Spring 1997

Phylogenetic diversity and community structure of sulfate-reducing bacteria in a salt marsh sediment

Juliette Nicole Rooney Varga
University of New Hampshire, Durham

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

PHYLOGENETIC DIVERSITY AND COMMUNITY STRUCTURE OF SULFATE-REDUCING BACTERIA IN A SALT MARSH SEDIMENT

BY

JULIETTE N. ROONEY VARGA
B.A., Colby College, 1991
M.S., Cornell University, 1994

DISSERTATION

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Doctor of Philosophy

in

Microbiology

May, 1997

This dissertation has been examined and approved.

Dissertation Director, Dr. Aaron B. Margolin,
Associate Professor of Microbiology

Dr. Mark E. Hines, Assistant Professor of
Biological Sciences

Dr. Steven H. Jones, Research Associate Professor
of Natural Resources and Marine Science

Dr. Richard P. Blakemore, Professor of
Microbiology

Dr. Robert M. Zsigray, Professor of Microbiology

Jan. 10, 1997
Date

DEDICATION

This dissertation is dedicated to my beloved husband and my family, who have given me the love, support, and encouragement that made this work possible.

ACKNOWLEDGEMENTS

I would like to thank my advisors, Dr. Mark E. Hines and Dr. Aaron B. Margolin for providing me with the opportunity, resources, and guidance I needed to conduct my doctoral research. I would also like to thank Dr. R. Devereux for welcoming me in his laboratory, contributing to my training in molecular techniques, and providing helpful comments both as my research progressed and on manuscript drafts; and Drs. B. Assmus and R. I. Amann for likewise welcoming me in their laboratories and providing me with training in *in situ* hybridization techniques. I thank Dr. Barbara Sharak Genthner, Bob Evans, Stephanie Friedman, Stephanie Willis, Shannon Hogan, Amy Moore, Amanda Clement, and Caroline Martorano for their collaboration and advice. This work was supported by the U.S. Environmental Protection Agency Cooperative Agreement #CR-820062 and the National Science Foundation Ecology Program grant #ANSF URE GCH6.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

PHYLOGENETIC DIVERSITY AND COMMUNITY STRUCTURE OF SULFATE-REDUCING BACTERIA IN A SALT MARSH SEDIMENT

by

Juliette N. Rooney Varga

University of New Hampshire, May, 1997

Phylogenetic diversity and community structure of sulfate-reducing bacteria (SRB) in a salt marsh sediment and rhizosphere of *Spartina alterniflora* were investigated. Uncultivated phylotypes were studied by selectively amplifying *Desulfobacteriaceae* 16S rRNA gene fragments from DNA extracted from salt marsh rhizosphere samples. An *in vitro* transcription technique was developed to synthesize reference RNAs containing sequences presumably identical to corresponding regions of the uncultivated organisms' 16S rRNAs. These reference RNAs were used in subsequent quantitative probing experiments. Oligonucleotide probes were designed to specifically target novel phylotypes and were tested for optimal hybridization wash conditions and target specificity. The newly designed probes were then applied together with eubacterial probes to determine the relative abundances of the novel phylotypes in the salt marsh sediment and rhizosphere. Lastly, 16S rRNA sequences of ten SRB isolates were analyzed and compared to sequences of other cultivated SRB and novel phylotypes retrieved directly from environmental samples.

Two novel phylotypes were retrieved from rhizosphere samples, with A01 sharing 89.1% sequence identity with *Desulfococcus multivorans* and 4D19 sharing 96.3% sequence identity with *Desulfosarcina variabilis*. Additionally, six sequences were found that were extremely closely related to *D. multivorans*. Synthetic reference RNAs were successfully used in the optimization and application of probes A01-183 and 4D19-189, which specifically targeted A01 and 4D19, respectively. Mean relative abundances of A01-183 and 4D19-189 targets were 7.5% and 3.4%, respectively, suggesting that the target organisms of A01-183 and, to a lesser extent, 4D19-189 played a dominant role in the salt marsh sediment and rhizosphere.

Phylogenetic analysis of SRB isolates placed all isolates within the Gram-negative mesophilic SRB group. Two isolates were members of the *Desulfovibrionaceae* family, with one a member of the genus *Desulfovibrio* and the other possibly representing a novel genus. The remaining eight isolates were members of the *Desulfobacteriaceae* family and were comprised of novel species within the genera *Desulfobulbus*, *Desulfobacter*, *Desulfobacterium*, and *Desulfoarculus*, as well as a novel genus most closely related to *Desulfobotulus sapovorans*. None of the SRB isolates appeared to be related to the phylotypes A01 or 4D19 at the species or genus level.

INTRODUCTION

In plant-inhabited ecosystems, the rhizosphere harbors intense microbial activity which greatly affects plant and ecosystem health. Key biogeochemical processes such as organic matter decomposition, mineralization, pollutant degradation, and nonsymbiotic nitrogen fixation occur at accelerated rates in the rhizosphere. The rhizosphere is the target for bacteria introduced into agricultural systems, and it may provide a habitat conducive for colonization of introduced bacteria which are not targeted to roots. In addition, in wet soils, sediments, estuaries, and lakes, the rhizosphere harbors the primary redox gradients that control precipitation and dissolution of geochemicals, control hydraulic conductivity, and determine whether microbes adhere to solids or are transported. While the rhizosphere is clearly an important and dynamic zone, its microbial community remains largely unexplored. Conventional techniques such as direct microscopic counts, viable counts, and most probable number determinations often give widely differing results. The presence of dead or inactive cells, the inability to distinguish bacteria from detritus and other particulate matter, damaging of cells prior to cultivation, or inappropriate conditions during cultivation all contribute to artifacts in conventional methods.

Many of the limitations of conventional microbiological techniques can be overcome by molecular techniques that use 16S rRNA as a phylogenetic descriptor. By now, the use of 16S rRNA as a phylogenetic molecule is well-established and, together with concurrent advances in molecular biological techniques, has dramatically altered the fields of microbial ecology and, more

generally, microbiology. With these techniques, it is possible to study the phylogenetic diversity, community composition, population dynamics, and microarchitecture of bacteria in their native habitats without relying on cultivation or morphology for identification. For both cultivated and uncultivated bacteria, comparative sequence analysis of 16S rRNAs has enabled the investigation of phylogenetic relationships among microorganisms in a manner that was not feasible through traditional microbiological methods. As a result, there have been drastic revisions in our understanding of bacterial evolution, new insights into the relationships between various phenotypic traits and phylogeny, and the emergence of a natural system of bacterial taxonomy that is founded in a phylogenetic framework.

The goal of the current dissertation research was to combine newly developed and currently available 16S rRNA-based approaches to study the phylogenetic diversity and community structure of a natural sediment and rhizosphere microbial community. To this end, the sulfate-reducing bacteria (SRB) community inhabiting a salt marsh sediment and rhizosphere was chosen as a model system for reasons that will be described in more detail in the following chapters. Briefly, sulfate reduction is the dominant terminal electron accepting process in salt marsh sediments, and the close interaction between sulfate reduction rates and plant phenology of *Spartina alterniflora* documented by Hines et al. (1989) indicated that SRB dynamics in the rhizosphere are ecologically important. In addition, the 16S rRNA phylogeny of many SRB has been determined (Devereux et al., 1989; 1990) and oligonucleotide probes are available for many of the major groups and genera (Devereux et al., 1992).

This dissertation was part of a larger, multifaceted project involving the use of molecular, microbiological, and biogeochemical techniques to investigate interactions between the salt marsh SRB community and the marsh plant *S.*

alterniflora. The researchers that were involved with this project were Dr. M. E. Hines (University of New Hampshire; UNH), Dr. R. Devereux (U.S. Environmental Protection Agency; EPA), Dr. B. R. Sharak Genthner (UNH), R. S. Evans (UNH), S. G. Willis (UNH), S. Friedman (UNH), and Amanda Clement (UNH). Briefly, this larger project consisted of several parts, including: 1) the investigation of SRB community structure and population dynamics over pertinent temporal and spatial scales in the rhizosphere and bulk sediment by applying group- and genus-specific probes to RNA extracted from environmental samples; 2) measurement of pertinent geochemicals, plant-related parameters, and sulfate reduction rates to place results in a biogeochemical framework; and 3) use of conventional microbiological techniques to isolate novel SRB from salt marsh samples, followed by physiological and phylogenetic characterization of isolates. One of the findings of this larger study was that the *Desulfobacteriaceae* family was quantitatively important in the salt marsh sediment and rhizosphere and appeared to contain previously undescribed species. Due to this result, emphasis was placed on the *Desulfobacteriaceae* family in my dissertation research.

The experimental approach used for my research, as well as closely related components of the larger research project, are shown in Fig. 1. The three major components of this work were: 1) an investigation of the phylogenetic diversity of the *Desulfobacteriaceae* SRB family by direct retrieval and analysis of 16S RNA genes from rhizosphere samples; 2) the design and application of 16S rRNA-targeted oligonucleotide probes that target novel phylotypes discovered in (1) to quantitatively investigate their population dynamics; and 3) use of comparative 16S rRNA sequence analysis to infer phylogenetic relationships of novel SRB isolates, provide an alternate route for studying phylogenetic diversity in the salt marsh SRB community, and reevaluate currently available probes.

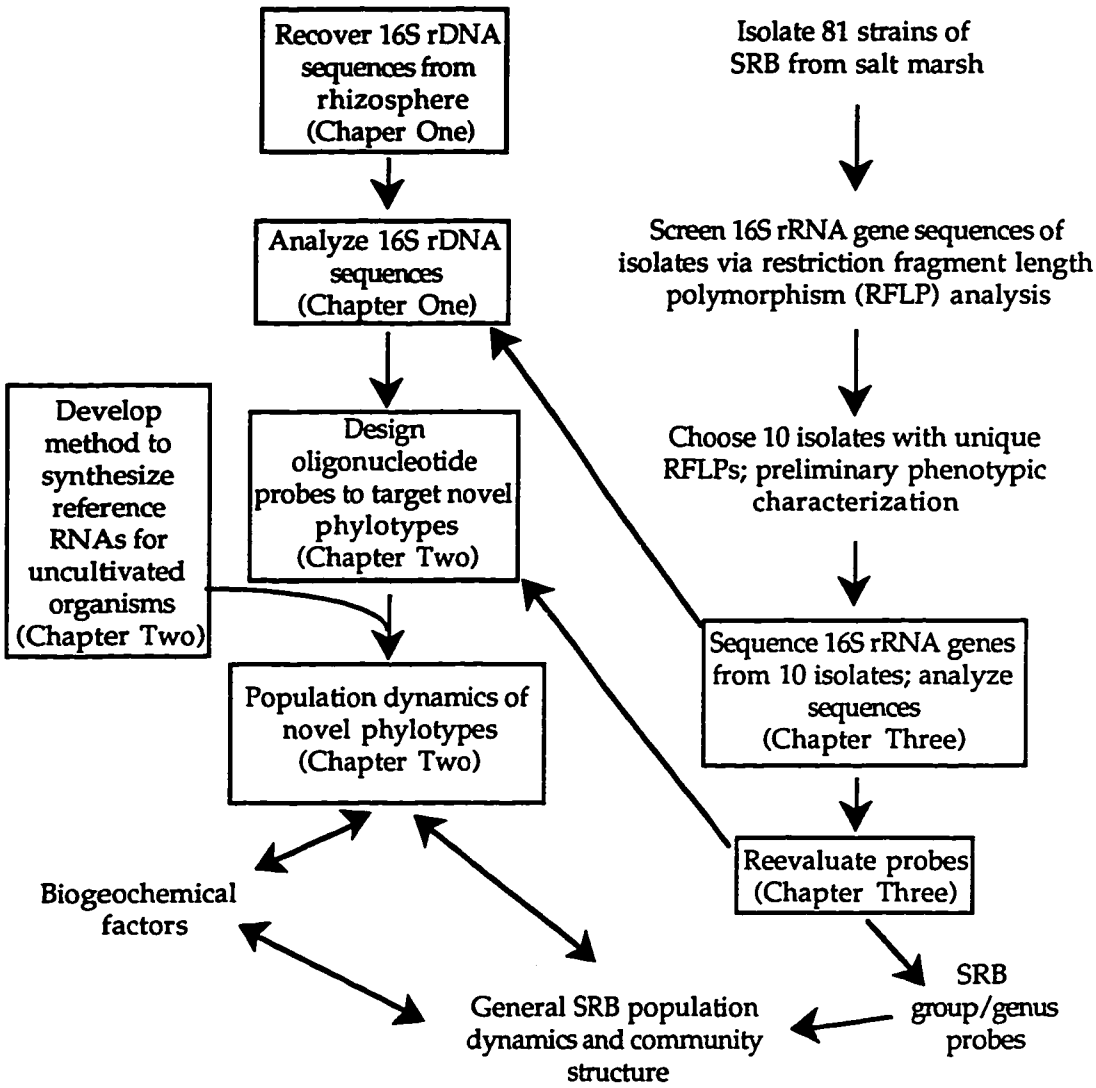


Fig. 1. Schematic diagram of experimental approach, showing components conducted in the current dissertation research (in boxes) as well as closely related work conducted by other researchers.

CHAPTER ONE

RETRIEVAL AND ANALYSIS OF *DESULFOBACTERIACEAE* 16S rDNA FROM THE RHIZOSPHERE OF *SPARTINA ALTERNIFLORA*

Introduction

In plant-inhabited ecosystems, the rhizosphere harbors intense microbial activity which, in turn, greatly affects plant and ecosystem health (Coleman et al., 1978; Teal et al., 1979; Paul and Clark, 1989; Anderson et al., 1993). Key biogeochemical processes such as organic matter decomposition, pollutant degradation (Anderson et al., 1993), and nonsymbiotic nitrogen fixation (Teal et al., 1979) occur at accelerated rates in the rhizosphere zone. Despite the ecological importance of the rhizosphere, rhizosphere microbial communities remain poorly understood due to steep environmental gradients over microscales, complex microbial interactions (Kluepfel, 1993), and shortcomings of conventional techniques to quantify and characterize natural microbial communities (Litchfield, 1976; Zarda et al., 1991). However, with the advent of molecular microbial ecology and, in particular, techniques based on comparative analysis of 16S rRNA sequences, it is now possible to investigate natural rhizosphere communities much more thoroughly.

In the current study, molecular phylogenetic techniques were used to investigate the sulfate-reducing bacteria (SRB) community in the rhizosphere of the salt marsh cordgrass, *Spartina alterniflora*. This community was chosen as a

model system because both its biogeochemical dynamics and the 16S rRNA phylogeny of SRB have been relatively well studied. Sulfate reduction is the dominant terminal electron accepting process (Howarth and Hobbie, 1982) and has been shown to be closely tied to plant phenology, suggesting that plant-SRB interactions in the *S. alterniflora* rhizosphere play an important role in salt marsh biogeochemical cycles (Hines et al., 1989; Hines, 1991). To date, the 16S rRNA phylogeny of SRB is one of the most complete, and hybridization probes are available for each of the major groups and several individual species (Devereux et al., 1989; Devereux et al., 1990; Devereux et al., 1992). The phylogenetic groups are also defined by distinct physiological features, in particular, the ability to use specific electron donors, the suite of which is rather limited by the group as a whole. Therefore, comparative rRNA methods may also provide information on the types of substrates used by rhizosphere bacteria.

The Salt Marsh Rhizosphere Microenvironment

The rhizosphere is frequently described as being comprised of three zones: the endorhizosphere, or interior of the root; the rhizoplane, or root surface; and the ectorhizosphere, or area around the root surface that is influenced by the root's presence (Paul and Clark, 1989). Plant roots release at least 20% of total plant dry weight into the rhizosphere (Kluepfel, 1993). Thus, the rhizosphere is rich in dissolved organic carbon compounds, such as amino acids, aliphatic acids, aromatic acids, amides, and sugars, as well as insoluble organic materials, such as cellulose, lignin, and proteins (Paul and Clark, 1989). The release of root exudates and sloughed off root cells results in intense microbial activity in root zones (Coleman et al., 1978). In fact, microbial counts in the rhizosphere have been reported that are up to 100 times higher than in root-free soil (Anderson et al., 1993), with their numbers dropping precipitously within 5 μm of plant roots

(Paul and Clark, 1989). Rhizosphere bacteria are not only more numerous, but also demonstrate higher metabolic activity than their non-rhizosphere counterparts (Paul and Clark, 1989).

A diverse array of microbial processes is found in salt marshes, and many of these are closely linked to the rhizosphere of the dominant cordgrass species, *Spartina alterniflora* (Howarth, 1993). The rhizosphere is particularly important in salt marshes for several reasons. Firstly, salt marshes are among the most productive ecosystems on Earth, with at least half of their productivity occurring belowground in the form of roots and rhizomes (Valiela et al., 1976; Howes et al., 1985; Blum, 1993) and most of this organic matter being decomposed *in situ* (Valiela et al., 1976). Secondly, *S. alterniflora* releases large amounts of dissolved organic matter into the rhizosphere, thereby fueling microbial activity (Howarth, 1993). During hypoxic and anoxic conditions, roots are unable to maintain aerobic respiration. The result is production of low molecular weight fermentation products which easily diffuse out of root cells (Mendelssohn and McKee, 1987; Hines et al., 1989; Hines et al., 1994). Lastly, the hollow internal channels of *S. alterniflora* provide a conduit for movement of oxygen into the otherwise anoxic sediments, resulting in steep redox gradients over microscales surrounding the roots. These redox microgradients may provide an ideal habitat for diverse microbial metabolisms such as sulfate reduction and sulfur oxidation, aerobic respiration, nitrification, denitrification, iron and manganese reduction and oxidation and methanogenesis (Kaplan et al., 1979; Giblin and Howarth, 1984; Luther et al., 1986).

Sulfate reduction is the main pathway of organic matter decomposition in salt marsh sediments (Hines et al., 1989; Vernberg, 1993). Evidence that it is linked to the rhizosphere stems from the fact that SRB utilize primarily low molecular weight alcohols and fatty acids (Howarth, 1993) - compounds which,

as mentioned above, are likely to be found in relatively high concentrations in the salt marsh rhizosphere. Specifically, malate, ethanol (Hines et al., 1989), and probably acetate (Hines et al., 1994) are produced by roots during anaerobic fermentation and can be directly utilized by SRB. Hines et al. (1989) found that sulfate reduction rates were closely linked to the physiological state of plants. They suggested that high sulfate reduction rates were related to increased release of dissolved organic matter from roots during vegetative growth of tall *S. alterniflora* plants.

SRB have traditionally been thought of as obligate anaerobes, and therefore may not be expected to proliferate in the potentially oxic rhizosphere microenvironment. However, several recent studies have provided evidence that SRB are actually capable of tolerating and even utilizing oxygen at low concentrations. For example, Cypionka et al. (1985) found several strains of SRB that tolerated varying exposures to aeration without loss of viability. In fact, certain strains of SRB have been found to utilize oxygen as a terminal electron acceptor with either lactate, hydrogen, hydrogen sulfide, or sulfide as an electron donor (Dilling and Cypionka, 1990). Marschall et al. (1993) reported superoxide dismutase activity, an enzyme that confers oxygen tolerance, in *Desulfovibrio desulfuricans*. In that same study, several strains of SRB grew optimally near an anoxic (sulfide-containing agar medium) - oxic (oxygen-containing atmosphere) interface. Thus, it seems likely that the rhizosphere, which may frequently exist as an interface between oxic and anoxic microenvironments, would in fact be inhabited by numerous SRB.

Use of Molecular Techniques to Study the Rhizosphere Microbial Community

While the rhizosphere is clearly an important and dynamic zone, its microbial community remains largely unexplored. Conventional techniques

such as direct microscopic counts, viable plate counts, and most probable number determinations frequently give widely differing results (Witzel, 1990). The presence of dead or inactive cells, inability to distinguish bacteria from detritus and other particulate matter, damaging of cells prior to cultivation, or inappropriate conditions during cultivation all contribute to the discrepancies observed among conventional techniques (Litchfield, 1976). Fatty acid analysis may also give biased results because many classifications in the fatty acid data base are based on clinical isolates which may differ substantially from their counterparts in environmental samples (Wagner et al., 1993). However, the advent of molecular approaches in microbial ecology has provided tools to begin a more thorough exploration of soil and sediment microbial community dynamics without introducing many of the biases associated with conventional techniques.

Largely as a result of the incorporation of concurrently advancing techniques in molecular biology and the conceptual development of 16S- (and 23S-) like ribosomal RNAs as phylogenetic descriptor molecules (Woese et al., 1985; Olsen et al., 1994a; Woese, 1994), the field of microbial ecology has undergone revolutionary advances in recent years. The use of 16S rRNA as a phylogenetic descriptor molecule is now well-established. The reasons for this are manifold: rRNA is ubiquitous and functionally identical in all life forms; it contains regions of highly conserved sequences allowing for sequence alignment of distantly related organisms; and it contains regions that are quite variable over evolutionary time, providing 'signature sequences' at the species or sub-species level (Woese, 1987). rRNA genes do not appear to be subject to horizontal gene transfer, so that the evolutionary history contained in an rRNA molecule should in fact be consistent with the evolutionary history of the organism possessing it (Woese, 1987). In addition, the large number of 16S rRNA sequences currently available in data bases

further enhances the utility of 16S rRNA in comparative phylogenetic analyses. In fact, 16S rRNA has become so central to the field of microbial ecology that while the definition of a prokaryotic species remains somewhat elusive (Witzel, 1990), 16S rRNA sequence analysis has become an important component in defining new species. There is now a general acceptance that a newly proposed species is indeed a separate species if the difference in 16S rRNA sequence between it and its closest relatives is greater than 1.5-2.5% (Stackebrandt and Goebel, 1994). It should be noted that 23S-like rRNA contain even more phylogenetic information than the smaller 16S rRNA. However, 16S rRNA molecules are sufficiently large to contain a significant amount of information without being so large as to make their analysis technically difficult.

Armed with basic molecular tools and available 16S rRNA sequence information, a microbial ecologist can retrieve 16S rRNA sequences from natural samples without prior cultivation (e.g., Fuhrman et al., 1992; Gordon and Giovannoni, 1996; Murray et al., 1996); probe natural samples for broad phylogenetic groups (likely to encompass currently uncultured bacteria), or specific species or strains (e.g., Giovannoni et al., 1988; Krumholz et al., 1995); analyze community microarchitecture and relative cellular activity with whole cell hybridization (e.g., DeLong et al., 1989; Amann et al., 1990; Assmus et al., 1995); or develop general measures of community composition for intercomparison of two or more communities (e.g., Muyzer et al., 1993; Moyer et al., 1994).

Retrieval of rRNA Sequences from Natural Communities

The selective recovery of 16S rRNA sequences can be seen as the exploratory phase of a molecular investigation of a natural microbial community, in which the probability of discovering novel phylotypes is high (Tiedje, 1993). While 16S rRNA

retrieval from environmental samples is a powerful tool in environmental microbiology, it is not without its limitations and problems. The pioneering studies in which this technique was first applied involved environments that did not contain the high concentrations of humic compounds or clays found in salt marsh sediments (Weller and Ward, 1989; Giovannoni et al., 1990; Ward et al., 1990). These substances co-purify with nucleic acid extracts and can interfere with hybridization efficiency and specificity as well as enzymatic manipulation (Picard et al., 1992; Tsai and Olson, 1992). Another potential problem in DNA extraction is unbiased, quantitative lysis of all cell types present. Recent studies that have addressed these issues are numerous. Lysis techniques currently in use include thermal shock; microwaving; sonication; lysozyme/protease treatment; bead-beating; and various combinations of these techniques (Moré et al., 1994). Purification techniques are similarly diverse, including purification with Sephadex columns (Abbaszadegan et al., 1993; Erb and Wagnerdobler, 1993), Elutip-d columns (Tsai and Olson, 1991; Picard et al., 1992); Bio-Gel polyacrylamide gel columns (Tsai and Olson, 1992), and Chelex columns (Abbaszadegan et al., 1993); treatment with polyvinylpolypyrolidine (PVPP) (Steffan et al., 1988); cesium chloride gradient and hydroxyapatite purification (Steffan et al., 1988); repeated washes with 70% ethanol (Bruce et al., 1992); and purification by electrophoresis in low-melt agarose (Herrick et al., 1993; Moré et al., 1994). Unfortunately, there is still no single method that yields sufficiently pure DNA from all microbial cell types in any environmental sample type. Instead, optimization and adaptation of various methods to the system of interest is necessary.

Objectives

In preliminary analyses using membrane hybridization with RNA extracted from marsh sediment samples and various probes for Gram-negative

mesophilic SRB (Devereux et al., 1992; Hines et al., in prep) found that the members of the *Desulfobacteriaceae* family (Widdel and Bak, 1992) targeted by probe 804 (Devereux et al., 1992) accounted for up to 20% of total eubacterial rRNA and appeared to be the most abundant group of SRB in the salt marsh sediment (Hines et al., in prep). However, the relative abundances of probed genera within the *Desulfobacteriaceae* accounted for only a small fraction of the relative abundance of the family as a whole, suggesting that other undescribed *Desulfobacteriaceae* species played a significant role in the salt marsh microbial community. Therefore, the objective of the current study was to investigate the phylogenetic diversity of the *Desulfobacteriaceae* and search for novel phylotypes by retrieving and analyzing 16S rDNA directly from rhizoplane bacterial communities.

Methods

Cultivation of Organisms

Organisms used in this study were kindly provided by B. Sharak-Genthner and S. Friedman. *Desulfococcus multivorans* (ATCC 33890) and *Desulfovibrio vulgaris* (ATCC 29579) were grown using anaerobic aseptic techniques described by Widdel and coworkers (Widdel, 1983; Widdel and Bak, 1992). First, the following stock solutions were prepared: nonchelated trace element solution (100 mM HCl, 7.5 mM $FeSO_4 \cdot 7H_2O$, 0.5 mM H_3BO_3, 0.8 mM $CoCl_2 \cdot 6H_2O$, 0.01 mM $CuCl_2 \cdot 2H_2O$); selinite-tungstate solution (10 mM NaOH, 0.02 mM $Na_2SeO_3 \cdot 5H_2O$, 0.02 mM $Na_2WO_4 \cdot 2H_2O$); 1.0 M $NaHCO_3$ solution; vitamin mixture (0.4 mg/l 4-aminobenzoic acid, 0.1 mg/l D(+)-biotin, 1 mg/l nicotinic acid, 0.5 mg/l calcium D(+)-pantothenate, and 1.5 mg/l pyridoxine dihydrochloride in 10 mM sodium phosphate buffer, pH 7.1); vitamin B_{12} solution (0.5 mg/l cyanocobalamine); thiamine solution (1 mg/l thiamine

chloride dihydrochloride in 25 mM sodium phosphate buffer, pH 3.4); and sulfide solution (0.20 M $Na_2S{\cdot}9H_2O$). The trace element solution and selenite-tungstate solution were flushed with 9:1 N_2: CO_2 to remove O_2 and autoclaved in bottles with fixed rubber stoppers. Similarly, the bicarbonate solution was flushed with CO_2 and also autoclaved in stoppered bottles. The vitamin mixture, vitamin B_{12} solution, and thiamine solution were filter-sterilized and stored at -20° C in dark bottles. The sulfide solution was prepared under a N_2 atmosphere and autoclaved in bottles with fixed stoppers. Freshwater basal medium was then prepared by adding 1.0 g NaCl, 0.4 g $MgCl_2{\cdot}6H_2O$, 0.1 g $CaCl_2{\cdot}2H_2O$, 4.0 g Na_2SO_4, 0.25 g NH_4Cl, 0.2 g KH_2PO_4, and 0.5 g KCl to 1.0 l distilled H_2O. The basal medium was flushed with 9:1 N_2: CO_2 gas, aliquoted into serum bottles that were also flushed with N_2/CO_2, and autoclaved with rubber stoppers fixed to bottles. The described stock solutions were then added to basal medium using sterile disposable syringes that were flushed with N_2/CO_2 gas, in the following amounts per l basal medium: 1.0 ml trace element solution; 1.0 ml selinite-tungstate solution; 30.0 ml $NaHCO_3$ solution; 1.0 ml vitamin mixture; 1.0 ml vitamin mixture; 1.0 ml thiamin solution; 1.0 ml vitamin B_{12} solution; 7.5 ml Na_2S solution. Sterile lactate was aseptically added (to a final concentration of 20 mM) into serum bottles containing about 100 ml medium and each serum bottle was inoculated with 1-2 ml of an active culture of *D. multivorans* or *D. vulgaris*. Cultures were grown for 7-12 days at room temperature.

Study Site and Sample Collection

Samples were collected from a tall-form, creekside stand of *S. alterniflora* in Chapman's Marsh in southeastern New Hampshire (Fig. 1.1). Iron and sulfur biogeochemistry (Hines et al., 1989; Hines, 1991), production and emission of biogenic sulfur gases (Morrison and Hines, 1990), acetate cycling in the

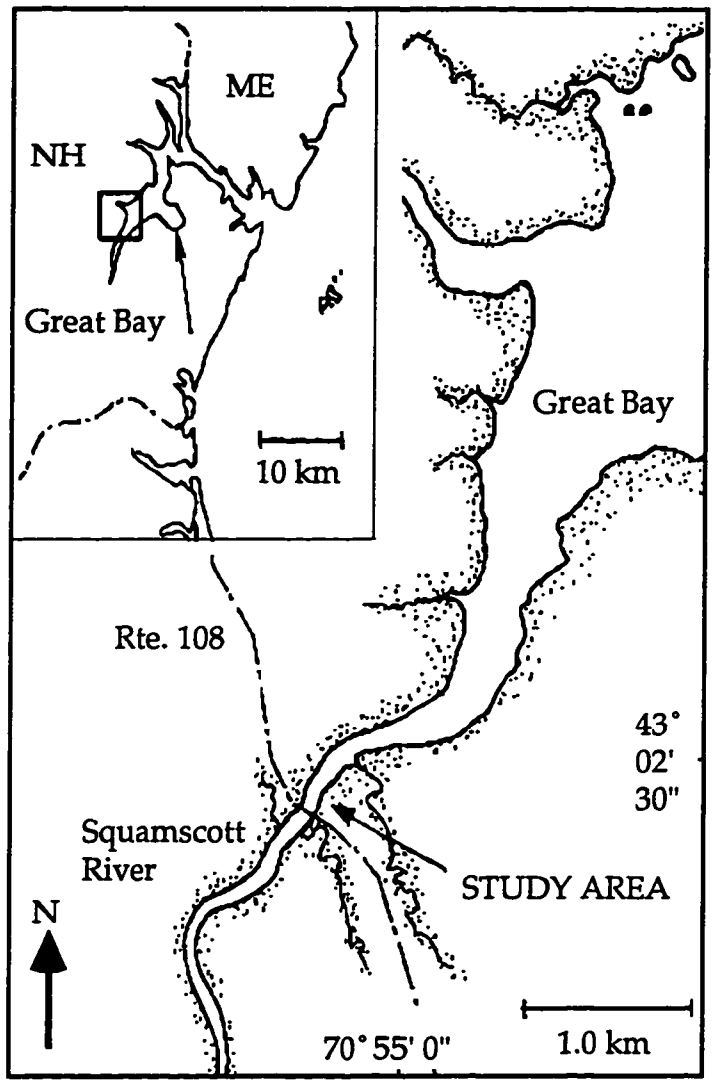


Fig. 1.1. Study site location in Chapman's Marsh, New Hampshire.

rhizosphere of *S. alterniflora* (Hines et al., 1994), and the effect of plant phenology on sulfate reduction (Hines et al., 1989) have been studied at this site. In order to avoid disturbing the vegetation and sediment, boardwalks were used to access sampling sites. Sediment cores (5 cm diameter) were collected using a handheld corer (Wildco Wildlife Supply Co., Saginaw, Michigan) equipped with a separate plastic liner for each sample, and were held anoxically on ice (Hines et al., 1989) for transport to the laboratory. Sediment cores were either processed within 1-2 h of sample collection or stored at -80° C until used for further manipulations. Cores for 16S rDNA sequence retrieval were collected on 22 August 1994 and 8 September 1994.

DNA Extraction and Purification

The upper 2.5 cm of each core were used for DNA extractions, as this depth zone has been shown to contain the majority of active roots and the highest sulfate reduction rates (Hines et al., 1989). Non-rhizosphere sediment was removed from roots in the upper 2.5 cm of each core by briefly rinsing roots with phosphate buffer (8.7 mM $Na_2HPO_4 \cdot H_2O$) that was adjusted with NaCl to the same salinity as sediment porewater (about 26 ppt). While most of the sediment was removed from roots through rinsing, microscopic observation of root hairs stained with DAPI (4',6-diamidino-2-phenylindole) (Hicks et al., 1992) showed that root hairs remained densely covered with rhizoplane bacteria. DNA extraction and purification procedures that were attempted included a freeze/thaw method adapted from Tsai and Olson (1991), with or without purification by Sephadex G-200 columns; and a bead-beating method modified from Moré et al. (1994) with a low-melt agarose electrophoresis purification step (Fig. 1.2). In the freeze-thaw method, about 5 g washed roots were added to 10 ml salinity-adjusted phosphate buffer (above), mixed well, and shaken at 75 RPM

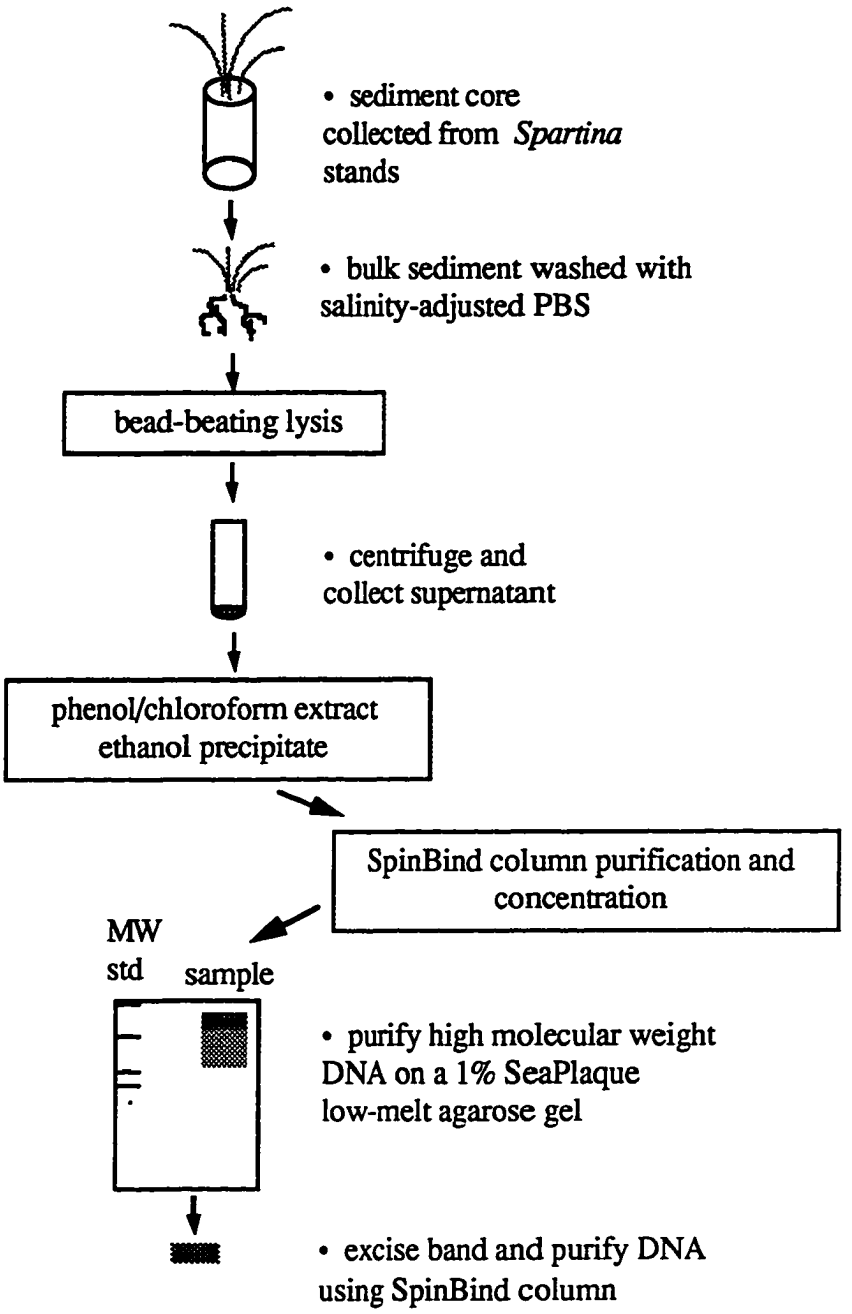


Fig. 1.2. Schematic diagram of method used for extraction and purification of DNA from rhizosphere samples.

for 30 min. The mixture was pelleted by centrifugation at 6000 x *g* for 10 min, and the supernatant fluid was decanted and discarded. 10 ml salinity-adjusted phosphate buffer was added to the pellet, shaken, and pelleted as described above. Cells were lysed by adding 8 ml lysis solution (0.15 M NaCl, 0.1 M EDTA [pH 8.0]) containing 15 mg lysozyme/ml and incubating for 30 min at 37° C and 75 RPM. 7.5 ml 0.1 M NaCl - 0.5 M Tris-HCl (pH 8.0) - 10% sodium dodecyl sulfate (SDS) were added. The samples were then subjected to three freeze-thaw cycles at -70° C and 65° C, and centrifuged at 6000 x *g* for 10 min. The supernatant fluid was transferred to a clean tube, 2-3 g PVPP were added, and the sample was mixed and incubated on ice for 30 min. PVPP was pelleted by centrifugation at 8000 x *g* for 8 min. The supernatant fluid was transferred to a clean tube, and the pellet was washed with an additional 8 ml phosphate-buffered saline solution (PBS; 130 mM NaCl; 10 mM sodium phosphate [pH 7.2]), which was then combined with the first supernatant fluid. Proteinase K was added to a final concentration of 50 µg/ml, and the sample was incubated at 37° C for 30 min with slow shaking. Proteins were removed from the solution by two extractions with Tris-buffered (pH 8.0) phenol. One-sixth volume 5 M NaCl and 1/9 volume CTAB solution (10% hexadecyltrimethyl-ammonium bromide in 0.7 M NaCl) were added, and the sample was mixed and incubated at 65° C for 5-7 min. The sample was then extracted twice with Tris-buffered (pH 8.0) phenol: chloroform: isoamyl alcohol (25: 24: 1) and twice with chloroform: isoamyl alcohol (24: 1). DNA was precipitated with 0.5 volume 7.5 M ammonium acetate and 1 volume isopropanol at -20° C overnight. DNA was pelleted by centrifugation at 6000 x *g* for 20 min, washed with 70% ethanol, dried at room temperature, and resuspended in 100-300 µl TE (10 mM Tris base, 1 mM EDTA, pH 8.0).

For the bead-beating lysis technique (Fig. 1.2.), 10 g (wet weight) rinsed roots, 10 g sterilized 0.1-mm-diameter zirconia/silica beads (BioSpec Products, Inc., Bartlesville, OK), and 10 ml extraction buffer (150 mM NaCl, 10 mM Tris-Cl pH 8.0, 100 mM EDTA, 4% SDS) were combined in a bead mill homogenizer cup (BioSpec Products, Inc.) that was packed in ice. The mixture was homogenized for 15 s and cooled for 1 min. This cycle was repeated a total of 5 times. The homogenized rhizosphere sample was then subjected to three freeze-thaw cycles at -80° C and 65° C, transferred to a centrifuge tube, and centrifuged for 8 min at 8,000 x *g*. The supernatant fluid was transferred to a clean tube and the pellet was washed with 3 ml 10 mM Tris-Cl (pH 8.0), centrifuged, and the resulting supernatant fluid was combined with the previous fraction. 2 g acid-washed PVPP were added to the supernatant fluid, which was then incubated on ice for 30 min and centrifuged at 8,000 x *g* for 8 min. As before, the resulting pellet was washed with 3 ml 10 mM Tris-Cl (pH 8.0), which was combined with the previous supernatant fluid after centrifugation. The supernatant fluid was then extracted sequentially with Tris-buffered (pH 8.0) phenol, phenol: chloroform: isoamyl alcohol (25: 24: 1), and chloroform: isoamyl alcohol (24: 1). DNA was precipitated with ethanol at -20° C overnight, and collected by centrifugation at 10,000 x *g* for 20 min. The pellet was dried and resuspended in approximately 400 µl TE. The extracted DNA was further purified using SpinBind cartridges (FMC BioProducts, Rockland, ME) and low-melt gel electrophoresis as described by Moré et al. (1994), except that electrophoresis was carried out for 1 h and EDTA was not added to the electrophoresis gel or running buffers in order to avoid inhibition of PCR by EDTA. High molecular weight DNA (> 6 kbp) was recovered from agarose gels by SpinBind cartridge purification as described by the manufacturer, except that EDTA was omitted from the wash buffer.

Genomic DNA was extracted from pure cultures of *D. multivorans* and *D. vulgaris* using a technique modified from Amann et al. (1992), as follows. Approximately 200 mg (wet weight) bacterial cells were pelleted by centrifugation at 10,000 x *g* 4° C for 20 min, resuspended in SE buffer (0.15 M NaCl, 0.1 M EDTA, pH 8.0) containing 1 mg/ml lysozyme, and incubated on ice for 30 min. Proteinase K and SDS were added to a concentration of 50 µg/ml and 1% (wt/vol.), respectively. Cells were lysed by freezing the cell suspension at -70° C followed immediately by thawing at 65° C and repeating the freeze-thaw cycle for a total of 3 times. After incubating the mixture at 37° C for 90 min, cellular debris was pelleted by centrifugation at 10,000 x *g* 4°C for 30 min and the resulting supernatant fluid was transferred to a sterile tube. The supernatant fluid was then extracted twice with an equal volume of phenol (saturated with TE [10 mM Tris, 1 mM EDTA, pH 8.0], 0.1 M NaCl, 1% SDS). One-sixth volume 5 M NaCl and 1/9 volume CTAB were added to the phenol-extracted supernatant fluid and, after incubating the solution at 65° C for 5 min, it was extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (24: 24: 1). Nucleic acids were precipitated by adding 1/2 volume 7.5 M ammonium acetate and 1 volume isopropanol and incubating overnight at -20° C. Nucleic acids were collected by centrifuging at 10,000 x *g* at 4° C for 20 min, washed with 70% ethanol, dried briefly, and then resuspended in sterile dH_2O. RNA was degraded by adding 2 µl *DNase*-free *RNase* A (Sigma, St. Louis, MO) and incubating at 37° C for 30 min. DNA was then reprecipitated, as before, washed with 70% ethanol, and resuspended in TE. DNA was extracted from mid-exponential phase cultures of *E. coli* using standard techniques (Sambrook et al., 1989).

