DNA Microarray Technology

for Biodiversity Inventories

of Sulfate Reducing Prokaryotes

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ABBREVIATIONS

apsA	gene encoding alpha subunit of adenosine-5`-phosphosulfate reductase
ApsA	alpha subunit of adenosine-5`-phosphosulfate reductase
BLAST	Basic Local Alignment Search Tool
bp	base pairs
Cy5	5,5'-disulfo-1,1'-di(X-carbopentynyl)-3,3,3',3'-tetramethyindole-Cy5.18-derivative, N-hydroxysuccimidester
Cy5-dCTP	5-amino-propargyl-2'-deoxycytidine 5'-triphosphate coupled to Cy5 fluorescent dye
cDNA	complementary deoxyribonucleic acid
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
dsrAB	genes encoding alpha and beta subunit of dissimilatory (bi)sulfite reductase
DsrAB	alpha and beta subunits of dissimilatory (bi)sulfite reductase
dCTP	deoxycytidine 5'-triphosphate
dNTPs	deoxynucleotide triphosphates
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures)
EDTA	ethylenediamine tetraacetic acid
FISH	fluorescence *in situ* hybridization
kb	kilobases
LSU	large-subunit
mg	milligram
ml	milliliter
ng	nanogram
OPD	Oligonucleotide Probe Database
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pmol	picomole
RDP	Ribosomal Database Project
rRNA	ribosomal ribonucleic acid
RSGP	reverse sample genome probing
SDS	sodium dodecyl sulfate
SSU	small-subunit
SRAs	sulfate-reducing archaea
SRBs	sulfate-reducing bacteria
SRPs	sulfate-reducing prokaryotes
SSC	standard sodium citrate
TMAC	tetramethylammonium chloride
µg	microgram
µl	microliter

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INTRODUCTION

I. Classification and phylogeny of sulfate-reducing prokaryotes

Taxonomic considerations concerning dissimilatory sulfate-reducing bacteria (SRBs) began in 1895 with Beijerink's first isolation of a strict anaerobic, sulfate-reducing bacterium, which he termed *Spirillum desulfuricans* (Beijerinck 1895). *Vibrio* (Baars 1930) was a synonymous genus name for *Spirillum desulfuricans* for which finally the genus *Desulfovibrio* (*D. desulfuricans* as the type species) was established by Kluyver and van Niel (1936). The early history on the classification of *Desulfovibrio desulfuricans* already reflected the problems of continual reclassifications and amendments that microbial taxonomists faced over years of research on SRB systematics.

In 1925, Elion was the first to describe the thermophilic sulfate-reducing bacterium *Vibrio thermodesulfuricans* (Elion 1925). The capability of some SRBs to form endospores was initially recognized for the thermophiles *Clostridium nigrificans* (Werkman and Weaver 1927) and *Sporovibrio desulfuricans* (Starkey 1938). Later, Campbell *et al.* demonstrated that both bacteria were members of the same species (Campbell *et al.* 1957). The continuous accumulation of newly described SRBs demanded thorough (re)classification of all existing strains. As a result, all non-sporulating SRBs were assigned to the vibrio-shaped genus *Desulfovibrio* (Postgate and Campbell 1966), whereas the endospore-forming species formed the new sausage-shaped genus *Desulfotomaculum* (Campbell and Postgate 1965). At that time it was thought that SRBs comprise a small and nutritionally limited guild, growing preferentially on electron donors such as lactate and pyruvate that are incompletely oxidized to acetate. This point of view changed considerably with the description of new types of SRBs capable of completely oxidizing acetate, higher fatty acids, or aromatic compounds (Bak and Widdel 1986a, Bak and Widdel 1986b, Brysch *et al.* 1987, Pfennig and Widdel 1981, Pfennig *et al.* 1981, Widdel 1980, Widdel *et al.* 1983, Widdel and Pfennig 1977, Widdel and Pfennig 1981a, Widdel and Pfennig 1981b, Widdel and Pfennig 1982). In addition, the novel genus *Thermodesulfobacterium* was established for thermophilic SRBs which were isolated from hot aquatic habitats in the Yellowstone National Park (USA), contained unusual ether lipids, and were phylogenetically distinct from previously known SRBs (Langworthy *et al.* 1983, Zeikus *et al.* 1983). Studies by Stetter *et al.* on hyperthermophiles led to first description of the archaeal genus *Archaeoglobus* and demonstrated that the capacity for dissimilatory sulfate reduction is not restricted to the bacterial domain of life (Achenbach-Richter *et al.* 1987, Burggraf *et al.* 1990, Stetter *et al.* 1987).

Traditional classification of sulfate-reducing prokayotes (SRPs) relied on (i) phenotypic characteristics such as nutrition and morphology and (ii) biochemical properties such as the presence of desulfoviridin, lipid fatty acids, or menaquinones. The discovery of ribosomal RNA (rRNA) as the ultimate universal molecular chronometer set the basis for modern prokaryotic phylogeny and taxonomy (Fox *et al.* 1980, Woese 1987). Together with the advent of the nucleic acid sequencing era, comparative 16S rRNA sequence analysis became decisive for the inference of natural relationships among prokaryotes, consequently for SRPs, too. Early applications of this novel taxonomic approach demonstrated that the delta subdivision of the phylum purple bacteria harbored bacteria with different phenotypes like sulfate-reducing bacteria (represented by *Desulfovibrio desulfuricans* and *Desulfobacter postgatei*), sulfur-reducing bacteria, myxobacteria and relatives, and bdellovibrios (Oyaizu and Woese 1985, Woese 1987). Later, all members of the phylum purple bacteria were reclassified into the new class *Proteobacteria* (Stackebrandt *et al.* 1988). A more comprehensive phylogenetic study of 20 nonsporeforming and two endospore-forming SRBs based on comparison of nearly complete 16S rRNA sequences was performed by Devereux *et al.* (1989). This study confirmed the classification of the genus *Desulfotomaculum* within the gram-positive bacteria as suggested previously by 16S rRNA oligonucleotide cataloging (Fowler *et al.* 1986). Among the nonsporeforming deltaproteobacterial species seven natural groups could be defined. Although this grouping was generally consistent with the existent physiology-based classification, the need for taxonomic revision was obvious. A similar study, focussing on phylogeny of *Desulfovibrio* species, revealed further misclassified species and strains (Devereux *et al.* 1990). Additionally, the monophyletic origin of the genuine *Desulfovibrio* group was recognized at a higher taxonomic level, what led to the provisional proposal of the family "*Desulfovibrionaceae*".

In the early nineties, several reviews summarized phylogenetic and taxonomic relationships among SRPs (Devereux and Stahl 1993, Stackebrandt *et al.* 1995, Widdel and Bak 1992). SRPs were roughly classified into four main groups according to cell wall properties and growth temperature requirements: the mesophilic gram-negative, the thermophilic gram-negative, the thermophilic gram-positive SRBs and the hyperthermophilic sulfate-reducing archaea (SRAs). All mesophilic SRBs known at that time could be assigned to the two provisionally proposed deltaproteobacterial families "*Desulfovibrionaceae*" (Devereux *et al.* 1990) and "*Desulfobacteriaceae*" (Widdel and Bak 1992). A few years later, a third major line of descent of SRBs within the delta-subclass of *Proteobacteria* was provisionally recognized as the family "*Desulfobulbusaceae*" (Rooney-Varga *et al.* 1998). Phylogenetically

independent from deltaproteobacterial SRBs are the genera *Thermodesulfobacterium* (phylum *Thermodesulfobacteria*) and *Thermodesulfovibrio* (phylum *Nitrospirae*) which encompass the thermophilic gram-negative members of the SRP guild. Use of transversion distances in phylogenetic tree reconstruction reduced biases introduced by differences in DNA G+C content of the analyzed microorganisms and demonstrated that these two thermophilic gram-negative genera were as dissimilar to each other as to mesophilic *Desulfovibrio* species (Henry *et al.* 1994). More recently the thermophilic gram-positive SRBs were subject of extensive phylogenetic analysis and reclassification (Stackebrandt *et al.* 1997). Accordingly, the genera *Desulfotomaculum* and *Desulfosporosinus*, which are phylogenetically independent from each other, were validly recognized to belong to the low DNA G+C content gram-positive bacterial line of descent (phylum *Firmicutes*). As aforementioned, the only SRAs recognized to date are members of the genus *Archaeoglobus* (phylum *Euryarchaeota*).
In conclusion, the phylogenetic backbone based on comparative 16S rRNA sequence analysis was generally supported by classical SRP taxonomy. However, phylogenetic inconsistencies, namely the poly- or paraphyletic origin of some SRP genera and species, pointed out particular misclassified SRPs which already were (for example Kuever *et al.* 2001, Loubinoux *et al.* 2002c, Sharak Genthner *et al.* 1997) or should be subject of further taxonomic revisions. Although considerable effort is put in the correct (re)classification of SRP genera and species, a valid hierarchical SRP taxonomy above the genus level is currently lacking. With the increasing number of newly described SRP species the necessity for higher order classification arose. Especially the provisional taxonomic trichotomy in "*Desulfovibrionaceae*", "*Desulfobacteriaceae*", and "*Desulfobulbusaceae*" among the deltaproteobacterial SRBs can not be regarded as sufficient anymore.

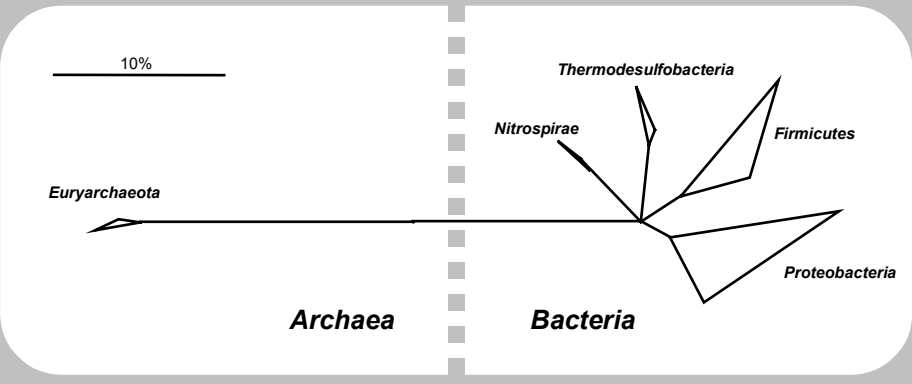

Figure 1. Schematic 16S rRNA-based phylogenetic tree showing all prokaryotic phyla which harbor recognized SRPs.

Although the latest edition of Bergey`s Manual of Systematic Bacteriology already proposed a substantial taxonomic ranking for all prokaryotes, this outline classification "is a work in progress" and must await further amendment (Garrity and Holt 2001). Today, 126 sulfate-reducing species belonging to 35 genera, which can be assigned to four bacterial phyla and one archaeal phylum (Figure 1), have been validly described (to date 19 september 2002, http://www.bacterio.cict.fr/) (Euzeby 1997).

II. Habitats and general ecological aspects of SRPs

SRPs constitute an essential biotic component of the global sulfur cycle. As already shown in the preceding chapter, SRPs form a rather heterogenous group from the viewpoint of modern rRNA-based taxonomy. However, their general geobiological importance legitimates grouping in a functional microbial guild. The unifying physiological trait of these microorganisms is their ability to use sulfate dissimilatively as terminal electron acceptor coupled with the generation of energy. This unique geobiological process is called dissimilatory sulfate reduction or anaerobic sulfate respiration and is exclusively restricted to the prokaryotic domains of life. Dissimilatory sulfate reduction is a very ancient process. Earliest geological records of microbial sulfate reduction date back in the early Archaean era, more than ~3.47 billion years ago (Shen *et al.* 2001).

SRPs are ubiquitous and inhabit mainly anoxic zones but also the oxic/anoxic interface of various environments. Findings from environmental studies that nonsporeforming SRPs existed in high numbers in oxic environments (Canfield and Des Marais 1991, Krekeler *et al.* 1997, Minz *et al.* 1999a, Ramsing *et al.* 1993, Teske *et al.* 1998) and could cope with oxygen stress came as a surprise, because, for a long time, all SRPs were considered to be obligate anaerobic microorganisms. Only endospore-forming SRPs of the genus *Desulfotomaculum* were thought to survive under long-term oxic conditions (Widdel 1988). Physiological and biochemical studies on the influence of oxygen on anaerobes revealed that several oxygen-scavenging mechanisms exist among different SRP species. For instance, some *Desulfovibrio* species are able to utilize oxygen directly via periplasmatic reduction. Despite a high respiration rate and energy coupling, it has been assumed that this process has only a protective function (Baumgarten *et al.* 2001, Cypionka 2000). Further protective mechanisms involve enzymes such as rubredoxin oxidoreductase (desulfoferredoxin) (Lumppio *et al.* 2001, Silva *et al.* 2001b) or neelaredoxin (Abreu *et al.* 2000, Silva *et al.* 2001a) that catalyze the removal of toxic superoxide which is formed in the presence of oxygen. In addition to

these physiological capacities, some SRPs also show behavioral responses to oxygen exposure such as flocculation (Sigalevich *et al.* 2000), simple migration to anoxic regions (Krekeler *et al.* 1997, Krekeler *et al.* 1998), or the formation of bands in oxygen-containing zones at concentrations of lesser than or equal to 20% air saturation. The latter behavior is driven by a complex interplay of positive and negative aerotaxis (Eschemann *et al.* 1999). Although all these protective mechanisms allow SRPs to survive oxygen stress, substantial aerobic growth in pure culture has not yet been observed. Thus, SRPs still remain anaerobic microorganisms but the dogma on their strict dependence on anoxic living conditions had to be reconsidered.

In terms of microbial abundance and ecoproductivity, anoxic marine environments in general and sediments in particular represent the most important habitats for SRPs. High sulfate concentrations in sea water (approximately 28 mM) promote growth and activity of SRPs. Jørgensen *et al.* have shown by using the $^{35}SO_4^{2-}$ radiotracer method (Sorokin 1972) that up to 50% of the organic carbon in marine sediments is mineralized via dissimilatory sulfate reduction (Jørgensen 1977, Jørgensen 1982). Because of their profound ecological importance in these systems, SRPs in marine sediments were subject of many extensive studies (Devereux and Mundfrom 1994, Knoblauch *et al.* 1999a, Knoblauch *et al.* 1999b, Llobet-Brossa *et al.* 1998, Ravenschlag *et al.* 2001). Worth mentioning is the anaerobic oxidation of methane in marine sediments as it is an extraordinary example for the versatility of the ecological processes that SRPs are involved in. Anaerobic methane oxidation has been known for a long time (Reeburgh 1982), but is was only recently demonstrated that microbial aggregates composed of SRPs and methane-oxidizing archaea catalyze this geobiological process (Boetius *et al.* 2000, DeLong 2000, Orphan *et al.* 2001, Thomsen *et al.* 2001). However, besides sediments there is a vast number of other ecological niches in marine environments that are colonized by SRP.

An example for a highly specialized niche for SRPs is the gutless marine oligochaete *Olavius algarvensis*. It harbors sulfate-reducing and sulfide-oxidizing bacterial endosymbionts which syntrophically catalyze a closed endosymbiotic sulfur cycle in the worm (Dubilier *et al.* 2001).

Another mutualistic relationship probably gives rise to the high numbers and activities of SRPs associated with marine macrophytes (Hines *et al.* 1999, Küsel *et al.* 1999, Rooney-Varga *et al.* 1997). SRPs inhabit the rhizosphere of marsh and sea grasses and it is hypothesized that they profit from the dissolved organic carbon exuded from the roots in exchange for fixed nitrogen (Nielsen *et al.* 2001).

Well-studied habitats that encompass a variety of phylogenetically diverse SRP groups are cyanobacterial microbial mats (Minz *et al.* 1999a, Risatti *et al.* 1994, Teske *et al.* 1998). The distinct SRP groups are mostly distributed in nonoverlapping depth intervals of the mat what led to the suggestion that they are responsible for specific interrelated metabolic functions in the community (Risatti *et al.* 1994).

In contrast to marine sediments, the main carbon mineralization process in freshwater sediments is methanogenesis. However, dissimilatory sulfate reduction, carried out by a diverse assemblage of SRPs (Li *et al.* 1999, Sass *et al.* 1998), may contribute with more than 20% to the total anaerobic mineralization (Ingvorson and Brock 1982). Further freshwater habitats where occurrence of SRPs has been demonstrated are waterlogged rice soils (Ouattara *et al.* 1999, Scheid and Stubner 2001, Stubner and Meuser 2000, Wind and Conrad 1995, Wind *et al.* 1999), groundwater from aquifers (Lehman *et al.* 2001), and wastewater treatment systems (De Smul and Verstraete 1999, Ito *et al.* 2002a, Ito *et al.* 2002b, Lens *et al.* 1995, Manz *et al.* 1998, Oude Elferink *et al.* 1998, Ramsing *et al.* 1993, Schramm *et al.* 1999), to name only a few.

The detection of SRPs in anthropogenically or naturally contaminated habitats (Anderson and Lovley 2000, Leu *et al.* 1998, Robertson *et al.* 2001, Voordouw *et al.* 1991) has attracted economic interests to SRPs. Some specialized SRPs have the metabolic capacity to degrade environmental pollutants such as oil (Harms *et al.* 1999, Rabus *et al.* 1996¸ Annweiler, 2001 #1592) and thus, these SRPs are promising candidates for the use in large-scale bioremediation attempts. In contrast to these beneficial aspects, there are cases where high metabolic activity of SRPs is undesired from an economical point of view. Such a negative aspect is that SRPs, among other microorganisms, are the driving force for microbiologically influenced metal corrosion. SRP growth is responsible for significant modifications of many physicochemical parameters at metal surfaces, including local changes in pH and redox potential values, variations in anion and cation concentrations and alteration of the composition and structure of corrosion products (Javaherdashti 1999, Videla 2000). This harmful activity of SRPs causes considerable economical damage to e.g. oil pipelines or other man-made metal constructions (Rao *et al.* 2000). Therefore, mechanisms to control or suppress colonization of such environments by SRPs are needed (Billman 1997, Jayaraman *et al.* 1999).

Besides their ecological and economical importance, SRPs also attracted attention as potential opportunistic pathogens in connection with human diseases such as periodontitis (Langendijk *et al.* 1999, Langendijk *et al.* 2000, Langendijk *et al.* 2001, Loubinoux *et al.* 2002a), pyogenic

liver abscesses (Schoenborn *et al.* 2001, Tee *et al.* 1996), inflammatory bowel diseases (Loubinoux *et al.* 2002b) or bacteremia (Loubinoux *et al.* 2000, McDougall *et al.* 1997).

III. Current molecular approaches to determine SRP community structure

Traditional approaches attempted to identify and quantify microbial biodiversity by means of cultivation. Especially for the isolation (for review see Widdel and Bak 1992) and enumeration (Vester and Ingvorsen 1998) of anaerobic SRPs, sophisticated media and culturing techniques were designed in order to mimic *in situ* growth conditions as perfectly as possible *in vitro*. However, the introduction of cultivation-independent molecular, primarily nucleic acid-based techniques in microbial ecology research led to the recognition that these cultivation approaches underlie significant quantitative (Staley and Konopka 1985) and qualitative biases (Wagner *et al.* 1993). Although molecular methods are generally not free from methodological errors (Martin-Laurent *et al.* 2001, Polz and Cavanaugh 1998, Speksnijder *et al.* 2001, Suzuki and Giovannoni 1996, von Wintzingerode *et al.* 1997), their vast application in SRP diversity research provided the basis for our today's view on the natural distribution and ecophysiological function of these microorganisms in the environment. The individual molecular methods which found widespread application in studies on natural SRP communities are summarized in the next paragraph. Because benefits and potential pitfalls of these methods have already been reviewed in detail elsewhere (Amann *et al.* 1995, Friedrich 2002, Klein *et al.* 2001, Muyzer *et al.* 1998, Muyzer *et al.* 1996, Voordouw 1998, Wagner *et al.* 1998), only general technical aspects in the context of SRP diversity research are presented.

Comparative sequence analysis

Comparative sequence analysis of rRNA (genes) following nucleic acid extraction from an environmental sample, the use of universal, bacterial or archaeal primers for PCR amplification of rRNA genes, and the setup of a rRNA gene library, has become the prime tool for molecular microbial ecologists to assess prokaryotic species richness independent from cultivation (Amann *et al.* 1995, Pace *et al.* 1986, Stackebrandt and Rainey 1995). Its numerous application in all kinds of habitats has dramatically improved our knowledge on the phylogenetic extent of microbial life in general (for example see Barns *et al.* 1996, Eder *et al.* 1999, Hugenholtz *et al.* 1998, Kuske *et al.* 1997, Ludwig *et al.* 1997). Nevertheless, the

application of this general rRNA gene library approach is limited in environments with a high prokaryotic diversity like sediments and soils (Torsvik *et al.* 2002) because hundreds of rRNA gene sequences must be sequenced to cover the whole microbial richness. A more focussed view on the diversity of certain microorganisms is possible by using primers that target phylogenetic groups at higher levels of specificity. A first application of this technique for SRPs has revealed unique environmental 16S rRNA sequences in a sandy marine sediment (Devereux and Mundfrom 1994). However, in contrast to phylogenetically and functionally homogeneous bacterial groups such as the betaproteobacterial ammonium oxidizers (Purkhold *et al.* 2000), the polyphyletic origin of SRPs (i) does not allow the design of a single 16S rRNA-targeted primer pair that is specific for all SRPs and (ii) complicates the unambiguous assignment of environmentally derived 16S rRNA sequences to this microbial guild.

16S rRNA aside, comparative amino acid sequence analyses of key enzymes of the dissimilatory sulfate reduction pathway, such as the siroheme dissimilatory (bi)sulfite reductase (EC 1.8.99.3) or the adenosine-5`-phosphosulfate reductase (EC 1.8.99.2), provide a bypass for this drawback of rRNA-based approaches. The genetic capacity of sulfate reduction can be directly deduced from these so-called "functional" phylogenetic marker molecules. The remarkable sequence conservation of the genes encoding dissimilatory (bi)sulfite (DsrAB) and adenosine-5`-phosphosulfate reductases (ApsA) (Hipp *et al.* 1997, Karkhoff-Schweizer *et al.* 1995) allowed the design of degenerated PCR primers for their detection in the environment (Friedrich 2002, Klein *et al.* 2001, Wagner *et al.* 1998). Analogous to the rRNA approach, *dsrAB* or *apsA* gene sequences can be PCR-amplified from environmental DNA, singularized by cloning, and phylogenetically classified by comparative analyses of nucleic acid and/or deduced amino acid sequences. One downside of the *dsrAB* and the *apsA* approach is that these genes were subject of several lateral transfer events as indicated by partly inconsistent phylogenetic tree topologies of SRP pure cultures inferred from 16S rRNA, DsrAB, and ApsA sequence analyses (Friedrich 2002, Klein *et al.* 2001, Stahl *et al.* 2002). This fact hampers exact identification of environmental sequences that are not closely related to known SRP lineages. Nevertheless, it has been proven for various environments that it is possible to reveal the presence of heretofore uncultured SRPs beyond the recognized lineages via *dsrAB* (Castro *et al.* 2002, Chang *et al.* 2001, Cottrell and Cary 1999, Dubilier *et al.* 2001, Joulian *et al.* 2001, Minz *et al.* 1999b, Nakagawa *et al.* 2002, Schramm *et al.* 1999, Thomsen *et al.* 2001) or *apsA* gene-based molecular metabolic diversity surveys (Deplancke *et al.* 2000).

Denaturing gradient gel electrophoresis

A very common PCR-based method, which was introduced in microbial ecology by Muyzer *et al.* (1993) and since then was often applied in SRP diversity research, is denaturing gradient gel electrophoresis (DGGE). The basic principle of this nucleic acid fingerprinting technique is the analytical resolution of PCR-amplified DNA fragments identical in length but different in sequence composition. Separation in DGGE is based on decreased electrophoretic mobility of a partially melted DNA fragment in polyacrylamide gels containing a linearly increasing gradient of DNA denaturants (for methodological details see Muyzer *et al.* 1998, Muyzer *et al.* 1996). The separated DNA bands can be identified either by comparative sequence analysis following extraction of the bands from the gel, reamplification by PCR, and cloning, or by hybridization with nucleic acid probes following blotting of bands on nylon membranes. The greatest advantage of DGGE is that the genetic diversity of many samples can be rapidly analyzed in parallel by a single gel run. However, besides general biases that underlie all analytical methods based on nucleic acid extraction and PCR-amplification (Bonnet *et al.* 2002, Martin-Laurent *et al.* 2001, Polz and Cavanaugh 1998, Speksnijder *et al.* 2001, Suzuki and Giovannoni 1996, von Wintzingerode *et al.* 1997), a DGGE-specific caveat is that only short PCR fragments of up to 500 bp can be well separated which limits phylogenetic information retrieved after sequencing of the individual bands. Furthermore, it has been shown that amplification of identical sequences by using degenerated primers leads to multiple bands in DGGE, which are solely caused by differences in the primer sequence (Nicolaisen and Ramsing 2002). This potential bias can be avoided if non-degenerated primers are used for DGGE.

Analyses of complex SRP communities by DGGE mainly used 16S rRNA genes as target molecules (Kleikemper *et al.* 2002, Koizumi *et al.* 2002, Santegoeds *et al.* 1998, Teske *et al.* 1998, Teske *et al.* 1996). Nevertheless, as aforementioned it strongly depends on the specificity of the rRNA gene-targeted PCR primer pair whether the obtained DGGE fingerprints can be directly linked to SRPs. For a subgroup of SRPs, an alternative to 16S rRNA (gene) as target molecule for DGGE is the gene encoding the large subunit of [NiFe] hydrogenase (Wawer and Muyzer 1995), an enzyme which plays an important role in the hydrogen metabolism of *Desulfovibrio* species. The [NiFe] hydrogenase gene is conserved among all *Desulfovibrio* spp. investigated so far, making it an ideal target for their PCR-based detection (Voordouw *et al.* 1990). The determination of diversity and expression of this

functional gene by DGGE in anaerobic bioreactors allowed to differentiate active from dormant members in natural assemblages of *Desulfovibrio* spp. (Wawer *et al.* 1997).

Hybridization with rRNA-targeted oligonucleotide probes

Integral part of the experimental setup of most ecological studies focussing on identification and abundance of SRPs in their natural habitats, is the application of rRNA-targeted oligonucleotide probes (for review on rRNA-targeted probes in general see Amann and Kühl 1998, Amann and Ludwig 2000, Amann and Schleifer 2001, Amann 1995, Amann *et al.* 1995, DeLong *et al.* 1989, Giovannoni *et al.* 1988, Stahl and Amann 1991). Today, a whole suite of empirically pretested probes targeting different taxonomic SRP groups is available for straightforward use in different hybridization formats (Daly *et al.* 2000, Devereux *et al.* 1992, Hristova *et al.* 2000, Manz *et al.* 1998). Thus, the composition of the SRP community can be analyzed with increasing taxonomic resolution if probes of hierarchical specificity are hybridized either in parallel with the same sample or separately with parallel subsamples, depending on the hybridization format chosen. Hybridization of whole cells and hybridization of extracted nucleic acids on a membrane are the two basic formats to use rRNA-targeted oligonucleotide probes for quantification.

Fluorescence *in situ* hybridization (FISH). The essence of FISH is that, using fluorescently tagged probes, it allows the specific visualization of morphologically intact organisms (hence, FISH is also referred to as whole-cell hybridization) directly in their natural environment. The simultaneous hybridization with three nested probes, each probe labeled with a different fluorophore, permits differentiation of up to seven distinct microbial populations within a single sample (Amann *et al.* 1996). Besides identification and spatial localization of microorganisms, absolute and/or relative numbers of visualized cells can be determined either by tedious manual counting (Glöckner *et al.* 1999) or semi-automatic quantification by using sophisticated digital image analyses (Bouchez *et al.* 2000, Daims *et al.* 2001, Juretschko *et al.* 2002, Schmid *et al.* 2000). Furthermore, FISH has the great potential that it can be directly combined with techniques such as microsensors (Ramsing *et al.* 1993) or microautoradiography (Ito *et al.* 2002a) to elucidate the ecophysiology of identified SRPs.

First application of FISH for SRP community analysis identified *Desulfovibrio vulgaris*-related bacteria in sulfidogenic biofilms established in anaerobic bioreactors (Amann *et al.* 1992). Further FISH studies monitored abundance and spatial organization of single deltaproteobacterial SRB populations in activated sludge (Manz *et al.* 1998), investigated

response of SRPs to oxygen stress under oligotrophic conditions in particle-free systems (Bade *et al.* 2000), or revealed the spatial structure of a consortium of *Archaea* and SRBs responsible for anaerobic methane oxidation (Boetius *et al.* 2000).

Quantitative membrane hybridization. In the dot-/slot-blot hybridization format, environmentally retrieved total rRNA is immobilized on a membrane by using a blotting apparatus with round (dot) or longitudinal (slot) cavities and subsequently hybridized with radioactively labeled phylogenetic probes. The amount of rRNA of a certain microbial population measured by a specific probe (as counts per minute) is normalized against the amount of rRNA measured by a probe of broader specificity e.g. a universal probe (Raskin *et al.* 1994, Stahl *et al.* 1988). However, this information on relative rRNA abundance can not be directly extrapolated into total cell numbers because the amount of rRNA per cell may vary drastically according to the physiological state of the cell (Kemp *et al.* 1993). This particular drawback of the membrane format can be partially overcome if total DNA is immobilized and the number of rRNA genes is quantified (Edgcomb *et al.* 1999). The number of target cells in a sample can be estimated by comparing the amount of a specific probe hybridized to extracted DNA to that obtained with a standard curve of genomic DNA for reference strains included on the same membrane. Nevertheless, one has to bear in mind that different species might have different genome and/or rRNA operon copy numbers (Fegatella *et al.* 1998, Klappenbach *et al.* 2000) leading to errors that can be greater than severalfold.

In practice, quantitative dot-/slot-blot hybridization has been preferentially applied in classical SRP environments such as cyanobacterial mats (Minz *et al.* 1999a, Risatti *et al.* 1994) or sediments (Hines *et al.* 1999, Li *et al.* 1999, Ravenschlag *et al.* 2001, Ravenschlag *et al.* 2000, Rooney-Varga *et al.* 1997, Sahm *et al.* 1999a, Sahm *et al.* 1999b), because high background fluorescence of theses habitats hampers application of FISH techniques.

DNA microarray technology. In a ground-breaking study by Guschin *et al.* (1997), a new hybridization format for rRNA-targeted oligonucleotides, the DNA microarray, was introduced to microbial ecology research. Only recently, this novel technology has been applied in an proof-of-principal attempt to directly detect rRNA from SRPs in soils (Small *et al.* 2001) and in an anaerobic toluene- and ethylbenzene-degrading enrichment (Koizumi *et al.* 2002). Further details on DNA microarrays and their application for determinative microbiological studies are presented in a separate chapter below.

Reverse sample genome probing

Another DNA hybridization method that was initially applied for identification of SRPs in oil field samples is reverse sample genome probing (RSGP) (Voordouw *et al.* 1993, Voordouw *et al.* 1992, Voordouw *et al.* 1991). In this approach total genomic DNA from cultured reference strains is denatured and immobilized on a membrane together with an internal control (concentration series of bacteriophage lambda DNA). The genomes of the different reference strains on the membrane (the so-called master filter) should generally cross-hybridize less than 1%. Total environmental DNA is mixed with a standard amount of lambda DNA, radioactively labeled by random prime labeling with [α–^{32}]P, and hybridized under stringent conditions to the master filter. After hybridization and washing, the amount of bound sample DNA is quantified. Subsequently, the fraction of environmental DNA composed of individual component DNA is calculated from the hybridization to the individual genomic pure culture standards relative to the lambda reference series (Voordouw *et al.* 1993).
The main advantage of this whole-genome probe array technique is that it provides information on complex microbial communities in a single assay. Although the actual RSGP does not require culturing, the most criticized aspect of this technique is that composition of the microbial community is displayed in terms of its culturable component (Voordouw 1998).

IV. DNA microarrays for determinative studies in microbiology

The DNA microarray (microchip) technology allows parallel analysis of many genes in a single assay and thus emblematize, as no other methodological means, the (post-) genomic era of “big science”. Very simply described, DNA microchips consist of up to thousands of diagnostic nucleic acid sequences (referred to as **probes**) tethered to a miniaturized solid support (usually a glass slide) in an arrayed order. Probes can be either oligonucleotides or PCR amplificates (usually cDNA). The identity (and amount) of labeled nucleic acid sequences that are subject of analysis (referred to as **target**) is revealed after hybridization to the microarray.
Initially microarrays containing probes for every single gene of an organism were developed to analyze gene expression on a genomic scale (Schena *et al.* 1995). Since then, these genomic microarrays have been widely applied in microbiological research to reveal genes involved in response to stress and environmental change, cellular response to bacterial infection, the cell-cycle, and dissection of regulatory circuitry. Moreover, it is possible to

compare different strains (genomotyping) or to identify potential drug target sites by microarray hybridization (for a summary of microbiological DNA microarray studies see Lucchini *et al.* 2001).

Beyond this genomic level-oriented research, DNA microarrays hold much potential for determinative studies in environmental and clinical microbiology (Cho and Tiedje 2001, Cho and Tiedje 2002, Guschin *et al.* 1997, Kingsley *et al.* 2002, Koizumi *et al.* 2002, Liu *et al.* 2001, Reyes-Lopez *et al.* 2003, Small *et al.* 2001, Urakawa *et al.* 2002, Volokhov *et al.* 2002, Wilson *et al.* 2002, Wu *et al.* 2001, Zhou and Thompson 2002). The scope of such studies was often restricted by conventional experimental formats such as FISH or dot-/slot-blot hybridization, which strongly limit the number of probes that can be applied and the number of samples that can be analyzed. The invention of DNA microarrays provided the basis for a hybridization format that allows greater sample throughput and highly parallel detection of complex microbial communities. Target nucleic acids for DNA microarray probes are basically the same as in conventional hybridization assays used for microbial identification: namely the small- and/or large-subunit rRNA genes or functional respectively virulence genes that are diagnostic for certain microbial groups.

Ribosomal RNA-based oligonucleotide microarrays developed so far can be divided in two categories according to the strategy of the underlying probe design. One strategy, namely the so-called “multiple probe concept” (Amann and Schleifer 2001, Behr *et al.* 2000), takes advantage of the fact that rRNA genes consist of highly conserved and variable sequence regions. Thus, it is possible to design multiple oligonucleotide probes to detect target groups at different (or same) phylogenetic levels by targeting rRNA regions of different (or same) sequence conservation. Consequently, the simultaneous application of a whole set of nested and parallel probes enhances the reliability of the detection of target organisms. The “multiple probe concept” proved to be fruitful for the application of rRNA-targeted oligonucleotide probes for e.g. whole cell (Amann *et al.* 1996, Juretschko *et al.* 2002), membrane (Raskin *et al.* 1994), or micro well plate hybridization (Behr *et al.* 2000) but can probably be utilized most excessively for DNA microarray hybridization (Liu *et al.* 2001), owing to the enormous number of potentially applicable probes. A different probe design strategy was the basis for a high-density microarray of small-subunit rRNA-targeted oligonucleotides developed by Wilson *et al.* (2002). Based on a subalignment of the small-subunit rRNA database (version 5.0) of the Ribosomal Database Project (RDP) almost all possible 20-mer probes for every single sequence in the database were designed, resulting in a total of 31179 oligonucleotides on the DNA microarray. After hybridization of a given environmental sample with the DNA

microarray, an RDP 16S rRNA sequence was regarded to be present if 22 or more of at least 24 probes specifically targeting this sequence were scored positive. This probe design strategy is totally devoted to high parallelism, the main feature of DNA microarrays, but it ignores the potential to specifically design rRNA-targeted probes for phylogenetically defined target groups.

If diagnostic DNA microarrays target functional genes (Wu *et al.* 2001) or virulence genes (Volokhov *et al.* 2002) a certain physiological property or pathogenic potential can be directly inferred upon identification as already pointed out in the preceding chapters. In comparison to rRNA-based microarrays, probe design for functional gene arrays has some general disadvantages. One is that sequence databases, the basis for adequate probe design, for functional genes of microbial groups of interest are currently still not as comprehensive as the respective small-subunit rRNA databases. Furthermore, development of nested oligonucleotide probes according to the "multiple probe concept" is more difficult due to the highly variable, third codon (wobble) position.

Another DNA microarray variant for detection and differentiation of microorganisms consists of random oligonucleotides that allow fingerprinting of microbial strains as shown for closely related *Xanthomonas* pathovars (Kingsley *et al.* 2002). Furthermore, random genome fragments can be immobilized on microarrays and used analogous to traditional whole genomic DNA-DNA hybridization for bacterial species determination (Cho and Tiedje 2001).

Although potential fields of application of diagnostic DNA microarrays are numerous, most microarrays developed so far for microbial identification were mainly used for method development and optimization. Moreover, with a single exception (Wilson *et al.* 2002) these microarrays consisted of a limited number of probes, not making use of the advantageous capacity of DNA microarrays for highly parallel identification. Consequently, diagnostic DNA microarrays are not yet routinely implemented in environmental and medical research.

V. Scope of this thesis

The initial part of this thesis should focus on the collection and alignment of all 16S rRNA sequences from isolated and yet uncultured SRPs available in public databases. Subsequently, a thorough reevaluation of natural relationships among SRPs based on comparative 16S rRNA sequence analysis should provide (i) a robust phylogenetic and taxonomic framework for the assignment of environmentally retrieved SRP sequences and (ii) the basis for the design of an encompassing oligonucleotide probe set that target SRPs at multiple hierarchical

and parallel levels of specificity and is suitable for application on diagnostic DNA microarrays. After *in silico* development of a comprehensive 16S rRNA-based oligonucleotide microarray for identification of SRPs (SRP-PhyloChip), specificity and sensitivity of this hybridization technique should be evaluated and optimized with suitable SRP pure cultures. Besides methodological development, a main aspect of this thesis will focus on the potential applicability of the developed microarray for routine detection of SRPs in environmental and medical samples. Therefore, results obtained by microarray hybridization in environmental or clinical studies should be confirmed by well-established molecular methods for SRP identification based on comparative sequence analyses of 16S rRNA genes and dissimilatory (bi)sulfite reductase genes (*dsrAB*).

VI. References

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MATERIALS AND METHODS

Pure cultures of SRP. Table 1 lists the SRP reference organisms that were used in this thesis. All strains were obtained as lyophilized cells or active cultures from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany). *Archaeoglobus veneficus* SNP6 (containing plasmid XY) was deposited in the DSMZ by K. O. Stetter, Lehrstuhl für Mikrobiologie, Universität Regensburg, Universitätsstr. 31, D-93053 Regensburg, Germany, as DSM 11195^T.

Table 1. SRP strains used in this thesis.

Species	Strain DSMZ number
Desulfovibrio cuneatus	DSM 11391^T
Desulfovibrio aminophilus	DSM 12254^T
Desulfovibrio gabonensis	DSM 10636^T
Desulfovibrio alcoholivorans	DSM 5433^T
Desulfovibrio termitidis	DSM 5308^T
Desulfovibrio zosterae	DSM 11974^T
Desulfovibrio halophilus	DSM 5663^T
Desulfovibrio longus	DSM 6739^T
"*Desulfovibrio aestuarii*"	DSM 1926^T
Desulfovibrio profundus	DSM 11384^T
Desulfomicrobium apsheronum	DSM 5918^T
Desulfomicrobium orale	DSM 12838^T
Desulfohalobium retbaense	DSM 5692^T
Desulfotalea arctica	DSM 12342^T
Desulforhopalus vacuolatus	DSM 9700^T
Desulfobulbus propionicus	DSM 2032^T
"*Desulfobotulus sapovorans*"	DSM 2055^T
Desulfococcus multivorans	DSM 2059^T
Desulfonema limicola	DSM 2076^T
Desulfonema ishimotonii	DSM 9680^T
Desulfobacterium indolicum	DSM 3383^T
Desulfosarcina variabilis	DSM 2060^T
Desulfofaba gelida	DSM 12344^T
Desulfofrigus oceanense	DSM 12341^T
"*Desulfobacterium niacini*"	DSM 2650^T
Desulfobacula toluolica	DSM 7467^T
Desulfotignum balticum	DSM 7044^T
Desulfobacter halotolerans	DSM 11383^T
Desulfobacter latus	DSM 3381^T
Syntrophobacter wolinii	DSM 2805^T
Thermodesulforhabdus norvegica	DSM 9990^T
Desulfomonile tiedjei	DSM 6799^T
Desulfobacca acetoxidans	DSM 11109^T
Desulfotomaculum aeronauticum	DSM 10349^T
Desulfotomaculum geothermicum	DSM 3669^T
Desulfotomaculum australicum	DSM 11792^T
Desulfotomaculum thermobenzoicum	DSM 6193^T
Desulfotomaculum acetoxidans	DSM 771^T
Desulfotomaculum halophilum	DSM 11559^T
Desulfosporosinus orientis	DSM 765^T
Thermodesulfovibrio islandicus	DSM 12570^T
Thermodesulfobacterium mobile (*T. thermophilum*)	DSM 1276^T
Archaeoglobus veneficus	DSM 11195^T

Sampling sites. Peridontal tooth pocket samples. Samples from five patients with adult periodontitis (Figure 2) were taken by inserting a sterile medium-sized paper point into a single periodontal tooth pocket. After sampling paper points were stored at -20°C.

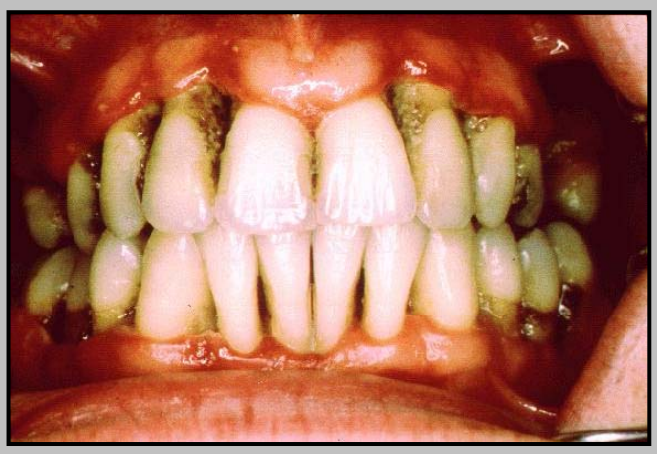

Figure 2. Patient with advanced adult periodontitis.

Solar Lake mat sample. A core (1 by 1cm; depth, 4cm) of a hypersaline cyanobacterial mat from the Solar Lake (Sinai, Egypt) (Figure 3) was sectioned horizontally at 200-µm intervals with a cryomicrotome (MIKROM HM500; Microm, Walldorf, Germany). Mat sections were stored at -80°C.

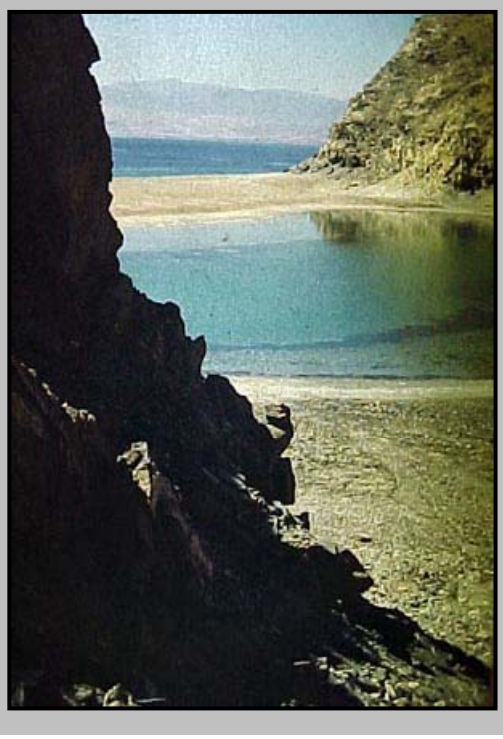

Figure 3. Hypersaline pond (Solar Lake) in the Sinai desert in Egypt.

Acidic fen soil samples. Two sites at the Lehstenbach catchment in the Fichtelgebirge mountains in northeastern Bavaria (Germany) were investigated. The catchment has an area of 4.2 km^2 with a highest elevation of 877 m a.s.l. Ninety percent of the area is stocked by Norway spruce (*Picea abies*, [L.] Karst.) of different ages. Upland soils in the catchment (i.e. aerated soils, which are not water saturated) have developed from weathered granitic bedrock and are predominantly Cambisols and Cambic Podsols (according to FAO-system). Considerable parts of the catchment (approx. 30%) are covered by minerotrophic fens or intermittent seeps. The annual precipitation in the catchment is 900 to 1160 mm $year^{-1}$ and the average annual temperature is 5°C.

The site Schlöppnerbrunnen I (SbI) is a fen (low moor) alternately covered with patches of *Sphagnum* mosses and with spruce stocking (Figure 4). The water saturated soil was classified as Fibric Histosol. The site Schlöppnerbrunnen II (SbII) is also a water saturated fen and completely overgrown by *Molinia caerula* grasses. The soil pH of both sites approximated 3.9 and 4.2, respectively. In the soil solution, the pH varied between 4 and 5 at site SbI and 4.5 to 6 at site SbII.

Figure 4. Fen at Schlöppnerbrunnen site I in the Lehstenbach catchment (Fichtelgebirge, Germany).

For subsequent DNA isolation from both sites, soil cores (3 cm in diameter) from four different depths (approximately 0-7.5, 7.5-15, 15-22.5, and 22.5-30 cm) were collected on 24 July 2001 and immediately cooled on ice. After transfer to the laboratory, the soil samples

were homogenized 1:1 (volume/volume) in 1x phosphate-buffered saline (PBS) and stored at -20°C.

DNA extraction. Genomic DNA was isolated from reference organisms by using the FastDNA kit (Bio101, Vista, Calif.). DNA from periodontal tooth pocket material, DNA from a cryosection of Solar Lake mat from the chemocline (1400 to 1600 µm from the mat surface), and DNA from fen soil homogenates were extracted by using a modification of the protocol of Griffiths *et al.* (2000). In contrast to the original protocol, precipitation of nucleic acids in the aqueous phase was performed with 0.1 volume sodium acetate (pH 5.2) and 0.6 volume of isopropanol for 2 h at room temperature.

Amplification of microbial genes by polymerase chain reaction (PCR).

PCR amplification of 16S rRNA genes. For subsequent DNA microarray hybridization, almost complete 16S rRNA gene fragments were amplified from DNA of reference pure cultures and clones of SRPs by using the bacterial primer pair 616V-630R (Table 2) or the cloning vector-specific primers M13F(-20) (5´-GTAAAACGACGGCCAG-3´) and M13R (5´-CAGGAAACAGCTATGAC-3´) (Invitrogen Corp., San Diego, Calif.), respectively. 16S rRNA gene fragments of *Archaeoglobus veneficus* were amplified by using the newly designed *Archaeoglobus* genus-specific forward primer ARGLO36F and the universal reverse primer 1492R (Table 2). Amplification of bacterial 16S rRNA gene fragments from environmental genomic DNA was performed by using the 616V-630R and the 616V-1492R primer pairs (Table 2).

To confirm DNA microarray results, specific amplification of 16S rRNA gene fragments of defined SRP groups was performed with periodontal tooth pocket DNA, Solar Lake mat DNA, and fen soil DNA by using previously described and newly designed primers (Table 2).

Table 2. 16S rRNA gene-targeted primers used and/or developed in this thesis.

