THE UNIVERSITY OF CALGARY

Molecular Biological Characterization of the Diversity of Sulfate-Reducing Bacteria

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOLOGICAL SCIENCES

CALGARY, ALBERTA

DECEMBER, 1994

THE UNIVERSITY OF CALGARY

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Molecular Biological Characterization of the Diversity of Sulfate-Reducing Bacteria" submitted by Anita J. Telang in partial fulfillment of the requirements for the degree of Master of Science.

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Abstract

Molecular biological techniques were used to characterize the diversity of sulfate-reducing bacteria (SRB) isolated from soil and waste water environments. Genomic DNA preparations obtained from liquid enrichment cultures of environmental SRB were characterized by their reaction to; (i) a specific gene probe aimed at the genus *Desulfovibrio,* (ii) a general 16S rRNA gene probe aimed at all SRB, and (iii) whole genome probes aimed at specific SRB. Considerable SRB diversity was observed within each environment and most SRB isolated from distinct environments were genomically different. Consequently, whole genome probes cannot be applied universally to characterize SRB at different environments.

Characterization of some DNA preparations with PCR and 16S rRNA gene sequencing demonstrated that sequences could be amplified from genomes present as minor components of individual DNA preparations. Sequences obtained were compared to databases and identified.

Acknowledgements

I have had much help along the way in my endeavour to learn to be a 'good scientist'. I would first like to gratefully acknowledge the financial support of the Alberta Heritage Fund for Medical Research. This work was done in collaboration with Dr. D. W. S. Westlake, Dr. J. Foght, and Dr. P. Fedorak at the University of Alberta. They provided many suggestions and much encouragement. The samples analyzed in this study were provided by various agencies as indicated in the Materials section. Sara Ebert and Neili Sifeldeen at the University of Alberta were responsible for most of the SRB enrichment cultures and DNA preparations. Yin Shen performed the ribotyping and whole genome analysis of the Digout samples. From Yin and from Hansje Voordouw I learned the value of precise, efficient and thorough analysis. They also helped make the lab an enjoyable and efficient place for learning molecular biology. My supervisor, Gerrit Voordouw, taught me many of the different, yet equally important skills necessary for doing 'good' science. His guidance and encouragement is deeply appreciated. I enjoyed expanding my horizons, scientific and non-scientific, with Harm Deckers, Rick Keon and Elaine Sihota over coffee or the occasional glass'o'Hog. Finally, even though they probably still do not have the first clue as to what I do, I would like to thank my Mom and Dad and the rest of my family, Telangs and Carrolls, for infusing some perspective into my education and for encouraging and supporting me. Particularly Kevin, who helped me to believe in myself and my work, and not only listened, but also tried to look interested.

To Mom and Dad

Table of Contents

Section		Page

List of tables

List of Figures

Abbreviations and symbols

Ace	acetate
am or AMD	acid mine drainage
ATP	adenosine triphosphate
Ben	Benzoate
bp	base pair
BSA	Bovine Serum Albumin
CANMET	Canada Centre for Mineral and Energy Technology
Ci	Curie
CTP	cytosine triphosphate
Dec	decanoate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
Eth	ethanol
g/L	grams per litre
HGT	high gelling temperature
IPTG	isopropyl-β-D-thiogalactoside
kb	kilobases
λ	lambda
Lac	lactate
LGT	low gelling temperature
min	minute
[NiFe]	Nickle Iron
OFPW	oil field production water

PCR	polymerase chain reaction
PEG	polyethylene glycol
Pro	propionate
rRNA	ribosomal ribonucleic acid
RSGP	reverse sample genome probing
SDS	sodium dodecyl sulfate
so	soil
SSC	standard sodium citrate
SRB	sulfate-reducing bacteria
TAE	Tris acetate EDTA
TBE	Tris boric acid EDTA
TE	Tris-EDTA
tp	oil sand tailings pond
TY	tryptone yeast
w/v	weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter 1. Introduction

1.1 Microbial Communities

In the laboratory, microbes are generally studied in pure cultures although this is rarely how they exist in the environment. Pure cultures provide important information about the metabolism of single organisms. The role that these organisms play in environmental processes may be deduced from such studies. Pure culture information, however, does not give a complete view and thus may be only partially relevant to the actual process in the environment (Stahl, 1993). For example, *Desulfovibrio* is thought to be involved in metal corrosion, but corrosion rates observed with pure cultures of *Desulfovibrio* are generally slower than those observed in field operations (Hamilton, 1985). This rate difference, which has been linked to the accumulation of FeS, may also be affected by the metabolism of other microbes. *Desulfovibrio* metabolism is only one component of the complex biochemical reaction that is metal corrosion, and an adequate explanation of metal corrosion can not be obtained by studying *Desulfovibrio* metabolism in isolation. More accurate models of environmental biological processes incorporate multiple components or organisms, describing these processes in terms of microbial communities rather than pure cultures (Stahl, 1993).

Microbial communities function as biological units. Just as many proteins within a microbe contribute to its metabolism as enzymes in metabolic pathways, different microbes complement the metabolism of each other to catalyze the food chain of a microbial community. The net effect of this is that an environmental biological process such as denitrification or the anaerobic degradation of organic

matter, as well as corrosion, sewage treatment, or bioremediation, must be considered in the context of the metabolism of the resident microbial community. Thus it is important to identify the community structure (the different microbes present in a single environmental community at a specific site) and the community composition (the numbers or percentages of different microbes at a particular site) in order to understand how the community is catalyzing the process of interest. In this way it may be possible to understand why differences in the nature, extent, and rate of a process occur under seemingly similar physical conditions (e.g. different oil field production plants with different rates of corrosion, or different contaminated sites bioremediating at different rates.).

Microbial communities can be studied in the environment using both traditional microbiological techniques and molecular biological ones. Culturing, followed by physiological and morphological analysis can provide some of the necessary information about the individual members of a community. However, these techniques are limited by the inability of many bacteria to grow as pure cultures, or even at all under laboratory conditions, and by the difficulties in recognizing differences between bacteria using physiological and morphological criteria (Postgate, 1984). The pure culture collections have been shown to be incomplete representations of environmental microbial diversity (Giovannoni *et al.*, 1990, Stahl *et al.*, 1985, Voordouw *et al.*, 1991, Ward *et al.*, 1990). Although at present more than 3000 species have been identified and characterized, the total number of different bacteria that are thought to be engaged in various environmental processes has been estimated at 1 million (Palleroni, 1994). Identifying and characterizing bacteria at the genomic or 16S rRNA level will reveal more diversity and allow a more thorough analysis of the community

(Wayne *et al.*, 1987). Furthermore, there is the potential to identify and characterize bacteria that are difficult to culture and to establish their role in the community.

1.2 The sulfate-reducing bacteria

Sulfate-reducing bacteria (SRB) exist in most soil and water environments (Postgate, 1984). They can form part of stable anaerobic communities, co-existing with other anaerobic bacteria such as *Clostridium*. These communities are vital to the sulfur cycle (Singleton, 1993). Alternatively, SRB can be dormant in an aerobic environment until conditions are anaerobic and reducing. In this scenario, SRB undergo a growth explosion, rapidly becoming the dominant members of a microbial community as their metabolic product, sulfide, depletes oxygen in the surrounding area and is toxic to many eubacteria. Aerobes and non-SRB anaerobes are unable to compete in the community, and are replaced by SRB (Postgate, 1984). This change in community structure frequently occurs as a result of pollution with organic matter.

1.2.1 SRB metabolism; environmental and industrial effects

The SRB metabolism generally relies on sulfate as the terminal electron acceptor while organic compounds or hydrogen are oxidized (Postgate, 1984). This reaction is referred to as dissimilatory sulfate reduction and produces sulfide, an important contaminant in many industrial operations. The net reaction is as follows:

$$4AH_2 + SO_4^{2-} + H^+ \text{ ----------> } 4A + HS^- + 4H_2O \quad (1)$$

In this reaction 'AH_2' represents a variety of molecules that can be oxidized, such as hydrogen, lactate, ethanol, etc. A significant proportion of carbon mineralization in anaerobic aquatic systems, soils and sediments occurs via this reaction (Herlihy and Mills, 1985), estimated at up to 50% in estuarine sediments where the sulfate concentration is intrinsically high (about 28 mM) (Jorgensen, 1982). The sulfate concentration is often the limiting factor for SRB growth in an anaerobic environment and in freshwater sediments, where the sulfate concentration is in the micromolar range (about 110 μM), carbon degradation occurs via methanogenesis rather than sulfate reduction.

Industry can contribute to the SRB community dominance by providing conditions where SRB thrive (Postgate, 1984). An accumulation of organic matter will decrease the oxygen concentration, setting up anaerobic microniches (Odom, 1993). Mine tailings waters often have high sulfate concentrations. The freshwater systems into which these sulfate rich tailings are discharged will have an alteration in their microbial community composition as conditions become favorable for SRB growth (Herlihy and Mills, 1985). A hydrogen layer can spontaneously form along metal surfaces such as pipelines. This electrochemical reaction is termed cathodic polarization and is generally stable, unless the hydrogen is removed. (Odom, 1993). This hydrogen layer is a source of electrons for SRB with hydrogenases, encouraging their growth. For each of the above cases, the resulting metabolism of SRB will have important effects. SRB, in concert with other anaerobic bacteria, will deplete the organic material, but will also produce noxious sulfide emissions, a problem in sewage treatment (Odom, 1993). Mining waste waters are generally acidic, and metalliferous in addition to the high sulfate concentration. SRB metabolism has been shown to

ameliorate acid mine drainage (AMD) by generating alkaline conditions and precipitating heavy metals as metal sulfides (Dvorak *et al.*, 1992, Hammak and Edenborn, 1992, Herlihy and Mills, 1985). Utilization of the hydrogen along a metal surface will result in cathodic depolarization, which is supplemented by the removal of ferrous ions as ferrous sulfide at the anode. Cathodic depolarization has been linked to metal corrosion, an economically detrimental effect in the oil industry, as well as in many others. It would be useful for industries to be able to analyze the SRB potential of a site, possibly avoiding problematic situations.

1.2.2 SRB phylogeny; more than a metabolic association

Dissimilatory sulfate reduction is not unique to the sulfate reducing eubacteria. A group of archaebacteria that also use sulfate as the terminal respiratory electron acceptor have been identified in extreme environments (Huber *et al.*, 1990, Stetter *et al.*, 1987, 1993). These archaeal microbes form a unique group that is taxonomically distinct from the eubacterial SRB (Stetter *et al.*, 1987). Furthermore, although most eubacterial SRB are Gram negative, the genus *Desulfotomaculum*, a Gram positive eubacteria also capable of dissimilatory sulfate reduction, has also been described (Postgate, 1984). The Gram negative SRB belong to the delta subdivision of the proteobacteria (Devereux *et al.*, 1989, Woese, 1987). This subdivision includes *bdellovibrio* and *myxococcus*, two aerobic groups, as well as the obligately anaerobic sulfur reducing bacteria and the sulfate reducing bacteria.

Taxonomic assignments of SRB based on physiological and morphological studies have generally been confirmed by 16S rRNA gene sequence analysis

(Devereux *et al.*, 1989). At least eight genera of SRB can be distinguished by these criteria, which fall into three monophyletic groups, the Gram positive *Desulfotomacula,* mentioned above, and two Gram negative groups for which the family names *Desulfovibrionaceae* and *Desulfobacteriaceae* have been suggested based on 16S rRNA sequence analysis (Widdel and Bak, 1992). A comparison of the 16S rRNA of species within the genus *Desulfovibrio* revealed that, although it was, with two exceptions, a monophylogenetic group, the diversity within the genus exceeded that normally seen within a eubacterial genus. The diversity was comparable to that between all the other identified genera of Gram negative SRB. Between *Desulfovibrio* species, 16S rRNA sequence similarities range as low as 87%, while species within the other genera have 16S rRNA sequence similarities of at least 92%. Between other genera 16S rRNA sequence similarities range from 81 to 90% (Devereux *et al.*, 1989, 1990). Thus Devereux *et al.* suggested that many '*Desulfovibrio*' could be reclassified into separate genera, but that almost all of these genera would be retained in one family, the *Desulfovibrionaceae* (Devereux *et al.*, 1990). All other Gram negative SRB genera (e. g. *Desulfobulbus, Desulfobacter, Desulfosarcina, Desulfococcus, Desulfobacterium,* as well as *Desulfovibrio sapovorans* and *Desulfovibrio baarsii*) would form the second family, *Desulfobacteriaceae* (Devereux and Stahl, 1993). Presently, the *Desulfovibrio* are still commonly referred to as a genus and will be considered as such for this work. 16S rRNA sequence analysis has also proved useful for redistributing some species, such as *Desulfomonas pigra* which is more closely related to the *Desulfovibrionaceae, and Desulfovibrio baculatus,* reclassified as *Desulfomicrobium baculatus.* Reassignment or reclassification always considers both the 16S rRNA sequence as well as

morphological and physiological information, such as nutritional requirements (Devereux *et al.*, 1989).

1.2.3 SRB communities; acquiring information

As obligate anaerobes, the SRB are difficult to culture (Postgate, 1984). Morphological and physiological criteria for distinguishing between species are limited (Postgate, 1984), although an enormous amount of novel information has been generated by Pfennig and Widdel (Widdel and Pfennig, 1984). The potential diversity of this group has been confirmed and further explored by application of 16S rRNA sequencing and probing (Stahl *et al.*, 1985, Devereux *et al.*, 1989). This work has provided a basis that allows SRB that have not been isolated in pure culture to be characterized and identified. As described below, SRB community structure and composition can be studied with 16S rRNA analysis, as well as with other molecular biological techniques such as hybridization with specific genes or whole genomes.

1.3 Nucleic Acid Hybridization Techniques

Large scale identification and characterization of SRB in a multitude of environmental samples using only traditional microbiological techniques would be too time-consuming and may not recognize the true diversity that exists within a community (Giovannoni *et al.*, 1990, Ogram and Sayler, 1988, Ward *et al.*, 1990). A more rapid analysis providing comparative information on a level where all bacteria, including the SRB, have more significant differences, would target the genome (Woese, 1987). Sequence comparison of the entire genomes of two organisms would completely define their level of (dis)similarity. Although

with present sequencing technology this is an unrealistic goal, it may become a tangible opportunity in the future. Currently it is necessary to establish more limited genomic profiles of organisms by comparing specific, representative features of their genomes (Ogram and Sayler, 1988). The presence or absence of specific genes (e. g. those for hydrogenases or desulfoviridin) can identify groups of related bacteria, whereas an analysis of more general or ubiquitous genes (e.g. those for ATP synthase, cytochromes, or 16S rRNA) will give more specific taxonomic information (Ogram and Sayler, 1988). It is also possible to compare the similarity of entire bacterial genomes by hybridization to resolve differences between species. Examples of each of these nucleic acid hybridization techniques are given below.

1.3.1 Single Gene Analysis

Preliminary investigation into the diversity of an SRB community could begin by focusing on the genus *Desulfovibrio*, which has been well studied, and thus can be readily identified (Voordouw, 1993). Identification of *Desulfovibrio* is facilitated by the availability of a specific gene probe, the [NiFe] hydrogenase gene probe, that will distinguish all members of this genus from other SRB (Voordouw *et al.*, 1990). Hydrogenases have been well characterized in the SRB and carry out the following reaction;

$$H_2 \text{ <----------> } 2H^+ + 2e^- \qquad (2)$$

Hydrogen is either consumed as a source of energy (see equation 1), or produced when the electrons from organic carbon sources being oxidized are donated to hydrogen ions. *Desulfovibrio* species can contain up to three different hydrogenases, the [Fe], the [NiFe], and the [NiFeSe] hydrogenases, so named

because of the metal ions associated with the enzyme (Voordouw *et al.*, 1990). The [NiFe] hydrogenase is found in all members of the genus *Desulfovibrio* and probes derived from the *Desulfovibrio* [NiFe] hydrogenase gene are specific to *Desulfovibrio* (Voordouw, 1990). Hydrogenases are not unique to the SRB and [NiFe] hydrogenase genes are also found in other microbes such as *Rhizobium* and *Escherichia coli.* The application of this gene probe is primarily to take a variety of environmental samples, enrich them for SRB on specific media, and screen genomic DNA isolated from the enrichments for the presence of the [NiFe] hydrogenase gene, and thus for *Desulfovibrio* (Voordouw *et al.*, 1990).

A 'yes or no' answer with respect to the presence of *Desulfovibrio* is obtained by dot blot hybridization of DNA isolated from the enrichment with the [NiFe] hydrogenase gene probe. Differentiation at the species level can be obtained by probing restriction digests of isolated DNA with this gene probe. Different species may have the gene on different size restriction fragments, thus giving different Southern blot patterns (Voordouw *et al.*, 1990). It should be noted that although the presence of a specific gene can be shown by such gene hybridizations, the presence of the corresponding protein (i. e. gene expression) would require physiological tests.

1.3.2 General gene probes

Other SRB genera have not been characterized to the same degree as *Desulfovibrio.* Consequently, characteristic, conserved single gene probes are not available. Different comparative data must be generated requiring more general methods or gene probes. Use of the 16S rRNA genes has resulted in successful phylogenetic characterization of a variety of microbes, even when culturing is

difficult, either by sequence comparison (Giovannoni *et al.*, 1990, Ward *et al.*, 1990), *in situ* hybridization with phylogenetically targeted, fluorescently labeled oligonucleotide probes (Amman *et al.*, 1990, 1992), or by fingerprinting with various restriction enzymes (Moyer *et al.*, 1994). rRNA molecules have regions that are highly homologous and regions of great variability. Thus, both general probes that will hybridize with most eubacterial genomes (Grimont and Grimont, 1986), or specific probes that will differentiate at the family, genus, or species level (Amman *et al.*, 1992) can be derived from 16S rRNA gene sequences. General probes will not provide useful information in a dot blot analysis, since all eubacterial genomes will hybridize. However, Southern blot analysis with general probes can provide comparative information. If restriction digested bacterial genomic DNAs are electrophoretically fractionated, fragments containing the rRNA gene operons will be resolved and can be located in a hybridization assay with a general 16S rRNA gene fragment homologous to highly conserved regions of the 16S rRNA molecule (Grimont and Grimont, 1986). Different species and strains will have their 16S rRNA genes on fragments of varying sizes, thus patterns of hybridizing bands will be generated that can be compared between species and strains and are indicative of genomically unique organisms (Grimont and Grimont, 1986). This technique is called ribotyping, and applications have included identifying genomic diversity (Grimont and Grimont, 1986), tracking the source of aeromonad infection by differentiating clinical and environmental isolates (Moyer et al., 1992), and determining the level of genomic homogeneity between and within *Vibrio* serovars (Olsen and Larsen, 1993).

The information provided by Southern blot hybridization of 16S rRNA genes can be expanded by sequencing all or a portion of these genes to allow

comparison with a reference database. The sample genome can thus be identified and phylogenetically grouped based on sequence similarities. This approach has been useful in demonstrating genomic diversity and in detecting previously uncultured microbes in the environment (Giovannoni *et al.*, 1990, Ward *et al.*, 1990). Sequence information is particularly valuable because it can be used to establish phylogenetic relationships between the unidentified microbes and known species (Ward *et al.*, 1990) and can be applied in the development of oligonucleotide probes with varying taxonomic specificities. For example, Amman *et al.* used the polymerase chain reaction (PCR) to amplify 16S rRNA gene fragments from a biofilm community. Sequence from the fragments was used to develop specific probes that would target SRB sequences. These probes were used *in situ* to visualize the cells in the biofilm from which these sequences had been obtained. This provided community structure and composition information, as hybridization between the probes and cells in the biofilm first confirmed the presence of specific SRB in the community, then allowed enumeration of these SRB with regards to the rest of the biofilm (Amman *et al.*, 1992).

1.3.3 Whole Genome Probes

Bacterial genomes have, as discussed above, regions that are similar between species, such as the 16S rRNA genes. Nevertheless, large sections of the genomes of different bacteria are highly dissimilar and this is the basis for using the entire chromosome as a probe for species differentiation by nucleic acid hybridization. Provided that highly stringent hybridization conditions are used, the entire genome of a bacterium can be used as a specific probe to detect that

bacterium in a sample (Voordouw *et al.*, 1991). Only dot blots provide useful information in this case, since probing restriction digests will give hybridizing smears that cannot be interpreted.

Much as the hydrogenase gene probe was used as a specific gene probe for *Desulfovibrio*, a given SRB genome can be identified in an environmental sample provided a specific genome probe is available (Voordouw *et al.*, 1991). It is thus possible to probe an environmental sample repeatedly with the genomes of suspected community members, provided those genomes are available in purified form. Alternatively, the sample can be labeled and used to probe a dot blot on which all of these suspected community members are represented (Voordouw *et al.*, 1991). This technique is referred to as Reverse Sample Genome Probing (RSGP) and was applied in the identification of genomic diversity in oil field production waters in Alberta (Voordouw *et al.*, 1992).

In a specific application of RSGP, SRB were enriched from oil field production waters and their genomes were tested for cross-hybridization. Chromosomal DNAs from non-cross-hybridizing isolates, defined as standards, were immobilized on a hybridization membrane as representatives of the SRB of the target environmental community. Samples were then taken from different sites of this same environment and enriched for SRB. Genomic DNA from these enrichments was labeled, and used to probe the hybridization membrane, referred to as the master filter. Two different SRB communities were demonstrated in seven western Canadian oil fields (Voordouw *et al.*, 1992). Qualitatively therefore, this technique can be useful in a broad survey of microbial populations at different sites in the same environment, rapidly giving an indication of the SRB community structure. A quantitative version of this

technique has been described and used to determine community composition (Voordouw *et al.*, 1993).

A combination of all three nucleic acid hybridization techniques (single gene, general gene and whole genome probing) can give useful definitive and comparative information on SRB. This information can be used to evaluate the genomic diversity of SRB in the environment, and to establish SRB community structures. Identification and characterization of SRB communities at a variety of environmental and industrial sites may eventually lead to better monitoring and better understanding of the reactions catalyzed by the community members.

Chapter 2. Objectives.

The primary goal of this research is to determine if it is feasible to develop a master filter similar to that used in the oil field production water analysis, but with a broader environmental scope. It is desirable to be able to analyze the SRB populations in a variety of waste water and soil environments rapidly. For this purpose, the diversity of SRB from these different environments must first be studied. Two alternatives are possible. The first is that genomically similar SRB can be enriched from a given, related, and different environments. This would allow generation of a single SRB master filter to analyze the relationship between an environmental process (e. g. metal corrosion) and the structure or composition of the SRB community. The other alternative is that the SRB isolated from different environments are always genomically different and thus not readily comparable. It would then be necessary to develop specific master filters for each environment to monitor its SRB community structure and composition.

A study of SRB diversity, aimed at determining the feasibility of designing a general SRB master filter for waste water and soil samples, must assess the relatedness of as many SRB genomes as possible from a variety of environments and ascertain if there are common representative SRB genomes. The strategy to complete this assessment was (i) to choose different environments for analysis: soil, acid mine drainage (AMD), oil sands production water, and sewage; (ii) to take samples from sites representing each environment; (iii) to enrich a variety of SRB on six different growth substrates; (iv) to obtain DNA preparations from these liquid enrichments and from colony purified SRB; and (v) to determine

genomic relatedness by methods specified in section 1.3. The detailed objectives for the last step of this strategy are detailed below.

2.1 Specific Objectives for the Characterization of DNA preparations.

DNA preparations will be characterized by analysis with; (i) a single gene probe, (ii) a general gene probe, and (iii) whole genome probes.

2.1.1 [NiFe] hydrogenase gene probe

The first objective in the genomic characterization of the DNA preparations will be to test for the presence of *Desulfovibrio*. Preparations will be probed with the [NiFe] hydrogenase gene probe using dot blots. Positives will be further tested on Southern blots to provide comparative data on different *Desulfovibrio* species present. These tests will allow the *Desulfovibrio* content of the different sites and environments to be determined and will serve as a first comparison for the SRB community structures present in different environments.

2.1.2 16S rRNA gene probe

The second objective will be to ribotype the DNA preparations using a general 16S rRNA gene probe. Descriptive codes may be assigned to the SRB yielding DNA preparations as an indication of their genomic relatedness. This preliminary identification step will indicate which DNA preparations contain similar rRNA gene operons. Preparations with a defined ribotype will be studied further with whole genome probing.

2.1.3 Whole genome probes

The third objective will be to assess total genomic homology of different DNA preparations. This will be done by preparing dot blots of DNA preparations and cross-hybridizing them with labeled genomic DNA from the same set of preparations. Homology between preparations suspected after ribotyping may be confirmed in this way, and homology between preparations that gave different ribotypes may also be established. Preparations giving clear ribotypes that do not cross-hybridize will be designated as standards after this step. SRB community structures will be established for the different sites and environments in terms of these standards. The feasibility of developing a general SRB master filter will be considered following evaluation of the results obtained with the three different probes.

2.2 Comparison to oil field production water standards

Further indication of the feasibility of developing one master filter for the analysis of diverse environmental sites will be obtained by assessing total genomic homology of standards identified in this study with those previously identified in the oil field production water analyses. DNA from soil and waste water standards will be cross-hybridized to dot blots of oil field production water standards.

2.3 Colony purification as a means to obtain stable standards

Most of the DNA preparations used in the initial genomic characterizations will be obtained from liquid culture enrichments, which may contain genomic material from a variety of organisms. For design of a reliable

RSGP master filter capable of giving specific SRB community structure or composition information it is necessary to obtain as many pure standard genomes as possible. Colony purified isolates will be obtained from some of the liquid culture enrichments and DNA from these colony purified SRB will be characterized by comparison to DNA from earlier enrichments.

2.4 Identification of standards by 16S rRNA gene sequencing

Standards selected following gene and genome probing, as in section 2.1, will be characterized further by sequencing a portion of their 16S rRNA genes. Homologies between sequences obtained for standards in this study and previously determined 16S rRNA gene sequences for known species will be detected by comparisons with a database of 16S rRNA sequences.

Chapter 3. Materials and methods

3.1 Materials

3.1.1 Biochemical reagents

All enzymes and their supplied dilution buffers were purchased from Pharmacia, Boehringer Mannheim, or Gibco BRL. Hybond-N membrane was purchased from Amersham. All deoxyoligonucleotides and random hexanucleotide primers were synthesized by the regional DNA Synthesis Laboratory (University of Calgary, Calgary, Canada). Lambda phage DNA (λ), deoxynucleotides and dideoxynulceotides were purchased from Pharmacia. Radiochemicals [α-^{32}P]-dCTP (3000 Ci/mmol, 10 mCi/ml), which was used for random hexamer labeling, and [γ-^{32}P]-dATP (3000 Ci/mmol, 10 mCi/ml), which was used for deoxyoligonucleotide 5′-end labeling, were purchased from ICN. The radiochemical [α-^{35}S]-dATP (400 Ci/mmol, 10 mCi/ml) used for dideoxy sequencing and for λ DNA 5′-end labeling was purchased from Amersham. Ficoll 400 was purchased from Pharmacia. Bovine serum albumin fraction V (BSA), polyvinylpyrrolidone (molecular weight 40 000 Da) (PVP), molecular biology-grade sodium dodecylsulfate (SDS) and salmon sperm DNA (sodium salt) were all purchased from Sigma. High and low gelling temperature (HGT and LGT) agarose, ethidium bromide and reagent grade phenol were obtained from Boehringer Mannheim. Bactotryptone and yeast extract were from Difco Laboratories. Molecular biology-grade dextran sulfate (sodium salt, molecular weight 500 000), Acrylamide, N, N′-methylenebisacrylamide, formamide, NNN′N′-tetramethylethylenediamine (TEMED), and agar-agar were purchased

from BDH. All other reagent-grade chemicals were obtained from Fisher or Sigma.

3.1.2 Buffers and frequently used solutions

TE buffer is 10 mM Tris-HCl, 0.1 mM EDTA, pH 8. 1 X SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2. 1 X TAE is 0.04 M Tris acetate, 0.002 M EDTA, pH 8.0. 1% (w/v) HGT and 0.7% (w/v) LGT agarose gels are prepared in 1 X TAE. 1 X TBE is 0.09 M Tris, 0.09 M boric acid and 0.002 M EDTA.

3.1.3 Bacterial strains and standards

All bacterial strains are described in Table 3-1 and were available in the laboratory.

3.1.4 Plasmid and vectors

Plasmid pHCAL2 is described in Table 3-1. Cloning vectors M13mp18 and M13mp19 are described in Table 3-1 and were obtained in the replicative form (RF) from Boehringer Mannheim.

3.1.5 Deoxyoligonucleotides

Two 33-mer primers with internal restriction sites, EUB-338 (CCGGATCCACTCCTACGGGAGGCAGCAG[G/A]GGAA), (*Bam*HI) and Univ-907R (CCAAGCTTCGGGCCCGTCAATT[C/T]CTTTGAGTTT) (*Hind*III) were used for 16S rRNA gene amplification with PCR (Amman *et al.*, 1992). Restriction sites are underlined. These primers were obtained in the 5' phosphorylated form. Four deoxyoligonucleotides, P75, P76, P77 and P78, were

Table 3-1. Bacterial strains, vectors and plasmids.

