Lehrstuhl für Mikrobiologie
der Technischen Universität München

Dissimilatory (bi-) sulfite reductase as a marker for phylogenetic and ecological studies of sulfate-reducing prokaryotes

Michael Dieter Klein

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München
zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften
(Dr. rer. nat.)

genehmigten Dissertation.

Vorsitzender:	**Univ.- Prof. Dr. Erwin Grill**
Prüfer der Dissertation:	**1. Univ.- Prof. Dr. Michael Wagner** **Universität Wien / Österreich**
	2. Univ.- Prof. Dr. Karl-Heinz Schleifer

Die Dissertation wurde am 21.10.2004 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 17.12.2004 angenommen.

Parts of this work have been published in advance:

Dubilier, N., C. Mulders, T. Ferdelman, D. de Beer, A. Pernthaler, M. Klein, M. Wagner, C. Erseus, F. Thiermann, J. Krieger, O. Giere and R. Amann (2001). "Endosymbiotic sulphate-reducing and sulphide-oxidizing bacteria in an oligochaete worm." Nature **411**(6835): 298-302.

Klein, M., M. Friedrich, A. J. Roger, P. Hugenholtz, S. Fishbain, H. Abicht, L. L. Blackall, D. A. Stahl and M. Wagner (2001). "Multiple lateral transfer events of dissimilatory sulfite reductase genes between major lineages of sulfate-reducing prokaryotes." Journal of Bacteriology **183**(20): 6028-6035.

Schmid, M., U. Twachtmann, M. Klein, M. Strous, S. Juretschko, M. Jetten, J. W. Metzger, K. H. Schleifer and M. Wagner (2000). "Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation." Syst Appl Microbiol **23**(1): 93-106.

In preparation:

Zverlov, V., Klein, M., Lücker, S., Friedrich, M.W., Kellermann, J., Stahl, D.A., Loy, A., and M. Wagner (2004). „Lateral Gene Transfer of Dissimilatory (Bi)Sulfite Reductase Revisited", submitted to J. Bacteriol.

Abbreviations:

AMP	Adenosine-mono-phosphate
APS	Adenosine – 5´phosphosulfate
ATP	Adenosinetriphosphate
bp	base pairs
DNA	Deoxyribonucleic acid
dsr	Gene coding for the dissimilatory sulfite reductase
Dsr	Dissimilatory sulfite reductase, the protein
dsrA/ DsrA	Partial sequence of *dsr* / Dsr alpha subunit as sequenced by the primers presented
dsrAB	approximately 1.9 kb long fragment of the *dsr* operon, encompassing most of the alpha and beta subunit of the *dsr*
DsrAB	Amino acid sequence inferred form *dsrAB*
dsrB /DsrB	Partial sequence of *dsr* / Dsr beta subunit as sequenced by the primers presented
et al.	et alteri und andere
g	Gravity
GC or GC%	Mol % Guanine + Cytosine
h	Hour
H^+	Proton
H_2	Hydrogen
Indel	Insertion / deletion events of certain sequences
IS	Insertion sequences
kb	Kilo base, 1000 nucleotide bases
kDa	Kilo Dalton
min	Minute
ml	milliliter
mM	milli molar
nm	Nanometer
PAGE	Polyacrylamidegelelectrophoresis
PAPS	Phospho-adenosine – 5´phosphosulfate
PCR	polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
S	Svedberg (s^{-1})
s	Second
S^{2-} or H_2S	Sulfide or hydrogen sulfide
SDS	Sodium- dodecyl- sulfate
SO_3^{2-}	Sulfite
SO_4^{2-}	Sulfate
SRB	Sulfate reducing bacteria/bacterium
SRP	Sulfate reducing prokaryotes/prokaryote
UTP	Uraciltriphosphate

A INTRODUCTION 1

A.1 The global sulfur cycle 1

A.2 Physiological traits of sulfate reducing prokaryotes 2

A.3 Habitats of sulfate reducing prokaryotes 4

A.4 Phylogeny of sulfate reducing prokaryotes 5

A.5 Detection of SRP using 16S rRNA as marker 7

A.6 The dissimilatory sulfite reductase – an alternative marker molecule for sulfate reducing prokaryotes 9

A.7 Extension of the DsrAB data base 11

A.8 The aims of this Ph.D. thesis 12

B MATERIAL AND METHODS 13

B.1 Reference strains, and clone maintenance 13

B.2 DNA extraction from pure cultures and environmental samples 15

B.3 Polymerase chain reaction amplification of *dsrAB* genes 16

B.4 Molecular cloning of *dsrAB* genes into pCRTM2.1 or pCR-XL-TOPO vectors and identification of dsrAB carrying clones 16

B.5 Sequencing of cloned *dsrAB* gene fragments 17

B.6 Gelretardation 17

B.7 Phylogenetic Analysis 17
B.7.1 Pure culture databank 17
B.7.2 Environmental databank 18
B.7.3 Alignment and phylogenetic analysis of 16rRNA gene data 18
B.7.4 Alignment and phylogenetic analysis of DsrAB data (amino acid based analysis) 19
B.7.5 Calculation of Distance matrices 19
B.7.6 Analysis of short environmental DsrAB sequence fragments 19
B.7.7 Alignment of DsrA to DsrB and phylogenetic analysis of this databank 20
B.7.8 Phylogenetic analysis of *dsrAB* gene sequences (nucleic acid based analysis) 21

B.8 Preparation of tissue of the *oligochaet Olavius algarvensis* for fluorescence in situ hybridization (FISH) 21

B.9 Fluorescence in situ hybridization 21

C RESULTS AND DISCUSSION 23

C.1 Establishment of reference strain DsrAB databank 23
C.1.1 Reevaluation of PCR primers for amplification of *dsrAB* gene fragments 23
C.1.2 Inconsistencies between Edman degradation/ inference from nucleotide sequencing 26
C.1.3 DsrAB sequence alignment and characteristic motives 27
C.1.4 Conservation profile of DsrAB 28

C.2 Comparison of 16S rRNA and DsrAB-based phylogeny for SRP reference strains 29

C.2.1 Comparative analysis of 16S rRNA gene and DsrAB sequence based phylogeny 29
C.2.2 Further analysis on *dsrAB* originating from *Desulfobacula toluolica* 35
C.2.3 Comparison of 16S rRNA gene sequence similarity and DsrAB identity values between SRP 41
C.2.4 Further reflections on lateral gene transfer..... 45
C.2.4.1 Comparison of G+C% content of dsrA and dsrB 45
C.2.4.2 Comparative analysis of genomic G+C% content and dsrAB G+C% content..... 46
C.2.4.3 Consequences of lateral gene transfer..... 47

C.3 Analysis of environmental DsrAB sequences 49
C.3.1 Global traits in DsrAB based environmental surveys..... 49
C.3.2 Consensus tree containing available pure culture and environmental DsrAB sequences of good sequence quality 50
C.3.3 Comparison of branching order of pure culture SRP in pure culture DsrAB and environmental DsrAB dendrogram 52
C.3.4 Classification of short environmental *dsr* sequences 54
C.3.5 Richness of SRP in different environmental habitats 65

C.4 Environmental SRP surveys with the DsrAB - approaches in the scope of this thesis.....66
C.4.1 Analysis of mixed populations of sulfate reducing prokaryotes with the DsrAB-approach and gelretardation 66
C.4.2 Combination of 16S rRNA and DsrAB approach for studying complex symbiosis 70
C.4.3 Analysis of metabolic features of sulfate reducing prokaryotes 72

C.5 Limitations as chances and outlook 75

D SUMMARY.....77

E ZUSAMMENFASSUNG.....79

F REFERENCES 81

G APPENDIX.....91

G.1 Accession numbers of Dsr and 16S rRNA gene sequences and index of figures and tables presenting results from analyses performed with these sequences 91

G.2 Publications which contain results from this Ph.D. thesis 99
G.2.1 Title: Multiple lateral transfer events of dissimilatory sulfite reductase genes between major lineages of sulfate-reducing prokaryotes 99
G.2.2 Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation 122
G.2.3 Endosymbiotic sulphate-reducing and sulphide-oxidizing bacteria in an oligochaete worm 137
G.2.4 Lateral Gene Transfer of Dissimilatory (Bi)Sulfite Reductase Revisited..... 151
G.2.5 Community-level genetic analysis: functional marker genes for the specific identification of sulphate-reducing prokaryotes..... 173

H NOTES OF THANKS 174

PREFACE

Why investigating sulfate-reducing prokaryotes?

Sulfur has always been considered a mythic and miraculous substance. Paracelsus described this element besides “sal” (representing solidity) and “mercurius” (representing volatility) as the third principal of existence, with sulfur representing the combustible (anima) aspect (Biedermann 1991). In our mythology and literature sulfur is often cited as the element of evil. The devil’s stench is described as sulfurous, yellowish, “malodor”. On the other hand the burning of sulfur was used to turn away foul creatures, pest or, in case of Odysseus in Homers Odyssey, bad spirits (Homer’s Odyssey, Book 22). Both descriptions point at hidden powers of this yellow element that have to be revealed. On earth, mighty deposits of elemental sulfur have been found in Italy, North-, Middle and South America and Japan, but nevertheless sulfur occurs on our planet predominately as inorganic sulfites or sulfates (Wiberg 1985). Adding to the picture of sulfur, as an element of fire and heat are the extreme environments were significant amounts of these sulfite and sulfate can be found. Here land and submarine volcanoes, as well as black - or white smokers, hot springs, arctic habitats, deep marine methane seeps and aquifers, halophilic cyanobacterial mats, and all kinds of contaminated sites have to be mentioned.

At these sites, organisms that thrive in the presence of various sulfur compounds have to cope with extreme conditions like high or low temperatures (ranging from below 0 C° up to and above 100 C°), extreme pH values (ranging form 0.5 to 9), the toxicity of some sulfur compounds like hydrogen sulfide (Hausmann 1995), or high salts concentrations (e.g. Tardy-Jacquenod 1998). Nature has developed possibilities for microorganisms not only to survive in these environments, but to gain energy by the transformation of sulfur compounds.

One group of microorganisms that is able to live from the reduction of the chemically quite inert sulfate by producing aggressive hydrogen sulfide is the guild of sulfate reducing prokaryotes.
In this context it is appropriate to use the term prokaryotes, since sulfate-reducers are found in the domain Bacteria, as well as within the domain Archaea.
The history of microbial sulfate reduction is quite long. There is good isotopic (Shen 2001) and molecular (Wagner 1998) evidence that microbial dissimilatory sulfate reduction is a very ancient process, at least in our perception. It is most likely older than 3.47 billion years. This makes sulfate-respiration an evolutionary very successful metabolic pathway, a pathway that enables the sulfate reducer to gain energy in environments where the redox potential is low, energy conservation is hard and growth is slow. This successful reduction of the highest oxidized state of sulfur in nature and the participation in the last steps of microbial decomposition near the endpoint of possible energy conservation is a reason for the widespread distribution of sulfate-reducers in various environments on planet earth.

In essence, the investigation of the evolutionary history and environmental distribution patterns of sulfate reducing prokaryotes in various and extreme environments was the main research focus of my Ph.D. thesis.

A Introduction

A.1 The global sulfur cycle

Life on earth is only possible through tightly interwoven material transformations in various cycles. Carbon, nitrogen, phosphorus and sulfur are essential components of all living organisms and thus represent the most important elements circulating within the biosphere. During this circulation sulfur can be found in various oxidation states and transformations occur both, biologically and chemically. Figure 1 display the biological sulfur cycle.

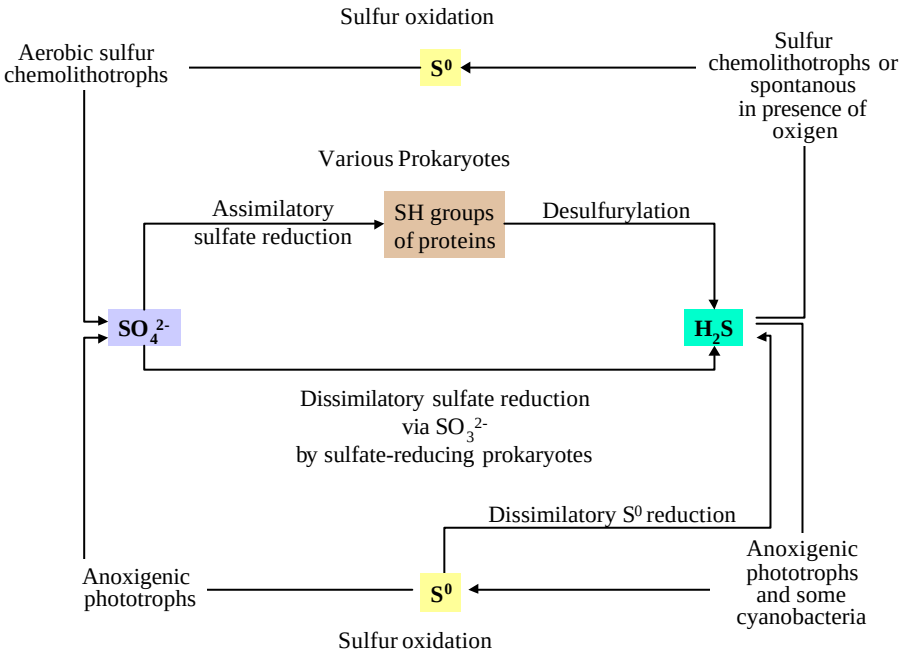


Figure1 Simplified biological sulfur cycle.

Sulfur is present in nature predominately as sulfates and sulfites (Wiberg 1985). Plants and microorganisms take up sulfate via aerobic and anaerobic assimilatory sulfate reduction, while animals are only able to take up reduced sulfur compounds with their diet. Sulfur is present in living cells mostly as integral part of amino acids (e.g. methionine, cysteine). Cysteine, which is the only amino acid that carries a highly reactive sulfhydryl-group (R-SH), can be found frequently in reaction centers of enzymes (Miserta 2000 and citations within). It is further involved in formation of three-dimensional structures of proteins through intra- and inter-chain disulfide links (Noiva 1994; Raina 1997). During the decomposition of sulfur containing

proteins by desulfurylation, carried out by a huge variety of fungi and prokaryotes (Lengeler 1999), sulfide is produced and released into the environment. Sulfide can be transformed either biologically or chemically in the presence of oxygen or, biologically in the absence of oxygen. Under oxygenic conditions, sulfide can be used by e.g. *Beggiatoa*, *Thiothrix* and *Thiobacilli* as electron donor and it is oxidized to sulfur. The same transformation of sulfide to elemental sulfur can occur spontaneously and abiotically (Widdel 1988; Brock 1994). The generated sulfur can further be oxidized to sulfate by colorless sulfur bacteria (see above) as well as by members of the archaeal genus *Sulfolobus*. Under oxygen depleted conditions sulfide can be oxidized to sulfur (e.g. by *Chlorobium*) and even to sulfate (e.g. by *Chromatium*) by phototrophic purple and green sulfur bacteria. Under anaerobic conditions sulfur can alternatively be reduced to hydrogen sulfide by the activity of dissimilatory sulfur reducers, for example by members of the bacterial genus *Desulfuromonas* or the archaeal genera *Desulfurococcus* and *Thermoproteus* (Widdel 1988).

This thesis deals with sulfate reducing prokaryotes which close the sulfur cycle by using sulfate (sulfite, thiosulfate) as electron acceptor, thereby gaining energy under anaerobic conditions from the dissimilatory reduction of sulfate to sulfide. In addition to this general life style, a set of metabolic features, described in more detail below, allows the sulfate-reducing prokaryotes to play an essential role in the anaerobic mineralization of organic compounds.

A.2 Physiological traits of sulfate reducing prokaryotes

Anaerobic sulfate respiration represents a very old, thus evolutionary successful metabolic lifestyle of prokaryotes. Molecular evidence has suggested that dissimilatory sulfate reduction is ancient (Wagner 1998) and geo-chemical data indicate the occurrence of microbial sulfate reduction 3.47 billion years ago (Shen 2001). Despite its long evolutionary history, the anaerobic sulfate respiration pathway seems to be restricted to a rather small group of very specialized microbes, summarized as the sulfate reducing prokaryotes (SRP). With the exception of *Archaeoglobus and* some *Syntrophobacter* species, the names of most SRP have the prefix "*Desulfo-*" or "*Thermodesulfo-*" as tribute to their sulfate reducing activity. SRP thrive mostly in anaerobic or microaerophilic habitats (e.g. see Widdel 1988; Widdel 1992a; Widdel 1992b; Widdel 1992c; Widdel 1994). They perform one of the last steps of anaerobic decomposition of organic compounds within the carbon cycle and reintroduce sulfate (via hydrogen sulfide) back into the sulfur cycle. SRP posses a chemo-organo-heterotrophic, or in some cases even a chemo-

litho-autotrophic lifestyle. Sulfates are thermodynamically very stable and thus comprise the most abundant sulfur compound in rocks and sediments. Sulfates play an important role as sulfur reservoir in aquatic, and especially in marine ecosystems. The sulfate concentrations range from 27 – 28 mM/l (˜ 2.7 g/l) in sea water and, depending of the depth, up to 30 mM/l in marine sediment (Widdel 1988; Jannasch 1995; see also Brock 1994). In freshwater, the sulfate concentration is much lower, ranging from 0.01 to 0.2 mM/l (Widdel 1988).

Because of the inertness toward transformations, sulfate has to be activated to adenosine—5'-phosphosulfate as first step in the assimilatory or dissimilatory reduction process (see Figure 2).

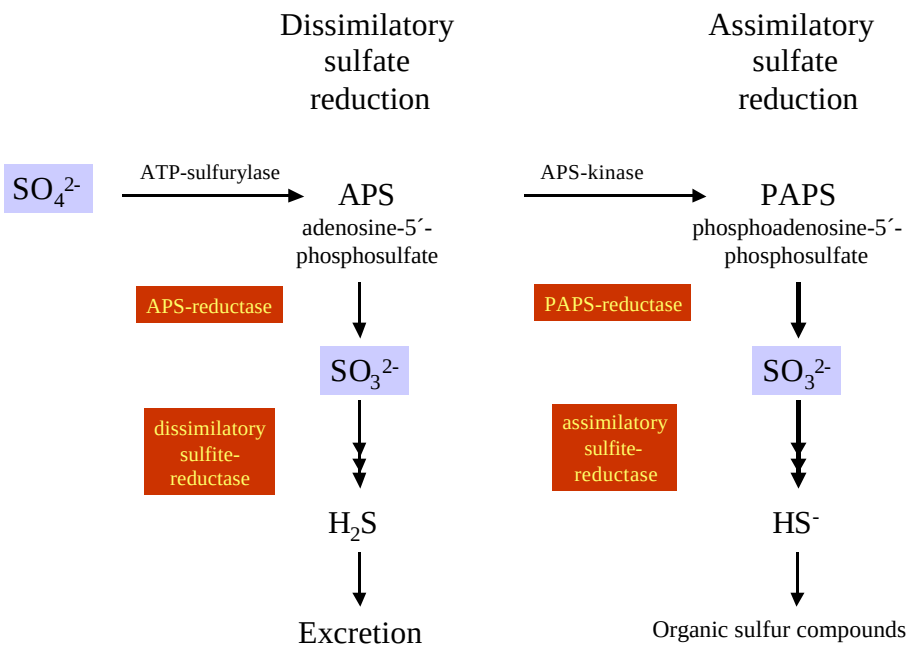


Figure 2 Dissimilatory and assimilatory sulfate reduction (simplified), according to Lengeler 1999 and Brock 1994.

The assimilatory sulfate reduction, which requires a second activation step catalyzed by APS-kinase, leads to incorporation of sulfur into the organism at the expense of energy under both, oxic and anoxic conditions. In case of the dissimilatory pathway, the energy conserving six-electron reduction is carried out only under anoxic conditions by the enzymes dissimilatory or (bi-) sulfite reductase (DSR). In this catabolic reaction sulfate serves as terminal electron acceptor. Hydrogen, carbonic acids (e.g. lactate, pyruvate, acetate), aromatic compounds (e.g. benzoate, indole), or other organic substances (e.g. ethanol, fatty acids) can serve as electron donors (Widdel 1981; Widdel 1992b; Widdel 1992c; Brock 1994; Widdel 1994). Additional metabolic features of sulfate reducers are the ability (i) to gain energy by fermentation (e.g. Lie 1999; Sonne-Hansen 1999), (ii) to fix nitrogen (e.g. Nazina 1979) and (iii) to fix carbon dioxide (e.g. Brysch 1987; Daumas 1988; Kuever 1993). Other conspicuous traits of SRP are the ability

(i) of reducing nitrate to nitrite by some *Desulfotomaculum*, *Desulfobulbus*, and *Desulfovibrio* strains (Mitchell 1986; Moura 1997; Pereira 1996b; Barton 1983), (ii) of anaerobic reductive dehalogenation of chlorinated aromatic or aliphatic substances by e.g. *Desulfomonile* (DeWeerd 1990) and (iii) of the extraordinary coupling of phosphite oxidation to phosphate with simultaneous reduction of sulfate to hydrogen sulfide under anaerobic conditions by an deltaproteobacterial sulfate reducing strain (Schink 2000). SRP have also been reported to live in symbiosis or, at least, in close association with *Eukarya*. They have been identified and isolated from the hindguts of termites (e.g. Breznak 1994), the digestion tracts of ruminants (e.g. Coleman 1960) and, as epibionts of the deep-sea marine worm *Alvinella pompejana* (Cottrell 1999). Beside free living and symbiotic lifestyles some SRP have the ability of syntrophic growth with other microorganisms. Prominent examples for this lifestyle are the interactions between green sulfur bacteria (Biebl 1978), *Marinobacter* species (Sigalevich 2000a; Sigalevich 2000b; Sigalevich 2000c), *Methanococcus maripaludis* (Pak 1998a; Pak 1998b), and various sulfate reducing partners. The latter syntrophic partnership is very interesting since an archaeal methanogene and a deltaproteobacterial microorganism share the same habitat and have interwoven substrate pathways.

A.3 Habitats of sulfate reducing prokaryotes

SRP can be found frequently in high numbers in and below the oxic/anoxic interfaces in marine- (e.g. Taylor 1985; Devereux 1994; Bale 1997; Finster 1997; Ravenschlag 1999), brackish- (e.g. Widdel 1981; Boschker 2001) and freshwater sediments (e.g. Drzyzga 1994; Sass 1998; Li 1999; Miskin 1999), within microbial mats (Caumette 1991; Krekeler 1997; Teske 1998), (Minz 1999a; Minz 1999b), in soils (e.g. Sexstone 1977; Großkopf 1998; Henckel 1999; Ouattara 1999; Wind 1999; Hristova 2000; Stubner 2000), and also free living in water columns (e.g. Tonolla 2000; Ramsing 1996; Teske 1996). The dogma that SRP are restricted to anaerobic habitats due to the toxicity of oxygen has been challenged recently by different studies. SRP have been reported to evade high oxygen pressure by formation of aggregates (Eschemann 1999), migration in response to oxygen-stress (Krekeler 1998; Minz 1999b) and some fast growing stains are even able to detoxify oxygen by respiration, forming ATP, but did not show growth (Dilling 1990; Cypionka 2000; Hansen 1994). Furthermore, SRP have also been detected in wastewater treatment plants within activated sludge (Schramm 1999; Manz 1998), but commonly growth in presence of too high oxygen concentrations does not take place (Gall 1996; Johnson 1997; Cypionka 2000). SRP seem to retreat into micro niches were oxygen

concentration is lower and, by producing hydrogen sulfide via dissimilatory sulfate reduction. This induces a chemical detoxification process of oxygen by reconstitution of the reduced sulfate (Widdel 1988).

SRP have also been isolated from human digestion tracts (Gibson 1991), from periodontal tooth pockets (Langendijk 2001), and from pyogenic liver abscesses (Schoenborn 2001). Thus, at least some SRP species might be opportunistic pathogens for humans. Further, sulfate reducing microbes posses economical relevance due to their ability to cause corrosion of metal built structures like oil production sites (e.g. Beeder 1995; Beeder 1996; Nilsen 1996b), tanks (Hagenauer 1997) or water tubes (e.g. Pereira 1996a and citations within).

It should be noted that no single strain of SRP is able to perform all the described anabolic and catabolic processes and that different types of SRP occur in different ecosystems.

A.4 Phylogeny of sulfate reducing prokaryotes

Comparative sequence analysis of ribosomal RNA genes comprises the gold standard for inference of phylogeny. A short summary of the current knowledge on 16S ribosomal RNA gene based SRP phylogeny is given in the section below.

The majority of SRP is affiliated with the *Deltaproteobacteria* (e.g. Woese 1987; Widdel 1992c) as depicted in Figure 3.

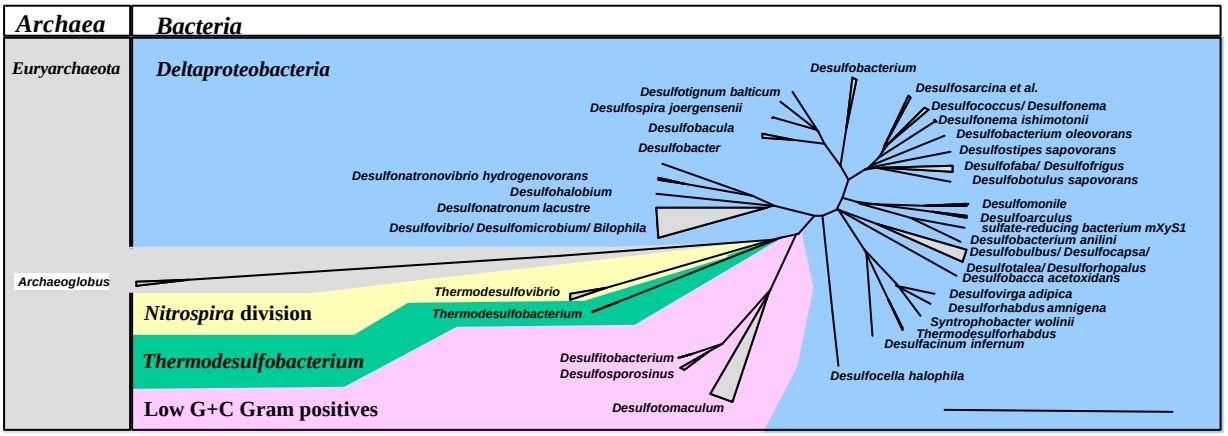


Figure 3 16S rRNA gene based neighbor joining tree containing all recognized phyla of sulfate-reducing prokaryotes. Different phyla are color coded. Length bar indicates 10 % estimated sequence divergence.

According to the taxonomy browser of the NCBI (http://www.ncbi.nlm.nih.gov) at least 35 different lineages of SRP within the class of *Deltaproteobacteria* are described (date: September

2004). With exception of the thermophilic genera *Thermodesulforhabdus* and *Desulfacinum* all deltaproteobacterial SRP are mesophilic or in some cases psychrophilic (Knoblauch 1999).

The second largest group of described SRP belongs to the spore forming, Gram- positive genera *Desulfotomaculum,* and *Desulfosporosinus* within the *Peptococcaceae* in the phylum *Firmicutes.* Based on 16S rRNA gene analyses these SRP were assigned into three major clusters (Stackebrandt 1997b). The composition and substructure of these clusters are depicted in Figure 4. Additional strains have been placed into this classification system (Kuever 1999; Pikuta 2000) and the supplementary subgroup If has been proposed by Kluever et al. (Kuever 1999).

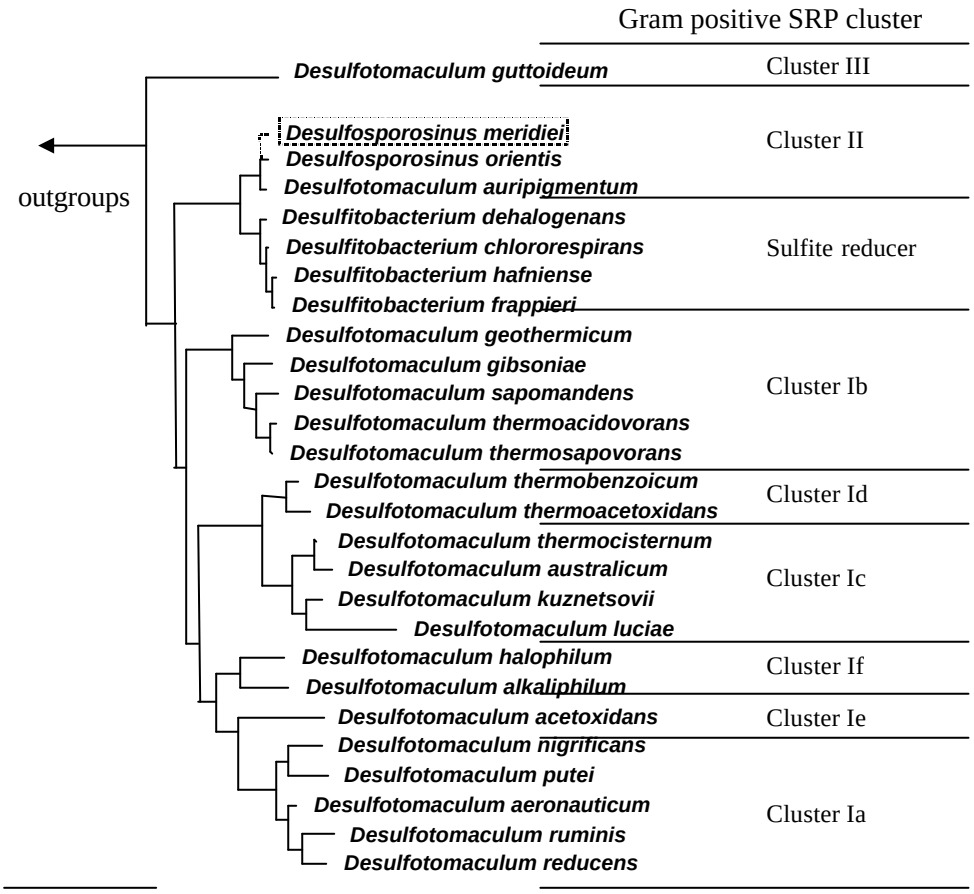


Figure 4 16S rRNA gene based neighbor joining tree containing Gram positive SRB as clustered by Stackebrandt et al. updated with newly described species and clusters (e.g. Cluster if by Kuever et al. 1999). Length bar indicates 10 % estimated sequence divergence

Cluster I contains most of the described *Desulfotomaculum* species. Their number and names are given in Figure 4. Cluster II contains *Desulfosporosinus orientis,* formally called *Desulfotomaculum orientis,* which was reclassified lately (Stackebrandt 1997a). Recently, second specie, *Desulfosporosinus meridiei* of the same genus has been described (Robertson 2001). However, up to now the sequence of its 16S rRNA gene has not been published. In

addition, "*Desulfotomaculum auripigmentum*" should also be also included in this group and possibly reclassified (Kuever 1999) as *Desulfosporosinus auripigmentum*. Cluster III contains *Desulfotomaculum guttoideum,* a possibly misclassify member of the *Clostridiaceae* (Stackebrandt 1997b).

Within the domain *Bacteria* two other phyla contain sulfate reducers. Within the *Nitrospira* phylum, the genus *Thermodesulfovibrio* and within the deep branching *Thermosdesulfobacteria* phylum the genus *Thermodesulfobacterium* are as well gaining energy by dissimilatory sulfate reduction.

In addition to the bacterial SRP lineages mentioned above, dissimilatory sulfate reduction is also found within the domain of *Archaea*. The euryarchaeotal order *Archaeoglobales* comprise, at present, the only recognized members of the archaeal domain which use the dissimilatory sulfate reduction pathway for energy conservation.

In conclusion, the ability to respire sulfate under anaerobic conditions via the dissimilatory sulfate reduction pathway has been observed in five different phyla within the *bacterial* and *archaeal* domains. Thus, the guild (guild - a collection of species that perform the same ecological function) of SRP represent a phylogenetically very inhomogeneous group.

A.5 Detection of SRP using 16S rRNA as marker

Since sulfate reducing prokaryotes comprise a group of microorganisms with economical, ecological and even medical significance (see above, and Widdel 1992b) it is important to establish a reliable system for their detection and identification. The classical approach for the identification of microorganisms was, and still is, the time consuming isolation of strains from the environment with selective cultivation media and subsequent characterization of the isolates based on their morphological, biochemical, and physiological traits. The major disadvantage of this approach has been summarized as the "great plate count anomaly" (Staley 1985). This term was coined to describe the huge deviation of cell numbers within environmental samples determined by cultivation (plate counts or MPN), and direct microscopic cell counts, whereby

the microscopic method yields cell numbers several magnitudes higher then the cultivation dependent methods. The expression of the “plate count anomaly” simply compiles all difficulties involved in cultivation of characterized and yet uncharacterized prokaryotes from complex samples. Missing data on cultivation conditions like nutrient requirements, optimal pH, toxicity of media or atmospheric compounds, and even essential interactions with other organisms (e.g. syntrophism) allows only cultivation of a rather small proportion of the naturally occurring microbial diversity (Staley 1985; Wagner 1993; Amann 1995b). In order to circumvent this problem, the application of culture independent molecular methods for SRP detection in environmental samples is necessary. The use of the full cycle 16S rRNA gene approach (Amann 1995b) allows the identification of the vast majority of prokaryotes including the SRP occurring in a complex environmental matrix (Juretschko 1998).

The 16S rRNA gene sequences of all described SRP strains have been deposited in public databases (see ARB, www.arb-home.de or RDP, www.rdp.cme.msu.edu) and provide a phylogenetic framework for the assignment of 16S rRNA gene sequences retrieved from new SRP isolates or directly obtained from environmental samples (Devereux 1994; Voordouw 1996; Leu 1998; Stubner 2000).

Beside comparative sequence analysis, the collected 16S rRNA gene data can be used for the design of specific oligonucleotide probes, which can be applied in different hybridization formats for the identification of SRP (DeLong 1989; Amann 1995b). Probes, targeting the 16S rRNA of known sulfate reducing prokaryotes have been used in e.g. slot blot (Sahm 1999b; Minz 1999b) and fluorescence *in* hybridization *situ* (FISH) experiments (e.g. Amann 1995a; Ramsing 1996; Manz 1998) to detect sulfate reducers in the environment. Recently, the application of DNA arrays, especially designed to detect 16S rRNA and 16S rRNA gene sequences of sulfate reducers has been introduced in microbial ecology (Loy 2002). With this approach the presence of different sulfate reduces of all known lineages can be detected

simultaneously by hybridization with over 130 genus, group, or even specie specific oligonucleotide probes (Loy 2002).

However, the major problem of 16S rRNA sequence based identification of SRP in the environment is still unsolved. The retrieved 16S rRNA sequences do not contain information on the physiology of the respective organisms. SRP are widespread in the phylogenetic tree, in both the domain Archaea and Bacteria. They are members of lineages which also contain organisms with other modes of energy conservation. Thus, unambiguous identification of an organism as SRP by its 16S rRNA sequence is only possible if it is very closely related to a recognized SRP which 16S rRNA sequence has already been deposited. Therefore, completely novel lineages of sulfate reducers in the environment would not even been recognized as SRP by the rRNA-approach.

Due to close relationship of SRP with other bacteria 16S rRNA targeted probes are frequently inefficient to discriminate between SRP and non-SRP. Additionally, the phylogenetic inhomogeneity of the guild of SRP does not allow the application of simple sets of oligonucleotide probes or primers for the hybridization/PCR-amplification of all SRP 16S rRNA genes (Devereux 1992; Castro 2000; Daly 2000; Manz 1998). Consequently, it is necessary to identify and exploit additional phylogenetically informative marker genes which allow one to specifically detect and identify SRP.

A.6 The dissimilatory sulfite reductase – an alternative marker molecule for sulfate reducing prokaryotes

In the dissimilatory sulfate reduction pathway there are three key enzymes that could possibly serve as marker molecules for SRP (see Figure 2). Since the ATP- sulfurylase, responsible for the activation of sulfate to APS (Figure 2), is also present in assimilatory sulfate reducers (Lengeler 1999) the genes coding for this enzyme are not suitable as marker molecule for sulfate reduction. The APS reductase on the other hand has also been discovered in chemotrophic and phototrophic strains (Hipp 1997 and citations within). Recently, more sequence data of APS

genes have become available (Speich 1994; Hipp 1997; Deplancke 2000; Dahl 2001; Friedrich 2002). However, the rather short length of the APS genes (approx. 900 bp) limits the phylogenetic information content of these molecules. Further, the APS genes seem to be prone to lateral gene transfer events (Friedrich 2002).

The genes coding for the dissimilatory (bi -) sulfite reductase (DSR) [E.C. number: 1.8.99.3] can be considered as potentially suitable phylogenetic markers for SRP. The DSR is present in all dissimilatory sulfate-reducing prokaryotes investigated so far. Based on spectrophotometric measurements of oxidized and reduced forms of the DSR, four different types, desulfoviridin (e.g. Seki 1985), desulforubidin (e.g. Lee 1973), desulfofuscidin (e.g. Fauque 1990) and P-582 (Akagi 1973), have been identified. These enzymes consist of two different polypeptides in a $\alpha_2\beta_2$ structure and contain sirohaem, non-haem iron and acid-labile sulfite (Fauque 1990; LeGall 1988). Recently a third subunit has been discovered and a $\alpha_2\beta_2\gamma_2$ structure of the DSR has been proposed for *Desulfovibrio* (Pierik 1992; Karkhoff-Schweizer 1993). The genes coding for the alpha and beta subunit of the dissimilatory sulfite reductase (DsrA and DsrB) are exclusively organized in a single operon. The coding sequences of the alpha subunit (*dsrA*) always precede the sequences coding for the beta subunit (*dsrB*) (Dahl 1993; Karkhoff-Schweizer 1995; Wagner 1998; Larsen 1999; Larsen 2000; Laue 2001). Based on *dsrAB* sequences from *Desulfovibrio vulgaris* and *Archaeoglobus fulgidus* published in earlier studies (Karkhoff-Schweizer 1995; Dahl 1993), the primer pair DSR1F and DSR4R targeting conserved regions within these genes has been constructed and has been confirmed to amplify a 1.9 kb large fragment, encompassing most of the *dsrA* and *dsrB* subunit genes of all tested SRP (Wagner 1998).

However, these conserved sequence motives, targeted by this primer pair might also be present in other commonly found sulfite reductase related gene sequences. For example, the archaeum *Pyrobaculum islandicum* does expresses a sulfite reductase type protein containing siroheam, but is lacking the ability to reduce sulfate. Its sulfite reductase belongs to a different enzyme family (Imhoff 1998; Pott 1998), only distantly related to the DSR. Phototrophic *Allochromatium vinosum* (Imhoff 1998; Pott 1998) and *Thiobacillus* species (Schedel 1979) express enzymes which share also sequence motives with the DSR of sulfate reducers. They are using a “reverse” reductase for the oxidation of sulfite and sulfur, respectively, and not for dissimilatory sulfate reduction. As further structural relatives have to be mentioned the assimilatory sulfite reductase of *Escherichia coli* and *Salmonella typhimurium* (Murphy 1973a;

Murphy 1973b), as well as the low-spin sulfite reductase of *Desulfovibrio vulgaris* (Huynh 1984), *Desulfuromonsa acetoxidans*, and *Methanosarcina barkeri* (Moura 1986) .
However, experimental evaluation of the specificity of the above mentioned primer pair showed that the dissimilatory sulfite reductase can be specifically amplified with this primer pair and that amplification of the above mentioned non target sequences does not occur (Wagner 1998).

Since the amplified *dsrAB* gene fragments (i) carry conserved as well as variable regions, (ii) code for an essential enzyme of the dissimilatory sulfate reduction pathway, and (iii) are present in all sulfate reducers examined so far, the DSR is a well-suited phylogenetic marker molecule for dissimilatory sulfate reducing microorganisms. The comparison of results from phylogenetic analyses of 16S rRNA genes and DsrAB databases yielded similar trees topologies (see Figure 5) and thus, it was suggested that comparative DsrAB sequence analysis allows specific culture independent detection and identification of SRP (Wagner 1998).

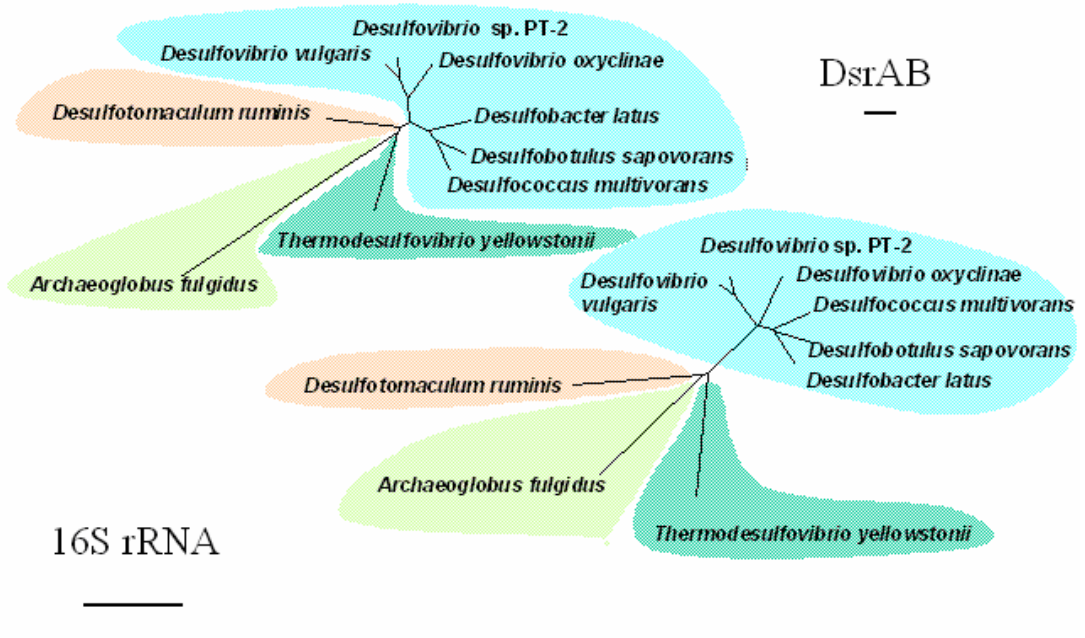


Figure 5 Comparison of 16S rRNA gene and dissimilatory sulfite reductase amino acid sequence based phylogenetic trees (according to Wagner 1998). Dendrograms have been calculated using distance methods. Bars indicate 10 % estimated sequence divergence.

A.7 Extension of the DsrAB data base

The main hindrance of using the DSR as phylogenetic marker for SRP diversity studies in environmental surveys was the lack of an exhaustive data base. At the beginning of this Ph.D.

thesis only *dsr* sequences from 9 SRP had been published (see Figure 4, (Karkhoff-Schweizer 1995; Wagner 1998; Dahl 1993). Consequently, SRP from the environment could only be identified based on their *dsrAB* sequences if they were closely related to SRP already present in the *dsrAB* data bank. Other *dsrAB* sequences retrieved from the environment could either originate from novel SRP lineages or represent known SRP not yet sequenced on the *dsrAB* level.

Recent studies using the *dsr* approach in various environments suffered from this problem by retrieving deep-branching *dsr* sequences from a deep-sea marine worm (Cottrell 1999), a cyanobacterial mat (Minz 1999b), a consortia able to degrade hydrocarbons (Pérez-Jiménez 2001) and marine sediment (Thomsen 2001) which were not closely related to any so far recognized *dsrAB* reference sequence. Additionally, phylogenetic analyses conducted in these studies were further complicated by the use of very short *dsr* sequence stretches with very limited phylogenetic information.

A.8 The aims of this Ph.D. thesis

The presented Ph.D. thesis had tree major goals. Firstly, the *dsr* data bank had to be extended significantly. The second part of the work was dedicated to the thorough comparison of the 16S-rRNA gene based SRP phylogeny with the SRP phylogeny based on the Dsr sequences. This analysis should reveal possible lateral gene transfer events affecting the *dsrAB* genes and the implications for the evolutionary history of the dissimilatory sulfite reduction pathway. The third part of the work was the application of the *dsrAB* approach for SRP diversity surveys in several ecosystems. The presence and distribution of SRP was investigated in the water columns of Mariager Fjord (Denmark) and the hypersaline Solar Lake (Egypt). Furthermore, the sulfate reducing symbionts and their role in a complex symbiosis within a gutless marine worm were also successfully investigated with the help of *dsrAB* approach.

The following chapters give an overview on the materials and methods used and the results obtained. Further, a discussion of the most important results is presented. Detailed information on the conducted investigations can be obtained from the published or submitted manuscripts in the appendix.

B Material and Methods

The following chapter gives a short overview about the materials and methods used during my Ph.D. thesis. More details can be found in the respective sections of the manuscripts provided in the appendix.

B.1 Reference strains, and clone maintenance

Table 1 contains the sulfate reducing pure cultures from which the *dsrAB* genes were retrieved and used to build up the reference data bank.

Table 1 Sequenced reference strains with DSM numbers, lengths of the determined *dsrAB* fragments, Gene Bank accession numbers of fragments as determined by Klein.

Species	Nucleotides	Accession number of *dsrAB*	Strain
Archaeoglobus veneficus	1862	AF482452	DSM11195
Thermodesulfovibrio islandicus	1806	AF334599	DSM12570
Thermodesulfobacterium mobile	2040	AF334598	DSM1276
Desulfotomaculum acetoxidans	1956	AF271768	DSM771
Desulfotomaculum thermosapovorans	1934	AF271769	DSM6562
Desulfotomaculum thermoacetoxidans	1940	AF271770	DSM5813
Desulfotomaculum thermocisternum	1925	AF074396	DSM10259
Desulfotomaculum nigrificans	1807	AF482466	DSM574
Desulfotomaculum guttoideum (reclassified as *Clostridium guttoideum* Stackebrandt 1997b)	No *dsrAB* sequence could be obtained		DSM4024
Desulfoacinum infernum	1862	AF482454	DSM9756
Desulfoarculus baarsii	1899	AF3345600	DSM2075
Desulfobacterium oleovorans	1931	AF482464	DSM 6200
Desulfobacula phenolica	1950	AF551758	DSM3384
Desulfobacula toluolica	1930	AF271773*	DSM7467
Desulfobulbus propionicus	1929	AF218452	DSM 2032
Desulfofaba gelida	1941	AF334593	DSM12344
Desulfofustis glycolicus	1912	AF482457	DSM9705
Desulfohalobium retbaense	1944	AF482458	DSM5692
Desulfomicrobium apsheronum	1902	AF482459	DSM5918
Desulfomonas pigra	1902	AF482462	DSM749
Desulfomonile tiedjei	1908	AF334595	DSM6799
Desulforhopalus vacuolatus	1971	AF334594	DSM9700
Desulfosarcina variabilis	1925	AF191907	DSM2060
Desulfospira joergensenii	1719	AF482467	DSM10085
Desulfotignum balticum	1946	AF482463	DSM7044
Desulfovibrio africanus	1980	AF271772	DSM2603

Species	Nucleotides	Accession number of *dsrAB*	Strain
Desulfovibrio desulfuricans El Agheila Z	1967	AF334592	DSM 1926
Desulfovibrio halophilus	1941	AF482461	DSM5663
Desulfovirga adipica	1880	AF334591	DSM12016
sulfate-reducing strain oXyS1	1916	AF482465	DSM13228
Thermodesulforhabdus norvegica	1946	AF334597	DSM9990

* *Desulfobacula toluolica* was re-sequenced by Zverlov *et al.* 2004 see below.

Pure cultures were obtained from the German Type Culture Collection (DSMZ), either as lyophilized cells, or as actively growing cultures. If necessary, the reference strains were cultured according to the recommendations of the DSMZ (Braunschweig, Germany) using the anaerobic cultivation techniques described by Widdel and Bak (Widdel 1992b).

TOP10 competent *Escherichia coli* cells (TA/TOPO TA Cloning Kits, Invitrogen GmbH, Karlsruhe, Germany) hosting plasmids (pCR2.1-TOPO or pCR-XL-TOPO vectors, Invitrogen) which carry the *dsrAB* genes were stored in glycerin stocks (1 part 50 % glycerin and 2 parts overnight culture) at –80°C. LB-medium (5g NaCl, 5g yeast extract, and 10g casein per l H_2O, pH 7.0) containing 100 µg/l Kanamycin or Ampicillin, was used for overnight cultures, incubation was carried out at 37°C.

The *dsrAB* clones listed in Table 2, originally cloned by Michael Wagner (Technical University of Munich, Germany), Nicole Dubilier (MPI Bremen, Germany), and Ronen Nahary / Yehuda Cohen (Hebrew University of Jerusalem, Israel), were also sequenced during my Ph.D. thesis. Additionally, environmental *dsrAB* sequences from Mariager Fjord were also sequenced:

Table 2 Additional *dsrAB* gene fragments sequenced.

***dsrAB* source**	**Autor**	**Accession number**
Desulfovibrio sp. strain PT-2	Wagner *et al.* [11]	Partial sequence U58114, U58115 completed
Desulfovibrio oxyclinae	Wagner *et al.* [11]	Partial sequence U58116, U58117 completed
Desulfobotulus sapovorans	Wagner *et al.* [11]	Partial sequence U58120, U58121 completed
Desulfococcus multivorans	Wagner *et al.* [11]	Partial sequence U58126, U58127 completed

***dsrAB* source**	**Autor**	**Accession number**
Desulfotomaculum ruminis	Wagner *et al.* (Wagner 1998)	Partial sequence U58118, U58119 completed
29 Solar Lake water column clones	Ronen Nahary, Yehuda Cohen, Hebrew University of Jerusalem	Recloned and completely sequenced by Michael Klein
Thermodesulfovibrio yellowstonii	Wagner *et al.* (Wagner 1998)	Partial sequence U58122, U58123 completed
27 Mariager Fjord water column clones	see Results and Discussion Michael Klein Natuschka Lee, Technische Universität München	14 completely sequenced and 13 partial sequences (approx. 1600 bp)
Olavius algarvensis deltaproteobacterial symbiont	Dubilier *et al.* (Dubilier 2001)	Completely sequenced by M ichael Klein

B.2 DNA extraction from pure cultures and environmental samples

Cells from actively growing cultures or environmental samples were harvested by centrifugation. The pellet was resuspended and adjusted to a final volume of 200µl. Lyophilized cells were resuspended in lysis buffer. The actual cell lysis was done (i) mechanically by bead beating (FastPrep FP120 bead beater and the FastDNA^{TM} Kit MH, BIO101, CA), or (ii) chemically by alkaline/lysozyme lysis (e.g. DNeasy, Quiagene, Hilden, Germany). Extracted DNA was analyzed qualitatively by horizontal agarose gel electrophoresis (1.5 % agarose) and quantitatively by OD measurement at 260 nm (Sambrook 1989). Aliquots of DNA were stored at –20°C until needed.

B.3 Polymerase chain reaction amplification of *dsrAB* genes

An approx. 1.9 kb *dsrAB* segment was PCR amplified as described (Wagner 1998). Additional degeneracies inferred from recently published (Larsen 1999; Larsen 2000; Morse 2000; Larsen 2001) *dsrAB* operon sequence data were introduced into the previously published primers (Wagner 1998) DSR1F and DSR4R (see results). PCR reactions were carried out using 20 to 100 ng DNA, 15 to 50 pM of each primer, 200 µM deoxynucleoside- triposphates each, 20 mM $MgCl_2$, and 2U of Taq Polymerease (Promega). Oligonucleotide primers were obtained from MWG-Biotech AG (Munich, Germany) or INTERACTIVA, The Virtual Laboratory (Ulm, Germany). Amplification started with an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 15s, primer annealing at 54°C for 20s, and elongation at 72°C for 2 min. The reaction was completed by a final elongation step at 72°C for 10 min. The successful PCR amplification of the *dsrAB* fragment was checked by running amplificates on horizontal agarose gel electrophoresis (1 % agarose, staining after gel run in ethidium bromide as described (Sambrook 1989).

B.4 Molecular cloning of *dsrAB* genes into pCRTM2.1 or pCR-XL-TOPO vectors and identification of dsrAB carrying clones

In addition PCR products were screened on low melting Agarose gels (2% NuSieve 3:1, FMC Bioproducts), stained with SYBRgreen®I nucleic acid stain (Hanse Analytik GmbH, Bremen, Germany), and bands with the size of 1.9 kb were punched out of the gel with glass capillaries. Subsequently, the gel was removed form the capillaries and dissolved in 50 µl steril deionized water at 80 °C for 10 minutes. 4 µl of this solution were taken for cloning. Ligation, transformation and cultivation of clones were carried out according to the manufacturer instructions (invitrogen, TOPO-TA or TOPO-XL cloning kit). Recombinant clones were cultured over night in LB media in the presence of the respective antibiotic (ampicillin, kanamycin, see above) to prevent cells from loosing the plasmids. 4 ml overnight cultures were harvested by centrifugation. Pellet was used for plasmid extraction by QIAprep spin Plasmid Isolation Kit (Quiagen, Hilden, Germany). Correct size of inserted *dsrAB* gene fragment was verified by restriction digestion of the plasmid and subsequent horizontal Agarose (1.5 % Agarose) gel electrophoresis as described (Sambrook 1989).

B.5 Sequencing of cloned *dsrAB* gene fragments

Purified plasmid DNA was sequenced with a 4200L automated Li-Cor Long Reader DNA Sequencer (MWG, Ebersberg, Germany) according to the manufacturer instructions. If needed, internal primers were designed to completely sequence the *dsrAB* fragments.

B.6 Gelretardation

The method of gelretardation was optimized to separate equal sized DNA fragments with different sequence composition. Gelretardation is a special horizontal Agarose gel electrophoresis. A DNA binding dye is added to the molten Agarose (1 ppm dye) prior casting of the gel. Two different types of dye are available which preferential binding to A+T% (HA Yellow, Hanse Analytik, Bremen, Germany) or G+C% (HA Red, Hanse Analytik, Bremen, Germany) rich sequence motives of the DNA fragments. These dyes are coupled with polyethylene glycol (PEG). The additional molecular weight (PEG) bound sequence specifically to the DNA affects the gel migration quality of the DNA and allows the discrimination of sequences with ≥ 1% G+C content variation (Wawer 1995b; Schmid 2000); and see Results and Discussion, and Appendix).

B.7 Phylogenetic Analysis

Comparative sequence analyses were carried out on alignments of (i) the 16S rRNA genes and (ii) the amino acid sequences inferred from the *dsrAB* gene sequences. All investigations were carried out using the programs implemented into the phylogenetic inference package ARB (www.arb-home.de, Ludwig 2004). For comparative analysis of 16S rRNA gene based with DsrAB amino acid based dendrograms identical data sets were used to avoid sampling artifacts.

B.7.1 Pure culture databank

Pure culture dendrograms are based on 82 reference strains with more then 1400 nucleic acid residues of the *dsrAB* genes in good sequence quality (less then 3.5 % ambiguous sites).

B.7.2 Environmental databank

A data bank including *dsrAB* sequences of more then 424 amino acid residues, and 1400 nucleic acid residues, respectively, of good quality (less then 3.5 % ambiguous sites) has been prepared. This data set contained 86 described SRP species and 73 environmental clone sequences. Other sequences present in the data set originated from not described isolates or represented strains that have been sequenced more then once. The last sequences were included in order to make sure that all sequences from one organism yield the same *dsrAB* sequence. A table containing the examined sequences and Gene-Bank accession numbers are given in the appendix.

B.7.3 Alignment and phylogenetic analysis of 16rRNA gene data

16S rRNA gene sequences were aligned into the existing alignment using the ARB software package. The alignment was manually refined. Dendrograms were constructed using the implemented treeing tools in ARB. For all reconstruction methods filters were used to remove positions with less then 50% eubacterial sequence homology in order to minimize biases from highly variable regions of the 16S rRNA gene sequences. For constructing evolutionary distance (ED) trees Neighbor Joining (Saitou 1987) was used with the Jukes-Cantor correction (Jukes 1969). Further, Maximum Parsimony (MP) trees were reconstructed using the PHYLIP program package (Felsenstein 1993). Bootstrap resamplings of the MP trees were performed with 100 replicates. Maximum Likelihood (ML) dendrograms were calculated with help of the fastDNAml program (Olsen 1994) from the PHYLIP (Felsenstein 1993) software package. For ML analysis a representative collection of species was chosen and trees were calculated with smaller data sets in order to meet the high computing expense of the calculation. Tree topologies resulting from the different calculation methods were compared and consensus trees were constructed by introducing multifurcations into trees where the branching order was not confirmed by all methods. Partial sequences were added into trees calculated exclusively with complete reference sequences. Integration of the partial sequences in the tree was done with the Parsimony Interactive tool implemented in ARB.

B.7.4 Alignment and phylogenetic analysis of DsrAB data (amino acid based analysis)

For Dsr based phylogeny *dsrAB* gene sequences were imported into ARB and translated into amino acids. Novel sequences were manually aligned into the existing alignment by using conserved sequence motives as guidelines. Dendrograms were reconstructed using 543 amino acid residues (327 alpha subunit, 216 beta subunit) excluding regions with major insertions or deletions (indel). Additional filters were used to calculate separate DsrA or DsrB trees. ED amino acid trees were calculated using FITCH (Felsenstein 1993) with global rearrangement and the Dayhoff PAM matrix as the amino acid replacement model. Protein MP treeing was performed with the program Protpars (PHYLIP, Felsenstein 1993). ML amino acid trees were calculated with a reduced data set (as described above) with the PROTML v2.3b3 program (MOLPHY, Adachi 1996) with the JTT amino acid replacement model.

In order to compile as much information in a single dendrogram as possible consensus tress were reconstructed:
Consensus trees were constructed for both molecular marker molecules by comparing tree topologies of Distance Method based and Maximum Parsimony (100 bootstrap resamplings) based dendrograms. Multifurcations were added into the Distance tree manually where the branching order of the species did not correspond in both trees.

B.7.5 Calculation of Distance matrices

Distance matrices for 16S rRNA gene and DsrAB sequences were calculated with the Neighbor joining method (Saitou 1987). The same filters as for the tree reconstructions were used in order to gain comparable results and cut out unaligned sequence stretches between DsrA and DsrB.

B.7.6 Analysis of short environmental DsrAB sequence fragments

Definition of phylogenetic units based on DsrAB sequences

39 phylogenetic units were defined as (i) monophyletic groups or lineages (ii) with the identical phylogenetic branching order obtained with both treeing methods (ED and MP) and (iii) intra group sequence identities of 77% on DsrAB amino acid level as calculated by the program

Matrix (H. Daims, Technical University of Munich, Department of Microbiology, 2001). The identity value of 77% indicates in this context that each sequences in this group shares 77 % sequence identity at least with one other sequence within the this group. The value of 77% amino acid identity value has been observed to reflect phylogenetic relationships determined by comparative DsrAB sequence analysis in good accordance.

The evaluation of the exact phylogenetic position of partial DsrAB sequences can not as easily been carried out as with partial 16S rRNA gene sequence. No Parsimony method is available for the addition of partial DsrAB protein sequences to existing trees which were build from complete sequences without changing the overall tree topology.
In order to affiliate environmental *dsrAB* sequences to these groups, each short sequence was processed individually. The Parsimony interactive option implemented in ARB was used to add the single sequence into the parsimony tree, which was used for building up the consensus tree. Nucleic acid sequences were added by the Parsimony Interactive algorithm since amino acid sequences can not be processed.
Further, a Neighbor Joining tree was reconstructed based on the amino acid sequences of the single environmental sequence and all sequences present in the consensus tree. For the calculation the INDEL filter and the Kimura correction were used.
The phylogenetic position of the environmental sequence within the Parsimony tree and the Neighbor Joining tree was compared.
If the phylogenetic position in both trees matched, the environmental sequence was affiliated with the respective lineage. If no close relative of the sequence could be detected the result was interpreted as "could not be affiliated due to missing neighbor". If the phylogenetic position did not match in both trees, the result was interpreted as "sequence could not affiliate due to incoherent results of phylogenetic analyses".

B.7.7 Alignment of DsrA to DsrB and phylogenetic analysis of this databank

In a separated data set DsrA was manually aligned to DsrB to infer the root of the DsrA and DsrB sub trees (for details see appendix). All tree calculation methods were applied as described above.

B.7.8 Phylogenetic analysis of *dsrAB* gene sequences (nucleic acid based analysis)

Phylogenetic calculations were also carried out with *dsrAB* gene sequences. Filters were generated for omitting the third codon position and indel regions from the calculation. This analysis was performed on both, the *dsrAB* data set and on separate *dsrA* or *dsrB* data sets using suitable filters. ED, MP, and ML methods were used as for the 16S rRNA tree reconstructions.

B.8 Preparation of tissue of the *oligochaet Olavius algarvensis* for fluorescence in situ hybridization (FISH)

As part of my Ph.D. thesis sulfate-reducing symbionts of *Olavius algarvensis* were studied using the *dsrAB* approach. Additionally, FISH was used in order to confirm the results. Paraffin imbedded cryosections of *Olavius algarvensis* were sent by Nicole Dubilier (see publication in appendix).

All following steps were carried out at RT if not mentioned otherwise. First step of the preparation of the tissue for FISH was the paraffin removal from the slides by incubating the slides three times in 100 % xylol for 10 min. The sample was rehydrated by an ethanol series with 95%, 80% and, 70 % ethanol for 10 min each. Subsequently, incubation of the slides were carried out for 12 min with 0.2 M HCl, 10 min with 20 mM Tris/HCl, 5 min with Proteinase K (0.5 µg/ml in 20 mM Tris/HCl, pH 8) at 37 °C to remove part of the tissue to allow better probe access during the hybridization. After a washing step for 10 min with 20 mM Tris/HCl, the tissue was further fixated 5 min with 4 % formalin (in 20 mM Tris/HCl, pH 8), and finally washed again for 10 min with 20 mM Tris/HCl. The slides were air dried and dehydrated with an ethanol series (50, 80, and 96 %) 1 min each.

B.9 Fluorescence in situ hybridization

FISH was carried out as described (Amann 1995a; Manz 1998; Wagner 1993). Fixed cells were spotted on slides, dried, and subsequently dehydrated in 50%, 80% and 98% ethanol for 5 minutes each.

All hybridizations were carried out at 46 °C for 90 min, followed by a stringent wash step at 48 °C for 10 min. The 23S rRNA and 16S rRNA targeted oligonucleotide probes were used at a concentration of 30 ng/µl. Probes labeled in Cy3, Cy5 and Fluos were ordered from MWG-

Biotech AG (Munich, Germany) or INTERACTIVA, The Virtual Laboratory (Ulm, Germany). In some experiments, the DNA-binding dye 4',6-diamino-2-phenylindole (DAPI; Sigma, Buchs, Switzerland) was used for the visualization all nucleic acids containing cells. In order to prevent bleaching effects during the microscopic examination cells and tissue was covered with Citifluor-AF1 (Citifluor Ltd, London) (10 min) prior to detection. The signals were recorded with a confocal laser-scanning microscope CLSM LSM510 (Zeiss, Germany). An Argon laser (430-514 nm), two Helium laser (543 nm and 633 nm), and an UV laser (351-364 nm) were used. Digital images were processed with the Zeiss CLSM software (Version 2.01 SP2).

C Results and Discussion

C.1 Establishment of reference strain DsrAB databank

The backbone of every environmental SRP diversity survey employing the Dsr-approach is the reference data bank containing the *dsrAB* sequences of SRP pure cultures. Only if all recognized SRP lineages are represented in this data base it is possible to decide whether a novel *dsrAB* sequence obtained from an environmental sample indeed indicates the existence of a novel SRP lineage. In 1998 only 9 pure culture *dsrAB* (partial) sequences were published. In order to providing a suitable sequence background for further environmental studies the existing data base was extended by 34 novel *dsrAB* sequences from members of various sulfate reducing lineages in this study.

Today, at least 91 *dsrAB* sequences from pure culture SRP and two *dsrAB* sequences of sulfite reducing bacteria, all with a minimum length of 1700 base pairs are publicly available. Seven entire *dsr* operon sequences are available at present (see Table 3 and publications cited within).