Short name[a]	Full name[b]	Annealing temp. [°C]	Sequence 5'-3'	Specificity	Reference
616V	S-D-Bact-0008-a-S-18	52	AGA GTT TGA TYM TGG CTC	most *Bacteria*	Juretschko *et al.* 1998
630R	S-D-Bact-1529-a-A-17	52	CAK AAA GGA GGT GAT CC	most *Bacteria*	Juretschko *et al.* 1998
1492R	S-*-Proka-1492-a-A-19	52, 60[c]	GGY TAC CTT GTT ACG ACT T	most *Bacteria* and *Archaea*	Modified from Kane *et al.* 1993
ARGLO36F	S-G-Arglo-0036-a-S-17	52	CTA TCC GGC TGG GAC TA	*Archaeoglobus* spp.	This thesis[d]
DSBAC355F	S-*-Dsb-0355-a-S-18	60	CAG TGA GGA ATT TTG CGC	most "*Desulfobacterales*" and "*Syntrophobacterales*"	Scheid and Stubner 2001
BACT11F	S-D-Bact-0011-a-S-17	67[f]	GTT TGA TCC TGG CTC AG	most *Bacteria*	Kane *et al.* 1993
TDSV1329R	S-G-Tdsv-1329-a-A-17	67	AGC GAT TCC GGG TTC AC	*Thermodesulfovibrio* spp.	This thesis
TDSBM1361R	S-F-Tdsbm-1361-a-A-16	67	ATT CAC GGC GGC ATG C	*Thermodesulfo-bacteriaceae*	This thesis

DSN61F	S-*-Dsn-0061-a-S-17	52	GTC GCA CGA GAA CAC CC	*Desulfonema limicola*, *Desulfonema ishimotonii*	This thesis[d]
DSN+1201R	S-*-Dsn-1201-a-A-17	52	GAC ATA AAG GCC ATG AG	*Desulfonema* spp. and some other *Bacteria*	This thesis[d]
DSB+57F	S-G-Dsb-0057-a-S-21	64	GCA AGT CGA ACG AGA AAG GGA	*Desulfobacter* spp., *Desulfobacula* spp., *Desulfospira* spp., *Desulfobacterium autotrophicum*	This thesis
DSB1243R	S-G-Dsb-1243-a-A-21	64	AGT CGC TGC CCT TTG TAC CTA	*Desulfobacter* spp.	This thesis
DSMON85F	S-G-Dsmon-0085-a-S-20	62	CGG GGT RTG GAG TAA AGT GG	*Desulfomonile* spp.	This thesis[e]
DSMON1419R	S-G-Dsmon-1419-a-A-20	62	CGA CTT CTG GTG CAG TCA RC	*Desulfomonile* spp.	This thesis[e]
DBACCA65F	S-S-Dbacca-0065-a-S-18	58	TAC GAG AAA GCC CGG CTT	*Desulfobacca acetoxidans*	This thesis[e]
DBACCA1430R	S-S-Dbacca-1430-a-A-18	58	TTA GGC CAG CGA CAT CTG	*Desulfobacca acetoxidans*	This thesis[e]
DSB140F	S-*-Dsb-0140-a-S-20	60	GAA TTG GGG ATA ACG TTG CG	*Desulfobacterium. cetonicum*, *Desulfosarcina variabilis*	This thesis
DSB1438R	S-*-Dsb-1438-a-A-18	60	CCG AAG GGT TAG CCC GAC	*Desulfobacterium. cetonicum*, *Desulfosarcina variabilis*	This thesis
DSV682F	S-*-Dsv-0682-a-S-19	58	GGT GTA GGA GTG AAA TCC G	"*Desulfovibrionales*", "*Desulfuromonadales*"	This thesis
DSV+1402R	S-*-Dsv-1402-a-A-18	58	CTT TCG TGG TGT GAC GGG	"*Desulfovibrionales*", "*Desulfuromonadales*", and some other *Bacteria*	This thesis
DVHO130F	S-*-Dvho-0130-a-S-18	58	ATC TAC CCG ACA GAT CGG	*Desulfovibrio halophilus*, *Desulfovibrio oxyclinae*	This thesis
DVHO1424R	S-*-Dvho-1424-a-A-18	58	TGC CGA CGT CGG GTA AGA	*Desulfovibrio halophilus*, *Desulfovibrio oxyclinae*	This thesis
DSM172F	S-G-Dsm-0172-a-S-19	56	AAT ACC GGA TAG TCT GGC T	*Desulfomicrobium* spp.	This thesis[d]
DSM1469R	S-G-Dsm-1469-a-A-18	56	CAA TTA CCA GCC CTA CCG	*Desulfomicrobium* spp.	This thesis[d]
SYBAC+282F	S-*-Sybac-0282-a-S-18	60	ACG GGT AGC TGG TCT GAG	"*Syntrophobacteraceae*" and some other *Bacteria*	This thesis[e]
SYBAC1427R	S-*-Sybac-1427-a-A-18	60	GCC CAC GCA CTT CTG GTA	"*Syntrophobacteraceae*"	This thesis[e]
DSBB280F	S-*-Dsb-0280-a-S-18	58	CGA TGG TTA GCG GGT CTG	"*Desulfobulbaceae*"	This thesis
DSBB+1297R	S-*-Dsb-1297-a-A-19	58	AGA CTC CAA TCC GGA CTG A	"*Desulfobulbaceae*" and some other *Bacteria*	This thesis

[a] Short name used in the reference or in this thesis.
[b] Name of 16S rRNA gene-targeted oligonucleotide primer based on the nomenclature of Alm *et al.* (1996).
[c] The annealing temperature was 52°C when the primer was used with forward primer 616V or ARGLO36F and the annealing temperature was 60°C when the primer was used with forward primer DSBAC355F.
[d] Appendix I, (Loy *et al.* 2002).
[e] Appendix III, (Loy *et al.* 2003b).
[f] The annealing temperature was 67°C when the primer was used with reverse primer TDSV1329R or TDSBM1361R.

Positive controls containing purified DNA from suitable reference organisms were included in all of the PCR amplification experiments along with negative controls (no DNA added). For 16S rRNA gene amplification, reaction mixtures (total volume, 50 µl) containing each primer at a concentration of 25 pM were prepared by using 10x *Ex Taq* reaction buffer and 2.5 U of *Ex Taq* polymerase (Takara Biomedicals, Otsu, Shiga, Japan). Additionally, 20 mM tetramethylammonium chloride (TMAC; Sigma, Deisenhofen, Germany) was added to each amplification mixture to enhance the specificity of the PCR (Kovárová and Dráber 2000). Thermal cycling was carried out by using an initial denaturation step of 94°C for 1 min, followed by 30 cycles of denaturation at 94°C for 40 s, annealing at temperatures ranging from 52°C to 64°C (depending on the primer pair [Table 2]) for 40 s, and elongation at 72°C for 1 min 30 s. Cycling was completed by a final elongation step of 72°C for 10 min.

PCR amplification of dissimilatory (bi)sulfite reductase (*dsrAB*) genes. An approximately 1.9-kb *dsrAB* fragment was amplified from SRP pure culture DNA or environmental DNA samples by using either the primers DSR1F and DSR4R described by Wagner *et al.* (1998) or the primers DSR1Fmix (equimolar mixture of DSR1F, DSR1Fa, and DSR1Fb) and DSR4Rmix (equimolar mixture of DSR4R, DSR4Ra, DSR4Rb, and DSR4Rc) which contained additional degeneracies (Table 3).

TABLE 3. Dissimilatory (bi)sulfite reductase gene-(*dsrAB*)-targeted primers used and/or developed in this thesis. The target site of all listed DSR1 and DSR4 primer versions was analyzed for those SRPs (n=8) for which complete *dsrAB* operons are available in GenBank (Benson *et al.* 2002). SRPs with a fully complementary target site to the respective primers are listed in the specificity column.

Primer[a]	Sequence 5`-3`	Specificity	Reference
DSR1F	ACS CAC TGG AAG CAC G	*Archaeoglobus fulgidus, Archaeoglobus profundus, Desulfovibrio vulgaris*	Wagner *et al.* 1998
DSR1Fa	ACC CAY TGG AAA CAC G	*Desulfotomaculum thermocisternum, Desulfobulbus rhabdoformis, Desulfobacter vibrioformis*	This thesis[b]
DSR1Fb	GGC CAC TGG AAG CAC G	*Thermodesulforhabdus norvegica*	This thesis[b]
DSR4R	GTG TAG CAG TTA CCG CA	*Archaeoglobus fulgidus, Desulfovibrio vulgaris, Desulfobulbus rhabdoformis*	Wagner *et al.* 1998
DSR4Ra	GTG TAA CAG TTT CCA CA	*Archaeoglobus profundus*	This thesis[b]
DSR4Rb	GTG TAA CAG TTA CCG CA	*Desulfobacter vibrioformis*	This thesis[b]
DSR4Rc	GTG TAG CAG TTT CCG CA	*Thermodesulforhabdus norvegica, Desulfotomaculum thermocisternum*	This thesis[b]

[a] Primer was used at non-stringent conditions by applying an annealing temperature of 48°C for PCR in order to target a wide diversity of SRPs.
[b] Appendix III, (Loy *et al.* 2003b).

Reaction mixtures were prepared as mentioned above for 16S rRNA gene amplification. Thermal cycling was carried out by using an initial denaturation step of 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 40 s, annealing at 48°C for 40 s, and elongation at 72°C for 1 min 30 s. Cycling was completed by a final elongation step of 72°C for 10 min.

Random prime fluorescence labeling of PCR amplificates. Prior to labeling, 16S rRNA gene PCR amplificates were purified by using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). Subsequently, the amount of DNA was determined spectrophotometrically by measuring the optical density at 260 nm. Purified PCR products were labeled with Cy5 by using the DecaLabel DNA labeling kit (MBI Fermentas, Vilnius, Lithuania). Reaction mixtures (total volume, 45 µl) containing 200 ng of purified PCR product and 10 µl of decanucleotides in reaction buffer were denatured at 95°C for 10 min and immediately placed on ice. After addition of 3 µl of the desoxynucleotide Mix C

(containing no dCTP), 1 µl Cy5-dCTP (Amersham Biosciences, Freiburg, Germany) and 1 µl Klenow fragment (Exo^-; 5 U μl^{-1}), the labeling reaction mixtures were incubated at 37°C for 45 min. For more efficient labeling, the addition of Mix C, Cy5-dCTP, and the Klenow fragment and incubation at 37°C for 45 min were repeated. Labeling was completed by addition of 4 µl of dNTP-Mix and incubation at 37°C for 60 min. To remove unincorporated desoxynucleotides and decanucleotides, the labeling mixture was purified with a QIAquick nucleotide removal kit (Qiagen) by using double-distilled water for DNA elution. Finally, the eluted DNA was vacuum-dried and stored in the dark at -20°C.

Microarray manufacture and processing. Oligonucleotides for microarray printing were obtained from MWG Biotech (Ebersberg, Germany). The sequence, specificity, and microarray position of each oligonucleotide probe are shown in Table 5. In addition, difference alignments for all probes generated with the latest ARB small-subunit rRNA database (http://www.arb-home.de) can be viewed at the probeBase website (http://www.probebase.net) (Appendix II, Loy *et al.* 2003a). The 5' end of each oligonucleotide probe was tailed with 15 dTTP molecules (T-spacer) to increase the on-chip accessibility of spotted probes to target DNA (Shchepinov *et al.* 1997, Southern *et al.* 1999). In addition, the 5'-terminal nucleotide of each oligonucleotide was aminated to allow covalent coupling of the oligonucleotides to aldehyde group-coated CSS-100 glass slides (CEL Associates, Houston, Tex.). The concentration of oligonucleotide probes before printing was adjusted to 50 pmol μl^{-1} in 50% dimethyl sulfoxide to prevent evaporation during the printing procedure. SRP-PhyloChips were printed by using a GMS 417 contact arrayer (Affymetrix, Santa Clara, Calif.). Spotted DNA microarrays were dried overnight at room temperature to allow efficient crosslinking. Slides were washed twice at room temperature in 0.2% sodium dodecyl sulfate (SDS) and then twice with double-distilled water with vigorous agitation to remove unbound oligonucleotides and the SDS. After air drying, the slides were incubated for 5 min in fresh sodium borohydride solution (1.0 g NaBH4 in 300 ml phosphate-buffered saline and 100 ml of absolute ethanol) to reduce all remaining reactive aldehyde groups on the glass. The reaction was stopped by adding ice-cold absolute ethanol. The reduced slides were washed three times with 0.2% SDS and double-distilled water, air dried, and stored in the dark at room temperature.

Reverse hybridization on microarrays. Vacuum-dried Cy5-labeled PCR products (400 ng) and 0.5 pmol of the Cy5-labeled control oligonucleotide CONT-COMP (Table 5) were

resuspended in 20 µl of hybridization buffer (5x SSC [1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate] , 1% blocking reagent [Roche, Mannheim, Germany], 0.1% n-lauryl sarcosine, 0.02% SDS, 5% formamide), denatured for 10 min at 95°C, and immediately placed on ice. Then the solution was pipetted onto an SRP-PhyloChip, covered with a cover slip, and inserted into a tight custom-made hybridization chamber (http://cmgm.stanford.edu/pbrown/mguide/HybChamber.pdf) containing 50 µl of hybridization buffer for subsequent equilibration. Hybridization was performed overnight at 42°C in a water bath. After hybridization, the slides were washed immediately under stringent conditions for 5 min at 55°C in 50 ml washing buffer (containing 3 M TMAC, 50 mM Tris-HCl, 2 mM EDTA, and 0.1% SDS). To record probe-target melting curves, the temperature of the washing step was varied from 42 to 80°C. After the stringent washing, the slides were washed twice with ice-cold double-distilled water, air dried, and stored in the dark at room temperature.

Scanning of microarrays. Fluorescence images of the SRP-PhyloChips were recorded by scanning the slides with a GMS 418 array scanner (Affymetrix). The fluorescence signals were quantified by using the ImaGene 4.0 software (BioDiscovery, Inc., Los Angeles, Calif.). A grid of individual circles defining the location of each spot on the array was superimposed on the image to designate each fluorescent spot to be quantified. The mean signal intensity was determined for each spot. In addition, the mean signal intensity of the local background area surrounding the spots was determined.

Selective enrichment of nucleic acids by a capture probe approach. Five microliters of aldehyde group-coated glass beads (diameter, 1 µm; Xenopore, Hawthorne, NJ) was incubated overnight with 5 µl of the appropriate capture probe (100 pmol μl^{-1}; tailed with 15 dTTP molecules, aminated with 5'-terminal nucleotide) at room temperature. Subsequently, the beads were washed once with 400 µl of 0.2% SDS and pelleted by centrifugation (1 min at 14.000 rpm), and the supernatant was decanted. After this step, the beads were washed twice with 400 µl double-distilled water, dried, and stored at room temperature prior to hybridization. A vacuum-dried bacterial 16S rRNA gene PCR product (obtained from DNA from the Solar Lake mat with the 616V-1492R primer pair) was resuspended with 200 µl of hybridization buffer (see above), denatured for 10 min at 95°C, and immediately cooled on ice. The hybridization solution and capture probe beads were mixed in a screw-cap tube and incubated overnight at 42°C on a shaker. Subsequently, the

beads were washed twice with 1.5 ml washing buffer (see above) at 55°C for 2.5 min. After the stringent washes, the beads were washed with 1.5 ml ice-cold double-distilled water and then with ice cold 70% ethanol. Beads with captured nucleic acids were vacuum dried and resuspended in 50 µl EB buffer (part of the QIAquick PCR purification kit; Qiagen) for storage at –20°C. Reamplification of bacterial 16S rRNA gene fragments from the captured nucleic acids was performed by using 5 µl of the resuspended beads for PCR performed by using the 616V-1492R primer pair and the protocols described above.

Cloning and sequencing. Prior to cloning the PCR amplification products were purified by low-melting-point agarose (1.5%) gel electrophoresis (NuSieve 3:1; FMC Bioproducts, Biozym Diagnostics GmbH, Oldendorf, Germany) and stained in SYBR Green I solution (10 µl 10.000x SYBR Green I stain in 100 µl TAE buffer [40 mM TRIS, 10 mM sodium acetate, 1 mM EDTA, pH 8.0]; Biozym Diagnostics GmbH) for 45 min. Bands of the expected size were excised from the agarose gel with a glass capillary and melted with 80 µl double-distilled water for 10 min at 80°C. Four microliters of each solution were ligated as recommended by the manufacturer (Invitrogen Corp.) either into the cloning vector pCR2.1 of the TOPO TA cloning kit (16S rRNA gene amplificates) or into the cloning vector pCR-XL-TOPO of the TOPO XL cloning kit (*dsrAB* gene amplificates). Nucleotide sequences were determined by the dideoxynucleotide method (Sanger *et al.* 1977) as described by Purkhold *et al.* (2000). In addition, internal *dsrAB* gene-targeted sequencing primers (Table 4) were used to complete the *dsrAB* sequences.

TABLE 4. Internal dissimilatory (bi)sulfite reductase gene-(*dsrAB*)-targeted sequencing primers

Primer[a]	Sequence 5`-3`	Specificity	Reference[b]
DSR978Fa	GGT CAT CGA CCT TTG TCC	Schlöppnerbrunnen I soil OTU 5	This thesis
DSR978Fb	CGT CGT CGG GAA GTG CCC	Schlöppnerbrunnen I soil OTU 8	This thesis
DSR978Fc	AGT AGT CGA CCT TTG CCC	Schlöppnerbrunnen I+II soil OTU 6	This thesis
DSR978Fd	TGT CAC CGA TCT CTG CCC	Schlöppnerbrunnen I soil OTU 1	This thesis
DSR978Fe	TGT TAC CGA CCT CTG CCC	Schlöppnerbrunnen II soil OTU 1 (dsrSbII-20)	This thesis
DSR978Ff	TGT CAC CGA TCT TTG CCC	Schlöppnerbrunnen II soil OTU 4 (dsrSbII-15)	This thesis
DSR978Fg	CGT CAC CAT TCT CTG CCC	Schlöppnerbrunnen II soil OTU 4 (dsrSbII-9)	This thesis
DSR978Fh	GGT CGT TGA CAT GTG TCC	Schlöppnerbrunnen II soil OTU 11	This thesis
DSR978Fi	GGT CTG CAA TCT CTG YCC	Schlöppnerbrunnen I+II soil OTU 2 and 3	This thesis
DSR978Fj	GGT TGT TGA CCT TTG CCC	Schlöppnerbrunnen I soil OTU 9	This thesis
DSR978Fk	CGT TTG CGA TCT CTG CCC	Schlöppnerbrunnen II soil OTU 7	This thesis
DSR860F	AGA TCC GGC GGG ACG ATG	Schlöppnerbrunnen I soil OTU 10	This thesis

[a] Internal sequencing primer used to complete *dsrAB* gene sequences retrieved from acidic fen sites Schlöppnerbrunnen I and II.
[b] Appendix III, (Loy *et al.* 2003b).

Phylogeny inference. All phylogenetic analyses were performed by using the undermentioned alignment and treeing tools implemented in the ARB program package (http://www.arb-home.de).

16S rRNA gene-based phylogeny. The new 16S rRNA sequences were added to an ARB alignment of about 16.000 small-subunit rRNA sequences by using the alignment tool ARB_EDIT. Alignments were refined manually by visual inspection. 16S rRNA phylogenetic analyses were exclusively performed with sequences having more than 1.300 bases by applying distance-matrix, maximum-parsimony, and maximum-likelihood methods and the aforementioned ARB treeing tools for nucleotide sequences. The composition of the 16S rRNA data sets varied with respect to the reference sequences and the alignment positions included. Variability of the individual alignment positions was determined by using the ARB_SAI tools and used as criterion to remove or include variable positions (50% conservation filter) for phylogenetic analyses. Parsimony bootstrap analyses based on 100 or 1000 resamplings were performed with PHYLIP. All phylogenetic consensus trees were drawn according to the recommendations of Ludwig *et al.* (1998).

***dsrAB* gene-based phylogeny.** New *dsrAB* sequences were added to an ARB alignment which contains all *dsrAB* sequences of recognized (Friedrich 2002, Klein *et al.* 2001) and uncultured SRPs available in GenBank (Benson *et al.* 2002). Deduced amino acid sequences were manually aligned by using the editor GDE 2.2 (S.W. Smith, C. Wang, P.M. Gillevet and W. Gilbert (1992) Genetic Data Environment and the Harvard Genome Database. Genome mapping and Sequencing, Cold Spring Harbor Laboratory). Nucleic acid sequences were aligned according to the amino acid alignment. During phylogenetic analyses of amino acid sequences, regions of insertions and deletions were removed from the DsrAB data set by using an amino acid alignment mask (indel filter) prepared in ARB. A total of 543 amino acid positions (alpha subunit, 327; beta subunit, 216) were used in DsrAB analyses. Distance-matrix (using FITCH with global rearrangements and randomized input order of species) and maximum-parsimony trees were calculated with the PHYLogeny Inference Package (PHYLIP, version 3.57c, J. Felsenstein, Department of Genetics, University of Washington, Seattle). In addition, the programs MOLPHY (version 2.3, Computer science monographs, no. 28.: Programs for molecular phylogenetics based on maximum-likelihood. J. Adachi and M. Hasegawa, Institute of Statistics and Mathematics, Tokyo, Japan) and TREE-PUZZLE (Strimmer and von Haeseler 1996) were used to infer maximum-likelihood trees with JTT-f as the amino acid replacement model. To perform *dsrAB* phylogenetic analysis on the nucleotide level filters were constructed which allowed to exclude regions of insertions and

deletions as well as the third codon position for phylogenetic analysis. Nucleic acid level phylogenetic analyses were performed by applying distance-matrix [using the Jukes-Cantor model (Jukes and Cantor 1969)], maximum-parsimony (PHYLIP program), and maximum-likelihood methods [fastDNAml program (Olsen *et al.* 1994)]. Parsimony bootstrap analyses for protein (DsrAB) and nucleotide level (*dsrAB*) phylogenetic analysis were performed with PHYLIP. For each calculation 100 or 1000 resamplings were performed. All phylogenetic consensus trees were drawn according to the recommendations of Ludwig *et al.* (1998).

Nomenclature of prokaryotes. Names of bacterial taxa were used in accordance with the prokaryotic nomenclature proposed in the taxonomic outline of the second edition of Bergey`s Manual of Systematic Bacteriology (http://www.cme.msu.edu/bergeys/) (Garrity and Holt 2001) and the Approved Lists of Bacterial Names (http://www.bacterio.cict.fr/) (Euzeby 1997) as recommended by Oren and Stackebrandt (2002).

Nucleotide sequence accession numbers. The sequences determined in this thesis are available in the GenBank database under accession numbers AY083010 to AY083027 and AY167444 to AY167462 (16S rRNA gene clones), and AY083028/AY083029 and AY167464 to AY167483 (*dsrAB* gene clones). The *dsrAB* gene sequence of *Desulfomicrobium orale* DSM 12838^T has been deposited under accession number AY083030.

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RESULTS AND DISCUSSION

I. Phylogeny of hitherto recognized SRPs based on comparative 16S rRNA sequence analyses

The last encompassing study that has revealed natural relationships among all known SRP genera already dates back eight years (Stackebrandt *et al.* 1995). Since that time microbial taxonomy was confronted with a tremendous increase in the description of novel SRPs. In total, 70 new species and 19 new genera of SRPs have been validly published between 1996 and 2002. The primary aim of the work presented in this chapter was to establish a thorough and robust phylogenetic framework for SRPs based on comparison of 16S rRNA gene sequences. Therefore, all 16S rRNA gene sequences from provisionally proposed and validly recognized SRPs available in GenBank were collected, aligned, and analyzed phylogenetically by using maximum-likelihood, maximum-parsimony, and distance-matrix treeing methods, each in combination with appropriate conservation filters (for details see figure legends in this chapter). As already pointed out in the introduction part of this thesis, above-genus level classification of SRPs that is currently in use is scarce and not up-to-date. Thus, latest taxonomic outlines proposed in the second edition of Bergey`s Manual of Systematic Bacteriology (http://www.cme.msu.edu/bergeys/) (Garrity and Holt 2001c) were used as guidance to assign SRPs into higher taxonomic ranks and to reveal potentially misclassified species.

SRBs of the class "*Deltaproteobacteria*" belonging to the bacterial phylum *Proteobacteria*

Most SRB species isolated so far belong to the orders "*Desulfobacterales*", "*Syntrophobacterales*", and "*Desulfovibrionales*" (Garrity and Holt 2001c) within the delta-class of *Proteobacteria*. Figure 5 depicts the 16S rRNA-based phylogeny of "*Deltaproteobacteria*" with emphasis on the affiliation of distinct SRB lineages. Independent from the treeing method applied, all analyzed species of the order "*Desulfovibrionales*" formed a stable monophyletic entity which was highly supported by parsimony bootstrap analysis (92%). In contrast, the phylogenetic position of distinct "*Desulfobacterales*" and "*Syntrophobacterales*" families relative to each other could not be resolved on the basis of 16S rRNA phylogeny as indicated by a polytomic tree topology (Figure 5).

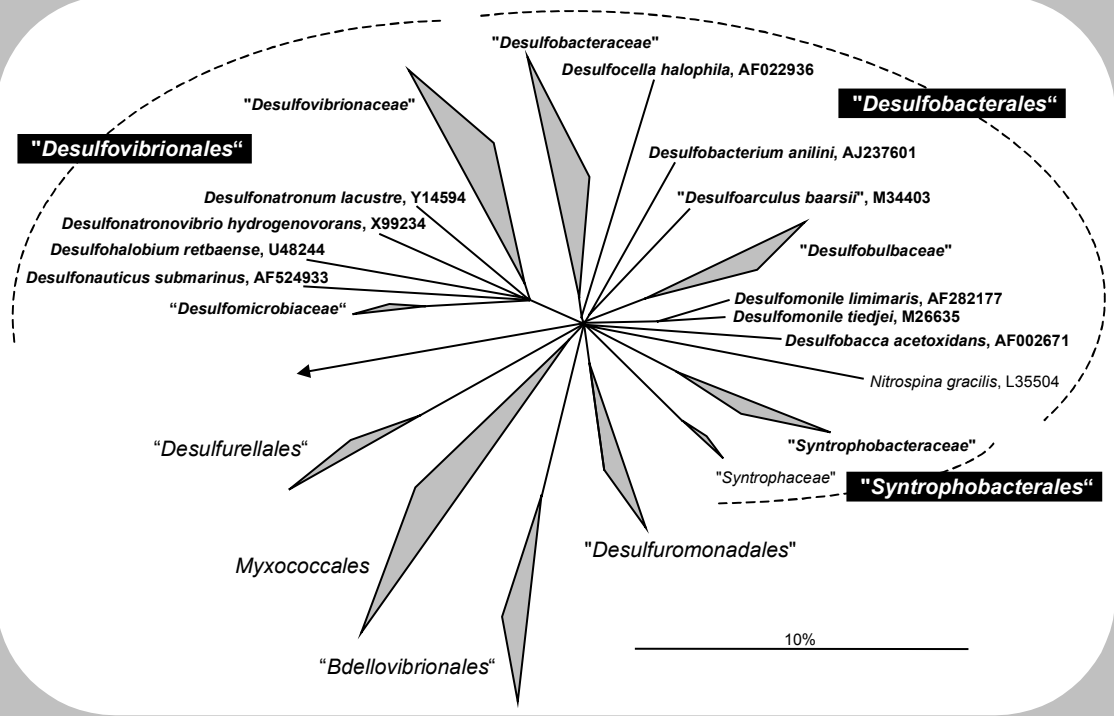

Figure 5. Radial 16S rRNA-based phylogenetic tree showing the affiliation of all named SRPs within the class "*Deltaproteobacteria*". The consensus tree is based on neighbor-joining analysis performed with a 50% conservation filter for the "*Deltaproteobacteria*". The bar indicates 10% estimated sequence divergence (distance inferred by neighbor-joining). Multifurcation connect branches for which a relative order could not be determined unambiguously by applying neighbor-joining, maximum-parsimony, and maximum-likelihood treeing methods.

The order "*Desulfovibrionales*". According to Bergey`s Manual of Systematic Bacteriology, the order "*Desulfovibrionales*" currently encompass four phylogenetic groups which correspond to the taxonomic rank of a family: the "*Desulfovibrionaceae*", the "*Desulfomicrobiaceae*", the "*Desulfohalobiaceae*", and the "*Desulfonatronumaceae*" (Garrity and Holt 2001c).

The family "*Desulfovibrionaceae*". The current version of the Approved Lists of Bacterial Names (Euzeby 1997) contains 37 entries on validly published *Desulfovibrio* species. Members of the mesophilic genus *Desulfovibrio* and the two non-sulfate-reducing species *Bilophila wadsworthia*, which has been found to be associated with human diseases (Baron *et al.* 1989, Kasten *et al.* 1992), and *Lawsonia intracellularis*, the etiological agent of proliferative enteropathy in animals (Smith and Lawson 2001) constitute the provisional family "*Desulfovibrionaceae*" (Garrity and Holt 2001c). All treeing methods applied indicated a common ancestry for all members of this family, although this was not substantially supported by parsimony bootstrap analysis (Figure 6).

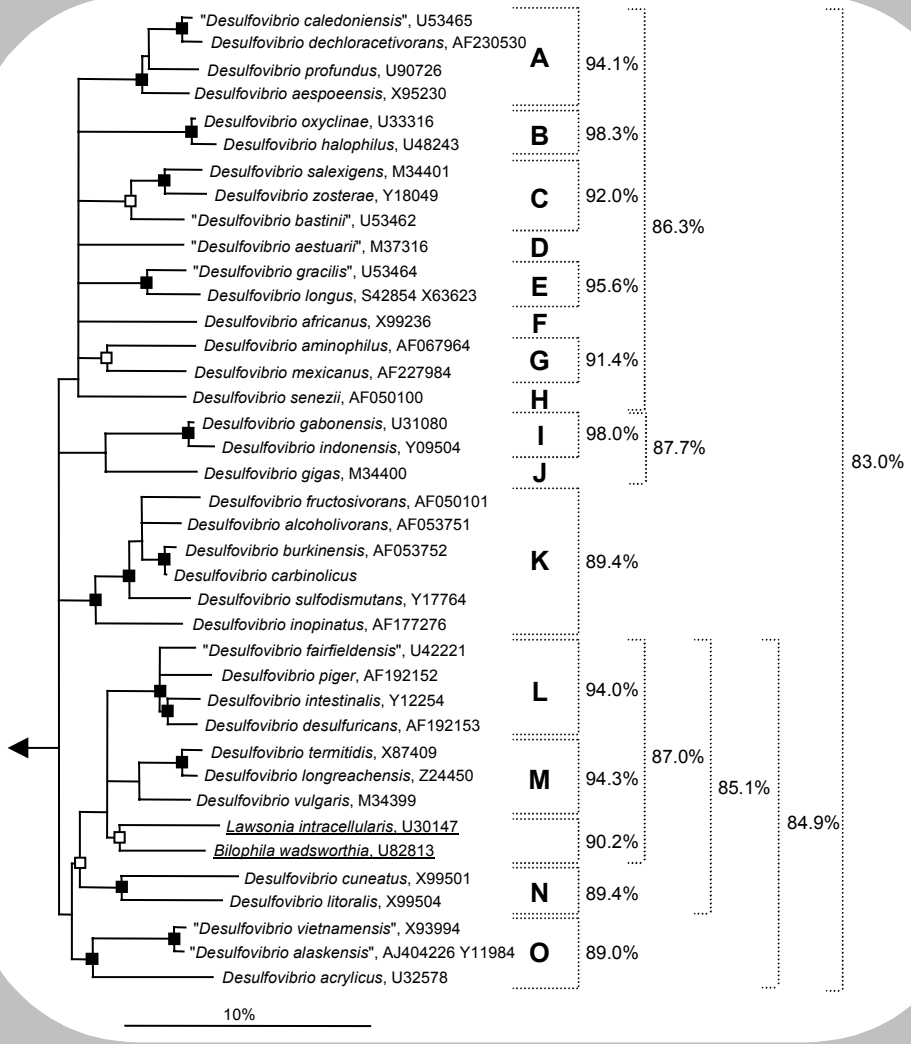

Figure 6. Phylogenetic 16S rRNA-based dendrogram showing the affiliation of all named SRPs of the deltaproteobacterial family "*Desulfovibrionaceae*". The consensus tree is based on neighbor-joining analysis performed with a 50% conservation filter for the "*Deltaproteobacteria*". The bar indicates 10% estimated sequence divergence (distance inferred by neighbor-joining). Multifurcation connect branches for which a relative order could not be determined unambiguously by applying neighbor-joining, maximum-parsimony, and maximum-likelihood treeing methods. Parsimony bootstrap values (100 resamplings) for branches are indicated by solid squares (>90%) or open squares (75 to 90%). Branches without squares had bootstrap values of less than 75%. Non-SRPs are underlined. Percentage value at dotted parenthesis depicts minimum 16S rRNA sequence similarities of two members of the parenthetical group.

In addition, it could be demonstrated that the genus *Desulfovibrio* is paraphyletic owing to the intermediate phylogenetic position of *Bilophila wadsworthia* and *Lawsonia intracellularis* among distinct *Desulfovibrio* spp. Furthermore, the vast diversity among *Desulfovibrio* spp. was categorized in 15 lineages (A to O). The individual *Desulfovibrio* lineages were phylogenetically well distinguishable (parsimony bootstrap values >71% and 16S rRNA

similarities >89%), although their phylogenetic position relative to each other could not always be resolved (Figure 6). This subgrouping of the genus *Desulfovibrio* was additionally supported by the existence of lineage-specific signature sequence segments (18-mer) within the 16S rRNA molecule that could be exploited as target sites for diagnostic oligonucleotide probes (Appendix I, Loy *et al.* 2002). Devereux *et al.* (1990) were the first to recognize the great phylogenetic depth and diversity within the genus *Desulfovibrio*. Correlation between 16S rRNA similarity and percentage of DNA-DNA homology have shown that differences between the *Desulfovibrio* lineages are equivalent to differences between other bacterial genera. These results have lead to the provisional recognition of *Desulfovibrio* spp. at the taxonomic rank of a family, the "*Desulfovibrionaceae*", (Devereux *et al.* 1990) to establish the basis for reclassification of distinct *Desulfovibrio* lineages into separate genera. However, Devereux *et al.* already have argued that it is not appropriate to reclassify *Desulfovibrio* spp. only based on 16S rRNA phylogeny. This statement still holds true today, because lineage-specific physiological properties of diagnostic value for the establishment of new genera are still lacking.

The family "*Desulfomicrobiaceae*". Although *Desulfomicrobium* spp. have been traditionally classified to the "*Desulfovibrionaceae*" (Devereux *et al.* 1990), they were provisionally reassigned to the novel family "*Desulfomicrobiaceae*", recently (Garrity and Holt 2001c). Phylogenetic treeing showed that "*Desulfomicrobiaceae*" comprise a closely related and monophyletic assemblage of *Desulfomicrobium* spp. and the presumably misnamed species *Desulfobacterium macestii* (Figure 7).

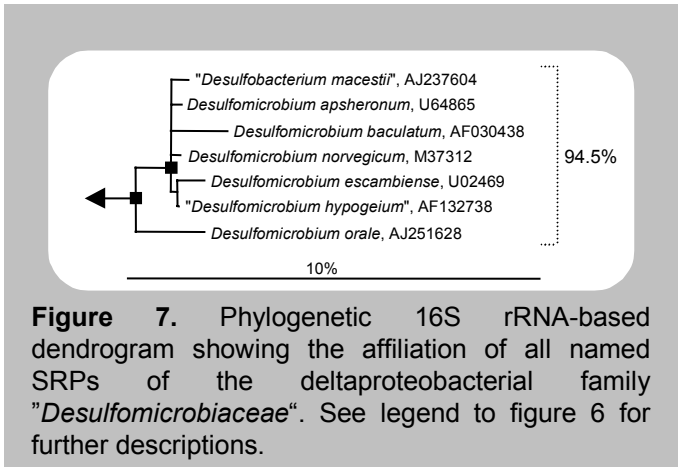

Figure 7. Phylogenetic 16S rRNA-based dendrogram showing the affiliation of all named SRPs of the deltaproteobacterial family "*Desulfomicrobiaceae*". See legend to figure 6 for further descriptions.

16S rRNA analysis could not separate *Desulfobacterium macestii* from the genus *Desulfomicrobium*, contrariwise *Desulfobacterium macestii* branched off clearly within this

genus (Figure 7). Based on this phylogenetic evidence, a reclassification of *Desulfobacterium macestii* into the genus *Desulfomicrobium* should be taken into consideration.

The family "*Desulfohalobiaceae*". Although the only two species of this family, *Desulfohalobium retbaense* and *Desulfonatronovibrio hydrogenovorans*, share the ability to tolerate high salinity (Zhilina *et al.* 1997), they represented two independent lines of descent within the radiation of the "*Desulfovibrionales*" (Figure 5). In addition, 16S rRNA dissimilarity of both species to each other (12.5%) was in the range of 16S rRNA dissimilarities of both species to other "*Desulfovibrionales*" (11.1-17.4%). Whether *Desulfonatronovibrio hydrogenovorans* is a true member of the "*Desulfohalobiaceae*" or represent a novel family must await further taxonomic analysis of yet to describe SRBs which are most closely related to *Desulfohalobium retbaense* or *Desulfonatronovibrio hydrogenovorans*.

The family "*Desulfonatronumaceae*". This family currently comprises only a single species: the extremely alkaliphilic *Desulfonatronum lacustre* (Pikuta *et al.* 1998), which was phylogenetically well distinguishable from other SRBs of the "*Desulfovibrionales*" (Figure 5).

Additional family-level diversity within the order "*Desulfovibrionales*". The 16S rRNA sequence of the newly described hydrothermal vent SRB *Desulfonauticus submarinus* (Audiffrin *et al.* 2003) was phylogenetically in the radiation of the "*Desulfovibrionales*" (Figure 5). Further assignment of *Desulfonauticus submarinus* to one of the proposed "*Desulfovibrionales*" families was not unambiguously possible due to the low 16S rRNA similarity of *Desulfonauticus submarinus* (82.8 to 86.7%) to other SRBs of this order. According to its 16S rRNA-based genealogy, *Desulfonauticus submarinus* is likely a member of a novel SRB family.

The order "*Desulfobacterales*". The order "*Desulfobacterales*" is subdivided at present into three provisional families, the "*Desulfobacteraceae*", the "*Desulfobulbaceae*", and the "*Nitrospinaceae*" (Garrity and Holt 2001c). The former two families include solely bacteria capable of anaerobic sulfate respiration.

The family "*Desulfobacteraceae*" (formerly "*Desulfobacteriaceae*"). Aside from *Desulfovibrio* and *Desulfomicrobium*, all remaining deltaproteobacterial SRBs have been traditionally grouped into the "*Desulfobacteraceae*" (Widdel and Bak 1992). Presently, this family comprises a phenotypically and phylogenetically diverse collection of meso- and psychrophilic SRB genera, most of which have a monophyletic origin (Figure 8).

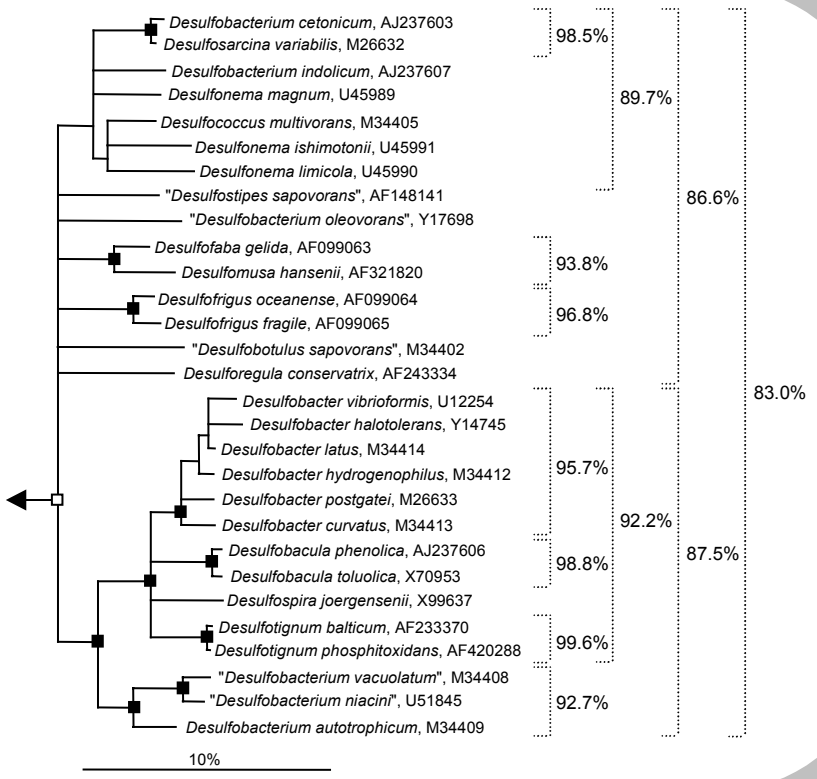

Figure 8. Phylogenetic 16S rRNA-based dendrogram showing the affiliation of all named SRPs of the deltaproteobacterial family "*Desulfobacteraceae*". See legend to figure 6 for further descriptions.

Thus, the "*Desulfobacteraceae*" already have a considerable taxonomic substructure, which is in contrast to the "*Desulfovibrionaceae*" which harbor only a single SRB genus (see above), although both families have the same evolutionary depth (83% minimum 16S rRNA similarity) (Figures 6 and 8). It should be stressed that members of the genera *Desulfobacterium* and *Desulfonema* are polyphyletic from a 16S rRNA-based point of view (Figure 8). Especially *Desulfobacterium* spp. are phylogenetically scattered among the "*Deltaproteobacteria*" (Figures 5, 7, 8, and 9) and should be subject of future revision. Moreover, whether *Desulfocella halophila* (Figure 5) represents the deepest branch within the "*Desulfobacteraceae*" tree or already a novel family can not be decided based only on 16S rRNA sequence data.

The family "*Desulfobulbaceae*" (formerly "*Desulfobulbusaceae*"). Recently, the taxonomic status of a family, the "*Desulfobulbaceae*", has been tentatively proposed for *Desulfobulbus* and related SRB genera (Rooney-Varga *et al.* 1998). Owing to the fact that the "*Desulfobulbaceae*" were phylogenetically far apart from other deltaproteobacterial families

(Figure 5), the considerable phylogenetic depth of this group (Figure 9), and similar phenotypic traits among members of this group (Rooney-Varga *et al.* 1998), authorized the proposal of a new family.

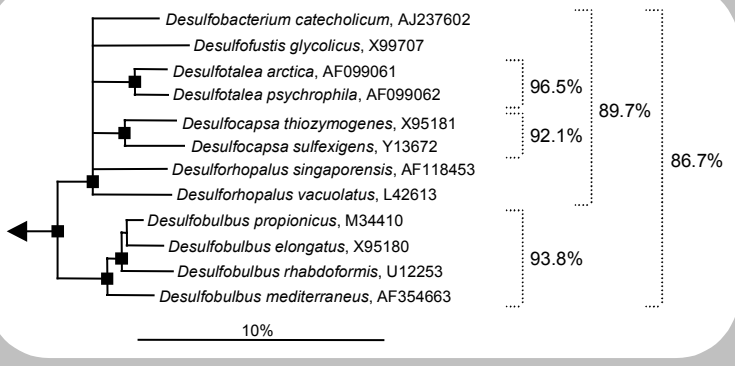

Figure 9. Phylogenetic 16S rRNA-based dendrogram showing the affiliation of all named SRPs of the deltaproteobacterial family "*Desulfobulbaceae*". See legend to figure 6 for further descriptions.

Additionally, the "*Desulfobulbaceae*" can be subdivided into two major lines of descent. One of them contains solely the genus *Desulfobulbus* whereas the other harbors the remaining "*Desulfobulbaceae*" genera (Figure 9). Considering potentially misclassified SRBs, the genus *Desulfotalea* (Knoblauch *et al.* 1999) is a descendant of the "*Desulfobulbaceae*" (Figure 9) and not of the "*Desulfobacteraceae*" as listed in the taxonomic outline of Bergey`s Manual of Systematic Bacteriology (Garrity and Holt 2001c).

The family "*Nitrospinaceae*". The marine nitrite-oxidizing bacterium *Nitrospina gracilis* and the sulfate-reducing genera *Desulfomonile* and *Desulfobacca* are currently listed as members of the family "*Nitrospinaceae*" (Garrity and Holt 2001c). However, arrangement of these three genera into a single family is not supported by current 16S rRNA data. *Nitrospina, Desulfomonile*, and *Desulfobacca* are not monophyletic (Figure 5) and, additionally, show considerable evolutionary distance to each other (16S rRNA similarities of 83.7 to 85.2%). Therefore, these bacteria should be reconsidered for family-level classification when additional, closely related isolates become available.

Additional family-level diversity within the order "*Desulfobacterales*". Although *Desulfobacterium anilini* and "*Desulfoarculus baarsii*" (still validly described as *Desulfovibrio baarsii*) have been formerly assigned to the "*Desulfobacteraceae*" (Widdel and Bak 1992), comparative 16S rRNA analysis clearly separated both species from each other and from other SRB families (Figure 5). The low maximal 16S rRNA similarity of

Desulfobacterium anilini and "*Desulfoarculus baarsii*" to other deltaproteobacterial SRBs (86.8% and 87.7%, respectively) and their isolated position in the phylogenetic tree indicates that they represent two novel families.

The order "*Syntrophobacterales*". The SRB family "*Syntrophobacteraceae*" and the non-SRB family "*Syntrophaceae*", which comprises the syntrophic genera *Syntrophus* and *Smithella*, are presently lumped together in the order "*Syntrophobacterales*" (Garrity and Holt 2001c). However, the topology of the deltaproteobacterial phylogenetic tree did not indicate a monophyletic origin of both families (Figure 5).

The family "*Syntrophobacteraceae*". The "*Syntrophobacteraceae*" line of descent gave rise to three phylogenetically well separated lineages, two of which harbored a single, thermophilic SRB genus: *Desulfacinum* and *Thermodesulforhabdus*, respectively (Figure 10). The third lineage was composed of the sulfate-reducing genera *Desulfovirga*, *Desulforhabdus*, and *Syntrophobacter*. Because it has been long thought that *Syntrophobacter* spp. are strictly syntrophic bacteria and can only be grown in co-culture with methanogens or SRPs (Boone and Bryant 1980), the finding that *Syntrophobacter wolinii* itself was capable of dissimilatory sulfate reduction in pure culture came as a surprise (Wallrabenstein *et al.* 1994).

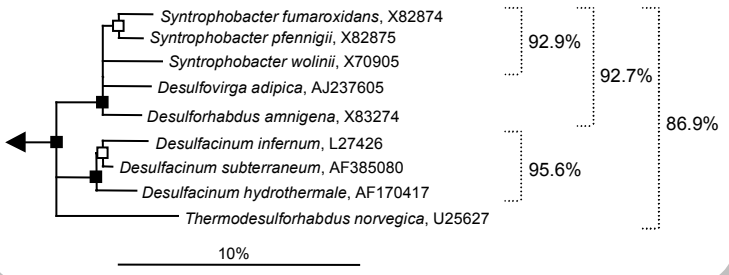

Figure 10. Phylogenetic 16S rRNA-based dendrogram showing the affiliation of all named SRPs of the deltaproteobacterial family "*Syntrophobacteraceae*". See legend to figure 6 for further descriptions.

SRBs of the class "*Clostridia*" belonging to the bacterial phylum *Firmicutes*

Because phylogeny of all endosporeforming, low DNA G+C content gram-positive SRBs was analyzed in detail recently (Stackebrandt *et al.* 1997) and just six new sulfate-reducing

Firmicutes have been validly described since then, only a brief overview on the present taxonomic status of endosporeforming SRBs is given below.

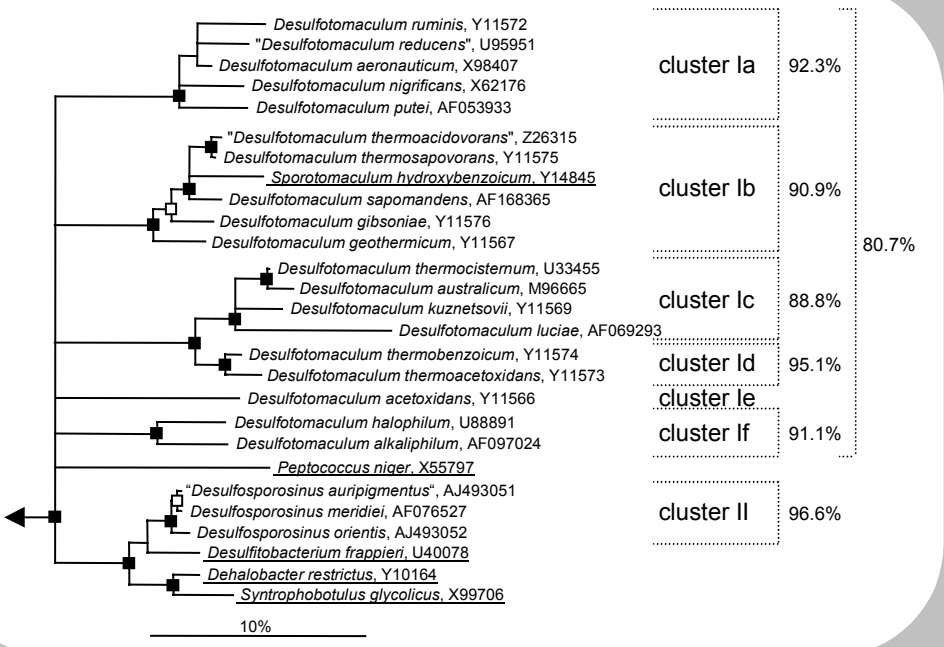

Figure 11. Phylogenetic 16S rRNA-based dendrogram showing the affiliation of all named SRPs of the low G+C gram-positive bacterial family *Peptococcaceae* (phylum *Firmicutes*). The consensus tree is based on maximum-likelihood analysis performed with a 50% bacterial conservation filter. The bar indicates 10% estimated sequence divergence (distance inferred by maximum-likelihood). Multifurcation connect branches for which a relative order could not be determined unambiguously by applying neighbor-joining, maximum-parsimony, and maximum-likelihood treeing methods. Parsimony bootstrap values (100 resamplings) for branches are indicated by solid squares (>90%) or open squares (75 to 90%). Branches without squares had bootstrap values of less than 75%. Cluster designation of low G+C gram-positive SRPs is according to Stackebrandt *et al*. (1997). Non-SRPs are underlined. Percentage value at dotted parenthesis depicts minimum 16S rRNA sequence similarities of two members of the parenthetical group.

The low DNA G+C content gram-positive SRB genera are represented only by the genera, *Desulfotomaculum* and *Desulfosporosinus*, which belong to two independent lineages within the phylum *Firmicutes* (Figure 11). Members of the genus *Desulfotomaculum* have been subdivided in six clusters (Ia-Ie) based on 16S rRNA data (Stackebrandt *et al.* 1997). This clustering could be confirmed by the results presented in this thesis and further extended to include cluster If comprising *Desulfotomaculum halophilum* and *D. alkaliphilum* (Figure 11) (Kuever *et al.* 1999). As already observed for *Desulfovibrio* spp., the different cluster might be treated taxonomically as individual genera owing to the low intercluster 16S rRNA similarity values (Stackebrandt *et al.* 1997).

The phylogenetically homogeneous genus *Desulfosporosinus* represents the formerly *Desulfotomaculum* cluster II (Figure 11) and currently encompass three species: *D. orientis*, *D. meridiei*, and "*D. auripigmentus*", which is still validly recognized as *Desulfotomaculum auripigmentum* (Stackebrandt, E., unpublished data).

SRBs of the class "*Nitrospira*" belonging to the bacterial phylum *Nitrospirae*

All members of the phylum *Nitrospirae*, although metabolically diverse, were also combined at lower taxonomic level to the family "*Nitrospiraceae*" (order "*Nitrospirales*" and class "*Nitrospira*", respectively) (Garrity and Holt 2001b). Consistent with the original definition of the phylum (Ehrich *et al.* 1995) and results from a recent study (Daims *et al.* 2001a), *Nitrospirae* can be differentiated in three main monophyletic lineages: the *Nitrospira* lineage, the *Leptospirillum* lineage, and the deeply branching "*Magnetobacterium*"-*Thermodesulfovibrio* lineage (Figure 12).

