Investigation of Sulfate-Reducing Bacteria in Landfill Sites Using Molecular Biological Tools

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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February 2000

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Abbreviations

bp	base pair
BSA	bovine serum albumen
DNA	deoxyribonucleic acid
dH_2O	deionised H_2O
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediamine tetra acetic acid
g	gram
g	1 x gravitational force
h	hour
IPTG	Isopropyl β-D-Thiogalactopyranoside
kb	kilobase
l	litre
LB	Luria-Bertani
M	moles per litre
mM	millimoles per litre
min	minute
ml	millilitre
µg	microgram
µl	microlitre
ng	nanogram
PCR	polymerase chain reaction
pmol	picomole
RDP	Ribosomal Database Project
RNA	ribonucleic acid
rDNA	ribosomal DNA aka rRNA gene
rRNA	ribosomal RNA
SDS	sodium dodecyl sulfate
SRB	sulfate-reducing bacteria

SSC	salt-sodium citrate
SSU	small ribosomal subunit aka 16S subunit in prokaryotes
Taq	*Thermus aquaticus*
TEMED	N, N, N', N'-tetramethyl-ethylenediamine
Tm	mid-point of denaturation curve as estimated by Suggs *et al* (1981) formula
TTGE	temporal thermal gradient electrophoresis
UV	ultra-violet
vol.	volume
v/v	volume/volume
w/v	weight/volume
X-gal	5-bromo-4chloro-3indolyl-β-D-galactoside

Standard abbreviations for bases

A	adenosine	**M**	C or A (amino)
T	thymine	**S**	G or C (strong)
G	guanine	**W**	A or T (weak)
C	cytosine	**B**	not A
U	uracil	**D**	not C
R	A or G (purine)	**H**	not G
Y	C or T (pyrimidine)	**V**	not T
K	G or T (keto)	**N**	unknown

Inter-conversions of mass and molarity for nucleic acids assume a molecular mass of 330 per nucleotide.

ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisor Dr. Alan McCarthy for his constant support and encouragement, not just as a postgraduate but also as an undergraduate all those years ago. Alan even interviewed me for my place at Liverpool University when I was just 17 years old. I would also like to thank my Case supervisor at the Centre for Applied Microbiology and Research in Porton Down, Prof. Richard Sharp.

I would like to give a special thank you to Dr. Michele van Dyke who worked with me on the landfill project and was a constant source of good ideas, encouragement and inspiration. Thank you Michele, it was a privilege to work with you.

The list of people who aided and abetted my journey through this Ph.D is endless and it is impossible for me to mention everyone. Thank you all anyway.

A big shout goes out to the cast and crew of the 8th floor Life Sciences Building and especially to the legend that is 8.02. Gold medals go to Michele, Rosie, Corrine, Paul, Jamie (for the computer, thanks mate), Heather (for the PEG precipitation, you saved my life), Ang (for sequencing) and to Joan & Mary.

I would also like to mention Dr. Ian Head for giving me the opportunity of two years lab experience up in Newcastle. Thank you Ian, I learnt an awful lot up there and had a fantastic time as well. Thanks also to Dr. Paul Riley who provided me with B&B in the Riley Hilton on my visits to Salisbury (winner for the past 1000 years of THE most boring town in the world award).

I would like to thank my family and friends for all the support they've given me, not just over the last three years. Thank you Leigh, Gavin, Mike, Chris, and Mark. Also, a special thank you to Laila for being with me and for helping me so much over the last year and a bit without even realising it. I love you.

Finally, to Mum and Dad. This would not have been possible without the 26 years of total love and support you have given me in everything I've ever done. I really don't know what I'd do without you. Thank you so much. I love you both.

To Laila, this is for both of us.

ABSTRACT

PCR primer sets specific for the 16S rRNA gene of six phylogenetic subgroups of sulfate-reducing bacteria (SRB) were designed. Their application in conjunction with subgroup-specific internal oligonucleotide probes enabled information to be obtained on the occurrence and distribution of SRB in environmental samples.