Amplification and Cloning of *Desulfobacteriaceae* 16S rDNA

A schematic diagram of the methods used to selectively recover *Desulfobacteriaceae* 16S rDNA fragments is shown in Fig. 1.3. Selective amplification of *Desulfobacteriaceae* 16S rDNA was carried out either directly from DNA extracted from the rhizosphere or by using nested PCR in which 16S rDNA was first amplified with eubacterial primers and then subsequently with *Desulfobacteriaceae*-specific primers. The second approach was intended as a means to dilute potential PCR inhibitors while simultaneously increasing target DNA concentration. Primers fD1 (5'-gggaattcgtcgacAGAGTTTGATCCTGGCTCA-3') and rP2 (5'-ggaagcttggatccACGGCTACCTTGTTACGACTT-3') (Weisburg et al., 1991) were used to amplify eubacterial 16S rDNA, while primers fD1 and r804 (5'-ggaagcttggatccCAACGTTTACTGCGTGGA-3') were used to amplify an 830 bp region of 16S rDNA from *Desulfobacteriaceae* (annealing sites are written in upper case letters). Primer r804 was derived from probe 804, which was designed to target all members of the *Desulfobacteriaceae* family except *Desulfobulbus* species and *Desulfoarculus baarsii* (Devereux et al., 1992). The PCR mixtures consisted of 50 mM KCl, 10 mM Tris-Cl pH 8.3, 2 mM $MgCl_2$, 200 µM each dNTP (dATP, dCTP, dGTP, dTTP), 0.2 µM each primer, and 1-2 µl DNA template in a total volume of 100 µl. The 'hot-start' method was used by heating the PCR mixture to 94° C for 2 min, and then adding 2 U *Taq* DNA polymerase to each reaction mixture. The "hot start" method was used, and for amplification with fD1 and rP2, 30 cycles were used, each consisting of 1 min at 92° C, 1 min at 37° C, and 2 min at 72° C, using a Perkin-Elmer DNA Thermal Cycler (Perkin Elmer-Cetus, Norwalk, CT) or Cyclogene (Model PHC-3, Techne, Cambridge, UK) thermal cycler. For fD1 and r804, the annealing temperature and magnesium chloride

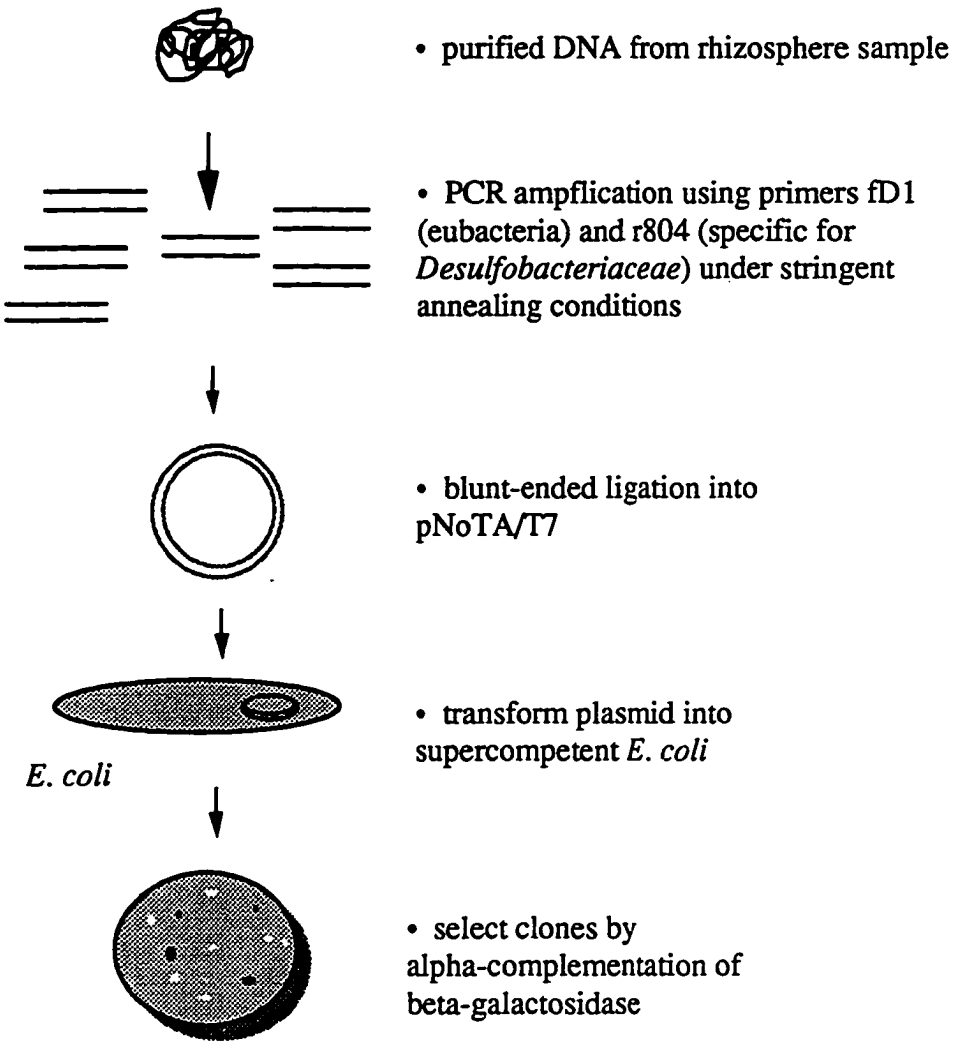


Fig. 1.3. Schematic diagram of method used for selective amplification of *Desulfobacteriaceae* 16S rDNA fragments.

concentration were optimized for specificity and product yield. Specific amplification of *Desulfobacteriaceae* 16S rDNA was ensured by using DNA extracted from the following species as control DNA templates: *D. multivorans* (positive control); *D. vulgaris* (negative control), and *E. coli* (negative control). Controlling for specificity was important in this reaction because the r804 primer has only two mismatches with several non-target bacteria. Optimal specificity and product yields were obtained by subjecting reaction mixtures to 40 PCR cycles, each consisting of 1 min at 92° C, 1 min at 65° C, and 1 min at 72° C, followed by 5 min at 72° C. PCR products were analyzed by electrophoresis on a 0.8% agarose gel using standard techniques.

Amplified 16S rDNA fragments were purified from the PCR mixture, ligated bidirectionally into plasmid vector pNoTA/T7 (Five Prime Three Prime, Inc., Boulder, CO) using blunt-ended ligation, and transformed into competent *E. coli* cells using the Prime PCR Cloning Kit (Five Prime Three Prime, Inc.) as described by the manufacturer. Because restriction digestion of PCR products was not necessary in this cloning procedure, any bias associated with internal restriction sites was avoided. Transformants were selected for ampicillin-resistance conferred by the pNoTA/T7 plasmid and colonies were screened for inserts by alpha-complementation using X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside) and IPTG (isopropyl-ß-D-thiogalactopyranoside) (Sambrook et al., 1989). Further screening to ensure that white colonies chosen for analysis contained the appropriate insert was carried out by using PCR to selectively amplify the cloned insert (Fig. 1.4). For this purpose, PCR conditions described above were used, except that the template consisted of 1-2 µl clone cells grown to mid-exponential phase in Luria-Bertani broth (10 g/l bacto-tryptone, 5 g/l bacto-yeast extract, 10 g/l NaCl, pH 7.0) with 100 µg/ml ampicillin.

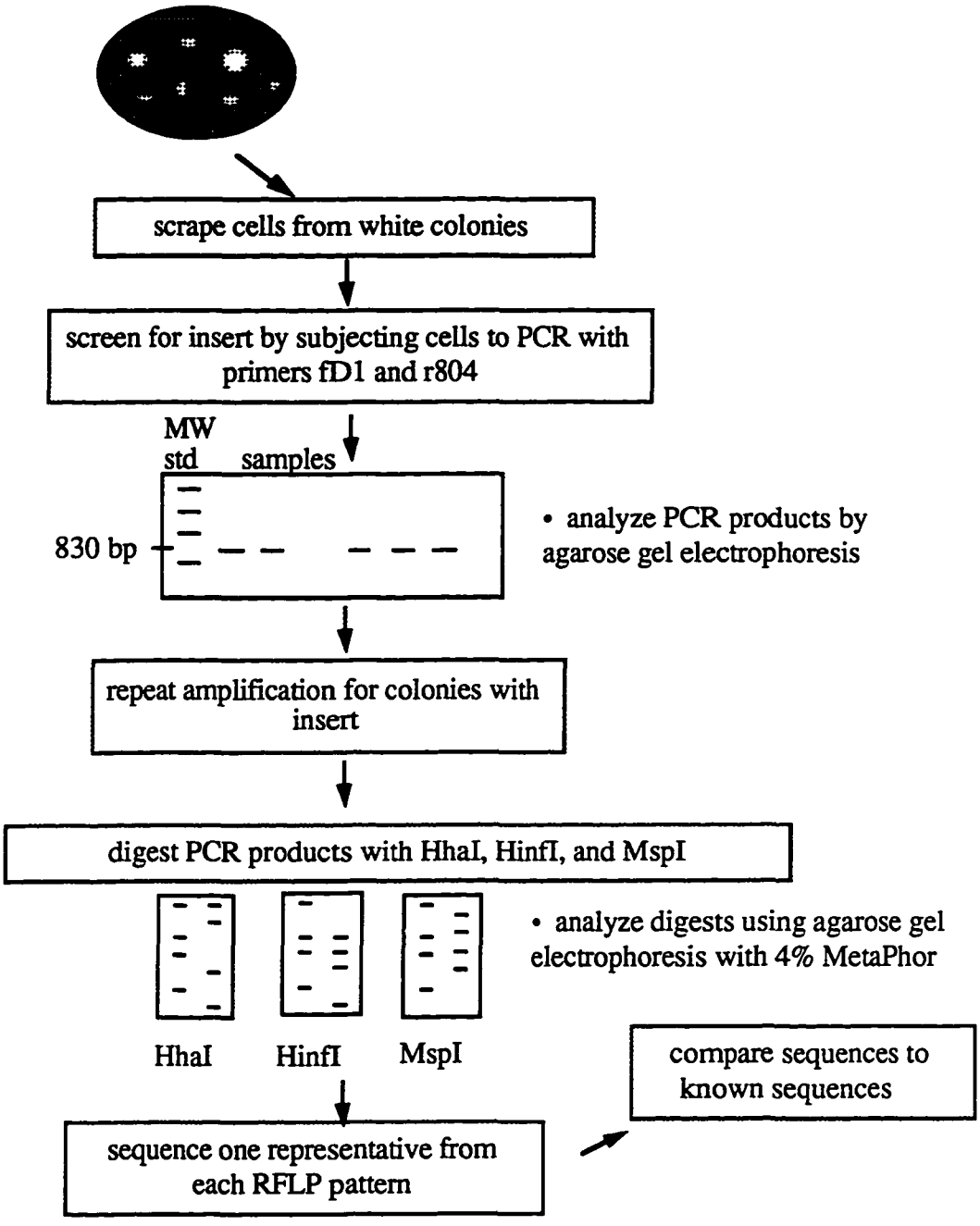


Fig. 1.4. Schematic diagram of method used to screen cloned inserts and place them into RFLP categories.

In order to avoid sequencing all the cloned inserts, clones that were found to contain the 830 bp insert were placed into categories using restriction fragment length polymorphism (RFLP) analysis (Fig. 1.4). Cloned inserts were amplified using whole cells as templates in PCR, as described above. The PCR products were then concentrated and desalted by ultrafiltration using Ultra-MC filter units with a 10,000 nominal molecular weight limit low-protein-binding regenerated cellulose membrane (Millipore, Inc., Bedford, MA) as recommended by the manufacturer. Each PCR product was digested separately with the tetrameric endonucleases *Msp*I, *Hha*I, and *Hin*fI (Sambrook et al., 1989). Restriction fragments and a molecular weight standard (pBR322 DNA digested with *Hae*III, Sigma Chemical Co., St. Louis, MO) were resolved by gel electrophoresis in 4% MetaPhor agarose (FMC Bioproducts) containing 0.2 μg/ml ethidium bromide and were visualized by UV excitation. Clones were categorized by comparing restriction patterns obtained.

Sequencing and Phylogenetic Analyses

Plasmid DNA from at least one representative clone from each RFLP category was purified using the Perfect Prep system (Five Prime Three Prime, Inc.) and sequenced using a PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit (Perkin-Elmer Cetus) and an ABI 373A automated sequencer (Applied Biosystems, Foster City, CA). Primers M13 -20, M13 reverse (Stratagene, Inc., La Jolla, CA), and R536 (5'-ACCGCGGCKGCTGGC-3') were used in sequencing reactions. Expected RFLP patterns for restriction endonucleases *Hha*I, *Hin*fI, and *Msp*I were generated for each cloned insert from sequence data using the program DNA* (DNASTAR, Inc., Madison, WI) and were compared to observed RFLP patterns (above).

Close phylogenetic relatives of the sequences were found by comparing them with Ribosomal Database Project (RDP) sequences using the programs SIMILARITY_RANK and SUGGEST_TREE (Maidak et al., 1994) and sequences in GenBank using BLASTN (Altschul et al., 1990). This allowed for the identification of the isolates' close relatives. The cloned sequences were aligned with 16S rRNA sequences of other *Desulfobacteriaceae*, *Myxococcus xanthus*, and *E. coli* using the ClustalW Multiple Sequence Alignment Program (version 1.5; (Thompson et al., 1994) and then using secondary structure characteristics to manually refine the automated alignments using the sequence editor, SEQAPP (Gilbert, 1989). GenBank accession numbers for the sequences used in this study, including those determined here, are shown in Table 1.1. Only base positions that were unambiguously aligned were used in subsequent analyses. This was effected by applying masks to the alignments to designate positions that were to be included in analyses.

Phylogenetic trees were constructed using maximum parsimony, neighbor-joining, and least-squares methods available in the phylogenetic analysis application package PHYLIP 3.57 (Felsenstein, 1989). The program SEQBOOT was used to generate 100 bootstrapped data sets from each alignment. For maximum parsimony analyses, trees were inferred from the bootstrapped data sets using the program DNAPARS, with the options for randomized input order of sequences and global rearrangements invoked. For neighbor-joining trees, the program DNADIST was used to calculate Jukes-Cantor corrected evolutionary distances for each of the 100 bootstrapped data sets. Subsequently, the program NEIGHBOR was used to infer neighbor-joining trees from evolutionary distances (once again with a randomized input order of sequences). For both parsimony and neighbor-joining methods, the program CONSENSE was used to identify a consensus tree from the 100 trees generated by the

Table 1.1. GenBank accession numbers for 16S rRNA sequences used in phylogenetic analyses, including sequences determined here.

Organism/Cloned Sequence	Accession no.
clone 2B14	U85478
clone 4D19	U85479
clone A01	U85480
Desulfoarculus baarsii str. 2st14, Konstanz	M34403
Desulfobacter curvatus str. AcRM3	M34413
Desulfobacter hydrogenophilus str. AcRS1	M34412
Desulfobacter latus str. AcRS2	M34414
Desulfobacter postgatei str. 2 ac 9	M26633
Desulfobacter sp. str. 4ac11	M34416
Desulfobacterium autotrophicum	M34409
Desulfobacterium vacuolatum	M34408
Desulfobacula toluolica	X70953
Desulfobulbus elongatus	X95180
Desulfobulbus propionicus str. 1 pr 3, Lindhorst	M34410
Desulfobulbus sp. str. 3pr10	M34411
Desulfocapsa thiozymogenes str. Bra2	X95181
Desulfococcus multivorans str. 1 be 1, Goettingen	M34405
Desulfomonile tiedjei	M26635
Desulfonema limicola	U45990
Desulfonema magnum	U45989
Desulfosarcina variabilis str. 3 be 13, Montpellier	M26632
Escherichia coli subsp. K-12	M87049
Myxococcus xanthus str. DK1622	M34114

bootstrapped data sets. Branch lengths for consensus trees were then obtained by using DNADIST to calculate Jukes-Cantor distances from the original data set (i.e., not from bootstrapped data sets) and the topologies of the parsimony and neighbor-joining consensus trees were used to construct trees with branch lengths based on evolutionary distances with the tree-building program, FITCH. This was done because branch lengths are not calculated by CONSENSE. Likewise, least-squares trees were inferred by first using DNADIST to calculate Jukes-Cantor distances directly from aligned sequences (with no bootstrapping) and then using the FITCH program to infer phylogenetic trees from evolutionary distances based on the Fitch-Margoliash least-squares method. For least-squares trees, the randomized input order and global rearrangements options were invoked, and trees resulting from 10 different input orders were evaluated. Bootstrapped data sets were not used in least squares phylogenetic analyses because the computational intensity of this method precluded analysis of multiple data sets with the available computer resources. For all tree-building algorithms, global rearrangements were carried out. The programs RETREE and DRAWGRAM were used to designate outgroup species and to plot trees. The cloned sequences were also checked for potential chimeras by using the CHECK_CHIMERA program of the RDP (Maidak et al., 1994).

Results and Discussion

DNA Purification and Amplification of 16S rDNA Fragments

Amplification of DNA extracted by the freeze/thaw method without further purification was achieved with the 'universal' prokaryotic primers, fD1 and rP2 by diluting root-associated DNA by a factor of 10^{-3} (Fig. 1.5). However, use of the crude PCR product in subsequent amplification with fD1 and r804

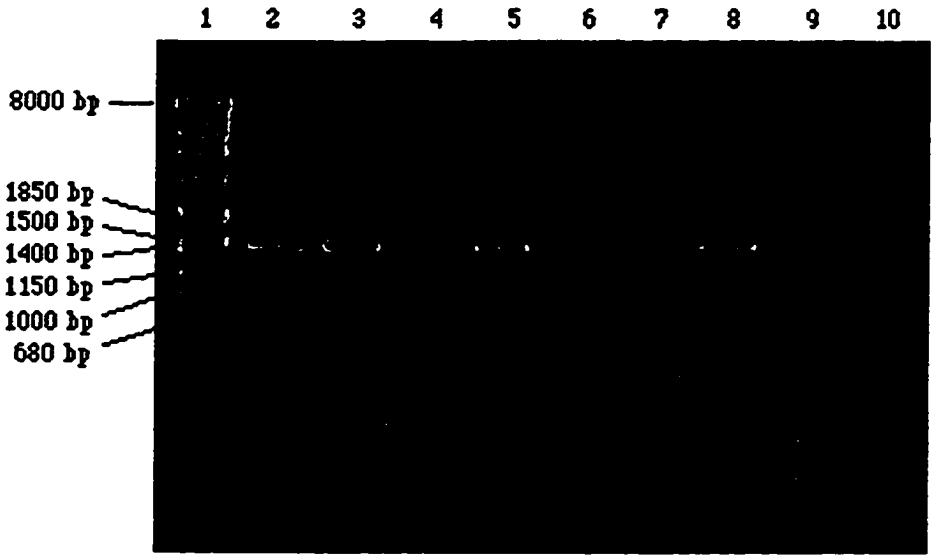


Fig. 1.5. PCR product from selective amplification of near-complete eubacterial 16S rRNA genes from DNA extracted from rhizosphere samples. Primers fD1 and rP2 were used in reactions. Lane 1 contains a molecular weight standard, with the length of each band given in base pairs. Lanes 2-10 contain PCR products from reactions with the following templates: 2: *E. coli* DNA (positive control); 3: *D. multivorans* DNA (positive control); 4: no DNA (negative control); 5 - 10: DNA extracted from the rhizopshere in the diluted to 10^{-3} (lanes 5 and 8), 10^{-4} (lanes 6 and 9), 10^{-5} (lanes 7 and 10).

resulted in non-specific amplification and background product formation, and direct amplification with fD1 and r804 from the extracted DNA was unsuccessful. The latter result was not surprising due to the expected decrease in target DNA and, hence, increase in the ratio of contaminating inhibitors compared to the universal amplification reaction. Because of these results, an alternate cell lysis technique (bead beating) that generates higher yields (Moré et al., 1994) and further purification of DNA were used in an attempt to achieve direct amplification of environmental DNA with fD1 and r804. PCR amplification was achieved with DNA extracted by the bead-beating technique and purified by the SpinBind/low-melt agarose electrophoresis technique (Fig. 1.2 and 1.6).

65 of 100 clones screened contained an insert of the expected size (approximately 830 bp), and were further analyzed for restriction fragment length polymorphisms (RFLPs). From RFLP analyses, 8 unique operational taxonomic units (OTUs) were found, based on a combination of 5 unique *Hha*I fragment patterns (Fig. 1.7), 4 unique *Hinf*I patterns (Fig. 1.8), and 3 unique *Msp*I patterns (Fig. 1.9). Moyer et al. (1996), using simulated RFLP data from 16S rRNA sequences available in the RDP, have shown that digestion with combinations of 3-4 tetrameric restriction endonucleases detected >99% of the different OTUs in their model data set. Therefore, it is likely that the three tetrameric restriction endonucleases used in this study were sufficient to screen clones for unique sequences.

Sequence Analysis and Consideration for Potential Chimeras

Comparison of expected RFLP patterns generated from sequence data to RFLP patterns determined empirically was used as a check against the quality of

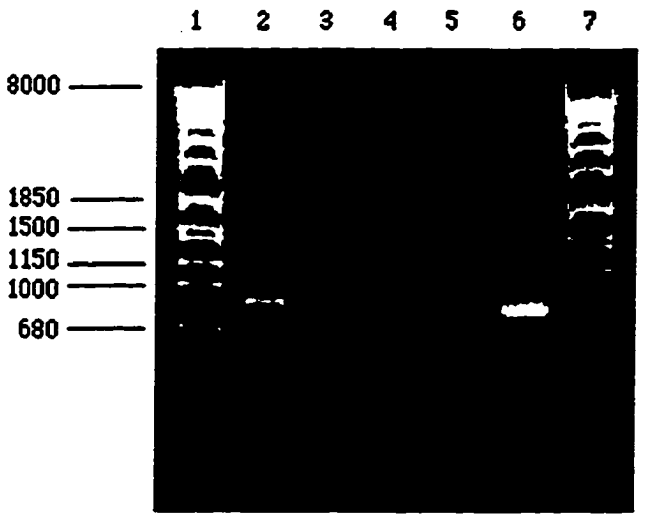


Fig. 1.6. PCR product from selective amplification of *Desulfobacteriaceae* 16S rRNA gene fragments from DNA extracted from rhizosphere samples. Primers fD1 and r804 were used in reactions. Lanes 1 and 7 contain molecular weight standards, with the length of each band given in base pairs. Lanes 2-6 contain PCR products from reactions with the following templates: 2. rhizosphere DNA diluted to 10^{-3}; 3. rhizosphere DNA diluted to 10^{-4}; 4. *E. coli* DNA (negative control for specificity); 5. *D. vulgaris* DNA (negative control for specificity); 6. *D. multivorans* DNA (positive control).

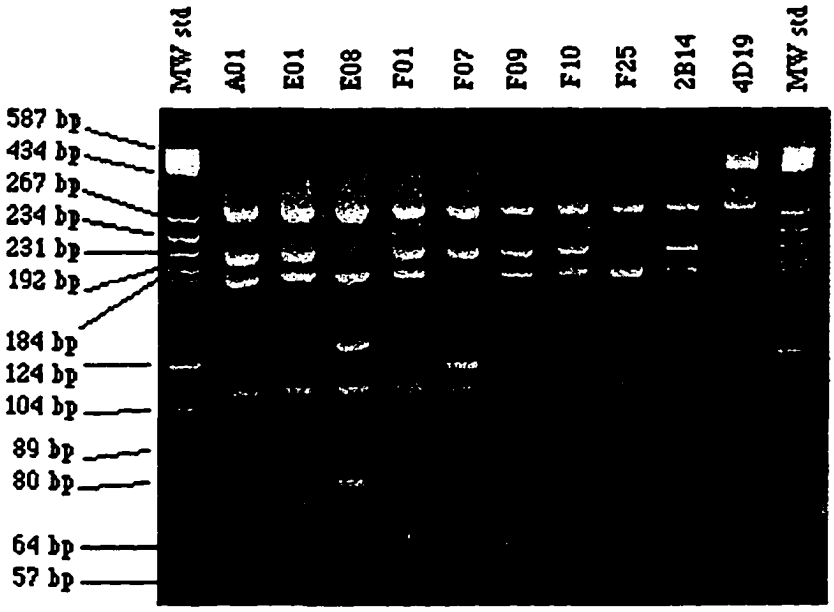


Fig. 1.7. Restriction fragment length polymorphisms generated by digesting PCR amplified cloned 16S rDNA fragments with *Hha*I. Clones A01, E01, F01, F09, F10, and 2B14 exhibited the same *Hha*I RFLP pattern, while patterns for clones E08, F07, F25, and 4D19 were unique.

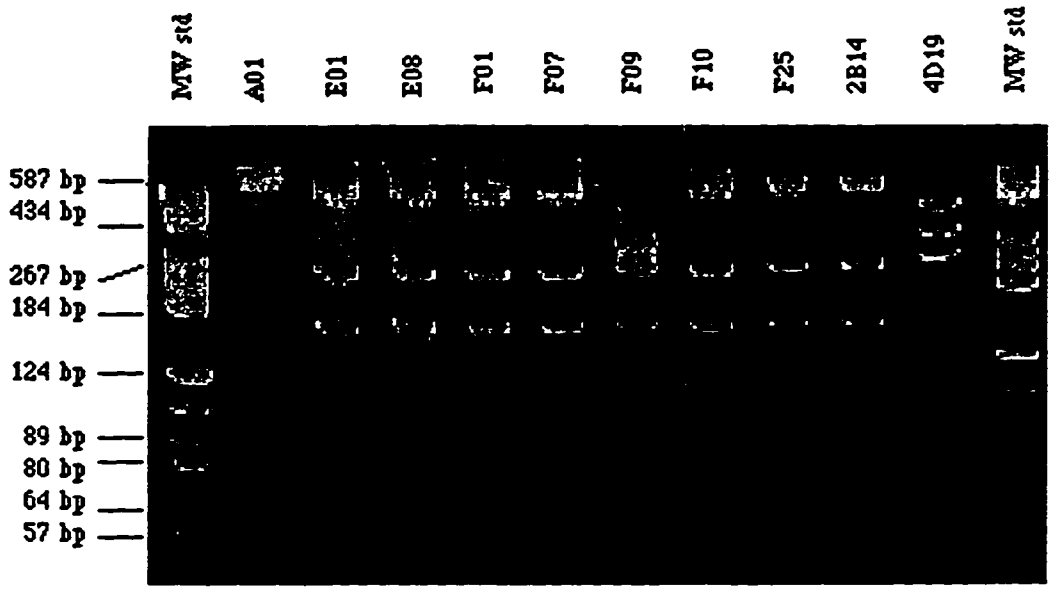


Fig. 1.8. Restriction fragment length polymorphisms generated by digesting PCR amplified cloned 16S rDNA fragments with *Hinfl*. Clones E01, E08, F01, F07, F10, F25, and 2B14 exhibited the same *Hinfl* RFLP pattern, while patterns for clones A01, F09, and 4D19 were unique.

both sequence and RFLP data. As expected, RFLP patterns generated from sequence data matched empirically determined patterns well.

Analysis of the cloned sequences using the CHECK_CHIMERA program of the RDP (Maidak et al., 1994) indicated that one of the eight sequences, A01, had some characteristics of a chimera. However, analysis of the predicted secondary structure of A01 showed complementary base-pairing for all 74 bp where the two potential chimera fragments were expected to form helices and both fragments shared higher identity with *D. multivorans* (88.3% and 90.2% for first and second fragments, respectively) than with *Desulfosarcina variabilis* (84.1% and 89.7%). I was therefore confident that A01 was not chimeric.

Phylogeny of Retrieved 16S rDNA Sequences

Phylogenetic trees constructed using maximum parsimony, neighbor-joining, and least squares methods exhibited similar topologies and placed all of the cloned sequences within the *Desulfobacteriaceae* near *D. multivorans* and *D. variabilis* (Fig. 1.10-1.12). Two sequences were unique: A01, which shared 89.1% identity with *D. multivorans*, and 4D19, which shared 96.1% identity with *D. variabilis*. The remaining 6 sequences (2B14, E08, F01, F07, F09, and F25) were very closely related to *D. multivorans* (sharing 99.0 to 99.7% identity), suggesting that they represented strains of this species. *D. multivorans* and *D. variabilis* are members of an SRB phylogenetic group whose members are capable of utilizing a wide array of electron donors for sulfate reduction (Devereux et al., 1989; Widdel and Bak, 1992). It is likely that the cloned sequences were derived from sulfate reducers that possess capabilities similar to those of *D. multivorans* and *D. variabilis*.

The algorithms used for phylogenetic tree inference were chosen in order to compare trees generated from methods with different underlying principles

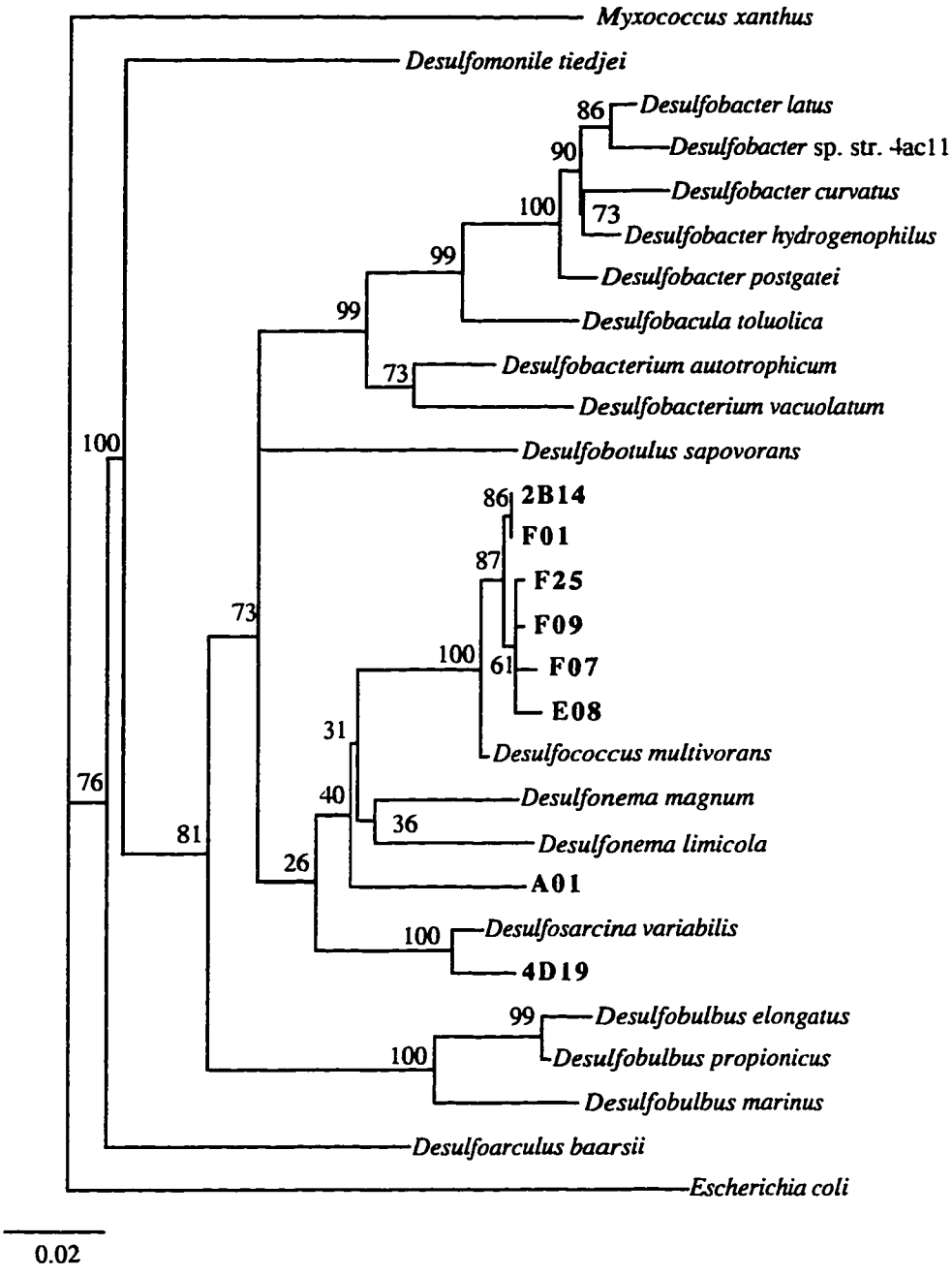


Fig. 1.10. Phylogenetic tree of cloned16S rRNA gene fragments and 16S rRNA sequences of members of the *Desulfobacteriaceae* family constructed using a maximum parsimony method. 688 base positions were considered in the analysis. Bootstrap values (out of 100 trees) are shown adjacent to nodes. The scale bar is in fixed nucleotide substitutions per sequence position.

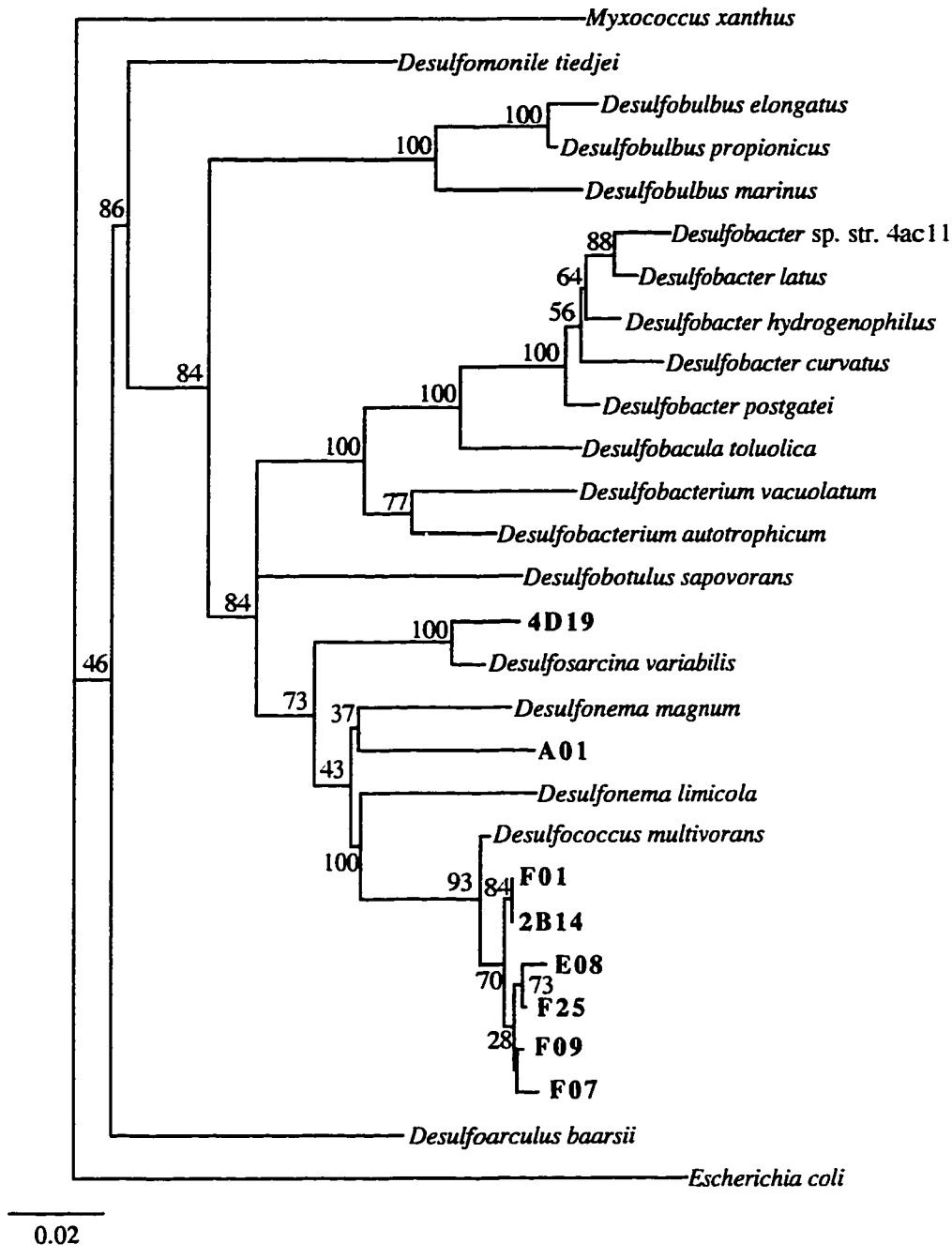


Fig. 1.11. Phylogenetic tree of cloned 16S rRNA gene fragments and 16S rRNA sequences from members of the *Desulfobacteriaceae* family constructed using a neighbor-joining algorithm. Jukes-Cantor evolutionary distances were calculated from 688 base positions. Bootstrap values (out of 100 trees) are shown adjacent to nodes. The scale bar is in fixed nucleotide substitutions per sequence position.

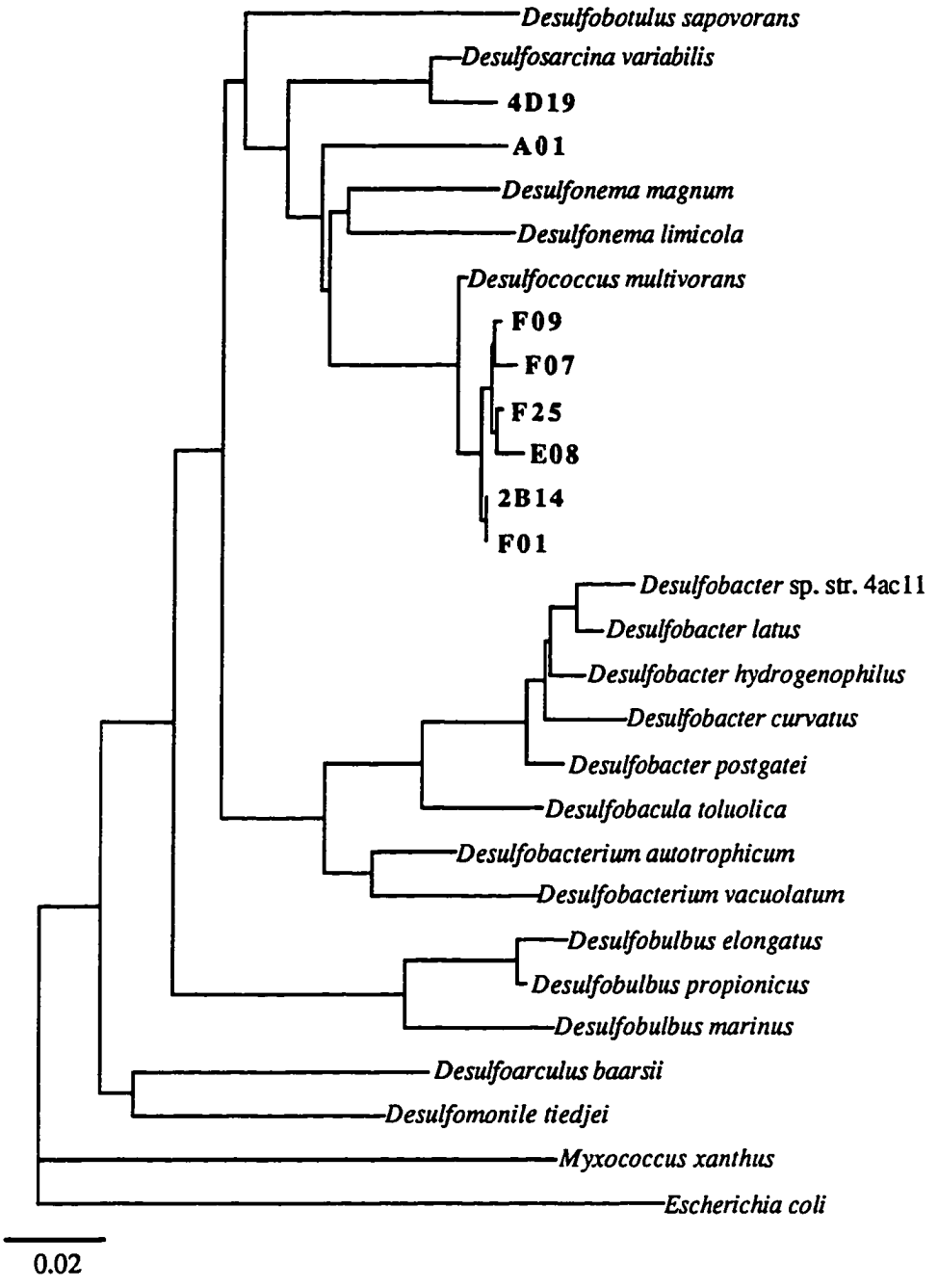


Fig. 1.12. Phylogenetic tree of cloned 16S rRNA gene fragments and 16S rRNA sequence of members of the *Desulfobacteriaceae* family constructed from evolutionary distances using the Fitch-Margoliash least squares method. Jukes-Cantor evolutionary distances were calculated from 688 base positions. The scale bar is in fixed nucleotide substitutions per sequence position.

and biases, as well as for practical reasons such as limited availability of computational power, which precluded use of maximum likelihood inference techniques. Although maximum likelihood inferences are gaining favor and are considered by some to be the most statistically valid phylogenetic analysis method (Felsenstein, 1981; Russek-Cohen and Jacobs, 1993; Olsen et al., 1994a), the less computationally intensive maximum parsimony, neighbor-joining, and least squares methods used here should be sufficiently accurate given the characteristics of the phylogenetic data set analyzed (Felsenstein, 1988; Felsenstein, 1989).