Strain, vector, or plasmid	genotype, comments and reference
E. coli TG2[a]	Δ(*lac-pro*) *sup*E *thi hsd*M *hsd*R *rec*A F' [*tra*D36 *pro*AB$^+$ *Lac*ZDM15Iq]
Desulfovibrio vulgaris	NCIMB 188399
D. multispirans	NCIMB 11312078
*Lac*1,2	*D. vulgaris* subsp. *oxamicus* (Voordouw *et al.*, 1990)
*Lac*3	*D. desulfuricans* G200 (Voordouw *et al.*, 1990)
*Lac*6	*Desulfovibrio sp.*(Voordouw *et al.*, 1991)
*Lac*15	*Desulfovibrio sp.*(Voordouw *et al.*, 1992)
*Pro*5	*Desulfobulbus sp.*(Voordouw *et al.*, 1992)
pHCAL2	Contains the *D. vulgaris* Miyazaki F *hyn*A gene and the 3' end of the *hyn*B gene on a 3.8 kbp *Eco*RI fragment cloned into pUC8 (Deckers *et al.*, 1990)
M13mp18RF, M13mp19RF	*E. coli* cloning and single strand vectors (Messing & Vieira, 1982)

[a] Constructed from *E. coli* JM101 by T. J. Gibson and M. D. Biggin at the Laboratory of Molecular Biology, MRC Centre, Cambridge, United Kingdom

targeted to hybridize to conserved regions of SRB 16S rRNA genes, and were used for sequencing. P75 is ACCGCGGC(G/T)GCTGGC, P76 is complementary to P75, P77 is GAT(A/C)TCTACG(G/A)ATTTCAC, and P78 is complementary to P77. Three deoxyoligonucleotides, P88, P89 and P90, were designed from 16S rRNA gene sequences obtained in this work and were targeted to hybridize to highly variable regions of the 16S rRNA genes (i. e. they were species specific). P88 is GGAGGACGTGTCTCTTTTG, P89 is GTTTGCGATACACAGTAAGC, and P90 is CCCTTGGATTCGAATAGGG.

3.1.6 Hybridization solutions and conditions

Hybridization solutions were for either stringent or non-stringent hybridization conditions. Stringent pre-hybridization and hybridization solution was; 6 X SSC, 0.2% (w/v) SDS, 0.1% (w/v) each of Ficoll 400, PVP and BSA and 0.01% (w/v) denatured salmon sperm DNA. The wash solutions for the stringent procedures were 1 X SSC and 1 X SSC, 0.2% SDS. The conditions for stringent procedures were; i) pre-hybridization at 68°C for 4 hours, ii) hybridization at 68°C, overnight. iii) 2 washes at 22°C in 1 X SSC for 5 min each, iv) 1 wash in 1 X SSC, 0.2% SDS at 68°C for 60 min.

Non-stringent pre-hybridization and hybridization solution was; 50% (v/v) formamide, 0.2% (w/v) each of PVP, Ficoll 400, and BSA, 0.05 M Tris hydrochloride (pH 7.5), 1 M NaCl, 0.1% (w/v) sodium pyrophosphate, 0.1% (w/v) SDS, 10% (w/v) dextran sulfate and 0.05% (w/v) denatured salmon sperm DNA. The wash solutions for the non-stringent procedures were: solution A, 0.3 M NaCl, 0.06 M Tris hydrochloride (pH 8.0), and 0.002 M EDTA; solution B, solution A with 0.5% (w/v) SDS; and solution C, 0.003 M Tris base. The

conditions for the non-stringent procedures were i) pre-hybridization at 42°C for 4 hours, ii) hybridization at 42°C overnight, iii) one wash in 100 mL solution A, 22°C for 5 min, iv) one wash in 100 mL solution B, 60°C for 30 min, v) one wash in 100 mL solution C, 22°C for 30 min.

For both sets of conditions, after the final wash, all filters were air dried on 3MM paper then wrapped in Saran Wrap prior to autoradiography.

3.1.7 *Escherichia coli* growth media and conditions

E. coli growth media recipes were as in Sambrook *et al.* (1989). Liquid tryptone yeast extract (TY) medium contained 10 g of bactotryptone, 5 g of yeast extract and 5 g of NaCl per liter of water, pH 7.4. Solid TY media had 1.5% (w/v) agar added to the above liquid media. When necessary for screening, TY plates had isopropyl-β-D-thiogalactoside (IPTG; 20 µL of 20 mg/mL per plate) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 40 µL of 20 mg/ml in dimethylformamide per plate). When necessary for growth of M13 phage infected *E. coli*, TY top agar (TY liquid media with 0.75% (w/v) agar) was used. All *E. coli* cultures were grown at 37°C. Liquid bacterial cultures were shaken at 250 rpm and liquid phage cultures were shaken at 300 rpm. *E. coli* TG2 was stored on M9 minimal glucose medium plates at 4°C. M9 minimal glucose plates contained 1 X M9 salts (Miller, 1972), 0.2% (w/v) glucose, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 2 µg/ml thiamin and 1.5% (w/v) agar. Other *E. coli* cultures were stored on TY plates at 4°C. Long term storage of *E. coli* was in glycerol at -70°C.

3.2 Environmental samples

The samples from which SRB were enriched were provided by a variety of sources and agencies (Table 3-2) and belonged to four environmental types, soil, acid mine drainage, oil sands production waters, and sewage. They were, but for two, from industrial sites. Two sample sets provided by personnel at the Canada Center for Mineral and Energy Technology (CANMET) in Ottawa represented two, separate laboratory microcosm experiments.

3.2.1 Acid mine drainage samples

CANMET supplied acid mine drainage (AMD) samples from a laboratory microcosm which was being used to evaluate a continuous-flow, mixed aerobic-anaerobic microbial process using cellulosic substrates for growth as a treatment for acidic, metal rich waters (Béchard *et al.*, 1993, 1994). Two bioreactors were set up with this process, the first supplemented with straw and sucrose (Béchard *et al.*, 1993) from which one water sample was taken, and the second with alfalfa hay (Béchard *et al.*, 1994) from which two water samples were taken. These samples were named CANMET AMD 1, 2, and 3 respectively.

Nine waste water samples of acid mine drainage origin were provided by Boojum Research Limited of Toronto. These samples, named Boojum, were taken from locations where different AMD treatment methods were being tested. Samples 1 and 2 were obtained from a nickel mine in Makela, Ontario, where a straw dam was installed to slow the flow of the acidic waters and to provide carbon and energy sources for the microbial population. Sample 1 was taken from the bottom of the pond formed by this dam and sample 2 was taken from the surface of the pond. Sample 3 was taken from the surface sediment at the

Table 3-2. Description of sample environments, sources, and the numbers of DNA preparations obtained.

Sample Name	Environment[a]	Sample source	N[b]	n[c]	x[d]
CANMET AMD	Acid mine drainage bioremediation test project (ww)	Two bioreactors containing acid mine drainage waters and 1) straw/sucrose or 2) alfalfa hay	3	18	10
Boojum	Acid mine drainage field remediation test projects (ww)	Mine tailings from: 1, 2 Nickel mine 3 Uranium mine 4 Zinc mine 5-9 Coal mine	9	54	5
Bell Creek	Acid mine drainage (so)	Copper mine tailings impacted swamp	1	6	1
Uranium mine	Acid mine drainage (ww)	Uranium mine tailings	1	4	1
AMD	Acid mine drainage (ww)	metal mine tailings	3	15	1
Suncor	oil sands production water (ww)	depths in a tailings pond; 23, 33, and 43 feet	3	22	4
Syncrude	oil sands production water (ww)	depth ranges in a tailings pond; 1-9, 6-10, 5-11, and 16-20, plus one additional pond sample	5	30	1

Table 3-2 continued.

Sample Name	Environment[a]	Sample source	N[b]	n[c]	x[d]
CANMET Soil	simulated pipeline digout microcosm (so)	soil taken from positions around a section of pipe in a laboratory	20	20	18
Digout	field pipeline digout (so)	soil taken from positions around an oil pipeline	14	-	43
Sewage B	sewage (ww)	anaerobic digestor at a sewage treatment plant	1	13	6
Gold Bar	sewage (ww)	anaerobic digestor or activated sludge	3	16	0
Capitol Region	sewage (ww)	wastewater from a sewage treatment plant	1	4	0

[a] Environments are either soil (so) or wastewater (ww).
[b] number of samples taken from the site.
[c] number of DNA preparations obtained from the samples after primary liquid enrichment.
[d] number of DNA preparations obtained from colony purified isolates.

outflow of a pond near Elliott Lake, Ontario, which was receiving AMD from a Uranium mine and had been amended with hay bales. Sample 4 represented a zinc mine near Selbaie, Quebec. This sample was taken from a pond in a bog that received AMD during spring run-off. Samples 5-9 were obtained from coal mining operations near Sydney, Nova Scotia. Sample 5 was taken from the middle of a porous bag containing alfalfa pellets that had been placed in a bog through which AMD was flowing. Sample 9 was from an untreated bog. The effluent from this bog flowed into test cells that were amended with hay bales. Sample 8 was collected from a test cell. Flow from the test cells then went into a bog supplemented with slow-release fertilizer, the source of sample 6. Finally, the flow from this bog went trough a ditch in which alfalfa pellets were added to help generate reducing conditions. Sample 7 was taken from this ditch.

One soil sample from a swamp in British Columbia that had been impacted by AMD from the Bell Creek copper mine was provided by EVS Environmental Consultants of North Vancouver. This was named Bell Creek. One water sample was obtained from a barrel containing AMD collected from a Uranium Mine. This sample is referred to as Uranium mine. Three water samples were taken from metal AMD tailings ponds and are called AMD.

3.2.2 Oil sands production water samples

The oil sands production waters samples were obtained from two companies, Suncor Inc. Oil Sands Group and Syncrude Canada Ltd., from separate oil sand extraction plants in northeastern Alberta. These companies use surface mining methods to obtain the oil sands that are subsequently extracted with hot water and NaOH to recover bitumen from the solids. The remaining

extracted solids are pumped into large tailings ponds to allow the solids to settle so that the clear surface water can be reused in the process (MacKinnon and Sethi, 1993). These ponds were the source of the samples. The Suncor tailings pond was sampled at three depths, 23, 33 and 43 feet, to provide Suncor 23', Suncor 33' and Suncor 43'. The Syncrude pond was sampled in depth ranges to provide Syncrude samples 1-9, 6-10, 5-11 and 16-20. The numbers reflect depths in feet. An additional Syncrude sample (pond) was obtained from the pond, but from an unspecified depth.

3.2.3 Soil samples

The second set of samples provided by CANMET was taken from a laboratory microcosm studying microbially influenced corrosion. The corrosion study was being carried out on a section of a gas pipeline that had been in service for 17 years prior to excavation and burial in the laboratory test chamber. The experimental design, conditions, and numbers of SRB in the chamber are described by MacLeod *et al.* (1992). Twenty samples were taken from various positions around the pipe and were both soil and disbondment samples. These samples were named CANMET Soil.

Soil samples were also obtained from Nova Husky during a field pipeline digout in March/April of 1993. Fourteen soil samples were taken from different locations around the pipe and named Digout.

3.2.4 Sewage samples

Sewage samples were obtained from two sewage treatment plants in Edmonton, Gold Bar and Capitol Region. Three Gold Bar samples were obtained

from an anaerobic digestor and one from activated sludge, representing three sampling trips. The sample from the first trip was designated Sewage B, and those from the next two trips were designated Gold Bar and either Anaerobic digestor (Gold Bar) or Activated sludge (Gold Bar). The Sewage B sample, the Gold Bar sample, and the Anaerobic digestor (Gold Bar) sample were obtained from anaerobic digestor #2 at 26 feet. The Activated sludge (Gold Bar) sample was obtained from activated sludge. One Capitol Region sample was obtained.

3.3 Methods

3.3.1 SRB enrichment from environmental samples

Anaerobic conditions were maintained throughout the culturing procedures primarily by innoculation and growth of cultures in an anaerobic hood with a mixed nitrogen, carbon dioxide, and hydrogen atmosphere (85%:10%:5% respectively). Sealed, anaerobic, still cultures were used for growth outside of the hood. SRB were enriched from all but the CANMET Soil samples at the University of Alberta, Edmonton by S. Ebert and N. Sifeldeen. The CANMET Soil samples were received at The University of Calgary as SRB enrichments in 20 mL Postgate B. Postgate media used for enrichment and routine culture of SRB from the CANMET Soil samples are described in Table 3-3 (Postgate, 1984). The Postgate B enrichment cultures had been grown for four weeks at 30°C at CANMET and were stored at 4°C. These cultures were used to prepare primary cultures in Postgate C media (1 mL culture into 100 mL of Postgate C) which were grown at 37°C until turbid, generally between two days and one week, at

Table 3-3. Postgate growth media for SRB enrichment

Chemical	Postgate B		Postgate C		Postgate E	
	g[a]	mM[b]	g[a]	mM[b]	g[a]	mM[b]
KH_2PO_4	0.5	3.7	0.5	3.7	0.5	3.7
NH_4Cl	1.0	20	1.0	20	1.0	20
$MgSO_4 \cdot 7H_2O$	1.0	4	0.03	0.12	1.8	7.3
sodium lactate	3.5	nc	8.6	nc	3.5	nc
yeast extract	1.0	nc	1.0	nc	1.0	nc
$FeSO_4 \cdot 7H_2O$	0.5	1.8	0.004	0.014	0.5	1.8
$CaSO_4$	1.0	7.3	-	-	-	-
Na_2SO_4	-	-	4.5	32	1.0	7.0
$CaCl_2 \cdot 2H_2O$	-	-	0.042	0.29	0.7	4.8
Na_3citrate	-	-	0.28	0.95	-	-
ascorbic acid	0.1	0.6	-	-	0.1	0.6
thioglycollic acid	0.1	0.9	-	-	0.1	0.9
agar	-	-	-	-	15	nc

[a] grams added per litre of solution. '-'=the chemical was not added.

[b] milliMolar concentration. nc=mM concentration has not been or can not be calculated.

which time DNA was harvested. Genomic analysis required growth of some secondary Postgate C cultures from the Postgate B original enrichments. After genomic characterization of the primary and secondary cultures, eight of the original Postgate B enrichments were chosen for colony purification (Table 4-2). 1 mL aliquots of the original Postgate B cultures were again grown in Postgate C media at 37°C until turbid. Aliquots of these enrichments were then streaked onto Postgate E plates anaerobically, and onto both Postgate E or TY plates aerobically. Plates were incubated at 37°C for one to two weeks. Two single colonies from each plate were restreaked onto the same media under the same conditions. This step was repeated once, then single colonies were inoculated into 5 mL of Postgate B medium. After growth at 37°C (generally two days to one week), 1 mL of the Postgate B cultures was used to inoculate 100 mL of Postgate C media. These scale-up cultures were grown as above to provide sufficient cell material for DNA extraction.

All samples processed at the University of Alberta were inoculated into 75 mLs of Widdel/Pfennig (W&P) medium (Pfennig *et al.*, 1981) which contained one of the following carbon and energy sources (g/L): acetate (0.9), ethanol (0.48), propionate (1.5), lactate (1.3), decanoate (0.5), or benzoate (0.42). All samples were treated as freshwater samples, therefore the medium contained only 1 g NaCl per litre. The inoculum was either 7.5 mL of a liquid sample, or 7.5 g of a sediment or soil sample. All cultures were incubated at room temperature and transferred approximately monthly until the solids that had accompanied the inoculum had been removed by dilution. The culture resulting from the final transfer was used to inoculate three bottles of W&P medium (75 mL each) to produce sufficient cell material for DNA extraction. These cultures are

collectively referred to as the primary enrichment culture. All primary enrichment cultures had aliquots stored at -70°C. These aliquots were the inoculum source for subsequent cultures required for additional genomic material, as was the case for the Sewage B, or for additional procedures described below. This SRB enrichment protocol has been described previously by Voordouw *et al.* (1991).

Several additional procedures were carried out on cultures from selected samples at the University of Alberta. A secondary CANMET AMD 2 lactate enrichment and a CANMET 1 benzoate enrichment were subjected to a $HgCl_2$ treatment. Aliquots of the secondary enrichments were plated onto both W&P media or Blood agar (Difco Manual, 1984) that either contained or did not contain mercuric chloride. Colonies were selected and inoculated into Hungate tubes containing W&P media. Sewage B benzoate and ethanol secondary enrichments were colony purified by repeated transfer between W&P plates and Hungate tubes containing W&P media. All Digout enrichments were also colony purified as follows: Aliquots from primary liquid enrichment cultures were plated onto W&P plates, three colonies (a, b, and c) were selected from each plate and inoculated into W&P medium in Hungate tubes. Scale-up from Hungate tubes for DNA extraction was into W&P media as described above (Voordouw *et al.*, 1991), and all conditions were anaerobic.

The viability as well as the genomic characteristics of the stored aliquots of primary cultures were verified following regrowth. A number of primary cultures which had their DNA well characterized were colony purified, as described for Sewage B. Aliquots of scale-up cultures from colony purified isolates were stored at -70°C as previously described. These scale-up cultures are

referred to as primary colony purified cultures and were used as the inoculum for subsequent cultures of colony purified isolates.

3.3.2 Genomic DNA extraction and purification

Genomic DNA from all cultures, except for those resulting from CANMET Soil samples, was prepared at the University of Alberta. DNA from enrichment cultures that grew poorly was extracted by the method of Somerville *et al.* (1989). Otherwise, DNA was extracted by a modified Marmur procedure (Voordouw *et al.*, 1991). DNA preparations are described in relation to the culture from which they were obtained (e. g. name of sample, carbon and energy source used, primary, secondary, primary colony purified, etc.; see Table 4-1). The total number of primary DNA preparations obtained for each set of samples is detailed in Table 3-2 along with the number of DNA preparations obtained from primary colony purified isolates.

The DNA was often found to be poorly suited for genomic analysis (e. g. incompletely digested, inadequately labeled in cross-hybridization experiments), thus all preparations were subjected to a cleaning procedure to remove salts and other contaminants. An equal volume of 20% (w/v) polyethylene glycol (PEG 8000), 2.5 M NaCl was added to the DNA preparations. After overnight precipitation at 6°C, DNA was recovered by centrifugation for fifteen minutes at 14 000 x g. The pellet was resuspended in 200 µL of TE buffer and DNase free RNase was added to a final concentration of 100 ng/µL. This was incubated at room temperature for thirty minutes, then Proteinase K was added to a final concentration of 100 ng/µL. This was again incubated at room temperature for sixty minutes, then extracted with one third of a volume of TE saturated phenol.

The preparations were vortexed for two minutes, then centrifuged at 14 000 x g for two minutes and the upper layer was transferred. 2.5 volumes of DNA precipitation mix (95% (w/v) ethanol, 2.5 M sodium acetate) was added and precipitation was carried out overnight at -20°C. The DNA was harvested by 10 minutes of centrifugation at 6°C, 14 000 x g, followed by one wash with 70% (w/v) ice-cold ethanol. The pellet was dried and resuspended in TE.

DNA concentrations were estimated by a fluorimetric method (Voordouw *et al.*, 1993). 20 mL of 1% HGT agarose to which 10 μg of ethidium bromide was added was poured into a plate. Plates were set overnight. DNA preparations were boiled for 2 min then cooled rapidly on ice. 2 μL aliquots of this denatured DNA was spotted in an ordered grid on the plate. λ DNA in a dilution series from 100 ng/μL to 5 ng/μL was also boiled and spotted as above. After 1 hour the intensity of the DNA preparation dots when viewed at 312 nm was compared to the intensity of the λ DNA dots and concentrations were assigned based on this comparison.

3.3.3 Dot Blot Preparation

Dot blots required for [NiFe] hydrogenase gene probing of the primary DNA preparations were assembled in five batches as the primary DNA preparations became available. Hybond-N membranes were divided into grids with 1 x 1 cm squares containing all primary DNA preparations to be analyzed plus squares for positive (three *Desulfovibrio* standards, *Lac*1,2, *Lac*6 and *Lac*15) and negative (no DNA) controls. Aliquots of all primary DNA preparations and the controls were diluted to 20 ng/μL with TE. These were first boiled for 2 min, then cooled on ice prior to spotting 2 μL volumes (40 ng) onto the membranes.

Dot blots were left to dry at room temperature for two to three hours. One blot was prepared for each set of preparations.

Dot blots were also required for whole genome probing. Fifty multi-environment filters were prepared containing DNA from 50 primary cultures (the selection criteria will be described in sections 4.1.2 and 4.1.3), two pure cultures (*Desulfovibrio vulgaris* NCIMB 188399 and *D. multispirans* NCIMB 11312078), and two standards from past work (*Lac*3 and *Lac*15). All DNA preparations were diluted to 20 ng/μL. A dilution series of λ phage DNA was also included on these filters as a positive control for labeling and hybridization (Voordouw *et al.*, 1993). Ordered grids with 1 x 1 cm squares were drawn on Hybond-N membrane. DNA preparations were boiled and cooled on ice and 2 μL volumes (40 ng) were spotted using a Hamilton repeat dispensing pipette (dispenses 2 μL volumes sequentially up to a total volume of 50 μL). The filters were left overnight to dry.

Separate dot blots were prepared for the analysis of sewage and soil DNA preparations. Sewage B derived DNA preparations, Sewage B secondary colony purified DNA preparations, and Anaerobic digestor (Gold Bar) and Activated sludge (Gold Bar) primary DNA preparations were spotted as for the multi-environment filters, but with only 25 filters being prepared. *Lac*15 was included as a standard. These filters were used for both [NiFe] hydrogenase gene and whole genome probing. The primary colony purified DNA preparations from Digout samples were spotted as above with only 40 filters being prepared for whole genome analysis. Twenty filters of CANMET Soil mixed primary/secondary DNA preparations and twenty filters of CANMET Soil primary colony purified DNA preparations were also prepared as above for

analysis with both the [NiFe] hydrogenase and whole genome probes. Both of these CANMET sets of filters contained *Lac*15 and two Digout preparations (777 and 776). The λ dilution series was not included on any of the sewage or soil filters.

After spots had dried (2 hours to overnight), dot blots were UV irradiated at 312 nm for 3 min to immobilize the DNA on the filter. Filters were then ready for pre-hybridization.

Dot blots containing DNA from oil field production water standards were available in the lab. Standards used on the filters were described by Voordouw *et al.* (1992, 1993). These dot blots had a dilution series of λ DNA spotted on them.

3.3.4 Southern Blot preparation

Southern blots of genomic DNA preparations digested with *Eco*RI were prepared for hybridization with the [NiFe] hydrogenase gene and for ribotyping. Restriction digest mixes were; 10 μL of undiluted DNA preparation, 6 μL of sterile H_2O, 2 μL of One Phor All buffer and 2 μL of *Eco*RI. These were digested at 37°C for five to six hours, then the enzyme was denatured at 68°C. 2 μL of bromophenol blue dye was added to each mix and the contents of each tube was loaded onto 1% (w/v) high gelling temperature (HGT) agarose gels with generally 8-12 digested preparations per gel. *Lac*15 DNA was also digested with each set of preparations and loaded on each gel. To provide molecular weight markers after Southern blotting, λ DNA was digested with *Hind*III for two hours, then the enzyme was denatured by incubation at 68°C for five minutes. This mix was end-labeled by the addition of; 6 μL of H_2O, 2 μL of One Phor All buffer, 1 μL of Klenow Polymerase and 1 μL of [α-35S]dATP. End-labeling proceeded for

one hour at 22°C and was quenched by the addition of 30 µL of TE. 10 µL of end-labeled λ DNA was combined with 10 µL of unlabeled, *Hind*III digested λ DNA and loaded on each gel. Electrophoresis was either at 100 V for 3.5 hours or at 18 V overnight in 1 X TAE buffer. DNA was visualized by staining with 50 µg ethidium bromide in 100 mL of 1 X TAE buffer. DNA was transferred by the method of Southern (Maniatis *et al.*, 1982) to Hybond-N membrane. All primary DNA preparations except for those from Anaerobic digestor (Gold Bar) and Activated sludge (Gold Bar) samples were digested, electrophoresed and transferred for ribotyping. Only primary DNA preparations that tested positive for the [NiFe] hydrogenase gene were blotted for hybridization with the [NiFe] hydrogenase gene probe.

3.3.5 Probe preparation

The [NiFe] hydrogenase gene probe was prepared from plasmid, pHCAL2 (Deckers *et al.*, 1990). pHCAL2 was digested with *Eco*RI and the 1.0 kb fragment was gel isolated.

The 16S rRNA gene fragment used for ribotyping was generated with PCR from *Lac*3 (*D. desulfuricans* G200). PCR conditions are detailed in section 3.3.7. A fragment of approximately 500 bp was obtained and was gel isolated.

Whole genome probes were genomic DNA preparations or purified genomic DNA from standards. These were generally used at a concentration of 20 ng/µL.

The random hexamer method for labeling was used in all three cases (Voordouw *et al.*, 1992). For the [NiFe] hydrogenase gene probe and the 16S rRNA gene probe, 3 µL of the purified fragment was diluted with 7 µL of water.

For whole genome probes, 10 μL of the 20 ng/μL dilutions were used. For some whole genome labeling reactions, 100 pg of λ DNA (10 mL of 10 pg/μL) was added to the genomic DNA. In all cases, DNAs were boiled for 2 min, then rapidly cooled on ice immediately prior to labeling. Labeling mixture was; denatured DNA (10 or 20 μL), 6 μL of primer extension mix (PE; Voordouw *et al.*, 1992), 2 μL of Klenow Polymerase (2 U/μL) and 2 mL of [α-^{32}P]-dCTP. Labeling was for four to six hours at 22°C.

Deoxyoligonucleotide probes were end-labeled as described in Sambrook *et al.* (1989).

3.3.6 Probe/Filter hybridization conditions

The [NiFe] hydrogenase gene probe was hybridized to dot blots and Southern blots. In both cases non-stringent conditions were used and autoradiography was generally for two days to one week. The 16S rRNA gene probe was hybridized to Southern blots only and non-stringent conditions were used. Autoradiography was generally for five to seven days. The whole genome probes were hybridized to dot blots only and stringent conditions were used. Autoradiography was generally for one to three days. Deoxyoligonucleotides were hybridized to Southern blots using stringent conditions. Autoradiography was generally from one to three days.

3.3.7 Cloning 16S rRNA gene fragments

Amplification of 16S rRNA gene fragments with lengths of approximately 500 bp used the Polymerase Chain Reaction (Saiki *et al.*, 1988) with genomic DNA and two general, eubacterial primers, EUB-338 and Univ-907R. The

amplification mix contained, in a total volume of 100 µL; 200 ng genomic DNA, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 0.8 mM each of dATP, dCTP, dGTP and dTTP, 1 µM of each primer and 2.5 Units of Taq polymerase. 60 µL of mineral oil was used as an overlay to prevent evaporation. PCR cycles were; denaturation at 94°C for 2 min, annealing at 64°C for 2 min and extension at 72°C for 2 min for 30 cycles, followed by a 9 min final extension at 72°C. Amplified product was gel purified and resuspended in final volume of 50 µL.

Cloning was either into the *Sma*I site of M13mp18 (blunt end cloning), or into *Hind*III, *Bam*HI digested M13mp18 and M13mp19 (sticky end cloning). Vector digestion with restriction enzymes, ligation of vector and PCR fragment, and transfection of ligation mix were essentially as described by Sambrook *et al.* (1989). For blunt end cloning, M13mp18 was also dephosphorylated and phenol extracted after digestion and prior to ligation (Sambrook *et al.*, 1989). Ligation mixes were transfected into *E. coli* TG2 and screened with α-complementation (Sambrook *et al.*, 1989). Phage isolation and the subsequent purification of single stranded DNA from phage particles used the procedure of Sanger *et al.* (1977) and Bankier and Barrell (1983) with one additional step. After phage particles were isolated but before the DNA was extracted from them, phage preparations were incubated with 15 µg of DNase free RNase at 22°C for 30 min. This was to remove contaminant rRNA from the cells. Extracted DNA from phage particles was resuspended in a final volume of 40 µL of TE.

For blunt-end clones, one of two screening procedures was used in addition to α-complementation to test for the presence of a 16S rRNA gene fragment insert; (i) hybridization between the radiolabeled *Lac*3 16S rRNA gene fragment and dot blots of unknown clones (prepared as in sections 3.3.3 and

3.3.5, with non-stringent hybridization solutions and conditions used), or (ii) hybridization between M13 clones known to contain a 16S rRNA gene fragment and the unknown clones in a gel shift assay. Since M13 clones isolated were single stranded, and the strand (positive, +, or negative, -) of the 16S rRNA gene fragment in the unknown clone was undetermined, testing with both strands of the confirmed positive was required. The hybridization reaction mix was; 6 X SSC, 2 μL of confirmed clone (either + or -), and 2 μL of unknown clone in a volume of 10 μL. Reaction mixtures were incubated at 68°C for one hour, then electrophoresed on 1.0 % HGT agarose gels with positive and negative controls (confirmed + and - strand clones, and confirmed + and + strand clones respectively). The shift of unknown clones when hybridized with each strand of a confirmed clone was compared to the shift of the positive and negative controls. Additional screening besides α-complementation was found to be unnecessary for sticky-end cloning.