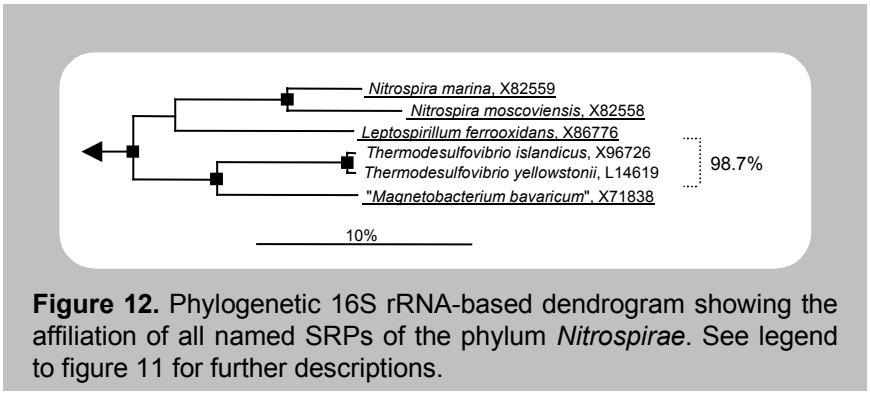

Figure 12. Phylogenetic 16S rRNA-based dendrogram showing the affiliation of all named SRPs of the phylum *Nitrospirae*. See legend to figure 11 for further descriptions.

Among the *Nitrospirae* solely the two described species of the thermophilic genus *Thermodesulfovibrio* (growth optimum at 65°C) have the ability to use sulfate as terminal electron acceptor in anaerobic respiration. For further details on physiology and phylogeny of this SRB genus the reader is referred to the *Thermodesulfovibrio* chapter in the first volume of Bergey`s Manual of Systematic Bacteriology published recently (Maki 2001).

SRBs of the class *Thermodesulfobacteria* belonging to the bacterial phylum *Thermodesulfobacteria*

The phylum *Thermodesulfobacteria*, like the phylum *Nitrospirae*, branches deeply in the mayor bacterial reference trees (Garrity and Holt 2001a). Furthermore, only one family,

Themodesulfobacteriaceae (order *Thermodesulfobacteriales* and class *Thermodesulfobacteria*, respectively) has been accepted in the phylum *Thermodesulfobacteria*. Until recently, this family consisted of a single, thermophilic sulfate-reducing genus, *Thermodesulfobacterium* (Hatchikian and Ollivier 2001, Widdel 1999). This has changed owing to the stable monophyletic affiliation and close evolutionary distance (16S rRNA similarities of 93.5 to 95.2%) of the newly discovered Fe(III)-reducer "*Geothermobacterium ferrireducens*" (Kashefi *et al.* 2002a) to *Thermodesulfobacterium* spp. (Figure 13). Thus, based on phylogenetic evidence, "*Geothermobacterium ferrireducens*" can be considered as member of the *Themodesulfobacteriaceae*.

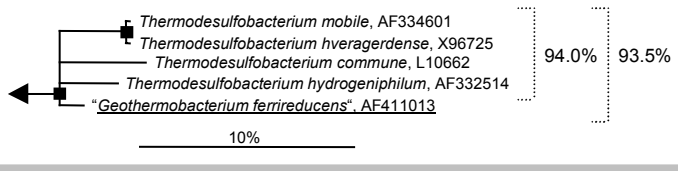

Figure 13. Phylogenetic 16S rRNA-based dendrogram showing the affiliation of all named SRPs of the phylum *Thermodesulfobacteria*. See legend to figure 11 for further descriptions.

SRAs of the class *Archaeoglobi* belonging to the archaeal phylum *Euryarchaeota*

Representatives of the hyperthermophilic genus *Archaeoglobus* (growth optimum around 80°C) are the only validly recognized sulfate-reducers of the domain *Archaea* to date (Huber and Stetter 2001). Regarding taxonomy, sulfate-reducing *Archaeoglobus* spp., Fe(II)-oxidizing *Ferroglobus placidus*, and Fe(III)-reducing *Geoglobus ahangari* were placed in the same family *Archaeoglobaceae* (order *Archaeoglobales* and class *Archaeoglobi*, respectively) (Kashefi *et al.* 2002b). Although a monophyletic origin for all descendants of the *Archaeoglobaceae* was verified by all phylogeny inference methods applied, this was not the case for *Archaeoglobus* spp., which were heterogeneously spread among non-SRAs of the *Archaeoglobaceae* (Figure 14).

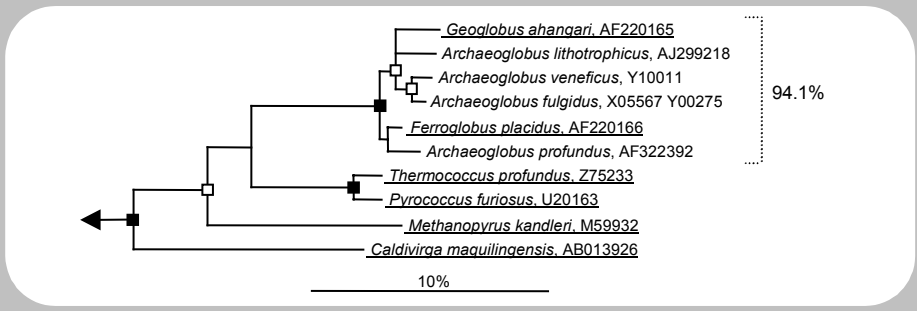

Figure 14. Phylogenetic 16S rRNA-based dendrogram showing the affiliation of all named SRPs of the domain *Archaea*. The consensus tree is based on maximum-likelihood analysis performed with a 50% archaeal conservation filter. The bar indicates 10% estimated sequence divergence (distance inferred by maximum-likelihood). Multifurcation connect branches for which a relative order could not be determined unambiguously by applying neighbor-joining, maximum-parsimony, and maximum-likelihood treeing methods. Parsimony bootstrap values (100 resamplings) for branches are indicated by solid squares (>90%) or open squares (75 to 90%). Branches without squares had bootstrap values of less than 75%. Non-SRPs are underlined. Percentage value at dotted parenthesis depicts minimum 16S rRNA sequence similarities of two members of the parenthetical group.

II. *In silico* design and evaluation of SRP-specific, 16S rRNA-targeted oligonucleotide probes suitable for DNA microarray application

Essential for the design of a set of rRNA-targeted oligonucleotides with hierarchical specificity is the knowledge of the phylogeny of the target groups. Hence, the 16S rRNA-based phylogenetic framework established for SRPs in the first part of this thesis served as reference throughout the whole, computer-assisted probe development procedure.

Initially, the specificities of previously described probes and primers for SRPs (Amann *et al.* 1990, Daims *et al.* 2000, Daly *et al.* 2000, Devereux *et al.* 1992, Fukui *et al.* 1999, Hristova *et al.* 2000, Manz *et al.* 1998, Rabus *et al.* 1996, Ravenschlag *et al.* 2000, Scheid and Stubner 2001, Stubner and Meuser 2000) were reevaluated with the current 16S rRNA data set by using the ARB PROBE_MATCH tool (Strunk and Ludwig, http://www.arb-home.de). Based on this analysis, 27 probes were considered to be suitable for inclusion on the DNA microarray (Table 5). These probes were, if necessary, adjusted to a length of 18 nucleotides (not including the T-spacer). Twenty-five of these probes exclusively target SRPs. Probes SRB385 (Amann *et al.* 1990) and SRB385Db (Rabus *et al.* 1996) were included on the microarray because they have been widely used in previous SRP research (Amann *et al.* 1992, Edgcomb *et al.* 1999, Li *et al.* 1999, Oude Elferink *et al.* 1998, Santegoeds *et al.* 1998, Teske *et al.* 1996), although both probes do target a considerable number of phylogenetically diverse non-SRPs. In addition, the existing SRP probe set was significantly extended by designing 111 probes targeting monophyletic groups of SRPs. (Table 5) (Appendix I and III, Loy *et al.* 2003b, Loy *et al.* 2002). *In silico* design of oligonucleotide probes was performed with ARB tools PROBE_DESIGN and PROBE_MATCH in order to retrieve lists of all potential, SRP-specific probes from the 16S rRNA database and, subsequently, to determine the most specific probes (Hugenholtz *et al.* 2001). The probes were selected to have a minimum G+C content of 50%, a length of 18 nucleotides (not including the T-spacer), and as many centrally located mismatches with the target sites on 16S rRNA genes of nontarget organisms as possible. Consistent with design formats used in previous microarray applications for identification of other bacterial groups (Guschin *et al.* 1997, Liu *et al.* 2001), a hierarchical set of oligonucleotides complementary to the 16S rRNA genes of the target microorganisms at multiple levels of specificity was developed according to the "multiple probe concept" (Amann and Schleifer 2001, Behr *et al.* 2000). Thus, several of these probes target the same SRPs, complementing several unique regions of the 16S rRNA gene, while others exhibit hierarchical specificity. For example, the genus *Desulfotalea* is specifically detected by five

probes and is also targeted by three probes with broader specificities (Table 5) (Appendix I, Loy *et al.* 2002). Altogether, all recognized SRPs for which 16S rRNA sequences have been published are covered by the probe set developed.

Table 5. 16S rRNA-targeted oligonucleotide probes.

Original probe name	Probe name	Full name[a]	Sequence 5'-3'	Microarray position	Specificity	Reference
-	CONT	-	AGG AAG GAA GGA AGG AAG	A1-F1, A48-F48	Control oligonucleotide	This thesis[g]
-	CONT-COMP	-	CTT CCT TCC TTC CTT CCT	-	Complementary to control oligonucleotide	This thesis[g]
-	NONSENSE	-	AGA GAG AGA GAG AGA GAG	F47	Nonbinding control	This thesis[g]
EUB338	EUB338	S-D-Bact-0338-a-A-18	GCT GCC TCC CGT AGG AGT	D25, F2	most *Bacteria*	Amann *et al.* 1990
EUB338II	EUB338II	S-*-BactP-0338-a-A-18	GCA GCC ACC CGT AGG TGT	F3	Phylum *Planctomycetes*	Daims *et al.* 1999
EUB338III	EUB338III	S-*-BactV-0338-a-A-18	GCT GCC ACC CGT AGG TGT	F4	Phylum *Verrucomicrobia*	Daims *et al.* 1999
UNIV1390	UNIV1389a	S-D-Univ-1389-a-A-18	ACG GGC GGT GTG TAC AAG	D26, F5	*Bacteria*, not "*Epsilonproteobacteria*"	Zheng *et al.* 1996[b]
UNIV1390	UNIV1389b	S-D-Univ-1389-b-A-18	ACG GGC GGT GTG TAC AAA	F6	*Eucarya*	Zheng *et al.* 1996[b]
UNIV1390	UNIV1389c	S-D-Univ-1389-c-A-18	ACG GGC GGT GTG TGC AAG	D34, F7	*Archaea*	Zheng *et al.* 1996[b]
ARCH915	ARCH917	S-D-Arch-0917-a-A-18	GTG CTC CCC CGC CAA TTC	D35	*Archaea*	Stahl and Amann 1991[b]
-	DELTA495a	S-C-dProt-0495-a-A-18	AGT TAG CCG GTG CTT CCT	C2, E2	most "*Deltaproteobacteria*"	This thesis[g]
-	DELTA495b	S-*-dProt-0495-b-A-18	AGT TAG CCG GCG CTT CCT	C3, E3	some "*Deltaproteobacteria*"	This thesis[g]
-	DELTA495c	S-*-dProt-0495-c-A-18	AAT TAG CCG GTG CTT CCT	C4, E4	some "*Deltaproteobacteria*"	This thesis[g]
S-*-Ntspa-712-a-A-21	NTSPA714	S-*-Ntspa-714-a-A-18	CCT TCG CCA CCG GCC TTC	D30	Phylum *Nitrospirae*, not *T. islandicus*	Daims *et al.* 2001a[b]
LGC354A	LGC354a	S-*-Lgc-0354-a-A-18	TGG AAG ATT CCC TAC TGC	A2	Probes LGC354a, LGC354b, and LGC354c target together the phylum *Firmicutes*, but not *Desulfotomaculum* and *Desulfosporosinus*	Meier *et al.* 1999
LGC354B	LGC354b	S-*-Lgc-0354-b-A-18	CGG AAG ATT CCC TAC TGC	A3	see above	Meier *et al.* 1999
LGC354C	LGC354c	S-*-Lgc-0354-c-A-18	CCG AAG ATT CCC TAC TGC	A4	see above	Meier *et al.* 1999
SRB385	SRB385	S-*-Srb-0385-a-A-18	CGG CGT CGC TGC GTC AGG	C5, E5	Many but not all deltaproteobacterial SRPs, *Aerothermobacter* spp., *Thermomonospora* spp., *Actinobispora* spp., *Actinomadura* spp., *Thermoanaerobacter* spp., *Frankia* spp., *Clostridium* spp., *Streptosporangium* spp., *Nitrospira* spp., *Geodermatophilus* spp., *Nocardiopsis* spp., and many more	Amann *et al.* 1990
SRB385Db	SRB385Db	S-*-Srb-0385-b-A-18	CGG CGT TGC TGC GTC AGG	C6, E6	Many but not all deltaproteobacterial SRPs, *Geobacter* spp., *Pelobacter* spp., *Campylobacter* spp., *Saccharopolyspora* spp., *Acetivibrio* spp., *Syntrophus* spp., *Clostridium* spp., *Nitrospina* spp., *Chlorobium* spp., and many more	Rabus *et al.* 1996
DSBAC355	DSBAC355	S-*-Dsbac-0355-a-A-18	GCG CAA AAT TCC TCA CTG	C7	most *"Desulfobacterales"* and *"Syntrophobacterales"*	Scheid and Stubner 2001
-	DSB706	S-*-Dsb-0706-a-A-18	ACC GGT ATT CCT CCC GAT	C8	*Desulfotalea* spp., *Desulfosarcina* sp., *Desulforhopalus* sp., *Desulfocapsa* spp., *Desulfofustis* sp., *Desulfobacterium* sp., *Desulfobulbus* spp., *Thermodesulforhabdus* sp.	This thesis[g]
DSS658	DSS658	S-*-Dsb-0658-a-A-18	TCC ACT TCC CTC TCC CAT	C11	*Desulfostipes* sp., *Desulfobacterium* sp., *Desulfofrigus* spp., *Desulfofaba* sp., *Desulfosarcina* sp., *Desulfomusa* sp.	Manz *et al.* 1998
DSR651	DSR651	S-*-Dsb-0651-a-A-18	CCC CCT CCA GTA CTC AAG	C10	*Desulforhopalus* sp., *Desulfobacterium* sp., *Desulfofustis* sp., *Desulfocapsa* sp., *Desulfobulbus* spp., *Spirochaeta* spp.	Manz *et al.* 1998

Original probe name	Probe name	Full name[a]	Sequence 5'-3'	Microarray position	Specificity	Reference
probe 804	DSB804	S-*-Dsb-0804-a-A-18	CAA CGT TTA CTG CGT GGA	C9	*Desulfobacter* spp., *Desulfobacterium* spp., *Desulfofrigus* spp., *Desulfofaba* sp., *Desulfosarcina* sp., *Desulfostipes* sp., *Desulfococcus* sp., *Desulfobotulus* sp., *Desulforegula* sp.	Devereux *et al.* 1992
-	DSB230	S-*-Dsb-0230-a-A-18	CTA ATG GTA CGC AAG CTC	B6	*Desulfotalea* spp., *Desulforhopalus* sp., *Desulfocapsa* spp., *Desulfofustis* sp., *Desulfobacterium* sp.	This thesis[g]
-	DSTAL131	S-G-Dstal-0131-a-A-18	CCC AGA TAT CAG GGT AGA	B9	*Desulfotalea* spp.	This thesis[g]
-	DSTAL213	S-G-Dstal-0213-a-A-18	CCT CCC GAT ACA ATA GCT	B8	see above	This thesis[g]
-	DSTAL645	S-G-Dstal-0645-a-A-18	CCA GTA CTC AAG CTC CCC	B10	see above	This thesis[g]
-	DSTAL732	S-G-Dstal-0732-a-A-18	TAT CTG GCC AGA TGG TCG	B12	see above	This thesis[g]
-	DSTAL835	S-G-Dstal-0835-a-A-18	GAA GCG ATT AAC CAC TCC	B11	see above	This thesis[g]
-	DSRHP185	S-*-Dsrhp-0185-a-A-18	CCA CCT TTC CTG TTT CCA	B7	*Desulforhopalus* spp.	This thesis[g]
-	DSBB228	S-G-Dsbb-0228-a-A-18	AAT GGT ACG CAG ACC CCT	B4	*Desulfobulbus* spp.	This thesis[g]
probe 660	DSBB660	S-G-Dsbb-0660-a-A-18	ATT CCA CTT TCC CCT CTG	B5	see above	Devereux *et al.* 1992[b]
DSB985	DSB986	S-*-Dsb-0986-a-A-18	CAC AGG ATG TCA AAC CCA	C28	*Desulfobacter* spp., *Desulfobacula* sp., *Desulfobacterium* sp., *Desulfospira* sp., *Desulfotignum* sp.	Manz *et al.* 1998[b]
-	DSB1030	S-*-Dsb-1030-a-A-18	CTG TCT CTG TGC TCC CGA	C27	see above	This thesis[g]
-	DSB1240	S-*-Dsb-1240-a-A-18	TGC CCT TTG TAC CTA CCA	C34	*Desulfobacter* spp., *Desulfotignum* sp.	This thesis[g]
DSB623	DSB623a	S-*-Dsb-0623-a-A-18	TCA AGT GCA CTT CCG GGG	C35	*Desulfobacter curvatus, Dsb. halotolerans, Dsb. hydrogenophilus, Dsb. postgatei, Dsb. vibrioformis*	Daly *et al.* 2000[b]
-	DSB623b	S-*-Dsb-0623-b-A-18	TCA AGT GCA CTT CCA GGG	C36	*Desulfobacter* sp. BG8, *Dsb.* sp. BG23	This thesis[g]
DSB623	DSBLA623	S-S-Dsb.la-0623-a-A-18	TCA AGT GCT CTT CCG GGG	C37	*Desulfobacter latus*	Daly *et al.* 2000[b]
-	DSBACL143	S-G-Dsbacl-0143-a-A-18	TCG GGC AGT TAT CCC GGG	C29	*Desulfobacula* spp.	This thesis[g]
-	DSBACL225	S-G-Dsbacl-0225-a-A-18	GGT CCG CAA ACT CAT CTC	C30	see above	This thesis[g]
-	DSBACL317	S-G-Dsbacl-0317-a-A-18	GAC CGT GTA CCA GTT CCA	C31	see above	This thesis[g]
-	DSBACL1268	S-G-Dsbacl-1268-a-A-18	AGG GAT TCG CTT ACC GTT	C32	see above	This thesis[g]
-	DSBACL1434	S-G-Dsbacl-1434-a-A-18	ATA GTT AGC CCA ACG ACG	C33	see above	This thesis[g]
DSF672	DSB674	S-*-Dsb-0674-a-A-18	CCT CTA CAC CTG GAA TTC	C20	*Desulfofrigus* spp., *Desulfofaba gelida*, *Desulfomusa hansenii*	Ravenschlag *et al.* 2000[b]
-	DSB220	S-*-Dsb-0220-a-A-18	GCG GAC TCA TCT TCA AAC	C25	*Desulfobacterium niacini, Dsbm. vacuolatum, Dsbm. autotrophicum, Desulfofaba gelida*	This thesis[g]
-	DSBM1239	S-*-Dsbm-1239-a-A-18	GCC CGT TGT ACA TAC CAT	C26	*Desulfobacterium niacini, Dsbm. vacuolatum, Dsbm. autotrophicum*	This thesis[g]
-	DSFRG211	S-G-Dsfrg-0211-a-A-18	CCC CAA ACA AAA GCT TCC	C22	*Desulfofrigus* spp.	This thesis[g]
-	DSFRG445	S-G-Dsfrg-0445-a-A-18	CAT GTG AGG TTT CTT CCC	C23	see above	This thesis[g]
-	DSFRG1030	S-G-Dsfrg-1030-a-A-18	TGT CAT CGG ATT CCC CGA	C24	see above	This thesis[g]
DCC868	DCC868	S-*-Dsb-0868-a-A-18	CAG GCG GAT CAC TTA ATG	C38	*Desulfosarcina* sp., *Desulfonema* spp., *Desulfococcus* sp., *Desulfobacterium* spp., *Desulfobotulus* sp., *Desulfostipes* sp., *Desulfomusa* sp.	Daly *et al.* 2000
-	DSSDBM194	S-*-DssDbm-0194-a-A-18	GAA GAG GCC ACC CTT GAT	C40	*Desulfosarcina variabilis, Desulfobacterium cetonicum*	This thesis[g]
-	DSSDBM217	S-*-DssDbm-0217-a-A-18	GGC CCA TCT TCA AAC AGT	C41	see above	This thesis[g]
-	DSSDBM998	S-*-DssDbm-0998-a-A-18	TTC GAT AGG ATT CCC GGG	C39	see above	This thesis[g]
-	DSSDBM1286	S-*-DssDbm-1286-a-A-18	GAA CTT GGG ACG GCT TTT	C42	see above	This thesis[g]
DSC193	DSC193	S-*-Dsb-0193-a-A-18	AGG CCA CCC TTG ATC CAA	C43	*Desulfosarcina variabilis*	Ravenschlag *et al.* 2000
-	DSBMIN218	S-S-Dsbm.in-0218-a-A-18	GGG CTC CTC CAT AAA CAG	C44	*Desulfobacterium indolicum*	This thesis[g]
DCC209	DCC209	S-S-Dcc.mv-0209-a-A-18	CCC AAA CGG TAG CTT CCT	B3	*Desulfococcus multivorans*	Ravenschlag *et al.* 2000
-	DSNISH179	S-S-Dsn.ish-0179-a-A-18	GGG TCA CGG GAA TGT TAT	C45	*Desulfonema ishimotonii*	This thesis[g]
-	DSNISH442	S-S-Dsn.ish-0442-a-A-18	CCC CAG GTT CTT CCC ACA	C46	see above	This thesis[g]
-	DSNISH1001	S-S-Dsn.ish-1001-a-A-18	CGT CTC CGG AAA ATT CCC	C47	see above	This thesis[g]
DNMA657	DSN658	S-*-Dsn-0658-a-A-18	TCC GCT TCC CTC TCC CAT	B2	*Desulfonema limicola, Dsn. magnum*	Fukui *et al.* 1999[b]
-	DSBOSA445	S-S-Dsbo.sa-0445-a-A-18	ACC ACA CAA CTT CTT CCC	C21	*Desulfobotulus sapovorans*	This thesis[g]
-	DSMON95	S-*-Dsmon-0095-a-A-18	GTG CGC CAC TTT ACT CCA	C18	*Desulfomonile* spp.	This thesis[g]
-	DSMON1421	S-*-Dsmon-1421-a-A-18	CGA CTT CTG GTG CAG TCA	C19	see above	This thesis[g]

Original probe name	Probe name	Full name[a]	Sequence 5'-3'	Microarray position	Specificity	Reference
-	DSMON999	S-*-Dsmon-0999-a-A-18	TTT CCA TAG CTG TCC GGG	B17	Uncultured *Desulfomonile*-related bacteria (Schlöppnerbrunnen I soil clones SbIDsmon2, 3, 4, 5, and 8; Cadagno Lake clones 618, 624, 626, 650, and 651)	This thesis[h]
-	DSMON1283	S-*-Dsmon-1283-a-A-18	CTG AGG ACC GAT TTG TGG	B18	Uncultured *Desulfomonile*-related bacteria (Schlöppnerbrunnen I soil clones SbIDsmon2, 3, 4, 5, and 8)	This thesis[h]
-	DSMON447	S-*-Dsmon-0447-a-A-18	ACT CAT GGA GGG TTC TTC	B19	Uncultured *Desulfomonile*-related bacteria (Schlöppnerbrunnen I soil clones SbIDsmon3, 5, and 8)	This thesis[h]
-	DSMON468a	S-*-Dsmon-0468-a-A-18	CCG TCA TTT CCA TGA GCT	B20	See above	This thesis[h]
-	DSMON446	S-*-Dsmon-0446-a-A-18	CTA GAA GAG GTT TCT TCC	B21	Uncultured *Desulfomonile*-related bacteria (Schlöppnerbrunnen I soil clones SbIDsmon2 and 4)	This thesis[h]
-	DSMON468b	S-*-Dsmon-0468-b-A-18	CCG TCA GTT CCT CTA GCT	B22	See above	This thesis[h]
-	SYBAC986	S-*-Sybac-0986-a-A-18	CCG GGG ATG TCA AGC CCA	C17	*Desulfovirga adipica*, *Desulforhabdus amnigena*, *Syntrophobacter* spp.	This thesis[g]
-	SYBAC697	S-*-Sybac-0697-a-A-18	CCT CCC GAT CTC TAC GAA	B13	See above	This thesis[h]
-	SYN835	S-*-Sybac-0835-a-A-18	GCA GGA ATG AGT ACC CGC	B14	See above	Scheid and Stubner 2001
-	SYBAC587a	S-*-Sybac-0587-a-A-18	CAT CAG ACT TTT CGG CCC	B15	Uncultured *Syntrophobacter*-related bacteria (Schlöppnerbrunnen I soil clones SbISybac13, 15, and 19; Schlöppnerbrunnen II soil clones SbIISybac12-1, 1-2, 3-2, and 13-2)	This thesis[h]
-	SYBAC587b	S-*-Sybac-0587-b-A-18	CAT CAG ACT TGC CGG CCC	B16	Uncultured *Syntrophobacter*-related bacteria (Schlöppnerbrunnen I soil clones SbISybac16; Schlöppnerbrunnen II soil clones SbIISybac25-1, 6-2, and 8-2)	This thesis[h]
-	DSACI175	S-G-Dsaci-0175-a-A-18	CCG AAG GGA CGT ATC CGG	C16	*Desulfacinum* spp.	This thesis[g]
-	DSACI207	S-G-Dsaci-0207-a-A-18	CGA ACA CCA GCT TCT TCG	C15	see above	This thesis[g]
-	TDRNO448	S-S-Tdr.no-0448-a-A-18	AAC CCC ATG AAG GTT CTT	C13	*Thermodesulforhabdus norvegica*	This thesis[g]
-	TDRNO1030	S-S-Tdr.no-1030-a-A-18	TCT CCC GGC TCC CCA ATA	C12	see above	This thesis[g]
-	TDRNO1443	S-S-Tdr.no-1443-a-A-18	GAC ACA ATC GCG GTT GGC	C14	see above	This thesis[g]
probe 687	DSV686	S-*-Dsv-0686-a-A-18	CTA CGG ATT TCA CTC CTA	E7	*"Desulfovibrionales"* and other "*Deltaproteobacteria*"	Devereux *et al.* 1992[b]
DSV1292	DSV1292	S-*-Dsv-1292-a-A-18	CAA TCC GGA CTG GGA CGC	E9	*Desulfovibrio litoralis*, *Dsv. vulgaris*, *Dsv. longreachensis*, *Dsv. termitidis*, *Dsv. desulfuricans*, *Dsv. fairfieldensis*, *Dsv. intestinalis*, *Dsv. inopinatus*, *Dsv. senezii*, *Dsv. gracilis*, *Dsv. halophilus*, *Bilophila wadsworthia*	Manz *et al.* 1998
DSV698	DSV698	S-*-Dsv-0698-a-A-18	TCC TCC AGA TAT CTA CGG	E8	*Desulfovibrio caledoniensis*, *Dsv. dechloracetivorans*, *Dsv. profundus*, *Dsv. aespoeensis*, *Dsv. halophilus*, *Dsv. gracilis*, *Dsv. longus*, *Dsv. salexigens*, *Dsv. zosterae*, *Dsv. bastinii*, *Dsv. fairfieldensis*, *Dsv. intestinalis*, *Dsv. piger*, *Dsv. desulfuricans*, *Dsv. termitidis*, *Dsv. longreachensis*, *Dsv. vietnamensis*, *Dsv. alaskensis*, *Bilophila wadsworthia*, *Lawsonia intracellularis*	Manz *et al.* 1998[b]
-	DVDAPC872	S-*-Dv.d.a.p.c-0872-a-A-18	TCC CCA GGC GGG ATA TTT	E33	*Desulfovibrio caledoniensis*, *Dsv. dechloracetivorans*, *Dsv. profundus*, *Dsv. aespoeensis*	This thesis[g]
-	DVHO130	S-*-Dv.h.o-0130-a-A-18	CCG ATC TGT CGG GTA GAT	E36	*Desulfovibrio halophilus*, *Dsv. oxyclinae*	This thesis[g]
-	DVHO733	S-*-Dv.h.o-0733-a-A-18	GAA CTT GTC CAG CAG GCC	E37	see above	This thesis[g]
-	DVHO831	S-*-Dv.h.o-0831-a-A-18	GAA CCC AAC GGC CCG ACA	E35	see above	This thesis[g]
-	DVHO1424	S-*-Dv.h.o-1424-a-A-18	TGC CGA CGT CGG GTA AGA	E38	see above	This thesis[g]

Original probe name	Probe name	Full name[a]	Sequence 5'-3'	Microarray position	Specificity	Reference
-	DVAA1111	S-*-Dv.a.a-1111-a-A-18	GCA ACT GGC AAC AAG GGT	E30	*Desulfovibrio africanus*, *Dsv. aminophilus*	This thesis[g]
-	DVGL199	S-*-Dv.g.l-0199-a-A-18	CTT GCA TGC AGA GGC CAC	E26	*Desulfovibrio gracilis*, *Dsv. longus*	This thesis[g]
-	DVGL445	S-*-Dv.g.l-0445-a-A-18	CCT CAA GGG TTT CTT CCC	E27	see above	This thesis[g]
-	DVGL1151	S-*-Dv.g.l-1151-a-A-18	AAC CCC GGC AGT CTC ACT	E28	see above	This thesis[g]
-	DVGL1421	S-*-Dv.g.l-1421-a-A-18	CGA TGT CGG GTA GAA CCA	E29	see above	This thesis[g]
DSD131	DSVAE131	S-S-Dsv.ae-0131-a-A-18	CCC GAT CGT CTG GGC AGG	E34	*Desulfovibrio aestuarii*	Manz *et al.* 1998
-	DSV820	S-*-Dsv-0820-a-A-18	CCC GAC ATC TAG CAT CCA	E25, E31	*Desulfovibrio salexigens*, *Dsv. zosterae*, *Dsv. fairfieldensis*, *Dsv. intestinalis*, *Dsv. piger*, *Dsv. desulfuricans*	This thesis[g]
-	DVSZ849	S-*-Dv.s.z-0849-a-A-18	GTT AAC TTC GAC ACC GAA	E32	*Desulfovibrio salexigens*, *Dsv. zosterae*	This thesis[g]
-	DVIG448	S-*-Dv.i.g-0448-a-A-18	CGC ATC CTC GGG GTT CTT	E15	*Desulfovibrio gabonensis*, *Dsv. indonesiensis*	This thesis[g]
-	DVIG468	S-*-Dv.i.g-0468-a-A-18	CCG TCA GCC GAA GAC ACT	E16	see above	This thesis[g]
-	DSV651	S-*-Dsv-0651-a-A-18	CCC TCT CCA GGA CTC AAG	E39	*Desulfovibrio fructosivorans*, *Dsv. alcoholivorans*, *Dsv. sulfodismutans*, *Dsv. burkinensis*, *Dsv. inopinatus*	This thesis[g]
-	DVFABS153	S-*-Dv.f.a.b.s-0153-a-A-18	CGG AGC ATG CTG ATC TCC	E40	*Desulfovibrio fructosivorans*, *Dsv. alcoholivorans*, *Dsv. sulfodismutans*, *Dsv. burkinensis*	This thesis[g]
-	DVFABS653	S-*-Dv.f.a.b.s-0653-a-A-18	CAC CCT CTC CAG GAC TCA	E41	see above	This thesis[g]
-	DVFABS1351	S-*-Dv.f.a.b.s-1351-a-A-18	GAG CAT GCT GAT CTC CGA	E42	see above	This thesis[g]
-	DVLVT139	S-*-Dv.l.v.t-0139-a-A-18	GCC GTT ATT CCC AAC TCA	E17	*Desulfovibrio termitidis*, *Dsv. longreachensis*, *Dsv. vulgaris*	This thesis[g]
-	DVLVT175	S-*-Dv.l.v.t-0175-a-A-18	AAA TCG GAG CGT ATT CGG	E18	see above	This thesis[g]
-	DVLT131	S-*-Dv.l.t-0131-a-A-18	TCC CAA CTC ATG GGC AGA	E22	*Desulfovibrio termitidis*, *Dsv. longreachensis*	This thesis[g]
-	DVLT986	S-*-Dv.l.t-0986-a-A-18	TCC CGG ATG TCA AGC CTG	E23	see above	This thesis[g]
-	DVLT1027	S-*-Dv.l.t-1027-a-A-18	TCG GGA TTC TCC GAA GAG	E21	see above	This thesis[g]
-	DSM194	S-G-Dsm-0194-a-A-18	GAG GCA TCC TTT ACC GAC	E11	*Desulfomicrobium* spp., *Desulfobacterium macestii*	This thesis[g]
DSV214	DSM213	S-G-Dsm-0213-a-A-18	CAT CCT CGG ACG AAT GCA	E10	see above	Manz *et al.* 1998[b]
-	DSHRE830	S-S-Dsh.re-0830-a-A-18	GTC CTA CGA CCC CAA CAC	E12	*Desulfohalobium retbaense*	This thesis[g]
-	DSHRE995	S-S-Dsh.re-0995-a-A-18	ATG GAG GCT CCC GGG ATG	E13	see above	This thesis[g]
-	DSHRE1243	S-S-Dsh.re-1243-a-A-18	TGC TAC CCT CTG TGC CCA	E14	see above	This thesis[g]
DFM228	DFMI227a	S-*-Dfmi-0227-a-A-18	ATG GGA CGC GGA CCC ATC	A5	*Desulfotomaculum putei*, *Dfm. gibsoniae*, *Dfm. geothermicum*, *Dfm. thermosapovorans*, *Dfm. thermoacidovorans*, *Dfm. thermobenzoicum*, *Dfm. thermoacetoxidans*, *Dfm. australicum*, *Dfm. kuznetsovii*, *Dfm. thermocisternum*, *Dfm. luciae*, *Sporotomaculum hydroxybenzoicum*	Daly *et al.* 2000[b]
DFM228	DFMI227b	S-*-Dfmi-0227-b-A-18	ATG GGA CGC GGA TCC ATC	A6	*Desulfotomaculum aeronauticum*, *Dfm. nigrificans*, *Dfm. reducens*, *Dfm. ruminis*, *Dfm. sapomandens*, *Dfm. halophilum*	Daly *et al.* 2000[b]
S-*-Dtm(cd)-0216-a-A-19	DFMI210	S-*-Dfmi-0210-a-A-18	CCC ATC CAT TAG CGG GTT	A7	some *Desulfotomaculum* spp. of clusters Ic and Id[c]	Hristova *et al.* 2000[b]
S-*-Dtm(bcd)-0230-a-A-18	DFMI229	S-*-Dfmi-0229-a-A-18	TAA TGG GAC GCG GAC CCA	A8	some *Desulfotomaculum* spp. of clusters Ib, Ic, and Id[c]	Hristova *et al.* 2000
-	DFMIa641	S-*-Dfmia-0641-a-A-18	CAC TCA AGT CCA CCA GTA	A9	*Desulfotomaculum* spp. (cluster Ia)[c]	This thesis[g]
-	DFMIb726	S-*-Dfmib-0726-a-A-18	GCC AGG GAG CCG CTT TCG	A10	*Desulfotomaculum* spp., *Sporotomaculum hydroxybenzoicum* (cluster Ib)[c]	This thesis[g]
-	DFMIc841	S-*-Dfmic-0841-a-A-18	GGC ACT GAA GGG TCC TAT	A11	*Desulfotomaculum* spp. (cluster Ic)[c]	This thesis[g]
-	DFMIc1012	S-*-Dfmic-1012-a-A-18	CGT GAA ATC CGT GTT TCC	A12	see above	This thesis[g]
-	DFMIc1119	S-*-Dfmic-1119-a-A-18	ACC CGT TAG CAA CTA ACC	A13	see above	This thesis[g]
-	DFMIc1138	S-*-Dfmic-1138-a-A-18	GGC TAG AGT GCT CGG CTT	A14	see above	This thesis[g]
-	DFMId436	S-*-Dfmid-0436-a-A-18	CTT CGT CCC CAA CAA CAG	A15	*Desulfotomaculum* spp. (cluster Id)[c]	This thesis[g]
-	DFMId625	S-*-Dfmid-0625-a-A-18	TTT CAA AGG CAC CCC CGC	A16	see above	This thesis[g]
-	DFMId996	S-*-Dfmid-0996-a-A-18	CAC AGG CTG TCA GGG GAT	A17	see above	This thesis[g]
-	DFMId1117	S-*-Dfmid-1117-a-A-18	CCG CTG GCA ACT AAC CGT	A18	see above	This thesis[g]
-	DFACE199	S-S-Df.ace-0199-a-A-18	GCA TTG TAA AGA GGC CAC	A20	*Desulfotomaculum* spp. (cluster Ie)[c]	This thesis[g]

Original probe name	Probe name	Full name[a]	Sequence 5'-3'	Microarray position	Specificity	Reference
-	DFACE438	S-S-Df.ace-0438-a-A-18	CTG TTC GTC CAA TGT CAC	A19	see above	This thesis[g]
-	DFACE995	S-S-Df.ace-0995-a-A-18	CAC AGC GGT CAC GGG ATG	A21	see above	This thesis[g]
D-acet1027r	DFACE1028	S-S-Df.ace-1028-a-A-18	CTC CGT GTG CAA GTA AAC	A22	see above	Stubner and Meuser 2000
-	DFACE1436	S-S-Df.ace-1436-a-A-18	TGC GAG TTA AGT CAC CGG	A23	see above	This thesis[g]
-	DFMIf126	S-*-Dfmlf-0126-a-A-18	CTG ATA GGC AGG TTA TCC	A24	*Desulfotomaculum* spp. (cluster If)[d]	This thesis[g]
-	DFMII1107	S-*-DfmII-1107-a-A-18	CTA AAT ACA GGG GTT GCG	A29	*Desulfosporosinus* spp., *Desulfotomaculum auripigmentum* (cluster II)[c]	This thesis[g]
-	DFMII1281	S-*-DfmII-1281-a-A-18	GAG ACC GGC TTT CTC GGA	A28	see above	This thesis[g]
-	TDSV601	S-*-Tdsv-0601-a-A-18	GCT GTG GAA TTC CAC CTT	D32	*Thermodesulfovibrio* spp.	This thesis[g]
S-*-Tdsulfo-0848-a-A-18	TDSV849	S-*-Tdsv-0849-a-A-18	TTT CCC TTC GGC ACA GAG	D33	see above	Daims *et al.* 2000
-	TDSV1326	S-*-Tdsv-1326-a-A-18	CGA TTC CGG GTT CAC GGA	D31	see above	This thesis[g]
-	TDSBM1282	S-P-Tdsbm-1282-a-A-18	TGA GGA GGG CTT TCT GGG	D27	*Thermodesulfobacterium* spp., *Geothermobacterium* sp.	This thesis[g]
-	TDSBM353	S-*-Tdsbm-0353-a-A-18	CCA AGA TTC CCC CCT GCT	D28	*Thermodesulfobacterium* spp.	This thesis[g]
-	TDSBM652	S-*-Tdsbm-0652-a-A-18	AGC CTC TCC GGC CCT CAA	D29	see above	This thesis[g]
-	ARGLO37	S-G-Arglo-0037-a-A-18	CTT AGT CCC AGC CGG ATA	D37	*Archaeoglobus* spp.	This thesis[g]
-	ARGLO276	S-G-Arglo-0276-a-A-18	GCC CGT ACG GAT CTT CGG	D38	see above	This thesis[g]
-	ARGLO576	S-G-Arglo-0576-a-A-18	CCA GCC CGG CTA CGG ACG	D39	see above	This thesis[g]
-	ARGLO972	S-G-Arglo-0972-a-A-18	CCC CGG TAA GCT TCC CGG	D40	see above	This thesis[g]
-	DSBM168[e]	S-*-Dsbm-0168-a-A-18	ACT TTA TCC GGC ATT AGC	-	*Desulfobacterium niacini, Dsbm. vacuolatum*	This thesis[g]
-	DVHO588[e]	S-*-Dv.h.o-0588-a-A-18	ACC CCT GAC TTA CTG CGC	-	*Desulfovibrio halophilus, Dsv. oxyclinae*	This thesis[g]
-	DVIG267[e]	S-*-Dv.i.g-0267-a-A-18	CAT CGT AGC CAC GGT GGG	-	*Desulfovibrio gabonensis, Dsv. indonesiensis*	This thesis[g]
-	DVLT1425[e]	S-*-Dv.l.t-1425-a-A-18	TCA CCG GTA TCG GGT AAA	-	*Desulfovibrio termitidis, Dsv. longreachensis*	This thesis[g]
-	DVGL228[e]	S-*-Dv.g.l-0228-a-A-18	CAG CCA AGA GGC CTA TTC	-	*Desulfovibrio gracilis, Dsv. longus*	This thesis[g]
-	ARGLO390[e]	S-G-Arglo-0390-a-A-18	GCA CTC CGG CTG ACC CCG	-	*Archaeoglobus* spp.	This thesis[g]
-	DVLVT194[f]	S-*-Dv.l.v.t-0194-a-A-18	AGG CCA CCT TTC CCC CGA	-	*Desulfovibrio termitidis, Dsv. longreachensis, Dsv. vulgaris*	This thesis[g]
-	DVLVT222[f]	S-*-Dv.l.v.t-0222-a-A-18	ACG CGG ACT CAT CCA TGA	-	see above	This thesis[g]
-	DVCL1350[f]	S-*-Dv.c.l-1350-a-A-18	GGC ATG CTG ATC CAG AAT	-	*Desulfovibrio cuneatus, Dsv. litoralis*	This thesis[g]
-	DFMIf489[f]	S-*-Dfmlf-0489-a-A-18	CCG GGG CTT ACT CCT ATG	-	*Desulfotomaculum* spp. (cluster If)[d]	This thesis[g]

[a] Name of oligonucleotide probe based on the nomenclature of Alm *et al.* (1996).
[b] Length of oligonucleotide probe was adapted to the microarray format (18-mer).
[c] Cluster designations of gram-positive, spore-forming SRPs according to Stackebrandt *et al.* (1997).
[d] *Desulfotomaculum halophilum* and *D. alkaliphilum* were assigned to new cluster If.
[e] Probe was removed from the SRP microarray because no positive signal could be detected after hybridization with fluorescently labeled 16S rRNA gene amplificate of the perfect-match reference strain.
[f] Probe was removed from the SRP microarray because it hybridized nonspecifically to many reference organisms that have mismatches in the 16S rRNA gene target site (see supplementary web material).
[g] Appendix I, (Loy *et al.* 2002).
[h] Appendix III, (Loy *et al.* 2003b).

III. Electronic forum for the maintenance and evaluation of rRNA-targeted oligonucleotide probes (http://www.probeBase.net)

All previously published and newly developed SRP-specific, rRNA-targeted oligonucleotide probes were compiled in a database that can be freely accessed via a world wide web-interface (http://www.probeBase.net) (Appendix II, Loy *et al.* 2003a). Each probe entry contains information on the probe sequence, target organisms, target molecule (16S or 23S rRNA), target site, G+C content, melting temperature, molecular weight, and the reference that originally described the oligonucleotide probe plus a direct link to the respective reference abstract at PubMed (Wheeler *et al.* 2002). In order to facilitate database searches, each probe entry includes additional hidden information on the taxonomic context of the probe target organisms. If a probe has been successfully applied for FISH, the probe name is highlighted, and the recommended formamide concentration in the hybridization buffer required for specific hybridization is provided.
Additionally, one has to keep in mind that with the increasing amounts of rRNA sequence data stored in public databases (Cole *et al.* 2003, Maidak *et al.* 2001, Wuyts *et al.* 2002) (Strunk and Ludwig, http://www.arb-home.de) the recognized specificity range for a probe might change. A good example is probe SRB385 which was designed to be specific for deltaproteobacterial SRBs (Amann *et al.* 1990), but nowadays with many more 16S rRNA sequence data available has been recognized to target various, phylogenetically unrelated bacterial groups (Table 5). Thus, prior to the application of a rRNA-targeted oligonucleotide probe, researchers are obliged to ascertain that the specificity proposed for this probe in the original publication is still valid. Probe match tools as implemented in the ARB program package or provided by the Ribosomal Database Project II (RDP-II) (Cole *et al.* 2003, Maidak *et al.* 2001) offer an option to check for up-to-date specificity of a probe when used in combination with the latest rRNA databases. Consequently, for each probe, probeBase offers direct links to the probe match tool at the RDP-II web site. Difference alignments for 16S rRNA-targeted probes are available that were generated using the PROBE_MATCH tool of the ARB software and the ARB database (release June 2002).

Microbiological research in general was confronted with an increase in interest in rRNA-targeted oligonucleotide probes during the past years. This is reflected in the widespread use of rRNA-targeted oligonucleotide probes in hybridization formats like dot-/slot-blot (Raskin *et al.* 1994) and FISH (Juretschko *et al.* 2002) to identify uncultured prokaryotes and to

quantitatively determine the composition of complex microbial communities (Daims *et al.* 2001b). Several recent studies also demonstrated the applicability of FISH for routine diagnostic purposes in the clinical laboratory (Hu *et al.* 2002, Jansen *et al.* 2000, Poppert *et al.* 2002). In addition, a suite of new techniques circling around rRNA-targeted probes has been developed. For example, the combination of FISH and microautoradiography can be used to determine the ecophysiology of microorganisms by visualizing *in situ* uptake and subsequent incorporation of a radioactively labeled substrate into individual microbial cells (Gray *et al.* 2000, Lee *et al.* 1999, Ouverney and Fuhrman 1999). As a consequence of the increased development and application of rRNA-targeted oligonucleotide probes during the past years, several hundred of ready-to-use domain-, phylum-, genus-, and species-specific probes are already available. Furthermore, it is obvious that this number of already available rRNA-targeted oligonucleotide probes will dramatically increase in the near future because of the continuous exploitation of highly parallel hybridization formats such as DNA microarray technology (Guschin *et al.* 1997, Liu *et al.* 2001, Small *et al.* 2001, Urakawa *et al.* 2002, Wilson *et al.* 2002). However, an overview over published probe sequences can only be obtained by a time-consuming, tedious literature search.

In order to account for the pronounced interest in rRNA-targeted oligonucleotide probes of the scientific community, the aim behind the setup of probeBase was not only to compile SRP-specific but all published rRNA-targeted probes. A similar probe database project started in 1996, when Alm *et al.* compiled the Oligonucleotide Probe Database (OPD) that listed 96 PCR primers and probes mainly targeting small-subunit (SSU) and large-subunit (LSU) rRNA (Alm *et al.* 1996). However, OPD has not been updated since 1997 and is now no longer available through the internet. ProbeBase (Appendix II, Loy *et al.* 2003a) closes this gap by providing a user-friendly web-interface to search for more than 850 published oligonucleotide probe sequences (to date February 2003) and annotated bibliographic and biological information. In addition, probeBase offers two main functions, <search probeBase> and <find probe set>, to search for suitable probes. Figure 15 gives a schematic overview of the basic structure and functions of probeBase.

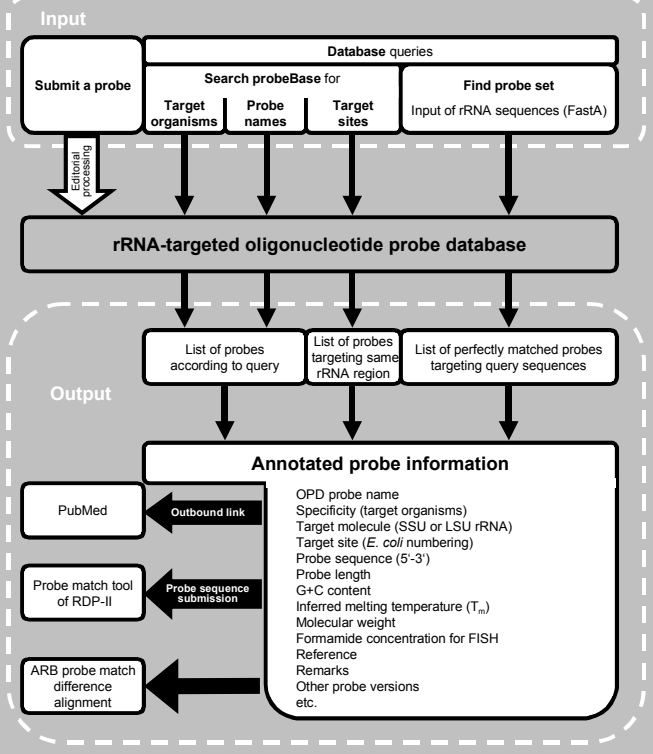

Figure 15. Concept and structure of probeBase.

By using the <search probeBase> function probeBase can either be searched for probe target organisms, for probe names, or for probe target sites. If probeBase is searched for oligonucleotide probes specific for certain target organisms, it returns a list of all oligonucleotide probes specific for the searched target organisms as well as probes targeting higher taxonomic levels. This list of probes supports researchers in the choice of an appropriate set of nested probes according to the "multiple probe concept" (see above) (Amann and Schleifer 2001, Behr *et al.* 2000, Mobarry *et al.* 1996). The simultaneous application of a set of hierarchical probes enhances the reliability of the detection of a particular microorganism.

The possibility to search for a given probe target site assists in the development of new oligonucleotide probes for FISH by providing information whether a searched target site has previously been found accessible for oligonucleotide probes in other microorganisms. Studies by Fuchs *et al.* have demonstrated that some regions on the 16S and 23S rRNA of *Escherichia coli* are virtually inaccessible for oligonucleotide probes if used for FISH (Fuchs *et al.* 2001, Fuchs *et al.* 1998). Unfortunately, these results can only be extrapolated to distantly related microorganisms within certain limits. However, if different probes targeting microorganisms affiliated with different evolutionary lineages but sharing the same target site

on the respective rRNA molecule have been successfully applied for FISH, it is very likely that the respective target site is generally accessible for oligonucleotide probes.