Analysis of multiple bootstrapped data sets (Felsenstein, 1985) was carried out for the computationally less intensive parsimony and neighbor-joining tree inference methods as a means of assessing the accuracy of each node (Fig. 1.10 - 1.11). Through analysis of simulated and experimental phylogenetic data sets, Hillis and Bull (1993) have shown that bootstrap proportions generally reflect very conservative estimates of accuracy. For example, in parsimony analyses under most conditions, bootstrap proportions of greater than 50% were much lower than the probability that the corresponding node was correct (Hillis and Bull, 1993). As a general rule, bootstrap proportions ≥70% corresponded to a probability of 95% that a given clade was accurate (Hillis and Bull, 1993). For parsimony analyses, bootstrap proportions were underestimates of accuracy only under conditions of highly unequal rates of change among different branches, extremely high rates of change (i.e., such that characters were randomized with respect to evolutionary history), or systematic biases in the data set (Hillis and Bull, 1993). However, under these conditions, parsimony methods are themselves inconsistent (Felsenstein, 1978; Felsenstein, 1988). Zarkikh and Li (1992a; 1992b) also evaluated bootstrapping as a method for estimating accuracy

in parsimony and neighbor-joining inference methods and likewise concluded that bootstrap proportions could serve as conservative estimates of accuracy.

The high bootstrap proportions found in both parsimony and neighbor-joining trees for the node connecting *D. multivorans* with 2B14, F01, F07, F08, F09, and F25 (Fig. 1.10 - 1.11) indicate the high probability of this node's accuracy. Similarly, bootstrap proportions indicated a high probability of the placement of 4D19 as a neighbor of *D. variabilis*, with bootstrap proportions of 100% for both parsimony and neighbor-joining trees (Fig. 1.10 - 1.11). Much lower bootstrap proportions and differences in branching patterns were observed for nodes connecting A01 with other members of the *Desulfococcus-Desulfosarcina-Desulfonema* assemblage (Fig. 1.10 - 1. 12), implying that although A01 appears to be a member of this assemblage, its branching order is uncertain.

Parsimony methods, such as the Fitch and Wagner parsimony method of DNAPARS (Felsenstein, 1989) used to infer the tree in Fig. 1.10, are based on the principle that the correct tree is that which minimizes the total number of evolutionary steps needed to explain the observed data set (Felsenstein, 1988; Swofford et al., 1996). Here, both local rearrangements (all possible rearrangements of internal branches) and global rearrangements (removal of each possible subtree followed by adding the subtree back in all possible places) in the program DNAPARS were carried out in the search for a tree topology that yielded the most parsimonious of all trees tested (Felsenstein, 1989). The algorithm used in DNAPARS functions by adding an OTU, evaluating local and global rearrangements, and then successively adding and evaluating remaining OTUs. Therefore, the input order of OTUs can affect the outcome (Felsenstein, 1989). In order to avoid biases based on OTU input order, a different random input order was used for each bootstrapped data set analyzed.

Although parsimony methods are widely used, they have been shown to be inconsistent under conditions of highly unequal branch lengths (Felsenstein, 1978). Under these conditions, parsimony methods tend to cluster longer branches together even if the resulting topologies are incorrect. This tendency is due to the increased number of mutations in longer branches resulting in fewer character sites that are informative (i.e., that reflect the true tree topology) and an increase in sites that are misinformative (i.e., that suggest an incorrect tree due to more than one change in character state at a given site) (Felsenstein, 1978; Felsenstein, 1988). However, the smaller the rate of overall evolutionary change in the data set, the more unequal the branch lengths must be in order to generate this inconsistency in parsimony analyses (Felsenstein, 1988). Here, the evolutionary distances among the sequences considered, with the exception of outgroup species *E. coli* and *M. xanthus*, were less than 0.20 (Table 1.2) and were therefore unlikely to be problematic in parsimony analyses. Moreover, comparison of the parsimony tree (Fig. 1.10) with trees generated by neighbor-joining and least squares methods (Fig. 1.10 - 1.12) reveal very similar topologies.

For both neighbor-joining and least squares methods, the input data set consisted of evolutionary distances calculated from sequence data (Table 1.2). Here, Jukes-Cantor evolutionary distances (Jukes and Cantor, 1969) were calculated in the program DNADIST (Felsenstein, 1989). These corrected evolutionary distances attempt to account for superimposed mutations that are likely to occur with increasing frequency as distances between sequences increase (Swofford et al., 1996).

The neighbor-joining method of Saitou and Nei (1987) was used to generate the tree in Fig. 1.11. This method begins with a star-like tree topology (i.e., one internal node connecting all OTUs). Using evolutionary distances, it successively links neighbors (i.e., OTUs or groups of OTUs connected by a single

Table 1.2. Jukes-Cantor evolutionary distances for partial 16S rRNA of 'molecular isolates' and members of the *Desulfobacteriaceae* family.

Organism	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.	19.	20.	21	22.	23.	24.	25.	26.	27.
1. *Escherichia coli*																											
2. *Myxococcus xanthus*	0.240																										
3. *Desulfoarculus baarsii*	0.201	0.182																									
4. *Desulfobacter curvatus*	0.252	0.215	0.180																								
5. *Desulfobacter hydrogenophilus*	0.248	0.212	0.177	0.025																							
6. *Desulfobacter latus*	0.244	0.217	0.177	0.031	0.018																						
7. *Desulfobacter postgatei*	0.248	0.206	0.185	0.031	0.020	0.021																					
8. *Desulfobacter* sp. str. 4ac11	0.266	0.232	0.191	0.036	0.024	0.017	0.031																				
9. *Desulfobacterium autotrophicum*	0.224	0.195	0.161	0.094	0.091	0.095	0.089	0.101																			
10. *Desulfobacterium vacuolatum*	0.245	0.217	0.172	0.098	0.088	0.099	0.093	0.099	0.052																		
11. *Desulfobotulus sapovorans*	0.226	0.217	0.155	0.145	0.134	0.145	0.131	0.144	0.102	0.128																	
12. *Desulfobacula toluolica*	0.260	0.208	0.178	0.069	0.062	0.064	0.053	0.065	0.073	0.082	0.122																
13. *Desulfobulbus elongatus*	0.246	0.212	0.161	0.165	0.155	0.157	0.147	0.164	0.158	0.176	0.165	0.146															
14. *Desulfobulbus propionicus*	0.240	0.211	0.149	0.156	0.152	0.151	0.142	0.157	0.145	0.164	0.154	0.139	0.012														
15. *Desulfobulbus marinus*	0.249	0.218	0.164	0.171	0.166	0.163	0.158	0.159	0.138	0.165	0.161	0.152	0.057	0.065													
16. *Desulfococcus multivorans*	0.213	0.203	0.144	0.134	0.129	0.128	0.124	0.141	0.093	0.121	0.101	0.125	0.159	0.140	0.146												
17. *Desulfomonile tiedjei*	0.219	0.189	0.116	0.185	0.181	0.183	0.175	0.190	0.152	0.174	0.170	0.161	0.163	0.150	0.164	0.125											
18. *Desulfonema limicola*	0.218	0.214	0.167	0.141	0.136	0.138	0.129	0.150	0.103	0.139	0.108	0.130	0.162	0.154	0.152	0.061	0.143										
19. *Desulfonema magnum*	0.226	0.222	0.152	0.147	0.139	0.141	0.127	0.146	0.108	0.128	0.097	0.126	0.163	0.152	0.150	0.064	0.139	0.065									
20. *Desulfosarcina variabilis*	0.219	0.207	0.147	0.134	0.129	0.138	0.119	0.143	0.094	0.113	0.107	0.124	0.139	0.120	0.137	0.076	0.127	0.088	0.077								
21. 2B14	0.219	0.201	0.150	0.137	0.137	0.137	0.131	0.146	0.100	0.128	0.109	0.128	0.164	0.143	0.150	0.008	0.132	0.071	0.069	0.079							
22. 4D19	0.224	0.216	0.154	0.145	0.139	0.147	0.129	0.152	0.102	0.123	0.118	0.131	0.151	0.130	0.146	0.083	0.139	0.092	0.082	0.020	0.087						
23. A01	0.227	0.211	0.131	0.148	0.136	0.139	0.137	0.151	0.114	0.133	0.124	0.134	0.158	0.144	0.144	0.074	0.124	0.088	0.070	0.085	0.075	0.086					
24. E08	0.229	0.209	0.160	0.148	0.143	0.143	0.136	0.155	0.108	0.139	0.116	0.137	0.173	0.153	0.157	0.017	0.139	0.078	0.076	0.088	0.010	0.092	0.074				
25. F01	0.219	0.201	0.150	0.137	0.137	0.137	0.131	0.146	0.100	0.128	0.109	0.128	0.164	0.143	0.150	0.008	0.132	0.071	0.069	0.079	0.000	0.087	0.075	0.010			
26. F07	0.226	0.206	0.157	0.144	0.142	0.142	0.135	0.154	0.101	0.129	0.112	0.127	0.170	0.150	0.154	0.014	0.137	0.075	0.074	0.084	0.009	0.092	0.078	0.012	0.009		
27. F09	0.218	0.200	0.147	0.135	0.134	0.134	0.128	0.145	0.099	0.127	0.108	0.125	0.163	0.144	0.147	0.009	0.131	0.070	0.068	0.080	0.006	0.086	0.072	0.008	0.006	0.006	
28. F25	0.224	0.204	0.155	0.142	0.140	0.141	0.134	0.153	0.102	0.131	0.111	0.129	0.169	0.148	0.153	0.012	0.135	0.074	0.072	0.082	0.006	0.089	0.073	0.006	0.006	0.007	0.005

node) that minimize total tree length. This method is algorithmic (Swofford et al., 1996); it produces one tree and does not evaluate alternative trees in order to optimize an objective criterion (such as maximum parsimony, least squares, or maximum likelihood methods do). However, it is computationally efficient and therefore amenable to the analysis of large data sets and bootstrapping analyses (Felsenstein, 1989).

The Fitch-Margoliash least squares method was used to infer the phylogenetic tree in Fig. 1.12 (Fitch and Margoliash, 1967; Felsenstein, 1989). This method calculates a least squares measure of the lack of fit between observed and expected distances given a certain tree topology and then seeks to minimize this criterion (Fitch and Margoliash, 1967). As in parsimony tree construction, both local and global rearrangements in the program FITCH were carried out in the search for a tree topology that yielded the lowest least squares measure of all trees tested (Felsenstein, 1989). Because input order of OTUs can affect the outcome (Felsenstein, 1989), trees constructed from 10 different random OTU input orders were evaluated and compared.

Potential Physiological and Ecological Characteristics of Novel Phylotypes

The physiological characteristics of the novel phylotypes' closest relatives suggest that the versatility of this group of SRB may contribute to their success in the salt marsh sediment. As its name suggests, the closest relative of A01, *D. multivorans*, is capable of utilizing a diverse array of electron donors including formate, lactate, ethanol, acetate, 3-16 C fatty acids (Widdel and Bak, 1992), secondary alcohols such as 2-propanol and 2-butanol (Hansen, 1993), and isobutyrate, (Hansen, 1993). Other electron donors utilized by members of the *Desulfococcus-Desulfosarcina-Desulfonema* assemblage include H_2, fumarate, malate, and benzoate (Widdel and Bak, 1992). All members of this group are

capable of complete oxidation of organic carbon to CO_2. Such nutritional versatility could be advantageous in a complex environment such as the salt marsh sediment and rhizosphere. In this habitat, potential electron donors for SRB include compounds directly released from roots, such as products of fermentative metabolism in roots during periods of hypoxia or anoxia (i.e., ethanol, malate, and probably acetate; Hines et al., 1989), as well as low molecular weight compounds such as fatty acids and amino acids released from areas of root necrosis or from sloughed root cells. Electron donors may also be indirectly supplied to SRB by fermentative and acetogenic bacteria that incompletely oxidize dissolved organic carbon released from roots and detritus. Acetate, for example, is thought to be an important intermediate produced by fermentors and subsequently utilized by SRB (Smith, 1993). Other compounds that have been found to stimulate sulfate reduction rates in salt marsh sediments, and therefore may be significant substrates for SRB, include lactate, ethanol, butanol, and formate (Smith, 1993).

Another physiological trait possessed by all members of the *Desulfococcus-Desulfosarcina-Desulfonema* assemblage is motility (Widdel and Bak, 1992), which would be advantageous in adapting to rapidly changing microscale gradients in redox potential and electron donor availability. In addition, aerobic respiration by *D. multivorans* has been reported (Dilling and Cypionka, 1990), suggesting that *D. multivorans'* close relatives (i.e., A01 and 4D19) may also be capable of aerobic respiration, or at least exhibit some tolerance of oxygen.

Conclusions

The phylogenetic diversity of the SRB community inhabiting the rhizosphere of the salt marsh cordgrass, *S. alterniflora*, was investigated by selectively retrieving and analyzing 16S rRNA gene fragments directly from

rhizosphere bacterial DNA. Due to the presence of high levels of humic compounds and clays in the salt marsh rhizosphere environment, it was necessary to attempt several methods of DNA extraction and purification. Successful DNA purification was achieved by using a bead-beating lysis technique followed by low-melt agarose gel electrophoresis. PCR was then used to selectively amplify *Desulfobacteriaceae* 16S rDNA sequences, which were cloned and analyzed. The eight gene fragments that were sequenced were all found to be members of the *Desulfococcus-Desulfosarcina-Desulfonema* assemblage. Two sequences appeared to represent novel *Desulfobacteriaceae* species: A01 which shared 89.1% identity with *D. multivorans* and 4D19 which shared 96.3% identity with *D. variabilis*. The remaining six sequences were very closely related to *D. multivorans*, sharing 99.0 - 99.7% identity with the published *D. multivorans* sequence. It is likely that the novel phylotypes found share physiological traits with their closest relatives, which utilize a diverse array of electron donors, are capable of complete oxidation of organic carbon to CO_2, and are capable of aerobic respiration.

CHAPTER TWO

POPULATION DYNAMICS OF UNCULTIVATED SULFATE-REDUCING BACTERIA IN A SALT MARSH SEDIMENT AND RHIZOSPHERE OF *SPARTINA ALTERNIFLORA*

Introduction

The advent of molecular microbial ecology has provided a glimpse of the extensive genetic diversity of soil microbial communities and, at the same time, has underscored the fact that their structure and dynamics are largely unknown. Attempts to describe the genetic diversity of natural soil or sediment communities have included DNA reassociation experiments (Torsvik et al., 1990; Torsvik et al., 1994), 16S rDNA retrieval and analysis (Borneman et al., 1996), fractionation of total bacterial DNA by G+C content (Holben and Harris, 1995), and cross-hybridization of bacterial DNA from two communities (Ritz and Griffiths, 1994), all of which have pointed to highly complex assemblages of bacterial populations. For example, Torsvik et al. (1990) estimated that 10^3-10^4 different genomic equivalents were present in one gram of soil, while Borneman et al. (1996) recovered 124 previously undescribed 16S rRNA gene sequences from an agricultural soil. These studies of genetic diversity have provided valuable but qualitative insights into microbial community composition. Several investigations of the quantitative significance of various phylogenetic groups in soils and sediments have been conducted, but these have generally included

culturing (e.g., Braun-Howland et al., 1993) or enrichment (Spring et al., 1993; Brink et al., 1994; Telang et al., 1994) steps, or have been limited to extremely broad phylogenetic groups, such as domains (e.g., Krumholz et al., 1995). As a result, very little is known about community structure and how it is influenced by different environmental conditions or different microhabitats.

In plant-inhabited soils and sediments, a particularly important microhabitat is the rhizosphere, or region of soil immediately surrounding and influenced by the roots of a plant. Key biogeochemical processes such as organic matter decomposition, pollutant degradation (Anderson et al., 1993; Anderson et al., 1994), and non-symbiotic nitrogen fixation (Teal et al., 1979) occur at accelerated rates in the rhizosphere and greatly influence plant health and ecosystem functions. In addition, in wet soils and sediments, the rhizosphere harbors the primary redox gradients which control precipitation and dissolution of geochemicals, hydraulic conductivity, and whether microbes adhere to solids or are transported. Despite its importance, very little is known about this subset of the total soil microbial community, or how it differs from its non-rhizosphere counterpart.

In the current study, a natural sediment and rhizosphere community was investigated by combining findings from a qualitative molecular phylogenetic survey of microbial diversity (Chapter One) with a quantitative study of the environmental significance of novel phylotypes. The sulfate-reducing bacteria (SRB) community inhabiting a salt marsh sediment was chosen as a model system because both the biogeochemical dynamics of this community and the physiology and 16S rRNA phylogeny of SRB have been relatively well studied. Sulfate reduction is the dominant terminal electron accepting process and accounts for more than half of the total decomposition (including aerobic) of organic matter in salt marshes (Howarth and Hobbie, 1982). In addition, the salt

marsh cordgrass, *Spartina alterniflora*, is the most thoroughly studied of marine wetland plants and considerable information is available on plant-sediment interactions, production of organic compounds by roots, and aspects of decomposition in sediments (Dacey and Howes, 1984; Howes et al., 1985; Mendelssohn and McKee, 1987; Hines et al., 1989; Hines, 1991). Sulfate reducing activity has been shown to be closely tied to plant phenology, suggesting that plant-SRB interactions in the *S. alterniflora* rhizosphere play an important role in salt marsh biogeochemical cycles (Hines et al., 1989; Hines, 1991). To date, the 16S rRNA phylogeny of SRB is one of the most complete, and hybridization probes are available for each of the major groups and several individual species (Devereux et al., 1989; Devereux et al., 1990; Devereux et al., 1992). The phylogenetic groups are also defined by distinct physiological features, in particular, the ability to use specific electron donors. Therefore, comparative rRNA methods may also provide information on the types of substrates used by rhizosphere bacteria.

As mentioned in Chapter One, previous 16S rRNA probing studies have shown that members of the *Desulfobacteriaceae* family targeted by probe 804 (Devereux et al., 1992) played a significant role in the salt marsh sediment and rhizosphere of *S. alterniflora* (Devereux et al., 1996; Hines et al., in prep). In Chapter One, direct retrieval of 16S rRNA gene fragments from rhizosphere samples was used to discover novel phylotypes A01 and 4D19, which were members of the *Desulfobacteriaceae* family. While this study showed that novel phylotypes existed in the salt marsh rhizosphere, the polymerase chain reaction (PCR)-based method used to recover sequences A01 and 4D19 cannot be used to describe the quantitative significance of the phylotypes. In fact, several studies have shown that the amplification of mixed populations of 16S rRNA genes results in PCR products that do not quantitatively reflect the distribution of 16S

rRNA genes present in the original sample (Reysenbach et al., 1992; Wilson and Blitchington, 1996). For example, Reysenbach et al. (1992) found that 16S rRNA genes from *Saccharomyces cerevisiae* were selectively amplified from a mixture of DNA purified from two strains of extremely thermophilic Archaea and from *S. cerevisiae* DNA. Similarly, Wilson and Blitchington (1996) found that the diversity of 16S rRNA genes present in amplicons from 35 cycles of PCR was lower than that of amplicons from nine cycles of PCR, indicating preferential amplification of certain 16S rDNA sequences. In addition, the potentially vast diversity of 16S rRNA genes present in a given sediment or rhizosphere sample (see Introduction) precludes quantitative assessment of their distribution via PCR/cloning methods due to the enormous number of cloned sequences that would have to be analyzed. Given these limitations of PCR-based approaches, I chose to directly probe 16S rRNA extracted from sediment and rhizosphere samples to quantitatively study the environmental significance and population dynamics of the novel phylotypes described in Chapter One. Thus, the objectives of this chapter were to: 1) develop a new technique to synthesize reference RNAs for the uncultivated phylotypes; 2) design and optimize 16S rRNA-targeted oligonucleotide probes that specifically target the novel phylotypes; and 3) apply the newly designed and currently available probes to quantitatively investigate the population dynamics of the targeted phylotypes in the marsh sediment and rhizosphere.

Methods

Study Site and Sample Collection

Samples were collected from a tall-form, creekside stand of *S. alterniflora* in Chapman's Marsh in southeastern New Hampshire as described in Chapter One. Sediment cores were either processed within 1-2 h of sample collection or stored

at -80° C until used for further manipulations. Cores were collected biweekly or monthly from 3 November 1993 to 5 October 1994.

Generation of RNA Standards by *In Vitro* Transcription

Reference RNAs containing the target sequences for the probes for novel phylotypes and for the eubacterial probe (EUB338; Table 2.1; Stahl et al., 1988) were generated using *in vitro* transcription with the cloned environmental sequences A01, 2B14, and 4D19 (Chapter One) serving as template DNA (Fig. 2.1). In order to generate a transcript that contained a sense 16S rRNA sequence, it was first necessary to unidirectionally sub-clone inserts into a plasmid vector that contained an RNA polymerase promoter located at the 5' end of the 16S rDNA insert. This was done by first cleaving the inserts from pNoTA using *Sal*I and *Bam*HI -- endonucleases whose recognition sequences had been incorporated into the 5' ends of primers fD1 and r804, respectively, that were used to amplify 16S rRNA gene fragments from environmental samples, as described in Chapter One. The cleaved insert was then separated from pNoTA DNA by gel electrophoresis in 2.5% NuSieve agarose (FMC Bioproducts, Inc., Rockland, ME) and cloned into pBluescript II KS + (pBS; Stratagene, Inc., La Jolla, CA) that had been previously digested with *Bam*HI and *Sal*I (Sambrook et al., 1989). pBS was transformed into competent *E. coli* cells and transformants were screened for inserts as described in Chapter One.

Plasmid DNA was isolated from transformants and linearized by digestion with *Xba*I, which cleaved pBS at the 3' end of insert sequences. Because the presence of unlinearized plasmid would lead to preferential production of RNA transcripts of the entire plasmid sequence, complete *Xba*I digestion was ensured by analyzing an aliquot of the digest mixture by gel electrophoresis in 0.8% agarose. *Xba*I and *RNase*s were then inactivated by treatment with 50

Table 2.1. 16S rRNA oligonucleotide probes and target groups

Targets	Probe	Probe Sequence	Target site[a]	Wash temp. °C
A01	A01-183	CCCCTAAGAAAATACGAT	183-201	40
A01	A01-267	CTAACCATCGCGGCCTTG	267-285	53
4D19	4D19-189	CCCTTGATCCAACATTCC	189-207	46
Most eubacteria	EUB338[b]	GCTGCCTCCCGTAGGAGT[c]	338-356	48
Most eubacteria and A01	A01-338	GCTGCCTCCCGTAGGMGT	338-356	48
Desulfococcus multivorans *Desulfosarcina variabilis* *Desulfobotulus sapovorans*	814[d]	ACCTAGTGATCAACGTTT	814-831	45

[a] *E. coli* numbering.
[b] Stahl *et al.* 1988.
[c] M refers to A or C
[d] Devereux *et al.* 1992.

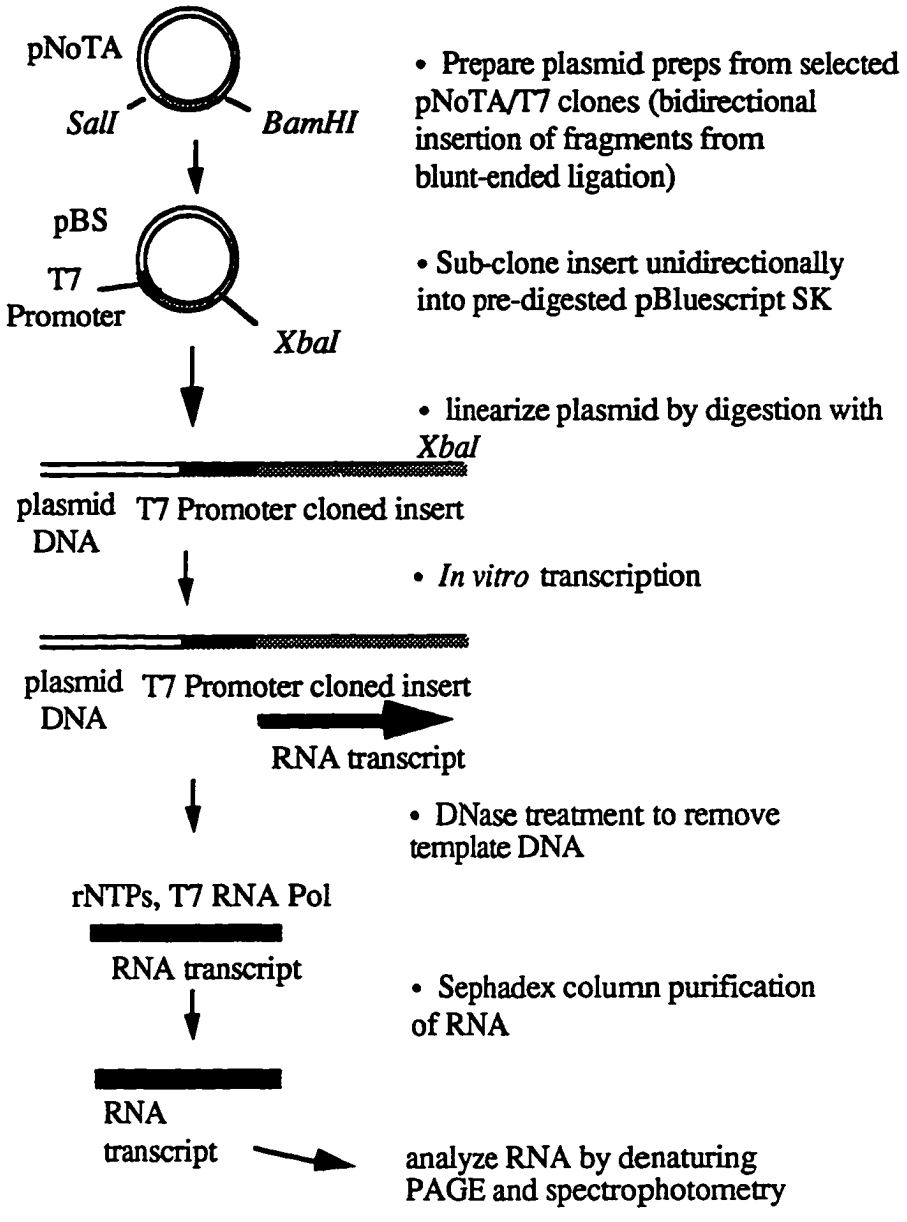


Fig. 2.1. Schematic diagram of method used to synthesize reference RNAs via *in vitro* transcription.

μg/ml proteinase K for 30 min at 37° C. The mixture was extracted twice with Tris-buffered phenol: chloroform: isoamyl alcohol (25: 24: 1; pH 8.0), and the linearized plasmid was precipitated with ethanol and resuspended in sterile dH_2O. The *in vitro* transcription reaction components were assembled in an *RNase*-free microfuge tube on ice, and contained the following: transcription buffer (40 mM Tris-HCl [pH 8.0], 8 mM $MgCl_2$, 50 mM NaCl, 2 mM spermidine, 30 mM DTT), 1 μg linearized pBS DNA, 400 μM each rNTP (rATP, rCTP, rGTP, rUTP), 30 mM DTT, 1 μl *RNase* inhibitor (Boehringer Mannheim Corp., Indianapolis, IN) and 10 U T7 RNA polymerase (Stratagene, Inc.) in a total volume of 25 μl. After incubating the reaction at 37° C for 1.5 h, an equal volume of sterile dH_2O was added and the template DNA was degraded by adding 1 μl *RNase*-free *DNaseI* (Stratagene, Inc.) and incubating at 37° C for 15 min. T7 RNA polymerase was then inactivated by heating the mixture to 75° C for 10 min and the RNA product was purified using NuClean R50 Sephadex spin columns (VWR Scientific, Inc., Bridgeport, NJ). The purified RNA transcripts were then analyzed by spectrophotometry, to determine their concentrations, and by denaturing polyacrylamide gel electrophoresis, to ensure that they were of the expected molecular weight (Sambrook et al., 1989).

Probe Design and Optimization

Oligonucleotide probes for novel *Desulfobacteriaceae* 16S rRNA sequences were designed by examining an alignment 16S rRNA gene fragments retrieved directly from Chapman's marsh *S. alterniflora* rhizosphere samples (Chapter One) and all previously described *Desulfobacteriaceae* 16S rRNA sequences available from the Ribosomal Database Project (RDP) (Maidak et al., 1994). Regions that contained sequences unique to the novel phylotypes were considered as potential probe target sites. Other factors that were also considered in probe design

included extent of mismatch between target and non-target sequences, probe G+C content, and predicted accessibility of the probe target site in *in situ* hybridization (Amann et al., 1995). Intended probe specificity was checked against the RDP using the CheckProbe utility (Maidak et al., 1994). Probe target sequences and specificity are shown in Fig. 2.2. Other oligonucleotide probes used in this study included 814 (complementary to *D. multivorans*, *Desulfosarcina variabilis*, and *Desulfobotulus sapovorans* 16S rRNA; Devereux et al., 1992); EUB338 (complementary to almost all known Bacteria 16S rRNAs; Stahl et al., 1988); and bacterial probe A01-338 (Table 2.1).

Optimal wash temperatures for the newly designed ^{32}P labeled specific probes were determined following hybridization with the target RNAs generated by *in vitro* transcription (above). Oligonucleotide probes were labeled with ^{32}P following Devereux et al. (1992) and purified from unincorporated ^{32}P using Nensorb 20 cartridges (Dupont Corp., Wilmington, DE) (Stahl and Amann, 1991). Reference RNAs were denatured by adding 3 volumes 2% glutaraldehyde in 50 mM sodium phosphate (pH 7.0) to 1 volume RNA solution and incubating at room temperature for 10 min (Stahl and Amann, 1991). Denatured RNA was then diluted to 125 µg/ml with dilution water (sterile dH_2O containing 0.0002% bromophenol blue and 1 µg/ml poly [A]). Using a slot blot device (Minifold II; Schleicher and Schuell, Inc., Keene NH) under slight vacuum, 12.5 ng (in a volume of 100 µl) of each RNA standard was applied to Immobilon-N membranes (Millipore Corp., Bedford, MA) that had been pre-wetted in 95% ethanol and rinsed in dH_2O. Membranes were then dried at room temperature and baked at 80° C for 1 h prior to hybridization.

For each optimization experiment, membranes loaded with reference RNAs were cut into 5 strips, each containing triplicate blots with 12.5 ng RNA. Each strip was then placed in a 14 ml disposable screw-cap tube and pre-wetted

Species/Sequence	A01-183
Target	CAUCGUAUUUUCUUAGGGG
4D19	UAUCUAAUAUUCCUUGGAA
Desulfococcus multivorans	AGUCAUUUAGGCUGUGGUU
Desulfosarcina variabilis	UAUCCAAAUAUCUUCGGAU
Desulfoarculus baarsii	GACCACGACAACUGCGGUU
Desulfobotulus sapovorans	UGUUGURUUUUCUUCGGGG
Desulfonema limicola	AGUCAUUA-AUACCCCGGU
Desulfonema magnum	UAUC-UUGAGAACUUCGGU

Species/Sequence	A01-267
Target	CAAGGCCGCGAUGGUUAG
4D19	CAAGGCGACGAUGGUUAG
Desulfococcus multivorans	CAAGGCGACGAUGGUUAG
Desulfosarcina variabilis	CAAGGCAACGAUGGUUAG
Desulfoarculus baarsii	YAAGGCCGCGAUGGGUAG
Desulfobotulus sapovorans	CAAGGCAGUGAUGGGNAG
Desulfonema limicola	CAAGGCAUCGAUGGUUAG
Desulfonema magnum	CAAGGCUUCGAUGGUUAG

Species/Sequence	4D19-189
Target	GGAAUGUUGGAUCAAGGG
A01	GGGGGAUGCGGUCAAGGU
Desulfococcus multivorans	GGUUUAGAUGAUGAAAGG
Desulfosarcina variabilis	GGAUUUUUGGAUCAAGGG
Desulfoarculus baarsii	CGGUUGUUGCGGNNAAAG
Desulfobotulus sapovorans	GGGAAUGC-AACCAAAGA
Desulfonema limicola	GGUAUUAAUGAUGAAAGA
Desulfonema magnum	GGUUUUUAAGAUCAAAGG

Fig. 2.2. Comparison of probes A01-183, A01-267, and 4D19-189 with aligned sequences from empirically tested non-target SRB and other closely related sequences. Mismatches with probe target sequence are shown in boldtype and underlined.

by adding 1.6 ml hybridization buffer (0.9 M NaCl, 50 mM sodium phosphate [pH 7.0], 5 mM EDTA, 10X Denhardt solution [Sambrook et al., 1989], 0.5% SDS, and 0.5 mg/ml poly[A]) and incubating at 40° C for 2 h in a rotating hybridization oven (Hybaid Instruments, Holbrook, NY). 20 µl ^{32}P-labeled probe was then added to each tube and hybridization was allowed to occur by incubating at 40° C for 14-16 h in the rotating incubator. After removing the hybridization solution, the membranes were washed by adding 1.6 ml wash buffer (1% SDS - 1X SSC [0.15 M NaCl, 0.015 M sodium citrate, pH 7.0]), and rotating at room temperature for 30 min. After removing the initial wash buffer, each of the 5 membrane strips was then subjected to a second wash by adding 1.6 ml fresh wash buffer and incubating at 40°, 45°, 50°, 55°, or 60° C, with rotation for 30 min. The membranes were then air-dried briefly and the remaining bound probe was quantified using a gas proportional radioisotope detection system (Ambis, Inc., San Diego, CA). Probe signals were corrected for background hybridization levels. It should be noted that it was necessary to omit poly(A) from hybridization buffers for hybridizations with one of the newly designed probes (A01-183) because the probe target contained a U-rich region (Fig. 2.2, Table 2.1) that appeared to be blocked by poly(A).

Once the optimal probe wash conditions had been roughly determined (above), wash conditions were then refined and probe specificity was tested empirically. This was accomplished by preparing reference RNA membranes for each probe, with each membrane containing triplicate 50 ng blots of *D. multivorans* RNA, *D. variabilis* RNA, and *Desulfoarculus baarsii* RNA, and triplicate 12.5 ng blots of each of the reference RNA transcripts. Reference RNAs from cultivated organisms were kindly provided by R. Devereux. The membranes were prepared and hybridized as described above, except that: prehybridizations, hybridizations, and washes were performed in screw-cap

hybridization tubes; 10 ml prehybridization, hybridization, and wash buffers were used; and approximately 400 µl ^{32}P-labeled probe was used. For each probe, 4 membrane wash temperatures were used, consisting of 2° C increments that bracketed the approximate T_d as determined above.

Application of Probes to Environmental RNA

RNA was extracted from sediment and rhizosphere samples using a technique modified from that of Devereux et al. (1992). For rhizosphere samples, excess bulk sediment was briefly rinsed from roots as described in Chapter One. For bulk sediment samples, RNA was extracted directly from sectioned cores containing both roots and sediment. Approximately 10 g bulk sediment or roots (wet weight), 10 g baked 0.1 mm-diameter zirconia/silica beads, and 1.275 ml phenol equilibrated with 50 mM NaAcetate/10 mM EDTA (pH 5.2) were combined in a 20 ml bead mill homogenizer cup (BioSpec Products, Inc., Bartlesville, OK). The mixture was homogenized for 15 s, allowed to cool on ice for 1 min, and re-homogenized for an additional 15 s. The sample was transferred to a 50 ml centrifuge tube and centrifuged at 8,000 x *g* for 8 min at 4° C. The supernatant fluid was transferred to a clean tube, and the pellet was resuspended in 5 ml 50 mM NaAcetate/10 mM EDTA (pH 5.2), vortexed, centrifuged as before, and the supernatant fluids were combined. The supernatant fluids were extracted with phenol (equilibrated with 50 mM NaAcetate/10 mM EDTA [pH 5.2]), phenol: chloroform (1: 1), and chloroform: isoamyl alcohol (24: 1). Nucleic acids were precipitated by adding 0.1 volume 3M NaAcetate and 2 volumes 95% ethanol, and incubating overnight at -20° C. Nucleic acids were then pelleted by centrifugation at 12,000 x *g*, 4° C for 30 min, the pellet was washed with 80% ethanol, dried, and resuspended in 100-500 µl

sterile dH_2O. Nucleic acids were further purified using Sephadex G25 spin columns (Moran et al., 1993) and were analyzed by spectrophotometry.

Serial dilutions of RNA extracted from salt marsh samples were applied to nylon membranes and hybridized with probes A01-183, 4D19-189, 814, EUB338, or A01-338. Probe signals from environmental RNA samples were compared with those from serial dilutions of reference RNAs that were immobilized on the same membrane. Reference RNAs consisted of RNA generated by *in vitro* transcription (above) for probes A01-183 and 4D19-189 or rRNA extracted from *D. sapovorans* for probe 814. For membranes hybridized with specific probes, a range of 0.78 to 12.5 ng/blot transcript reference RNA was applied to membranes, while for membranes hybridized with the bacterial probes 0.78 to 100 ng/blot transcript RNA was used. The corresponding amounts of reference RNAs (extracted from pure cultures of *D. sapovorans*) used for probe 814 were 1.56 to 25 ng/blot for membranes probed with 814 and 1.56 to 200 ng/blot for membranes probed with EUB338. The relative abundances of the specific probe targets as a function of total eubacterial rRNA were determined by first quantifying radioactive signal per blot and correcting for background. Next, the following equation was used to calculate relative abundances (RA):

$$RA = \frac{(m_{SS})(m_{SR})^{-1}}{(m_{ES})(m_{ER})^{-1}} \times 100\%$$

where m_{SS} is the slope of specific probe signal per unit sample RNA; m_{SR} is the slope of the specific probe signal per unit reference RNA; m_{ES} is the slope of the eubacterial probe signal per unit sample RNA; and m_{ER} is the slope of the eubacterial probe signal per unit reference RNA. Samples for which the slope of probe signal per unit RNA was not linear (i.e., $R^2 < 0.90$) were omitted from analyses.

Results and Discussion

In Vitro Generation of Reference RNAs

In order to quantitatively determine the relative abundance of a specific rRNA target as a function of total eubacterial rRNA, it is necessary to use reference RNA containing both the specific and eubacterial target sequences. For probes targeting cultivated bacteria, a source of reference RNA is readily available from pure cultures of the organism. However, for probes designed to specifically target yet uncultivated bacteria, an alternate source of RNA standard must be used. Here, an RNA standard was generated using *in vitro* transcription with the cloned environmental sequences as template DNA. The RNA transcript thus produced (Fig. 2.3) contained a sense RNA sequence presumably identical with positions 9 to 822 (*E. coli* numbering) of the uncultivated bacterium's 16S rRNA. This region includes the target sequences of both the EUB338 probe and the newly designed probes.

The use of *in vitro* RNA synthesis is an attractive alternative to conventional extraction of reference RNA from pure cultures, even for cultivable organisms. Once the reference rDNA sequence has been cloned, *in vitro* transcription can be used to rapidly generate μg quantities of highly purified RNA. Because other cellular RNAs are not present in the purified transcription product, the concentration of actual target sequences is easily determined by measuring the nucleic acid concentration of the purified product. In addition, it is much easier to cultivate *E. coli* clones containing the target sequence than slow-growing or fastidious organisms that may contain the desired reference RNA.

One precaution that should be taken when using this approach, especially for previously undescribed phylotypes, is comparison of the cloned sequence to probe target sites to ensure that expected target sequences are present. For example, the EUB338 target in clone A01 was found to contain a G_{340} residue,

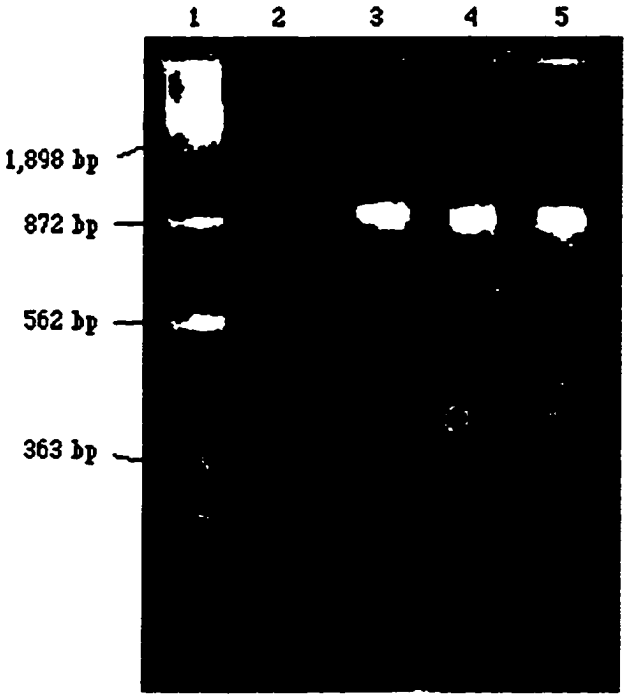


Fig. 2.3. Characterization of synthetic RNA generated via *in vitro* transcription of cloned 16S rRNA gene fragments. An RNA molecular weight standard (lane 1) and RNA transcripts from clones A01 (lane 3), 4D19 (lane 4), and 2B14 (lane 5) were analyzed by denaturing polyacrylamide gel electrophoresis.

resulting in a G-A mismatch with the published EUB338 probe. This mismatch may be due to an error in nucleotide incorporation by *Taq* polymerase, although it would also be tolerated by predicted secondary structure models as it results in a non-canonical base pair, G-A, that is common in 16S rRNA (Woese et al., 1983). In order to account for this mismatch, a modified EUB338 probe (5'-GCTGCCTCCCGTAGGMGT-3', where M is A or C; A01-338) was used for hybridizations in which A01 RNA was used as a standard. The EUB338 target site in the remaining seven 16S rDNA fragments that were sequenced contained the expected EUB338 probe target.

Design and Optimization of Oligonucleotide Probes

Oligonucleotide probes were designed that targeted unique sequences of the two novel phylotypes, A01 and 4D19, described in Chapter One (Fig. 2.2, Table 2.1). Probe targets for both A01-183 and 4D19-189 are within the 180-220 region of the 16S rRNA molecule -- a region that is highly variable in sequence and also is somewhat variable in length (Woese et al., 1983). For example, A01 and 4D19 sequences contained 16 and 17 more bases, respectively, than *E. coli* 16S rRNA in the 180-220 region. It was thought that the variability of this region would result in high specificity of the probes for their intended targets. In addition, the number and position of mismatches of each probe with currently available 16S rRNA sequences (Fig. 2.2) indicate high probe specificity, especially for probe A01-183 which has 4 mismatches with its closest known non-target relatives.