3.3.8 Sequencing of positive clones.

Clones were sequenced by essentially the dideoxy-chain termination procedure of Sanger *et al.* (1977), with either Sequenase or T7 polymerase (Tabor and Richardson, 1987), using the protocol of the appropriate manufacturer. Primers used were either the M13 Universal primer, or one of P75, P76, P77 or P78. The P75/P77 primer set was hybridized to clones with the negative strand insert (defined as the reverse orientation with regards to the 16S rRNA gene) while the P76/P78 primers were hybridized to clones with the positive strand insert. Sequence compilation and analysis was with the Staden programs

(Staden, 1984). Sequences were compared to databases either with Fasta of the GCG package (Devereux *et al.*, 1984) or with Blast searches (Altschul *et al.*, 1990).

Chapter 4. Results

4.1 DNA preparations

All liquid enrichment cultures of samples processed at the University of Alberta yielded 180 DNA preparations for genomic characterization (Table 4-1). All DNA preparations were assigned chronological numbers as they were received from Edmonton. Subsequent cultures yielded 65 DNA preparations and colony purifications yielded 40 preparations. These were also assigned chronological numbers. No DNA preparations were obtained from original liquid enrichments of the Digout samples. Instead, genomic DNA was prepared from 43 colony purified isolates only.

Twenty primary CANMET Soil DNA preparations representing all twenty samples (Postgate B enrichments) were obtained from Postgate C liquid cultures. Ten secondary Postgate C enrichment cultures were required to provide sufficient genomic DNA for whole genome analysis of all 20 samples. Postgate B enrichments chosen for colony purification are described in Table 4-2. Colony purification used five secondary cultures and three tertiary cultures inoculated from the Postgate B enrichments. No growth was observed from any of the eight cultures on TY plates. Growth was observed from six of the eight cultures on aerobic Postgate E plates, only (the tertiary culture from S-6-31-3 and the secondary culture from 10-6-1 did not produce colonies. All eight selected cultures gave colonies on anaerobic Postgate E plates with lactate as the carbon and lactate and/or hydrogen as the energy source. Two of the cultures (the secondary culture from 5-3-2 and the tertiary culture from 10-9-1) grew successfully on Postgate E plates using acetate as the carbon and acetate and/or

Table 4-1 Hybridization results for all DNA preparations

#	Preparation description	type	[NiFe]	16S code
71	CANMET AMD 1 lactate	pc	+	amLac3
72	CANMET AMD 2 lactate	pc	+	amLac4
145	CANMET AMD 2-2Bh lactate	cp	-	-
146	CANMET AMD 2-2B lactate	cp	+	-
147	CANMET AMD 2-4Bh lactate	cp	+;4.9	amLac4
148	CANMET AMD 2-2A lactate	cp	-	amLac4;?
149	CANMET AMD 2-1B lactate	cp	+	amLac5
150	CANMET AMD 2-2Aa lactate	cp	-	amLac3;amLac4
151	CANMET AMD 2-2Ah lactate	cp	+;4.9	amLac4
73	CANMET AMD 3 lactate	pc	-	amLac5
215	CANMET AMD 1 lactate (71-1)	sc	nd	-
216	CANMET AMD 2 lactate (72-1)	sc	nd	-
217	CANMET AMD 3 lactate (73-1)	sc	nd	-
314	CANMET AMD 2a lactate	cp	nd	
126	CANMET AMD 1 acetate	pc	-	amLac2
127	CANMET AMD 2 acetate	pc	-	amLac6?;amAce2?
128	CANMET AMD 3 acetate	pc	-	-
231	CANMET AMD 1 acetate (126-1)	sc	nd	-
239	CANMET AMD 2 acetate (127-1)	sc	nd	-
240	CANMET AMD 3 acetate (128-1)	sc	nd	-
269	CANMET AMD 3 acetate (128-2)	sc	nd	
152	CANMET AMD 1 benzoate	pc	+	-
153	CANMET AMD 2 benzoate	pc	-	nb
154	CANMET AMD 3 benzoate	pc	-	nb
163	CANMET AMD 1-1h benzoate	pc	-	-
164	CANMET AMD 1 propionate	pc	+	-
197	CANMET AMD 2 propionate	pc	-	-
165	CANMET AMD 3 propionate	pc	+	amPro1
227	CANMET AMD 3 propionate (165-1)	sc	nd	-
172	CANMET AMD 1 decanoic	pc	-	-

Table 4-1 continued.

#	filter used/Cross-hybridizations	std. name
71	mf/amLac3:ofpwf/nh	amLac3
72	mf/amLac4	Lac30
145	mf/amLac4 (145, 146, 150)	Lac30; amLac11
146	mf/sh (145, 150): ofpwf/nh	Lac11
147	mf/amLac4 (145, 150): ofpw/(Lac30)	Lac30
148	mf/amLac4 (145, 150): ofpw/(Lac30)	Lac30
149	amLac5: ofpwf/nh	amLac5
150	mf/150(amLac4, 145, 146)	Lac30; amLac11
151	mf/amLac4 (145, 150)	Lac30
73		
215	mf/(amLac2,amBen1,amLac3,amPro4)	na
216	mf/(145,150,amLac4)	Lac30
217		na
314		
126		na
127		na
128	mf/sh	amAce4
231		na
239		na
240	mf/(66,amAce4,tpEth2,amPro1)	na
269	mf/(amPro1,tpPro5, 21,128)	na
152		na
153		na
154		na
163		na
164		na
197		na
165	mf/amPro1(203,204,tpEth2,tpPro1/5): ofpw/(Pro5)	amPro1
227	mf/(204,tpEth2,amPro1,tpPro1/5,)	amPro1
172		na

Table 4-1 continued.

#	Preparation description	type	[NiFe]	16S code
173	CANMET AMD 2 decanoic	pc	-	-
174	CANMET AMD 3 decanoic	pc	+	-
180	CANMET AMD 1 ethanol	pc	+	-
181	CANMET AMD 2 ethanol	pc	+	-
182	CANMET AMD 3 ethanol	pc	-	-
270	CANMET AMD 1 ethanol (180-1)	sc	nd	
228	CANMET AMD 2 ethanol (182-1)	sc	nd	-
253	BSR (CANMET AMD)	cp	nd	
76	Boojum 1 lactate	pc	+: 2.4; 1.6	amLac6c
75	Boojum 2 lactate	pc	+	amLac6b
77	Boojum 3 lactate	pc	+	amLac7
78	Boojum 4 lactate	pc	+	amLac8
79	Boojum 5 lactate	pc	-	amLac9
80	Boojum 6 lactate	pc	+	nb
81	Boojum 7 lactate	pc	-	-
82	Boojum 8 lactate	pc	+: 1.5	-
74	Boojum 9 lactate	pc	+: 1.6	amLac6a
220	Boojum 1 lactate (76-1)	sc	nd	-
219	Boojum 2 lactate (75-1)	sc	nd	-
221	Boojum 3 lactate (77-1)	sc	nd	-
222	Boojum 4 lactate (78-1)	sc	nd	-
223	Boojum 5 lactate (79-1)	sc	nd	-
218	Boojum 9 lactate (74-1)	sc	nd	-
251	Boojum 4a lactate (78a)	cp	nd	
252	Boojum 2 lactate (75-2)	sc	nd	
280	Boojum 2a lactate (75a)	cp	nd	
281	Boojum 2b lactate (75b)	cp	nd	
285	Boojum 2c lactate (75c)	cp	nd	
283	Boojum 2a lactate (75a-1)	sc	nd	

Table 4-1 continued.

#	filter used/Cross-hybridizations	std. name
173		na
174		na
180	mf/sh	amEth2
181		na
182		na
270	mf/(amLac2, amBen1, amPro4, amEth2)	amEth2; ?
228		na
253	mf/(amLac2)	amLac2
76	mf/amLac6c/a(amLac2): ofpwf/nh	amLac6a; amLac2
75	mf/amLac6b(amLac2,amPro4): ofpwf/nh	amLac6b; amLac2
77	mf/amLac7	amLac7
78	mf/amLac8	amLac8
79		na
80		na
81		na
82		na
74	mf/amLac6a/c(amLac2)	amLac6a; amLac2
220	mf/(amLac6c)	amLac6a
219	mf/(amLac6b, amPro4)	amLac6b
221	mf/(amDec1)	amDec1
222	mf/(amLac2,amBen1,amLac6a,amPro1/4)	na
223		na
218	mf/(amLac2,amBen1,amLac6a/c,amAce2,amPro1/4)	na
251	mf/(amLac2, amBen1, amLac6a/c, amPro4)	na
252	mf/(amLac6b,amPro4)	amLac6b
280	mf/(21, amLac6b, amPro4)	amLac6b
281	mf/(amLac6b, amPro4)	amLac6b
285	mf/(amLac6b, amPro4)	amLac6b
283	mf/(amLac6b, amPro4)	amLac6b

Table 4-1 continued.

#	Preparation description	type	[NiFe]	16S code
282	Boojum 2b lactate (75b-1)	sc	nd	
286	Boojum 4a lactate (78a-1)	sc	nd	
83	Boojum 1 benzoate	pc	-	-
84	Boojum 2 benzoate	pc	-	-
85	Boojum 3 benzoate	pc	-	-
86	Boojum 4 benzoate	pc	+	-
87	Boojum 5 benzoate	pc	-	-
88	Boojum 6 benzoate	pc	+	-
89	Boojum 7 benzoate	pc	+	-
90	Boojum 8 benzoate	pc	+	-
91	Boojum 9 benzoate	pc	+	-
277	Boojum 8a benzoate (90a)	cp	nd	
288	Boojum 8a benzoate (90a-1)	sc	nd	
114	Boojum 1 acetate	pc	-	-
115	Boojum 2 acetate	pc	-	-
138	Boojum 3 acetate	pc	+	amAce3
116	Boojum 4 acetate	pc	-	-
117	Boojum 5 acetate	pc	-	-
118	Boojum 6 acetate	pc	-	-
119	Boojum 7 acetate	pc	-	-
120	Boojum 8 acetate	pc	-	-
121	Boojum 9 acetate	pc	-	amAce2
242	Boojum 3 acetate (138-1)	sc	nd	-
246	Boojum 9 acetate (121-1)	sc	nd	-
129	Boojum 1 decanoic	pc	+	-
130	Boojum 2 decanoic	pc	-	nb
131	Boojum 3 decanoic	pc	-	-
132	Boojum 4 decanoic	pc	-	amDec2
133	Boojum 5 decanoic	pc	-	amDec2
134	Boojum 6 decanoic	pc	+	-

Table 4-1 continued.

#	filter used/Cross-hybridizations	std. name
282	mf/(amLac6b, amPro1, amPro4)	na
286	mf/(amLac2, amBen1, ,amLac6,amPro4,amPro1,amEth2)	na
83		na
84		na
85		na
86		na
87		na
88		na
89		na
90	mf/sh	amBen2
91		na
277	mf/(90)	amBen2
288	mf/(amLac6b, amPro4)	amLac6b
114		na
115		na
138	mf/amAce3	amAce3
116		na
117		na
118		na
119		na
120		na
121	mf/amAce2	amAce2
242	mf/(amAce3)	amAce3
246	mf/(amAce2)	amAce2
129		na
130		na
131		na
132		na
133		na
134		na

Table 4-1 continued.

#	Preparation description	type	[NiFe]	16S code
135	Boojum 7 decanoic	pc	+	-
136	Boojum 8 decanoic	pc	+	amDec2
137	Boojum 9 decanoic	pc	-	amDec1
237	Boojum 4 decanoic (132-1)	sc	nd	-
234	Boojum 5 decanoic (133-1)	sc	nd	-
236	Boojum 6 decanoic (134-1)	sc	nd	-
233	Boojum 8 decanoic (136-1)	sc	nd	-
243	Boojum 9 decanoic (137-1)	sc	nd	-
166	Boojum 1 propionate	pc	+	nb
167	Boojum 2 propionate	pc	+	-
168	Boojum 3 propionate	pc	+	nb
169	Boojum 4 propionate	pc	+	-
170	Boojum 5 propionate	pc	+	amPro2
198	Boojum 6 propionate	pc	-	-
171	Boojum 7 propionate	pc	+	-
194	Boojum 8 propionate	pc	+	-
195	Boojum 9 propionate	pc	+	-
235	Boojum 5 propionate (170-1)	sc	nd	-
266	Boojum 6 propionate (198-1)	sc	nd	
294	Boojum 5b propionate (170b)	cp	nd	
187	Boojum 1 ethanol	pc	+	-
188	Boojum 2 ethanol	pc	+	nb
189	Boojum 3 ethanol	pc	-	-
190	Boojum 4 ethanol	pc	-	-
192	Boojum 7 ethanol	pc	+	-
193	Boojum 9 ethanol	pc	+	-
199	Boojum 5 ethanol	pc	+	amPro3
200	Boojum 8 ethanol	pc	+	amPro4
247	Boojum 8 ethanol (200-1)	sc	nd	-
273	Boojum 8a ethanol (200a)	cp	nd	

Table 4-1 continued.

#	Cross-hybridizations	std. name
135		na
136		na
137	mf/amDec1;ofpwf/nh	amDec1
237		na
234		na
236		na
233		na
243	mf/(amDec1)	amDec1
166		na
167		na
168		na
169		na
170	mf/amPro2(Lac15)	amPro2
198	mf/sh(amPro4)	na
171		na
194		na
195		na
235	mf/(amPro2,amAce3,tpBen1,amPro1)	na
266	mf/(amAce3)	amAce3
294	mf/(Lac15 family/Pro5 family)	na
187		na
188		na
189		na
190		na
192		na
193		na
199		na
200	mf/amPro4(13,139,198,amLac2,am6b,tpLac1/2/3,tpEth2,Lac15)	amPro4
247	mf/(amAce3)	amAce3
273	mf/(amAce1, 184)	amAce3

Table 4-1 continued.

#	Preparation description	type	[NiFe]	16S code
287	Boojum 8a ethanol (200a-2)	sc	nd	
191	Boojum 6 ethanol	pc	+	-
51	Bell Creek lactate T=1	pc	+	amLac2
52	Bell Creek lactate	pc	+	nb
210	Bell Creek lactate (51-2)	sc	nd	-
279	Bell Creek a lactate (51a)	cp	nd	
284	Bell Creek a lactate (51a-1)	sc	nd	
53	Bell Creek benzoate	pc	-	amBen1
245	Bell Creek benzoate (53-1)	sc	nf	-
54	Bell Creek acetate	pc	+	-
186	Bell Creek ethanol	pc	+	-
196	Bell Creek propionate	pc	-	-
19	U mine acetate A	pc	-	amAce1
20	U mine acetate B	pc	-	amAce1
21	U mine acetate C	pc	-	-
113	U Mine acetate	pc	-	amAce1;?
241	U Mine acetate (113-1)	sc	nd	-
275	U mine acetate a (113a)	cp	nd	
289	U mine acetate a (113a-1)	sc	nd	
37	Acid Mine Drainage ace T=1	pc	-	nb
38	Acid Mine Drainage acetate	pc	-	nb
39	Acid Mine Drainage acetate	pc	-	nb
125	Acid Mine Drainage acetate	pc	-	amAce1
238	Acid Mine Drainage acetate (39-1)	sc	nd	-
33	Acid Mine Drainage lactate T=1	pc	-	nb
32	Acid Mine Drainage lactate	pc	-	-
34	Acid Mine Drainage lactate A	pc	-	-

Table 4-1 continued.

#	Cross-hybridizations	std. name
287	mf/(amAce1, amLac6b, 156, 180, amPro4)	na
191		na
51	mf/amLac2(amBen1,amPro4,amLac6)	amLac2; ?
52		na
210		na
279	mf/(amLac2)	amLac2
284	mf/(amPro4)	amPro4; ?
53	mf/amBen1(amLac2)	amBen1; ?
245	mf/(tpBen1,amAce3,amPro1)	na
54		na
186		na
196		na
19	mf/amAce1 (21,184)	amAce1
20	mf/amAce1 (21, 184)	amAce1
21	mf/sh(203,204,tpPro1/5,amAce1)	na
113	mf/amAce1(21,184)	amAce1
241	mf/amAce1	amAce1
275	mf/(amAce1, 184)	amAce1
289	mf(21, amAce1, tpPro1, amPro1, 201, 202)	amAce1; ?
37		na
38		na
39		na
125	mf/amAce1 (21)	amAce1
238		na
33		na
32		na
34		na

Table 4-1 continued.

#	Preparation description	type	[NiFe]	16S code
35	Acid Mine Drainage lactate B	pc	+	amLac1
36	Acid Mine Drainage lactate (32-1)	pc	+	-
112	Acid Mine Drainage propionate T=1	pc	-	-
31	Acid Mine Drainage propionate	pc	-	nb
40	Acid Mine Drainage benzoate T=1	pc	-	-
41	Acid Mine Drainage benzoate	pc	-	-
42	Acid Mine Drainage ethanol T=1	pc	-	nb
43	Acid Mine Drainage ethanol	pc	-	amEth1
44	Suncor 33' lactate T=1	pc	-	-
45	Suncor 23' lactate T=1	pc	-	-
46	Suncor 43' lactate T=1	pc	+;2.3	-
47	Suncor 23' lactate	pc	+:1.5	tpLac1a
48	Suncor 33' lactate	pc	+:1.5	tpLac1b
49	Suncor 43' lactate	pc	+;2.3;1.5	tpLac2
206	Suncor 33' lactate (48-1)	sc	nd	-
207	Suncor 33';43 lactate (48-1;49-1)	sc	nd	-
208	Suncor 43' lactate (49-1)	sc	nd	-
50	Suncor 43' benzoate	pc	+;1.8	tpBen1
155	Suncor 23' benzoate	pc	+;2.5	nb
156	Suncor 33' benzoate	pc	+	-
209	Suncor 43' benzoate (50-1)	sc	nd	-
225	Suncor 33' benzoate (156-1)	sc	nd	-
267	Suncor 33a benzoate (156a)	cp	nd	
292	Suncor 33a benzoate (156a-1)	sc	nd	
315	Suncor 43a benzoate (50a)	cp	nd	
158	Suncor 23' propionate (phenol)	pc	+	tpPro1
159	Suncor 33' propionate (spool)	pc	+	nb
160	Suncor 43' propionate (spool)	pc	+	nb
161	Suncor 23' propionate (spool)	pc	+	-

Table 4-1 continued.

#	Cross-hybridizations	std. name
35		na
36		na
112		na
31		na
40		na
41		na
42		na
43		na
44		na
45		na
46		na
47	mf/tpLac1a(13,139,156,184,tpLac1/2/3,Lac15,tpEth2,amPro4);ofpwf/(Lac15)	tpLac1
48	mf/tpLac1b(13,139?,156,184,tpLac1/2/3,Lac15,tpEth2,amPro4)	tpLac1
49	mf/tpLac2(13,139?,156,184,tpLac1/3,Lac15,tpEth2,amPro4)	tpLac2
206	mf/(13,139,156,tpLac1/2/3,tpEth2,amPro4,Lac15)	tpLac1
207	mf/(13,139,156,tpLac1/2/3,tpEth2,amPro1/4,Lac15)	tpLac1
208	mf/(13,139,156,tpLac1/2/3,tpEth2,amPro4,Lac15)	tpLac2
50	mf/tpBen1: ofpwf/nh	tpBen1
155		na
156	mf/sh(184, Lac15 family): ofpwf/nh	na
209	mf/nh	na
225	mf/(156,amPro1)	na
267	mf/(156, Lac15 family)	na
292	mf/(tpLac1/2,amLac2,amBen1,tpEth2,156,amPro1/4,Lac15)	na
315		na
158	mf/tpPro1(21,184,204,amPro1,tpPro5,tpEth1):ofpwf/(Pro5)	tpPro1
159		na
160		na
161		na

Table 4-1 continued.

#	Preparation description	type	[NiFe]	16S code
162	Suncor 43' propionate (filter)	pc	-	-
226	Suncor 23' propionate (158-1)	sc	nd	-
183	Suncor 23' ethanol	pc	-	-
184	Suncor 33' ethanol	pc	+	-
185	Suncor 43' ethanol	pc	-	tpEth1
229	Suncor 33' ethanol (184-1)	sc	nd	-
230	Suncor 43' ethanol (185-1)	sc	nd	-
250	Suncor 33' ethanol (184a)	cp	nd	
278	Suncor 43'a ethanol (185a)	cp	nd	
295	Suncor 43a ethanol (185a-1)	sc	nd	
122	Suncor 23' acetate	pc	-	-
123	Suncor 33' acetate	pc	-	amAce2?
124	Suncor 43' acetate	pc	-	tpAce1
254	Suncor 23' acetate (122-1)	sc	nd	-
232	Suncor 33' acetate (123-1)	sc	nd	-
248	Suncor 43' acetate (124-1)	sc	nd	-
274	Suncor 33'a acetate (123a)	cp	nd	
290	Suncor 33a acetate (123a-1)	sc	nd	
157	Suncor 23' decanoic	pc	+	-
144	Suncor 33' decanoic	pc	-	-
55	Syncrude 11-5 lactate	pc	+: 12	-
56	Syncrude 9-1 lactate	pc	+: 4.4;1.5	-
57	Syncrude pond lactate	pc	+	tpLac3a
58	Syncrude 16-20 lactate	pc	+: 8.1	tpLac3a
59	Syncrude 6-10 lactate	pc	+	tpLac3b
211	Syncrude pond lactate (57-1)	sc	nd	-
212	Syncrude 16-20 lactate (58-1)	sc	nd	-
213	Syncrude 6-10 lactate (59-1)	sc	nd	-
60	Syncrude 16-20 benzoate	pc	+	-

Table 4-1 continued.

#	Cross-hybridizations	std. name
162		na
226	mf/(21,204,tpPro1/5,amPro1)	tpPro1
183		na
184	mf/sh(156,amAce1,tpLac1/2,tpPro1,tpEth1)	na
185	mf/tpEth1(184,tpPro1)	na
229	mf/(139,tpLac1/2/3,tpEth2,amPro1/4,tpPro5,Lac15)	na
230	mf/(139,tpLac1/2/3,tpEth1/2,Lac15)	na
250	mf/(tpLac1/2)	na
278	mf/(tpBen1, 184,156)	na
295	mf/(amLac2,amBen1,amLac6c)	na
122		na
123		na
124		na
254		na
232		na
248		na
274	mf/(Lac15 family)	na
290	mf/(Lac15 family)	na
157		na
144		na
55		na
56		na
57	mf/tpLac3(13,Lac15,tpLac1/2)	tpLac3a
58	mf/tpLac3(13,204,Lac15,tpLac1/2,tpEth2,amPro4): ofpwf/(Lac15)	tpLac3a
59	mf/tpLac3(13,139,204,Lac15,,amPro4,tpPro5,tpLac1/2,tpEth2)	tpLac3b
211	mf/(13,139,204,tpLac1/2/3,tpEth2,amPro1/4,Lac15)	tpLac3a
212	mf/(204,tpLac1/2/3,tpEth2,amPro1/4,tpPro5,Lac15)	tpLac3a
213	mf/(204,tpLac2/3,tpEth2,amPro1/4,tpPro5,Lac15)	tpLac3b
60		na

Table 4-1 continued.

#	Preparation description	type	[NiFe]	16S code
61	Syncrude 6-10 benzoate	pc	-	-
62	Syncrude 9-1 benzoate	pc	-	-
63	Syncrude 11-5 benzoate	pc	-	-
64	Syncrude pond benzoate	pc	-	-
65	Syncrude 16-20 acetate	pc	+	-
66	Syncrude 6-10 acetate	pc	+	-
67	Syncrude 11-5 acetate	pc	+	-
68	Syncrude 9-1 acetate	pc	+	-
69	Syncrude pond acetate	pc	-	-
214	Syncrude 9-1 acetate (68-1)	sc	nd	-
139	Syncrude 6-10 ethanol	pc	+;2.5	-
140	Syncrude 16-20 ethanol	pc	+;<2.0	tpEth2
141	Syncrude pond ethanol	pc	+;2.0;<2.0	tpEth2
142	Syncrude 9-1 ethanol	pc	+;<2.0	-
143	Syncrude 11-5 ethanol	pc	+;<2.0	-
224	Syncrude 16-20 ethanol (140-1)	sc	nd	-
244	Syncrude pond ethanol (141-1)	sc	nd	-
249	Syncrude 6-10 ethanol (139-1)	sc	nd	-
265	Syncrude 6-10 ethanol (139-1)	sc	nd	
276	Syncrude pond a ethanol (141a)	cp	nd	
291	Syncrude pond A ethanol (141a-1)	sc	nd	
293	Syncrude 6-10a ethanol (139a)	sc	nd	
296	Syncrude 6-10b ethanol (139b)	sc	nd	
175	Syncrude 6-10 decanoic	pc	+	-
176	Syncrude 9-1 decanoic	pc	-	nb
177	Syncrude 11-5 decanoic	pc	+	soLac2?
178	Syncrude 16-20 decanoic	pc	+	-
179	Syncrude pond decanoic	pc	-	-
201	Syncrude 6-10 propionate	pc	-	tpPro5a;tpLac1
202	Syncrude 9-1 propionate	pc	+	tpPro5b

Table 4-1 continued.

#	Cross-hybridizations	std. name
61		na
62		na
63		na
64		na
65		na
66	mf/sh(139?)	tpAce2
67		na
68		na
69		na
214		na
139	mf/sh(13,amPro4,tpLac1/2/3b,Lac15)	na
140	mf/tpEth2(13,tpPro5,tpLac1/2/3,Lac15):ofpwf/(Lac15)	tpEth2
141	mf/tpEth2(13,139,204,amPro1/4,tpLac1/2/3,tpPro5,Lac15)	tpEth2; ?
142		na
143		na
224	mf/(139,156,185,tpLac1/2/3,tpEth2,amPro1/4,tpEth1)	tpEth2; ?
244	mf/(139,tpLac1/2/3,amLac2,tpEth2,amPro1/4,tpPro5,Lac15)	tpEth2; ?
249	mf/(139,tpLac1/2/3b,tpEth2,amPro4,Lac15)	na
265	mf/(Lac15 family)	na
276	mf/(amLac4)	Lac30
291	mf/(amLac4)	Lac30
293	mf/(tpLac1/2/3,139,198,amPro4)	na
296	mf/(tpLac3,amLac6b,amPro1/4)	amLac6b; ?
175		na
176		na
177		na
178		na
179		na
201	mf/tpPro5(21,203,204,tpLac3b,amPro1,tpEth2,tpPro1): ofpw/(Pro5)	tpPro5; ?
202	mf/tpPro5(21,204,tpLac3b,tpEth2,tpPro1,amPro1): ofpw/(Pro5)	tpPro5b; ?

Table 4-1 continued.

#	Preparation description	type	[NiFe]	16S code
203	Syncrude 11-5 propionate	pc	+	-
204	Syncrude 16-20 propionate	pc	-	-
205	Syncrude pond propionate	pc	+	-
324	Syncrude 6-10a propionate (201a)	cp	+	
92	CANMET Soil 10-9-1; lactate	pc	-	soLac3
93	CANMET Soil S-10-31-2; lactate	pc	+	soLac4
94	CANMET Soil S-9-31-3; lactate	pc	+	nb
95	CANMET Soil 10-6-1; lactate	pc	+	nb
96	CANMET Soil 10-3-2; lactate	pc	+	soLac5
97	CANMET Soil 5-12-1; lactate	pc	+	nb
98	CANMET Soil S-7-31-2; lactate	pc	+: 4.0; <.5	soLac2
99	CANMET Soil S-7-31-4; lactate	pc	+: 1.9	-
100	CANMET Soil 10-12-2; lactate	pc	+	-
101	CANMET Soil 10-3-3; lactate	pc	+	-
102	CANMET Soil 8-12-1; lactate	pc	+	-
103	CANMET Soil 7-3-2; lactate	pc	+	-
104	CANMET Soil 7-9-2; lactate	pc	+	-
105	CANMET Soil S-6-31-3; lactate	pc	-	nb
106	CANMET Soil 7-6-1; lactate	pc	+	-
107	CANMET Soil 7-12-2; lactate	pc	+	-
108	CANMET Soil S-8-31-3; lactate	pc	+	-
109	CANMET Soil S-5-31-2; lactate	pc	+	-
110	CANMET Soil 5-9-2; lactate	pc	+	-
111	CANMET Soil 5-3-2; lactate	pc	-	-
255	CANMET Soil 10-9-1-2 lactate (92-1)	sc	nd	
256	CANMET Soil S-9-31-3-2 lactate (94-1)	sc	nd	
257	CANMET Soil S-7-31-2-2 lactate (98-1)	sc	nd	
258	CANMET Soil 8-12-1-2 lactate (102-1)	sc	nd	
259	CANMET Soil 7-12-2-2 lactate (107-1)	sc	nd	

Table 4-1 continued.