C.1.1 Reevaluation of PCR primers for amplification of *dsrAB* gene fragments

Since the primer pair DSR1F and DSR4R was designed only on an alignment of the *dsr* sequences of *Archaeoglobus fulgidus* and *Desulfovibrio vulgaris* (Karkhoff-Schweizer 1995; Wagner 1998) reevaluation of the specificity and target range of this primer pair became possible and necessary with the publication of additional *dsr* operon sequences (Larsen 1999; Larsen 2000; Larsen 2001). Inspection of the primer target sites in the different operon sequences revealed that additional degeneracies (DSR1Fa and DSR1Fb, DSR4Ra to DSR4Rc) had to be introduced in the original primer sequences to achieve full match between the primers and the novel sequences (this thesis). The mixture of all these primer variations does fully match the *dsr* operon sequences of *Archaeoglobus fulgidus, Archaeoglobus profundus, Desulfotomaculum thermocisternum, Desulfobacter vibrioformis, Desulfobulbus rhabdoformis, Thermodesulforhabdus norvegica, Desulfovibrio vulgaris* and the *dsrAB* sequence of *Desulfovibrio desulfuricans* (Table 3).

Table 3 Modifications of DSR1F and DSR4R primers based on *dsr* operons / *dsrAB* sequences published after 1997.

Forward Primer	**Sequence 5‘ → 3‘**	**Comment**
DSR1F	AC(GC) CAC TGG AAG CAC G	(Wagner 1998) targeting *Archaeoglobus fulgidus, Desulfovibrio vulgaris* and *Archaeoglobus profundus* (Larsen 1999)
DSR1F a	ACC CA(CT) TGG AAA CAC G	DSR1F modified according to (Larsen 1999; Larsen 2000) targeting *Desulfobacter vibrioformis, Desulfobulbus rhabdoformis* and *Desulfotomaculum thermocisternum* and *Desulfovibrio desulfuricans* (Morse 2000)
DSR1F b	GGC CAC TGG AAG CAC G	DSR1F modified according to operon sequence of *Thermodesulforhabdus norvegica* (AJ277293)(Larsen 2001)
DSR1Fconsensus	(AG)(CG)(GC)CA(CT)TGGAA(AG)CACG	all published *dsr* operons
Reverse Primer	**Sequence 5‘ → 3‘**	**Comment**
DSR4R	GTG TAG CAG TTA CCG CA	(Wagner 1998) targeting *Archaeoglobus fulgidus, Desulfovibrio vulgaris* and *Desulfobulbus rhabdoformis*(Larsen 2000)
DSR4R a	GTG TAA CAG TTT CCA CA	DSR4R modified according to *Archaeoglobus profundus* (Larsen 1999)
DSR4R b	GTG TAA CAG TTA CCG CA	DSR4R modified according to operon sequence of *Desulfobacter vibrioformis* (Larsen 2000)
DSR4R c	GTG TAG CAG TTT CCG CA	DSR4R modified according to operon sequence of *Desulfotomaculum thermocisternum* (Larsen 1999), the operon sequence of *Thermodesulforhabdus norvegica* (AJ277293) (Larsen 2001) and the *dsrAB* sequences of *Desulfovibrio desulfuricans* (Morse 2000)
DSR4Rconsensus	GTGTA(GA)CAGTT(AT)CC(AG)CA	All published *dsr* operons

In order to retrieve *dsrAB* gene sequences from novel reference strains the above listed primers were used in a hierarchical approach, beginning with DSR1F/DSR4R. If amplification failed, an equimolar mixture of the seven primer variations described above was applied. With this primer combination, *dsrAB* gene fragments of all reference strains could be amplified. The only exception was *Desulfotomaculum guttoideum*. No *dsrAB* amplificat could be obtained for this microorganism. *Desulfotomaculum guttoideum* is, according to recent published data, misclassified and should be reclassified as *Clostridium* specie (Stackebrandt 1997b). Thus, the primer combinations can be regarded as applicable. Full length sequences of the amplified approx. 1.9-kb gene fragments were determined with help of species or group specific internal sequencing primers, or by restriction digestion and subcloning of the *dsrAB* gene fragments.

Table 4 Sulfate reducing reference strains within the *dsrAB* data set, species printed in bold were sequenced by M.Klein

Archaea
- *Euryarchaeota, Archaeoglobi, Archaeoglobales, Archaeoglobaceae*
  - *Archaeoglobus veneficus*

Bacteria
- *Nitrospira* division
  - *Thermodesulfovibrio islandicus*
- *Thermodesulfobacterium* division
  - *Thermodesulfobacterium mobile*
- *Firmicutes, Bacillus/Clostridium* group (Gram-positives)
  - *Desulfotomaculum acetoxidans*
  - *Desulfotomaculum thermosapovorans*
  - *Desulfotomaculum thermoacetoxidans*
  - *Desulfotomaculum thermocisternum*
  - *Desulfotomaculum nigrificans*
- *Proteobacteria*, delta subdivision
  - *Desulfoacinum infernum*
  - *Desulfoarculus baarsii*
  - *Desulfobacca acetoxidans*
  - *Desulfobacterium oleovorans*
  - *Desulfobacula phenolica*
  - *Desulfobacula toluolica*
  - *Desulfobulbus propionicus*
  - *Desulfofaba gelida*
  - *Desulfofustis glycolicus*
  - *Desulfohalobium retbaense*
  - *Desulfomicrobium apsheronum*
  - *Desulfomonas pigra*
  - *Desulfomonile tiedjei*
  - *Desulforhopalus vacuolatus*
  - *Desulfosarcina variabilis*
  - *Desulfospira joergensenii*
  - *Desulfotignum balticum*
  - *Desulfovibrio africanus*
  - *Desulfovibrio desulfuricans* El Agheila Z
  - *Desulfovibrio halophilus*
  - *Desulfovirga adipica*
  - Sulfate-reducing strain oXyS1
  - *Thermodesulforhabdus norvegica*

Resulting *dsrAB* sequences were aligned manually into the existing alignment and translated into DsrAB sequences for further analysis.

C.1.2 Inconsistencies between Edman degradation/ inference from nucleotide sequencing

For some reference species Edman degradation had been carried out to infer partial DsrA and DsrB protein sequences (Hatchikian 1983; Fauque 1990; Steuber 1995; Morse 2000). The deduced amino acid sequences of the PCR amplified *dsr* genes were compared to the respective resulting amino acid sequence fragments predicted by Edman degradation (Figure 6).

Thermodesulfobacterium commune strain YSRA-1 (according to this study and (Hatchikian 1983):	
N-terminus of DsrB deduced from nucleotide sequencing	G IEKFKELDP
N-terminus of DsrB deduced from Edman degradation	T/S IEKFKELDP
Thermodesulfobacterium mobile DSM 1276 (according to this study and (Fauque 1990):	
N-terminus of DsrB deduced from nucleotide sequencing	G IEKFK
N-terminus of DsrB deduced from Edman degradation	G IEKFK
Desulfovibrio desulfuricans strain Essex 6 (according (Steuber 1995) and (Morse 2000):	
N-terminus of DsrB deduced from nucleotide sequencing	A FISSGYNP
N-terminus of DsrB deduced from Edman degradation	A FIPTGYNP

Figure 6 N-terminal sequences from the dissimilatory sulfite reductase beta subunit as determined by nucleotide sequencing and Edman degradation, respectively.

For *Thermodesulfobacterium commune* and *Desulfovibrio desulfuricans* (Morse 2000), the sequence determined by Edman degradation did not fully match the respective DsrB sequence fragment inferred from gene sequencing. This inconsistency can either be explained by Taq polymerase induced nucleotide sequence errors (two nucleotide changes in close neighborhood) or erroneous results from Edman degradation. Another theoretical and very unlikely explanation could be that in the respective organisms' changes in the genetic code occurred. It is known that some organisms like *Candida albicans* or *Mycoplasma genitatlium* have altered genetic codes (O'Sullivan 2001 and citations within).

However, sense to sense changes in the genetic code are very rare (O'Sullivan 2001) and only one well-established example is known. In asoporogenic yeast *Candida cylindracea* (Kawaguchi 1989) CUG codes for serine instead of leucin. Most cases of codon reassignment in bacteria lead to formation of additional stop codons as shown for *Micrococcus* spp. and *Mycoplasma* spp.(O'Sullivan 2001) and citations within), where in *Micrococcus* AGA functions as stop codon and does not code for arginine and CGG functions as stop codon and does not code for arginine in *Mycoplasma*.

Additionally, in case of *Thermodesulfobacterium mobile*, the amino acid sequences inferred from the nucleic acid sequence and by Edman degradation, respectively, match completely. These findings lead to the conclusion that codon reassignment is not the cause for the inconsistencies between the sequences found.

Another possible explanation would be the presence of multiple *dsrAB* operons within these organisms. If the *dsrAB* sequences had been derived from the less expressed operon and the Edman degradations were carried out on proteins synthesized from the higher expressed operon the predicted amino acid sequence form translation could not match the amino acid sequence by Edman degradation.

C.1.3 DsrAB sequence alignment and characteristic motives

The present data set, including also sequences from other authors, covers now all known SRP containing divisions and even the majority of sulfate reducing lineages within the *Deltaproteobacteria*. All inferred DsrAB sequences contained the complete $[Fe_4S_4]$-sirohaem binding site motif (Cys-X_5-Cys)-X_n-(Cys-X_3-Cys) (Crane 1995), as well as the Cys-Pro and Cys-X_2-Cys- X_2-Cys motif required for linking $[Fe_4S_4]$ clusters (Dahl 1993) in the alpha subunit (see also Klein 2001). The sirohaem-binding motif is truncated in the beta subunit as it is also known for other dissimilatory sulfite reductases (Hipp 1997) while the $[Fe_4S_4]$ linking clusters of the beta subunits are not amplified with the applied primer pairs.

Sequence insertions and deletions can be identified in the alignment as additional /missing bases or sequence stretches restricted to certain lineages. The insertion/deletion (indel) events should be considered as a single mutation event and not as a number of independent mutations events corresponding to the number of bases (Gupta 1998). Thus, the regions of insertions and deletion events were removed prior to phylogenetic analyses leaving 543 amino acid residues out of approx. 625 for calculation.

The difficulties observed during the inference of phylogeny (Ludwig 1998) of partial environmental sequences (see below) led to the conclusion that it is essential to determine at least the complete sequences of one representative of each environmental SRP lineage. Only this approach allows to unequivocally determining the phylogenetic position of the environmental sequence cluster.

C.1.4 Conservation profile of DsrAB

The first investigation was carried out to gain knowledge on the global conservation of the DsrAB sequence. Conservation values were calculated in ARB and exported to Excel. Values were blotted for each amino acid residue on the y-axis (Figure 7). This figure is based on a data set of 86 reference strains on the x-axis (see Table in appendix) which were also used for the calculation of the environmental consensus dendrogram (see below).

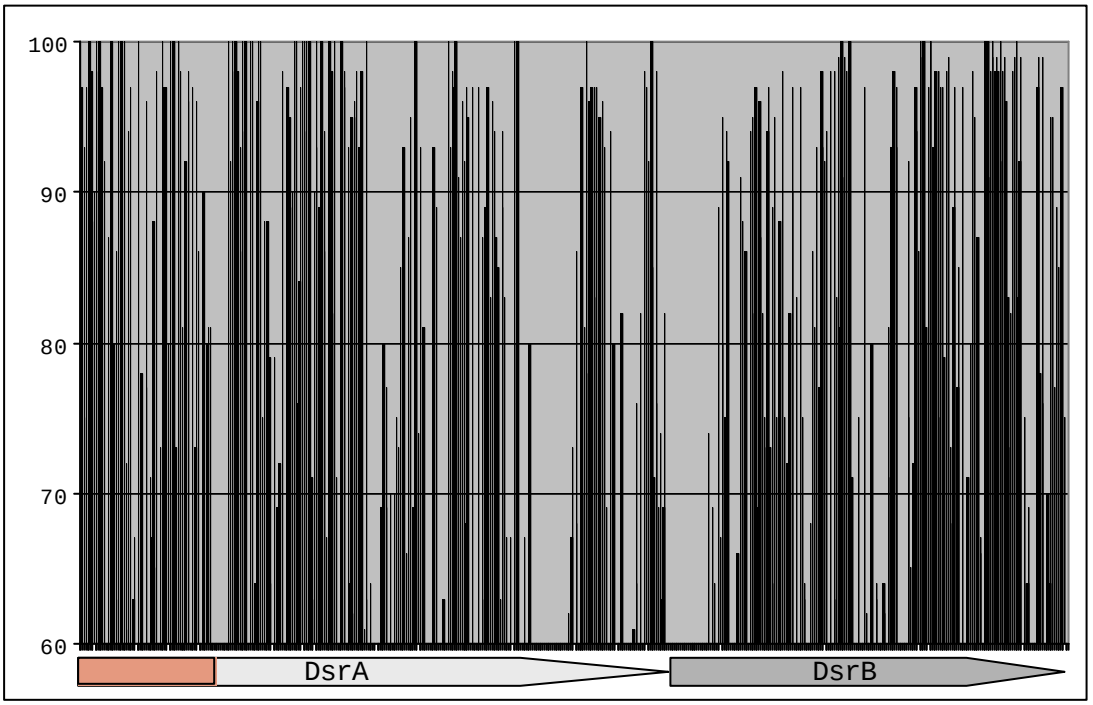


Figure 7 Conservation blot of DsrA and DsrB sequence, x axis amino acid residues, y axis % conservation as calculated in ARB, arrows indicate Dsr subunits, hatched part of DsrA which was used for phylogenetic analysis by Cottrell *et al.*1999.

The level of conservation was calculated with the tool implemented in ARB (ARB_NT/SAI/Functions: Create SAI from Sequence/Filter by base frequency). The non-coding sequence region between *dsrA* and *dsrB* was removed from the alignment prior to calculation. Only conservation values above 60 were considered. In the alignment four different character

states have to be distinguished. The character states can be (i) a known amino acid, (ii) an unknown amino acid, or (iii) an insertion, or (iv) no information is available on this alignment position. State (i) contains all amino acids, including stop codons. The second state is represented by an X since the coding region of this amino acid is ambiguous and a correct translation is not possible. Concerning character state (iii), the insertions were treated as other bases, since insertions do contain phylogenetic information as it they are also subjects of evolutionary changes (Gupta 1998). The last character state, alignment positions of unknown character state were ignored for the calculation of the conservation profile.

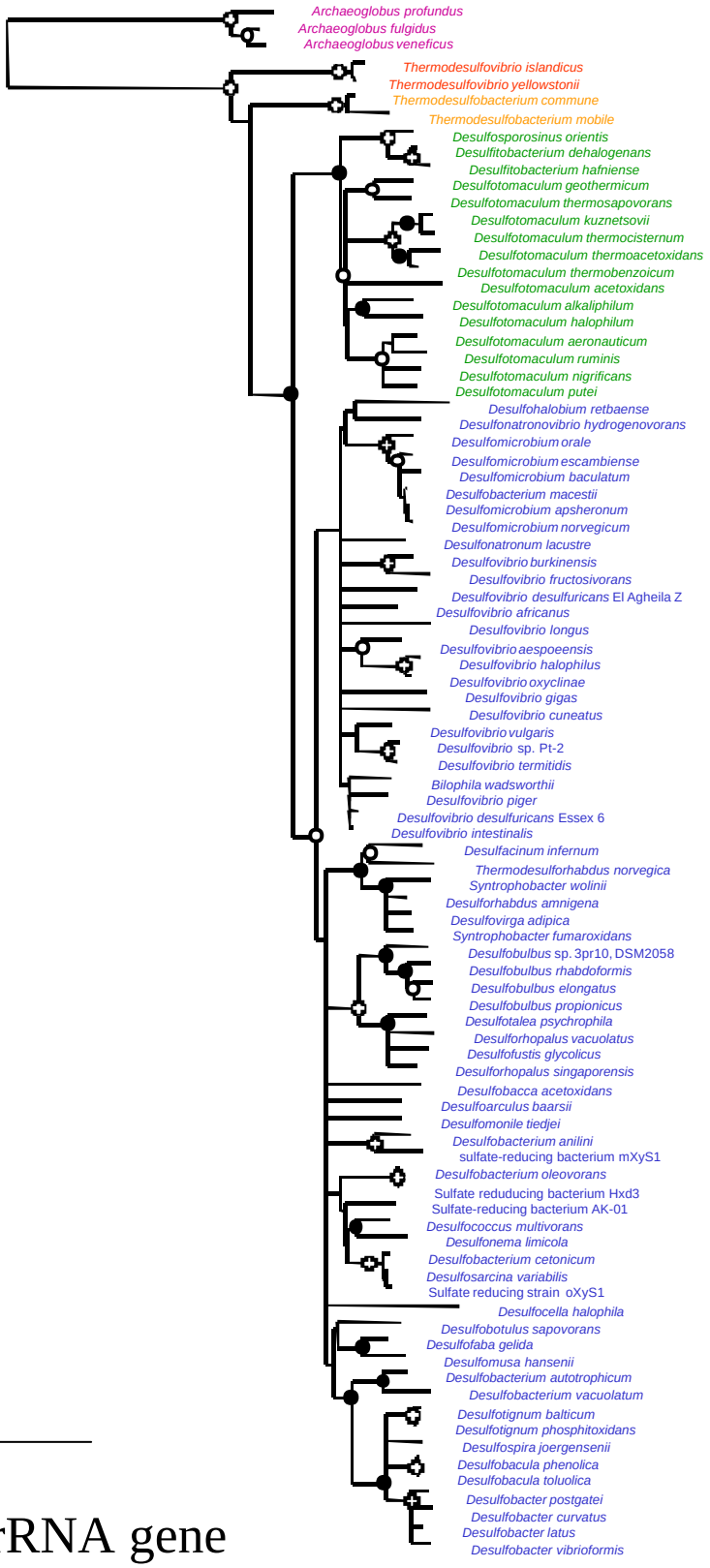
In the case of the DsrAB sequences 59 out of 654 considered sites were 100% conserved. This equals 9% of all sites examined. Cottrell *et al.* only used the hatched sequence part of DsrA for phylogenetic analyses (Cottrell 1999). Within the hatched part even 16% of the amino acid positions were 100% conserved, and 40 % were conserved in more than 90% of the sequences, limiting the phylogenetic information content significantly. Thus, phylogenetic analysis should be carried out at least with the entire DsrA fragment (see also Chapter Analysis of short environmental sequences) but most reliable data will be obtained by analyzing the entire DsrAB fragment.

C.2 Comparison of 16S rRNA and DsrAB-based phylogeny for SRP reference strains

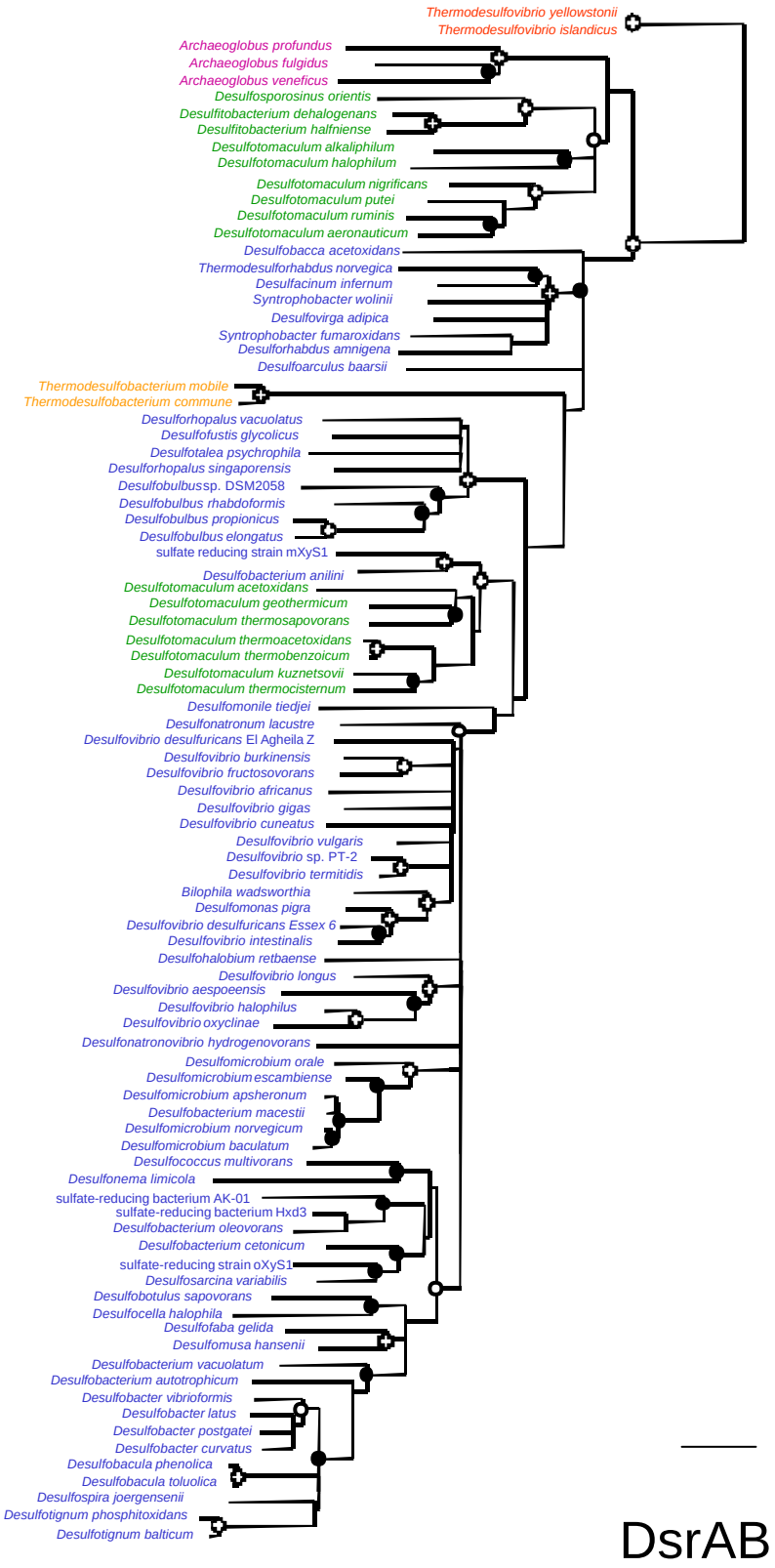
C.2.1 Comparative analysis of 16S rRNA gene and DsrAB sequence based phylogeny

Comparative phylogenetic analyses were carried out on 16S rRNA gene and DsrAB databases. In total 91 SRP pure culture sequences of good sequence quality, being present in both databases, were used to calculate Distance method based and Parsimony method based dendrograms on 16S rRNA / DsrAB gene sequences, respectively. The deduced consensus dendrograms are presented in Figure 8. Comparison of the resulting dendrograms revealed consistent, as well as inconsistent branching orders.

Figure 8 on the following pages: Deduced consensus tree based on comparative 16S rRNA gene sequence and DsrAB sequence analysis. Bar indicates 10 percent estimated sequence deviation. phylogenetic groups are color coded: *Thermodesulfovibrio* red, *Archaeoglobus* magenta, *Thermodesulfobacterium* yellow, Gram-Positives (*Desulfotomaculum*, *Desulfitobacterium*, *Desulfosporosinus*) green, and *Deltaproteobacteria* blue; parsimony bootstrap support of the respective branch is indicated by open cycles for bootstrap support above 75, closed above 90, and cross 100.



16S rRNA gene


Thermodesulfovibrio yellowstonii
Thermodesulfovibrio islandicus
Archaeoglobus profundus
Archaeoglobus fulgidus
Archaeoglobus veneficus
Desulfosporosinus orientis
Desulfitobacterium dehalogenans
Desulfitobacterium halfniense
Desulfotomaculum alkaliphilum
Desulfotomaculum halophilum
Desulfotomaculum nigrificans
Desulfotomaculum putei
Desulfotomaculum ruminis
Desulfotomaculum aeronauticum
Desulfobacca acetoxidans
Thermodesulforhabdus norvegica
Desulfacinum infernum
Syntrophobacter wolinii
Desulfovirga adipica
Syntrophobacter fumaroxidans
Desulforhabdus amnigena
Desulfoarculus baarsii
Thermodesulfobacterium mobile
Thermodesulfobacterium commune
Desulforhopalus vacuolatus
Desulfofustis glycolicus
Desulfotalea psychrophila
Desulforhopalus singaporensis
Desulfobulbus sp. DSM2058
Desulfobulbus rhabdoformis
Desulfobulbus propionicus
Desulfobulbus elongatus
sulfate reducing strain mXyS1
Desulfobacterium anilini
Desulfotomaculum acetoxidans
Desulfotomaculum geothermicum
Desulfotomaculum thermosapovorans
Desulfotomaculum thermoacetoxidans
Desulfotomaculum thermobenzoicum
Desulfotomaculum kuznetsovii
Desulfotomaculum thermocisternum
Desulfomonile tiedjei
Desulfonatronum lacustre
Desulfovibrio desulfuricans El Agheila Z
Desulfovibrio burkinensis
Desulfovibrio fructosovorans
Desulfovibrio africanus
Desulfovibrio gigas
Desulfovibrio cuneatus
Desulfovibrio vulgaris
Desulfovibrio sp. PT-2
Desulfovibrio termitidis
Bilophila wadsworthia
Desulfomonas pigra
Desulfovibrio desulfuricans Essex 6
Desulfovibrio intestinalis
Desulfohalobium retbaense
Desulfovibrio longus
Desulfovibrio aespoeensis
Desulfovibrio halophilus
Desulfovibrio oxyclinae
Desulfonatronovibrio hydrogenovorans
Desulfomicrobium orale
Desulfomicrobium escambiense
Desulfomicrobium apsheronum
Desulfobacterium macestii
Desulfomicrobium norvegicum
Desulfomicrobium baculatum
Desulfococcus multivorans
Desulfonema limicola
sulfate-reducing bacterium AK-01
sulfate-reducing bacterium Hxd3
Desulfobacterium oleovorans
Desulfobacterium cetonicum
sulfate-reducing strain oXyS1
Desulfosarcina variabilis
Desulfobotulus sapovorans
Desulfocella halophila
Desulfofaba gelida
Desulfomusa hansenii
Desulfobacterium vacuolatum
Desulfobacterium autotrophicum
Desulfobacter vibrioformis
Desulfobacter latus
Desulfobacter postgatei
Desulfobacter curvatus
Desulfobacula phenolica
Desulfobacula toluolica
Desulfospira joergensenii
Desulfotignum phosphitoxidans
Desulfotignum balticum
DsrAB

In general, recognized families of SRP formed distinct lineages in both consensus trees. Exceptions of this finding were observed for the genus *Desulfovibrionaceae* and *Desulfotomaculum*.
Furthermore, different branching orders were observed for the genera *Archaeoglobus*, *Thermodesulfovibrio*, and *Thermodesulfobacterium*.

The exact branching order within the *Desulfovibrionaceae* is not clearly resolved, neither by 16S rRNA gene based, nor by DsrAB sequence based phylogeny. The formation of the lineage containing the genera *Desulfovibrio* and *Bilophila* is only very weakly supported by bootstrap values on 16S rRNA gene sequence level and is even less supported when examining DsrAB based dendrograms. The branching order within this family is also dependent on the tree calculation method applied.

In contradiction to the 16S rRNA gene sequence based dendrogram the genus *Desulfotomaculum* formed two polyphyletic groupings within the DsrAB based dendrograms. For the following discussion the nomenclature, as described recently by Stackebrandt *et al.* (Stackebrandt 1997a), is used. Stackebrandt *et al.* defined several subgroups within the *Desulfotomaculum* family based on 16S rRNA gene sequence data. Recently, more sequences have been added to this classification system (Kuever 1999; Pikuta 2000). Accordingly, cluster Ia contains *Desulfotomaculum nigrificans, Desulfotomaculum putei, Desulfotomaculum aeronauticum, and Desulfotomaculum ruminis*. Cluster Ib consists of *Desulfotomaculum thermosapovorans* and *Desulfotomaculum geothermicum*, cluster Ic *Desulfotomaculum thermocisternum, Desulfotomaculum kuznetsovii*, and *Desulfotomaculum luciae* (specie not in tree, short sequence). Cluster Id contains *Desulfotomaculum thermoacetoxidans* and *Desulfotomaculum thermobenzoicum*. *Desulfotomaculum acetoxidans* is the only recognized member of cluster Ie. Additionally, a novel cluster If was defined containing *Desulfotomaculum alkaliphilum* and *Desulfotomaculum halophilum* (Kuever 1999; Pikuta 2000).
Subcluster Ia and If showed consistent phylogenetic positioning in both dendrograms. Subcluster Ia and If were monophyletic with the other Gram-Positive sulfate and sulfite reducers *Desulfosporosinus* and *Desulfitobacterium*, respectively. This branching was supported with a strong bootstrap value above 90% within the 16S rRNA gene based dendrogram, and a bootstrap value above 75% within the DsrAB sequence based dendrogram. In contrast subclusters Ib to Id were monophyletic with the other *Desulfotomaculum* species when analyzing 16S rRNA data, but formed a monophyletic cluster within the *Deltaproteobacteria* when analyzing DsrAB

sequence data. The DsrAB sequences of this cluster branched robustly with the deltaproteobacterial DsrAB sequences of *Desulfobacterium anilini* and strain mXyS1. Amino acid sequence identities of DsrAB between members of the *Desulfotomaculum* cluster Ib to Id and *Desulfobacterium aniline*/strain mXyS1 ranged between 73% and 83%. In comparison to this *Desulfobacterium anilini* and strain mXyS1 share an amino acid identity of 87%.

As member of the domain *Archaea* the genus *Archaeoglobus* represents the deepest branching lineage within the 16S rRNA gene based dendrogram. Within the DsrAB based dendrogram the genus *Thermodesulfovibrio* showed the longest branch and should therefore be considered the deepest branching lineage. This finding is also supported by paralogous rooting (Klein 2001). The alignment of DsrA to DsrB is possible since both subunits originate from a common ancestor. The resulting dendrogram presented *Thermodesulfovibrio* as the deepest branching lineages in both subunit dendrograms.

Thermodesulfobacterium commune and Thermodesulfobacterium mobile did not branch deeply within the DsrAB based dendrogram, as it would be expected from 16S rRNA phylogeny. Their branching within the *Deltaproteobacteria* was clearly confirmed by all phylogenetic analyses.

These inconsistencies could theoretically result from (i) lateral gene transfer of 16S rRNA genes, (ii) or lateral gene transfer of *dsrAB* genes. Although the existence of lateral gene transfer of rRNA genes is very contentious, a few lateral gene transfer events of 16S rRNA gene genes have been described (Stratz 1996; Yap 1999). Since the ribosomal RNA operon is an integral component of the information system of cells, i.e. tightly interwoven into transcription and translation processes, the complexity theory suggest a low likelihood of lateral exchange of 16S rRNA gene operons (Jain 1999).

Lateral transfer of genes involved in energy generation is more likely and has been observed in various studies (among many others: Herrick 1997; Jain 1999; Nelson 1999; Garcia-Vallve 2000; Nesbø 2001). Therefore, the deviation between the 16S rRNA gene and DsrAB sequence based phylogeny of the branching orders of (i) *Archaeoglobus and Thermodesulfovibrio*, (ii) *Thermodesulfobacterium*, and (ii) members of the *Desulfotomaculum* can most parsimoniously be explained by such an inter-species/ inter-domain lateral transfer of *dsr* genes and/or *dsr* operons, respectively.

The three cases of potential lateral gene transfer are considered separately. Two cases share certain DsrAB sequence characteristics, which support the lateral gene transfer from a deltaproteobacterial donor to Gram-Positive *Desulfotomaculum* strains, and *Thermodesulfobacterium* as acceptors. The most important means of detecting lateral gene transfer is comparative sequence analysis of several marker molecules. For the SRP the deviating phylogenetic position determined by DsrAB sequence comparison of the strains described above from their 16S rRNA phylogenetic positioning hints at lateral gene transfer.

Insertions and deletions within the DsrAB amino acid sequences (excluded in the phylogenetic analyses) were investigated as additional signposts of lateral gene transfer events (Gupta 1998). In total, three insertions were unique to the *Deltaproteobacteria*: one in the alpha subunit and two in the beta subunit (Fig. 3, Klein 2001). These insertions were also found in the Deltaproteobacterium-like DsrAB sequences of the seven *Desulfotomaculum* species, and two *Thermodesulfobacterium* species, thus supporting the suggested lateral transfers. These insertions were missing in *Archaeoglobus*, supporting that its *dsrAB* genes were not acquired from deltaproteobacterial SRP.

It appears likely that the dissimilatory sulfite reductases of the *Archaeoglobales* originate from a bacterial donor, because the evolutionary distance between *Archaeoglobus* species and the bacterial sulfate reducers is much shorter in the DsrAB tree than in the 16S rRNA tree. Consistent with this hypothesis, the sulfate-reducing phenotype is currently restricted to the genus *Archaeoglobus* within the archaeal domain. Further support for a lateral transfer of bacterial *dsrAB* genes to the *Archaeoglobales* was obtained by phylogenetic analysis of an alignment of DsrA against the DsrB amino acid sequences. Such analysis can be used to root the Dsr subunit trees (Gogarten 1989; Iwabe 1989, and see Fig. 2 in reference Klein 2001 for details), since the subunits are paralogs that arose from ancestral *dsr* gene duplication (Dahl 1993). Independent of the treeing method used the root of the Dsr trees was consistently indicated between *Thermodesulfovibrio* species and all other analyzed SRP, including the *Archaeoglobales*.

C.2.2 Further analysis on *dsrAB* originating from *Desulfobacula toluolica*

After the detection of multiple lateral transfers affecting the *dsrAB* genes (Klein 2001) we decided to examine the most recent transfer event in more detail. According to our data *Desulfobacula toluolica* received its *dsr* operon during the latest transfer event so far detected (Klein 2001).

Remark:

In respect of the results presented below, the position of *Desulfobacula toluolica* DsrAB sequence, as determined by Klein *et al*. 2001, between *Desulfobacterium anilini* and strain mXyS1, is not shown in the dendrograms presented above. The alleged *Desulfobacula toluolica* DsrAB sequence has been removed and replaced by a novel, confirmed sequence next to *Desulfobacula phenolica* (see below).

Genetic traces of transfer mechanisms, if existing, should be present upstream or downstream of the xenologous *dsr* operon acquired during the latest transfer event.

Details on work performed by M. Klein for the section C.2.2 see appendix. For methodotical details see Zverlov *et al.* 2004 (submitted to Journal of Bacteriology, May, 2004) also in the appendix.

In order to verify the phylogenetic position of the *dsrAB* genes of *Desulfobacula toluolica* and to reveal the transfer mechanism cell material of *Desulfobacula toluolica* and its closest 16S rRNA neighbor *Desulfobacula phenolica* was obtained from the DSMZ, or cultivated, respectively. Additionally, *Desulfobacter latus* was cultivated as a control. High quality DNA was extracted in high concentrations of all three organisms by lysozym treatment, chloroform extraction, and subsequent isopropanol precipitation of aqueous phase.

A digoxigenin- labelled *dsrA*-targeted polynucleotide probe was generated by PCR amplification of a *dsrA* gene fragment of *Desulfobacula toluolica*. In the PCR reaction the degenerated primer pair DsrA415F (5´-TAT CA(AG) GAT GAG CT(GT) CAT CG(CT) CC-3´) and DsrA542R (5´-AC(CT) GC(AGT) TCC TGA TCA AT(AGC) CGG ATA T-3´) was used for amplification of a 152 bp long DNA fragment.

During the PCR reactions approx. 25% of dTTPs were replaced by DIG labelled dUTPs. The resulting polynucleotide probe was used in Southern blot hybridization experiments. DNA extracts from the cultures described above were digested with different enzymes (see Figure 8) and subsequently blotted and hybridized. *dsr* like sequences were detected by the probe described above in all three species (data only shown for *Desulfobacula toluolica* in Figure 8). Visualization was carried out by colorimetric detection using anti- digoxigenin antibodies tagged with alkaline phosphatase and substrate NBT/BCIP.

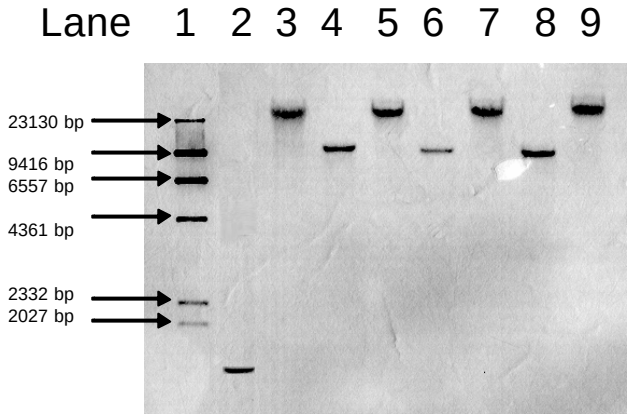


Figure 8 Southern blot hybridization of *Desulfobacula toluolica* DNA with polynucleotide probes (see above), Lane 1 l Hind III digested, Lanes 2 to 9 digested *Desulfobacula toluolica* DNA with the following enzymes EcoRI (2), BamHI (3), HindIII (4), KpnI (5), Pst1 (6), Sal1 (7), Sma1 (8), Xba1 (9).

All enzymes, except EcoRI (Lane 2 in Figure 8), posses a single restriction site within *dsrAB*. Thus, DNA fragments resulting from these restriction digestions are rather big in contrast to the fragment resulting form EcoRI digestions. The *dsrAB* gene fragment harbors two restriction sites for EcoRI. The southern blot of DNA fragements from *Desulfobacula toluolica* showed that this organism carries a singe *dsrA* copy in its genome.

For preparation of a λ - library DNA of *Desulfobacula toluolica* was partially digested with MboI (isochizomer of Sau3A), and ligated into a λBlueStar™ Vector. *dsrA* containing plaques were identified by plaque hybridization (Sambrook 1989) with the DIG labelled 152 bp polynucleotide probe described above. *dsrA*- containing λBlueStar™ Vectors were subjected to Cre recombinase- mediated excision of plasmid. Plasmids form *E. coli* BM25.8 cells were recovered and transformed into *E. coli* DH5 α. From six clones overlapping fragments were sequenced from purified plasminds form *E coli* DH5α cells. An 8868 kb long sequence stretch

containing the *dsr* operon and flanking regions was obtained by primer walking (Gene Bank accession number: AJ457136).

Open reading frames were identified and compared to genes in public databases by BLAST search (Altschul 1990) (see figure below).

Figure 9 Organization of Dsr operon, Dsr Dissimilatory sulfite reductase subunit A, B, D and N

The operon structure of *dsrA, dsrB, dsrD,* and *dsrN* was compared to the *dsr* operons of *Archaeoglobus fulgidus, Archaeoglobus profundus, Desulfovibrio vulgaris, Desulfotomaculum thermocisternum, Desulfobulbus rhabdoformis, Desulfobacter vibrioformis, Thermodesulforhabdus norvegica, Desulfovibrio desulfuricans,* and *Bilophila wadsworthia*. The operon organization with the succession of *dsrA, dsrB, dsrD,* and *dsrN* matched the operon organization of *Desulfovibrio vulgaris,* and *Desulfobacter vibrioformis*. This finding is in good accordance with the phylogenetic position of the DsrAB sequence of *Desulfobacula toluolica* close to *Desulfobacter vibrioformis.*

The sequence of the novel *dsrAB* fragment from *Desulfobacula toluolica* was aligned to the previously determined *dsrAB* sequence of *Desulfobacula toluolica* (Klein 2001). Surprisingly, both sequences were highly different (lessthen 66% nucleic acid similarity).

In order to confirm the expression of the novel deduced *dsrAB* genes the alpha and beta subunit of the Dsr complex were purified form pure culture and Edman degradation was performed on the alpha subunit. These experiments were carried out by Vladimir Zverlov *et al.* (Technical University of Munich) as follows:

The pure culture cells of *Desulfobacula toluolica* were harvested by centrifugation and lyzed by ultrasonic treatment. The lysate was centrifuged and supernatant was purified via HiTrap Q HP ion exchange columns. Fractions showing maximum absorption at 390 nm ($A_{280}/A_{390} < 4$) were pooled and concentrated by ultrafiltration holding back proteins bigger then 10 kDa. Concentrate was purified by applying 10%- polyacrylamide gel electrophoresis (PAGE), avoiding

denaturation (data not shown). Brown Dsr band was excised and extracted from the gel. DsrA and DsrB subunits from extract were separated by denaturing SDS-PAGE into two different bands. Proteins were blotted on polyvinylidene difluoride membrane and stained with Coomassie Blue (see figure 10).

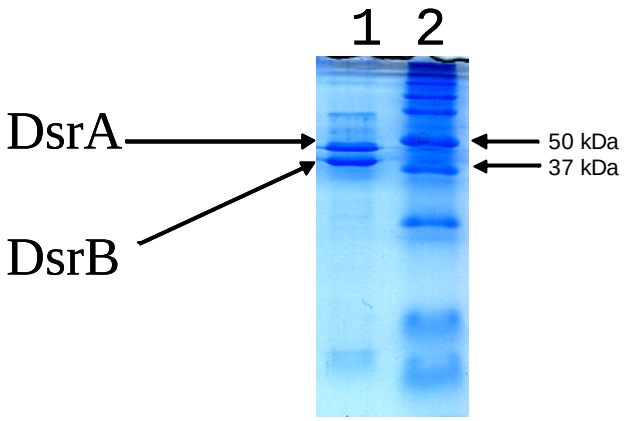


Figure 10 Quantitative denaturing SDS-PAGE of DsrA and DsrB, 15% SDS - PAGE, stained with colloid comassie blue, lane 1 purified Dsr Proteins, lane 2 molecular weight marker

The bands of DsrA and DsrB band are visible between 50 and 37 kDa (see arrows Figure 10).

Bands of DsrA and DsrB were excised from the gel after de-staining and the N- terminus of each protein was determined by Edman degradation on a pulsed liquid phase sequencer. The discovered amino acid sequences matched exactly to the deduced amino acid residues retrieved by sequencing of the Dsr-A subunit:

DsrA N terminus as determined by Edman degradation:	**A K H E T P F L**
DsrA N-terminus as determined by sequencing and translating into amino acids:	**A K H E T P F L**

Due to technical problems it was not possible to determine the protein sequence of DsrB lacking cell material for further experiments.

To answer the question whether both *dsrAB* sequences, the one determined by Klein *et al.* (Klein 2001), and the one determined by Zverlov *et al.* are present in the strain *Desulfobacula toluolica* DSM7467 the following PCR experiments were carried out. The primer pair DSR1F and DSR4R, as well as the newly designed primers Dsr1.9rev and Dsr1.9-d were used to specifically amplify *dsr* fragments.

Dsr1.9rev (5'- GTA GCA GTT ACC GCA (A/G)(A/T)A CAT GC-3') and

Dsr1.9-d (5'- ACC CAC TGG AA(A/G) CAC GG(C/T) GG-3')

are specific primers for conservative sequence motives of the old and new *dsrAB* sequence of *Desulfobacula toluolica*, *Desulfobacula phenolica* and *Desulfobacter latus* were tested with fresh DNA extract of *Desulfobacula toluolica.*

Regrettably, no original DNA from with the *Desulfobacula toluolica* sequenced by Klein was available. Thus, for the following PCR experiments *Desulfobacula toluolica* DNA, purified by Zverlov, has been used as template. The DNA originated from the same culture of which the Dsr subunit has been purified and Edman degradation has been performed.

In the first PCR experiment amplification of *dsrAB* was carried out with the original primer pair DSR1F and DSR4R as published by Wagner *et al*. (Wagner 1998). As shown in figure 11 the expected amplificat of 1.9 kb size could not be amplified out of fresh *Desulfobacula toluolica* DNA (shown in lane 4).

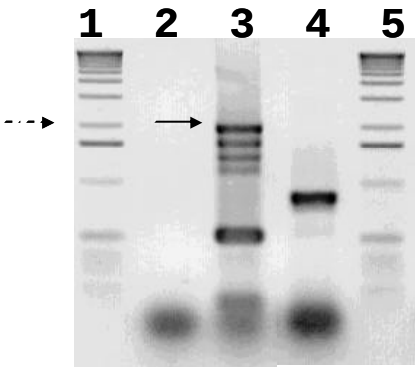


Figure 11 Graph of agarose gel loaded with PCR products amplified with DSR1F / DSR4R primer pair (Wagner 1998). Lane 1 and 5 molecular weight standard, dashed arrow: band at 2000 bp, black arrow: specific product 1.9 kb, lane 2 negative control, lane 3 positive control with DNA from *Desulfobacter latus,* lane 4 amplificats of DNA of *Desulfobacula toluolica* used for PCR.

The next experiment was designed to show that the *dsrAB* operon sequence determined by Zverlov matches the *dsrAB* sequences present in the fresh *Desulfobacula toluolica* DNA. A specific primer pair was designed, targeting *dsrAB* and amplifying an approx. 800 bp long sequence stretch (Figure 12 below).

The specificity of the primer pair was tested by BLAST search.

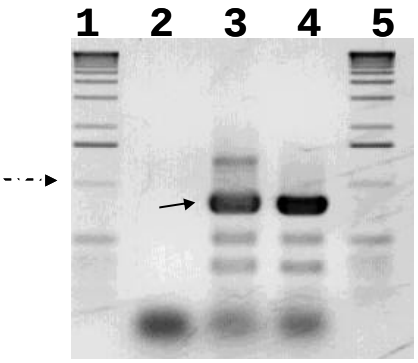


Figure 12 Agarose gel with PCR products amplified with primers specific for *D. toluolica dsr* (recognized from operon sequence). 1 and 5 molecular weight standard, dashed arrow: band at 1000 bp, arrow: specific product, lane 2 negative control, lane 3 and 4 PCR products amplified from two different *D. toluolica* DNA extractions.

The PCR reaction yielded the expected product of the right size. Thus, the primer pair designed on the operon sequence of *Desulfobacula toluolica* successfully amplified the expected fragment of the *dsrA* gene from the DNA extracts.

In a last experiment a primer pair was designed targeting specifically sequence motives on the original, old *dsrAB* gene sequence of *Desulfobacula toluolica* still present in a plasmid (see figure 13 below).

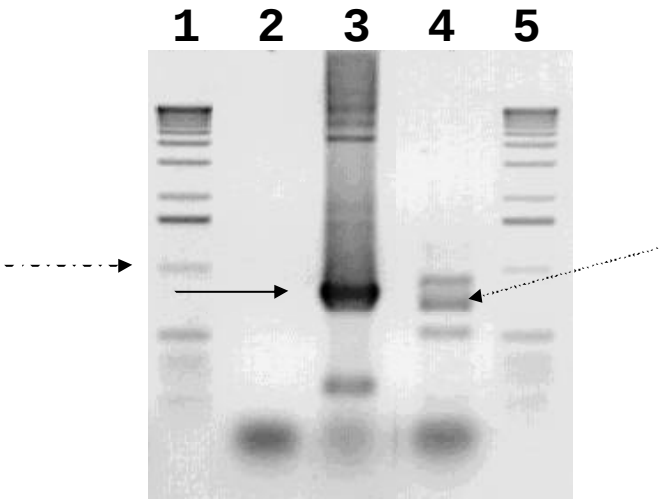


Figure 13 Agarose gel with PCR amplificats with primer specific for contamination of *D. toluolica* culture (recognized from old plasmid). Lane 1 and 5 molecular weight standard, dashed arrow: band at 1000 bp, arrow: specific product, lane 2 negative control, lane 3 positive control with old *D. toluolica* plasmid DNA, lane 4 fresh DNA from novel *D. toluolica* culture.

The primer pair (see figure 14) specific for the "old" *Desulfobacula toluolica* clone sequence did not yield a specific *dsrAB* amplification product from the new DNA from *Desulfobacula toluolica*. The lower band marked with the dotted arrow in figure 13 was sequenced but was not related to *dsrAB* sequences. On the other hand PCR with *Desulfobacula toluolica dsr* operon specific primers yielded a fragment of the expected size.

Subsequent, close examination of the priming site of DSR1F and DSR4R along with their variations (see Results and Discussion above) lead to the discovery of multiple mismatches between these primers and the *dsr* operon sequence of *Desulfobacula toluolica* (see Figure 14 below).

Primer DSR1F	AC(G	C)	CA	C	TGG	AA	G	CA	C	G
Alleged *D. toluolica*	AC	C	CA	C	TGG	AA	G	CA	C	G
D. phenolica	AC	C	CA	C	TGG	AA	A	CA	C	G
D. toluolica operon	AC	C	CA	T	TGG	AA	A	CA	T	G

Primer DSR4R	GTG	TAG	CAG	TTA	CC	G	CA
Alleged *D. toluolica*	GTG	TAG	CAG	TTA	CC	G	CA
D. phenolica	GTG	TAG	CAG	TTA	CC	G	CA
D. toluolica operon	GTG	TAG	CAG	TTA	CC	A	CA

Figure14 Primer sequences of DSR1F/4R and from alleged *Desulfobacula toluolica, Desulfobacula phenolica* and the operon sequence of *Desulfobacula toluolica,* boxes indicate mismatches.

Because of multiple mismatches between the primers DSR1F and DSR4R primers and their targeting sequences within the *dsrAB* sequences of *Desulfobacula toluolica* (as determined by Zverlov) the *dsrAB* genes of this organisms could not be amplified. The obtained "old" *D. toluolica dsrAB* sequence obviously originated from a culture or DNA contamination. Thus, the statement that *Desulfobacula toluolica* carries a xenologous *dsrAB* sequence (Klein *et al*. 2001) has to be corrected.

C.2.3 Comparison of 16S rRNA gene sequence similarity and DsrAB identity values between SRP

In addition to the analyses described above (see also Klein 2001) estimated DsrAB identity values and 16S rRNA gene similarities were calculated for all available SRP reference strains for which the respective marker molecule sequences are of sufficient quality The resulting

identity/similarity values (DsrAB and 16S rRNA gene sequence) for each pair of SRP were blotted against each other (Figure 15).

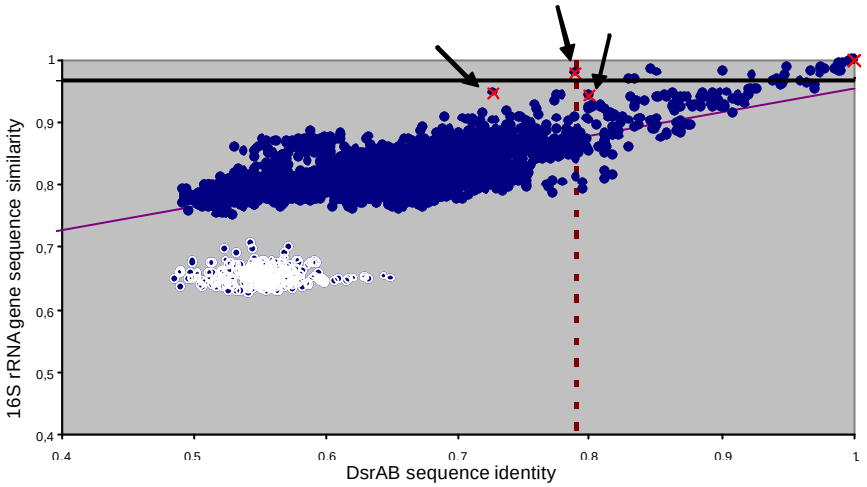


Figure 15 DsrAB identity values blotted versus 16S rRNA gene sequence similarity values of pairs of pure culture SRP; horizontal line on the y – axis at 0,97 16S rRNA similarity indicates the threshold for species assignment on the 16S rRNA level (Stackebrandt 1994); transverse line constitutes the trend line of all species pairs consisting exclusively of organisms with an orthologous sulfite reductase; filled dots represent all pairs, open dots represent identity/similarity pairs with one partner as a member of *Archaeoglobus*, arrows indicate identity/similarity pairs for data point having both partners as members of *Archaeoglobus*, all available sequence positions have been compared i.e. insertions and deletions were not removed from the data set.

Figure 15 shows that SRP pairs possessing DsrAB sequences with less than 79% sequence identity always share less than 97% sequence similarity on the 16S rRNA gene level and can thus be considered as separate species (Stackebrandt 1994). This threshold value should be used as guideline for interpretation of environmentally retrieved *dsrAB* sequences. However, since two bacteria which have more than 97% sequence similarity on the 16S rRNA gene level may or may not be members of the same species (Fox 1992) this approach will lead to underestimation of SRP species richness in ecosystems deduced from *dsrAB* clone libraries.

Consistent with the phylogenetic analysis, the DsrAB identity values between members of the genus *Archaeoglobus* and bacterial SRPs are much higher than expected from 16S rRNA gene sequence similarities, reflecting that *Archaeoglobus* possesses a bacterial sulfite reductase. Therefore, specie pairs, having *Archaeoglobus* as one partner, form a separate cluster (at the lower left) in figure 15. Furthermore, the DsrAB identity values within the genus *Archaeoglobus* (arrows in figure 15) are higher than expected from 16S rRNA gene sequence similarity data. This finding indicates that the sulfite reductase genes have, compared to the 16S rRNA gene, a higher mutation rate within the genus *Archaeoglobus* than in other bacterial SRP. Probably, the

bacterial sulfite reductase genes received by *Archaeoglobus* had to be adapted by their new hosts, thus evolving faster than orthologous sulfite reductases. The similarity/identity values between members of *Archaeoglobus* are presented in Table 5:

Table 5: Estimated similarity/identity values in (%) of *Archaeoglobus* species, DsrAB identity values are presented in the upper square, 16S rRNA gene similarity values in the lower square

	Archaeoglobus fulgidus	*Archaeoglobus veneficus*	*Archaeoglobus profundus*
			DsrAB identity values
Archaeoglobus fulgidus		79%	80%
Archaeoglobus veneficus	98%		73%
Archaeoglobus profundus	94%	95%	
	16S rRNA similarity values		

In the following evaluation DsrAB identity values and 16S rRNA gene sequence similarity values of *Thermodesulfobacterium* are examined in more detail (figure 16 below).

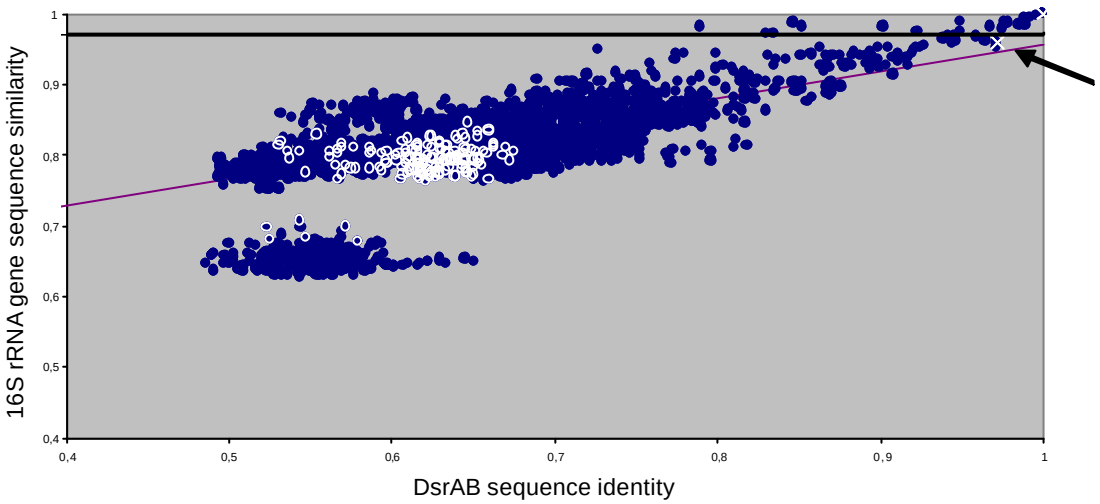


Figure 16 DsrAB identity values blotted versus 16S rRNA gene sequence similarity values of pairs of pure culture SRP; horizontal line at 0,97 16S rRNA gene similarity indicates species border on 16S rRNA level (Stackebrandt 1994); transverse line constitutes the trend line of all species pairs consisting exclusively of organisms with an orthologous sulfite reductase; filled dots represent all pairs, open dots represent identity/similarity pairs with one partner is a member of *Thermodesulfobacterium*, arrow indicate identity/similarity pairs with both partners are members of *Thermodesulfobacterium*.

The clustering of *Thermodesulfobacterium* similarity and identity pairs reveal an unexpected finding. As shown earlier, *Thermosdesulfobacteria* have accepted *dsrAB* genes from a proteobacterial donor. Since *Thermodesulfobacterium* is no close relative of *Deltaproteobacteria* on 16S rRNA gene level one would expect to find the similarity / identity pairs significantly below the trend line. Nevertheless, the similarity and identity values can be found next to the trend line (figure 16). This finding indicates that the lateral *dsrAB* gene transfer from a

proteobacterial donor to *Thermodesulfobacterium* has taken place long time ago, and the 16S rRNA and the *dsrAB* genes of *Thermodesulfobacterium* evolved simultaneously. This explains also the larger distance on DsrAB level between *Thermodesulfobacterium* and *Deltaproteobacteria.*

The similarity/identity pair of *Thermodesulfobacterium commune* and *Thermodesulfobacterium mobile* with each other is indicated by an arrow in figure 16. The dot is placed very close to the trend line. The phylogenetic divergence between these two strains in thus comparable between both marker molecules. It can be hypothesized that the lateral gene transfer event that brought the *dsr* gene into the genus *Thermodesulfobacterium* took place before the speciation of the ancestral *Thermodesulfobacterium* into the species *Thermodesulfobacterium commune* and *Thermodesulfobacterium mobile.*

As shown above *Desulfotomaculum* holds a special position, since part of these Gram- positives carry *dsrAB* genes from deltaproteobacterial donors and other stains do not. In figure 17 and the subsequent text the identity / similarity pairs of different *Desulfotomaculum* clusters are examined.

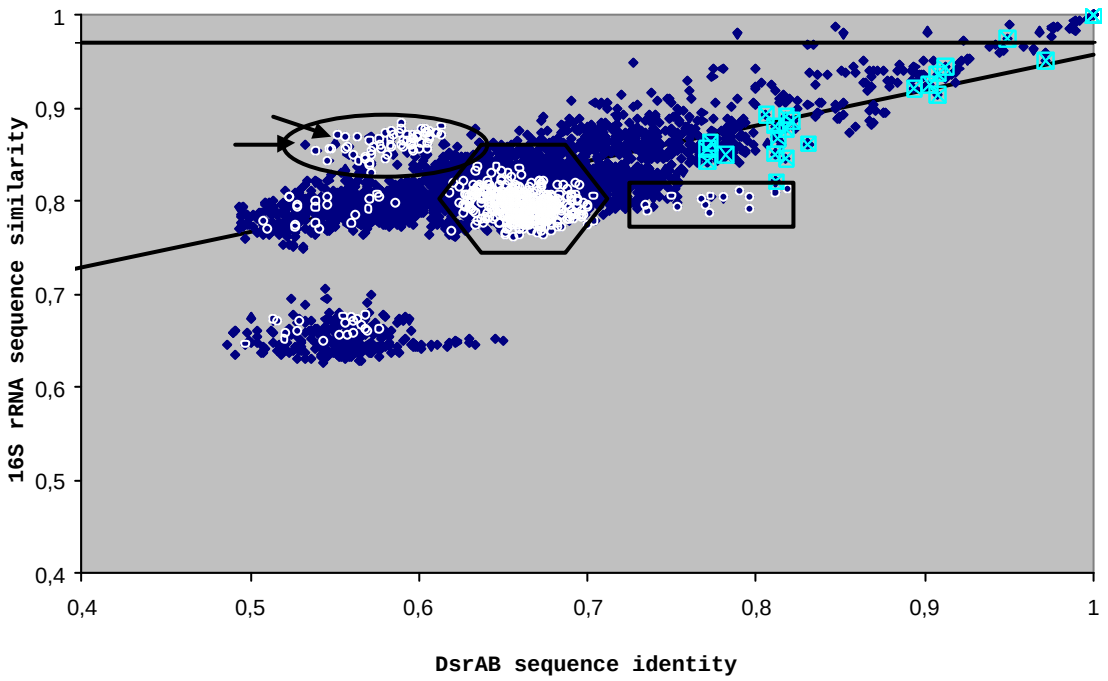


Figure 17 DsrAB identity values blotted versus 16S rRNA gene sequence similarity values of pairs of pure culture SRP; horizontal line at 0,97 16S rRNA similarity indicates species border on 16S rRNA level (Stackebrandt 1994); transverse line constitutes the trend line of all species pairs consisting exclusively of organisms with an orthologous sulfite reductase; filled dots represent all pairs, open dots represent identity/similarity pairs with one partner is a member of *Desulfotomaculum* with a xenologous *dsr*, crossed diamonds indicate identity/similarity pairs with both partners are members of *Desulfotomaculum with* xenologous *dsr.*

Three clusters of dots will be discussed in more detail. Cluster I as indicated in figure 17 by the ellipsoid consists mainly of similarity/identity pairs with a xenologous and an orthologous Gram-Positive partner, respectively. Other diamonds in this ellipsoid are pairs with *Desulfobacca acetoxidans* as a partner. This cluster contains SRP which have unusually low DsrAB sequence identities compared to their 16S rRNA sequence similarities. This finding can be clearly explained by the lateral gene transfer of *dsr*, which affected only a part of the *Desulfotomaculum* strains and thus, caused members of this genus to possess very different sulfite reductases. In good accordance to this, the pairs consisting of a xenologous *Desulfotomaculum* and a deltaproteobacterial partner (marked by the hexagon) mostly exhibit a slightly too low 16S rRNA similarity compared to their DsrAB identity values. Pairs boxed in the square consist of a xenologous *Desulfotomaculum* partner and either *Desulfobacterium anilini* or strain mXyS1, their closest DsrAB neighbors. The position of the square clearly below the trend line indicates that these organisms have unusually similar DsrAB sequences compared to their 16S rRNA similarities. This finding is consistent with the phylogenetic analysis and suggests that *Desulfobacterium anilini*/strain mXyS1 represent the donor lineage from which the sulfite reductase was transferred to the *Desulfotomaculum* species.

C.2.4 Further reflections on lateral gene transfer

C.2.4.1 Comparison of G+C% content of dsrA and dsrB

DsrAB genes were subject to multiple lateral gene transfer events. Theoretically, SRPs might in addition be able to exchange and replace the gene for one subunit of the sulfite reductase and keep the gene for the other subunit. Such events would result in organisms carrying *dsrA* and *dsrB* genes of different origin. A rapid method for detection of replacements affecting only one subunit is to blot the G+C% contents of the alpha subunits versus the beta subunits. Diverging dots from the trend line would indicate such events. In figure 18 such a blot is presented:

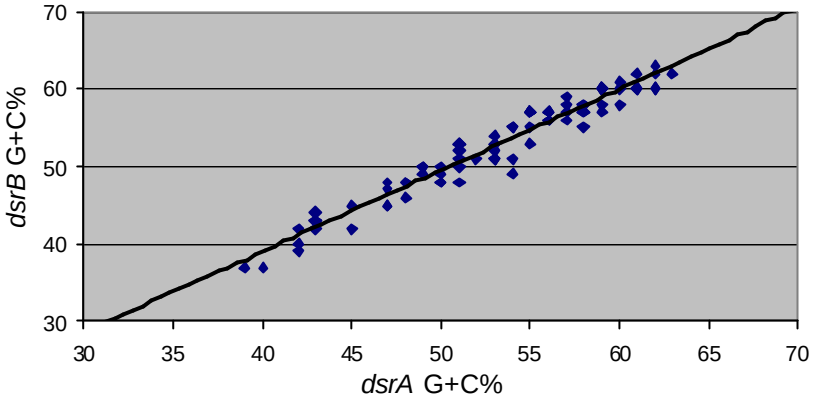


Figure 18 Blot of the G+C% content of *dsrA* versus *dsrB*, line represents trend line; intergenic spacer was removed for calculation.

