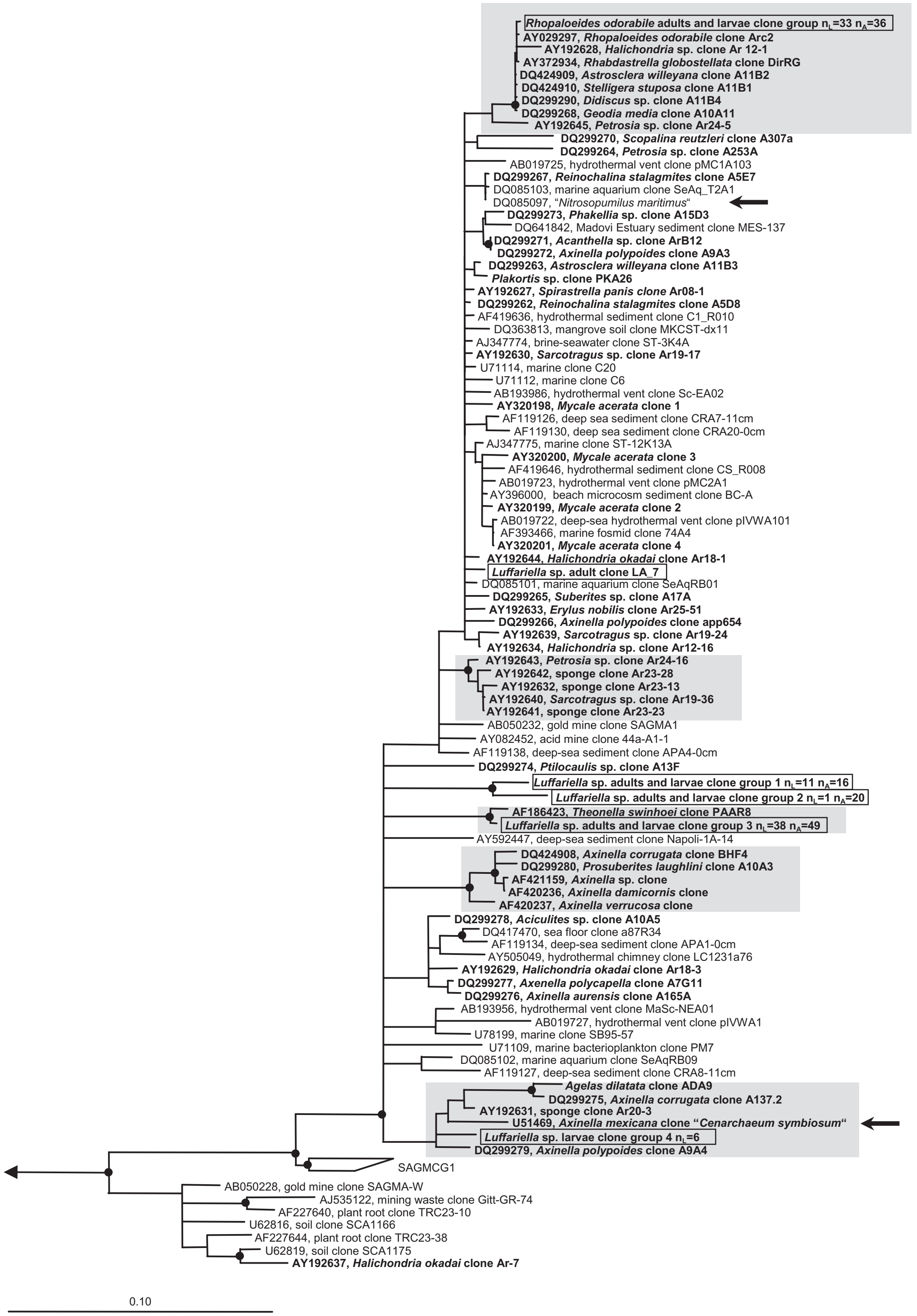
Rhopaloeides odorabile adults and larvae clone group n_L=33 n_A=36
AY029297, Rhopaloeides odorabile clone Arc2
AY192628, Halichondria sp. clone Ar 12-1
AY372934, Rhabdastrella globostellata clone DirRG
DQ424909, Astrosclera willeyana clone A11B2
DQ424910, Stelligera stuposa clone A11B1
DQ299290, Didiscus sp. clone A11B4
DQ299268, Geodia media clone A10A11
AY192645, Petrosia sp. clone Ar24-5
DQ299270, Scopalina reutzleri clone A307a
DQ299264, Petrosia sp. clone A253A
AB019725, hydrothermal vent clone pMC1A103
DQ299267, Reinochalina stalagmites clone A5E7
DQ085103, marine aquarium clone SeAq_T2A1
DQ085097, "Nitrosopumilus maritimus"
DQ299273, Phakellia sp. clone A15D3
DQ641842, Madovi Estuary sediment clone MES-137
DQ299271, Acanthella sp. clone ArB12
DQ299272, Axinella polypoides clone A9A3
DQ299263, Astrosclera willeyana clone A11B3
Plakortis sp. clone PKA26
AY192627, Spirastrella panis clone Ar08-1
DQ299262, Reinochalina stalagmites clone A5D8
AF419636, hydrothermal sediment clone C1_R010
DQ363813, mangrove soil clone MKCST-dx11
AJ347774, brine-seawater clone ST-3K4A
AY192630, Sarcotragus sp. clone Ar19-17
U71114, marine clone C20
U71112, marine clone C6
AB193986, hydrothermal vent clone Sc-EA02
AY320198, Mycale acerata clone 1
AF119126, deep sea sediment clone CRA7-11cm
AF119130, deep sea sediment clone CRA20-0cm
AJ347775, marine clone ST-12K13A
AY320200, Mycale acerata clone 3
AF419646, hydrothermal sediment clone CS_R008
AB019723, hydrothermal vent clone pMC2A1
AY396000, beach microcosm sediment clone BC-A
AY320199, Mycale acerata clone 2
AB019722, deep-sea hydrothermal vent clone pIVWA101
AF393466, marine fosmid clone 74A4
AY320201, Mycale acerata clone 4
AY192644, Halichondria okadai clone Ar18-1
Luffariella sp. adult clone LA_7
DQ085101, marine aquarium clone SeAqRB01
DQ299265, Suberites sp. clone A17A
AY192633, Erylus nobilis clone Ar25-51