The second function, the <find probe set> tool of probeBase can be used to rapidly retrieve all published probes targeting one or several query rRNA gene sequences without prior comparative sequence analysis. A set of up to 150 sequences, provided by the researcher as rRNA or DNA sense strand sequence in 5'-3' orientation (FastA format) can be searched simultaneously for the presence of the perfect match target sites of all probes deposited at probeBase. The output is a table sequentially listing (i) each single query sequence with all perfectly matching probes found in probeBase and (ii) each possible probe with all perfectly matching query sequences. Using this probeBase feature researchers will, for example, easily be able to determine a set of already published probes that target the microbial sequences in a certain environmental rRNA gene clone library. This probe set might then be used in subsequent hybridization experiments to confirm the presence of the organisms detected in the rRNA gene clone library *in situ* and to gain insight in the actual abundance of these microorganisms in the investigated environment.

IV. Specificity and sensitivity of a 16S rRNA-based oligonucleotide microarray for detection of all recognized SRPs (SRP-PhyloChip) as evaluated with pure cultures

Prior to hybridization, the SRP-specific probes were spotted onto glass slides by using a pattern roughly reflecting the phylogeny of the SRPs (Table 5). In addition, universal, bacterial, and archaeal probes as well as a nonsense probe (NONSENSE, with a sequence having at least four mismatches to every known 16S rRNA sequence) were immobilized on the microarray for hybridization control purposes (Table 5). Furthermore, another nonsense probe (CONT) (Table 5) was spotted at the beginning and the end of each probe row of the microarray. During hybridization, a fluorescently labeled oligonucleotide fully complementary to this probe was added for control of hybridization efficiency and for straightforward localization of the probe spot rows in the microarray readout. The number of 16S rRNA-targeted oligonucleotide probes used in this thesis is significantly higher than the number of probes used in previous applications of microarrays for bacterial identification (Guschin *et al.* 1997, Koizumi *et al.* 2002, Liu *et al.* 2001, Reyes-Lopez *et al.* 2003, Small *et al.* 2001). This difference had important implications for the strategy which was selected for optimizing the hybridization conditions to ensure maximum specificity of the probes.

In the first step, temperature-dependent dissociation of several probe-target duplexes with perfect matches or mismatches was measured by hybridizing the SRP-PhyloChip with fluorescently labeled 16S rRNA gene amplificates of *Desulfovibrio halophilus*, *Desulfomicrobium apsheronum*, and *Desulfohalobium retbaense* under increasingly stringent conditions. For each data point, a separate microarray with nine replicate spots of each probe was hybridized, washed, and analyzed. Figure 16 shows representative melting curves of probe-target duplexes for two of the SRP-specific probes and for the bacterial probe EUB338 with the labeled 16S rRNA gene amplificates of the three reference organisms. Positive hybridization signals were recorded with probe EUB338 for the three SRPs when wash temperatures between 42°C and 60°C were used. However, the EUB338 hybridization signal intensities varied significantly for the three reference organisms, indicating that there were variations in the efficiency of the fluorescence labeling of the PCR amplificates (Figure 16C). Clear discrimination between perfectly matched and mismatched duplexes was achieved for most but not all of the probes investigated (Figure 16 A and B and Figure 17). When a wash temperature of 42°C was used, the fluorescence intensity of probe-target hybrids with mismatches was almost always lower than the fluorescence intensity of completely matched

hybrids (Figure 17 A). Unexpectedly, the difference in signal intensity between completely matched and mismatched duplexes was not significantly increased by gradually increasing the wash temperature to 80°C (Figure 17). Comparable dissociation temperatures (T_d) between 58 and 62°C, at which 50% of the starting duplexes remained intact, were observed for the different duplexes.

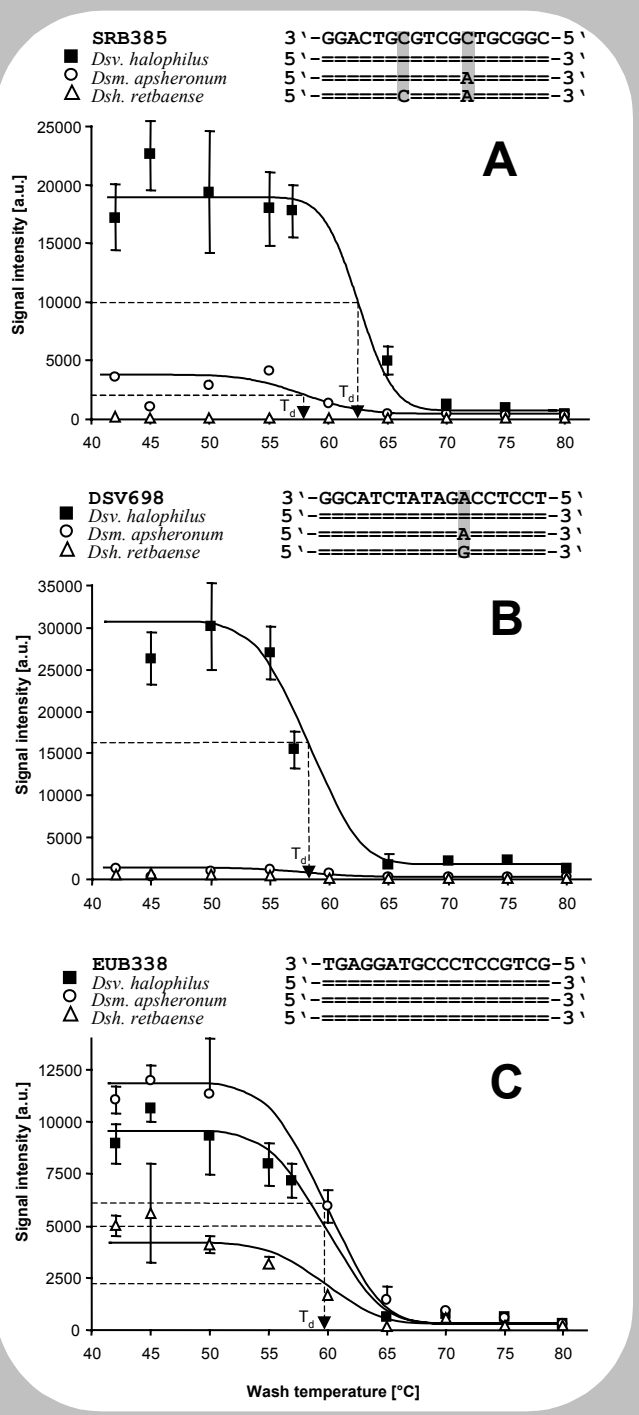

Figure 16. Melting curves for probe SRB385 (A), probe DSV698 (B), and EUB338 (C) after hybridization with fluorescently labeled PCR-amplified 16S rRNA gene fragments of *Desulfovibrio halophilus*, *Desulfomicrobium apsheronum*, and *Desulfohalobium retbaense*. For each probe the difference alignment with these reference SRPs is shown. The observed dissociation temperature (T_d) is indicated for each probe. Each data point represents the mean signal intensity value for 10 probe spots (local background was subtracted for each measurement). The error bars indicate the standard deviations. For each wash temperature and reference organism a separate microarray hybridization was performed. a.u., arbitrary units.

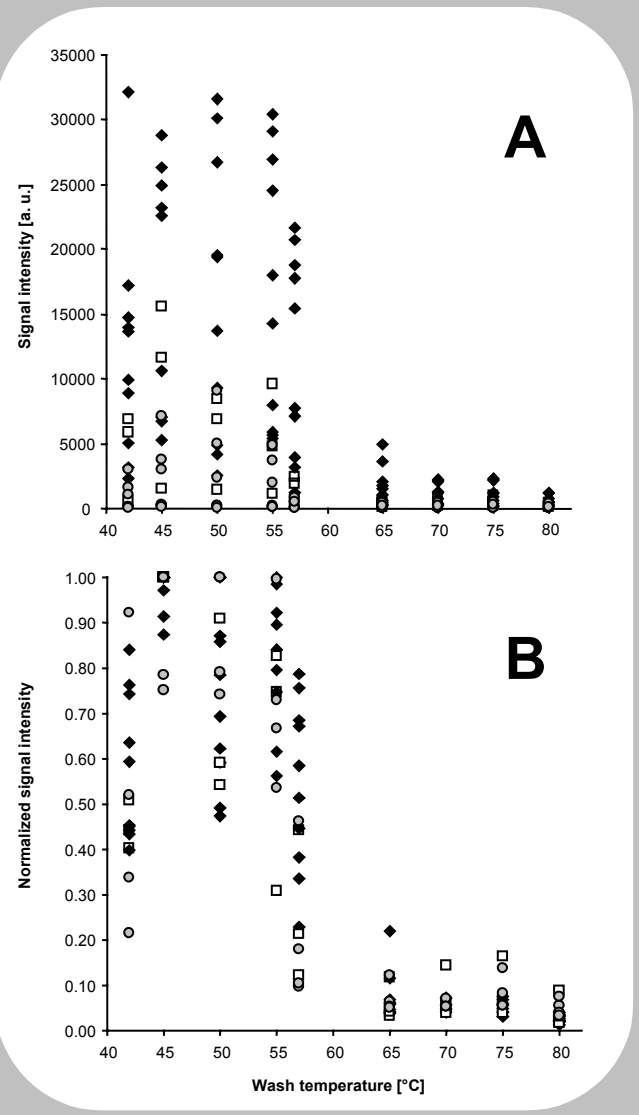

Figure 17. Hybridization intensities of probes forming perfect-match (diamonds), one-mismatch (squares), and two-mismatch duplexes (circles) after hybridization with fluorescently labeled PCR-amplified 16S rRNA gene fragments of *Desulfovibrio halophilus* at different stringencies. (A) Mean signal intensities (for 10 spots, with local background subtracted) for each probe and wash temperature. (B) Normalized mean signal intensity values for each probe and wash temperature. Mean intensity values were normalized for each probe separately by assuming that the highest value observed at the different wash temperatures had a value of 1.00. In panel B, probes which showed no hybridization signals already at low stringencies are not shown.

This congruence probably reflects the fact that all probes of the SRP-PhyloChip are the same length (18 nucleotides) and the fact that the wash buffer contained 3 M tetramethylammonium chloride to equalize A:T and G:C base pair stability (Maskos and Southern 1992). Because our setup did not allow us to determine nonequilibrium online melting curves (Liu *et al.* 2001, Urakawa *et al.* 2002), it was not feasible (due to the high number of probes used) to record melting curves for each probe with perfectly matched and suitably mismatched target nucleic acids. Based on the recorded melting curves of selected probes, a wash temperature of 55°C was chosen for all further experiments as the best compromise between signal intensity and stringency. A further increase in stringency significantly reduced the signal intensity of some probes after hybridization with the perfectly matched target molecules (Figure 17 A) and thus decreased the sensitivity of the microarray.

In the next step, an SRP-PhyloChip with duplicate spots for each probe was evaluated by using 42 SRP reference organisms to determine a threshold value above which a probe hybridization signal was considered positive. For each SRP-specific probe, this set of reference organisms contained an SRP which has a 16S rRNA gene with a perfectly matched target site. For each reference organism, fluorescently labeled, PCR-amplified 16S rRNA gene fragments were hybridized separately with the microarray by using 55°C as the wash temperature. The array readout was quantitatively analyzed by digital image analysis to determine a signal-to-noise ratio for each probe according to the following formula:

$$T = [I_P - (I_N - I_{NLB})] \times I_{PLB}^{-1}$$

where T is the signal-to-noise ratio of the probe, I_P is the mean pixel intensity of both specific probe spots, I_N is the mean pixel intensity of both NONSENSE probe spots (note that I_N-I_{NLB} must always have a lower value than I_P), I_{NLB} is the mean pixel intensity of the local background area around both NONSENSE probe spots, and I_{PLB} is the mean pixel intensity of the local background area around both specific probe spots.

Spots for which the signal-to-noise ratio was equal to or greater than 2.0 were considered positive in the pure culture evaluation experiments and all subsequent analyses. Furthermore, the signal-to-noise ratio of each probe was divided by the signal-to-noise ratio of the bacterial EUB338 probe recorded on the same microarray in order to compare the duplex yields of the different SRP-specific probes. To do this, the following formula was used:

$$R = T \times \{[I_{EUB} - (I_N - I_{NLB})] \times I_{EUBLB}^{-1}\}^{-1}$$

where R is the normalized signal-to-noise ratio of the probe, I_{EUB} is the mean pixel intensity of all EUB338 probe spots, and I_{EUBLB} is the mean pixel intensity of the local background area around all EUB338 probe spots.

The individual hybridization results for each of the 142 probes with each of the reference organisms can be downloaded via the internet (supplementary material at http://www.microbial-ecology.net/) (Appendix I and III, Loy *et al.* 2003b, Loy *et al.* 2002). Consistent with data from quantitative FISH experiments performed with different 16S rRNA-targeted oligonucleotide probes for *Escherichia coli* (Fuchs *et al.* 1998), the normalized signal-to-noise ratios of the probes ranged from 0.3 for probe DFACE1028 with *Desulfotomaculum acetoxidans* to 17.6 for probe DSBAC355 with *Syntrophobacter wolinii*,

demonstrating that different probes exhibit very different signal intensities after hybridization with their perfectly matched target sequences (factor up to 59). In addition, six of the probes evaluated (listed separately in Table 5) did not show a positive hybridization signal with any of the reference organisms, including the perfect-match target SRP, and thus were excluded from the microarray in subsequent experiments. Dramatic differences in duplex yield arising from different regions of the target were also observed in other microarray applications (Milner *et al.* 1997, Southern *et al.* 1994) and probably reflect either accessibility differences for the different probe target sites due to secondary structures of the target DNA or different steric hindrances of the different nucleic acid hybrids formed on the microarrays after hybridization. Furthermore, four probes (listed separately in Table 5) were found to be not suitable for SRP diversity surveys due to their nonspecific binding to many nontarget organisms under stringent hybridization conditions (see supplementary web material).

Under the conditions used, 75 of the probes found to be suitable for the SRP-PhyloChip hybridized exclusively to their target organisms. The other probes hybridized to rRNA gene amplificates with perfectly matched target sites, as well as to some rRNA genes with target sites having between one and six mismatches. In summary, of the 5248 individual probe-target hybridization reactions performed (by hybridizing the reference organisms with the final SRP-PhyloChip), 5050 (96%) gave the expected results by either showing a detectable signal with the appropriate perfect-match target or showing no signal with target sequences containing mismatches (Appendix I, Loy *et al.* 2002).

Finally, the SRP-PhyloChip was hybridized in independent experiments with different amounts (1, 5, 10, 25, 50, 100, 200, and 400 ng) of PCR amplified, labeled 16S rRNA gene fragments of *Desulfovibrio halophilus*. The same hybridization pattern was observed when 50 to 400 ng of labeled nucleic acids was used. When less than 50 ng of added nucleic acid was used, the signal-to-noise ratios of the hybridization signals were less than 2.0.

The evaluation of the microarray with SRP pure cultures demonstrated (i) that false-negative hybridization never occurred (within the detection limit of the microarray method) but (ii) that some of the probes still hybridized to nontarget organisms under the hybridization and washing conditions used, leading to false-positive results (see supplementary web material). As expected, the nucleotide composition of the mismatch, the mismatch position (Stahl and Amann 1991, Urakawa *et al.* 2002), and possibly other variables, such as the influence of an adjacent nucleotide stacking interaction (Fotin *et al.* 1998), were the major factors determining the duplex yields of probes with mismatched target nucleic acids. Most of the mismatched duplexes with signal intensities above the threshold value (used to differentiate

between positive and negative hybridization results) had a signal intensity (and normalized signal-to-noise ratio) lower than that of the corresponding perfect-match duplex (Figure 17 A). However, this difference cannot be exploited for interpretation of microarray hybridization results for environmental samples because a low hybridization signal of a probe can be caused not only by mismatched duplex formation but also by low abundance of the perfect-match target nucleic acid.

Misinterpretation of microarray hybridization patterns caused by the nonperfect specificity of some of the probes could be avoided at least partially by using the "multiple probe concept". While hybridization patterns consistent with the hierarchical or parallel specificity of the probes increase the reliability of detection, inconsistent probe hybridization patterns must be interpreted with caution. In complex samples, inconsistent hybridization patterns can be caused either by nonspecific binding of one or several probes or by previously unrecognized prokaryotes with unusual combinations of perfect-match probe target sites in their 16S rRNA gene sequences.

V. Application of the SRP-PhyloChip for analysis of complex environmental and medical samples

To evaluate the applicability of the SRP-PhyloChip for clinical and environmental determinative studies, three different habitats each containing a diverse assemblage of microorganisms were analyzed.

In the first experiment, tooth pocket samples from five patients suffering from adult periodontitis (Loesche and Grossman 2001) were investigated (Appendix I, Loy *et al.* 2002). Previous culturing studies have suggested that SRPs, among other well-known, anaerobic bacteria (Loesche and Grossman 2001), may be etiologically involved in destructive periodontal diseases (Langendijk *et al.* 1999, Langendijk *et al.* 2000). Hydrogen sulfide (H_2S), main end product of metabolically active SRPs, is a biologically active reagent with a strongly cytotoxic effect (Ratcliff and Johnson 1999, Rizzo 1967). Elevated concentration of H_2S do correlate with increased periodontal pocket depth (Persson 1992), reaching H_2S concentrations far beyond the toxic level (Ratcliff and Johnson 1999). Sulfate, that serves as terminal electron acceptor and promotes growth of SRPs, might theoretically derive from serum and/or be liberated from glycosaminoglycans in the connective tissue (van der Hoeven *et al.* 1995).

While for three of the five patients analyzed none of the SRP-specific probes on the microarray showed a positive signal (data not shown), probe hybridization patterns indicative of the presence of members of the genus *Desulfomicrobium* were obtained for the other two patients (Figure 18 A). This result was confirmed independently by PCR analysis of the DNA obtained from the tooth pockets of the five patients by using primers specific for the 16S rRNA gene of members of the genus *Desulfomicrobium* (primer pair DSM172F-DSM1469R [Table 2]). Consistent with the microarray results, specific PCR amplificates were obtained for two of the five patients. Amplificates from both of these patients were cloned and sequenced. Comparative analysis of six clones demonstrated that the amplified sequences were almost identical to each other and to the corresponding 16S rRNA gene fragment of *Desulfomicrobium orale* (99.6 to 99.9% sequence similarity) (Figure 19).

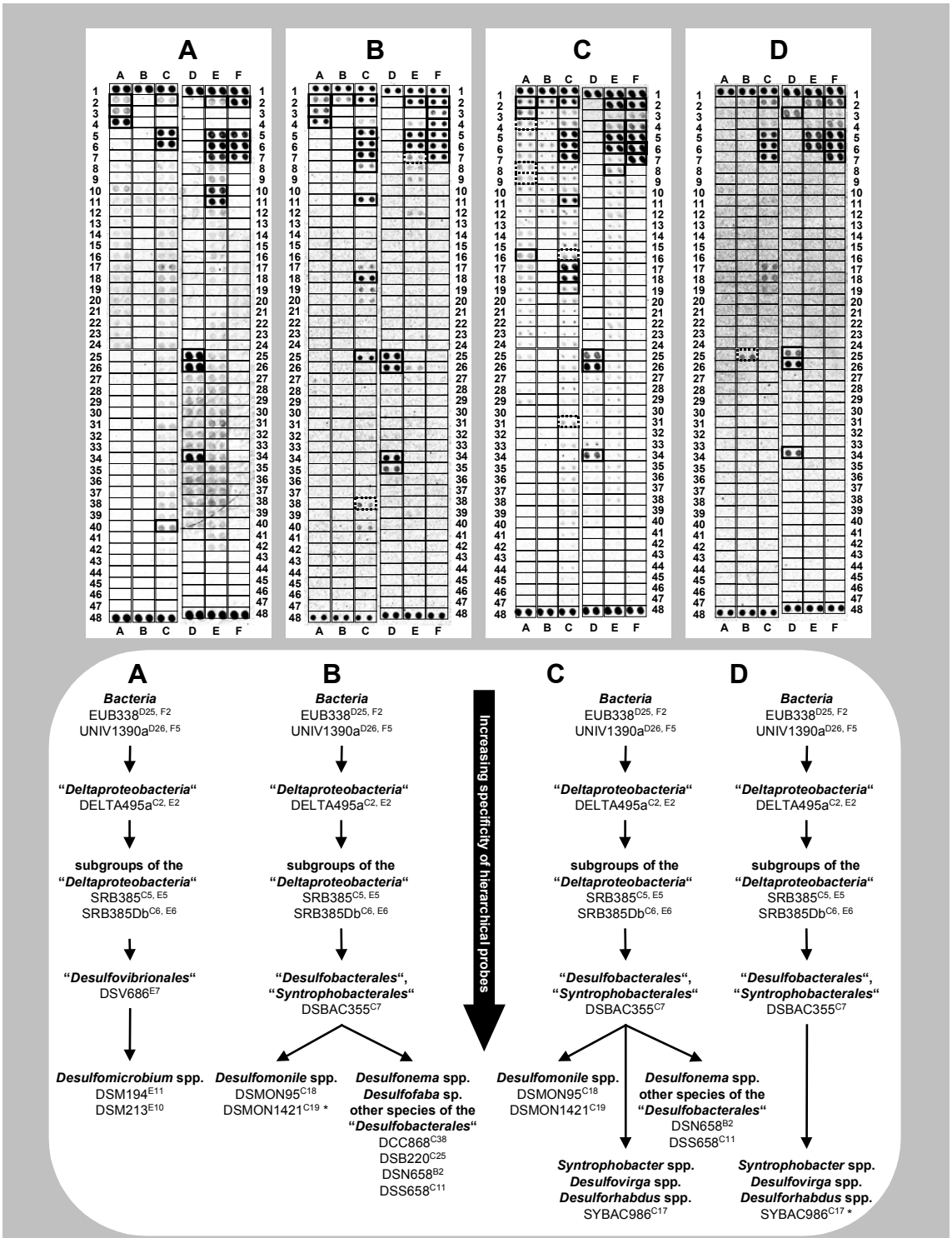

Figure 18. Use of the SRP-PhyloChip for surveys of SRP diversity in periodontal tooth pockets (A), in the chemocline of a cyanobacterial microbial mat (B), and in 22.5-30 cm depth of two acidic fen soils SbI (C) and SbII (D), respectively. Upper panel: On the microarray each probe was spotted in duplicate. For each microarray position, the probe sequence and specificity are shown in Table 5. Probe spots having a signal-to-noise ratio equal to or greater than 2.0 are indicated by boldface boxes and were considered to be positive. The dotted boldface boxes indicate that only one of the duplicate spots had a signal-to-noise ratio equal to or greater than 2.0. Lower panel: The flow charts illustrate the presence of distinct SRP groups in the analyzed samples as inferred from positive signals of sets of probes with nested and/or parallel specificity. The asterisk indicate that the mean signal-to-noise ratios of duplicate probe spots were only slightly below the threshold value of 2.0.

Furthermore, the composition of the SRP communities in the tooth pockets of the patients were analyzed by using the genes encoding the dissimilatory (bi)sulfite reductase (*dsrAB*) as marker (Klein *et al.* 2001, Wagner *et al.* 1998). Approximately 1.9-kb *dsrAB* fragments could be PCR amplified from two of the five patients by using the primer pair DSR1F-DSR4R (Table 3), and these fragments were cloned and sequenced (Figure 19).

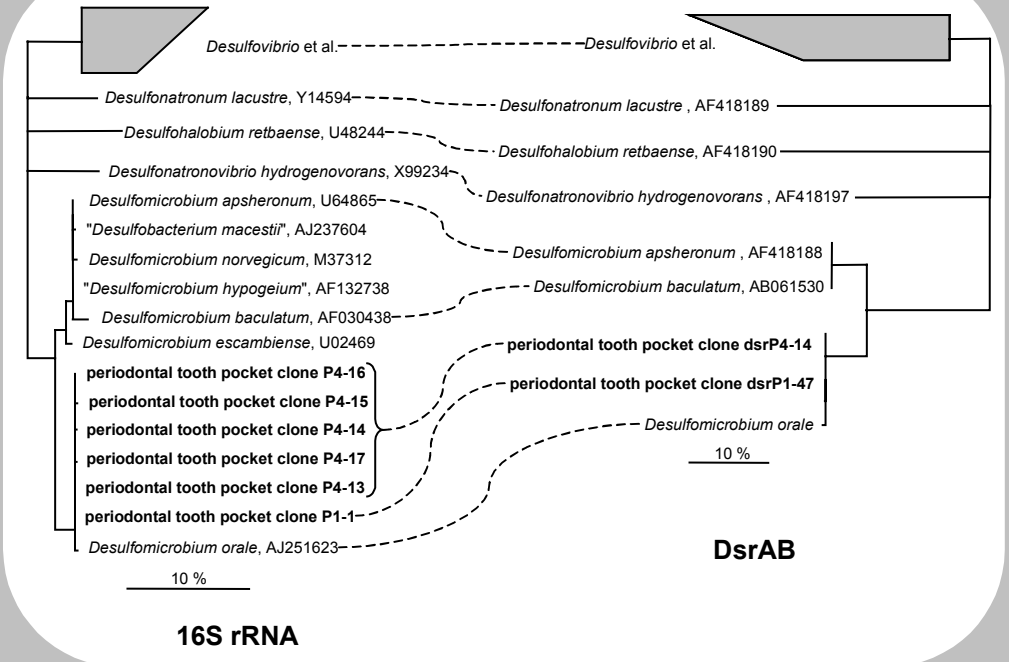

Figure 19. 16S rRNA- and DsrAB-based phylogenetic trees showing the affiliation of clone sequences retrieved from periodontal tooth pockets. Clones obtained from the tooth pockets are indicated by boldface type. 16S rRNA and the DsrAB consensus trees are based on maximum-likelihood analyses. 16S rRNA tree calculations were performed with a 50% conservation filter for the *Bacteria*. Multifurcations connect branches for which a relative order could not be determined unambiguously after different treeing methods and filters were used. The bar indicates 10% estimated sequence divergence.

All 19 clones analyzed (6 clones from patient 1 and 13 clones from patient 4) had sequences almost identical to each other and to the *dsrAB* sequence of *Desulfomicrobium orale* (99.2 to 99.7% amino acid identity), which was also determined in this thesis (Appendix I, Loy *et al.* 2002). Colonization of the tooth pockets analyzed by this SRP species is consistent with a previous report of isolation of *Desulfomicrobium orale* from periodontal tooth pockets (Langendijk *et al.* 2001).

In the second experiment, the SRP-PhyloChip was used to investigate the SRP community in the chemocline of a hypersaline, cyanobacterial mat from Solar Lake (Appendix I, Loy *et al.* 2002). This particular habitat was chosen in order to test whether SRP-PhyloChip hybridization leads to similar results compared to previous studies that investigated the composition of the SRP community of the Solar Lake mat (Minz *et al.* 1999a, Minz *et al.* 1999b, Teske *et al.* 1998).

Comparable to marine sediments, SRPs represent the dominant and highly active, anaerobic microbiota of cyanobacterial mats (Jørgensen 1982). Cyanobacterial mats are chemically stratified habitats where photosynthetic oxygen production, sulfide production from anaerobic sulfate respiration, and sulfide oxidation overlap vertically and create steep, opposing gradients of oxygen and sulfide (Teske *et al.* 1998). According to the diurnal rhythm these gradients are subject of frequent fluctuations with substantial modulation by cloud cover and season (De Wit *et al.* 1989, van Gemerden 1993, Visscher *et al.* 1992). Different SRP phylotypes were found to be restricted to defined depth intervals within cyanobacterial mats (Minz *et al.* 1999a, Risatti *et al.* 1994). It is assumed that the establishment of such a sequentially ordered SRP community structure is closely linked to the flow of energy and carbon in the mat system and to specific physiological properties [e.g. oxygen tolerance (Minz *et al.* 1999a, Minz *et al.* 1999b, Teske *et al.* 1998)] of members of the distinct phylogenetic groups, respectively (Risatti *et al.* 1994).

The SRP-PhyloChip hybridization patterns of fluorescently labeled 16S rRNA gene PCR amplificates obtained from the mat chemocline were more complex than those obtained from the tooth pockets (Figure 18 B). The probe hybridization patterns indicated that bacteria related to the genera *Desulfonema* and *Desulfomonile* were present. Furthermore, probe DSB220 showed signals above the threshold value which could have resulted from SRPs related to the genus *Desulfofaba.* However, the signal of probe DSB674, which also targets this genus, was below the threshold value. To confirm these results, 16S rRNA gene PCRs specific for most members of the "*Desulfobacterales"* (including the genera *Desulfonema* and *Desulfofaba*) and the "*Syntrophobacterales"* (primer pair DSBAC355F-1492R), as well as for some *Desulfonema* species (primer pair DSN61F-DSN+1201R) were performed (Table 2). Cloning and sequencing of the PCR amplificates confirmed that *Desulfonema*- and *Desulfomonile*-related organisms were present in the mat chemocline (Figure 20). In contrast to the microarray results, no sequences affiliated with the genus *Desulfofaba* were retrieved. The failure to detect *Desulfofaba*-like bacteria with the PCR assay might mean that a relatively limited number of 16S rRNA gene clones was sequenced or that the microarray

hybridization pattern indicative of *Desulfofaba* was caused by the presence of bacteria that have not been recognized yet.

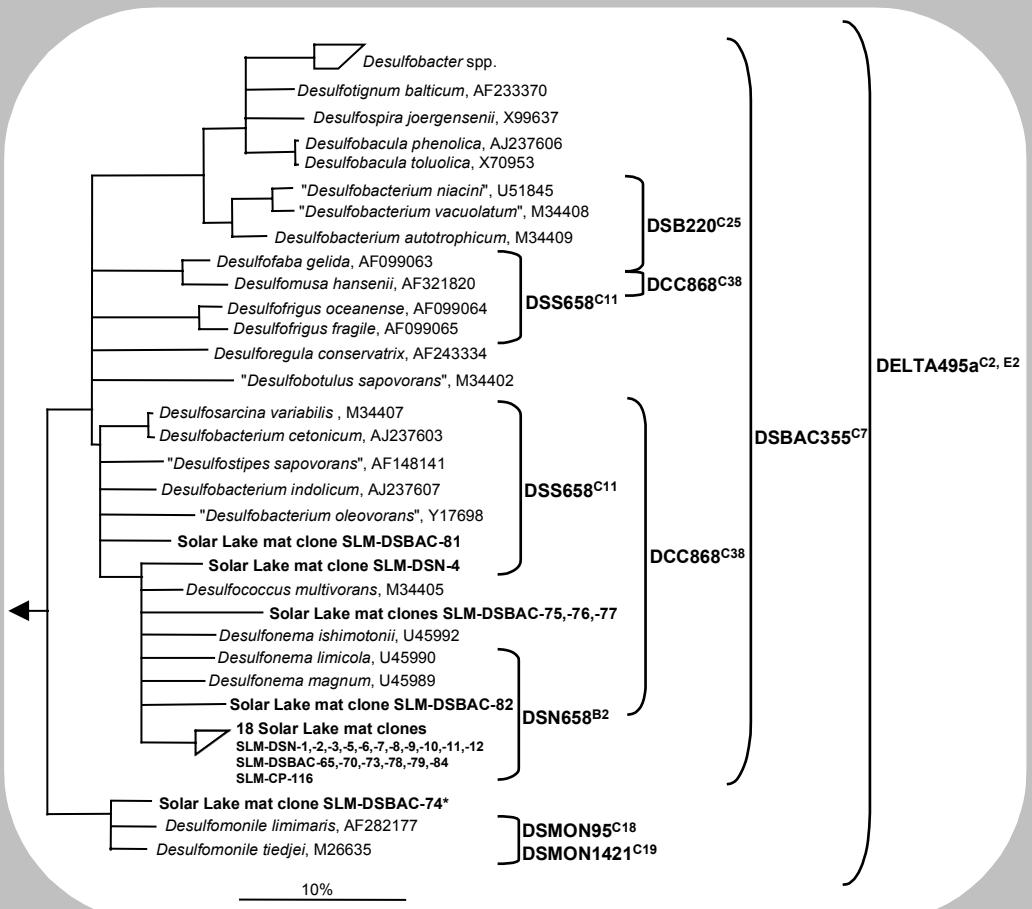

Figure 20. Evaluation of the microarray results by amplification, cloning, and comparative sequence analysis of 16S rRNA gene fragments by using primers specific for some *Desulfonema* species (SLM-DSN clones) and most members of the "*Desulfobacterales*" and "*Syntrophobacterales*" (SLM-DSBAC clones). Clone SLM-CP-116 was obtained from the mat chemocline by amplification, cloning, and sequencing after enrichment using probe DSN658 as the capture probe. 16S rRNA gene clones obtained from the chemocline of the Solar Lake mat are indicated by boldface type. The tree is based on a maximum-likelihood analysis performed with a 50% conservation filter for the *Bacteria*. Multifurcations connect branches for which a relative order could not be determined unambiguously after different treeing methods and filters were used. The bar indicates 10% estimated sequence divergence. The brackets indicate the perfect-match target organisms of the probes. The microarray position is indicated after each probe name. The amplified and sequenced 16S rRNA gene fragment of Solar Lake mat clone SLM-DSBAC-74 (indicated by an asterisk) is outside the target site for probe DSMON95 and has one mismatch (located at position 16) within the target site for probe DSMON1421.

In addition, glass beads coated with probe DSN658 (Table 5) were used to enrich *Desulfonema*-related 16S rRNA gene sequences from bacterial 16S rRNA gene amplificates from the mat chemocline. After enrichment, reamplification, and cloning, 1 of 12 cloned sequences did indeed possess the target site of probe DSN658 and was identical to

Desulfonema-related sequences obtained by the specific PCR assay described above (Figure 20). The remaining 11 cloned sequences did not possess the probe DSN658 target site and were unrelated to recognized SRPs (data not shown). The detection of *Desulfonema*-like bacteria in the chemocline of the Solar Lake mat is consistent with findings of previous studies based on comparative sequence analysis of *dsrAB* genes (Minz *et al.* 1999b), quantitative membrane hybridization (Minz *et al.* 1999a), and DGGE (Teske *et al.* 1998), and further supports the importance of these filamentous SRPs in the oxic/anoxic interface of hypersaline mat ecosystems.

The third experiment aimed to identify SRPs in two acidic fen soils in a forested catchment (Lehstenbach, Bavaria) (Appendix III, Loy *et al.* 2003b), where the SRP community structure has not yet been investigated.

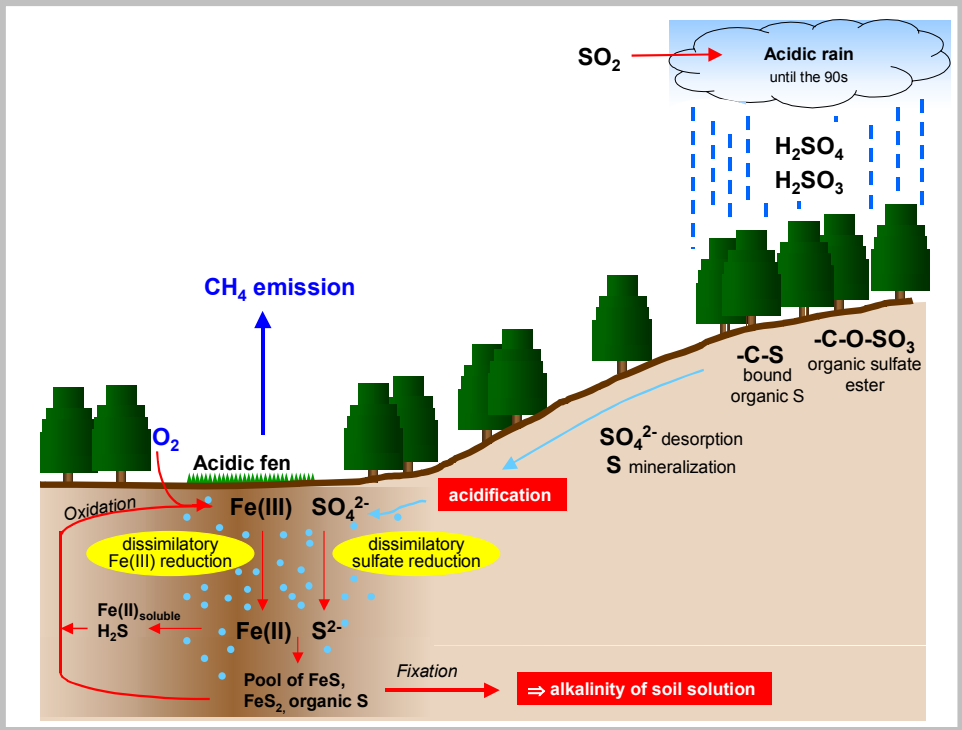

Figure 21. Biogeochemical processes in the Lehstenbach catchment (Fichtelgebirge, Germany).

In contrast to the well-studied sulfate-reducing communities in marine (Llobet-Brossa *et al.* 1998, Ravenschlag *et al.* 2001, Ravenschlag *et al.* 2000, Sahm *et al.* 1999a, Sahm *et al.* 1999b) and freshwater habitats (Li *et al.* 1999), knowledge of the distribution, diversity, and

function of SRPs in terrestrial ecosystems is generally scarce. Biogeochemical analyses based on $\delta^{34}S$ values and ^{35}S radiolabeling have shown that dissimilatory sulfate reduction is an ongoing process at the fen sites in the Lehstenbach catchment (Alewell and Gehre 1999, Alewell and Novak 2001). Owing to air pollution this catchment has seen a major deposition of sulfate through acidic rainfall (in form of sulfuric and sulfurous acid) until the 1990s, when efficient air filtration systems have been introduced in factories in former East Germany. With each rainfall, sulfate is leached out of the upland aerated soils into the lower situated fens. It has been hypothesized that dissimilatory sulfate reduction in these mainly anaerobic and waterlogged soils contributes to the retention of sulfur in this ecosystem (Alewell and Gehre 1999, Alewell and Giesemann 1996, Novak *et al.* 1994). Sulfate reduction in these fens is a sink for sulfate and protons, thus decreasing acidity of soil solution and the adjacent groundwater (Figure 21).

Initially, the SRP-PhyloChip was used to screen for members of already recognized SRP lineages at the sites SbI and SbII. Bacterial 16S rRNA genes were separately amplified from each DNA extract retrieved from the four soil depths, fluorescently labeled with Cy5, and hybridized with the SRP-PhyloChip. At site SbI highly similar microarray hybridization patterns were observed from the four different soil depths indicating low changes in SRP richness over depth (Appendix III, Loy *et al.* 2003b). Positive signals of nested probes indicated the presence of (i) *Desulfomonile* spp., (ii) *Desulfonema* or related species of the order "*Desulfobacterales*" and (iii) bacteria belonging to the *Syntrophobacter-Desulfovirga-Desulforhabdus* line of descent of the family "*Syntrophobacteraceae*" (order "*Syntrophobacterales*") (Figure 18 C). For confirmation of the microarray results, 16S rRNA gene PCRs specific for *Desulfomonile* spp. (primer pair DSMON85F-DSMON1419R), for some *Desulfonema* species (primer pair DSN61F-DSN+1201R) as well as for members of the "*Syntrophobacteraceae*" (primer pair SYBAC+282F-SYBAC1427R) were performed with DNA from each soil depth (Table 2). With each of the *Desulfomonile*- and "*Syntrophobacteraceae*"-specific primer pairs increasing amounts of PCR products of the expected size were retrieved with increasing soil depth (data not shown). Cloning and comparative sequence analysis of the PCR amplificates from 22.5-30 cm depth confirmed that *Desulfomonile* spp. and *Syntrophobacter wolinii*-related bacteria were present at site SbI (Figure 22).

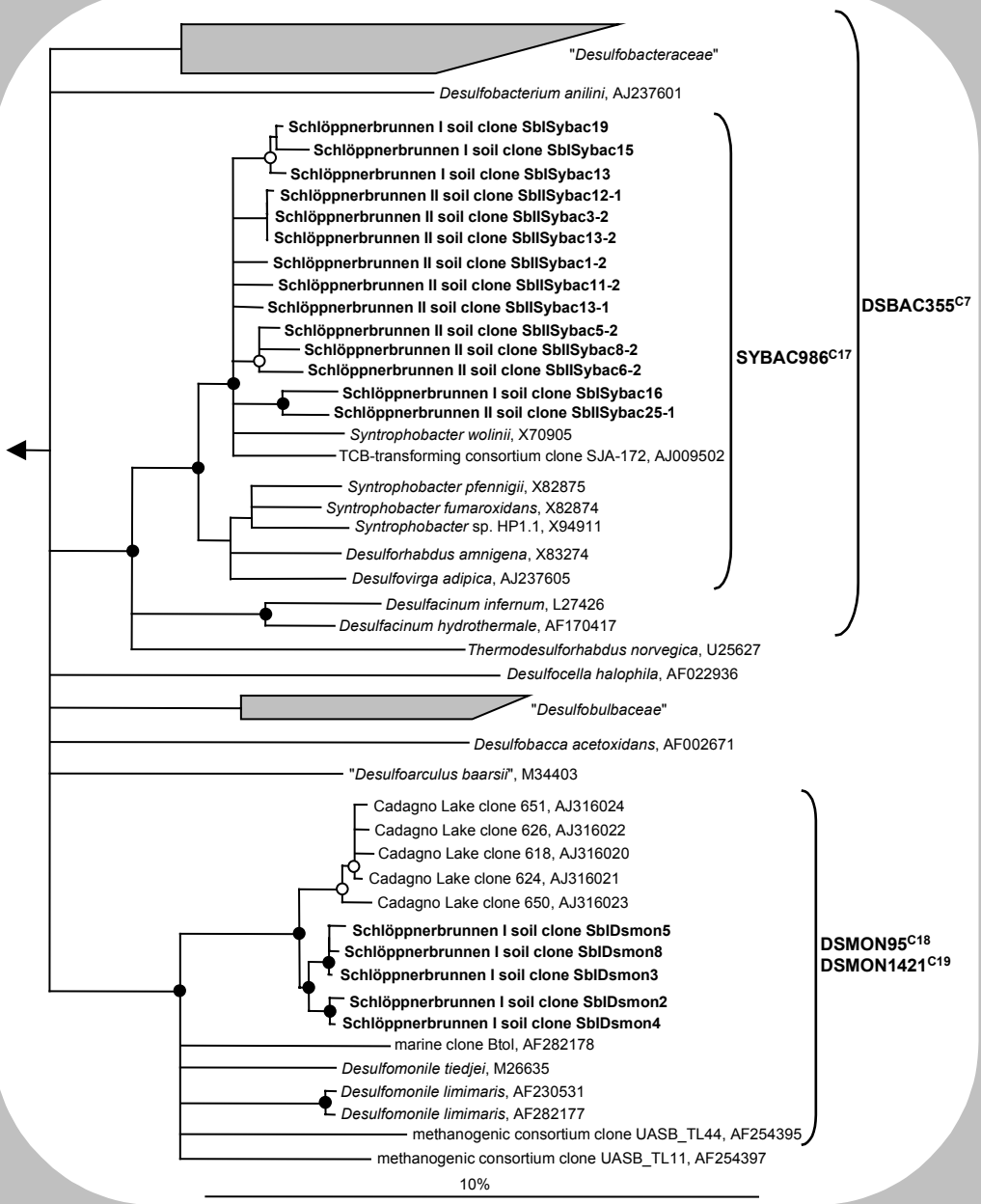

Figure 22. 16S rRNA gene phylogenetic dendrogram showing the affiliation of clone sequences from Schlöppnerbrunnen soil sites I and II (indicated by boldface type). Clone sequences were retrieved from soil DNA by PCR amplification, cloning, and sequencing of 16S rRNA gene fragments by using primers specific for the family "*Syntrophobacteraceae*" (Sybac clones) and for the genus *Desulfomonile* (Dsmon clones). The consensus tree is based on neighbor-joining analysis performed with a 50% conservation filter for the "*Deltaproteobacteria*". The bar indicates 10% estimated sequence divergence (distance inferred by neighbor-joining). Multifurcation connect branches for which a relative order could not be determined unambiguously by applying neighbor-joining, maximum-parsimony, and maximum-likelihood treeing methods. Parsimony bootstrap values (100 resamplings) for branches are indicated by solid circles (>90%) or an open circle (75 to 90%). Branches without circles had bootstrap values of less than 75%. Parentheses indicate the perfect-match target organisms of the probes. The microarray position is depicted after each probe name. Cadagno Lake clones are not sequenced at the target site for probe DSMON1421. The sequence of methanogenic consortium clone UASB_TL11 (AF254397) has three mismatches in the target site for probe DSMON95 and one terminal mismatch in the target site for probe DSMON1421.

However, no PCR amplificates were obtained with primers DSN61F and DSN+1201R. Thus, presence of *Desulfonema* or related species of the family "*Desulfobacteraceae*" in SbI samples could not be confirmed. Positive signals of probes DSN658 and DSS658 were probably caused by cross-hybridization with uncultured members of the genus *Desulfomonile* (see supplementary web material) (Appendix III, Loy *et al.* 2003b).

Similar to site SbI, the microarray hybridization patterns of site SbII showed no profound variation over depth (Appendix III, Loy *et al.* 2003b). However, microarray fingerprints at site SbII were less complex than at site SbI (Figure 18 C and D). Only probes targeting SRPs at higher taxonomic levels of specificity were unambiguously positive (e.g. probes DELTA495a and DSBAC355). However, the mean signal-to-noise ratios of the duplicate SYBAC986 probe spots at depths 7.5-15 cm, 15-22.5 cm, and 22.5-30 cm were just below the threshold value of 2.0 (1.88, 1.95, and 1.70, respectively) (Appendix III, Loy *et al.* 2003b) (Figure 18 D). In order to verify the presence or absence of *Syntrophobacter-Desulfovirga-Desulforhabdus*-related bacteria at site II, "*Syntrophobacteraceae*"-16S rRNA genes were separately amplified from each soil depth DNA by using the primer pair SYBAC+282F-SYBAC1427R. Increasing amounts of PCR product were retrieved with increasing soil depth (data not shown). According to site I, subsequent cloning and sequence analysis of the PCR product from 22.5-30 cm depth confirmed the presence of *Syntrophobacter wolinii*-related bacteria at site SbII (Figure 22). Hence, signal intensities of SRP-PhyloChip probe SYBAC986 below the threshold value were either caused (i) by low *in situ* abundances of *Syntrophobacter wolinii*-related bacteria at site SbII or (ii) by a reduced DNA recovery and/or PCR amplificability (by using standard bacterial primers [Table 2]) of DNA obtained from these fen soil bacteria.

Additionally, the microarray results were verified by comparative sequence analysis of *dsrAB* genes. In total, 29 and 24 *dsrAB* clones were retrieved from the deepest soil depth (22.5-30 cm) of site SbI and site SbII, respectively, following *dsrAB* gene amplification by using primer pair DSR1Fmix-DSR4Rmix (Table 3), cloning and sequencing. The phylogenetic affiliation of deduced DsrAB amino acid sequences from Schlöppnerbrunnen fen soils is depicted in Figure 23. One *dsrAB* clone from each fen soil sample was most closely related to *Syntrophobacter wolinii* whereas another clone from SbI could be affiliated with *Desulfomonile tiedjei*. Although *dsrAB* analysis nicely confirmed results founded on 16S rRNA gene surveys, it additionally revealed the presence of a great number of *dsrAB* sequences from yet uncultured SRP lines of descent, indicating that biodiversity of SRPs in fens is not well represented by cultured SRPs (Figure 23) (Appendix III, Loy *et al.* 2003b).

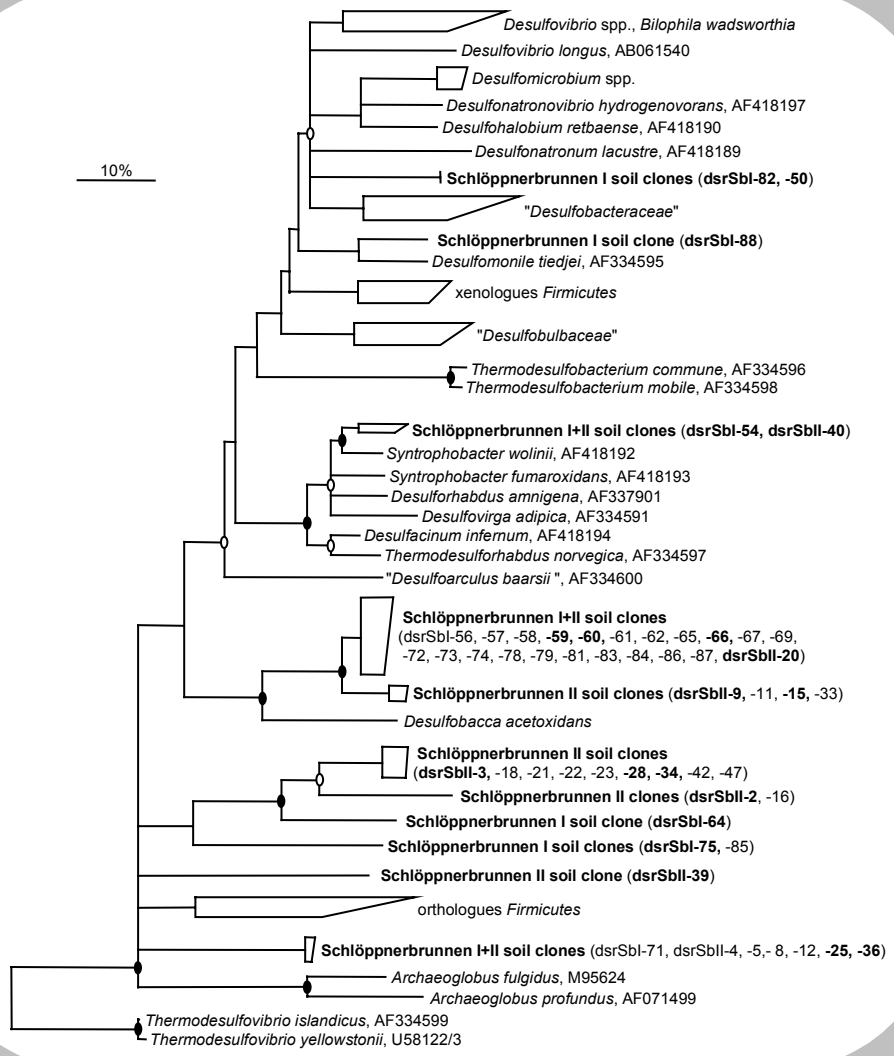

Figure 23. Phylogenetic dendrogram based on deduced DsrAB amino acid sequences showing the affiliation of Schlöppnerbrunnen fen soil clones (indicated in boldface type). Completely sequenced Schlöppnerbrunnen *dsrAB* clones (>1750 bases) are indicated in boldface type in parenthesis. The consensus tree is based on distance-matrix analysis and amino acid sequences deduced from *dsrAB* gene sequences greater than 1750 bases. DsrAB sequences deduced from *dsrAB* gene sequences shorter than 1750 bases (indicated by dashed branches) were added to the distance-matrix tree without changing the overall tree topology by using the ARB treeing tool PARSIMONY_INTERAKTIV. The bar indicates 10% estimated sequence divergence (distance inferred by distance-matrix analysis). Multifurcation connect branches for which a relative order could not be determined unambiguously by applying distance-matrix, maximum-parsimony, and maximum-likelihood treeing methods. Parsimony bootstrap values (100 resamplings) for branches are indicated by solid circles (>90%) or an open circle (75 to 90%). Branches without circles had bootstrap values of less than 75%.