#	Cross-hybridizations	std. name
203	mf/sh(21,204,amPro1,tpPro5)	na
204	mf/sh(21,203,tpLac3,amPro1,tpPro1/5,tpEth2): ofpw/(Pro5)	na
205		na
324		na
92	mf/soLac3(soLac2,soLac5,soLac4,101)	na
93	mf/soLac4(soLac2,soLac3): cf/(family IV, soLac1)	na
94	nd	na
95	cf/ (family I, soLac1)	na
96	mf/soLac5 (soLac3): cf/(family III, soAce1)	na
97	cf/(family I)	na
98	mf/soLac2 (soLac3,soLac4)	na
99	cf/(family IV, soLac1)	na
100	cf/(family III, soAce1)	na
101	mf/sh(soLac3): cf/(family IV)	na
102	nd	na
103	nd	na
104	nd	na
105	nd	na
106	nd	na
107	nd	na
108	nd	na
109	nd	na
110	cf/(family VI)	na
111	cf/(family I, soLac1)	na
255	cf/(family VI)	na
256	nd	na
257	cf/(family VI)	na
258	cf/(family II)	na
259	cf/(family VI)	na

Table 4-1 continued.

#	Preparation description	type	[NiFe]	16S code
260	CANMET Soil 7-3-2-2 lactate (103-1)	sc	nd	
261	CANMET Soil S-6-31-3-2 lactate (105-1)	sc	nd	
262	CANMET Soil 7-6-1-2 lactate (106-1)	sc	nd	
263	CANMET Soil S-31-8-3-2 lactate (108-1)	sc	nd	
264	CANMET Soil S-5-31-2-2 lactate (109-1)	sc	nd	
400	CANMET Soil 10-9-1a lactate (92a)	cp	-	
401	CANMET Soil 10-9-1an acetate (92b)	cp	-	
402	CANMET Soil 10-9-1 an lactate (92c)	cp	-	
403	CANMET Soil S-10-31-2 a lactate (93a)	cp	-	
404	CANMET Soil S-10-31-2 an lactate (93b)	cp	-	
405	CANMET Soil S-10-31-2 an lactate (93c)	cp	-	
406	CANMET Soil 10-6-1 an lactate (95a)	cp	-	
407	CANMET Soil 10-6-1 an lactate (95b)	cp	-	
408	CANMET Soil 10-3-2 a lactate (96a)	cp	-	
409	CANMET Soil 10-3-2 an lactate (96b)	cp	-	
410	CANMET Soil 7-3-2 a lactate (103a)	cp	-	
411	CANMET Soil 7-3-2 an lactate (103b)	cp	-	
412	CANMET Soil S-6-31-3 an lactate (105a)	cp	-	
413	CANMET Soil 5-9-2 a lactate (110a)	cp	-	
414	CANMET Soil 5-9-2 an lactate (110b)	cp	-	
415	CANMET Soil 5-3-2 a lactate (111a)	cp	-	
416	CANMET Soil 5-3-2 an acetate (111b)	cp	-	
417	CANMET Soil 5-3-2 an lactate (111c)	cp	+	
751	Digout lactate 5a	cp	nd	no digestion
757	Digout lactate 14b	cp	nd	soLac1 (soAce1)
758	Digout lactate 1c	cp	nd	soLac1
771	Digout lactate 3a	cp	nd	soLac1
772	Digout lactate 3c	cp	nd	soLac1 (soAce1)
773	Digout lactate 6a	cp	nd	soLac1

Table 4-1 continued.

#	Cross-hybridizations	std. name
260	cf/(family II, soAce1)	na
261	cf/(family V)	na
262	cf/(family VI)	na
263	cf/(family VI)	na
264	nd	na
400	ccpf/nh	na
401	ccpf/nh	na
402	ccpf/nh	na
403	ccpf/nh	na
404	ccpf/nh	na
405	ccpf/sh(103a, 95a, 96b, 105a)	na
406	ccpf/sh(95b)	na
407	ccpf/sh(95a)	na
408	ccpf/nh	na
409	ccpf/sh(93c, 103a)	na
410	ccpf/sh(93c, 95a, 96b, 105a)	na
411	ccpf/sh(110b)	na
412	ccpf/sh(93c, 96b, 103a)	na
413	ccpf/nh	na
414	ccpf/nh	na
415	ccpf/nh	na
416	ccpf/sh	na
417	ccpf/sh:ofpwf/nh	na
751	Dof/soLac1	soLac1
757	Dof/soLac1 (soAce1)	soLac1/soAce1
758	Dof/soLac1	soLac1
771	Dof/soLac1 (soAce1)	soLac1/soAce1
772	Dof/soLac1 (soAce1)	soLac1/soAce1
773	Dof/soLac1 (soAce1)	soLac1/soAce1

Table 4-1 continued.

#	Preparation description	type	[NiFe]	16S code
774	Digout lactate 6c	cp	nd	soLac1; soLac1b (soAce1)
775	Digout lactate 5c	cp	nd	soLac1
776	Digout lactate 13a	cp	(+)	soLac1; soAce1
777	Digout lactate 14a	cp	+	soLac1
778	Digout lactate 14b	cp	nd	soLac1 (soAce1)
783	Digout lactate 4c	cp	nd	soLac1
784	Digout lactate 7c	cp	nd	soLac1
785	Digout lactate 8c	cp	nd	soLac1 (soAce1)
786	Digout lactate 9c	cp	nd	soLac1
819	Digout lactate 7a	cp	nd	soLac1
820	Digout lactate 11b	cp	nd	soLac1
822	Digout lactate 7b	cp	nd	nb
821	Digout lactate 11c	cp	nd	soLac1
779	Digout acetate 3b	cp	nd	soLac1; soAce1
780	Digout acetate 5b	cp	nd	soAce1;(soLac1)
781	Digout acetate 7b	cp	nd	soLac1; soAce1
800	Digout acetate 3a	cp	nd	(soAce1) soLac1
801	Digout acetate 5c	cp	nd	(soLac1)
802	Digout acetate 11a	cp	nd	(soLac1)
803	Digout acetate 11b	cp	nd	(soLac1; soAce1)
823	Digout acetate 2c	cp	nd	nb
824	Digout acetate 7a	cp	nd	nb
825	Digout acetate 8c	cp	nd	nb
826	Digout acetate 10c	cp	nd	nb
782	Digout propionate 5b	cp	nd	soAce1
799	Digout propionate 8b	cp	nd	nd
816	Digout propionate 5a	cp	nd	(soAce1)
818	Digout propionate 11c	cp	nd	soLac1 (soAce1)
817	Digout propionate 11b	cp	nd	(soLac1)

Table 4-1 continued.

#	Cross-hybridizations	std. name
774	Dof/soLac1 (soAce1)	soLac1/soAce1
775	Dof/soLac1	soLac1
776	Dof/soLac1, soAce1: cf sh(family II, III):ccpf/sh	soLac1/soAce1
777	Dof/soLac1: cf/sh(family I, IV):ccpf/sh	soLac1
778	Dof/soLac1, soAce1	soLac1/soAce1
783	Dof/soLac1	soLac1
784	Dof/soLac1	soLac1
785	Dof/soLac1 (soAce1)	soLac1/soAce1
786	Dof/soLac1	soLac1
819	Dof/soLac1	soLac1
820	Dof/soLac1	soLac1
822	Dof/nh	na
821	Dof/soLac1	soLac1
779	Dof/soLac1, soAce1	soLac1/soAce1
780	Dof/soAce1	soAce1
781	Dof/soLac1, soAce1	soLac1/soAce1
800	Dof/soAce1, soLac1	soAce1/soLac1
801	Dof/soLac1	soLac1
802	Dof/soAce1, soLac1	soAce1/soLac1
803	Dof/soAce1, soLac1	soAce1/soLac1
823	Dof/nh	na
824	Dof/nh	na
825	Dof/nh	na
826	Dof/nh	na
782	Dof/soAce1	soAce1
799	Dof/nd	na
816	Dof/soAce1 (soLac1)	soAce1/soLac1
818	Dof/soLac1, soAce1	soLac1/soAce1
817	Dof/soLac1 (soAce1)	soLac1/soAce1

Table 4-1 continued.

#	Preparation description	type	[NiFe]	16S code
795	Digout benzoate 4a	cp	nd	soAce1; soLac1
796	Digout benzoate 5b	cp	nd	soLac1; soAce1
797	Digout benzoate 6a	cp	nd	soLac1; soAce1
798	Digout benzoate 13a	cp	nd	soLac1
814	Digout benzoate 6c	cp	nd	no dig
792	Digout decanoate 2c	cp	nd	soAce1; soLac1
793	Digout decanoate 8a	cp	nd	soLac1
794	Digout decanoate 13c	cp	nd	nb
815	Digout decanoate 10c	cp	nd	soAce1 (soLac1)
1	sewage B EtOH 1	pc	-	nb
2	sewage B EtOH	pc	+: 7.9	-
3	sewage B EtOH	pc	-	nb
4	sewage B EtOH	pc	-	-
15	sewage B ethanol a	cp	-	swEth1
16	sewage B ethanol b	cp	+	swEth1
17	sewage B ethanol a (15-1)	sc	+	swEth1?
18	sewage B ethanol b (16-1)	sc	+	nb
316	sewage B ethanol a (15-2)	sc	+	
317	sewage b ethanol a (b)	cp	+	
318	sewage b ethanol b (b)	cp	+	
319	sewage b ethanol b (16-2)	sc	+	
7	sewage B benzoate	pc	-	-
8	sewage B benzoate	pc	-	nb
9	sewage B benzoate	pc	-	swBen1
10	sewage B benzoate	pc	+: 3.7	-
11	sewage B benzoate	pc	-	-
320	sewage b benzoate (8-2)	sc	-	
12	sewage B benzoate a(b)	cp	-	nb
321	sewage B benzoate a (b) (12-1)	sc	-	

Table 4-1 continued.

#	Cross-hybridizations	std. name
795	Dof/soAce1, soLac1	soAce1/soLac1
796	Dof/soLac1, soAce1	soLac1/soAce1
797	Dof/soLac1, soAce1	soLac1/soAce1
798	Dof/soLac1	soLac1
814	Dof/soAce1, soLac1	soAce1/soLac1
792	Dof/soAce1, soLac1	soAce1/soLac1
793	Dof/soLac1	soLac1
794	Dof/soLac1	soLac1
815	Dof/soAce1 (soLac1)	soAce1/soLac1
1		na
2		na
3		na
4		na
15		na
16		na
17		na
18		na
316	swf/sh(317-319, 322,324, 332, 333)	swEth1
317	swf/sh(316,318,319, 322,324, 332, 333)	swEth1
318	swf/sh(316,317,319, 322, 324, 332, 333): mf/(Lac15 family)	swEth1
319	swf/sh(316-318, 322, 324, 332, 333)	swEth1
7		na
8		na
9		na
10		na
11		na
320	swf/sh: mf/nh	swBen2
12		na
321	swf/sh: mf/(13, 66, tpBen1)	na

Table 4-1 continued.

#	Preparation description	type	[NiFe]	16S code
13	sewage B benzoate b(b)	cp	-	-
268	sewage B benzoate b (b) (13-1)	sc	nd	
323	sewage B benzoate b (b) (13-2)	sc	-	
5	sewage B decanoate	pc	-	-
6	sewage B decanoate	pc	-	swDec1
322	sewage b decanoic (5-1)	sc	+	
14	sewage B acetate	pc	-	nb
325	activated sludge propionate (Gold Bar)	pc	-	
326	activated sludge lactate (Gold Bar)	pc	-	
327	activated sludge ethanol (Gold Bar)	pc	-	
328	activated sludge decanoic (Gold Bar)	pc	-	
329	activated sludge benzoate (Gold Bar)	pc	-	
330	activated sludge acetate (Gold Bar)	pc	-	
331	anaerobic digestor propionate (Gold Bar)	pc	-	
332	anaerobic digestor lactate (Gold Bar)	pc	+	
333	anaerobic digestor ethanol (Gold Bar)	pc	+	
334	anaerobic digestor decanoic (Gold Bar)	pc	-	
335	anaerobic digestor benzoate (Gold Bar)	pc	-	
336	anaerobic digestor acetate (Gold Bar)	pc	-	
22	Gold Bar lactate	pc	-	-
23	Gold Bar propionate	pc	-	swEth1?
24	Gold Bar benzoate	pc	-	nb
25	Gold Bar ethanol	pc	-	swEth2
27	Capitol Region acetate	pc	-	swAce1
28	Capitol Region propionate	pc	-	-
29	Capitol Region lactate	pc	+	-
30	Capitol Region decanoate	pc	-	nb

Table 4-1 continued.

#	Cross-hybridizations	std. name
13	mf/sh (139,Lac15,tpLac1/2/3,tpEth2,amPro4)	na
268	mf/(Lac15 family)	na
323	swf/sh: mf/nh	swBen3
5		na
6		na
322	swf/sh(316-319, 324, 332, 333)	swEth1
14		na
325	swf/sh(326-330, 333, 335)	swPro1
326	swf/sh(325, 327-330, 333, 335)	swPro1
327	swf/sh(325, 326, 328-330, 333, 335, 336): mf/nh	swPro1; ?
328	swf/sh(325-327, 329-331, 333, 334-336)	swPro1; ?
329	swf/sh(325-328, 330, 331, 333-336)	swPro1; ?
330	swf/sh(325-329, 331, 333, 335, 336)	swPro1; ?
331	swf/sh(325-330, 332-336): mf/Pro5 family	soPro1; ?
332	swf/sh(316-319, 329, 331, 333): mf/Lac15 family	swEth1; ?
333	swf/sh(316-319, 325-332, 334-336): mf/Lac15 family	swPro1; swEth1
334	swf/sh(325-330, 335,336): mf/Pro5 family	swPro1; ?
335	swf/sh(325-331, 333, 334, 336)	swPro1; ?
336	swf/sh(325-331, 333, 334, 335)	swPro1; ?
22		na
23		na
24		na
25		na
27		na
28		na
29		na
30		na

Table 4-1 legend.

All columns for a given row are separated into two sections on two, concurrent pages (#, preparation description, type, [NiFe], and 16S code, and #, filter used/cross-hybridizations, and standard name). The number for each preparation (#) is given for each section of the table for clarity. Thus, all information for a given preparation (or row) can be obtained by combining pages in sets of two.

column headings:

#: chronological number assigned to the DNA preparation as it was received in Calgary.

Preparation description: Names indicate sample source (see section 3.2), sample (see section 3.2), and carbon and energy source. For CANMET AMD, sample names were assigned as follows; S = soil sample (also assigned 31 for 31 inches below pipe), otherwise disbondment sample, first number indicates environmental cell from which the sample was taken, second number indicates clockwise position around the pipe from which the sample was taken, and the last number indicates the original dilution of the enrichment sample from which the latest enrichment (the Postgate B bottles received in Calgary) have been inoculated. Sample names for the other environments are described in the text. For all DNA preparations, small case letters indicate preparations obtained from colony purified isolates. Numbers in parentheses; first number indicates corresponding primary preparation, second number indicates subsequent culture

(1 for secondary, 2 for tertiary, etc.). For CANMET AMD; 'A' or no letter =W&P media, 'B' = Blood media, 'h' = $HgCl_2$ added, first number is sample number, second number is colony number, BSR was colony purified at CANMET. Preparations are grouped by environment (AMD, oil sand production water, soil, and sewage), sample set, carbon and energy source, and culture type.

type: Describes the culture from which the DNA preparation was obtained; either pc (primary liquid enrichment culture), sc (subsequent liquid enrichment culture), or cp (colony purified).

[NiFe]: Reaction of the preparation with the [NiFe] hydrogenase gene probe on dot and Southern blots; either + (hybridization), - (no hybridization), nd (preparation was not tested). Numbers indicate the size of the hybridizing band observed on a Southern blot in kb; < (band was smaller than this value, but could not be calculated accurately).

16S code= ribotype code assigned to preparations that successfully hybridized with the 16S rRNA gene probe (see section 4.2.2.). Codes indicate environment (*am* (acid mine drainage), *tp* (tailings pond), *so* (soil), *sw* (sewage)), carbon and energy source (*Lac*=lactate, etc.), and and identifying number. Other; nb (no band), - (not tested with probe), ? (pattern was unclear).

filter used/Cross-hybridization= Whole genome analysis. Indicates filter used (swf=sewage filter, mf=multi-environment filter, cf=CANMET Soil filter, ccpf=CANMET Soil colony purified isolates filter, dof= Digout filter), and DNA

preparations to which the probe cross-hybridized (sh = self-hybridization, otherwise ribotype code name or preparation number is given). '*Lac*15 family' or '*Pro*5' family indicates multiple, unspecified hybridizations to these family members.

std. name=the standard name assigned to the preparation based on the whole genome analysis (see 16S code).

Table 4-2. CANMET Soil samples chosen for colony purification

Sample[a]	family[b]	N[c]	n[d]	post-family[e]
5-3-2 (111)	I	3	2	I
10-6-1 (95)	I, II	2	2	I, II
7-3-2-2 (260)	II	2	2	II
S-10-31-2 (93)	II,IV	3	1	II
10-3-2 (96)	III	2	1	II
S-6-31-3-2 (261)	V	1	1	II
10-9-1 (255)	VI	3	0	nd
5-9-2 (110)	VI	2	1	II

a Naming/numbering is described in Table 4-1. Number corresponds to the DNA preparation used for the whole genome analysis.

b Hybridization group to which the DNA preparation used for whole genome analysis belonged. Preparations belonging to more than one family had complex cross-hybridizations.

c Number of colony purified isolates obtained.

d Number of colony purified isolates for which whole genome analysis was successful.

e Hybridization group to which the colony purified isolates belonged. nd=not determined.

hydrogen as the energy source. Genomic DNA was prepared from Postgate C cultures of eighteen colony purified isolates.

All DNA preparations are described in Table 4-1. In this table, DNA preparations have been ordered by (i) environmental category: acid mine drainage, oil sands production water, soil, and sewage; (ii) sample set: CANMET AMD, Boojum, Bell Creek, Uranium Mine, and AMD, Suncor and Syncrude, CANMET Soil and Digout, and Sewage B, Anaerobic digestor (Gold Bar), Activated sludge (Gold Bar), Gold Bar, and Capitol Region; (iii) carbon and energy source, and (iv) DNA preparation type: primary, secondary, primary colony purified, secondary colony purified.

4.2 Genomic Characterization of DNA preparations

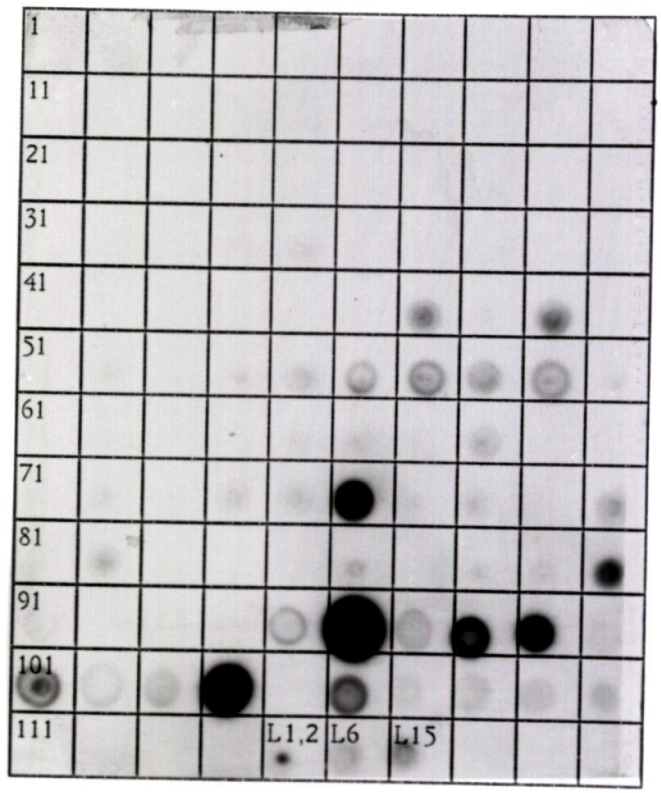
4.2.1 [NiFe] hydrogenase gene probes; dot and Southern blots

The [NiFe] hydrogenase gene probe is a useful tool for identifying *Desulfovibrio* in DNA preparations. Dot blot hybridizations successfully identified DNA preparations containing *Desulfovibrio*. An example of typical data obtained is shown in Figure 4-1A. DNA preparations tested are indicated in Table 4-1. A breakdown of the distribution of *Desulfovibrio* positive DNA preparations, both by site and by carbon and energy source used in the liquid culture enrichment is presented in Table 4-3. Hybridization with the [NiFe] hydrogenase gene probe was observed for 51% of the DNA preparations tested. Positive DNA preparations were further analyzed on Southern blots. Approximate hybridizing band sizes were estimated by comparison to the labeled lambda internal standard (Figure 4-1B). The results for both dot and Southern blot analysis for all DNA preparations are compiled in Table 4-1.

Figure 4-1. Hybridization with the [NiFe] hydrogenase gene probe of:
(A) Dot blot of DNA preparations 1-111 inclusive. Oil field production water standards *Lac*1,2 (L1,2), *Lac*6 (L6) and *Lac*15 (L15), all *Desulfovibrio sp.*, are indicated. Numbering corresponds to the DNA preparation spotted and proceeds sequentially along each line, with ten DNA preparations spotted per line, except for line 111 where only DNA preparation 111 and the three standards are spotted.

(B) Southern blot of *Eco*RI digested genomic DNA from DNA preparations 141, 142, 143, 146, 147, 148, and 151. '+' is digested *Lac*15 DNA, B is no digested DNA, and λ is end-labeled lambda DNA digested with *Hind*III.

A.


1
11
21
31
41
51
61
71
81
91
101
111
L1,2
L6
L15

B.

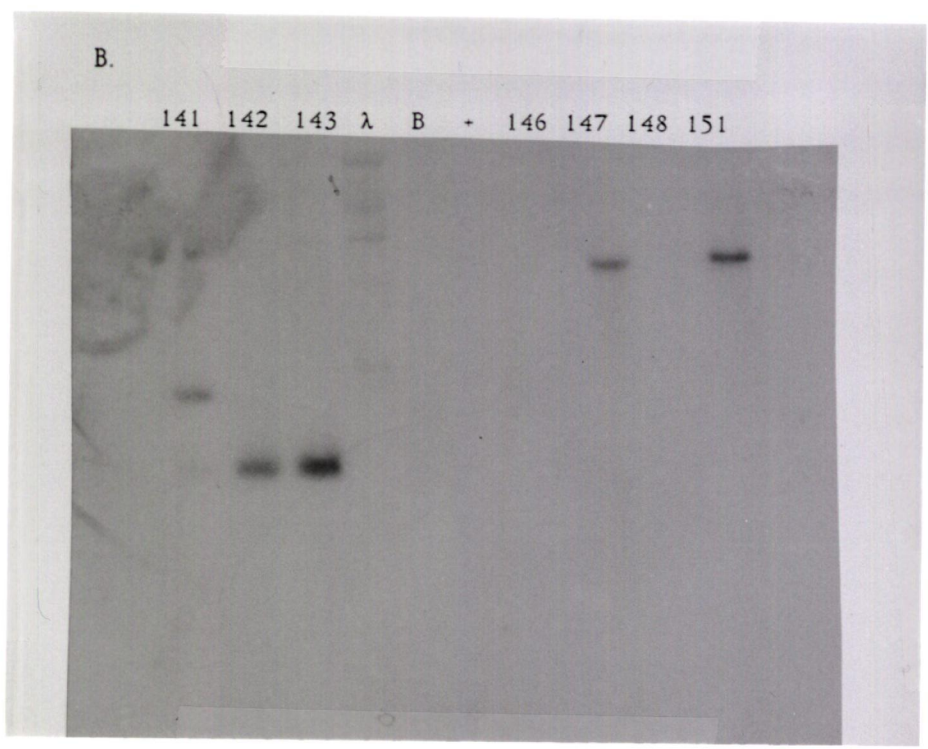

141 142 143 λ B + 146 147 148 151

Table 4-3. Distribution of *Desulfovibrio* in DNA preparations.

Site[a]	Total		Carbon Sources: Lactate		Carbon Sources: Other	
	N[b]	%[c]	n[b]	%[c]	m[b]	%[c]
CANMET AMD	26	46	10	60	16	38
Boojum 1,2	12	58	2	100	10	50
Boojum 3	6	50	1	100	5	40
Boojum 4	6	50	1	100	5	40
Boojum 5-9	30	63	4	50	26	65
Bell Creek	6	67	2	100	4	50
Uranium mine	4	0	0	-	4	0
AMD	15	13	5	40	10	0
Suncor	22	59	6	67	16	56
Syncrude	30	70	5	100	25	64
CANMET Soil[e]	20	85	20	85	0	--
Digout[d]	43	50	-	-	-	-
Sewage B	26	38	0	-	26	38
Gold Bar[f]	16	13	3	34	13	8
Capitol Region	4	20	1	100	3	0
Total/Average	266	51	60	73	163	43

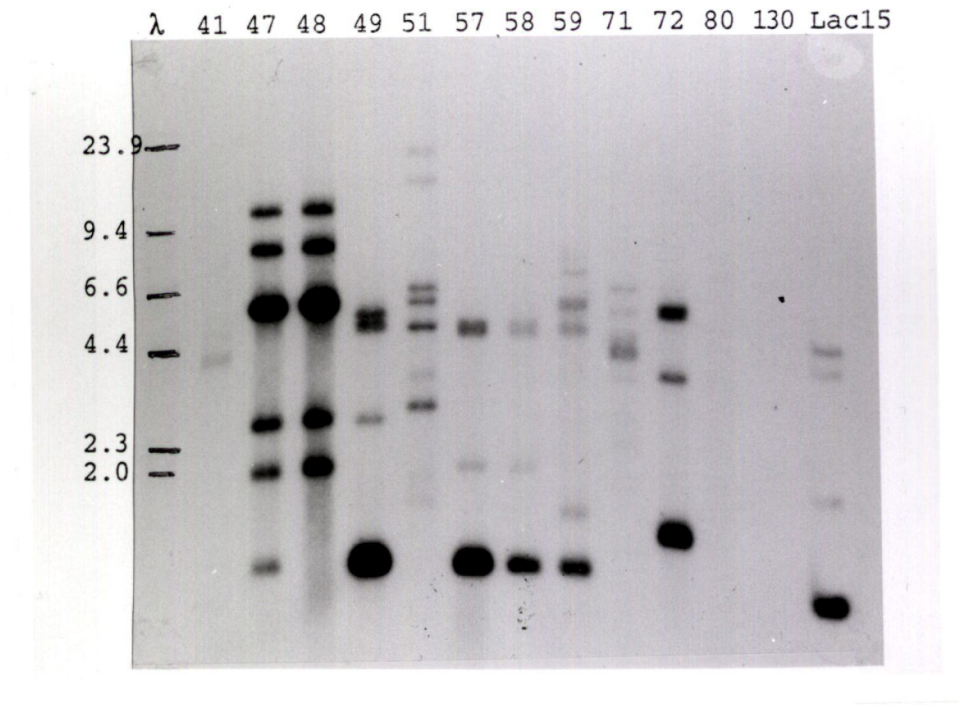
a Sites are as described in text and in Table 3-1.
b The number of [NiFe] positive DNA preparations.
c The percentage of [NiFe] positive DNA preparations.
d Individual preparations were not tested. Of the two standards, only *soLac*1 was [NiFe] positive. These numbers were not used in further calculations.
e Values for the primary set of liquid enrichment cultures only.
f Includes Gold Bar, Anaerobic digestor, and Activated sludge.