No clear deviation can be observed. Lateral gene transfer of a single subunit gene with significantly higher or lower G+ C% content can not be observed. However, based on this analysis, it can neither be ruled out that a subunit gene transfer took place between organisms with similar G+C% content, nor that a transfer has taken place long ago and the G+C% ameliorated towards the novel host genome G+C% content.

This finding, that no subunit lateral transfer took place was further confirmed by separate phylogenetic analysis of *dsrA* and *dsrB*. Subunit trees have been calculated and examined for species with deviating phylogenetic positions (data not shown, see also Klein 1998). Again no hints at lateral gene transfer of single subunits were found.

C.2.4.2 Comparative analysis of genomic G+C% content and dsrAB G+C% content

A blot of the G+C% contents of the *dsrAB* gene fragments against the host genomic G+C% contents was examined (Figure 19).

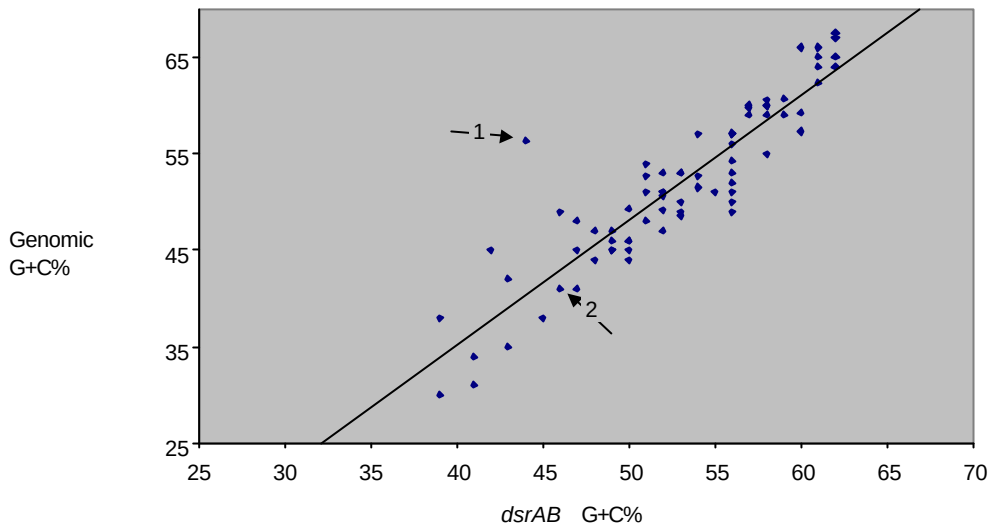


Figure 19: Blot of *dsrAB* G+C% versus genomic G+C% of the same SRP, line represents trend line, arrow 1 marks *Desulfotomaculum halophilum*, arrow 2 *Desulfotomaculum alkaliphilum.*

The majority of G+C% pairs in figure 19 can be found near the trend line. For those SRP which carry a xenologous sulfite reductase this finding indicates that either the respective donor strains had a comparable genomic G+C% content and/or that the sulfite reductase transfer occurred a long time ago and that the G+C% content of the sulfite reductase genes was ameliorated towards the new host genome. The only conspicuous finding affects *Desulfotomaculum halophilum*, marked by arrow 1 in Figure 19. The *dsrAB* G+C% content of *Desulfotomaculum halophilum* is 44 and thus very low compared to its genomic G+C% content of 56. The closest neighbor of *Desulfotomaculum halophilum* on 16S rRNA gene sequence level and DsrAB level, respectively, is *Desulfotomaculum alkaliphilum*. Both share a 16S rRNA gene sequence similarity of 92,7 %. Nevertheless, *Desulfotomaculum alkaliphilum* has no unusual G+C% content of its sulfite reductase genes (*dsrAB* G+C%: 46; genomic G+C%: 41). The DsrAB sequence identity between the two SRP is 82 %.

The two *Desulfotomaculum* strains form monophyletic clusters in ribosomal RNA gene based and DsrAB based phylogenetic dendrograms. Therefore lateral gene transfer is not adequate explanation for the unusually low G+C% content of the *dsrAB* genes of *D. halophilum*.

C.2.4.3 Consequences of lateral gene transfer

Future investigation on acceptors of lateral *dsr* genes should investigate the close genetic neighborhood of the *dsr* operons in order to reveal the present of insertion sequence (IS) elements, or other hints at the integration of mobile genetic elements (e.g. genes encoding

transposase). IS elements have already been identified in *Desulfovibrio vulgaris* and *Desulfomicrobium norvegicum* (Fu 1998) and could thus be found in other sulfate reducing strains, including the donor strain of xenologous *dsr* genes. Notably, IS elements are also very far distributed within the *Archaea* and there also have been detected within members of *Archaeoglobus* (DiRuggiereo 2000). Additional knowledge on genes that flank the *dsrAB* genes, and that might have been transferred in the same event, could help to clarify the question of the origin of the *dsr* donor lineages and the extend of the transfers. Questions are, for example, weather the complete *dsr* operon has been transferred, co-transfer of genes encoding the APS reductase took place (Friedrich 2002), or cytochromes involved in energy conservation during sulfate respiration were also moved. It has been observed for eukaryotic transposable elements that these elements formed the basis for gene duplication (Kidwell 2001). A possible scenario could be that the genetic mechanisms, which lead to the gene duplication of the ancestral *dsr* gene, were also responsible for the lateral transfer of the descendant genes.

In the search for the donor strains of laterally transferred *dsr* genes the structure of the *dsr* operon could restrict the number of possible candidates. The sulfite reductase found in *Desulfobacterium anilini* is closely related to the xenologous Gram- positive *dsrAB* genes. Thus, the investigation of the neighboring genes of the sulfite reductase could help to understand how the *dsr* operon, obviously originating from a Gram- negative bacterium could be functionally expressed in the Gram- positive strains. Examination of the most recent lateral transfer is in this case the most promising way to answer this questions, because reshuffling of the genome can extinct the tracks of older lateral gene transfer events and separated the originally group of genes transferred (e.g. Kidwell 2001) and citations within). However, 7.3 kb upstream of the *dsrABD* genes in *Archaeoglobus fulgidus* (genome at www.tigr.org) a gene coding putatively a histidine kinase can be found. In a recent study (Kim 2001) the authors stated that *Archaeoglobus fulgidus* bears a histidine kinase originating from a bacterial source that has been acquired via lateral gene transfer. It seams possible that the *dsrAB* genes and the histidine kinase could have been acquired in the same transfer event or at least frequent transfer of genetic material between *Archaea* and *Bacteria* is possible. *Archaeoglobus* carries also another gene of bacterial origin, a gyrase. This type II DNA topoisomerase which specifically introduces negative supercoils into DNA (Moreira 1998) was possibly transferred from the *Bacteria* towards the *Archaea* via lateral gene transfer. However, no evidence can be found that these lateral gene transfers have taken place in one event and were in connection with sulfate reducing bacteria.

It has been hypothesized that *Archaeoglobus* was the donor of a glutamate synthase Glts for *Thermotoga maritima* (Nesbø 2001) and had close "genetic" contact with a non sulfate reducing bacterium. In conclusion, it can be stated that members of *Archaea* and *Bacteria* had and have frequent genetic contact and functioning genes can be exchanged.

It is known that functional genetic elements of sizes up to 500 kb can be transferred in a single event as shown in the case of the symbiosis island of *Mesorhizobium* sp. strain R7A (Sullivan 2001). In this case genes coding for a *nif*-specific regulator, several synthetases (e.g. quinolinate-biotin-, and dethiobiotin-synthetase) and several other enzymes were transported. The transfer of this symbiosis island converts nonsymbiontic mesorhizobia to symbionts able to nudulate and fix nitrogen with *Lotus corniculatus* (Sullivan 2001). A transmission process like that could have represented the transport mechanism of the *dsr* genes and other genes of the sulfate reduction pathway over the domain borders form a bacterial donor towards an archaeal acceptor, or vice versa.

C.3 Analysis of environmental DsrAB sequences

C.3.1 Global traits in DsrAB based environmental surveys

Since the publication of Wagner *et al.* (Wagner 1998) who demonstrated that the Dsr approach is suitable to detect SRP, its application in various environmental studies has been shown (see below). Although DsrAB sequences of all, at the present known, SRP lineages have been added to the data base, regularly novel evolutionary lineages are recognized. How to cope with this problem holds a key position in this work. This thesis is the first to summarize the results form environmental SRP surveys based on the Dsr approach.

The major tendencies of DsrAB based environmental SRP surveys can be summarized as (i) soil studies, exhibited the highest mean species richness of SRP i.e. the highest number of different DsrAB sequences were found per study. Further, (ii) the biggest differences of richness could be observed between studies in aquatic habitats, (iii) the highest total number of detected SRP lineages was also observed in aquatic habitats, and (iv) 62 % of the environmentally derived DsrAB sequences could be clearly related to lineages containing at least one pure culture sequence, 38 % were placed in exclusively environmental lineages (sequences which could not be affiliated in regard of different phylogenetic positions resulting from different tree calculation methods, were ignored for calculation of this percentage value).

C.3.2 Consensus tree containing available pure culture and environmental DsrAB sequences of good sequence quality

The consensus tree summarizes the stable and reproducible results from different tree calculations algorithms performed on the DsrAB data set containing pure culture and environmental DsrAB sequences and compiles this information in a single dendrogram. Figure 20 shows the consensus tree constructed as described in the Material and Methods section.

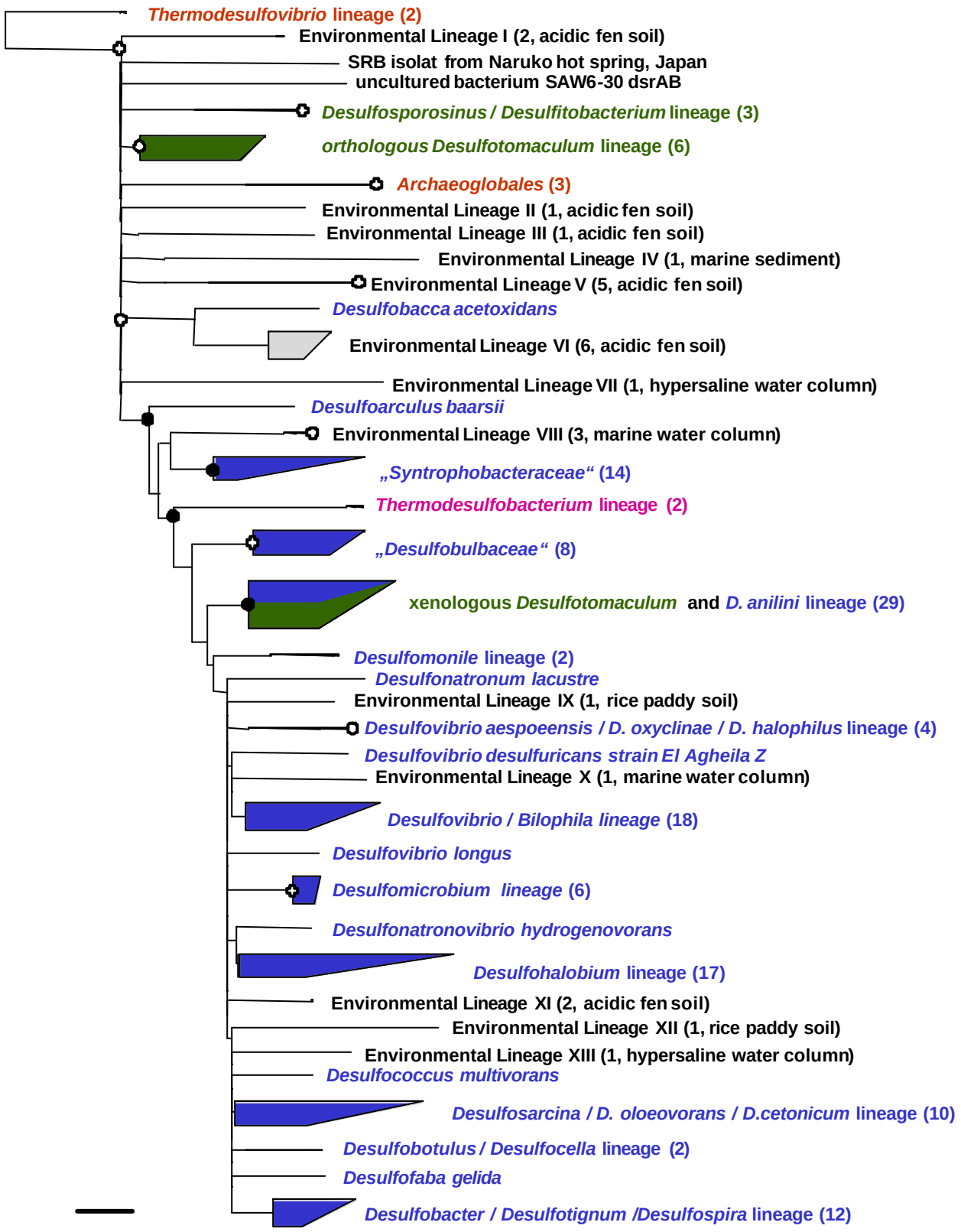


Figure 20 DsrAB consensus dendrogram with pure cultures and environmental sequences of good sequence quality (see Material and Methods section for details), open cycles, closed cycles and crosses indicate bootstrap support for the branching point higher then 75, 90 and equal 100, respectively; bar indicates 10% estimated sequence deviation. Number behind lineages indicates number of species in the respective group.

In total 39 phylogenetic units have been defined using phylogenetic monophyly, and an intra-group-DsrAB-identity-value of 77 % (see Material and Method section) or higher as criteria. Twenty-four of these lineages contain at least one DsrAB sequence derived form a described pure culture. Fifteen environmental lineages have been defined, consisting exclusively of environmental DsrAB sequences from uncultured SRP. Environmental lineages X to XI are clearly affiliated with the *Deltaproteobacteria* and thus represent novel lines of evolution within this group. The phylogenetic affiliation of the other environmental lineages remains unresolved but they are not closely related to any recognized SRPs.

C.3.3 Comparison of branching order of pure culture SRP in pure culture DsrAB and environmental DsrAB dendrogram

The inclusion of environmental sequences into the database of DsrAB sequences had consequences for the stability of the deduced dendrograms. The changes induced by addition of the environmental sequences to the data set were examined in figure 21. While most of the lineages are well supported, the branching order of many lineages differs if different treeing methods are applied. In this case multifurcations were used to indicate that, for the respective lineages, no consistent tree topology was observed, when using different methods for phylogeny inference. Most likely, long- branch attraction caused several of these inconsistencies. A more robust phylogenetic analysis will thus have to await the inclusion of more high quality *dsrAB* sequences for each of the environmental lineages.

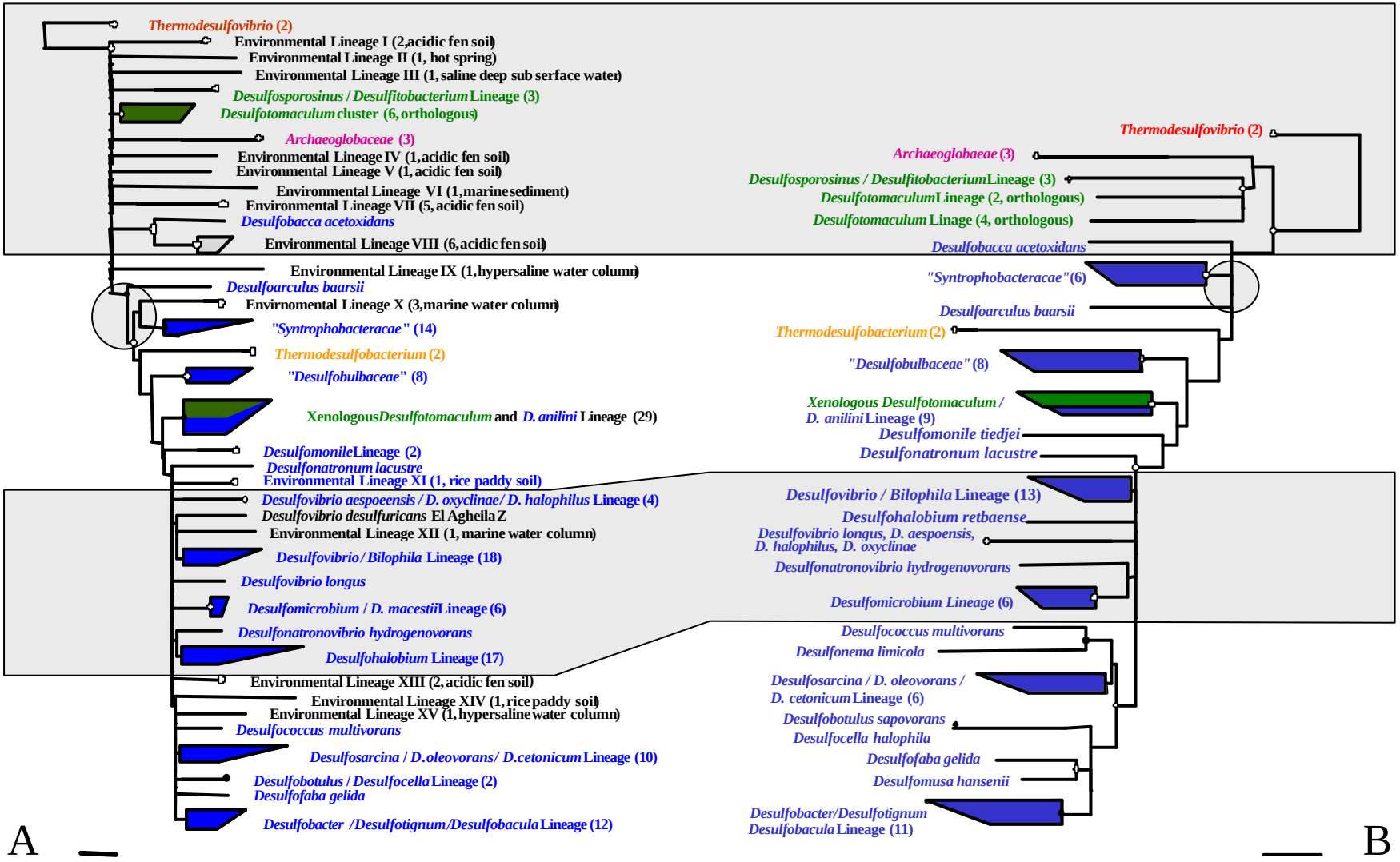


Figure 21 Comparison of topology of DsrAB based consensus dendrograms; A: Pure culture dendrogram with additional environmental sequences and B: Pure culture dendrogram without environmental sequences; bar indicates 10 % estimated sequence deviation; big gray cycles and boxes indicate changed branching orders; open cycles, closed cycles and crosses indicate bootstrap support for the branching point higher then 75, 90 and equal 100, respectively.

C.3.4 Classification of short environmental *dsr* sequences

Table 6 summarizes the affiliations of the short (less then 1401 nucleotides or bad sequence quality) environmental *dsr* sequences. The columns present examined habitats and provide the respective references. Sequences were assigned to the phylogenetic lineages as defined above. The numbers within the table indicate the number of sequences obtained in the respective study which were related to a certain lineage. In the last two columns the total number of retrieved clones per lineage and the absolute number of studies containing sequences of a certain lineage are presented. The last six lines of the table give (i) the number of clones that were impossible to affiliate due to missing corresponding described species or cloned *dsrAB* sequences, (ii) the number of lineages found per study, (iii) the number of clones that were impossible to affiliate due to contradictory results of the phylogenetic analyses (iv) the total number of clones sequenced per study, (v) the number of lineages found per habitat, and (vi) the ratio of lineages containing at least a single pure culture and lineages without any recognized affiliation to a pure culture. The accession numbers or citations of all examined *dsrAB* sequences are given in the appendix.

Table 6 Phylogenetic affiliation of short environmental DsrAB gene fragments.

Environmental Habitat	Soil			Sediment					Water							"Symbiotic/Associated"					
	Acidic fen soil	Pristine	Rice paddy soil	Methanogenic sediment	Deep sea cold methane seep sediment	Estuarine sediment	Estuarine Sediment	Sulfidogenic consortia from polluted sediment	Marine water column	Hypersaline water column	Boreholes Ultradeep Gold Mines and Deep Basalt Aquifers	Groundwater (uranium mill tailings site)	Cu-Pb-Zn mine	Hyper-saline microbial mat	Hot spring microbial streamer	*Alvinella pompejana* epibiont	Symbiont of termite gut	*Olavius algarvensis* endosymbiont	Peridontal pockets	Bioreactor	Lineages in studies
Reference	Loy *et al.*	Castro *et al.*	Friedrich *et al.*	Thomsen *et al.*	Fukuba *et al.*	Joulian *et al.*	Leloup *et al.*	Perez-Jimenez *et al.*	Klein *et al.*	Klein *et al.*	Baker *et al.*	Chang *et al.*	Naka-gawa *et al.*	Minz *et al.*	Naka-gawa *et al.*	Cottrell *et al.*	Klein *et al.*	Dubelier *et al.*	Loy *et al.*	Wagner *et al.*	
Orthologous *Desulfotomaculum* lineage												1									1
Desulfobacca acetoxidans		2					1					1									3
"Syntrophobacteracae"	2	3	1					1		5			5		3						7
Thermodesulfobacterium lineage															3						1
"Desulfobulbaceae"		1			4		1					7				5					5
Xenologous *Desulfotomaculum* and *D. anilini* lineage		10	3			1		8	16	1	31	2	10	1							10
Desulfomonile lineage	1											2		2							3
Desulfovibrio aespoeensis / oxyclinae lineage										1											1
Desulfovibrio/ Bilophila lineage			2					1	3								1			4	5
Desulfovibrio longus						1															1
Desulfomicrobium lineage						13													19		2
Desulfohalobium lineage										19											1
Desulfosarcina/ D. oleovorans/ D. cetonicum lineage		2		13	2	11	9	6	2							1		1			9
Desulfobacter/ Desulfotignumg /Desulfospira lineage								4													1
Desulfosporosinus / Desulfitobacterium lineage																					
Archaeoglobales																					
Desulfoarculus baarsii																					
Desulfonatronum lacustre																					
Desulfovibrio desulfuricans strain El Agheila Z																					
Desulfonatronovibrio hydrogenovorans																					
Desulfococcus multivorans														14							
Desulfofaba gelida																					
Environmental Lineage I	7	2					1					5									4
Environmental Lineage II															1						1
Environmental Lineage III		1		3							3										3
Environmental Lineage IV	1	4										3									3
Environmental Lineage V	2																				1
Environmental Lineage VI				3																	1
Environmental Lineage VII	24	6										5									3
Environmental Lineage VIII	12											2									2
Environmental Lineage IX				1			2			1		1									4
Environmental Lineage X									3												1
Environmental Lineage XI			1									1									2
Environmental Lineage XII									1												1
Environmental Lineage XIII	2											1									2
Environmental Lineage XIV			1																		1
Environmental Lineage XV										1				4							2
Not affiliable, no related described species available		5		20	8					1	2	29	1	2							
Number of Lineages found per study	8	10	5	5	3	4	5	5	5	7	3	13	3	5	3	2	1	1	1	1	
Sequences not monophylotic in both trees		1		4			2	3				11		2		5					
Sum of clones sequenced per study	59	47	13	49	17	30	21	28	30	36	39	84	19	30	10	13	2	2	19	4	
Number of Lineages found per habitat	16			13					20							4					
Pure culture lineages/unaffiliated lineages in habitat	7/9			9/4					9/11							4/0					

Environmental sequences were assignable to members of the *Deltaproteobacteria*-, *Thermodesulfobacterium*-, and *Desulfotomaculum*-lineages.

In the following paragraph the results which are summarized in table 6 will be discussed in more detail. However, some important remarks on the interpretation of table 6 have to be considered previously.

High numbers of retrieved *dsr* gene sequences related to a certain lineage are no prove for a significant ecological role of the microorganisms harboring these *dsr* genes, since the presence of genes is not necessarily connected with expression of the respective genes. A high number of retrieved *dsr* genes do also not hint at a high abundance of the organisms. None of the studies summarized in Table 6 was carried out quantitatively, in respect of coverage values or rarefaction analyses. Sequences of *dsr* genes were also retrieved form environmental enrichment cultures, growing on various hydrocarbon sources. Thus, the sampling introduced the first biases in the determination of the SRP diversity. Each following step, beginning with the DNA extraction introduces further biases. Furthermore, one should keep in mind that the Dsr – approach is PCR dependent. It is very likely that the PCR will not reproduce the abundance of the different environmental *dsr* sequences correctly. It is known that certain genes can be amplified more efficiently than others (e.g. Hansen 1998; Polz 1998; Suzuki 1998). This preferred amplification can be, but must not be, independent of the copy number of the respective DNA molecules in the DNA template mixture (Suzuki 1996; Polz 1998). The selective amplification of certain target molecules is strongly influenced by (i) the of the target molecule, and (ii) G+C% composition the, (iii) the numbers of PCR cycles and (iv) the target DNA flanking regions (Suzuki 1996; von Wintzingerode 1997; Hansen 1998; Polz 1998; Bonnet 2002).

Quantification of microbial communities from analyses of clone libraries is thus not possible. It is only admissible to draw qualitative conclusions. The number of studies comprising a certain lineage of *dsr* sequences allows an estimation of the distribution of this lineage in a certain habitat.

Accordingly members of the *Desulfosarcina /D. oleovorans /D. cetonicum* (Dss/Do/Dc) lineage and the xenologous Gram-Positive/*Desulfobacterium anilini* (xG+/Dai) lineage are the most widespread SRP in the examined studies. Subsequently, the most widespread lineages will be discussed in more detail.

Firstly, *Desulfosarcina* related environmental *dsr* sequences will be discussed.

dsrAB sequences related to the Dss/Do/Dc lineage have been retrieved from all sediments analyzed in this study. With the exception of the hot spring streamer, the hyper-saline microbial

mat (Minz 1999b) and the bio-reactor Dss/Do/Dc lineage related sequences were found in every kind of habitat (water, sediment, soil), even as symbiont of a marine worm (see Table 6).

In order to evaluate these findings the results were compared to findings of experiments employing other techniques then the *dsr* approach. In the following paragraph results from mostly 16S rRNA gene sequence based environmental SRP surveys are discussed.

Environmental 16S rRNA gene sequence based studies (FISH, PCR) and cultivation dependent approaches (besides the studies summarized in Table 6) confirm the wide distribution of Dss/Do/Dc related organisms in sediments (Loka-Bharathi 1991; Devereux 1994; Devereux 1996; Gray 1996; Rooney-Varga 1997; Crump 1999; Ravenschlag 1999; Sahm 1999b; So 1999; Urakawa 1999; Bowman 2000; Ravenschlag 2000; Orphan 2001; Hayes 2002; Inagaki 2002; Michaelis 2002; Orphan 2002). The most common probes to detect *Desulfosarcina*-like organisms in FISH studies were S-*-Dsb-0804-a-A-18 (nomenclature according to (Alm 1996), probe design by (Devereux 1992), and S-*-Dsb-0658-a-A-18 (Manz 1998). These probes however do not exclusively detect members of the Dss/Do/Dc lineage. These probes targets also 16S rRNA from *Desulfobacter* spp., *Desulfobacterium* spp., *Desulfofrigus* spp., *Desulfofaba* sp., *Desulfostipes* sp., *Desulfococcus* sp., *Desulfobotulus* sp., *Desulfomusa* sp. and *Desulforegula* sp. (Loy 2003). Since it is also possible that *Desulfosarcina* like non–sulfate-reducing *Deltaproteobacteria* could be detected, it is not possible to state that the retrieved signals and/ or rRNA gene sequences originate from sulfate reducing bacteria.

Subsequently the picture of the Dss/Do/Dc lineage will be completed by considering the biology of the pure cultures which define this lineage.

The Dss/Do/Dc lineage contains the reference strains *Desulfosarcina variabilis*, *Desulfobacterium oleovorans*, and *Desulfobacterium cetonicum*. The DsrAB sequences of strain oXyS1 (DSM13228, (Harms 1999), *Desulfosarcina* CME1 (Joulian 2001), and strain AK-01(So 1999) were also clearly affiliated with the Dss/Do/Dc lineage.
The type strains have been isolated form marine mud (*Desulfosarcina variabilis*; Widdel 1980), mud from an oil/water separator (*Desulfobacterium oleovorans*; Aeckersberg 1991) and from oil recovery water (*Desulfobacterium cetonicum*; Galushko 1991).

The reference strains and strain oXyS1 have been described as effectively degrading various hydrocarbons. They oxidize e.g. aliphatic alcohols, aromatic hydrocarbons (o-Xylene), fatty acids, and alkanes (e.g. Widdel 1980; Heider 1998; Harms 1999; So 1999).

The presence of sequences related to the Dss/Do/Dc lineage might reflect the residence of organisms capable of oxidizing a large variety of hydrocarbons. Their occurrence could also be interpreted as the presence of such organic compounds in the examined ecosystems. Furthermore, the wide distribution of *Desulfosarcina* like organisms, especially in coherence with anaerobic methane oxidation (Boetius 2000; Orphan 2001; Thomsen 2001; Michaelis 2002; Nauhaus 2002; Valentine 2002) indicate a major environmental importance of these organisms.

The monophyletic branch of the xenologous Gram- positives and *Desulfobacterium anilini* related environmental *dsr* sequences are of special interest. This branch contains Gram-positive SRP acceptors of xenologous *dsr* genes of proteobacterial origin. Furthermore, the branch contains two deltaproteobacterial reference strains, *Desulfobacterium aniline* and strain mXyS1.

This second most frequently retrieved group, abridged xG+/Dai, was found most widespread in water samples. The presence of this group was less distinctive in soil and sediment samples. The bootstrap support for this lineage was 99%. Retrieval of sequences related to this lineage has to be interpreted with caution since this lineage encloses acceptors of laterally transferred *dsr* genes (see above). Most of the environmental clones can be affiliated to the monophyletic branch of xenologous *Desulfotomaculum dsrAB* sequences or to the monophyletic branch of *Desulfobacterium anilini*/ mXyS1 lineage (Figure 22):

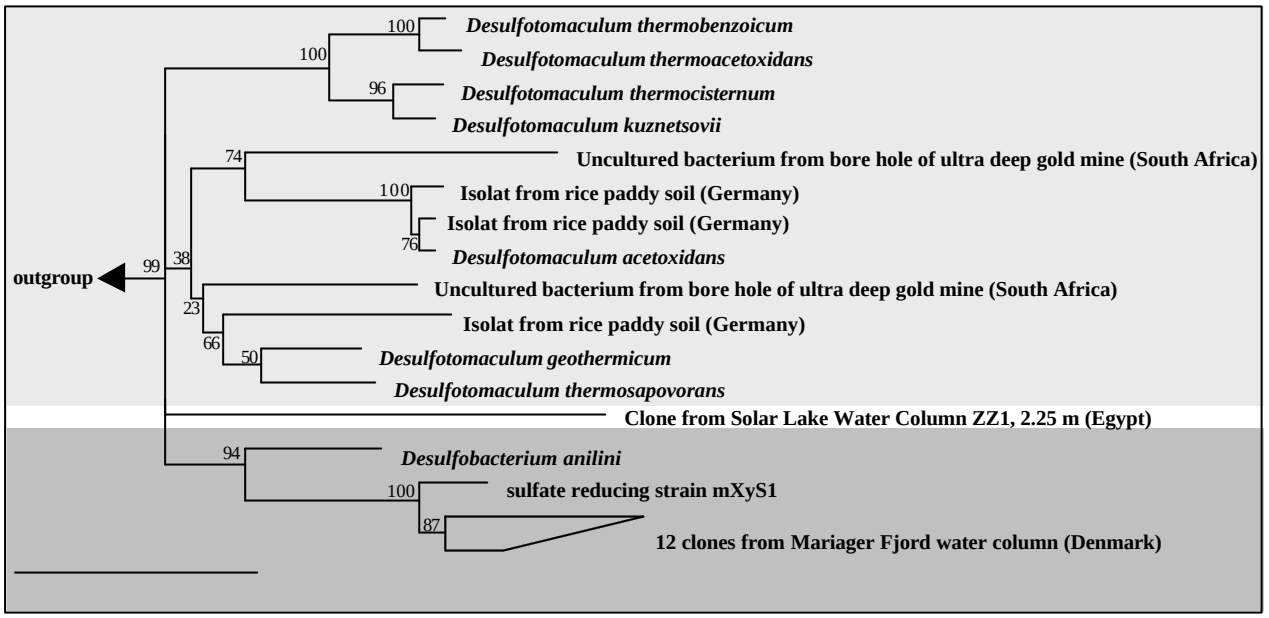


Figure 22 Consensus tree based on Protein Parsimony (100 bootstraps replications) and Fitch-Margoliash DsrAB dendrograms (calculated with indel filter). Outgroup species were chosen form main DsrAB lineages. Bar indicates 10 % sequence identity. Light shaded box marks sub lineages containing xenolog Gram-Positives and related environmental sequences, white bar indicates environmental sequence which can not be affiliated to one of the other groups, dark shaded lower box indicate monophyletic sub lineages of the Dai lineage.

Besides *dsrAB* sequences from pure cultures, environmental clones of good sequence quality were used for computing the dendrogram. Seventeen environmental clones grouped with the xG+/Dai lineage. All twelve Mariager Fjord clones were affiliated with the deltaproteobacterial strains *Desulfobacterium anilini* and mXyS1. The bootstrap support for this branching order was 94%. The minimum identity of the DsrAB sequences in this cluster was 77%.

Environmental clones related to *Desulfobacterium anilini* and stain mXyS1 were detected in hydrocarbon rich deep surface water (aquifers), soil samples (pristine, eutrophic, methanogenic), brackish water sediments, chemocline of marine water column, hypersaline water column, and within a Cu-Pb-Zn mine.

DsrAB sequences related to the *Desulfotomaculum* strains were derived from environmental clones and isolates originating ground water from three different study sites, from rice paddy soil and sediment samples.

59 % of the environmental sequences affiliated with the xG+/Dai lineage could be assigned to the xenologous *Desulfotomaculum* lineage, and 41 % with the *Desulfobacterium anilini* lineage,

respectively. Both lineages contain described species isolated from aqueous sites. These sites were often polluted with heavy hydrocarbons load. The group represents a metabolically variable lineage of SRP. Interestingly, there was only a single study (Chang 2001) which retrieved an environmental *dsrAB* gene sequence that was related to the orthologous Gram-positive *Desulfotomaculum* strains. The clone originated from ground water and was most closely related to *Desulfotomaculum aeronauticum.*

In this context, findings of experiments employing other techniques then the *dsr* approach and concerning *Desulfobacterium anilini* and strain mXyS1 were reviewed.

16S rRNA and 16S rRNA gene sequence based studies detected *Desulfobacterium anilini* related organisms and mXyS1 related organisms in marine sediment (Galushko 1999; Bowman 2000; Hayes 2002; Inagaki 2002).

Desulfobacterium anilini was isolated from marine sediment (North Sea, Germany, Schnell 1989), and mXyS1 was recovered from the seawater phase in an oil tank (Harms 1999). Both strains are able to degrade various hydrocarbons. They are able to oxidize organic compounds completely.

Gram- positive *Desulfotomaculum* strains of the xG+/Dai lineage were also detected by *dsr* independent studies in various sulfidogenic environments.

Members of the xenologous Gram- positive *Desulfotomaculum* were detected in polluted aquifer/ground water from an oil-storage cavity (Dojka 1998; Watanabe 2002), and sandstone core, drilling mud and production water from oil fields (Leu 1998). These strains were also found in samples and enrichment cultures from rice fields and rice root soil (Wind 1999; Stubner 2000; Stubner 2002), from marine sediment (Isaksen 1994), from sediment of freshwater lakes (Bak 1991; Fukui 1996; Scholten 2000), and from brackish sediment (Boschker 2001). Additionally, a 16S rRNA gene sequence related to *Desulfotomaculum thermosapovorans* was retrieved from fluid sample from ocean crust (Cowen 2003). Generally, *Desulfotomaculum* targeted 16S rRNA oligonucleotide probes are not so commonly used as it is the case for deltaproteobacterial SRP targeted FISH probes.

The following section provides information on xenologous *dsr* carrying Gram- positive reference strains with regard to temperature preference and oxidation capacity.

With one exception, all xenologous *Desulfotomaculum* strains present in the pure culture tree and *Desulfotomaculum luciae*, which also belongs to this xenologous group (according to DsrAB based dendrograms, data not shown), represent thermophilic spore forming SRB. The only exception is the mesophilic *Desulfotomaculum acetoxidans*. The pure cultures have been isolated from piggery waste (*Desulfotomaculum acetoxidans*, (Widdel 1977), rice hulls and peanut shells compost *(Desulfotomaculum thermosapovorans*, (Fardeau 1995), geothermal ground-water (*Desulfotomaculum geothermicum*, (Daumas 1988) and *Desulfotomaculum kuznetsovii*, (Nazina 1989), sludge from a thermophilic anaerobic reactors (*Desulfotomaculum thermoacetoxidans* and *Desulfotomaculum thermobenzoicum*, (Min 1990; Tasaki 1991), hot spring sediment (*Desulfotomaculum luciae*, (Liu 1997), and formation water from an oil reservoir (*Desulfotomaculum thermocisternum*, (Nilsen 1996b). With the exception of *Desulfotomaculum thermocisternum* and *Desulfotomaculum thermosapovorans* (no data found for *Desulfotomaculum luciae*) all other strains are complete oxidizers.

The spore forming sulfate reducers are usually found more frequently in habitats with alternating oxic and anoxic cycles. They are less frequently found than Gram-Negative SRP in habitats that are permanently or usually anoxic. Their spores allow them to survive dryness and oxic conditions.

The xenologous *Desulfotomaculum* were found in three of five aqueous habitats. No xenologous *Desulfotomaculum* strains were detected in the water column of a marine habitat and a hypersaline habitat.

Syntrophobacteracae contain the sulfate reducing *Syntrophobacter wolinii* and *Syntrophobacter fumaroxidans* as well as *Thermodesulforhabdus norvegica, Desulfovirga adipica, Desulforhabdus amnigenus,* and *Desulfacinium infernum:*

The presence of *Syntrophobacteracae* in all three soil studies implicate that this SRP lineage is widely distributed in soil ecosystems. Members of the *Syntrophobacteracae* were also found in the hyper saline water column of Solar Lake, the hydrothermal water of a Cu-Pb-Zn mine, within a hot spring microbial streamer and at petroleum contaminated sediment.

With other methods member of the *Syntrophobacteracae* were found in comparable habitats.

Members of this lineage were detected in oil field water, oil reservoirs, and from crude oil (*Thermodesulforhabdus norvegicus* related, (Nilsen 1996a; Tanaka 2002), in a marine hydrothermal vent (*Desulfacinum* related Sievert 2000), in bio reactors feed with waste water (*Syntrophobacter spp*., Santegoeds 1999), and associated with rice roots (*"Syntrophobacteriacae"*, Wind 1999; Scheid 2001).

The biology of the pure cultures of the *"Syntrophobacteracae"* has to be regarded in respect of the syntrophic lifestyle of some of its members.

Within the lineage of *Syntrophobacteracae* syntrophic and non-syntrophic organisms can be found. Non-syntrophic SRP are the two thermophilic organisms, *Desulfacinum infernum* and *Thermodesulforhabdus norvegicus* and the mesophilic *Desulforhabdus amnigenus* and *Desulfovirga adipica* strains. Real syntrophs are only *Syntrophobacter fumaroxidnas* and *Syntrophobacter wolinii*.

Additionally five environmental sequences from the hyper saline Solar Lake water column, two sequences from fen soil and a sequence from rice paddy soil can be found in this lineage.

Desulfacinum infernum and *Thermodesulforhabdus norvegicus* were isolated from enrichment cultures inoculated with formation water from North Sea oil fields (Beeder 1995; Rees 1995), *Desulfovirga adipica*, *Syntrophobacter wollini*, and *Syntrophobacter fumaroxidans* from wastewater digesters (Boone 1980; Harmsen 1998; Tanaka 2000).

Desulfobulbus/Desulforhophalus/Desulfofustis related environmental *dsr* sequences were found in soil, sediment and water samples. In addition *Desulfobulbus/Desulforhophalus* like sequences were retrieved from *Alvinella pompejana* exosymbionts.

Findings of experiments employing other techniques are presented in the following section.

In the literature *Desulfobulbus* / *Desulforhopalus/ Desulfofustis* related organisms have been described to thrive in marine sediments (Devereux 1994; Gray 1996; Ravenschlag 1999; Sahm 1999a; Sahm 1999b; Ravenschlag 2000; Wieringa 2000; Orphan 2001; Purdy 2001; Hayes

2002; Purdy 2002), the marine water column (Teske 1996), the water column of a meromictic alpine lake (Bosshard 2000), deep-sea cold seep sediment (Inagaki 2002), in an estuarine environment (Crump 1999), in rice paddy soil (Wind 1995), from a shallow oil field (Voordouw 1996), and from a landfill leachate (Daly 2000).

Regarding the reference strains, *Desulfobulbus propionicus* was isolated from freshwater mud (Widdel 1980), *Desulfobulbus rhabdoformisfrom* oil field water (Lien 1998), *Desulfobulbus elongatus* from a digester (Samain 1984), *Desulfobulbus* DSM2058 and *Desulfofustis glycolicus* from marine mud (Widdel 1980; Friedrich 1995), *Desulforhopalus singaporensis* marine mud from a marsh (Lie 1999), and *Desulforhopalus vacuolatus* from an estuary (Isaksen 1996).

Members of the *Desulfobulbus/Desulforhopalus/Desulfofustis* lineage have been found consistently in the same habitats by the DsrAB approach, by cultivation and 16S rRNA gene sequence based studies.

An interesting metabolic feature, which is found in this lineage, is the ability to gain energy from the disproportion of sulfur. *Desulfobulbus propionicus* and the related strains *Desulfocapsa thiozymogenes* and *Desulfocapsa sulfoexigens*, grow well by disproportion of sulfur.

Within the lineage of *Desulfovibrio/Bilophila* the highest number of sequenced species (reference strains) can be found. In nature members of this last lineage that can be found in a multitude of *dsrAB* based environmental surveys contains a significant number of species belonging to the genus *Desulfovibrio*. *Desulfovibrio* related strains were found in soil, sediment, water samples as well as associated with a termite.

Since *Desulfovibrio* represents the longest know, thus best characterized genera of SRP, cultivation conditions and ribosomal RNA data as basis for hybridization experiments are well known. Because of this, a long history of publications, which detected *Desulfovibrio* sp. in all kinds of habitats, is available. More recently described genera of SRP are thus underrepresented in literature and the presence of *Desulfovibrio* sp. in such a high number of publications should not be taken as a sign for absolute environmental dominance of *Desulfovibrio* genera.

The following cited publications should be regarded as examples and the presented list is far from being complete. *Desulfovibrio* related strain were retrieved from underground oil storage

cavities or other oil contaminated sites (Rabus 1996; Voordouw 1996; Tanaka 2002; Watanabe 2002), from a salt marsh sediment (Rooney-Varga 1998), from marine sediment (Ravenschlag 1999; Sahm 1999a; Ravenschlag 2000; Wieringa 2000; Purdy 2001; Tanaka 2002), in a landfill leachate (Daly 2000), from rice soil or rice roots (Wind 1995; Wind 1999; Scheid 2001), microbial mats (Risatti 1994; Wawer 1995a; Krekeler 1997), in an estuary (Crump 1999; Purdy 2002)(Boyle 1999) in a marine water column (Teske 1996), from an alkaline aquifer (Fry 1997), in a freshwater lake (Bak 1991; Overmann 1999), and from sulfidogenic bio reactors (Kane 1993; Santegoeds 1999). Moreover *Desulfovibiro* sp. was found associated with termites (Brauman 1990; Frohlich 1999; Cypionka 2000).

The description of all characterized pure cultures belonging to the *Desulfovibrionaceae* is beyond the scope of this thesis. In summary, members of this family incompletely oxidize electron donors to acetate. Additionally, growth in the absence of an external electron acceptor is possible by fermentation of pyruvate and in several cases also of malate or fumarate (Widdel 1992b).

In table 6 a number of reference strain containing lineages are present which do not yet comprehend environmental DsrAB sequences**.**

Although members of the *Archaeoglobus* and the *Desulfosporosinus/ Desulfitobacterium* lineages were previously detected in the environment by 16S rRNA gene sequence based analyses and isolation based methods (Nilsen 1996a; Dojka 1998; Robertson 2000), no related DsrAB sequences were retrieved from environmental sampling sites so far.
Other lineages of which no environmental related sequences were retrieved by the Dsr -approach are *Desulfoarculus baarsii, Desulfonatronum lacustre, Desulfovibrio desulfuricans* strain El Agheila Z, *Desulfonatronovibrio hydrogenovorans, Desulfococcus multivorans,* and *Desulfofaba gelida.*
Failure to detect certain lineages can be a consequence of (i) the choice of the sampling site (for example mesophilic sampling sites will not allow the detection of thermophiles), (ii) homogeneity of samples, sampled area and time of sampling, (iii) unknown cultivation conditions if samples should be pre-enriched, (iv) difficulties with DNA extractability from certain prokaryotes, (v) individual cell numbers, or (vi) PCR induced biases.

C.3.5 Richness of SRP in different environmental habitats

The number of detected lineages allows estimating the minimum richness of SRP in a certain habitat. The richness of SRP arises from the numbers of detected SRP lineages per study. In order to receive estimation on the mean richness of a habitat the mean value of the detected SRP lineages of the different studies for the soil-, sediment-, and water- habitats were calculated. The highest value was calculated for the soil habitat whereas the highest total number of detected lineages was observed in water samples. An interesting fact in this context is the ratio of detected lineages that contain DsrAB sequences from reference strains and lineages which consist exclusively of environmental sequences. In the soil habitat a total of 16 distinguishable lineages was detected, 7 lineages containing pure culture DsrAB sequence (PC lineage), and 9 lineages which contain exclusively environmental DsrAB sequences (E lineage). The ratio PC versus E lineages is 9 to 4 (total 13 lineages) in the sediment habitat and 9 to 11 (total 21 lineages) in the aqueous habitat.

The study with the highest number of recognized SRP lineages was carried out by Chang *et al.* on groundwater at a uranium mill tailing site (Chang 2001). At least 12 different lineages were detected. One could hypothesize the presence of even more SRP lineages since 29 short *dsr* sequences could not yet be affiliated to existing lineages. It is well known that certain sulfate reducers, e.g. members of the genera *Desulfovibrio*, are able to reduce Uranium (VI) to Uranium (IV) (Lovley 1992; Abdelouas 2000; Spear 2000). In the study of Chang *et al.* a *dsrAB* gene fragment of an orthologous *Desulfotomaculum* species was retrieved. Strikingly, this study was the only one in which a *dsrAB* sequence of this lineage was obtained (see above). This result is consistent with the finding that orthologous *Desulfotomaculum* related species are able to grow with Uranium (VI) as electron acceptor (Tebo 1998). The reason for the considerable diversity of SRP in the study of Chang *et al.* may be found in the pronounced gradients of sulfate and uranium concentrations at the different sampling sites.

C.4 Environmental SRP surveys with the DsrAB- approaches in the scope of this thesis

C.4.1 Analysis of mixed populations of sulfate reducing prokaryotes with the DsrAB-approach and gelretardation

The molecular analysis of microbial communities has become a widespread method for studying diversity (Hugenholtz 1998 and citations within). A very powerful tool for monitoring natural diversity is amplification of a molecular marker gene by polymerase chain reaction (PCR), followed by subsequent cloning, sequencing and phylogenetic analysis of the sequences. A major goal of environmental diversity surveys is to recover/identify all microorganisms from a chosen ecosystem. The main problem in this context is the various biases introduced by PCR. For example, independent of the real distribution, some template DNA molecules can be preferentially amplified and thus are over represented in the clone library. For example, PCR amplification of *dsrAB* genes from the water column of Mariager Fjord (see below) resulted in a single band of nucleic acid fragments when examined on a horizontal agarose gel electrophoresis. This single band does however not consist of a unique sequence type, but rather of a mixture of very different DNA fragments. Direct cloning approaches on this mixture lead only to the recovery of a single sequence type. To more efficiently harvest the actual diversity of gene sequences within a PCR amplificat, a method able of separating the mixed DNA fragments prior to cloning and sequencing was needed. Molecular microbiologists have used for this purpose different genetic fingerprint methods like denaturing gradient gel electrophoresis (DGGE) (Muyzer 1993) and temperature gradient gel electrophoresis (TGGE) (Zoetendal 1998). The separation principal is based on the sequence specific DNA melting and in gel mobility behavior of double stranded DNA in a denaturant or temperature gradient. These separation systems allow detection of community compositions as well as specific retrieval of sequence fragments for subsequent cloning. However, they also exhibit some severe limitations. Beside the detection of heteroduplexes (Ferris 1997) and co-migration of different DNA fragments in a single band (Vallaeys 1997), the most important limitation of these techniques is the relatively small size of separable DNA fragments (approx. 400 bp) (Muyzer 1998). Thus, the application of DGGE or TGGE on the 1,9 kb sized *dsrAB* fragment is not possible.

Gelretardation as a good alternative for separation of DNA fragments up to 1440 bp long has been successfully used on an artificial mixture of sequences (Wawer 1995b). In order to obtain a

suitable method for separating the longer *dsrAB* fragment, the gelretardation method was optimized for environmental samples.

After optimization of the running conditions (running buffer, type agarose, concentration of agarose gel, type of retardation dye, running time, staining, extraction of DNA fragments from gel) the gelretardation system was used on natural samples (Schmid 2000) in this study. DNA was retrieved from a trickling bed reactor. Genes coding for the ammonia monooxygenase alpha subunit (*amoA*) originating from ammonia oxidizing bacteria were partially PCR amplified. In order to retrieve the whole spectrum of *amoA* genes present in the sample the PCR amplificats were subjected to an agarose gelretardation electrophoresis (Figure 16, B). Each band was excised from the gel, cloned and sequenced.

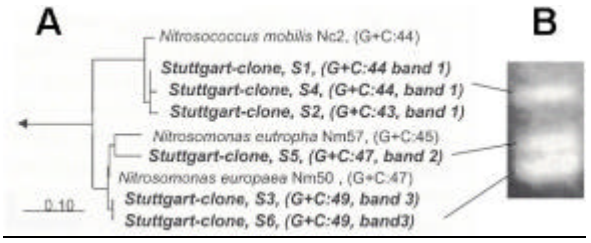


Figure 23 A: *amoA* amino acid based dendrogram of ammonia oxidizers from trickling bed reactor biofilm, arrow indicates position of outgroup, scale bar indicates 10 % estimated sequence deviation B: Graph of gelretardation of *amoA* PCR amplificats (approx. 490 base pairs), lines indicate corresponding sequences in the dendrogram.

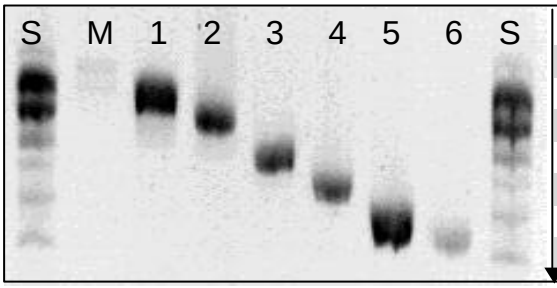
Three different types of *amoA* gene sequences were obtained, which affiliate with *Nitrosococcus mobilis*, *Nitrosomonas eutropha*, and *Nitrosomonas europaea*. Clones originating from a specific band at the gelretardation were always affiliated to the same pure culture. The gelretardation run was able to separate sequences differing in 2 % G+C content.

After the successful application of the gelretardation in a natural complex sample, the method was used to separate the larger (approx. 1900 base pair) *dsrAB* gene fragments amplified from DNA retrieved from the chemocline of Mariager Fjord (Dk).

The high similarity of Mariager Fjord *dsrAB* clones retrieved by direct cloning which formed a tight cluster in phylogenetic analyses and contained 9 out of 12 clones was inconsistent with a much higher SRP diversity observed within Mariager Fjord in previous studies (Ramsing 1996; Teske 1996). Thus, the gelretardation method was used to separate the *dsrAB* PCR amplificats

from Mariager Fjord prior to cloning. All *dsrAB* PCR amplificats from the Mariager Fjord chemocline were detectable as a single band at 1.9-kb on a conventional horizontal agarose gel electrophoreses due to their equal size (data not shown). After the *dsrAB* PCR amplificats were separated via gelretardation, seven clearly distinguishable bands were observed, each representing *dsrAB* fragments of unique A+T% composition (Figure 24). DNA was extracted from each band, cloned, and sequenced.

A



B

Band	Clone	G+C mol%	bp
M	MAFM12G	54	1929
1	MAF17G	53	1930
2	MAF28G	53	1934
3	MAF36G	56	1896
4	MAF411G	59	1928
5	MAF53G	59	1949
6	MAF65G	63	1933

Figure 24 A: Graph of gelretardation, S original *dsrAB* PCR product, lanes M to 6 PCR amplificats from clones carrying *dsrAB* fragments derived from the corresponding bands of lane S; B: Table giving information on band from which clones were obtained, the G+C mol% and the lengths of *dsrAB* fragments.

The sequences of the clones MAFM12G/MAF17G and MAF411G/MAF53G showed only minor variations in their A+T% content (A+T% content was determined after sequencing), but were separated nevertheless. Possible explanations of this finding are slight length variations (MAF411G 1928 bp /MAF53G 1949 bp) or different distribution of the A+T% rich regions within the clone sequences.

The nucleic acid sequences and the deduced amino acid sequences were aligned to the Mariager Fjord *dsrAB* clones obtained by direct cloning and to *dsrAB* sequences of pure culture SRP in the database and phylogenetically analyzed. ED and MP calculation methods placed the gelretardation clones into 5 different clusters (figure 25 below).

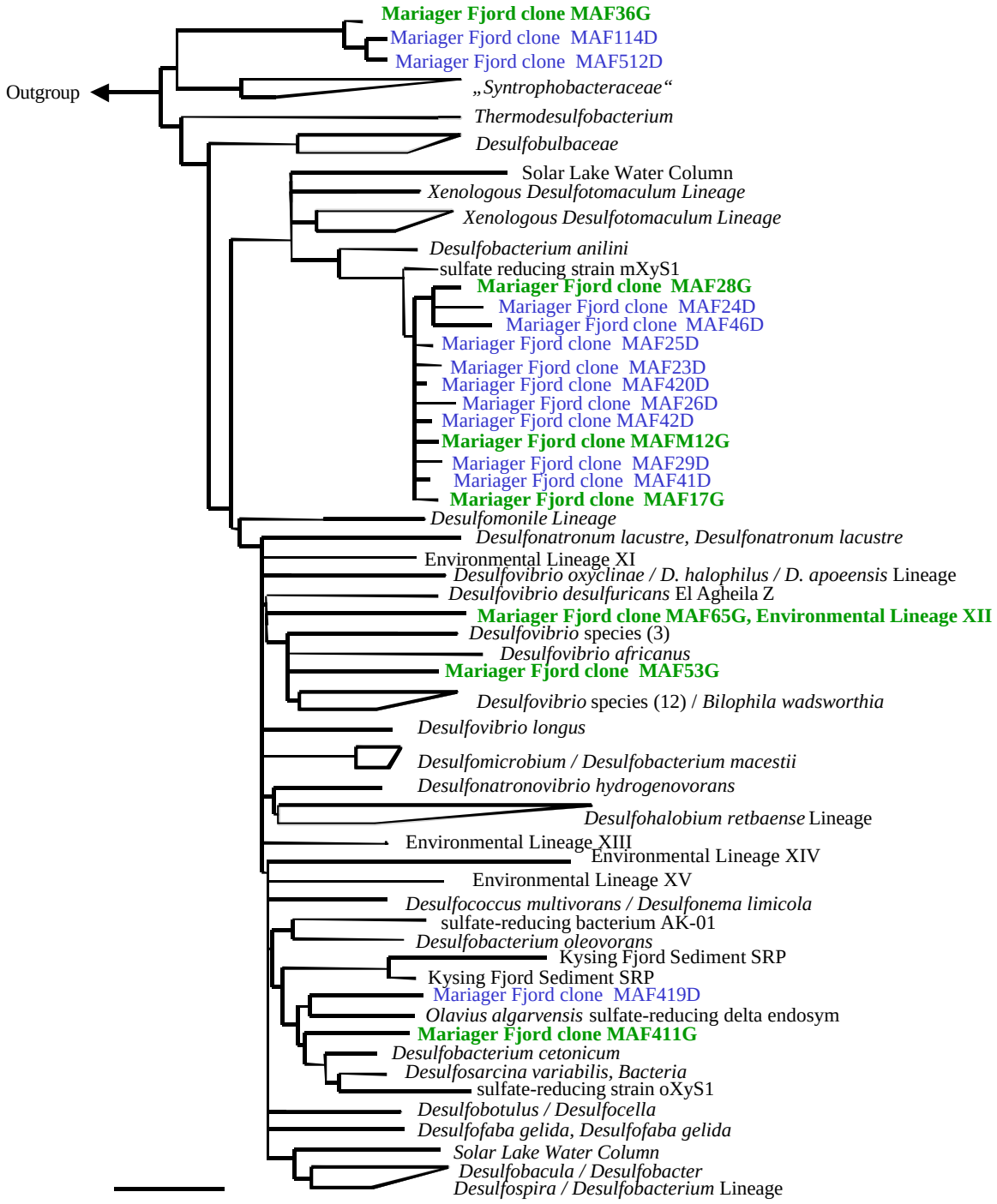


Figure 25 DsrAB based consensus dendrogram (as described above); species were grouped for transperency; directly cloned *dsrAB* sequences from Mariager Fjord are labeled blue, clones from gelretardation green, scale bar indicates 10% estimated sequence deviation.

The clones MAFM12G, MAF17G and MAF28G clustered with most of the directly cloned *dsrAB* sequences. In total, this cluster contains 12 out of 19 Mariager Fjord clones with in-cluster DsrAB amino acid identity values between 82% and 96%. The clone sequence of MAF36G groups together with the clones MAF512D (amino acid identity values of 94%) and MAF114D (amino acid identity values of 95%), respectively. The sequence of MAF411G was

placed in a cluster including *Desulfosarcina variabilis* cluster and clone MAF419D (69 to 82% identities on amino acid level). Clone MAF53G was clearly placed within the *Desulfovibrio/Bilophila* lineage, whereas clone MAF65G was not clearly affiliable to an existing lineage and was therefore considered as a separate environmental lineage.

The implementation of gelretardation in the *dsrAB* approach significantly reduces the effort to harvest the diversity hidden in a *dsrAB* PCR amplificat by providing direct access to different sequence types prior cloning. The application of gelretardation lead to the detection of three, so far unrecognized, *dsrAB* clone sequences in Mariager Fjord. The resolution of the gelretardation method was found to be less then 1 % difference in A+T%. If these encouraging findings will be confirmed in future studies, the gelretardation technique could be used as a supplement or substitution for the more cost and time intensive DGGE technique. The easy procedure and the low instrumental expense make the gelretardation a very useful tool for microbial ecology.

C.4.2 Combination of 16S rRNA and DsrAB approach for studying complex symbiosis

Microscopy can reveal the presence of different partners in complex symbiotic relationships. The limitation of light microscopy, in this context, is the inability of differentiating between prokaryotes with identical or similar morphology.

Examples are the bacterial symbionts of the gutless marine worm *Olavius algarvensis.* Distinguishable only by size and spatial allocation, two microorganisms below the cuticle of the worm were recognized (Dubilier 2001). The first step towards a more comprehensive understanding of this symbiosis was the identification of the involved bacteria symbionts by molecular biological methods. Fluorescence in situ hybridization (done partially by Michael Klein) and analysis of the 16S rRNA genes allowed the assignment of the symbionts too other gammaproteobacterial thioautotrophic symbionts of other marine oligochaetes and too deltaproteobacterial sulfate reducers, respectively (figure 26).

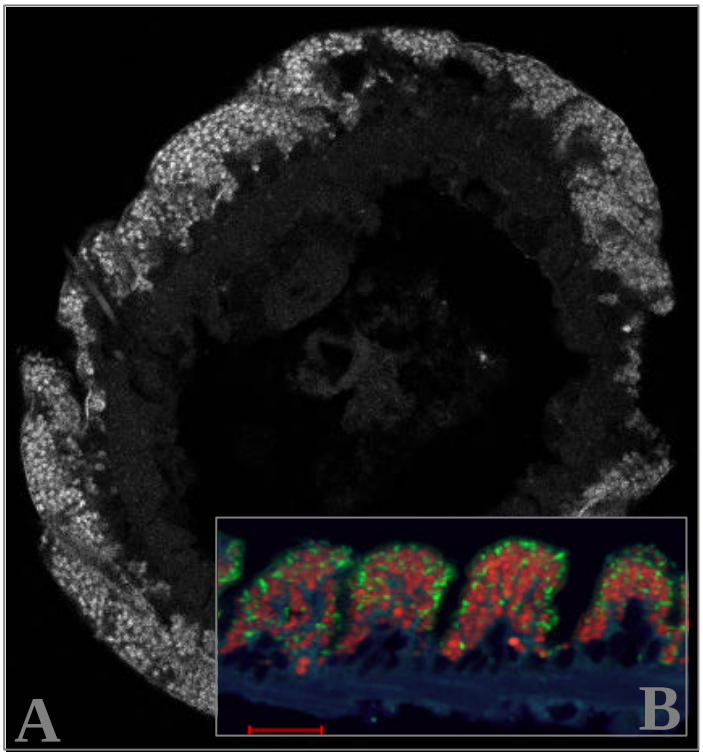


Figure 26 Fluorescence in situ hybridization of endosymbionts in *Olavius algarvensis* with oligonucleotide probes labeled with different fluorochromes. A. Cross-section though entire worm. B. gammaproteobacterial symbionts labeled in green, deltaproteobacterial symbionts in red, Scale bar 10 µm, graph taken by Michael Klein.

However, the phylogenetic affiliation of the symbionts by their 16S rRNA genes alone did not allow inference of their actual metabolic activities within their host. Thus, the ability of the gammaproteobacterial symbiont to fix CO_2 was proven by immunocytochemical labeling with antibodies directed against form I of the key enzyme of the Calvin – Benson cycle, ribuolose-1,5-bisphosphate carboxylase/oxygenase (RubisCO). The thiotrophic nature of these bacteria was further substantiated by electron microscopic spectroscopy, where the presence of sulfur containing membrane-bound vesicles was observed. The assumption drawn form the 16S rRNA analysis that the deltaproteobacterial symbiont is able to reduce sulfate was proven by PCR amplification of the dissimilatory sulfite reductase gene *dsrAB*. Subsequently the retrieved amplificats were sequenced along with the *dsrAB* genes from *Desulfosarcina variabilis* (both done by Michael Klein). Comparative sequence analysis of the deduced amino acids of these DsrAB sequences (done by Michael Klein) placed the deltaproteobacterial symbiont close to *Desulfosarcina variabilis* (consistent with the retrieved 16S rRNA sequence). Thus, we were able to demonstrate that the deltaproteobacterial symbiont carries a key enzyme of the

dissimilatory sulfite reduction. The evidence, that the deltaproteobacterial symbiont indeed carries out dissimilatory sulfate reduction and produces hydrogen sulfide as end product was confirmed by an additional experiment. Silver needles were inserted into individual living and formalin fixed worms. Worms were incubated in radiolabelled $^{35}SO_4^{2-}$ under microaerobic and aerobic conditions. After exposure of the needles to autoradiographic film, blots from the needles inserted in live worms under microaerobic conditions were the only to show a positive signal from ^{35}S-labelled sulfide that had precipitated on the needles (Dubilier 2001). This finding indicates that sulfate is reduced during dissimilatory sulfite reduction by the deltaproteobacterial symbiont of *O. algarvensis* under microaerobic conditions.