In summary, an encompassing 16S rRNA gene-targeting oligonucleotide microarray suitable for SRP diversity analyses of complex environmental and clinical samples was developed. The microarray was used to screen samples in order to rapidly obtain indications of the presence of distinct lineages of SRPs. However, keeping in mind that (i) most environmental microbial communities contain a high percentage of bacteria not yet sequenced on the 16S rRNA level and (ii) not all probes on the microarray are absolutely specific under the monostringent conditions used (see preceding chapter), the SRP-PhyloChip experiments should always be supplemented with microarray-independent techniques to confirm the phylogenetic affiliations of the SRPs detected. Subsequently, the information obtained after microarray hybridization was used to select or develop appropriate PCR-based techniques (Tables 2 and 3) for verification of the microarray results and for retrieval of sequence information for phylogenetic analysis.

It should be emphasized that the SRP-PhyloChip developed in this thesis allows to obtain a phylogenetically informative, high-resolution fingerprint of the entire SRP diversity in a given sample within 48 h (including all experimental work from DNA extraction to hybridization pattern interpretation) and, thus, nicely complements the molecular tool box of previously available methods for cultivation-independent identification of SRPs (Devereux *et al.* 1992, Friedrich 2002, Manz *et al.* 1998, Risatti *et al.* 1994, Voordouw *et al.* 1991, Wagner *et al.* 1998).

VI. Conclusions and perspectives: the PhyloChip approach

Ribosomal RNA-based oligonucleotide microarrays designed according to the "multiple probe concept" (so-called "PhyloChips") perfectly fit into the methodological concept of cultivation-independent analysis of complex microbial communities in the environment (Amann *et al.* 1995) (Figure 24).

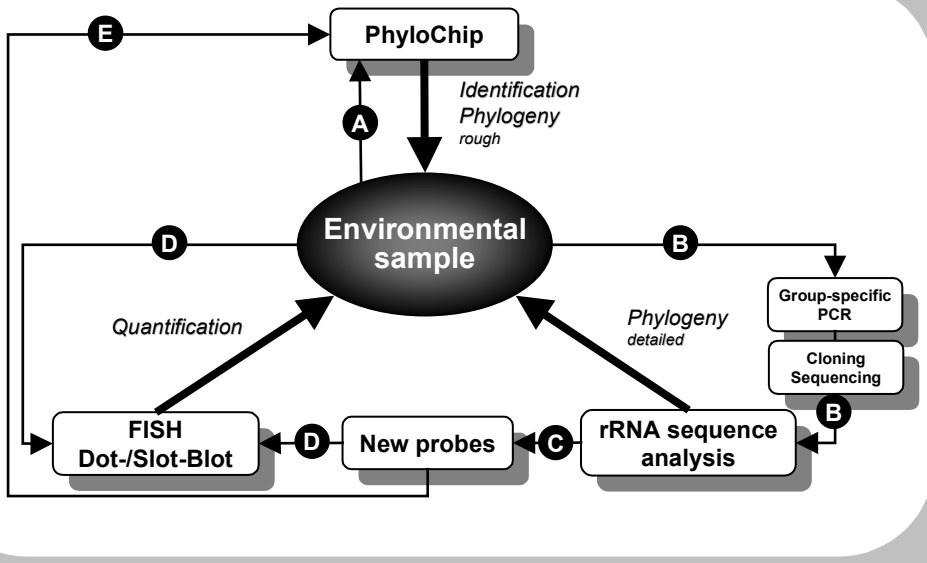

Figure 24. Flow chart showing the basic principal of the PhyloChip approach for cultivation-independent determination of microbial community composition. (A) Phylogenetic inventory by PhyloChip hybridization. (B) Selection/design of appropriate rRNA-targeted primers for PCR, subsequent cloning, and comparative sequence analysis. (C) Design of novel, clone sequence-specific probes. (D) Abundance of specific microbial groups revealed in quantitative experiments such as fluorescence *in situ* hybridization and/or membrane hybridization by using probes selected according to results obtained from initial experiments. (E) Addition of clone-specific probes on the PhyloChip.

A core part of the so-called "full-cycle rRNA approach" (Amann *et al.* 1995) is the establishment and phylogenetic processing of rRNA gene libraries by using domain-specific or universal primers for PCR, subsequent cloning, and comparative sequence analysis. This time-consuming and tedious analytical step might be omitted if PhyloChips are used for phylogenetic inventory of the microbial community instead. As accomplished for the microbial guild of sulfate-reducing prokaryotes in this thesis, PhyloChips can be developed for virtually every functional or phylogenetic organism group of interest. One might also design PhyloChips that specifically allow identification of all microorganisms which are present in a certain environmental habitat (keyword "Habitat-PhyloChip"). Moreover, considering high-density microarrays with more than tens of thousand immobilized

oligonucleotides, it would theoretically be possible to develop a single PhyloChip for detection of all members of the entire bacterial and/or archaeal domain.

PhyloChip hybridization of an environmental sample reveals a phylogenetically informative, diversity fingerprint of the microbial community (Figure 24 A). The degree of phylogenetic resolution of this hybridization pattern depends on the specificity range of the respective rRNA-targeted probes on the microarray. The diversity microarray fingerprint allows researchers to preselect the relevant microbial groups whose phylogeny, abundance, and ecophysiological function should be further characterized. Consequently, the genealogy of the identified microbial groups can be analyzed in greater detail by designing/applying **group-specific PCR primers** for retrieval of rRNA gene sequences from the analyzed sample (Figure 24 B). Furthermore, based on the obtained rRNA sequence data, **clone-specific probes** can be designed (Figure 24 C) and applied in subsequent, more precise hybridization experiments (Figure 24 D and E). It should be pointed out that the microarray technique as described in this thesis does not allow to obtain quantitative data on the composition of microbial communities due to the recognized biases introduced by using PCR for rRNA gene amplification (Polz and Cavanaugh 1998, Suzuki and Giovannoni 1996, von Wintzingerode *et al.* 1997). In addition, the signal intensity respectively duplex yield of a probe on the microarray is dependent not only on the actual abundance of its perfect-match target nucleic acid in the PCR amplificate mixture but also on a variety of other factors, including the labeling efficiency of the specific target nucleic acid, the secondary structure of the target region, and the inherent variations associated with microarray fabrication. Nevertheless, in the course of this integrated and encompassing PhyloChip-based approach, probes specific for the microorganisms identified in the initial experiments can be applied in **quantitative hybridization experiments** such as FISH (Daims *et al.* 2001b, Juretschko *et al.* 2002) and/or membrane hybridization (Raskin *et al.* 1994) (Figure 24 D).

Although quantitative data can not be readily collected by using the microarray technology developed in this thesis, PhyloChips have great potential for rapid screening of microbial diversity in complex samples. This methodological option should be integrated in the pool of rRNA-based techniques and is suggested to be of particular value if large numbers of samples are to be analyzed to study temporal or spatial variations in microbial diversity.

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SUMMARY

The ability of some prokaryotes to use sulfate as terminal electron acceptor for energy generation purposes (dissimilatory sulfate reduction) is essential for a central part of the biogeochemical cycling of sulfur on planet earth. Hence, the community composition and the ecophysiology of sulfate-reducing prokaryotes (SRPs) in the environment raises great scientific interest. However, the polyphyletic origin and the vast biodiversity of SRPs recognized so far hampers their encompassing, cultivation-independent identification by well-established rRNA-based techniques.

In this thesis, the natural genealogy of all provisionally proposed and validly recognized SRPs was thoroughly determined based on comparative analyses of 16S rRNA sequences. SRP phylogeny generally confirmed the hierarchical classification of taxa above the genus level as proposed by the second edition of Bergey`s Manual of Systematic Bacteriology but also indicated individual, potentially misnamed SRP species. Furthermore, the updated 16S rRNA database and the emended phylogenetic information for SRPs was used to develop and evaluate an encompassing set of 142 phylogenetic oligonucleotide probes *in silico*. 16S rRNA-targeted probes were designed in accordance with the "multiple probe concept" to target SRPs at multiple hierarchical (and identical) phylogenetic levels of specificity. The applicability of the developed probe set for DNA microarray hybridization was evaluated according to its specificity and sensitivity with more than 40 SRP pure cultures. Subsequently, the tested SRP microarray was successfully applied to reveal the SRP diversity in periodontal tooth pockets, a hypersaline cyanobacterial mat, and acidic fen soils. In parallel, microarray results were independently verified by comparative analyses of 16S rRNA and dissimilatory (bi)sulfite reductase (*dsrAB*) gene sequences retrieved from these habitats. The results of this thesis demonstrated for the first time that DNA microarrays for microbial identification can be routinely applied to rapidly screen for the prokaryotic biodiversity in any given environmental or clinical sample.

In addition, all rRNA-targeted oligonucleotide probes published so far, including the SRP-specific probes from this thesis, were compiled in a database which is freely accessible through the world wide web at http://www.probebase.net. Besides additional biological and bibliographical information available for each probe entry, probeBase offers online tools assisting in database search and in successful design of new probes for fluorescence *in situ* hybridization.

ZUSAMMENFASSUNG

Ein zentraler Teil des Schwefelkreislaufes der Erde beruht ausschließlich auf der Aktivität bestimmter Prokaryonten, die die Fähigkeit besitzen Sulfat als terminalen Elektronenakzeptor zur Energiegewinnung zu nutzen (dissimilatorische Sulfatreduktion). Folglich erweckt die Zusammensetzung der Gemeinschaft sulfatreduzierender Prokaryonten (SRPs) in der Umwelt und ihre ökologische Funktion großes wissenschaftliches Interesse. Die polyphyletische Herkunft der SRPs und ihre enorme Biodiversität erschwert allerdings eine umfassende, kultivierungsunabhängige Identifizierung mit Hilfe etablierter rRNS-basierender Techniken.

Die vorliegende Arbeit liefert eine umfassende Bestimmung der natürlichen Abstammung aller sowohl vorläufig und als auch bereits valide beschriebener SRPs auf der Basis vergleichender 16S rRNS-Sequenzanalyse. Im Allgemeinen wurde die hierarchische Klassifizierung von SRP Taxa, wie sie in der zweiten Ausgabe des Bergey's Manual of Systematic Bacteriology vorgeschlagen wurde, durch die SRP Phylogenie bestätigte. Im Speziellen wies sie allerdings auf einzelne, potentiell falsch benannte SRP Arten hin. Des Weiteren fungierte die aktualisierte 16S rRNS-Datenbank und die erweiterte phylogenetische Information bezüglich SRPs als Grundlage für die *in silico* Entwicklung und Evaluierung eines umfassenden Sondensatzes, bestehend aus 142 so genannten phylogenetischen Oligonukleotidsonden. Die 16S rRNS-gerichteten Sonden wurden entsprechend des „Mehrfachsondenkonzepts" entworfen, um SRPs auf mehreren hierarchischen (und identischen), phylogenetischen (taxonomischen) Ebenen spezifisch nachweisen zu können. Die Eignung des entwickelten Sondensatzes für die DNS-Mikroarray-Hybridisierung wurde mit mehr als 40 SRP Reinkulturen in Bezug auf Sensitivität und Spezifität überprüft. Anschließend wurde der ausgetestete SRP-Mikroarray erfolgreich zur Aufklärung der SRP-Diversität in parodontalen Zahntaschen, einer hypersalinen, cyanobakteriellen Matte und sauren Moorböden eingesetzt. Anhand vergleichender Analyse von 16S rRNS- und dissimilatorische (Bi)Sulfitreduktase (*dsrAB*)-Gensequenzen, die aus diesen Habitaten gewonnen wurden, konnten die Mikroarray-Ergebnisse unabhängig voneinander bestätigt werden. Die Ergebnisse der vorliegenden Arbeit demonstrieren zum ersten Mal, dass DNS-Mikroarrays, die zur mikrobiellen Identifizierung entwickelt wurden, routinemässig zur schnellen Aufklärung prokaryontischer Biodiversität in beliebigen Umwelt- oder klinischen Proben eingesetzt werden können.

Darüber hinaus wurden alle bis zum heutigen Zeitpunkt publizierten, rRNS-gerichteten Oligonukleotidsonden, u.a. die SRP-spezifischen Sonden dieser Arbeit, in einer über das Internet frei zugänglichen Datenbank (http://www.probebase.net) zusammengetragen. Neben zusätzlicher biologischer und bibliographischer Information zu jeder Sonde bietet probeBase über das Internet abrufbare Funktionen an, die bei der Suche in der Datenbank und bei der erfolgreichen Entwicklung neuer Sonden für die Fluoreszenz *in situ* Hybridisierung assistieren.

APPENDIX I

Oligonucleotide Microarray for 16S rRNA Gene-Based Detection of All Recognized Lineages of Sulfate-Reducing Prokaryotes in the Environment

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Oligonucleotide Microarray for 16S rRNA Gene-Based Detection of All Recognized Lineages of Sulfate-Reducing Prokaryotes in the Environment

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For cultivation-independent detection of sulfate-reducing prokaryotes (SRPs) an oligonucleotide microarray consisting of 132 16S rRNA gene-targeted oligonucleotide probes (18-mers) having hierarchical and parallel (identical) specificity for the detection of all known lineages of sulfate-reducing prokaryotes (SRP-PhyloChip) was designed and subsequently evaluated with 41 suitable pure cultures of SRPs. The applicability of SRP-PhyloChip for diversity screening of SRPs in environmental and clinical samples was tested by using samples from periodontal tooth pockets and from the chemocline of a hypersaline cyanobacterial mat from Solar Lake (Sinai, Egypt). Consistent with previous studies, SRP-PhyloChip indicated the occurrence of *Desulfomicrobium* spp. in the tooth pockets and the presence of *Desulfonema*- and *Desulfomonile*-like SRPs (together with other SRPs) in the chemocline of the mat. The SRP-PhyloChip results were confirmed by several DNA microarray-independent techniques, including specific PCR amplification, cloning, and sequencing of SRP 16S rRNA genes and the genes encoding the dissimilatory (bi)sulfite reductase (*dsrAB*).

Anaerobic respiration with sulfate is a central component of the global sulfur cycle and is exhibited exclusively by prokaryotes (53). Sulfate-reducing prokaryotes (SRPs) are thus of major numerical and functional importance in many ecosystems, including marine sediments (14, 29, 30, 38, 54) and cyanobacterial microbial mats (46, 56, 70). Recently, SRPs were also identified as unculturable symbionts of gutless marine oligochetes (15) and as uncultured components of microbial aggregates catalyzing anaerobic methane oxidation (4, 10, 48, 72). In addition, some SRPs have been implicated in human disease (32, 35, 39, 43, 60, 69). More than 130 species of SRPs have been described so far, and they comprise a phylogenetically diverse assemblage of organisms consisting of members of at least four bacterial phyla and one archaeal phylum (11, 12, 66). The polyphyletic affiliation of SRPs and the fact that several SRPs are closely related to microorganisms which cannot perform anaerobic sulfate reduction for energy generation hamper cultivation-independent detection of these organisms by established 16S rRNA-based methods because many different PCR primer sets or probes would be required to target all members of this microbial guild. Consequently, previous environmental microbiology research on the composition of SRP communities performed by using specific 16S rRNA gene-targeting PCR systems or probes has focused on a few selected genera or groups (16, 24, 36, 41, 49, 50, 56, 59, 68, 71).

Nucleic acid microarrays, which have recently been introduced for bacterial identification in microbial ecology (5, 23, 37, 62, 73, 77), provide a powerful tool for parallel detection of 16S rRNA genes (23, 37, 62, 73) and thus might be particularly useful for environmental studies of phylogenetically diverse microbial groups. However, most microarrays developed so far for bacterial identification consist of a limited number of probes and are mainly used for method development and optimization. In this study, we developed and successfully used a microarray consisting of 132 16S rRNA-targeted oligonucleotide probes covering all recognized lineages of SRPs for high-resolution screening of clinical and environmental samples. For periodontal tooth pockets and a hypersaline microbial mat, microarray SRP diversity fingerprints were found to be consistent with results obtained by using well-established molecular methods for SRP community composition analysis.

MATERIALS AND METHODS

Pure cultures of SRPs. Table 1 lists the 42 reference organisms that were obtained as lyophilized cells or active cultures from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and were used to evaluate our microarray (SRP-PhyloChip). *Archaeoglobus veneficus* SNP6^T (containing plasmid XY) was deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen by K. O. Stetter, Lehrstuhl fur Mikrobiologie, Universität Regensburg, Regensburg, Germany, as DSM 11195^T.

Solar Lake mat sample. A core (1 by 1cm; depth, 4 cm) of a hypersaline cyanobacterial mat from Solar Lake (Sinai, Egypt) was sectioned horizontally at 200-μm intervals with a cryomicrotome (MIKROM HM500; Microm, Walldorf, Germany). The mat sections were stored at −80°C.

Peridontal tooth pocket samples. Samples from five patients with adult periodontitis were taken by inserting a sterile medium-sized paper point into a single periodontal tooth pocket. After sampling the paper points were stored at −20°C.

DNA extraction. Genomic DNA was isolated from reference organisms with a FastDNA kit (Bio 101, Vista, Calif.). DNA from periodontal tooth pocket material and DNA from a cryosection of Solar Lake mat from the chemocline (1,400 to 1,600 μm from the mat surface) were extracted by using a modification of the protocol of Griffiths et al. (22). In contrast to the original protocol, precipitation of nucleic acids in the aqueous phase was performed with 0.1 volume of sodium acetate (pH 5.2) and 0.6 volume of isopropanol for 2 h at room temperature.

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TABLE 1. SRP strains used in this study

Species	Strain
Desulfovibrio cuneatus	DSM 11391^T
Desulfovibrio aminophilus	DSM 12254^T
Desulfovibrio gabonensis	DSM 10636^T
Desulfovibrio alcoholivorans	DSM 5433^T
Desulfovibrio termitidis	DSM 5308^T
Desulfovibrio zosterae	DSM 11974^T
Desulfovibrio halophilus	DSM 5663^T
Desulfovibrio longus	DSM 6739^T
"*Desulfovibrio aestuarii*"	DSM 1926^T
Desulfovibrio profundus	DSM 11384^T
Desulfomicrobium aspheronum	DSM 5918^T
Desulfomicrobium orale	DSM 12838^T
Desulfohalobium retbaense	DSM 5692^T
Desulfotalea arctica	DSM 12342^T
Desulforhopalus vacuolatus	DSM 9700^T
Desulfobulbus propionicus	DSM 2032^T
"*Desulfobotulus sapovorans*"	DSM 2055^T
Desulfococcus multivorans	DSM 2059^T
Desulfonema limicola	DSM 2076^T
Desulfonema ishimotonii	DSM 9680^T
Desulfobacterium indolicum	DSM 3383^T
Desulfosarcina variabilis	DSM 2060^T
Desulfofaba gelida	DSM 12344^T
Desulfofrigus oceanense	DSM 12341^T
"*Desulfobacterium niacini*"	DSM 2650^T
Desulfobacula toluolica	DSM 7467^T
Desulfotignum balticum	DSM 7044^T
Desulfobacter halotolerans	DSM 11383^T
Desulfobacter latus	DSM 3381^T
Thermodesulforhabdus norvegica	DSM 9990^T
Desulfomonile tiedjei	DSM 6799^T
Desulfobacca acetoxidans	DSM 11109^T
Desulfotomaculum aeronauticum	DSM 10349^T
Desulfotomaculum geothermicum	DSM 3669^T
Desulfotomaculum australicum	DSM 11792^T
Desulfotomaculum thermobenzoicum	DSM 6193^T
Desulfotomaculum acetoxidans	DSM 771^T
Desulfotomaculum halophilum	DSM 11559^T
Desulfosporosinus orientis	DSM 765^T
Thermodesulfovibrio islandicus	DSM 12570^T
Thermodesulfobacterium mobile (*Thermodesulfobacterium thermophilum*)	DSM 1276^T
Archaeoglobus veneficus	DSM 11195^T

PCR amplification of 16S rRNA and *dsrAB* genes. For subsequent DNA microarray hybridization, almost complete 16S rRNA gene fragments were amplified from DNA of pure cultures of SRPs by using the 616V-630R primer pair (Table 2). 16S rRNA gene fragments of *A. veneficus* were amplified by using the newly designed *Archaeoglobus* genus-specific forward primer ARGLO36F and the universal reverse primer 1492R (Table 2). Amplification of bacterial 16S rRNA gene fragments from periodontal tooth pocket or Solar Lake mat genomic DNA was performed by using the 616V-630R and 616V-1492R primer pairs (Table 2).

To confirm DNA microarray results, specific amplification of 16S rRNA gene fragments of defined SRP groups was performed with periodontal tooth pocket DNA and Solar Lake mat DNA by using previously described and newly designed primers (Table 2). In addition, an approximately 1.9-kb *dsrAB* fragment was amplified from periodontal tooth pockets by using primers DSR1F and DSR4R under the conditions described by Wagner et al. (76).

Positive controls containing purified DNA from suitable reference organisms were included in all of the PCR amplification experiments along with negative controls (no DNA added). For 16S rRNA gene amplification, reaction mixtures (total volume, 50 μl) containing each primer at a concentration of 25 pM were prepared by using 10× *Ex Taq* reaction buffer and 2.5 U of *Ex Taq* polymerase (Takara Biomedicals, Otsu, Shiga, Japan). Additionally, 20 mM tetramethylammonium chloride (Sigma, Deisenhofen, Germany) was added to each amplification mixture to enhance the specificity of the PCR (31). Thermal cycling was carried out by using an initial denaturation step of 94°C for 1 min, followed by 30 cycles of denaturation at 94°C for 40 s, annealing at temperatures ranging from 52 to 60°C (depending on the primer pair [Table 2]) for 40 s, and elongation at 72°C for 1 min 30 s. Cycling was completed by a final elongation step of 72°C for 10 min.

Fluorescence labeling of PCR amplificates. Prior to labeling, PCR amplificates were purified by using a QIAquick PCR purification kit (Qiagen, Hilden, Germany). Subsequently, the amount of DNA was determined spectrophotometrically by measuring the optical density at 260 nm. Purified PCR products were labeled with Cy5 by using a DecaLabel DNA labeling kit (MBI Fermentas, Vilnius, Lithuania). Reaction mixtures (total volume, 45 μl) containing 200 ng of purified PCR product and 10 μl of decanucleotides in reaction buffer were denatured at 95°C for 10 min and immediately placed on ice. After addition of 3 μl of deoxynucleotide Mix C (containing no dCTP), 1 μl of Cy5-dCTP (Amersham Biosciences, Freiburg, Germany), and 1 μl of the Klenow fragment (Exo^-; 5 U μl^{-1}), the labeling reaction mixtures were incubated at 37°C for 45 min. For more efficient labeling, the addition of Mix C, Cy5-dCTP, and the Klenow fragment and incubation at 37°C for 45 min were repeated. Labeling was completed by addition of 4 μl of dNTP-Mix and incubation at 37°C for 60 min. To remove unincorporated deoxynucleotides and decanucleotides, the labeling mixture was purified with a QIAquick nucleotide removal kit (Qiagen) by using double-distilled water for DNA elution. Finally, the eluted DNA was vacuum dried and stored in the dark at −20°C.

Microarray manufacture and processing. Oligonucleotides for microarray printing were obtained from MWG Biotech (Ebersberg, Germany). The sequence, specificity, and microarray position of each oligonucleotide probe are

TABLE 2. 16S rRNA gene-targeted primers

Short name[a]	Full name[b]	Annealing temp (°C)	Sequence 5′-3′	Specificity	Reference
616V	S-D-Bact-0008-a-S-18	52	AGA GTT TGA TYM TGG CTC	Most *Bacteria*	26
630R	S-D-Bact-1529-a-A-17	52	CAK AAA GGA GGT GAT CC	Most *Bacteria*	26
1492R	S-*-Proka-1492-a-A-19	52, 60[c]	GGY TAC CTT GTT ACG ACT T	Most *Bacteria* and *Archaea*	Modified from reference 27
ARGLO36F	S-G-Arglo-0036-a-S-17	52	CTA TCC GGC TGG GAC TA	*Archaeoglobus* spp.	This study
DSBAC355F	S-*-Dsb-0355-a-S-18	60	CAG TGA GGA ATT TTG CGC	Most "*Desulfobacterales*" and "*Syntrophobacterales*"	59
DSM172F	S-G-Dsm-0172-a-S-19	56	AAT ACC GGA TAG TCT GGC T	*Desulfomicrobium* spp.	This study
DSM1469R	S-G-Dsm-1469-a-A-18	56	CAA TTA CCA GCC CTA CCG	*Desulfomicrobium* spp.	This study
DSN61F	S-*-Dsn-0061-a-S-17	52	GTC GCA CGA GAA CAC CC	*Desulfonema limicola, Desulfonema ishimotonii*	This study
DSN+1201R	S-*-Dsn-1201-a-A-17	52	GAC ATA AAG GCC ATG AG	*Desulfonema* spp. and other *Bacteria*	This study

[a] Short name used in the reference or in this study.
[b] Name of 16S rRNA gene-targeted oligonucleotide primer based on the nomenclature of Alm et al. (1).
[c] The annealing temperature was 52°C when the primer was used with forward primer 616V or ARGLO36F, and the annealing temperature was 60°C when the primer was used with forward primer DSBAC355F.

TABLE 3. 16S rRNA-targeted oligonucleotide probes

Original probe name	Short name	Full name[a]	Sequence (5′–3′)	Microarray position	Specificity	Reference
	CONT		AGG AAG GAA GGA AGG AAG	A1-F1, A48-F48	Control oligonucleotide	This study
	CONT-COMP		CTT CCT TCC TTC CTT CCT		Complementary to control oligonucleotide	This study
	NONSENSE		AGA GAG AGA GAG AGA GAG	F47	Nonbinding control	This study
EUB338	EUB338	S-D-Bact-0338-a-A-18	GCT GCC TCC CGT AGG AGT	D25, F2	Most *Bacteria*	2
EUB338II	EUB338II	S-*-BactP-0338-a-A-18	GCA GCC ACC CGT AGG TGT	F3	Phylum *Planctomycetes*	6
EUB338III	EUB338III	S-*-BactV-0338-a-A-18	GCT GCC ACC CGT AGG TGT	F4	Phylum *Verrucomicrobia*	6
UNIV1390	UNIV1389a	S-D-Univ-1389-a-A-18	ACG GGC GGT GTG TAC AAG	D26, F5	*Bacteria,* not "*Epsilonproteobacteria*"	78[b]
UNIV1390	UNIV1389b	S-D-Univ-1389-b-A-18	ACG GGC GGT GTG TAC AAA	F6	*Eucarya*	78[b]
UNIV1390	UNIV1389c	S-D-Univ-1389-c-A-18	ACG GGC GGT GTG TGC AAG	D34, F7	*Archaea*	78[b]
ARCH915	ARCH917	S-D-Arch-0917-a-A-18	GTG CTC CCC CGC CAA TTC	D35	*Archaea*	67[b]
	DELTA495a	S-C-dProt-0495-a-A-18	AGT TAG CCG GTG CTT CCT	C2, E2	Most "*Deltaproteobacteria*"	This study
	DELTA495b	S-*-dProt-0495-b-A-18	AGT TAG CCG GCG CTT CCT	C3, E3	Some "*Deltaproteobacteria*"	This study
	DELTA495c	S-*-dProt-0495-c-A-18	AAT TAG CCG GTG CTT CCT	C4, E4	Some "*Deltaproteobacteria*"	This study
S-*-Ntspa-712-a-A-21	NTSPA714	S-*-Ntspa-714-a-A-18	CCT TCG CCA CCG GCC TTC	D30	Phylum *Nitrospira,* not *Thermodesulfovibrio islandicus*	7
LGC354A	LGC354a	S-*-Lgc-0354-a-A-18	TGG AAG ATT CCC TAC TGC	A2	Probes LGC354a, LGC354b, and LGC354c target together the phylum *Firmicutes* but not *Desulfotomaculum* and *Desulfosporosinus*	44
LGC354B	LGC354b	S-*-Lgc-0354-b-A-18	CGG AAG ATT CCC TAC TGC	A3	See above	44
LGC354C	LGC354c	S-*-Lgc-0354-c-A-18	CCG AAG ATT CCC TAC TGC	A4	See above	44
SRB385	SRB385	S-*-Srb-0385-a-A-18	CGG CGT CGC TGC GTC AGG	C5, E5	Many but not all deltaproteobacterial SRPs, *Aerothermobacter* spp., *Thermomonospora* spp., *Actinobispora* spp., *Actinomadura* spp., *Thermoanaerobacter* spp., *Frankia* spp., *Clostridium* spp., *Streptosporangium* spp., *Nitrospira* spp., *Geodermatophilus* spp., *Nocardiopsis* spp., and many more	2
SRB385Db	SRB385Db	S-*-Srb-0385-b-A-18	CGG CGT TGC TGC GTC AGG	C6, E6	Many but not all deltaproteobacterial SRPs, *Geobacter* spp., *Pelobacter* spp., *Campylobacter* spp., *Saccharopolyspora* spp., *Acetivibrio* spp., *Syntrophus* spp., *Clostridium* spp., *Nitrospina* spp., *Chlorobium* spp., and many more	52
DSBAC355	DSBAC355	S-*-Dsbac-0355-a-A-18	GCG CAA AAT TCC TCA CTG	C7	Most "*Desulfobacterales*" and "*Syntrophobacterales*"	59
	DSB706	S-*-Dsb-0706-a-A-18	ACC GGT ATT CCT CCC GAT	C8	*Desulfotalea* spp., *Desulfosarcina* sp., *Desulforhopalus* sp., *Desulfocapsa* spp., *Desulfofustis* sp., *Desulfobacterium* sp., *Desulfobulbus* spp., *Thermodesulforhabdus* sp.	This study
DSS658	DSS658	S-*-Dsb-0658-a-A-18	TCC ACT TCC CTC TCC CAT	C11	*Desulfostipes* sp., *Desulfobacterium* sp., *Desulfofrigus* spp., *Desulfofaba* sp., *Desulfosarcina* sp., *Desulfomusa* sp.	41
DSR651	DSR651	S-*-Dsb-0651-a-A-18	CCC CCT CCA GTA CTC AAG	C10	*Desulforhopalus* sp., *Desulfobacterium* sp., *Desulfofustis* sp., *Desulfocapsa* sp., *Desulfobulbus* spp., *Spirochaeta* spp.	41
probe 804	DSB804	S-*-Dsb-0804-a-A-18	CAA CGT TTA CTG CGT GGA	C9	*Desulfobacter* spp., *Desulfobacterium* spp., *Desulfofrigus* spp., *Desulfofaba* sp., *Desulfosarcina* sp., *Desulfostipes* sp., *Desulfococcus* sp., *Desulfobotulus* sp., *Desulforegula* sp.	13

	DSB230	S-*-Dsb-0230-a-A-18	CTA ATG GTA CGC AAG CTC	B6	*Desulfotalea* spp., *Desulforhopalus* sp., *Desulfocapsa* spp., *Desulfofustis* sp., *Desulfobacterium* sp.	This study
	DSTAL131	S-G-Dstal-0131-a-A-18	CCC AGA TAT CAG GGT AGA	B9	*Desulfotalea* spp.	This study
	DSTAL213	S-G-Dstal-0213-a-A-18	CCT CCC GAT ACA ATA GCT	B8	See above	This study
	DSTAL645	S-G-Dstal-0645-a-A-18	CCA GTA CTC AAG CTC CCC	B10	See above	This study
	DSTAL732	S-G-Dstal-0732-a-A-18	TAT CTG GCC AGA TGG TCG	B12	See above	This study
	DSTAL835	S-G-Dstal-0835-a-A-18	GAA GCG ATT AAC CAC TCC	B11	See above	This study
	DSRHP185	S-*-Dsrhp-0185-a-A-18	CCA CCT TTC CTG TTT CCA	B7	*Desulforhopalus* spp.	This study
	DSBB228	S-G-Dsbb-0228-a-A-18	AAT GGT ACG CAG ACC CCT	B4	*Desulfobulbus* spp.	This study
probe 660	DSBB660	S-G-Dsbb-0660-a-A-18	ATT CCA CTT TCC CCT CTG	B5	See above	13[b]
DSB985	DSB986	S-*-Dsb-0986-a-A-18	CAC AGG ATG TCA AAC CCA	C28	*Desulfobacter* spp., *Desulfobacula* sp., *Desulfobacterium* sp., *Desulfospira* sp., *Desulfotignum* sp.	41[b]
	DSB1030	S-*-Dsb-1030-a-A-18	CTG TCT CTG TGC TCC CGA	C27	See above	This study
	DSB1240	S-*-Dsb-1240-a-A-18	TGC CCT TTG TAC CTA CCA	C34	*Desulfobacter* spp., *Desulfotignum* sp.	This study
DSB623	DSB623a	S-*-Dsb-0623-a-A-18	TCA AGT GCA CTT CCG GGG	C35	*Desulfobacter curvatus, Desulfobacter halotolerans, Desulfobacter hydrogenophilus, Desulfobacter postgatei, Desulfobacter vibrioformis*	9[b]
	DSB623b	S-*-Dsb-0623-b-A-18	TCA AGT GCA CTT CCA GGG	C36	*Desulfobacter* sp., strain BG8, *Desulfobacter* sp. strain BG23	This study
DSB623	DSBLA623	S-S-Dsb.la-0623-a-A-18	TCA AGT GCT CTT CCG GGG	C37	*Desulfobacter latus*	9[b]
	DSBACL143	S-G-Dsbacl-0143-a-A-18	TCG GGC AGT TAT CCC GGG	C29	*Desulfobacula* spp.	This study
	DSBACL225	S-G-Dsbacl-0225-a-A-18	GGT CCG CAA ACT CAT CTC	C30	See above	This study
	DSBACL317	S-G-Dsbacl-0317-a-A-18	GAC CGT GTA CCA GTT CCA	C31	See above	This study
	DSBACL1268	S-G-Dsbacl-1268-a-A-18	AGG GAT TCG CTT ACC GTT	C32	See above	This study
	DSBACL1434	S-G-Dsbacl-1434-a-A-18	ATA GTT AGC CCA ACG ACG	C33	See above	This study
DSF672	DSB674	S-*-Dsb-0674-a-A-18	CCT CTA CAC CTG GAA TTC	C20	*Desulfofrigus* spp., *Desulfofaba gelida, Desulfomusa hansenii*	55[b]
	DSB220	S-*-Dsb-0220-a-A-18	GCG GAC TCA TCT TCA AAC	C25	*Desulfobacterium niacini, Desulfobacterium vacuolatum, Desulfobacterium autotrophicum, Desulfofaba gelida*	This study
	DSBM1239	S-*-Dsbm-1239-a-A-18	GCC CGT TGT ACA TAC CAT	C26	*Desulfobacterium niacini, Desulfobacterium vacuolatum, Desulfobacterium autotrophicum*	This study
	DSFRG211	S-G-Dsfrg-0211-a-A-18	CCC CAA ACA AAA GCT TCC	C22	*Desulfofrigus* spp.	This study
	DSFRG445	S-G-Dsfrg-0445-a-A-18	CAT GTG AGG TTT CTT CCC	C23	See above	This study
	DSFRG1030	S-G-Dsfrg-1030-a-A-18	TGT CAT CGG ATT CCC CGA	C24	See above	This study
DCC868	DCC868	S-*-Dsb-0868-a-A-18	CAG GCG GAT CAC TTA ATG	C38	*Desulfosarcina* sp., *Desulfonema* spp., *Desulfococcus* sp., *Desulfobacterium* spp., *Desulfobotulus* sp., *Desulfostipes* sp., *Desulfomusa* sp.	9
	DSSDBM194	S-*-DssDbm-0194-a-A-18	GAA GAG GCC ACC CTT GAT	C40	*Desulfosarcina variabilis, Desulfobacterium cetonicum*	This study
	DSSDBM217	S-*-DssDbm-0217-a-A-18	GGC CCA TCT TCA AAC AGT	C41	See above	This study
	DSSDBM998	S-*-DssDbm-0998-a-A-18	TTC GAT AGG ATT CCC GGG	C39	See above	This study
	DSSDBM1286	S-*-DssDbm-1286-a-A-18	GAA CTT GGG ACG GCT TTT	C42	See above	This study
DSC193	DSC193	S-*-Dsb-0193-a-A-18	AGG CCA CCC TTG ATC CAA	C43	*Desulfosarcina variabilis*	55
	DSBMIN218	S-S-Dsbm.in-0218-a-A-18	GGG CTC CTC CAT AAA CAG	C44	*Desulfobacterium indolicum*	This study
DCC209	DCC209	S-S-Dcc.mv-0209-a-A-18	CCC AAA CGG TAG CTT CCT	B3	*Desulfococcus multivorans*	55
	DSNISH179	S-S-Dsn.ish-0179-a-A-18	GGG TCA CGG GAA TGT TAT	C45	*Desulfonema ishimotonii*	This study
	DSNISH442	S-S-Dsn.ish-0442-a-A-18	CCC CAG GTT CTT CCC ACA	C46	See above	This study
	DSNISH1001	S-S-Dsn.ish-1001-a-A-18	CGT CTC CGG AAA ATT CCC	C47	See above	This study
DNMA657	DSN658	S-*-Dsn-0658-a-A-18	TCC GCT TCC CTC TCC CAT	B2	*Desulfonema limicola, Desulfonema magnum*	20[b]
	DSBOSA445	S-S-Dsbo.sa-0445-a-A-18	ACC ACA CAA CTT CTT CCC	C21	*Desulfobotulus sapovorans*	This study

	DSMON95	S-*-Dsmon-0095-a-A-18	GTG CGC CAC TTT ACT CCA	C18	*Desulfomonile* spp.	This study
	DSMON1421	S-*-Dsmon-1421-a-A-18	CGA CTT CTG GTG CAG TCA	C19	See above	This study
	SYBAC986	S-*-Sybac-0986-a-A-18	CCG GGG ATG TCA AGC CCA	C17	*Desulfovirga adipica, Desulforhabdus amnigena, Syntrophobacter* spp.	This study
	DSACI175	S-G-Dsaci-0175-a-A-18	CCG AAG GGA CGT ATC CGG	C16	*Desulfacinum* spp.	This study
	DSACI207	S-G-Dsaci-0207-a-A-18	CGA ACA CCA GCT TCT TCG	C15	See above	This study
	TDRNO448	S-S-Tdr.no-0448-a-A-18	AAC CCC ATG AAG GTT CTT	C13	*Thermodesulforhabdus norvegica*	This study
	TDRNO1030	S-S-Tdr.no-1030-a-A-18	TCT CCC GGC TCC CCA ATA	C12	See above	This study
	TDRNO1443	S-S-Tdr.no-1443-a-A-18	GAC ACA ATC GCG GTT GGC	C14	See above	This study
Probe 687	DSV686	S-*-Dsv-0686-a-A-18	CTA CGG ATT TCA CTC CTA	E7	"*Desulfovibrionales*" and other "*Deltaproteobacteria*"	13[b]
DSV1292	DSV1292	S-*-Dsv-1292-a-A-18	CAA TCC GGA CTG GGA CGC	E9	*Desulfovibrio litoralis, Desulfovibrio vulgaris, Desulfovibrio longreachensis, Desulfovibrio termitidis, Desulfovibrio desulfuricans, Desulfovibrio fairfieldensis, Desulfovibrio intestinalis, Desulfovibrio inopinatus, Desulfovibrio senezii, Desulfovibrio gracilis, Desulfovibrio halophilus, Bilophila wadsworthia*	41
DSV698	DSV698	S-*-Dsv-0698-a-A-18	TCC TCC AGA TAT CTA CGG	E8	*Desulfovibrio caledoniensis, Desulfovibrio dechloracetivorans, Desulfovibrio profundus, Desulfovibrio aespoeensis, Desulfovibrio halophilus, Desulfovibrio gracilis, Desulfovibrio longus, Desulfovibrio salexigens, Desulfovibrio zosterae, Desulfovibrio bastinii, Desulfovibrio fairfieldensis, Desulfovibrio intestinalis, Desulfovibrio piger, Desulfovibrio desulfuricans, Desulfovibrio termitidis, Desulfovibrio longreachensis, Desulfovibrio vietnamensis, Desulfovibrio alaskensis, Bilophila wadsworthia, Lawsonia intracellularis*	41[b]
	DVDAPC872	S-*-Dv.d.a.p.c-0872-a-A-18	TCC CCA GGC GGG ATA TTT	E33	*Desulfovibrio caledoniensis, Desulfovibrio dechloracetivorans, Desulfovibrio profundus, Desulfovibrio aespoeensis*	This study
	DVHO130	S-*-Dv.h.o-0130-a-A-18	CCG ATC TGT CGG GTA GAT	E36	*Desulfovibrio halophilus, Desulfovibrio oxyclinae*	This study
	DVHO733	S-*-Dv.h.o-0733-a-A-18	GAA CTT GTC CAG CAG GCC	E37	See above	This study
	DVHO831	S-*-Dv.h.o-0831-a-A-18	GAA CCC AAC GGC CCG ACA	E35	See above	This study
	DVHO1424	S-*-Dv.h.o-1424-a-A-18	TGC CGA CGT CGG GTA AGA	E38	See above	This study
	DVAA1111	S-*-Dv.a.a-1111-a-A-18	GCA ACT GGC AAC AAG GGT	E30	*Desulfovibrio africanus, Desulfovibrio aminophilus*	This study
	DVGL199	S-*-Dv.g.l-0199-a-A-18	CTT GCA TGC AGA GGC CAC	E26	*Desulfovibrio gracilis, Desulfovibrio longus*	This study
	DVGL445	S-*-Dv.g.l-0445-a-A-18	CCT CAA GGG TTT CTT CCC	E27	See above	This study
	DVGL1151	S-*-Dv.g.l-1151-a-A-18	AAC CCC GGC AGT CTC ACT	E28	See above	This study
	DVGL1421	S-*-Dv.g.l-1421-a-A-18	CGA TGT CGG GTA GAA CCA	E29	See above	This study
DSD131	DSVAE131	S-S-Dsv.ae-0131-a-A-18	CCC GAT CGT CTG GGC AGG	E34	*Desulfovibrio aestuarii*	41
	DSV820	S-*-Dsv-0820-a-A-18	CCC GAC ATC TAG CAT CCA	E25, E31	*Desulfovibrio salexigens, Desulfovibrio zosterae, Desulfovibrio fairfieldensis, Desulfovibrio intestinalis, Desulfovibrio piger, Desulfovibrio desulfuricans*	This study
	DVSZ849	S-*-Dv.s.z-0849-a-A-18	GTT AAC TTC GAC ACC GAA	E32	*Desulfovibrio salexigens, Desulfovibrio zosterae*	This study

	DVIG448	S-*-Dv.i.g-0448-a-A-18	CGC ATC CTC GGG GTT CTT	E15	*Desulfovibrio gabonensis, Desulfovibrio indonesiensis*	This study
	DVIG468	S-*-Dv.i.g-0468-a-A-18	CCG TCA GCC GAA GAC ACT	E16	See above	This study
	DSV651	S-*-Dsv-0651-a-A-18	CCC TCT CCA GGA CTC AAG	E39	*Desulfovibrio fructosivorans, Desulfovibrio alcoholivorans, Desulfovibrio sulfodismutans, Desulfovibrio burkinensis, Desulfovibrio inopinatus*	This study
	DVFABS153	S-*-Dv.f.a.b.s-0153-a-A-18	CGG AGC ATG CTG ATC TCC	E40	*Desulfovibrio fructosivorans, Desulfovibrio alcoholivorans, Desulfovibrio sulfodismutans, Desulfovibrio burkinensis*	This study
	DVFABS653	S-*-Dv.f.a.b.s-0653-a-A-18	CAC CCT CTC CAG GAC TCA	E41	See above	This study
	DVFABS1351	S-*-Dv.f.a.b.s-1351-a-A-18	GAG CAT GCT GAT CTC CGA	E42	See above	This study
	DVLVT139	S-*-Dv.1.v.t-0139-a-A-18	GCC GTT ATT CCC AAC TCA	E17	*Desulfovibrio termitidis, Desulfovibrio longreachensis, Desulfovibrio vulgaris*	This study
	DVLVT175	S-*-Dv.l.v.t-0175-a-A-18	AAA TCG GAG CGT ATT CGG	E18	See above	This study
	DVLT131	S-*-Dv.l.t-0131-a-A-18	TCC CAA CTC ATG GGC AGA	E22	*Desulfovibrio termitidis, Desulfovibrio longreachensis*	This study
	DVLT986	S-*-Dv.l.t-0986-a-A-18	TCC CGG ATG TCA AGC CTG	E23	See above	This study
	DVLT1027	S-*-Dv.l.t-1027-a-A-18	TCG GGA TTC TCC GAA GAG	E21	See above	This study
	DSM194	S-G-Dsm-0194-a-A-18	GAG GCA TCC TTT ACC GAC	E11	*Desulfomicrobium* spp., *Desulfobacterium macestii*	This study
DSV214	DSM213	S-G-Dsm-0213-a-A-18	CAT CCT CGG ACG AAT GCA	E10	See above	41[b]
	DSHRE830	S-S-Dsh.re-0830-a-A-18	GTC CTA CGA CCC CAA CAC	E12	*Desulfohalobium retbaense*	This study
	DSHRE995	S-S-Dsh.re-0995-a-A-18	ATG GAG GCT CCC GGG ATG	E13	See above	This study
	DSHRE1243	S-S-Dsh.re-1243-a-A-18	TGC TAC CCT CTG TGC CCA	E14	See above	This study
DFM228	DFMI227a	S-*-DfmI-0227-a-A-18	ATG GGA CGC GGA CCC ATC	A5	*Desulfotomaculum putei, Desulfotomaculum gibsoniae, Desulfotomaculum geothermicum, Desulfotomaculum thermosapovorans, Desulfotomaculum thermoacidovorans, Desulfotomaculum thermobenzoicum, Desulfotomaculum thermoacetoxidans, Desulfotomaculum australicum, Desulfotomaculum kuznetsovii, Desulfotomaculum thermocisternum, Desulfotomaculum luciae, Sporotomaculum hydroxybenzoicum*	9[b]
DFM228	DFMI227b	S-*-Dfml-0227-b-A-18	ATG GGA CGC GGA TCC ATC	A6	*Desulfotomaculum aeronauticum, Desulfotomaculum nigrificans, Desulfotomaculum reducens, Desulfotomaculum ruminis, Desulfotomaculum sapomandens, Desulfotomaculum halophilum*	9[b]
S-*-Dtm(cd)-0216-a-A-19	DFMI210	S-*-Dfml-0210-a-A-18	CCC ATC CAT TAG CGG GTT	A7	Some *Desulfotomaculum* spp. of clusters Ic and Id[c]	25[b]
S-*-Dtm(bcd)-0230-a-A-18	DFMI229	S-*-Dfml-0229-a-A-18	TAA TGG GAC GCG GAC CCA	A8	Some *Desulfotomaculum* spp. of clusters Ib, Ic, and Id[c]	25
	DFMIa641	S-*-DfmIa-0641-a-A-18	CAC TCA AGT CCA CCA GTA	A9	*Desulfotomaculum* spp., (cluster Ia)[c]	This study
	DFMIb726	S-*-Dfmlb-0726-a-A-18	GCC AGG GAG CCG CTT TCG	A10	*Desulfotomaculum* spp., *Sporotomaculum hydroxybenzoicum* (cluster Ib)[c]	This study
	DFMIc841	S-*-DfmIc-0841-a-A-18	GGC ACT GAA GGG TCC TAT	A11	*Desulfotomaculum* spp. (cluster Ic)[c]	This study
	DFMIc1012	S-*-DfmIc-1012-a-A-18	CGT GAA ATC CGT GTT TCC	A12	See above	This study
	DFMIc1119	S-*-DfmIc-1119-a-A-18	ACC CGT TAG CAA CTA ACC	A13	See above	This study
	DFMIc1138	S-*-DfmIc-1138-a-A-18	GGC TAG AGT GCT CGG CTT	A14	See above	This study
	DFMId436	S-*-DfmId-0436-a-A-18	CTT CGT CCC CAA CAA CAG	A15	*Desulfotomaculum* spp. (cluster Id)[c]	This study

	DFMId625	S-*-DfmId-0625-a-A-18	TTT CAA AGG CAC CCC CGC	A16	See above	This study
	DFMId996	S-*-DfmId-0996-a-A-18	CAC AGG CTG TCA GGG GAT	A17	See above	This study
	DFMId1117	S-*-DfmId-1117-a-A-18	CCG CTG GCA ACT AAC CGT	A18	See above	This study
	DFACE199	S-S-Df.ace-0199-a-A-18	GCA TTG TAA AGA GGC CAC	A20	*Desulfotomaculum* spp. (cluster Ie)[c]	This study
	DFACE438	S-S-Df.ace-0438-a-A-18	CTG TTC GTC CAA TGT CAC	A19	See above	This study
	DFACE995	S-S-Df.ace-0995-a-A-18	CAC AGC GGT CAC GGG ATG	A21	See above	This study
D-acet1027r	DFACE1028	S-S-Df.ace-1028-a-A-18	CTC CGT GTG CAA GTA AAC	A22	See above	68
	DFACE1436	S-S-Df.ace-1436-a-A-18	TGC GAG TTA AGT CAC CGG	A23	See above	This study
	DFMIf126	S-*-DfmIf-0126-a-A-18	CTG ATA GGC AGG TTA TCC	A24	*Desulfotomaculum* spp. (cluster If)[d]	This study
	DFMII1107	S-*-DfmII-1107-a-A-18	CTA AAT ACA GGG GTT GCG	A29	*Desulfosporosinus* spp., *Desulfotomaculum auripigmentum* (cluster II)[c]	This study
	DFMII1281	S-*-DfmII-1281-a-A-18	GAG ACC GGC TTT CTC GGA	A28	See above	This study
	TDSV601	S-*-Tdsv-0601-a-A-18	GCT GTG GAA TTC CAC CTT	D32	*Thermodesulfovibrio* spp.	This study
S-*-Tdsulfo-0848-a-A-18	TDSV849	S-*-Tdsv-0849-a-A-18	TTT CCC TTC GGC ACA GAG	D33	See above	8
	TDSV1326	S-*-Tdsv-1326-a-A-18	CGA TTC CGG GTT CAC GGA	D31	See above	This study
	TDSBM1282	S-P-Tdsbm-1282-a-A-18	TGA GGA GGG CTT TCT GGG	D27	*Thermodesulfobacterium* spp., *Geothermobacterium* sp.	This study
	TDSBM353	S-*-Tdsbm-0353-a-A-18	CCA AGA TTC CCC CCT GCT	D28	*Thermodesulfobacterium* spp.	This study
	TDSBM652	S-*-Tdsbm-0652-a-A-18	AGC CTC TCC GGC CCT CAA	D29	See above	This study
	ARGLO37	S-G-Arglo-0037-a-A-18	CTT AGT CCC AGC CGG ATA	D37	*Archaeoglobus* spp.	This study
	ARGLO276	S-G-Arglo-0276-a-A-18	GCC CGT ACG GAT CTT CGG	D38	See above	This study
	ARGLO576	S-G-Arglo-0576-a-A-18	CCA GCC CGG CTA CGG ACG	D39	See above	This study
	ARGLO972	S-G-Arglo-0972-a-A-18	CCC CGG TAA GCT TCC CGG	D40	See above	This study
	DSBM168[e]	S-*-Dsbm-0168-a-A-18	ACT TTA TCC GGC ATT AGC		*Desulfobacterium niacini, Desulfobacterium vacuolatum*	This study
	DVHO588[e]	S-*-Dv.h.o-0588-a-A-18	ACC CCT GAC TTA CTG CGC		*Desulfovibrio halophilus, Desulfovibrio oxyclinae*	This study
	DVIG267[e]	S-*-Dv.i.g-0267-a-A-18	CAT CGT AGC CAC GGT GGG		*Desulfovibrio gabonensis, Desulfovibrio indonesiensis*	This study
	DVLT1425[e]	S-*-Dv.l.t-1425-a-A-18	TCA CCG GTA TCG GGT AAA		*Desulfovibrio termitidis, Desulfovibrio longreachensis*	This study
	DVGL228[e]	S-*-Dv.g.l-0228-a-A-18	CAG CCA AGA GGC CTA TTC		*Desulfovibrio gracilis, Desulfovibrio longus*	This study
	ARGLO390[e]	S-G-Arglo-0390-a-A-18	GCA CTC CGG CTG ACC CCG		*Archaeoglobus* spp.	This study
	DVLVT194[f]	S-*-Dv.l.v.t-0194-a-A-18	AGG CCA CCT TTC CCC CGA		*Desulfovibrio termitidis, Desulfovibrio longreachensis, Desulfovibrio vulgaris*	This study
	DVLVT222[f]	S-*-Dv.l.v.t-0222-a-A-18	ACG CGG ACT CAT CCA TGA		See above	This study
	DVCL1350[f]	S-*-Dv.c.l-1350-a-A-18	GGC ATG CTG ATC CAG AAT		*Desulfovibrio cuneatus, Desulfovibrio litoralis*	This study
	DFMIf489[f]	S-*-DfmIf-0489-a-A-18	CCG GGG CTT ACT CCT ATG		*Desulfotomaculum* spp. (cluster If)[d]	This study

[a] Name of oligonucleotide probe based on the nomenclature of Alm et al. (1).
[b] Length of oligonucleotide probe was adapted to the microarray format (18-mer).
[c] Cluster designation(s) of gram-positive, spore-forming SRPs according to Stackebrandt et al. (65).
[d] *Desulfotomaculum halophilum* and *Desulfotomaculum alkaliphilum* were assigned to new cluster If.
[e] Probe was removed from the SRP microarray because no positive signal could be detected after hybridization with fluorescently labeled 16S rRNA gene amplificate of the perfect-match reference strain.
[f] Probe was removed from the SRP microarray because it hybridized nonspecifically to many reference organisms that have mismatches in the 16S rRNA gene target site (see supplementary web material).

shown in Table 3. In addition, difference alignments for all probes generated with the latest ARB small-subunit rRNA database (http://www.arb-home.de) can be viewed at the probeBase website (http://www.probebase.net). The 5′ end of each oligonucleotide probe was tailed with 15 dTTP molecules (T-spacer) to increase the on-chip accessibility of spotted probes to target DNA (61, 63). In addition, the 5′-terminal nucleotide of each oligonucleotide was aminated to allow covalent coupling of the oligonucleotides to aldehyde group-coated CSS-100 glass slides (CEL Associates, Houston, Tex.). The concentration of oligonucleotide probes before printing was adjusted to 50 pmol μl^{-1} in 50% dimethyl sulfoxide to prevent evaporation during the printing procedure. SRP-PhyloChips were printed by using a GMS 417 contact arrayer (Affymetrix, Santa Clara, Calif.). Spotted DNA microarrays were dried overnight at room temperature to allow efficient cross-linking. Slides were washed twice at room temperature in 0.2% sodium dodecyl sulfate (SDS) and then twice with double-distilled water with vigorous agitation to remove unbound oligonucleotides and the SDS. After air drying, the slides were incubated for 5 min in a fresh sodium borohydride solution (1.0 g of $NaBH_4$ in 300 ml of phosphate-buffered saline and 100 ml of absolute ethanol) to reduce all remaining reactive aldehyde groups on the glass. The reaction was stopped by adding ice-cold absolute ethanol. The reduced slides were washed three times (with 0.2% SDS and double-distilled water), air dried, and stored in the dark at room temperature.