4.2.2 16S rRNA gene probe; ribotyping preparations

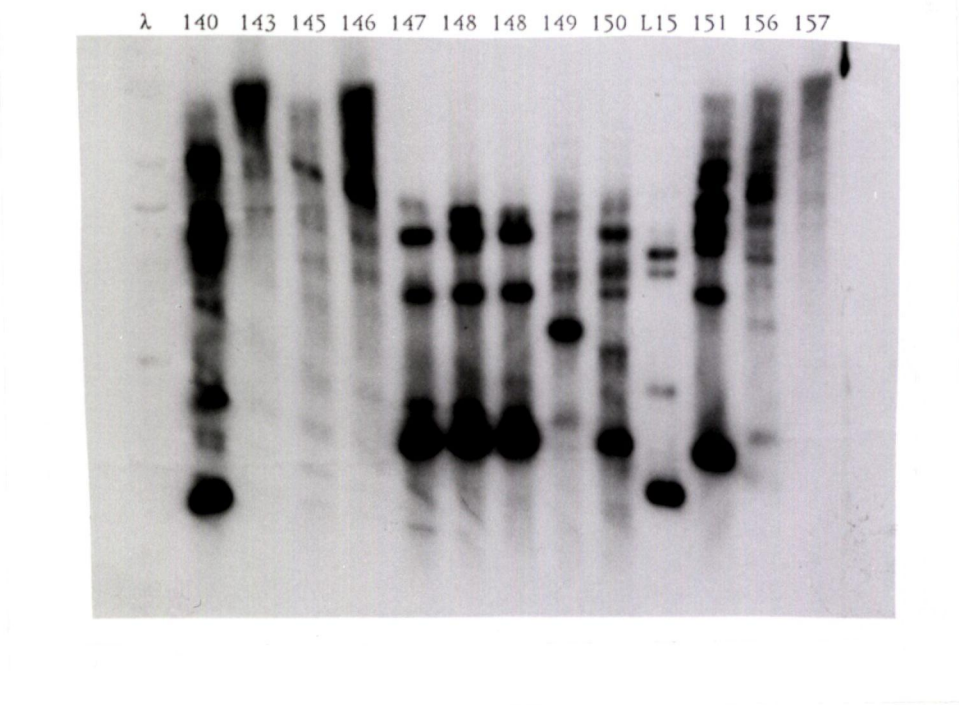
In order to obtain meaningful comparative data, the 16S rRNA gene probe was used exclusively for hybridization with Southern blots. All DNA preparations with numbers ranging from 1-205 were analyzed. Patterns of bands (ribotypes) were obtained for 62 of these DNA preparations, some of which are shown in Figure 4-2. These 62 DNA preparations were assigned descriptive codes. Patterns were occasionally highly complex, indicating multiple 16S rRNA operons, possibly from multiple species, or non-distinct, indicating genomic degradation or incomplete digestion (Figure 4-2B, 143 and 157). These DNA preparations were not assigned descriptive codes, and were generally not analyzed with whole genome probes as the quality of the genomic DNA appeared poor and unsuitable for further analysis. Codes were assigned as follows; two letters indicating the environment from which the sample was obtained (*tp*=tailings pond, *am*=acid mine drainage, *so*=soil, *sw*=sewage, no letters=oil field production water standard), three letters indicating the carbon and energy source on which the sample was originally enriched(*Lac*=lactate, *Ace*=acetate, etc.), a unique number for each unique pattern, and, if necessary, a letter distinguishing highly similar, but not identical patterns. For example, in Figure 4-2A, DNA preparation 47 was assigned the code *tpLac*1a (tailings pond lactate 1), and the 'a' was assigned to recognize the difference between preparation 47, which gave a band of approximately 500 bp, from preparation 48, which had no band at that position. Preparation 48 was thus assigned the code *tpLac*1b. Similarly, preparations 72, 147, 148 were all assigned the code *amLac*4 due to the identical patterns that were observed. Preparation 150 gave a highly similar pattern to 72, 147 and 148, but had several additional bands. Thus it was

Figure 4-2. Southern blots of *Eco*RI digested DNA preparations hybridized with the 16S rRNA gene probe. (A) DNA preparations 41, 47, 48, 49, 51, 57, 58, 59, 71, 72, 80, and 130. (B) DNA preparations 140, 143, 145-151, 156, and 157. For both (A) and (B), Lac15 or L15 is *Eco*RI digested *Lac*15 genomic DNA and λ is end-labeled, HindIII digested lambda DNA. The positions and sizes of the λ bands are indicated in (A).

A.


λ 41 47 48 49 51 57 58 59 71 72 80 130 Lac15
23.9
9.4
6.6
4.4
2.3
2.0

B.


λ 140 143 145 146 147 148 148 149 150 L15 151 156 157

assigned the code *amLac*4; ?, to indicate the presence of additional genomic material. The CANMET soil samples in particular gave very complex ribotypes (numerous bands, often resulting in smears) thus, although codes were assigned for a few of the preparations, they were not expected to reflect the presence of only one genome. Coded preparations with sufficient genomic material for large scale cross-hybridization analysis were selected for multi-environment whole genome probing studies, described below, to determine to what extent 16S rRNA gene fingerprints correlated with overall genomic homology.

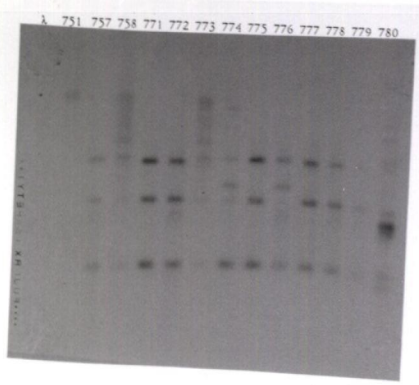
The colony purified Digout preparations (numbers 751-823) were also tested, but gave only three different ribotypes for 43 DNA preparations. Some of these ribotypes were clearly mixtures of the two distinct ribotypes. Each distinct ribotype was assigned a code; either *soLac*1 or *soAce*1 (Figure 4-3A, lanes 777 and 780 respectively). Preparations that gave both ribotypes were coded *soLac*1; *soAce*1 (Figure 4-3A, lane 776).

4.2.3 Whole genome comparisons; defining standards

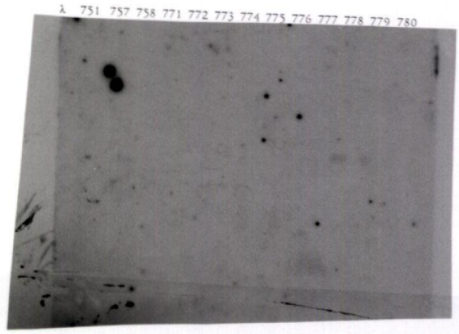
A multi-environment filter was prepared from 50 original DNA preparations, including 37 preparations with codes derived from ribotypes. This dot blot was initally probed with each of the DNA preparations represented on it, and was later hybridized with DNA preparations from secondary liquid enrichment cultures or colony purified isolates for which primary DNA preparations had been present on the filter, as well as with standards defined in other cross-hybridization studies. The primary analysis required the preparation of 50 multi-environment filters, thus only 37 of the 62 ribotyped and coded preparations could be used (i. e. there was insufficient genomic material for

Figure 4-3. Southern blot of Digout DNA preparations 751, 757, and 771-780 hybridized with; (A) the general16S rRNA gene probe, (B) P88 (*Bacteroides sp.* specific probe), or (C) P90 (*Desulfovibrio sp.* specific probe. The same filter was used for all three hybridization reactions. λ is *Hind*III digested lambda DNA.

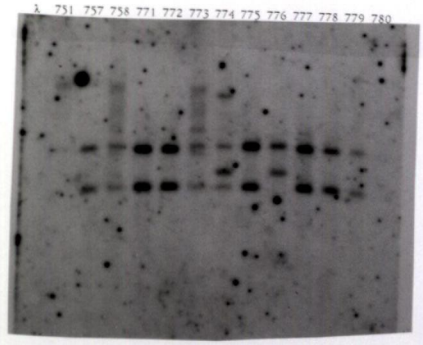
A.


λ 751 757 758 771 772 773 774 775 776 777 778 779 780

B.

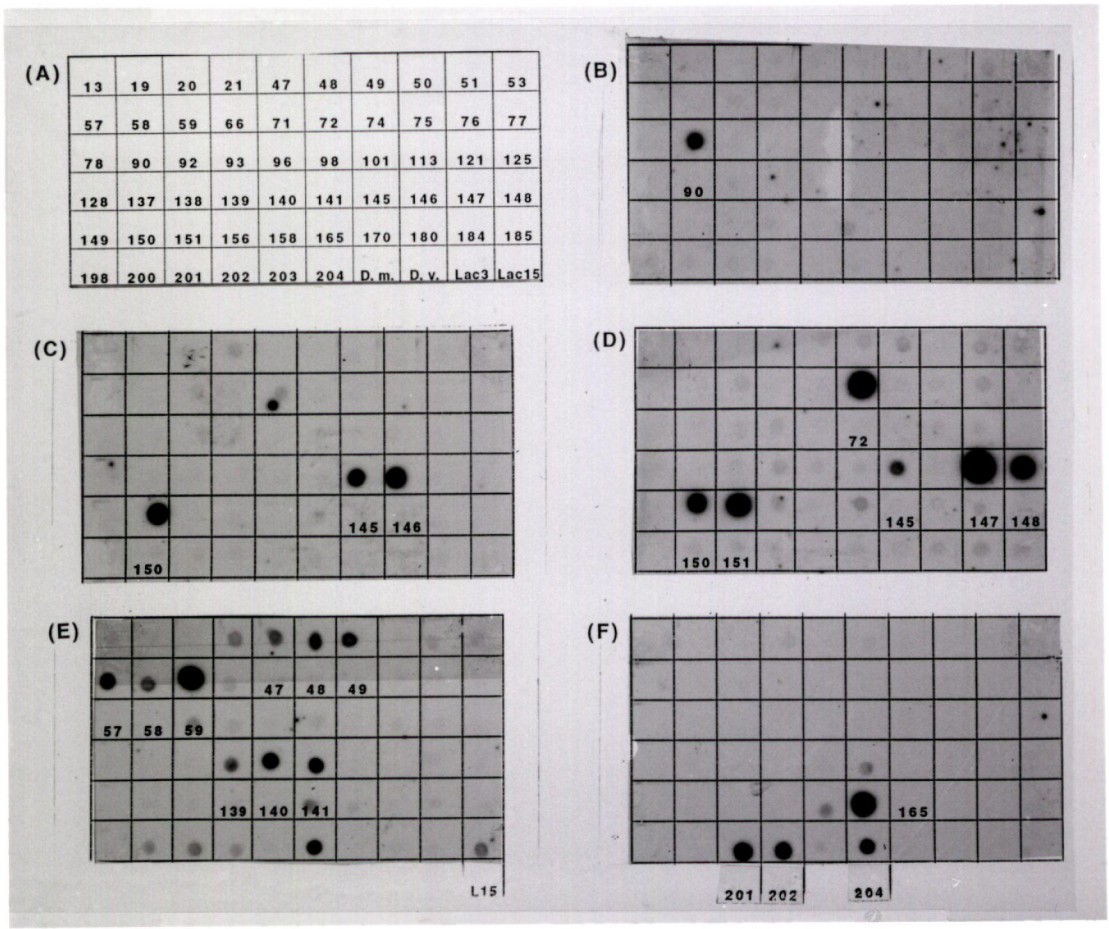

λ 751 757 758 771 772 773 774 775 776 777 778 779 780

C.


λ 751 757 758 771 772 773 774 775 776 777 778 779 780

analysis in some cases because preparation of 50 filters for cross-hybridization and subsequent DNA preparation labeling required at least 2100 ng of genomic DNA.). Additional DNA preparations were selected on the basis of DNA availability, and attempts were made to include multiple representatives from each environment (for example, preparations 145 and 146 from CANMET AMD were not assigned ribotype codes, but were nevertheless included on the filter). The spatial distribution of DNA preparations used in the construction of the multi-environment filter is shown in Figure 4-4A. Cross-hybridization patterns were evaluated qualitatively. For example, DNA preparation 90 (no ribotype code assigned) hybridized only to self (Figure 4-4B). The standard name *amBen*2 was selected for this preparation, as it adhered to the defining criteria for a standard (non-cross-hybridization to other standards). The ribotype code name was adopted as the standard name for all DNA preparations that had previously been assigned a ribotype code and only displayed self-hybridization, or hybridization with other preparations of the same ribotype. Mixtures of standards were also identified. For example, DNA preparation 146 (no ribotype code assigned) hybridized with self, and with 145 (no ribotype code assigned) and 150 (*amLac*4; ?) (Figure 4-4C). DNA preparation 72 (*amLac*4) hybridized with self, 145 (no ribotype code assigned), 147 (*amLac*4), 148 (*amLac*4), 150 (*amLac*4; ?), and 151 (*amLac*4) (Figure 4-4D). The standard name *amLac*4 was therefore adopted for preparations 72, 147, 148, and 151. Preparations 145 and 150 were mixtures of the standards *amLac4* and a unique genome also present in preparation 146. Preparation 146 was assigned the standard name *amLac*11. This approach allowed the assignment of standard names to mixed DNA

Figure 4-4. Multi-environment filter hybridized with five DNA preparations. (A) spatial relationship of DNA preparations. Numbers correspond to those given in Table 4-1. D. m. is *Desulfovibrio multispirans*, D. v. is *Desulfovibrio vulgaris*. Probes used were; (B) DNA preparation 90, (C) DNA preparation 146, (D) DNA preparation 72, (E) DNA preparation 59 (*Lac*15 family member), and (F) DNA preparation 165 (*Pro*5 family member). Numbers corresponding to cross-hybridizing dots are indicated in the square below the dot, except in the case of (E), where preparation 204 is not indicated, and (F), where preparation 165 is indicated on the right side.


(A)
13 19 20 21 47 48 49 50 51 53
57 58 59 66 71 72 74 75 76 77
78 90 92 93 96 98 101 113 121 125
128 137 138 139 140 141 145 146 147 148
149 150 151 156 158 165 170 180 184 185
198 200 201 202 203 204 D. m. D. v. Lac3 Lac15
(B)
90
(C)
145 146
150
(D)
72
145
147 148
150 151
(E)
47 48 49
57 58 59
139 140 141
L15
(F)
165
201 202
204

preparations without ribotype codes, such as 145 (*amLac*4; *amLac*11), and the resolution of complex ribotypes, such as 150 (*amLac*4; *amLac*11).

Cross-hybridization between DNA preparations could not always be explained strictly in terms of a mixture, as in the case of *amLac*4 and *amLac*11. Two sets of cross-hybridizing genomes that gave distinctly different ribotypes were observed. These sets were named the *Lac*15 family and the *Pro*5 family for the oil field production water standard DNA with which they also all cross-hybridized. The term family is not used in a phylogenetic sense, as it is most likely that these are all strains of the same species. For example, when DNA preparation 59 (*tpLac*2b) was labeled and used as a probe to the multi-environment filter, it cross-hybridized to self, 47 (*tpLac*1a), 48 (*tpLac*1b), 57 and 58 (*tpLac*2a), 139, 140 and 141 (*tpEth*2), 204 and *Lac*15 (Figure 4-4E). While cross-hybridization to *tpLac*2a was anticipated due to the similarity between the two patterns, cross-hybridization to the other DNA preparations was not expected, nor could it be resolved in terms of mixtures of standards. Additional members of this family were observed when all preparations had been used as probes to the filter; 156, 170, 184 and 200 (*amPro*4). Another example was that of preparations 158 (*tpPro*1), 165 (*amPro*1), 201 (*amPro*5a), 202 (*amPro*5b), 203 and 204, which all cross-hybridized to some extent (Figure 4-4F), again despite different ribotypes. Several preparations from this set, when tested against the oil field production water master filter, were found to cross-hybridize to *Pro*5, (*Desulfobulbus propionicus*). Preparations belonging to these two families were assigned standard names based on ribotype, despite the cross-hybridizations.

Cross-hybridization results for each of the environments (AMD, oil sand production water, soil and sewage) are presented individually in Figure 4-5.

Representative standards from each environment are displayed in Figure 4-6 (e. g. *amLac*4 is shown only once, only one member of the *Lac*15 or *Pro*5 family is displayed per site).

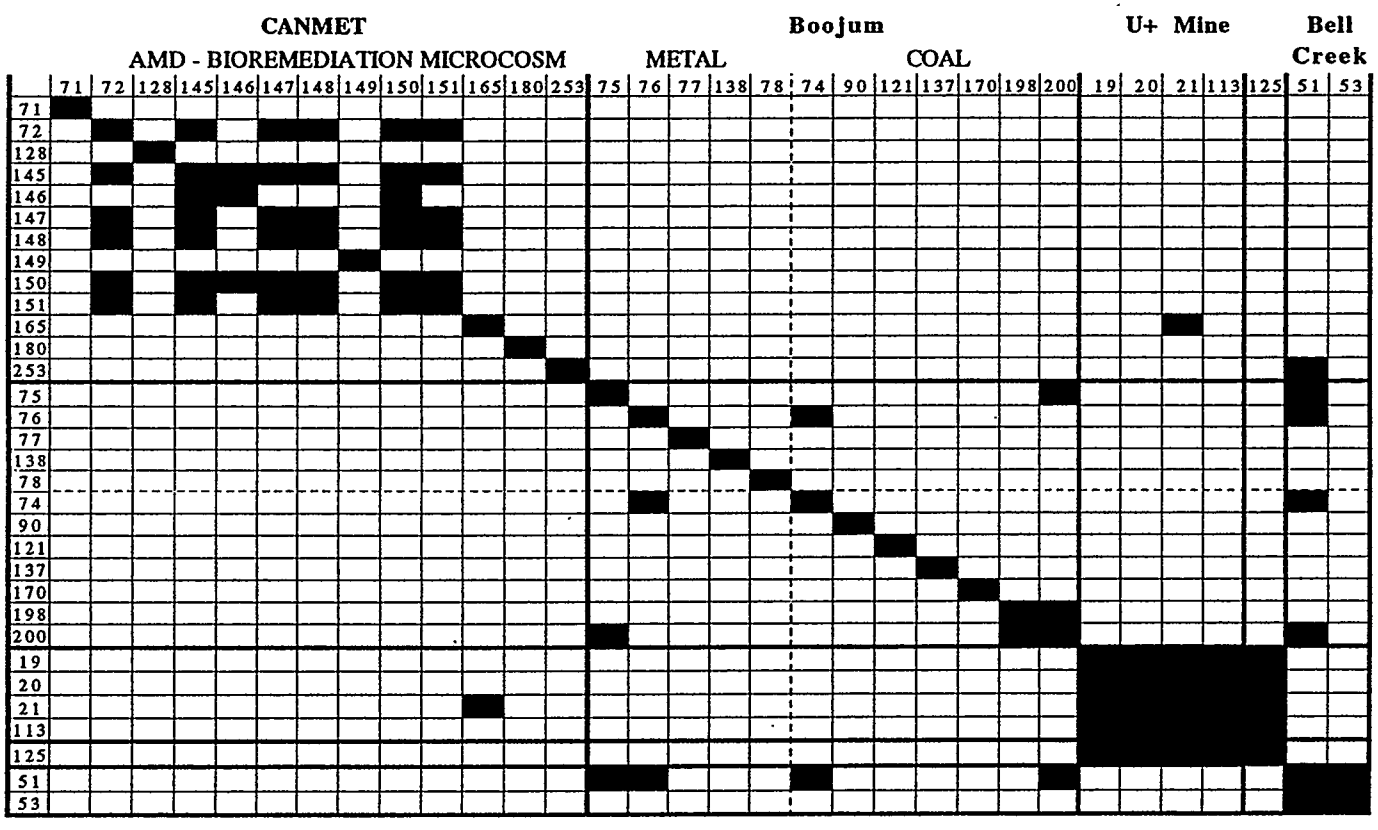
4.2.4 Whole genome analysis of subsequent enrichments and colony purified isolates; maintaining standards

Genomic DNA preparations of subsequent enrichment cultures or colony purified isolates for which the DNA preparation from the primary enrichment culture was present on the multi-environment filter generally had the same cross-hybridization patterns as the primary preparation. In many cases complex patterns could be resolved after colony purification. For example, DNA preparation 75 was ribotype coded *amLac*6b, and cross-hybridized to preparations 51 (*amLac*2) and 200 (*amPro*4). After colony purification, preparations 280, 281, and 285 resulted (also designated as 75a, b, and c respectively in Table 4-1). These all cross-hybridized with the primary *amLac*6b DNA preparation (75) and with *amPro*4, but not with *amLac*2. Secondary liquid enrichments of these colony purified isolates gave genomic DNA that also only cross-hybridized with *amLac*6b and *amPro*4. Since it was possible to repeatedly subculture *amLac*6b, it can be considered a stable standard.

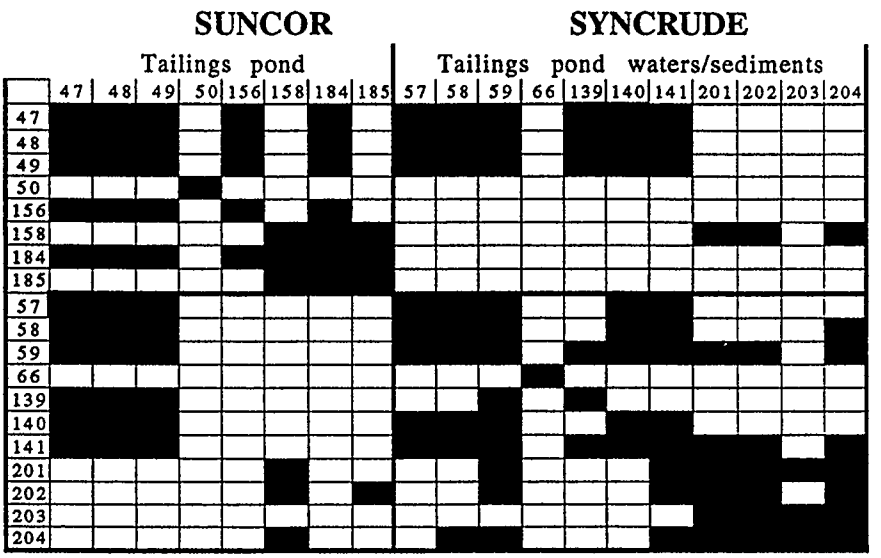
Occasionally DNA preparations from a secondary enrichment culture or colony purified isolate did not cross-hybridize with that from the primary preparation although some similarities to the whole genome cross-hybridization pattern for the primary DNA preparation were maintained. For example, the primary ethanol liquid enrichment culture from sample Suncor 33′ gave DNA preparation 184 (no ribotype code assigned) that cross-hybridized with self, 113

Figure 4-5. Hybridization patterns for genomic dot blots of (A) AMD DNA preparations, (B) oil sands production water DNA preparations, (C) sewage DNA preparations, (D) soil DNA preparations, and (E) CANMET Soil colony purified isolate DNA preparations. (A) and (B) were wholly derived from the multi-environment filter. (C) was derived from the sewage filter. (D) was partially derived from the multi-environment filter and partially from the CANMET Soil and Digout filters. (E) was wholly derived from the CANMET colony purified isolate filter. Bell Creek DNA preparations are included in both (A) and (D). DNA preparations are organized by site, family (for (D) and (E) only), then numerically. Numbers correspond to those given in Table 4-1. All DNA preparations tested are indicated. Hybridization is indicated by shaded squares.

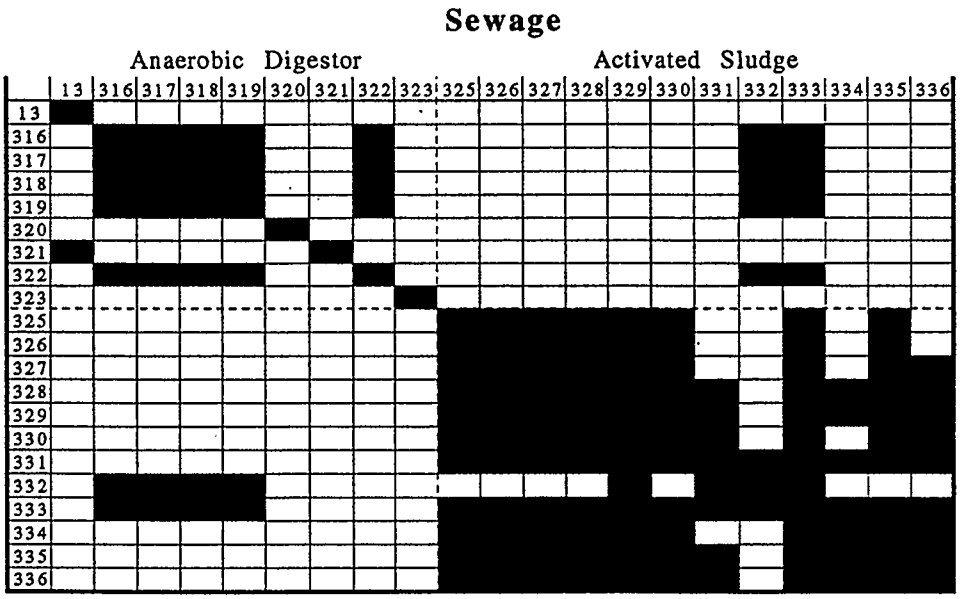
A


CANMET
AMD - BIOREMEDIATION MICROCOSM
Boojum
METAL
COAL
U+ Mine
Bell Creek
71 72 128 145 146 147 148 149 150 151 165 180 253 75 76 77 138 78 74 90 121 137 170 198 200 19 20 21 113 125 51 53

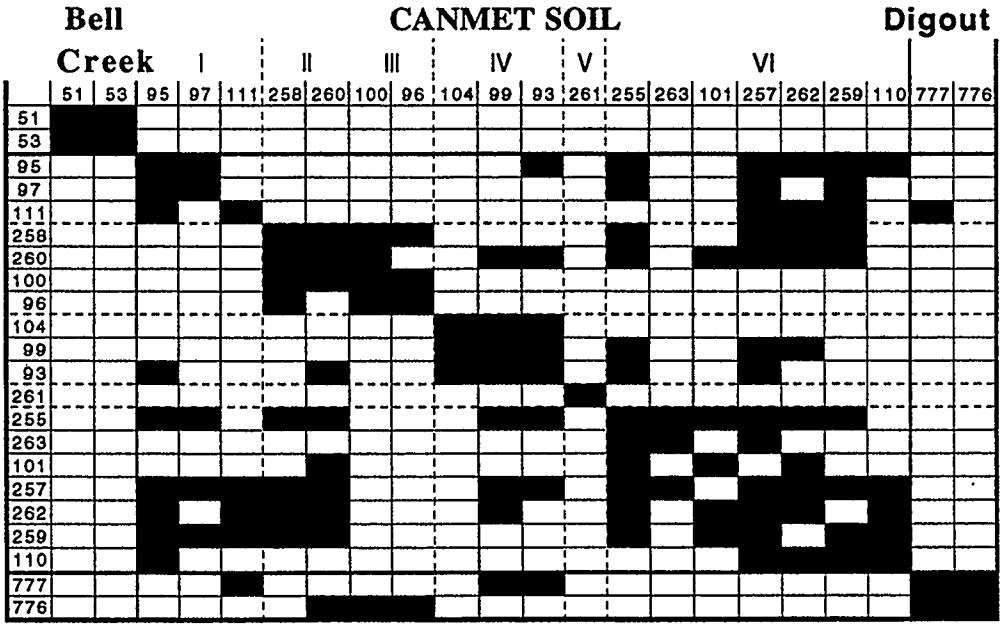
B


SUNCOR
SYNCRUDE
Tailings pond
Tailings pond waters/sediments
47 48 49 50 156 158 184 185 57 58 59 66 139 140 141 201 202 203 204

C


Sewage
Anaerobic Digestor
Activated Sludge
13 316 317 318 319 320 321 322 323 325 326 327 328 329 330 331 332 333 334 335 336

D


Bell Creek
CANMET SOIL
Digout
I
II
III
IV
V
VI
51 53 95 97 111 258 260 100 96 104 99 93 261 255 263 101 257 262 259 110 777 776

E

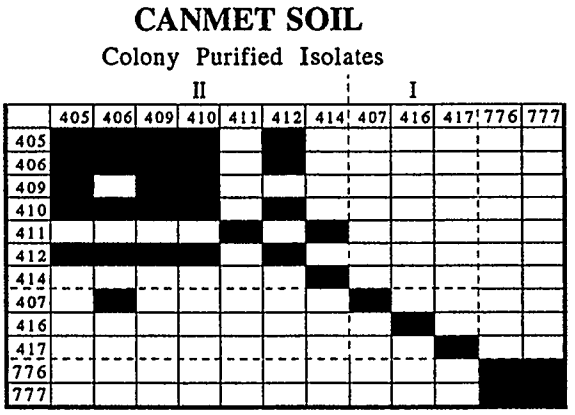

CANMET SOIL
Colony Purified Isolates
II
I
405 406 409 410 411 412 414 407 416 417 776 777

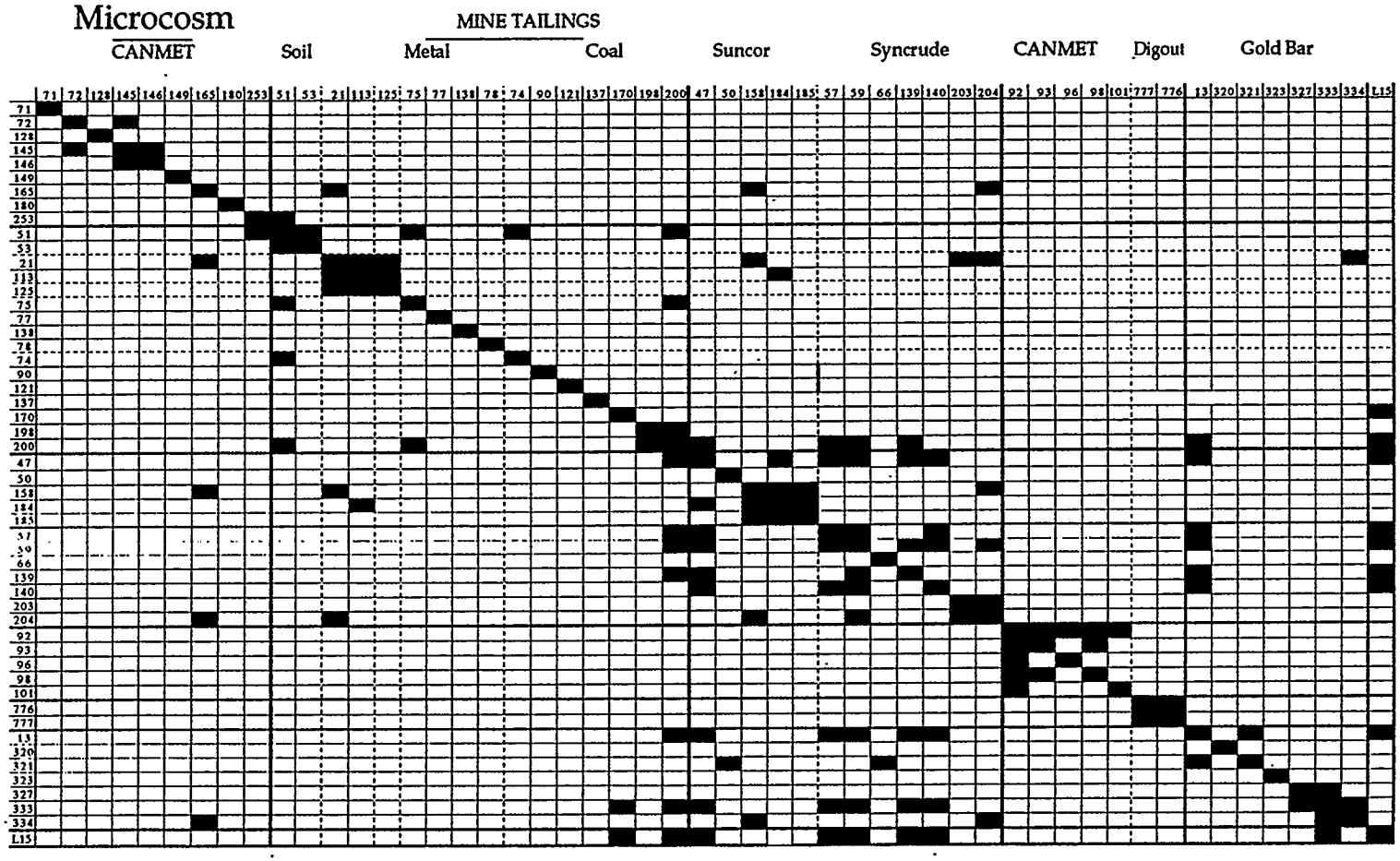
Figure 4-6. Hybridization patterns obtained from the multi-environment filter. DNA preparations are grouped by process (AMD, oil sand production water, soil and sewage) and organized by site, then numerically. The hybridization pattern for *Lac*15 (L15) is also shown. Only preparations tested against the multi-environment filter and representative preparations are shown (i. e. only one member of the *Lac*15 family per site and only one preparation from a cross-hybridizing group per site). Preparations that were only hybridized to the filter and were not spotted on the filter (i. e. most sewage preparations) are not symmetric across the self-hybridization diagonal, with patterns shown only along rows and not columns.