DQ299266, Axinella polypoides clone app654
AY192639, Sarcotragus sp. clone Ar19-24
AY192634, Halichondria sp. clone Ar12-16
AY192643, Petrosia sp. clone Ar24-16
AY192642, sponge clone Ar23-28
AY192632, sponge clone Ar23-13
AY192640, Sarcotragus sp. clone Ar19-36
AY192641, sponge clone Ar23-23
AB050232, gold mine clone SAGMA1
AY082452, acid mine clone 44a-A1-1
AF119138, deep-sea sediment clone APA4-0cm
DQ299274, Ptilocaulis sp. clone A13F
Luffariella sp. adults and larvae clone group 1 n_L=11 n_A=16
Luffariella sp. adults and larvae clone group 2 n_L=1 n_A=20
AF186423, Theonella swinhoei clone PAAR8
Luffariella sp. adults and larvae clone group 3 n_L=38 n_A=49
AY592447, deep-sea sediment clone Napoli-1A-14
DQ424908, Axinella corrugata clone BHF4
DQ299280, Prosuberites laughlini clone A10A3
AF421159, Axinella sp. clone
AF420236, Axinella damicornis clone
AF420237, Axinella verrucosa clone
DQ299278, Aciculites sp. clone A10A5
DQ417470, sea floor clone a87R34
AF119134, deep-sea sediment clone APA1-0cm
AY505049, hydrothermal chimney clone LC1231a76
AY192629, Halichondria okadai clone Ar18-3
DQ299277, Axenella polycapella clone A7G11
DQ299276, Axinella aurensis clone A165A
AB193956, hydrothermal vent clone MaSc-NEA01
AB019727, hydrothermal vent clone pIVWA1
U78199, marine clone SB95-57
U71109, marine bacterioplankton clone PM7
DQ085102, marine aquarium clone SeAqRB09
AF119127, deep-sea sediment clone CRA8-11cm
Agelas dilatata clone ADA9
DQ299275, Axinella corrugata clone A137.2
AY192631, sponge clone Ar20-3
U51469, Axinella mexicana clone "Cenarchaeum symbiosum"
Luffariella sp. larvae clone group 4 n_L=6
DQ299279, Axinella polypoides clone A9A4
SAGMCG1
AB050228, gold mine clone SAGMA-W
AJ535122, mining waste clone Gitt-GR-74
AF227640, plant root clone TRC23-10
U62816, soil clone SCA1166
AF227644, plant root clone TRC23-38
U62819, soil clone SCA1175
AY192637, Halichondria okadai clone Ar-7
0.10