Reverse hybridization on microarrays. Vacuum-dried Cy5-labeled PCR products (400 ng) and 0.5 pmol of the Cy5-labeled control oligonucleotide CONTCOMP (Table 3) were resuspended in 20 μl of hybridization buffer (5× SSC, 1% blocking reagent [Roche, Mannheim, Germany], 0.1% *n*-lauryl sarcosine, 0.02% SDS, 5% formamide [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]), denatured for 10 min at 95°C, and immediately placed on ice. Then the solution was pipetted onto an SRP-PhyloChip, covered with a coverslip, and inserted into a tight custom-made hybridization chamber (http://cmgm.stanford.edu/pbrown/mguide/HybChamber.pdf) containing 50 μl of hybridization buffer for subsequent equilibration. Hybridization was performed overnight at 42°C in a water bath. After hybridization, the slides were washed immediately under stringent conditions for 5 min at 55°C in 50 ml of washing buffer (containing 3 M tetramethylammonium chloride, 50 mM Tris-HCl, 2 mM EDTA, and 0.1% SDS). To record probe-target melting curves, the temperature of the washing step was varied from 42 to 80°C. After the stringent washing, the slides were washed twice with ice-cold double-distilled water, air dried, and stored in the dark at room temperature.

Scanning of microarrays. Fluorescence images of the SRP PhyloChips were recorded by scanning the slides with a GMS 418 array scanner (Affymetrix). The fluorescence signals were quantified by using the ImaGene 4.0 software (BioDiscovery, Inc., Los Angeles, Calif.). A grid of individual circles defining the location of each spot on the array was superimposed on the image to designate each fluorescent spot to be quantified. The mean signal intensity was determined for each spot. In addition, the mean signal intensity of the local background area surrounding the spots was determined.

Selective enrichment of nucleic acids by a capture probe approach. Five microliters of aldehyde group-coated glass beads (diameter, 1 μm; Xenopore, Hawthorne, N.J.) was incubated overnight with 5 μl of the appropriate capture probe (100 pmol μl^{-1}; tailed with 15 dTTP molecules; aminated with 5′-terminal nucleotide) at room temperature. Subsequently, the beads were washed once with 400 μl of 0.2% SDS and pelleted by centrifugation (1 min at 14,000 rpm; Hettich Zentrifuge type 1000, Tuttlingen, Germany), and the supernatant was decanted. After this step, the beads were washed twice with 400 μl of double-distilled water, dried, and stored at room temperature prior to hybridization. A vacuum-dried bacterial 16S rRNA gene PCR product (obtained from DNA from the Solar Lake mat with the 616V-1492R primer pair) was resuspended in 200 μl of hybridization buffer (see above), denatured for 10 min at 95°C, and immediately cooled on ice. The hybridization solution and capture probe beads were mixed in a screw-cap tube and incubated overnight at 42°C on a shaker. Subsequently, the beads were washed twice with 1.5 ml of washing buffer (see above) at 55°C for 2.5 min. After the stringent washes, the beads were washed with 1.5 ml of ice-cold double-distilled water and then with ice-cold 70% ethanol. Beads with captured nucleic acids were vacuum dried and resuspended in 50 μl of EB buffer (part of the QIAquick PCR purification kit; Qiagen) for storage at −20°C. Reamplification of bacterial 16S rRNA gene fragments from the captured nucleic acids was performed by using 5 μl of the resuspended beads for PCR performed by using the 616V-1492R primer pair and the protocols described above.

Cloning, sequencing, and phylogeny inference. Prior to cloning, the PCR amplification products were purified by low-melting-point agarose (1.5%) gel electrophoresis (NuSieve 3:1; FMC Bioproducts, Biozym Diagnostics GmbH, Oldendorf, Germany) and stained in a SYBR Green I solution (10 μl of 1,0000× SYBR Green I stain in 100 μl of TAE buffer [40 mM Tris, 10 mM sodium acetate, 1 mM EDTA; pH 8.0]; Biozym Diagnostics GmbH) for 45 min. Bands of the expected size were excised from the agarose gel with a glass capillary and melted with 50 μl of double-distilled water for 10 min at 80°C. Four microliters of each solution was ligated as recommended by the manufacturer into the cloning vector pCR2.1 supplied with a TOPO TA cloning kit (Invitrogen Corp., San Diego, Calif.). Nucleotide sequences were determined by the dideoxynucleotide method (57) as described by Purkhold et al. (51). The new 16S rRNA sequences were added to an alignment of about 16,000 small-subunit rRNA sequences by using the alignment tool of the ARB program package (O. Strunk and W. Ludwig, http://www.arb-home.de). Alignments were refined by visual inspection. Phylogenetic analyses were performed by using distance matrix, maximum-parsimony, and maximum-likelihood methods and the appropriate tools of the ARB program package and the fastDNAml program (34). The compositions of the data sets varied with respect to the reference sequences and the alignment positions included. Variability in the individual alignment positions was determined by using the appropriate tool of the ARB package and was used as a criterion to remove or include variable positions for phylogenetic analyses. Phylogenetic consensus trees were drawn by following the recommendations of Ludwig et al. (40). The new *dsrAB* sequences were translated into amino acids and added to an alignment of 62 DsrAB sequences of SRPs (18, 28). Phylogenetic analyses were performed by using the procedures described by Klein et al. (28).

Nucleotide sequence accession numbers. The sequences determined in this study are available in the GenBank database under accession numbers AY083010 to AY083027 (16S rRNA gene clones) and AY083028 to AY083029 (*dsrAB* gene clones). The *dsrAB* gene sequence of *Desulfomicrobium orale* DSM 12838^T has been deposited under accession number AY083030.

RESULTS

SRP phylogeny. As the basis for development of the SRP-PhyloChip, a thorough reevaluation of the phylogeny of SRPs was performed. All 16S rRNA sequences of SRPs which are available in public databases (as of October 2001) were collected, aligned, and analyzed phylogenetically by using maximum-parsimony, maximum-likelihood, and neighbor-joining methods. Figures 1 and 2 illustrate the phylogeny of the delta-proteobacterial SRPs. Figure 3 shows the phylogeny of SRPs affiliated with the *Firmicutes*, *Nitrospira*, *Thermodesulfobacteria*, and *Euryarchaeota* phyla (phylum names according to the taxonomic outline in the second edition of *Bergey's Manual of Systematic Bacteriology*, 2nd ed. [21]).

Probe design. Initially, the specificities of previously described probes and primers for SRPs (2, 8, 9, 13, 20, 25, 41, 52, 55, 59, 68) were reevaluated with the current 16S rRNA data set containing more than 16,000 entries. Based on this analysis, 26 probes were considered to be suitable for inclusion on the SRP-PhyloChip (Table 3). These probes were, if necessary, adjusted to a length of 18 nucleotides (not including the T-spacer). Twenty-four of these probes exclusively target SRPs. Probes SRB385 (2) and SRB385Db (52) were included on the chip because they have been widely used in previous SRP research (3, 16, 36, 49, 58, 71), although both of these probes target a considerable number of phylogenetically diverse non-SRPs. In addition, we significantly extended the SRP probe set by designing 102 probes targeting monophyletic groups of SRPs (Fig. 1 to 3 and Table 3). These probes were designed to have a minimum G+C content of 50%, a length of 18 nucleotides (not including the T-spacer), and as many centrally located mismatches with the target sites on 16S rRNA genes of nontarget organisms as possible. Several of these probes target the same SRPs, complementing several unique regions of the 16S rRNA gene, while others exhibit hierarchical specificity.

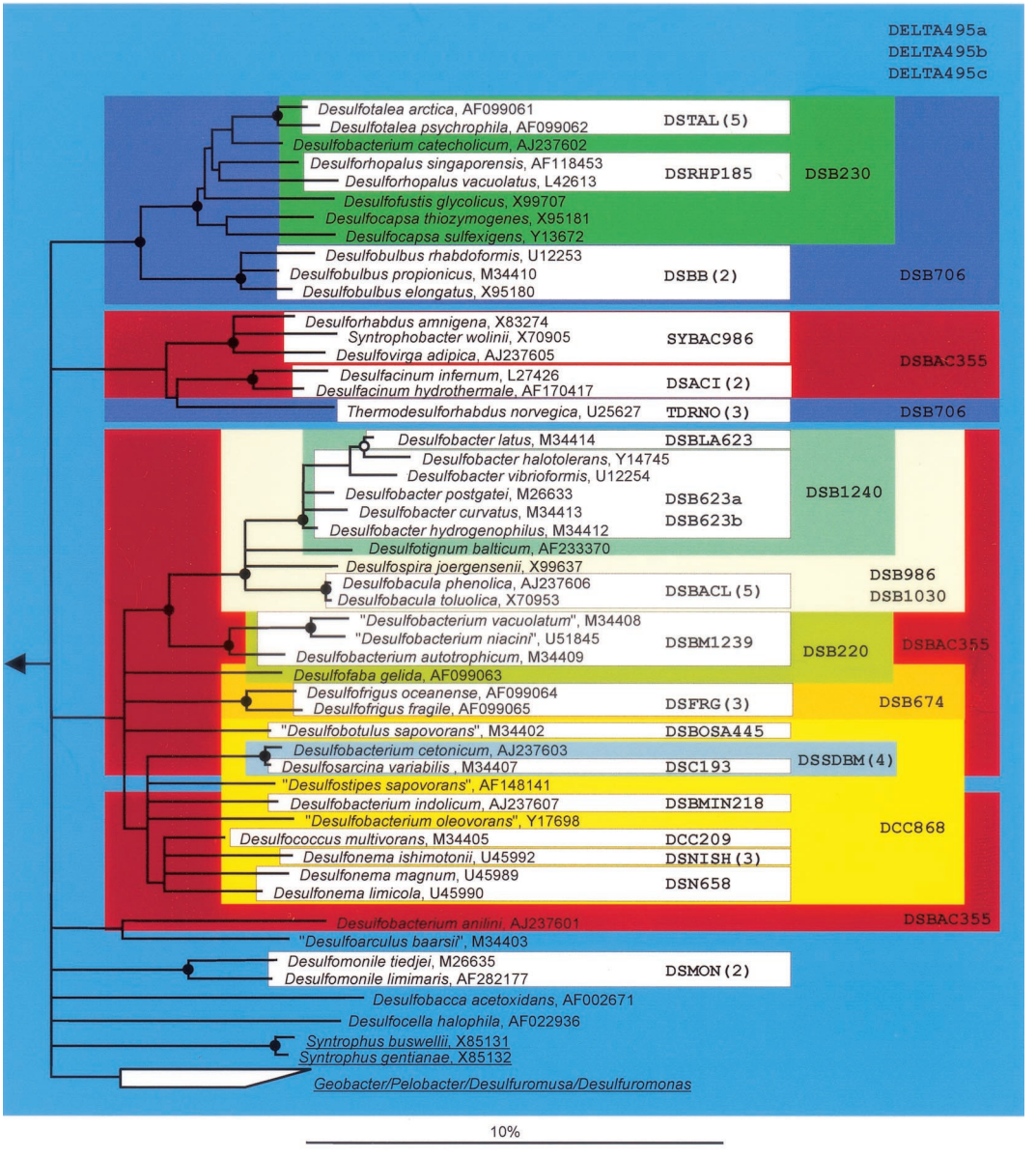

FIG. 1. Phylogenetic affiliations of SRPs belonging to the orders "*Desulfobacterales*" and "*Syntrophobacterales*" of the class "*Deltaproteobacteria*." The 16S rRNA consensus tree was constructed from comparative sequence analysis data by using maximum-parsimony, maximum-likelihood, and neighbor-joining methods and applying filters excluding all alignment positions which are not conserved in at least 50% of all bacterial and deltaproteobacterial 16S rRNA sequences. A collection of organisms representing all major lineages of the *Archaea* and *Bacteria* was used as an outgroup. Multifurcations connect branches for which a relative order could not be determined unambiguously. Non-SRPs are underlined. Parsimony bootstrap values (1,000 resamplings) for branches are indicated by solid circles (>90%) or an open circle (75 to 90%). Branches without circles had bootstrap values of less than 75%. The bar indicates 10% estimated sequence divergence (distance inferred by neighbor joining by using a 50% bacterial conservation filter). The colored boxes show the specificities (perfect-match target organisms) of the SRP-PhyloChip probes (indicated by short names). The numbers of probes with identical specificities for the target organisms are indicated in parentheses. Probes SRB385Db, DSS658, DSR651, and DSB804 are not shown to enhance clarity.

For example, the genus *Desulfotalea* is specifically detected by five probes and is also targeted by three probes with broader specificities (Fig. 1 and Table 3). Altogether, all 134 recognized SRPs for which 16S rRNA sequences have been published are covered by the probe set which we developed. The probes were spotted onto glass slides by using a pattern roughly reflecting the phylogeny of the SRPs (Table 3). In addition, universal, bacterial, and archaeal probes, as well as a nonsense probe (NONSENSE, with a sequence having at least four mismatches with every known 16S rRNA sequence), were immobilized on the microarray for hybridization control purposes (Table 3). Furthermore, another nonsense probe (CONT) (Table 3) was spotted at the beginning and end of each probe row of the microarray. During hybridization, a fluorescently labeled oligonucleotide fully complementary to this probe was added for control of hybridization efficiency and for straightforward localization of the probe spot rows in the microarray readout.

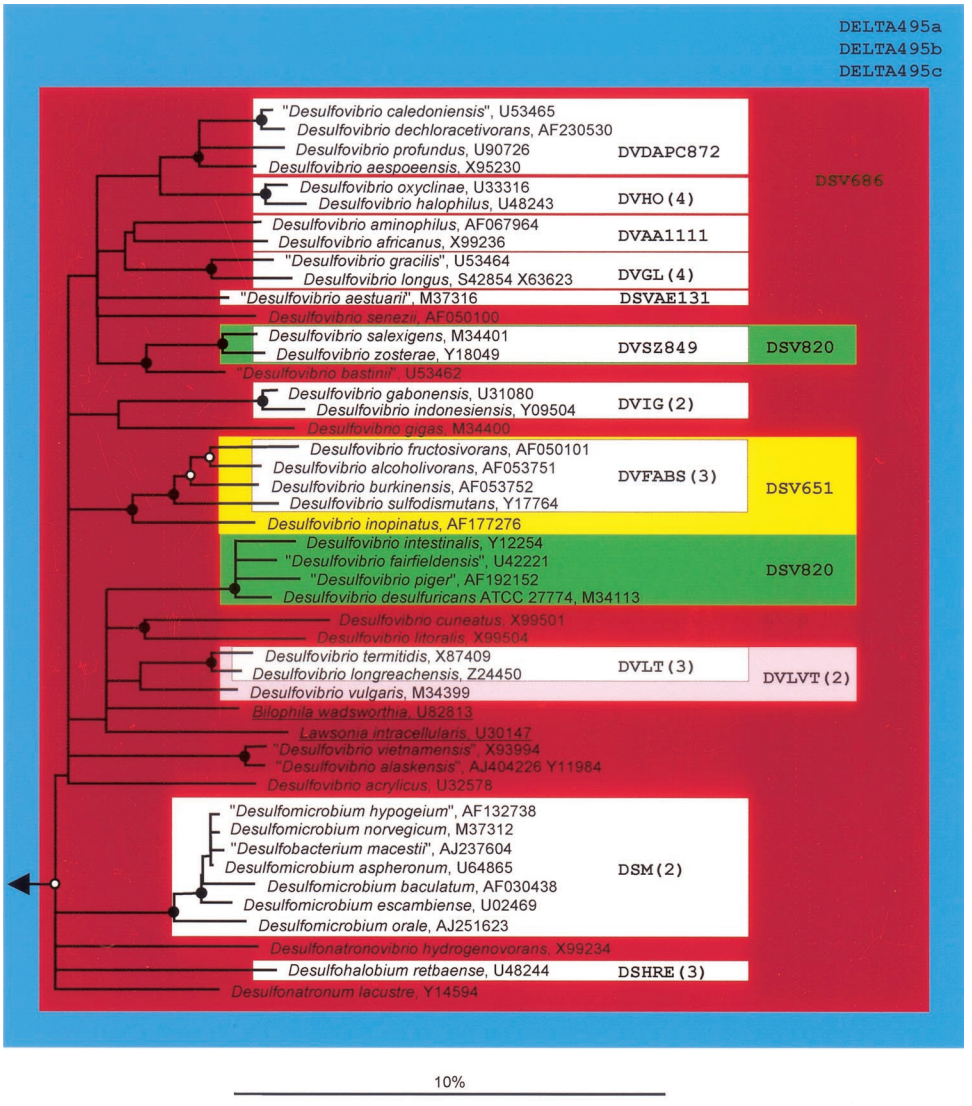

FIG. 2. Phylogenetic affiliations of SRPs belonging to the order "*Desulfovibrionales*" of the class "*Deltaproteobacteria*." The 16S rRNA consensus tree was constructed as described in the legend to Fig. 1. Non-SRPs are underlined. The colored boxes show the specificities (perfect-match target organisms) of the SRP-PhyloChip probes (indicated by short names). The numbers of probes with identical specificities for the target organisms are indicated in parentheses. Probes SRB385, DSV1292, and DSV698 are not shown to enhance clarity.

Evaluation of the SRP-PhyloChip with pure cultures. In the first step, the SRP-PhyloChip was hybridized with fluorescently labeled 16S rRNA gene amplificates of *Desulfovibrio halophilus*, *Desulfomicrobium aspheronum*, and *Desulfohalobium retbaense* under increasingly stringent conditions. For each data point, a separate microarray with nine replicate spots of each probe was hybridized, washed, and analyzed. Figure 4 shows representative melting curves of probe-target duplexes for two of the SRP-specific probes and for bacterial probe EUB338 with the labeled 16S rRNA gene amplificates of the three reference organisms. Positive hybridization signals were recorded with probe EUB338 for the three SRPs when wash temperatures between 42 and 60°C were used. However, the EUB338 hybridization signal intensities varied significantly for the three reference organisms, indicating that there were variations in the efficiency of the fluorescence labeling of the PCR amplificates (Fig. 4C). Clear discrimination between perfectly matched and mismatched duplexes was achieved for most but not all of the probes investigated (Fig. 4A and B and 5). When a wash temperature of 42°C was used, the fluorescence intensity of probe-target hybrids with mismatches was almost always lower than the fluorescence intensity of completely matched hybrids (Fig. 5A). Unexpectedly, the difference in signal intensity between completely matched and mismatched duplexes was not significantly increased by gradually increasing the wash temperature to 80°C (Fig. 5). Based on the recorded melting curves, a wash temperature of 55°C was selected for all further experiments.

In the next step, an SRP-PhyloChip with duplicate spots for each probe was evaluated by using 41 SRP reference organisms. For each SRP-specific probe, this set of reference organisms contained an SRP which has a 16S rRNA gene with a

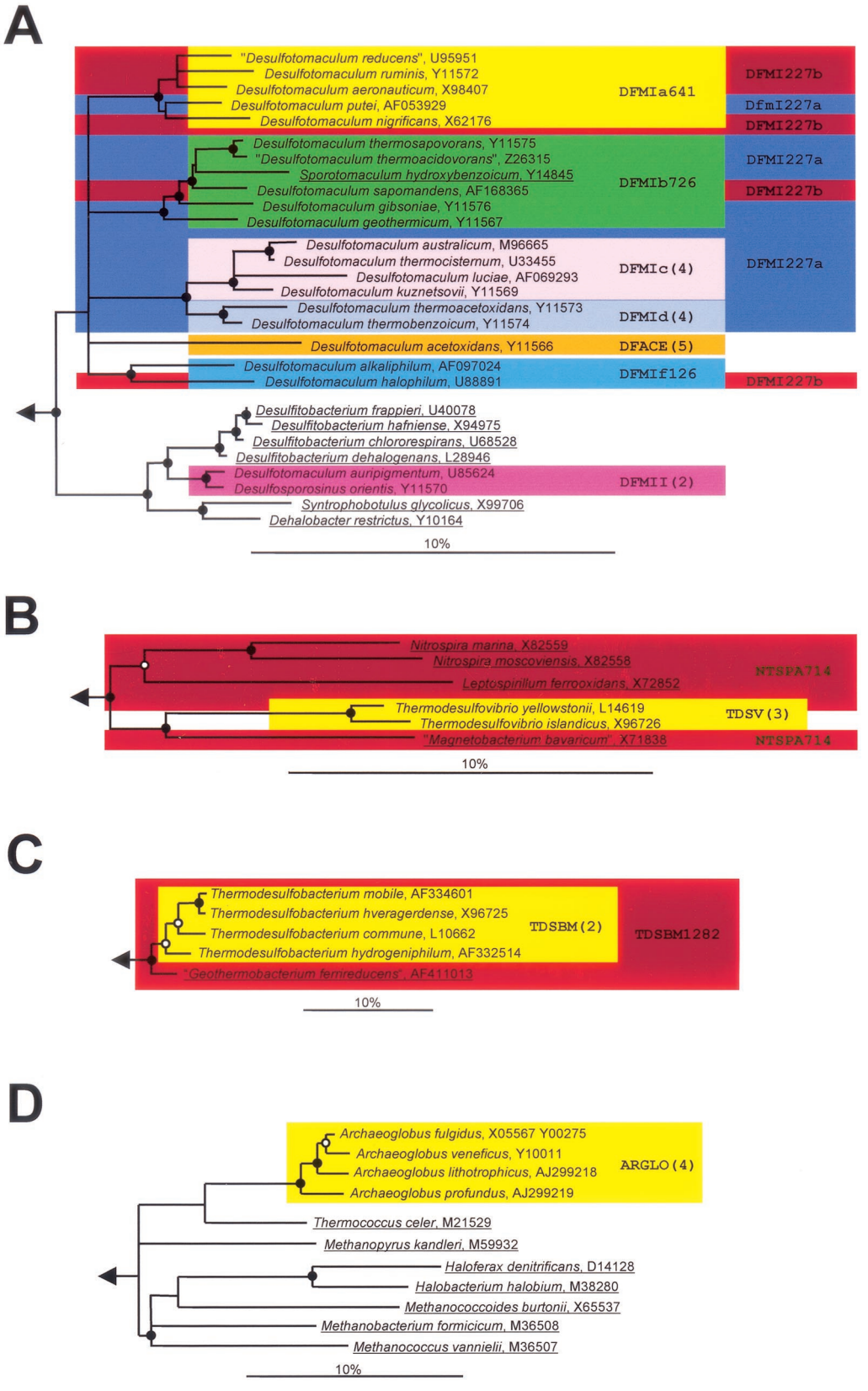

FIG. 3. (A) Phylogenetic affiliations of SRPs belonging to the family *Peptococcaceae* of the phylum *Firmicutes* (low-G+C-content gram-positive bacteria). (B) Phylogenetic affiliations of SRPs belonging to the genus *Thermodesulfovibrio* of the phylum *Nitrospira*. (C) Phylogenetic affiliations of SRPs belonging to the phylum *Thermodesulfobacteria*. (D) Phylogenetic affiliations of SRPs of the genus *Archaeoglobus* belonging to the phylum *Euryarchaeota*. In all panels non-SRPs are underlined. The 16S rRNA consensus trees were constructed as described in the legend to Fig. 1. The colored boxes show the specificities (perfect-match target organisms) of the SRP-PhyloChip probes (indicated by short names). The numbers of probes with identical specificities for the target organisms are indicated in parentheses. In panel A probes DFMI210 and DFMI229 are not shown to enhance clarity.

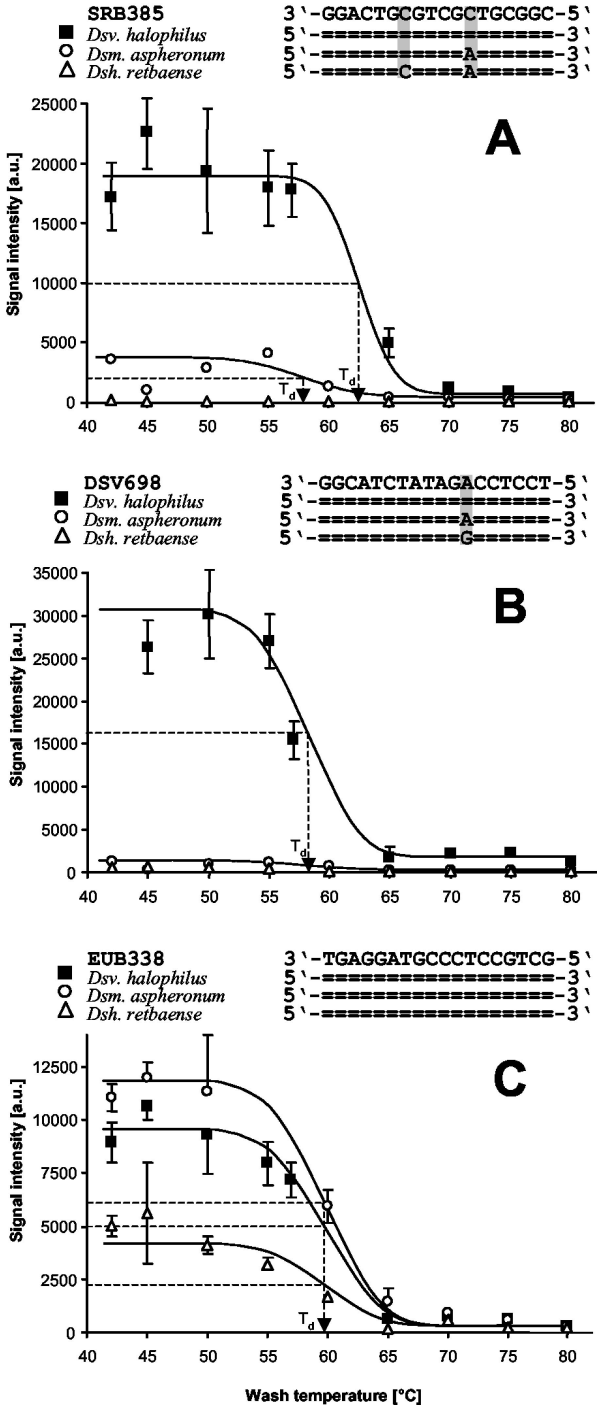

FIG. 4. Melting curves for probe SRB385 (A), probe DSV698 (B), and probe EUB338 (C) after hybridization with fluorescently labeled PCR-amplified 16S rRNA gene fragments of *Desulfovibrio halophilus*, *Desulfomicrobium aspheronum*, and *Desulfohalobium retbaense*. For each probe the difference alignment with these reference SRPs is shown. The observed dissociation temperature (T_d) is indicated for each probe. Each data point represents the mean signal intensity value for 10 probe spots (local background was subtracted for each measurement). The error bars indicate the standard deviations. For each wash temperature and reference organism a separate microarray hybridization was performed. a.u., arbitrary units.

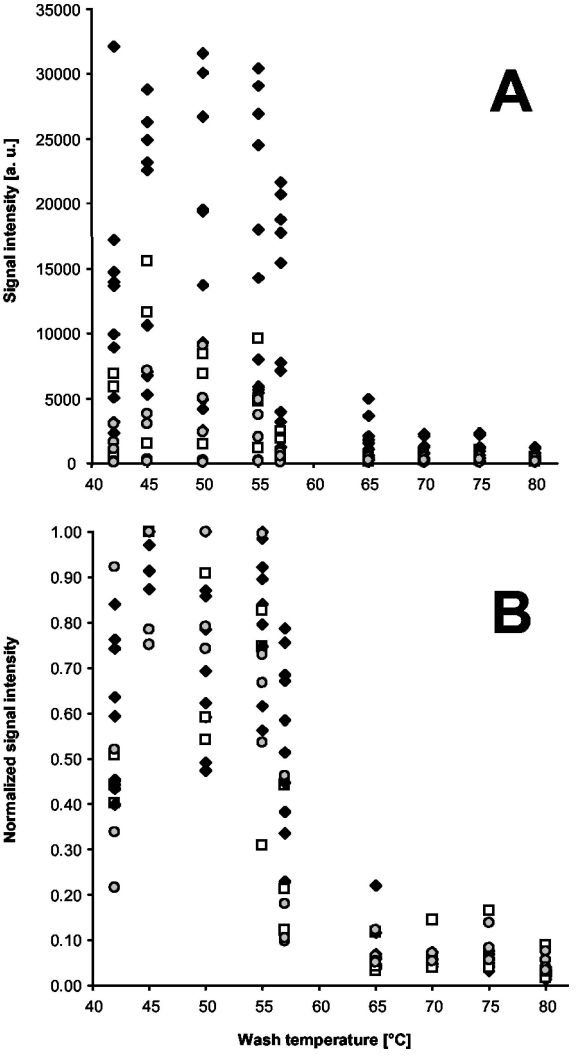

FIG. 5. Hybridization intensities of probes forming perfect-match (diamonds), one-mismatch (squares), and two-mismatch (circles) duplexes after hybridization with fluorescently labeled PCR-amplified 16S rRNA gene fragments of *Desulfovibrio halophilus* at different stringencies. (A) Mean signal intensities (for 10 spots, with local background subtracted) for each probe and wash temperature. (B) Normalized mean signal intensity values for each probe and wash temperature. Mean intensity values were normalized for each probe separately by assuming that the highest value observed at the different wash temperatures had a value of 1.00. In panel B, probes which showed no hybridization signals at low stringencies are not shown.

perfectly matched target site. For each reference organism, fluorescently labeled, PCR-amplified 16S rRNA gene fragments were hybridized separately with the microarray by using 55°C as the wash temperature. The array readout was quantitatively analyzed by digital image analysis to determine a signal-to-noise ratio for each probe according to the following formula:

$$T = [I_P - (I_N - I_{NLB})] \times I_{PLB}^{-1}$$

where T is the signal-to-noise ratio of the probe, I_P is the mean pixel intensity of both specific probe spots, I_N is the mean pixel intensity of both NONSENSE probe spots (note that I_N −

I_{NLB} must always have a lower value than I_P), I_{NLB} is the mean pixel intensity of the local background area around both NONSENSE probe spots, and I_{PLB} is the mean pixel intensity of the local background area around both specific probe spots.

Spots for which the signal-to-noise ratio was equal to or greater than 2.0 were considered positive in the pure-culture evaluation experiments and all subsequent analyses. Furthermore, the signal-to-noise ratio of each probe was divided by the signal-to-noise ratio of the bacterial EUB338 probe recorded on the same microarray in order to compare the duplex yields of the different SRP-specific probes. To do this, the following formula was used:

$$R = T \times \{[I_{EUB} - (I_N - I_{NLB})] \times I_{EUBLB}{}^{-1}\}^{-1}$$

where R is the normalized signal-to-noise ratio of the probe, I_{EUB} is the mean pixel intensity of all EUB338 probe spots, and I_{EUBLB} is the mean pixel intensity of the local background area around all EUB338 probe spots.

The normalized signal-to-noise ratios of the probes ranged from 0.3 for probe DFACE1028 with *Desulfotomaculum acetoxidans* to 16.9 for probe DSBAC355 with *Desulfobacula toluolica*, demonstrating that different probes exhibit very different signal intensities after hybridization with their perfectly matched target sequences.

The individual hybridization results for each of the 132 probes with each of the reference organisms can be downloaded from our website (http://www.microbial-ecology.de/srpphylochip/). Six of the probes evaluated (listed separately in Table 3) did not show a positive hybridization signal with any of the reference organisms, including the perfect-match target SRP, and thus were excluded from the microarray in subsequent experiments. In addition, four probes (listed separately in Table 3) were found to be not suitable for SRP diversity surveys due to their nonspecific binding to many nontarget organisms under stringent hybridization conditions (see supplementary web material). Under the conditions used, 75 (59%) of the probes found to be suitable for the SRP-PhyloChip hybridized exclusively to their target organisms. The other probes hybridized to rRNA gene amplificates with perfectly matched target sites, as well as to some rRNA genes with target sites having between one and six mismatches. In summary, of the 5,248 individual probe-target hybridization reactions performed (by hybridizing the 41 reference organisms with the final SRP-PhyloChip), 5,050 (96%) gave the expected results by either showing a detectable signal with the appropriate perfect-match target or showing no signal with target sequences containing mismatches.

Subsequently, the SRP-PhyloChip was hybridized in independent experiments with different amounts (1, 5, 10, 25, 50, 100, 200, and 400 ng) of PCR-amplified, labeled 16S rRNA gene fragments of *Desulfovibrio halophilus*. The same hybridization pattern was observed when 50 to 400 ng of labeled nucleic acids was used. When less than 50 ng of added nucleic acid was used, the signal-to-noise ratios of the hybridization signals were less than 2.0.

SRP-PhyloChip analyses of complex samples. To evaluate the applicability of the SRP-PhyloChip for medical and environmental studies, two different samples, both containing diverse microbial communities, were analyzed. In the first experiment, tooth pocket samples from five patients suffering from adult periodontitis were investigated. While for three of the patients none of the SRP-specific probes showed a positive signal (data not shown), probe hybridization patterns indicative of the presence of members of the genus *Desulfomicrobium* were obtained for the other two patients (Fig. 6A). This result was confirmed independently by PCR analysis of the DNA obtained from the tooth pockets of the five patients by using primers specific for the 16S rRNA gene of members of the genus *Desulfomicrobium* (Table 2). Consistent with the microarray results, specific PCR amplificates were obtained for two of the five patients. Amplificates from both of these patients were cloned and sequenced. Comparative analysis of six clones demonstrated that the amplified sequences were almost identical to each other and to the corresponding 16S rRNA gene fragment of *Desulfomicrobium orale* (99.6 to 99.9% sequence similarity) (Fig. 6B). Furthermore, the compositions of the SRP communities in the tooth pockets of the patients were analyzed by using the genes encoding the dissimilatory (bi)sulfite reductase as a marker (28, 76). Approximately 1.9-kb *dsrAB* fragments could be PCR amplified from two of the five patients, and these fragments were cloned and sequenced. All 19 clones analyzed (6 clones from patient 1 and 13 clones from patient 4) had sequences almost identical to each other and to the *dsrAB* sequence of *Desulfomicrobium orale* (99.2 to 99.7% amino acid identity), which was also determined in this study.

In the second experiment, the SRP-PhyloChip was used to investigate the SRP community in the chemocline of a hypersaline cyanobacterial mat from Solar Lake. The SRP-PhyloChip hybridization patterns of fluorescently labeled 16S rRNA gene PCR amplificates obtained from the chemocline were more complex than those obtained from the tooth pockets (Fig. 7A). The probe hybridization patterns indicated that bacteria related to the genera *Desulfonema* and *Desulfomonile* were present. Furthermore, probe DSB220 showed signals above the threshold value which could have resulted from SRPs related to the genus *Desulfofaba.* However, the signal of probe DSB674, which also targets this genus, was below the threshold value. To confirm these results, 16S rRNA gene PCRs specific for most members of the "*Desulfobacterales*" (including the genera *Desulfonema* and *Desulfofaba*) and the "*Syntrophobacterales*" (primers DSBAC355F and 1492R [Table 2]), as well as for some *Desulfonema* species (primers DSN61F and DSN+1201R [Table 2]), were performed. Cloning and sequencing of the PCR amplificates confirmed that *Desulfonema*- and *Desulfomonile*-related organisms were present in the mat chemocline (Fig. 7B). In contrast to the microarray results, no sequences affiliated with the genus *Desulfofaba* were retrieved. In addition, we used glass beads coated with probe DSN658 to enrich *Desulfonema*-related 16S rRNA gene sequences from bacterial 16S rRNA gene amplificates from the mat chemocline. After enrichment, reamplification, and cloning, 1 of 12 cloned sequences did indeed possess the target site of probe DSN658 and was identical to *Desulfonema*-related sequences obtained by the specific PCR assay described above (Fig. 7B). The remaining 11 cloned sequences did not possess the probe DSN658 target site and were unrelated to recognized SRPs (data not shown).

Software-assisted interpretation of microarray readouts. Interpretation of experiments performed with the SRP-Phylo-

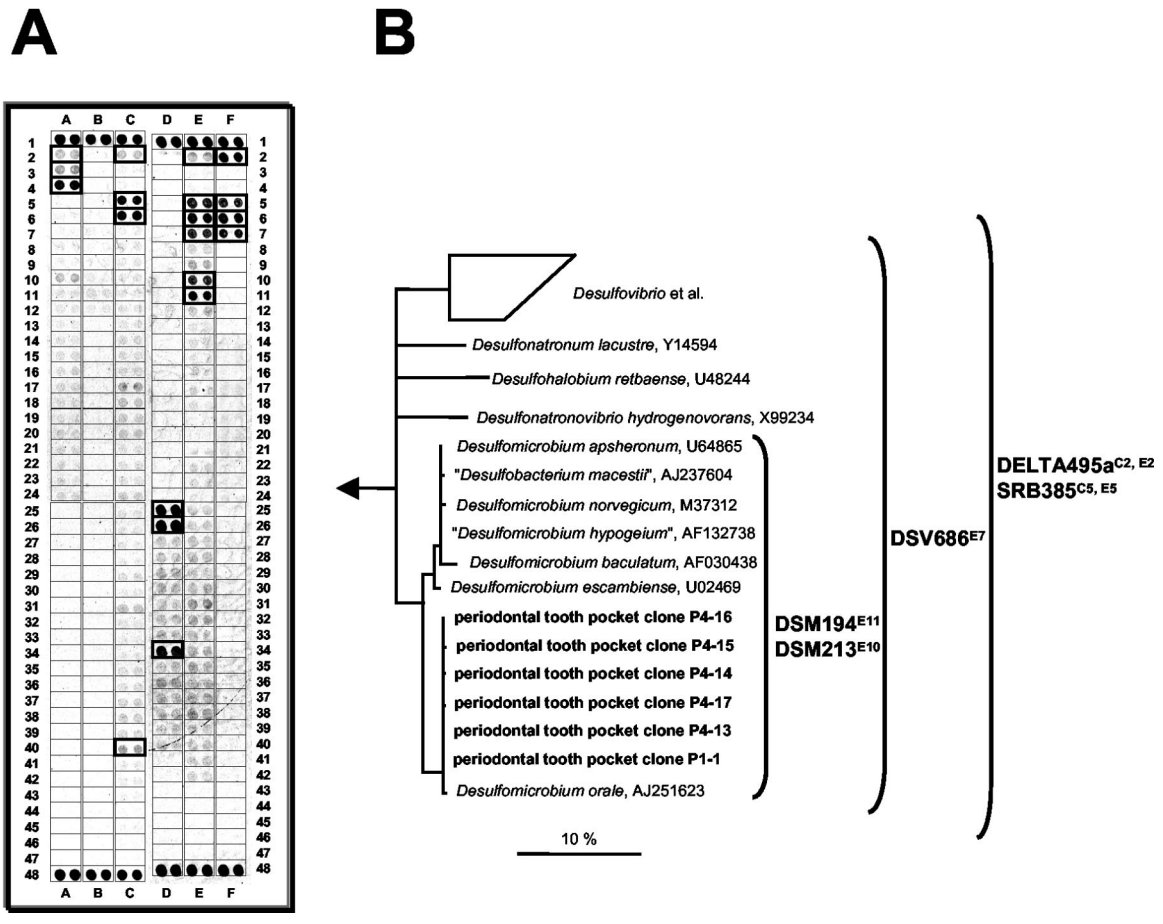

FIG. 6. (A) Use of the SRP-PhyloChip for surveys of SRP diversity in periodontal tooth pockets. On the microarray each probe was spotted in duplicate. For each microarray position, the probe sequence and specificity are shown in Table 3. Probe spots having a signal-to-noise ratio equal to or greater than 2.0 are indicated by boldface boxes and were considered to be positive. (B) Evaluation of the microarray results by amplification, cloning, and comparative sequence analysis of 16S rRNA gene fragments by using *Desulfomicrobium*-specific primers for PCR. 16S rRNA gene clones obtained from the tooth pockets are indicated by boldface type. The tree is based on a maximum-likelihood analysis performed with a 50% conservation filter for the *Bacteria*. Multifurcations connect branches for which a relative order could not be determined unambiguously after different treeing methods and filters were used. The bar indicates 10% estimated sequence divergence. The brackets indicate the perfect-match target organisms for the probes. The microarray position is indicated after each probe name.

Chip requires translation of more or less complex probe hybridization patterns into a list of SRPs which might be present in the sample analyzed. In principle, this task can be performed manually by using Table 3 and Fig. 1 to 3 as guides, but this procedure is tedious and sometimes not straightforward when it is performed with complex hybridization patterns. Consequently, we developed a software tool termed ChipChecker, which, after the microarray readout file (output from the ImaGene software) is imported, automatically creates a list of SRPs that potential occur in a sample. To do this, the software determines for each hybridization experiment which probes were positive (signal-to-noise ratio greater than the threshold; default signal-to-noise ratio, $\geq$2.0) and compares this result automatically with a list which specifies for each recognized SRP all fully complementary probes. Only those SRPs for which all perfect-match probes show a positive signal are listed. The ChipChecker software can easily be adapted for interpretation of other DNA microarrays and is available together with additional information for free download (http://wwwbode.cs.tum.edu/~meierh/download_chipchecker.html).