Combining all these facts, the symbiotic interactions between the two bacteria and *Olavius algarvensis* can be summarized as follows: a syntropohic sulfur cycle is maintained between the two bacterial symbionts by exchanging substrate (appendix). Toxic metabolites of the host are detoxified by the *Deltaproteobacterium* and used for sulfate reduction. The reduced sulfur compounds constitute a environmental independent sulfur source for the gammaproteobacterial symbiont and *Olavius algarvensis*.

C.4.3 Analysis of metabolic features of sulfate reducing prokaryotes

The retrieval of the *dsr* genes allows to specific detection of members of the guild of SRP in environmental samples. Beside the comparison of 16S rRNA gene and DsrAB based phylogeny the resemblance of metabolic peculiarities of SRP is very interesting. The question is whether a DsrAB based dendrogram can also be used as “phenogram”, connecting certain lineages of SRP with specific metabolic abilities.

For addressing this question oxidation capacities of reference strains were taken form publications and assigned to the respective strain in a DsrAB based dendrogram (dendrogram not shown, see table 7 below). It could be observed that cluster of phylogenetic related strains share e.g. the same oxidation capacity.

No clear connection of the type of dissimilatory sulfite reductase and the phylogenetic position, optimal growth temperature or the oxidation capacity can be observed. Moreover, no relation between the isotope fractionations (Detmers 2001) and the type or phylogenetic position of dissimilatory sulfite reductases or the termophilic /mesophilic lifestyle was found.

Comparison of oxidation capacity and isotope fractionation revealed a very global trend. Incomplete lactate oxidizing strains fractionated between 2.0‰ and 17‰ whereas complete acetate oxidizing species fractionated between 18.0‰ and 22.0‰. It is however striking that most of the acceptor strains of *dsrAB* genes from a deltaproteobacterial donor via lateral gene transfer (seven *Desulfotomaculum* species, three *Deltaproteobacteria*, and the two *Thermodesulfobacterium* strains) are complete oxidizers. With the exception of the *Thermodesulfobacterium* strains, they share a broad range of degradation capacities (Min 1990; Tasaki 1991; Widdel 1992a; Fardeau 1995; Nilsen 1996b; Liu 1997) and short doubling times (Stefan Spring personal communication). The Gram-Positive strains with an orthologous sulfite reductase on the other hand are growing very slowly (Stefan Spring personal communication) and on a very narrow substrate spectrum. In a future project the investigation of the question weather the genes necessary for sulfate respiration were transferred towards the Gram-positive xenologous *dsrAB* bearing strains in combination with other, e.g. degradation genes like the naphthalene dioxygenase gene or genes involved in 2,4-Dichlorophenoxyacetic acid degradation (Herrick 1997; McGowan 1998) could be of great interest.

Table 7 (on next page) Overview of metabolic features of sulfate reducing prokaryotes, values in brackets were taken form sources others than the first publication of a novel SRP (e.g. optimal growth temperatures were taken from www.dsmz.de). Vertical bars in red indicate incomplete oxidation of substrate, green indicates complete oxidation to CO_2. Blue bar indicates mesophilic organisms, yellow bar thermophilic organisms.

Organismus	Oxidation capacity	Growth temperature	Type of Dsr	Isotope fractination Detmers *et. al.* 2001
Thermodesulfovibrio islandicus	incomplete	65		
Thermodesulfovibrio yellowstonii	incomplete	65	Desulforubidin	-17,00
Archaeoglobus fulgidus	complete	83	Desulfoviridin	-17,00
Archaeoglobus profundus	complete	82		
Archaeoglobus veneficus	complete	80		
Desulfosporosinus orientis	incomplete	30	P582	
Desulfitobacterium dehalogenans	incomplete	37		
Desulfitobacterium halfniense	incomplete	37		
Desulfotomaculum alkaliphilum	complete	50-55		
Desulfotomaculum halophilum	(incomplete)	35		
Desulfotomaculum nigrificans	incomplete	55	P582	
Desulfotomaculum putei	incomplete	50-65		
Desulfotomaculum ruminis	incomplete	28-37	P582	
Desulfotomaculum aeronauticum	incomplete	37		
Desulfobacca acetoxidans	complete	37		-18,00
Thermodesulforhabdus norvegica	complete	60		
Desulfacinum infernum	(complete)	60		
Desulforhabdus amnigena	complete	37		
Desulfovirga adipica	complete	35		
Syntrophobacter fumaroxidans	incomplete	37		
Syntrophobacter wolinii	incomplete	(35)		
Desulfoarculus baarsii	complete	37		23,20
Thermodesulfobacterium commune	incomplete	70	Desulfofuscidin	-5,00
Thermodesulfobacterium mobile	incomplete	65	Desulfofuscidin	
Desulfobulbus elongatus	(incomplete)	(35)		-5,50
Desulfobulbus propionicus	incomplete	39	Desulforubidin	
Desulfobulbus rhabdoformis	incomplete	31	Desulforubidin	
Desulfobulbus sp. DSM2058	incomplete	29	Desulforubidin	
Desulfofustis glycolicus	incomplete	28	Desulforubidin	
Desulforhopalus singaporensis	incomplete	31		
Desulforhopalus vacuolatus	incomplete	18		
Desulfotalea psychrophila	(incomplete)	10		-4,30
sulfate reducing strain mXyS1	complete	30		
Desulfobacterium anilini	complete	(35)	P582	
Desulfotomaculum acetoxidans	complete	37	P582	
Desulfotomaculum geothermicum	complete	54		-12,50
Desulfotomaculum kuznetsovii	complete	50-65		
Desulfotomaculum thermoacetoxidans	complete	55-60		
Desulfotomaculum thermobenzoicum	complete	(60)		
Desulfotomaculum thermocisternum	incomplete	62		-15,00
Desulfotomaculum thermosapovorans	incomplete	50		
Desulfomonile tiedjei	complete	37	Desulfoviridin	
Desulfonatronum lacustre	incomplete	37-40		-18,70
Desulfomonas pigra	(incomplete)	(37)	Desulfoviridin	
Bilophila wadsworthia	(incomplete)	(30)	Desulfoviridin	
Desulfovibrio africanus	incomplete	30-36	Desulfoviridin	
Desulfovibrio burkinensis	(incomplete)	(35)	(Desulfoviridin)	
Desulfovibrio cuneatus	(incomplete)	(28)	(Desulfoviridin)	
Desulfovibrio desulfuricans El Agheila Z	(incomplete)	30	(Desulfoviridin)	
Desulfovibrio desulfuricans Essex 6	incomplete	30	Desulfoviridin	
Desulfovibrio fructosovorans	(incomplete)	(37)	(Desulfoviridin)	
Desulfovibrio gigas	(incomplete)	(34)	(Desulfoviridin)	
Desulfovibrio intestinalis	(incomplete)	(30)	(Desulfoviridin)	
Desulfovibrio termitidis	(incomplete)	(35)	(Desulfoviridin)	
Desulfovibrio vulgaris	incomplete	30-36	Desulfoviridin	
Desulfohalobium retbaense	(incomplete)	(35)	Desulfofuscidin	-10,60
Desulfovibrio longus	(incomplete)	(35)	(Desulfoviridin)	
Desulfovibrio oxyclinae	(incomplete)	(30)	Desulfoviridin	-4,50
Desulfovibrio halophilus	(incomplete)	(30-35)	Desulfoviridin	-2,00
Desulfovibrio aspoeensis	incomplete	(30)	Desulfoviridin	
Desulfonatronovibrio hydrogenovorans		37		-5,50
Desulfomicrobium apsheronum	(incomplete)	(30)	Desulforubidin	
Desulfomicrobium baculatum	(incomplete)	(30)	Desulforubidin	-12,70
Desulfomicrobium escambiense	(incomplete)	(30)	(Desulforubidin)	
Desulfomicrobium norvegicum	(incomplete)	(30)	(Desulforubidin)	
Desulfomicrobium orale	(incomplete)	(37)	(Desulforubidin)	
Desulfobacterium macestii	(incomplete)	(35)	(Desulforubidin)	
Desulfonema limicola	(complete)	(30)	Desulfoviridin	
Desulfococcus multivorans	complete	35	Desulfoviridin	
Desulfosarcina variabilis	complete	33	Desulforubidin	-15,00
sulfate-reducing strain oXyS1	complete	32	Desulfoviridin	
Desulfobacterium oleovorans	complete	(28-30)		
Desulfobotulus sapovorans	incomplete	34	P582	-16,50
Desulfocella halophila	incomplete	34		-8,10
Desulfofaba gelida	incomplete	7	Desulforubidin	
Desulfomusa hansenii	(incomplete)	20		
Desulfobacterium vacuolatum	complete	25-30		
Desulfobacterium autotrophicum	complete	20-26		-32,70
Desulfobacter curvatus	complete	28-32		
Desulfobacter latus	complete	29-32	Desulforubidin	
Desulfobacter postgatei	complete	28-33		
Desulfobacter vibrioformis	complete	33	Desulforubidin	
Desulfobacula phenolica	complete	28		-36,70
Desulfobacula toluolica	complete	28		-28,50
Desulfospira joergensenii	complete	26-30		-25,70
Desulfotignum balticum	complete	28-32		-23,10
Desulfotignum phosphitoxidans	complete	30	Desulforubidin	

In summary, metabolic abilities seem to be reflected phylogenetically only in very tight groups of closely related SRP.

C.5 Limitations as chances and outlook

Novel *dsrAB* sequences isolated form the environment can form unaffiliated novel lineages within phylogenetic trees. In any case where no closely related *dsrAB* sequence from a pure culture reference strain is present, phylogenetic assignment of the environmental sequence is not possible.

Today, their is no possible way of linking environmental 16S rRNA and *dsrAB* sequences if these sequences have not been isolated from systems harboring simple microbial communities like the sub-cuticle of a marine worm which has been described above. In other words, in many ecosystems it is possible to identify SRP by sequencing the *dsr* genes and to quantify cells by 16S rRNA based fluorescence in situ hybridization, but linking this information is nearly impossible.

A new approach to circumvent this problem lies in the field of environmental genomics (Rondon 2000), where quite large DNA fragments can be cloned and subsequently sequenced (Rondon 2000). The genomes of at least some sulfate reducing prokaryotes have been demonstrated to be of relatively small size, e.g. *Archaeoglobus fulgidus* genome size is only 2,18 Mb (Klenk 1997) and *Desulfovibrio vulgaris* is 3,2 Mb (partial genome at www.ncbi.nlm.nih.gov) to 3,6 Mb (Devereux 1997, as determined by pulsed field gelelectrophoresis). The simultaneous detection of a novel *dsrAB* gene and the corresponding 16S rRNA gene (or another marker molecule suitable for phylogeny) on a single DNA fragment cloned from the environment in a bacterial artificial chromosome (BAC, Rondon 2000) would allow phylogenetic analyses of novel *dsrAB* lineages without cultivation. Additionally, the 16S rRNA gene information could be used for further, e.g. quantitative oligonucleotide probe based analyses. In order to increase the probability of retrieving both desired genes at one yeast artificial chromosomes (YACs) could be used as an alternative application for of BACs. YACs are able to take up DNA inserts of up to 1 Mb (Burke 1987). The evaluation of this approach has to be done in future experiments and it would be highly useful for the identification of so far unaffiliated environmental clones as well as the recognition of novel sulfate reducing lineage's. Another promising progress for fast screening of environmental SRP communities is the combination of the *dsrAB* approach with microarray technology. Here the development of SRP chips (Loy 2002) that can contain ribosomal DNA/RNA targeted oligonucleotide probes as well as *dsr* or messenger *dsr* targeted

oligonucleotide probes might evens allow not only to determine the SRP community composition but also to identify those organisms which were actively respiring with sulfate at the time of sampling.

Complete genome sequencing and subsequent “in silico” analyses have become a valuable tool r in molecular biology and ecology. The dimens ion of the use of these novel techniques can not yet been fully assessed. These inventive ways of investigating the history and tracking of phylogenetic relationships of SRP can now be used to clear the question of the donor strain of lateral *dsrAB* gene transfers by comparative analysis of *dsr* operon structures. Additionally, other genes that are also involved in dissimilatory sulfite reduction e.g. genes encoding APS reductase (Friedrich 2002) can be surveyed. The novel approaches will also help to discover the mode of inter-species DNA transfer.

In conclusion, the *dsr* approach is an essential tool for studying the ecology and phylogenetic relationship of sulfate reducing prokaryotes. Therefore, it could be appropriate to call for the deposition of the *dsrAB* sequences when novel sulfate reducing strains are described.

D Summary

All members of the guild of sulfate reducing prokaryotes (SRP) gain energy via dissimilatory sulfate reduction. The phylogenetic inhomogeneity of this group does not allow to target 16S ribosomal RNA genes of all its members with a simple set of oligonucleotide probes or PCR primers. It is further impossible to identify novel lineages of SRP by their ribosomal RNA solely. Thus, the suitability of the dissimilatory (bi-) sulfite reductase (DSR), a key enzyme of the dissimilatory sulfate reduction pathway, as alternative phylogenetic marker molecule for SRP was investigated. For this purpose, the evolutionary history of the DSR was studied using a large collection of pure culture SRP and correspondence with the 16S rRNA gene based phylogeny was inspected.

In addition, cultivation independent SRP diversity surveys were carried out based on comparative amino acid sequence analysis of environmentally retrieved *dsr* clones in order to test whether cultured representatives of SRP adequately represent the natural diversity of this guild.

In order to build up an encompassing DSR reference data base, existing primers were optimized to PCR amplify a 1.9 kb *dsrAB* fragment from 30 pure culture reference strains. These stains represented all lineages of SRP recognized at this time. *dsrAB* gene fragments were cloned and sequenced. Subsequent comparative phylogenetic sequence analyses of all available DsrAB pure culture sequences and their corresponding 16S rRNA gene sequences lead to the discovery of at least three presumptive lateral *dsr* gene transfer events from (i) a deltaproteobacterial donor to the genus *Thermodesulfobacterium*, (ii) a deltaproteobacterial donor related to *Desulfobacterium anilini* to certain *Desulfotomaculum* strains, and (iii) a bacterial donor to *Archaeoglobus*. Although these events complicate the interpretation of *dsrAB*-based SRP diversity studies, the Dsr-approach represents the best available method for simultaneous detection of recognized and novel SRP in environmental samples.

In this thesis, the Dsr-approach was applied to investigate SRP diversity in the water column of Mariager Fjord (Denmark), and Solar Lake (Egypt), as well as tissue material from a marine worm. The sequences originating form these studies were analyzed along with 550 publicly available environmental *dsrAB* sequences. 13 environmental SRP lineages without closely related isolated or sequenced SRP reference strains were identified suggesting that many environmentally important SRP lineages have not yet been successfully cultured. Further, a detailed inspection of all available environmental *dsrAB*

sequences revealed characteristic SRP lineages in different ecosystems like soil, sediment, marine water, hypersaline water, and in symbiotic relationships.

In conclusion, this thesis showed that lateral gene transfer was significantly influencing the evolutionary history of the DSR. The generally accepted opinion of strict vertical transmission of the key enzyme of dissimilatory sulfate reduction has to be adjusted. Nevertheless, it could be demonstrated that the Dsr – approach is a valid tool for investigating the diversity and biogeography of SRPs.

E Zusammenfassung

Alle Mitglieder der Gilde der sulfatreduzierenden Prokaryonten (SRP) gewinnen ihre Energie mittels dissimilatorischer Sulfatreduktion.

Wegen der phylogenetischen Uneinheitlichkeit dieser Gruppe können die ribosomalen 16S- rRNA- Gene ihrer Mitglieder nicht mit einem einfachen Satz von Oligonukleotidsonden oder PCR- Primern erfasst werden. Ferner ist es nicht möglich neue Linien von SRP alleine anhand ihrer ribosomalen RNA- Gene zu erkennen.

Aus diesen Gründen wurde die Eignung der dissimilatorischen (Bi-) Sulfitreduktase (DSR), einem Schlüsselenzym der dissimilatorischen Sulfatreduktion, als alternatives phylogenetisches Markermolekül für SRP untersucht.

Hierzu wurde die evolutionäre Geschichte der DSR anhand einer großen Auswahl von SRP Reinkulturen näher betrachtet und die Übereinstimmung mit der 16S- rRNA- Gen basierenden Phylogenie überprüft.

Zusätzlich dazu wurden kultivierungsunabhängige SRP- Diversitätsstudien auf der Grundlage vergleichender Aminosäuresequenzanalysen von umweltstämmigen *dsr*-Klonen vorgenommen, um festzustellen, ob die kultivierten Repräsentanten der SRP das natürliche Vorkommen dieser Gilde widerspiegeln.

Um eine umfassende Referenzdatenbank aufzubauen wurden existierende Primer so optimiert, dass ein 1.9 kb großes *dsrAB* –Genfragment von 30 Reinkulturreferenzstämmen mittels PCR amplifiziert werden konnte. Diese untersuchten Stämme repräsentierten alle, zum Beginn der Doktorarbeit bekannten, SRP- Linien. Die *dsrAB*- Genfragmente wurden kloniert und sequenziert. Anschließende vergleichende phylogenetische Analysen aller verfügbaren DsrAB- Sequenzen und ihrer entsprechenden 16S- rRNA- Gensequenzen führte zur Entdeckung von mindestens drei (mutmaßlichen) lateralen *dsr*-Genstransferereignissen von (i) einem deltaproteobakteriellen Donor zu *Thermodesulfobacterium*, (ii) einem mit *Desulfobacterium anilini* verwandten, deltaproteobakteriellen Donor zu bestimmten *Desulfotomaculum* Stämmen und (iii) einem bakteriellen Donor zu *Archaeoglobus*.

Obwohl diese Ereignisse die Interpretation von *dsrAB*- basierenden SRP-Diversitätsstudien komplizieren, bleibt der *dsr* Ansatz die am besten geeignete Methode zum gleichzeitigen Nachweis von bekannten und neuen SRP in Umweltproben.

In der vorliegenden Arbeit wurde der Dsr-Ansatz verwendet um die Artenvielfalt der SRP in der Wassersäule des Mariager Fjords (Dänemark), des Solar Lakes (Ägypten) sowie in Gewebematerial eines marinen Wurms zu erforschen.

Dsr- Sequenzen dieser Studien wurden zusammen mit 550 publizierten Dsr-Umweltsequenzen analysiert. Auf diese Weise wurden 13 Umwelt- SRP- Linien identifiziert, welche keinem nahe verwandten Isolat oder sequenzierten Reinkulturstamm zugeordnet werden konnten. Dies legt den Schluss nahe, dass viele wichtige Umwelt- SRP-Linien bis jetzt noch nicht erfolgreich kultiviert wurden.

Detaillierte Untersuchungen aller zur Verfügung stehenden *dsrAB*- Sequenzen zeigten auf, dass charakteristische SRP- Linien in verschiedenen Ökosystemen wie Boden, Sediment, Meerwasser und in hypersalinem Wasser vorhanden sind.

Zusammenfassend wurde mit dieser Doktorarbeit gezeigt, dass lateraler Gentransfer einen großen Einfluss auf die evolutionäre Geschichte der DSR hatte. Die Lehrmeinung der strikten vertikalen Weitergabe der Gene des Schlüsselenzyms der dissimilatorischen Sulfatreduktion muss somit überdacht werden. Dennoch erwies sich der Dsr-Ansatz als eine wertvolle Methode zur Untersuchung von Diversität und Biogeographie der SRP.

F References

Abdelouas, A., W. Lutze, W. Gong, E. Nuttall, B. Strietelmeier and B. Travis (2000). "Biological reduction of uranium in groundwater and subsurface soil." Sci Total Environ **250**(1-3).

Adachi, J. and M. Hasegawa (1996). Computer science monographs, no. 28 MOLPHY programs for molecular phylogenetics based on maximim likelihood. Tokyo, Japan.

Aeckersberg, F., F. Bak and F. Widdel (1991). "Anaerobic oxidation of saturated hydrocarbons to CO2 by a new type of sulfate-reducing bacteria." Arch Microbiol **156**: 5-14.

Akagi, J. M. and V. Adams (1973). "Isolation of a bisulfite reductase activity from Desulfotomaculum nigrificans and its identification as the carbon monoxide-binding pigment P582." J Bacteriol **116**(1): 392-6.

Alm, E. W., D. B. Oerther, N. Larsen, D. A. Stahl and L. Raskin (1996). "The Oligonucleotide Probe Database." Applied and Environmental Microbiology **62**: 3557-3559.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers and D. J. Lipman (1990). "Basic local alignment search tool (BLAST)." J. Mol. Biol. **215**: 290-296.

Amann, R. (1995a). In situ identification of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes. Molecular Microbial Ecology Manual. A. D. C. Akkman, J. D. v. Elsas and F. J. Bruijin. Dortrecht. **3.3.6:** 1-15.

Amann, R. I., W. Ludwig and K. H. Schleifer (1995b). "Phylogenetic identification and in situ detection of individual microbial cells without cultivation." Micobiol. Rev. **59**: 143-169.

Bak, F. and N. Pfennig (1991). "Sulfate-Reducing Bacteria in Littoral Sediment of Lake Constance." FEMS Microbiol. Ecol. **85**: 43–52.

Baker, B., D. Moser, B. MacGregor, S. Fishbain, M. Wagner, N. Fry, B. Jackson, N. Speolstra, S. Loos, K. Takai, B. Lollar, J. Fredrickson, D. Balkwill, T. Onstott, C. Wimpee and D. Stahl (2003). "Related assemblages of sulphate-reducing bacteria associated with ultradeep gold mines of South Africa and deep basalt aquifers of Washington State." Environ Microbiol. **5**(4): 267-77.

Bale, S. J., K. Goodman, P. A. Rochelle, J. R. Marchesi, J. C. Fry, A. J. Weightman and R. J. Parkes (1997). "Desulfovibrio profundus sp. nov., a novel barophilic sulfate-reducing bacterium from deep sediment layers in the Japan Sea." Int J Syst Bacteriol **47**(2): 515-21.

Barton, L. L., J. LeGall, J. M. Odom and J. Harry D Peck (1983). "Energy coupling to nitrite respiration in the sulfate-reducing bacterium *Desulfovibrio gigas*." J Bacteriol **153**(2): 867-871.

Beeder, J., R. K. Nilsen, T. Thorstenson and T. Torsvik (1996). "Penetration of Sulfate Reducers through a Porous North Sea Oil Reservoir." Applied and Environmental Microbiology **62**(9): 3551-3553.

Beeder, J., T. Torsvik and T. Lien (1995). "Thermodesulforhabdus norvegicus gen. nov., sp. nov., a novel thermophilic sulfate-reducing bacterium from oil field water." Arch Microbiol **164**(5): 331-6.

Biebl, H. and N. Pfennig (1978). "Growth yields of green sulfur bacteria in mixed cultures with sulfur and sulfate reducing bacteria." Archieves of Microbiology **117**: 9-16.

Biedermann, H. (1991). Lexikon der magischen Künste. Munich, Germany, Wilhelm HeyneVerlag Gmbh & Co. KG.

Boetius, A., K. Ravenschlag, C. Schubert, D. Rickert, F. Widdel, A. Gieseke, R. Amann, B. Jorgensen, U. Witte and O. Pfannkuche (2000). "A marine microbial consortium apparently mediating anaerobic oxidation of methane." Nature **5**(407): 623-626.

Bonnet, R., A. Suau, J. Dore, G. R. Gibson and M. D. Collins (2002). "Differences in rDNA libraries of faecal bacteria derived from 10- and 25-cycle PCRs." Int J Syst Evol Microbiol **52**(3): 757-763.

Boone, D. R. and M. P. Bryant (1980). "Propionate-degrading bacterium Syntrophobacter wolinii sp. nov., from methanogenic ecosystems." Appl. Environ. Microbiol. **40**: 626-632.

Boschker, H. T., W. de Graaf, M. Koster, L. Meyer-Reil and T. E. Cappenberg (2001). "Bacterial populations and processes involved in acetate and propionate consumption in anoxic brackish sediment." FEMS Microbiol Ecol **35**(1): 97-103.

Bosshard, P. P., Y. Santini, D. Grüter, R. Stettler and R. Bachofen (2000). "Bacterial diversity and community composition in the chemocline of the meromictic alpine Lake Cadagno as revealed by 16S rDNA analysis." FEMS Microbiology Ecology **31**(2): 173-182.

Bowman, J. P., S. M. Rea, S. A. McCammon and T. A. McMeekin (2000). "Diversity and community structure within anoxic sediment from marine salinity meromictic lakes and a coastal meromictic marine basin, Vestfold Hills, Eastern Antarctica." Environmental Microbiology **2**(2): 227-237.

Boyle, A. W., C. D. Phelps and L. Y. Young (1999). "Isolation from estuarine sediments of a Desulfovibrio strain which can grow on lactate coupled to the reductive dehalogenation of 2,4, 6-tribromophenol." Appl Environ Microbiol **65**(3): 1133-40.

Brauman, A., J. Koenig, J. Dutreix and G. JL. (1990). "Characterization of two sulfate-reducing bacteria from the gut of the soil-feeding termite, Cubitermes speciosus." Antonie Van Leeuwenhoek **58**(4): 271-275.

Breznak, J. A. and A. Brune (1994). "Role of microorganisms in the digestion of lignocellulose by termites." Annu. Rev. Entomol. **39**: 453–487.

Brock, T. D., M. T. Madigan, J. M. Martinko and J. Parker (1994). Biology of Microorganisms. New Jersey, Prentice-Hall International, Inc.

Brysch, K., C. Schneider, G. Fuchs and F. Widdel (1987). "Lithoautotrophic growth of sulfte-reducing bacteria, and decription of *Desulfobacter autotrophicum* gen. nov., sp. nov." Arch Microbiol **148**: 264-274.

Burke, D. T., G. F. Carle and M. V. Olson (1987). "Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors." Science **236**: 806-812.

Castro, H., K. Reddy and O. A (2002). "Composition and Function of Sulfate-Reducing Prokaryotes in Eutrophic and Pristine Areas of the Florida Everglades dagger." Appl Environ Microbiol **68**(12): 6129-6137.

Castro, H. F., N. H. Williams and A. Ogram (2000). "Phylogeny of sulfate-reducing bacteria." FEMS Microbiology Ecology **31**: 1-9.

Caumette, P., Y. Cohen and R. Matheron (1991). "Isolation and characterization of Desulfovibrio halophilus sp. nov., a halophilic sulfate-reducing bacterium isolated from Solar Lake (Sinai)." System. Appl. Microbiol. **14**: 33-38.

Chang, Y.-J., A.-D. Peacock, P.-E. Long, J.-R. Stephen, J.-P. McKinley, S.-J. Macnaughton, A. K. M. A. Hussain, A.-M. Saxton and D.-C. White (2001). "Diversity and characterization of sulfate-reducing bacteria in groundwater at a uranium mill tailings site." Applied-and-Environmental-Microbiology **67**(7): 3149-3160.

Coleman, G. S. (1960). "A sulfate-reducing bacterium from the sheep rumen." J. Gen. Microbiol. **22**: 423-436.

Cottrell, M. T. and S. C. Cary (1999). "Diversity of Dissimilatory Bisulfite Reductase Genes of Bacteria Associated with the Deep-Sea Hydrothermal Vent Polychaete Annelid Alvinella pompejana." Appl. Environ. Microbiol. **65**: 1127-1132.

Cowen, J. P., S. J. Giovannoni, F. Kenig, H. P. Johnson, D. Butterfield, M. S. Rappe, M. Hutnak and P. Lam (2003). "Fluids from Aging Ocean Crust That Support Microbial Life." Science **299**(5603): 120-123.

Crane, B. R., L. M. Siegel and E. D. Getzoff (1995). "Sulfite Reductase Structure at 1.6 A: Evolution and Catalysis for Reduction of Inorganic Anions." Science **270**(6): 59-67.

Crump, B. C., E. V. Armbrust and J. A. Baross (1999). "Phylogenetic Analysis of Particle-Attached and Free-Living Bacterial Communities in the Columbia River, Its Estuary, and the Adjacent coastal Ocean." Applied and Environmental Microbiology **65**(7): 3192-3204.

Cypionka, H. (2000). "Oxygen respiration by *Desulfovibrio* species." Annu Rev Microbiol **54**: 827-48.

Dahl, C., N. M. Kredich, R. Deutzmann and H. G. Trüper (1993). "Dissimilatory sulfite reductase form *Archaeoglobus fulgidus*: physico-chemical properties of the enzyme and cloning, sequencing and analysis of the reductase gene." Journal of General Microbiology **139**: 1817-1828.

Dahl, C. and H. G. Truper (2001). "Sulfite reductase and APS reductase from Archaeoglobus fulgidus." Methods Enzymol **331**: 427-41.

Daly, K., R. J. Sharp and A. J. McCarthy (2000). "Development of oligonucleotide probes and PCR primers for detecting phylogenetic subgroups of sulfate-reducing bacteria." Microbiology **146**(Pt 7): 1693-705.

Daumas, S., R. Cord-Ruwisch and J. L. Garcia (1988). "Desulfotomaculum geothermicum sp. nov., a thermophilic, fatty acid-degrading, sulfate-reducing bacterium isolated with H2 from geothermal ground water." Antonie Van Leeuwenhoek **54**(2): 165-78.

DeLong, E. F., G. S. Wickham and N. R. Pace (1989). "Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells." Science **243**(4896): 1360-3.

Deplancke, B., K. R. Hristova, H. A. Oakley, V. J. McCracken, R. Aminov, R. I. Mackie and H. R. Gaskins (2000). "Molecular ecological analysis of the succession and diversity of sulfate-reducing bacteria in the mouse gastrointestinal tract." Appl Environ Microbiol **66**(5): 2166-74.

Detmers, J., V. Brüchert, K. S. Habicht and J. Kuever (2001). "Diversity of Sulfur Isotope Fractionations by Sulfate-Reducing Prokaryotes." Applied and Environmental Microbiology **67**: 888-894.

Devereux, M., M. D. Kane, J. Winfrey and D. A. Stahl (1992). " Genus- and Group-Specific Hybridization Probes for Determinative Studies of Sulfate-Reducing Bacteria." System. Appl. Microbiol. **15**: 601-609.

Devereux, R., M. Hines and D. Stahl (1996). "S Cycling: Characterization of Natural Communities of Sulfate-Reducing Bacteria by 16S rRNA Sequence Comparisons." Microb Ecol **32**: 283-292.

Devereux, R. and G. W. Mundfrom (1994). "A Phylogenetic Tree of 16S rRNA Sequences from Sulfate-Reducing Bacteria in a Sandy Marine Sediment." Appl. Environ. Microbiol. **60**: 3437-3439.

Devereux, R., S. G. Willis and M. E. Hines (1997). "Genome sizes of Desulfovibrio desulfuricans, Desulfovibrio vulgaris, and Desulfobulbus propionicus estimated by pulsed-field gel electrophoresis of linearized chromosomal DNA." Curr Microbiol **34**(6): 337-9.

DeWeerd, K. A., L. Mandelco, R. S. Tanner, C. R. Woese and J. M. Suflita (1990). "*Desulfomonile tiedjei* gen. nov. and sp. nov., a novel anaerobic, dehalogenating, sulfate-reducing bacterium." Archives of Microbiology **154**: 23-30.

Dilling, W. and H. Cypionka (1990). "Aerobic respiration in sulfate-reducing bacteria." FEMS Micobiol. Lett. **71**: 123-128.

DiRuggiereo, J., D. Dunn, D. L. Maeder, R. Holley-Shanks, J. Chatard, R. Horlacher, F. T. Robb, W. Boos and R. B. Weiss (2000). "Evidence of recent lateral gene transfer among hyperthermophilic Archaea." Molecular Microbiogy **38**(4): 684-693.

Dojka, M. A., P. Hugen, S. K. Haack and N. R. Pace (1998). "Microbail Diversity in a Hydrocarbon- and Chlorinated-Solvent-Contaminated Aquifer Undergoing Intrinsic Bioremediation." Applied and Environmental Microbiology **64**(10): 3869-3877.

Drzyzga, O., S. Jannsen and K. H. Blotevogel (1994). "Mineralization of monofluorobenzoate by a diculture under sulfate-reducing conditions." FEMS Microbiol Lett **116**(2): 215-9.

Dubilier, N., C. Mulders, T. Ferdelman, D. de Beer, A. Pernthaler, M. Klein, M. Wagner, C. Erseus, F. Thiermann, J. Krieger, O. Giere and R. Amann (2001). "Endosymbiotic sulphate-reducing and sulphide-oxidizing bacteria in an oligochaete worm." Nature **411**(6835): 298-302.

Eschemann, A., M. Kühl and H. Cypionka (1999). "Aerotaxis in *Desulfovibrio*." Environ Microbiol **1**: 489-495.

Fardeau, M.-L., B. Ollivier, B. K. C. Patel, P. Dwivedi, M. Ragot and J.-L. Garcia (1995). "Isolation and Characterization of a Thermophlic Sulfate-Reducing Bacterium, *Desulfotomaculum thermosapovorans* sp. nov." International Journal of Systematic Bacteriology **45**(2): 218-221.

Fauque, G., A. R. Lino, M. Czechowski, L. Kang, D. V. DerVartanian, J. J. Moura, J. LeGall and I. Moura (1990). "Purification and characterization of bisulfite reductase (desulfofuscidin) from Desulfovibrio thermophilus and its complexes with exogenous ligands." Biochim Biophys Acta **1040**(1): 112-8.

Felsenstein, J. (1993). PHYLIP (Phylogeny Inference Package). Department of Genetics, University of Washington, Seattle, Distributed by the author.

Ferris, M. H. and D. M. Ward (1997). "Seasonal distributions of dominant 16S rDNA-defined populations in a hot spring micobial mat examined by denaturing gradient gel electrophoresis." Appl Environ Microbiol **63**: 1375-1381.

Finster, K., W. Liesack and B. Tindall (1997). "*Desulfospira joergensenii*, gen.nov., sp. nov., a new sulfate-reducing bacterium isolated from marine surface sediment." Systematic and Applied Microbiology **20**: 201-208.

Fox, G., J. Wisotzkey and P. Jurtshuk, Jr (1992). "How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity." Int J Syst Bacteriol **42**(1): 166-170.

Friedrich, M. and B. Schink (1995). "Isolation and characterization of a desulforubidin-containing sulfate-reducing bacterium growing with glycolate." Archives of Microbiology **164**(4): 271-279.

Friedrich, M. W. (2002). "Phylogenetic analysis reveals multiple lateral transfers of adenosine-5'-phosphosulfate reductase genes among sulfate-reducing microorganisms." J Bacteriol **184**(1): 278-89.

Frohlich, J., H. Sass, H. D. Babenzien, T. Kuhnigk, A. Varma, S. Saxena, C. Nalepa, P. Pfeiffer and H. Konig (1999). "Isolation of *Desulfovibrio intestinalis* sp. nov. from the hindgut of the lower termite Mastotermes darwiniensis." Can J Microbiol **45**(2): 145-52.

Fry, N. K., J. K. Fredrickson, S. Fishbain, M. Wagner and D. A. Stahl (1997). "Population Structure of Microbial Communities Associated with Two Deep, Anaerobic, Alkaline Aquifers." Applied and Environmental Microbiology **63**(4): 1498-1504.

Fu, R. and G. Voordouw (1998). "ISD1, an Insertion Element from the Sulfate-Reducing Bacterium *Desulfovibrio vulgaris* Hildenborough: Structure, Transposition, and Distribution." Appl Environ Microbiol **64**: 53-61.

Fukuba, T., M. Ogawa, T. Fujii and T. Naganuma (2003). "Phylogenetic diversity of dissimilatory sulfite reductase genes from deep-sea cold seep sediment." Mar Biotechnol (NY) **5**(5): 458-68.

Fukui, M. and S. Takii (1996). "Microdistribution of sulfate-reducing bacteria in sediments of a hypertrophic lake and their response to the addition of organic matter." Ecological-Research **11**(3): 257-267.

Gall, J. L. and A. V. Xavier (1996). "Anaerobes Response to Oxygen: Sulfate-reducing Bacteria." Anaerobe **2**: 1-9.

Galushko, A., D. Minz, B. Schink and F. Widdel (1999). "Anaerobic degradation of naphthalene by a pure culture of a novel type of marine sulphate-reducing bacterium." Environ Microbiol **1**(5): 415-420.

Galushko, A. and E. Rozanova (1991). "Desulfobacterium cetonicum sp. nov.: a sulfate-reducing bacterium which oxidizes fatty acids and ketones." Microbiology (Engl. Transl. of Mikrobiologiya) **60**: 742-746.

Garcia-Vallve, S., A. Romeu and J. Palau (2000). "Horizontal gene transfer in bacterial and archaeal complete genomes [In Process Citation]." Genome Res **10**(11): 1719-25.

Gibson, G. R., J. H. Cummings and G. T. Macfarlane (1991). "Growth and activities of sulphate-reducing bacteria in gut contents of healthy subjects and patients with ulcerative colitis." FEMS Microbiol Ecol **86**: 103-112.

Gogarten, J. P., H. Kibak, P. Dittrich, L. Taiz, E. J. Bowman, B. J. Bowman, M. F. Manolson, R. J. Poole, T. Date, T. Oshima and et al. (1989). "Evolution of the vacuolar H+-ATPase: implications for the origin of eukaryotes." Proc Natl Acad Sci U S A **86**(17): 6661-5.

Gray, J. P. and R. P. Herwig (1996). "Phylogenetic Analysis of the Bacterial Communities in Marine Sediments." Appl. Environ. Microbiol. **62**: 4049-4059.

Großkopf, R., P. H. Janssen and W. Liesack (1998). "Diversity and Structure of the Methanogenic Community in Anoxic Rice Paddy Soil Microcosms as Examined by Cultivation and Direct 16S rRNA Gene Sequence Retrieval." Applied and Environmental Mircobiology **64**(3): 960-969.

Gupta, R. S. (1998). "Protein phylogenies and signature sequences: A reappraisal of evolutionary relationships among archaebacteria, eubacteria, and eukaryotes." Microbiol Mol Biol Rev **62**(4): 1435-91.

Hagenauer, A., H. Hippe and F. A. Rainey (1997). "*Desulfotomaculum aeronauticum* sp. nov., a sporeforming, thiosulfate-reducing bacterium form corroded aluminium alloy in an aircraft." Systematic and Applied Microbiology **20**: 65-71.

Hansen, M. C., T. Tolker-Nielsen, Michaelgivskov and S. Molin (1998). "Biased 16S rDNA PCR amplification caused by interference from DNA flanking the template region." FEMS Microbiology Ecology **26**: 141-149.

Hansen, T. A. (1994). "Metabolism of sulfate-reducing prokaryotes." Antonie van Leeuwenhoek **66**: 165-185.

Harms, G., K. Zengler, R. Rabus, F. Aeckersberg, D. Minz, R. Rossello-Mora and F. Widdel (1999). "Anaerobic Oxidation of o-Xylene, m-Xylene, and Homologous Alkylbenzenes by New Types of Sulfate-Reducing Bacteria." Appl. Environ. Microbiol. **65**: 999-1004.

Harmsen, H., B. Van Kuijk, C. Plugge, A. Akkermans, W. De Vos and A. Stams (1998). "Syntrophobacter fumaroxidans sp. nov., a syntrophic propionate- degrading sulfate-reducing bacterium." Int J Syst Bacteriol **48**(4): 1383-1387.

Hatchikian, E. C. and J. G. Zeikus (1983). "Characterization of a new type of dissimilatory sulfite reductase present in Thermodesulfobacterium commune." J Bacteriol **153**(3): 1211-20.

Hausmann, K. and B. P. Kremer (1995). Extremophile, Mikoorganismen in ausgefallenen Lebensräumen. Weinheim, Germany, VCH Verlagsgesellschaft.

Hayes, L. and D. Lovley (2002). "Specific 16S rDNA Sequences Associated with Naphthalene Degradation under Sulfate-Reducing Conditions in Harbor Sediments." Microb Ecol **43**(1): 134-145.

Heider, J., A. M. Spormann, H. R. Beller and F. Widdel (1998). "Anaerobic bacterial metabolism of hydrocarbons." FEMS Microbiology Reviews **22**(5): 459-473.

Henckel, T., M. Friedrich and R. Conrad (1999). "Molecular analyses of the methane-oxidizing microbial community in rice field soil by targeting the genes of the 16S rRNA, particulate methane monooxygenase, and methanol dehydrogenase." Appl Environ Microbiol **65**: 1980-90.

Herrick, J. B., K. G. Stuart-Keil, W. C. Ghiorse and E. L. Madsen (1997). "Natural horizontal transfer of a naphthalene dioxygenase gene between bacteria native to a coal tar-contaminated field site." Appl Environ Microbiol **63**(6): 2330-7.

Hipp, W. M., A. S. Pott, N. Thum-Schmitz, I. Faath, C. Dahl and H. G. Trüper (1997). "Towards the phylogeny of APS reductases and sirohaem sulfite reductases in sulfate-reducing and sulfur-oxidizing prokaryotes." Microbiology **143**: 2891-2902.

Hristova, K. R., M. Mau, D. Zheng, R. I. Aminov, R. I. Mackie, H. R. Gaskins and L. Raskin (2000). "Desulfotomaculum genus- and subgenus-specific 16S rRNA hybridization probes for environmental studies." Environ Microbiol **2**(2): 143-59.

Hugenholtz, P., B. N. Goebel and N. R. Pace (1998). "Impact of Culture-Independent Studies on the Emerging Phylogenetic View of Bacterial Diversity." Journal of Bacteriology **180**(18): 4765-4774.

Huynh, B. H., L. Kang, D. V. Der Vartanian, H. D. J. Peck and J. LeGall (1984). "Characterization of a sulfite reductase from *Desulfovibrio vulgaris*. Evidence for the presence of a low-spin siroheme and an exchange-coupled siroheme-[4Fe-4S] unit." Journal of Biological Chemistry **259**: 15373-15376.

Imhoff, J. F., J. Suling and R. Petri (1998). "Phylogenetic relationships among the Chromatiaceae, their taxonomic reclassification and description of the new genera Allochromatium, Halochromatium, Isochromatium, Marichromatium, Thiococcus, Thiohalocapsa and Thermochromatium." Int J Syst Bacteriol **48 Pt 4**(12): 1129-43.

Inagaki, F., Y. Sakihama, A. Inoue, C. Kato and K. Horikoshi (2002). "Molecular phylogenetic analyses of reverse-transcribed bacterial rRNA obtained from deep-sea cold seep sediments." Environmental Microbiology **4**(5): 277-286.

Isaksen, M.-F., F. Bak and B.-B. Jorgensen (1994). "Thermophilic sulfate-reducing bacteria in cold marine sediment." FEMS-Microbiology-Ecology **14**(1): 1-8.

Isaksen, M. F. and A. Teske (1996). "Desulforhopalus vacuolatus gen. nov., sp. nov., a new moderately psychrophilic sulfate-reducing bacterium with gas vacuoles isolated from a temperate estuary." Arch. Microbiol. **166**(3): 160-168.

Iwabe, N., K. Kuma, M. Hasegawa, S. Osawa and T. Miyata (1989). "Evolutionary relationship of archaebacteria, eubacteria, and eukaryotes inferred from phylogenetic trees of duplicated genes." Proc Natl Acad Sci U S A **86**(23): 9355-9.

Jain, R., M. C. Rivera and J. A. Lake (1999). "Horizontal gene transfer among genomes: the complexity hypothesis." Proc Natl Acad Sci USA **96**: 3801-3806.

Jannasch, H. W. (1995). Die Mikroben der heißen Tiefseequellen. Extremophile, Mikoorganismen in ausgefallenen Lebensräumen. K. Hausmann and B. P. Kremer. Weinheim, Germany, VCH Verlagsgesellschaft**:** 69-86.

Johnson, M. S., I. B. Zhulin, M. E. R. G. puzan and B. L. Taylor (1997). "Oxygen-Dependent Growth of the Obligate Anaerobe *Desulfovibrio vulgaris* Hildenborough." J. Bacteriol. **179**: 5598-5601.

Joulian, C., N. B. Ramsing and K. Ingvorsen (2001). "Congruent Phylogenies of Most Common Small-Subunit rRNA and Dissimilatory Sulfite Reductase Gene Sequences Retrieved from Estuarine Sediments." Appl. Environ. Microbiol. **67**(7): 3314-3318.

Jukes, T. H. and C. R. Cantor (1969). Mamalian Protein Metabolism. Mamaliean Protein Metabolism. H. N. Munro. New York, Academic Press**:** 21-132.

Juretschko, S., G. Timmermann, M. Schmid, K. H. Schleifer, A. Pommerening-Roser, H. P. Koops and M. Wagner (1998). "Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations." Appl Environ Microbiol **64**(8): 3042-51.

Kane, M. D., L. K. Poulsen and D. A. Stahl (1993). "Monitoring the Enrichment and Isolation of Sulfate-Reducing Bacteria by Using Oligonucleotide Hybridization Probes Designed from Environmentally Derived 16S rRNA Sequences." Applied and Environmental Microbiology **59**(3): 682-686.

Karkhoff-Schweizer, R. R., M. Bruschi and G. Voordouw (1993). "Expression of the gamma-subunit gene of desulfoviridin-type dissimilatory sulfite reductase and of the alpha- and beta-subunit genes is not coordinately regulated." Eur J Biochem **211**(3): 501-7.

Karkhoff-Schweizer, R. R., D. P. W. Huber and G. Voordouw (1995). "Conservation of the Genes for Dissimilatory Sulfite Reductase from Desulfovibrio vulgaris and Archaeoglobus fulgidus Allows Their Detection by PCR." Appl. Environ. Microbiol. **61**: 290-296.

Kawaguchi, Y. (1989). "The code CUIG is read as serine in an asoporogneic yeast *Candida cylindracea*." Nature **341**: 164-166.

Kidwell, M. G. and D. R. Lisch (2001). "Perspective: transposable elements, parasitic DNA, and genome evolution." Evolution **55**(1): 1-24.

Kim, D.-j. and S. Forst (2001). "Genomic analysis of the histidine kinase family in bacteria and archaea." Microbiology **147**: 1197-1212.

Klein, M. (1998). Untersuchungen zur Phylogenie und Ökologie Sulfat-reduzierender Prokaryonten im Mariager Fjord (Dänemark) anhand vergleichender Analyse ihrer dissimilatorischen Sulfit-Reduktasen. Lehrstuhl für Mikrobiologie. München, Technische Universität München.

Klein, M., M. Friedrich, A. J. Roger, P. Hugenholtz, S. Fishbain, H. Abicht, L. L. Blackall, D. A. Stahl and M. Wagner (2001). "Multiple lateral transfer events of dissimilatory sulfite reductase genes between major lineages of sulfate-reducing prokaryotes." Journal of Bacteriology **183**(20): 6028-6035.

Klenk, H., R. Clayton, J. Tomb, O. White, K. Nelson, K. Ketchum, R. Dodson, M. Gwinn, E. Hickey, J. Peterson, D. Richardson, A. Kerlavage, D. Graham, N. Kyrpides, R. Fleischmann, J. Quackenbush, N. Lee, G. Sutton, S. Gill, E. Kirkness, B. Dougherty, K. McKenney, M. Adams, B. Loftus, J. Venter and e. al. (1997). "The

complete genome sequence of the hyperthermophilic sulphate-reducing archaeon Archaeoglobus fulgidus." Nature **390**: 364-70.

Knoblauch, C., K. Sahm and B. B. Joergensen (1999). "Psychrophilic sulfate-reducing bacteria isolated form permanently cold Arctic marine sediments: description of *Desulfofrigus oceanense* gen. nov., sp. nov., *Desulfofrigus fragile* sp. nov., *Desulfofaba gelida* gen. nov., sp. nov., *Desulfotalea psychrophila* gen. nov., sp. nov. and *Desulfotalea arctica* sp. nov." International Journal of Systematic Bacteriology **49**: 1631-1643.

Krekeler, D., P. Sigalevich, A. Teske, H. Cypionka and Y. Cohen (1997). "A sulfate-reducing bacterium from the oxic layer of a microbial mat from Solar Lake (Sinai), *Desulfovibrio oxyclinae* sp. nov." Arch. Micobiol. **167**: 369-375.

Krekeler, D., A. Teske and H. Cypionka (1998). "Strategies of sulfate-reducing bacteria to escape oxygen stress in a cyanobacterial mat." FEMS Microbiol. Ecol. **25**: 89-96.

Kuever, J., J. Kulmer, S. Jannsen, U. Fischer and K. H. Blotevogel (1993). "Isolation and characterization of a new spore-forming sulfate-reducing bacterium growing by complete oxidation of catechol." Arch Microbiol **159**(3): 282-8.

Kuever, J., F. A. Rainey and H. Hippe (1999). "Description of Desulfotomaculum sp. Groll as Desulfotomaculum gibsoniae sp. nov." Int J Syst Bacteriol **49**(Pt 4): 1801-1808.

Langendijk, P., E. Kulik, H. Sandmeier, J. Meyer and J. van der Hoeven (2001). "Isolation of Desulfomicrobium orale sp. nov. and Desulfovibrio strain NY682, oral sulfate-reducing bacteria involved in human periodontal disease." Int J Syst Evol Microbiol **51**: 1035-1044.

Larsen, T. Lien and N. K. Birkeland (2000). "Characterization of the desulforubidin operons from Desulfobacter vibrioformis and Desulfobulbus rhabdoformis." FEMS Microbiol Lett **186**(1): 41-6.

Larsen, O., T. Lien and N. Birkeland (2001). "A novel organization of the dissimilatory sulfite reductase operon of Thermodesulforhabdus norvegica verified by RT-PCR." FEMS Microbiol Lett **203**(1): 81-85.

Larsen, O., T. Lien and N. K. Birkeland (1999). "Dissimilatory sulfite reductase from Archaeoglobus profundus and Desulfotomaculum thermocisternum: phylogenetic and structural implications from gene sequences." Extremophiles **3**(1): 63-70.

Laue, H., M. Friedrich, J. Ruff and A. M. Cook (2001). "Dissimilatory Sulfite Reductase (Desulfoviridin) of the Taurine-Degrading, Non-Sulfate-Reducing Bacterium *Bilophila wadsworthia* RZATAU Contains a Fused DsrB-DsrD Subunit." Journal of Bacteriology **183**(5): 1727-1733.

Lee, J. P., C. S. Yi, J. LeGall and H. D. Peck, Jr. (1973). "Isolation of a new pigment, desulforubidin, from Desulfovibrio desulfuricans (Norway strain) and its role in sulfite reduction." J Bacteriol **115**(1): 453-5.

LeGall, J. and G. Fauque (1988). Dissimilatory reduction of sulfur compounds. Biology of anaerobic microorganisms. A. J. B. Zehnder. New York, J Wiley & Sons**:** 587-639.

Lengeler, J. W., G. Drews and H. G. Schlegel (1999). Biology of the prokaryotes. Stuttgart, Germany, Thieme Verlag.

Leu, J.-Y., C. P. McGovern-Traa, A. J. R. Porter, W. J. Harris and W. A. Hamilton (1998). "Identification and Phylogenetic Analysis of Thermophilic Sulfate-Reducing Bacteria in Oil Field Samples by 16S rDNA Gene cloning and Sequencing." Anaerobe **4**: 165-174.

Li, J.-h., K. J. Purdy, S. Takii and H. Hayashi (1999). "Seasonal changes in ribosomal RNA of sulfate-reducing bacteria and sulfate reducing activity in a freshwater lake sediment." FEMS Microbiology Ecology **28**: 31-39.

Lie, T. J., M. L. Clawson, W. Godchaux and E. R. Leadbetter (1999). "Sulfidogenesis from 2-aminoethanesulfonate (taurine) fermentation by a morphologically unusual sulfate-reducing bacterium, Desulforhopalus singaporensis sp. nov." Appl Environ Microbiol **65**(8): 3328-34.

Lien, T., M. Madsen, I. H. Steen and K. Gjerdevik (1998). "*Desulfobulbus rhabdoformis* sp. nov., a sulfate reducer from a water-oil separation system." International Journal of Systematic Bacteriology **48**: 469-474.

Liu, Y., T. M. Karnauchow, K. F. Jarrell, D. L. Balkwill, G. R. Drake, D. Ringelberg, R. Clarno and D. R. Boone (1997). "Description of two new thermophilic *Desulfotomaculum* spp., *Desulfotomaculum putei* sp. nov., from a deep terrestrial subsurface, and *Desulfotomaculum luciae* sp. nov., from a hot spring." International Journal of Systematic Bacteriology **47**: 615-621.

Loka-Bharathi, P. A., S. Oak and D. Chandramohan (1991). "Sulfate-Reducing Bacteria From Mangrove Swamps II. Their Ecology and Physiology." Oceanologica-Acta. **14**(2): 163-172.

Lovley, D. and E. Phillips (1992). "Reduction of uranium by Desulfovibrio desulfuricans." Appl Environ Microbiol **58**(3): 850-856.

Loy, A., M. Horn and M. Wagner (2003). "probeBase: an online resource for rRNA-targeted oligonucleotide probes." Nucl. Acids. Res. **31**(1): 514-516.

Loy, A., A. Lehner, N. Lee, J. Adamczyk, H. Meier, J. Ernst, K. Schleifer and M. Wagner (2002). "Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment." Appl Environ Microbiol **68**(10): 5064-5081.

Ludwig, W., O. Strunk, S. Klugbauer, N. Klugbauer, M. Weizenegger, J. Neumaier, M. Bachleitner and K. H. Schleifer (1998). "Bacterial phylogeny based on comparative sequence analysis." Electrophoresis **19**: 554-568.

Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Förster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. König, T. Liss, R. Lüßmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode and K.-H. Schleifer (2004). "ARB: a software environment for sequence data." Nucleic Acids Research **32**(4): 1363-1371.

Manz, W., M. Eisenbrecher, T. R. Neu and U. Szewzyk (1998). "Abundance and spatial organization of Gram-negative sulfate-reducing bacteria in activated sludge investigated by in situ pobing with specific 16S rRNA targeted oligonucleotiedes." FEMS Micorbiolog Ecology **25**: 43-61.

McGowan, C., R. Fulthorpe, A. Wright and J. M. Tiedje (1998). "Evidence for Interspecies Gene Transfer in the Evolution of 2,4-Dichlorophenoxyacetic Acid Degraders." Applied and Environmental Microbiology **64**(10): 4089-4092.

Michaelis, W., R. Seifert, K. Nauhaus, T. Treude, V. Thiel, M. Blumenberg, K. Knittel, A. Gieseke, K. Peterknecht, T. Pape, A. Boetius, R. Amann, B. B. Jorgensen, F. Widdel, J. Peckmann, N. V. Pimenov and M. B. Gulin (2002). "Microbial Reefs in the Black Sea Fueled by Anaerobic Oxidation of Methane." Science **297**(5583): 1013-1015.

Min, H. and a. S. H. Zinder (1990). "Isolation and characterization of a thermophilic sulfate-reducing bacterium *Desulfotomaculum thermoacetoxidans* sp. nov." Archives of Microbiology **153**: 399-404.

Minz, D., S. Fishbain, S. J. Green, G. Muyzer, Y. Cohen, B. E. Rittmann and D. A. Stahl (1999a). "Unexpected Population Distribution in a Microbial Mat Community: Sulfate-Reducing Bacteria Localized to the Highly Oxic Chemocline in Contrast to a Eukaryotic Preference for Anoxia." Applied and Environmental Microbiology **65**(10): 4659-4665.

Minz, D., J. L. Flax, S. J. Green, G. Muyzer, Y. Cohen, M. Wagner, B. E. Rittmann and D. A. Stahl (1999b). "Diversity of sulfate-reducing bacteria in oxic and anoxic regions of a microbial mat characterized by comparative analysis of dissimilatory(bi-) sulfite reductase genes." Applied and Envirionmental Microbiology.

Miserta, A. and P. Csutora (2000). "Relationship between the occurrence o f cysteine in proteins and the complexity of organisms." Mol. Biol. Evol. **17**(8): 1232-1239.

Miskin, I. P., P. Farrimond and I. M. Head (1999). "Identification of novel bacterial lineages as active members of microbial populations in a freshwater sediment using a rapid RNA extraction procedure and RT-PCR." Microbiology **145**: 1977-87.

Mitchell, G. J., J. G. Jones and J. A. Cole (1986). "Distribution and regulation of nitrate and nitrite reduction by *Desulfovibrio* and *Desulfotomaculum* species." Archieves of Microbiology **144**: 35-40.

Moreira, D. and P. López-García (1998). "Symbiosis Between Methanogneic Archaea and δ-Proteobacteria as the Origin of Eukaryotes: The Syntrophic Hypothesis." J Mol Evol **47**: 517-530.

Morse, R., G. R. Gibson and M. D. Collins (2000). "Secondary structure analysis of the dissimilatory sulphite reductase in Desulfovibrio desulfuricans." Lett Appl Microbiol **30**(5): 375-8.

Moura, I., S. Bursakov, C. Costa and J. J. G. Moura (1997). "Nitrate and Nitrite Utilization in Sulfate-Reducing Bacteria." Anaerobe **3**: 279-290.

Moura, I., A. R. Lino, J. J. G. Moura, A. V. Xavier, G. Fauque, H. D. J. Peck and L. J (1986). "Low-spin sulfite reductases: a new homologous group of non-heme iron-sirohaem proteins in anaerobic bacteria." Biochemical and Biophysical Research communications **141**: 1032-1041.

Murphy, M. J. and L. M. Siege (1973a). "Siroheme and sirohydrochlorin: the basis for a new type of porphyrin-related prostetic group common to both assimilatory and dissimilatory sulfite reductases." Journal of Biological Chemistry **248**: 6911-6919.

Murphy, M. J., L. M. Siegel, H. Kamin and D. Rosenthal (1973b). "Reduced nicotinamide adenine dinucleotide phosphate-sulfite reductase of enterobacteriaceae. II. Identification of a new class of heme prostetic group: an iron-tetrahydroporphyrin (isobacteriochlorin type) with eight carboxylic groups." Journal of Biolgical Chemistry **248**: 2801-2814.

Muyzer, G., E. C. de Waal and A. G. Uitterlinden (1993). "Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes encoding for 16S rRNA." Appl Environ Microbiol **59**: 695-700.

Muyzer, G. and K. Smalla (1998). "Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology." Antonie van Leeuwenhoek **73**: 127-141.

Nakagawa, T. and M. Fukui (2003). "Molecular characterization of community structures and sulfur metabolism within microbial streamers in Japanese hot springs." Appl Environ Microbiol. **69**(12): 7044-57.

Nakagawa, T., S. Hanada, A. Maruyama, K. Marumo, T. Urabe and M. Fukui (2002). "Distribution and diversity of thermophilic sulfate-reducing bacteria within a Cu-Pb-Zn mine (Toyoha, Japan)." FEMS Microbiol. Ecol. **41**: 199-209.

Nauhaus, K., A. Boetius, M. Krüger and F. Widdel (2002). "In vitro demonstration of anaerobic oxidation of methane coupled to sulphate reduction in sediment from a marine gas hydrate area." Environmental Microbiology **4**(5): 296-305.

Nazina, T. N., A. Ivanova, L. Kanchaveli and E. Rozanova (1989). "A new sporeforming thermophilic methylotrophic sulfate-reducing bacterium, Desulfotomaculum kuznetsovii sp. nov." Microbiology (Engl. Transl. of Mikrobiologiya) **57**: 659-663.

Nazina, T. N., E. P. Rozanova and T. A. Kalininskaia (1979). "Fixation of molecular nitrogen by sulfate-reducing bacteria from petroleum strata." Mikrobiologiia **48**(1): 133-6.

Nelson, K. E., R. A. Clayton, S. R. Gill, M. L. Gwinn, R. J. Dodson, D. H. Haft, E. K. Hickey, J. D. Peterson, W. C. Nelson, K. A. Ketchum, L. McDonald, T. R. Utterback, J. A. Malek, K. D. Linher, M. M. Garrett, A. M. Stewart, M. D. Cotton, M. S. Pratt, C. A. Phillips, D. Richardson, J. Heidelberg, G. G. Sutton, R. D. Fleischmann, J. A. Eisen, W. White, S. L. Salzberg, H. O. Smith, J. C. Venter and C. M. Fraser (1999). "Evidence for lateral gene transfer between Archaea and Bacteria from genome sequence of *Thermotoga maritima*." Nature **399**: 323-329.

Nesbø, C. L., S. L'Haridon, K. O. Stetter and W. F. Doolittle (2001). "Phylogenetic 'Analyses of Two "Archael" Genes in *Thermotoga maritima* Reveals Multiple Transfers Between Archaea and Bacteria." Mol Biol Evol **18**(3): 362-375.

Nilsen, R.-K., J. Beeder, T. Thorstenson and T. Torsvik (1996a). "Distribution of thermophilic marine sulfate reducers in North sea oil field waters and oil reservoirs." Applied-and-Environmental-Microbiology **62**(5): 1793-1798.

Nilsen, R. K., T. Torsvik and T. Lien (1996b). "*Desulfotomaculum thermocisternum* sp. nov., a Sulfate-Reducer Isolated from a Hot North Sea Oil Reservoir." International Journal of Systematic Bacteriology **46**(2): 397-402.

Noiva, R. (1994). "Enzymatic catalysis of disulfide formation." Protein Expr Purif **5**: 1-13.

Olsen, G. J., H. Matsuda, R. Hagstrom and R. Overbeek (1994). “fastDNAmL: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood.” Comput Appl Biosci **10**(1): 41-8.

Orphan, V. J., K.-U. Hinrichs, W. Ussler, III, C. K. Paull, L. T. Taylor, S. P. Sylva, J. M. Hayes and E. F. Delong (2001). “Comparative Analysis of Methane-Oxidizing Archaea and Sulfate-Reducing Bacteria in Anoxic Marine Sediments.” Appl. Environ. Microbiol. **67**(4): 1922-1934.

Orphan, V. J., C. H. House, K.-U. Hinrichs, K. D. McKeegan and E. F. DeLong (2002). “Multiple archaeal groups mediate methane oxidation in anoxic cold seep sediments.” PNAS **99**(11): 7663-7668.

O'Sullivan, J. M., J. B. Davenport and M. F. Tuite (2001). “Codon reassignment and the evolving genetic code: problems and pitfalls in post-genome analysis.” TRENDS in Genetics **17**(1): 20-22.

Ouattara, A. S., B. K. Patel, J. L. Cayol, N. Cuzin, A. S. Traore and J. L. Garcia (1999). “Isolation and characterization of Desulfovibrio burkinensis sp. nov. from an African ricefield, and phylogeny of Desulfovibrio alcoholivorans.” Int J Syst Bacteriol **49**: 639-43.

Overmann, J., M. J. L. Coolen and C. Tuschak (1999). “Specific detection of different phylogenetic groups of chemocline bacteria based on PCR and denturing gradient gel electrophoresis of 16S rRNA gene fragments.” Arch Microbiol **172**: 83-94.

Pak, K. R. and R. Bartha (1998a). “Mercury Methylation and Demethylation in Anoxic Lake Sediments and by Strictly Anaerobic Bacteria.” Applied and Environmental Microbiology **64**(3): 1013-1017.

Pak, K. R. and R. Bartha (1998b). “Mercury Methylation by Interspecies Hydrogen and Acetate Transfer between Sufidogens and Methanogens.” Applied and Environmental Microbiology **64**(6): 1987-1990.

Pereira, A. S., R. Franco, M. J. Feio, C. Pinto, J. Lampreia, M. A. Reis, J. Calvete, I. MOura, I. Beech, A. R. Lino and J. J. G. Moura (1996a). “Characterization of representative enzymes from a sulfate reducing bacterium implicated in the corrosion of steel.” Biochemical and Biophysical Research Communications **221**: 414-421.

Pereira, I. C., I. A. Abreu, A. V. Xavier, J. LeGall and M. Teixeira (1996b). “Nitrite Reductase from *Desulfovibrio desulfuricans* (ATCC 277774) - A Heterooligomer Heme Protein with Sulfite Reductase Activity.” Biochemical and Biophysical Reseacrch Communications **224**: 611-618.