Fig. 2. 16S rRNA gene-based phylogenetic consensus tree displaying the affiliation of sponge-associated *Crenarchaeota* within Marine Group I. Shaded boxes represent sponge-specific monophyletic clusters *sensu* Hentschel and colleagues (Hentschel *et al.*, 2002). Other boxes signify *Rhopaloeides odorabile* or *Luffariella variabilis* sequences, with n_L = number of identical sequences obtained from the respective larvae and n_A = number of identical sequences obtained from adult sponges. Arrows indicate the two known ammonia-oxidizing archaea, '*Candidatus* Cenarchaeum symbiosum' and '*Cand.* Nitrosopumilus maritimus'. Filled circles indicate parsimony bootstrap values of 90–100%. Bar, 10% sequence divergence. The 16S rRNA gene was amplified using the Arch21F/Arch958R primers (DeLong, 1992) with the following PCR cycling conditions: 94°C for 3 min; 35 cycles of 94°C for 40 s, 55°C for 40 s, 72°C for 1 min; 72°C for 20 min. 879 characters were used for tree construction.

rule can also be applied for AOA, these findings indicate that at the species level more AOA occur in sponges than in the marine water column (note that 302 marine water column sequences and only 83 sponge-related *amoA* sequences are available; Fig. 3b), while in the latter environment there is apparently an enormous strain-level diversity of a few species.

Elucidating the mechanism(s) by which symbionts are acquired by a host is an important goal in symbiosis research. Numerous microscopy- and molecular-based studies have demonstrated transmittance of diverse microbial assemblages in sponge reproductive stages (eggs, sperm, larvae) (Usher *et al.*, 2001; 2005; Ereskovsky *et al.*, 2005; Enticknap *et al.*, 2006; Schmitt *et al.*,