The empirically determined probe T_ds for A01-183 and 4D19-189 were 40° C and 46° C, respectively (Fig. 2.4-2.5). These T_ds are sufficiently close for simultaneous hybridization with both probes. In addition, empirical specificity tests of both probes showed no detectable probe remained bound to non-target

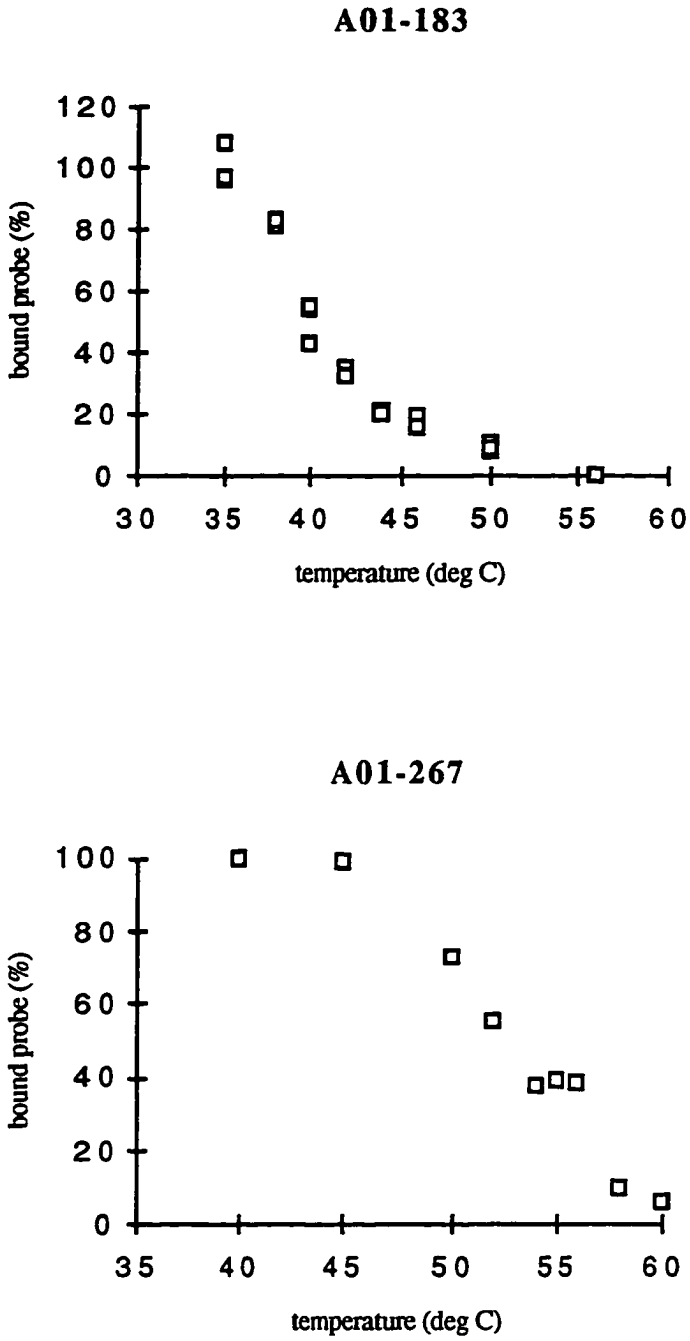


Fig. 2.4. T_d determinations for probes A01-183 (top) and A01-267 (bottom) designed to specifically target phylotype A01. The amount of probe remaining bound to target RNA after washing membranes at various temperatures was normalized to the average probe signal after washing at 35° C (top) or 40° C (bottom).

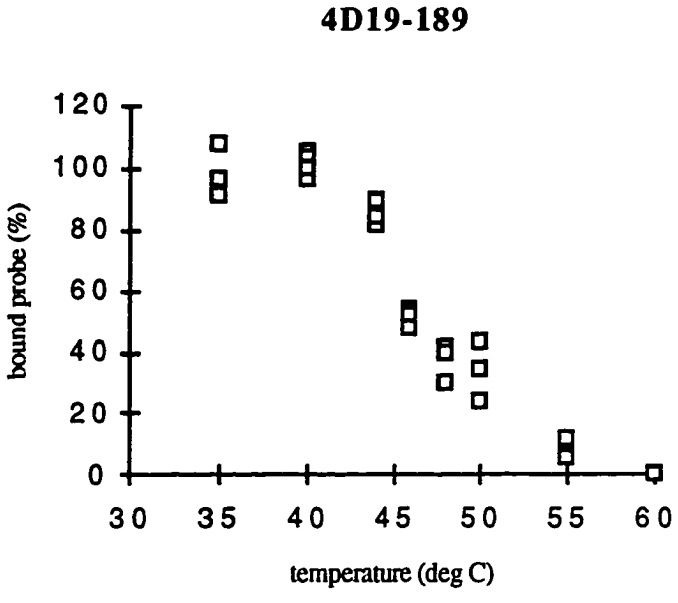


Fig. 2.5. T_d determinations for probe 4D19-189 designed to specifically target phylotype 4D19. The amount of probe remaining bound to target RNA after washing membranes at various temperatures was normalized to the average probe signal after washing at 40° C.

reference RNAs after membranes were washed at their respective T_ds, thereby supporting the high level of probe specificity intended (Fig. 2.6).

Probe A01-267 was designed to target a moderately conserved region of the 16S rRNA molecule (Stahl and Amann, 1991; Ward et al., 1992) and contained only one mismatch with 16S rRNA from *D. baarsii* (Fig. 2.2). This second probe for A01 was designed to allow for the detection of A01 and, potentially, other undescribed organisms that are closely related to A01. It was intended for use as a probe within which A01-183 should be nested, as well as a means to detect A01 and relatives in the case that the relative abundance of A01 itself (as targeted by the highly specific probe A01-183) were below the detection limit. An analogous probe to specifically target a moderately conserved region of 4D19 16S rRNA could not be identified. Unfortunately, empirical tests of the specificity of A01-267 revealed that its single mismatch was insufficient to confer specificity for A01 alone. After washing membranes hybridized with A01-267 at its T_d (53° C; Fig. 2.4; Table 2.1), the *D. baarsii* signal was greater than 10% of the corresponding A01 signal. Therefore, A01-267 was not used to probe environmental RNAs.

Although *in situ* hybridization (ISH) was not used in the current study, considerations for potential use of the new probe in ISH formats were taken into account. One consideration was the accessibility of the probe targets in ribosomes of fixed, whole cells (Amann, 1995; Amann et al., 1995). While I did not empirically test for ISH accessibility, other probes targeting the same regions as A01-183, A01-267, and 4D19-189 have been successfully used in ISH, suggesting that these sites are accessible (Amann et al., 1995). In addition, the probes were designed to have similar predicted T_ds, so that it would be possible to simultaneously hybridize with both probes. Such dual hybridizations can be used to either enhance detection of target cells (Lee et al., 1993) or to simultaneously visualize different target cells (Amann et al., 1995).

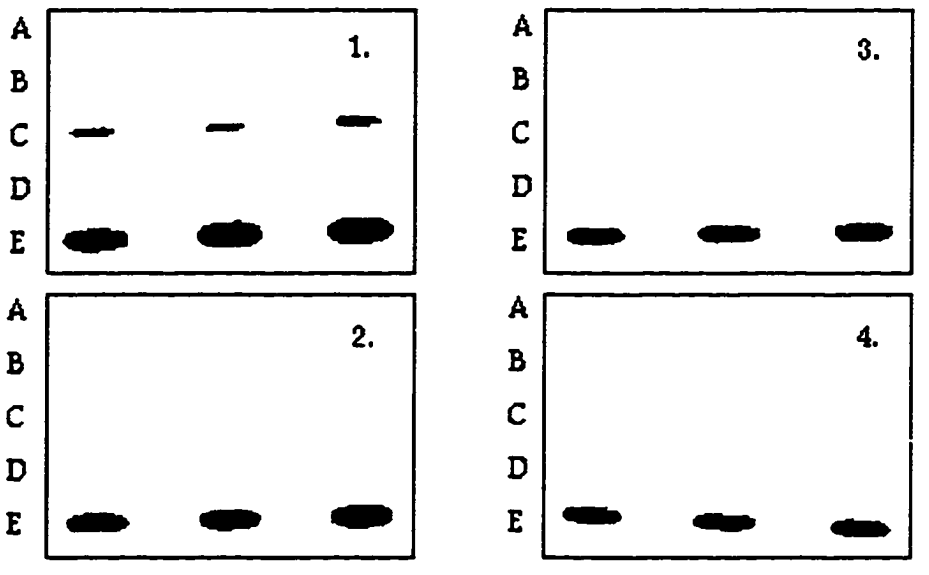


Fig. 2.6. Digital images of hybridization results from tests of probe specificity and optimal wash temperature for probe 4D19-189. ^{32}P-labeled probe 4D19-189 was hybridized to triplicate blots of: A. *D. multivorans* RNA; B. *D. baarsii* RNA; C. *D. variabilis* RNA; D. A01 synthetic RNA; E. 4D19 synthetic RNA. Membranes were washed at the following temperatures: 1. 44° C; 2. 46° C; 3. 48° C; 4. 50° C.

Relative Abundances of A01-183, 4D19-189, and 814

Examples of membranes containing serial dilutions of reference RNA and environmental RNA samples hybridized with specific probe A01-183 and eubacterial probe EUB338 are shown in Fig. 2.7 and Fig. 2.8, respectively. The method used for determining relative abundances of specific rRNAs as a function of eubacterial rRNA was first described by Giovannoni et al. (1990) and has several advantages over more conventional means of determining sample concentrations via standard curves. As described in the methods section, linear relationships between probe signal and amount of RNA were determined for both the sample and standard RNAs and for both specific and eubacterial probe targets. Because slopes (i.e., signal per unit RNA) were used instead of individual sample points, it was not necessary to accurately measure the 16S rRNA concentration of either standards or samples. This was particularly advantageous for samples that may have contained humics, nucleic acids other than 16S rRNA, or other compounds that interfere with accurate spectrophotometric determination of 16S rRNA concentration. Likewise, reference RNAs may contain other cellular nucleic acids (especially if they consist of total RNA extracted from a cultivated target organism) that preclude accurate spectrophotometric measurement of 16S rRNA target concentration. In addition, this technique corrects for non-specific binding that results in a positive y-intercept in the relationship between probe signal and unit sample RNA, although it does not correct for non-specific binding that is proportional to sample RNA concentration. It provides a check on data quality for each sample by testing for the linearity of probe signal response per unit target. Lastly, because sample and reference RNAs are hybridized under the same conditions, any differences in specific and eubacterial probe labeling and hybridization efficiencies can be corrected.

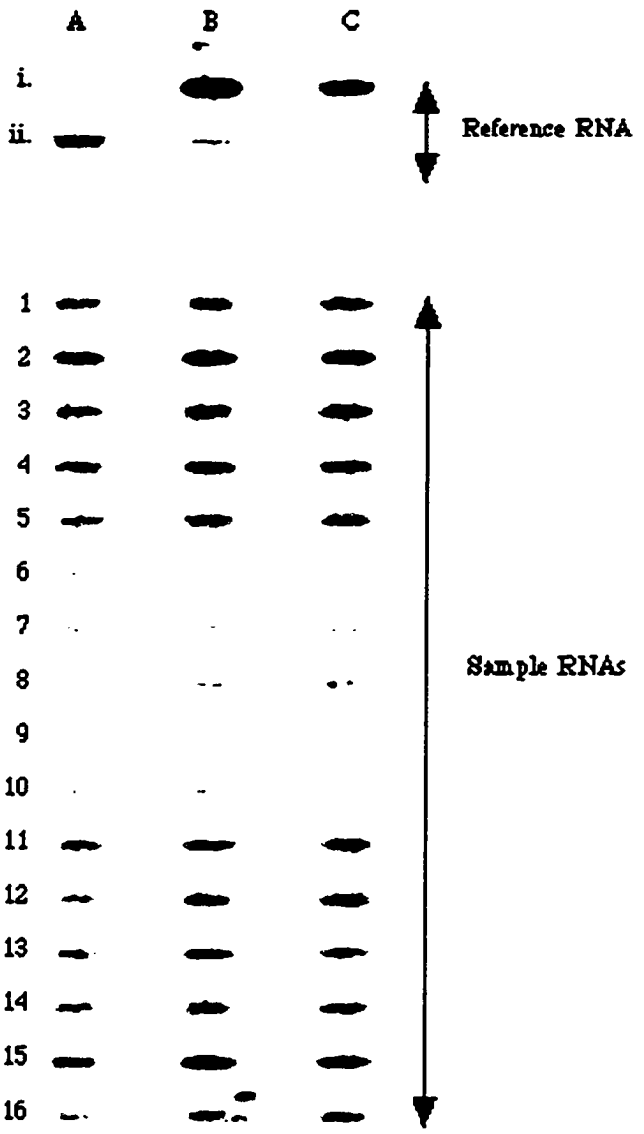


Fig. 2.7. Hybridization of ^{32}P-labeled probe A01-183 to A01 reference RNA and nucleic acids extracted from rhizosphere and bulk sediment samples. Membranes hybridized with specific probes contained the following amounts of reference RNA per blot: 0.78 ng (row ii, column C), 1.56 ng (ii, B), 3.13 ng (ii, A), 6.25 ng (i, C), and 12.50 ng (i, B). In addition, they contained approximately 450 ng (column A), 900 ng (B), and 1,800 ng (C) per blot of each sample RNA (rows 1-16).

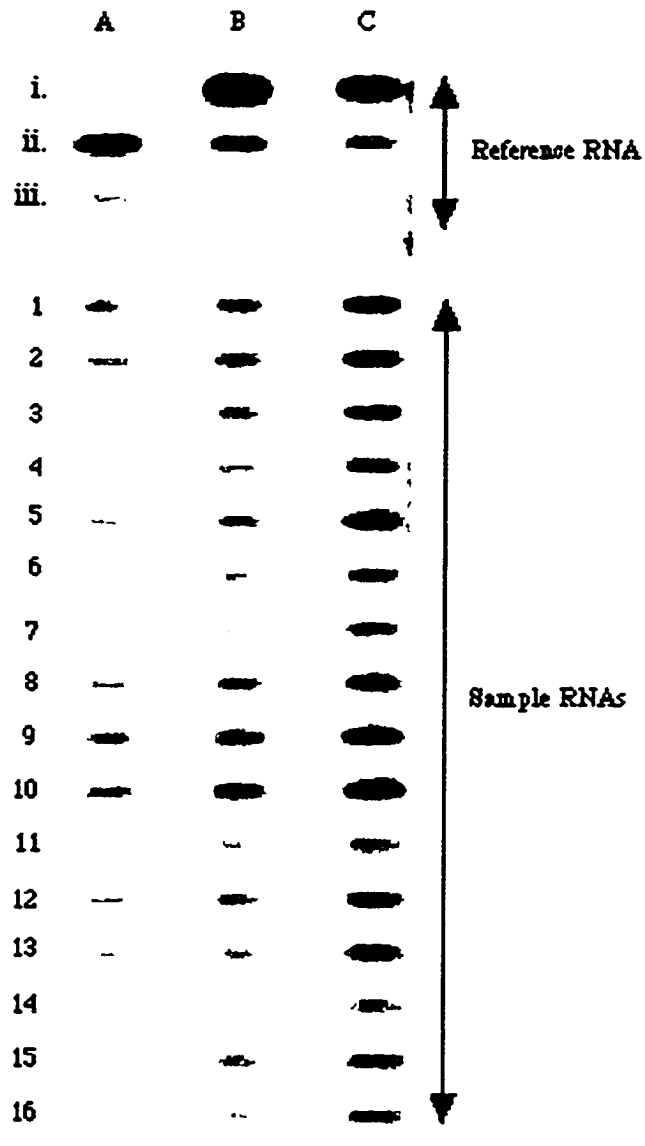


Fig. 2.8. Hybridization of ^{32}P-labeled probe EUB338 to 4D19 reference RNA and nucleic acids extracted from rhizosphere and bulk sediment samples. Membranes hybridized with eubacterial probes contained the following amounts of reference RNA per blot: 0.78 ng (row iii, column C), 1.56 ng (iii, B), 3.13 ng (iii, A), 6.25 ng (ii, C), 12.5 ng (ii, B), 25 ng (ii, A), 50 ng (i, C), and 100 ng (i, B). In addition, they contained approximately 50 ng (column A), 100 ng (B), and 200 ng (column C) per blot of each sample RNA (1-16).

Over all depths and sampling dates, the mean relative abundance of A01-183 was 7.5± 3.5% eubacterial rRNA (Table 2.2). The corresponding mean for 4D19-189 relative abundance was 3.4 ± 2.1% (Table 2.2). If the A01-183 and 4D19-189 probes are indeed specific for A01 and 4D19, respectively (i.e., if they do not target other unknown phylotypes), then this finding may provide an important insight into sediment and soil microbial communities. As discussed above, while molecular studies of soil/sediment microbial communities have suggested extremely high complexity, with up to 10^4 species present in a gram of soil (Torsvik et al., 1990), the community structure or quantitative distribution of individual phylotypes remains poorly understood. Here, the rather high relative abundances of A01-183 and, to a lesser extent, 4D19-189 suggest that while the overall sediment community may be highly diverse, there are a small number of well-adapted species in the sediment habitat that play a significant role in microbial community dynamics.

As discussed in Chapter One, the physiological traits of the novel phylotype's close relatives suggest that the versatility of this group of SRB may contribute to their success in the salt marsh sediment and rhizosphere environment. A diverse array of electron donors is utilized by A01 and 4D19's close relatives, including formate, lactate, ethanol, acetate, secondary alcohols, hydrogen, fumarate, malate, benzoate, and 3-16 C fatty acids (Widdel and Bak, 1992; Hansen, 1993). Other phenotypic traits possessed by this group of bacteria include motility (Widdel and Bak, 1992), the capacity for complete oxidation of organic carbon to CO_2 (Widdel and Bak, 1992), and the ability to utilize O_2 as an electron acceptor (Dilling and Cypionka, 1990).

Relative abundances of 814 target rRNA were quite low (mean 3.1%; Table 2.2), and were lower than the sum of A01-183 and 4D19-189 relative abundances for all sampling dates and treatments for which data points from all three probes

Table 2.2. Mean relative abundances of probe targets in bulk sediment and rhizosphere samples.

Treatment	Probe	Mean RA[a]	Standard Deviation	n
Sed[c] (0-2 cm)	A01-183	4.1	2.3	3
	4D19-189	2.9	2.4	8
	814	1.1	0.5	4
	804[b]	4.9	2.1	10
Sed[c] (2-4 cm)	A01-183	7.5	2.5	5
	4D19-189	2.8	1.3	5
	814	3.2	1.5	7
	804[b]	13.6	2.7	9
Sed[c] (6-8 cm)	A01-183	11.4	1.1	2
	4D19-189	4.1	2.6	4
	814	3.8	4.3	4
	804[b]	19.2	3.1	4
Rhiz[d] (0-4 cm)	A01-183	7.8	3.9	9
	4D19-189	3.8	2.0	9
	814	3.7	2.0	6
	804[b]	22.8	8.8	9
Total	A01-183	7.5	3.5	19
	4D19-189	3.4	2.1	26
	814	3.1	2.3	21
	804[b]	14.2	8.8	32

[a] Relative abundance.
[b] 804 relative abundances measured by Hines et. al (in prep).
[c] Bulk sediment samples taken from depths indicated in parantheses.
[d] Rhizosphere samples taken from a depth of 0-4 cm.

were available. This result suggests that probe 814, which (Devereux et al., 1992) designed to target the *Desulfococcus-Desulfosarcina-Desulfobotulus* group, did not target the novel phylotypes A01 and 4D19, which are also members of this group. Unfortunately, it was impossible to directly determine whether the novel phylotypes contained the 814 target (*E. coli* positions 814-831) because the cloned environmental 16S rDNA fragments consisted of base positions 9-822 (Chapter One). However, recently published 16S rRNA sequences from other organisms that fall within the *Desulfococcus-Desulfosarcina-Desulfobotulus* group provide evidence that the 814 probe does not target all group members. For example, 16S rRNA sequences from the genus *Desulfonema*, which is a close relative of *Desulfosarcina* and *Desulfococcus*, contain a mismatch with 814 in the center of the probe-target hybrid. Similarly, environmental clones A34 and A52 (Devereux and Mundfrom, 1994), also closely related to *Desulfococcus* and *Desulfosarcina*, contained the 804 target sequence but had a mismatch with the 814 probe. Thus, it is quite possible that while the novel phylotypes were targeted by 804 and were monophyletic with 814-targeted organisms, they did not contain the 814 target.

Seasonal and Spatial Trends in Relative Abundances

Although it is impossible to assess the statistical significance of seasonal patterns in relative abundances given the limited size of the data set, several interesting trends were apparent. In the rhizosphere, the relative abundance of A01-183 and, to a lesser extent, 4D19-189, exhibited a seasonal trend that was similar to the trend in 804 relative abundances observed by Hines et al. (in prep) (Fig. 2.9). As mentioned in Chapter One, probe 804 was designed by Devereux et al. (1992) to target most members of the *Desulfobacteriaceae* family. Rhizosphere relative abundances increased immediately after the onset of vegetative plant

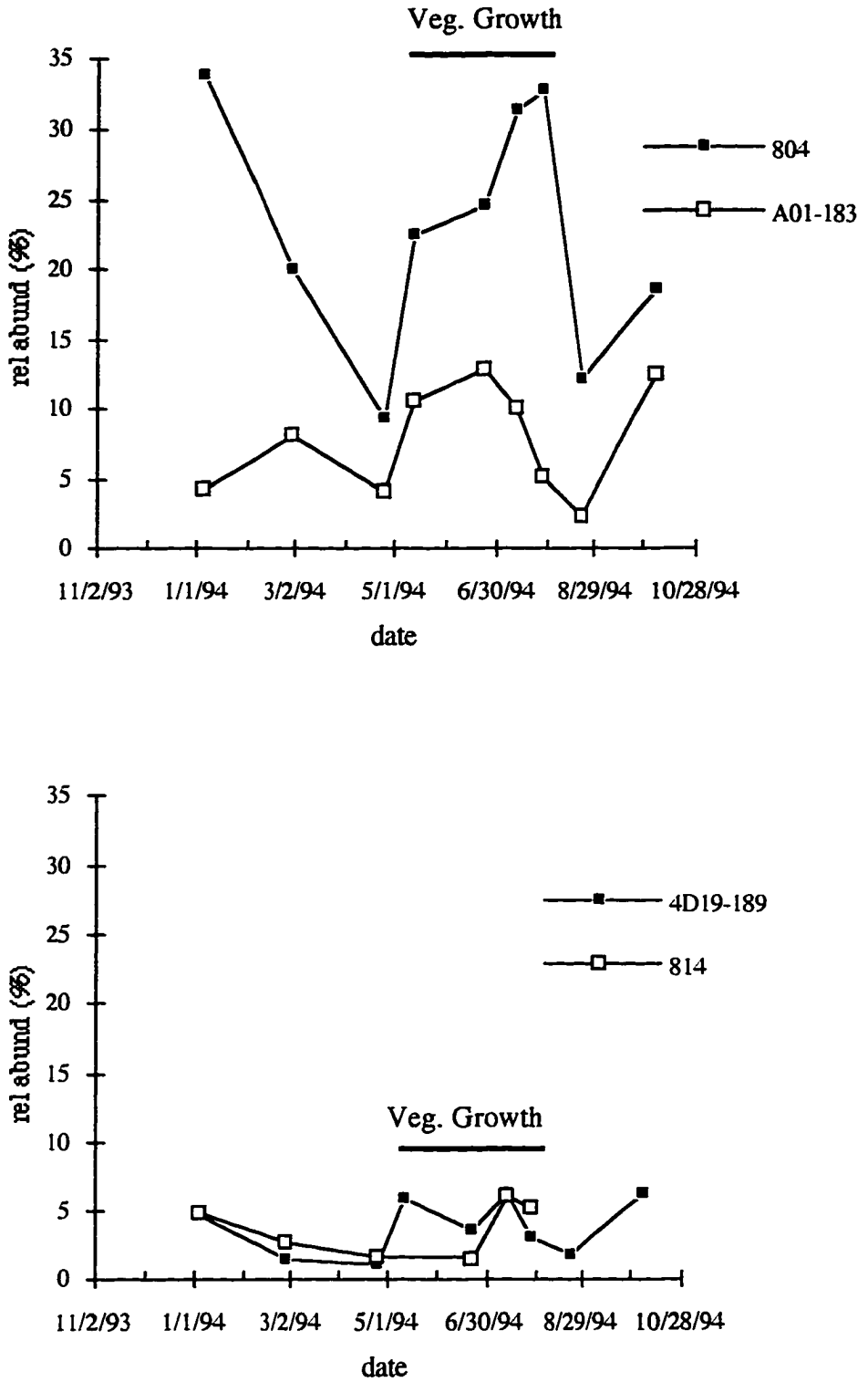


Fig. 2.9. Seasonal trends in rhizosphere relative abundances for 804, A01-183 (top), 4D19-189, and 814 (bottom) target rRNA in the salt marsh. The period of vegetative growth for *S. alterniflora* is indicated.

growth and then decreased as the plants began to flower (Fig. 2.9). A similar trend in sulfate reduction rates (SRR) has also been observed by Hines et al. at the same study site (Hines et al., 1989; Hines et al., in prep) and at the same times (Hines et al., in prep) as the current study.

It is likely that the trends of increased *Desulfobacteriaceae* relative abundances and SRR during the period of vegetative plant growth were a direct result of dissolved organic carbon (DOC) released from roots and rhizomes during this period. First, this seasonal pattern was only observed in the rhizosphere and was not found in bulk sediment relative abundances of A01-183, 4D19-189, 814 (Fig. 2.10-2.11), or 804 (Hines et al., in prep), suggesting a direct influence of roots. Second, the physiological changes in *S. alterniflora* during the vegetative growth period provide evidence for its role in influencing rhizosphere bacterial dynamics. As *S. alterniflora* enters the vegetative growth period, it remobilizes nonstructural carbohydrates from rhizome stores and translocates new photosynthate (Lytle and Hull, 1980) to rapidly growing roots and rhizomes. This increased supply of soluble carbohydrates, along with lysates from sloughed off root cap cells (Brady, 1990) from rapidly growing young roots results in increasing amounts of DOC leaking from plant roots and rhizomes (Hines et al., 1989). When plants reach the reproductive growth stage, carbon is reallocated to flowering structures, carbohydrates are immobilized in rhizomes (Lytle and Hull, 1980), and, therefore, release of DOC into the rhizosphere is thought to rapidly decrease (Hines et al., 1989). Here decreases in A01-183 (Fig. 2.9) and 804 (Hines et al., in prep) relative abundances in the rhizosphere correspond with the onset of *S. alterniflora* flowering and decreases in SRR (Hines et al., in prep).

Although not coincident with general seasonal ecological patterns in the salt marsh, increased relative abundances of 804 (Hines et al., in prep), A01-183,

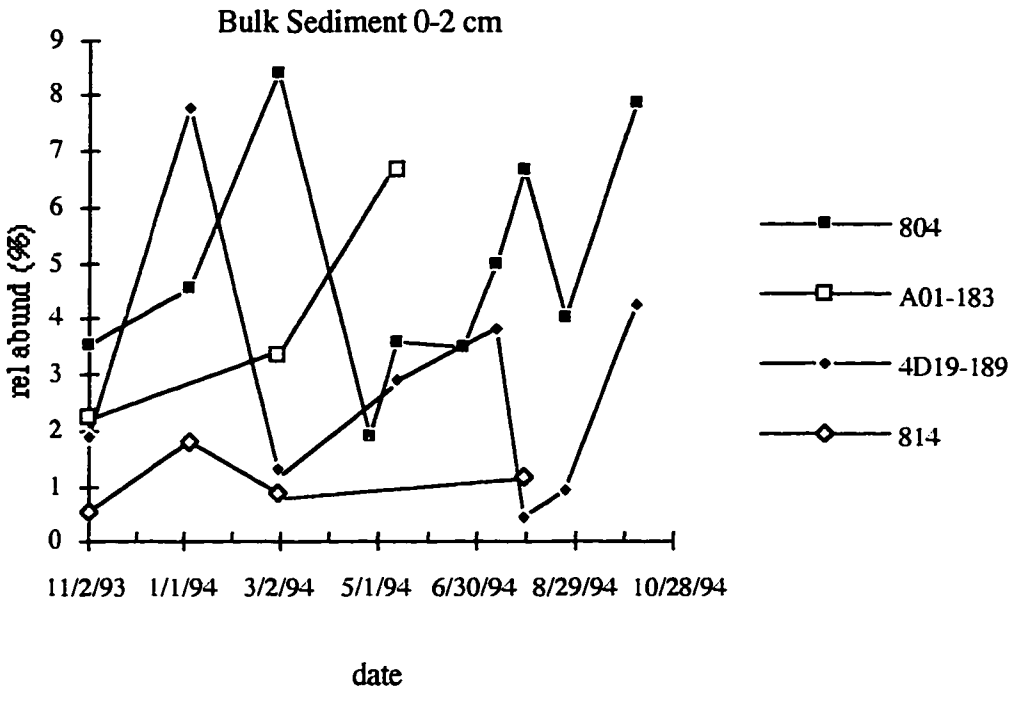


Fig. 2.10. Relative abundances of 804[a], A01-183, 4D19-189, and 814 probe target rRNA in 0-2 cm depth bulk sediment.
[a]Measured by Hines et al. (in prep).

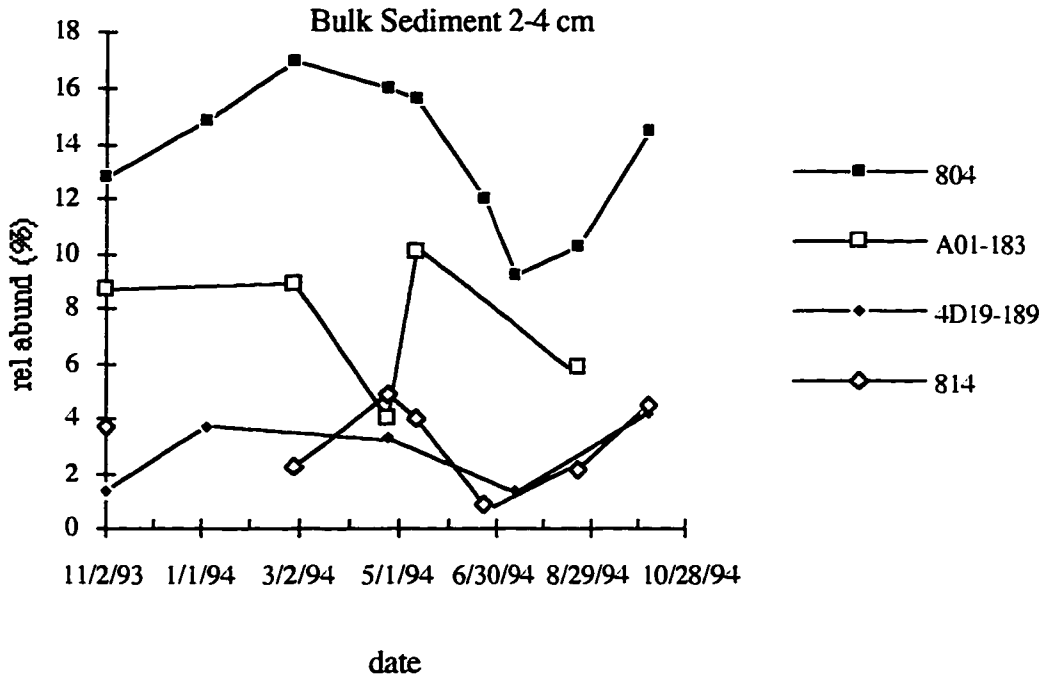


Fig. 2.11. Relative abundances of 804[a], A01-183, 4D19-189, and 814 probe target rRNA in 2-4 cm depth bulk sediment.
[a]Measured by Hines et al. (in prep).

and 4D19-189 (Fig. 2.9) probe targets observed in the rhizosphere on 5 October 1994 did coincide with increased SRR on the same date (Hines et al., in prep). This apparent stimulation of the SRB community may be due to either fortuitous availability of organic substrates at the sampling location or perhaps initial degradation of senescing root hairs.

The lack of any clear seasonal patterns in bulk sediment relative abundances (Fig. 2.10-2.11) was somewhat surprising given the strong seasonal variation in SRR (Hines et al., 1989; Hines et al., in prep). However, it is important to keep in mind that these data are relative and not absolute abundances, and therefore only reflect relative increases in target rRNA compared with total eubacterial rRNA. Thus, a lack of clear seasonal trends suggests that varying abundances of other eubacteria masked the seasonal trends in abundances of target SRB. Environmental factors that may affect fermentative, acetogenic, and sulfate-reducing communities in similar manners include temperature and general availability of organic carbon (as opposed to availability of specific substrates that may disproportionately affect one group). In addition, the fact that several data points were omitted from analyses (due to nonlinear relationships between probe signal and amount of RNA/blot) may have obscured a seasonal trend if it were present.

Depth profiles in A01-183 and 804 relative abundances were measured in samples from 3 depths (0-2 cm, 2-4 cm, and 6-8 cm), taken on 12 May 1994, at the beginning of the plant growing season. Biogeochemical measurements of sulfate reduction rates and total reduced sulfur were also available from samples taken on the same date and adjacent to the samples from which RNA was extracted (Hines et al., in prep). As shown in Fig. 2.12, relative abundance of A01-183 target increased with depth, as did total reduced sulfur. However, sulfate reduction rates were highest in the upper sediments and decreased with depth,

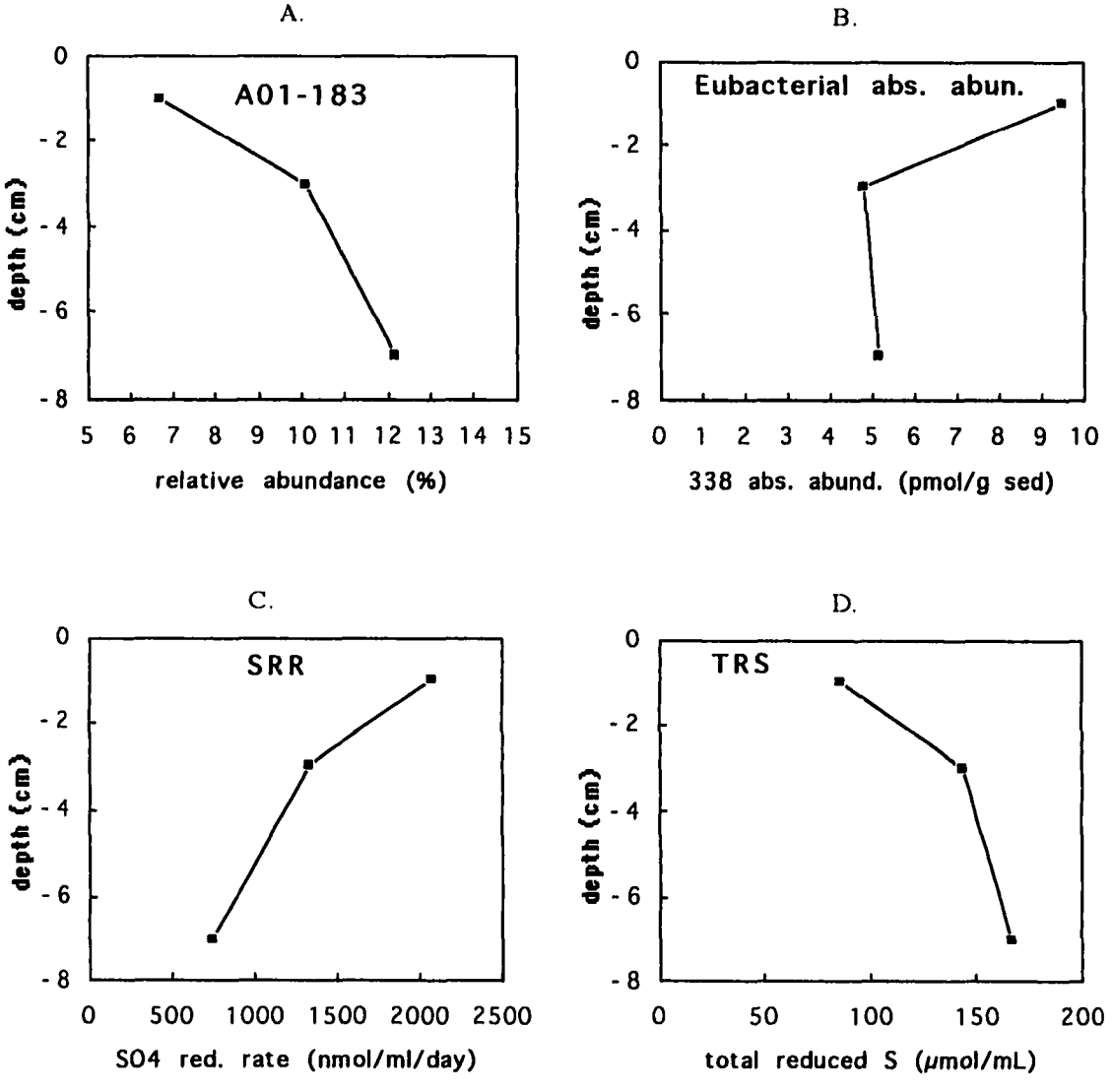


Fig. 2.12 Depth profiles of (A) A01-183 relative abundances, (B) eubacterial absolute abundance, (C) sulfate reduction rates[a], and (D) total reduced sulfur[a] measured on 5/12/94.

[a] Measured by Hines et al. (in prep).

suggesting that the SRB community was more active in the upper than lower sediment. In addition, absolute abundance of eubacterial rRNA, expressed as picomoles 338 target/g sediment, was highest in the upper sediment. While absolute abundance values should be interpreted with caution due to sample-to-sample variability in RNA extraction efficiency, this trend of higher amounts of eubacterial rRNA in the upper sediment also indicates a more active community compared to the lower sediment. Taken together, these data suggest that (1) while the upper sediment harbors a more active SRB community, other eubacterial groups are also active in the upper sediment and (2) A01 is relatively better adapted to the lower sediments than the upper sediments.

Conclusions

The quantitative significance and population dynamics of novel phylotypes A01 and 4D19, discovered in a qualitative survey of SRB phylogenetic diversity (Chapter One), was investigated. A new method for generating reference RNA for uncultivated phylotypes was developed and applied in quantitative probing experiments. Probes directed against the novel phylotypes were used to show that A01 and, to a lesser extent, 4D19 played significant roles in the salt marsh rhizosphere and sediment communities. In addition, seasonal trends in A01-183 relative abundances suggested a direct influence of plant phenology on rhizosphere bacterial dynamics.

CHAPTER THREE

PHYLOGENETIC ANALYSIS OF SULFATE-REDUCING BACTERIA ISOLATED FROM SALT MARSH SEDIMENTS

Introduction

Until the 1970's, dissimilatory sulfate-reducing bacteria (SRB) were thought to be comprised of a few species that were capable of utilizing only lactate or pyruvate as energy sources (Barton and Tomei, 1995). However, SRB are now known to be both physiologically and phylogenetically diverse. As of 1993, close to 100 substrates for sulfate reduction had been described, including hydrogen, carbon monoxide, monocarboxylic acids (e.g., acetate, propionate, butyrate, and higher fatty acids up to C_{20}), dicarboxylic acids (e.g., malate, succinate, and fumarate), alcohols (e.g., ethanol, methanol, propanol, etc.), amino acids, sugars, aromatic compounds, and several xenobiotic compounds (Hansen, 1993). In addition, the capacity of many SRB to fix molecular nitrogen (Lespinat et al., 1987; Widdel, 1987); grow fermentatively in the absence sulfate (Hansen, 1993) and utilize nitrate, iron, chlorinated aromatics, and oxygen as electron acceptors (Barton et al., 1983; Cypionka et al., 1985; Lovley et al., 1993; Stackebrandt et al., 1995) has been demonstrated.

SRB that have been isolated to date are distributed among three major eubacterial lines of descent (i.e., the ∂ proteobacteria, the Gram-positive Bacteria, and the thermophilic Gram-negative genus *Thermodesulfobacterium* (Widdel and Bak, 1992; Stackebrandt et al., 1995), and one archaeon genus, *Archaeglobus*,

(Thauer and Kunow, 1995). However, the majority of characterized SRB are members of the Gram-negative nonsporeforming mesophilic SRB and it appears that this group is the most widely distributed in nature (Widdel and Bak, 1992). The Gram-negative mesophilic SRB form a phylogenetically coherent group within the ∂ proteobacteria (Fowler et al., 1986; Devereux et al., 1989). The ∂ subdivision was originally defined by Woese (Woese et al., 1985; Woese, 1987) to include three main phylogenetic subgroups with widely differing physiological traits: the Gram-negative nonsporeforming mesophilic anaerobic sulfate and sulfur-reducing bacteria; the small predatory bdellovibrios; and six representatives of the order Myxococcales. Since Woese's original description of the ∂ subdivision, the genera *Pelobacter* (Stackebrandt et al., 1989) and *Geobacter* (Lovley et al., 1993; Lonergan et al., 1996), both obligate anaerobes that are closely related to the sulfur-reducer genus *Desulfuromonas*, have also been placed within the ∂ proteobacteria. Recently, a thermophilic Gram-negative SRB has been isolated that is also a member of this group (Beeder et al., 1995).

The phylogenetic relationships of the ∂ proteobacteria SRB, as determined by 16S rRNA sequence analysis, were among the first to be studied in detail with phylogenetic trees inferred first through analysis of 16S rRNA oligonucleotide cataloging techniques (Fowler et al., 1986) and subsequently by analysis of near complete 16S rRNA sequences obtained by reverse transcriptase sequencing of rRNA (Devereux et al., 1989; Devereux et al., 1990). This group of SRB was found not only to be phylogenetically coherent, but also to generally form phylogenetic groups that were consistent with various phenotypic traits. As a result of 16S rRNA analyses, it has been proposed that the Gram-negative mesophilic SRB be divided into two families, each representing a separate lineage within the group as a whole. The first of these is the *Desulfovibrionaceae* (Devereux et al., 1990), which includes the genera *Desulfovibrio* and

Desulfomicrobium, while the second proposed family is the *Desulfobacteriaceae* (Widdel and Bak, 1992). The latter encompasses the genera *Desulfobulbus*, *Desulfobacter*, *Desulfobacterium*, *Desulfococcus*, *Desulfosarcina*, *Desulfomonile*, *Desulfonema*, *Desulfobotulus*, and *Desulfoarculus* (Widdel and Bak, 1992). 16S rRNA sequence analysis has also enabled the identification of signature sequences at the group and genus levels, as well as the design of oligonucleotide probes that specifically target individual groups or genera (Devereux et al., 1992). These probes have been applied, in turn, to the investigation of the phylogenetic diversity, community structure, and population dynamics of SRB in their natural habitats, including salt marsh sediments (Devereux et al., 1996; Harmsen et al., 1996; Hines et al., in prep; Chapter Two).