Pérez-Jiménez, J. R., L. Y. Young and L. J. Kerkhof (2001). “Molecular characterization of sulfate-reducing bacteria in anaerobic hydrocarbon-degrading consortia and pure cultures using the dissimilatory sulfite reductase (dsAB) genes.” FEMS Microbiology Ecology **35**: 145-150.

Pierik, A. J., M. G. Duyvis, J. M. van Helvoort, R. B. Wolbert and W. R. Hagen (1992). “The third subunit of desulfoviridin-type dissimilatory sulfite reductases.” Eur J Biochem **205**(1): 111-5.

Pikuta, E., A. Lysenko, N. Suzina, G. Osipov, B. Kuznetsov, T. Tourova, V. Akimenko and K. Laurinavichius (2000). “Desulfotomaculum alkaliphilum sp. nov., a new alkaliphilic, moderately thermophilic, sulfate-reducing bacterium.” Int J Syst Evol Microbiol **50 Pt 1**: 25-33.

Polz, M. F. and C. M. Cavanaugh (1998). “Bias in Template-to-Product Ratios in Multitemplate PCR.” Appl. Environ. Microbiol. **64**: 3724-3730.

Pott, A. S. and C. Dahl (1998). “Sirohaem sulfite reductase and other proteins encoded by genes at the dsr locus of Chromatium vinosum are involved in the oxidation of intracellular sulfur.” Microbiology **144**(Pt 7): 1881-94.

Purdy, K. J., T. M. Embley and D. B. Nedwell (2002). “The distribution and activity of sulphate reducing bacteria in estuarine and coastal marine sediments.” Antonie van Leeuwenhoek **81**(1-4): 181-187.

Purdy, K. J., D. B. Nedwell, T. M. Embley and S. Takii (2001). “Use of 16S rRNA-targeted oligonucleotide probes to investigate the distribution of sulphate-reducing bacteria in estuarine sediments.” FEMS Microbiology Ecology **36**(2-3): 165-168.

Rabus, R., M. Fukui, H. Wilkes and F. Widdel (1996). “Degradative Capacities and 16S rRNA-Targeted Wohle-Cell Hybridization of Sulfate-Reducing Bacteria in an Anaerobic Enrichment Culture utilizing Alkylbenzenes from Crude Oil.” Applied and Environmental Microbiology **62**(10): 3605-3613.

Raina, S. and D. Missiakas (1997). “Making and breaking disulfide bond.” Annu Rev Microbiol **51**: 179-202.

Ramsing, N. B., H. Fossing, T. G. Ferdelman, F. Andersen and B. Thamdrup (1996). “Distribution of bacterial populations in a stratified fjord (Mariager Fjord, Denmark) quantified by in situ hybridization and related to chemical gradients in the water Column.” Appl. Environ. Microbiol. **62**: 1391-1404.

Ravenschlag, K., K. Sahm, C. Knoblauch, B. B. Jorgensen and R. Amann (2000). “Community Structure, Cellular rRNA Content, and Activity of Sulfate-Reducing Bacteria in Marine Arctic Sediments.” Appl. Environ. Microbiol. **66**(8): 3592-3602.

Ravenschlag, K., K. Sahm, J. Pernthaler and R. Aman (1999). “High Bacterial Diversity in Permanently Cold Marine Sediments.” Applied and Environmental Microbiology **65**: 3982-3989.

Rees, G. N., G. S. Grassia, A. J. Sheehy, P. P. Dwivedi and B. K. C. Patel (1995). “*Desulfacinum infernum* gen. nov., sp. nov., a thermophilic sulfate-reducing bacterium from a petroleum reservoir.” Int J Syst Bacteriol **4**(5): 85-89.

Risatti, J. B., W. C. Capman and D. A. Stahl (1994). “Community structure of a microbial mat: The phylogenetic dimension.” Proc. Natl. Acad. Sci. USA **91**: 10173-10177.

Robertson, W. J., J. P. Bowman, P. D. Franzmann and B. J. Mee (2001). “Desulfosporosinus meridiei sp. nov., a spore-forming sulfate-reducing bacterium isolated from gasolene-contaminated groundwater.” International-Journal-of-Systematic-and-Evolutionary-Microbiology **51**(1): 133-140.

Robertson, W. J., P. D. Franzmann and B. J. Mee (2000). “Spore-forming, Desulfosporosinus-like sulphate-reducing bacteria from a shallow aquifer contaminated with gasoline.” J Appl Microbiol **88**(2): 248-59.

Rondon, M. R., P. R. August, A. D. Bettermann, S. F. Brady, T. H. Grossman, M. R. Liles, K. A. Loiacono, B. A. Lynch, I. A. Macneil, C. Minor, C. L. Tiong, M. Gilman, M. S. Osburne, J. Clardy, J. Handelsman and R. M. Goodman (2000). “Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms.” Applied and Environmental Microbiology **66**(6): 2541–2547.

Rooney-Varga, J., B. Genthner, R. Devereux, S. Willis, S. Friedman and M. Hines (1998). "Phylogenetic and physiological diversity of sulphate-reducing bacteria isolated from a salt marsh sediment." Syst Appl Microbiol **21**(4): 557-568.

Rooney-Varga, J. N., R. Devereux, R. S. Evans and M. E. Hines (1997). "Seasonal changes in the relative abundance of uncultivated sulfate-reducing bacteria in a salt marsh sediment and in the rhizosphere of Spartina alterniflora." Appl Environ Microbiol **63**(10): 3895-901.

Sahm, K., C. Knoblauch and R. Amann (1999a). "Phylogenetic Affilition and Quantification of Psychrophilic Sulfate-Reducing Isolates in Marine Arctic Sediments." Applied and Environmental Microbiology **65**: 3976-3981.

Sahm, K., B. J. MacGregor, B. B. Joergensen and D. A. Stahl (1999b). "Sulphate reduction and vertical distribution of sulphate-reducing bacteria quantified by rRNA slot-blot hybridization in a coastal marine sediment." Environ. Micobiol. **1**: 65-74.

Saitou, N. and M. Nei (1987). "The neighbor-joining method: a new method for reconstructing phylogenetic trees." Mol Biol Evol **4**(4): 406-25.

Samain, E., H. C. Dubourguier and G. Albagnac (1984). "Isolation and characterization of Desulfobulbus elongatus sp. nov. from a mesophilic industrial digester." System. Appl. Microbiol. **5**: 391-401.

Sambrook, J., E. F. Fritsch and T. Maniatis (1989). Molecular cloining: A laboratory manual. New York.

Santegoeds, C. M., L. R. Damgaard, G. Hesselink, J. Zopfi, P. Lens, G. Muyzer and D. de Beer (1999). "Distribution of Sulfate-Reducing and Methanogenic Bacteria in Anaerobic Aggregates Determined by Microsensor and Molecular Analyses." Appl. Environ. Microbiol. **65**(10): 4618-4629.

Sass, H., M. Berchtold, J. Branke, H. Konig, H. Cypionka and H. D. Babenzien (1998). "Psychrotolerant sulfate-reducing bacteria from an oxic freshwater sediment, description of Desulfovibrio cuneatus sp. nov. and Desulfovibrio litoralis sp. nov." Syst Appl Microbiol **21**(2): 212-9.

Schedel, M. and H. G. Truper (1979). "Purification of Thiobacillus denitrificans siroheme sulfite reductase and investigation of some molecular and catalytic properties." Biochim Biophys Acta **568**(2): 454-66.

Scheid, D. and S. Stubner (2001). "Structure and diversity of Gram-negative sulfate-reducing bacteria on rice roots." FEMS Microbiology Ecology **36**(2-3): FEMS Microbiology Ecology.

Schink, B. and M. Friedrich (2000). "Phosphite oxidation by sulphate reduction." Nature **406**: 37.

Schmid, M., U. Twachtmann, M. Klein, M. Strous, S. Juretschko, M. Jetten, J. W. Metzger, K. H. Schleifer and M. Wagner (2000). "Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation." Syst Appl Microbiol **23**(1): 93-106.

Schnell, S., F. Bak and N. Pfennig (1989). "Anaerobic degradation of aniline and dihydroxybenzenes by newly isolated sulfate-reducing bacteria and description of Desulfobacterium anilini." Arch Microbiol **152**(6): 556-63.

Schoenborn, L., H. Abdollahi, W. Tee, M. Dyall-Smith and P. H. Janssen (2001). "A member of the delta subgroup of proteobacteria from a pyogenic liver abscess is a typical sulfate reducer of the genus desulfovibrio." J Clin Microbiol **39**(2): 787-90.

Scholten, J. C. and A. J. Stams (2000). "Isolation and characterization of acetate-utilizing anaerobes from a freshwater sediment." Microb Ecol **40**(4): 292-299.

Schramm, A., C. M. Santegoeds, H. K. Nielsen, H. Ploug, M. Wagner, M. Pribyl, J. Wanner, R. Amann and D. D. Beer (1999). "On the Occurrence of Anoxic Microniches, Denitrification, and Sulfate Reduction in Aerated Activated Sludge." Applied and Environmental Microbiology **65**: 4189-4196.

Seki, Y., Y. Nagai and M. Ishimoto (1985). "Characterization of a dissimilatory-type sulfite reductase, desulfoviridin, from Desulfovibrio africanus Benghazi." J Biochem (Tokyo) **98**(6): 1535-43.

Sexstone, A. J. and R. M. Atlas (1977). "Response of microbial populations in Arctic tundra soils to crude oil." Can. J. Microbiol. **23**: 1327-1333.

Shen, Y., R. Buick and D. E. Canfield (2001). "Isotopic evidence for microbial sulphate reduction in the early Archaean era." Nature **410**: 77-81.

Sievert, S. and J. Kuever (2000). "Desulfacinum hydrothermale sp. nov., a thermophilic, sulfate-reducing bacterium from geothermally heated sediments near Milos Island (Greece)." Int J Syst Evol Microbiol **50**(3): 1239-1246.

Sigalevich, P., M. V. Baev, A. Teske and Y. Cohen (2000a). "Sulfate Reduction and Possible Aerobic Metabolism of the Sulfate-Reducing Bacterium *Desulfivibiro oxyclinae* in a Chemostat Coculture with *Marinobacter* sp. Strain MB under Exposure to Increasing Oxygen Concentrations." Applied and Environmental Microbiology **66**(11): 5013-5018.

Sigalevich, P., M. V. Baev, A. Teske and Y. Cohen (2000b). "Sulfate reduction and possible aerobic metabolism of the sulfate-reducing bacterium Desulfovibrio oxyclinae in a chemostat coculture with Marinobacter sp. Strain MB under exposure to increasing oxygen concentrations." Appl Environ Microbiol **66**(11): 5013-8.

Sigalevich, P. and Y. Cohen (2000c). "Oxygen-dependent growth of the sulfate-reducing bacterium Desulfovibrio oxyclinae in coculture with Marinobacter sp. Strain MB in an aerated sulfate-depleted chemostat." Appl Environ Microbiol **66**(11): 5019-23.

So, C. M. and L. Y. Young (1999). "Isolation and Characterization of a Sulfate-Reducing Bacterium That Anaerobically Degrades Alkanes." Appl. Environ. Microbiol. **65**(7): 2969-2976.

Sonne-Hansen, J. and B. K. Ahring (1999). "Thermodesulfobacterium hveragerdense sp. nov., and Thermodesulfovibrio islandicus sp. nov., two thermophilic sulfate reducing bacteria isolated from a Icelandic hot spring." Syst Appl Microbiol **22**(4): 559-64.

Spear, J. R., L. A. Figueroa and B. D. Honeyman (2000). "Modeling Reduction of Uranium U(VI) under Variable Sulfate Concentrations by Sulfate-Reducing Bacteria." Appl. Environ. Microbiol. **66**(9): 3711-3721.

Speich, N., C. Dahl, P. Heisig, A. Klein, F. Lottspeich, K. O. Stetter and H. G. Truper (1994). "Adenylylsulphate reductase from the sulphate-reducing archaeon Archaeoglobus fulgidus: cloning and characterization of the genes and comparison of the enzyme with other iron-sulphur flavoproteins." Microbiology **140**(Pt 6): 1273-84.

Stackebrandt, E. and B. M. Goebel (1994). "Taxonomic Note: A Place for DNA-DNA Reassociation and 16S rRNA Sequence Analysis in the Present Species Definition in Bacteriology." International Journal of Systematic Bacteriology : 846-849.

Stackebrandt, E., C. Sproer, F. A. Rainey, J. Burghardt, O. Pauker and H. Hippe (1997a). "Phylogenetic analysis of the genus *Desulfotomaculum*: evidence for the misclassification of *Desulfotomaculum guttoideum* and description of *Desulfotomaculum orientis* as *Desulfosporosinus orientis* gen. nov., comb. nov." Int J Syst Bacteriol **47**(4): 1134-9.

Stackebrandt, E., C. Sproer, F. A. Rainey, J. Burghardt, O. Päuker and H. Hippe (1997b). "Phylogenetic analysis of the genus *Desulfotomaculum*: evidence for misclassification of *Desulfotomaculum guttoideum* and decription of *Desulfotomaculum orientis* as *Desulfosporosinus orientis* gen. nov., comb. nov." International Journal of Systematic Bacteriology **47**(4): 1134-1139.

Staley, J. T. and A. Konopka (1985). "Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats." Annu Rev Microbiol **39**: 321-346.

Steuber, J., A. F. Arendsen, W. R. Hagen and P. M. H. Kroneck (1995). "Molecular properties of the dissimilatory sulfite reductase from *Desulfovibrio desulfuricans* (Essex) and comparison with the enzyme from *Desulfovibrio vulgaris* (Hildenborough)." European Journal of Biochemistry **233**: 873-879.

Stratz, M., M. Mau and K. Timmis (1996). "System to study horizontal gene exchange among microorganisms without cultivation of recipients." Mol Microbiol **22**(2): 207-215.

Stubner, S. (2002). "Enumeration of 16S rDNA of Desulfotomaculum lineage 1 in rice field soil by real-time PCR with SybrGreenTM detection." Journal-of-Microbiological-Methods **50**(2): 155-164.

Stubner, S. and K. Meuser (2000). "Detection of Desulfotomaculum in an Italian rice paddy soil by 16S ribosomal nucleic acid analyses." FEMS Microbiol Ecol **34**(1): 73-80.

Sullivan, J. T., S. D. Brown, R. R. Yocum and C. W. Ronson (2001). "The *bio* operon on the acquired symbiosis island of *Mesorhizobium* sp. strain R7A includes a novel gene involved in pimeloyl-CoA synthesis." Microbiology **147**: 1315-1322.

Suzuki, M. and S. Giovannoni (1996). "Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR." Appl. Environ. Microbiol. **62**(2): 625-630.

Suzuki, M., M. S. Rape and S. J. Giovannoni (1998). "Kinetic Bias in Estimates of Coastal Picoplankton Community Structure Obtained by Measurements of Small-Subunit rRNA Gene PCR Amplicon Length Heterogeneity." Appl. Environ. Microbiol. **64**: 4522-4529.

Tanaka, K., E. Stackebrandt, S. Tohyama and T. Eguchi (2000). "*Desulfovirga adipica* gen. nov., sp. nov., an adipate-degrading, Gram-negative, sulfate-reducing bacterium." International Journal of Systematic and Evolutionary Microbiology **50**: 639-644.

Tanaka, Y., M. Sogabe, K. Okumura and R. Kurane (2002). "A highly selective direct method of detecting sulphate-reducing bacteria in crude oil." Lett Appl Microbiol **35**(3): 242-246.

Tardy-Jacquenod, C., M. Magot, B. K. Patel, R. Matheron and P. Caumette (1998). "Desulfotomaculum halophilum sp. nov., a halophilic sulfate-reducing bacterium isolated from oil production facilities." Int J Syst Bacteriol **48 Pt 2**: 333-8.

Tasaki, M., Y. Kamagata, K. Nakamura and E. Mikami (1991). "Isolation and characterization of a thermophilic benzoate-degrading, sulfate-reducing bacterium, *Desulfotomaculum thermobenzoicum* sp. nov." Arch Microbiol **155**: 348-352.

Taylor, J. and R. J. Parkes (1985). "Identifiying different populations of sulfate reducing bacteria within marine sediment systems, using fatty acid biomarkers." J. Gen. Microbiol. **131**: 631-642.

Tebo, B. M. and A. Y. Obraztsova (1998). "Sulfate-reducing bacterium grows with Cr(VI), U(VI), Mn(IV), and Fe(III) as electron acceptors." FEMS Microbiology Letters **162**(1): 193-198.

Teske, A., N. B. Ramsing, K. Habicht, M. Fukui, J. Klüver, B. B. Joergensen and Y. Cohen (1998). "Sulfate-reducing bacteria and their activities in cyanobacterial mats of Solar Lake (Sinai, Egypt)." Appl. Environ. Microbiol. **64**: 2943-2951.

Teske, A., C. Wawer, G. Muyzer and N. B. Ramsing (1996). "Distribution of Sulfate-Reducing Bacteria in a Stratified Fjord (Mariager Fjord, Denmark) as Evaluated by Most-Probable-Number Counts and Denaturing Gradient Gel Electrophoresis of PCR-Amplified Ribosomal DNA Fragments." Appl. Environ. Microbiol. **62**: 1405-1415.

Thomsen, T. R., K. Finster and N. B. Ramsing (2001). "Biogeochemical and Molecular Signatures of Anerobic Methane Oxidation in a Marine Sediment." Applied and Environmental Microbiology **67**(4): 1646-1656.

Tonolla, M., A. Demarta, S. Peduzzi, D. Hahn and R. Peduzzi (2000). "In Situ Analysis of Sulfat-Reducing Bacteria Related to *Desulfocapsa thiozymognes* in the Chemocline of Meromictic Lake Cadagno (Switzerland)." Applied and Environmental Microbiology **66**(2): 820-824.

Urakawa, H., K. Kita-Tsukamoto and K. Ohwada (1999). "Microbial diversity in marine sediments from Sagami Bay and Tokyo Bay, Japan, as determined by 16S rRNA gene analysis." Microbiology **145**(11): 3305-3315.

Valentine, D. L. (2002). "Biogeochemistry and microbial ecology of methane oxidation in anoxic environments: a review." Antonie van Leeuwenhoek **81**(1-4): 271-282.

Vallaeys, T., E. Topp, G. Muyzer, V. Macheret, G. Laguerre and G. Soulas (1997). "Evaluation of denaturing gradient gel electrophoresis in the detection of 16S rDNA sequence variation in rhizobia and methanotrophs." FEMS Microbiol Ecol **24**: 279-285.

von Wintzingerode, F., U. Gobel and E. Stackebrandt (1997). "Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis." FEMS Microbiol Rev **21**(3): 213-29.

Voordouw, G., S. M. Armstrong, M. F. Reimer, B. Fouts, A. J. Telang, Y. Shen and D. Gevertz (1996). "Characterization of 16S rRNA Genes from Oil Field Microbial Communities Indicates the Presence of a Variety of Sulfate-Reducing, Fermentative, and Sulfide-Oxidizing Bacteria." Applied and Environmental Microbiology **62**(5): 1623-1629.

Wagner, M., R. Amann, H. Lemmer and K.-H. Schleifer (1993). "Probing activated sludge with proteobacteria-specific oligonucleotides: inadequacy of culture-dependent methods for describing microbial community structures." Appl. Envrion. Microbiol. **59**: 1520-1525.

Wagner, M., A. J. Roger, J. L. Flax, G. A. Brusseau and D. A. Stahl (1998). "Phylogeny of Dissimilatory Sulfite Reductases Supports an Early Origin of Sulfate Respiration." Appl. Environ. Microbiol. **180**: 2975-2982.

Watanabe, K., Y. Kodama and N. Kaku (2002). "Diversity and abundance of bacteria in an underground oil-storage cavity." BMC Microbiology **2**(1): 23.

Wawer, C. and G. Muyzer (1995a). "Genetic Diversity of Desulfovibrio spp. in Environmental Samples Analyzed by Denaturing Gradient Gel Electrophoresis of [NiFe] Hydrogenase Gene Fragments." Applied and Environmental Microbiology **61**: 2203-2210.

Wawer, C., H. Rüggeberg, G. Meyer and G. Muyzer (1995b). "A simple and rapid electrophoresis method to detect sequence variantion in PCR-amplified DNA fragments." Nucleic Acids Research **23**(23): 4928-4929.

Wiberg, N. (1985). Der Schwefel. Lehrbuch der Anorganischen Chemie. Berlin, Walter de Gruyter**:** 471-524.

Widdel, F. (1980). Anaerober Abbau von Fettsäuren und Benzoesäure durch neu isolierte Arten sulfatreduzierender Bakterien, Univ. Göttingen, Germany.

Widdel, F. (1988). Microbiology and ecology of sulfate- and sulfur-reducing bacteria. New York, N.Y, Wiley & Sons.

Widdel, F. (1992a). The Genus *Desulfotomaculum*. The Prokarytes, 2nd ed. A. Balows, H. G. Trüper, M. Dworkin, W. Harder and K.-H. Schleifer. New York, Springer-Verlag; 1792-1799.

Widdel, F. and F. Bak (1992b). Gram-negative mesophilic sulfate-reducing bacteria. The Prokarytes, 2nd ed. A. Balows, H. G. Trüper, M. Dworkin, W. Harder and K.-H. Schleifer. New York, Springer-Verlag; 3352-3378.

Widdel, F. and T. A. Hansen (1992c). The dissimilatory sulfate- and sulfur bacteria. The Prokarytes, 2nd ed. A. Balows, H. G. Trüper, M. Dworkin, W. Harder and K.-H. Schleifer. New York, Springer-Verlag; 583-624.

Widdel, F. and N. Pfennig (1977). "A new anaerobic, sporing, acetate-oxidizing, sulfate-reducing bacterium, Desulfotomaculum (emend.) acetoxidans." Arch Microbiol **112**(1): 119-22.

Widdel, F. and N. Pfennig (1981). "Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. I. Isolation of new sulfate-reducing bacteria enriched with acetate from saline environments. Description of Desulfobacter postgatei gen. nov., sp. nov." Arch Microbiol **129**(5): 395-400.

Widdel, F. and N. Pfennig (1994). Dissimilatory sulfate- or sulfur-reducing bacteria. Bergey's manual of systematic bacteriology , vol. 1. N. R. Krieg and J. G. Holt. Baltimore, Williams & Wilkins. **-:** 663-679.

Wieringa, E. B. A., J. Overmann and H. Cypionka (2000). "Detection of abundant sulphate-reducing bacteria in marine oxic sediment layers by a combined cultivation and molecular approach." Environmental Microbiology **2**(4): 417-427.

Wind, T. and R. Conrad (1995). "Sulfur compounds, potential turnover of sulfate and thiosulfate, and numbers of sulfate-reducing bacteria in planted and unplanted paddy soil." FEMS-Microbiology -Ecology **18**(4): 257-266.

Wind, T., S. Stubner and R. Conrad (1999). "Sulfate-reducing bacteria in rice field soil and on rice roots." Syst Appl Microbiol **22**(2): 269-79.

Woese, C. R. (1987). "Bacterial evolution." Microbiol Rev. **51**: 221-271.

Yap, W. H., Z. Zhang and Y. Wang (1999). "Distinct Types of rRNA Operons Exist in the Genome of the Actinomycete Thermomonospora chromogena and Evidence for Horizontal Transfer of an Entire rRNA Operon." J. Bacteriol. **181**(17): 5201-5209.

Zoetendal, E. G., A. D. L. Akkermans and W. M. de Vos (1998). "Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific comunities of active bacteria." Appl Environ Microbiol **64**: 3854-3859.

G Appendix

G.1 Accession numbers of Dsr and 16S rRNA gene sequences and index of figures and tables presenting results from analyses performed with these sequences

The following sequences have been analyzed in table 6. In some cases two accession numbers are given for the *dsrAB* fragment referring to the subunits *dsrA* and *dsrB*, nevertheless, partial sequences were aligned to each other and phylogenetic calculation was carried out on the complete *dsrAB* fragments. Details on origin and studies performed on these sequences please see publications given in brackets, or GeneBank entries, respectively.

Sequences derived form acidic fen soil by Loy *et al.*: Publication submitted. Alexander Loy, Technische Universität Wien, Austria
AY167464-83.

Sequences analyzed from the study of Castro *et al.* (Castro 2002):
AY096038, AY096039, AY096040, AY096041, AY096042, AY096043, AY096044, AY096045, AY096046, AY096047, AY096048, AY096049, AY096050, AY096051, AY096052, AY096053, AY096054, AY096055, AY096056, AY096057, AY096058, AY096059, AY096060, AY096061, AY096062, AY096063, AY096064, AY096065, AY096066, AY096067, AY096068, AY096069, AY096070, AY096071, AY096072, AY096073, AY096074.

Sequences derived form rice paddy soil by Friedrich *et al.* are unpublished. Michael Friedrich, Max-Plank-Institut Marburg, Deutschland.

Sequences analyzed from the study of Thomsen *et al.* (Thomsen 2001):
AF388210, AF388211, AF388212, AF388213, AF388214, AF388215, AF388216, AF388217, AF388218, AF388219, AF388220, AF388221, AF388222, AF388223, AF388224, AF388225, AF388226, AF388227, AF388228, AF388229, AF388230, AF388231, AF388232, AF388233, AF388234, AF388235, AF388236, AF388237, AF388238, AF388239, AF388240, AF388241, AF388242, AF388243, AF388244, AF388245, AF388246, AF388247, AF388248, AF388249, AF388250, AF388251, AF388252, AF388253, AF388254, AF388255, AF388256, AF388257, AF388258, AF388259, AF388260, AF388261, AF388262, AF388263, AF388264, AF388265, AF388266, AF388267, AF388268, AF388269, AF388270, AF388271, AF388272, AF388273, AF388274, AF388275, AF388276, AF388277, AF388278, AF388279, AF388280, AF388281, AF388282, AF388283, AF388284, AF388285, AF388286, AF388287, AF388288, AF388289, AF388290, AF388291, AF388292, AF388293, AF388294, AF388295, AF388296, AF388297, AF388298, AF388299, AF388300, AF388301.

Sequences analyzed from the study of Fukuba *et al.* (Fukuba 2003):
AB036433, AB036434, AB036435, AB036436, AB036437, AB036438, AB036439, AB036440, AB036441, AB036442, AB036443, AB036444, AB036445, AB039915.

Sequences analyzed from the study of Joulian *et al.* (Joulian 2001):
AF360643, AF360644, AF360645, AF360646, AF360647, AF360648, AF360649, AF360650, AF360651, AF360652, AF360653, AF360654, AF360655, AF360656, AF360657, AF360658, AF360659, AF360660, AF360661, AF360662, AF360663, AF360664, AF360665, AF360666, AF360667, AF360668, AF360669, AF360670, AF360671, AF360672, AF360673, AF360674, AF360675, AF360676, AF360677, AF360678, AF360679, AF360680, AF360681, AF360682, AF360683, AF360684, AF360685, AF360686, AF360687, AF360688, AF360689, AF360690, AF360691, AF360692, AF360693, AF360694.

Sequences analyzed from the study of LeLoup *et al.* have not been part of a publication so far. Leloup, Microbiologie, Universite de Rouen, Mont Saint Aignan, France.

Sequences analyzed from the study of Perez-Jimenez *et al.* (Pérez-Jiménez 2001):
AF327301, AF327302, AF327303, AF327304, AF327305, AF327306, AF327307, AF327308, AF327309, AF327310, AF327311, AF327312, AF327313, AF327314, AF327315, AF327316, AF327317, AF327318, AF327319, AF327320, AF327321, AF327322, AF327323.

Publications on sequences form Mariager Fjord and Solar Lake water column are in preparation by Klein and Lee et al. Sequences are unpublished so far. Sequence derived form termite gut is also unpublished. Termite guts were kindly provided by Cora Beier.

Sequences analyzed from the study of Baker *et al.* (Baker 2003):
AF510672, AF510673, AF510674, AF510675, AF510676, AF510677, AF510678, AF510679, AF510680, AF510681, AF510682, AF510683, AF510684, AF510685, AF510686, AF510687, AF510688, AF510689, AF510690, AF510691, AF510692, AY101579, AY101580, AY101581, AY116459, AY116460, AY116461, AY116462, AY116463, AY116464, AY116465, AY116466, AY116467, AY116468, AY116469, AY116470, AY135358.

Sequences analyzed from the study of Chang *et al.* (Chang 2001):
AY015493, AY015494, AY015495, AY015497, AY015498, AY015499, AY015500, AY015501, AY015502, AY015503, AY015504, AY015505, AY015506, AY015507, AY015508, AY015509, AY015510, AY015511, AY015512, AY015513, AY015514, AY015515, AY015516, AY015517, AY015518, AY015519, AY015520, AY015521, AY015522, AY015523, AY015524, AY015525, AY015526, AY015527, AY015528, AY015529, AY015530, AY015531, AY015532, AY015533, AY015534, AY015535, AY015536, AY015537, AY015538, AY015539, AY015540, AY015541, AY015542, AY015543, AY015544, AY015545, AY015546, AY015547, AY015548, AY015549, AY015550, AY015551, AY015552, AY015553, AY015554, AY015555, AY015556, AY015557, AY015558, AY015559, AY015560, AY015561, AY015562, AY015563, AY015564, AY015565, AY015566, AY015567, AY015568, AY015569, AY015577, AY015578, AY015579, AY015580, AY015581, AY015582, AY015583, AY015584, AY015585, AY015586, AY015587, AY015588, AY015589, AY015590, AY015591, AY015592, AY015593, AY015594, AY015595, AY015596, AY015597, AY015598, AY015599, AY015600, AY015601, AY015602, AY015603, AY015604, AY015605, AY015606, AY015607, AY015608, AY015609, AY015610, AY015611, AY015612, AY015613, AY015614, AY015615.

Sequences analyzed from the study of Nakagawa and Fukui. (Nakagawa 2003):
AB079482, AB079483, AB079484, AB079485, AB079486, AB079487, AB079488, AB079489, AB079490, AB079491, AB079492, AB079493, AB079494, AB079495, AB079496, AB079497, AB081522, AB081523, AB081531, AB081532, AB081533, AB089186.

Sequences analyzed from the study of Minz *et al.* (Minz 1999b):
AF179309, AF179310, AF179311, AF179312, AF179313, AF179314, AF179315, AF179316, AF179317, AF179318, AF179319, AF179320, AF179321, AF179322, AF179323, AF179324, AF179325, AF179326, AF179326, AF179327, AF179328, AF179329, AF179330; AF179331, AF179332, AF179333, AF179334, AF179335, AF179336, AF179337, AF179338, AF179339, AF179340, AF179341, AF179342, AF179343, AF179344, AF179345, AF179346, AF179346, AF179347, AF179348, AF190885, AF190886, AF190887, AF190888, AF190889.

Sequences analyzed from the study of Nakagawa *et al.* (Nakagawa 2002):
AB079482, AB079483, AB079484, AB079485, AB079486, AB079487, AB079488, AB079489, AB079490, AB079491, AB079492, AB079493, AB079494, AB079495, AB079496, AB079497, AB081522, AB081523, AB081531, AB081532, AB081533, AB089186.

Sequences analyzed from the study of Cottrel and Cary (Cottrell 1999):
AF139066, AF139067, AF139068, AF139069, AF139070, AF139071, AF139072, AF139073, AF139074, AF139075, AF139076.

Sequence analyzed from the study of Dubilier *et al.* (Dubilier 2001):
AF244995.

Sequences derived form sulfidogenic bioreactors by Wagner *et al.* are unpublished so far. Michael Wagner, Universität Wien, Austria.

Sequence analyzed from the study of Loy *et al* (periodontal pockets) (Loy 2002):
AY083028, AY083029.

The table presented on the following pages contains an overview over the *dsr* and 16 S rRNA gene sequences analyzed in this study:

SRP / Dsr origin	Accession number	Figure 5	Figure 7	Figures 15, 16, 17	Figures 18 and 19	Figure 21 rk part	Figures 20, 21, 22, 25
Archaeoglobus fulgidus	M95624	x	x	x	x	x	x
Archaeoglobus profundus	AF071499		x	x	x	x	x
Archaeoglobus veneficus	AF482452		x	x	x	x	x
Bilophila wadsworthia	AF269147		x	x	x	x	x
contamination of Desulfobacula toluolica	AF271773					x	x
Desulfacinum infernum	AF418194		x	x	x	x	x
Desulfitobacterium dehalogenans	AF337903		x	x	x	x	x
Desulfitobacterium halfniense	NZ_AAAW00000000		x	x	x	x	x
Desulfoarculus baarsii	AF334600		x	x	x	x	x
Desulfobacca acetoxidans	AF482453		x	x	x	x	x
Desulfobacter curvatus	AF418199		x	x	x	x	x
Desulfobacter halotolerans	AF388210, AF388256					x	x
Desulfobacter latus	U58124, U58125	x	x	x	x	x	x
Desulfobacter postgatei	AF418198		x	x	x	x	x
Desulfobacter vibrioformis	AJ250472		x	x	x	x	x
Desulfobacterium anilini	AF482455		x	x	x	x	x
Desulfobacterium autotrophicum	AF418182		x	x	x	x	x
Desulfobacterium cetonicum	AF420282		x		x	x	x
Desulfobacterium macestii	AB061533		x	x		x	x
Desulfobacterium oleovorans	AF482464		x	x		x	x
Desulfobacterium oleovorans	AF418201						x
Desulfobacterium vacuolatum	AF418203		x	x			x
Desulfobacula phenolica	AF551758		x	x			x
Desulfobacula toluolica	ARB_6D2D6AA9		x	x	x	x	x
Desulfobotulus sapovorans	U58120, U58121	x	x	x	x	x	x
Desulfobulbus elongatus	AJ310430		x	x	x	x	x
Desulfobulbus elongatus	AF418202						x
Desulfobulbus propionicus	AF218452		x	x	x	x	x
Desulfobulbus rhabdoformis	AJ250473		x	x	x	x	x
Desulfobulbus sp. DSM2058	AF337902		x	x		x	x
Desulfocella halophila	AF418200		x	x	x	x	x
Desulfocella halophila	AF388211, AF388257						x
Desulfococcus multivorans	U58126, U58127	x	x	x	x	x	x
Desulfococcus multivorans, strain G? tingen, d	AJ277107						x
Desulfofaba gelida	AF334593		x	x	x	x	x
Desulfofustis glycolicus	AF418191		x	x	x	x	x
Desulfohalobium retbaense	AF418190		x	x	x	x	x
Desulfohalobium retbaense	AF482458						x
Desulfomicrobium apsheronum	AB061529		x	x	x	x	x
Desulfomicrobium apsheronum	AF418188						x
Desulfomicrobium baculatum	AF482463		x	x	x	x	x
Desulfomicrobium baculatum	AB061530						x
Desulfomicrobium escambiense	AB061531		x	x	x	x	x
Desulfomicrobium norvegicum	AB061532		x	x	x	x	x
Desulfomicrobium orale	AY083030		x	x	x	x	x
Desulfomonas pigra	AB061534		x	x	x	x	x
Desulfomonas pigra	AF418184						x
Desulfomonile tiedjei	AF334595		x	x	x	x	x
Desulfonatronovibrio hydrogenovorans	AF418197			x	x	x	x
Desulfonatronum lacustre	AF418189		x	x	x	x	x
Desulfonema limicola	U58128, U58129		x	x			x

SRP / Dsr origin	Accession number	Figure 5	Figure 7	Figures 15, 16, 17	Figures 18 and 19	Figure 21 rk part	Figures 20, 21, 22, 25
Desulforhabdus amnigena	AF337901		x	x	x	x	x
Desulforhopalus singaporensis	AF418196		x	x	x	x	x
Desulforhopalus vacuolatus	AF334594		x	x	x	x	x
Desulfosarcina variabilis	AF191907		x	x	x	x	x
Desulfosarcina variabilis, strain Montpellier	AJ310429						x
Desulfospira joergensenii	AF482467		x	x	x	x	x
Desulfosporosinus orientis	AF271767		x	x	x	x	x
Desulfotalea psychrophila	S. Behrens (MPI Bremen) unpublished		x				x
Desulfotignum balticum	AF482463		x	x	x	x	x
Desulfotignum balticum	AF420287						x
Desulfotignum balticum	AF420286						x
Desulfotignum balticum	AF420285						x
Desulfotignum balticum	AF420284						x
Desulfotignum phosphitoxidans	AF420283		x		x	x	x
Desulfotomaculum acetoxidans	AF271768		x	x	x	x	x
Desulfotomaculum acetoxidans, UMTRA	AY015493, AY015580						x
Desulfotomaculum aeronauticum	AF273033		x	x	x	x	x
Desulfotomaculum aeronauticum, UMTRA	AY015497, AY015578						x
Desulfotomaculum alkaliphilum	AF418195		x	x	x	x	x
Desulfotomaculum geothermicum	AF273029		x	x	x	x	x
Desulfotomaculum halophilum	M. Friedrich Unpublished		x	x	x	x	x
Desulfotomaculum kuznetsovii	AF273031		x	x	x	x	x
Desulfotomaculum kuznetsovii	AJ310431						x
Desulfotomaculum luciae, UMTRA	AY015494, AY015579		x				x
Desulfotomaculum nigrificans	AF482466		x	x	x	x	x
Desulfotomaculum nigrificans, UMTRA	AY015499, AY015581						x
Desulfotomaculum putei	AF273032		x	x	x	x	x
Desulfotomaculum putei, UMTRA	AY015498, AY015577						x
Desulfotomaculum ruminis	U58118, U58119	x	x	x	x	x	x
Desulfotomaculum thermoacetoxidans	AF271770		x	x	x	x	x
Desulfotomaculum thermobenzoicum	AF273030		x	x	x	x	x
Desulfotomaculum thermobenzoicum	AJ310432						x
Desulfotomaculum thermocisternum	AF074396		x	x	x	x	x
Desulfotomaculum thermosapovorans	AF271769		x	x	x	x	x
Desulfovibrio aespoeensis	AF492838		x			x	x
Desulfovibrio africanus	AF271772		x	x	x	x	x
Desulfovibrio africanus	AB061535						x
Desulfovibrio burkinensis	AB061536		x	x	x	x	x
Desulfovibrio burkinensis	AF418186						x
Desulfovibrio cuneatus	AB061537		x	x	x	x	x
Desulfovibrio desulfuricans El Agheila Z	AF334592		x	x			x
Desulfovibrio desulfuricans Essex 6	AJ249777		x	x	x	x	x
Desulfovibrio desulfuricans subsp. aestuarii,	AJ289157						x
Desulfovibrio desulfuricans subsp. desulfuric	AY015495						x
Desulfovibrio desulfuricans, DSM642, Essex 6,	AF273034						x
Desulfovibrio fructosovorans	AB061538		x	x	x	x	x
Desulfovibrio fructosovorans	AF418187						x
Desulfovibrio halophilus	AF482461		x	x	x	x	x
Desulfovibrio intestinalis	AB061539		x	x	x	x	x
Desulfovibrio intestinalis	AF418183						x
Desulfovibrio longus	AB061540		x	x	x	x	x
Desulfovibrio oxyclinae	U58116, U58117	x	x	x			x
Desulfovibrio simplex	AB061541		x				x
Desulfovibrio simplex, strain XVI DSM 4141, d	U78738						x
Desulfovibrio sp. P1B2	U58116, U58117		x				x
Desulfovibrio sp. PT-2	U58114, U58115	x					x
Desulfovibrio termitidis	AF418185		x	x	x	x	x
Desulfovibrio termitidis	AB061542						x
Desulfovibrio vulgaris	U16723	x	x	x	x	x	x

SRP / Dsr origin	Accession number	Figure 5	Figure 7	Figures 15, 16, 17	Figures 18 and 19	Figure 21 rk part	Figures 20 and 21, 22, 25
Desulfovibrio vulgaris oxamicus	AB061543						x
Desulfovirga adipica	AF334591		x	x	x	x	x
Mariager Fjord clone MAF114D	Klein et al. unpublished						x
Mariager Fjord clone MAF17G	Klein et al. unpublished						x
Mariager Fjord clone MAF23D	Klein et al. unpublished						x
Mariager Fjord clone MAF24D	Klein et al. unpublished						x
Mariager Fjord clone MAF25D	Klein et al. unpublished						x
Mariager Fjord clone MAF26D	Klein et al. unpublished						x
Mariager Fjord clone MAF28G	Klein et al. unpublished						x
Mariager Fjord clone MAF29D	Klein et al. unpublished						x
Mariager Fjord clone MAF419D	Klein et al. unpublished						x
Mariager Fjord clone MAF41D	Klein et al. unpublished						x
Mariager Fjord clone MAF420D	Klein et al. unpublished						x
Mariager Fjord clone MAF46D	Klein et al. unpublished						x
Mariager Fjord clone MAF512D	Klein et al. unpublished						x
Mariager Fjord clone MAF53G	Klein et al. unpublished						x
Mariager Fjord clone MAF36G	Klein et al. unpublished						x
Mariager Fjord clone MAF411G	Klein et al. unpublished						x
Mariager Fjord clone MAF42D	Klein et al. unpublished						x
Mariager Fjord clone MAF65G	Klein et al. unpublished						x
Mariager Fjord clone MAFM12G	Klein et al. unpublished						x
Olavius algarvensis sulfate-reducing delta en	AF244995						x
rice paddy soil Marburg	M. Friedrich Unpublished						x
rice paddy soil Marburg auf Acetat gewachsen	M. Friedrich Unpublished						x
rice paddy soil Marburg auf Aceton gewachsen	M. Friedrich Unpublished						x
rice paddy soil Marburg auf Butyrat gewachsen	M. Friedrich Unpublished						x
rice paddy soil Marburg auf Caproat gewachsen	M. Friedrich Unpublished						x
rice paddy soil Marburg auf Lactat gewachsen	M. Friedrich Unpublished						x
rice paddy soil Marburg auf Propionat gewachs	M. Friedrich Unpublished						x
rice paddy soil Marburg auf Succhrose gewachs	M. Friedrich Unpublished						x
Schlöpnerbrunnen I soil clone dsrSbI-50	AY167464						x
Schlöpnerbrunnen I soil clone dsrSbI-54	AY167478						x
Schlöpnerbrunnen I soil clone dsrSbI-59	AY167473						x
Schlöpnerbrunnen I soil clone dsrSbI-60	AY167466						x
Schlöpnerbrunnen I soil clone dsrSbI-64	AY167474						x
Schlöpnerbrunnen I soil clone dsrSbI-66	AY167480						x
Schlöpnerbrunnen I soil clone dsrSbI-75	AY167476						x
Schlöpnerbrunnen I soil clone dsrSbI-82	AY167482						x
Schlöpnerbrunnen I soil clone dsrSbI-88	AY167475						x
Schlöpnerbrunnen II soil clone dsrSbII-15	AY167471						x
Schlöpnerbrunnen II soil clone dsrSbII-2	AY167483						x
Schlöpnerbrunnen II soil clone dsrSbII-20	AY167470						x
Schlöpnerbrunnen II soil clone dsrSbII-25	AY167481						x
Schlöpnerbrunnen II soil clone dsrSbII-28	AY167479						x
Schlöpnerbrunnen II soil clone dsrSbII-3	AY167467						x
Schlöpnerbrunnen II soil clone dsrSbII-34	AY167468						x
Schlöpnerbrunnen II soil clone dsrSbII-36	AY167469						x
Schlöpnerbrunnen II soil clone dsrSbII-39	AY167472						x
Schlöpnerbrunnen II soil clone dsrSbII-40	AY167465						x
Schlöpnerbrunnen II soil clone dsrSbII-9	AY167477						x

SRP / Dsr origin	Accession number	Figure 5	Figure 7	Figures 15, 16, 17	Figures 18 and 19	Figure 21 rk part	Figures 20 and 21, 22, 25
Solar Lake Water Column S23, 5.0 m	Lee und Klein unpublished						x
Solar Lake Water Column AA17, 4.5 m	Lee und Klein unpublished						x
Solar Lake Water Column AA7, 4.5 m	Lee und Klein unpublished						x
Solar Lake Water Column AM57, 4.5 m	Lee und Klein unpublished						x
Solar Lake Water Column C22, 5.5 m	Lee und Klein unpublished						x
Solar Lake Water Column C4, 5.5 m	Lee und Klein unpublished						x
Solar Lake Water Column E20, 6.0 m	Lee und Klein unpublished						x
Solar Lake Water Column FII, 4.75 m	Lee und Klein unpublished						x
Solar Lake Water Column M14, 4.75 m	Lee und Klein unpublished						x
Solar Lake Water Column M37, 4.75 m	Lee und Klein unpublished						x
Solar Lake Water Column P77, 4.25 m	Lee und Klein unpublished						x
Solar Lake Water Column Q20, 6.0 m	Lee und Klein unpublished						x
Solar Lake Water Column Q32, 6.0 m	Lee und Klein unpublished						x
Solar Lake Water Column Q8, 6.0 m	Lee und Klein unpublished						x
Solar Lake Water Column R9, 4.75 m	Lee und Klein unpublished						x
Solar Lake Water Column S30, 5.0 m	Lee und Klein unpublished						x
Solar Lake Water Column AA30, 4.5 m	Lee und Klein unpublished						x
Solar Lake Water Column AH11, 3.5 m	Lee und Klein unpublished						x
Solar Lake Water Column AJ55, 4.0 m	Lee und Klein unpublished						x
Solar Lake Water Column BB6, 4.5 m	Lee und Klein unpublished						x
Solar Lake Water Column J45, 6.0 m	Lee und Klein unpublished						x
Solar Lake Water Column M12, 4.75	Lee und Klein unpublished						x
Solar Lake Water Column Y39, 6.0 m	Lee und Klein unpublished						x
Solar Lake Water Column ZZ1, 2.25 m	Lee und Klein unpublished						x
Solar Lake Water Column, R18, 4.75 m	Lee und Klein unpublished						x
SRB Pure Culture 32-1, 2.75 m	Lee und Klein unpublished						x
sulfate reducing strain mXyS1	AF482456		x	x	x	x	x
sulfate-reducing bacterium AK-01	AF327301			x			x
sulfate-reducing bacterium Na82	AB077818						x
sulfate-reducing strain oXyS1	AF482465		x	x	x	x	x
Syntrophobacter fumaroxidans	AF418193		x	x	x	x	x
Syntrophobacter wolinii	AF418192		x	x			x
Thermodesulfobacterium commune	AF334596		x	x	x	x	x
Thermodesulfobacterium mobile	AF334598		x	x	x	x	x
Thermodesulforhabdus norvegica	AF334597		x	x	x	x	x
Thermodesulfovibrio islandicus	AF334599		x	x	x	x	x
Thermodesulfovibrio yellowstonii	U58122, U58123	x	x	x	x	x	x
uncultured bacterium SAE-33 dsrAB	AY101580						x
uncultured bacterium SAW6-28 dsrAB	AY101579						x
uncultured bacterium SAW6-30 dsrAB	AY101581						x
Uncultured SRB, KYF-128	AF388259, AF388213						x
Uncultured SRB, KYF-135	AF388214, AF388260						x
Uncultured SRB, KYF-136	AF388215, AF388261						x
Desulfovibrio gigas	M62784			x			

SRP / Origin of 16S rRNA genes	Accession number	Figure 3 and 4	Figure 5	Figures 15,16,17
Archaeoglobus fulgidus	X05567 Y	x	x	x
Archaeoglobus profundus	AJ299219	x		x
Archaeoglobus veneficus	Y10011	x		x
Bilophila wadsworthia 2	U82813	x		x
delta proteobacterium oXyS1	Y17286	x		x
Desulfacinum infernum	L27426	x		x
Desulfitobacterium dehalogenans	L28946	x		x
Desulfitobacterium frappieri	U40078	x		
Desulfitobacterium hafniense	X94975	x		x
Desulfoarculus sp. BG74	U85477	x		x
Desulfobacca acetoxidans	AF002671	x		x
Desulfobacter curvatus	M34413	x		x
Desulfobacter latus	M34414	x	x	x
Desulfobacter postgatei	M26633	x		x
Desulfobacter vibrioformis	U12254	x		x
Desulfobacterium anilini	AJ237601	x		x
Desulfobacterium autotrophicum	M34409	x		x
Desulfobacterium macestii	AJ237604	x		x
Desulfobacterium oleovorans	Y17698	x		x
Desulfobacterium vacuolatum	M34408	x		x
Desulfobacula phenolica	AJ237606	x		x
Desulfobacula toluolica	X70953	x		x
Desulfobotulus sapovorans	M34402	x	x	x
Desulfobulbus elongatus	X95180	x		x
Desulfobulbus propionicus	M34410	x		x
Desulfobulbus rhabdoformis	U12253	x		x
Desulfobulbus species	M34411	x		x
Desulfocella halophila	AF022936	x		x
Desulfococcus multivorans	M34405	x	x	x
Desulfofaba gelida	AF099063	x		x
Desulfofustis glycolicus	X99707	x		x
Desulfohalobium retbaense	U48244	x		x
Desulfomicrobium apsheronum	U64865	x		x
Desulfomicrobium baculatum	AF030438	x		x
Desulfomicrobium escambiense	U02469	x		x
Desulfomicrobium norvegicum	M37312	x		x
Desulfomicrobium orale	AJ251623	x		x
Desulfomonile tiedjei	M26635	x		x
Desulfonatronovibrio hydrogenovorans	X99234	x		x
Desulfonatronum lacustre	Y14594	x		x
Desulfonema limicola	U45990	x		x
Desulforhabdus amnigena	X83274	x		x
Desulforhopalus singaporensis	AF118453	x		x
Desulforhopalus vacuolatus	L42613	x		x
Desulfosarcina variabilis	M34407	x		x
Desulfospira joergensenii	X99637	x		x
Desulfosporosinus orientis	Y11570	x		x
Desulfotalea psychrophila	AF099062	x		
Desulfotignum balticum	AF233370	x		x
Desulfotomaculum acetoxidans	Y11566	x		x
Desulfotomaculum aeronauticum	X98407	x		x
Desulfotomaculum alkaliphilum	AF097024	x		x
Desulfotomaculum auripigmentum	U85624	x		
Desulfotomaculum australicum	M96665	x		
Desulfotomaculum geothermicum	Y11567	x		x
Desulfotomaculum gibsoniae	Y11576	x		
Desulfotomaculum guttoideum	Y11568	x		
Desulfotomaculum halophilum	U88891	x		x
Desulfotomaculum kuznetsovii	Y11569	x		x
Desulfotomaculum luciae	AF069293	x		
Desulfotomaculum nigrificans	X62176	x		x
Desulfotomaculum putei	AF053933	x		x
Desulfotomaculum reducens	U95951	x		
Desulfotomaculum ruminis	Y11572	x	x	x
Desulfotomaculum sapomandens	AF168365	x		
Desulfotomaculum thermoacetoxidans	Y11573	x		x
Desulfotomaculum thermoacidovorans	Z26315	x		
Desulfotomaculum thermobenzoicum	Y11574	x		x
Desulfotomaculum thermocisternum	U33455	x		x
Desulfotomaculum thermosapovorans	Y11575	x		x

G.2 Publications which contain results from this Ph.D. thesis

G.2.1 Title: Multiple lateral transfer events of dissimilatory sulfite reductase genes between major lineages of sulfate-reducing prokaryotes

The *dsrAB* genes from 30 sulfate reducing prokaryotes were PCR amplified, cloned and sequenced. For 20 SRP 16S rRNA gene and DsrAB based phylogeny was consistent. The remaining ten SRP carried *dsrAB* genes, which according to phylogenetic data must have been acquired by lateral gene transfer events. At least four independent lateral *dsrAB* gene transfer events were recognized and the histories of sulfate reducing prokaryotes were discussed in an evolutionary context. Michael Klein derived 16 full *dsrAB* sequences from SRP pure cultures and the full 16S-rDNA sequence of *Thermodesulfobacterium mobile*. In addition, Michael Klein did the alignment of the dsrAB sequences within the data bank and of dsrA against dsrB. Furthermore, Michael Klein contributed to the writing of this paper and, in part, to the phylogenetic analyses. This paper was published in the Journal of Bacteriology in October 2001 (Klein 2001).

Multiple lateral transfer events of dissimilatory sulfite reductase genes between major lineages of sulfate-reducing prokaryotes

Michael Klein[1], Michael Friedrich[2], Andrew J. Roger[3], Philip Hugenholtz[4], Susan Fishbain[5], Heike Abicht[1], Linda L. Blackall[4], David A. Stahl[6], and Michael Wagner[1]*

[1] Lehrstuhl für Mikrobiologie, Technische Universität München, Am Hochanger 4, D-85350 Freising, Germany
[2] Max-Planck-Institute for terrestrial Microbiology, Department of Biogeochemistry, Karl-von-Frisch-Str., D-35043-Marburg, Germany
[3] Department of Biochemistry and Molecular Biology, Dalhousie University; Halifax, Nova Scotia, B3H 4H7, Canada.
[4] Advanced Wastewater Management Centre, Department of Microbiology and Parasitology, The University of Queensland, Brisbane 4072, Australia.
[5] Department of Civil Engineering, Northwestern University, Evanston, Illinois 60208-3109
[6] Civil and Environmental Engineering, 302 More Hall, Box 352700, University of Washington, Seattle, WA 98195-2700, USA

*corresponding author: PD Dr. Michael Wagner, Lehrstuhl für Mikrobiologie, Technische Universität München, Am Hochanger 4, D-85350 Freising, Germany. Tel: +498161715444; Fax: +498161715475; E-mail: wagner@mikro.biologie.tu-muenchen.de

Keywords: dissimilatory sulfite reductase/lateral gene transfer/Archaea/Bacteria/phylogeny

Running title: Lateral transfer of dissimilatory sulfite reductases

ABSTRACT

A large fragment of the dissimilatory sulfite reductase genes (*dsrAB*) was PCR-amplified and fully sequenced from 30 reference strains, representing all recognized lineages of sulfate-reducing bacteria. In addition, the sequence of the *dsrAB* gene homologs of the sulfite-reducer *Desulfitobacterium dehalogenans* was determined. In contrast to previous reports, comparative analysis of all available DsrAB sequences produced a tree topology partially inconsistent with the corresponding 16S rRNA phylogeny. For example, the DsrAB sequences of several *Desulfotomaculum* species (low G+C Gram positive division) and two members of the genus *Thermodesulfobacterium* (a separate bacterial division) were monophyletic with δ-proteobacterial DsrAB sequences. The most parsimonious interpretation of these data is that *dsrAB* genes from ancestors of as-yet unrecognized sulfate-reducers within the δ-*Proteobacteria* were laterally transferred across divisions. A number of insertions and deletions in the DsrAB alignment independently support these inferred lateral acquisitions of *dsrAB* genes. Evidence for a *dsrAB* lateral gene transfer event also was found within the δ-*Proteobacteria,* affecting *Desulfobacula toluolica*. The root of the *dsr* tree was inferred to be within the *Thermodesulfovibrio* lineage by paralogous rooting of the alpha and beta subunits. This rooting suggests that the *dsrAB* genes in *Archaeoglobus* species also are the result of an ancient lateral transfer from a bacterial donor. Although these findings complicate the use of *dsrAB* genes to infer phylogenetic relationships among sulfate-reducers in molecular diversity studies, they establish a framework to resolve the origins and diversification of this ancient respiratory lifestyle among organisms mediating a key step in the biogeochemical cycling of sulfur.

INTRODUCTION

Sirohaem dissimilatory sulfite reductases (EC 1.8.99.3) catalyze the reduction of sulfite to sulfide, an essential step in the anaerobic sulfate-respiration pathway. Consequently, this enzyme has been found in all dissimilatory sulfate-reducing prokaryotes (SRPs) investigated so far. Furthermore, sirohaem dissimilatory sulfite reductase-like enzymes have been detected in the hyperthermophilic archaeon *Pyrobaculum islandicum* capable of using sulfite as terminal electron acceptor (24), the phototrophic bacterium *Allochromatium vinosum* (11,13), and the obligate chemolithotrophic species *Thiobacillus denitrificans* (33). In the latter two organisms the dissimilatory sulfite reductase has a proposed function in sulfide oxidation.

Sirohaem sulfite reductases consist of at least two different polypeptides in an $\alpha_2\beta_2$ structure. The genes encoding the two subunits are found adjacent to each other in the respective genomes (e.g. 3,16,18,19,37) and probably arose from duplication of an ancestral gene (3). Comparative amino acid sequence analysis of the dissimilatory sulfite reductase genes (*dsrAB*) has recently been used to investigate the evolutionary history of anaerobic sulfate- (sulfite-) respiration (11,18,19,37). The presence of *dsrAB* homologs in at least five highly divergent prokaryotic lineages and overall phylogenetic congruence of the *dsrAB* tree with the 16S rRNA gene tree suggested that the dissimilatory sulfite reductases of extant SRPs evolved vertically from common ancestral protogenotic genes (37). The remarkable degree of conservation of the *dsrAB* genes also provided a basis for culture-independent molecular diversity studies of natural sulfate-reducing assemblages using PCR primers broadly specific for a large fragment of all known *dsrAB* genes (1,23). However, recently one contradiction between the *dsrAB* and 16S rRNA phylogenies was recognized in that the *dsrAB* sequences of *Desulfotomaculum thermocisternum* (18) and *Desulfotomaculum ruminis* are not monophyletic (19). This

finding could indicate that, in addition to vertical transmission, lateral gene transfer is involved in the evolution of SRPs.

In the present study we have investigated this question further by phylogenetic analysis of the *dsrAB* genes from a wide range of cultivated SRPs. We find a clear case for multiple lateral transfer events of the *dsrAB* genes between major lineages of Bacteria, and likely between the domains Bacteria and Archaea, suggesting genes involved in primary metabolic functions, such as sulfate-respiration, may be more prone to lateral transfer than previously thought.

MATERIALS AND METHODS

Bacterial strains. The investigated reference strains of sulfate- and sulfite-reducing bacteria are listed in Table 1. If necessary, strains were cultured as recommended by the German type culture collection (DSMZ, Braunschweig, Germany**).**

DNA isolation and PCR amplification. Genomic DNA of the reference organisms investigated was obtained from logarithmically growing or lyophilized cells by either using the FastPrep FP120 bead beater and the FastDNA^{TM} Kit MH (BIO101, CA) or another direct lysis technique (25) modified as described previously (10). An approx. 1.9 kb *dsrAB* segment was PCR amplified as described (37). Since amplification of the *dsrAB* gene fragment was not possible for all investigated reference strains additional degeneracies were introduced in the previously published primers DSR1F and DSR4R (DSR1Fdeg: 5'-ACSCAYTGGAARCACG-3'; DSR4Rdeg: 5'-GTGTARCAGTTDCCRCA-3') making them fully complementary to the respective target sites of recently published *dsrAB* sequences (18,19). However, it should be noted that many "non

dsrAB" amplificates of approximately 1.9 kb size were obtained using the degenerated primers.

Cloning and sequencing of *dsrAB* gene fragments. If not mentioned otherwise *dsrAB* PCR products of the sulfite- and sulfate-reducing reference strains were ligated into pCR2.1-TOPO or pCR-XL-TOPO vectors (Invitrogen, CA). Clones with approximate 1.9 kb inserts were recovered with the QIAprep spin kit (Qiagen, Hilden, Germany) and sequenced with a 4200L automated Li-Cor Long Reader DNA Sequencer (MWG, Ebersberg, Germany). *DsrAB* PCR products of the *Desulfotomaculum* species *D. aeronauticum*, *D. putei*, *D. geothermicum*, *D. kuznetsovii*, and *D. thermobenzoicum* were directly sequenced. In addition *dsr* sequences of *Desuforhabdus amnigenus*, *Desulfobulbus sp.* and *Desulfitobacterium dehalogenans* were determined by directly sequencing as well as sequencing of the cloned PCR product. Previously published (37) partial *dsrAB* sequences of *Desulfotomaculum ruminis*, *Thermodesulfovibrio yellowstonii*, *Desulfobacter latus*, *Desulfobotulus sapovorans*, *Desulfococcus multivorans*, and *Desulfovibrio sp.* PT-2 were completed by re-sequencing of the original clones.

16S rRNA of *Thermodesulfobacterium mobile.* The 16S rRNA gene sequence of *T. mobile* was obtained as described previously (15).

Phylogeny inference. Phylogenetic analyses were performed on alignments of the 16S rDNA nucleotide and the inferred amino acid sequences of the *dsrAB* genes. Regions of ambiguous positional homology were removed from the 16S rDNA data set using the Lane mask (17) and a DsrAB amino acid alignment mask prepared in ARB (http://www.arb-home.de). A total of 1,335 nucleotides and 543 amino acid positions (alpha subunit, 327; beta subunit 216) were used in 16S rDNA and DsrAB analyses, respectively. For paralogous rooting DsrA

sequences were aligned against DsrB and trees were calculated based on 173 amino acid positions including positions with insertions and deletions. Phylogenetic analyses were performed with PAUP* version 4.0b2a (35), ARB, or PHYLIP version 3.57c (5). Evolutionary distance (ED) analyses were conducted on the 16S rDNA data set using the Kimura 2 parameter and general time reversible substitution model corrections with and without rate correction. Rate heterogeneities were corrected using a gamma distribution model (the shape parameter, alpha, was estimated to be 0.52 using a parsimony based approximation in PAUP*). ED analysis of the DsrAB data set was performed using a Dayhoff PAM correction and neighbor joining. Maximum parsimony (MP) trees were constructed for both data sets using the default settings in PAUP*. Maximum likelihood (ML) analysis of the 16S rDNA dataset was performed in the ARB package using the fastDNAml program (29). Bootstrap resampling of the ED and MP trees was performed for all analyses to provide confidence estimates for the inferred topologies. 1000 or 2000 replicates were used in all cases with the exception of the ED analysis of the DsrAB dataset where 100 replicates were calculated.

RESULTS

Dissimilatory sulfite reductase phylogeny. A DNA fragment approximately 1.9 kb in size, encompassing most of the alpha and beta subunit genes of the dissimilatory sulfite reductase, was amplified from 30 sulfite-, and sulfate-reducing bacteria (Tab. 1).