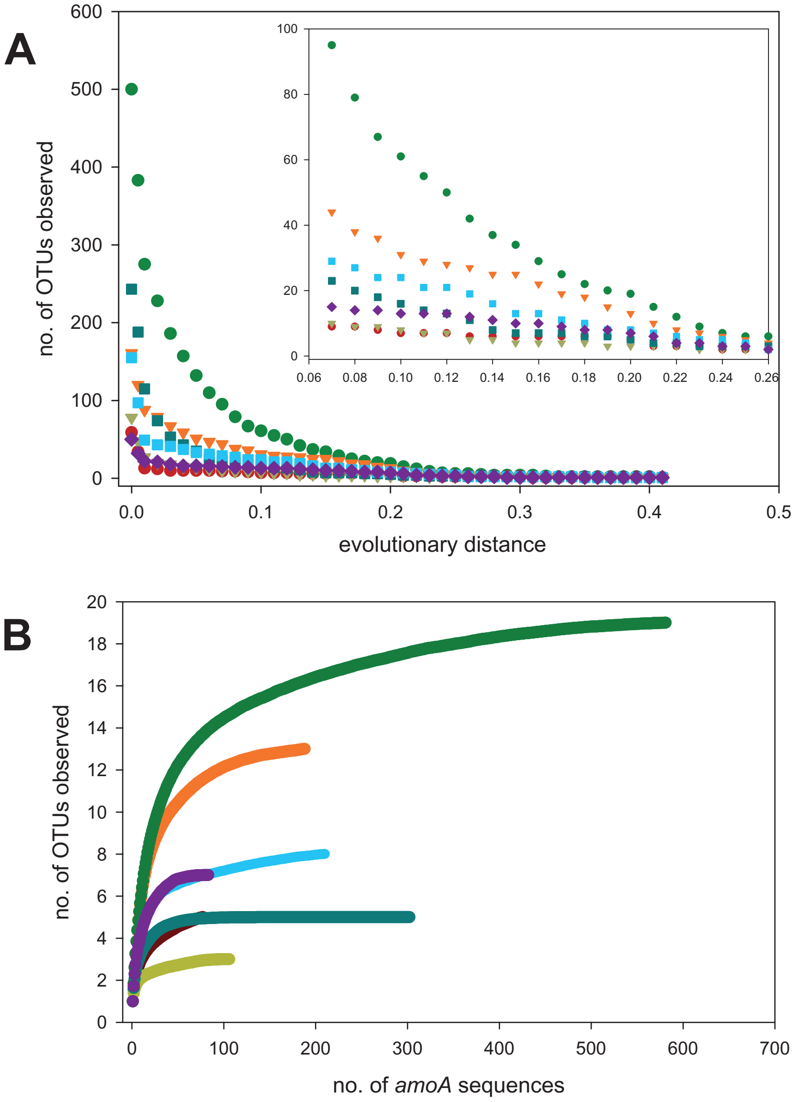

Fig. 3. Archaeal *amoA* diversity in various environments.
A. The number of *amoA* operational taxonomic units (OTUs) is plotted against the evolutionary distance threshold used for OTU definition. The inset displays a selected region of the graph to enhance clarity.
B. Rarefaction analysis of the *amoA* sequences using an evolutionary distance threshold of 20% (i.e. 80% similarity) for OTU definition. The analysis was performed using DOTUR (Schloss and Handelsman, 2005). Symbols represent the following environments: (◆) sponge, (■) coral, (■) water column, (▼) thermal environments, (▼) soil, (●) activated sludge, (●) sediment. The 45 archaeal *amoA* sequences from the metagenome projects were excluded from these analyses owing to a relatively low sequence quality which would create microdiversity artefacts.

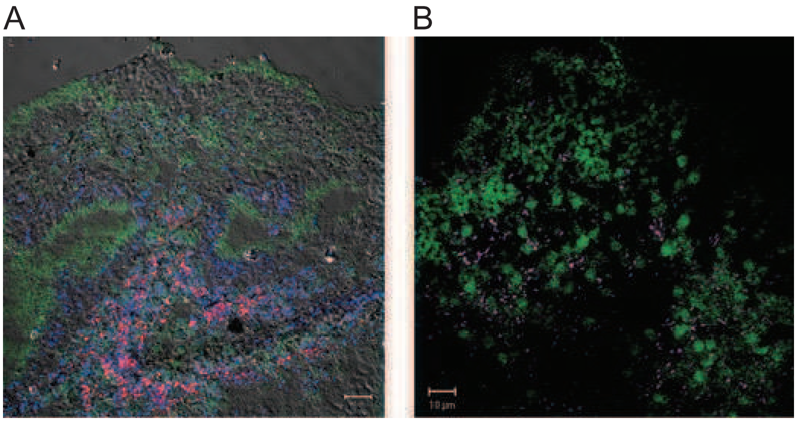

Fig. 4. FISH visualization of *Crenarchaeota* in *Luffariella variabilis* larvae.
A. Blue – *Archaea* detected by Cy5-labelled Arch915 probe (Stahl and Amann, 1991). Red – Bacteria [Cy3-labelled with the probe set EUB338(I–III) (Daims *et al.*, 1999)]. Green – background fluorescence from sponge larva.
B. Blue – *Archaea* (Cy5-labelled with probe Arch915). Red – *Crenarchaeota* [Cy3-labelled with probe CREN512 (Jurgens *et al.*, 2000)]. Note that pink signal indicates cohybridization of Arch915 and CREN512 probes. Green – background fluorescence from larva.

2007; Sharp *et al.*, 2007). In the tropical sponge *Corticium* sp., this assemblage includes archaea (Sharp *et al.*, 2007). However, to our knowledge there exists no information as to whether AOA are transmitted via the reproductive stages. To address this issue, we collected both adult and larval samples from two viviparous Great Barrier Reef sponges, *L. variabilis* (Ettinger-Epstein *et al.*, 2007) and *Rhopaloeides odorabile* (Whalan *et al.*, 2007). The latter species harbours a dense and diverse microbial community including archaea (Webster *et al.*, 2001a,b), whereas nothing is known about the microbiology of *L. variabilis*. Larvae and corresponding individual parent sponges were sampled during the 2005–2006 austral summer, with larvae collected by placing larval traps over each individual sponge following the method of Lindquist and colleagues (1997). Larvae were collected and either frozen (for DNA analyses), or preserved in 3% paraformaldehyde or 96% ethanol [for fluorescence *in situ* hybridization (FISH); detailed in Daims *et al.* 2005] within 2 h of release.