While SRB are known to play ecologically important roles in such diverse habitats as freshwater ponds, oil production facilities, animal intestines, and rice paddies, their primary habitats are thought to be estuarine and marine sediments (Gibson, 1990; Widdel and Bak, 1992; Smith, 1993). It has been inocula from estuarine and marine sediments that have provided the greatest variety of SRB isolates (Widdel and Bak, 1992) In particular, SRB have been found to exhibit high activity and to play an extremely important role in organic carbon remineralization, belowground geochemistry, and plant-microbe interactions in salt marsh environments (Hines et al., 1989; Howarth, 1993). Primary productivity is extremely high in salt marshes (Howes et al., 1985; Blum, 1993), and most of the organic matter produced is decomposed *in situ* (Valiela et al., 1976). As the dominant terminal electron accepting process, it has been estimated that sulfate reduction accounts for up to 50% of organic carbon remineralization in these ecosystems (Howarth and Hobbie, 1982). In an environment such as the organic-rich salt marsh sediment where SRB play a key role in ecosystem function, sulfate reduction rates are among the highest in any

natural system, and SRB are not limited by sulfate availability, it is likely that diverse populations of SRB exist. In fact, as discussed in the previous chapters and by Hines et al. (in prep), the application of 16S rRNA probes to study SRB community structure in this ecosystem has suggested that as yet undescribed species are present in significant numbers.

The primary goal of the current chapter was to use comparative 16S rRNA analysis of novel SRB isolates from salt marsh sediments in order to investigate phylogenetic diversity of the salt marsh SRB community. 16S rRNA sequence analysis is generally recognized as the definitive method for determining an organism's phylogeny and plays an increasingly important role in characterizing and defining new taxa (Trüper and Schleifer, 1992). Although it should be viewed as one component of a polyphasic approach to defining taxonomic relationships, 16S rRNA sequence analysis has many advantages over methods based on phenotypic or other molecular traits. In fact, many phenotypic traits that were once thought to be central in defining taxonomic relationships of bacteria have since been found to hold little or no phylogenetic information (Woese, 1992). Examples include cell shape, cell aggregation patterns, bacterial appendages, electron donor utilization patterns, autotrophy, and heterotrophy (Fox et al., 1980; Woese, 1987). In general, these morphological and physiological traits tend to incompletely define phylogenetic groups, which almost always contain members that lack a given trait (Woese, 1992). This is not surprising when viewed in light of Woese's (1992) observation that: "the human and the frog... are separated by less evolutionary distance - about 5% in rRNA sequence terms - than separates most *species* of the genus *Bacillus*."

Molecular approaches other than those involving rRNA are certainly important in describing phylogenetic relationships, but none shares the phylogenetic breadth of 16S rRNA sequence analysis. While 16S rRNA sequence

analysis can be used to compare taxa at levels ranging from species to domain, techniques such as DNA: rRNA hybridization and cytochrome *c* sequence analysis are limited to analyzing phylogenetic relationships within the range of genus or species to class or order (De Ley, 1992). DNA-DNA reassociation and phenotypic traits are useful for describing relationships at the strain to genus levels (De Ley, 1992; Stackebrandt, 1992; Stackebrandt and Goebel, 1994), and should therefore be used in combination with 16S rRNA sequence analysis when characterizing novel strains. Similarly, DNA GC content can be phylogenetically informative below the genus and species levels. However, GC content can be misleading because while closely related organisms possess similar GC contents, distant relatives can also have similar GC contents (Trüper and Schleifer, 1992).

As stated above, the objective of the current chapter was to investigate the phylogenetic diversity of novel SRB isolates using 16S rRNA sequence analysis. This approach also allows the comparison of the isolates' phylogenies to the 16S rRNA sequences retrieved directly from marsh rhizosphere samples (Chapter One). The SRB strains used here were isolated by B. Sharak Genthner from the same study site described in Chapter One. Isolates were obtained by direct dilution of sediment samples in liquid media, without preceding enrichment, followed by isolation of colonies on solid media. This method was used in order to avoid preclusion of slow-growing species by opportunistic organisms (Widdel and Bak, 1992). Electron donors for isolations were chosen to include those that were likely to be present in significant concentrations in the salt marsh rhizosphere (i.e., malate, ethanol, and acetate) (Nedwell and Abram, 1979; Smith and ap Rees, 1979; Mendelssohn and McKee, 1987; Hines et al., 1994). Additional electron donors (butyrate, propionate) used for isolations were chosen such that all currently known genera would be capable of utilizing at least one out of the suite of electron donors used (Sharak Genthner, Pers. Comm.). Out of 81 isolates,

ten were chosen for 16S rRNA sequence analysis and preliminary phenotypic characterization. These ten isolates were selected on the basis of unique restriction fragment length patterns (RFLP) generated by digesting amplified 16S rRNA genes with tetrameric restriction enzymes (Willis et al., 1995). 16S rRNA genes from isolates BG14 and BG50 were sequenced by R. Devereux and included in the phylogenetic analyses described here. 16S rRNA genes from isolates BG6, BG33, BG72, BG74 were amplified by S. Friedman. 16S rRNA genes of isolates BG8, BG18, BG23, and BG25 were amplified and 16S rRNA genes of all the isolates except BG14 and BG50 were sequenced as part of the current dissertation research. In addition, B. Sharak Genthner carried out preliminary phenotypic characterizations of the isolates. It was hoped that comparative 16S rRNA sequence analysis of the isolates would provide a phylogenetic framework within which further phenotypic characterization could be facilitated. In addition, phylogenetic analysis of novel isolates should provide a foundation for expanding and refining current understanding of ecology and evolution of SRB and allow for the evaluation and revision of currently available 16S rRNA-directed probes that were intended to target specific groups, genera, or species.

Methods

Cultivation of Organisms

Organisms used in this study were kindly provided by B. Sharak-Genthner and S. Friedman. Isolates BG8, BG18, BG23, and BG25 were grown using the anaerobic asceptic technique described in Chapter One, except that brackish water basal medium was used instead of freshwater medium and electron donors propionate and butyrate were used instead of lactate. Brackish water basal medium was prepared by adding 7.0 g NaCl, 1.2 g $MgCl_2 \cdot 6H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 4.0 g Na_2SO_4, 0.25 g NH_4Cl, and 0.5 g KCl to 1.0 l dH_2O. The basal

medium was flushed with 9:1 N_2: CO_2 gas, aliquoted into serum bottles that were also flushed with N_2/CO_2, and autoclaved with rubber stoppers fixed to bottles. Sterile propionate was asceptically added to medium for cultivation of BG8 (to 0.2 M) and butyrate was likewise added to medium for cultivation of BG18, BG23, and BG25 (to 0.1 M).

DNA Extraction

DNA was extracted from cultures of isolates BG8, BG18, BG23, and BG25, as follows. Approximately 200 mg (wet weight) bacterial cells were pelleted by centrifugation at 10,000 x *g* at 4° C for 20 min, resuspended in SE buffer (0.15 M NaCl, 0.1 M EDTA, pH 8.0) containing 1 mg/ml lysozyme, and incubated on ice for 30 min. Proteinase K and sodium dodecyl sulfate (SDS) were added to a concentration of 50 µg/ml and 1% (wt/vol), respectively. Cells were lysed by freezing the cell suspension at -70° C followed immediately by thawing at 65° C and repeating the freeze-thaw cycle for a total of 3 times. After incubating the mixture at 37° C for 90 min, cellular debris was pelleted by centrifugation at 10,000 x *g* 4°C for 30 min and the resulting supernatant fluid was transferred to a sterile tube. The supernatant fluid was then extracted twice with an equal volume of phenol (saturated with TE [10 mM Tris, 1 mM EDTA, pH 8.0], 0.1 M NaCl, 1% SDS). 1/6 volume 5 M NaCl and 1/9 volume CTAB (10% hexadecyltrimethyl-ammonium bromide in 0.7 M NaCl) were added to the phenol-extracted supernatant fluid and, after incubating the solution at 65° C for 5 min, it was extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (24: 24: 1). Nucleic acids were precipitated by adding 1/2 volume 7.5 M ammonium acetate and 1 volume isopropanol and incubating overnight at -20° C. Nucleic acids were collected by centrifuging at 10,000 x *g* at 4° C for 20 min, washed with 70% ethanol, dried briefly, and then resuspended in

sterile dH_2O. RNA was degraded by adding 2 µl *DNase*-free *RNase*A (Sigma, St. Louis, MO) and incubating at 37° C for 30 min. DNA was then reprecipitated, as before, washed with 70% ethanol, and resuspended in TE.

Amplification, Cloning, and Sequencing of 16S rRNA Genes

Primers fD1 and rP2 (Table 3.1) (Weisburg et al., 1991) were used to amplify near-complete 16S rRNA gene (16S rDNA) sequences from the extracted DNA of isolates BG8, BG18, BG23, and BG25. These primers were also used to amplify near-complete 16S rDNA from washed cell suspensions of isolates BG6, BG33, BG72, and BG74. It should be noted that amplifications from washed cell suspensions were carried out by S. Friedman. The PCR mixtures consisted of 50 mM KCl, 10 mM Tris-Cl pH 8.3, 2 mM $MgCl_2$, 200 µM each dNTP (dATP, dCTP, dGTP, dTTP), 0.2 µM each primer, and 1-2 µl DNA template in a total volume of 100 µl. The 'hot-start' method was used by heating the PCR mixture to 94° C for 2 min, and then adding 2 U *Taq* DNA polymerase to each reaction mixture. The reaction mixtures were then subjected to 27 cycles, each consisting of 1 min at 92° C, 1 min at 40° C, and 1 min at 72° C, followed by 5 min at 72° C, using a Perkin Elmer Thermal Cycler (Perkin Elmer-Cetus, Norwalk, CT). PCR products were analyzed by electrophoresis in 0.8% agarose using standard techniques (Sambrook et al., 1989).

Amplified 16S rDNA sequences from isolates BG6, BG8, BG18, BG23, BG33, and BG72 were ligated bidirectionally into plasmid vector pNoTA/T7 (Five Prime Three Prime, Inc., Boulder, CO) using blunt-ended ligation, and transformed into competent *Escherichia coli* cells using the Prime PCR Cloning Kit (Five Prime Three Prime, Inc.) as described by the manufacturer. Because restriction digestion of PCR products was not necessary in this cloning procedure, any bias associated with internal restriction sites was avoided.

Table 3.1. Primers used for amplification and sequencing of 16S rRNA genes of isolates.

Primer	Sequence[a]	Reference
926F	5'-AAACTYAAAKGAATTGRCGG-3'	Britschgi et al., 1994
1115F	5'-CAACGAGCGCAACCCT-3'	Dorsch & Stackebrandt, 1992
M13-20	5'-GTAAAACGACGGCCAGT-3'	
357R	5'-CTGCTGCCTCCCGTA-3'	Dorsch & Stackebrandt, 1992
536R	5'-ACCGCGGCKGCTGGC-3'	Devereux, et al., 1989
690R	5'-GATMTCTACGRATTTCAC-3'	Devereux et al., 1989
907R	5'-CCGTCAATTCMTTTRAGTTT-3'	Lane et al., 1985
1100R	5'-AGGGTTGCGCTCGTTG-3'	Devereux et al., 1990
RM13	5'-GGAAACAGCTATGACCATG-3'	
fD1	5'-GGGAATTCGTCGACAGAGTTTGATCCTGGCTCA-3'	Weisburg et al., 1991
rP2	5'-GGAAGCTTGGATCCACGGCTACCTTGTTACGACTT-3'	Weisburg et al., 1991

[a]Mixed base positions were K: G and T; M: A and C; R: A and G; and Y: C and T.

Transformants were selected for by ampicillin-resistance conferred by the pNoTA/T7 plasmid and colonies were screened for inserts by alpha-complementation using X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside) and IPTG (isopropyl-ß-D-thiogalactopyranoside) (Sambrook et al., 1989). Clones were screened to ensure they contained an insert of the expected size (~1,500 bp) by digesting isolated plasmid DNA with *XbaI* (Sambrook et al., 1989), a restriction endonuclease that cleaves plasmid pNoTA on both sides of inserted DNA (Five Prime Three Prime, Inc.) Plasmid DNA was isolated from clones for sequencing using the Qiagen midi-prep system (Qiagen, Inc., Chatsworth, CA) as described by the manufacturer.

16S rDNA fragments from isolates BG25 and BG72 were sequenced directly from PCR products (Meltzer, 1993). This technique was used because inconsistent yields of plasmid DNA from clones containing the partial 16S rDNA sequences from these isolates to pursuit of an alternate route for obtaining DNA template for sequencing reactions. Amplified 16S rDNA sequences were purified from the PCR mixture by electrophoresis in 1% SeaPlaque agarose (FMC Bioproducts, Rockland, ME) containing 0.2 µg/ml ethidium bromide in 40 mM Tris, 20 mM glacial acetic acid (pH 8.4). After briefly visualizing the electrophoresed PCR products by UV illumination, the ~1,500 bp products were excised from the gel. DNA was recovered by melting the agarose matrix at 65° C and then incubating with 5 U ß-agarase at 37° C for 1-4 h.

Partial 16S rRNA genes were sequenced using a PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit (Perkin-Elmer Cetus) and an ABI 373A automated sequencer (Applied Biosystems, Foster City, CA). Primers that anneal to conserved regions of 16S rRNA genes were used in sequencing reactions and are listed in Table 3.1. In addition, primers M13 -20, and M13 reverse (Table 3.1; Stratagene, Inc., La Jolla, CA) were used for sequencing cloned

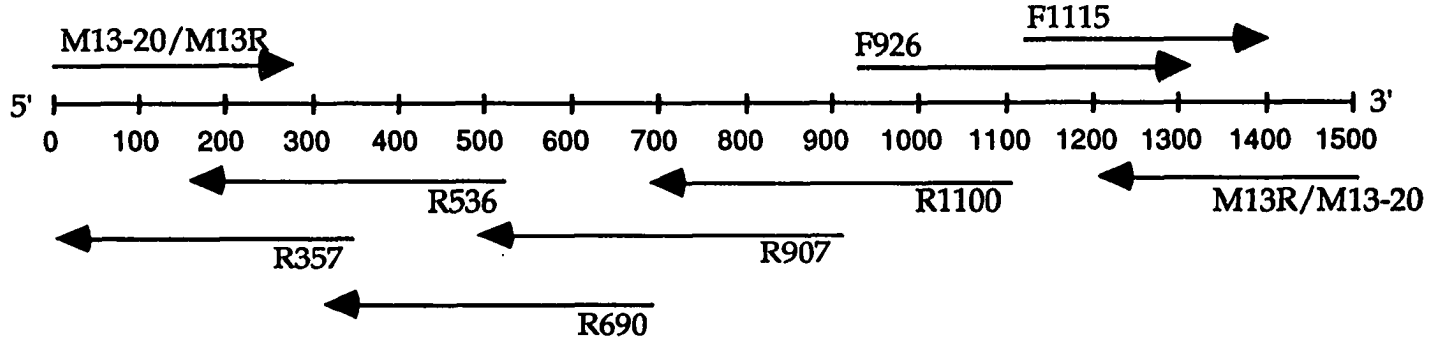
16S rDNA (i.e., BG6, BG8, BG18, BG23, BG33, and BG74). A schematic diagram of sequencing primer annealing sites and reactions is shown in Fig. 3.1. The near-complete 16S rDNA sequences of two other salt marsh isolates, BG14 and BG50 (also isolated by B. Sharak Genthner as described above) were provided by R. Devereux and were included in phylogenetic analyses.

Phylogenetic Analyses

The partial 16S rDNA sequences from all ten isolates were compared to sequences in both GenBank, using BLASTN, and in the Ribosomal Database Project (RDP), using the functions SUGGEST_TREE and SIMILARITY_RANK (Maidak et al., 1994). This allowed for the identification of the isolates' close relatives. Isolate 16S rDNA sequences were then manually aligned, using the sequence editor SeqApp, to aligned 16S rDNA sequences available from the RDP. 16S rDNA sequences of close relatives to the isolates that were not yet available in the RDP's aligned databases were obtained from GenBank and also aligned manually. GenBank accession numbers for the sequences used in this study, including those determined here, are given in Table 3.2. Only base positions that were unambiguously aligned were used in subsequent analyses. This was effected by applying masks to the alignments to designate positions that were to be included in analyses.

Phylogenetic trees were constructed using maximum parsimony, neighbor-joining, and least-squares methods available in the phylogenetic analysis application package PHYLIP 3.57 (Felsenstein, 1989) as described in Chapter One. Sequence identity values were determined by comparing a given isolate's 16S rRNA sequence with those of its closest relative(s) and omitting any base positions that contained ambiguous base positions or that were ambiguously aligned. This allowed for the maximum number of bases to be

Fig. 3.1. Schematic diagram of sequencing primers and reactions used to obtain near-complete sequences of 16S rRNA genes from SRB isolates.*



*See Table 3.1 for primer sequences and references.

Table 3.2. GenBank accession numbers for 16S rRNA sequences used in phylogenetic analyses.

Organism	Accession no.
Strain BG6	U85468
Strain BG8	U85469
Strain BG14	U85470
Strain BG18	U85471
Strain BG23	U85472
Strain BG25	U85473
Strain BG33	U85474
Strain BG50	U85475
Strain BG72	U85476
Strain BG74	U85477
Bdellovibrio bacteriovorus str. 109J	M59297
Desulfoarculus baarsii str. 2st14, Konstanz	M34403
Desulfobacter curvatus str. AcRM3	M34413
Desulfobacter hydrogenophilus str. AcRS1	M34412
Desulfobacter latus str. AcRS2	M34414
Desulfobacter postgatei str. 2 ac 9	M26633
Desulfobacter sp. str. 3ac10	M34415
Desulfobacter sp. str. 4ac11	M34416
Desulfobacterium autotrophicum	M34409
Desulfobacterium niacini	M34406
Desulfobacterium vacuolatum	M34408
Desulfobacula toluolica	X70953
Desulfobulbus elongatus	X95180
Desulfobulbus propionicus str. 1 pr 3, Lindhorst	M34410
Desulfobulbus sp. str. 3pr10	M34411
Desulfocapsa thiozymogenes str. Bra2	X95181
Desulfococcus multivorans str. 1 be 1, Goettingen	M34405
Desulfohalobium retbaense	U48244
Desulfomicrobium baculatus	M37311
Desulfomicrobium escambium	U02469
Desulfomicrobium sp. str. Norway 4	M37312
Desulfomonile tiedjei	M26635
Desulfonema limicola	U45990
Desulfonema magnum	U45989
Desulforhabdus acetothermus	U25627
Desulforhopalus vacuolatus	L42613
Desulfosarcina variabilis str. 3 be 13, Montpellier	M26632
Desulfotomaculum nigrificans	X62176
Desulfovibrio africanus	M37315
Desulfovibrio caledoniensis	U53465
Desulfovibrio desulfuricans	M34113
Desulfovibrio desulfuricans str. E1 Agheila Z.	M37316

Table 3.2. Continued.

Organism	Accession no.
Desulfovibrio desulfuricans str. Essex 6	M37313
Desulfovibrio fairfieldensis	U42221
Desulfovibrio gabonensis	U31080
Desulfovibrio gigas	M34400
Desulfovibrio halophilus	U48243
Desulfovibrio longreachii str. 16910a	Z24450
Desulfovibrio longus str. SEBR 2582	X63623
Desulfovibrio pigra	M34404
Desulfovibrio salixigens	M34401
Desulfovibrio sapovorans str. 1pa3, Lindhorst	M34402
Desulfovibrio sp. str. MIT 87-599	U07570
Desulfovibrio sp. str. PT-2	M98496
Desulfovibrio vulgaris subsp. vulgaris str. Hildenborough	M34399
Desulfuromonas acetoxidans	M26634
Escherichia coli subsp. K-12	M87049
Geobacter metallireducens str. GS-15	L07834
Myxococcus xanthus str. DK1622	M34114
Pelobacter acetylenicus str. WoACY1	X70955
Syntrophobacter wolinii	X70905

compared between closest relatives, while evolutionary distances and phylogenetic relationships necessarily included only those base positions that were unambiguously aligned across all sequences included in a given tree.

Results and Discussion

Quality of Sequence Data

Both direct sequencing of purified PCR products and sequencing of cloned PCR products yielded high quality sequence data with about 1-2 ambiguous bases per 1500 bases. The primers used (Table 3.1) allowed for a significant amount of overlapping data between sequencing reactions (Fig. 3.1). Advantages of direct sequencing of PCR products included reduced processing time and a lower probability of error in sequence data due to errors in nucleotide incorporation by *Taq* polymerase (about 1 in 2×10^4; Watson et al. 1992) (because an entire population, instead of only one, PCR amplicons is analyzed; Meltzer, 1993). However, cloning of amplified 16S rDNA prior to sequencing also had advantages over direct sequencing. Namely, M13 primers that anneal to plasmid DNA flanking the cloned inserts can be used in addition to primers that anneal to the 16S rRNA itself. Here, as a result of using M13 primers, 1,527 bases of 16S rDNA sequence data were obtained from cloned 16S rDNA, while 1,460 bases were obtained from direct sequencing of amplified 16S rDNA.

Phylogenetic Analysis of Isolates

Analysis of nearly complete 16S rRNA gene sequences from the ten new SRB isolates revealed that they were all members of the ∂ subdivision of the proteobacteria and were closely related to other known SRB within this group (Fig. 3.2-3.4; Table 3.3). This result lends support to the hypothesis that the Gram-negative, nonsporeforming, mesophilic SRB are a phylogenetically

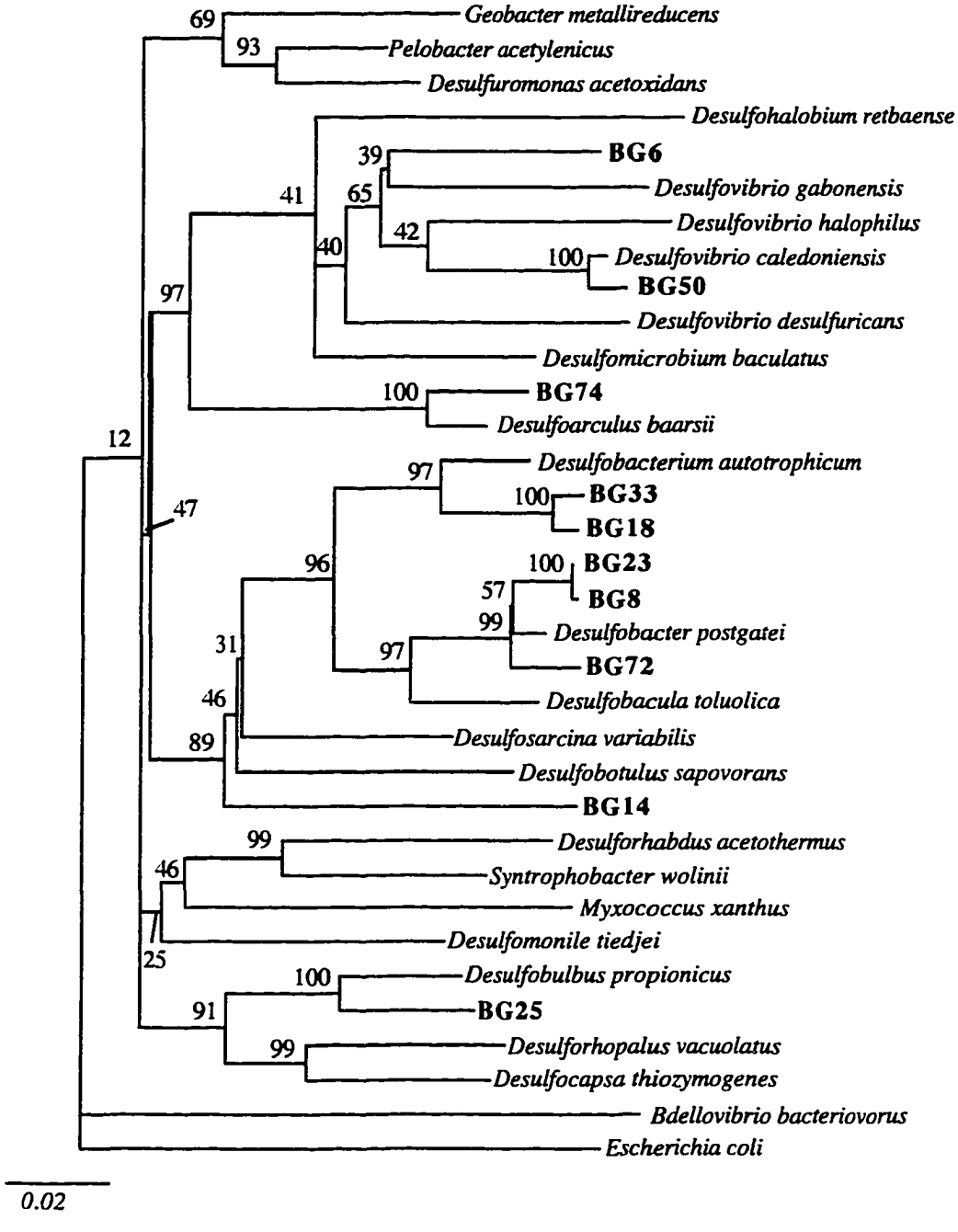


Fig. 3.2. 16S rRNA phylogenetic tree of SRB isolates, related SRB, and other representatives of the ∂ proteobacteria constructed using a maximum parsimony method. 1,033 base positions were considered in the analysis. Bootstrap values (out of 100 trees) are shown adjacent to nodes. The scale bar is in fixed nucleotide substitutions per sequence position.

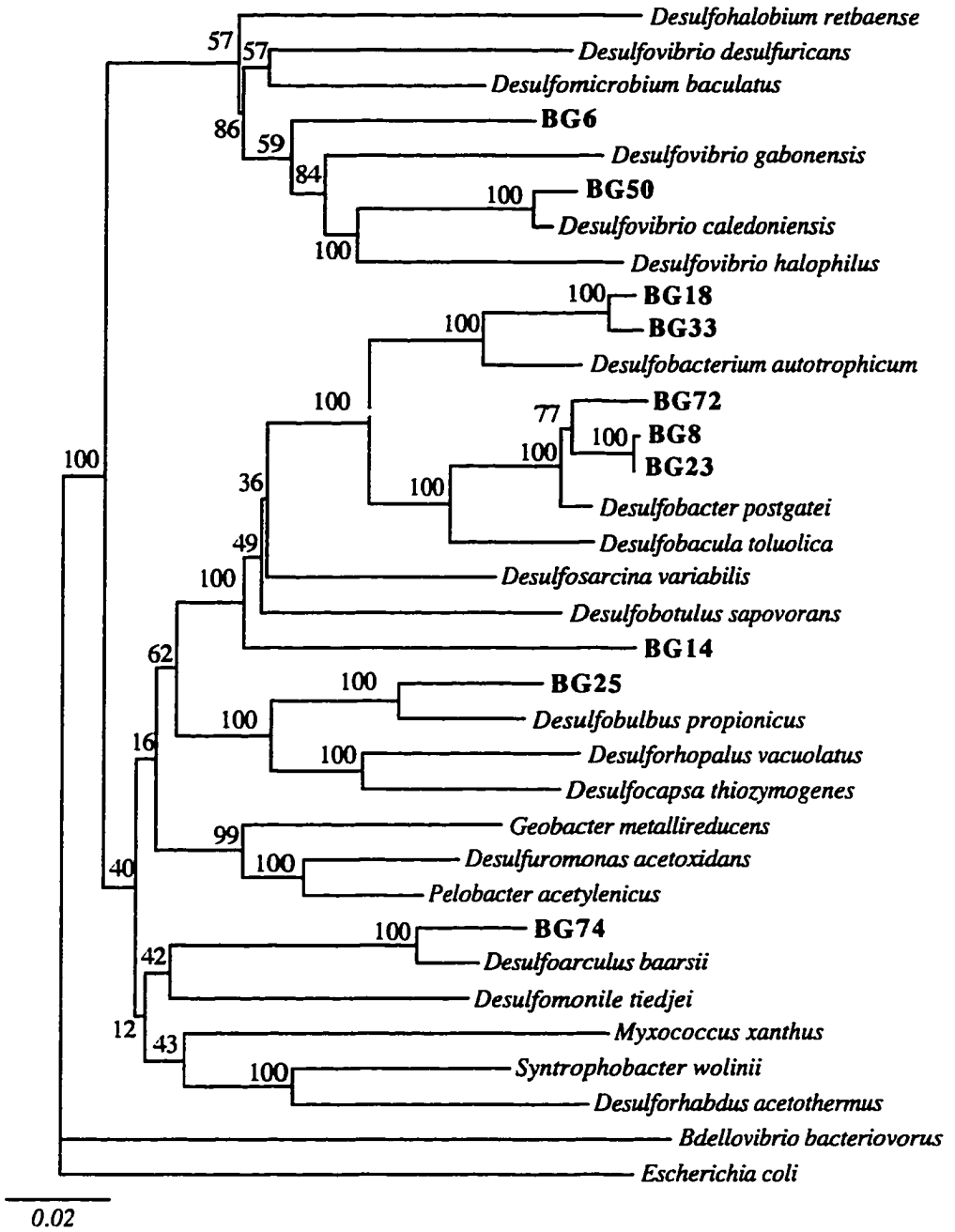


Fig. 3.3. 16S rRNA phylogenetic tree of SRB isolates, related SRB, and other representatives of the ∂ proteobacteria constructed from evolutionary distances using a neighbor-joining algorithm. Jukes-Cantor evolutionary distances were calculated from 1,033 base positions. Bootstrap values (out of 100 trees) are shown adjacent to nodes. The scale bar is in fixed nucleotide substitutions per sequence position.

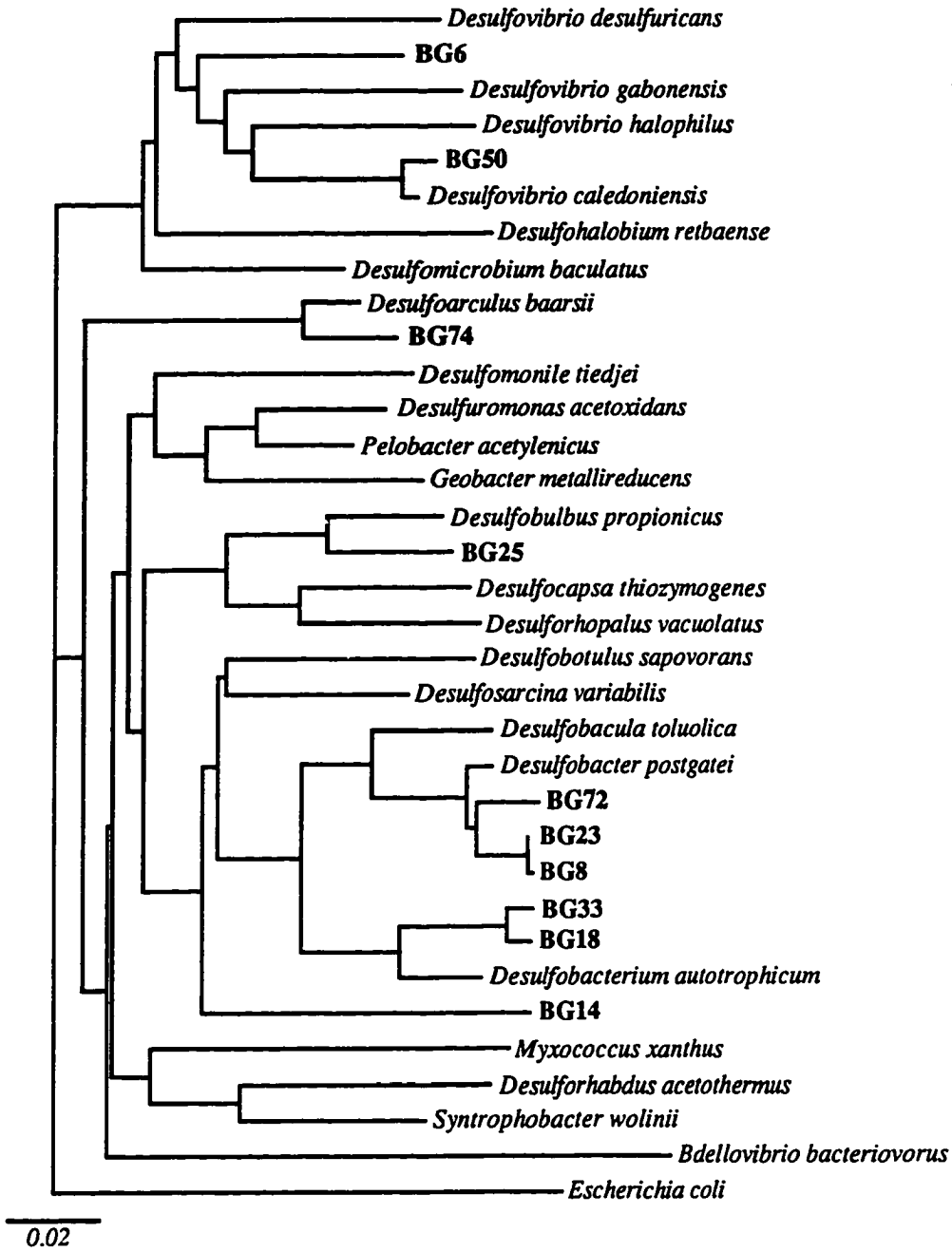


Fig. 3.4. 16S rRNA phylogenetic tree of SRB isolates, related SRB, and other representatives of the ∂ proteobacteria constructed from evolutionary distances using the Fitch-Margoliash least squares method. Jukes-Cantor evolutionary distances were calculated from 1,033 base positions. Bootstrap values (out of 100 trees) are shown adjacent to nodes. The scale bar is in fixed nucleotide substitutions per sequence position.

Table 3.3. Jukes-Cantor evolutionary distances for 16S rRNA sequences from various members of the ∂ proteobacteria and new SRB isolates.

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.
1. *Escherichia coli*										
2. *Myxococcus xanthus*	0.204									
3. *Bdellovibrio bacteriovorus*	0.225	0.202								
4. *Desulfuromonas acetoxidans*	0.190	0.138	0.186							
5. *Syntropherbacter wolinii*	0.215	0.148	0.227	0.136						
6. *Pelobacter acetylenicus*	0.175	0.130	0.189	0.053	0.125					
7. *Geobacter metallireducens*	0.181	0.144	0.186	0.091	0.148	0.082				
8. *Desulforhabdus acetothermus*	0.201	0.152	0.221	0.162	0.099	0.150	0.157			
9. *Desulfohalobium retbaense*	0.207	0.197	0.233	0.172	0.170	0.166	0.175	0.174		
10. *Desulfovibrio desulfuricans*	0.200	0.187	0.222	0.160	0.179	0.153	0.165	0.181	0.152	
11. *Desulfovibrio caledoniensis*	0.191	0.177	0.218	0.158	0.168	0.157	0.174	0.184	0.128	0.113
12. *Desulfovibrio halophilus*	0.199	0.201	0.242	0.166	0.186	0.161	0.191	0.196	0.151	0.121
13. *Desulfovibrio gabonensis*	0.210	0.196	0.238	0.170	0.168	0.160	0.170	0.166	0.139	0.124
14. *Desulfomicrobium baculatus*	0.186	0.176	0.198	0.142	0.160	0.129	0.141	0.166	0.125	0.098
15. *Desulfomonile tiedjei*	0.196	0.140	0.181	0.112	0.127	0.101	0.122	0.145	0.193	0.179
16. *Desulfobulbus propionicus*	0.203	0.162	0.203	0.122	0.137	0.127	0.141	0.139	0.191	0.177
17. *Desulfocapsa thiozymogenes*	0.183	0.163	0.210	0.146	0.140	0.128	0.135	0.143	0.178	0.180
18. *Desulforhopalus vacuolatus*	0.201	0.172	0.216	0.144	0.154	0.122	0.132	0.168	0.190	0.186
19. *Desulfobacterium autotrophicum*	0.213	0.178	0.200	0.134	0.153	0.136	0.149	0.179	0.192	0.177
20. *Desulfosarcina variabilis*	0.202	0.169	0.203	0.133	0.131	0.121	0.133	0.155	0.177	0.178
21. *Desulfobotulus sapovorans*	0.212	0.177	0.212	0.142	0.154	0.125	0.131	0.174	0.194	0.181
22. *Desulfobacula toluolica*	0.226	0.181	0.213	0.133	0.164	0.128	0.154	0.186	0.209	0.184
23. *Desulfobacter postgatei*	0.217	0.180	0.218	0.133	0.155	0.127	0.150	0.178	0.199	0.178
24. *Desulfoarculus baarsii*	0.186	0.165	0.211	0.141	0.130	0.124	0.144	0.152	0.169	0.160
25. BG6	0.207	0.178	0.219	0.150	0.171	0.144	0.152	0.180	0.141	0.112
26. BG8	0.225	0.196	0.229	0.143	0.166	0.140	0.163	0.181	0.210	0.190
27. BG14	0.238	0.198	0.231	0.148	0.160	0.143	0.157	0.185	0.195	0.188
28. BG18	0.231	0.200	0.215	0.149	0.176	0.147	0.164	0.198	0.211	0.191
29. BG23	0.224	0.195	0.229	0.143	0.165	0.140	0.163	0.180	0.210	0.190
30. BG25	0.203	0.158	0.195	0.133	0.142	0.131	0.152	0.152	0.178	0.187
31. BG33	0.234	0.204	0.211	0.146	0.179	0.149	0.163	0.203	0.212	0.186
32. BG50	0.188	0.183	0.218	0.159	0.168	0.160	0.174	0.185	0.131	0.122
33. BG72	0.236	0.193	0.227	0.144	0.166	0.138	0.161	0.188	0.211	0.190
34. BG74	0.196	0.174	0.206	0.139	0.141	0.138	0.151	0.161	0.177	0.155

Table 3.3. Continued.

	11.	12.	13.	14.	15.	16.	17.	18.	19.	20.	21.	22.	23.	24.	25.	26.	27.	28.	29.	30.	31.	32.	33.
12.	0.088																						
13.	0.094	0.112																					
14.	0.108	0.125	0.125																				
15.	0.183	0.176	0.184	0.168																			
16.	0.169	0.191	0.176	0.165	0.125																		
17.	0.180	0.192	0.191	0.154	0.145	0.106																	
18.	0.169	0.178	0.191	0.157	0.151	0.109	0.079																
19.	0.163	0.195	0.194	0.138	0.143	0.148	0.162	0.156															
20.	0.164	0.167	0.171	0.143	0.119	0.124	0.136	0.147	0.096														
21.	0.167	0.186	0.181	0.156	0.152	0.150	0.156	0.151	0.107	0.098													
22.	0.170	0.189	0.189	0.158	0.146	0.139	0.161	0.148	0.085	0.118	0.130												
23.	0.165	0.198	0.183	0.152	0.152	0.136	0.150	0.151	0.097	0.106	0.130	0.056											
24.	0.152	0.150	0.149	0.153	0.117	0.145	0.165	0.159	0.150	0.124	0.133	0.150	0.151										
25.	0.105	0.100	0.099	0.107	0.177	0.167	0.164	0.173	0.165	0.150	0.164	0.177	0.172	0.138									
26.	0.180	0.210	0.199	0.165	0.163	0.153	0.156	0.161	0.099	0.119	0.144	0.062	0.020	0.155	0.193								
27.	0.190	0.196	0.195	0.164	0.160	0.158	0.155	0.153	0.138	0.133	0.126	0.144	0.136	0.166	0.170	0.150							
28.	0.190	0.204	0.209	0.167	0.158	0.157	0.174	0.167	0.047	0.106	0.117	0.090	0.093	0.169	0.183	0.100	0.144						
29.	0.180	0.210	0.199	0.165	0.162	0.153	0.157	0.161	0.098	0.118	0.144	0.061	0.019	0.155	0.193	0.001	0.150	0.098					
30.	0.176	0.191	0.185	0.173	0.133	0.053	0.097	0.118	0.148	0.128	0.158	0.138	0.138	0.154	0.172	0.150	0.161	0.147	0.150				
31.	0.189	0.204	0.210	0.162	0.166	0.154	0.169	0.167	0.048	0.109	0.118	0.091	0.095	0.170	0.183	0.102	0.147	0.011	0.102	0.151			
32.	0.011	0.092	0.102	0.116	0.186	0.176	0.182	0.173	0.166	0.167	0.170	0.173	0.167	0.158	0.113	0.182	0.193	0.193	0.182	0.183	0.191		
33.	0.181	0.213	0.200	0.159	0.160	0.155	0.166	0.166	0.095	0.116	0.135	0.062	0.023	0.159	0.187	0.027	0.143	0.096	0.026	0.155	0.099	0.183	
34.	0.167	0.159	0.158	0.156	0.124	0.154	0.175	0.176	0.162	0.130	0.154	0.163	0.164	0.034	0.143	0.164	0.174	0.178	0.164	0.161	0.178	0.167	0.174

coherent group (Devereux et al., 1996) and is consistent with the general observation that most SRB isolated to date are members of this group (Stackebrandt et al., 1995). Other members of the ∂ Proteobacteria include the '*Geobacteriaceae*' family (Lonergan et al., 1996), bdellovibrios, and myxobacteria (Woese, 1987) and exhibit widely differing phenotypic characteristics from the Gram-negative SRB (De Ley, 1992). As mentioned above, two distinct lineages of SRB are known to exist within this group, the first being defined by the family *Desulfovibrionaceae* (Devereux et al., 1990) and the second by the proposed family *Desulfobacteriaceae* (Widdel and Bak, 1992). All of the SRB isolates in the current study appear to be members of these two lineages (Fig. 3.2-3.4).