Acid Mine Drainage

Oil Sands Production Water

Soil

Sewage



(*amAce*1), and with the *Lac*15 family. Genomic DNA preparations from both the secondary culture (229) and a colony purified isolate (250) failed to cross-hybridize to preparation 184 or to *amAce*1 but continued to cross-hybridize to members of the *Lac*15 family.

In several cases, DNA preparations from the secondary culture or colony purified isolate resulted in an entirely new cross-hybridization pattern, or no cross-hybridization to the multi-environment filter at all. The primary liquid enrichment culture obtained from Boojum 8 ethanol gave DNA preparation 200 (*amPro*4), which had extensive cross-hybridizations indicating it to be a genomic consortium (Table 4-1). The secondary liquid enrichment culture gave DNA preparation 247, which cross-hybridized only to *amAce*3. Colony purification from the secondary liquid enrichment culture resulted in DNA preparation 273 that had yet another cross-hybridization pattern. Instead of cross-hybridizing to *amPro*4, *amLac*6b, *amLac*2, or members of the *Lac*15 family, or to *amAce*3, 273 cross-hybridized only to 113 (*amAce*1). A secondary enrichment culture of the colony purified isolate gave DNA preparation 287, which again cross-hybridized to *amAce*1, but also cross-hybridized to *amPro*4, *amLac*6b, as well as to 180 and 156. Thus, this set of preparations can not be considered stable. Similar results were also see for preparation 90 (*amBen*2). The DNA preparation from the colony purified isolate (277) cross-hybridized only with *amBen*2, but the DNA preparation from the secondary culture of the colony purified isolate (288) failed to cross-hybridize to *amBen*2, cross-hybridizing to *amLac*6b and *amPro*4 instead. Apparantly *amBen*2 is also an unstable standard. Standards that could not be repeatedly subcultured were considered unstable. Table 4-4 indicates all the DNA preparations obtained from colony purified isolates for which primary

Table 4-4. Colony purified isolates and their stability.

#[a]	expected standard[b]	observed standard[c]	standard resolution[d]	subsequent cultures[e]
275	*amAce*1	*amAce*1	nc	*amAce*1
279	*amLac*2	*amLac*2	yes	*amPro*4
145	*amLac*4	*amLac*4/*amLac*11	nc	na
146	*amLac*4	*amLac*11	nc	na
147	*amLac*4	*amLac*4	nc	na
148	*amLac*4	*amLac*4	nc	na
149	*amLac*4	*amLac*5	nc	na
150	*amLac*4	*amLac*4/*amLac*11	nc	na
151	*amLac*4	*amLac*4	nc	na
253	ne	*amLac*2	nc	na
251	*amLac*8	*amLac*6a/*amBen*1	nc	unstable
280	*amLac*6b	*amLac*6b	yes	*amLac*6b
281	*amLac*6b	*amLac*6b	yes	*amLac*6b
285	*amLac*6b	*amLac*6b	yes	na
277	*amBen*2	*amBen*2	nc	*amLac*6b
294	*amPro*2	(*Lac*15)	no	na
273	*amPro*4	*amAce*1	yes	unstable
267	(156)	(*Lac*15)	nc	unstable
315	*tpBen*1	nh	nc	na
250	(*Lac*15)	(*Lac*15)	nc	na
278	*tpEth*1	*tpBen*1	nc	unstable
276	*tpEth*2	*amLac*4	nc	*amLac*4
293	*tpEth*2+	*tpEth*2	yes	na
296	*tpEth*2+	*amLac*6b	yes	na

Table 4-4. continued.

[a] Chronological number assigned to the preparation; grouped by site.

[b] Standard observed in the DNA preparation from the primary liquid culture. ne=no expected result. () indicates an uncoded standard either with self-hybridization only, or with hybridization to a family. + indicates genomic mixture.

[c] standard or preparations on the multi-environment filter to which the DNA preparation from the colony purified isolate cross-hybridized. nh=no hybridization.

[d] yes=the cross-hybridization pattern from the colony purified isolate was less complex than that of the primary preparation. no= the pattern is the same as, or more complex than the primary preparation. nc=pattern for the primary preparation was not complex.

[e] na= no subsequent culture available. Standard names are given unless the cross-hybridization pattern was complex and unstable.

DNA preparations were represented on the multi-environment filter, and their stability in subsequent cultures. The results for all DNA preparations, (primary, secondary, colony purified, etc.) that were tested against the multi-environment filter are presented in Table 4-1.

4.2.5 Whole genome comparisons; individual sites and colony purified isolates.

Individual dot blots prepared from CANMET Soil primary and secondary DNA preparations demonstrated the presence of six cross-hybridizing families (Figure 4-5D). Cross-hybridization was observed between Digout preparation 776 (*soAce*1) and some members of family II and III, and Digout preparation 777 (*soLac*1) and members of family I and IV (Figure 4-5D). Only preparation 261, a DNA preparation from a secondary enrichment of sample S-6-31-3, had no cross-hybridization to any of the other preparations. One sample from each of the families was used for the colony purification procedure, except in the case of the more complex families, (II, and VI), where two samples were used (Table 4-2). Cross-hybridizations observed for the filter with colony purified CANMET Soil DNA preparations are indicated in Figure 4-5E. Four standards were identified from the CANMET Soil colony purified isolate filter, which grouped into only two of the previous six families (I and II, Table 4-2) and also all failed to cross-hybridize to either *soLac*1 or *soAce*1 (Figure 4-5E). Five primary DNA preparations for which a ribotype code had been assigned were also included on the multi-environment filter. These did not cross-hybridize with any of the preparations from other environments (Figure 4-6).

Cross-hybridizations for the sewage single-environment filter that included preparations obtained from colony purified isolates (6 of 20 genomic DNA preparations represented colony purified isolates) are indicated in Figure 4-5C. Ten preparations containing only one of four standards (*swEth*1 (preparations 316-319, and 322), *swBen*2 (320), *swBen*3 (323), *swBen*4 (321), and *swPro*1 (325,326)) were identified from the sewage filter. Of these ten, 316-319, 321, and 323 had been derived from colony purified isolates (Table 4-1). Ten DNA preparations containing mixtures of standards were also identified (327-336). None of these ten had been derived from colony purified isolates or from Sewage B. Representative preparations of each of the standards from the sewage filter (318, 320, 321, 323, 327, 331, 332, 333, and 334) were tested against the multi-environment filter (Table 4-5). Of the five standards observed from the sewage filter, *swEth*1 belonged to the *Lac*15 family, *swPro*1 belonged to the *Pro*5 family, *swBen*4 cross-hybridized to preparations 13 (a primary Sewage B DNA preparation), *tpAce*1, and *tpBen*1, while *swBen*2 and *swBen*3 showed no cross-hybridization.

Digout, with genomic DNA exclusively from colony purified isolates, had results that correlated with the ribotyping (Figure 4-3A), in that only two standards (*soLac*1 and *soAce*1, preparations 777 and 776 respectively) were identified (Figure 4-5D). Neither of these Digout preparations cross-hybridized to the multi-environment filter (Figure 4-6). These two standards showed some cross-hybridization to the CANMET Soil filter (Figure 4-5D), but not to the CANMET Soil colony purified isolate filter (Figure 4-5E), as discussed above.

Table 4-5. Cross-hybridization of DNA preparations from colony purified sewage and soil standards to the multi-environment filter.

#[a]	preparation	standard[b]	hybridization[c]
776	Soil lactate 13A	*soLac*1; *soAce*1	nh
777	Soil lactate 14A	*soLac*1	nh
318	Sewage B ethanol b	*swEth*1	*Lac*15 family
320	Sewage b benzoate	*swBen*2	nh
323	Sewage B benzoate b(b)	*swBen*3	nh
321	Sewage B benzoate a(b)	*swBen*4	*swBen*1, *tpAce*1, *tpBen*1
327	Activated sludge ethanol	*swPro*1;?	nh
331	Anaerobic digestor propionate	*swPro*1;+	*Pro*5 family
332	Anaerobic digestor lactate	*swEth*1;?	*Lac*15 family
333	Anaerobic digestor ethanol	*swPro*1;*swEth*1	*Lac*15 family
334	Anaerobic digestor decanoate	*swPro*1;*swEth*1	*Pro*5 family

a Chronological number assigned to the DNA preparation

b standard name assigned based on cross-hybridization andribotype. na= no name assigned.

c cross-hybridizations are indicated. nh= no cross-hybridizations

4.3 Comparison to oil field production water standards

Several standards from each environment were selected for hybridization with the oil field production water master filter. Some of the results are shown in Figure 4-7B and C. The spatial organization of the standards represented on this master filter is shown in Figure 4-7A. Results for all DNA preparations tested are summarized in Table 4-6. Typically, there was no cross-hybridization between standards isolated in this study and oil field production water standards. For example, when preparation 149 (*amLac*5) was used as a probe, no cross-hybridizations were observed, only the internal control (lambda DNA) self-hybridized (Figure 4-6B). Exceptions generally occurred with the *Lac*15 and *Pro*5 families. When preparation 58 (*tpLac*2a) was used as a probe, hybridization was observed to *Lac*15 and *Lac*15 family members (Figure 4-6C). The only hybridization between a standard identified in this study and a previously identified and characterized standard was between *amLac*4 (preparations 147 and 148) and *Lac*30, a standard isolated from river water in Edmonton (Table 4-6).

4.4 Identification of standards by 16S rRNA gene sequencing.

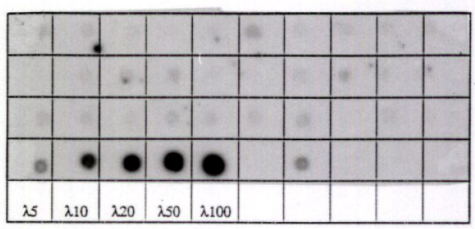
Eight standards were selected for identification. PCR amplification from many primary DNA preparations was, for the most part, unsuccesful. This may have been due to DNA degradation over time. Thus, fresh DNA preparations from either secondary enrichments or colony purified isolates were used for PCR, provided that their whole genome hybridization patterns corresponded with that of the primary preparation. Standards selected are detailed in Table 4-7, along with the numbers of M13 clones obtained and sequenced.

Figure 4-7. Oil field production water master filter hybridizations. (A) Spatial relationship of standards. Probes used were (B) DNA preparation 149 and (C) DNA preparation 59. A lambda DNA dilution series was included on this filter, indicated by λ5-λ100 and lambda DNA was added to both preparations prior to labeling (see Methods section 3.3.5). Hybridizing dots for standards and for lambda are indicated in the square below the dot.

(A)

Lac1/2	Lac3	Lac4	Lac5	Lac6	Lac7	Lac10	Lac12	Lac15	Lac17
Lac21	Lac22	Lac23	Lac24	Lac25		Lac30	Eth3	Eth5	Eth6
Ben1	Ben4	Dec6	Pro4	Pro5	Pro12	Ace1	Ace9	Ben7	
λ5	λ10	λ20	λ50	λ100		Ace8			

(B)



(C)

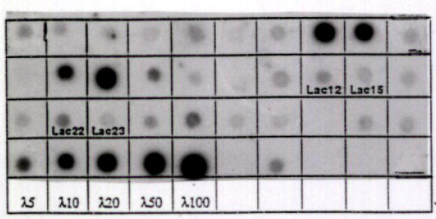


Table 4-6. Hybridizations to oil field production water standards.

#	Preparation	standard	hybridizations
147	CANMET AMD 2-4Bh lactate	*amLac*4	*Lac*30
148	CANMET AMD 2-2A lactate	*amLac*4	*Lac*30
149	CANMET AMD 2-1B lactate	*amLac*5	nh[a]
71	CANMET AMD 1 lactate	*amLac*3	nh
146	CANMET AMD 2-2B lactate	*amLac*11	nh
165	CANMET AMD 3 propionate	*amPro*1	*Pro*5
76	Boojum 1 lactate	*amLac*6c	nh
75	Boojum 2 lactate	*amLac*6b	nh
137	Boojum 9 decanoate	*amDec*1	nh
125	AMD acetate	*amAce*1	nh
47	Suncor 23' lactate	*tpLac*1a	*Lac*15 family
50	Suncor 43' benzoate	*tpBen*1	nh
156	Suncor 33' benzoate	na	nh
158	Suncor 23' propionate	*tpPro*1	*Pro*5
185	Suncor 43' ethanol	*tpEth*1	nh
58	Syncrude 16-20 lactate	*tpLac*2	*Lac*15 family[b]
140	Syncrude pond ethanol	*tpEth*2	*Lac*15 family
417	CANMET Soil 111_{AN} lactate	na	nh

Note: headings are as for Table 4-5.

[a] Figure 4-7B.

[b] Figure 4-7C.

Table 4-7. Standards selected for identification by sequencing

Standard[a]	#[b]	blunt clones[b]	sticky clones[c]	sequenced[d]
*Lac*30	151	5	16	9
na	253	3	9	5
*amBen*2	277	5	7	6
*amLac*6b	252	1	10	5
*amLac*2	279	5	6	5
*amAce*1	275	0	7	7
*soLac*1	777	1	0	1
*soAce*1	776	4	0	4

a na= not assigned

b Chronological number assigned to the DNA preparation used for PCR.

c number of blunt or sticky end clones with 16S rRNA gene fragment inserts, as determined by screening.

d Number of clones for which sequence information was obtained.

4.4.1 Cloning and sequencing of soil standards

Preparations 776 and 777 were chosen for PCR amplification for reasons of DNA availability and the differences in ribotype (Figure 4-3A) and cross-hybridization on dot blots (results not shown). Clones and sequences from 776 were named for *soAce*1, while clones and sequence for 777 were named for *soLac*1. Blunt end cloning was used exclusively to obtain potential *soAce*1 and *soLac*1 clones (Table 4-7). Four clones with identical sequence information were obtained for 776, two in each orientation (defined with repsect to the 16S rRNA gene); sA1-1 and sA1-2 in the reverse orientation, and sA1-3 and sA1-4 in the forward orientation. sA1-1, sA1-3, and sA1-4 were sequenced using all five primers (Universal, P75, and P77, or Universal, P76 and P78), while sequence for sA1-2 was obtained only with Universal and P76. The 535 bp fragment that resulted from PCR amplification of 776 was sequenced in its entirety with these four clones. Only one clone was obtained for 777 (sL1-1, in the forward orientation), thus to attempt to complete the sequence and to obtain sequence on both strands, a clone-turn around was performed (sL1-1t). This resulted in the loss of some sequence due to an internal *Hind*III restriction site near the 3' end of the fragment (near the Univ-907R PCR primer hybridization site). Two of the internal primers could not be used to obtain sequence from sL1-1 or sL1-1t, as only P76 consistently hybridized while P78 had poor hybridization (P75 and P77 did not hybridize to sL1-1t). The sequence obtained for the fragment amplified by PCR from 777 was thus incomplete at 529 bp.

4.4.1.1 Sequence analysis of soil standards

The sequences obtained for 776 and 777 are compiled in Figure 4-8A and B respectively. Sequence corresponding to the hybridization region of P75/P76 is underlined with a dotted line in Figure 4-8, and sequence corresponding to the hybridization region of P77/P78 is underlined with a single solid line. Sequence alignments between sequence obtained from 776 (named *soAce*1seq), 777 (named *soLac*1seq) and other *Desulfovibrio* sequences are shown in Figure 4-9. Fasta searches and Blast searches indicated that the sequence amplified from 776 was closely related to sequences of *Desulfovibrio* species, while the sequence amplified from 777 was more closely related to the genus *Bacteroides* than to any SRB (Table 4-8).

4.4.1.2 Verification of soil standards; Southern blot and PCR analysis

The unexpected observation of a *Bacteroides* sequence obtained by PCR from DNA preparation 777 (*soLac*1) prompted the question whether *Bacteroides* was a major or minor component in DNA preparation 777. A minor component that had not been observed in the ribotype or whole genome analysis could be preferentially amplified during PCR. Three deoxyoligonucleotides were designed targeting non-conserved regions of the SRB 16S rRNA gene sequences, as judged by alignment with several SRB 16S rRNA gene sequences (Figure 4-8, double underlined, Figure 4-9, underlined). Two deoxyoligonucleotides were designed for *Bacteroides* (P88 and P89, designed from regions 99-117 and 466-485 respectively, as in Figure 4-8B), and one was designed to target the observed *Desulfovibrio sp.* sequence (p90 from region 91-109, as in Figure 4-8A). P88, P89 and P90 were hybridized to the Southern blots used in the ribotyping of the

Figure 4-8. Alignment of contiguous sequences obtained from M13 clones of the 16S rRNA gene PCR product amplified from Digout preparation (A) 776 and (B) 777. Sequences are named by clone (sA1-1 to sA1-4 for 776 and sL1-1 or sL1-1t for 777), number (distinguishing between multiple sequences of the same primer/clone combinations), and primer used to obtain the sequence (U=universal primer, or one of P75-P78). Pads (*) have been inserted to improve alignment. The consensus sequence is indicated. '-' indicates ambiguous positions. The hybridization positions for the internal sequencing primers are indicated in bold type; P75/P76 with a dotted underline and P77/P78 with a solid underline. In (B), these regions do not have sequence identical to that of the primers (see Materials, section 3.1.5). The sequence from which specific deoxyoligonucleotides were designed are indicated with a double underline. In (B), the *Hind*III internal restriction site is indicated, in bold, at position 496-501.

A

```
                          10        20        30        40        50
 16  sA1-3.7U     TATTGCGCAATGGGCGAAA*CGTGACGCAGCGACGCCTCGTGAGGGATGA
-19  sA1-1.5p75   TATTGCGCAATGGGCGAAAGCCTGACGCAGCGACGCCTCGTGAGGGATGA
-13  sA1-1.4p75   TATTGCGCAATGGGCGAAAGCCTGACGCAGCGACGCCTCGTGAGGGA**A
 -2  sA1-4p75     TATTGCGCAATGGGCGAAAGCCTGACGCAGCGACGCCTCGTGAGGGATGA
 -5  sA1-1.3p75   TATTGCGCAAT*GGCGAAAGCCTGACGCAGCGACGCCTCGTGAGGGATGA
 -4  sA1-1.2p75   TATTGCGCAATGGGCGAAAGCCTGACGCAGCGACGCCTCGTGAGGGATGA
 10  sA1-3.2U     TATTGCGCAATGGGCGAAA*CGTGACGCAGCGACGCCTCGTGAGGGATGA
  3  sA1-3.1U     TATTGCGCAATGGGCGAAA*CGTGACGCAGCGACGCCTCGTGAGGGATGA
     CONSENSUS    TATTGCGCAATGGGCGAAA-C-TGACGCAGCGACGCCTCGTGAGGGATGA

                          60        70        80        90       100
 16  sA1-3.7U     AGGTCTTCGGATCGTAAACCTCTGTCAAGAGGGAAGAAACCCTTGGATTC
-19  sA1-1.5p75   AGGTCTTCGGATCGTAAACCTCTGTCAAGAGGGAAGAAACCCTTGGATTC
 -2  sA1-4p75     AGG
 -5  sA1-1.3p75   AGGTCTTCGGATCGTAAACCTCTGTCAAGAGGGAAGAAACCCTTGGATTC
 -4  sA1-1.2p75   AGGTCTTCGGATCGTAAACCTCTGTCAAGAGGGAAGAAACCCTTGGATTC
 10  sA1-3.2U     AGGTCTTCGGATCGTAAACCTCTGTCAAGAGG*AAGAAACCCTT*GA*TC
  3  sA1-3.1U     AGGTCTTCGGATCGTAAACCTCTGTCAAGA
     CONSENSUS    AGGTCTTCGGATCGTAAACCTCTGTCAAGAGGGAAGAAACCCTTGGATTC

                         110       120       130       140       150
 16  sA1-3.7U     GAATAGGGTCCTTGGCTGACGGTACCTCAAAAGGAAGCACCGCTAACTC*
-19  sA1-1.5p75   GAATAGGGTCCTT
 -5  sA1-1.3p75   GAATAGGGTCCTTGG
 -4  sA1-1.2p75   GAATAGGGTCCTTGG
 10  sA1-3.2U     GAATA
-15  sA1-4p77      AATAGGGTCCTTGGCTGACGGTACCTCAAAAG*AAGCACCGCTAACTCC
 -6  sA1-1.1p77                                  AAG*AAGCACCGCTAACTCC
     CONSENSUS    GAATAGGGTCCTTGGCTGACGGTACCTCAAAAG-AAGCACCGCTAACTCC

                         160       170       180       190       200
 16  sA1-3.7U     GTGCCAGCAGCCGCG*TAATACGGAGGTGC*AGC
-15  sA1-4p77     GTGCCAGCAGCCGCGGTAATACGGAGGTGCAAGCGTTAATCGGAATCACT
 -6  sA1-1.1p77   GTGCCAGCAGCCGCGGTAATACGGAGGTGCAAGCGTTAATCGGAATCACT
 -7  sA1-1.2p77                                        TAATCGGAATCACT
     CONSENSUS    GTGCCAGCAGCCGCGGTAATACGGAGGTGC-AGCGTTAATCGGAATCACT

                         210       220       230       240       250
-15  sA1-4p77     GGGCGTAAAGCGCTCGTAGGCGGTTTGG
 -6  sA1-1.1p77   GGGCGTAAAGCGCTCGTAGGCGGTTTGGTCAGTCAGATGTGAAA*GCCCT
 -7  sA1-1.2p77   GGGCGTAAAGCGCTCGTAGGCGGTTTGGTCAGTCAGATGTGAAA*GCCCT
 14  sA1-3.4p76      CGTAAAGCGCTCGTAGGCGGTTTGGTCAGTCAGATGCGAAACG*CCT
  8  sA1-3.2p76               TCGTAGGCGGTTTGGTCAGTCAGATGCGAAACG*CCT
 12  sA1-3.1p76                                        TGCGAAACG*CCT
     CONSENSUS    GGGCGTAAAGCGCTCGTAGGCGGTTTGGTCAGTCAGATG-GAAA-G-CCT
```

Figure 4-8A continued.

```
                          260        270        280        290        300
 -6   sA1-1.1p77   CGGCTTAACCGGGGAACTGCATTTGATACTGCCAGACTTGAGTGTCGGAG
 -7   sA1-1.2p77   CGGCTTAACCGGGGAACTGCATTTGATACTGCCAGACTTGAGTGTCGGAG
 14   sA1-3.4p76   CGGCTTAACCGGGGAACTGCATTTGATACTGCCAGACTTGAGTGTCGGAG
  8   sA1-3.2p76   CGGCTTAACCGGGGAACTGCATTTGATACTGCCAGACTTGAGTGTCGGAG
 12   sA1-3.1p76   CGGCTTAACCGGGGAACTGCATTTGATACTGCCAGACTTGAGTGTCGGAG
 18   sA1-5p76               GGGAACTGCATTTGATACTGCCAGACTTGAGTGTCGGAG
      CONSENSUS    CGGCTTAACCGGGGAACTGCATTTGATACTGCCAGACTTGAGTGTCGGAG


                        310        320        330        340        350
 14   sA1-3.4p76   AG
  8   sA1-3.2p76   AGAGGGTGGCGAATTCCGGTGTAGGAGTGAAATCCGTAGATATCGAGAC
 18   sA1-5p76     AGAGGGTGGCGAATTCCGGTGTAGGAGTGAAATCCGTAGATATCGAGAAC
-17   sA1-4.1U                                              ATCGAGAAC
      CONSENSUS    AGAGGGTGGCGAATTCCGGTGTAGGAGTGAAATCCGTAGATATCGAGAAC


                          360        370        380        390        400
 18   sL1-2p76     ACCAGTGCGAAGGCGCCACCTGGACGACAACTGACGCTGAGGAGC*AAAG
-17   sA1-4.1U     ACCAGTGCGAAGGCGCCACCT*GAC*ACAACTGACGCTGAGGAGCGAAAG
 20   sA1-3.4p78                 ACCTGGACGACAACTGTCACTGAGGAGCGAAAG
 11   sA1-3.2p78                     GGACGACAACTGACGCTGAGGAGCGAAAG
      CONSENSUS    ACCAGTGCGAAGGCGCCACCTGGACGACAACTGACGCTGAGGAGCGAAAG


                          410        420        430        440        450
-17   sA1-4.1      CGTGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCGGTAAACGAT
 20   sA1-3.4p78   AG*GGGAGCAAACAGGATTAGATACCCTGGTAGTC*ACGCG*TAAACGAT
 11   sA1-3.2p78   CGTGGGA-CAAACAGGATTAGATACCCT*GTAGT*CACGC*GTAAACGAT
 -1   sA1-1.3U                   TTAGATACCCTGGTAGTCCACGCGGTAAACGAT
 -9   sA1-4.2U                            CCCTGGTAGTCCA*GCGGTAAAC*AT
      CONSENSUS    -G-GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCGGTAAACGAT


                          460        470        480        490        500
-17   sL1-4.1U     GGATGCTGGGTGTCG*GGGGTTTA*CTTCGGTGCCGCAGTTAACGCGTTA
 20   sA1-3.4p78   GGATGCTGG*TGTCA*GGGGTT*A*CTTCGGTGCCGCAGTTAACGCGTTA
 11   sA1-3.2p78   GGATG
 -1   sA1-1.3U     GGATGCTGGGTGTCG*GGGGTTTAGCTTCGGTGCCGCAGTTAACTCGTTA
 -9   sA1-4.2U     *GATGCTGGGTGTCGAGGGGTTTA*CTTCGGTG*CGCAGTTAACGCGTTA
      CONSENSUS    GGATGCTGGGTGTCG*GGGGTTTA*CTTCGGTGCCGCAGTTAACGCGTTA


                          510        520        530
-17   sA1-4.1U     AGCATCCCGCCT
 20   sA1-3.4p78   AGCATCCCGCCTGGGGAGTAC*GGTCGCAAGGCTG
 -1   sA1-1.3U     AGCATCCCGCCTGGGGAGTAC*GGTCGCAAGGCTG
 -9   sA1-4.2U     AGCATCCCGCCTGGGGAGTACTGGTCGCAAGGCTG
      CONSENSUS    AGCATCCCGCCTGGGGAGTAC-GGTCGCAAGGCTG
```