DISCUSSION

Microarray design and hybridization strategy. In this study an encompassing DNA microarray for analysis of SRP diversity in complex samples was developed and evaluated. A total of 132 previously described and newly designed probes for the detection of 16S rRNA genes of SRPs were immobilized on the microarray. Consistent with design formats used in previous microarray applications for identification of other bacterial groups (23, 37), a hierarchical set of oligonucleotides complementary to the 16S rRNA genes of the target microorganisms at multiple levels of specificity was developed. However, the number of 16S rRNA-targeted oligonucleotide probes used in this study is significantly higher than the numbers of probes used in previous applications of chips for bacterial identification (23, 37, 62). This difference had important implications for the strategy which we selected for optimizing the hybridization conditions to ensure maximum specificity of the probes. Initially, temperature-dependent dissociation of several probe-target duplexes with perfect matches or mismatches was mea-

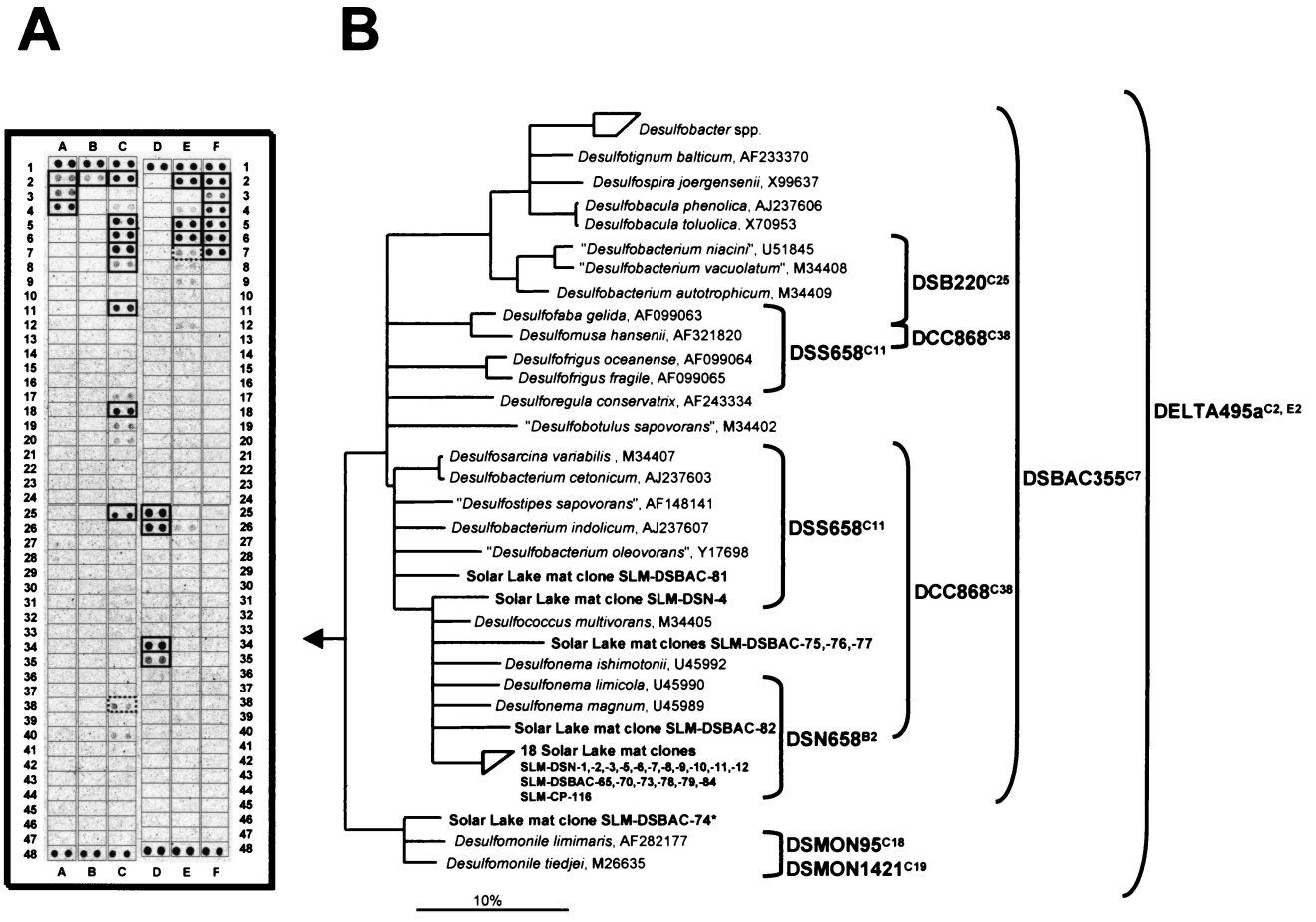

FIG. 7. (A) Use of the SRP-PhyloChip for surveys of SRP diversity in the chemocline of a cyanobacterial microbial mat. On the microarray each probe was spotted in duplicate. For each microarray position, the probe sequence and specificity are shown in Table 3. Probe spots having a signal-to-noise ratio equal to or greater than 2.0 are indicated by boldface boxes and were considered to be positive. The dotted boldface boxes indicate that only one of the duplicate spots had a signal-to-noise ratio equal to or greater than 2.0. (B) Evaluation of the microarray results by amplification, cloning, and comparative sequence analysis of 16S rRNA gene fragments by using primers specific for some *Desulfonema* species (SLM-DSN clones) and most members of the "*Desulfobacterales*" and "*Syntrophobacterales*" (SLM-DSBAC clones). Clone SLM-CP-116 was obtained from the mat chemocline by amplification, cloning, and sequencing after enrichment by using probe DSN658 as the capture probe. 16S rRNA gene clones obtained from the chemocline of the Solar Lake mat are indicated by boldface type. The tree is based on a maximum-likelihood analysis performed with a 50% conservation filter for the *Bacteria*. Multifurcations connect branches for which a relative order could not be determined unambiguously after different treeing methods and filters were used. The bar indicates 10% estimated sequence divergence. The brackets indicate the perfect-match target organisms of the probes. The microarray position is indicated after each probe name. The amplified and sequenced 16S rRNA gene fragment of Solar Lake mat clone SLM-DSBAC-74 (indicated by an asterisk) is outside the target site for probe DSMON95 and has one mismatch (located at position 16) within the target site for probe DSMON1421.

sured by using labeled 16S rRNA gene amplificates of three SRP reference organisms (Fig. 4 and 5). Comparable dissociation temperatures between 58 and 62°C, at which 50% of the starting duplexes remained intact, were observed for the different duplexes. This congruence probably reflects the fact that all probes of the SRP-PhyloChip are the same length (18 nucleotides) and the fact that the wash buffer contained 3 M tetramethylammonium chloride to equalize A · T and G · C base pair stability (42). Because our setup did not allow us to determine nonequilibrium online melting curves (37), it was not feasible (due to the high number of probes used) to record melting curves for each probe with perfectly matched and suitably mismatched target nucleic acids. Based on the recorded melting curves of selected probes, a wash temperature of 55°C was chosen for all further experiments as the best compromise between signal intensity and stringency. A further increase in stringency significantly reduced the signal intensity of some probes after hybridization with the perfectly matched target molecules (Fig. 5A) and thus decreased the sensitivity of the microarray.

Evaluation of the SRP-PhyloChip with more than 40 SRP reference strains was used to determine a threshold value above which a probe hybridization signal was considered positive. In addition, for each probe the signal intensity after hybridization with a perfectly matched target was compared to the signal intensity of the EUB338 probe on the same microarray (normalized signal-to-noise ratio). Consistent with data from quantitative fluorescence in situ hybridization experiments performed with different 16S rRNA-targeted oligonucleotide probes for *Escherichia coli* (19), (i) some of the probes

used in the first version of the SRP-PhyloChip did not hybridize to their perfect-match targets and (ii) the signal intensities measured for the other probes on the SRP-PhyloChip varied significantly, by factors of up to 56. Dramatic differences in duplex yield arising from different regions of the target were also observed in other microarray applications (45, 64) and probably reflect either accessibility differences for the different probe target sites due to secondary structures of the target DNA or different steric hindrances of the different nucleic acid hybrids formed on the microarrays after hybridization.

The evaluation of the microarray with SRP pure cultures demonstrated (i) that false-negative hybridization never occurred (within the detection limit of the microarray method) but (ii) that some of the probes still hybridized to nontarget organisms under the hybridization and washing conditions used, leading to false-positive results (see supplementary web material). As expected, the nucleotide composition of the mismatch, the mismatch position (67, 73), and possibly other variables, such as the influence of an adjacent nucleotide stacking interaction (17), were the major factors determining the duplex yields of probes with mismatched target nucleic acids. Most of the mismatched duplexes with signal intensities above the threshold value (used to differentiate between positive and negative hybridization results) had a signal intensity (and normalized signal-to-noise ratio) lower than that of the corresponding perfect-match duplex (Fig. 5). However, this difference cannot be exploited for interpretation of microarray hybridization results for environmental samples because a low hybridization signal of a probe can be caused not only by mismatched duplex formation but also by low abundance of the perfect-match target nucleic acid.

Misinterpretation of microarray hybridization patterns caused by the nonperfect specificity of some of the probes could be avoided at least partially by using the multiple-probe concept. While hybridization patterns consistent with the hierarchical or parallel specificity of the probes increase the reliability of detection, inconsistent probe hybridization patterns must be interpreted with caution. In complex samples, inconsistent hybridization patterns can be caused either by nonspecific binding of one or several probes or by previously unrecognized prokaryotes with unusual combinations of perfect-match probe target sites in their 16S rRNA gene sequences.

Microarray-based SRP diversity surveys of complex samples. In this study, periodontal tooth pocket material and a cyanobacterial microbial mat were used to demonstrate the suitability of using the microarray developed for SRP diversity analysis of medical and environmental samples. For the tooth pocket material of two patients suffering from adult periodontitis the SRP-PhyloChip hybridization pattern indicated the presence of members of the genus *Desulfomicrobium*. Colonization of the tooth pockets analyzed by these SRPs, which is consistent with a previous report of isolation of *Desulfomicrobium orale* from periodontal tooth pockets (33), was independently confirmed by retrieval of 16S rRNA and *dsrAB* gene sequences of *Desulfomicrobium orale*, demonstrating the reliability of the microarray results.

The microarray hybridization patterns obtained by reverse hybridization of 16S rRNA gene fragments amplified from the chemocline of a Solar Lake microbial mat suggested that several phylogenetically different SRPs, including bacteria related to the genera *Desulfonema*, *Desulfomonile*, and *Desulfofaba*, were present. By using specific PCR assays, 16S rRNA gene sequences related to sequences of members of the genera *Desulfonema* and *Desulfomonile* were obtained from the mat material analyzed, while the presence of *Desulfofaba*-like organisms could not be confirmed. The failure to detect *Desulfofaba*-like bacteria with the PCR assay might mean that a relatively limited number of 16S rRNA gene clones was sequenced or that the microarray hybridization pattern indicative of *Desulfofaba* was caused by the presence of bacteria that have not been recognized yet. The detection of *Desulfonema*-like bacteria in the chemocline of the Solar Lake mat is consistent with findings of previous studies (46, 47, 70) and further supports the importance of these SRPs in hypersaline mat ecosystems.

In conclusion, we developed an encompassing 16S rRNA gene-targeting oligonucleotide microarray suitable for SRP diversity analyses of complex environmental and clinical samples. The microarray was used to screen samples in order to rapidly obtain indications of the presence of distinct lineages of SRPs. Subsequently, this information was used to select appropriate PCR-based techniques for confirmation of the microarray results and for retrieval of sequence information for phylogenetic analysis. In contrast to previously available tools for cultivation-independent SRP identification (13, 18, 41, 56, 75, 76), the SRP-PhyloChip allowed us to obtain a phylogenetically informative, high-resolution fingerprint of the SRP diversity in a given sample within 48 h (including all experimental work from DNA extraction to hybridization pattern interpretation). However, keeping in mind that (i) most environmental microbial communities contain a high percentage of bacteria not yet sequenced on the 16S rRNA level and (ii) not all probes on the microarray are absolutely specific under the conditions used, the SRP-PhyloChip experiments should always be supplemented with microarray-independent techniques to confirm the phylogenetic affiliations of the SRPs detected. Furthermore, it should be noted that the microarray approach described here did not allow us to obtain quantitative data on the compositions of SRP communities because of the recognized biases introduced by using PCR for 16S rRNA gene amplification (74). In addition, the duplex yield of a probe on the microarray is dependent not only on the actual abundance of its perfect-match target nucleic acid in the PCR amplificate mixture but also on a variety of other factors, including the labeling efficiency of the specific target nucleic acid, the secondary structure of the target region, and the inherent variations associated with microarray fabrication. Despite these limitations, the microarray which we developed has great potential for rapid screening of SRP diversity in complex samples. The SRP diversity microarray fingerprint technique should allow workers to identify the probes which have relevance for further characterization of a sample by PCR or quantitative hybridization experiments. This option should be particularly valuable if large numbers of samples are to be analyzed to study temporal or spatial variations in SRP diversity.

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APPENDIX II

probeBase: An Online Resource for rRNA-Targeted Oligonucleotide Probes

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probeBase: an online resource for rRNA-targeted oligonucleotide probes

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ABSTRACT

Ribosomal RNA-(rRNA)-targeted oligonucleotide probes are widely used for culture-independent identification of microorganisms in environmental and clinical samples. ProbeBase is a comprehensive database containing more than 700 published rRNA-targeted oligonucleotide probe sequences (status August 2002) with supporting bibliographic and biological annotation that can be accessed through the internet at http://www.probebase.net. Each oligonucleotide probe entry contains information on target organisms, target molecule (small- or large-subunit rRNA) and position, G + C content, predicted melting temperature, molecular weight, necessity of competitor probes, and the reference that originally described the oligonucleotide probe, including a link to the respective abstract at PubMed. In addition, probes successfully used for fluorescence *in situ* hybridization (FISH) are highlighted and the recommended hybridization conditions are listed. ProbeBase also offers difference alignments for 16S rRNA-targeted probes by using the probe match tool of the ARB software and the latest small-subunit rRNA ARB database (release June 2002). The option to directly submit probe sequences to the probe match tool of the Ribosomal Database Project II (RDP-II) further allows one to extract supplementary information on probe specificities. The two main features of probeBase, 'search probeBase' and 'find probe set', help researchers to find suitable, published oligonucleotide probes for microorganisms of interest or for rRNA gene sequences submitted by the user. Furthermore, the 'search target site' option provides guidance for the development of new FISH probes.

INTRODUCTION

Comparative sequence analysis of ribosomal RNA (rRNA) gene sequences has become the gold standard to infer prokaryotic phylogeny and is widely used in microbial ecology. For example, the application of rRNA-targeted oligonucleotide probes in different hybridization formats such as dot blot (1) and fluorescence *in situ* hybridization (FISH) (2) allows one to identify uncultured prokaryotes and to quantitatively determine the composition of complex microbial communities (3). Several recent studies also demonstrated the applicability of FISH to routine diagnostic purposes in the clinical laboratory (4–6). In addition, a suite of new techniques circling around rRNA-targeted probes has been developed. rRNA-based phylogenetic DNA microarrays (so-called 'PhyloChips') (7–11) consisting of collections of oligonucleotide probes that detect the target microorganisms at multiple taxonomic levels of specificity are now increasingly being developed and applied for diagnostics and environmental microbiology. Furthermore, the combination of FISH and microautoradiography can be used to determine the ecophysiology of microorganisms by visualizing *in situ* uptake and subsequent incorporation of a radioactively labelled substrate into individual microbial cells (12). As a consequence of the apparent increase in interest in rRNA-targeted oligonucleotide probes during the past years, several hundred ready-to-use domain-, phylum-, genus-, and species-specific probes are already available. However, an overview over published probe sequences can only be obtained by a time-consuming, tedious literature search. Additionally, one has to keep in mind that with the increasing amounts of rRNA sequence data stored in public databases (13,14) (Strunk,O. and Ludwig,W., 1993–2002, ARB—a software environment for sequence data, http://www.arb-home.de) the recognized specificity range for a probe might change. Thus, prior to the application of a rRNA-targeted oligonucleotide probe, researchers are obliged to ascertain that the specificity proposed for this probe in the original publication is still valid. Probe match tools as implemented in the ARB program package (Strunk,O. and Ludwig,W., 1993–2002, ARB—a software environment for sequence data, http://www.arb-home.de) or provided by the Ribosomal Database Project II (RDP-II) (13) offer an option to check for up-to-date specificity of a probe when used in combination with the latest rRNA databases. The pronounced interest of the scientific community in rRNA-targeted oligonucleotide probes is documented by the average 742 user sessions (347 different users) per month recorded for the probe match tool of RDP-II in 2001, making this tool one of the most frequently used software features of the RDP-II website (James R. Cole, personal communication). While

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oligonucleotide databases for, for example, viral (VirOligo) (15) or human genes (Molecular Probe Data Base) (16) are available, an up-to-date resource for rRNA-targeted oligonucleotide probe sequences for the identification of prokaryotes is currently lacking. In 1996, Alm and coworkers compiled the Oligonucleotide Probe Database (OPD) that listed 96 PCR primers and probes mainly targeting small-subunit (SSU) and large-subunit (LSU) rRNA (17). However, OPD has not been updated since 1997 and is now no longer available through the internet. ProbeBase closes this gap by providing a user-friendly web-interface to search for published oligonucleotide probe sequences and annotated information. Using probeBase, it is possible to search for suitable probes by submitting the name of a target organism or by indicating a certain probe target site. In addition, the 'find probe set' tool can be used to rapidly retrieve all published probes perfectly matching rRNA gene query sequences.

ORGANIZATION OF probeBase

A schematic overview of the structure and organization of probeBase is shown in Figure 1. ProbeBase currently comprises more than 700 published rRNA-targeted oligonucleotide probes (status August 2002). Each probe entry contains information on the probe sequence, target organisms, target molecule (SSU or LSU rRNA), target site, G + C content, melting temperature, molecular weight, and the reference that originally described the oligonucleotide probe. In order to facilitate database searches, each probe entry includes additional hidden information on the taxonomic context of the probe target organisms. If a probe has been successfully applied for FISH, the probe name is highlighted, and the recommended formamide concentration in the hybridization buffer required for specific hybridization is provided. For each probe, probeBase offers direct links to the probe match tool at the RDP-II web site and to the respective reference abstract at PubMed (18). Difference alignments for SSU rRNA-targeted probes are available that were generated using the probe match tool of the ARB software and the ARB database (release June 2002) (Strunk,O. and Ludwig,W., 1993–2002, ARB—a software environment for sequence data, http://www.arb-home.de). Probe entries can also contain supplementary information, such as remarks on the application of the probe or the sequence of a possible competitor probe that has to be used together with the probe to ensure its specificity. In addition, probeBase offers a comprehensive and interactive list 'Coverage of group-specific probes' [modified from (19)] showing the coverage of the main prokaryotic lines of descent by general group-specific probes.

Search probeBase

ProbeBase can either be searched for probe target organisms, for probe names, or for probe target sites. If probeBase is searched for oligonucleotide probes specific for certain target organisms, it returns a list of all oligonucleotide probes specific for the searched target organisms as well as probes targeting higher taxonomic levels. This list of probes supports researchers in the choice of an appropriate set of nested probes according to the 'multiple probe concept' (20). This approach

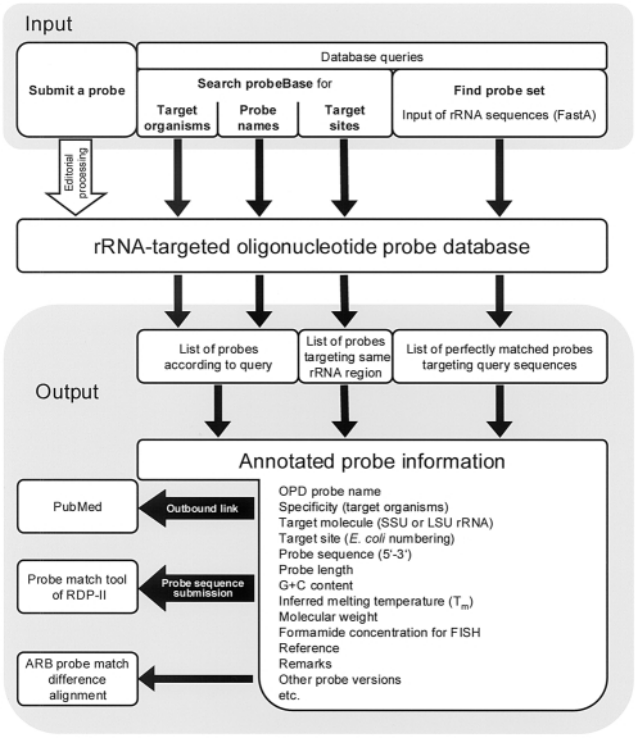

Figure 1. Concept and structure of probeBase.

takes advantage of the option to design and apply rRNA-targeted probes for phylogenetic groups at different taxonomic levels (e.g. phylum-, order-, family-, genus-, or species-specific probes). The simultaneous application of a set of hierarchical probes enhances the reliability of the detection of a particular microorganism.

The option to search for a given probe target site assists in the development of new oligonucleotide probes for FISH by providing information on whether a searched target site has previously been found accessible for oligonucleotide probes in other microorganisms. Studies by Fuchs and coworkers have demonstrated that some regions on the 16S and 23S rRNA of *Escherichia coli* are virtually inaccessible for oligonucleotide probes if used for FISH (21,22). Unfortunately, these results can only be extrapolated to distantly related microorganisms within certain limits. However, if different probes targeting microorganisms affiliated with different evolutionary lineages but sharing the same target site on the respective rRNA molecule have been successfully applied for FISH, it is very likely that the respective target site is generally accessible for oligonucleotide probes.

Find probe set

The 'find probe set' tool of probeBase can be used to rapidly retrieve all published probes targeting one or several query rRNA gene sequences without prior comparative sequence analysis. A set of up to 150 sequences, provided by the researcher as rRNA or DNA sense strand sequence in 5′–3′ orientation (FastA format) can be searched simultaneously for the presence of the perfect match target sites of all probes

deposited at probeBase. The output is a table sequentially listing (i) each single query sequence with all perfectly matching probes found in probeBase and (ii) each possible probe with all perfectly matching query sequences. Using this probeBase feature researchers will, for example, easily be able to determine a set of already published probes that target the microbial sequences in a certain environmental rRNA gene clone library. This probe set might then be used in subsequent hybridization experiments to confirm the presence of the organisms detected in the rRNA gene clone library *in situ* and to gain insight into the actual abundance of these microorganisms in the investigated environment.

AVAILABILITY

ProbeBase is maintained and updated by the Microbial Ecology Group staff at the Lehrstuhl für Mikrobiologie of the Technische Universität München, Bavaria, Germany. Free access to probeBase is provided via the world wide web at http://www.probebase.net. Researchers are kindly invited and encouraged to deposit their newly designed probe sequences and supplementary information at probeBase. Submission might either be performed through the probe submission form 'submit a probe' accessible at the main page or by Email. For queries concerning probeBase and for alternative probe submission contact probebase@microbial-ecology.net.

CITING probeBase

If you use probeBase as a tool in your published research or if you have deposited your newly designed rRNA-targeted oligonucleotide probes at probeBase, we ask that this paper be cited.

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APPENDIX III

Diversity of Sulfate-Reducing Prokaryotes in an Acidic Fen: Comparison of 16S rRNA Gene-Based Oligonucleotide Microarray Hybridization with Dissimilatory (Bi)Sulfite Reductase Gene Surveys

Submitted to APPLIED AND ENVIRONMENTAL MICROBIOLOGY

Diversity of Sulfate-Reducing Prokaryotes in an Acidic Fen: Comparison of 16S rRNA Gene-Based Oligonucleotide Microarray Hybridization with Dissimilatory (Bi)Sulfite Reductase Gene Surveys

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The richness of sulfate-reducing prokaryotes (SRPs) in two acidic fen soils (Schlöppnerbrunnen I and II, Lehstenbach, Fichtelgebirge, Germany) was determined cultivation-independently by combination of 16S rRNA gene-based oligonucleotide microarray analyses and phylogenetic surveys of dissimilatory (bi)sulfite reductase genes (*dsrAB*). Microarray hybridization and subsequent evaluation by SRP group-specific 16S rRNA gene amplification, cloning, and comparative sequence analysis uncovered the occurrence of bacteria affiliated with the deltaproteobacterial genera *Syntrophobacter* and *Desulfomonile*. Detailed sequence analysis of *dsrAB* genes identified in total eleven distinct operational taxonomic units (OTUs) thereby reconfirming the presence of *Syntrophobacter wolinii*- and *Desulfomonile*-related species. Five of these eleven OTUs were of deltaproteobacterial origin (two OTUs most closely related to *Desulfobacca acetoxidans*) whereas six deeply branching OTUs could not be unambiguously affiliated to any DsrAB amino acid sequence from yet recognized SRPs. Both investigated Schlöppnerbrunnen soil samples showed exactly the same total SRP richness (seven *dsrAB* gene OTUs). However, the same three OTUs were identified at both sampling sites whereas four were exclusively found at one site, respectively. This molecular study further proves the power of the 16S rRNA gene-based microarray technology in identifying prokaryotes of known SRP lineages but simultaneously substantiates the necessity of alternative identification approaches that allow the diversity assessment of yet unknown SRPs.

Dissimilatory sulfate reduction, which is carried out exclusively by prokaryotic organisms, is one of the most important organic carbon mineralization processes in anaerobic aquatic environments especially in marine sediments (26, 27). In contrast to the well-studied sulfate-reducing communities in marine (39, 56-59) and freshwater habitats (38), little is known about the distribution and diversity of sulfate-reducing prokaryotes (SRPs) in terrestrial ecosystems. The contribution of terrestrial SRPs, compared to aquatic SRPs, to sulfur cycling in terms of substrate turnover might be of minor importance on a global scale. Nevertheless, their ecological importance for the biodegradation of environmental pollutants such as oil (21, 37, 55, 73) or for the operativeness of a particular terrestrial ecosystem can be profound. Based on $\delta^{34}S$ values and ^{35}S radiolabeling, it has been shown that dissimilatory sulfate reduction is an ongoing process at some fen sites in a forested catchment in Bavaria, Germany (Lehstenbach, Fichtelgebirge) (1, 3). Owing to air pollution the catchment has seen a major deposition of sulfate through acidic rainfall (in form of sulfuric and sulfurous acid) until the 90s, when efficient air filtration systems have been introduced in factories in former East Germany. With each rainfall, sulfate is leached out of the upland aerated soils in the lower situated fens. It is hypothesized that dissimilatory sulfate reduction in these mainly anaerobic and waterlogged soils contributes to the retention of sulfur in this ecosystem (1, 2, 51). Sulfate reduction in these fens is a sink for sulfate and protons, thus decreasing acidity of soil solution and the adjacent groundwater. The primary aim of this study is to establish a detailed inventory of all SRPs inhabiting two selected fen sites at the Lehstenbach catchment at a single point in time, to get insights into the phylogenetic origin of these microorganisms, and to provide a robust basis for further characterization of their ecophysiological role in time and space.

Recently, an 16S rRNA-based oligonucleotide DNA microarray for SRPs (SRP-PhyloChip) has been introduced in determinative and environmental SRP research (43). We used the SRP-PhyloChip to rapidly screen for members of all known SRP lineages at the Lehstenbach catchment. However, SRPs constitute a polyphyletic microbial guild and thus, 16S rRNA-based approaches do not easily allow do directly link the sulfate reducing ability to a certain phylogenetic position. Molecular metabolic diversity surveys based on key enzymes for certain

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physiological traits, such as the adenosine-5`-phosphosulfate (EC 1.8.99.2) or the siroheme dissimilatory (bi)sulfite (EC 1.8.99.3) reductases for dissimilatory sulfate reduction, provide a bypass for this drawback of rRNA-based approaches. Therefore, we additionally used the genes encoding the alpha and beta subunits of the dissimilatory (bi)sulfite reductase (*dsrAB*) as target for PCR, subsequent cloning and comparative DsrAB sequence analysis (32, 66, 74). Heretofore unknown diversity of sulfate- respectively sulfite-reducing microorganisms can be directly seized via this molecular metabolic survey (11-14, 28, 46, 50, 53, 62, 69).

MATERIALS AND METHODS

Site description and soil sample collection. Two sites at the Lehstenbach catchment in the Fichtelgebirge mountains in northeastern Bavaria (Germany) were investigated. The catchment has an area of 4.2 km^2 with a highest elevation of 877 m a.s.l. Ninety percent of the area is stocked by Norway spruce (*Picea abies*, [L.] Karst.) of different ages. Upland soils in the catchment (i.e. aerated soils, which are not water saturated) have developed from weathered granitic bedrock and are predominantly Cambisols and Cambic Podsols (according to FAO-system). Considerable parts of the catchment (approx. 30%) are covered by minerotrophic fens or intermittent seeps. The annual precipitation in the catchment is 900 to 1160 mm $year^{-1}$ and the average annual temperature is 5°C.

The site Schlöppnerbrunnen I (SbI) is a fen (low moor) alternately covered with patches of *Sphagnum* mosses and with spruce stocking. The water saturated soil was classified as Fibric Histosol. The site Schlöppnerbrunnen II (SbII) is also a water saturated fen and completely overgrown by *Molinia caerula* grasses. The soil pH of both sites approximated 3.9 and 4.2, respectively. In the soil solution, the pH varied between 4 and 5 at SbI and 4.5 to 6 at SbII.

For subsequent DNA isolation from both sites, soil cores (3 cm in diameter) from four different depths (approximately 0-7.5, 7.5-15, 15-22.5, and 22.5-30 cm) were collected on 24 July 2001 and immediately cooled on ice. After transfer to the laboratory, the soil samples were homogenized 1:1 (volume/volume) in 1x phosphate-buffered saline (PBS) and stored at -20°C. For incubation experiments, peat samples from three different depths (approximately 0-10, 10-20, and 20-30 cm) were obtained in December 2001 in sterile airtight vessels, transported to the laboratory and processed within 4 h. From each site, soil solution of the upper 40 cm was sampled with dialysis chambers (23) every two months during the time period from July 2001 to November 2002. The chamber consisted of forty 1-cm cells covered with a cellulose acetate membrane of 0.2 µm pore diameter. Prior to installation, the chamber was filled with anoxic, deionized water. The chambers stayed 2 weeks in the water saturated fens for equilibration. At the sampling date, the chamber was closed airtight, transported to the laboratory, and sampled by syringes.

Incubation experiments: Anoxic microcosms. 30 g (fresh weight) of peat samples were placed into 125-ml infusion flasks (Merck ABS, Dietikon, Switzerland) inside of an anaerobic chamber (100% N_2 gas phase). 60 ml of anoxic, deionized water was added to ensure liquid sampling by sterile, argon-flushed syringes. The bottles were closed with rubber stoppers and screw caps and were incubated in the dark at 15°C. Sulfate was added from a sterile anoxic stock solution (0.5 M K_2SO_4) to reach a final concentration of 500 µM. Microcosm experiments were performed in triplicate.

Analytical methods. The pH was measured with a U457-S7/110 combination pH electrode (Ingold, Steinbach, Germany). Sulfate was determined by ion chromatography (34). The concentration of CH_4 in the headspace was measured with a Hewlett-Packard Co. (Palo Alto, CA, USA) 5980 series II gas chromatograph (34). Peat samples were analyzed for total reduced inorganic sulfur (TRIS) and acid volatile sulfur (AVS) according to published protocols (71). TRIS is generally considered to comprise pyrite (FeS_2), amorphous FeS, and S^0. AVS refers to amorphous FeS.

DNA extraction. DNA from soil homogenates was extracted by using a modification of the protocol of Griffiths et al. (20). In contrast to the original protocol, precipitation of nucleic acids in the aqueous phase was performed with 0.1 volume sodium acetate (pH 5.2) and 0.6 volume of isopropanol for 2 h at room temperature.

TABLE 1. 16S rRNA gene-targeted primers

Short name[a]	Full name[b]	Annealing temp. [°C]	Sequence 5'-3'	Specificity	Reference
616V	S-D-Bact-0008-a-S-18	52	AGA GTT TGA TYM TGG CTC	most *Bacteria*	30
630R	S-D-Bact-1529-a-A-17	52	CAK AAA GGA GGT GAT CC	most *Bacteria*	30
1492R	S-*-Proka-1492-a-A-19	52, 60[c]	GGY TAC CTT GTT ACG ACT T	most *Bacteria* and *Archaea*	Modified from 31
ARGLO36F	S-G-Arglo-0036-a-S-17	52	CTA TCC GGC TGG GAC TA	*Archaeoglobus* spp.	43
DSBAC355F	S-*-Dsb-0355-a-S-18	60	CAG TGA GGA ATT TTG CGC	most "*Desulfobacterales*" and "*Syntrophobacterales*"	61
DSMON85F	S-G-Dsmon-0085-a-S-20	62	CGG GGT RTG GAG TAA AGT GG	*Desulfomonile* spp.	This study
DSMON1419R	S-G-Dsmon-1419-a-A-20	62	CGA CTT CTG GTG CAG TCA RC	*Desulfomonile* spp.	This study
SYBAC+282F	S-*-Sybac-0282-a-S-18	60	ACG GGT AGC TGG TCT GAG	"*Syntrophobacteraceae*" and some other *Bacteria*	This study
SYBAC1427R	S-*-Sybac-1427-a-A-18	60	GCC CAC GCA CTT CTG GTA	"*Syntrophobacteraceae*"	This study
DBACCA65F	S-S-Dbacca-0065-a-S-18	58	TAC GAG AAA GCC CGG CTT	*Desulfobacca acetoxidans*	This study
DBACCA1430R	S-S-Dbacca-1430-a-A-18	58	TTA GGC CAG CGA CAT CTG	*Desulfobacca acetoxidans*	This study
DSN61F	S-*-Dsn-0061-a-S-17	52	GTC GCA CGA GAA CAC CC	*Desulfonema limicola*, *Desulfonema ishimotonii*	43
DSN+1201R	S-*-Dsn-1201-a-A-17	52	GAC ATA AAG GCC ATG AG	*Desulfonema* spp. and some other *Bacteria*	43

[a] Short name used in the reference or in this study.
[b] Name of 16S rRNA gene-targeted oligonucleotide primer based on the nomenclature of Alm et al. (4).
[c] The annealing temperature was 52°C when the primer was used with forward primer 616V or ARGLO36F and the annealing temperature was 60°C when the primer was used with forward primer DSBAC355F.

PCR amplification of 16S rRNA and *dsrAB* genes. All PCRs were performed with five nanograms of DNA. For subsequent DNA microarray hybridization, amplification of bacterial 16S rRNA gene fragments from fen soil genomic DNA was performed by using primer pairs 616V-630R and 616V-1492R (Table 1). 16S rRNA gene fragments from reference pure cultures and clones were amplified by using the bacterial primer pair 616V-630R or the cloning vector-specific primers M13F(-20) (5´-GTAAAACGACGGCCAG-3´) and M13R (5´-CAGGAAACAGCTATGAC-3´) (Invitrogen Corp., San Diego, Calif.), respectively. For confirmation of microarray results, 16S rRNA gene fragments of defined SRP groups were directly amplified from soil DNA by using previously published and newly designed primers (Table 1). In addition, an approximately 1.9-kb *dsrAB* gene fragment was amplified from fen soil DNA by using the degenerated primers DSR1Fmix (equimolar mixture of DSR1F, DSR1Fa, and DSR1Fb) and DSR4Rmix (equimolar mixture of DSR4R, DSR4Ra, DSR4Rb, and DSR4Rc) (Table 2). Positive controls containing purified DNA from suitable reference organisms were included in all of the PCR amplification experiments along with negative controls (no DNA added). For 16S rRNA and *dsrAB* gene amplification, reaction mixtures containing 25 pM of each primer were prepared in a total volume of 50 µl by using 10x Ex Taq™ reaction buffer and 2.5 U of *Ex Taq* polymerase (Takara Biomedicals, Japan). Additionally, 20 mM tetramethylammonium chloride (Sigma, Deisenhofen, Germany) was added to each amplification mixture to enhance the specificity of the PCR reaction (33). Thermal cycling was carried out by an initial denaturation step at 94°C for 1 min, followed by 30 (16S rRNA genes) or 35 cycles (*dsrAB* genes) of denaturation at 94°C for 40 s, annealing from 48°C to 62°C (depending on the primer pair; Table 1 and 2) for 40 s, and elongation at 72°C for 1 min 30 s. Cycling was completed by a final elongation step at 72°C for 10 min.

DNA microarray technology. Fluorescence labeling of PCR amplificates, manufacturing and processing of SRP-PhyloChips, reverse hybridization on microarrays, scanning and image analysis of microarrays were performed as described by Loy et al. (43). Oligonucleotides for printing of the SRP-PhyloChips were obtained from MWG Biotech (Ebersberg, Germany). The sequence, specificity, and microarray position of all newly designed oligonucleotide probes are depicted in Table 3. In addition, the novel SRP-PhyloChip probes were deposited at probeBase (http://www.probebase.net) (42), where probe-target difference alignments, generated with the PROBE_MATCH tool of the ARB program package (Strunk and Ludwig, http://www.arb-home.de), can be viewed for each probe.

Cloning and sequencing. Prior to cloning the PCR amplification products were purified by low-melting-point agarose (1.5%) gel electrophoresis (NuSieve 3:1; FMC Bioproducts, Biozym Diagnostics GmbH, Oldendorf, Germany) and stained in SYBR Green I solution (10 µl 10.000x SYBR Green I stain in 100 µl TAE buffer [40 mM TRIS, 10 mM sodium acetate, 1 mM EDTA, pH 8.0]; Biozym Diagnostics GmbH) for 45 min. Bands of the expected size were excised from the agarose gel with a glass capillary and melted with 80 µl double-distilled water for 10 min at 80°C. Four microliters of each solution were ligated as recommended by the manufacturer (Invitrogen Corp.) either into the cloning vector pCR2.1 of the TOPO TA cloning kit (16S rRNA gene amplificates) or into the cloning vector pCR-XL-TOPO of the TOPO XL cloning kit (*dsrAB* gene amplificates). Nucleotide sequences were determined by the dideoxynucleotide method (60) as described by Purkhold et al. (54). In addition, internal *dsrAB* gene-targeted sequencing primers (Table 2) were used to complete the *dsrAB* sequences.

TABLE 2. Dissimilatory (bi)sulfite reductase gene-(*dsrAB*)-targeted primers. The target site of all listed DSR1 and DSR4 primer versions was analyzed for those SRPs (n=8) for which complete *dsrAB* operons are available in GenBank (8). SRPs with a fully complementary target site to the respective primers are listed in the specificity column.

Primer	Sequence 5`-3`	Specificity	Reference
DSR1F[a]	ACS CAC TGG AAG CAC G	*Archaeoglobus fulgidus, Archaeoglobus profundus, Desulfovibrio vulgaris*	74
DSR1Fa[a]	ACC CAY TGG AAA CAC G	*Desulfotomaculum thermocisternum, Desulfobulbus rhabdoformis, Desulfobacter vibrioformis*	This study
DSR1Fb[a]	GGC CAC TGG AAG CAC G	*Thermodesulforhabdus norvegica*	This study
DSR4R[a]	GTG TAG CAG TTA CCG CA	*Archaeoglobus fulgidus, Desulfovibrio vulgaris, Desulfobulbus rhabdoformis*	74
DSR4Ra[a]	GTG TAA CAG TTT CCA CA	*Archaeoglobus profundus*	This study
DSR4Rb[a]	GTG TAA CAG TTA CCG CA	*Desulfobacter vibrioformis*	This study
DSR4Rc[a]	GTG TAG CAG TTT CCG CA	*Thermodesulforhabdus norvegica, Desulfotomaculum thermocisternum*	This study
DSR978Fa[b]	GGT CAT CGA CCT TTG TCC	Schlöppnerbrunnen I soil OTU 5	This study
DSR978Fb[b]	CGT CGT CGG GAA GTG CCC	Schlöppnerbrunnen I soil OTU 8	This study
DSR978Fc[b]	AGT AGT CGA CCT TTG CCC	Schlöppnerbrunnen I+II soil OTU 6	This study
DSR978Fd[b]	TGT CAC CGA TCT CTG CCC	Schlöppnerbrunnen I soil OTU 1	This study
DSR978Fe[b]	TGT TAC CGA CCT CTG CCC	Schlöppnerbrunnen II soil OTU 1 (dsrSbII-20)	This study
DSR978Ff[b]	TGT CAC CGA TCT TTG CCC	Schlöppnerbrunnen II soil OTU 4 (dsrSbII-15)	This study
DSR978Fg[b]	CGT CAC CAT TCT CTG CCC	Schlöppnerbrunnen II soil OTU 4 (dsrSbII-9)	This study
DSR978Fh[b]	GGT CGT TGA CAT GTG TCC	Schlöppnerbrunnen II soil OTU 11	This study
DSR978Fi[b]	GGT CTG CAA TCT CTG YCC	Schlöppnerbrunnen I+II soil OTU 2 and 3	This study
DSR978Fj[b]	GGT TGT TGA CCT TTG CCC	Schlöppnerbrunnen I soil OTU 9	This study
DSR978Fk[b]	CGT TTG CGA TCT CTG CCC	Schlöppnerbrunnen II soil OTU 7	This study
DSR860F[b]	AGA TCC GGC GGG ACG ATG	Schlöppnerbrunnen I soil OTU 10	This study

[a] Primer was used at non-stringent conditions by applying an annealing temperature of 48°C for PCR in order to target a wide diversity of SRPs.

[b] Internal sequencing primer used to complete *dsrAB* gene sequences retrieved from acidic fen sites Schlöppnerbrunnen I and II.

Phylogeny inference. All phylogenetic analyses were performed by using the alignment and treeing tools implemented in the ARB program package.

New 16S rRNA sequences obtained from the fen samples were added to an ARB alignment of about 16.000 small-subunit rRNA sequences (including all sequences from recognized SRPs and clone sequences from yet uncultured prokaryotes from sulfate-reducing environments) by using the alignment tool ARB_EDIT. Alignments were refined by visual inspection. 16S rRNA phylogenetic analyses were exclusively performed with sequences having more than 1150 bases by applying distance-matrix, maximum-parsimony, and maximum-likelihood methods according to the guidelines proposed by Ludwig et al. (45). The composition of the 16S rRNA data sets varied with respect to the reference sequences and the alignment positions included. Variability of the individual alignment positions were determined by using the ARB_SAI tools and used as criterion to remove or include variable positions for phylogenetic analyses.

New *dsrAB* sequences obtained from fen samples and from *Desulfobacca acetoxidans* were added to an ARB alignment which contains all *dsrAB* sequences of recognized (17, 32) and uncultured SRPs available in GenBank (8). Deduced amino acid sequences were manually aligned by using the editor GDE 2.2 (S.W. Smith, C. Wang, P.M. Gillevet and W. Gilbert (1992) Genetic Data Environment and the Harvard Genome Database. Genome mapping and Sequencing, Cold Spring Harbor Laboratory). Nucleic acid sequences were aligned according to the amino acid alignment. For phylogeny inference of DsrAB amino acid sequences, insertions and deletions were removed from the data set by using a suitable alignment mask (indel filter) leaving a total of 543 amino acid positions (alpha subunit, 327; beta subunit, 216) for comparative analyses. Distance-matrix (using FITCH with global rearrangements and randomized input order of species) and maximum-parsimony trees were calculated with the PHYLogeny Inference Package (PHYLIP, version 3.57c, J. Felsenstein, Department of Genetics, University of Washington, Seattle). In addition, the programs MOLPHY (version 2.3, Computer science monographs, no. 28.: Programs for molecular phylogenetics based on maximum-likelihood. J. Adachi and M. Hasegawa, Institute of Statistics and Mathematics, Tokyo, Japan) and TREE-PUZZLE (67) were used to infer maximum-likelihood trees with JTT-f as the amino acid replacement model.

Parsimony bootstrap analysis for nucleic acid (16S rRNA) and protein (DsrAB) trees were performed with PHYLIP. For each calculation 100 bootstrap resamplings were analyzed. All phylogenetic consensus trees were drawn according to the recommendations of Ludwig et al. (45).

Bacterial nomenclature. Names of bacterial taxa were used in accordance with the prokaryotic nomenclature proposed in the taxonomic outline of the second edition of Bergey`s Manual of Systematic Bacteriology (18).

Nucleotide sequence accession numbers. The sequences determined in this study were deposited at GenBank under accession numbers AY167444 to AY167462 (16S rRNA gene clones) and AY167464 to AY167483 (*dsrAB* gene clones).

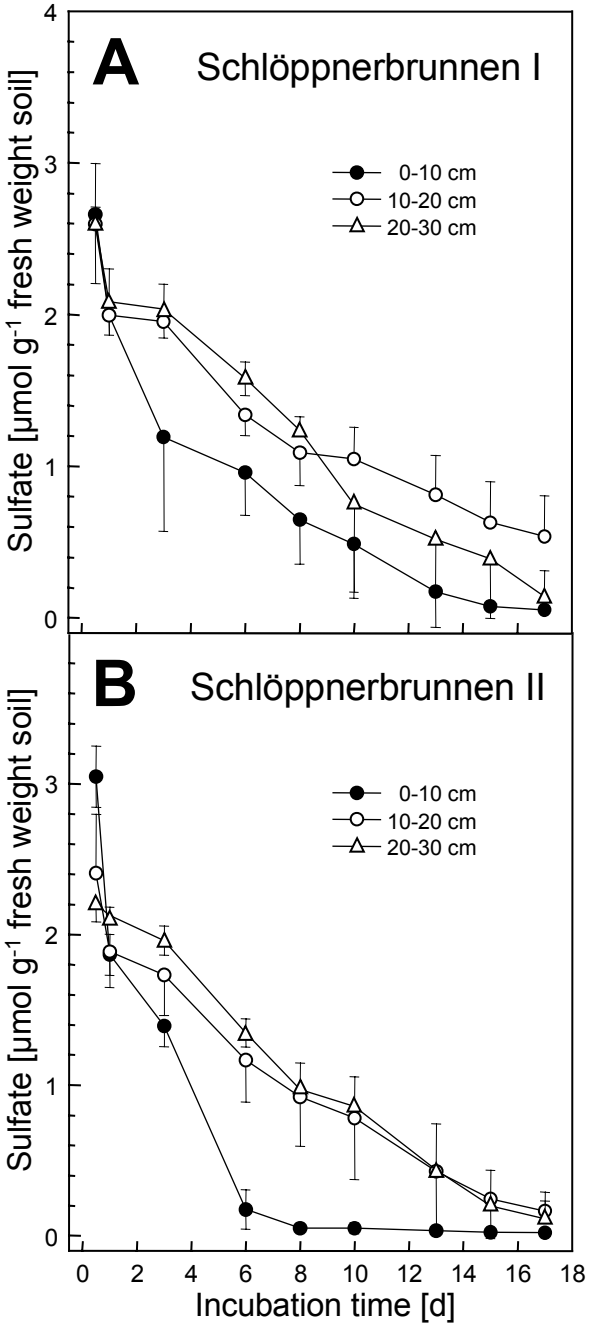

Figure 1. Consumption of supplemental sulfate (500 µM) in anoxic microcosms of soil samples obtained from Schlöppnerbrunnen sites I (A) and II (B). Presented are the averages standard ± deviation of triplicates.

RESULTS

Biogeochemical studies. At sites SbI and SbII, the concentration of sulfate in the soil solution varied over the year with minimum concentrations of 20 µM in late autumn and maximum concentrations of 200 µM after the snow melt in February. At site SbI, TRIS concentrations in soil samples obtained from 0-10, 10-20, and 20-30 cm depth in December approximated 0.05 µmol g (fresh wt soil)$^{-1}$. AVS concentrations increased with increasing soil depth from 0.01, over 0.04 to 0.05 µmol g (fresh wt soil)$^{-1}$. At site SbII, TRIS concentrations reached 0.29, 0.47, and 0.53 µmol g (fresh wt soil)$^{-1}$ with increasing soil depth, and AVS concentrations approximated 0.05, 0.06, and 0.05 µmol g (fresh wt soil)$^{-1}$.

In anoxic soil microcosms supplemented with sulfate (500 µM), sulfate was rapidly consumed within 8 to 17 days of incubation (Figure 1). At the end of the experiment, the concentrations of TRIS and AVS were enhanced in the soil samples compared to unsupplemented controls indicating that the consumption of sulfate was linked to dissimilatory reduction of sulfate. In soil samples obtained from the three depths of site SbI, TRIS and AVS concentrations averaged 0.67 and 0.12 µmol g (fresh wt soil)$^{-1}$ at the end of incubation,

respectively; in samples of SbII, TRIS and AVS concentrations averaged 0.86 and 0.28 µmol g (fresh wt soil)$^{-1}$. However, the reduced S-recovery approximated only 21%. Thus, a part of the reduced S might be lost in the headspace as H_2S due to the low soil pH and the potential low availability of soluble Fe(II). Potential rates of sulfate reduction with increasing depth approximated 0.14, 0.11, and 0.14 µmol g (fresh wt soil)$^{-1}$ d^{-1} at site SbI and 0.41, 0.13, and 0.13 µmol g (fresh wt soil)$^{-1}$ d^{-1} at site SbII. In soil microcosms, rates of CH_4 formation decreased with increasing depth in the absence of supplemental sulfate from 0.07, 0.04, and 0.04 µmol g (fresh wt soil)$^{-1}$ d^{-}1 to 0.016, 0.013, and 0.014 µmol g (fresh wt soil)$^{-1}$ d^{-1} in the presence of supplemental sulfate at site SbI and from 0.19, 0.09, and 0.09 µmol g (fresh wt soil)$^{-1}$ d^{-}1 to 0.057, 0.031, and 0.023 µmol g (fresh wt soil)$^{-1}$ d^{-1} in the presence of supplemental sulfate at site SbII. Thus, rates of CH_4 formation in the presence of supplemental sulfate approximated 31% of the rates of the unsupplemented controls.

SRP-PhyloChip analyses and evaluation. Initially, the recently developed SRP-PhyloChip (43) was used to screen for members of already recognized SRP lineages at the fen sites SbI and SbII. Bacterial 16S rRNA genes were separately amplified from each DNA extract retrieved from the four soil depths and fluorescently labeled with Cy5. For PCR amplification primer sets 616V-1492R and 616V-630R were used and PCR amplificates were mixed prior to labeling. As expected, *Archaeoglobus*-specific 16S rRNA gene amplification from the soil DNAs by using primer pair ARGLO36F-1492R (Table 1) did not yield positive PCR products. For each site and soil depth, two separate microarrays with duplicate spots for each probe were hybridized with Cy5-labeled bacterial PCR amplificates, washed at 55°C, and scanned. The array readouts were quantitatively analyzed by digital image analysis to determine a signal-to-noise ratio for each probe spot. Spots for which the signal-to-noise ratio was equal to or greater than 2.0 were considered positive (43).

At site SbI highly similar microarray hybridization patterns were observed from the four different soil depths indicating low changes in richness of recognized SRPs over depth (Figure 2A). Positive signals of probes with nested specificity (designed according to the multiple probe concept) indicated the presence of (i) *Desulfomonile* spp., (ii) *Desulfonema* or related species of the order "*Desulfobacterales*", and (iii) bacteria belonging to the *Syntrophobacter-Desulfovirga-Desulforhabdus*

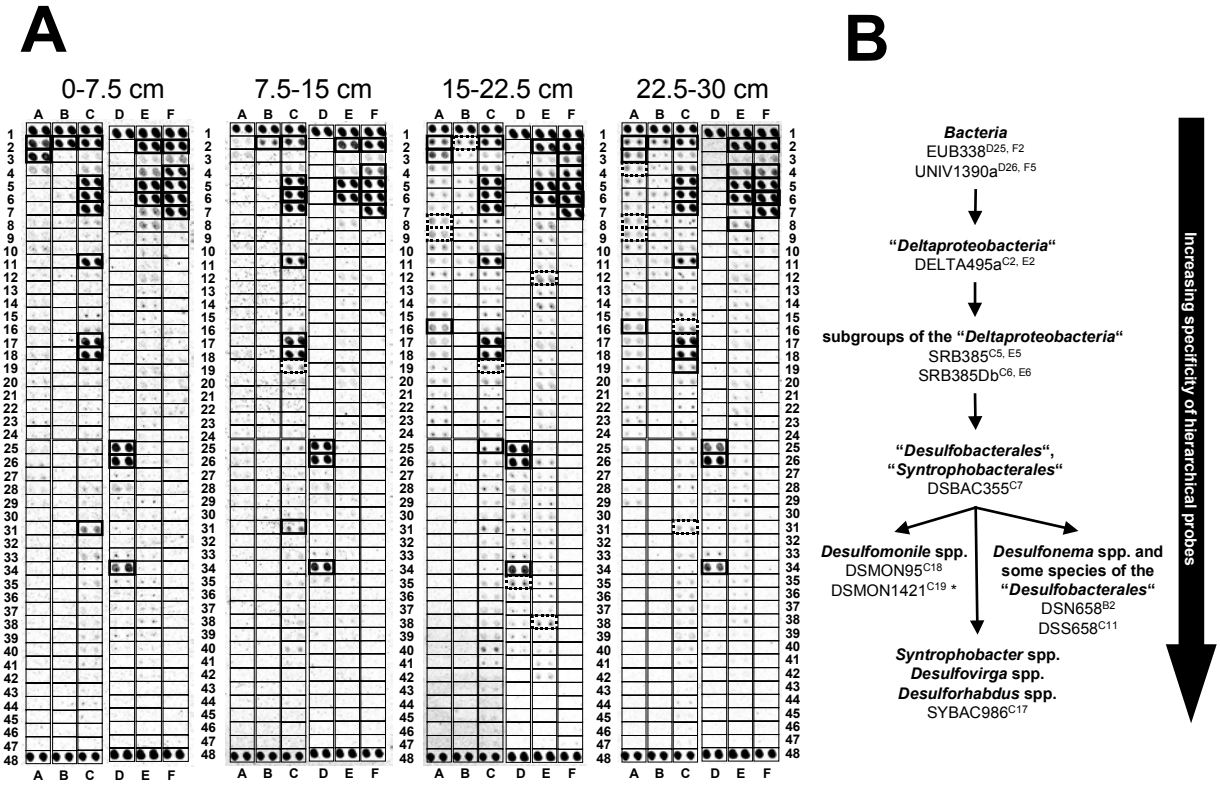

Figure 2. (A) Use of the SRP-PhyloChip for surveys of SRP diversity in four different horizontal soil sections of Schlöppnerbrunnen site I. On the microarray each probe was spotted in duplicate. The specificity and microarray position of each probe are according to Loy et al. (43) or are shown in Table 3. Probe spots having a signal-to-noise ratio equal to or greater than 2.0 are indicated by boldface boxes and were considered to be positive. The dotted boldface boxes indicate that only one of the duplicate spots had a signal-to-noise ratio equal to or greater than 2.0. (B) The flow chart illustrates the presence of distinct SRP groups in Schlöppnerbrunnen I soil as inferred from positive signals of sets of probes with nested and/or parallel specificity. Probe DSMON1421 was only unambiguously positive in the 22.5-30 cm soil section (indicated by an asterisk).