Phylogenetic analysis of known SRB sequences enabled six generic/suprageneric subgroups of SRB to be differentiated: *Desulfotomaculum*; *Desulfobulbus*; *Desulfobacterium*; *Desulfobacter*; *Desulfococcus-Desulfonema-Desulfosarcina*; *Desulfovibrio-Desulfomicrobium.*

The proliferation of SRB in landfill sites interferes with methanogenesis and waste stabilization, but relatively little is known about the composition of SRB populations in this environment. DNA was extracted from samples of landfill leachate from several municipal waste landfill sites and used as template in polymerase chain reactions (PCR) with SRB group-specific primer sets. Group-specific oligonucleotide probes were then used to confirm that the PCR products contained the target SRB 16S rDNA. Both 'direct' and 'nested' PCR protocols were used to amplify SRB 16S rDNA from landfill leachates. Three of the six SRB groups could be detected using the 'direct' PCR approach (*Desulfotomaculum*, *Desulfobacter* and the *Desulfococcus-Desulfonema-Desulfosarcina* group). When 'nested' PCR was applied, an additional two groups could be detected (*Desulfobulbus* and the *Desulfovibrio-Desulfomicrobium* group). Only *Desulfobacterium*, a predominantly marine genus, could not be detected in any landfill leachate samples using either 'direct' or 'nested' PCR.

Genetic diversity within the SRB subgroups detected in landfill was investigated by temporal thermal gradient electrophoresis (TTGE) and sequence analysis of cloned SRB 16S rDNA fragments. PCR products amplified from landfill leachate using the SRB group-specific primers were reamplified with eubacterial primers containing a GC clamp and analysed by TTGE. This generated profiles of banding patterns that could be used to indicate the genetic diversity within the SRB subgroups.

Consequently, profiles contained few bands (<5) suggesting that genetic diversity within each respective SRB population present in landfill leachate was limited. TTGE profiles also demonstrated differences in SRB community structure between the landfill sites. Sequence analysis of cloned SRB 16S rDNA fragments revealed that they were all members of the δ-subclass of the Proteobacteria and all clustered within the specific subgroups for which the PCR primers and oligonucleotide probes were designed. This therefore demonstrates the validity of the primers and probes designed to specifically amplify and detect populations of SRB in environmental samples. Construction of phylogenetic trees showed that the sequences formed novel lineages within subgroups and may represent centres of variation that could be as yet undescribed species of SRB.

CHAPTER 1. Introduction

1.1 Overview

Dissimilatory sulfate reduction is an integral part of the biological sulfur cycle (LeGall and Fauque, 1988; Fauque *et al*, 1991) and is a key process in the mineralization of organic matter in natural environments (Hines *et al*, 1997).

The principal micro-organisms involved, the sulfate-reducing bacteria (SRB), are a physiologically diverse group of anaerobic bacteria that utilize sulfate as a terminal electron acceptor in the degradation of organic compounds (Gibson, 1990; Odom and Singleton, 1993).

Landfill sites are normally associated with methanogenesis. However, SRB have the ability to compete with methanogenic bacteria for available electron donors, which can inhibit methane production and have serious implications for the efficiency of waste degradation (Gurijala & Suflita, 1993; Harvey *et al*, 1997).

The isolation of SRB in enrichment cultures can prove difficult due to the anaerobic nature of these organisms. Therefore, the application of molecular biological tools can provide invaluable information on the structure and diversity of SRB communities in environmental samples, that would not be possible using culture-dependent methods.

1.2. Molecular Microbial Ecology

Molecular microbial ecology is the study of the ecology, diversity and community structure of micro-organisms in natural environments by application of molecular biological techniques. The main advantage of using molecular techniques is that

ecological questions that traditional methodologies have been unable to answer may now be addressed (Wagner *et al*, 1993; Reeves *et al*, 1995; Kampfer *et al*, 1996).