B

```
                          10         20         30         40         50
9   sL1-1.2U     TATTGGTCAATGGGCGATTACGTGTAATCCAGCCAAGTCGCGTGTAAGGA
1   sL1-1.4U     TATTGGTCAATGGGCGATTACGTG*AATCCAGCCAAGTCGCGTGTAAGGA
    CONSENSUS    TATTGGTCAATGGGCGATTACGTG-AATCCAGCCAAGTCGCGTGTAAGGA

                          60         70         80         90        100
9   sL1-1.2U     AGAAGGATCTATGGTTCGTAAACTTCTTTTGCAGGGGAAATAAAG*TGGA
1   sL1-1.4U     AGAAGGATCTATGGTTCGTAAACTTCTTTTGCAGGGGAAATAAAGATGGA
    CONSENSUS    AGAAGGATCTATGGTTCGTAAACTTCTTTTGCAGGGGAAATAAAG-TGGA

                         110        120        130        140        150
9   sL1-1.2U     GGACGTGTCTCTTTTGTATGTATCCCTGAGAATAAGGATCGGCTAACTCG
1   sL1-1.4U     GGACGTGTCTCTTTTGTATGTATCCCTGAGAATAAGGATCGGCTAACTCG
    CONSENSUS    GGACGTGTCTCTTTTGTATGTATCCCTGAGAATAAGGATCGGCTAACTCG

                         160        170        180        190        200
9   sL1-1.2U     TGCCAGCAGCCGCGTAATACGAGGATCGAGCGTTATCCGGAGTTATTGGG
1   sL1-1.4U     TGCCAGCAGCCGCGTAATACGAGGA
2   sL1-1.1p76                                AGCGTTATCCGGA*TTATTGGG
4   sL1-1.2p76                                AGCGTTATCCGGA*TTATTGGG
    CONSENSUS    TGCCAGCAGCCGCGTAATACGAGGATCGAGCGTTATCCGGA-TTATTGGG

                         210        220        230        240        250
9   sL1-1.2U     T
2   sL1-1.1p76   TTTAAAGGGTGCGTAGGTGGTTTAATAAGTCAGCGGTGAAAGTTTGCCAG
4   sL1-1.2p76   TTTAAAGGGTGCGTAGGTGGTTTAATAAGTCAGCGGTGAAAGTTTGCCAG
5   sL1-1.3p76                                GTCAGCGGTGAAAGTTTGCCAG
    CONSENSUS    TTTAAAGGGTGCGTAGGTGGTTTAATAAGTCAGCGGTGAAAGTTTGCCAG

                         260        270        280        290        300
2   sL1-1.1p76   GCTTAACTGTAAAA
4   sL1-1.2p76   GCTTAACTGTAAAA
5   sL1-1.3p76   GCTTAACTGTAAAAATGCCGTTGAAACTGTTAGACTTGAGTGTAAATGAG
6   sL1-1.4p76                TGCCGTTGAAACTGTATGACTTGAGTGTAAATGAG
    CONSENSUS    GCTTAACTGTAAAAATGCCGTTGAAACTGT--GACTTGAGTGTAAATGAG

                         310        320        330        340        350
5   sL1-1.3p76   GTAGGCGGAATGCGTGGTGTAGCGGTGAAATGCTTAGATATCACGCAGAA
6   sL1-1.4p76   GTAGGCGGAATGCGTGGTGTAGCGGTGAAATGCTTAGATATCACGCAGAA
-8  sL1-1t.2U                    GTAGCGGTGAAATGCTTAGATATCACGCAGAA
    CONSENSUS    GTAGGCGGAATGCGTGGTGTAGCGGTGAAATGCTTAGATATCACGCAGAA

                         360        370        380        390        400
5   sL1-1.3p76   CTCGATTGCGAAGGCAGCTTACCAAGCTACAACTGACACTGAAGCACGAA
6   sL1-1.4p76   CTCGATTGCGAAGGCAGCTTACCAAGCTACAACTGACACTGAAGCACGAA
-8  sL1-1t.2U    CTCGATTGCGAAG*CAGC*TAC*AAGCTACA*CTGACACTGAAGCACGAA
-3  sL1-1.5U                 GCAGCTT*C*AAGCT*CAACTGAC*CT*AAGCAC*AA
-7  sL1-1t.1U                                          GAAGCACGAA
    CONSENSUS    CTCGATTGCGAAGGCAGCTTAC-AAGCTACAACTGACACTGAAGCACGAA
```

Figure 4-8B continued.

```
                          410        420        430        440        450
 5  sL1-1.3p76   AGCGTGGGGATCAAACA
 6  sL1-1.4p76   AGCT****GATCAAACAGG
-8  sL1-1t.2U    AGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCAGTAAAC
-3  sL1-1.5U     AGCGTGGGGATCACACAGGATTAGATACCCTGGTAGTCCACGCAGTAAAC
-7  sL1-1t.1U    AGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCAGTAAAC
10  sL1-1.1p78                                                  TAAAC
    CONSENSUS    AGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCAGTAAAC


                          460        470        480        490        500
-8  sL1-1t.2U    GATGATTACTAGCTGTTTGCGATACACAGTAAGCGGCAC
-3  sL1-1.5U     GATGATATCTAGCTGTTTGCA
-7  sL1-1t.1U    GATGATTACTAGCTGTTTGCGATACACAGTAA
10  sL1-1.1p78   GATGATTACTAGCTGTTTGCGATACACAGTAAGCGGCACAGCGAAAGCTT
    CONSENSUS    GATGATTACTAGCTGTTTGCGATACACAGTAAGCGGCACAGCGAAAGCTT

                          510        520
10  sL1-1.1p78   AAGTAATCACCTGGGGAGTACGCCGGCA
    CONSENSUS    AAGTAATCACCTGGGGAGTACGCCGGCA
```

Figure 4-9. Alignment of consensus sequences from the 16S rRNA gene PCR products of 776 (soAce1seq), 777 (soLac1seq), and various SRB standards. SRB sequences were available from previous work and were obtained by the same methods used here (Fouts, unpublished observations). Ambiguous positions are indicated with a '-'. and pads (*) have been added to improve alignment. The consensus sequence is also given. Regions of high ambiguity (many dashes) correspond to non-conserved regions of the 16S rRNA gene. The hybridization positions of the deoxyoligonucleotides designed for specific recognition of soAce1seq and soLac1seq are indicated in bold.

```
                          10         20         30         40         50
20  soLac1seq   TATTGGT-AATGGGCGA-TACG-G-AA-CCAGCCAAGTCGCGTG-AAGGA
 1  eth3seq     TATTGCGCAATGGGGGAAACCCTG*ACGC*AGCGACGCCATGTG*AGGGA
14  lac10se1    TATTGCGCAATGGGCGAACA-TGGAACGCGAGCGACGGCGCGTGTAGGGA
 3  lac26seq    TATTGCGCAATGGGCGAAAC*GTG*ACGC*AGCAACGCCGTGTG*AGGGA
 7  lac3se1     TATTGCGCAATGGGCGAA*C**TGGACGC*AGCG*CGCCGCGTGGAGGGA
13  lac8se1     TATTGCGCAATGGGGGAAAC**TG*ACGC*AGCGACGC*GTGTG*AGGGA
11  lac6se2     TATTGCGCAATGGGGGAAACC*TG*ACGC*AGCGACGCCGCGTGTAGG*A
16  lac12se1    TATTGCGCAATGGGCGAA*C*TGGGACGGAACAAA*GC***GTGTGAGGG
 2  lac24seq    TATTGC-CAACGGGGGAAACC*TG*AC-C*AGCGACG-CGCG-GC**-GA
19  soAce1seq   TATTGCGCAATGGGCGAAA-C-TG*ACGC*AGCGACGCCTCGTG*AGGGA
10  lac5se1               TGG*CG*****TTG**CGC*AGCGACGCCGCGTG*AGGGA
 5  lac15se2                                                TG*AGGGA
    CONSENSUS   TATTGCGCAATGGG-GAA----TG-ACGC-AGC-ACGCC--GTG-AGGGA

                          60         70         80         90        100
20  soLac1seq   AGAAGGATCTATGGTTCGTAAACTTCTTTTGCAGGGGAA-TAAAG-TGGA
 1  eth3seq     CGAAGG*CTTTCGGGTCGTAAACCTCTGTCAGGAGGGAAGAAACTGTTTG
14  lac10se1    AG-AGGCTCTCCGG*TCGTAAACCTCTGTCAGAAGGGAAGAAACC*TTGA
 3  lac26seq    TGAAGG*CTTTCGGGTCGTAAACCTCTGTCGGAAGGGAAGAACGGGCATT
 7  lac3se1     TGAAGGCCTT*CGGGTCGTAAACTCCTGTCAAGAGGGAAGAACCGTATGC
13  lac8se1     AGAAGG*CTTTCGGGTCGTAAACCTCTGTCGGGAAGGAAGAACCCCCCAA
11  lac6se2     AGAAGGCCTT*CGCGTCGTAAACTACTGTCAAGAGGGAAGAAACCG*TAG
16  lac12se1    TGAAGG*CTTTCGGGTCGTAAAC*TCTGTCGGAAGGGAAGAAAGGGTGGT
 2  lac24seq    AGAAGGCCTT*CGGGTCGTAAACCGCTGTCAGGAGGGAAGAAACTGTTAG
19  soAce1seq   TGAAGGTCTT*CGGATCGTAAACCTCTGTCAAGAGGGAAGAAACCCTTGG
10  lac5se1     AG*AGGCCTT*CGGGTCGTAAACCTCTGTCAGGAGGGAAGAA*CCCCGAG
 5  lac15se2    TGAAGG-CTTTCGGGTCGTAAACCTCTGTCGGAAGGGAAGAACGGGCATT
    CONSENSUS   -GAAGG-CTT-CGGGTCGTAAAC-TCTGTC---AGGGAAGAA--------

                          110        120        130        140        150
20  soLac1seq   GGACGTGTCTCTTTTG-ATGT*A***-CCCT*GAGAATAAGGATCGGCTA
 1  eth3seq     AGGCTAATACCCTCTTTCACTGACGGTACCTCCAGAGGAAGCACCGGCTA
14  lac10se1    GGTCGAATAGGCTTCTTGGCTGACGGTACCTTCAGAGGAAGC
 3  lac26seq    GGTCTAATAGGCCTTTGTTTTGACGGTAGGTTTAGAGGAAGCACCGGCTA
 7  lac3se1     GGAATA*TACGCCGCATGCCTGACGGTACCTCTAGAGGAAGCACCGGCTA
13  lac8se1     GTCCGAATAGGGC**TTGGCTGACGGTACTTCCAAAGGA*GCAC**GCTT
11  lac6se2     GCATTAATACGGCTATGCGCTGACGGTACCTCTAGAGGAAGCACCGGCTA
16  lac12se1    GGTCT*ATAGGCCTCA
 2  lac24seq    GGTATAATACG-CCTTTCACTGACGGTACCTCCAAAGGAAGCACCGGCTA
19  soAce1seq   ATTCGAATAGGGTCCTTGGCTGACGGTACCTCAAAAG-AAGCACCG*CTA
10  lac5se1     GATGCGATAGTGTCTTCGGCTGACGGTACCTCCAG
 5  lac15se2    GGTCTAATA
    CONSENSUS   G----AATA----------CTGACGGTACCT--A-AGGAAGCACCGGCTA
```

Figure 4-9 continued.

```
                        160        170        180        190        200
 20  soLac1seq    ACTC*GTG-CAGCAGCCGCG*TAATAC-*AGGAT*CGAGCGTTA*TC-G-
  1  eth3seq      ACTCCGTGCCAGCAGCCGCGGA--ATCG**GGGTGCGAGCGTTAATCGGA
  3  lac26seq     ACTCCGT-CCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTATTCGGA
  7  lac3se1      ACTCC
 13  lac8se1      *CTC*GTGCCAGCAGCCG*G*TA*T
 11  lac6se2      ACTC*G
  2  lac24seq     ACTCCGTGCCAGCAGCCGCGGTAATACGGAGAGTGCGAGCGTTAATCGGA
 19  soAce1seq    ACTC-GTGCCAGCAGCCGCG-TAATACGGAG*GTGC-AGCGTTAATCGGA
     CONSENSUS    ACTC-GTGCCAGCAGCCGCG-TAATACG-AG-GTGC-AGCGTTA-TCGGA

                        210        220        230        240        250
 20  soLac1seq    ATT-AT-GG-TTAAAGGGTGCGTAGGTGGTTTAATAAGTCAGCGGTGAAA
  1  eth3seq      ATCACTGGGCGTAAAGCGCGCGTA---GGCGCGATAAGTCAGGCGTGAAA
  3  lac26seq     ATTACTGGGCGTAAAGCGCACGTAGGCGGCCTTGTAAGTCAGGGGTGAA*
  2  lac24seq     ATCACTGGGCGTAAAGCGCACGTAGGCGGCGCATCAAGTCAGGCGTGAAA
 19  soAce1seq    ATCACTGGGCGTAAAGCGCTCGTAGGCGGTTTGGTCAGTCAGATG-GAAA
     CONSENSUS    AT-ACTGGGCGTAAAGCGC-CGTAGG-GG-----TAAGTCAG--GTGAAA

                        260        270        280        290        300
 20  soLac1seq    GTTTGCCAGCTTAACTGTAAAAATGCCGTTGAAACTGT--GACTTGAGTG
  1  eth3seq      *GCCCTCGGCTCAACCGGGGAATTGCGCTTGATACTGTCGTGCTTGAGTC
  3  lac26seq     *TCC*ACGGCTCAACCGTG*AACTGCCTTTGAA*CTGCAG*GCTTCAATC
  2  lac24seq     *GCC*TCGGCTCAAGCGGG*AATTGCGTTTGAAACTGGTGTGCTGGAGTC
 19  soAce1seq    -G-CCTCGGCTTAACCGGGGAACTGCATTTGATACTGCCAGACTTGAGTG
     CONSENSUS    ---C--CGGCT-AACCG-G-AA-TGC--TTGA-ACTG-----CTTGAGT-

                        310        320        330        340        350
 20  soLac1seq    TAAATGA*GGTAGGCGGAATGCGTGGTGTAGCG*GTGAAATGCTTAGATA
  1  eth3seq      GCGGAGA**GGGTGGCGGAATTCCAGGTGTAGGAGTGAAATCCGTAGATA
  3  lac26seq     TGA
  2  lac24seq     TCGGAGA**GG*
 19  soAce1seq    TCGGAGAGAGGGTGGC*GAATTCC*GGTGTAGGAGTGAAATCCGTAGATA
-17  lac17se2                      ATTCCAGGTGTAGGAGTGAAATCCGTAGATA
 -6  lac3se2                                                CTAGATA
     CONSENSUS    ---GAGA-*GG--GGC-GAATTCC-GGTGTAGGAGTGAAATCC-TAGATA

                        360        370        380        390        400
 20  soLac1seq    TCACGCAGAAC*TCGATTGCGAAGGCAGCTTAC-AAGCTACAACTGACAC
  1  eth3seq      TCTGGAGGAACACCAGTGGCGAAG*CGGCCACCTGGACGAGTACTGACGC
 19  soAce1seq    TC**GA*GAACACCAGTG*CGAAGGCG*CCACCTGGACGACAACTGACGC
-17  lac17se2     TCTGGAGGAACATCAGTGGCGAAGGCGGCCACCTGGACCGGTATTGACGC
 -6  lac3se2      TCT*GA*GAAC*TC*GTG*CGAAG*CGGCCACCTGGACTGACACTGACGC
-12  lac6se1          GA*GAACACC*GTG*CGAAG*CGGCCAACTGGACGAGTACTGACGC
-18  lac21se1                           AGGCGGCCATCTGGACTGTCACTGACGC
     CONSENSUS    TC--GA-GAAC--C-GTG-CGAAG-CGGCCA-CTGGAC----ACTGACGC
```

Figure 4-9 continued.

```
                          410        420        430        440        450
 20  soLac1seq   TGAAGCACGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCC
  1  eth3seq     TGAGGTGCGAAA*CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC
 19  soAce1seq   TGAGGAGCGAAAG-G-GGG*AGCAAACAGGATTAGATACCCTGGTAGTCC
-17  lac17se2    TGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC
 -6  lac3se2     TGAGGTGCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCC
-12  lac6se1     TGAGGTACGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCC
-18  lac21se1    TGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC
 -9  lac5se3          TACGAA*GCGTGGG*AGCAAACAGGATTAGATACCCTGGTAGTCC
 -4  lac15se1            AA*GTGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC
 -8  lac4se1                                    TTAGATACCCTGGTAGTCC
     CONSENSUS   TGAGGT-CGAAAGCGTGGG-AGCAAACAGGATTAGATACCCTGGTAGTCC


                          460        470        480        490        500
 20  soLac1seq   ACGCAGTAAACGATGATTACTAGCTGTTTGCGATACACAGTAAGCGGCAC
  1  eth3seq     ACGCCGTAAACGATGGATGCTAGCTGTCGGG*CCTTAACCGGTTCGGTGT
 19  soAce1seq   ACGCGGTAAACGATGGATGCTGGGTGTCG*GGGGTTTA*C**TTCGGTGC
-17  lac17se2    ACGCTGTAAACGATGGATGCTAGATGTCGGGAGTATTC****TTCG*TGT
 -6  lac3se2     ACGCCGTAAACGATGGATACTAGGTGTCGGGGACTTGATC**CTCGGTGC
-12  lac6se1     ACGCTGTAAACGATGGATATTAGGTGTCGGGGTTTTAC****TTCGGTGC
-18  lac21se1    ACGCTGTAAACGATGGACGCTAGGTGTCGGGGGGTCGCCCC**TCGGCGC
 -9  lac5se3     ACGCTGTAAACGATGGACGTTAGGTGTCGGGGGCTTGACCC**TCGGTGC
 -4  lac15se1    ACACCGTAAACGATGGATACTAGGTGTCGGGGACTTGATC**TTCGGTGC
 -8  lac4se1     ACGCTGTAAACGATGGACGTTAGGTGTCGGGGGCTTGACCC**TCGGTGC
     CONSENSUS   ACGC-GTAAACGATGGA---TAG-TGTCGGGG--TT-----*-TCGGTGC

                          510        520        530        540        550
 20  soLac1seq   AGCG****AAAGCTTAAGTAATC*ACCTGGGGAGTACGCCGGCA
  1  eth3seq     CGAAGTTAACGCGATAAGCATCCCACCTGGGGAGTACGGTCGCAAGGCTG
 19  soAce1seq   CGCAGTTAACGCGTTAAGCATCCCGCCTGGGGAGTACGGTCGCAAGGCTG
-17  lac17se2    CGTAGTTAACGCGTTAAGCATCC*GCC*GGGAAGTA*GGTCG*AAGGCT*
 -6  lac3se2     CGTA**TAACGCGTTAAGTATCC*GCCTGGGGAGTACGGCCGCAAGGCTG
-12  lac6se1     CGCAGTTAACGCGTTAAATATCCCGCCTGGGGAGTACGG
-18  lac21se1    CGAAGCTAACGCGTTAAGCCTCCCGCCTGGGGAGTACGGTCGCAAGGCTG
 -9  lac5se3     CGGACGTAACGCGTTAAACGTCCCGCCTGGGGAGTACGGTCGCAAGGCTG
 -4  lac15se1    CGTACGTAACGCGTTAAGTATCCCGCCTGGGGAGTACGGTCGCAAGGCTG
 -8  lac4se1     CGCACGTAACGCGTTAAACGTCCCGCCTGGGGAGTACGGTCGCAAGGCTG
     CONSENSUS   CG-A--TAACGCGTTAA---TCCCGCCTGGGGAGTACGGTCGCAAGGCTG
```

Table 4-8. Homologies between 16S rRNA gene sequences in databases and 776 and 777 sequences as determined by Fasta or Blast searches.

preparation	search	score	Identity
776	Fasta	488	*Desulfovibrio desulfuricans*
776	Fasta	468	*Desulfovibrio sp.*
776	Blast	318	*Desulfovibrio longus*
776	Blast	310	*Desulfovibrio sp.*
777	Fasta	485	*Bacteroides distasonis*
777	Fasta	465	*Bacteroides forsythus*
777	Blast	564	*Bacteroides distasonis*
777	Blast	528	*Bacteroides forsythus*

Note: The consensus sequences were used for the searches. Only the top two highest scoring isolates are given.

Digout DNA preparations and the results for one of the filters with both P88 and P90 is shown in Figure 4-3B and C respectively. Generally, P88 and P89 did not have observable cross-hybridization to any of the DNA preparations (Figure 4-3B for P88) while P90 cross-hybridized to most preparations (Figure 4-3C). P90 gave a pattern of hybridizing bands that corresponded to major bands observed for the *soLac*1 ribotype (as in Figure 4-3A). Both P88 and P90 however, could be used with Univ-907R to amplify approximately 400 bp 16S rRNA gene fragments from preparations 776 and 777. PCR results with the *Bacteroides* specific primer (P88) and the *Desulfovibrio sp.* specific primer (P90) are detailed in Table 4-9.

4.4.2 Cloning and sequencing of waste water standards

A larger number of M13 clones were analyzed following PCR amplification of other standards (Table 4-7). The sequence lengths obtained were smaller for these clones (generally only 100-200 nucleotides). Clearly overlapping sequences were combined in contigs. The consensus sequence for these contigs were used to search the databases. If the sequence from a clone did not overlap with any other contigs, it was considered a consensus and used to search the databases. Best matches between consensus sequences and 16S rRNA gene sequences stored in the databases are given in Table 4-10.

Table 4-9. Summary of PCR products obtained with either P88 or P90, and Univ-907R.

#	standard	primer	PCR product	Identification
776	*soAce*1/*soLac*1	P88	yes	*Bacteroides*
776	*soAce*1/*soLac*1	P90	yes	*Desulfovibrio*
777	*soLac*1	P88	yes	*Bacteroides*
777	*soLac*1	P90	yes	*Desulfovibrio*
780	*soAce*1	p88	no	-
780	*soAce*1	P90	yes	*Desulfovibrio*
151	*Lac*30	P88	yes	*Bacteroides*
151	*Lac*30	P90	no	-

Note: # represents the preparation used for PCR amplification. PCR fragments observed by gel electrophoresis only and were approximately 400 bp in length.

Table 4-10. Best match between a consensus and a data base 16S rRNA gene sequence.

Contig[a]	sequence length, orientation[b]	best matches[c]
*amLac*4.6 (1)	210,f	*Bacteroides forsythus*
*amLac*4.7 (1)	108,f	*Desulfovibrio africanus*
BSR.2[d] (2)	156, r	*Desulfovibrio sp.*
BSR.1[d] (3)	162, f	*Desulfomicrobium* (*D. desulfuricans*)
*amBen*2.4 (1)	192, r	*Desulfobacula toluolica*
*amBen*2.3 (1)	231, r	*Flexibacter canadensis*
*amBen*2.2[d] (1)	96, f	*Desulfobulbus. propionicus*
amLac6b.2 (2)	174, f	*Desulfovibrio sp.*
amLac6b.1 (2)	163, r	*D. desulfuricans* (*Desulfovibrio sp.*)
*amLac*2.1 (3)	142, f	*Desulfovibrio baarsi*
*amLac*2.2 (1)	119, r	*Clostridium bifermentans*
*amLac*2.5 (1)	125, r	*Clostridium novyi* (*C. botulinum*)
*amAce*1.1 (2)	184, r	*D. propionicus*
*amAce*1.3 (1)	112, f	*D. propionicus*
*amAce*1.4 (1)	149, f	*F. canadensis* (*Clostridium*)
*amAce*1.5 (2)	157, r	*Bacteroides distasonis*
*amAce*1.7 (1)	128, r	*Desulfomicrobium*

a Preparations used for amplification are given in Table 4-7. Standard names are followed by a number corresponding to the data base numbering (gel number) of the consensus. The number of sequences used to obtain the consensus are given in parentheses.

b length does not include the PCR primers. r=reverse, f=forward (with respect to the 16S rRNA gene).

c highest scoring match given. Parentheses indicate organisms with close scores.

d Fasta search results given instead of blast search results.

Chapter 5. Discussion

5.1 Genomic characteristics of DNA preparations

5.1.1 Presence of *Desulfovibrio* in DNA preparations

Desulfovibrio species were enriched from all environments, sites, and samples, with the exception of Gold Bar and Uranium Mine (Table 4-2). Genomic DNA from *Desulfovibrio* was present in a significant proportion of the liquid culture enrichments as determined by hybridization with the [NiFe] hydrogenase gene (51%, Table 4-2). This number does not indicate that *Desulfovibrio* is necessarily a significant fraction of the community composition however, in view of the relative ease with which *Desulfovibrio* species can be cultured from the environment. *Desulfovibrio* has been historically recognized as one of the easiest SRB to culture (Postgate, 1984). In order to culture the other members of the SRB and acquire an understanding of their diversity in the environment, a broad scope enrichment protocol using a carbon and energy source other than lactate is required. This is demonstrated by the observation that *Desulfovibrio* was present in 73% of DNA preparations from lactate enrichment cultures, but in only 43% of DNA preparations from other carbon and energy sources (acetate, propionate, benzoate, ethanol, or decanoate) (Table 4-2). Therefore, for the CANMET Soil set of DNA preparations, the percentage of *Desulfovibrio* containing enrichments (85% for the DNA preparations from primary liquid enrichments) is most likely an overestimate of the contribution *Desulfovibrio* makes to the community composition, because only lactate was used as the carbon and either lactate or hydrogen as the energy source. Similarly, the absence of *Desulfovibrio* from the Uranium mine samples may be an underestimate because these samples were enriched only on acetate. A better measure of SRB diversity from both of these

sites would have been obtained if they had been enriched on more than one carbon and energy source.

For the samples for which a variety of carbon and energy sources were used, several trends emerge with regards to the presence of *Desulfovibrio* in SRB communities. The AMD soil sample (Bell Creek) had a higher percentage of liquid enrichment cultures containing *Desulfovibrio* (67%) than did the AMD waste water samples (13-63%). Water environments that had been exposed to hydrocarbons (oil sands production waters and coal mine tailings ponds) also had higher average percentages of [NiFe] positive DNA preparations (65% and 63% respectively) than those that had not been in contact with hydrocarbons, such as sewage (28%), or metal AMD (46%). The higher percentage could be partly attributed to the prevalence of the *Lac*15 family in these environments.

Contrary to results obtained for oil field production waters (Voordouw *et al.*, 1992), colony purification yielded many non-*Desulfovibrio* isolates, as only 42% of colony purified isolates were either [NiFe] positive, or cross-hybridized with known [NiFe] positive DNA preparations (results not shown). This was most striking for colony purifications for CANMET Soil samples, where the percentage of [NiFe] positive DNA preparations from colony purified isolates was only 6% (results not shown). These results were unexpected, since *Desulfovibrio* is generally quite easily colony purified and grows vigorously on plates incubated under a hydrogen atmosphere, irrespective of the carbon source used (Voordouw *et al.*, 1992).

The Southern blot results with the [NiFe] hydrogenase probe were used to support observed genomic homology. All samples that cross-hybridized and had similar ribotypes also had corresponding hybridizing bands when probed

with the [NiFe] hydrogenase gene probe. For example, preparations 147 and 151 both had a 4.9 kb hybridizing band (Figure 4-1B) and both were later determined to be *amLac*4 (*Lac*30). A band of that size was not observed for any of the other DNA preparations that had hybridizing bands. This, in addition to other genomic characteristics, confirmed that preparations 147 and 151 represented the same standard. Similarly, preparations 74 (*amLac*6a) and 76 (*amLac*6c) both had a band at 1.6 kb, although *amLac*6c had an additional band at 2.4 kb (Table 4-1). They had different ribotypes but cross-hybridized consistently to each other (Table 4-1). Based on this combined information, preparations 74 and 76 were both assigned to standard *amLac*6a, with DNA preparation 76 having additional genomic material which complicated the ribotype and added the second [NiFe] hybridizing fragment. Colony purification might resolve standard *amLac*6a from the corresponding liquid enrichment cultures.

The genomic diversity of the *Lac*15 family members was also confirmed by [NiFe] hydrogenase gene probing of Southern blots. For example, *tpLac*1a and *tpLac*1b had one band at 1.5 kb, *tpLac*2 had two bands, at 1.5 kb and 2.3 kb, *tpLac*3a had one band at 8.1 kb (Table 4-1). The observation that some differently coded preparations from within a sample set (e. g. *tpLac*1 and *tpLac*2, both from Suncor) have some shared bands suggests that these preparations have some genomic DNAs in common (i. e. *tpLac*2 contains not only *tpLac*2, but also some *tpLac*1). This could account for the cross-hybridization between these preparations. However, recognizing that [NiFe] hydrogenase gene fragments of different sizes also occurred in preparations within the*Lac*15 family members demonstrates that this conclusion must be made with caution. It is possible that *tpLac*1 and *tpLac*2 are simply very similar isolates.

5.1.2 Ribotypes to estimate diversity

Ribotyping can explore a diversity of genomes that could not be distinguished otherwise (Grimont and Grimont, 1985, Moyer *et al.* 1994). Genomic differences between SRB which could not be resolved with whole genome probing were identified by ribotyping in this study (e. g. the *Lac*15 and *Pro*5 cross-hybridization families). The significance of different ribotypes in genomes that cross-hybridize must still be established. These organisms are often only different strains of the same species and have numerous physiological similarities (e. g. antigenic properties, isoenzymes, Moyer *et al.*, 1992).