We PCR-amplified the archaeal *amoA* and 16S rRNA genes from DNA extracts of freeze-dried adult and larval samples of both sponge species. Phylogenetic analyses revealed the same crenarchaeotal 16S rRNA phylotype [previously reported from this sponge (Webster *et al.*, 2001a)] in both adults and larvae of *R. odorabile* (Fig. 2). Similarly, a single, identical *amoA* sequence type was recovered from *R. odorabile* adults and corresponding larvae (Fig. 1). Both *amoA*- and 16S rRNA-based analyses indicated a more diverse AOA community in *L. variabilis* (Figs 1 and 2). Again, in most cases the same sequence types were found in adults and larvae, suggesting that AOA are present in the larvae of these two sponges. To confirm that archaea were present *within* the larvae, and did not represent surface contaminants from the surrounding seawater, we performed FISH with archaeal and crenarchaeal 16S rRNA-targeted oligonucleotide probes. By examining 6 μm sections of larvae which had been embedded in Neg-50 (Microm, Richard-Allan Scientific) and frozen, we were able to visualize *Bacteria* and *Crenarchaeota* in larvae from *L. variabilis*. Most of these microorganisms were located in the central part of the larvae (Fig. 4). However, because of unexpectedly rapid deterioration of the fixed larvae samples at –20°C we were unable to perform quantitative FISH or to use FISH for either the study of *R. odorabile* larvae, or for the application of newly designed probes targeting specific sequence types in *L. variabilis*. Unlike many marine invertebrates, the larvae of sponges are lecithotrophic, or non-feeding (Maldonado, 2004; Mariani *et al.*, 2006). Sponge larvae thus rely upon finite stored nutrients for nourishment, and there exists no evidence that they are capable of feeding upon microorganisms from the surrounding seawater. We are therefore confident that larval transmission of AOA is indeed occurring, and that it is not merely diet-derived archaea that were detected in the larvae by FISH.

Taken together, our data suggest that AOA may be frequently associated with marine sponges. Their apparent transmission in the larval stages of at least one host species is suggestive of a possible symbiotic role for these microorganisms. Further work is needed to determine the contribution of AOA to the nitrification process within sponges, and to establish whether – as shown for some other environments (Leininger *et al.*, 2006; Wuchter *et al.*, 2006) – archaea and not bacteria are the numerically dominant ammonia oxidizers in marine sponges.

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Part IV

Summary

Summary

In the present thesis, functional groups of microorganisms were examined in two extremely different ecosystems known for their highly complex microbial community structure.

The first part of this work deals with the abundance, community dynamics, and biogeography of *dsrAB*-carrying microorganisms in terrestrial wetlands. The dissimilatory (bi-) sulfite reductase (Dsr) is present in microorganisms that reduce sulfate, sulfite or some organosulfonates or in anaerobic syntrophs. Microorganisms with phylogenetically novel variants of the dissimilatory (bi-)sulfite reductase genes *dsrAB* were identified earlier in the Schlöppnerbrunnen fen (Lehstenbach catchment, Germany). Using newly developed molecular tools to analyze 16S rRNA and *dsrAB* gene sequences, a temporally stable but spatially variable composition of the putative sulfate reducing populations could be detected in the highly diverse and *Acidobacteria* dominated fen. Thereby, the proportion of yet uncharacterized deep-branching *dsrAB* sequences outnumbered the known syntrophic *dsrAB* lineages. Furthermore, denaturing gradient gel electrophoresis (DGGE) and clone library-based analyses of eight additional wetlands confirmed the widespread occurrence of these deep-branching *dsrAB* lineages in peatlands. The *dsrB*-DGGE pattern was not correlated with geographic distance, but observed variability could be largely explained by measured environmental conditions.