Phylogeny of Members of the *Desulfovibrionaceae* Family

Both 16S rRNA phylogenetic tree topology (Fig. 3.2-3.4 and Fig. 3.5-3.7) and evolutionary distances (Table 3.3-3.4) placed isolates BG6 and BG50 within the *Desulfovibrionaceae* family. The *Desulfovibrionaceae* form a physiologically coherent group and traditional taxonomy of this group, as determined by phenotypic traits, has been shown to correspond quite well with 16S rRNA phylogenetic relationships (Devereux et al., 1990). The *Desulfovibrionaceae* are characterized by their inability to completely oxidize lactate to CO_2 or to utilize fatty acids as growth substrates (Devereux et al., 1990). Organic substrates most commonly utilized by *Desulfovibrionaceae* members are lactate (the organic carbon source that many of the 'classical' desulfovibrios were enriched on), pyruvate, ethanol, and frequently malate and fumarate (Widdel and Bak, 1992). While utilization of H_2 as an electron donor is quite common within this family, growth on H_2 is not autotrophic as it requires acetate in addition to CO_2 (Widdel and Bak, 1992). Other phenotypic characteristics of this family include the presence of the isoprenoid quinones of the MK-6 type (Stackebrandt et al., 1995) and

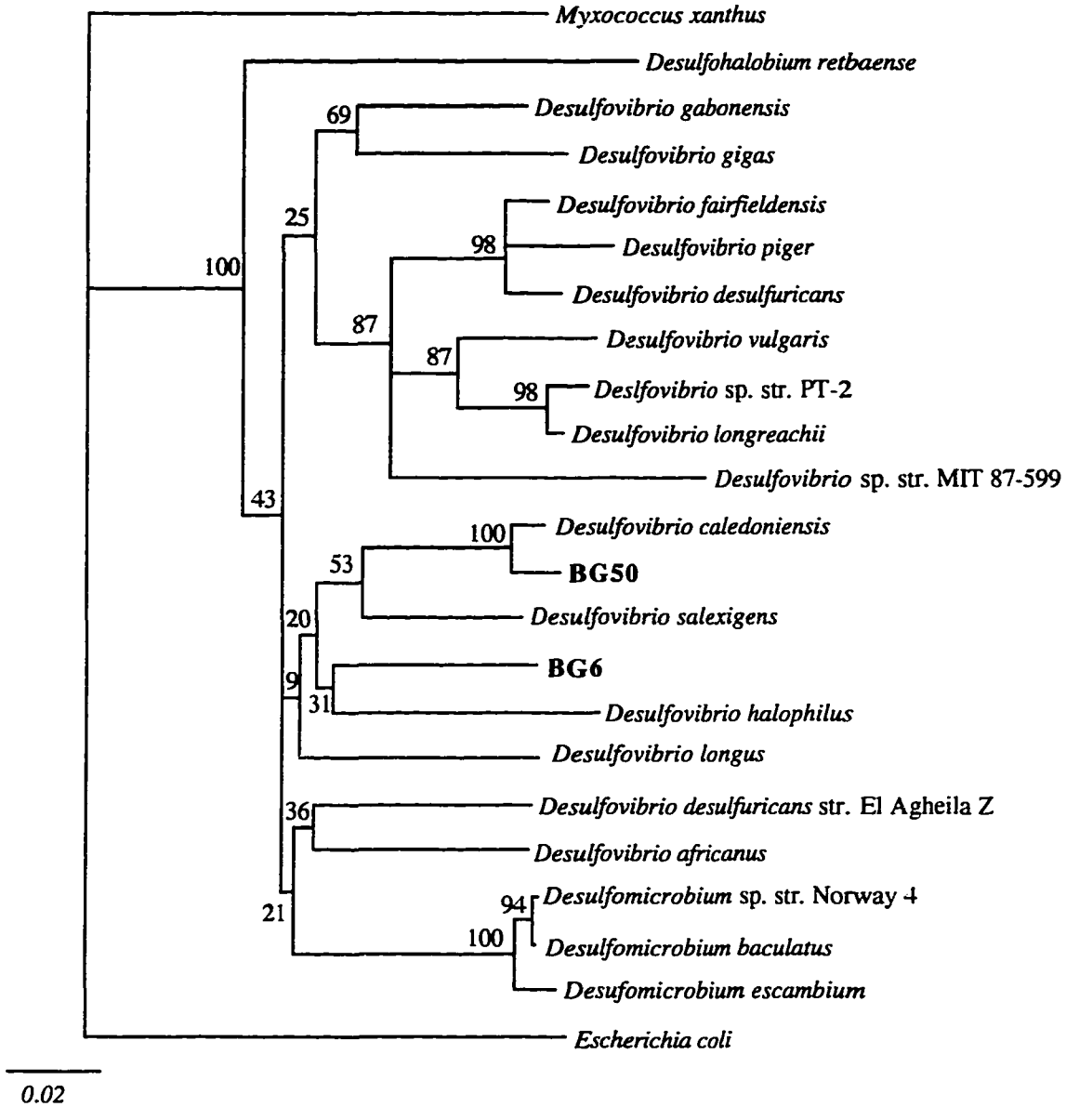


Fig. 3.5. 16S rRNA phylogenetic tree of isolates BG6 and BG50 and members of the *Desulfovibrionaceae* family constructed using a maximum parsimony method. 867 base positions were considered in the analysis. Bootstrap values (out of 100 trees) are shown adjacent to nodes. The scale bar is in fixed nucleotide substitutions per sequence position.

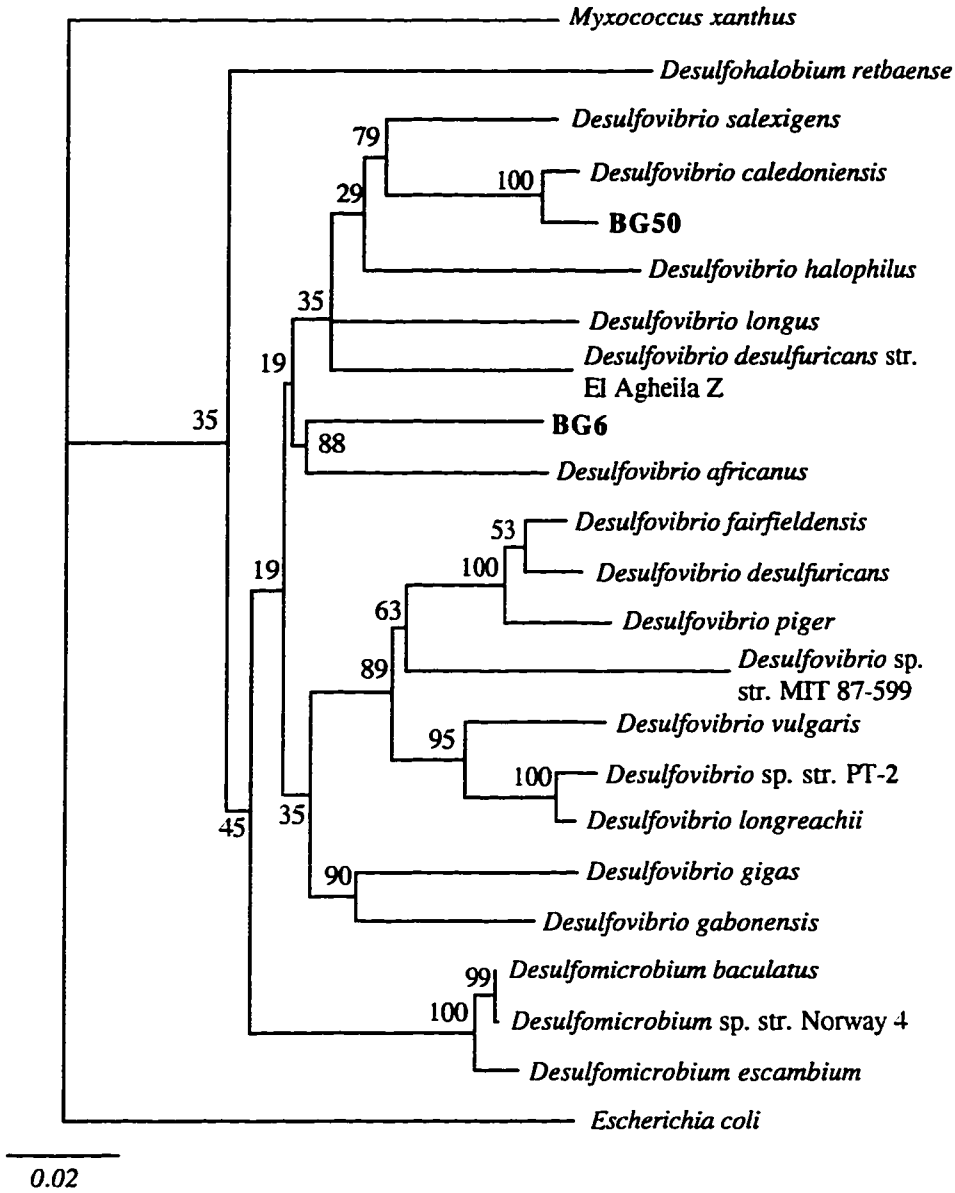


Fig. 3.6. 16S rRNA phylogenetic tree of isolates BG6 and BG50 and members of the *Desulfovibrionaceae* family constructed from evolutionary distances using a neighbor-joining algorithm. Jukes-Cantor evolutionary distances were calculated from 867 base positions. Bootstrap values (out of 100 trees) are shown adjacent to nodes. The scale bar is in fixed nucleotide substitutions per sequence position.

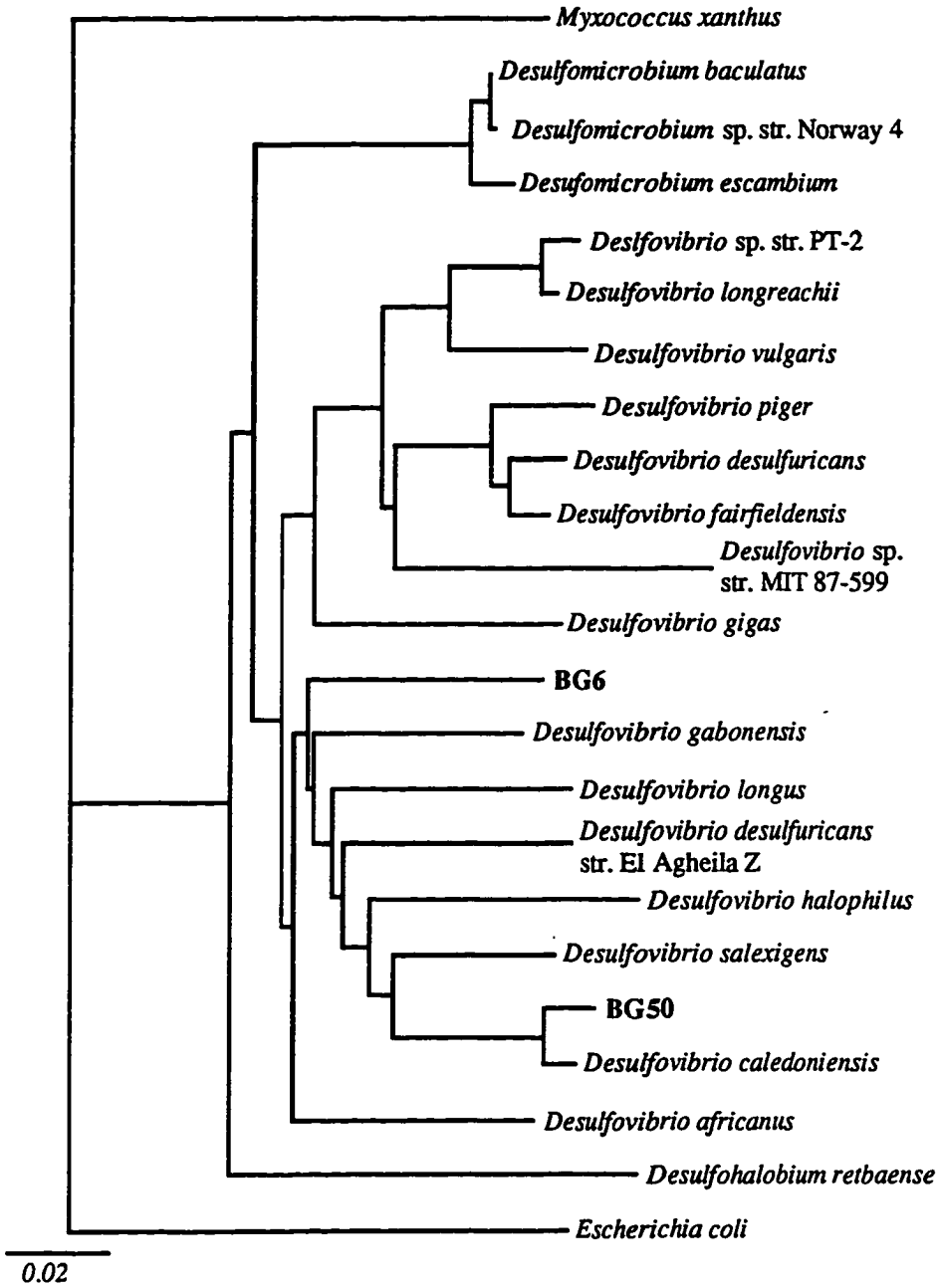


Fig. 3.7. 16S rRNA phylogenetic tree of isolates BG6 and BG50 and members of the *Desulfovibrionaceae* family constructed from evolutionary distances using the Fitch-Margoliash least squares method. Jukes-Cantor evolutionary distances were calculated from 867 base positions. The scale bar is in fixed nucleotide substitutions per sequence position.

Table 3.4. Jukes-Cantor evolutionary distances for 16S rRNA sequences of *Desulfovibrionaceae* isolates and relatives.

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.	19.	20.	21.	22.
1. *Escherichia coli*																						
2. *Myxococcus xanthus*	0.201																					
3. *Desulfovibrio africanus*	0.203	0.189																				
4. *Desulfovibrio caledoniensis*	0.200	0.185	0.127																			
5. *Desulfovibrio desulfuricans*	0.204	0.207	0.110	0.121																		
6. *Desulfovibrio desulfuricans* str. El	0.195	0.206	0.094	0.104	0.119																	
7. *Desulfomicrobium* sp. str. Norway 4	0.195	0.181	0.102	0.117	0.102	0.110																
8. *Desulfovibrio fairfieldensis*	0.210	0.213	0.116	0.125	0.020	0.126	0.103															
9. *Desulfovibrio gigas*	0.209	0.206	0.114	0.127	0.101	0.126	0.118	0.092														
10. *Desulfovibrio halophilus*	0.213	0.213	0.123	0.095	0.130	0.107	0.133	0.143	0.146													
11. *Desulfovibrio longreachii*	0.211	0.215	0.121	0.119	0.075	0.132	0.115	0.071	0.101	0.130												
12. *Desulfovibrio longus*	0.207	0.202	0.114	0.108	0.101	0.106	0.122	0.109	0.123	0.111	0.113											
13. *Desulfovibrio salexigens*	0.214	0.190	0.103	0.075	0.124	0.086	0.114	0.125	0.124	0.096	0.120	0.094										
14. *Desulfovibrio* sp. str. MIT 87-599	0.198	0.206	0.154	0.150	0.105	0.172	0.147	0.096	0.144	0.173	0.103	0.152	0.145									
15. *Desulfovibrio* sp. str. PT-2	0.219	0.225	0.126	0.124	0.084	0.138	0.126	0.078	0.107	0.133	0.012	0.119	0.123	0.109								
16. *Desulfovibrio gabonensis*	0.223	0 208	0.102	0.095	0.113	0.105	0.128	0.108	0.082	0.115	0.103	0.112	0.090	0.145	0.105							
17. *Desulfovibrio vulgaris*	0.212	0.230	0.107	0.117	0.089	0.115	0.129	0.094	0.128	0.136	0.054	0.113	0.108	0.120	0.054	0.108						
18. *Desulfohalobium retbaense*	0.222	0.214	0.164	0.147	0.168	0.153	0.141	0.162	0.139	0.176	0.164	0.166	0.144	0.167	0.162	0.144	0.161					
19. *Desulfomicrobium baculatus*	0.190	0.181	0.101	0.116	0.103	0.107	0.001	0.105	0.120	0.134	0.113	0.123	0.117	0.143	0.124	0.126	0.122	0.139				
20. *Desulfomicrobium escambium*	0.194	0.188	0.105	0.117	0.109	0.111	0.014	0.109	0.131	0.140	0.116	0.134	0.125	0.152	0.124	0.130	0.125	0.144	0.013			
21. *Desulfovibrio piger*	0.218	0.223	0.123	0.141	0.043	0.137	0.115	0.031	0.106	0.151	0.073	0.116	0.136	0.109	0.077	0.122	0.089	0.170	0.118	0.120		
22. BG6	0.204	0.180	0.097	0.104	0.120	0.106	0.105	0.124	0.121	0.101	0.112	0.126	0.101	0.140	0.116	0.094	0.124	0.147	0.108	0.108	0.134	
23. BG50	0.199	0.194	0.128	0.018	0.122	0.107	0.129	0.126	0.128	0.109	0.119	0.105	0.076	0.153	0.123	0.094	0.115	0.147	0.126	0.133	0.140	0.122

cytochrome c_3 (Postgate and Campbell, 1966). The presence of the pigment and bisulfite reductase desulfoviridin is characteristic of most *Desulfovibrionaceae*, with the exception of *Desulfomicrobium* species which possess the bisulfite reductase desulforubidin (Devereux et al., 1990; Sharak Genthner et al., 1994). In addition, most of the members of the genus *Desulfovibrio* exhibit a vibroid morphology, although rod-shaped cells also occur within this genus (Postgate and Campbell, 1966; Devereux et al., 1990).

Of the two *Desulfovibrionaceae* isolates, BG6 branched at a deeper level (Fig. 3.5-3.7). Percent sequence identities shared by BG6 and its closest relatives were 89.0% with *Desulfovibrio desulfuricans* str. El Agheila Z (1,204 base positions compared), 88.9% with *Desulfovibrio gabonensis* (1,400 base positions compared), and 88.7% with *Desulfovibrio africanus* (1,163 base positions compared). Corresponding Jukes-Cantor-corrected evolutionary distances (867 base positions compared) used for distance-based phylogenetic trees were 0.106, 0.094, and 0.097 (Table 3.4). The observed level of divergence between BG6 and its closest relatives corresponds to a level of DNA relatedness of about 7% and would generally be considered sufficient to place BG6 in a distinct genus (Devereux et al., 1990). However, the genus *Desulfovibrio* is unusually phylogenetically diverse (Devereux et al., 1990) and may be determined to be inclusive of BG6 after further phenotypic characterization. The branching order of BG6 and other deep-branching *Desulfovibrionaceae* remains unclear, as reflected in bootstrapping values and differences in branching order among parsimony, neighbor-joining, and least-squares trees (Fig. 3.5-3.7). Many of the *Desulfovibrionaceae* 16S rRNA sequences were determined by reverse transcriptase sequencing, and the resulting data are missing tracts of sequence information where reverse transcription was prematurely terminated (probably as a result of modified bases in template rRNA) (Devereux et al., 1989). As a result, the current analysis of the

Desulfovibrionaceae isolates was limited to 867 base positions. It is possible that remaining uncertainties, such as the branching order of BG6 and other deeply branching members of the family, would be resolved if improved sequence data were available for certain desulfovibrios.

Preliminary physiological characterization of BG6 (Sharak Genthner in prep) also lends support to its placement within the *Desulfovibrionaceae* and possibly to the genus *Desulfovibrio*. Its morphology consisted of chains of vibroid cells with pointed ends (Sharak Genthner pers. comm.) and it exhibited excellent growth on ethanol, fumarate, and pyruvate; good growth on lactate and malate; slight growth on H_2 and formate as electron donors; and no growth acetate, benzoate, butyrate, or propionate (Table 3.5). In particular, its inability to utilize acetate and fatty acids such as propionate and butyrate as electron donors is consistent with other *Desulfovibrionaceae*. Its relatives *D. desulfuricans* str. El Agheila Z., *D. africanus*, and *D. gabonensis* share similar patterns of substrate utilization (Table 3.5). BG6 does not appear to share the salt requirement of its moderately halophilic relative, *D. gabonensis* (5-8% salinity) (Tardy-Jacquenod et al., 1996), as all of the isolates were grown under brackish salinity conditions (about 8 ppt). However, salt tolerance generally varies throughout the genus *Desulfovibrio* and has not been found to be a phylogenetically coherent characteristic.

Further characterization of BG6 should include the determination of major menaquinones, DNA GC content, presence of desulfoviridin, and ability to utilize higher fatty acids and to completely oxidize organic substrates. If BG6 is, indeed, a member of this phylogenetically diverse genus, it would be expected to possess desulfoviridin and MK-6 type menaquinones (Widdel and Bak, 1992). In addition, it would be expected to be incapable of utilizing higher fatty acids or of completely oxidizing organic substrates to CO_2 (Devereux et al., 1990). Other

Table 3.5. Electron donors utilized for sulfate reduction by new SRB isolates and selected relatives.

Organism	A	B	E	F	FU	H	L	M	P	S	FA	References
Desulfovibrio												
africanus	-	nr	+	+	-	+	+	+	+	nr	-	Postgate, 1984 a,b
desulfuricans	-	nr	+	+	+	+	+	+	+	nr	-	Postgate, 1984 a,b
gabonensis	-	-	+	+	+	-	+	+	+	+	-	Tardy-Jacquenod et al., 1996
gigas	-	nr	+	+	+	+	+	+	+	nr	-	Postgate, 1984 a,b
longus	-	-	-	+	-	+	+	-	+	-	-	Magot et al., 1992
salexigens	-	nr	+	+	-	+	+	+	+	nr	-	Postgate, 1984 a,b
vulgaris	-	nr	+	+	+	+	+	+	+	nr	-	Widdel, 1992
BG6	-	-	+	+	+	+	+	+	+	-	-	Sharak Genthner, Pers. Comm.
BG50	-	-	+	+	+	+	+	+	+	+	-	Sharak Genthner, Pers. Comm.
Desulfomicrobium												
baculatum	-	nr	-	+	-	+	+	+	+	nr	-	Sharak Genthner et al., 1994
escambium	-	-	+	+	-	+	+	-	+	-	-	Sharak Genthner et al., 1994
Desulfobulbus												
elongatus	-	nr	+	-	-	+	+	-	nr	nr	3	Widdel, 1992
marinus	-	-	+	+	-	+	+	-	+	nr	3	Widdel, 1982
propionicus	-	nr	+	-	-	+	+	-	+	-	3	Widdel, 1982
2pr4	-	nr	+	-	nr	+	+	nr	+	nr	3-4	Widdel, 1982
BG25	-	-	+	+	+	-	-	+	+	-	4	Sharak Genthner, Pers. Comm.
Desulfocapsa												
thiozymogenes	-	-	+	-	-	-	-	-	-	-	-	Janssen et al., 1996
Desulforhopalus												
vacuolatus	-	-	+	-	-	+	+	-	+	nr	3	Isaksen & Teske, 1996
Desulforhabdus												
acetothermus	+	-	+	-	+	-	+	+	+	+	4-18	Beeder et al., 1995
Desulfobacterium												
autotrophicum	+	nr	+	+	+	+	+	+	+	+	3-16	Brysch et al., 1987
indolicum	+	-	+	+	+	-	-	+	+	+	3	Bak & Widdel, 1986
niacini	+	-	+	+	+	-	+	+	+	+	3-16	Imhoff-Stuckle & Pfennig, 1983
vacuolatum	+	-	+	+	+	+	+	+	nr	+	3-16	Widdel, 1992
BG18	-	+	+	+	+	+	+	+	+	+	4	Sharak Genthner, Pers. Comm.
BG33	+	+	+	+	+	+	+	+	+	+	3-4	Sharak Genthner, Pers. Comm.
Desulfobacter												
curvatus	+	-	+	-	-	+	-	-	+	nr	-	Widdel, 1987
hydrogenophilus	+	-	+	-	-	+	-	-	nr	nr	-	Widdel, 1987
latus	+	-	-	-	-	-	-	-	nr	nr	-	Widdel, 1987
postgatei	+	-	-	-	-	-	-	-	-	-	-	Widdel & Pfennig, 1982
3ac10	+	nr	+	-	nr	-	+	nr	-	nr	-	Widdel & Pfennig, 1982
4ac11	+	nr	-	-	nr	-	+	nr	-	nr	-	Widdel & Pfennig, 1982
BG8	-	-	+	-	+	+	+	+	+	+	3-4	Sharak Genthner, Pers. Comm.
BG23	-	-	+	+	+	+	-	+	+	-	3-4	Sharak Genthner, Pers. Comm.
BG72	+	-	-	-	-	+	-	-	-	-	-	Sharak Genthner, Pers. Comm.

Table 3.5. Continued.

Organism	A	B	E	F	FU	H	L	M	P	S	FA	References
Desulfococcus												
multivorans	+	+	+	+	-	-	+	-	nr	nr	3-16	Widdel, 1992
Desulfosarcina												
variabilis	+	+	+	+	+	+	+	-	nr	nr	3-14	Widdel, 1992
Desulfonema												
limicola	+	-	-	+	+	+	+	-	+	+	3-14	Widdel, 1983
magnum	+	-	-	-	+	-	-	+	-	+	3-10	Widdel, 1983
BG14	-	-	-	-	-	+	-	-	+	-	4	Sharak Genthner, Pers. Comm.
Desulfobotulus												
sapovorans	-	-	-	-	-	-	+	-	-	nr	4-16	Widdel, 1992; Fauque, 1995
Desulfoarculus												
baarsii	+	-	-	+	-	-	-	-	nr	nr	3-18	Widdel, 1992
BG74	-	-	-	-	-	-	-	-	-	-	4	Sharak Genthner, Pers. Comm.

A: acetate; B: benzoate; E: ethanol; F: formate (not necessarily autotrophic growth); FU: fumarate; H: hydrogen (not necessarily autotrophic); L: lactate; M: malate; P: pyruvate; S: succinate; FA: fatty acids; nr: not reported.

traits that would affiliate it with this genus would be an inability to grow autotrophically on H_2 or formate (Devereux et al., 1990).

Isolate BG50 shared 98.1% sequence identity (1,422 base positions compared) with its closest relative (Fig. 3.5-3.7), *Desulfovibrio caledoniensis*, a halophilic SRB isolated from an oil field brine. Other SRB that clustered near BG50 include *D. salexigens*, *Desulfovibrio halophilus*, and *Desulfovibrio longus* (Fig. 3.5-3.7; Table 3.4). 16S rRNA sequence divergence between BG50 and *D. caledoniensis* corresponds to the species level and DNA relatedness of $\leq 50\%$ (Devereux et al., 1990; Amann et al., 1992). Thus, BG50 should be considered a novel species within *Desulfovibrio* genus.

At the time of writing, a description of *D. caledoniensis*' physiological characteristics had not yet been published. However, physiological characteristics of BG50 and relatives for which data are available are consistent with its placement within the genus *Desulfovibrio*. BG50 exhibited a vibroid morphology, good growth on ethanol, fumarate, lactate, malate, and succinate; slight growth on H_2 (heterotrophic) and pyruvate; and no growth on acetate, benzoate, butyrate, and propionate (Table 3.5). This substrate utilization pattern and morphology are very similar to that of other *Desulfovibrio* species (Table 3.5) and is consistent with the abilities of most *Desulfovibrio* species to utilize lactate, ethanol, pyruvate, as well as the frequently observed ability to utilize fumarate and malate. Also consistent with a characteristic trait of *Desulfovibrio* species was BG50's inability to utilize acetate and fatty acids propionate and butyrate (Table 3.5).

As shown in Table 3.6, BG6 and BG50 were the only isolates enriched on ethanol and were also the only members of *Desulfovibrionaceae* isolated. When attempting to isolate ethanol-utilizing *Desulfobulbus* species, Laanbroek et al. (1982) also found that ethanol-sulfate enrichments yielded *Desulfovibrio* species,

Table 3.6. Electron donors and sources of inocula used for enrichment and isolation of the SRB isolates and selected close relatives.

Species	Energy Source for Isolation	Source of Organism	Reference
Desulfovibrio			
africanus	lactate	well water	Fauque, 1995
desulfuricans	lactate	soil	Fauque, 1995
gabonensis	lactate	oil field water	Tardy-Jacquenod et al., 1996
gigas	lactate	pond water	Fauque, 1995
halophilus	lactate	benthic microbial mat	Fauque, 1995
longus	lactate	oil-producing well	Magot et al., 1992
piger	lactate	human faeces	Fauque, 1995
vulgaris	lactate	estuarine mud	Fauque, 1995
BG6	ethanol	salt marsh sediment	Sharak Genthner, Pers. Comm.
BG50	ethanol	salt marsh sediment	Sharak Genthner in prep
Desulfomicrobium			
escambium	pyruvate	marine sediment	Sharak Genthner et al., 1994
baculatum	lactate	manganese ore	Fauque, 1995
Desulfobulbus			
marinus	propionate	marine mud flat	Widdel & Pfennig, 1982
propionicus	propionate	freshwater mud	Widdel & Pfennig, 1982
2pr4	propionate	fresh water sediment	Widdel & Pfennig, 1982
BG25	butyrate	salt marsh sediment	Sharak Genthner, Pers. Comm.
Desulforhopalus	lactate+		
vacuolatus	thiosulfate	estuarine mud	Isaksen et al., 1996
Desulfocapsa	acetate+		
thiozymogenes	thiosulfate*	fresh water sediment	Janssen et. al., 1996
Desulforhabdus			
acetothermus	acetate	oil fields water	Beeder et al., 1995

* Thiosulfate was used as an electron donor and acceptor (via disproportionation) and acetate was used as a carbon source.

Table 3.6. Continued.

Species	Energy Source for Isolation	Source of Organism	Reference
Desulfobacter			
curvatus	acetate	marine sediment	Widdel, 1987
hydrogenophilus	acetate	isovalerate enrichment	Widdel, 1987
latus	acetate	marine sediment	Widdel, 1987
postgatei	acetate	marine mud	Widdel & Pfennig, 1982
3ac10	acetate	marine/brackish sediment	Widdel & Pfennig, 1982
4ac11	acetate	marine/brackish sediment	Widdel & Pfennig, 1982
BG8	propionate	salt marsh sediment	Sharak Genthner, Pers. Comm.
BG23	butyrate	salt marsh sediment	Sharak Genthner, Pers. Comm.
BG72	acetate	salt marsh sediment	Sharak Genthner, Pers. Comm.
Desulfobacterium			
autotrophicum	hydrogen	marine and freshwater mud	Brysch et al., 1987
indolicum	indole	marine mud/ sewage sludge	Bak and Widdel, 1986
niacini	nicotinate	marine mud	Imhoff-Stuckle & Pfennig, 1983
vacuolatum	isobutyrate	marine mud	Brysch et al., 1987
BG18	butyrate	salt marsh sediment	Sharak Genthner, Pers. Comm.
BG33	benzoate	salt marsh sediment	Sharak Genthner, Pers. Comm.
Desulfococcus			
multivorans	benzoate	sewage digestor	Fauque, 1995
Desulfosarcina			
variabilis	benzoate	marine mud	Fauque, 1995
Desulfonema			
limicola	acetate	marine sediment	Widdel, 1983
magnum	benzoate	marine sediment	Widdel, 1983
Desulfobotulus			
sapovorans	butyrate	freshwater mud	Fauque, 1995
BG14	butyrate	salt marsh sediment	Sharak Genthner, Pers. Comm.
Desulfoarculus			
baarsii	stearate	freshwater mud	Jansen et al., 1984
BG74	butyrate	salt marsh sediment	Sharak Genthner, Pers. Comm.

apparently because they were able to outcompete other ethanol-utilizers, even though the desulfovibrios were not necessarily more numerous in the environment. It is possible that here, too, *Desulfovibrio* species had a competitive advantage when isolated on ethanol, as probing studies have indicated that *Desulfovibrionaceae* exhibit lower relative abundances in Chapman's marsh sediment than other ethanol-utilizers such as *Desulfobulbus* species (Devereux et al., 1996; Hines et al., in prep).

Phylogeny of Members of the *Desulfobacteriaceae* Family

Phylogenetic analysis placed the remaining isolates (BG8, BG14, BG18, BG23, BG25, BG33, BG72, and BG74) within the second Gram-negative mesophilic SRB family, the *Desulfobacteriaceae* (Fig. 3.2-3.4 and Fig. 3.8-3.10). This family encompasses a phenotypically diverse group of sulfate reducers, although its phylogenetic diversity is approximately equivalent to the family *Desulfovibrionaceae*. Metabolic traits possessed by various members include both complete and incomplete oxidation of organic compounds; the ability to utilize fatty acids; autotrophic growth on H_2 and formate; diverse morphologies that include vibrios, cocci, rods, and filaments; the bisulfite reductases desulforubidin and desulfoviridin; and menaquinones MK-5, MK-5 (H_2), MK-7, MK-7 (H_2), and MK-9 (Widdel and Bak, 1992). The recently isolated genera *Desulfocapsa* (Janssen et al., 1996), *Desulforhopalus* (Isaksen and Teske, 1996), and *Desulforhabdus* (Beeder et al., 1995) have been found to be members of the ∂ proteobacteria SRB and although they have not yet been assigned to the *Desulfobacteriaceae*, they are related to its members (Fig. 3.2-3.4) and may eventually be recognized as part of this family.

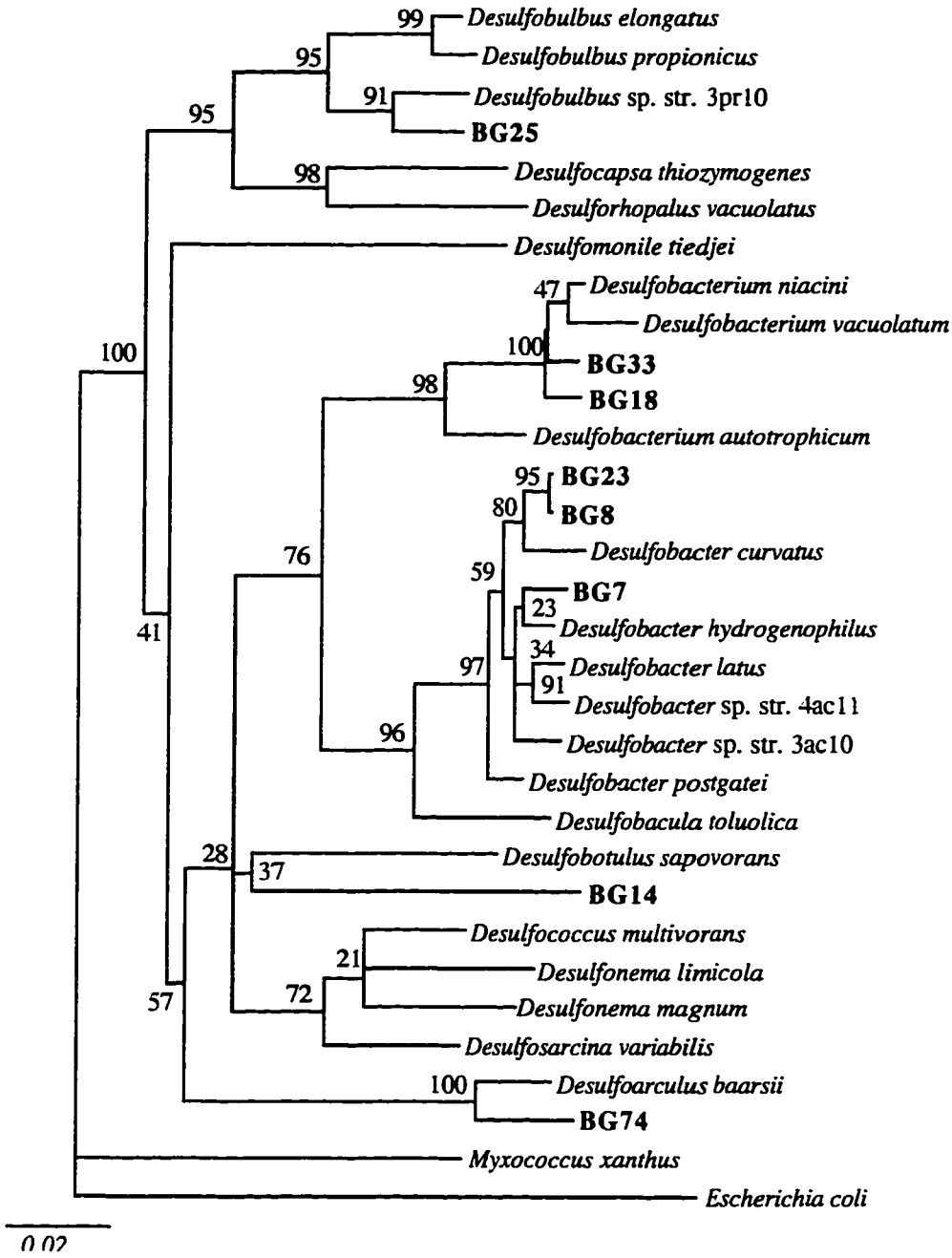


Fig. 3.8. 16S rRNA phylogenetic tree of isolates BG8, BG14, BG18, BG23, BG25, BG33, BG72, and BG74 and members of the *Desulfobacteriaceae* family constructed using a maximum parsimony method. 896 base positions were considered in the analysis. Bootstrap values (out of 100 trees) are shown adjacent to nodes. The scale bar is in fixed nucleotide substitutions per sequence position.

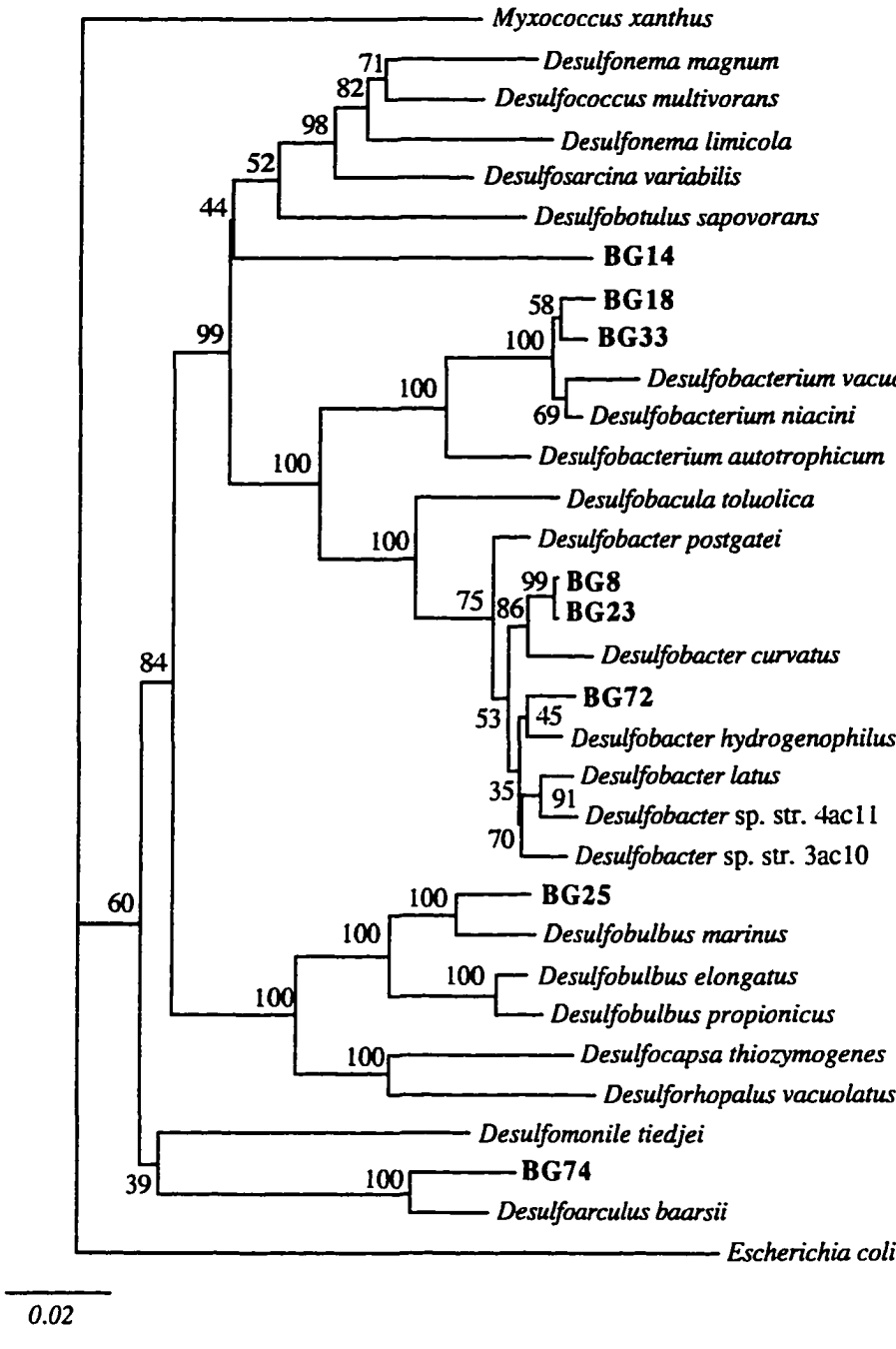


Fig. 3.9. 16S rRNA phylogenetic tree of isolates BG8, BG14, BG18, BG23, BG25, BG33, BG72, and BG74 and members of the *Desulfobacteriaceae* family constructed from evolutionary distances using a neighbor-joining algorithm. Jukes-Cantor evolutionary distances were calculated from 896 base positions. Bootstrap values (out of 100 trees) are shown adjacent to nodes. The scale bar is in fixed nucleotide substitutions per sequence position.