Table 1. Physiological and biochemical properties of the sulfite- and sulfate-reducing prokaryotes investigated

Species*	Strain†	Oxid.‡	Topt [°C]	G+C Content (mol%) Genome§	dsrAB	Genome§ dsrAB	dsrAB3rd¶	Genome§ dsrAB3rd¶	Accession number dsrAB	16S rDNA
Archaea										
Euryarchaeota										
Archaeoglobus profundus	DSM5631^T	C	82	41	47	0,87	50	0,82	AF071499	AF297529
Archaeoglobus fulgidus	DSM4304^T	C	83	46	50	0,92	59	0,78	M95624	X05567 Y00275
Bacteria										
Nitrospira division										
Thermodesulfovibrio yellowstonii	DSM11347^T	I	65	30	39	0,77	28	1,07	U58122/3	L14619
Thermodesulfovibrio islandicus	DSM12570^T	I	65	38	39	0,97	29	1,31	AF334599	X96726
Thermodesulfobacterium division										
Thermodesulfobacterium commune	DSM2178^T	I	70	34	41	0,83	33	1,03	AF334596	L10662
Thermodesulfobacterium mobile	DSM1276^T	I	65	31	41	0,76	34	0,91	AF334598	AF334601
Firmicutes; Bacillus/Clostridium group										
Desulfotomaculum ruminis	DSM2154^T	I	28	49	46	1,07	45	1,09	U58118/9	Y11572
Desulfotomaculum aeronauticum	DSM10349^T	I	37	44	48	0,92	52	0,85	AF273033	X96407
Desulfotomaculum putei	DSM12395^T	I	50-65	47	52	0,90	61	0,77	AF273032	AF053929
Desulfotomaculum geothermicum	DSM3669^T	C	54	50	53	0,94	65	0,77	AF273029	X80789
Desulfotomaculum thermosapovorans	DSM6562^T	I	50	51	52	0,98	62	0,82	AF271769	Y11575
Desulfotomaculum kuznetsovii	DSM6115^T	C	60-65	49	56	0,88	74	0,66	AF273031	Y11569
Desulfotomaculum thermocisternum	DSM10259^T	I	62	57	55	1,04	67	0,85	AF074386	U33455
Desulfotomaculum thermobenzoicum	DSM6193^T	C	62	53	56	0,95	74	0,72	AF273030	L15628
Desulfotomaculum thermoacetoxidans	DSM5813^T	C	55-60	50	56	0,89	73	0,68	AF271770	Y11573
Desulfotomaculum acetoxidans	DSM771^T	C	37	38	45	0,84	41	0,93	AF271768	Y11566
Desulfosporosinus orientis	DSM765^T	I	30	45	42	1,07	30	1,50	AF271767	Y11570
Desulfitobacterium dehalogenans	DSM9161^T	I	37	45	47	0,96	46	0,98	AF337903	L28946
Desulfitobacterium hafniense	DSM10664^T	I	37	47	48	0,98	47	1,00	n.d.	X94975
Proteobacteria delta subdivision										
Desulfobacter vibrioformis	DSM8776^T	C	33	47	49	0,96	55	0,85	AJ250473	U12254
Desulfobacter latus	DSM3381^T	C	29-32	44	49	0,90	54	0,81	U58124/5	M34414
Desulfobacula toluolica	DSM7467^T	C	28	42	53	0,79	66	0,64	AF271773	X70953
Desulfofaba gelida	DSM12344^T	I	7	53	52	1,02	61	0,87	AF334593	AF099063
Desulfobotulus sapovorans	DSM2055^T	I	34	53	52	1,02	59	0,90	U58120/1	M34402
Desulfosarcina variabilis	DSM2060^T	C	33	51	55	0,93	68	0,75	AF191907	M34407
Desulfococcus multivorans	DSM2059^T	C	35	57	56	1,02	72	0,79	U58126/7	M34405
Desulfovibrio vulgaris	DSM644^T	I	30-36	65	61	1,07	82	0,79	U16723	M34399
Desulfovibrio sp. PT-2	ATCC49975	I	30	65	62	1,05	86	0,76	U58114/5	M96496
Desulfovibrio desulfuricans Essex 6	DSM642^T	I	30	59	60	0,98	79	0,75	AJ249777	AF192153
Desulfovibrio africanus	DSM2603^T	I	30-36	65	62	1,05	86	0,76	AF271772	X99236
Desulfovibrio desulfuricans El Agheila Z	DSM1926	n.d.	30	n.d.	56	n.d.	72	n.d.	AF334592	M37316
Desulfoarculus baarsii	DSM2075^T	C	37	66	62	1,06	85	0,78	AF334600	M34403
Desulfomonile tiedjei	DSM6799^T	C	37	49	53	0,92	63	0,78	AF334595	M26635
Desulfobulbus rhabdoformis	DSM8777^T	I	31	51	52	0,98	55	0,93	AJ250473	U12253
Desulfobulbus propionicus	DSM2032^T	I	39	60	57	1,05	72	0,83	AF216452	M34410
Desulfobulbus sp. 3pr10	DSM2055	I	29	n.d.	48	n.d.	44	n.d.	AF337902	M34411
Desulforhopalus vacuolatus	DSM9700^T	I	18	48	47	1,02	46	1,04	AF334594	L42613
Desulfovirga adipica	DSM12016^T	C	35	60	57	1,05	75	0,80	AF334591	AJ237605
Desulforhabdus amnigena	DSM10338^T	C	37	53	52	1,02	57	0,93	AF337901	X83274
Thermodesulforhabdus norvegica	DSM9990^T	C	60	51	53	0,96	63	0,81	AF334597	U25627

* Sulfate-reducing prokaryotes with a putative xenologous DsrAB are labeled in bold; † DSMZ or ATCC strain number, T type strain; ‡ Oxidation: C complete or I incomplete; § Quotient of genomic and *dsrAB* G+C content and quotient of genomic and *dsrAB* 3rd position G+C content, respectively. Accuracy of the genomic G+C content values might vary due to different determination methods applied; ¶ G+C content (mol%) of third codon position; n.d. no data

Complete sequences of the PCR products were obtained. Compiled sequences were entered into the *dsrAB* database, translated into amino acids, and manually aligned. Previously published partial length *dsr* sequences of *Desulfovibrio oxyclinae* (37, U58116/7), *Desulfovibrio simplex* (11, U78738), *Desulfovibrio gigas* (U80961), *Desulfonema limicola* (37; U58128/9), and *Desulfobacterium autotrophicum* (Y15478) were not included to avoid resolution loss in phylogenetic analyses. Comparative sequence analyses were performed based on each subunit and both subunits combined. No major differences were noted between the individual and combined subunit tree topologies regardless of the inference method used, indicating a shared evolutionary history for the alpha and beta subunits. Consistent with these findings, the G+C contents of *dsrA* and *dsrB* were almost identical for each organism (data not shown).

Consequently, detailed phylogenetic analyses were performed on a combined (DsrAB) data set in order to include the maximum number of 543 comparable amino acid positions. For comparison, trees were calculated from the 16S rRNA genes of the identical set of organisms to avoid sampling artifacts (Fig. 1). Since the 16S rDNA sequence of *Thermodesulfobacterium mobile* was not available, it was determined in this study (1520 nucleotides). In Figure 1, the *Archaeoglobus* sequences were used as the outgroup for the 16S rRNA tree as they are the only representatives of the archaeal domain in an otherwise bacterial tree. In contrast, the *Thermodesulfovibrio* sequences (bacterial *Nitrospira* division), were used as the outgroup in the DsrAB analyses since paralogous outgrouping of the alpha and beta subunits suggests that the root of the Dsr tree is along the *Thermodesulfovibrio* line of descent (Fig. 2). Therefore, it appears likely that the dissimilatory sulfite reductases of the *Archaeoglobales* have a bacterial origin (see Discussion).

Overall, highly similar orderings of taxa, shaded grey in Fig. 1, were found between the 16S rRNA and DsrAB trees with all treeing methods. However, major incongruencies were found between DsrAB and 16S rRNA based analysis for seven members of the genus *Desulfotomaculum*, for both species of the genus *Thermodesulfobacterium*, and for the δ−proteobacterium *Desulfobacula toluolica* (color coded; Fig. 1). In contrast to relationships inferred using the rRNA, the genus *Desulfotomaculum*, a member of the low G+C Gram positive division (34), is not monophyletic in the DsrAB tree. *Desulfotomaculum aeronauticum*, *D. ruminis*, and *D. putei* form a clearly separated grouping together with *Desulfosporosinus orientis* based on their DsrAB sequences, while the other seven *Desulfotomaculum* species cluster together with *Desulfobacula toluolica* within the δ−proteobacterial radiation. Similarly, *Thermodesulfobacterium commune* and *T. mobile* comprise a division-level lineage by rRNA analysis but branch within the δ−*Proteobacteria* according to their DsrAB

sequences. A final discrepancy recognized is the inconsistent branching point of *Desulfobacula toluolica*. By 16S rRNA comparison, this species is closely related to *Desulfobacter latus* and *Desulfobacter vibrioformis*, while its DsrAB sequence is robustly associated with the *Desulfotomaculum* group in the δ–*Proteobacteria* (Fig. 1). The most parsimonious interpretation is that these significant topological conflicts reflect lateral transfer of the DsrAB genes (see Discussion). Points of inferred lateral gene transfer (LGT) are indicated in Fig.1 by circled letters on the 16S rRNA tree.

Additional evidence for lateral transfer of dissimilatory sulfite reductase. Insertions and deletions within the DsrAB amino acid sequences (excluded in the phylogenetic analyses) were investigated as additional signposts of the deduced evolutionary relationships, particularly with respect to inferred LGT events. In total, three insertions were unique to the δ–*Proteobacteria*, one in the alpha subunit and two in the beta subunit (Fig. 3). These insertions were also found in the δ-proteobacterial-like DsrAB sequences of the seven *Desulfotomaculum* species, and two *Thermodesulfobacterium* species, thus independently supporting the suggested LGT events.

Sizable differences in G+C content of the host genomes and acquired genes has been used to infer recent LGT events (21). A variation of more than 10% between the *dsrAB* G+C content and the respective genomic G+C content was found in *Thermodesulfobacterium mobile*, *Thermodesulfobacterium commune*, *Thermodesulfovibrio yellowstonii*, *Desulfobacula toluolica*, *Desulfotomaculum acetoxidans*, *Desulfotomaculum kuznetsovii*, *Desulfotomaculum thermoacetoxidans*; and *Archaeoglobus profundus* (Tab. 1). In seven of these eight organisms, LGT of *dsrAB* was predicted by comparison of tree topologies (Fig. 1). We attempted but failed to refine this analysis using the approach of Lawrence and Ochman (21) to identify atypical sequence

characteristics (data not shown) since this method produces unreliable estimates for samples containing fewer than 1,500 codons as described previously (21).

Dissimilatory sulfite reductase homolog of *Desulfitobacterium dehalogenans.* The conserved *dsrAB* primers also amplified a fragment of the expected length from *Desulfitobacterium dehalogenans*, a bacterium capable of sulfite- but not sulfate-reduction (36). Comparative sequence analysis of the amplicon demonstrated a specific relationship to the dissimilatory sulfite reductase of *Desulfosporosinus orientis* consistent with their 16S rRNA-based relationship (Fig. 1). Furthermore, the recently completed genome sequence of *Desulfitobacterium hafniense* (http://www.jgi.doe.gov) contains a *dsrAB* sequence highly similar to the one of *Desulfitobacterium dehalogenans* (97.0% amino acid identity). As expected from the close relationship of both species by 16S rDNA comparison (96.7% similarity), their DsrAB sequences group together independent of treeing methods applied.

DISCUSSION

In this study we investigated the phylogeny of the dissimilatory sulfite reductase from a study set of reference species encompassing all described lineages of SRPs in order to clarify whether - in addition to vertical transmission - *dsr* genes have also been laterally transferred. Using degenerated PCR primers, DNA fragments with strong sequence similarities over their entire length to previously published *dsrAB* sequences were obtained from all investigated SRPs and from the sulfite-reducer *Desulfitobacterium dehalogenans.*

DSR sequence motifs. The newly determined *dsrAB*-like sequences contain the essential cluster-binding residues typical for dissimilatory sulfite reductases. In particular, all alpha-subunit sequences contain the complete (Cys-X_5-Cys)-X_n-(Cys-X_3-Cys) motif required for coupling of the [Fe_4S_4]-sirohaem cofactor (2). As for other

dissimilatory sulfite reductases (11) this cys motif is truncated in the beta subunit of the newly determined DsrAB sequences. In contrast to the prediction of Dahl *et al.* (3) the DsrB subunit of *Thermodesulfobacterium mobile* and *Thermodesulfobacterium commune* (4,9) does not contain a complete sirohaem-[Fe_4S_4] binding site that could explain the measured binding of four sirohaems per $\alpha_2\beta_2$ molecule (versus two sirohaems for typical sulfite reductases). Furthermore, all DsrA sequences possess the Cys-Pro and Cys-X_2-Cys-X_2-Cys motif required for linking [Fe_4S_4] clusters (3). Since the reverse PCR primers used for amplification target part of the [Fe_4S_4] cluster binding motif of DsrB only the Cys-Pro signature is present in all deduced DsrB sequences. The absolute conservation of functionally important protein sequences and the absence of frameshift or nonsense mutations suggests that the characterized genes are transcribed and translated, and function as dissimilatory sulfite reductases. The sequenced dissimilatory sulfite reductase genes of *Thermodesulfobacterium mobile* are most likely functionally expressed since the highly variable N-terminal sequence of the beta-subunit is identical to the one determined by Edman degradation (4). Comparison of the 10 N-terminal amino acids of the beta-subunit determined by Edman degradation of the dissimilatory sulfite reductase of *Thermodesulfobacterium commune* (9) to the sequence deduced in our study revealed a single amino acid difference at position 1 [Thr/Ser predicted by Edman - Gly (GGA codon) found in our study]. This inconsistency is either caused by an experimental artifact (mistake in the Edman degradation determination or at least two Taq-induced mutations in the *dsrAB* clone of *T. commune*) or by the presence of more than one type of *dsrAB* genes in this organism. Differences between the deduced N-terminal sequence and that determined by N-terminal polypeptide sequencing were also reported for the DsrB protein of *Desulfovibrio desulfuricans* (26).

DSR homologs. Additional homologs to the investigated *dsrAB* genes may exist in some of the analyzed strains. This is not the case for *Desulfitobacterium hafniense* and

Archaeoglobus fulgidus since no additional *dsrAB* homologs are present in their complete genome sequences. Under the assumption that the PCR primers applied would amplify all putative *dsr* copies, we have indirect evidence that the *Desulfotomaculum* species, *D. aeronauticum*, *D. putei*, *D. geothermicum*, *D. kuznetsovii*, and *D. thermobenzoicum* do not contain multiple *dsrAB* copies which differ in sequence since the respective *dsrAB* PCR amplificates could be sequenced directly. For the other analyzed SRPs, knowledge of the copy number of *dsrAB*-like genes must await an extensive southern hybridization or complete genome sequence analysis which was beyond the scope of this study.

DSR phylogeny and lateral transfer. The core of our study was a direct comparison between 16S rRNA and DsrAB trees of the respective SRPs (Fig. 1). In this analysis it is an explicit assumption that the 16S rRNA phylogeny reflects the organismal phylogeny (38), that is, that these highly conserved genes have undergone no lateral transfer in the organisms studied. Accepting this supposition, seven *Desulfotomaculum* species, two *Thermodesulfobacterium* species and *Desulfobacula toluolica* possess non-orthologous *dsrAB* genes as demonstrated by major inconsistencies between the DsrAB and 16S rRNA trees. These inconsistencies most likely reflect lateral transfer of *dsrAB* genes rather than the occurrence of *dsrAB* paralogs which diverged after an initial *dsr* operon duplication since all non-orthologous *dsrAB* genes are phylogenetically affiliated with the (presumably orthologous) *dsrAB* genes of the δ-*Proteobacteria* (Fig. 1). Furthermore, organisms distantly related by 16S rRNA sequence relationship, such as *Desulfobacula toluolica* and several *Desulfotomaculum* species, contain similar non-orthologous *dsrAB* genes. This close relatedness of *dsrAB* genes between species belonging to different bacterial divisions is unlikely to be the product of convergent evolution and can more reasonably be explained by multiple lateral acquisitions from a common donor lineage within the δ-*Proteobacteria*. Consistent with this inference, all

putative xenologous *dsrAB* sequences have insertions typical for the δ-*Proteobacteria* (Fig. 3).

Five independent LGT events (red circles; Fig. 1) of *dsrAB* genes have been postulated to explain the observed discrepancies between the 16S rDNA and DsrAB topologies. It should be noted that for SRPs (i) which do not have close phylogenetic relatives in the current *dsrAB* dataset or (ii) whose positions in the deduced phylogenetic trees vary dependent upon the treeing method used, our analysis can not rule out that their characterized *dsr*AB sequences are xenologs. Within the δ-*Proteobacteria* these limitations apply to *Desulfoarculus baarsii* and *Desulfomonile tiedjei*. Furthermore, the characterized DsrAB sequences of *Archaeoglobus* and *Thermodesulfovibrio* species and the "authentic" *Desulfotomaculum* and *Desulfitobacterium* species possibly could originate from a progenitor of the δ-*Proteobacteria* or from other as yet unidentified SRPs. In fact, it seems likely that the genus *Archaeoglobus* inherited *dsrAB* genes from a bacterial donor because (i) the evolutionary distance between *Archaeoglobus* species and the bacterial sulfate-reducers is much shorter in the DsrAB tree than in the 16S rRNA tree and (ii) the sulfate-reducing phenotype is currently restricted to the genus *Archaeoglobus* within the archaeal domain. Further support for a lateral transfer of the *dsrAB* genes to the *Archaeoglobales* was obtained by a phylogenetic analysis on an alignment of the alpha- against the beta-subunit amino acid sequences. Such analysis can be used to root the Dsr subunit trees (7,14, Fig. 2), since the subunits are paralogs arising from an ancestral *dsr* gene duplication (3). Independent of the treeing method used, the root was consistently indicated between the DsrAB of the *Thermodesulfovibrio* species and the DsrABs of all other analyzed SRPs including the *Archaeoglobales*. This is inconsistent with the 16S rRNA phylogeny and points to a bacterial origin of the *Archaeoglobales dsrAB* genes (c in the DsrAB tree; Fig. 1). However, the results from the paralogous rooting should not be overemphasized since the alignment of the Dsr

subunits against each other is (i) relatively short (173 amino acid positions) and (ii) contains several regions which can not unambiguously be aligned (caused by the relatively low sequence similarities of the subunits to each other). Furthermore, no evidence for lateral transfer of the *Archaeoglobus fulgidus dsrAB* genes was indicated by atypical sequence characteristic analysis (28, Jeffrey Lawrence pers. comm.) suggesting that, if the genes are xenologs, they have completely ameliorated towards their host genome and were the result of an ancient LGT event.

DSR donor lineages. The *dsrAB* gene donors were members of at least two distinct evolutionary lineages within the δ-*Proteobacteria* (a1-a4 & b in the DsrAB tree; Fig. 1). Donor lineage a contributed *dsrAB* genes to two phylogenetically remote groups of bacteria, *Desulfobacula toluolica* (δ-*Proteobacteria*) and several *Desulfotomaculum* species (Low G+C Gram positives) (a1-a4 in the 16S rDNA tree; Fig. 1), suggesting that this lineage is particularly adept at donating *dsrAB* and possibly other genes. The specific identities of the donor lineages is unknown based on the current data, since no orthologous *dsrAB* genes were identified within the putative xenolog groups. It is however striking that *Desulfobacula toluolica* and all but two *Desulfotomaculum* species which received the xenologous *dsrAB* are oxidizing their characteristic substrates completely to CO_2 while the "authentic" *Desulfotomaculum* and *Desulfitobacterium* species are exclusively incomplete oxidizers. One possible explanation for this feature is that the *dsrAB* donor was a complete oxidizer which bestowed this metabolic capability to the *Desulfotomaculum* species and *Desulfobacula*, which subsequently was lost in *Desulfotomaculum thermosapovorans* and *Desulfotomaculum thermocisternum* (Tab. 1). Furthermore, most of the recipients of xenologous *dsrAB* are thermophilic (Tab. 1) which could indicate a thermophilic lifestyle of the donor species.

DSR recipient lineages. *Desulfobacula toluolica* is the recipient of the most recent putative LGT event so far identified (a1; Fig. 1) since its close relatives,

Desulfobacter latus and *Desulfobacter vibrioformis,* contain orthologous *dsrAB* genes. This is also supported by no identifiable amelioration of the 3rd codon position G+C content of the xenologous *dsr* genes towards the mean G+C content of the host *D. toluolica* genome (Tab. 1). The evolution of the genus *Desulfotomaculum* was affected by LGT events of *dsrAB* genes, too. The number of LGT events within this genus is difficult to predict since the sub-clustering of its members is not always well supported in the 16S rDNA tree (Fig. 1). Based on the presented 16S rDNA tree it is most parsimonious to postulate at least three LGT events within this genus (a2-a4 in the 16S rDNA tree of Fig. 1). Alternatively, one could hypothesize that a single lateral *dsrAB* gene transfer event occurred to the common ancestor of the genera *Desulfotomaculum, Desulfosporosinus,* and *Desulfitobacterium* (which did not displace the orthologous *dsr* genes) followed by a subsequent xenolog gene loss on at least two independent occasions from the ancestors of the "authentic" *Desulfotomaculum,* and *Desulfosporosinus/Desulfitobacterium* species, respectively.

In conclusion this study demonstrates that the genes encoding the dissimilatory sulfite reductase are subject to frequent LGT events within and across bacterial divisions and possibly even between the bacterial and archaeal domains. This finding was unexpected since the dissimilatory sulfite reductase represents an essential enzyme for anaerobic sulfate- and sulfite-respiration which acts (at least for the SRPs) in concert with other enzymes. One possible explanation for the observed widespread lateral distribution of dissimilatory sulfite reductases could be that the genes encoding this enzyme are part of a mobilizable metabolic island similar to the genes required for anaerobic nitrate respiration of *Thermus thermophilus* (31). More generally our findings add to the accumulating evidence that lateral gene transfer is a potent mechanism shaping the composition of prokaryotic genomes (e.g. 6,8,20,2122,27,32). On the other hand our data also demonstrate that the DsrAB phylogeny of most SRPs analyzed is still consistent

with the 16S rRNA phylogeny. This observation and the paralogous rooting of the Dsr tree still support an early and thermophilic origin of sulfate respiration.

The use of functional genes including *dsrAB* as molecular markers for defined physiological groups of bacteria has become increasingly popular in investigations of complex microbial communities (e.g. 1,23,30,39). If the functional genes are exploited for phylogenetic analysis of the respective bacteria, lateral gene transfer can complicate the interpretation. This has previously been demonstrated for the *nifH* gene encoding the nitrogenase reductase of nitrogen fixing bacteria (12) and was shown here to also hold true for the dissimilatory sulfite reductase genes. Therefore, the phylogenetic DsrAB framework established in our study provides an essential basis to better interpret environmental diversity surveys of SRPs based on comparative DsrAB sequence analysis.

ACKNOWLEDGMENTS

MK and HA were supported by a grant from the Deutsche Forschungsgemeinschaft to MW in the framework of the project “Degradation of marine pollutants by cyanobacterial mats – an interdisciplinary approach”. MF was supported by the Max-Planck-Gesellschaft. We wish to thank Bianca Wagner (Marburg, Germany) for excellent technical assistance. AJR was funded by NSERC grant: 2277085-00. DAS and SF were supported by NSF grant DEB-9714303 PH is funded by the Cooperative Research Centre for Waste Management and Pollution Control Ltd., a center established and supported under the Australian Government's Cooperative Research Centres Program. We thank Kathrin Riedel for helpful discussions.

REFERENCES

1.**Cottrell, M. T., and S. C. Cary.** 1999. Diversity of dissimilatory bisulfite reductase genes of bacteria associated with the deep-sea hydrothermal vent polychaete annelid *Alvinella pompejana*. Appl. Environ. Microbiol. **65:**1127-1132.

2.**Crane, B. R., L. M. Siegel, and E. D. Getzoff.** 1995. Sulfite reductase structure at 1.6 A: Evolution and catalysis for reduction of inorganic anions. Science. **270**(6)**:**59-67.

3.**Dahl, C., N. M. Kredich, R. Deutzmann, and H. G. Trüper.** 1993. Dissimilatory sulfite reductase form *Archaeoglobus fulgidus*: physico-chemical properties of the enzyme and cloning, sequencing and analysis of the reductase gene. J. Gen. Microbiol. **139:**1817-1828.

4.**Fauque, G., A. R. Lino, M. Czechowski, L. Kang, D. V. DerVartanian, J. J. Moura, J. LeGall, and I. Moura.** 1990. Purification and characterization of bisulfite reductase (desulfofuscidin) from *Desulfovibrio thermophilus* and its complexes with exogenous ligands. Biochim. Biophys. Acta. **1040**(1)**:**112-8.

5.**Felsenstein, J.** 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle

6.**Garcia-Vallve, S., A. Romeu, and J. Palau.** 2000. Horizontal gene transfer in bacterial and archaeal complete genomes. Genome Res. **10**(11)**:**1719-25.

7.**Gogarten, J. P., H. Kibak, P. Dittrich, L. Taiz, E. J. Bowman, B. Bowman, M. Manolson, R. Poole, T. Date, T. Oshima, J. Konishi, K. Denda, M. Yoshida.** 1989. Evolution of the vacuolar H^+-ATPase: implications for the origin of eukaryotes. Proc. Natl. Acad. Sci. USA. **86**(17)**:**6661-5.

8.**Goldman, B. S., and R. G. Kranz.** 1998. Evolution and horizontal transfer of an entire biosynthetic pathway for cytochrome c biogenesis: *Helicobacter*, *Deinococcus*, *Archae* and more. Mol. Microbiol. **27**(4)**:**871-3.

9.**Hatchikian, E. C., and J. G. Zeikus.** 1983. Characterization of a new type of dissimilatory sulfite reductase present in *Thermodesulfobacterium commune*. J. Bacteriol. **153**(3)**:**1211-20.

10. **Henckel, T., M. Friedrich, and R. Conrad.** 1999. Molecular analyses of the methane-oxidizing microbial community in rice field soil by targeting the genes of the 16S rRNA, particulate methane monooxygenase, and methanol dehydrogenase. Appl. Environ. Microbiol. **65:**1980-90.

11. **Hipp, W. M., A. S. Pott, N. Thum-Schmitz, I. Faath, C. Dahl, and H. G. Trüper.** 1997. Towards the phylogeny of APS reductases and sirohaem sulfite reductases in sulfate-reducing and sulfur-oxidizing prokaryotes. Microbiology. **143:**2891-2902.

12. **Hurek, T., T. Egener, and B. Reinhold-Hurek.** 1997. Divergence in nitrogenases of *Azoarcus* spp., *Proteobacteria* of the beta subclass. J. Bacteriol. **179**(13)**:**4172-4178.

13. **Imhoff, J. F., J. Suling, and R. Petri.** 1998. Phylogenetic relationships among the *Chromatiaceae*, their taxonomic reclassification and description of the new genera *Allochromatium*, *Halochromatium*, *Isochromatium*, *Marichromatium*, *Thiococcus*, *Thiohalocapsa* and *Thermochromatium*. Int. J. Syst. Bacteriol. **48 Pt 4**(12)**:**1129-43.

14. **Iwabe, N., K. Kuma, M. Hasegawa, S. Osawa, and T. Miyata.** 1989. Evolutionary relationship of archaebacteria, eubacteria, and eukaryotes inferred from phylogenetic trees of duplicated genes. Proc. Natl. Acad. Sci. USA. **86**(23)**:**9355-9.

15. **Juretschko, S., G. Timmermann, M. Schmid, K. H. Schleifer, A. Pommerening-Roser, H. P. Koops, and M. Wagner.** 1998. Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. Appl. Environ. Microbiol. **64**(8)**:**3042-51.

16. **Karkhoff-Schweizer, R. R., D. P. W. Huber, and G. Voordouw.** 1995. Conservation of the genes for dissimilatory sulfite reductase from *Desulfovibrio vulgaris* and *Archaeoglobus fulgidus* allows their detection by PCR. Appl. Environ. Microbiol. **61:**290-296.

17. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115-175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic Acid Techniques in Bacterial Systematics. John Wiley & Sons, New York.

18. **Larsen, O., T. Lien, and N.-K. Birkeland.** 2000. Characterization of the desulforubidin operons from *Desulfbacter vibrioformis* and *Desulfobulbus rhabdoformis*. FEMS Microbiol. Lett. **186:**41-46.

19. **Larsen, O., T. Lien, and N.-K. Birkeland.** 1999. Dissimilatory sulfite reductase from *Archaeglobus profundus* and *Desulfotomaculum thermocisternum*: phylogenetic and structural implications from gene sequences. Extremophiles. **3:**63-70.

20. **Lawrence, J. G.** 1999. Gene transfer, speciation, and the evolution of bacterial genomes. Curr. Opin. Microbiol. **2:**519-523.

21. **Lawrence, J. G., and H. Ochman.** 1997. Amelioration of bacterial genomes: rates of change and exchange. J. Mol. Evol. **44:**383-397

22. **Logsdon, J. M., and D. M. Faguy.** 1999. *Thermotoga* heats up lateral gene transfer. Curr. Biol. **9**(19)**:**R747-51.

23. **Minz, D., J. L. Flax, S. J. Green, G. Muyzer, Y. Cohen, M. Wagner, B. E. Rittmann, and D. A. Stahl.** 1999. Diversity of sulfate-reducing bacteria in oxic and anoxic regions of a microbial mat characterized by comparative analysis of dissimilatory(bi-) sulfite reductase genes. Appl. Environ. Microbiol. **65:**4666-4671.

24. **Molitor, M., C. Dahl, I. Molitor, U. Schäfer, N. Speich, R. Huber, R. Deutzmann, and H. G. Trüper.** 1998. A dissimilatory sirohaem-sulfite-reductase-type protein from the hyperthermophilic archaeon *Pyrobaculum islandicum*. Microbiology. **144:**529-541.

25. **More, M. I., J. B. Herrick, M. C. Silva, W. C. Ghiorse, and E. L. Madsen.** 1994. Quantitative cell lysis of indigenous microorganisms and rapid extraction of microbial DNA from sediment. Appl. Environ. Microbiol. **60**(5)**:**1572-80.

26. **Morse, R., G. R. Gibson, and M. D. Collins.** 2000. Secondary structure analysis of the dissimilatory sulphite reductase in *Desulfovibrio desulfuricans*. Lett. Appl. Microbiol. **30**(5)**:**375-8.

27. **Nelson, K. E., R. A. Clayton, S. R. Gill, M. L. Gwinn, R. J. Dodson, D. H. Haft, E. K. Hickey, J. D. Peterson, W. C. Nelson, K. A. Ketchum, L. McDonald, T. R. Utterback, J. A. Malek, K. D. Linher, M. M. Garrett, A.

M. Stewart, M. D. Cotton, M. S. Pratt, C. A. Phillips, D. Richardson, J. Heidelberg, G. G. Sutton, R. D. Fleischmann, J. A. Eisen, O. White, S. L. Salzberg, H. O. Smith, J. C. Venter and C. M. Fraser. 1999. Evidence for lateral gene transfer between *Archaea* and bacteria from genome sequence of *Thermotoga maritima*. Nature. **399**(6734)**:**323-9.

28. **Ochman, H., S. Elwyn, and N. A. Moran.** 1999. Calibrating bacterial evolution. Proc. Natl. Acad. Sci. USA. **96**(22)**:**12638-12643.
29. **Olsen, G. J., Matsuda, H., Hagstrom, R., and Overbeek, R** 1994. fastDNAml: A tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. Comput. Appl. Biosci. **10**: 41-48
30. **Purkhold, U., A. Pommerening-Roser, S. Juretschko, M. C. Schmid, H. P. Koops, and M. Wagner.** 2000. Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: implications for molecular diversity surveys. Appl. Environ. Microbiol. **66**(12)**:**5368-82.
31. **Ramirez-Arcos, S., L. A. Fernandez-Herrero, I. Marin, and J. Bergenguer.** 1998. Anaerobic growth, a property horizontally transferred by an Hfr-like mechanism among extreme thermophiles. J.Bacteriol. **180**(12)**:**3137-3143.
32. **Sassanfar, M., J. E. Kranz, P. Gallant, P. Schimmel, and K. Shiba.** 1996. A eubacterial *Mycobacterium tuberculosis* tRNA synthetase is eukaryote-like and resistant to a eubacterial-specific antisynthetase drug. Biochemistry. **35**(31)**:**9995-10003.
33. **Schedel, M., and H. G. Trüper.** 1979. Purification of *Thiobacillus denitrificans* siroheme sulfite reductase and investigation of some molecular and catalytic properties. Biochim Biophys. Acta. **568**(2)**:**454-66.
34. **Stackebrandt, E., C. Sproer, F. A. Rainey, J. Burghardt, O. Päuker, and H. Hippe.** 1997. Phylogenetic analysis of the genus *Desulfotomaculum*: evidence for misclassification of *Desulfotomaculum guttoideum* and description of *Desulfotomaculum orientis* as *Desulfosporosinus orientis* gen. nov., comb. nov. Int. J. Syst. Evol. Bacteriol. **47**(4)**:**1134-1139.
35. **Swofford, D. L.** 2001. *PAUP*: Phylogenetic Analysis Using Parsimony (*And Other Methods). Version 4*. Sinauer Associates, Sunderland, MA.
36. **Utkin, I., C. Woese, and J. Wiegel.** 1994. Isolation and characterization of *Desulfitobacterium dehalogenans* gen. nov., sp. nov., an anaerobic bacterium which reductively dechlorinates chlorophenolic compounds. Int. J. Syst. Evol. Bacteriol. **44:**612-619.
37. **Wagner, M., A. J. Roger, J. L. Flax, G. A. Brusseau, and D. A. Stahl.** 1998. Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration. Appl. Environ. Microbiol. **180:**2975-2982.
38. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221-271.
39. **Zani, S., M. T. Mellon, J. L. Collier, and J. P. Zehr.** 2000. Expression of *nifH* genes in natural microbial assemblages in Lake George, New York, detected by reverse transcriptase PCR. Appl. Environ. Microbiol. **66**(7)**:**3119-24.

FIGURES AND FIGURE LEGENDS

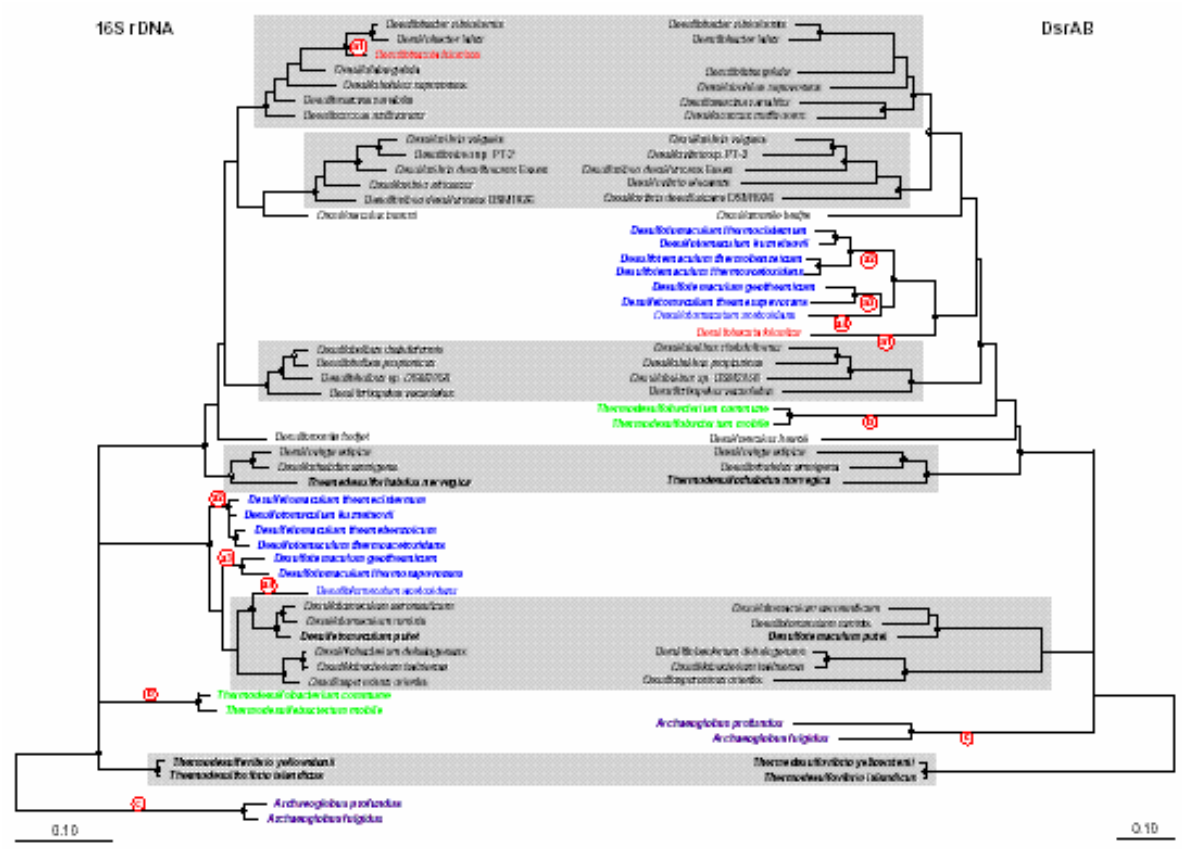


Figure 1. Comparison of 16S rRNA (ML) and DsrAB (ED) trees for the sulfate- and sulfite-reducing prokaryotes investigated. Branch points supported by phylogenetic analysis (bootstrap support >90% in all ED and MP methods) are indicated by filled circles. Open circles at branch points indicate >75% bootstrap support in most or all analyses, while branch points without circles were not resolved (bootstrap values <75%) as specific groups in the different analyses. Both trees are collapsed back at the division level. Thermophilic prokaryotes are bolded. Consistent monophyletic groups between both trees are grey-shaded. Microorganisms affected by putative LGT events of the *dsrAB* genes are color coded. *DsrAB* recipient and donor lineages are indicated by circled letters (a-c)

sitting above or below the branch, respectively. The bars represent 0.1 changes per nucleotide/amino acid, respectively.

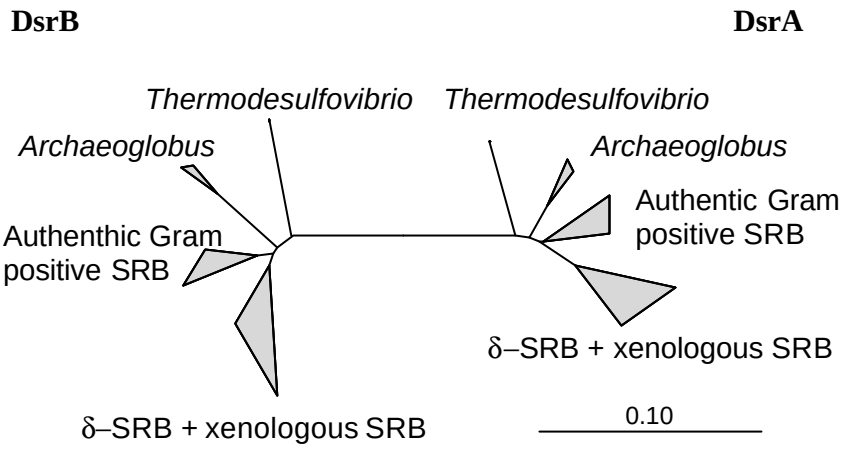


Figure 2. Unrooted amino acid tree (ED) based on an alignment of DsrA to DsrB. The dissimilatory sulfite reductases of *Allochromatium vinosum* and *Pyrobaculum islandicum* were excluded from the analysis since they likely are members of different enzyme families. (10, 23). The bar represents 0.1 changes per amino acid. Bootstrap analysis were performed using the Phylip parsimony method with 100 resamplings (5). Branch points with parsimony bootstrap support >85% are indicated by filled circles. Open circles at branch points indicate >50% bootstrap support, while branch points without circles either have parsimony bootstrap values <50% (authentic Gram positive SRB DsrB; δ-SRB+xenologous SRB DsrB) or are not obtained with the parsimony method (*Archaeoglobus* and authentic Gram positive SRB DsrA sequences form a monophyletic cluster in the parsimony method).

Organism	Amino acid positions (according to *Desulfovibrio vulgaris*)		
	256 DsrA 276	119 DsrB 131	238 DsrB 249
δ-*Proteobacteria*-like			
D. propionicus	ANDSEYPSNAGAHKGRDWGKFDI	KSH-----GTTFP	PAKATNSAGEEVK
D.amnigena	GGE--LVPHAGAG-GTEKRAFDI	RSR-----GNWFP	PKKVDD-----KK
T. norvegica	AGE--LVPNGGAH-GFEKRPLDI	KSR-----GNWFP	PKKVEI-DGKEYK
D. vulgaris	AGE--FKPNAGAHSGRDWGKFDI	ASRKFDGGSLKFP	PTKLEIGD-KKVN
D. variabilis	GGE--FAPNGGAHSGRNWGAFDI	ESRKFDGGSFKFP	PAKVTVGD-KELK
D. vibrioformis	ENDPAYPANAGAHKGKDWGPFDI	ASRKFPGGSLKFP	PAKVTLPNGTEVK
**D. toluolica*	GGE--IAPNAGAHARKDWGAFDI	NSRKHVTGSYKFP	PTKTES----GKK
**D.acetoxidans*	NGE--YAPNAGAHAGKDWGKFDI	NSRKFVSGSFKFP	PDKTAD----GKK
**D. thermosapovorans*	AGD--VVPNGGAHKGRDWGKFDI	ESRKFVSGSYKFP	PDKTPE----GGK
**D. geothermicum*	AGE--YVPNAGAHAGRDWGKFDI	QSRKFVGGSYKFP	PDKTPE----GKK
**D. thermocisternum*	AGD--IVPRGGAHRGRDWGKFDI	WSRKFVSGSYKFP	PDKTPE----GRR
**D. kuznetsovii*	AGD--IVPRGGSHKGRDWGKFDI	WSRKFVSGSYKFP	PDKTPE----GRR
**D. thermobenzoicum*	AGD--IVPRGGAQKGRDWGKFDI	AGRKFASGSYKFP	PDKTPE----GRR
**D. thermoacetoxidans*	AGD--IVPRGGARR-QDWGKFDI	AGRKFASGSYKFP	PDKTPE----GRR
**T. commune*	RGE--LKPNAGAFSDRDWGPFDI	KNRKHPNGSYKFS	PA-TAEVGGKKKK
**T. mobile*	RGE--LKPNAGAFSDRDWGPFDI	KNRKHPNGSYKFP	PA-TAEVDGKKKK
Low G+C Gram positives			
D. orientis	SEG----------FDI	DAK-------GLR	PDP-------KNK
D. aeronauticum	ADG----------LDI	KAM-------GVP	PNP-------KLK
D. putei	AEG----------FDI	NAM-------GVP	PNP-------KLK
D. ruminis	AAG----------FDI	QER-------GIP	PNP-------KLK
D. hafniense	AEG----------LNI	GAK-------GLP	PNP-------KEK
D. dehalogenans	AEV----------XNI	GAK-------GLP	PNP-------KEN
***Nitrospira* division**			
T. yellowstonii	KKG----------MNI	KAK-------KYM	PDP-------AKK
T. islandicus	KKG----------MNI	KAK-------KYM	PDP-------AKK
Archaea			
A. profundus	KAG----------VDI	QEK------VGFP	PDM-------KRK
A. fulgidus	SW----------MDI	QER------VGFP	PDM-------KNK

Figure 3. Amino acid alignment of DsrA and DsrB showing insertions supporting the δ-proteobacterial origin of the putative laterally transferred sulfite reductases (labeled with an asterix). It should be noted that the presumably xenologous DsrA and DsrB of *Archaeoglobus* do not show the typical δ-proteobacterial insertions.

G.2.2 Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation

The paper describes the identification of novel anaerobic ammonia oxidizing bacteria (ANAMOX) and classical "aerobic" ammonia oxidizing bacteria in a trickling filter biofilm. During this investigation Michael Klein optimized and applied a gelretardation method for the recovery of PCR fragments amplified from the functional gene ammonia-mono oxigenase (*amoA*) from the environmental sample. This optimized method was subsequently combined with the *dsrAB* approach on Mariager Fjord water column samples.

This chapter was published in the Journal of Systematic and Applied Microbiology in 2000 (Schmid 2000).

System. Appl. Microbiol. 23, 93–106 (2000)
© Urban & Fischer Verlag
http://www.urbanfischer.de/journals/sam

SYSTEMATIC AND APPLIED MICROBIOLOGY

Molecular Evidence for Genus Level Diversity of Bacteria Capable of Catalyzing Anaerobic Ammonium Oxidation

MARKUS SCHMID[1], ULF TWACHTMANN[2], MICHAEL KLEIN[1], MARC STROUS[3], STEFAN JURETSCHKO[3], MIKE JETTEN[3], JÖRG W. METZGER[2], KARL-HEINZ SCHLEIFER[1], and MICHAEL WAGNER[1*]

[1] Lehrstuhl für Mikrobiologie, Technische Universität München, Freising, Germany
[2] Institut für Siedlungswasserbau, Wassergüte- und Abfallwirtschaft, Lehrstuhl für Hydrochemie und Hydrobiologie, Universität Stuttgart, Germany
[3] Department of Biotechnology, Delft University of Technology, Delft, The Netherlands

Received December 21, 1999

Summary

Recently, a bacterium capable to oxidize ammonium anaerobically at a high rate was identified as novel member of the *Planctomycetales* (STROUS, M., FUERST, J. A., KRAMER, E. H. M., LOGEMANN, S., MUYZER, G., VAN DE PAS-SCHOONEN, K. T., WEBB, R. I., KUENEN, J. G., and JETTEN, M. S. M.: Nature 400, 446–449, 1999). Here we investigated the microbial community structure of a trickling filter biofilm with a high anaerobic ammonium oxidation activity. Fluorescence *in situ* hybridization (FISH) with a set of nine probes designed for specific identification of the recently described anaerobic ammonium oxidizer demonstrated that only one probe hybridized to bacteria within the biofilm. For phylogenetic characterization of putative biofilm anaerobic ammonium oxidizers a full-cycle 16S rDNA approach was performed by using a *Planctomycetales*-specific forward primer for PCR amplification. Of the twenty-five 16S rDNA fragments (1364 bp in length) amplified from the biofilm, nine were affiliated to the *Planctomycetales*. Comparative analysis showed that these sequences were more than 98.9% similar to each other but only distantly related to the previously recognized anaerobic ammonium oxidizer (below 91% similarity) and all other organisms represented in public 16S rRNA databases (similarities of below 79%). The retrieved sequences and the previously recognized anaerobic ammonium oxidizer represent two well-separated groups of a deep-branching lineage within the *Planctomycetales*. Quantitative FISH analysis with a newly designed specific probe showed that the novel bacterium, provisionally classified as "*Candidatus* Kuenenia stuttgartiensis" constituted the dominant fraction of the biofilm bacteria. *In situ* probing revealed that ammonia-oxidizing bacteria of the beta-subclass of Proteobacteria were also present, albeit in significant smaller amounts, within the anoxic biofilm. Comparative sequence analysis of a stretch of the gene encoding ammonia-monooxygenase (*amoA*) demonstrated the occurrence of the DNA of at least three different populations of beta-subclass ammonia oxidizers within the biofilm.

Key words: Anaerobic ammonium oxidation – diversity of *Planctomycetales* – beta-subclass ammonia oxidizers – trickling filter – nitrogen removal – biofilm – quantitative fluorescence in situ hybridization

Introduction

Twenty-two years after Broda's remarkable theoretical consideration that lithotrophic microorganisms which exclusively gain energy by using ammonium as inorganic electron donor for denitrification should be able to exist in nature (BRODA, 1977), a novel member of the *Planctomycetales* capable of ammonium oxidation with nitrite as the electron acceptor under anoxic conditions was identified (STROUS et al., 1999). In addition to this autotrophic yet uncultured organism, anaerobic ammonium-oxidizing activity has been reported for some of the classical "aerobic" ammonia oxidizers of the beta-subclass of *Proteobacteria* (BOCK et al., 1995; DE BRUIJN et al., 1995; KUAI and VERSTRAETE, 1998; POTH, 1986; POTH and FOCHT, 1995; SCHMIDT and BOCK, 1997). However, the anaerobic ammonium oxidation rate measured for enrichments of the novel planctomycete is more than twenty times higher than for pure cultures of the beta-subclass ammonia oxidizers (JETTEN et al., 1999).

Combined with recently developed nitrification systems which transform ammonium to nitrite with only

0723-2020/00/23/01-093 $ 12.00/0

minor nitrate formation (ABELING and SEYFRIED, 1992; HELLINGA et al., 1998; LOGEMANN et al., 1998) the process of anaerobic ammonium oxidation allows for efficient denitrification in the absence of available organic carbon sources. Thus, implementation of this combined process appears to be a promising alternative for treatment of sewage with low C/N ratios in waste water treatment plants. Here we present data on the microbial community structure of an anoxic (anaerobic in the presence of nitrite) trickling filter biofilm showing high rates of anaerobic ammonium oxidation. A novel member of the *Planctomycetales* (FUERST, 1995) which is distantly related to the previously recognized anaerobic ammonium oxidizer (STROUS et al., 1999) was shown to dominate the biofilm by applying the full-cycle rRNA approach. Fluorescence in situ hybridization (FISH) with rRNA-targeted probes demonstrated that beta-subclass ammonia oxidizers represented 27% of the area of those bacteria detectable by FISH within this biofilm. *AmoA* gene fragments retrieved from the biofilm clustered together with the *Nitrosomonas europaea*-lineage and *Nitrosococcus mobilis*, respectively.

Materials and Methods

Reactor operation and chemical analysis

A two-stage semi-technical trickling filter reactor system for the treatment of effluent from anaerobic sludge digestion is maintained at the Institute for Sanitary Engineering, Water Quality and Solid Waste Management at the University of Stuttgart. A scheme of the reactor system and important parameters of the trickling filters are presented in Figure 1. The plant was designed for efficient nitrogen removal from the sludge liquor (typically containing between 500–2500 mg NH_4^+-N l^{-1}) via coupled partial aerobic nitrification (nitritation) and anaerobic ammonium oxidation. The ammonium conversion rate of trickling filter 1 is regulated via the influent load of ammonium so that 60% of the ammonium is oxidized to nitrite. The resulting ratio of 1 : 1.3 of ammonium to nitrite in the effluent of trickling filter 1 is optimal for the subsequent anaerobic ammonium oxidation process in trickling filter 2 (STROUS et al., 1998; VAN DE GRAAF et al., 1996; VAN DE GRAAF et al., 1997). Nitrite oxidation to nitrate by nitrite oxidizers does not occur in trickling filter 1 most likely due to ammonia-inhibition. The effluent of trickling filter 1 containing a mixture of ammonium and nitrite is used as influent for the anoxic trickling filter 2 designed for anaerobic ammonium oxidation. The key parameters of trickling filter 2 are given in Table 1. The concentrations of ammonium, nitrite and nitrate in the influent and effluent of trickling filter 2 were determined photometrically (Merck, Spectroquant Darmstadt). Total nitrogen bounded (TNb; encompassing all inorganic and organic nitrogen compounds) was measured by a high temperature digestion process with subsequent chemiluminescence detection.

Biofilm sampling

In December 1998 and April 1999, biofilm samples were removed together with their plastic support material (NOR-PAC, Norddeutsche Seekabelwerke GmbH, Nordenham, Germany) from trickling filter 2 of the semitechnical plant (Figure 1). Several pieces of the support material were immediately immersed in a fixative containing 4% (w/v) paraformaldehyde (4) and stored at 4 °C for 8h. Subsequently, the biofilm was detached from its support material by gently mixing, washed with PBS (130 mM sodium chloride, 10 mM sodium phosphate, pH 7.2), transferred into 50% (v/v) PBS/EtOH and stored at –20 °C until use. Several other pieces of support material were transported on dry ice to the lab and stored at –20 °C for subsequent nucleic acid extraction. After sampling trickling filter 2 was rinsed with dinitrogen gas to reestablish anoxic conditions.

DNA extraction from biofilm

Biofilm material was removed from the plastic support by gently mixing in 10 ml of DNA extraction buffer (100 mM Tris/HCl [pH 8.0]; 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, 1% CTAB) (ZHOU et al., 1996). The plastic support was removed and the biofilm material was pelleted by centrifugation for 10 min at 5000 g (Hettich, Tuttlingen, Germany). Total genomic DNA was extracted as described previously for activated sludge by JURETSCHKO et al. (1998).

PCR amplification of 16S rDNA

For preferential PCR amplification of 16S rDNA of members of the *Planctomycetales* the recently published phylum-specific probe Pla46 (*E. coli* positions 46–63; NEEF et al., 1998) was used as unlabeled derivative as forward primer in combination with the universal reverse primer 1390R (*E. coli* positions 1390–1407; ZHENG et al., 1996). PCR was performed in a 96 micro well plate (Biorad, München, Germany) with the gradient cycler (Eppendorf, Hamburg, Germany). Reaction mixtures were prepared in a total volume of 50 µl containing 2 mM $MgCl_2$, 10 nmol of each deoxynucleoside triphosphate, 15 pmol of each primer, 100 ng of template DNA and 1.5 U of Taq DNA polymerase (Promega, Madison). Thermal cycling was carried out with an initial denaturation of 4 min at 94°C, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at different temperatures (see below) for 50 s, and elongation at 72 °C for 3 min. Cycling was completed by a final elongation step at 72 °C for 10 min. Negative controls (no DNA added) were included in all sets of amplifications. Optimum annealing temperature for the used primer set in combination with the DNA retrieved from the biofilm was determined by using the annealing temperature gradient function of the thermal cycler. 12 different annealing temperatures between 44 and 64 °C were tested. The presence and size of amplification products were determined by agarose (1%) gel electrophoresis of 5 µl aliquots of the PCR products.

PCR amplification of the *amoA* gene fragment

For PCR amplification of a stretch of the *amoA* gene the primer set amoA-1F and amoA-2R (ROTTHAUWE et al., 1997) was used. Amplification was performed in a total volume of

Table 1. Key parameters of trickling filter 2 during the sampling period.

O_2 [mg l^{-1}]	Temp. [°C]	pH	TOC (Infl. and Effl.) [mg l^{-1}]	$Q_{Influent}$ [l d^{-1}]	$Q_{Recycling}$ [l d^{-1}]
0.00–0.08	23–26	7.7–8.4	5–10	42–55	200–370

50 µl with a thermal capillary cycler (Idaho Technology, Idaho Falls). Reactions contained 2 mM $MgCl_2$ (Idaho Technology), 1× BSA (Idaho Technology), 10 nmol of each deoxynucleoside triphosphate, 15 pmol of each primer, 100 ng of template DNA and 1.5 U of Taq DNA polymerase (Promega). The thermal cycling profile used for amplification was as follows: 30 s initial denaturation at 94 °C; then 30 cycles consisting of 15 s at 94 °C for denaturing, 20 s annealing at 55 °C, 40 s at 72 °C for elongation, and a final elongation for 3 min at 72 °C. Negative controls (no DNA added) were included in all sets of amplifications. The presence and size of amplification products were determined by agarose (1%) gel electrophoresis of 5 µl aliquots of the PCR products.

Gel retardation of *amo*A amplificates

The gel retardation of amplified *amo*A fragments was performed in a Hoefer HE33 submarine gel electrophoresis unit (Pharmacia Biotech, Freiburg, Germany) with cooled base using a modification of the protocol published by Wawer et al., 1995. The 2% agarose gel consisted of 35 ml 0.5× TBE (0.0445 M Tris/HCl, 0.0445 M boric acid, 0.001 M EDTA, pH 8.3) and 0.7 g Nusieve 3:1 agarose. After cooling down the agarose mixture to 70 °C, 35 µl of the DNA ligand bisbenzimide, to which long chains of polyethylene glycol 6000 are covalently coupled (Hanse Analytik Yellow; Hanse Analytik, Bremen, Germany), was added to the gel. The dye bisbenzimide binds preferentially to A + T rich sequence motifs in the DNA and retards them

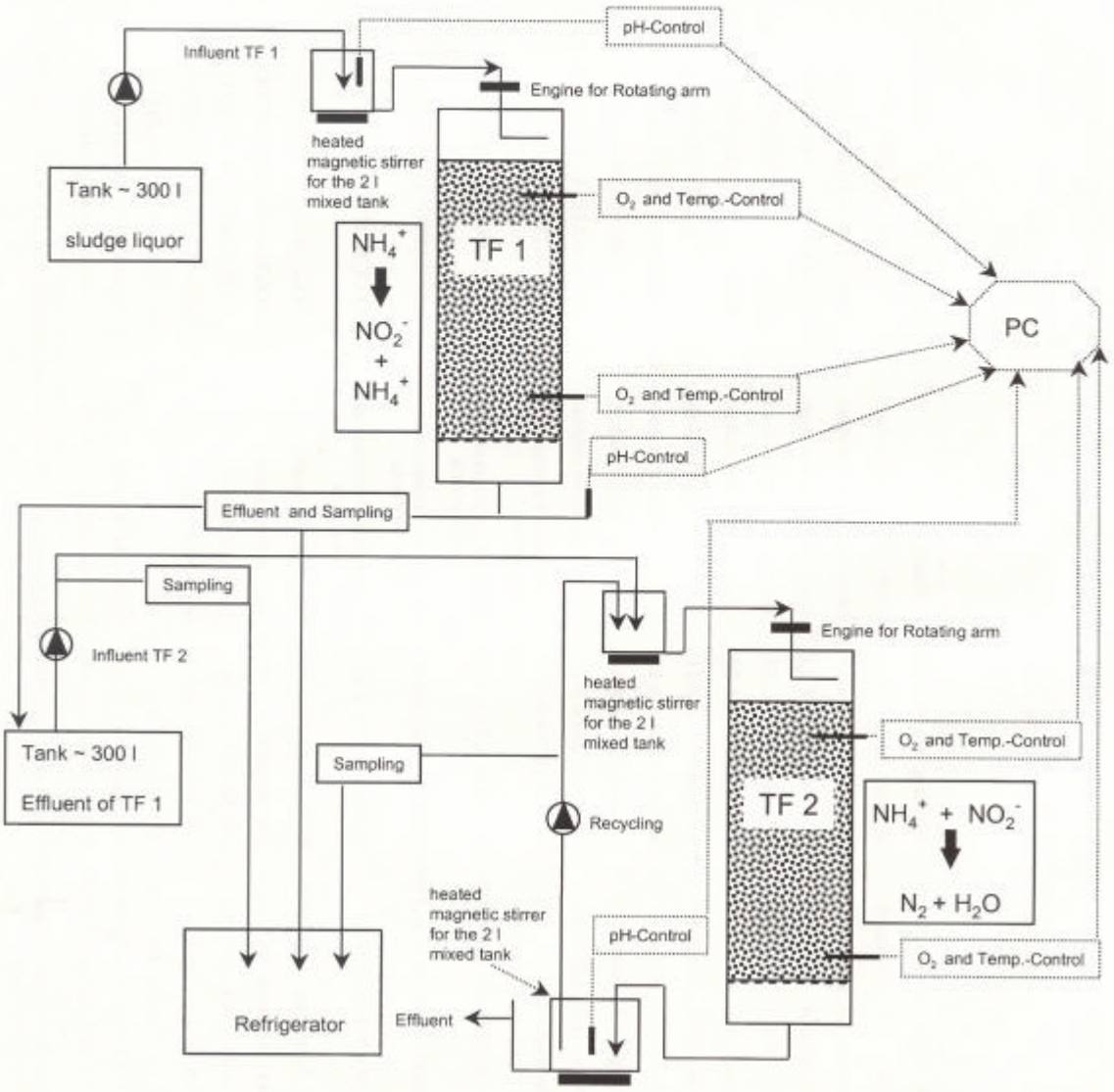


Fig. 1. Scheme of the semi-technical scale plant at the University of Stuttgart.

Table 2. Probe sequences, target sites, formamide concentrations in the hybridization buffer required for specific *in situ* hybridization and observed probe hybridization with the trickling filter 2 biofilm.

Trivial name (reference)	OPD[a] designation	Specificity	Sequence 5'-3'	Target site[b]	% Formamide/ mM [NaCl]	Hybrid-ization with biofilm
Pla 46 (NEEF et al., 1998)	S-P-Planc-0046-a-A-18	*Planctomycetales*	GACTTGCATGCCTAATCC	46–63	25/159	+
– (STROUS et al., submitted)	S-*-Amx-0156-a-A-18	Anaer. ammonium oxidizer	CGGTAGCCCCAATTGCTT	156–173	40/56	–
– (STROUS et al., submitted)	S-*-Amx-0223-a-A-18	Anaer. ammonium oxidizer	GACATTGACCCCTCTCTG	223–240	40/56	–
– (STROUS et al., submitted)	S-*-Amx-0432-a-A-18	Anaer. ammonium oxidizer	CTTAACTCCCGACAGTGG	432–449	40/56	–
– (STROUS et al., submitted)	S-*-Amx-0613-a-A-22	Anaer. ammonium oxidizer	CCGCCATTCTTCCGTTAAGCGG	613–634	40/56	–
– (STROUS *et al.*, submitted)	S-*-Amx-0820-a-A-22	Anaer. ammonium oxidizer	AAAACCCCTCTACTTAGTGCCC	820–841	40/56	+
– (STROUS et al., submitted)	S-*-Amx-0997-a-A-21	Anaer. ammonium oxidizer	TTTCAGGTTTCTACTTCTACC	997–1017	20/225	–
– (STROUS et al., submitted)	S-*-Amx-1015-a-A-18	Anaer. ammonium oxidizer	GATACCGTTCGTCGCCCT	1015–1032	60/14	–
– (STROUS et al., submitted)	S-*-Amx-1154-a-A-18	Anaer. ammonium oxidizer	TCTTGACGACAGCAGTCT	1154–1171	20/225	–
– (STROUS et al., submitted)	S-*-Amx-1240-a-A-23	Anaer. ammonium oxidizer	TTTAGCATCCCTTTGTACCAACC	1240–1262	60/14	–
– (this study)	S-*-Kst-1275-a-A-20	"*Candidatus* Kuenenia stuttgartiensis"	TCGGCTTTATAGGTTTCGCA	1275–1294	25/159	+
Nso 190 (MOBARRY et al., 1996)	S-P-Betao-1225-a-A-19	Ammonia oxidizers of the beta- Proteobacteria	CGATCCCCTGCTTTTCTCC	190–208	55/20	+
Nso 1225 (MOBARRY et al., 1996)	S-P-Betao-1225-a-A-20	Ammonia oxidizers of the beta-Proteobacteria	CGCCATTGTATTACGTGTGA	1225–1244	35/80	+
Neu (WAGNER et al., 1995)	S-*-Neu-0653-a-A-18	Halophilic and halotolerant *Nitrosomonas* sp.	CCCCTCTGCTGCACTCTA	653–670	40/56	+
NmV (JURETSCHKO et al., 1998)	S-S-Ncmob-0174-a-A-18	*Nitrosococcus mobilis*	TCCTCAGAGACTACGCGG	174–191	35/80	–
Nsv 443 (MOBARRY et al., 1996)	S-F-Nsp-0444-a-A-19	*Nitrosospira*-cluster	CCGTGACCGTTTCGTTCCG	444–462	30/112	–
NIT 3 (WAGNER et al., 1996)	S-G-Nb-1035-a-A-18	genus *Nitrobacter*	CCTGTGCTCCATGCTCCG	1035–1048	35/80	–
– (DAIMS et al., in press)	S-G-Ntspa-0662-a-A-18	genus *Nitrospira*	GGAATTCCGCGCTCCTCT	662–679	35/80	–
Eub 338 (AMANN et al., 1990)	S-D-Bact-0338-a-A-18	*Bacteria*	GCTGCCTCCCGTAGGAGT	338–355	0/900	+
Eub 338 II (DAIMS et al., 1999)	S-D-Bact-0338-b-A-18	Refer to DAIMS et al., 1999	GCAGCCACCCGTAGGTGT	338–355	0/900	+
Eub 338 III (DAIMS et al., 1999)	S-D-Bact-0338-c-A-18	Refer to DAIMS et al., 1999	GCTGCCACCCGTAGGTGT	338–355	0/900	+

[a]Oligonucleotide probe database (ALM et al., 1996), [b]16S rRNA position, *E. coli* numbering (BROSIUS et al., 1978), [c]Formamid in the hybridization buffer and [NaCl] in the washing buffer, respectively.

compared to DNA sequences with low A+T content. After poring, the gel was solidified in the dark. The electrophoresis was performed applying a voltage of 100V for 80 min with 0.5× TBE as running buffer. After the run, the gel was stained with SYBR Green I (FMC BioProducts, Rockwell, US) nucleic acid stain (5 µl in 50 ml 0.5× TBE) for 1 h in the dark. The bands on the gel were visualized using UV illumination (364nm) and cut out with a capillary. The gel fragments were resuspended in 300 µl sterile H_2O for 10 min at 80 °C and reamplified using the PCR protocol described above.

Cloning and Sequencing

The biofilm-derived 16S rDNA and *amoA* PCR products were cloned directly by using the TOPO TA Cloning kit following the instructions of the manufacturer (Invitrogen, Groningen, The Netherlands). Plasmid-DNA was isolated with the Quiaprep spin miniprep kit (Quiagen, Hilden, Germany). Plasmids with an insert of the expected size were identified by agaraose (1.0%) gel electrophoresis after EcoRI digestion (5 U, Eco RI-buffer for 3 h at 37 °C). Sequencing was done nonradioactively by using the Thermo Sequenase fluorescent labeled primer cycle sequencing kit according to the instructions of the manufacturer (Amersham, Freiburg, Germany). The reaction mixtures were analyzed with an infrared automated DNA sequencer (model LiCor Longreadir DNA 4200, MWG - Biotech, Ebersberg, Germany). The complete sequences of the *amoA* fragments and the 16S rDNA fragments were determined by using M13 forward and reverse primers targeting vector sequences adjacent to the multiple cloning site.