It has been suggested that a database of ribotypes could be used for identification purposes (Grimont and Grimont, 1985). The assignment of codes keeps the database organized and designates relationships between genomic DNAs. Standards isolated in the current work can be compared and assigned to the database using both cross-hybridization and ribotype data. For example, cross-hybridization results suggested that *amLac*4 and *Lac*30 were highly homologous. The ribotypes of these two DNAs were compared and found to be identical. Thus *amLac*4 has been named as standard *Lac*30 since they are identical by both comparison methods. The genomic stability of *Lac*15 and *Pro*5 family members could be monitored only by ribotype and not by cross-hybridization, ensuring that the same member of the family is repeatedly subcultured. A ribotype database can be regarded as a simplified version of a 16S rRNA gene sequence database, with the advantage that a ribotype can be much easier to obtain more rapidly than a 16S rRNA gene sequence.

5.1.3 Whole genome cross-hybridizations

Development of a multi-environment RSGP master filter hinges on defining standards that are representative of the environments in question. Whole genome analysis is the critical step for defining standards. If physical conditions at environmental sites can be linked to specific community structures or compositions, then a combination of site-specific, environment-specific, and general standards should allow for inter-community characterization. The site-specific standards could serve as markers of local conditions in an environment, such as defined substrates, environment-specific standards could serve as indicators of the general physical conditions that characterize an environment, such as acidic and metaliferrous, as in AMD, or high levels of hydrocarbons, as in oil field production waters, while general standards could serve as controls, demonstrating the presence of SRB and the successful application of the procedure.

The large number of unique ribotypes obtained for most environments studied here suggested significant genomic diversity and indicated that few genomes were in the environment-specific or general categories. A possible exception was the soil samples, where very few different ribotypes (only two for the Digout preparations) were observed. In the sections below, the possibility that a given standard occurs at multiple environmental sites or in more than one environmental type will be discussed extensively.

5.1.3.1 Genomic diversity at AMD sites

As shown in Figure 4-6, there was little genomic homology between AMD sites, except for genomes belonging to the *Lac*15 and *Pro*5 families. The SRB

diversity within the AMD microcosm (CANMET AMD) was extensive despite its artificial nature (Figure 4-5A). Eight standards were observed, with six appearing in only one DNA preparation from this set each (*amLac*3, *amLac*5, *amPro*1, 128, 180, and 253), while *amLac*4 and *amLac*11 were found in several preparations, either individually or as mixtures (Figure 4-5A). The DNA preparation obtained directly from CANMET (253) cross-hybridized to *amLac*2, an AMD standard isolated from soil (Bell Creek) (Figure 4-5A, Figure 4-6). None of the thirteen DNA preparations analyzed for CANMET AMD cross-hybridized with DNA preparations obtained from other water AMD sites with the exception of *amPro*1, which was a member of the *Pro*5 family.

The Boojum set of AMD samples represented four different sites that could be classified into two groups. The first group, Boojum 1-4, consisted of metal mine tailings and, although physical similarities to the CANMET AMD environment might be expected, it was found that isolates from CANMET AMD and Boojum 1-4 did not cross-hybridize (Figure 4-5A). Each of the standards isolated from the metal mine tailings was site specific, except for *amLac*6a and *amLac*6b, which were environment representative because they cross-hybridized to DNA preparations from the second Boojum group (Figure 4-5A). The second group consisted of coal mine tailings pond samples (Boojum 5-9). DNA preparations isolated from these samples were also not homologous to CANMET AMD preparations (Figure 4-5A). Both groups of Boojum AMD samples yielded eight unique standard DNA preparations, five DNA preparations with limited cross-hybridization (74, 75, 76, 198 and 200), and two preparations containing members of the *Lac*15 family (170 and 200) (Figure 4-5A). Cross-hybridization outside the *Lac*15 family occurred between i) 74 and 76, with ribotypes *amLac*6a

and *amLac*6c respectively, ii) 75, *amLac*6b, and 200, *amPro*4, and iii) 198 and 200, *amPro*4. Ribotypes *amLac*6a and *amLac*6c, isolated from coal and metal mine tailings respectively, have been discussed previously with regard to their hybridization with the [NiFe] hydrogenase probe (see section 5.1.1). They represent a single standard which is apparently environment representative, because *amLac*6a is found in both metal and coal mine tailings. Although DNA preparation 200 cross-hybridized to both 198 and 75, the latter two did not cross-hybridize to each other (Figure 4-5A). Furthermore, neither 198 nor 75 were members of the *Lac*15 family, while 200 was. This, together with the complex ribotype obtained for preparation 200, indicated that it was a consortium containing the genomic DNAs of *amLac*6b, 198, and of a member of the *Lac*15 family. Comparison of the *amPro*4 and the *amLac*6b ribotype showed several similar bands (results not shown). Since DNA preparations 200 and 75 were obtained from coal and metal mine tailings respectively, their common standard (*amLac*6b) can also be considered environment-representative. The common genome in 198 and 200 would be site-specific as both these preparations were derived from coal mine tailings samples. Colony purification of 200 yielded yet another, different standard (*amAce*1, Table 4-5), indicating that the consortium in the liquid enrichment culture was even more heterogeneous than previously indicated from cross-hybridization results. The SRB diversity observed in the liquid enrichment cultures of the Boojum samples is extensive, considering that only 22% of the preparations were studied. If the number of unique genomes per preparation in this subset is representative of all the DNA preparations, the number of site-specific genomes would be extremely high (projected to be 45, or approximately 11 site-specific genomes per site).

The third set of AMD samples were taken from Uranium mine waste water. One unique standard, *amAce*1 was identified in DNA preparations 19, 20, 21, and 113, Figure 4-5A) and one preparation had cross-hybridizations to the *Pro*5 family as well as to *amAce*1 (21, Figure 4-6). The diversity of SRB within this site will not be discussed, as only four DNA preparations, all from cultures with the same carbon and energy source (acetate), were analyzed. Standard *amAce*1 was also observed in the Acid Mine Drainage samples (125) and in coal mine tailings (273), indicating that it is an environment-representative standard. However, no homologies were observed between Boojum 3 standards, which were also obtained from Uranium mine tailings, and *amAce*1.

Only DNA preparation 125 from the AMD set was analyzed on the multi-environment filter. This preparation had the same ribotype as *amAce*1 and cross-hybridized only to *amAce*1 and *amAce*1-containing preparations (Figure 4-5A)

The fifth AMD set of samples was from soil instead of waste water (Bell Creek). Of two genomic DNA preparations on the multi-environment filter, only preparation 51 (*amLac*2) cross-hybridized to AMD preparations outside of the Bell Creek site (preparations 74 ,75 and 76, *amLac*6a, b, and c; 200, *amPro*4; 253). Preparation 51 also cross-hybridized to the other Bell Creek DNA preparation on the filter (53, *amBen*1). From this limited set, one site-specific genome was obtained (*amBen*1).

Thus, for waste water AMD sites, three different AMD communities were observed, one for the microcosm, one for the metal mine tailings and one for the coal mine tailings (Figure 4-5A). Most of the genomic DNA preparations isolated from each site contained site-specific standard genomes. Four environment-representative and potentially environment-specific genomes

(*amLac*6a, *amLac*6b, *amAce*1, and that in 253 and 51) were observed. Two members of the *Lac*15 family were observed in DNA preparations from AMD coal mine tailings ponds only. Two members of the *Pro*5 family was obtained, one from the microcosm and one from the Uranium mine samples.

5.1.3.2 Genomic diversity at oil sands production water sites

DNA preparations from the oil sands production water environment had many cross-hybridizations to DNA preparations from other samples from these sites (Figure 4-5B). This was due to the prevalence of the *Lac*15 and *Pro*5 families. No cross-hybridizations between DNA preparations from Suncor or Syncrude sites were observed that did not belong to either of these two families. One site specific DNA preparation (50, *tpBen*1) was obtained from the Suncor samples, and one from the Syncrude samples (66, *tpAce*2) (Figure 4-5B). Thus the communities between these sites had related organisms and few site-specific standards were obtained. Site-specific standards may be present, but may not be as amenable to culturing as were *Lac*15 and *Pro*5 families.

5.1.3.3 Genomic diversity at soil sites

The first soil site, Bell Creek, has been discussed above in the context of AMD. There were no homologous genomes isolated from Bell Creek and either of the other two soil environments examined, which both represented soils in contact with pipelines (Figure 4-5D). Six cross-hybridizing groups (termed families), which were mixtures of multiple genomes, were isolated from the CANMET Soil samples. Only two standards were isolated from the Digout samples, *soLac*1 and *soAce*1. Two representative DNA preparations of each of

these standards were tested against the CANMET Soil family members. Cross-hybridization was observed between 776, the *soAce*1 preparation, and members of CANMET family III (*soLac*5) and CANMET Soil preparation 103. Preparation 777, or *soLac*1, cross-hybridized with members of CANMET Soil family IV (*soLac*4) and family I (*soLac*7). This indicates that similar genomes can be isolated from various soil samples that may be useful as environment-specific standards. In addition, a number of other DNA preparations were obtained from CANMET Soil (some members of families I, II, and IV, and families V and VI) that did not cross-hybridize to either *soAce*1 or *soLac*1, indicating that site specific standards can also be isolated. A soil specific RSGP filter may be possible if the individual members seen in the CANMET families can be colony purified and demonstrated to be significant in the environment. The success of colony purification of CANMET Soil samples will be discussed in section 5.1.3.7.

5.1.3.4 Genomic diversity at a sewage site

An alternative approach was taken for the sewage environment, as standards were only compared to the multi-environment filter after preliminary investigation into the diversity present in a large number of DNA preparations. This approach allowed the differences between many preparations obtained from a site to be determined (i. e. the diversity obtainable within a site) prior to the analysis of the diversity between sites and environments. Two sets of cross-hybridizing genomes were observed, encompassing most of the DNA preparations obtained on all the carbon and energy sources. This contrasts with the diversity expected from the Boojum samples after partial analysis of the samples. As for oil field production water samples, this limited diversity was

due to the dominance of the *Lac*15 and *Pro*5 families in liquid enrichment cultures and from colony purified isolates. One set of sewage standards belonged to the *Lac*15 family (*swEth*1), and a second set belonged to the *Pro*5 family (*swPro*1) (Table 4-1). Several preparations were obtained that cross-hybridized to both families. Three preparations containing unique genomes were either enriched or colony purified using benzoate as a carbon and energy source (*swBen*2, *swBen*3, and *swBen*4) (Figure 4-5C, Table 4-1). Benzoate is a poor electron donor for many characterized SRB and is not commonly used by species of *Desulfovibrio* or *Desulfobulbus*, and thus may not readily sustain members of the *Lac*15 or *Pro*5 families (Devereux *et al.*, 1989). Genomic DNA from a member of the *Lac*15 family (preparation 156) and a member of the *Pro*5 family (preparation 329) had been extracted from benzoate enrichment cultures, although this may have been primarily due to *Desulfovibrio* and *Desulfobulbus* utilization of hydrogen as an energy source. Benzoate generally resulted in unique, standard genomes that were not consortia or members of hybridizing families (Table 4-1).

5.1.3.5 Genomic diversity between environments

There was considerable SRB diversity between environments, as demonstrated in Figure 4-6. There were generally no cross-hybridizations between preparations from different environments, except in the case of the *Lac*15 or *Pro*5 families. Most environment-representative standards discussed above were thus environment-specific. Only two multi-environment (general) standards that were not members of either the *Lac*15 or *Pro*5 families was obtained in this work. These were *Lac*30 (identified initially as *amLac*4 in this study), originally isolated from river sediments and cultured in this work from

an AMD microcosm, and *amAce*1, which was identified in both AMD (Uranium mine and AMD) and oil sands production water (Suncor). No environmentally ubiquitous standards were obtained, as neither the *Lac*15 or *Pro*5 families were found in soil, and the *Lac*15 family was not found in metal mine tailings samples.

5.1.3.6 Hybridization families

The two hybridization families observed have been previously identified as *Desulfomicrobium* species (*Lac*15) and *Desulfobulbus* species (*Pro*5). *Lac*15 family members were not found in metal mine tailings waters or in soil. They predominated in oil sands production waters and in sewage (Figure 4-6), which is not surprising since *Lac*15 was originally isolated from sewage. The standard *Lac*15, as determined by ribotype, was not seen in any of the preparations. *Lac*15 family members were also isolated from the coal mine tailings pond from the samples that contained either alfalfa pellets or hay. One common factor between these sets of samples is that they may all contain higher levels of hydrocarbons or other higher molecular weight carbon sources. This should not affect the SRB populations directly however, as most SRB are not generally considered capable of metabolizing long chain hydrocarbon molecules. These sites also have in common, in contrast with AMD, a more moderate pH and absence of substantial concentrations of heavy metal ions. These physical features may be the conditions required for growth of *Lac*15 family members, although this does not explain why there are no *Lac*15 members in soil. Different members of the family (as determined by ribotype and [NiFe] hydrogenase band fragments) were found at the different sites and environments, and different members were also found within one site. The same family member was never found in more than

one site. The overall genomic homology suggests that these different ribotypes could be different strains of the same species.

The *Pro*5 family members were also found in waste waters only, but were isolated from the AMD microcosm, the Uranium mine sample, oil sands production waters, and from sewage. Almost all of these *Pro*5 family members were cultured on propionate, the carbon and energy source *Desulfobulbus* thrives on, except for *swPro*1, which was also cultured on other carbon and energy sources (Table 4-1). All of the *Pro*5 isolates had different ribotypes, indicating that, as for the *Lac*15 family, there were a number of different strains of this species. Again, the different strains were site specific.

Both *Pro*5 and *Lac*15 families appear to be general waste water standards. They would thus be useful in a waste water RSGP analysis. General standards, while not diagnostic in relating community structure to environmental conditions, could be diagnostic in relating community composition to conditions. The *Lac*15 and *Pro*5 standards so far appear to be somewhat structurally diagnostic, as they are not present in all environments. Furthermore, the site specific nature of the different strains may be a useful feature in a combined RSGP/ribotype analysis. It would be useful to have only *Lac*15 and *Pro*5 as standards on a master filter. RSGP could identify a member of these families, then the ribotype could be analyzed to provide more specific information, such as which strain is present. If a suitable ribotype database is available, such information could be useful in comparing community structure in terms of different strains.

5.1.3.7 Culture bias and colony purification

Culture bias was exploited in the enrichment of SRB from environmental samples. The basis of this study was the selective enrichment of one group of organisms using specific media, which suggests that the standards enriched may not provide a complete picture of SRB diversity, much as this study, focusing only on the SRB, does not provide a complete picture of bacterial diversity in the environment. Culture bias was made particularly obvious in the *Desulfovibrio* analysis (see section 5.1.1) and in the Digout and CANMET Soil colony purifications, as described below.

It is desirable to have genomic DNA preparations of colony purified strains to prevent complicated cross-hybridizations such as those observed in the whole genome probing steps above, which used DNA from liquid enrichment cultures that may have been genomic mixtures. Colony purification, while essential prior to spotting a standard on an RSGP master filter, will affect the overall diversity observed. Colony purification reduced or limited the number of standards obtained from the CANMET Soil and Digout samples. From 13 Digout liquid enrichment cultures and 43 DNA preparations of colony purified isolates, only two different SRB could be isolated, as determined by ribotype and cross-hybridizations (Figure 4-3A, Table 4-1). Similarly, colony purification from the six CANMET Soil families resulted in the isolation of members from only two of these families (Table 4-2). The genomic diversity present in the original DNA preparations was thus only partially recovered. This can be partly explained by the use of only lactate and hydrogen for the primary enrichment and only lactate, hydrogen and acetate for the subsequent colony purification. This may also have affected the growth of the *Pro*5 family, which generally requires propionate,

although the absence of the *Lac*15 family cannot be correspondingly explained. *Lac*15 and *Pro*5 were not found in any of the other Soil sites however, so their absence from CANMET Soil is not unexpected. One of the colonies isolated did not belong to 'classical' SRB, as it could grow aerobically as well as anaerobically, as shown by the cross-hybridization between DNAs from aerobic and anaerobic cultures (Figure 4-5E, Table 4-1). A more rigorous, broad approach must be taken, first to obtain SRB in liquid culture enrichment, then to ensure that the diversity of genomes present in the original cultures is maintained.

Loss of diversity is not always a consequence of colony purification. Colony purification of the CANMET AMD 2 sample resolved three standards (*Lac*30, *amLac*5 and *amLac*11, preparations 145-151) where previously only *Lac*30 had been observed (preparation 72). The benefits of using different types of media to obtain SRB were demonstrated in this experiment, as both Blood and W&P plates were used for the colony purification. On the W&P plates, only *Lac*30 and an *Lac*30/*amLac*11 mixture were cultured. Blood media was required for purification of the *amLac*11 standard (preparation 146) and a third standard, *amLac*5, in addition to purification of *Lac*30 (preparations 145 and 147). Blood plates present a richer medium, explaining why, after an initial enrichment for SRB, more SRB were recovered from the liquid enrichments than from W&P plates alone. BSR, which was the colony purified isolate obtained directly from CANMET, was also found to be a *Desulfovibrio* and was genomically distinct from the other CANMET AMD DNA preparations. Thus in the case of the CANMET AMD samples, colony purification was a useful exercise, increasing the variety of SRB cultured from the samples.

Mercuric chloride ($HgCl_2$) can inhibit non-SRB growth on plates. It was postulated that it could be useful in an SRB enrichment protocol, reducing contamination from other species. The Hg^{2+} ion is toxic to cells, but SRB can precipitate Hg^{2+} as HgS, preventing it from entering the cell. Prior to general inclusion in the enrichment protocol, the effects of $HgCl_2$ on the diversity of SRB were first investigated. $HgCl_2$, either on the Blood or W&P plates, appeared to select for the *Lac*30 standard, which is a *Desulfovibrio*, either as only pure *Lac*30, or the *Lac*30/*amLac*11 mixtures. Since only *Lac*30 has consistent growth, $HgCl_2$ also seems to affect the SRB growth, thus it may not be a useful addition to a colony purification protocol designed to recover as many different SRB as possible.

Colony purification from liquid cultures for which DNA preparations had been coded and assigned standard names generally resulted in purified, standard genomes that gave the same or similar cross-hybridization patterns as the first DNA preparation from the liquid culture (Table 4-4). Furthermore, complex cross-hybridization patterns were often resolved after colony purification, and there appeared to be little loss of diversity after colony purification. The appearance of different standards following colony purification, some of which were present in negligible amounts in the primary liquid enrichment culture demonstrates how conditions (in this example, solid vs. liquid) can affect the dominant species in a consortium. For example, *amLac*6b, *Lac*30, *amLac*11 and some members of the *Lac*15 and *Pro*5 families are representative of just a few standards in this study well adapted to both liquid growth and to colony formation. In contrast, colony purification from the liquid enrichment culture of Syncrude pond ethanol, which had originally produced the *Lac*15 family member,*tpEth*2 (140), gave *Lac*30 (276), which had not been observed in the cross-

hybridization patterns of previous Syncrude pond ethanol DNA preparations (Table 4-1, Figure 4-6). *Lac*30 must have been present in the liquid enrichment culture as a minor component. When conditions changed and colony formation was required, *tpEth*2 was unable to compete or grow, thus the minor community component of the liquid culture, *Lac*30, became the major community component in solid culture. Growth conditions are an important consideration when attempting to culture a diverse range of organisms. If an organism is a major community component in waste water, it would be expected to have good growth in liquid culture but it may not be as successful at forming colonies if that is not its usual growth type. This was demonstrated by the drift that occurred from waste water DNA preparation 170, which had the ribotype *amPro*2, but also cross-hybridized to the *Lac*15 family. Following colony purification, the cross-hybridization to *amPro*2 was lost, while that to the *Lac*15 family was retained. The *Lac*15 family member component of the consortium appeared to be favored when conditions changed from liquid enrichment culture to solid media.

One assumption initially made with respect to the colony purified isolates was that they would be pure, representing only one genome. This assumption was belied by several sets of preparations from colony purified isolates, notably the*Lac*30/*amLac*11 mixtures. Although these had been colony purified, two preparations were obtained that contained both genomes. Similarly, the *soLac*1 and *soAce*1 colony purified isolates were also frequently obtained as mixed genomic preparations, as observed from cross-hybridization data. Molecular biological verification of the genomic homogeneity of the DNA preparations obtained from colony purification protocols will ensure that contamination does not go unrecognized and does not contribute misleading information. This

contamination of genomic DNA from colony purified isolates will be discussed further with regards to the *soLac*1/*soAce*1 sequencing results in section 5.4.2.

5.2 Potential for RSGP analysis

The SRB diversity observed here is not unexpected, since these environments and sites are both regionally and physically distinct. However, this observed diversity impedes the development of a multi-environment RSGP master filter, since so few environment representative genomes and multi-environment (general) standards were isolated, although many site specific standards were obtained (Figure 4-6). At present, RSGP analysis must remain limited to a targeted environment and can not be applied across environments. Characterization of more SRB isolates may provide additional environment representative or general standards, but the trend in this research suggests that the number of samples, genomic DNA preparations, and ultimately SRB standards that would be required is large. This analysis has provided standards from the AMD sites that could make it possible to generate site or AMD specific RSGP filters for monitoring changes in SRB community structure and composition at one site, or at a number of related AMD sites, much like the oil field production water master filter (Voordouw *et al.*, 1992). A necessary first step prior to this would be the screening of total environmental DNA from the AMD sites analyzed here against the multi-environment whole genome filter prepared here to discover if the genomic DNAs isolated in this study are representative of the environmental SRB communities.

5.3 Identification of standards

5.3.1 Digout standards; *soLac*1 and *soAce*1

From the Southern blot and whole genome analysis, it appeared that preparation 777 was a pure culture with only *soLac*1, while preparation 776 was a mixture of *soLac*1 and *soAce*1 (Figure 4-3A). 776 was selected for use and it was hoped that a number of different clones, some *soLac*1 and some *soAce*1, would be obtained. Unfortunately, only four clones were obtained for 776, and only one for 777. The scarcity of blunt-end generated clones can be attributed to a 'T' overhang that is commonly left in PCR using *Taq* polymerase. Before sequencing other standards, blunt-end cloning was thus modified with the addition of a kinase/end-filling step (Moyer *et al.* 1994) and supplemented with sticky-end cloning to provide more clones (Table 4-7). These procedures were not carried out on *soLac*1 and *soAce*1 however, as their two sets of clones gave different sequences, leading to the speculation that the *soLac*1 and *soAce*1 species had been cloned fortuitously in spite of the restricted number of clones.

Sequence comparison of these sequences and *Desulfovibrio* standard sequences allowed the quality of the sequences to be assessed. Comparable conserved and variable sequence regions were observed between *soAce*1, *soLac*1 and various standard sequences (Figure 4-9). Furthermore, analysis of these sequences in terms of two and three dimensional structure allowed changes in the sequence at different regions to be reconciled. This is particularly important for determining if sequence differences are intrinsic to the 16S rRNA genes, or if they reflect changes introduced through PCR. Analysis of the stem loop structure that forms by the pairing of positions 821-826 and 884-879 (*E. coli* numbering, for Figure 4-7 this region corresponds to 549-554 and 509-504) has the

sequence CAGCTG--GTCGAC, with the dashes indicating the non-paired region (Gutell *et al.*, 1994). For the *soAce*1 contig, the following sequence is found in those regions (in Figure 4-8A, positions 453-458 and 507-502); GATGCT--CTACGA, which could be capable of forming a similar stem loop structure. Sequence in the same region for the *soLac*1 contig was (in Figure 4-8B, positions 453-460 and 509-502); TGATTACT-ACTAATGA. This would also be capable of forming a stem loop structure. Thus, although the sequence in these regions cannot be compared to any conserved sequence in a database, the changes have been demonstrated to be conservative by secondary structure verification. Both of the contigs also contained positions 570, 571 and 866,867 (*E. coli* numbering; for Figure 4-7 these positions are approximately 211,212, and 509, 510), which are important in the three dimensional structure of the 16S rRNA molecule and as such, are highly conserved within eubacterial groupings (Woese, 1987). For the delta division of the proteobacteria, the division to which the SRB belong, the sequence at those positions is invariably GT-AC. This was the case for the 776 sequence. The 777 consensus read TT-AA, however, which is sequence indicative of *Bacteroides* or *Flavobacterium*. This anomaly will be discussed in the context of the overall sequence homology of the *soLac*1 sequence to database sequences.

Sequence comparison with the database suggested that the 776 sequence, hoped to be [NiFe] negative *soAce*1, was a *Desulfovibrio* sequence, while the 777 sequence, which was from the [NiFe] positive preparation 777, was closely related to a *Bacteroides* sequence. The tertiary sequence analysis demonstrating a conservative change in two residues had suggested that this may be the case. This unusual result prompted the decision to test the Southern blots employed in the ribotyping of the Digout preparations with deoxyoligonucleotides designed

specifically from the 777 and 776 contigs (Figure 4-3B, C). The alignment of the sequences in Figure 4-9 indicated useful, non-homologous regions for developing specific deoxyoligonucleotides that would recognize only the species sequenced here. Undetectable cross-hybridization between P88 and P89 and the Southern blots, including one containing preparation 777 (Figure 4-3B for P88), demonstrated that the *Bacteroides* species was not a major genomic component of these digout preparations, and thus was not *soLac*1. Cross-hybridization was observed between P90 and the ribotype Southerns, but the bands observed corresponded to those attributed to *soLac*1 (Figure 4-3C). Thus the *soLac*1 genomic component of 776 had been amplified, cloned and sequenced, while the major component that was present (*soAce*1) had not been sequenced at all. Thus the identity of *soLac*1 was resolved as was the discrepancy between the identity of the sequence and the [NiFe] hydrogenase results for *soAce*1 (the *Desulfovibrio* present in 776 as a minor component would not necessarily give a positive result with the [NiFe] hydrogenase probe. The major component could still be non-*Desulfovibrio* and thus [NiFe] negative). It remained unclear whether the sequence obtained from 777 was PCR contamination or if it represented a minor component of the colony purified preparation 777. PCR with P88 and P90 was used to test these options as well as to verify that the *soLac*1 sequence obtained from 776 was also present in 777, as was originally expected (Table 4-9). As the 776 *soLac*1 sequence could be amplified from both 777 and 776, but not from a non-Digout preparation, *Lac*30 (151), the 776 sequence was attributed to *soLac*1 (Table 4-9). This sequence could also be amplified from a Digout DNA preparation that had shown only the *soAce*1 ribotype and cross-hybridization (780, Figure 4-3A), further demonstrating the sensitivity of PCR in detecting

genomic material that is not evident by other methods (Table 4-9). The *Bacteroides* sequence in 777 was amplified from both 776, 777 and from another preparation, *Lac*30 (151), but not from preparation 780 (Table 4-9). Thus *Bacteroides* is a minor component of the DNA preparations that is not detectable on a Southern blot but can be amplified with PCR. This demonstrates the dangers of using PCR, as it can introduce a bias similar to culture bias, by amplifying minor genomic contributors to a genomic mixture. It also reinforces the power of PCR as a technique for amplification of 16S rRNA genes from total environmental genomic DNA to obtain not only the major community members, but also minor community members as well as organisms that may have been difficult to culture or identify by other means (Giovannoni et al. 1990).

5.3.2 Other standards

Although the other standards were not studied in as much detail, the information obtained was still useful for providing a perspective on the diversity of SRB, and on the success of colony purification. In most cases, each different clone from a given preparation gave a different sequence, frequently not even corresponding to homologous organisms (i. e. different 16S rRNA gene operons). The colony purification methods employed in this study, as expected from previous results, were not foolproof methods for obtaining genomic DNA pure enough to amplify only one set of 16S rRNA genes. DNA preparations of colony purified standards that had cross-hybridized only to other preparations with the same standard still often had 16S rRNA sequence from more than one organism. Two standards only gave a single set of sequences; 253 (BSR) and 252 (*amLac*6b), which was a secondary enrichment and not a colony purified isolate. Standards

giving more than one 16S rRNA sequence included 151 (*amLac*4), 277 (*amBen*2), 275 (*amAce*1), and 279 (*amLac*2). The extra genomic material indicated was generally from a non-SRB, such as *Clostridium, Bacteroides* or *Flexibacter.* One possible SRB mixture was discovered in *amAce*1, which had sequences homologous to both *Desulfobulbus* and *Desulfomicrobium.* This is not unexpected since SRB are frequently found in consortia with *Clostridium* and other bacteria (Postgate, 1984). It may thus be that the interactions uncovered here represent stable symbiotic associations that will be difficult to separate by colony purification.

5.4 SRB diversity in the environment

Microbial diversity in the environment is rapidly becoming acknowledged as an untapped resource (Palleroni, 1994). The SRB are no exception to the rule that microbial diversity is extensive. Molecular biological methods used here have been particularly useful in i) probing microbial diversity by identifying precise genomic differences between homologous organisms, ii) recognizing some of the difficulties in studying diversity, such as culture biases and the difficulties in obtaining pure cultures, and iii) compensating for some of these difficulties by recognizing genomic heterogeneity and monitoring the purity of cultures. The standards isolated here surely represent a fraction of the SRB present in environmental communities. The importance of these standards in the environment could be assessed in future work with quantitative RSGP to discover community composition in terms of these standards. At present, an analysis of community structure may be best accomplished by identifying more

of the standards and establishing phylogenetic relationships, particularly among the *Lac*15 and *Pro*5 family members.

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