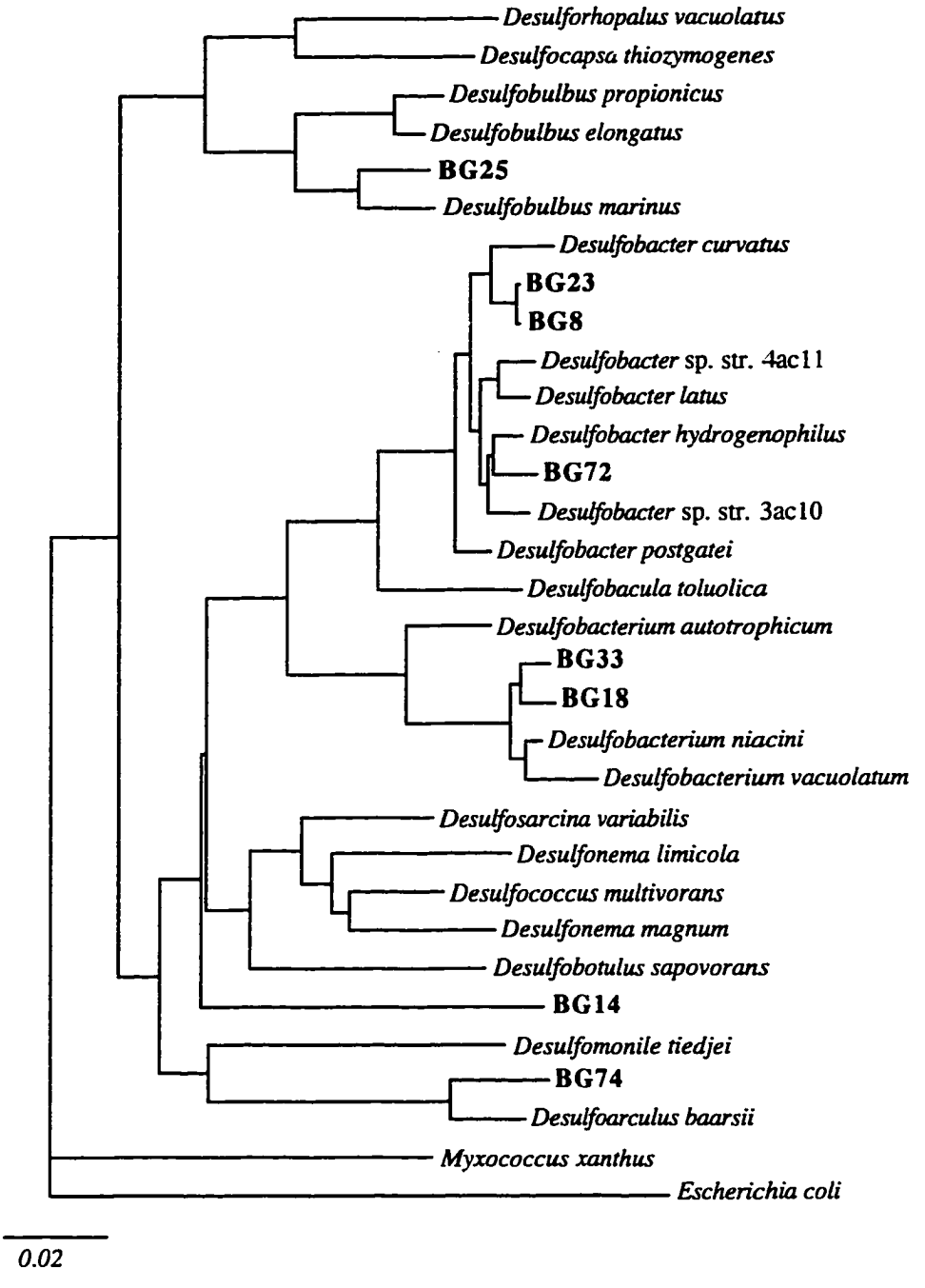


Fig. 3.10. 16S rRNA phylogenetic tree of isolates BG8, BG14, BG18, BG23, BG25, BG33, BG72, and BG74 and members of the *Desulfobacteriaceae* family constructed from evolutionary distances using the Fitch-Margoliash least squares method. Jukes-Cantor evolutionary distances were calculated from 896 base positions. The scale bar is in fixed nucleotide substitutions per sequence position.

Isolate BG25 was found to be closely related to members of the genus *Desulfobulbus* and to form a monophyletic group with more distantly related genera *Desulfocapsa* and *Desulforhopalus* (Fig. 3.8-3.10). BG25 and its closest relative, *Desulfobulbus marinus*, shared 95.6% sequence identity (1,208 base positions compared). In addition, BG25 appears to have arisen within the bifurcation of *D. marinus* and other *Desulfobulbus* species (Fig. 3.8-3.10). Therefore, both percent sequence identity and tree topology suggest that BG25 is a novel species within the genus *Desulfobulbus*. However, BG25 differs from other members of the genus *Deulfobulbus* in several phenotypic traits that were heretofore considered characteristic of the genus. The genus *Desulfobulbus* was considered to be comprised of ellipsoidal to rod-shaped SRB that are capable of incomplete oxidation of propionate and that develop preferentially in enrichments with propionate as a sole energy and carbon source and sulfate as an electron acceptor (Widdel, 1982). BG25, however, was vibroid/sigmoidal in cell shape (Sharak Genthner, pers. comm.), was incapable of utilizing propionate (Table 3.5), and was enriched on butyrate (Table 3.6). In addition, BG25 was incapable of growth on two electron donors, hydrogen and lactate, that other characterized *Desulfobulbus* species utilize and was capable of growth on two electron donors, fumarate and malate, that are not utilized by other *Desulfobulbus* species (Table 3.5) (Widdel, 1982). BG25 was similar to *Desulfobulbus* species in its inability to oxidize acetate and shared the ability to utilize ethanol and pyruvate with all other *Desulfobulbus* species and its ability to utilize butyrate and formate with *Desulfobulbus* sp. strain 2pr4 and *Desulfobulbus marinus*, respectively (Table 3.5) (Widdel, 1982; Sharak Genthner, pers. comm.). Other phenotypic traits that are characteristic of the genus *Desulfobulbus* include MK-5 (V-H2) as the major menaquinone and lipid fatty acids with straight unbranched C chains (Widdel and Bak, 1992). However, preliminary analysis of phenotypic

traits show that, by inclusion of BG25 in the *Desulfobulbus* genus, the morphology and nutritional characteristics of this genus are more diverse than originally thought.

Phylogenetic analysis of isolates BG18 and BG33 revealed that both fell within the bifurcation of *Desulfobacterium autotrophicum* and *Desulfobacterium vacuolatum*, suggesting that the two isolates are members of the genus *Desulfobacterium* (Fig. 3.8-3.10). BG18 shared a sequence identity with its two closest relatives of 96.4% (1,102 base positions compared) with *Desulfobacterium niacini* and 98.0% (1,525 base positions compared) with BG33. Similarly, BG33 shared 96.6% (over 1,102 base positions) sequence identity with *D. niacini*. Evolutionary distances between BG18, BG33, and other *Desulfobacteriaceae* are shown in Table 3.7. The level of sequence divergence observed suggests that each isolate represents a novel species.

The genus *Desulfobacterium* was defined by Bak and Widdel (1986) to consist of nonsporeforming completely oxidizing sulfate-reducers that utilize a number of fatty acids, that may grow autotrophically, and that are widespread in marine sediments. Many members of the genus are nutritionally versatile. Morphologies found within this genus include the ovoid shape of the type strain (*D. autotrophicum*) (Brysch et al., 1987), curved cell shapes, and the spherical shape of the bacterium *D. niacini* (Imhoff-Stuckle and Pfennig, 1983). The morphologies of both BG18 and BG33 were ovoid (Sharak Genthner, pers. comm.) and were therefore consistent with those of other members of the genus. "Nicks and gaps" that probably represented vacuoles were also present in BG18 (Sharak Genthner, pers. comm.). If verified as vacuoles, BG18 would be the second species in this genus, after *D. vacuolatum*, that possesses vacuoles.

Electron donors that are commonly utilized by members of this genus include acetate, ethanol, formate, fumarate, malate, succinate, and fatty acids

Table 3.7. Jukes-Cantor evolutionary distances for 16S rRNA sequences from SRB isolates BG8, BG14, BG18, BG23, BG25, BG33, BG72, and BG74 and their relatives within the *Desulfobacteriaceae* family.

Organism	1.	2.	3.	4.	5.	6.	7.	8.	9.
1. *Escherichia coli*									
2. *Myxococcus xanthus*	0.196								
3. *Desulfocapsa thiozymogenes*	0.190	0.151							
4. *Desulforhopalus vacuolatus*	0.212	0.159	0.074						
5. *Desulfoarculus baarsii*	0.182	0.168	0.165	0.166					
6. *Desulfomonile tiedjei*	0.195	0.143	0.150	0.155	0.122				
7. *Desulfobotulus sapovorans*	0.211	0.163	0.154	0.150	0.122	0.138			
8. *Desulfosarcina variabilis*	0.206	0.156	0.135	0.144	0.113	0.116	0.098		
9. *Desulfobacterium vacuolatum*	0.238	0.184	0.172	0.161	0.164	0.159	0.122	0.113	
10. *Desulfobacula toluolica*	0.222	0.159	0.158	0.144	0.148	0.134	0.118	0.113	0.098
11. *Desulfococcus multivorans*	0.187	0.159	0.131	0.143	0.108	0.110	0.080	0.056	0.120
12. *Desulfonema magnum*	0.212	0.166	0.132	0.147	0.123	0.127	0.096	0.058	0.130
13. *Desulfonema limicola*	0.209	0.169	0.142	0.160	0.137	0.127	0.088	0.068	0.125
14. *Desulfobulbus propionicus*	0.199	0.152	0.102	0.099	0.136	0.127	0.142	0.121	0.163
15. *Desulfobulbus elongatus*	0.199	0.152	0.097	0.098	0.144	0.132	0.144	0.123	0.165
16. *Desulfobulbus marinus*	0.197	0.154	0.099	0.100	0.146	0.129	0.153	0.128	0.151
17. *Desulfobacterium niacini*	0.232	0.179	0.164	0.152	0.162	0.153	0.111	0.107	0.017
18. *Desulfobacterium autotrophicum*	0.206	0.151	0.151	0.145	0.146	0.134	0.096	0.092	0.050
19. *Desulfobacter postgatei*	0.206	0.154	0.131	0.138	0.143	0.136	0.114	0.096	0.104
20. *Desulfobacter curvatus*	0.219	0.174	0.142	0.152	0.149	0.149	0.131	0.115	0.110
21. *Desulfobacter* sp. str. 3ac10	0.222	0.174	0.149	0.154	0.150	0.150	0.123	0.111	0.109
22. *Desulfobacter hydrogenophilus*	0.214	0.162	0.143	0.148	0.134	0.142	0.109	0.104	0.096
23. *Desulfobacter* sp. str. 4ac11	0.225	0.178	0.140	0.152	0.147	0.148	0.119	0.117	0.108
24. *Desulfobacter latus*	0.210	0.171	0.144	0.163	0.137	0.147	0.119	0.112	0.106
25. BG8	0.213	0.170	0.140	0.149	0.148	0.145	0.125	0.109	0.109
26. BG18	0.227	0.174	0.171	0.159	0.165	0.156	0.111	0.106	0.029
27. BG33	0.228	0.179	0.167	0.159	0.165	0.162	0.112	0.106	0.023
28. BG14	0.229	0.173	0.143	0.140	0.149	0.148	0.111	0.119	0.140
29. BG23	0.212	0.169	0.141	0.150	0.148	0.143	0.125	0.108	0.109
30. BG25	0.201	0.144	0.094	0.106	0.156	0.134	0.161	0.126	0.149
31. BG72	0.221	0.166	0.148	0.155	0.147	0.142	0.119	0.106	0.105
32. BG74	0.197	0.166	0.169	0.175	0.034	0.123	0.134	0.113	0.165

Table 3.7. Continued.

	10.	11.	12.	13.	14.	15.	16.	17.	18.	19.	20.	21.	22.	23.	24.	25.	26.	27.	28.	29.	30.	31.
11.	0.115																					
12.	0.122	0.046																				
13.	0.133	0.055	0.067																			
14.	0.136	0.135	0.142	0.151																		
15.	0.137	0.140	0.146	0.152	0.014																	
16.	0.140	0.130	0.138	0.152	0.058	0.050																
17.	0.093	0.109	0.124	0.123	0.154	0.154	0.142															
18.	0.079	0.096	0.116	0.095	0.144	0.146	0.133	0.044														
19.	0.049	0.107	0.117	0.124	0.122	0.117	0.128	0.092	0.089													
20.	0.058	0.121	0.129	0.131	0.143	0.139	0.140	0.099	0.094	0.028												
21.	0.057	0.118	0.128	0.131	0.139	0.134	0.140	0.100	0.090	0.021	0.026											
22.	0.054	0.111	0.121	0.121	0.132	0.126	0.135	0.087	0.082	0.022	0.028	0.015										
23.	0.057	0.117	0.125	0.126	0.138	0.133	0.132	0.099	0.088	0.029	0.027	0.019	0.019									
24.	0.062	0.110	0.121	0.123	0.135	0.133	0.136	0.095	0.088	0.026	0.027	0.019	0.019	0.013								
25.	0.054	0.114	0.125	0.127	0.139	0.134	0.137	0.097	0.090	0.018	0.017	0.023	0.022	0.021	0.019							
26.	0.092	0.105	0.124	0.115	0.153	0.153	0.141	0.014	0.044	0.092	0.103	0.102	0.089	0.098	0.093	0.097						
27.	0.098	0.106	0.130	0.119	0.152	0.152	0.144	0.013	0.043	0.095	0.107	0.105	0.091	0.102	0.098	0.102	0.012					
28.	0.133	0.121	0.119	0.130	0.145	0.147	0.153	0.136	0.130	0.115	0.134	0.138	0.132	0.137	0.129	0.129	0.136	0.137				
29.	0.053	0.113	0.124	0.126	0.139	0.135	0.137	0.097	0.088	0.017	0.019	0.022	0.021	0.022	0.020	0.001	0.095	0.102	0.129			
30.	0.132	0.135	0.137	0.154	0.057	0.050	0.027	0.146	0.142	0.120	0.136	0.135	0.132	0.130	0.133	0.132	0.142	0.148	0.145	0.132		
31.	0.055	0.110	0.120	0.122	0.139	0.135	0.136	0.094	0.084	0.019	0.030	0.019	0.015	0.022	0.022	0.024	0.094	0.097	0.127	0.023	0.133	
32.	0.153	0.111	0.122	0.142	0.146	0.149	0.154	0.161	0.152	0.149	0.157	0.160	0.143	0.156	0.144	0.151	0.164	0.163	0.151	0.151	0.156	0.156

(Table 3.5). Autotrophic growth on hydrogen is also frequently observed within the genus (Brysch et al., 1987; Widdel and Bak, 1992). Like its closest relatives, BG33 was quite nutritionally versatile and was capable of utilizing acetate, ethanol, formate, fumarate, malate, and succinate, as well as benzoate, butyrate, propionate, and pyruvate (Table 3.5). Benzoate utilization is not common among *Desulfobacterium* species, however, other members are known to utilize aromatic compounds such as phenol and indole (Bak and Widdel, 1986; Widdel and Bak, 1992). BG18 differed from BG33 only in its inability to utilize acetate and propionate (Table 3.5). While its inability to utilize acetate was unusual among *Desulfobacterium* species, it should be noted that other members of the genus are only capable of slight growth on acetate (Widdel and Bak, 1992). Further characterization of BG18 and BG33 should include determination of their ability to completely oxidize organic carbon to CO_2. However, 16S rRNA analysis, preliminary nutritional analysis, and morphology of isolates BG18 and BG33 enable placement of both strains within the genus *Desulfobacterium* without significant modification of the genus' defining characteristics.

Isolates BG8, BG23, and BG72 clustered within the genus *Desulfobacter* (Fig. 3.8-3.10), with isolates BG8 and BG23 most closely related to *Desulfobacter curvatus*, sharing sequence identities of 96.6% and 96.4%, respectively (over 1,337 base positions compared). BG8 and BG23 were extremely closely related to each other, with 99.8% sequence identity (over 1,337 base positions). BG72 was most closely related to *Desulfobacter postgatei* and *Desulfobacter hydrogenophilus*, sharing 97.1% (over 1,371 base positions) and 97.0% (over 1,349 base positions) sequence identity, respectively with those two species. Thus, based on 16S rRNA analysis alone, it appears that BG8 and BG23 represent two strains of a novel species, while BG72 represents a second novel species, all within the genus *Desulfobacter*.

Morphologies exhibited by the three *Desulfobacter* isolates were straight thick rod-shaped cells for BG8 and vibroid to sigmoidal cells for BG23 and BG72 (B. Sharak Genthner, pers. comm.). While these morphologies were consistent with other *Desulfobacter* species (which include oval-, rod-, and vibrio-shaped cells) (Widdel, 1987), preliminary nutritional characterization revealed some marked differences between the isolates BG8 and BG23 and their *Desulfobacter* relatives. The characteristic traits of *Desulfobacter* species include their ability to utilize acetate (which is commonly used to enrich them as shown in Table 3.6) more effectively than other completely oxidizing SRB and their lack of nutritional versatility (Table 3.5) (Widdel and Bak, 1992). BG8 and BG23, however, did not utilize acetate, and did utilize several other electron donors such as fumarate, malate, and propionate that are not commonly used by *Desulfobacter* species (Table 3.5). BG23 also utilized butyrate and formate, which are not utilized by other characterized *Desulfobacter* species. BG72's substrate utilization patterns were more similar to other *Desulfobacter* species and consisted of utilization of acetate and hydrogen, but not any other electron donors tested thus far (Table 3.5). Interestingly, BG8 and BG23 were isolated on propionate and butyrate, respectively, while other *Desulfobacter* species, including BG72, have been isolated on acetate (Table 3.6). While butyrate and propionate utilization has been reported among *Desulfobacterium, Desulfococcus, Desulfosarcina, Desulfonema, Desulfobotulus, Desulfoarculus, Desulfobulbus,* and *Desulforhabdus* species (Table 3.5), butyrate has not been commonly used for enrichment/isolation of SRB and propionate has only been used for isolation of *Desulfobulbus* species (Table 3.6). Perhaps it was use of these substrates that enabled the isolation of these novel phenotypes within the *Desulfobacter* genus.

Phylogenetic analysis of BG74 revealed that its closest relative was *Desulfoarculus baarsii* (formerly *Desulfovibrio baarsii*) (Fig. 3.8-3.10). BG74 and *D.*

baarsii shared 95.3% sequence identity (1,350 base positions compared) and therefore can be assumed to be distinct species, probably within the same genus. At the time of writing, *D. baarsii* was the only known member of the genus *Desulfoarculus* and was considered a separate lineage within the *Desulfobacteriaceae* family (Devereux et al., 1989; Stackebrandt et al., 1995). *Desulfoarculus* is characterized by its ability to carry out complete oxidation of organic carbon, its vibroid morphology, and its ability to utilize C_1-C_{18} fatty acids but few other electron donors (Widdel and Bak, 1992).

Phenotypic traits shared by the two species include their apparent nutritional limitations - like *D. baarsii*, BG74 is incapable of utilizing benzoate, ethanol, fumarate, hydrogen, lactate, and malate (Table 3.5). In fact, of all the substrates tested so far, BG74 was only capable of growth on butyrate, the electron donor used for its isolation (Table 3.6). Once again this trait is consistent with *D. baarsii*'s ability to utilize fatty acids. Further characterization of BG74 should include tests of its abilities to completely oxidize organic carbon and to utilize higher fatty acids, which, as mentioned above, are considered characteristic of the genus *Desulfoarculus*.

Phylogenetic analysis of isolate BG14 separated it from other members of the *Desulfobacteriaceae* family at the genus level (Fig. 3.8-3.10). It shared 85.3% sequence identity with its closest relative, *Desulfobotulus sapovorans* (1,315 base positions compared). However, the branching order of BG14, *D. sapovorans*, *Desulfomonile tiedjei*, *D. baarsii*, and the *Desulfobacter-Desulfobacterium* and *Desulfococcus-Desulfosarcina* lineages remains unclear, as reflected in bootstrap values for phylogenetic trees constructed by maximum parsimony and neighbor-joining methods (Fig. 3.8-3.9). Phenotypic traits of BG14 include its capacity to utilize butyrate, hydrogen, and pyruvate, but no other electron donors tested (Table 3.5).

Comparison of Isolates to Novel Phylotypes A01 and 4D19

Phylogenetic trees were constructed to infer relationships between SRB isolates and phylotypes A01, 2B14, and 4D19 (Fig. 3.11-3.13). As discussed in Chapter One, 16S rRNA gene fragments A01, 2B14, and 4D19 were selectively amplified from Chapman's marsh rhizosphere samples using a primer derived from the *Desulfobacteriaceae*-directed oligonucleotide probe, 804 (Devereux et al., 1992). However, none of the isolates, including those that contained the 804 target site, were related to A01 or 4D19 at the species or genus level (Fig. 3.11-3.13; Table 3.8). The isolate most closely related to A01, 2B14, and 4D19 was BG14 (which contained a mismatch with 804) and had evolutionary distances of 0.163, 0.138, and 0.146 with A01, 2B14, and 4D19, respectively (Table 3.8). The lack of overlap between the two methods of surveying SRB phylogenetic diversity (i.e., direct retrieval of 16S rRNA gene fragments and 16S rRNA analysis of novel isolates) provides further evidence for the diversity of SRB inhabiting the salt marsh sediment.

The fact that phylotype A01, which was shown to have a relative abundance (as a function of total eubacterial rRNA) of about 7.5% in quantitative probing studies) (Chapter Two), was not isolated may be due to either lack of appropriate conditions for its cultivation or simply to the non-quantitative nature of isolation procedures (i.e., only a small number of strains from a potentially highly diverse community are isolated). In either case, oligonucleotide probes applied to monitoring enrichment cultures (e.g., Kane et al., 1993) could be used to aid in the isolation of specific yet uncultivated organisms, such as A01.

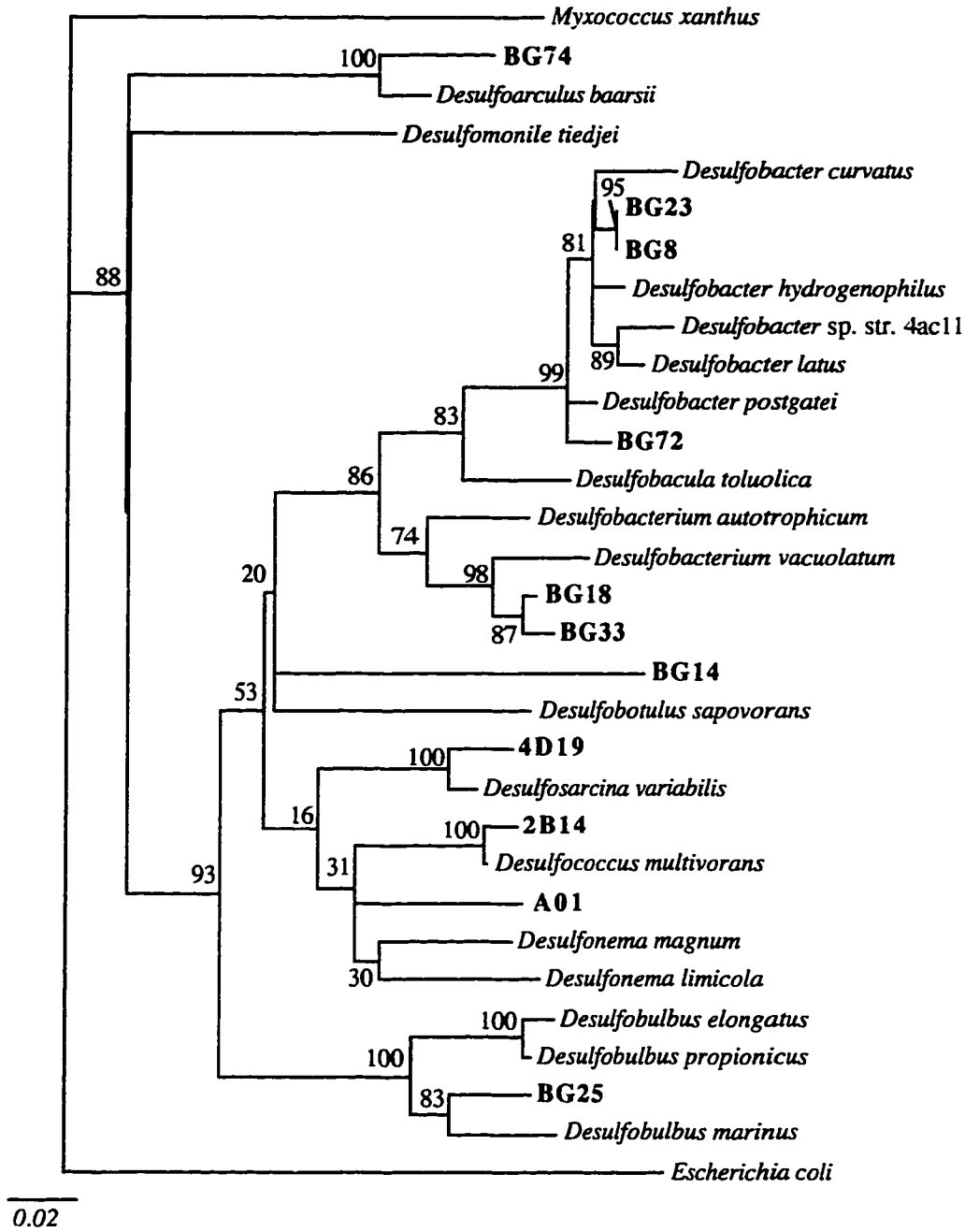


Fig. 3.11. 16S rRNA phylogenetic tree of isolates BG8, BG14, BG18, BG23, BG25, BG33, BG72, and BG74; phylotypes A01, 2B14, and 4D19; and members of the *Desulfobacteriaceae* family constructed using a maximum parsimony method. 679 base positions were considered in the analysis. Bootstrap values (out of 100 trees) are shown adjacent to nodes. The scale bar is in fixed nucleotide substitutions per sequence position.

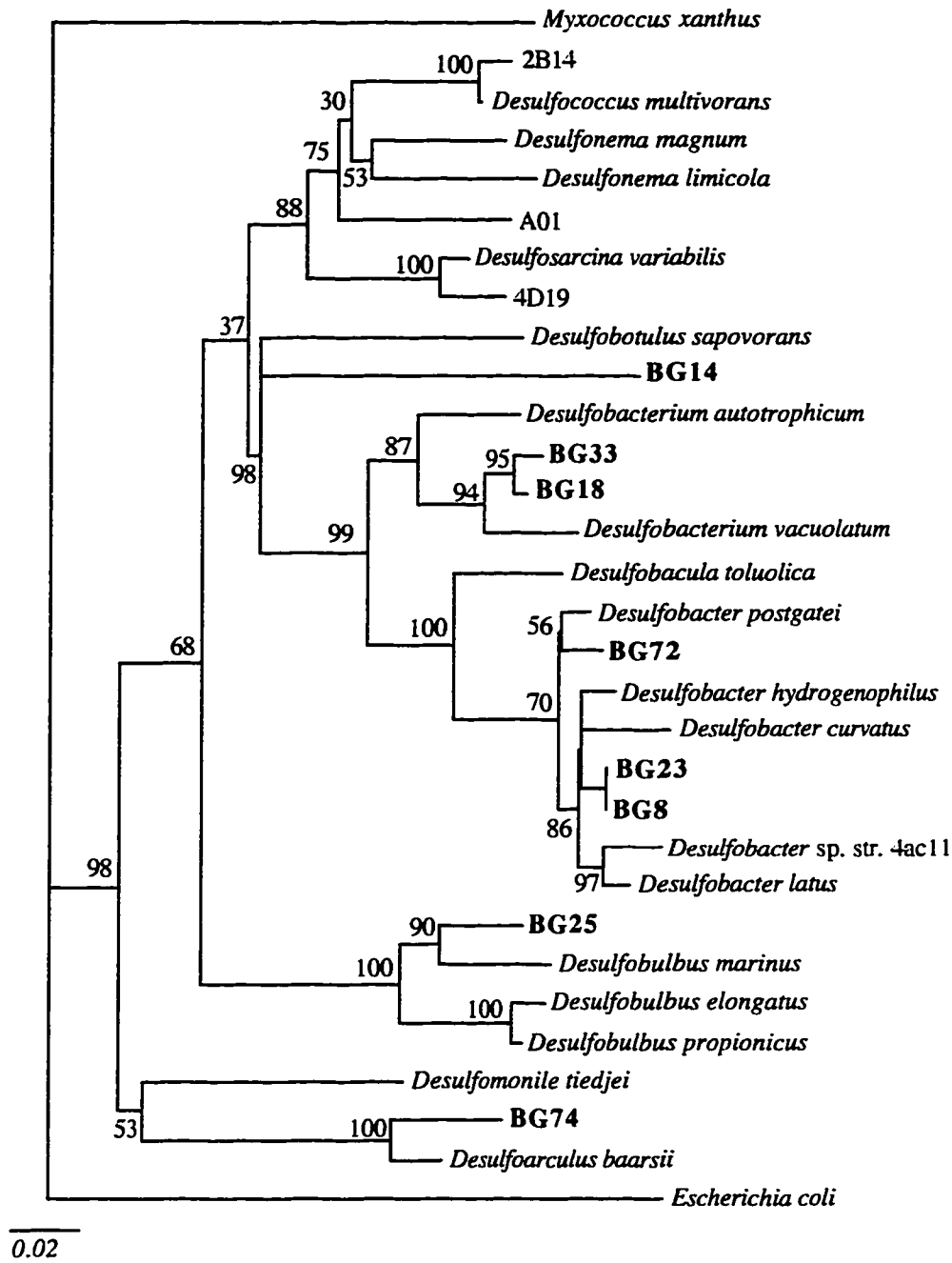


Fig. 3.12. 16S rRNA phylogenetic tree of isolates BG8, BG14, BG18, BG23, BG25, BG33, BG72, and BG74; phylotypes A01, 2B14, and 4D19; and members of the *Desulfobacteriaceae* family constructed from evolutionary distances using a neighbor-joining algorithm. Jukes-Cantor evolutionary distances were calculated from 679 base positions. Bootstrap values (out of 100 trees) are shown adjacent to nodes. The scale bar is in fixed nucleotide substitutions per sequence position.

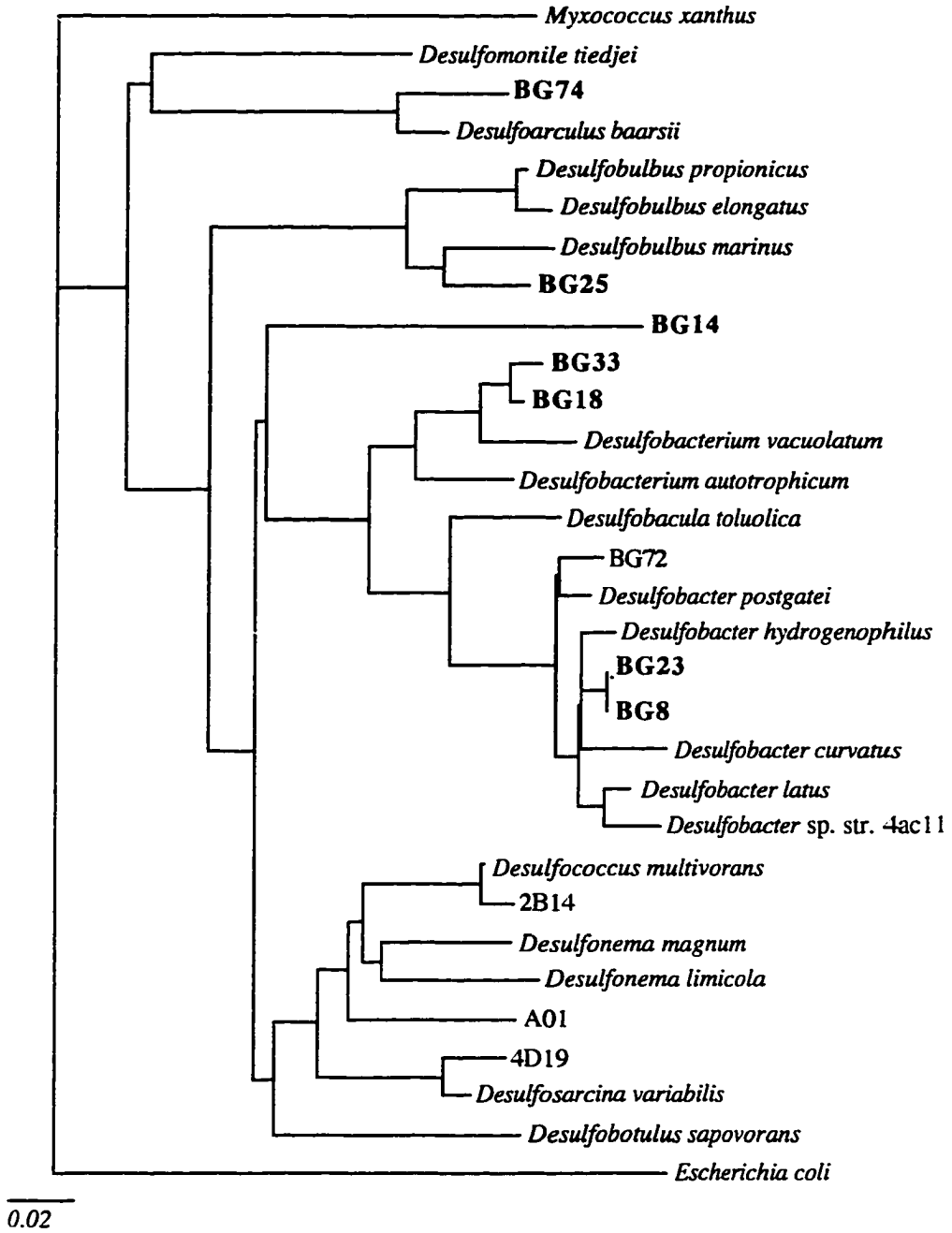


Fig. 3.13. 16S rRNA phylogenetic tree of isolates BG8, BG14, BG18, BG23, BG25, BG33, BG72, and BG74; phylotypes A01, 2B14, and 4D19; and members of the Desulfobacteriaceae family constructed from evolutionary distances using the Fitch-Margoliash least squares method. Jukes-Cantor evolutionary distances were calculated from 679 base positions. Bootstrap values (out of 100 trees) are shown adjacent to nodes. The scale bar is in fixed nucleotide substitutions per sequence position.

Table 3.8. Jukes-Cantor evolutionary distances for 16S rRNA sequences of isolates BG8, BG14, BG18, BG23, BG25, BG33, BG72, and BG74; phylotypes A01, 4D19, and 2B14; and relatives in the *Desulfobacteriaceae* family.

Organism	1.	2.	3.	4.	5.	6.	7.	8.	9.
1. *Escherichia coli*									
2. *Myxococcus xanthus*	0.229								
3. *Desulfoarculus baarsii*	0.196	0.184							
4. *Desulfobacter curvatus*	0.250	0.210	0.174						
5. *Desulfobacter hydrogenophilus*	0.246	0.207	0.171	0.025					
6. *Desulfobacter latus*	0.241	0.212	0.172	0.032	0.019				
7. *Desulfobacter postgatei*	0.245	0.201	0.179	0.031	0.020	0.022			
8. *Desulfobacter* sp. str. 4ac11	0.264	0.228	0.185	0.036	0.025	0.017	0.031		
9. *Desulfobacterium autotrophicum*	0.219	0.188	0.153	0.094	0.090	0.095	0.089	0.100	
10. *Desulfobacterium vacuolatum*	0.243	0.212	0.166	0.099	0.089	0.101	0.094	0.101	0.051
11. *Desulfobotolus sapovorans*	0.225	0.208	0.147	0.140	0.129	0.139	0.126	0.139	0.096
12. *Desulfobacula toluolica*	0.254	0.200	0.169	0.067	0.059	0.062	0.051	0.063	0.073
13. *Desulfobulbus elongatus*	0.238	0.205	0.152	0.156	0.147	0.149	0.138	0.156	0.147
14. *Desulfobulbus propionicus*	0.236	0.207	0.144	0.150	0.146	0.145	0.136	0.151	0.137
15. *Desulfobulbus marinus*	0.240	0.213	0.154	0.164	0.158	0.155	0.151	0.152	0.132
16. *Desulfococcus multivorans*	0.209	0.196	0.136	0.135	0.129	0.128	0.124	0.141	0.094
17. *Desulfomonile tiedjei*	0.211	0.187	0.115	0.176	0.172	0.174	0.167	0.181	0.145
18. *Desulfonema limicola*	0.214	0.208	0.160	0.142	0.136	0.138	0.129	0.150	0.104
19. *Desulfonema magnum*	0.221	0.213	0.146	0.141	0.134	0.136	0.122	0.141	0.104
20. *Desulfosarcina variabilis*	0.217	0.201	0.143	0.131	0.125	0.134	0.116	0.140	0.089
21. A01	0.222	0.204	0.127	0.141	0.129	0.131	0.130	0.144	0.108
22. 2B14	0.215	0.195	0.143	0.138	0.137	0.138	0.131	0.147	0.102
23. 4D19	0.222	0.209	0.150	0.141	0.136	0.143	0.125	0.149	0.096
24. BG8	0.248	0.211	0.176	0.023	0.012	0.017	0.017	0.023	0.088
25. BG14	0.259	0.219	0.172	0.154	0.155	0.146	0.136	0.163	0.141
26. BG18	0.246	0.207	0.167	0.092	0.079	0.085	0.076	0.088	0.049
27. BG23	0.248	0.211	0.176	0.023	0.012	0.017	0.017	0.023	0.088
28. BG25	0.233	0.190	0.161	0.154	0.148	0.147	0.138	0.146	0.136
29. BG33	0.244	0.210	0.167	0.101	0.087	0.094	0.085	0.097	0.047
30. BG72	0.252	0.206	0.184	0.034	0.023	0.025	0.015	0.033	0.092
31. BG74	0.218	0.194	0.034	0.195	0.188	0.193	0.196	0.208	0.171

Table 3.8. Continued.

	10.	11.	12.	13.	14.	15.	16.	17.	18.	19.	20.	21.	22.	23.	24.	25.	26.	27.	28.	29.	30.
11.	0.123																				
12.	0.080	0.118																			
13.	0.167	0.160	0.137																		
14.	0.158	0.151	0.131	0.009																	
15.	0.156	0.155	0.146	0.056	0.060																
16.	0.121	0.096	0.125	0.149	0.132	0.138															
17.	0.165	0.160	0.152	0.149	0.142	0.152	0.117														
18.	0.140	0.102	0.130	0.152	0.147	0.144	0.062	0.136													
19.	0.123	0.094	0.121	0.155	0.146	0.144	0.060	0.136	0.061												
20.	0.109	0.103	0.117	0.132	0.115	0.130	0.070	0.122	0.083	0.076											
21.	0.126	0.117	0.127	0.145	0.137	0.136	0.068	0.122	0.083	0.068	0.081										
22.	0.128	0.103	0.128	0.153	0.135	0.144	0.008	0.125	0.072	0.065	0.074	0.070									
23.	0.119	0.114	0.124	0.144	0.125	0.139	0.077	0.134	0.086	0.081	0.020	0.082	0.082								
24.	0.097	0.134	0.057	0.149	0.148	0.156	0.127	0.175	0.135	0.137	0.125	0.136	0.135	0.135							
25.	0.154	0.134	0.144	0.160	0.165	0.177	0.126	0.167	0.131	0.115	0.144	0.163	0.138	0.146	0.153						
26.	0.029	0.106	0.069	0.146	0.141	0.142	0.107	0.156	0.130	0.113	0.096	0.113	0.116	0.105	0.081	0.142					
27.	0.097	0.134	0.057	0.150	0.148	0.156	0.127	0.175	0.136	0.137	0.125	0.136	0.135	0.135	0.000	0.153	0.081				
28.	0.148	0.152	0.130	0.058	0.053	0.041	0.129	0.154	0.143	0.141	0.125	0.133	0.135	0.135	0.146	0.156	0.141	0.146			
29.	0.033	0.115	0.073	0.143	0.135	0.139	0.109	0.162	0.127	0.118	0.100	0.113	0.118	0.107	0.086	0.148	0.009	0.086	0.137		
30.	0.097	0.129	0.055	0.146	0.145	0.150	0.122	0.166	0.130	0.121	0.117	0.131	0.128	0.126	0.019	0.139	0.079	0.019	0.143	0.088	
31.	0.179	0.170	0.183	0.166	0.152	0.175	0.138	0.131	0.172	0.159	0.154	0.140	0.144	0.157	0.196	0.179	0.175	0.196	0.164	0.178	0.204

Evaluation of SRB Probes

A series of oligonucleotide probes intended to specifically target various groups or genera within the Gram-negative mesophilic SRB were designed by Devereux et al. (1992) and reevaluated here against 16S rRNA sequences of the new SRB isolates (Fig. 3.14). Several probes were found to contain a single mismatch with isolates that are members of the target group or genus. Probe 129 was designed to target the genus *Desulfobacter* and while it has no mismatches with *Desulfobacter* isolates BG8 and BG23, it does have a single mismatch with *Desulfobacter* isolate BG72. Although only a single base pair, this mismatch is fairly centrally located within the target site (Fig. 3.14) and may be sufficient to significantly destabilize the probe-target hybrid (Stahl and Amann, 1991; Ward et al., 1992). Probe 221, for the genus *Desulfobacterium,* has one mismatch with *Desulfobacterium* isolates BG18 and BG33. This mismatch, however, is very close to the 3' end of the target site and may not have a significant effect on probe-target hybridization. In both cases, adding a mixed based position to the probe sequence allows for inclusion of the new isolates in the probe target groups, while maintaining specificity for the group in question. Specifically, changing probe 129 to 5'-CAGGCTTGAAGSCAGATT-3' (where S is C or G) results in a probe that targets both previously characterized *Desulfobacter* species and isolates BG8, BG23, and BG72. By using the RDP CheckProbe utility (Maidak et al., 1994), the modified probe was checked against all 16S rRNA sequences in the unaligned RDP database and found to be specific for *Desulfobacter* species. Similarly, probe 221 could be modified to 5'-TSCGCGGACTCATCTTCAAA-3', and once again, the CheckProbe utility was used to show that this modified probe targeted characterized *Desulfobacterium* and new *Desulfobacterium* isolates to the exclusion of other 16S rRNA molecules.