Phylogenetic analysis

The 16S rDNA and *amoA* sequences retrieved in this study were added to the 16S rDNA and *amoA* sequence databases of the Technischen Universität München (currently encompassing more than 16.000 small subunit rRNA sequences and about 200 *amoA* sequences), respectively, by use of the ARB program package (STRUNK and LUDWIG, 1997). 16S rRNA sequences were aligned automatically using the respective tool of the ARB package. Subsequently, the alignments were corrected by visual inspection considering the secondary structure of the 16S rRNA. Deduced amino acid sequences for *amoA* were aligned manually by pooling the amino acids into six groups with the GDE 2.2 sequence editor implemented in the ARB software package. Nucleic acid sequences of the *amoA* gene fragments were then aligned in accordance with the amino acid alignment. Phylogenetic analysis of 16S rRNA sequences were performed by applying neighbor-joining, ARB parsimony and maximum likelihood analysis (fast DNAml, MAIDAK et al., 1996) to different data sets. Bootstrapping was performed using the PHYLIP parsimony tool (100x resampling) (Phylogeny Inference Package Version 3.5c, University of Washington, Seattle). Checks for chimeric sequences were conducted by independently subjecting the first 5' 454 base positions, the middle 455 base positions, or the last 454 3' base positions for phylogenetic analysis. The reconstruction of phylogenetic trees based on comparative analysis of the AmoA amino acid sequences was performed using protein maximum likelihood with the JTT-f amino acid replacement model (PROTML 2.2; ADACHI and HASEGAWA, 1996), Parsimony, and FITCH (using the Dayhoff PAM 001 matrix as the amino replacement model, randomized input order, and global rearrangements) methods (PHYLIP 3.5c; FELSENSTEIN, 1993) implemented in the ARB software package.

Probe design, fluorescence *in situ* hybridization, DAPI-staining, microscopy, and quantification of probe target bacteria

For the probes used in this study, sequences, target sites and optimal formamide concentrations in the hybridization buffers are displayed in Table 2. Probe S-*-Kst-1275-a-A-20 specific for the retrieved biofilm sequences related to the anaerobic ammonium oxidizer was designed using the probe design tool of the ARB package (Table 2). Probes were purchased as Cy3, Cy5 and 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) labeled derivatives from Interactiva (Ulm, Germany). Hybridizations were performed as described by AMANN (1995). Simultaneous hybridization with probes requiring different stringency was realized by a successive-hybridization procedure (WAGNER et al., 1994). Optimal hybridization conditions for probe S-*-Kst-1275-a-A-20 were determined by using the hybridization and wash buffers described by MANZ et al. (1992). An *in situ* probe dissociation curve was recorded by measuring the relative fluorescence intensity of biofilm bacteria after hybridization with probe S-*-Kst-1275-a-A-20 at different stringencies as described by DAIMS et al. (1999). Dual staining of cells with 4,6-diamidino-2-phenylindole (DAPI) and fluorescent oligonucleotides was performed as previously described (JURETSCHKO et al., 1998). Surprisingly, probe S-*-Kst-1275-a-A-20 positive bacteria did only show a very weak DAPI-conferred fluorescence. Thus, we post-stained biofilm material hybridized with Cy3-labeled probes with SYBR Green I (FMC Bioproducts, Rockland). For preparation of a working solution SYBR Green I was diluted with ddH_2O 10.000-fold. 20 µl of the SYBR Green I working solution were applied to each well of the microscopic slide and incubated in the dark for 10min at room temperature. Slides were washed briefly with ddH_2O, air-dried and embedded in Citifluor (Citifluor Ltd., Canterbury, UK). For image acquisitions a Zeiss LSM 510 scanning confocal microscope (Zeiss, Jena, Germany) equipped with a UV laser (351 and 364 nm), an Ar ion laser (458 and 488 nm) and two HeNe lasers (543 and 633nm) was used together with the standard software package delivered with the instrument (version 2.1). The EUB/SYBR Green I ratio was determined by using an equimolar mixture of the probes EUB338, EUB338-II, and EUB338-III (30 ng of each probe labeled with Cy3; DAIMS et al., 1999) by applying the digital image analysis procedure described below. For quantification of biofilm bacteria related to the anaerobic ammonium oxidizer or beta-subclass ammonia oxidizers Cy3 labeled probes S-*-Kst-1275-a-A-20 or Nso1225 were used together with the Cy5 labeled bacterial probe set (EUB338, EUB338-II, EUB338-III) for simultaneous hybridization. The ratio of the area of cells stained with SYBR Green I or the specific probe, respectively, vs. the ratio of the area of those cells labeled with the bacterial probes was determined for random confocal optical biofilm sections (1 µm) by digital image analysis using the Carl Zeiss Vision KS400 software package together with a newly developed macro (R.A.M., Relative Area Measurement – the macro is available on request). For each probe 20 different microscopic fields completely covered with biofilm material (magnification ×400) were analyzed.

Nucleotide sequence accession numbers

The sequences obtained in this study are available in GenBank under accession no. AF202655–AF202663 (16S rRNA of "*Candidatus* Kuenenia stuttgartiensis") and AF202649–AF202654 (anoxic biofilm *amoA* clones).

Results

Anaerobic ammonium oxidation in trickling filter 2

The semi-technical plant displayed in Figure 1 was inoculated with activated sludge of the denitrification and aeration tank (ratio 1:1) of the waste water treatment

Table 3. Average nitrogen balances of trickling filter 2 during the biofilm sampling period.

	Influent [mg l^{-1}]	Effluent [mg l^{-1}]	Degradation [%]	Formation [%]	Load conversion g $(m^2 \cdot d)^{-1}$	Load conversion g $(m^2 \cdot d)^{-1}$
NH_4^+-N	280	33	88.3		45.6	0.42
NO_2^--N	336	13	96.2		58.7	0.54
NO_3^--N	7	71		11.3[a]	11.6	0.11
ΣN_{anorg}	623	117	81.2		92.6	0.85
TNb	634	127	80.0		92.6	0.85

[a] It should be noted that nitrate formation is consistent with anaerobic ammonium-oxidizing activity (STROUS et al., 1998; VAN DE GRAAF et al., 1996, VAN DE GRAAF et al., 1997)

plant (designed for 10,000 inhabitants and population equivalents) of the Institute for Sanitary Engineering (Stuttgart, Germany). Sludge liquor containing up to 690 mg l^{-1} N ammonium was used as influent for trickling filter 1. During the start-up phase semisynthetic waste water was successfully used as influent for trickling filter 2 in order to establish anaerobic ammonium-oxidizing activity. Thereafter, the effluent of trickling filter 1 was used as influent for trickling filter 2. In the experimental period more than 88% of the ammonium and 96% of the nitrite present in the influent were removed in trickling filter 2. Furthermore, an average production of 64 mg l^{-1} of nitrate (11.3% formation) was observed (Table 3). Thus, the average total nitrogen removal in trickling filter 2 was 81.2%.

Biofilm bacteria related to the anaerobic ammonium oxidizer

Nine 16S-rRNA targeted oligonucleotide probes designed for specific *in situ* detection of the previously recognized anaerobic ammonium oxidizer (STROUS et al., submitted) were used for *in situ* hybridization of biofilm samples of trickling filter 2. While high numbers of densely clustered cells were specifically labeled after hybridization with probe S-*-Amx-0820-a-A-22, no signals could be detected after application of the other eight probes (Table 2). Simultaneous application of the *Planctomycetales*-specific probe Pla46 (NEEF et al., 1998) demonstrated that bacteria detectable with probe S-*-Amx-0820-a-A-22 made up more than 99% of the area

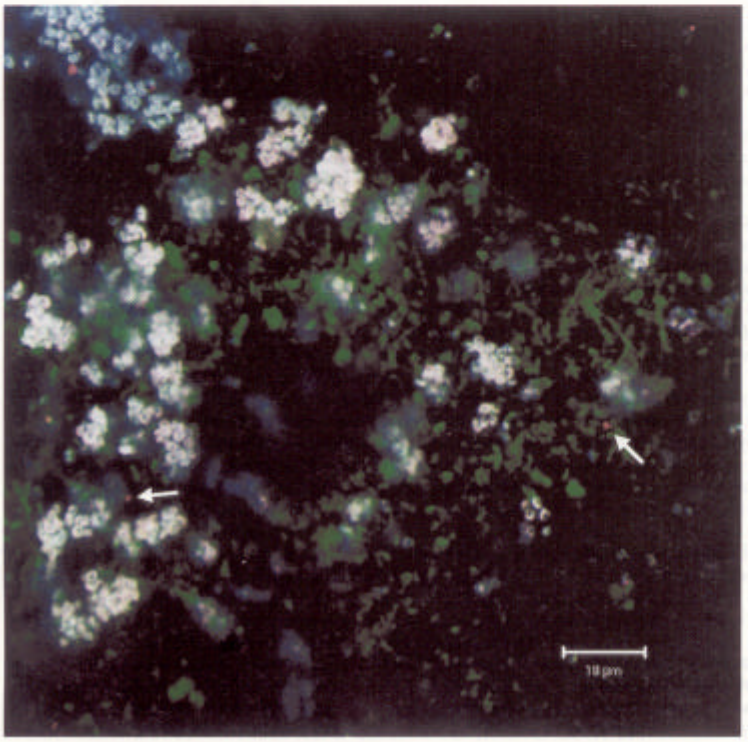


Fig. 2. *In situ* identification of "*Candidatus* Kuenenia stuttgartiensis" in biofilm of trickling filter 2 by simultaneous hybridization with Cy3-labeled probe Pla46, Fluos-labeled probe Eub338 and Cy5-labeled probe S-*-Kst-1275-a-A-20. "*Candidatus* Kuenenia stuttgartiensis" appears white because of the overlapping labels. Planctomycetes other than "*Candidatus* Kuenenia stuttgartiensis" are labeled red (locations indicated by arrows). Please note that for illustration purposes probe EUB338 was used without the probe modifications EUB338-II and EUB338-III. As all members of the genus *Isosphaera*, "*Candidatus* Kuenenia stuttgartiensis" possess a single mismatch within the EUB338 probe-target site which does, however, not hamper probe EUB338 binding under the hybridization conditions applied in this study (DAIMS et al., 1999).

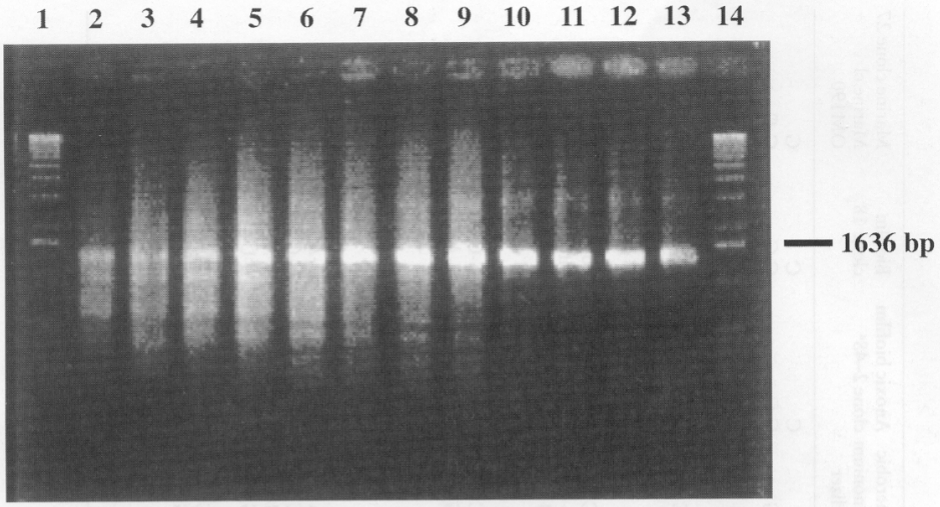


Fig. 3. PCR amplification of trickling filter 2 derived 16S rDNA with the primers Pla46 and 1390R using different annealing temperatures. Lanes: 1 and 14, 1-kb DNA ladder, 2: 44 °C, 3: 45.8 °C, 4:47.6 °C, 5: 49.5 °C, 6: 51.3 °C, 7: 53.1 °C, 8: 54.9 °C, 9: 56.7 °C, 10: 58.6 °C, 11: 60.4 °C, 12: 62.2 °C, 13: 64 °C.

of those cells stained with probe Pla46 (data not shown). Both, the probe S-*-Amx-0820-a-A-22-positive bacteria and the cells stained exclusively with probe Pla46 had a coccoid morphology (average diameter is 1.5 µm) and showed a ring-shaped hybridization signal indicative for members of the *Planctomycetales* (Neef et al., 1998; Strous et al., 1999). Pla46-positive cells which did not hybridize with probe S-*-Amx-0820-a-A-22 occurred as single cells within the biofilm (Figure 2).

To establish a phylogenetic inventory of members of the *Planctomycetales* present within trickling filter 2 probe Pla46 was used as forward primer in combination with the universal reverse primer 1390R for PCR 16S rDNA amplification. The effect of different annealing temperatures on yield and specificity of the PCR reaction was analyzed (Figure 3). At annealing temperatures from 44 °C to 56.7 °C relatively large amounts of unspecific amplificates were observed. Use of higher annealing temperatures up to 64 °C resulted in specific product formation. The PCR product obtained with an annealing temperature of 58.6 °C was used for subsequent direct cloning to cover a maximum diversity of 16S rDNA sequences. Surprisingly, only nine of the twenty-five clones analyzed were affiliated with the *Planctomycetales*. These nine sequences (1363–1365 bp in length – primers not counted) were highly similar to each other (more than 98.8% sequence similarity) but only distantly related to all other sequences presently represented in public

Table 4. Overall sequence similarities for the retrieved biofilm 16S rRNA sequences and representative species of the *Planctomycetales* and *Chlamydia trachomatis*, respectively.

rDNA source	% Similarity to rDNA of:								
	C. trachomatis	*P. marina*	*P. limnophilus*	*G. obscuriglobus*	*I. pallid*	Marine agg. 27	Mar. Pic. 190	Biof. 18	An.amm. ox.
Pirellula marina	72.4								
Planctomyces limnophilus	71.8	82.4							
Gemmata obscuriglobus	70.9	81.0	80.8						
Isosphera pallida	72.1	78.1	79.5	78.9					
Marine aggregate clone 27	72.7	77.8	76.0	75.4	75.8				
Marine picopl. clone OM190	73.1	78.2	76.4	76.1	75.3	97.9			
Biofilm clone18	71.4	75.0	73.0	72.2	71.9	77.7	76.9		
Anaerobic ammonium oxidizer	72.7	76.1	75.9	75.7	74.7	77.6	77.0	75.0	
Anoxic biofilm-clone 2-48[a]	74.2	77.3	75.5	74.8	73.8	78.6	78.0	74.5	90.4

[a] The molecular isolate 2–48 was selected as representative of the nine almost identical 16S rDNA sequences (>98.9%) retrieved from the anoxic biofilm

Table 5. 16S rRNA signature nucleotides for the subdivisions of the order *Planctomycetales*.

Position(s)	Reference	*Pirellula* group	*Planctomyces* group	*Gemmata* group	*Isosphaera*	Anaerobic ammonium oxidizer	**Anoxic biofilm clone 2–48**[a]	Biofilm clone 18	Marine clone 27 Marine cl OM190
169	LIESACK and STACKEBRANDT, 1992	G	G	G	G	C	C	C	G
291–309	LIESACK and STACKEBRANDT, 1992	G-C	G-C	G-C	G-C	C-G	C-G	C-G	G-C
331	LIESACK and STACKEBRANDT, 1992	U	U	U	U	U	U	U	U
353	WOESE, 1987; LIESACK and STACKEBRANDT, 1992	U	U	U	U	U	U	U	U
538	LIESACK and STACKEBRANDT, 1992	$A_{U,G}$[b]	A_{U}[b]	A	A	G	G	U	G
570–588	WOESE, 1987; LIESACK and STACKEBRANDT, 1992	U-A	U-A	U-A	U-A	G-C	G-C	A-U	A-U
659	LIESACK and STACKEBRANDT, 1992	$A_{G,U}$[b]	A	A	A or G	C	C	A	C
686	LIESACK and STACKEBRANDT, 1992	G	G	G	G	G	G	U	G
768	LIESACK and STACKEBRANDT, 1992	A	A	A	A	A	A	A	A
784–798	LIESACK and STACKEBRANDT, 1992	G-C	G-C	G-C	G-C	G-C	G-C	no sequence information	G-C
772–807	LIESACK and STACKEBRANDT, 1992	A-U	A-U	A-U	A-U	A-U	A-U	A-U	A-U
811	LIESACK and STACKEBRANDT, 1992	C	C	C	C	C	C	C	C
819	LIESACK and STACKEBRANDT, 1992	A	A	A	A	A	A	A	A
833–853	LIESACK and STACKEBRANDT, 1992	G-C	G-C_{A-U}[b]	G-C	G-C	G-C	G-C	G-C	A-U,
826–874	LIESACK and STACKEBRANDT, 1992	C-G	C-G	C-G	C-G	C-G	C-G	U-A	C-G
933	WOESE, 1987; LIESACK and STACKEBRANDT, 1992	A	A	A	A	G	G	A	A
955	WOESE, 1987; LIESACK and STACKEBRANDT, 1992	C	C	C	C	C	C	U	U
983:1[c]	WOESE, 1987; LIESACK and STACKEBRANDT, 1992	U	U	U	U	–	–	C	–
1109	WOESE, 1987	A	A	A	A	A	A	C	A
1384	WOESE, 1987	U	U	U	U	C	C	U	U
114–313	FUERST et al., 1997	A-U	G-C	G-C	U-A	U-A	U-A	U-A	U-A
115–312	FUERST et al., 1997	G-C	U-A	C-G	G-C	G-C	G-C	G-C	G-C
668–738	FUERST et al., 1997	U-A_{A-U}[b]	U-A	C-G	G-C	A-U	A-U	U-A	U-A
680–710	FUERST et al., 1997	A-U	C-G	C-G_{U-A}[b]	C-G	C-G	C-G	A-U	C-G
812	FUERST et al., 1997	G	G	C	G	G	G	C	G
822-878	FUERST et al., 1997	A-U_{U-A}[b]	A-U	G-C	G-C	G-C	G-C	U-A	G-C
948–1233	WOESE, 1987; FUERST et al., 1997	G-C	C-G	C-G	C-G	C-G	C-G	C-G	C-G
976	FUERST et al., 1997	G	G	G	A	G	G	G	G
1100	FUERST et al., 1997	U	G,A	U	U	U	U	U	U
1361:1[c]	FUERST et al., 1997	–	C	C	C	–	–	C	C

[a] The molecular isolate 2–48 was selected as representative of the nine almost identical 16S rDNA sequences (>98.9%) retrieved from the anoxic biofilm
[b] Lowercase letters indicate nucleotides present in less than 85% of members of main line of descent
[c] Colon indicates inserted nucleotide at position 983 and 1361, respectively

16S rRNA databases. Among those the highest similarity values were calculated for the previously recognized anaerobic ammonium oxidizer (90.2%–90.5%). Other members of the *Planctomycetales* and all other organisms displayed very low sequence similarities below 79% (Table 4). The nine biofilm 16S rDNA sequences share most but not all signature nucleotides and nucleotide pairs (Table 5) characteristic of planctomycetes (FUERST et al., 1997; LIESACK and STACKEBRANDT, 1992; WOESE, 1987). Consistent results were obtained after application of different treeing methods for phylogenetic analysis of the *Planctomycetales*-related sequences retrieved from the biofilm. Neighbor-joining, maximum-likelihood and maximum parsimony analysis agreed that these sequences form a grouping with the deep-branching anaerobic ammonium oxidizer within the *Planctomycetales* (Figure 4). Selection of different sets of outgroup organisms and exclusion of highly variable positions prior to treeing analysis (by use of a 50% conservation filter for the *Planctomycetales* and the *Bacteria*, respectively) resulted in identical assignment of the biofilm sequences (data not shown). Bootstrap support for the clustering of the biofilm-retrieved sequences with the anaerobic ammonium oxidizer and for the monophyly of the *Planctomycetales* and the anaerobic ammonium oxidizer related sequences is highly significant (Figure 4). Interestingly, three environmentally derived 16S rRNA sequences from a trickling filter biofilm (VAN DER MEER et al., 1998), marine coastal picoplankton (RAPPE et al., 1996) and a marine aggregate (DE LONG et al., 1993) group with the anaerobic ammonium oxidizer and the biofilm sequences if maximum likelihood (only in combination with a filter selecting those sequence positions which share the same nucleotides in at least 50% of the available bacterial sequences) or maximum parsimony methods (only without sequence filter) are applied. However, these methods used with other sequence filters as well as all neighbor joining analyses suggest that these three previously published sequences form a separate lineage which branches not as deep as the anaerobic ammonium oxidizer lineage.

The oligonucleotide probe S-*-Kst-1275-a-A-20 was designed complementary to a specific target region shared between the nine (anaerobic ammonium oxidizer related) 16S rDNA biofilm sequences. Probe S-*-Kst-1275-a-A-20 had at least three mismatches with respect to all other available 16S rRNA sequences (Figure 5). Since no pure culture is available to determine the optimal hybridization stringency for probe S-*-Kst-1275-a-A-20, an *in situ* probe dissociation curve was recorded with fixed biofilm samples using increasingly stringent

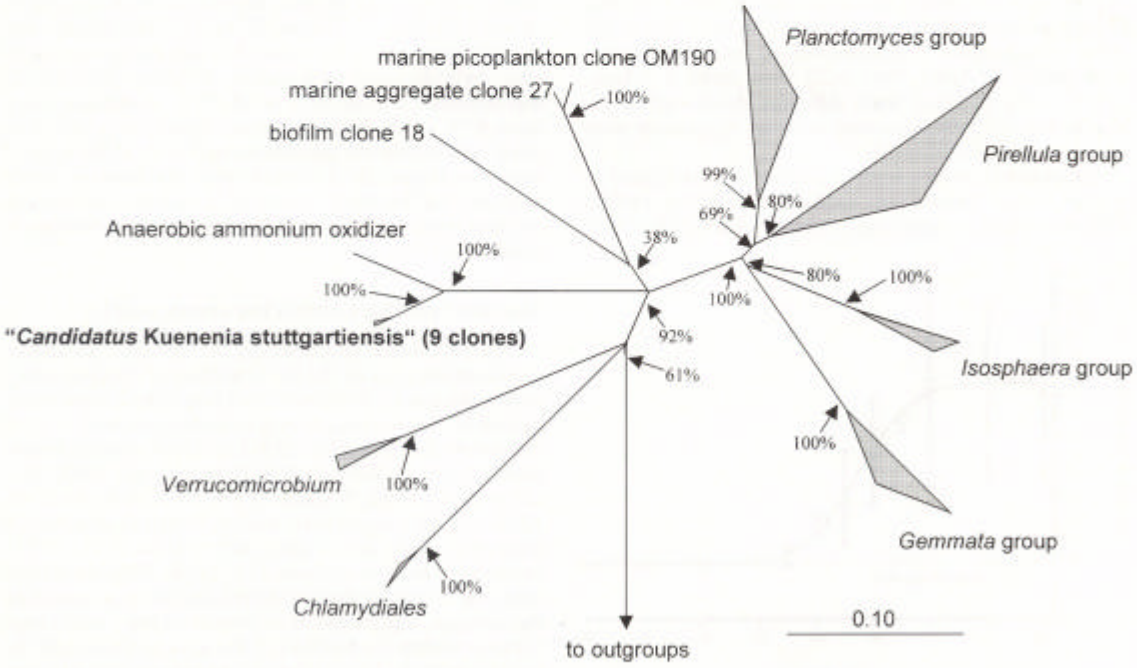


Fig. 4. Phylogenetic tree reflecting the relationships of the Stuttgart trickling filter 2 16S rDNA clones, the previously recognized anaerobic ammonium oxidizer (STROUS et al., 1999; AJ131819), the other *Planctomycetales*, and other reference organisms. The triangles indicate phylogenetic groups. The tree is based on the results of maximum likelihood analysis on different data sets. Multifurcations connect branches for which a relative order could not unambiguously be determined applying different treeing methods. Parsimony bootstrap values for branches are reported. Missing bootstrap values indicate that the branch in question was not recovered in the majority of bootstrap replicates by the parsimony method. The bar represents 10% estimated sequence divergence.

```
Probe sequence                          3'-ACGCTTTGGATATTTCGGCT-5'
Target sequence                         5'-UGCGAAACCUAUAAAGCCGA-3'
  "Candidatus Kuenenia stuttgartiensis"   ....................
  Anaerobic ammonium oxidizer             A........C.....A..A.
  Paenibacillus curdlanolyticus           A.....U............G
  Desulfotomaculum thermosapovorans       A.....C..G.........G
  Thermoanaerobacter lacticus             A........C.G.......G
  Actinopolyspora halophila               A........CGG.......G
  Halomonas halodurans                    A.....U..C.........G
  Brachybacterium alimentarium            A.....U....A.......G
  Clavibacter michiganense                A.....U..C.N.......G
  Mycobacterium ulcerans                  A.....U...U........G
  Legionella adelaidensis                 A.....U.U..G.......G
  Sporotomaculum hydroxybenzoicum         A.....C..G.G.......G
  Thermoanaerobacter brockii              A........C.A.......G
  Corynebacterium flavescens              A.....U.G.G........G
```

Fig. 5. Difference alignment for probe S-*-Kst-1275-a-A-20. 16S rRNA sequences at the target site of the probe are displayed for representative reference organisms.

conditions (Figure 6). Probe S-*-Kst-1275-a-A-20 yielded strong signals up to 25% (v/v) formamide in the hybridization buffer followed by a decline at 30% (v/v) formamide. Signal intensities dropped to the level of autofluorescence after increasing the formamide concentration in the hybridization buffers to more than 50% (v/v). Cells with highly similar morphology were detected by probe S-*-Kst-1275-a-A-20 under conditions of different stringency (data not shown). Considering strength and position of the mismatches in other known non-target organisms a formamide concentration of 25% (v/v) was chosen as optimal stringency for S-*-Kst-1275-a-A-20. After simultaneous hybridization of biofilm material with probe S-*-Kst-1275-a-A-20, and probe S-*-Amx-0820-a-A-22 (labeled with different dyes) exclusively double-labeled cells occurring in dense aggregates were observed (data not shown).

Quantitative *in situ* analysis of the bacteria related to the anaerobic ammonium oxidizer within the biofilm was performed by applying probe S-*-Kst-1275-a-A-20 with 25% formamide in the hybridization buffer and 159 mM NaCl in the washing buffer (see Materials and Methods). Digital image analysis of confocal biofilm sections simultaneously hybridized with the Cy3-labeled EUB338 probe mixture (DAIMS et al., 1999) and the Cy5-labeled probe S-*-Kst-1275-a-A-20 demonstrated that bacteria related to the anaerobic ammonium oxidizer occupied 49% (+/–12; 95% confidence limit; the relatively high standard deviation is caused by the unequal, cluster-like distribution of the probe target bacteria within the biofilm) of the area of those bacterial cells detectable by *in situ* hybridization (Figure 2). In a control experiment, the relative area of the novel biofilm *Planctomycetales* after hybridization with probe S-*-Amx-0820-a-A-22 was determined to be 50% (+/–8; 95% confidence limit). Since 85% (+/–8; 95% confidence limit) of the total bacterial cells within the biofilm stained with SYBR Green I were simultaneously detectable with the bacterial probe mixture, the bacteria related to anaerobic ammonium oxidizer constitute almost half of the bacterial biomass in trickling filter 2.

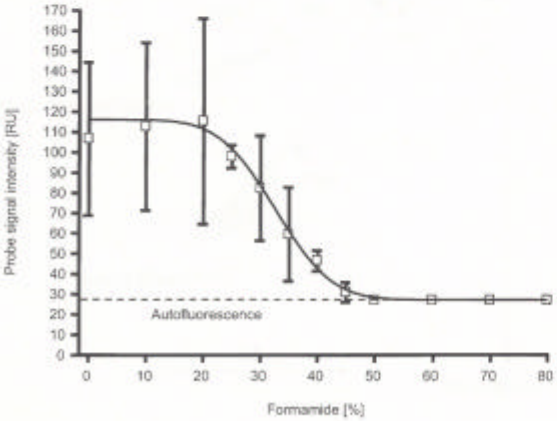


Fig. 6. Probe binding profile of probe S-*-Kst-1275-a-A-20. The relative strength of hybridization was determined at increasing concentrations of formamide in the hybridization buffer and decreasing concentrations of NaCl in the washing buffer by quantification of intensities of the fluorescence signals.

"Aerobic" nitrifiers within the anoxic biofilm

The abundance and diversity of "aerobic" ammonia-oxidizing bacteria of the beta-subclass of *Proteobacteria* within the anoxic biofilm of trickling filter 2 were investigated by fluorescence *in situ* hybridization with a set of previously developed 16S rRNA-targeted oligonucleotide probes listed in Table 2 (JURETSCHKO et al., 1998; MOBARRY et al., 1996; WAGNER et al., 1995; WAGNER et al., 1996) and by comparative sequence analysis of retrieved fragments of the *amoA* gene (ROTTHAUWE et al., 1997). In confocal biofilm sections 27% (+/–8; 95% confidence limit; the relatively high standard deviation is caused by the unequal, cluster-like distribution of the probe target bacteria within the biofilm) of the area of those cells detectable with the bacterial probe set was occupied by ammonia oxidizers identified as halophilic or halotolerant members of the genus *Nitrosomonas* by simultaneous hybridization with probes Nso 1225, Nso 190 and NEU. No signals were observed after hybridization with the *Nitrosococcus mobilis* specific probe NmV or the *Nitrosospira* cluster-specific probe Nsv 443. In addition, no

nitrite-oxidizing bacteria of the genera *Nitrobacter* and *Nitrospira* could be visualized after hybridization with the probes NIT3 (WAGNER et al., 1996) and S-G-Ntspa-0662-a-A-18 (DAIMS et al., in press), respectively.

For 16S rRNA-independent high resolution diversity analysis of beta-subclass ammonia oxidizers of trickling filter 2 the *amoA* approach was performed (ROTTHAUWE et al., 1997). *AmoA* gene fragments were successfully amplified from trickling filter 2 biofilm derived DNA. After gel electrophoretic separation of the equal-sized

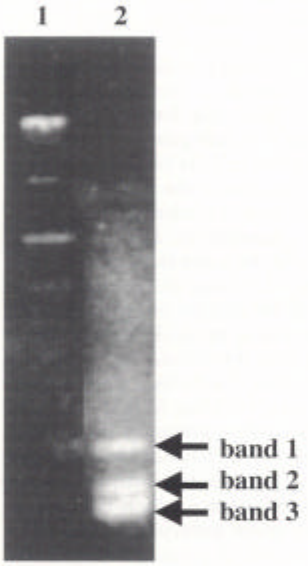


Fig. 7. Separation of trickling filter 2 retrieved *amoA* PCR amplificates according to their GC content by gel retardation. Lane 1: 1-kb DNA ladder (please note that the migration pattern is strongly altered due to the gel retardation); Lane 2: 5 µl PCR product. Bands 1, 2 and 3 were excised, cloned and sequenced (See Figure 8).

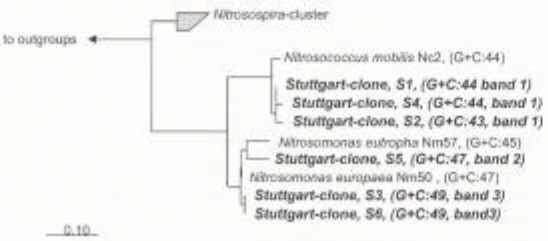


Fig. 8. Phylogenetic Fitch-Margoliash tree reflecting the relationships of the trickling filter 2 ammonia oxidizers based on deduced AmoA amino acid sequences. GC contents and the respective gel retardation band are given for each biofilm clone in brackets. The bar indicates 10% estimated sequence divergence.

amoA fragments according to their GC content by using gel retardation, three distinct bands were observed, cloned and sequenced (Figure 7). Phylogenetic analysis of the deduced amino acid sequences of the molecular isolates representing the respective bands demonstrated their grouping with *Nitrosococcus mobilis* (band 1; clones S1, S2, and S4, GC content 43–44%, identity values between 95.7 and 97.2% on the amino acid level to the respective *amoA* fragment of *N. mobilis*), *Nitrosomonas eutropha* (band 2, clone S5, GC content 47%, identity value of 95.0 % on the amino acid level to the respective *amoA* fragments of *N. eutropha*), and *Nitrosomonas europaea* (band 3, clones S3 and S6, GC content 49%, identity values of 98.6–99.3% on the amino acid level to the respective *amoA* fragment of *N. europaea*) (Figure 8).

Discussion

Genus-level diversity of anaerobic ammonium oxidizers

On thermodynamical grounds Broda predicted the existence of lithotrophic prokaryotes mediating the anaerobic oxidation of ammonia to nitrogen in nature (BRODA, 1977). Recently, this hypothesis was verified by the identification of a novel, deep-branching planctomycete capable of catalyzing the above mentioned process (STROUS et al., 1999). Our data provide evidence that an additional genus within the *Planctomycetales* capable to anaerobically oxidize ammonium does exist. The suggested novel genus was identified based on nine highly similar 16S rRNA sequences retrieved from a trickling filter biofilm with high anaerobic ammonium-oxidizing activity. Different phylogenetic analyses consistently demonstrated that these sequences group as a clearly separated cluster with the previously described anaerobic ammonium oxidizer. The moderate sequence similarities between the molecular biofilm isolates and the anaerobic ammonium oxidizer (below 91%) indicate the presence of two different genera (LUDWIG et al., 1998). Consistent with these findings, the biofilm retrieved sequences do not possess fully complementary target sites for eight out of nine 16S rRNA-targeted oligonucleotide probes previously designed for specific *in situ* detection of the anaerobic ammonium oxidizer (STROUS et al., submitted).

Our results do not provide direct evidence that the identified novel planctomycetes occurring in the trickling filter biofilm do actually perform anaerobic ammonium oxidation. However, there are two independent experimental results which strongly support the conclusion that these organisms are indeed anaerobic ammonium oxidizers. Firstly, it should be noted that the influent of trickling filter 2 contained significant amounts of ammonium (280 mg l^{-1}) and nitrite (336 mg l^{-1}) but was very low in total organic carbon (5–10 mg l^{-1}). Analysis of the chemical composition of the influent and effluent of the analyzed trickling filter showed that its biofilm possessed a high anaerobic ammonium-oxidizing activity at the

time of sampling. Taken together it appears very likely that the numerically dominant biofilm microorganism, the novel planctomycete-like bacterium which accounts for almost half of the prokaryotic biofilm biomass, are chemolithoautotrophs gaining energy from the anaerobic ammonium oxidation. Secondly, the previously described anaerobic ammonium oxidizer is the closest known relative of the novel biofilm planctomycete representing together the deepest recognized line of descent within the *Planctomycetales*. Since no other recognized members of the *Planctomycetales* which are capable to perform anaerobic ammonium oxidation have been described, it is tempting to speculate that the common ancestor of the anaerobic ammonium oxidizer and the novel *Candidatus* genus either developed or acquired (by lateral gene transfer from a yet unknown bacterium) this physiological ability. In this context three environmentally derived 16S rRNA sequences, obtained from a marine aggregate (DE LONG et al., 1993), marine picoplankton (RAPPE et al., 1996), and one from a rotating contactor disk biofilm (VAN DER MEER et al., 1998), should also be considered. Some but not all treeing methods suggest that these two sequences do also group with the anaerobic ammonium oxidizer and the novel biofilm planctomycete. Future studies are required to show whether these sequences also represent bacteria performing anaerobic ammonium oxidation. If such is the case a widespread environmental distribution of bacteria with this physiological trait would be expected.

Based on the results of our study we propose, according to MURRAY and SCHLEIFER (1994) provisional classification of the novel biofilm planctomycete as "*Candidatus* Kuenenia stuttgartiensis". The short description of "*Candidatus* Kuenenia stuttgartiensis" is as follows: deep-branching within the *Planctomycetales*; not cultivated; Gram-reaction not applicable; coccus, approximately 1.5 µm in diameter; basis of assignment, 16S rDNA sequences (accession numbers AF202655–AF202663) and oligonucleotide probe complementary to unique region of 16S rRNA S-*-Kst-1275-a-A-20 (5'-TCGGCTTTATAGGTTTCGCA-3'); free-living (anoxic biofilm); anaerobic ammonium oxidizer, mesophilic; SCHMID *et al.*, this study.

Significance of classical "aerobic" ammonia oxidizers in anoxic habitats

Several studies have demonstrated that "aerobic" ammonia oxidizers of the beta-subclass of *Proteobacteria* can survive extended periods of anaerobiosis (ABELIOVICH, 1987; BLACKBURN, 1983; JETTEN et al., 1999). In addition, a surprising metabolic versatility of different *Nitrosomonas* strains under oxygen limitation and/or anoxic conditions including anaerobic oxidation of ammonium with nitrite as electron acceptor has been reported (BOCK et al., 1995; DE BRUIJN et al., 1995; POTH, 1986; POTH and FOCHT, 1985, SCHMIDT and BOCK, 1997). Quantitative FISH showed that halophilic or halotolerant ammonia oxidizers of the genus *Nitrosomonas* constitute about one fourth of the microbial biomass of the anoxic trickling filter biofilm. Comparative sequence analysis of biofilm derived *amoA* fragments indicated the presence of DNA originating from ammonia oxidizers closely related to *Nitrosomonas europaea, Nitrosomonas eutropha*, and *Nitrosococcus mobilis* within the biofilm. It should be noted that, due to the reamplification of *amoA* fragments after gel-retardation, two rounds of 30 PCR cycles were performed prior to cloning of *amoA* fragments. Thus Taq polymerase induced sequence errors might account for some of the differences in the deduced amino acid sequences between the cloned *amoA* fragments and those of the cultured ammonia oxidizers. While the detection of *amoA* fragments from *Nitrosomonas europaea*- and *Nitrosomonas eutropha*-like bacteria is congruent with the FISH results, *Nitrosococcus mobilis* cells were not detectable *in situ*. Possible explanations of this discrepancy are that *Nitrosococcus mobilis*-like cells are not detectable by FISH since they (I) occur in low numbers below the FISH detection limit, (II) possess a low cellular ribosome content, (III) were subjected to a mutation in the NmV probe-target site, or (IV) were lysed within reactor 2 and their extracellular DNA served as template for *amoA* amplification. At present it is difficult to judge the significance of the relatively high *in situ* abundance of classical ammonia oxidizers in the anoxic biofilm for anaerobic ammonium oxidation. It appears to be most likely that aerobic ammonia oxidizers have been transferred via the sewage from the aerobic trickling filter 1 with high ammonia-oxidizing activity to the anoxic trickling filter 2. The high cellular ribosome content of these ammonia oxidizers in trickling filter 2 observed by FISH is not necessarily an indicator for substantial physiological activity since beta-subclass ammonia oxidizers maintain high rRNA concentrations per cell even under unfavorable conditions like starvation (MORGENROTH, in press; MORITA, 1993) or chemical inhibition (WAGNER et al., 1995). Consequently, the high ribosome content of trickling filter 2 beta-subclass ammonia oxidizers could just reflect their previous aerobic activity in trickling filter 1. However, the relatively high numbers of the "aerobic" ammonia oxidizers observed *in situ* in trickling filter 2 suggest that they might be able to grow under anoxic conditions or under transient periods of microaerophilic conditions (up to 0.08 mg O_2 l^{-1} was detectable in trickling filter 2; Table 1). Nevertheless, classical ammonia oxidizers constituted only approx. one half of the biomass of "*Candidatus* Kuenenia stuttgartiensis" within the trickling filter 2. Keeping in mind that beta-subclass ammonia oxidizers analyzed to date possess (at least in pure culture) specific rates for anaerobic ammonium oxidation which are more than 20fold lower that the rate measured for the anaerobic ammonium oxidizer affiliated with the *Planctomycetales* (JETTEN et al., 1999) we conclude that "*Candidatus* Kuenenia stuttgartiensis" is most likely responsible for a large part of the anaerobic ammonium oxidation in trickling filter 2. Future studies will show whether members of the *Planctomycetales* are also responsible for the nitrogen loss without corresponding decrease of BOD which has been recently reported for two rotating biological contactor systems treating landfill leachate

(HELMER and KUNST, 1998; HELMER et al., 1999; SIEGRIST et al., 1998). In a more general perspective these results demonstrate that there is a principal possibility to actually integrate the process of anaerobic ammonium oxidation in modern waste water treatment for inexpensive N-removal from waste water with high ammonium concentrations and low BOD. We just begin to elucidate the actual natural genetic diversity of bacteria capable to perform anaerobic ammonium oxidation. How this diversity also reflects differences in physiology remains unresolved but will ultimately be important in both, our fundamental understanding of nitrogen cycling and the design of highly efficient waste water treatment plants.

Acknowledgements

This work was supported by Sonderforschungsbereich 411 from the Deutsche Forschungsgemeinschaft (Research Center for Fundamental Studies of Aerobic Biological Wastewater treatment). U.T. was supported by a grant from the BMBF (02WA9626/2). M.S. JETTEN was supported by the Dutch Foundation for Applied Research (STW). We thank SIBYLLE SCHADHAUSER and KATINKA VAN DE PAS-SCHOONEN for excellent technical assistance and the participants at the "Mikrobiologischen Großpraktikum II 1999" (TU München) for their participation in the experiments.

References

ABELING, U., and SEYFRIED, C. F.: Anaerobic-aerobic treatment of high-strength ammonium wastewater – nitrogen removal via nitrite. Wat. Sci. Tech. 26(5–6), 1007–1015 (1992).

ABELIOVICH, A.: Nitrifying bacteria in waste water reservoirs. Appl. Environ. Microbiol. 53, 754–760 (1987).

ADACHI, J., and HASEGAWA, M.: Computer science monographs, no. 28.MOLPHY version 2.3: programs for molecular phylogenetics based on maximum likelihood. Institute of Statistics and Mathematics, Tokyo, Japan (1996).

ALM, E., OERTHER, D. B., LARSEN, N., STAHL, D. A., and RASKIN, L.: The oligonucleotide database. Appl. Environ. Microbiol. 62, 3557–3559 (1996).

AMANN, R.: *In situ* identification of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes, p.1–15. *In* A.D.C. Akkermans, J.D. van Elsas, and F.J. de Bruijn (ed.), Molecular microbial ecology manual. Kluwer Academic Publishers, Dordrecht, The Netherlands (1995).

AMANN, R. I., BINDER, B. J., OLSEN, R. J., CHISHOLM, S. W., DEVEREUX, R., and STAHL, D. A.: Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56, 1919–1925 (1990).

BLACKBURN, T. H.: The microbial nitrogen cycle. *In* W. E. KRUMBEIN (ed.), Microbial geochemistry. Blackwell Scientific Publications, Ltd., Oxford (1983).

BOCK, E., SCHMIDT, I., STÜVEN, R., and ZART, D.: Nitrogen loss caused by denitrifying *Nitrosomonas* cells using ammonium or hydrogen as electron donors and nitrite as electron acceptor. Arch. Microbiol. 163, 16–20 (1995).

BRODA, E.: Two kinds of lithotrophs missing in nature. Z. Allg. Mikrobiol. 17, 491–493 (1977).

BROSIUS, J., PALMER, M. L., KENNEDY, P. J., and NOLLER, H. F.: Complete sequence of a 16S ribosomal RNA gene from *Escherichia coli*. Proc. Natl. Acad. Sci. USA. 75, 4801–4805 (1978).

DAIMS, H., BRÜHL, A., AMANN, R., SCHLEIFER, K.-H., and WAGNER, M.: The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. 22, 434–444 (1999).

DAIMS, H., NIELSEN, P. H., NIELSEN, J.L., JURETSCHKO, S., and WAGNER, M. Novel *Nitrospira*-like bacteria as dominant nitrite-oxidizers in biofilms from wastewater treatment plants: Diversity and *in situ* physiology. Wat. Sci. Technol., in press.

DE BRUIJN, P., VAN DE GRAAF, A. A., JETTEN, M. S. M., ROBERTSON, L. A., and KUENEN, J. G.: Growth of *Nitrosomonas europaea* on hydroxylamine. FEMS Microbiol. Lett. 125, 179–184 (1995).

DE LONG, E. F., FRANKS, D. G., and ALLDREDGE, A. L.: Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. Limnol. Oceanogr. 38(5), 924–934 (1993).

FELSENSTEIN, J.: PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle (1993).

FUERST, J. A.: The planctomycetes: emerging models for microbial ecology, evolution and cell biology. Microbiology 141, 1493–1506 (1995).

FUERST, J. A., GWILLIAM, H. G., LINDSAY, M., LICHANSKA, A., BELCHER, C., VICKERS, J. E., and HUGENHOLTZ, P.: Isolation and molecular identification of Planctomycete bacteria from postlarvae of the Giant Tiger Prawn, *Penaeus monodon*. Appl. Environ. Microbiol. 63, 254–262 (1997).

HELLINGA C, SCHELLEN, A. A. J. C., MULDER, J. W., VAN LOOSDRECHT, M. C. M., and HEIJNEN, J. J.: The SHARON process: an innovative method for nitrogen removal from ammonium-rich waste water. Wat. Sci. Tech. 37, 135–142 (1998).

HELMER, C., and KUNST, S.: Simultaneous nitrification/denitrification in an aerobic biofilm system. Water Sci. Technol. 37(4–5), 183–187 (1998).

HELMER, C., KUNST, S., JURETSCHKO, S., SCHMID, M. C., SCHLEIFER, K.-H., and WAGNER, M.: Nitrogen loss in a nitrifying biofilm system. Wat. Sci. Tech. 39(7), 13–21 (1999).

JETTEN, M. S. M., STROUS, M., VAN DE PAS-SCHOONEN, K. T., SCHALK, J., VAN DONGEN, U. G. J. M., VAN DE GRAAF, A., LOGEMANN, S., MUYZER, G., VAN LOOSDRECHT, M. C. M., and KUENEN, J. G.: The anaerobic oxidation of ammonium. FEMS Microbiol. Rev. 22, 421–437 (1999).

JURETSCHKO, S., TIMMERMANN, G., SCHMID, M., SCHLEIFER, K.-H., POMMERENING-RÖSER, A., KOOPS, H.-P., and WAGNER, M.: Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. Appl. Environ. Microbiol. 64, 3042–3051 (1998).

KUAI, L., and VERSTRAETE, W.: Ammonium removal by the oxygen-limited autotrophic nitrification-denitrification system. Appl. Environ. Microbiol. 64, 4500–4506 (1998).

LIESACK, W., and STACKEBRANDT, E.: Occurrence of novel groups of the domain *Bacteria* as revealed by analysis of genetic material isolated from an australian terrestrial environment. J. Bacteriol. 174, 5072–5078 (1992).

LOGEMANN, S., SCHANTL, J., BIJVANK, S., VAN LOOSDRECHT, M., KUENEN, J. G., and JETTEN, M.: Molecular microbial diversity in a nitrifying reactor system without sludge retention. FEMS Microbiol. Ecol. 27, 239–249 (1998).

LUDWIG, W., STRUNK, O., KLUGBAUER, S., KLUGBAUER, N., WEIZEGGER, M., NEUMAIER, J., BACHLEITNER, M., and SCHLEIFER, K.-H.: Bacterial phylogeny based on comparative sequence analysis. Electrophoresis 19, 554–568 (1998).

MAIDAK, B. L., OLSEN, G. J., LARSEN, N., OVERBECK, R., MC CAUGHEY, M. J., and WOESE, C. R.: The ribosomal database project (RDP). Nucleic Acid Res. 24, 82–85 (1996).

MANZ, W., AMANN, R., LUDWIG, W., WAGNER, M., and SCHLEIFER, K.-H.: Phylogenetic oligonucleotide probes for the major subclasses of *Proteobacteria*: problems and solutions. Syst. Appl. Microbiol. 15, 593–600 (1992).

MOBARRY, B. K., WAGNER, M., URBAIN, V., RITTMANN, B. E., and STAHL, D. A.: Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl. Environ. Microbiol. 62, 2156–2162 (1996).

MORGENROTH, E., OBERMAYER, A., ARNOLD, E., BRÜHL, A., WAGNER, M., and WILDERER, P. A.: Effect of long term idle periods on the performance of sequencing batch reactors. Wat. Sci. Technol., in press.

MORITA, R. Y. (1993) Bioavailability of energy and starvation state. *In* S. Kjelleberg (ed.) Starvation in bacteria. Plenum Press, New York.

MURRAY, R.G.E., and SCHLEIFER, K.-H.: Taxonomic notes: A proposal for recording the properties of putative taxa of procaryotes. Int. J. System. Bacteriol. 44, 174–176 (1994).

NEEF, A., AMANN, R. I., SCHLESNER, H., and SCHLEIFER, K.-H.: Monitoring a widespread bacterial group: *in situ* detection of planctomycetes with 16S rRNA-targeted probes. Microbiology 144, 3257–3266 (1998).

POTH, M.: Dinitrogen gas production from nitrite by a *Nitrosomonas* isolate. Appl. Environ. Microbiol. 52, 957–959 (1986).

POTH, M., and FOCHT, D. D.: ^{15}N kinetic analysis of N_2O production by *Nitrosomonas europaea*: an examination of nitrifier denitrification. Appl. Environ. Microbiol. 49, 1134–1141 (1985).

RAPPE, M.S., KEMP, P.F., and GIOVANNONI, S.J.: Genbank direct submission. Accession number: U70712. (1996).

ROTTHAUWE, J. H., WITZEL, K.-P., and LIESACK, W.: The ammonia monooxygenase structural gene *amo*A as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl. Environ. Microbiol. 63, 4704–4712 (1997).

SCHMIDT, I., and BOCK, E.: Anaerobic ammonia oxidation with nitrogen dioxide by *Nitrosomonas eutropha*. Arch. Microbiol. 167, 106–111 (1997).

SIEGRIST, H., REITHAAR, S., and LAIS, P.: Nitrogen loss in a nitrifying rotating contractor treating ammonium rich leachate without organic carbon. Water Sci. Technol. 37(4–5), 589–591 (1998).

STROUS, M., FUERST, J. A., KRAMER, E. H. M., LOGEMANN, S., MUYZER, G., VAN DE PAS-SCHOONEN, K. T., WEBB, R. I., KUENEN, J. G., and JETTEN, M. S. M.: Missing lithotroph identified as new planctomycete. Nature. 400, 446–449 (1999).

STROUS, M., HEIJNEN, J. J., KUENEN, J. G., and JETTEN, M. S. M.: The sequencing batch reactor as a powerful tool to study very slowly growing micro-organisms. Appl. Microbiol. Biotechnol. 50, 589–596 (1998).

STROUS, M., VAN DE PAS-SCHOONEN, K.T., KRAMER, E. H. M., RAGHOEBARSING, A., MUYZER, G., WAGNER, M., KUENEN, J. G., and JETTEN, M. S. M.: New planctomycete responsible for the anaerobic ammonium oxidation (Anammox) process. Submitted to Environmental Microbiology

STRUNK, O., and LUDWIG W.: ARB software program package; http://www.biol.chemie.tu-muenchen.de/pup/ARB/. (1997).

VAN DE GRAAF, A. A., DE BRUIJN, P., ROBERTSON, L. A., JETTEN, M. S. M., and KUENEN, J. G.: Autotrophic growth of anaerobic ammonium-oxidizing microorganisms in a fluidized bed reactor. Microbiology. 142, 2187–2196 (1996).

VAN DE GRAAF, A. A., DE BRUIJN, P., ROBERTSON, L. A., JETTEN, M. S. M., and KUENEN, J. G.: Metabolic pathway of anaerobic ammonium oxidation on the basis of N-15 studies in a fluidized bed reactor. Microbiology. 143, 2415–2421 (1997).

VAN DER MEER, J. R., VAN DER MEER, J., WERLEN, C., and SIEGRIST, H.: Genbank direct submission. Accession number: AJ224943. (1998).

WAGNER, M., AMANN, R., KÄMPFER, P., ÆMUS, B., HARTMANN, A., HUTZLER, P., SPRINGER, N., and SCHLEIFER, K.-H.: (1994) Identification and *in situ* detection of gram-negative filamentous bacteria in activated sludge. Syst. Appl. Microbiol. 17, 405–417.

WAGNER, M., RATH, G., AMANN, R., KOOPS, H.-P., and SCHLEIFER, K.-H.: *In situ* identification of ammonia oxidizing bacteria. Syst. Appl. Microbiol. 18, 251–264 (1995).

WAGNER, M., RATH, G., FLOOD, J., KOOPS, H.-P., and AMANN, R. *In situ* analysis of nitrifying bacteria in sewage treatment plants. Water. Sci. Technol. 34(1–2), 237–244 (1996).

WAWER, C., RÜGGEBERG, H., MEYER, G., and MUYZER, G.: A simple and rapid electrophoresis method to detect sequence variation in PCR-amplified DNA fragments. Nucleic Acid Res. 23, 4928–4929 (1995).

WOESE, C. R.: Bacterial evolution. Microbiol. Rev. 51, 221–271 (1987).

ZHENG, D., ALM, E. W., STAHL, D. A., and RASKIN, L.: Characterization of universal small-subunit rRNA hybridization probes for quantitative molecular microbial ecology studies. Appl. Environ. Microbiol. 62, 4504–4513 (1996).

ZHOU, J., BRUNS, M. A., and TIEDJE, J. M.: DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. 62, 316–322 (1996).

Corresponding author:
MICHAEL WAGNER, Lehrstuhl für Mikrobiologie, Technische Universität München, Am Hochanger 4, D - 85350 Freising, Germany
Phone: +49 8161 71 5444; Fax: +49 8161 71 5475;
e-mail: wagner@mikro.biologie.tu-muenchen.de

G.2.3 Endosymbiotic sulphate-reducing and sulphide-oxidizing bacteria in an oligochaete worm

The unusual symbiosis of a sulfate reducing and a sulfide-oxidizing bacterium and a gutless marine worm was investigated with molecular biological methods. The two bacterial symbiosis partner were identified by 16S rDNA analysis and their localization inside the worm were ascertained with fluorescence in situ hybridization and detection with confocal laser scanning and electron microscopy. Michael Klein contributed to the in situ detection of the symbionts and sequenced the *dsrAB* genes from the sulfate reducing partner and *Desulfosarcina variabilis*, a close relative. Michael Klein as well carried out the subsequent phylogenetic analysis of these sequences. He further contributed to the writing of the respective sections.

This report was published in Nature 2001 (Dubilier 2001).

Endosymbiotic sulphate-reducing and sulphide-oxidizing bacteria in an oligochaete worm

Nicole Dubilier[1], Caroline Mülders[1], Tim Ferdelman[1], Dirk de Beer[1], Annelie Pernthaler[1], Michael Klein[2], Michael Wagner[2], Christer Erséus[3], Frank Thiermann[4], Jens Krieger[4], Olav Giere[4] & Rudolf Amann[1]

[1]Max Planck Institute of Marine Microbiology, Celsiusstrasse 1, D-28359 Bremen, Germany

[2] Department of Microbiology, Technical University of Munich, D-85350 Freising, Germany

[3] Department of Invertebrate Zoology, Swedish Museum of Natural History, S-10405 Stockholm, Sweden

[4] Zoological Institute and Zoological Museum, University of Hamburg, D-20146 Hamburg, Germany

Abstract

Stable associations of more than one species of symbiont within a single host cell or tissue are assumed to be rare in metazoans because competition for space and resources between symbionts can be detrimental to the host[1]. In animals with multiple endosymbionts, such as mussels from deep-sea hydrothermal vents[2] and reef-building corals[3], the costs of competition between the symbionts are outweighed by the ecological and physiological flexibility gained by the hosts. A further option for the coexistence of multiple symbionts within a host is if these benefit directly from one another, but such symbioses have not been previously described. Here we show that in the gutless marine oligochaete *Olavius algarvensis* , endosymbiotic sulphate-reducing bacteria produce sulphide that can serve as an energy source for sulphide- oxidizing symbionts of the host. Thus, these symbionts do not compete for resources but rather share a mutalistic relationship with each other in an endosymbiotic sulphur cycle, in addition to their symbiotic relationship with the oligochaete host.

Olavius algarvensis [4] is a small tubifcid worm (0.2mm x 20-30mm) that is found in the Mediterranean at sediment depths of 5-15 cm in coarse-grained sands surrounding beds of sea grass. As in other gutless oligochaetes[5,6,] two bacterial morphotypes occur in immediate proximity to one another just below the cuticle between extensions of the epidermal cells (Fig. 1).

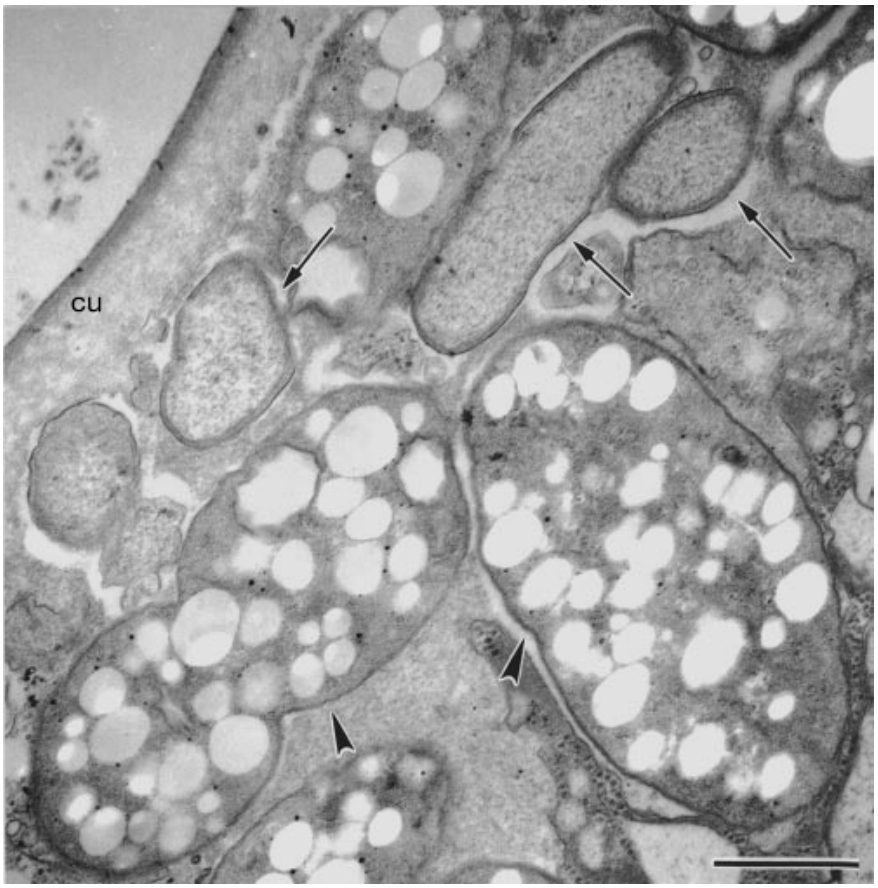


Figure 1 Transmission electron micrograph of bacterial endosymbionts in *O. algarvensis* . The symbionts occur just below the cuticle (cu) between extensions of the epidermal cells. The larger bacterium (arrowheads) contain numerous globules whereas the smaller bacteria (arrows) do no show any cytoplasmic inclusions. Scale Bar, 1 µm.

The larger morphotype (2.5µm x 1.5µm) contains numerous intracellular globules, whereas the smaller (1.1µm x 0.7 µm) has no conspicuous inclusions. We determined the phylogenetic identity of the *O. algarvensis* symbionts by using comparative 16S ribosomal RNA sequencing. We identified two dominant clone groups in the hosts, with minimal variations in the 16S rRNA sequences within

each clone group (0.1±1.2%). Phylogenetic analyses revealed that the 16S rRNA sequences from these two groups are derived from the γ- and δ-subclasses of the Proteobacteria (Fig. 2a, b). The γ-proteobacterial sequence isolated from *O. algarvensis* consistently falls in a cluster with endosymbionts from other gutless oligochaetes such as *Olavius loisae*[7] and *Inanidrilus leukodermatus*[8] (96±97% sequence identity) in all treeing methods used. The δ-proteobacterial sequence is always placed within a subgroup of free-living sulphate-reducing bacteria (*Desulfococcus/Desulfonema/Desulfosarcina*) by all inference methods, with *Desulfosarcina variabilis* consistently identified as its closest relative (93% sequence identity). Fluorescence *in situ* hybridization (FISH) confirmed that the γ– and δ-proteobacterial 16S rRNA sequences originated from the symbiotic bacteria in *O. algarvensis* (Fig. 3). The FISH signal from the probe specific to the γ-subclass of the Proteobacteria (GAM42a) and a species-specific probe based on the *O. algarvensis* γ-sequence (OalgGAM445) clearly originated from the larger bacterial symbiont, whereas the general *Desulfosarcina/Desulfococcus* probe (DSS658) and a probe targeting the *O. algarvensis* δ-sequence (OalgDEL136) consistently labeled the smaller bacterial symbiont. The thioautotrophic nature (that is, sulphur-oxidizing, CO2- fixing metabolism) of the γ-symbionts in *O. algarvensis* is suggested by their close evolutionary relationship to symbionts already characterized as thioautotrophic8,9. This assumption is corroborated by our results from immunocytochemical labeling with an antiserum directed against form I of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the key CO_2 fixing enzyme. The antiserum consistently labeled the larger g-symbionts but not the smaller δ-symbionts (see Supplementary Information). Further evidence for a thioautotrophic metabolism of the γ-symbionts is the high concentration of elemental sulphur in *O. algarvensis* (3.2 ± 1.7% dry weight; n = 5). Such large amounts of S^0 are characteristic for hosts with sulphide-oxidizing symbionts[10]. This corresponds well with electron microscopic spectroscopy studies that show the presence of sulphur in globules of the γ-symbionts (J.K., unpublished results). The close evolutionary relationship of the δ-symbionts of *O. algarvensis* to free-living sulphate-reducing bacteria (SRB) suggests that these are also sulphate reducers. SRB have been described from termite guts[11] and the intestines of some mammals[12] and there is indirect evidence that they may occur as epibionts on some marine ciliates[13] and invertebrates[14,15]. However, SRB as endosymbionts

have not been previously found in marine invertebrates and it has been suggested that such symbioses are unlikely because sulphide, their metabolic endproduct, is toxic to most aerobic organisms. We therefore used several methods to show that the d-symbionts of *O. algarvensis* are indeed SRB and can actively respire sulphate in the worms. The enzyme dissimilatory sulphite reductase (DSR) catalyses the reduction of (bi)sulphite to sulphide and is a good indicator for dissimilatory sulphate respiration, as it is only known to occur in sulphate-reducing prokaryotes[16]. Using specific primers, we successfully amplified the gene encoding DSR from *O. algarvensis* ; no amplification products were obtained from negative controls with another gutless oligochaete host *(I. leukodermatus*) that does not harbor δ-proteobacterial symbionts. Comparative phylogenetic analyses (Fig. 2c) consistently showed that the DSR sequence from *O. algarvensis* is most closely related to *D. variabilis* (79% DNA sequence identity, 82% amino-acid identity), the free-living SRB most closely related to the d-symbiont of *O. algarvensis* on the basis of 16S rRNA analyses (Fig. 2b). Previous studies have shown that 16S rRNA phylogenies of SRB agree well with their DSR phylogenies[16], indicating that the DSR sequence isolated from *O. algarvensis* originated from the δ-symbiont of this host and thus that this symbiont is a sulphate reducer. To show that sulphate is actively reduced in *O. algarvensis* , we inserted silver needles through individual worms and incubated these in radiolabeled $^{35}SO_4^{2-}$ under microaerobic and aerobic conditions. After exposure of the needles to an autoradiographic film, blots from the needles inserted in live worms under microaerobic conditions showed a positive signal from ^{35}S-labelled sulphide that had precipitated on the needles, whereas under the same conditions a needle inserted in a formalin-fixed worm remained unlabelled (data not shown). This indicates that sulphate is reduced to sulphide during dissimilatory sulphate respiration by the δ-symbionts of *O. algarvensis* under microaerobic conditions. Sulphate respiration appears to be inhibited at high O_2 concentrations, on the basis of the absence of a sulphide precipitate on needles inserted in worms incubated under aerobic conditions. We determined the sulphate reduction rates (SRRs) of the symbionts by incubating *O. algarvensis* in $^{35}SO_4^{2-}$ under microaerobic conditions (Table 1). In live worms, we measured SRRs of 53 - 534 pmol per worm per day, whereas SRRs in heat-killed worms under the same conditions were below detection limits. Sulphate was reduced to sulphide despite the absence of an external electron donor in the

incubation medium. Endogenous electron donors that could have been used by the sulphate-reducing symbionts are fermentation products from the host such as succinate, propionate and acetate. These substrates accumulate during anaerobic metabolism under low oxygen concentrations in other marine tubificids[17] and many other aquatic invertebrates[18]. Under fully aerobic conditions, SRRs in live worms were below detection limits, indicating, as in the silver needle experiments, that high oxygen concentrations inhibit sulphate reduction. This corresponds well with observations on SRB in pure cultures, where most species are temporarily oxygen tolerant but not able to respire sulphate in the presence of high oxygen concentrations[19]. On the basis of the numbers of d-symbionts in *O. algarvensis* as estimated by FISH, SRRs in these hosts (0.07 - 0.36 fmol per cell per day) are lower than those of SRB in pure cultures with saturating substrate concentrations (0.2-50 fmol per cell per day)20 but in the same range as those estimated for free living SRB in marine sediments (0.01-0.09 fmol per cell per day)[21]. SRRs in the worms on a volumetric basis are extremely high (690 - 19,600 nmol cm^{-3} per day) and comparable with rates measured in microbial mats (2,880-43,200 nmol cm^{-3} per day)[22]. To estimate the importance to the sulphide-oxidizing symbionts of internally produced sulphide compared with the import of external sulphide from the sediment, we compared the fluxes from these two sulphide sources. Dissolved sulphide concentrations in pore waters of *O. algarvensis* collection sites were extremely low: <14-76 nM (26±21, n = 9) at 5-15 cm sediment depth, with no trend with sediment depth or location. Correspondingly, sulphide flux from the environment into the worm was <50-270 pmol per worm per day (93±75, n = 9). Internal sulphide production from the sulphate-reducing symbionts on the basis of SRRs was 120-1,530 pmol per worm per day (640±780, n = 3). Thus, internal sulphide production is typically considerably higher than sulphide flux from the sediment, indicating that under prevalent conditions this symbiosis appears to be independent of an external source of sulphide. The coexistence of sulphate-reducing and sulphide-oxidizing bacteria as endosymbionts in *O. algarvensis* indicates that these are engaged in a syntrophic sulphur cycle in which oxidized and reduced sulphur compounds are recycled between the two symbionts (Fig. 4). For net growth of the symbiotic association, uptake of organic or inorganic sources of carbon and electron donors from the environment is required. As sulphide flux calculations indicate that the electron donor for the sulphide oxidizers is typically

supplied internally, external reductants must be imported through the sulphate reducers. Given the metabolic diversity of SRB, in particular within the *Desulfosarcina* group, where both chemoorganotrophic and chemoautotrophic metabolism occurs, dissolved organic carbon and hydrogen are possible sources of reducing power. Migration of the worms between oxidized and reduced sediments, as described for other gutless oligochaetes[23], would provide the host and its sulphide-oxidizing symbionts with oxygen and the sulphate reducers with reductants. The benefits of this endosymbiotic sulphur cycle to its partners are clear. Cycling of oxidized and reduced sulphur compounds between the two symbionts would result in increased protein yields, as shown for continuous cultures with free-living SRB and sulphide-oxidizing bacteria[24]. Furthermore, fermentation products of the host that accumulate during anaerobic metabolism would provide the sulphate reducers with an ideal energy source, aid the hosts in the removal of these undesirable endproducts and recycle metabolites that would otherwise be lost to the symbiosis. A further advantage for the host and its thioautotrophic symbiont is that they are not limited by the external presence of reduced sulphur compounds, given the endogenous production of sulphide by the sulphate-reducing symbiont. Thus the uptake of a sulphate reducer may have enabled these hosts to colonize new habitats and extend their geographic distribution.