In the second part of the thesis, the use of DNA microarray based methods uncovered significant variations in detected signal intensities of these nucleic acid based hybridization assays. DNA microarrays are regularly used in science as well as in clinical diagnosis for high-throughput multiplex analyte detection. By a simplified array format, systematically arranged spatial gradients were detected, which halved the number of detected targets from the edge to the center of the assay. Additionally, experimental data were confirmed by simulation of passive mass transport indicating diffusion limitations of the target molecules as causing mechanism.

In the last part of this work the microbial communities inhabiting marine sponges were investigated. A comprehensive analysis of all publicly available sponge derived 16S rRNA sequences and further 184 sequences of three hitherto unstudied sponges confirmed the presence of monophyletic, sponge-specific 16S rRNA sequence clusters, with approximately one-third of all sponge-derived sequences falling into such clusters. Furthermore, phylogenetic

analyses based on 16S rRNA and ammonia monooxygenase genes and fluorescence in situ hybridization revealed the presence of putative ammonia-oxidizing archaea (AOA) in a diverse range of sponges from the western Pacific, Caribbean and Mediterranean. The presence of AOA in sponge larvae suggests they are vertically transmitted and thus might be of importance for ammonia detoxification within the sponge.

Zusammenfassung

In der vorliegenden Arbeit wurden funktionelle Gruppen von Mikroorganismen in zwei äußerst unterschiedlichen Ökosystemen, die beide eine komplexe mikrobielle Gemeinschaftsstruktur aufweisen, untersucht.

Der erste Teil dieser Arbeit befasst sich mit der Häufigkeit, Populationsdynamik und Verbreitung von *dsrAB*-tragenden Mikroorganismen in terrestrischen Feuchtgebieten. Mikroorganismen, die Sulfat, Sulfit oder Organosulfonate reduzieren beziehungsweise in syntrophen Gemeinschaften leben, können eine dissimilatorische (Bi-) Sulfit-Reduktase (Dsr) besitzen. Neue, tiefzweigende *dsrAB*-Sequenzen wurden bereits in früheren Studien im Untersuchungsgebiet Schlöppnerbrunnen (Bayern, Deutschland) nachgewiesen. Unter Verwendung verschiedener molekularbiologischer Techniken zur Analyse von 16S-rRNS- und *dsrAB*-Gensequenzen, konnte in diesem hoch diversen und von *Acidobacteria* dominierten Moor eine zeitlich stabile, räumlich jedoch variable Zusammensetzung der potentiell sulfatreduzierenden Populationen gezeigt werden. Hierbei waren die bisher nicht charakterisierten, tiefzweigenden *dsrAB*-Sequenzen signifikant häufiger als die bekannten syntrophen DsrAB-Linien. Das verbreitete Vorkommen dieser tiefzweigenden DsrAB-Linien wurde mittels *dsrB* basierter denaturierender Gradientengelelektrophorese (DGGE) in acht weiteren Feuchtgebieten bestätigt. Hierbei konnte das detektierte *dsrB*-DGGE Bandenmuster nicht durch die geographische Distanz der Feuchtgebiete, sondern durch die vorherrschenden Umweltbedingungen erklärt werden.

Im zweiten Teil dieser Arbeit deckte die Anwendung von DNS-Mikroarray basierten Methoden signifikante Schwankungen der detektierten Fluoreszenzsignale in diesen nukleinsäurebasierten Hybridisierungsassays auf. DNS-Mikroarrays werden regelmäßig in der Wissenschaft sowie zur klinischen Diagnose als Hochdurchsatzanalyse komplexer Proben verwendet. Ein vereinfachtes Arraylayout ermöglichte den Nachweis von systematisch angeordneten räumlichen Gradienten in der Signalintensität, wobei die Anzahl an detektierten Zielmolekülen vom Rand zur Mitte des Mikroarrays halbieren wurde. Modellsimulationen wiesen auf Limitierungen im Massetransport der Zielmoleküle als mögliche Ursache hin.