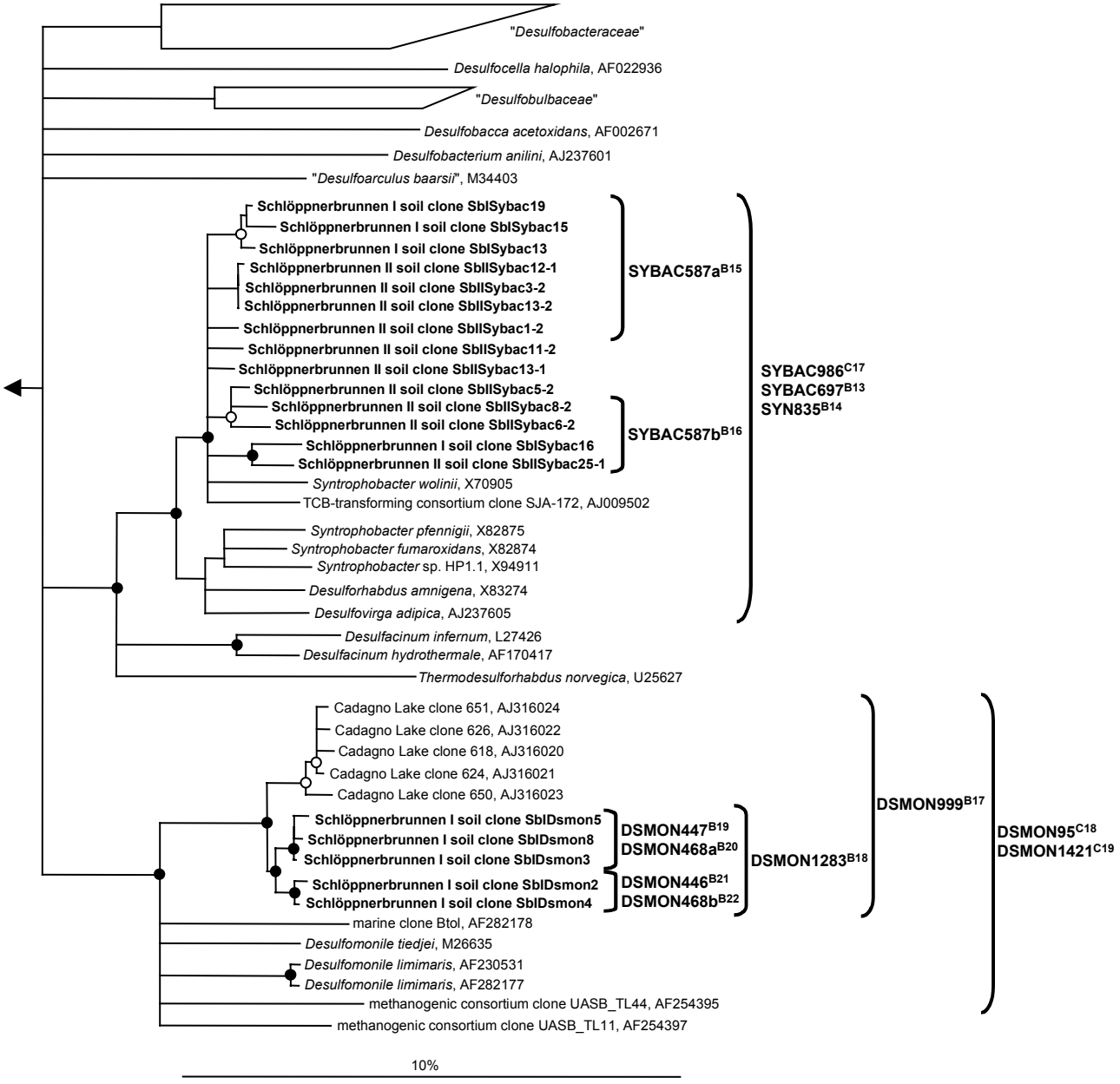

Figure 3. 16S rRNA gene phylogenetic dendrogram showing the affiliation of clone sequences from Schlöppnerbrunnen soil sites I and II (indicated by boldface type). Clone sequences were retrieved from soil DNA by PCR amplification, cloning, and sequencing of 16S rRNA gene fragments by using primers specific for the family "*Syntrophobacteraceae*" (Sybac clones) and for the genus *Desulfomonile* (Dsmon clones). The consensus tree is based on neighbor-joining analysis performed with a 50% conservation filter for the "*Deltaproteobacteria*". The bar indicates 10% estimated sequence divergence (distance inferred by neighbor-joining). Polytomy connect branches for which a relative order could not be determined unambiguously by applying neighbor-joining, maximum-parsimony, and maximum-likelihood treeing methods. Parsimony bootstrap values (100 resamplings) for branches are indicated by solid circles (>90%) or an open circle (75 to 90%). Branches without circles had bootstrap values of less than 75%. Parentheses indicate the perfect-match target organisms of the probes. The microarray position is depicted after each probe name. Cadagno Lake clones are not sequenced at the target site for probe DSMON1421. The sequence of methanogenic consortium clone UASB_TL11 (AF254397) has three mismatches in the target site for probe DSMON95 and one terminal mismatch in the target site for probe DSMON1421.

line of descent of the family "*Syntrophobacteraceae*" (order "*Syntrophobacterales*") (Figure 2B). For confirmation of the microarray results of site SbI, 16S rRNA gene PCRs specific for *Desulfomonile* spp. (primer pair DSMON85F-DSMON1419R), for some *Desulfonema* species (primer pair DSN61F-DSN+1201R), as well as for members of the "*Syntrophobacteraceae*" (primer pair SYBAC+282F-SYBAC1427R) were performed with DNA from each soil depth. No PCR amplificates were obtained with primers DSN61F and DSN+1201R. Therefore, presence of *Desulfonema* spp. at site SbI could not be confirmed. However, with each of the *Desulfomonile*- and "*Syntrophobacteraceae*"-specific primer pairs increasing amounts of PCR products of the expected size were retrieved with increasing soil depth (data not shown). Cloning and sequencing of the PCR amplificates from 22.5-30 cm depth confirmed that

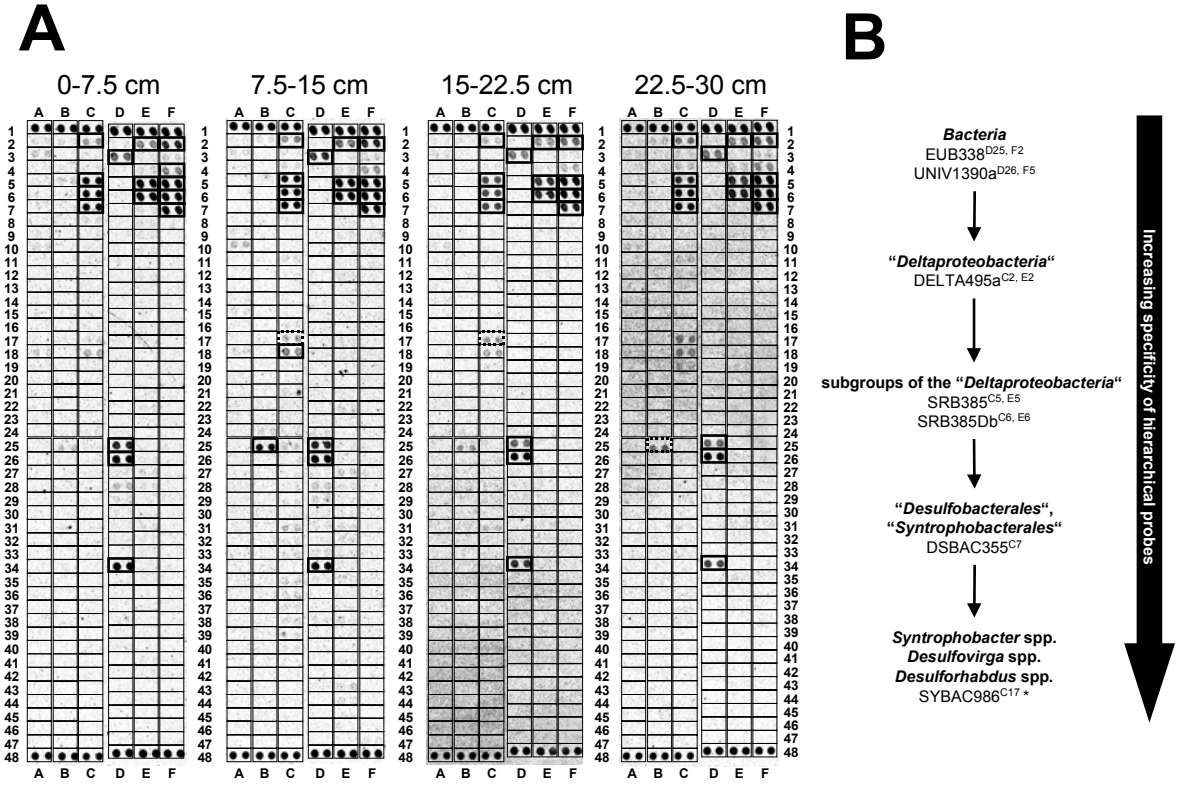

Figure 4. (A) Use of the SRP-PhyloChip for surveys of SRP diversity in four different horizontal soil sections of Schlöppnerbrunnen site II. On the microarray each probe was spotted in duplicate. The specificity and microarray position of each probe are according to Loy et al. (43) or are shown in Table 3. Probe spots having a signal-to-noise ratio equal to or greater than 2.0 are indicated by boldface boxes and were considered to be positive. The dotted boldface boxes indicate that only one of the duplicate spots had a signal-to-noise ratio equal to or greater than 2.0. (B) The flow chart illustrates the presence of distinct SRP groups in Schlöppnerbrunnen II soil as inferred from positive signals of sets of probes with nested and/or parallel specificity. The asterisk indicate that the mean signal-to-noise ratios of the duplicate SYBAC986 spots at sections 7.5-15 cm, 15-22.5 cm, and 22.5-30 cm were just below the threshold value of 2.0 (1.88, 1.95, and 1.70, respectively).

Desulfomonile spp. and *Syntrophobacter wolinii*-related bacteria were present at site SbI (Figure 3). Similar to site SbI, the microarray hybridization patterns of site SbII showed no profound variation over soil depth. However, microarray fingerprints at site SbII were less complex than at site SbI (Figure 4A). Only probes targeting SRPs at higher taxonomic levels were unambiguously positive (e.g. probes DELTA495a and DSBAC355). However, the mean signal-to-noise ratios of the duplicate SYBAC986 probe spots (specific for members of the *Syntrophobacter-Desulfovirga-Desulforhabdus* lineage) at depths 7.5-15 cm, 15-22.5 cm, and 22.5-30 cm were just below the threshold value of 2.0 (1.88, 1.95, and 1.70, respectively) (Figure 4B). In order to verify the presence or absence of *Syntrophobacter-Desulfovirga-Desulforhabdus*-related bacteria at site SbII, "*Syntrophobacteraceae*"-16S rRNA genes were separately amplified from each soil section DNA by using the primer pair SYBAC+282F-SYBAC1427R. Increasing amounts of PCR product were retrieved with increasing soil depth (data not shown). Consistent with site SbI, subsequent cloning and sequence analysis of the PCR product from 22.5-30 cm depth confirmed the presence of *Syntrophobacter wolinii*-related bacteria at site SbII (Figure 3).

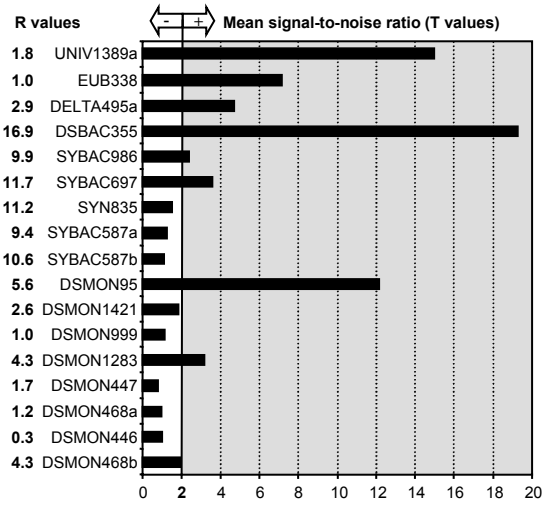

Figure 5. Hybridization results of the SRP-PhyloChip (amended version) with Cy5-labeled 16S rRNA gene amplificates obtained with DNA pooled from all four Schlöppnerbrunnen I soil sections. Horizontal bars indicate mean signal-to-noise ratios of relevant and Schlöppnerbrunnen clone-specific probes. Probe spots having a mean signal-to-noise ratio equal to or greater than 2.0 were considered to be positive. Mean normalized signal-to-noise ratios (R values) of relevant probes as determined with pure cultures (see supplementary web material) are depicted in boldface type next to probe names.

TABLE 3. Habitat specific 16S rRNA-targeted oligonucleotide probes added to the SRP-PhyloChip.

Probe name	Full name[a]	Sequence 5'-3'	Microarray position	Specificity[b]	Reference
SYBAC697	S-*-Sybac-0697-a-A-18	CCT CCC GAT CTC TAC GAA	B13	Genera *Syntrophobacter*, *Desulforhabdus*, and *Desulfovirga*	This study
SYN835	S-*-Sybac-0835-a-A-18	GCA GGA ATG AGT ACC CGC	B14	See above	61
SYBAC587a	S-*-Sybac-0587-a-A-18	CAT CAG ACT TTT CGG CCC	B15	Uncultured *Syntrophobacter*-related bacteria (Schlöppnerbrunnen I soil clones SbISybac13, 15, and 19; Schlöppnerbrunnen II soil clones SbIISybac12-1, 1-2, 3-2, and 13-2)	This study
SYBAC587b	S-*-Sybac-0587-b-A-18	CAT CAG ACT TGC CGG CCC	B16	Uncultured *Syntrophobacter*-related bacteria (Schlöppnerbrunnen I soil clones SbISybac16; Schlöppnerbrunnen II soil clones SbIISybac25-1, 6-2, and 8-2)	This study
DSMON999	S-*-Dsmon-0999-a-A-18	TTT CCA TAG CTG TCC GGG	B17	Uncultured *Desulfomonile*-related bacteria (Schlöppnerbrunnen I soil clones SbIDsmon2, 3, 4, 5, and 8; Cadagno Lake clones 618, 624, 626, 650, and 651)	This study
DSMON1283	S-*-Dsmon-1283-a-A-18	CTG AGG ACC GAT TTG TGG	B18	Uncultured *Desulfomonile*-related bacteria (Schlöppnerbrunnen I soil clones SbIDsmon2, 3, 4, 5, and 8)	This study
DSMON447	S-*-Dsmon-0447-a-A-18	ACT CAT GGA GGG TTC TTC	B19	Uncultured *Desulfomonile*-related bacteria (Schlöppnerbrunnen I soil clones SbIDsmon3, 5, and 8)	This study
DSMON468a	S-*-Dsmon-0468-a-A-18	CCG TCA TTT CCA TGA GCT	B20	See above	This study
DSMON446	S-*-Dsmon-0446-a-A-18	CTA GAA GAG GTT TCT TCC	B21	Uncultured *Desulfomonile*-related bacteria (Schlöppnerbrunnen I soil clones SbIDsmon2 and 4)	This study
DSMON468b	S-*-Dsmon-0468-b-A-18	CCG TCA GTT CCT CTA GCT	B22	See above	This study

[a] Name of oligonucleotide probe based on the nomenclature of Alm et al. (4).
[b] Oligonucleotide probe difference alignments are available at probeBase (http://www.probeBase.net) (42).

Additionally, 16S rRNA gene PCRs were conducted for both Schlöppnerbrunnen sites with primer pair DSBAC355F-1492R (Table 1), specific for most members of the "*Desulfobacterales*" and the "*Syntrophobacterales*" in order to screen for SRPs of these orders which are not covered by the specific primer pairs described above. For the deepest soil section from each site, twelve clone sequences were determined, but none was closely related to recognized SRP-16S rRNA sequences (data not shown).

Clone-specific probe design and microarray application. Specific 18-mer oligonucleotide probes targeting the 16S rRNA gene Schlöppnerbrunnen soil clones at different taxonomic levels of specificity were designed *in silico* by using the ARB PROBE_DESIGN and PROBE_MATCH tools (Figure 3, Table 3). Altogether, one previously published and nine newly designed Schlöppnerbrunnen soil clone-specific 16S rRNA-targeted probes were included on the SRP-PhyloChip for increasing resolution of detection. For subsequent evaluation of the new probes for microarray application, the extended SRP-PhyloChip was hybridized with fluorescently labeled 16S rRNA gene amplificates from four selected Schlöppnerbrunnen soil clones (SbISybac13, SbISybac16, SbIDsmon2, and SbIDsmon3) and from *Syntrophobacter wolinii* DSM 2805^T. For each reference clone or organism a separate microarray with triplicate spots for each probe was used. The mean signal-to-noise ratio (T values) and the mean normalized signal-to-noise ratio (R values) of all replicate spots for each probe were determined by using the hybridization conditions and formulas described before (43).

The individual hybridization results for each of the 142 SRP-PhyloChip probes with the five reference 16S rRNA genes are available as supplementary material on our website (http://www.microbial-ecology.net). Mean normalized signal-to-noise ratios of all probes showing a positive signal (mean signal-to-noise ratios ≥ 2.0) varied from 0.3 to 17.6 for perfect-match duplexes and from 0.1 to 4.3 for not fully complementary probe-target hybrids. In these experiments, the ten novel probes on the extended SRP-PhyloChip showed a positive signal with their perfectly matched target reference thereby proving their suitability for application on microarrays. The probe duos DSMON468a/DSMON447 and DSMON468b/DSMON446 each target one of the two *Desulfomonile*-affiliated SbI clone subclusters (Figure 3, Table 3). Under the hybridization conditions applied, each probe duo highly discriminated against the non perfectly matched 16S rRNA gene amplificate of the reference clone from the other subcluster (see supplementary material at http://www.microbial-ecology.net). High discriminatory capacity was also observed for probes SYBAC587a and SYBAC587b each targeting different subgroups of the *Syntrophobacter wolinii*-affiliated Schlöppnerbrunnen soil clones.

TABLE 4. Operational taxonomic units (OTUs) of sulfate- respectively sulfite-reducing prokaryotes based on comparative sequence analyses of *dsrAB* genes retrieved from acidic fen soil at the sampling sites Schlöppnerbrunnen I and II. OTUs are listed and sequentially numbered according to the number of clones retrieved.

OTU[a]	Number of clones		*dsrAB* clones[b]	Next related *dsrAB* sequence in GenBank as determined by BLAST search (accession number/ amino acid identity)	Inferred phylogeny[c]
	dsrSbI	dsrSbII			
1	21	1	dsrSbI-56, -57, -58, **-59, -60,** -61, -62, -65, **-66,** -67, -69, -72, -73, -74, -78, -79, -81, -83, -84, -86, -87, **dsrSbII-20**	uranium mill tailings clone UMTRAdsr828-17 (AY015508, AY015597/ 85.9-88.6%)	*Desulfobacca acetoxidans*-related, *"Deltaproteobacteria"*
2	-	9	**dsrSbII-3,** -18, -21, -22, -23, **-28, -34,** -42, -47	Everglades clone F1SU-12 (AY096051/ 82.5-84.0%)	Unaffiliated to known SRPs
3	1	6	dsrSbI-71, dsrSbII-4, -5,- 8, -12, **-25, -36**	uranium mill tailings clone UMTRA826-5 (AY015548, AY015614/ 87.5-87.8%)	Unaffiliated to known SRPs
4	-	4	**dsrSbII-9,** -11, **-15,** -33	uranium mill tailings clone UMTRAdsr828-17 (AY015508, AY015597/ 87.4-88.8%)	*Desulfobacca acetoxidans*-related, *"Deltaproteobacteria"*
5	2	-	**dsrSbI-82, dsrSbI-50**	only distantly related sequences in GenBank	*"Deltaproteobacteria"*
6	1	1	**dsrSbI-54, dsrSbII-40**	*Syntrophobacter wolinii* (AF418192/ 86.8-87.5%)	*Syntrophobacter wolinii*-related, *"Deltaproteobacteria"*
7	-	2	**dsrSbII-2**, -16	Everglades clone F1SU-12 (AY096051/ 83.1%)	Unaffiliated to known SRPs
8	2	-	**dsrSbI-75**, -85	only distantly related sequences in GenBank	Unaffiliated to known SRPs
9	1	-	**dsrSbI-88**	*Desulfomonile tiedjei* (AF334595/ 84.9%)	*Desulfomonile*, *"Deltaproteobacteria"*
10	1	-	**dsrSbI-64**	uranium mill tailings clone UMTRAdsr626-20 (AY015569, AY015611/ 89.9%)	Unaffiliated to known SRPs
11	-	1	**dsrSbII-39**	uranium mill tailings clone UMTRAdsr624-8 (AY015519, AY015596/ 92.4%)	Unaffiliated to known SRPs
Sum ∑	29	24			
Coverage C[d]	86%	88%			

[a] *dsrAB* clones sharing a deduced DsrAB sequence identity equal to or greater than 90% were grouped in an OTU.
[b] Completely sequenced *dsrAB* clones (>1750 bases) are indicated in boldface type.
[c] Phylogeny of *dsrAB* clones as inferred from Figure 6.
[d] Homologous coverage C was calculated according to $C = [1 - (n1 \times N^{-1})] \times 100$ %, with n1 as number of OTUs containing only one sequence, and N as total number of *dsrAB* gene clones analyzed (19, 29, 63).

Finally, fluorescently labeled 16S rRNA gene PCR product, amplified from an equimolar DNA mixture from all four SbI depths, was hybridized with the extended SRP-PhyloChip containing the habitat specific probes. Mean signal-to-noise ratios of all relevant probes are depicted in Figure 5. Only three of the ten newly added Schlöppnerbrunnen soil clone-specific probes showed positive signals probably reflecting (i) low abundance of amplified 16S rRNA genes from the *Desulfomonile*- and the *Syntrophobacter wolinii*-related SRPs among the total bacterial 16S rRNA gene amplificate and/or (ii) differences in the duplex yield of the individual probes as indicated by their different mean normalized signal-to-noise ratios with fully matched targets (see supplementary web material).

***dsrAB* gene diversity survey.** In order to independently verify the results of the 16S rRNA-based SRP diversity survey (microarray and 16S rRNA comparative sequence analysis) and to reveal whether yet unrecognized SRPs also contributed to the SRP richness at both Schlöppnerbrunnen sites, the genes encoding the alpha and beta subunits of the dissimilatory (bi)sulfite reductase (*dsrAB*) were used as target molecules for diversity analysis. The *dsrAB* genes were separately amplified from each soil depth DNA from both Schlöppnerbrunnen sites by using the primer pair DSR1Fmix-DSR4Rmix. As observed for SRP-16S rRNA gene amplification, *dsrAB* gene PCR yielded higher amounts of amplificate with increasing soil depth (data not shown). Subsequently, for both sites the PCR products retrieved from the deepest soil core (22.5-30 cm depth) were used for construction of *dsrAB* gene clone libraries. 41 and 35 clones of 42 (library dsrSbII) and 48 (library dsrSbII) randomly picked clones, respectively, had an insert of the expected size (1.9-kb). However, partial sequencing followed by BLAST search (5) revealed that only 29 clones from library dsrSbI and 24 clones from library dsrSbII contained *dsrAB* gene sequences. Preliminary phylogeny inference based on the partial DsrAB amino acid sequences grouped the 53 Schlöppnerbrunnen clones in eleven clusters. Subsequently, at least one *dsrAB* clone sequence per cluster was fully determined (in total 20 clones). In the next step, all *dsrAB* clones having a deduced DsrAB amino acid sequence identity equal to or greater than 90% with each other were grouped into an operational taxonomic unit (OTU) leading to a

total of eleven OTUs for both libraries. Table 4 lists the OTUs with the respective clones and the most closely related *dsrAB* sequences available in GenBank. Three OTUs contained *dsrAB* clones from both fen sites while eight OTUs consisted exclusively of clones from site SBI or SbII (four each) (Table 4).

The affiliation of deduced DsrAB sequences from Schlöppnerbrunnen fen soils is depicted in Figure 6. OTU 1, which comprised besides one dsrSbII clone most of the dsrSbI clones, and the dsrSbII-specific OTU 4 each displayed highest sequence identity to a groundwater clone from an uranium mill tailings site (Table 4). These clones formed a stable monophyletic group with *Desulfobacca acetoxidans*. Consistent with the DNA microarray data, one *dsrAB* clone from each fen sites represented OTU 6, which was most closely related to *Syntrophobacter wolinii* within the deltaprotebacterial family "*Syntrophobacteraceae*". A further deltaproteo-bacterial lineage was OTU 9, which consisted of only a single clone from site SbI and could be affiliated with *Desulfomonile tiedjei* (Figure 6). Another two clones from site SbI, representing OTU 5, formed an independent branch within a monophyletic deltaproteobacterial cluster consisting of the family "*Desulfobacteraceae*" and different groups of the order "*Desulfovibrionales*". The remaining six OTUs formed three deeply branching evolutionary lines of descent clearly different from any cultured SRP lineage (Figure 6). One of these three deeply branching lines of descent housed OTUs 2, 7, 8, and 10, whereas OTUs 3 and 11 represented the other two lines of descent. OTUs 2 and 7 consisted exclusively of dsrSbII clones. In contrast, only dsrSbI clones were present in OTUs 8 and 10. Interestingly, each of these three deep branching lines of descent contain also at least one *dsrAB* clone retrieved from an uranium mill tailings groundwater (12). Furthermore, a *dsrAB* clone related to OTU 10 was recently retrieved from an Everglades soil sample (11) (Figure 6). In summary, five of the eleven OTUs identified were affiliated with deltaproteobacterial SRPs, whereas the remaining six OTUs branched off deeply in the phylogenetic DsrAB tree and represented yet unknown SRPs.

***Desulfobacca acetoxidans*-related SRPs.** As mentioned above, DsrAB phylogeny surveys identified *Desulfobacca acetoxidans*-related SRPs at both Schlöppnerbrunnen sites. A specific 16S rRNA-based PCR assay was used to retrieve 16S rRNA sequences of *Desulfobacca acetoxidans*-related bacteria at the fen sites. DNA from each soil depth of both sites was separately amplified with the newly designed *Desulfobacca acetoxidans*-specific primer pair DBACCA65F-DBACCA1430R. PCRs were carried out at low stringency (annealing temperature 58°C) to allow for amplification of 16S rRNA genes of *Desulfobacca acetoxidans*-related SRPs even if they would have mismatches in the primer target sites. However, none of the PCRs yielded amplificates of the expected size (1.4-kb) (data not shown).

DISCUSSION

Biogeochemical studies. The occurrence of TRIS and AVS in peat samples of both sites and $\delta^{34}S$ values and ^{35}S tracer studies in peat samples performed from site SbI (1, 3) indicated that the dissimilatory reduction of sulfate is an on-going process in both acidic fens of the Lehstenbach catchment. However, minimum concentration of sulfate in the soil solution obtained in autumn approximated limit concentration sufficient for dissimilatory sulfate reduction (40), and maximum concentrations never exceeded 200 µM. Thus, the low concentrations of sulfate seem to be seasonally a limiting factor for the activity of sulfate-reducing prokaryotes in these fens. In general, fens of the Lehstenbach catchment emit CH_4 with rates that approximate 0.02 to 15 mmol CH_4 m^{-2} d^{-1} (24). In anoxic microcosms supplemented with sulfate, methanogenesis was inhibited in the presence of sulfate confirming that sulfate-reducing prokayrotes might outcompete methanogens in peatlands (9, 76). Potential rates of sulfate consumption were twice as high as those of methane formation in the absence of sulfate indicating a rapid microbial turnover of sulfate if sulfate is available and that the reduction of sulfate is not a marginal process in these fens. However, the ecophysiological role of sulfate reducers in acidic fens has to be further resolved.

H_2 appears to be an important substrate for acid-tolerant methanogens, and hydrogenotrophic methanogens may be symbiotically associated with hydrogen-producing anaerobes (24).

Assessing SRP diversity: 16S rRNA and *dsrAB* gene approaches. The basic rRNA (gene) approach, which is characterized by the use of universal, bacterial or archaeal primers for PCR amplification, the setup of a rRNA gene library, and comparative sequence analysis, is the gold standard tool for molecular microbial ecologists to assess prokaryotic species richness independent from cultivation (6, 65). Its various application in all kinds of habitats has dramatically improved our knowledge on the phylogenetic extent of microbial life in general {for example see (7, 15, 25, 35, 44)}. A more focussed view on the diversity of certain microorganisms is possible when primers are used that target monophyletic groups at higher specificity. However, the rRNA (gene) approach has its limits when a microbial group of interest is defined by a unique physiological property but is not monophyletic like it is the case for SRPs. At first, because of the polyphyletic nature of the sulfate-reducing microbial guild many different primers would be needed to specifically target monophyletic groups and subgroups of known SRPs, thereby making a traditional rRNA approach tedious and time-consuming. Furthermore, if an organism, exclusively

characterized by an environmental rRNA sequence, belongs to a new lineage it remains unknown whether it has a certain ecophysiological ability such as e.g. dissimilatory sulfate reduction. Only if the environmental rRNA sequence is closely and monophyletically related to rRNA sequences from already cultured organisms, which all exhibit this ecophysiological function, it is likely that this yet uncultured organism shows similar properties. In the presented study, we specifically addressed and avoided this major disadvantages of traditional rRNA-based diversity approaches by combining (i) a 16S rRNA-targeted oligonucleotide microarray for highly parallel pre-screening for SRPs (43) and (ii) the *dsrAB* gene approach which is directly linked to the process of dissimilatory sulfate reduction (32, 66, 74).

The results obtained from SRP-PhyloChip hybridizations were decisive for the selection of appropriate 16S rRNA gene-targeted primer sets to confirm the microarray results. The confirmation is essential because under the monostringent hybridization and washing conditions applied not every single probe does hybridize only with its perfectly matched target sequence (43). For example, presence of *Desulfonema* or related species of the family "*Desulfobacteraceae*" in SbI samples indicated by positive signals of probes DSN658 and DSS658 could not be confirmed by specific PCR assays and were probably caused by cross-hybridization with uncultured members of the genus *Desulfomonile* (see supplementary material at http://www.microbial-ecology.net). The 16S rRNA sequences retrieved from Schlöppnerbrunnen soil after SRP group-specific amplification with the selected primer pairs not only confirmed the microarray results but, as pointed out above, also allowed to establish the phylogenetic fine structure within these Schlöppnerbrunnen-specific SRP groups (Figure 3).

The applicability of the *dsrAB* gene approach for SRP diversity establishment has been previously proven by various studies which detected SRPs in a hypersaline cyanobacterial mat (46), marine and estuarine sediments (28, 69), groundwater at a uranium mill tailings site (12), a Cu-Pb-Zn mine (50), wetland soils (11), activated sludge (62), and deep-sea hydrothermal vent worms (13, 14). However, it has to be noted that the report of new SRP lineages by some of these studies was partly in consequence of the incompleteness of *dsrAB* gene reference sequences from recognized SRP pure cultures used for comparative sequence analysis. Lately, the framework of *dsrAB* sequences from reference cultures has been extensively increased to include numerous representatives from all major SRP lineages (17, 32). In addition, we complemented this sequence collection with the *dsrAB* gene sequence of *Desulfobacca acetoxidans* which is the only cultured member of an individual SRP lineage in the 16S rRNA tree apart from other recognized deltaproteobacterial SRPs (Figure 6) (43, 52).

Some of the previous *dsrAB* gene-based environmental studies inferred SRP phylogeny only from partial sequence data (either part of the *dsrA* or *dsrB* gene). Ludwig et al. (45) emphasized that the information content of phylogenetic marker molecules is limited and different parts of the primary structure provide information on different phylogenetic levels. Thus, tree calculations should always be based on complete sequence data to allow reliable phylogenetic positioning, especially when no closely related sequences are present. Nevertheless, it is practicable to pre-screen *dsrAB* gene clone libraries for highly similar clones e.g. by restriction digest with endonucleases such as *Msp*I (12, 50), *Hae*II (53), *Hha*I (11), or *Mbo*I (13) to avoid redundant sequencing. Our strategy to circumvent extensive sequencing was to initially cluster all partially sequenced *dsrAB* gene clones into OTUs (according to a deduced DsrAB sequence identity equal to or greater than 90%) and subsequently, determine the full sequence of at least one representative clone per OTU. After sequencing, simple statistical evaluation of the clone library such as rarefaction analysis (22, 41, 70) or calculation of the homologues coverage (19, 29, 63) helps to determine if enough clones have been sequenced to cover most of the expected diversity in the clone library. As almost 90% of the expected OTU diversity in each Schlöppnerbrunnen *dsrAB* gene clone library was harvested, it was unlikely that continued sequencing of additional clones would have revealed the presence of many novel OTUs. Finally, the full *dsrAB* sequences from Schlöppnerbrunnen fens and from the SRP pure culture backbone allowed a solid phylogenetic reconstruction based on deduced DsrAB sequences (Figure 6).

SRP richness and phylogeny in acidic fen soils. In contrast to other studies that dealt with SRPs in acidic habitats (16, 36), the polyphasic molecular approach applied in our study enabled us to identify SRPs at high resolution. According to the biogeochemical characteristics of the investigated fen sites, the SRP communities inhabiting those sites are presumably adapted to acidic conditions and low sulfate concentrations.

The SRP-PhyloChip fingerprints of soil samples from two acidic fens in the Lehstenbach catchment indicated no remarkable qualitative changes in SRP community composition over depth. All PCR experiments with the deepest soil section yielded highest amount of amplificate indicating higher abundance of SRP-DNA in the total DNA extract from this soil depth than from the other soil sections. Therefore, we focussed the establishment of the 16S rRNA and *dsrAB* gene clone libraries on the 22.5-30 cm soil section. Although SbI is not in direct vicinity of SbII, both sites showed exactly the same total SRP richness (in terms of numbers of *dsrAB* gene OTUs). However, the qualitative composition of the SRP richness was not identical. From the seven OTUs identified per sampling site, three (OTUs 1, 3, and 6) were identified at both sites whereas the other four were restricted to one site, respectively.

Owing to several lateral gene transfer events of *dsrAB* genes among major lineages of SRPs, DsrAB-based identification and phylogeny of yet uncultured SRPs is limited to some extent (32, 66). Unambiguous identification is only possible if DsrAB sequences from uncultured SRPs are in close monophyletic relation to recognized SRPs whose DsrAB phylogeny is consistent with their 16S rRNA

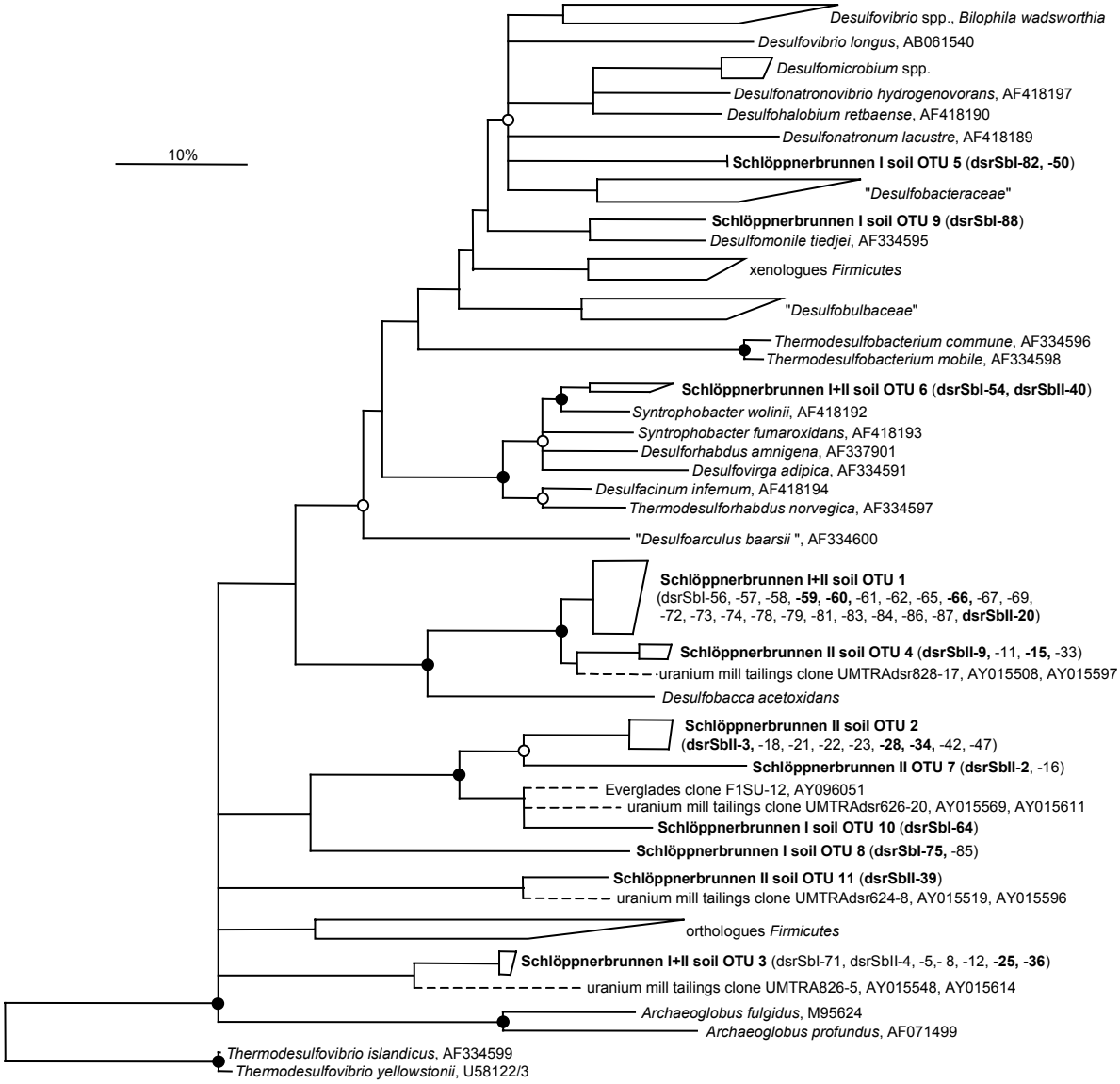

Figure 6. Phylogenetic dendrogram based on DsrAB amino acid sequences deduced from *dsrAB* gene sequences greater than 1750 bases showing the affiliation of operational taxonomic units (OTUs) from Schlöppnerbrunnen fen soils (indicated in boldface type). All clones assigned to the respective OTU are depicted in parentheses. Completely sequenced Schlöppnerbrunnen *dsrAB* clones (>1750 bases) are indicated in boldface type. The consensus tree is based on distance-matrix analysis. DsrAB sequences deduced from *dsrAB* gene sequences shorter than 1750 bases (indicated by dashed branches) were added to the distance-matrix tree without changing the overall tree topology by using the ARB treeing tool PARSIMONY_INTERAKTIV. The bar indicates 10% estimated sequence divergence (distance inferred by distance-matrix analysis). Polytomy connect branches for which a relative order could not be determined unambiguously by applying distance-matrix, maximum-parsimony, and maximum-likelihood treeing methods. Parsimony bootstrap values (100 resamplings) for branches are indicated by solid circles (>90%) or an open circle (75 to 90%). Branches without circles had bootstrap values of less than 75%.

phylogeny. This held true for five Schlöppnerbrunnen OTUs (OTUs 1, 4, 5, 6, and 9) that could be assigned to the class "*Deltaproteobacteria*". In contrast, in consequence of their isolated position within the DsrAB tree the remaining six Schlöppnerbrunnen OTUs (OTUs 2, 3, 7, 8, 10, and 11) neither could be identified nor could their exact phylogeny be unambiguously inferred. Even more, it could not be clarified whether these six OTUs were of bacterial or archaeal origin because they displayed similar evolutionary distances to *Archaeoglobus* spp. and to *Thermodesulfovibrio* spp. (Figure 6). Additionally, some prokaryotes that possess a siroheme sulfite reductase can reduce sulfite but not sulfate (32, 49). Therefore these unknown *dsrAB* gene sequences do not have to originate coercively from SRPs.

Among those OTUs which belonged to the "*Deltaproteobacteria*" were the *Desulfobacca acetoxidans*-related OTUs 1 and 4. The sulfate-reducing bacterium *D. acetoxidans* has been isolated from granular sludge of a laboratory-scale upflow anaerobic sludge bed reactor fed with acetate and an excess of sulfate (52). It was the most abundant SRP in this sludge, seemed to be specialized in acetate consumption, and was able to outcompete acetate-degrading methanogens.

As accomplished before for SRPs from estuarine sediments (28), we were able to retrieve congruent phylogenies of DsrAB and 16S rRNA sequences from *Syntrophobacter wolinii*- and *Desulfomonile*-related SRPs from the fens. This parallel identification fortified the phylogenetic position and metabolic potential of these uncultured Schlöppnerbrunnen bacteria. Stackebrandt and Goebel (64) have proposed that prokaryotes having 16S rRNA dissimilarities greater than 3% to each other represent distinct genomospecies. The dissimilarities of *Syntrophobacter*-related 16S rRNA clones retrieved from both sampling sites to the 16S rRNA sequence of *Syntrophobacter wolinii* ranged from 3.6 to 5.5% and most likely represent more than one different genomospecies because dissimilarities among those clones ranged from 0.1 to 5.2%. The propionate-degrading bacterium *Syntrophobacter wolinii* was first isolated in syntrophic anaerobic co-cultures with methanogens or SRPs (10). *Syntrophobacter wolinii* oxidizes propionate to acetate and CO_2. The generated electrons are then transferred via hydrogen and/or formate to methanogens that form methane by CO_2 reduction. This syntrophic lifestyle is reflected by the fact that propionate oxidation is exergonic only when hydrogen and/or formate are continuously removed by the methanogens. The finding that *Syntrophobacter wolinii* is capable of dissimilatory sulfate reduction, in other words to grow slowly on propionate with sulfate as terminal electron acceptor, led to the first isolation of this bacterium in pure culture (75) and disproved the obligate character of syntrophy originally proposed for this bacterium (10).

The *Desulfomonile*-related 16S rRNA gene clones, solely found in SbI soil, had 16S rRNA sequence dissimilarities to *Desulfomonile tiedjei* and *D. limimaris* that varied from 5.2 to 7.5%. Therefore, these sequences at least represent a new genomospecies within the genus *Desulfomonile*. Whether the two clone subcluster of *Desulfomonile*-related 16S rRNA gene sequences from SbI soil (Figure 3) reflect the presence of (i) two strains of the same species or (ii) two different species could not be elucidated. The most intriguing metabolic feature of cultivated members of the genus *Desulfomonile* is their capability of reductive dehalogenation (47, 68). Thus, they play an important role in the biodegradation of certain environmental pollutants such as highly chlorinated polychlorinated biphenyls, perchloroethene, and chlorobenzenes (48). Although the PCR-based methods used in this study suffer from well recognized biases (72) and generally do not allow any quantitative statements about the actual SRP community composition *in situ*, it should be noted that *Syntrophobacter wolinii*- and *Desulfomonile*-related sequences were among the least abundant once in the *dsrAB* gene libraries.

In summary, an extensive phylogenetic SRP inventory was performed for two acidic fen sites within the Lehstenbach catchment. The global diversity of SRPs known up to now is phylogenetically restricted to four bacterial phyla and one archaeal phylum. Despite this fact, the extent of yet unknown SRP diversity revealed by the present study further indicates that the distribution of the ability to anaerobically respire sulfate or sulfite for energy generation purposes within the bacterial and archaeal domains is not yet entirely recognized. Furthermore, the raw data collected here should make it easier for future studies on the microbial ecology of the Lehstenbach catchment to focus on questions such as: What is the influence of seasonal change on the SRP community composition or which SRPs are really active and responsible for the sulfate respectively sulfite turnover in those fens? Additionally, for monitoring successful enrichment of yet unknown Schlöppnerbrunnen-SRPs, specific *dsrAB* gene-targeted primers can be designed or the clone-specific 16S rRNA-targeted probes developed in this study can be used in fluorescence *in situ* hybridization assays.

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LIST OF PUBLICATIONS

Contributions to the manuscripts presented in this dissertation are listed in parentheses

1. **Juretschko S, Loy A, Lehner A, and Wagner M** [2002] The microbial community composition of a nitrifying-denitrifying activated sludge from an industrial sewage treatment plant analyzed by the full-cycle rRNA approach. *Syst Appl Microbiol*. **25:** 84-99.

2. **Loy A, Daims H, and Wagner M** [2002] Activated sludge - Molecular techniques for determining community composition, p. 26-43. *In* Bitton G (ed.), *The Encyclopedia of Environmental Microbiology*. John Wiley & Sons, Inc., New York.

3. **Loy A, Lehner A, Lee N, Adamczyk J, Meier H, Ernst J, Schleifer K-H, and Wagner M** [2002] Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. *Appl Environ Microbiol*. **68:** 5064-5081.
   [*concept by M.W. and A.Loy, probe design and experimental work by A.Loy, technical advice by A.L., J.A., and N.L., programming by H.M. and J.E., writing by M.W. and A.Loy with editorial help of the co-authors*]

4. **Wagner M, and Loy A** [2002] Bacterial community composition and function in sewage treatment systems. *Curr Opin Biotechnol*. **13:** 218-227.

5. **Wagner M, Loy A, Nogueira R, Purkhold U, Lee N, and Daims H** [2002] Microbial community composition and function in wastewater treatment plants. *Antonie Van Leeuwenhoek*. **81:** 665-680.

6. **Loy A, Horn M, and Wagner M** [2003] probeBase: an online resource for rRNA-targeted oligonucleotide probes. *Nucleic Acids Res*. **31:** 514-516.
   [*concept by M.H. and A.Loy, programming by M.H., acquisition of data by A.Loy and M.H., writing by A.Loy and M.H. with editorial help of M.W.*]

7. **Loy A, Küsel K, Lehner A, Drake HL, and Wagner M** [2003] Diversity of sulfate-reducing prokaryotes in an acidic fen: Comparison of 16S rRNA gene-based oligonucleotide microarray hybridization with dissimilatory (bi)sulfite reductase gene surveys. Submitted to *Appl Environ Microbiol*.
   [*concept A.Loy, K.K., and M.W., microarray experiments and comparative sequence analyses of 16S rRNA and dsrAB genes by A.Loy, biogeochemical analyses by K.K., writing A.Loy with editorial help of the co-authors*]

Parts of this thesis have been presented at national and international meetings

ORAL PRESENTATIONS

Speaker is underlined

Lehner A, Loy A, Adamczyk J, Lee N, Schöpfer A, and Wagner M [2001] Development of phylogenetic DNA Micro Arrays (PhyloChips) - Rapid tools for microbial community profiling and functional analysis. Jahrestagung der Vereinigung für Allgemeine und Angewandte Mikrobiologie e.V. (VAAM). Oldenburg, Germany, 25.-28. March 2001.

Lehner A, Loy A, Adamczyk J, Lee N, Schöpfer A, Wagner M [2001] Development and application of phylogenetic DNA-Microarrays (PhyloChips) for microbial community profiling and functional analysis. Ninth International Conference on Microbial Ecology (ISME-9). Amsterdam, Netherlands. 26.-31. August 2001.

Loy A, Adamczyk J, Lehner A, Lee N, Stubner S, Küsel K, Drake H, and Wagner M [2002] The SRP-PhyloChip - a 16S rRNA gene-based oligonucleotide microarray as biodiversity screening tool for sulfate-reducing prokaryotes in the environment. Jahrestagung der Vereinigung für Allgemeine und Angewandte Mikrobiologie e.V. (VAAM). Göttingen, Germany. 24.-27. March 2002.

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Loy A, Lehner A, Lee N, Stubner S, Küsel K, Drake H, and Wagner M [2002] The SRP-PhyloChip – a 16S rRNA gene-based oligonucleotide microarray as biodiversity screening tool for sulfate-reducing prokaryotes in the environment. Joint meeting of the three divisions of International Union of Microbiological Societies (IUMS) - The world of microbes. Paris, France. 27. July - 01. August 2002.

POSTER PRESENTATIONS

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