	Probe 687 (*Desulfovibrionaceae*)	Probe 804 (mixed *Desulfobacteriaceae*)
Target	AGGAGUGAAAUCCGUA	UCCACGCAGUAAACGUUG
BG6	AGGAGUGAAAUCCGUA	UCCACGCUGUAAACGAUG
BG8	AGAGGUGAAAUUCGUA	UCCACGCAGUAAACGUUG
BG14	AGCGGUGAAAUGCGUA	UCCAUGCAGUAAACGUUG
BG18	AGAGGUGAAAUUCGUA	UCCACGCAGUAAACGUUG
BG23	AGAGGUGAAAUUCGUA	UCCACGCAGUAAACGUUG
BG25	AGAGGUGAAAUUCGUA	UCCACGCCGUAAACGAUG
BG33	AGAGGUGAAAUUCGUA	UCCACGCAGUAAACGUUG
BG50	AGGAGUGAAAUCCGUA	UCCACGCUGUAAACGAUG
BG72	AGAGGUGAAAUUCGUA	UCCACGCAGUAAACGUUG
BG74	AGAGGUGAAAUUCGUA	UCCACGCCGUAAACGGUG

	Probe 660 (*Desulfobulbus*)	Probe 129 (*Desulfobacter*)
Target	CAGAGGGGAAAGUGGAAUUC	AAUCUGCCUUCAAGCCUG
BG6	UGGAGAGGGUGGCGGAAUNC	AAUCUCCCUGGAAAUUCG
BG8	GGGAGAGGAGAGAGGAAUUC	AAUCUGCCUUCAAGCCUG
BG14	GGUAGAGGAAAGCGGAAUUC	AAUCUGUCUCCGAAUCCG
BG18	GGGAGAGGAAAGGGGAAUUC	AAUCUACCUUCAAAUCGG
BG23	GGGAGAGGAGAGAGGAAUUC	AAUCUGCCUUCAAGCCUG
BG25	CAGAGGGGAAAGUGGAAUUC	AACCUACCUCCAUGUUUG
BG33	GGGAGAGGAAAGGGGAAUUC	AAUCUACCUUCAAAUCGG
BG50	CGGAGAGGUUGGCGGAAUUC	AAUCUGCCCUGAAGAUCG
BG72	GGGAGAGGAAAGCGGAAUUC	AAUCUGGCUUCAAGCCUG
BG74	UGGAGAGGAGAGUGGAAUUC	AAUCUACCUAAAGGUACG

	Probe 221 (*Desulfobacterium*)	Probe 814 (*Desulfococcus-Desulfosarcina-Desulfobotulus*)
Target	UUUGAAGAUGAGUCCGCGCA	AAACGUUGAUCACUAGGU
BG6	UUUCCAGAUGAGUCCGCGUC	AAACGAUGGAUGCUAGGU
BG8	UUUGGGGAUGAGUUUGCGUA	AAACGUUGUACACUCGGU
BG14	AUCGGAGGUGAGCUUGCGUC	AAACGUUGAUUACUAGGU
BG18	UUUGAAGAUGAGUCCGCGGA	AAACGUUGUAUGCUAGGU
BG23	UUUGGGGAUGAGUUUGCGUA	AAACGUUGUACACUCGGU
BG25	CAUGGAGAGGGGUCUGCGUA	AAACGAUGUCAACUAGAU
BG33	UUUGAAGAUGAGUCCGCGGA	AAACGUUGUAUGCUAGGU
BG50	CUUUUGGAUGAGUCCGCGUC	AAACGAUGGAUAUUAGGU
BG72	UUUGGGGAUGAGUUUGCGUA	AAACGUUGUACACUCGGU
BG74	CCCUUAGACGAGCCCGCGUC	AAACGGUGUCCACUAGGU

Fig. 3.14. Comparison of probe 687, 804, 660, 129, 221, and 814 target sites with aligned 16S rRNA sequences of SRB isolates. Mismatches with the probe target site are shown in boldtype and underlined.

Probe 814, designed to target the *Desulfococcus-Desulfosarcina-Desulfobotulus* assemblage contains one centrally located mismatch with BG14. However, while this assemblage constitutes BG14's closest relatives, as discussed above, BG14 appeared to represent a separate lineage and should therefore not necessarily be included in the group originally intended to be targeted by probe 814. Probe 804 was intended to target all members of the *Desulfobacteriaceae* family except *Desulfobulbus* and *Desulfoarculus*. Isolates BG8, BG18, BG23, BG33, and BG72, all members of this group, contained the target sequence. Isolate BG14 and *Desulfoarculus* isolate BG74 each had single mismatches with 804 that were centrally located. The former finding may be lend support to the placement of BG14 in a separate lineage, while the latter is consistent with expected specificity of probe 804.

The target sequence for the *Desulfobulbus* probe 660 was found in *Desulfobulbus* isolate BG25, as was the target sequence for the *Desulfovibrionaceae* probe 687 in *Desulfovibrionaceae* isolates BG6 and BG50 (Fig. 3.14). It should be noted that the latter probe was recently found to also target members of the *Geobacteriaceae* family (Lonergan et al., 1996) and therefore should no longer be considered specific for its originally intended targets. Additionally, evaluation of the eubacterial probe, EUB338 (Stahl et al., 1988) revealed that all isolates appear to contain its target site (Fig. 3.15).

Probes A01-183 and 4D19-189, described in Chapter Two, were also evaluated against new SRB 16S rRNA sequences (Fig. 3.16). As described in Chapter Two, these probes were designed to target hypervariable regions of 16S rRNAs that were retrieved directly from DNA extracted from salt marsh rhizosphere samples. Not surprisingly, none of the new SRB isolates contained

	Probe 338 (Bacteria)
Target	ACUCCUACGGGAGGCAGC
BG6	ACUCCUACGGGAGGCAGC
BG8	ACUCCUACGGGAGGCAGC
BG14	ACUCCUACGGGAGGCAGC
BG18	ACUCCUACGGGAGGCAGC
BG23	ACUCYUACGGGAGGCAGC
BG25	ACUCCUACGGGAGGCAGC
BG33	ACUCCUACGGGAGGCAGC
BG50	ACUCCUACGGGAGGCAGC
BG72	ACUCNUACGGGAGGCAGC
BG74	ACUCCUACGGGAGGCAGC

Fig. 3.15. Comparison of probe 338 target site to 16S rRNA sequences of SRB isolates.

	Probe A01-183	Probe 4D19-189
Target	CAUCGUAUUUUCUUAGGGG	GGAAUGUUGGAUCAAGGG
BG6	GCUCCCAAUUUAUUUUGGG	AUUUUGGGGGGAAAGGCG
BG8	AGUCGUUUCACAUAAGUGG	AAGUGGAUUGAUGAAAGA
BG14	AGUUGUAUUGACUGCGGUU	GGUUGAUACAAUGAAAGG
BG18	AGUCGUGGGAACUUUGGUU	GGUUUUUAAGAUGAAAGG
BG23	AGUCGUUUCACAUAAGUGG	AAGUGGAAUGAUGAAAGA
BG25	GCUUGCUUUUCAUAAGUUU	AAGUUUUGCAAGCAAAGG
BG33	AGUCGUGAGAACUUUGGUU	GGUUUUUAAGAUGAAAGG
BG50	UCUGCAUAUUUAACUUUAU	ACUUUAUGUGGGAAAGAU
BG72	AGUUGAUUCACAUAAGUGG	AAGUGGAUUAAUGAAAGA
BG74	GACCACGGUUUCUGCGGAU	GGAUUCUGUGGUAAAAGG

Fig. 3.16. Comparison of probe A01-183 and 4D19-189 target sites to 16S rRNA sequences of SRB isolates. Mismatches with the probe target sequences are shown in boldtype and underlined.

the probe target sites, with 7 to 15 mismatches found between probe target sequences and their respective 16S rRNA sites.

As clearly evident from the above discussion, continual reevaluation of 16S rRNA-directed probes is necessary as new sequences become available. As the database of 16S rRNA sequences expands, it may actually become more difficult to design oligonucleotide probes that specifically target all members of a given phylogenetic group. However, there are several possible avenues for overcoming this problem. As discussed above, in cases where the original probe is not inclusive of all members of its intended target group, refining probes by adding mixed base positions may be appropriate. In cases where a given probe is not found to be specific for its originally intended target (e.g., probe 687, which also targets *Geobacteriaceae*), multiple probes that may not be specific for their target individually, but are specific as a group, may be used. This approach is limited to *in situ* or whole cell hybridization formats, in which probes could be labeled with different fluorophores and only those cells to which all probes hybridized would be considered true targets. In still other cases it may be necessary to improve probe specificity by using unlabeled oligonucleotide competitors that block non-target sites and facilitate discrimination based on single mismatches (e.g., Manz et al., 1992).

There are several implications of probe reevaluation on the interpretation of studies in which these probes were used. Hines et al. (in prep) used the suite of SRB probes described above to investigate SRB community structure and population dynamics in the sediment and *S. alterniflora* rhizosphere at Chapman's marsh. They found that the group targeted by 804 played a significant role in the SRB community, but that probes targeting genera within this group (i.e., probes 129, 221, and 814) only accounted for a small percentage of the total 804 signal. Likewise, I found that the relative abundance of 814 was

quite low (3±2.3%), as described in Chapter Two. Although quantitative information on the relative abundances of the SRB isolates described here is not available, several of them are targeted by 804 but not by narrower phylogenetic probes and could therefore have contributed to the 'extra' 804 signal. Specifically, BG72, BG18, and BG33 contained the 804 target sequence but did not contain their respective genus-specific target sequences (Fig. 3.14).

Conclusions

Phylogenetic analysis of 10 new SRB isolates from a salt marsh sediment placed all strains within the ∂ proteobacteria group of SRB and within the families *Desulfovibrionaceae* and *Desulfobacteriaceae*. BG6 and BG50 were both members of the family *Desulfovibrionaceae*, with BG6 possibly representing a novel genus and BG50 representing a novel species within the genus *Desulfovibrio*. Preliminary phenotypic characterization of BG6 and BG50 (Sharak Genthner, Pers. Comm.) was consistent with their phylogenetic relationships to other members of the *Desulfovibrionaceae* family.

Isolates that were members of the *Desulfobacteriaceae* family were distributed among the genera *Desulfobulbus* (BG25), *Desulfobacter* (BG8, BG23, and BG72), *Desulfobacterium* (BG18 and BG33), *Desulfoarculus* (BG74) and a novel genus represented by isolate BG14. Based on 16S rRNA analysis, all isolates were distinct from characterized *Desulfobacteriaceae* at the species or genus levels.

Although only limited phenotypic data for the isolates were available at the time of writing, it appears that placement of isolates BG25 in the genus *Desulfobulbus* and BG8 and BG23 in *Desulfobacter* will require some modification of the defining characteristics of these genera. With the addition of BG25 in the genus *Desulfobulbus*, *Desulfobulbus* now includes species that do not utilize propionate (previously considered a characteristic trait) and also includes the

vibrio cell morphology. In addition, the genus *Desulfobacter* can no longer be considered to consist of acetate utilizers that do not utilize fatty acids, as isolates BG8 and BG23 fall within this genus and do not utilize acetate but do utilize propionate and butyrate.

Comparison of the *Desulfobacteriaceae* isolates' 16S rRNA sequences to 'molecular isolates' originating from the same study site revealed that none of the isolates shared more than about 86% sequence identity with environmental clones, and thereby provided further evidence of the diversity of SRB inhabiting salt marsh sediments. Evaluation of currently available probes for SRB (Devereux et al., 1992; Chapter Two) against the isolates' 16S rRNA sequences revealed that, with the addition of mixed base positions to probes 129 and 221, SRB-directed probes could still be used against their originally intended targets.

LITERATURE CITED

Abbaszadegan, M., M. S. Huber, C. P. Gerba and I. L. Pepper. 1993. Detection of enteroviruses in groundwater with the polymerase chain reaction. *Appl Environ Microbiol.* 59: 1318-1324.

Altschul, S. F., W. Gish, W. Miller, E. Myers and D. J. Lipman. 1990. Basic Local Alignment Search Tool. *J Mol Biol.* 215: 403-410.

Amann, R. I. 1995. Fluorescently labelled, rRNA-targeted oligonucleotide probes in the study of microbial ecology. *Mol Ecol.* 4: 543-554.

Amann, R. I., C. Lin, R. Key, L. Montgomery and D. A. Stahl. 1992. Diversity among *Fibrobacter* isolates: towards a phylogenetic classification. *Syst Appl Microbiol.* 15: 23-31.

Amann, R. I., L. Krumholz and D. A. Stahl. 1990. Fluorescent-oligonulceotide probing of whole cells for determinative phylogenetic, and environmental studies in microbiology. *J Bact.* 172: 762-770.

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

Anderson, T. A., E. A. Guthrie and B. T. Walton. 1993. Bioremediation in the rhizosphere. *Environ Sci Technol.* 27: 2630-2636.

Anderson, T. A., E. L. Kruger and J. R. Coats. 1994. Enhanced degradation of a mixture of 3 herbicides in the rhizosphere of a herbicide-tolerant plant. *Chemosphere.* 28: 1551-1557.

Assmus, B., P. Hutzler, G. Kirchhof, R. Amann, J. R. Lawrence and A. Hartmann. 1995. *In situ* localization of *Azospirillum brasilense* in the rhizosphere of wheat using fluorescently labeled, rRNA-targeted oligonucleotide probes and scanning confocal laser microscopy. *Appl Environ Microbiol.* 61: 1013-1019.

Bak, F. and F. Widdel. 1986. Anaerobic degradation of indolic compounds by sulfate-reducing enrichment cultures and description of *Desulfobacterium indolicum* gen. nov., sp. nov. *Arch Microbiol.* 146: 170-176.

Barton, L. L. and F. A. Tomei. 1995. Characteristics and Activities of Sulfate-Reducing Bacteria, p. 1-32. In L. L. Barton (ed.), Sulfate-Reducing Bacteria, Plenum Press, New York.

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

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: 331-336.

Blum, L. K. 1993. *Spartina alterniflora* root dynamics in a Virginia marsh. *Mar Ecol-Progr Ser.* 102: 169-178.

Borneman, J., P. W. Skroch, K. M. O'Sullivan, J. A. Palus, N. G. Rumjanek, J. L. Jansen, J. Nienhuis and E. W. Triplett. 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. *Appl Environ Microbiol.* 62: 1935-1943.

Brady, N. C. 1990. The Nature and Property of Soils. Macmillan Publishing Company, New York. 621 pp.

Braun-Howland, E. B., P. A. Vescio and S. A. Nierzwicki-Bauer. 1993. Use of a simplified cell blot technique and 16S rRNA-directed probes for identification of common environmental isolates. *Appl Environ Microbiol.* 59: 3219-3224.

Brink, D. E., I. Vance and D. C. White. 1994. Detection of *Desulfobacter* in oil field environments by non-radioactive DNA probes. *Appl Microbiol Biotechnol.* 42: 469-475.

Britschgi, T. B. and R. D. Fallon. 1994. PCR-Amplification of mixed 16S rRNA genes from an anaerobic, cyanide-degrading consortium. *FEMS Microbiol Ecol.* 13: 225-231.

Brock, T. D., M. T. Madigan, J. M. Martinko and J. Parker. 1994. Biology of Microorganisms. Prentice-Hall International, London. 909 pp.

Bruce, K. D., W. D. Hiorns, J. L. Hobman, A. M. Osborn, P. Strike and D. A. Ritchie. 1992. Amplification of DNA from native populations of soil bacteria by using the polymerase chain reaction. *Appl Environ Microbiol.* 58: 3413-3416.

Brysch, K., C. Schneider, G. Fuchs and F. Widdel. 1987. Lithoautotrophic growth of sulfate-reducing bacteria, and description of *Desulfobacterium autotrophicum* gen. nov., sp. nov. *Arch Microbiol.* 148: 264-278.

Coleman, D. C., C. V. Cole, H. W. Hunt and D. A. Klein. 1978. Trophic interactions in soils as they affect energy and nutrient dynamics. I. Introduction. *Microb Ecol.* 4: 345-349.

Cypionka, H., F. Widdel and N. Pfennig. 1985. Survival of sulfate-reducing bacteria after oxygen stress, and growth in sulfate-free oxygen-sulfide gradients. *FEMS Microbiol Ecol.* 31: 39-45.

Dacey, J. W. H. and B. L. Howes. 1984. Water uptake by roots controls water table movement and sediment oxidation in short *Spartina alterniflora* marsh. *Science.* 224: 487-490.

De Ley, J. 1992. The Proteobactera: Ribosomal RNA Cistron Similarities and Bacterial Taxonomy, p. 2111-2140. In A. Balows, H. G. Trüper, M. Dworkin, W. Harder and K.-H. Schleifer (ed.), The Prokaryotes, Springer-Verlag, New York.

DeLong, E. F., G. S. Wickman and N. R. Pace. 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. *Science.* 243: 1360-1363.

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., M. D. Kane, J. Winfrey and D. A. Stahl. 1992. Genus- and group-specific hybridization probes for determinative and environmental studies of sulfate-reducing bacteria. *System Appl Microbiol.* 15: 601-609.

Devereux, R., M. Delaney, F. Widdel and D. A. Stahl. 1989. Natural relationships among sulfate-reducing eubacteria. *J Bact.* 171: 6689-6695.

Devereux, R., M. E. Hines and D. A. Stahl. 1996. S cycling: Characterization of natural communities of sulfate-reducing bacteria by 16S rRNA sequence comparisons. *Microb Ecol.* 32: 283-292.

Devereux, R., S.-H. He, C. L. Doyle, S. Orkland, D. A. Stahl, J. LeGall and W. B. Whitman. 1990. Diversity and origin of *Desulfovibrio* species: Phylogenetic definition of a family. *J Bact.* 172: 3609-3619.

Dilling, W. and H. Cypionka. 1990. Aerobic respiration in sulfate-reducing bacteria. *FEMS Micriobiol Letters.* 71: 123-128.

Dorsch, M. and E. Stackebrandt. 1992. Some modifications in the procedure of direct sequencing of PCR amplified 16S rDNA. *J Microbiol Methods.* 16: 271-279.

Erb, R. W. and I. Wagnerdobler. 1993. Detection of polychlorinated biphenyl degradation genes in polluted sediments by direct DNA extraction and polymerase chain reaction. *Appl Environ Microbiol.* 59: 4065-4073.

Fauque, G. D. 1995. Ecology of Sulfate-Reducing Bacteria, p. 217-242. In L. L. Barton (ed.), Sulfate-Reducing Bacteria, Plenum Press, New York.

Felsenstein, J. 1978. Cases in which parsimony and compatibility methods will be positively misleading. *Syst Zool.* 27: 27-33.

Felsenstein, J. 1981. Evolutionary trees from DNA sequences: A maximum likelihood approach. *J Mol Evol.* 17: 368-376.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution.* 39: 783-791.

Felsenstein, J. 1988. Phylogenies from molecular sequences: Inference and reliability. *Annu Rev Genet.* 22: 521-565.

Felsenstein, J. 1989. PHYLIP - phylogeny inference package. *Cladistics.* 5: 164-166.

Fitch, W. M. and E. Margoliash. 1967. Construction of phylogenetic trees. *Science.* 155: 279-284.

Fowler, V. J., F. Widdel, N. Pfennig, C. R. Woese and E. Stackebrandt. 1986. Phylogenetic relationships of sulfate- and sulfur-reducing eubacteria. *System. Appl. Microbiol.* 8:32-41.

Fox, G. E., E. Stackebrandt, R. B. Hespell, J. Gibson, J. Maniloff, T. A. Dyer, R. S. Wolfe, W. E. Balch, R. S. Tanner, L. J. Magrum, L. B. Zablen, R. Blakemore, R. Gupta, L. Bonen, B. J. Lewis, D. A. Stahl, K. R. Luehrsen, K. N. Chen and C. R. Woese. 1980. The phylogeny of prokaryotes. *Science.* 209: 457-463.

Fuhrman, J. A., K. McCallum and A. A. Davis. 1992. Novel major archaebacterial group from marine plankton. *Nature.* 356: 148-149.

Giblin, A. E. and R. W. Howarth. 1984. Porewater evidence for a dynamic sedimentary iron cycle in salt marshes. *Limnol Oceanogr.* 29: 47-63.

Gibson, G. R. 1990. Physiology and ecology of sulphate-reducing bacteria. *J. Appl. Bacteriol.* 69:769-797.

Gilbert, D. G. 1989. IUBio archive of molecular and general biology software and data. An Internet resource available at ftp, gopher, http://iubio.indiana.edu.

Giovannoni, S. J., E. F. DeLong, G. J. Olsen and N. R. Pace. 1988. Phylogenetic group-specific oligonucleotide probes for identification of single microbial cells. *J Bact.* 170: 720-726.

Giovannoni, S. J., T. B. Britschgi, C. L. Moyer and K. G. Field. 1990. Genetic diversity in Sargasso Sea bacterioplankton. *Nature.* 345: 60-63.

Gordon, D. A. and S. J. Giovannoni. 1996. Detection of stratified microbial populations related to *Chlorobium* and *Fibrobacter* species in the Atlantic and Pacific Oceans. *Appl Environ Microbiol.* 62: 1171-1177.

Hansen, T. A. 1993. Carbon Metabolism of Sulfate-Reducing Bacteria, p. 21-40. In J. M. Odom and R. Singleton Jr. (ed.), The Sulfate-Reducing Bacteria: Contemporary Perspectives, v. Springer-Verlag, New York.

Harmsen, H. J. M., A. D. L. Akkermans, L. J. M. Stams and W. M. De Vos. 1996. Population dynamics of propionate-oxidizing bacteria under methanogenic and sulfidogenic conditions in anaerobic granular sludge. *Appl Environ Microbiol.* 62: 2163-2168.

Herrick, J. B., E. L. Madsen, C. A. Batt and W. C. Ghiorse. 1993. Polymerase chain reaction amplification of napthalene-catabolic and 16S rRNA gene sequences from indigenous sediment bacteria. *Appl Environ Microbiol.* 59: 687-694.

Hicks, R. E., R. I. Amann and D. A. Stahl. 1992. Dual staining of natural bacterioplankton with 4',6-diamidino-2-phenylindole and fluorescent oligonucleotide probes targeting kingdom-level 16S rRNA sequences. *Appl Environ Microbiol.* 58: 2158-2163.

Hillis, D. M. and J. J. Bull. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Syst Biol.* 42: 182-192.

Hines, M. E. 1991. The role of certain infauna and vascular plants in the mediation of redox reactions in marine sediments, p. 275-286. In J. Berthelin (ed.), Diversity of Environmental Biogeochemistry, v. 6. Elsevier, Amsterdam.

Hines, M. E., R. S. Evans, B. R. Sharak Genthner, S. G. Willis, S. Friedman and R. Devereux. in prep. Molecular phylogenetic and biogeochemical studies of sulfate-reducing bacteria in the rhizosphere of *Spartina alterniflora.*

Hines, M. E., G. T. Banta, A. E. Giblin, J. E. Hobbie and J. T. Tugel. 1994. Acetate concentrations and oxidation in salt marsh sediments. *Limnol Oceanogr.* 39: 140-148.

Hines, M. E., S. L. Knollmeyer and J. B. Tugel. 1989. Sulfate reduction and other sedimentary biogeochemistry in a northern New England salt marsh. *Limnol Oceanogr.* 34: 578-590.

Holben, W. E. and D. Harris. 1995. DNA-based monitoring of total bacterial community structure in environmental samples. *Mol Ecol.* 4: 627-631.

Howarth, R. W. 1993. Microbial processes in salt-marsh sediments, p. 239-260. In T. E. Ford (ed.), Aquatic Microbiology: an Ecological Approach, Blackwell Scientific Publications, Boston.

Howarth, R. W. and J. E. Hobbie. 1982. The regulation of decomposition and heterotrophic microbial activity in salt marsh soils: a review, p.103-127. In V. S. Kennedy (ed.), Estuarine Comparisons, Academic Press, New York.

Howes, B. L., J. W. H. Dacey and J. M. Teal. 1985. Annual carbon mineralization and belowground production of *Spartina alterniflora* in a New England salt marsh. *Ecol.* 66: 595-605.

Imhoff-Stuckle and Pfennig. 1983. Isolation and characterization of a nicotinic acid-degrading sulfate-reducing bacterium, *Desulfococcus niacini* sp. nov. *Arch Microbiol.* 136: 194-198.

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: 160-168.

Jansen, K., R. K. Thauer, F. Widdel and G. Fuchs. 1984. Carbon assimilation pathways in sulfate reducing bacteria. Formate, carbon dioxide, carbon monoxide, and acetate assimilation by *Desulfovibrio baarsii. Arch Microbiol.* 138: 257-262.

Janssen, P. H., A. Schuhmann, F. Bak and W. Liesack. 1996. Disproportionation of inorganic sulfur compounds by the sulfate-reducing bacterium *Desulfocapsa thiozymogenes* gen. nov., sp. nov. *Arch. Microbiol.* 166:184-192.

Jukes, T. H. and C. R. Cantor. 1969. Evolution of Protein Molecules, p. 21-132. In H. N. Munro (ed.), Mammalian Protein Metabolism, Academic Press, New York.

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. *Appl Environ Microbiol.* 59: 682-686.

Kaplan, W., I. Valiela and J. M. Teal. 1979. Denitrification in a salt marsh ecosystem. *Limnol Oceanogr.* 24: 726-734.

Kluepfel, D. A. 1993. The behavior and tracking of bacteria in the rhizosphere. *Annu Rev Phytopathol.* 31: 441-472.

Krumholz, L. R., J. L. Hollenback, S. J. Roskes and D. B. Ringelberg. 1995. Methanogenesis and methanotrophy within a *Sphagnum* peatland. *FEMS Microbiol Ecol.* 18: 215-224.

Laanbroek, H. J., T. Abee and I. L. Voogd. 1982. Alcohol conversions by *Desulfobulbus propionicus* Lindhorst in the presence and absence of sulfate and hydrogen. *Arch Microbiol.* 133: 178-184.

Lane, D., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin and N. R. Pace. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc Natl Acad Sci USA.* 82: 6955-6959.

Lee, S. H., C. Malone and P. F. Kemp. 1993. Use of multiple 16S rRNA-targeted fluorescent probes to increase signal strength and measure cellular RNA from natural planktonic bacteria. *Mar Ecol Prog Ser.* 101: 193-201.

Lespinat, P. A., Y. M. Verlier, G. D. Fauque, R. Toci, G. Denariaz and J. LeGall. 1987. The relationship between hydrogen metabolism, sulfate reduction, and nitrogen fixation in sulfate reducers. *J Ind Microbiol.* 1: 383-388.

Litchfield, C. D. 1976. Marine Microbiology. Dowden, Hutchinson & Ross, Inc., Stroudsburg, Pennsylvania. 517 p.

Lonergan, D. J., H. L. Jenter, J. D. Coates, E. J. P. Phillips, T. M. Schmidt and D. R. Lovley. 1996. Phylogenetic analysis of dissimilatory Fe(III)-reducing bacteria. *J Bacteriol.* 178: 2402-2408.

Lovley, D. R., S. J. Giovannoni, D. C. White, J. E. Champine, E. J. P. Phillips, Y. A. Gorby and S. Goodwin. 1993. *Geobacter metallireducens* gen. nov. sp. nov., a microorganism capable of coupling the complete oxidation of organic compounds to the reduction of iron and other metals. *Arch. Microbiol.* 159:336-344.

Luther, G. W. I., T. M. Church, J. P. Scudlark and M. Cosman. 1986. Inorganic and organic sulfur cycling in salt-marsh pore waters. *Science.* 232: 746-749.

Lytle, R. W., Jr. and R. J. Hull. 1980. Annual carbohydrate variaton in culms and rhizomes of the smooth cordgrass (*Spartina alterniflora* Loisel.). *Agron J.* 72: 942-946.

Magot, M., P. Caumette, J. M. Desperrier and R. Matheron. 1992. *Desulfovibrio longus* sp. nov., a sulfate-reducing bacterium isolated from an oil-producing well. *Int J Syst Bacteriol.* 42: 398-403.

Maidak, B. L., N. Larsen, M. J. McCaughey, R. Overbeek, G. J. Olsen, K. Fogel, J. Blandy and C. R. Woese. 1994. The Ribosomal Database Project. *Nucleic Acids Res.* 22: 3485-3487.

Manz, W., R. Amann, W. Ludwig, M. Wagner and K.-H. Schleifer. 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: Problems and solutions. *System Appl Microbiol* 15: 593-600.

Marschall, C., P. Frenzel and H. Cypionka. 1993. Influence of oxygen on sulfate reduction and growth of sulfate-reducing bacteria. *Arch Microbiol.* 159: 168-173.

Meltzer, S. J. 1993. Direct Sequencing of Polymerase Chain Reaction Products, p. 137-140. In B. A. White (ed.), PCR Protocols, v. 15. Humana Press, Inc., Totowa, NJ.

Mendelssohn, I. A. and K. L. McKee. 1987. Root metabolic response of *Spartina alterniflora* to hypoxia, p. 239-353. In R. M. M. Crawford (ed.), Plant Life in Aquatic and Amphibious Habitats, British Ecol. Soc. Spec. Publ. No. 5.

Moran, M. A., V. L. Torsvik, T. Torsvik and R. E. Hodson. 1993. Direct extraction and purification of rRNA for ecological studies. *Appl Environ Microbiol.* 59: 915-918.

Moré, 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: 1572-1580.

Morrison, M. C. and M. E. Hines. 1990. The variability of biogenic sulfur flux from a temperate salt marsh on short time and space scales. *Atmos Environ.* 24: 1771-1779.

Moyer, C. L., F. C. Dobbs and D. M. Karl. 1994. Estimation of diversity and community structure through restriction fragment length polymorphism distribution analysis of bacterial 16S rRNA genes from a microbial mat at an active, hydrothermal vent system, Loihi Seamount, Hawaii. *Appl Environ Microbiol.* 60: 871-879.

Moyer, C. L., J. M. Tiedje, F. C. Dobbs and D. M. Karl. 1996. A computer-simulated restriction fragment length polymorphism analysis of bacterial small-subunit rRNA genes: Efficacy of selected tetrameric restriction enzymes for studies of microbial diversity in nature. *Appl Environ Microbiol.* 62: 2489-2493.

Murray, A. E., J. T. Hollinbaugh and C. Orrego. 1996. Phylogenetic compositions of bacterioplankton from two California estuaries compared by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Appl Environ Microbiol.* 62: 2676-2680.

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 coding for 16S rRNA. *Appl Environ Microbiol.* 59: 695-700.

Nedwell, D. B. and J. W. Abram. 1979. Relative influence of temperature and electron donor and electron acceptor concentrations on bacterial sulfate reduction in salt marsh sediment. *Microb Ecol.* 5: 67-72.

Olsen, G. J., C. R. Woese and R. Overbeek. 1994a. The winds of (evolutionary) change: Breathing new life into microbiology. *J Bacteriol.* 176: 1-6.

Olsen, G. J., H. Matsuda, R. Hagstron and R. Overbeek. 1994b. fastDNAml: A tool for construction of phylogenetic trees using maximum likelihood. *CABIOS.* 10: 41-48.

Paul, E. A. and F. E. Clark. 1989. Soil Microbiology and Biochemistry. Academic Press, Inc., San Diego, California. 275 p.

Picard, C., C. Ponsonnet, E. Paget, X. Nesme and P. Simonet. 1992. Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. *Appl Environ Microbiol.* 58: 2717-2722.

Postgate, J. R. 1984a. Genus *Desulfovibrio*, p. 666-672. In N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, v. 1. Williams and Wilkins, Baltimore.

Postgate, J. R. 1984b. The Sulphate-Reducing Bacteria. Cambridge University Press, London. 208 p.

Postgate, J. R. and L. L. Campbell. 1966. Classification of *Desulfovibrio* species, the nonsporulating sulfate-reducing bacteria. *Bacteriol Rev.* 30: 732-738.

Reysenbach, A.-L., L. J. Giver, G. S. Wickham and N. R. Pace. 1992. Differential amplification of rRNA genes by polymerase chain reaction. *Appl Environ Microbiol.* 58: 3417-3418.

Ritz, K. and B. S. Griffiths. 1994. Potential application of a community hybridization technique for assessing changes in the population structure of soil microbial communities. *Soil Biol Biochem.* 26: 963-971.

Russek-Cohen, E. and D. Jacobs. 1993. Statistics, biodiversity, and microorganisms. Microbial Diversity and Ecosystem Function: IUBS/IUMS Workshop, Egham, UK, University Press.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol.* 4: 406-425.

Sambrook, J., E. F. Fritsch and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Sharak Genthner, B. R. Pers. Comm. 1996.

Sharak Genthner, B. R., G. Mundfrom and R. Devereux. 1994. Characterization of *Desulfomicrobium escambium* sp. nov. and proposal to assign *Desulfovibrio desulfuricans* strain Norway 4 to the genus *Desulfomicrobium. Arch Microbiol.* 161: 215-219.

Smith, A. M. and T. ap Rees. 1979. Pathways of carbohydrate fermentation in the roots of marsh plants. *Planta.* 146: 327-334.

Smith, D. W. 1993. Ecological actions of sulfate-reducing bacteria, p. 161-188. In J. M. Odom and R. Singleton Jr. (ed.), The Sulfate-Reducing Bacteria: Contemporary Perspectives, Springer-Verlag, New York.

Spring, S., R. Amann, W. Ludwig, K.-H. Schleifer, H. van Gemerden and N. Petersen. 1993. Dominating role of an unusual magnetotactic bacterium in the microaerobic zone of a freshwater sediment. *Appl Environ Microbiol.* 59: 2397-2403.

Stackebrandt, E. 1992. Unifying Phylogeny and Phenotypic Diversity, p. 19-47. In A. Balows, H. G. Trüper, M. Dworkin, W. Harder and K.-H. Schleifer (ed.), The Prokaryotes, Springer-Verlag, New York.

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. *Int J Syst Bacteriol.* 44: 846-849.

Stackebrandt, E., D. A. Stahl and R. Devereux. 1995. Taxonomic Relationships, p. 49-87. In L. L. Barton (ed.), Sulfate-Reducing Bacteria, Plenum Press, New York.

Stackebrandt, E., U. Wehmeyer and B. Schink. 1989. The phylogenetic status of *Pelobacter acidigallici, Pellobacter venetianus,* and *Pelobacter carbinolicus. Syst Appl Microbiol.* 11: 257-260.

Stahl, D. A. and R. Amann. 1991. Development and Application of Nucleic Acid Probes in Bacterial Systematics, p. 205-248. In E. Stackbrandt and M. Goodfellow (ed.), Sequencing and Hybridization Techniques in Bacterial Systematics, John Wiley & Sons, Chichester, UK.

Stahl, D. A., B. Flesher, H. R. Mansfield and L. Montgomery. 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. *Appl Environ Microbiol.* 54: 1079-1084.

Steffan, R. J., J. Goksøyr, A. K. Bej and R. M. Atlas. 1988. Recovery of DNA from soils and sediments. *Appl Environ Microbiol.* 54: 2908-2915.

Swofford, D. L., G. J. Olsen, P. J. Waddell and D. M. Hillis. 1996. Phylogenetic Inference, p. 407-514. In D. M. Hillis, C. Moritz and B. K. Mable (ed.), Molecular Systematics, Sinauer Associates, Inc., Sunderland, Massachusetts, U.S.A.

Tardy-Jacquenod, C., M. Magot, F. Laigret, M. Kaghad, B. K. C. Patel, J. Guezennec, R. Matheron and P. Caumette. 1996. *Desulfovibrio gabonensis* sp. nov., a new moderately halophilic sulfate-reducing bacterium isolated from an oil pipeline. *Int J System Bacteriol.* 46: 710-715.

Teal, J. M., I. Valiela and D. Berlo. 1979. Nitrogen fixation by rhizosphere and free-living bacteria in a salt marsh. *Limnol Oceanogr.* 24: 126-132.

Telang, A. J., G. Voordouw, S. Ebert, N. Sifeldeen, J. M. Foght, P. M. Fedorak and D. W. S. Westlake. 1994. Characterization of the diversity of sulfate-reducing bacteria in soil and mining waste water environments by nucleic acid hybridization techniques. *Can J Microbiol.* 40: 955-964.

Thauer, R. K. and J. Kunow. 1995. Sulfate-Reducing Archaea, p. 33-48. In L. L. Barton (ed.), Sulfate-Reducing Bacteria, Plenum Press, New York.

Thompson, J. D., D. G. Higgins and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673-4680.

Tiedje, J. M. 1993. Approaches to the comprehensive evaluation of prokaryote diversity of a habitat. Microbial Diversity and Ecosystem Function: IUBS/IUMS Workshop, Egham, UK, University Press.

Torsvik, V., J. Goksøyr and F. L. Daae. 1990. High diversity in DNA of soil bacteria. *Appl Environ Microbiol.* 56: 782-787.

Torsvik, V., J. Goksoyr, F. L. Daae, R. Sorheim, J. Michalsen and K. Salte. 1994. Use of DNA analysis to determine the diversity of microbial communities, p. 39-48. In K. Ritz, J. Dighton and K. E. Giller (ed.), Beyond the Biomass, John Wiley & Sons Ltd, Chichester, UK.

Trüper, H. G. and K.-H. Schleifer. 1992. Prokaryote Characterization and Identification, p. 126-148. In A. Balows, H. G. Trüper, M. Dworkin, W. Harder and K.-H. Schleifer (ed.), The Prokaryotes, Springer-Verlag, New York.

Tsai, Y. L. and B. H. Olson. 1992. Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. *Appl Environ Microbiol.* 58: 2292-2295.

Tsai, Y.-L. and B. H. Olson. 1991. Rapid method for direct extraction of DNA from soil and sediments. *Appl Environ Microbiol.* 57: 1070-1074.

Valiela, I., J. M. Teal and N. Y. Perrson. 1976. Production and dynamics of experimentally enriched salt marsh vegetation: belowground biomass. *Limnol Oceanogr.* 21: 245-252.

Vernberg, F. J. 1993. Salt-marsh processes - a review. *Environ Toxicol Chem.* 12: 2167-2195.

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

Ward, D. M., R. Weller and M. M. Bateson. 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature.* 345: 63-65.

Ward, D., M. Bateson, R. Weller and A. Ruff-Roberts. 1992. Ribosomal RNA analysis of microorganisms as they occur in nature, p. 219-286. In K. Marshall (ed.), Advances in Microbial Ecology, v. 12. Plenum Press, New York.

Ward, N., F. A. Rainey, B. Goebel and E. Stackebrandt. 1993. Identifying and culturing the 'unculturables': A challenge for microbiologists. Microbial

Diversity and Ecosystem Function: IUBS/IUMS Workshop, Egham, UK, University Press.

Watson, J. D., M. Gilman, J. Witkowski and M. Zoller. 1992. Recombinant DNA. W.H. Freeman and Company, New York. 626 p.

Weisburg, W. G., S. M. Barns, D. A. Pelleitier and D. J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol.* 173: 697-703.

Weller, R. and D. M. Ward. 1989. Selective recovery of 16S rRNA sequences from natural microbial communities in the form of cDNA. *Appl Environ Microbiol.* 55: 1818-1822.

Widdel, F. 1983. Methods for enrichment and pure culture isolation of filamentous gliding sulfate-reducing bacteria. *Arch Microbiol.* 134: 282-285.

Widdel, F. 1987. New types of acetate-oxidizing, sulfate-reducing *Desulfobacter* species, *D. hydrogenophilus* sp. nov., *D. latus* sp. nov., and *D. curvatus* sp. nov. *Arch. Microbiol.* 148:286-291.

Widdel, F. and F. Bak. 1992. Gram-Negative Mesophilic Sulfate-Reducing Bacteria, p. 583-624. In A. Balows, H. G. Trüper, M. Dworkin, W. Harder and K.-H. Schleifer (ed.), The Prokaryotes, Springer-Verlag, New York.

Widdel, F. and N. Pfennig. 1982. 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: 395-400.

Widdel, F., G.-W. Kohring and F. Mayer. 1983. Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids III. Characterization of the filamentous *Desulfonema limicola* gen. nov. sp. nov. and *Desulfonema magnum* sp. nov. *Arch Microbiol.* 134: 286-294.

Widdel, F. 1982. Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. II. Incomplete oxidation of propionate by *Desulfobulbus propionicus* gen. nov., sp. nov. *Arch Microbiol.* 131: 360-365.

Willis, S.G., B.R. Sharak Genthner, S. Friedman, M.E. Hines, and R. Devereux. 1995. Digestion of 16S rDNA PCR products with restriction endonucleases recognizing 4-bp sequences provides a method for rapid phylogenetic assignment of bacteria from anaerobic enrichment cultures. Abstr. Gen. Meeting Am. Soc. Microbiol. Q149, p. 425.

Wilson, K. H. and R. B. Blitchington. 1996. Human colonic biota studied by ribosomal DNA sequence analysis. *Appl Environ Microbiol.* 62: 2273-2278.

Witzel, K.-P. 1990. Approaches to bacterial population dynamics, p. 96-128. In R. J. Chróst and J. Overbeck (ed.), Aquatic Microbial Ecology: Biochemical and Molecular Approaches, Springer-Verlag, New York.

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

Woese, C. R. 1992. Prokarote Systematics: The Evolution of a Science, p. 3-18. In A. Balows, H. G. Trüper, M. Dworkin, W. Harder and K.-H. Schleifer (ed.), The Prokaryotes, Springer-Verlag, New York.

Woese, C. R. 1994. There must be a prokaryote somewhere: Microbiology's search for itself. *Microbiol Rev.* 58: 1-9.

Woese, C. R., E. Stackebrandt, T. J. Macke and G. E. Fox. 1985. A phylogenetic definition of the major eubacterial taxa. *System Appl Microbiol.* 6: 143-151.

Woese, C. R., R. Gutell, R. Gubta and H. F. Noller. 1983. Detailed analysis of the higher-order structure of 16S-like ribosomal ribonucleic acids. *Microbiol Rev.* 47: 621-669.

Zarda, B., R. Amann, G. Wallner and K.-H. Schleifer. 1991. Identification of single bacterial cells using digoxigenin-labelled rRNA-targeted oligonucleotides. *J Gen Microbiol.* 137: 2823-2830.

Zarkikh, A. and W.-H. Li. 1992a. Statistical properties of bootstrap estimation of phylogenetic variability from nucleotide sequences: I. Four taxa with a molecular clock. *Mol Biol Evol.* 9: 1119-1147.

Zarkikh, A. and W.-H. Li. 1992b. Statistical properties of bootstrap estimation of phylogenetic variability from nucleotide sequences: I. Four taxa without a molecular clock. *Mol Biol Evol.* 35: 356-366.