Im letzten Teil dieser Arbeit wurden die mikrobiellen Gemeinschaften in marinen Schwämmen untersucht. Eine umfassende Analyse aller öffentlich verfügbaren 16S-rRNS-Sequenzen aus

Schwämmen und weitere 184 Sequenzen von drei bisher nicht untersuchten Schwämmen, bestätigte das Vorhandensein monophyletischer, Schwamm-spezifischer 16S-rRNS Gensequenz-Cluster, wobei etwa ein Drittel der analysierten Sequenzen zu diesen Clustern zugeordnet werden konnte. Darüber hinaus zeigten 16S-rRNS- und Ammonium Monooxygenase-Gen als auch Fluoreszenz in situ Hybridisierung basierte Analysen, die Anwesenheit von potentiell ammoniumoxidierenden *Archaea* (AOA) in marinen Schwämmen aus dem westlichen Pazifik, der Karibik und des Mittelmeers. Das zusätzliche Vorhandensein dieser AOAs in den entsprechenden Schwamm-Larven weist darauf hin, dass AOAs in Schwämmen vertikal übertragen werden.

APPENDIX

Publications

Sebastian Lücker, Doris Steger, Kasper Urup Kjeldsen, Barbara J. MacGregor, Michael Wagner and Alexander Loy. 2007. Improved 16S rRNA-targeted probe set for analysis of sulfate-reducing bacteria by fluorescence in situ hybridization. *J. Microbiol. Methods.* **69**: 523-528.

Michael Taylor, Regina Radax, Doris Steger and Michael Wagner. 2007. Sponge-Associated Microorganisms: Evolution, Ecology, and Biotechnological Potential. *Microbiol. Mol. Biol. Rev.* **71**: 295-347

Doris Steger, Piers Ettinger-Epstein, Stephen Whalan, Ute Hentschel, Rocky de Nys, Michael Wagner and Michael Taylor. 2008. Diversity and mode of transmission of ammonia-oxidizing archaea in marine sponges. *Environ. Microbiol.* **10**: 1087-1094.

Alexander Loy, Michael Pester and Doris Steger. Phylogenetic Microarrays for Cultivation-Independent Identification and Metabolic Characterization of Microorganisms in Complex Samples. Book chapter in *PCR Mutation Detection Protocols*, Springer-Verlag. *In review.*

Doris Steger, Cecilia Wentrup, Michael Wagner and Alexander Loy. Dynamics of an unusual sulfate reducing population within a highly diverse microbial fen community. *To be submitted.*

Doris Steger, Roman Stocker, Michael Wagner und Alexander Loy. Spatial gradients govern nucleic acid surface hybridizations. *In preparation.*

Oral presentations

Doris Steger, Michael Wagner and Alexander Loy. Diversity of *apsA* genes in a shallow hydrothermal vent system. *5th International meeting on Novel Techniques In Microbial Ecology*, Lunz am See, Austria, June 2005

Doris Steger, Cecilia Wentrup, Manuel Hofer, Michael Wagner and Alexander Loy. RING-FISH and qPCR in Schlöppnerbrunnen samples. *6th International workshop on New Techniques In Microbial Ecology*, Sæby, Denmark, October 2007

Doris Steger, Cecilia Wentrup, Manuel Hofer, Michael Pester, Michael Wagner and Alexander Loy. New insights in the sulfate reducing community of an acidic fen system. *VAAM Jahrestagung*, Frankfurt, Germany, March 2008

Poster presentations

Doris Steger, Stephanie Füreder, Stephan Duller, Michael Wagner, Monika Bright and Alexander Loy. Shallow Water Hydrothermal Vents at the Island of Dominica (Caribbean Sea) are Diversity Hot Spots of Sulfur Prokaryotes. *ChEss workshop: Atlantic Equatorial Belt*, Barcelona, Spain, March 2006

Michael Taylor, Doris Steger, Regina Radax and Michael Wagner. Marine benthic eukaryotes as reservoirs of microbial diversity. *International Census of Marine Microbes*, Noordwijkerhout, the Netherlands, June 2006

Doris Steger, Regina Radax, Steven Whalan, Piers Ettinger-Epstein, Rocky de Nys, Michael Wagner and Michael Taylor. Ammonia-oxidizing archaea are widespread in marine sponges. *11th International symposium on Microbial Ecology*, Vienna, Austria, August 2006.

Regina Radax, Doris Steger, Michael Wagner and Michael Taylor. Phylogeny of all known sponge-associated prokaryotes: do sponge-specific clusters really exist? *11th International symposium on Microbial Ecology*, Vienna, Austria, August 2006.

Curriculum vitae

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