Methods

For more details see Supplementary Information.

Specimens

O. algarvensis was collected in 1998-2000 from sediments at 6-8m water depth in a bay off Capo di San Andrea (Elba, Italy) by SCUBA divers. *I. leukodermatus* specimens used as negative controls for the DSR amplifications were collected in Bermuda in 1997.

Pore water sulphide

Pore water was collected at 5, 10 and 15 cm depth at the *O. algarvensis* collection sites by SCUBA divers with immediate fixation of the samples in zinc acetate. In June 1999, October 1999 and January 2000, 1-2ml of pore water per sample was collected and total sulphide concentrations were below the detection limit of 0.4 mM in all samples. In June 2000 the detection limit was lowered to 14 nM by collecting greater amounts of pore water (40-60ml per sampling site) using samplers connected to evacuated serum vials containing zinc chloride. Concentrations of total sulphide were determined colorimetrically[25].

Transmission electron microscopy and immunocytochemistry

O. algarvensis individuals were fixed and prepared for electron microscopy as described4. For Rubisco immunocytochemistry, specimens were treated as described in ref. 9. In each worm (n = 5) 50-100 symbionts were examined for labeling response.

DNA analyses

Three *O. algarvensis* individuals (and two *I. leukodermatus* specimens for DSR controls) were prepared singly for polymerase chain reaction (PCR) as described in ref. 7. DNA was isolated from *D. variabilis* DSM 2060 as described[16]. Amplifications were performed with primers specific for the bacterial 16S rRNA genes (8F and 1507R) or the DSR genes of SRB (DSR1F and DSR4R)[16]. PCR products were cloned and grouped using amplified ribosomal DNA restriction analysis (ARDRA). Two or three clones per individual from dominant ARDRA

groups were partially sequenced and at least one clone per individual from each ARDRA group was sequenced fully in both directions. Alignments, treeing and phylogenetic analyses (distance, parsimony and maximum likelihood) were performed with the ARB program (http://www.mikro.biologie.tu-muenchen.de/pub/ARB/).

FISH

Five worms were fixed and prepared for FISH as described[7]. Sections were hybridized as described[7] with Cy3 and Cy5 labeled group-specific probes (GAM42a and DSS658) as well as two specific probes designed for this study (OalgGAM445: 5´-CTCGAGATCTTTCTTCCC-3´; OalgDEL136: 5´-GTTATCCCCGACTCGGGG-3´). Specificity of the probes was tested with reference strains as described[7].

$^{35}SO_4^{2-}$ incubations

For silver needle experiments worms were incubated in $Na^{35}SO_4^{2-}$ and 0.2-µm pore-size filtered seawater from the collection site. The medium was solidified with agar and the worms paralyzed with lidocaine (2mgml-1) to prevent excessive movements during insertion with silver needles (99.999% pure 50µM Ag wire, tapered to a <1 µm tip). Incubations were run for 2-3 h under microaerobic (2-4mM O_2) and aerobic (200µM O_2) conditions with monitoring of oxygen concentrations with microsensors (two replicate experiments per O_2 concentration with one worm per incubation). In a control experiment at 2-4 µM O_2 with a dead worm, the specimen was fixed in 4% formalin in seawater and subsequently washed in filtered seawater. After removal, the needles were washed in 50mM Na_2SO_4 solution and exposed to autoradiography film. Results were similar in replicate experiments.

For determination of SRRs we incubated five worms per experiment for 2-3 h in seawater with $Na^{35}SO_4^{2-}$ using agar or sand as substrates. Sand incubations were prepared and run in the same manner as the agar experiments (see above), but worms were not paralyzed and moved freely in sand from the collection site that had been washed and combusted at 480 °C. Oxygen concentrations were not monitored during the sand incubations. For control experiments, specimens were

heat killed in water at 70 °C for 10 min. SRRs were determined using the one-step acidic Cr-II method to separate reduced ^{35}S (ref. 26).

Elemental sulphur analyses

S_0 was extracted individually from five worms with methanol and quantified by high-performance liquid chromatography as described[27].

Flux calculations

Sulphide flux (Q) from the environment was calculated using the following equation[28]: $Q = 2pID_{eff}C_p/ln(1+2d/d)$, where the length of the worm (l) is 1 cm, the effective diffusion coefficient of total sulphide in sediment (D_{eff}) is 1.39 x $10^{-9}m^2 s^{-1}$, C_p the concentration of total sulphide in the pore water, the mass boundary layer (d) is 100 µm and the diameter (d) of the worm is 200 µm (see Supplementary Information). All assumptions are conservative and result in an overestimation of sulphide flux from the sediment (see Supplementary Information). Internal sulphide production from the symbionts is based on SRRs measured in worms incubated in sand (Table 1), assuming that all sulphide produced is consumed by the sulphide-oxidizing symbionts. SRRs in the worms are assumed to be underestimated, given that no external electron donor was used and experimental conditions are suboptimal in comparison to the natural environment.

Received 25 September 2000; accepted 26 February 2001.

1 Maynard Smith, J. & Szathmáry, E. The Major Transitions in Evolution (Oxford Univ. Press, Oxford 1995).

2 Distel, D. L., Lee, H. K.-W. & Cavanaugh, C. M. Intracellular coexistence of methano- and thioautotrophic bacteria in a hydrothermal vent mussel. Proc. Natl Acad. Sci. USA 92, 9598-9602 (1995).

3 Rowan, R., Knowlton, N., Baker, A. & Jara, J. Landscape ecology of algal symbionts creates variation in episodes of coral bleaching. Nature 388, 265-266 (1997).

4 Giere,O., Erséus, C. & Stuhlmacher, F. A new species of *Olavius* (*Tubificidae, Phallodrilinae*) from the Algarve Coast in Portugal, the first East Atlantic gutless oligochaete with symbiotic bacteria. Zool. Anzeiger 237, 209-214 (1998).

5 Giere, O. & Langheld, C. Structural organization, transfer and biological fate of endosymbiotic bacteria in gutless oligochaetes. Mar. Biol. 93, 641-650 (1987).

6 Giere,O., Nieser, C.,Windoffer, R. & Erséus, C. A comparative structural study on bacterial symbioses of Caribbean gutless *Tubifidae (Annelida, Oligochaeta*). Acta Zool. 76, 281-290 (1995).

7 Dubilier, N. et al. Phylogenetic diversity of bacterial endosymbionts in the gutless marine oligochaete *Olavius loisae* (*Annelida*). Mar. Ecol. Prog. Ser. 178, 271-280 (1999).

8 Dubilier, N., Giere, O., Distel, D. L. & Cavanaugh, C. M. Characterization of chemoautotrophic bacterial symbionts in a gutless marine worm (*Oligochaeta, Annelida*) by phylogenetic 16S rRNA sequence analysis and in situ hybridization. Appl. Environ. Microbiol. 61, 2346-2350 (1995).

9 Krieger, J., Giere, O. & Dubilier, N. Localization of RubisCO and sulfur in endosymbiotic bacteria of the gutless marine oligochaete *Inanidrilus leukodermatus (Annelida).* Mar. Biol. 137, 239-244 (2000).

10 Vetter, R. D. & Fry, B. Sulfur contents and sulfur-isotope compositions of thiotrophic symbioses in bivalve molluscs and vestimentiferan worms. Mar. Biol. 132, 453-460 (1998).

11 Kuhnigk, T., Branke, J., Krekeler, D., Cypionka, H. & König, H. A feasible role of sulfate-reducing bacteria in the termite gut. System. Appl. Microbiol. 19, 139-149 (1996).

12 Morvan, B., Bonnemoy, F., Fonty, G. & Gouet, P. Quantitative determination of H_2-utilizing acetogenic and sulfate-reducing bacteria and methanogenic archaea from digestive tracts of different mammals. Curr. Microbiol. 32, 129-133 (1996).

13 Fenchel, T. & Ramsing, N. B. Identification of sulphate-reducing ectosymbiotic bacteria from anaerobic ciliates using 16S rRNA binding oligonucleotide probes. Arch. Microbiol. 158, 394-397 (1992).

14 Bussmann, I. & Reichardt,W. Sulfate-reducing bacteria in temporarily toxic sediments with bivalves. Mar. Ecol. Prog. Ser. 78, 97-102 (1991).

15 Cottrell, M. T. & Cary, C. S. Diversity of dissimilatory bisulfite reductase genes of bacteria associated with the deep-sea hydrothermal vent polychaete *Alvinella pompejana*. Appl. Environ. Microbiol. 65, 1127-1132 (1999).

16 Wagner, M., Roger, A. J., Flax, J. L., Brusseau, G. A. & Stahl, D. A. Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration. J. Bacteriol. 180, 2975-2982 (1998).

17 Dubilier, N., Giere, O. & Grieshaber, M. K. Morphological and ecophysiological adaptations of the marine oligochaete *Tubificoides benedii* to sulfidic sediments. Am. Zool. 35, 163-173 (1995).

18 Grieshaber, M. K., Hardewig, I., Kreutzer, U. & Pörtner, H.-O. Physiological and metabolic responses to hypoxia in invertebrates. Rev. Physiol. Biochem. Pharmacol. 125, 43-147 (1994).

19 Barton, L. L. Sulfate-Reducing Bacteria (Plenum, New York, 1995).

20 JØrgensen, B. B. A comparison of methods for the quantification of bacterial sulfate reduction in coastal marine sediments. III. Estimation from chemical and bacteriological field data. Geomicrobiol. J. 1, 49-64 (1978).

21 Sahm, K.,MacGregor, B. J., JØrgensen, B. B.& Stahl,D. A. Sulphate reduction and vertical distribution of sulphate-reducing bacteria quantified by rRNA slot-blot hybridization in a coastal marine sediment. Environ. Microbiol. 1, 65-74 (1999).

22 Canfield, D. E. & Des Marais, D. J. Biogeochemical cycles of carbon, sulfur, and free oxygen in a microbial mat. Geochim. Cosmochim. Acta 57, 3971-3984 (1993).

23 Giere, O., Conway, N. M., Gastrock, G. & Schmidt, C. `Regulation' of gutless annelid ecology by endosymbiotic bacteria. Mar. Ecol. Prog. Ser. 68, 287-299 (1991).

24 van den Ende, F. P.,Meier, J. & van Gemerden, H. Syntrophic growth of sulfate-reducing bacteria and colorless sulfur bacteria during oxygen limitation. FEMS Microbiol. Ecol. 23, 65-80 (1997).

25 Cline, J. D. Spectrophotometric determination of hydrogen sulÆde in natural waters. Limnol. Oceanogr. 14, 454-458 (1969).

26 Fossing, H. & JØrgensen, B. B. Measurements of bacterial sulfate reduction in sediments: Evaluation of a single-step chromium reduction method. Biogeochemistry 8, 205-222 (1989).

27 Ferdelman, T. G. et al. Sulfate reduction and methanogenesis in a *Thioploca*-dominated sediment off the coast of Chile. Geochim. Cosmochim. Acta 61, 3065-3079 (1997).

28 Crank, J. The Mathematics of Diffusion (Oxford Univ. Press, New York, 1975).

Supplementary information is available on Nature's World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of Nature.

Acknowledgements

We thank F. Widdel and B. Barker JØrgensen for reviewing this manuscript; E. Llobet-Brossa and A. Boetius for discussions; the Hydra Institute for Marine Sciences on Elba for logistical support during collection of the worms; and J. Wulf, D. Lange, G. Eickert and A. Eggers for technical support. The Max-Planck Society and the German Research Foundation (DFG) provided financial support.

Correspondence and requests for materials should be addressed to N.D. (e-mail: ndubilie@mpi-bremen.de). GenBank accession numbers: 16S rRNA: γ-Proteobacteria symbiont AF328856, δ-Proteobacteria symbiont AF328857; DSR: δ-Proteobacteria symbiont AF244995, *D. variabilis* AF191907.

G.2.4 Lateral Gene Transfer of Dissimilatory (Bi)Sulfite Reductase Revisited

For this publication Michael Klein sequenced the 1.9 kb *dsrAB* fragments of 5 reference strains, performed the PCR control experiments and contributed to the writing.

Paper is submitted to Journal of Bacteriology Mai, 2004.

Lateral Gene Transfer of Dissimilatory (Bi)Sulfite Reductase Revisited

VLADIMIR ZVERLOV[1,6], MICHAEL KLEIN[1], SEBASTIAN LÜCKER[2], MICHAEL W. FRIEDRICH[4], JOSEF KELLERMANN[3], DAVID A. STAHL[5], ALEXANDER LOY[2], and MICHAEL WAGNER[2]*

[1] Department of Microbiology, Technical University of Munich, D-85350 Freising, Germany

[2] Department of Microbial Ecology, Institute of Ecology and Conservation Biology, University of Vienna, A-1090 Vienna, Austria

[3] Max-Planck-Institute for Biochemistry, D-82152 Martinsried, Germany

[4] Max-Planck-Institute for Terrestrial Microbiology, D-35043 Marburg, Germany

[5] Department of Civil and Environmental Engineering, University of Washington, Seattle, WA 98195, USA

[6] Russian Academy of Science, Institute of Molecular Genetics, Kurchatov Square, 123182 Moscow, Russia

* corresponding author; Address for correspondence:

Abteilung Mikrobielle Ökologie
Institut für Ökologie und Naturschutz
Universität Wien
Althanstr. 14
A-1090 Wien
Austria

Phone: +43 1 4277 54390
Fax: +43 1 4277 54389
Email: wagner@microbial-ecology.net

Short title: LATERAL GENE TRANSFER OF *dsrAB*

Abstract

Multiple lateral transfers of dissimilatory (bi)sulfite reductase genes (*dsrAB*) between major lineages of sulfate-reducing prokaryotes (SRPs) influenced the evolutionary history of this ancient enzyme, yet nothing is known about transfer mechanisms or identity of donor lineages. In this study an 8.9-kb genome fragment of the deltaproteobacterial SRP *Desulfobacula toluolica* carrying the entire *dsr* operon was sequenced in order to search for genetic traces indicative of the lateral gene transfer mechanism. However, in contrast to previously published data (Klein, M., M. Friedrich, A. J. Roger, P. Hugenholtz, S. Fishbain, H. Abicht, L. L. Blackall, D. A. Stahl, and M. Wagner. 2001. Multiple lateral transfers of dissimilatory sulfite reductase genes between major lineages of sulfate-reducing prokaryotes. J. Bacteriol. 183:6028-6035.), D*. toluolica* was found to possess an orthologous dsr operon. This result was confirmed by Southern hybridization, DsrAB protein purification in combination with N-terminal sequencing, and by *dsrAB* sequence analysis of its closest known relative *D. phenolica*. In addition, *Desulfobacterium anilini* and strain mXyS1 were identified by screening of *dsrAB* sequences of 16 SRP reference cultures as members of the putative donor lineage for those Gram-positive *Desulfotomaculum* species which laterally acquired a deltaproteobacterial (bi-) sulfite reductase.

INTRODUCTION

Dissimilatory (bi)sulfite reductase catalyzes the energy generating step during the anaerobic respiration of sulfite or sulfate and thus represents a key enzyme of all sulfite- and sulfate-reducing prokaryotes (11, 22, 33). Recently, the genes encoding the alpha- and beta-subunits of this enzyme (*dsrAB*) have been used to infer the evolutionary history of dissimilatory (bi)sulfite reductases. For this purpose, a *dsrAB* database containing 74 entries for described sulfate-reducing prokaryotes (SRPs) (representing all known major evolutionary lineages of this guild) and 2 for sulfite-reducing microorganisms has been established (9, 12, 14-17, 23, 31, 32). Comparison of 16S rRNA- and DsrAB-based phylogenetic trees revealed congruent topologies for many SRP lineages, suggesting an ancient origin of dissimilatory (bi)sulfite reductase (32). This finding is consistent with isotopic evidence for biological sulfate reduction at 3.47 Gyr ago (30). However, we now recognize that the distribution of *dsrAB* among sulfate-reducing species reflects a combination of divergence through speciation (vertical descent) and acquisition via lateral gene transfer from distantly related prokaryotes (15). For example, the archaeal SRPs of the genus *Archaeoglobus*, the deep-branching thermophilic SRPs of the genus *Thermodesulfobacterium*, as well as a large number of thermophilic Gram-positive *Desulfotomaculum* species, possess laterally acquired (bi)sulfite reductases. In addition, the deltaproteobacterial SRP *Desulfobacula toluolica* was postulated to have relatively recently acquired a xenologous (bi)sulfite reductase, since its close relatives, including *Desulfobacter latus*, have orthologous (bi)sulfite reductase genes. In the *dsrAB* tree, the putative xenologous *dsrAB* sequence of *D. toluolica* formed a well-supported monophyletic cluster with the xenologous *dsrAB* sequences of *Desulfotomaculum* species. Therefore, it was speculated that *D. toluolica* and the *Desulfotomaculum* species received

their *dsrAB* genes from a common but yet unidentified deltaproteobacterial donor lineage (15).

Descriptions of the evolutionary history of sulfate-reducing microorganisms will remain incomplete until a more comprehensive description of their contemporary diversity is available. For example, all xenologous *dsrAB* sequences so far identified have no close relationship to sequences from species having the orthologous versions of the enzyme. This suggests that the donor lineages have yet to be described or, alternatively, are no longer extant. Development of a more complete census of SRP diversity is being facilitated by the now widespread use of *dsrAB* gene fragments as marker molecules for PCR-based, cultivation-independent characterization of natural samples (2, 3, 5, 20, 25). These analyses support the general utility of this molecular approach. For example, several environmentally retrieved *dsrAB* sequences are closely related to the putative *dsrAB* sequence of *D. toluolica* (2, 13, 26). They have also revealed the existence of many novel sulfite- and sulfate-reducing prokaryotes only distantly related to recognized members of cultured guilds. Thus, the diversity of sulfite- and sulfate-reducing prokaryotes has not yet been circumscribed by traditional cultivation approaches (1, 4, 8, 19), as is needed for a more complete accounting of the evolution of this important functional assemblage of microorganisms.

The objectives of this study were to better resolve the evolutionary history and mechanisms of lateral gene transfer of *dsrAB* by more fully characterising described species and by more extensive sequence analysis of a *dsr* operon, and flanking genomic regions, encoding xenologous *dsrAB* genes. We sequenced a chromosomal fragment of *D. toluolica* containing the entire *dsr* operon and its flanking genomic regions in order to (i) determine which additional genes might have been co-transferred with the *dsrAB* genes, and (ii) reveal genetic traces indicative of the responsible transfer mechanism. Unexpectedly, our findings demonstrated that *D. toluolica* contains a single orthologous

dsr operon different in sequence from the previously published *dsrAB* gene fragment of this organism (15). In addition, we show by comparative sequence analyses of the *dsrAB* genes of *Desulfobacterium anilini* and the SRP strain mXyS1 that they might represent the donor lineage for those Gram-positive SRPs which carry deltaproteobacterial *dsr* genes.

MATERIALS AND METHODS

SRP strains. Cultures of *Desulfobacula toluolica* (DSM 7467), *Desulfobacula phenolica* (DSM 3384), *Desulfospira joergensenii* (DSM 10085), *Desulfonema ishimotonii* (DSM 9680), *Desulfonema limicola* (DSM 2076), *Desulfobacterium anilini* (DSM 4660), the sulfate-reducing strain mXyS1 (DSM 12567), *Desulfotalea arctica* (DSM 12342), *Desulfovibrio halophilus* (DSM 5663), *Desulfovibrio oxyclinae* (DSM 11498), *Desulfovibrio zosterae* (DSM 11974), *Desulfovibrio aminophilus* (DSM 12254), *Desulfovibrio gabonensis* (DSM 10636), *Desulfovibrio carbinolicus* (DSM 3852), *Desulfotomaculum nigrificans* (DSM 574), *Desulfotomaculum halophilum* (DSM 11559), and *Sporotomaculum hydroxybenzoicum* (DSM 5475) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany). The strain *Archaeoglobus veneficus* SNP6 (DSM 11195) (containing plasmid XY), had been deposited in the DSMZ by Prof. Dr. K. O. Stetter, Lehrstuhl für Mikrobiologie, Universität Regensburg, Universitätsstr. 31, D-93053 Regensburg, Germany. To confirm the identity of these species, PCR-amplified 16S rRNA genes were cloned into *E. coli*, sequenced and analyzed as described previously (27).

Extraction of high-molecular weight DNA. 0.5 g (wet weight) of cells were harvested and washed twice in 0.9% NaCl solution. Cells were lysed by incubation with lysozyme buffer (20mM Tris-HCl pH 7.5; 25 mM EDTA pH 8.0; 75 mM NaCl; 1 mg/ml lysozyme) for 30 min at room temperature. Subsequently, lysates were mixed gently with

0.1 volume 10% SDS and 1 mg ml^{-1} proteinase K, and incubated for 1-2 h at room temperature with occasional gentle agitation. Nucleic acids were separated from other cell compounds by gentle agitation with 0.33 volume of 5 M NaCl and 1 volume of chloroform for 30 min at room temperature. After centrifugation (10 min at 10,000 g), the upper aqueous phase was transferred into a new reaction tube. One volume of isopropanol was added to precipitate nucleic acids from the solution. High-molecular weight DNA was carefully extracted from the solution, washed with 70% ethanol, dried, and resuspended in 500 µl double-distilled water for two days at 4°C. Dissolved DNA was stored at –20°C.

PCR amplification and cloning of *dsrAB* genes. PCR amplification was performed with 50-100 ng of DNA. An approximately 1.9-kb *dsrAB* fragment was amplified from genomic DNA by using the primers DSR1F and DSR4R (32) or their variants (19).

Negative controls (no DNA) were included in all PCR amplification experiments. PCR reaction mixtures containing 25 pM of each primer were prepared in a total volume of 50 µl by using the Expand High Fidelity PCR system (Roche, Mannheim, Germany) according to the manufacturer's instructions. Thermal cycling was carried out by an initial denaturation step at 94°C for 1 min, followed by 30 cycles of denaturation at 94°C for 20 s, annealing at 52°C for 30 s, and elongation at 72°C for 2 min. Cycling was completed by a final elongation step at 72°C for 7 min. The presence and sizes of the amplification products were determined by agarose (1%) gel electrophoresis. Ethidium bromide-stained bands were digitally recorded by using a video documentation system (Cybertech, Hamburg, Germany).

PCR products were purified and ligated into the cloning vector pCR-XL-TOPO of the TOPO XL cloning kit (Invitrogen GmbH, Karlsruhe, Germany) as described previously (19).

Polynucleotide probe. For the generation of a digoxigenin-labeled, *dsrA*-targeted polynucleotide probe, an approximately 150 bp *dsrA* fragment was amplified from *D. toluolica* pure culture DNA by using the degenerate PCR primers DsrA415F (5'-tatca(ag)gatgagct(gt)catcg(ct)cc-3') and DsrA542R (5'-ac(ct)gc(agt)tcctgatcaat(agc)cggatat-3'). PCR reaction mixtures containing 25 pM of each primer were prepared in a total volume of 50 µl by using the PCR DIG probe synthesis kit (Roche) according to the manufacturer's instructions. Thermal cycling and analysis of PCR products were carried out as mentioned above.

Southern hybridization. Analysis of genomic DNA by Southern hybridization was carried out according to standard (28) or supplier protocols. Restriction enzymes *Eco*RI, *Bam*HI, *Xba*I, *Sma*I, *Sal*I, *Pst*I, *Kpn*I, and *Hind*III (Fermentas GmbH, St. Leon-Rot, Germany) were used separately for digestion of 8-10 µg of genomic DNA. Agarose gel-separated DNA fragments were blotted onto a nylon membrane (Biodyne A transfer membrane, Pall GmbH, Dreieich, Germany) and hybridized with a digoxigenin-labeled, *dsrA*-targeted polynucleotide probe. Subsequently, *dsrA*-positive fragments were visualized by colorimetric detection by using anti-digoxigenin antibodies tagged with alkaline phosphatase and the substrate NBT/BCIP according to the instructions of the DIG nucleic acid detection kit (Roche).

Cloning of a *dsr* operon-containing DNA fragment from *D. toluolica*. Genomic DNA of *D. toluolica* was partially digested with *Mbo*I (isochizomer of *Sau*3A) (Fermentas GmbH) to yield maximal amounts of fragments at approximately 15-kb length. DNA fragments (10 to 15-kb) were extracted from the agarose gel by using the QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany) and ligated to *Bam*HI-digested λBlueSTAR arms according to the λBlueSTAR *Bam*H I Arms kit instruction manual (Novagen, Darmstadt, Germany). *DsrA*-containing phages were identified by plaque hybridization (28) with a digoxigenin-labeled, *D. toluolica dsrA*-targeted polynucleotide

probe. Immobilization of λ plaques on nylon membranes (Roche), hybridization, and subsequent colorimetric detection were performed according to standard protocols (28) as described above. *DsrA*-containing λBlueSTAR phages were subjected to Cre recombinase-mediated excision of plasmids (λBlueSTAR *Bam*H I arms kit, Novagen). Plasmids from *E. coli* BM25.8 cells were recovered with the QIAprep spin miniprep kit (Qiagen) and transformed into *E. coli* DH5α. Finally, plasmid DNA was purified from *E. coli* DH5α cells for sequencing.

DNA sequencing. Cloned *dsrAB* sequences were determined with a 4200L automated Li-Cor Long Reader DNA sequencer (MWG, Ebersberg, Germany) by using the vector-specific M13 primers. The complete sequence and flanking regions of the *dsr* operon of *D. toluolica* were sequenced by using the promoter-specific primers T3 (5'-AATTAACCCTCACTAAAGGG-3') and T7 (5'-TAATACGACTCACTATAGGG-3'), and by subsequent primer walking.

16S rRNA and DsrAB phylogeny. Phylogenetic analyses were performed on alignments of 16S rRNA gene sequences or the deduced amino acid sequences of *dsrAB* as outlined previously (15, 19).

Purification of DsrA and DsrB of *D. toluolica* and N-terminal sequencing. 0.5 g (wet weight) of *D. toluolica* cells were washed with Tris-HCl (10 mM, pH 7.6), resuspended in 5 ml Tris-HCl (10 mM, pH 7.6), and lysed by ultrasonic treatment. The lysate was centrifuged twice at 18,000 g for 20 min to clear the cell extract. The supernatant was applied to a HiTrap Q HP ion exchange column (1 ml, Amersham Biosciences) equilibrated with Tris-HCl (10 mM, pH 7.6). Proteins were eluted at 1 ml min^{-1} with an increasing linear gradient to 0.5 M NaCl and collected in 1 ml fractions. Fractions that showed maximal absorption at 390 nm ($A_{280}/A_{390} < 4$) (7) were pooled and applied to 2 ml Vivaspin concentrators (30,300 MWCO PES; Vivascience AG, Hannover, Germany) for ultrafiltration. Concentrated proteins were separated by nondenaturing 10%-

polyacrylamide gel electrophoresis (PAGE). The Dsr protein band was identified by its brown colour, and was extracted from the gel in 2 ml Tris-HCl (10 mM, pH 7.6). For N-terminal amino acid sequence analysis proteins were separated on a 10% SDS-polyacrylamide gel and subsequently transferred onto a polyvinylidene difluoride membrane as described previously (6). The blotted protein bands were stained with Coomassie blue for 1 min. After de-staining, the bands were excised and sequenced on a pulsed liquid phase sequencer Procise 492 (Applied Biosystems, Foster City, USA).

Nucleotide sequence accession numbers. Sequences determined in this study were deposited at GenBank under accession numbers AJ457136 (*dsr* operon sequence of *D. toluolica*), AF482452, AF482455, AF482456, AF482461, AF482466, AF551758, AF551759, AY626024-AY626034 (*dsrAB* sequences), and AY626035 (16S rRNA sequence of *D. carbinolicus*).

RESULTS AND DISCUSSION

Southern hybridization of DNA restriction fragments from *D. toluolica* with a polynucleotide probe targeting an approximately 150 nucleotide region of *dsrA* showed that *D. toluolica* contains a single *dsr* operon in its genome (Figure 1). After cloning of genomic DNA of *D. toluolica* into the lambda vector, phages containing the *dsrA* gene were identified by plaque hybridization with the same polynucleotide probe as used for Southern hybridization. An 8.9 kb insert of *D. toluolica* DNA in a phage clone which hybridized with the *dsrA* polynucleotide probe was sequenced by primer walking. Annotation revealed that this fragment contained the entire *dsr* operon consisting of the genes *dsrA*, *dsrB*, *dsrD* and *dsrN* (Figure 2A). While *dsrA* and *dsrB* encode the alpha- and beta-subunit, respectively, of the dissimilatory (bi)sulfite reductase (14), *dsrN* is similar to *cbiA*, a cobyrinic acid a,c-diamide synthase, and has been suggested to be responsible for

the amidation of the siroheme cofactor of the (bi)sulfite reductase (16). Structural similarities of the crystallized DsrD protein (encoded by *dsrD*) to DNA-binding proteins indicates a possible role of DsrD in the regulation of *dsr* gene transcription (21). The *dsrABDN* operon structure of *D. toluolica* has previously been detected in other deltaproteobacterial SRPs (Figure 2B). Surprisingly, comparative sequence analysis of *dsrA* and *dsrB* of *D. toluolica* revealed that these genes were clearly different (less than 66% nucleic acid similarity) from the *dsrAB* gene fragments of this organism which were previously published (15). Sequence analysis of the target sites of the PCR primers used for *D. toluolica dsrAB* gene fragment amplification by Klein et al. (15) revealed that *dsrA* has three mismatches with primer DSR1F and *dsrB* has one mismatch with primer DSR4R. Therefore, the *dsrAB* gene fragment of *D. toluolica* could not be amplified with these primers (data not shown). Thus, the *dsrAB* sequence of *D. toluolica* reported previously (15) most likely originated from a laboratory contamination and the *dsr* operon sequence reported in the present paper is the actual *dsr* sequence of *D. toluolica*. This conclusion is further supported by the fact that the newly determined *dsrAB* sequence of *D. toluolica* phylogenetically clusters together with *dsrAB* sequences of the genus *Desulfobacter* (Figure 3). Since this affiliation is consistent with the respective 16S rRNA gene tree topology, *D. toluolica* contains an orthologous *dsr* operon. Two additional experiments were performed to further support this finding. Firstly, the sequence of a 1.9-kb *dsrAB* PCR fragment of *Desulfobacula phenolica*, the closest known relative of *D. toluolica*, was determined and found to be almost identical (97.5% and 99.5% *dsrA* and *dsrB* nucleic acid similarity, respectively) to the respective gene sequences of *D. toluolica* (Figure 3). Secondly, the DsrA and DsrB enzyme subunits were purified from cell extracts of *D. toluolica* (Figure 4) and N-terminal sequencing of the DsrA subunit (AKHETPFL) revealed 100% accordance with the respective amino acid stretch predicted from the *dsrA* gene sequence.

The sequence of *D. toluolica* extends the number of available *dsr* operon sequences from SRPs to eight. In addition complete *dsrAB* sequences are available from *D. desulfuricans* (24) and the *Desulfovibrio*-related human pathogen *Bilophila wadsworthia* (18). These sequences can be used to validate the commonly-used PCR primers for *dsrAB* amplification from SRP pure cultures and from environmental or clinical samples (Table 1). With the exception of *D. toluolica*, the primers DSR1F and DSR4R (32) and their recently published variants (19) perfectly match the available complete *dsrAB* genes. For maximum coverage of SRPs, it is recommended to add primer set DSR1Fc (5'-ACC CAT TGG AAA CAT G-3') and DSR4Rd (5'-GTG TAG CAG TTA CCA CA-3'), targeting *dsrAB* of *D. toluolica*, to the primer variant mixture. Furthermore, PCR annealing stringency should be kept low in environmental *dsrAB* diversity surveys because it is likely that additional sequence variants in the *dsrAB* PCR primer binding sites exist.

After the correction of the *dsrAB* sequence of *D. toluolica*, no *dsrAB* sequence of a deltaproteobacterial SRP has been published which is closely related to the xenologous *dsrAB* sequences of a group of Gram-positive SRP of the genus *Desulfotomaculum* (15). In order to identify the deltaproteobacterial SRP lineage which may have acted as *dsrAB* donor for these Gram-positive SRPs, a PCR-based *dsrAB* screening of 16 SRPs was initiated. Interestingly, the *dsrAB* sequences of the deltaproteobacterial SRPs *Desulfobacterium anilini* (29) and strain mXyS1 (10) formed a well-supported monophyletic branch with the xenologous *dsrAB* sequences of the Gram-positive SRPs (Figure 3). This affiliation, which is consistently inferred using different treeing methods, suggests that *D. anilini* and strain mXyS1 either acquired their (bi)sulfite reductase genes from the same unknown donor lineage as the Gram-positive SRPs, or that these two organisms are members of the *dsrAB* lineage which served as donor for the Gram-positive

SRPs. Since *D. anilini* and strain mXyS1, which are marine mesophiles, form an independent lineage within the *"Deltaproteobacteria"* in the 16S rRNA and *dsrAB* trees (apart from the xenologous Gram-positive bacteria) (Figure 3), this lineage is the most parsimonious *dsrAB* donor candidate for the Gram-positive SRPs.

As *Sporotomaculum hydroxybenzoicum* forms a monophyletic branch in the 16S rRNA tree together with *Desulfotomaculum* species known to have received deltaproteobacterial *dsrAB*, it was not unexpected that *S. hydroxybenzoicum* also contains a xenologous *dsrAB* sequence. The phylogenetic affiliations of the 13 remaining novel *dsrAB* sequences were found to be largely congruent with the respective 16S rRNA phylogeny of the organisms (Figure 3). This observation lends additional weight to our current perception that the dissimilatory (bi)sulfite reductase is an ancient enzyme whose evolutionary history is largely consistent with vertical transmission but has also been influenced by periodic lateral gene transfer events.

ACKNOWLEDGMENTS

The authors thank Natuschka Lee for database maintenance, Gerda Harms for valuable discussion and Michael Taylor for critical review of the manuscript. Christian Baranyi, Stephan Duller, and Sibylle Schadhauser are acknowledged for their excellent technical assistance. This research was supported by grants of the bmb+f (01 LC 0021 subproject 2 in the framework of the BIOLOG program) and of the DFG (in the framework of the project "Degradation of marine pollutants by cyanobacterial mats - an interdisciplinary approach") to MW, and by a Marie Curie Intra-European Fellowship (VENTSULFURMICDIV) within the 6th European Community Framework Programme to AL. DAS was supported by Grant DEB-0213186 from the US National Science Foundation.

REFERENCES

1. **Baker, B. J., D. P. Moser, B. J. MacGregor, S. Fishbain, M. Wagner, N. K. Fry, B. Jackson, N. Speolstra, S. Loos, K. Takai, B. S. Lollar, J. Fredrickson, D. Balkwill, T. C. Onstott, C. F. Wimpee, and D. A. Stahl.** 2003. Related assemblages of sulphate-reducing bacteria associated with ultradeep gold mines of South Africa and deep basalt aquifers of Washington State. Environ. Microbiol. **5:**267-277.
2. **Castro, H., K. R. Reddy, and A. Ogram.** 2002. Composition and function of sulfate-reducing prokaryotes in eutrophic and pristine areas of the Florida Everglades. Appl. Environ. Microbiol. **68:**6129-6137.
3. **Chang, Y. J., A. D. Peacock, P. E. Long, J. R. Stephen, J. P. McKinley, S. J. Macnaughton, A. K. Hussain, A. M. Saxton, and D. C. White.** 2001. Diversity and characterization of sulfate-reducing bacteria in groundwater at a uranium mill tailings site. Appl. Environ. Microbiol. **67:**3149-3160.
4. **Dhillon, A., A. Teske, J. Dillon, D. A. Stahl, and M. L. Sogin.** 2003. Molecular characterization of sulfate-reducing bacteria in the Guaymas Basin. Appl. Environ. Microbiol. **69:**2765-2772.
5. **Dubilier, N., C. Mulders, T. Ferdelman, D. de Beer, A. Pernthaler, M. Klein, M. Wagner, C. Erseus, F. Thiermann, J. Krieger, O. Giere, and R. Amann.** 2001. Endosymbiotic sulphate-reducing and sulphide-oxidizing bacteria in an oligochaete worm. Nature **411:**298-302.
6. **Eckerskor, C., and F. Lottspeich.** 1989. Internal amino acid sequence analysis of proteins separated by gel electrophoresis after tryptic digestion in polyacrylamide matrix. Chromatographia **28:**92-94.
7. **Fauque, G., A. R. Lino, M. Czechowski, L. Kang, D. V. DerVartanian, J. J. Moura, J. LeGall, and I. Moura.** 1990. Purification and characterization of bisulfite reductase (desulfofuscidin) from *Desulfovibrio thermophilus* and its complexes with exogenous ligands. Biochim. Biophys. Acta **1040:**112-118.
8. **Fishbain, S., J. G. Dillon, H. L. Gough, and D. A. Stahl.** 2003. Linkage of high rates of sulfate reduction in Yellowstone hot springs to unique sequence types in the dissimilatory sulfate respiration pathway. Appl. Environ. Microbiol. **69:**3663-3667.
9. **Friedrich, M. W.** 2002. Phylogenetic analysis reveals multiple lateral transfers of adenosine-5'-phosphosulfate reductase genes among sulfate-reducing microorganisms. J. Bacteriol. **184:**278-289.

10. **Harms, G., K. Zengler, R. Rabus, F. Aeckersberg, D. Minz, R. Rossello-Mora, and F. Widdel.** 1999. Anaerobic oxidation of o-xylene, m-xylene, and homologous alkylbenzenes by new types of sulfate-reducing bacteria. Appl. Environ. Microbiol. **65:**999-1004.

11. **Hatchikian, E. C., and J. G. Zeikus.** 1983. Characterization of a new type of dissimilatory sulfite reductase present in *Thermodesulfobacterium commune*. J. Bacteriol. **153:**1211-1220.

12. **Hipp, W. M., A. S. Pott, N. Thum-Schmitz, I. Faath, C. Dahl, and H. G. Trüper.** 1997. Towards the phylogeny of APS reductases and sirohaem sulfite reductases in sulfate-reducing and sulfur-oxidizing prokaryotes. Microbiology **143:**2891-2902.

13. **Joulian, C., N. B. Ramsing, and K. Ingvorsen.** 2001. Congruent phylogenies of most common small-subunit rRNA and dissimilatory sulfite reductase gene sequences retrieved from estuarine sediments. Appl. Environ. Microbiol. **67:**3314-3318.

14. **Karkhoff-Schweizer, R. R., D. P. Huber, and G. Voordouw.** 1995. Conservation of the genes for dissimilatory sulfite reductase from *Desulfovibrio vulgaris* and *Archaeoglobus fulgidus* allows their detection by PCR. Appl. Environ. Microbiol. **61:**290-296.

15. **Klein, M., M. Friedrich, A. J. Roger, P. Hugenholtz, S. Fishbain, H. Abicht, L. L. Blackall, D. A. Stahl, and M. Wagner.** 2001. Multiple lateral transfers of dissimilatory sulfite reductase genes between major lineages of sulfate-reducing prokaryotes. J. Bacteriol. **183:**6028-6035.

16. **Larsen, O., T. Lien, and N. K. Birkeland.** 2000. Characterization of the desulforubidin operons from *Desulfobacter vibrioformis* and *Desulfobulbus rhabdoformis*. FEMS Microbiol. Lett. **186:**41-46.

17. **Larsen, O., T. Lien, and N. K. Birkeland.** 2001. A novel organization of the dissimilatory sulfite reductase operon of *Thermodesulforhabdus norvegica* verified by RT-PCR. FEMS Microbiol. Lett. **203:**81-85.

18. **Laue, H., M. Friedrich, J. Ruff, and A. M. Cook.** 2001. Dissimilatory sulfite reductase (desulfoviridin) of the taurine-degrading, non-sulfate-reducing bacterium *Bilophila wadsworthia* RZATAU contains a fused DsrB-DsrD subunit. J Bacteriol **183:**1727-33.

19. **Loy, A., K. Küsel, A. Lehner, H. L. Drake, and M. Wagner.** 2004. Microarray and functional gene analyses of sulfate-reducing prokaryotes in low sulfate, acidic fens reveal stable depth diversity of recognized genera and site-specific novel lineages. Appl. Environ. Microbiol. **submitted**.

20. **Loy, A., A. Lehner, N. Lee, J. Adamczyk, H. Meier, J. Ernst, K.-H. Schleifer, and M. Wagner.** 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.

21. **Mizuno, N., G. Voordouw, K. Miki, A. Sarai, and Y. Higuchi.** 2003. Crystal structure of dissimilatory sulfite reductase D (DsrD) protein-possible interaction with B- and Z-DNA by its winged-helix motif. Structure (Camb) **11:**1133-40.

22. **Molitor, M., C. Dahl, I. Molitor, U. Schäfer, N. Speich, R. Huber, R. Deutzmann, and H. G. Trüper.** 1998. A dissimilatory sirohaem-sulfite-reductase-type protein from the hyperthermophilic archaeon *Pyrobaculum islandicum*. Microbiology **144:**529-541.

23. **Mori, K., H. Kim, T. Kakegawa, and S. Hanada.** 2003. A novel lineage of sulfate-reducing microorganisms: *Thermodesulfobiaceae* fam. nov., *Thermodesulfobium narugense*, gen. nov., sp. nov., a new thermophilic isolate from a hot spring. Extremophiles **7:**283-290.

24. **Morse, R., G. R. Gibson, and M. D. Collins.** 2000. Secondary structure analysis of the dissimilatory sulphite reductase in *Desulfovibrio desulfuricans*. Lett. Appl. Microbiol. **30:**375-378.

25. **Nakagawa, T., J. Ishibashi, A. Maruyama, T. Yamanaka, Y. Morimoto, H. Kimura, T. Urabe, and M. Fukui.** 2004. Analysis of dissimilatory sulfite reductase and 16S rRNA gene fragments from deep-sea hydrothermal sites of the Suiyo Seamount, Izu-Bonin Arc, Western Pacific. Appl. Environ. Microbiol. **70:**393-403.

26. **Perez-Jimenez, J. R., L. Y. Young, and L. J. Kerkhof.** 2001. Molecular characterization of sulfate-reducing bacteria in anaerobic hydrocarbon-degrading consortia and pure cultures using the dissimilatory sulfite reductase (*dsrAB*) genes. FEMS Microbiol. Ecol. **35:**145-150.

27. **Purkhold, U., M. Wagner, G. Timmermann, A. Pommerening-Röser, and H. P. Koops.** 2003. 16S rRNA and amoA-based phylogeny of 12 novel betaproteobacterial ammonia-oxidizing isolates: extension of the dataset and proposal of a new lineage within the nitrosomonads. Int. J. Syst. Evol. Microbiol. **53:**1485-1494.

28. **Sambrook, J., E. J. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

29. **Schnell, S., F. Bak, and N. Pfennig.** 1989. Anaerobic degradation of aniline and dihydroxybenzenes by newly isolated sulfate-reducing bacteria and description of *Desulfobacterium anilini*. Arch. Microbiol. **152:**556-563.

30. **Shen, Y., R. Buick, and D. E. Canfield.** 2001. Isotopic evidence for microbial sulphate reduction in the early Archaean era. Nature **410:**77-81.

31. **Stahl, D. A., S. Fishbain, M. Klein, B. J. Baker, and M. Wagner.** 2002. Origins and diversification of sulfate-respiring microorganisms. Antonie van Leeuwenhoek **81:**189-195.

32. **Wagner, M., A. J. Roger, J. L. Flax, G. A. Brusseau, and D. A. Stahl.** 1998. Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration. J. Bacteriol. **180:**2975-2982.

33. **Wolfe, B. M., S. M. Lui, and J. A. Cowan.** 1994. Desulfoviridin, a multimeric -dissimilatory sulfite reductase from *Desulfovibrio vulgaris* (Hildenborough). Purification, characterization, kinetics and EPR studies. Eur. J. Biochem. **223:**79-89.

FIGURE LEGENDS

Figure 1. Southern hybridization of genomic DNA fragments from *D. toluolica* using a *dsrA*-targeted polynucleotide probe. Fragments were generated by using the restriction enzymes *Eco*RI, *Bam*HI, *Hind*III, *Kpn*I, *Pst*I, *Sal*I, *Sma*I, and *Xba*I, respectively. First lane contains *Hind*III-digested lambda DNA as molecular weight marker.

Figure 2. (A) Schematic map showing the genetic organisation of a *dsr* operon-containing 8.9-kb genomic fragment of *D. toluolica*. Restriction sites of common endonucleases and sequence motifs similar to *E. coli* sigma 70 promoters are shown. Fully-sequenced open reading frames: *dsrA* and *dsrB*, alpha and beta subunits of the dissimilatory (bi)sulfite reductase; *dsrD*, dissimilatory (bi)sulfite reductase D; *dsrN*, putative siroheme amidase; *dapA*, dihydrodipicolinate synthase; ?ORF, unidentified open reading frame. (B) Genetic organisation of all known *dsr* operons from SRPs and *Bilophila wadsworthia*. Prokaryotes which are able to use sulfate as electron acceptor for anaerobic respiration are indicated in bold face type. Open reading frames: *dsrC*, gamma subunit of the dissimilatory (bi)sulfite reductase; *fdx*, ferredoxin. Accession numbers: *D. vulgaris*, AE017285; *B. wadsworthia*, AF269147; *D. vibrioformis*, AJ250472; *D. toluolica*, AJ457136; *D. rhabdoformis*, AJ250473; *T. norvegica*, AJ277293; *D. thermocisternum*, AF074396; *A. fulgidus*, NC_000917; and *A. profundus*, AF071499.

Figure 3. Comparison of 16S rRNA- and DsrAB-based phylogenetic consensus trees. Sequences determined in this study are in bold. 16S rRNA phylogenetic analyses were performed on alignment positions conserved in at least 50% of all *Bacteria*. Alignment regions of insertions and deletions were omitted in DsrAB amino acid sequence analyses. Polytomic nodes connect branches for which a relative order could not be determined

unambiguously by using distance-matrix, maximum-parsimony, and maximum-likelihood methods. Filled circles indicate branch points highly supported by maximum-parsimony bootstrap analysis (>90% in 1000 re-samplings). Open circles at nodes indicate 75-90%, while nodes without circles showed <75% bootstrap support. The bars represent 10% sequence divergence as estimated from maximum-likelihood and distance-matrix analysis for the 16S rRNA and DsrAB trees, respectively. Parentheses indicate SRPs which harbour laterally acquired *dsrAB* genes. Consistent groups between both trees are shaded grey. Note that the apparently inconsistent positions of SRP groups that are labelled by an asterisk are not well-resolved in the respective trees and can thus not be interpreted as indicators of lateral gene transfer events. An ungrouped version of this figure can be downloaded from our web site (http://www.microbial-ecology.net/supplements.html).

Figure 4. Denaturing polyacrylamide gel electrophoresis analysis of the dissimilatory (bi)sulfite reductase purified from *D. toluolica*. Predicted size of DsrA and DsrB according to the determined gene sequences are 49.9 kDa and 42.5 kDa, respectively. MWM: molecular weight marker.

TABLES

Table 1. DSR1F and DSR4R primer binding sites as recognized from completely sequenced *dsrAB* genes of SRPs.

dsrAB-containing prokaryotes	*dsrA*–targeted forward primer binding site (5'-3')[1]	Perfectly matching forward primer	Forward primer reference
D. vulgaris, AE017285	ACC CAC TGG AAG CAC G	DSR1F	32
D. desulfuricans, AJ249777	ACC CAT TGG AAA CAC G	DSR1Fa	19
B. wadsworthia, AF269147	ACG CAC TGG AAG CAC G	DSR1F	32
D. vibrioformis, AJ250472	ACC CAC TGG AAA CAC G	DSR1Fa	19
D. toluolica, AJ457136	ACC CAT TGG AAA CAT G	DSR1Fc	This study
D. rhabdoformis, AJ250473	ACC CAT TGG AAA CAC G	DSR1Fa	19
T. norvegica, AJ277293	GGC CAC TGG AAG CAC G	DSR1Fb	19
D. thermocisternum, AF074396	ACC CAC TGG AAA CAC G	DSR1Fa	19
A. fulgidus, NC_000917	ACG CAC TGG AAG CAC G	DSR1F	32
A. profundus, AF071499	ACG CAC TGG AAG CAC G	DSR1F	32

[1] Highly conserved nucleic acid positions are shaded in grey.

dsrAB-containing prokaryotes	*dsrB*–targeted reverse primer binding site (5'-3')[1]	Perfectly matching reverse primer	Reverse primer reference
D. vulgaris, AE017285	TGC GGT AAC TGC TAC AC	DSR4R	32
D. desulfuricans, AJ249777	TGC GGA AAC TGC TAC AC	DSR4Rc	19
B. wadsworthia, AF269147	TGC GGT AAC TGC TAC AC	DSR4R	32
D. vibrioformis, AJ250472	TGC GGT AAC TGT TAC AC	DSR4Rb	19
D. toluolica, AJ457136	TGT GGT AAC TGC TAC AC	DSR4Rd	This study
D. rhabdoformis, AJ250473	TGC GGT AAC TGC TAC AC	DSR4R	32
T. norvegica, AJ277293	TGC GGA AAC TGC TAC AC	DSR4Rc	19
D. thermocisternum, AF074396	TGC GGC AAC TGC TAC AC	DSR4Rc	19
A. fulgidus, NC_000917	TGC GGT AAC TGC TAC AC	DSR4R	32
A. profundus, AF071499	TGT GGA AAC TGT TAC AC	DSR4Ra	19

Figure 1. Zverlov et al.

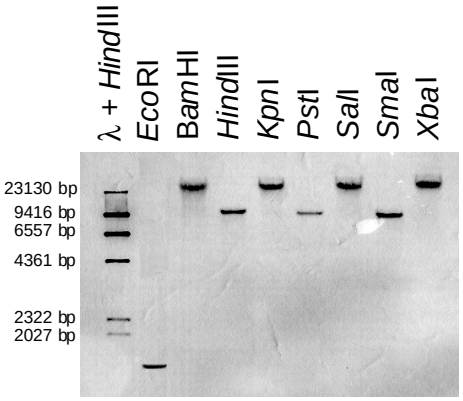


Figure 2. Zverlov et al.

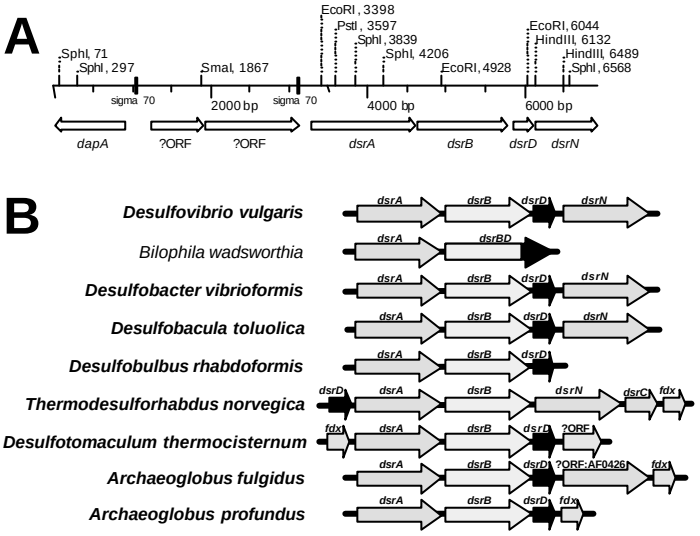


Figure 3. Zverlov et al.

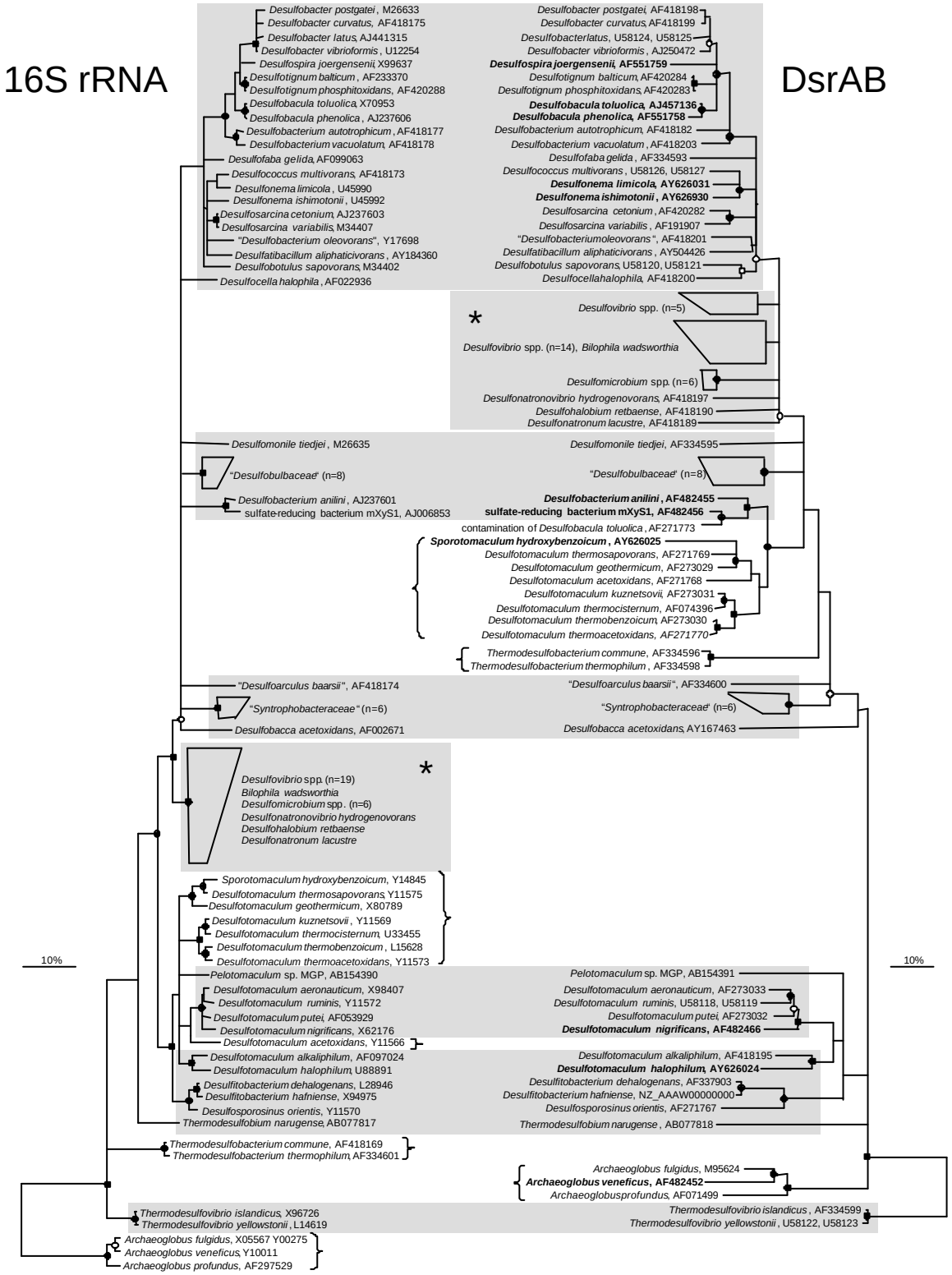
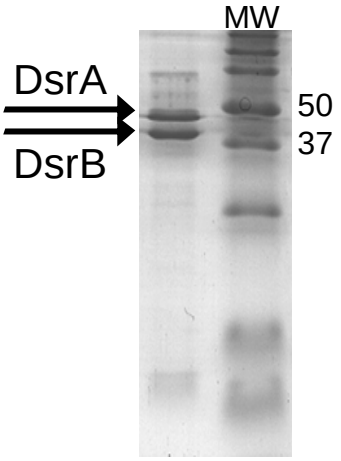


Figure 4. Zverlov et al.



G.2.5 Community-level genetic analysis: functional marker genes for the specific identification of sulphate-reducing prokaryotes

Michael Wagner, Alexander Loy, Michael Klein, and Michael W. Friedrich

In preparation for Methods in Enzymology

Michael Klein prepared the gelretardation and clonened and sequenced *dsrAB* fragments from Mariager Fjord and Solar Lake.

Notes of Thanks

Die vorgelegte Arbeit wurde am Lehrstuhl für Mikrobiologie der Technischen Universität München, unter der Leitung des Ordinarius Herrn Professor Dr. Karl-Heinz Schleifer erstellt.

Bleibt mir als letztes all jenen meinen Dank auszudrücken, die mir während meiner Zeit am Lehrstuhl für Mikrobiologie wissenschaftlich und menschlich zur Seite gestanden sind.

Mein besonderer Dank gilt

Herrn Professor Schleifer für die Möglichkeit an seinem Lehrstuhl diese Arbeit erstellen zu können, für sein Interesse an und seine Unterstutzung bei der Bearbeitung meines Themas.

Herrn Professor Michael Wagner für die Lenkung meiner Arbeit, viele fruchtbare Diskussionen, Anregungen und Hilfe bei großen und kleinen Problemen.

Dr. „Big Mike“ Schmid für seine Freundschaft, Hilfe und sein Wissen, was mir alles zusammen in den vergangenen Jahren so manches mal über Schwierigkeiten innerhalb und außerhalb der Universitätswelt hinweggeholfen hat.

meinen Mitstreitern Dr. Markus Schmid (himself), Dr. Matthias Horn (die Couch), Dr. Alexander Loy (der Espresso), Dr. Holgi Daims (Mr. Linux, aber nicht mit meinem Rechner!) und Dr. Natuschka Lee (*dsr* ohne Ende) für lange Tage und Nächte im Dienste der Wissenschaft, aber auch dafür, daß das Leben neben der Arbeit nicht zu kurz gekommen ist.

all den Wissenschaftlern, welche ich durch meine Arbeit kennen lernen durfte und mit welchen es ein Vergnügen war zusammenzuarbeiten: Professor Yehuda Cohen, Dr. Dirk deBeer, Dr. Stefan Grötchl, Dr. Michael Friedrich, Professor David Stahl,

Dr. Philipp Hugenholtz, Dr. Niels B. Ramsing, Dr. Tom Fritsche, Dr. Andreas Teske, Dr. Trine Thomsen, Dr. Nicole Dubilier Dr. Vladimir Zverlov, Dr. Wolfgang Ludwig, und Professor Dr. Leo Eberl.

Was wäre ein Labor ohne die Menschen, die es zum Leben erwecken. Danke für Hilfe, Unterstützung, eine tolle Atmosphäre und das Gefühl eine Familie zurückzulassen: Justyna Adamczyk, Birgit Kraus, Kirsten Hofacker, Claudia Schulz, Tanja Linner, Beatrix Schlatter, Cora Beier, Lotte Bjerrum, Andreas Brühl, Astrid Collingro, Dr. Stefan Juretschko, Dr. Angelika Lehner, Regina Nogueira, Dr. Uli Purkhold, Stephan Schmitz-Esser, Kilian Stöcker, Dr. Andreas Schöpfer, Dr. Marit Harzenetter, Dr. Heike Abicht und Dr. Marion Walcher, sowie Sibylle Schadhauser, Jutta Elgner und Josef Reischenbeck.

Selbstverständlich möchte ich mich, ohne alle Namen nennen zu können, bei allen Ehemaligen und den Mitgliedern der Arbeitsgruppen Eberl und Ludwig in gleicher Weise bedanken.

Zuletzt möchte ich noch meinen Freunden, meiner Familie und besonders meiner Frau Irene für viel Verständnis während langer Tage und Nächte, an denen ich an dieser Arbeit gesessen bin, Danke sagen. Danke auch für die unermüdliche Unterstützung in den vergangenen Jahren.

Macht’s gut und danke für den Fisch…
(Douglas Adams)