Traditional microbial ecology relies on the laboratory cultivation of axenic cultures from environmental samples. Not only can this be extremely difficult with regard to certain types of micro-organisms, including sulfate-reducing bacteria, but only a very small fraction of the microbes in the natural environment have been cultivated at all (Head *et al*, 1998). Furthermore, the study of micro-organisms using culture-dependent methods is inherently biased. Laboratory enrichment would be expected to favour those species that exhibit the fastest growth rate under artificial conditions, irrespective of their activity in the natural environment. This could lead to misconceptions about the ecological importance of certain species in natural environments. For example, ammonia oxidation in the environment has generally been equated with the activity of *Nitrosomonas* spp., due to the relative ease with which members of this genus can be grown in culture. However, recent work applying molecular biological techniques to the study of ammonia-oxidizers in environmental samples has revealed that it is *Nitrosospira* spp., which do not grow as readily in culture, that are possibly of more ecological significance than *Nitrosomonas* spp. (Hiorns *et al*, 1995).

Molecular biological methods circumvent this requirement for laboratory cultivation and provide a means of directly studying microbial ecology and diversity in environmental samples via the extraction and analysis of nucleic acids. Molecular methods also offer the possibility of identifying previously unknown species. This has allowed many new insights to be gained into the composition and structure of microbial communities that were not possible using culture-dependent methods. Whole groups of uncultivated and

unculturable micro-organisms that may be ecologically significant are now known only from nucleic acid sequences (Ward *et al*, 1990a, 1990b; Schmidt *et al*, 1991; Spring *et al*, 1992; Gordon and Giovannoni, 1996; Felske *et al*, 1997). Even well-studied habitats contain large numbers of micro-organisms that have never been cultured, and the same is almost certainly true of most, if not all, microbial communities in natural environments (Ward *et al*, 1990a, 1990b). The application of nucleic acid-based techniques such as the polymerase chain reaction, oligonucleotide hybridization, cloning, sequencing and more recent developments such as gradient gel electrophoresis are now commonplace in the study of microbial communities in natural environments.

However, although molecular biological techniques allow microbial communities to be assessed directly, there are certain limitations and problems that have to be appreciated and, where possible, minimized or eradicated. Whereas traditional microbiological methods have bias towards organisms that are most readily cultured, so molecular biological methods can introduce their own biases. The biases involved with the application of molecular techniques relate to the extraction of nucleic acids from environmental samples, PCR amplification of DNA and analysis of PCR products using techniques such as gradient gel electrophoresis. These limitations and their implications for the analysis of microbial diversity and community structure in relation to this study are discussed in Section 1.8.

Despite the limitations, molecular biological techniques have permitted major advances in our understanding and knowledge of microbial ecology in natural environments. The great potential of these techniques is that they are not just limited to the identification of specific microbial populations but can also be used to complement other methods to

assess functional capabilities and ecological significance of microbial populations in environmental processes (Head *et al*, 1998).

1.3. The Biological Sulfur Cycle

The reduction of inorganic sulfate to organic or inorganic sulfide and the subsequent oxidation of sulfide back to sulfate is known as the biological sulfur cycle (Peck and Lissolo, 1988; Widdel, 1988). The biological sulfur cycle consists of an assimilatory and a dissimilatory component. The assimilatory part includes sulfate and sulfide assimilation, as well as release of sulfur from dead and living organic substances by decomposition and excretion. The dissimilatory part of the sulfur cycle includes oxidative processes like chemotrophic and phototrophic sulfide and sulfur oxidation and reductive processes such as microbial sulfate and sulfur reduction (Fauque, 1995) (Fig.1.1, p.5).

1.4. Sulfate Reduction as a Microbiological Process

Sulfate reduction describes the biological reduction of sulfate (SO_4^{2-}) to sulfide (S^{2-}). The sulfate anion is very stable chemically, and its reduction does not occur spontaneously in nature under normal environmental conditions (Brock and Madigan, 1991). Therefore, the reduction of sulfate to sulfide in the environment is entirely mediated by biological activity.

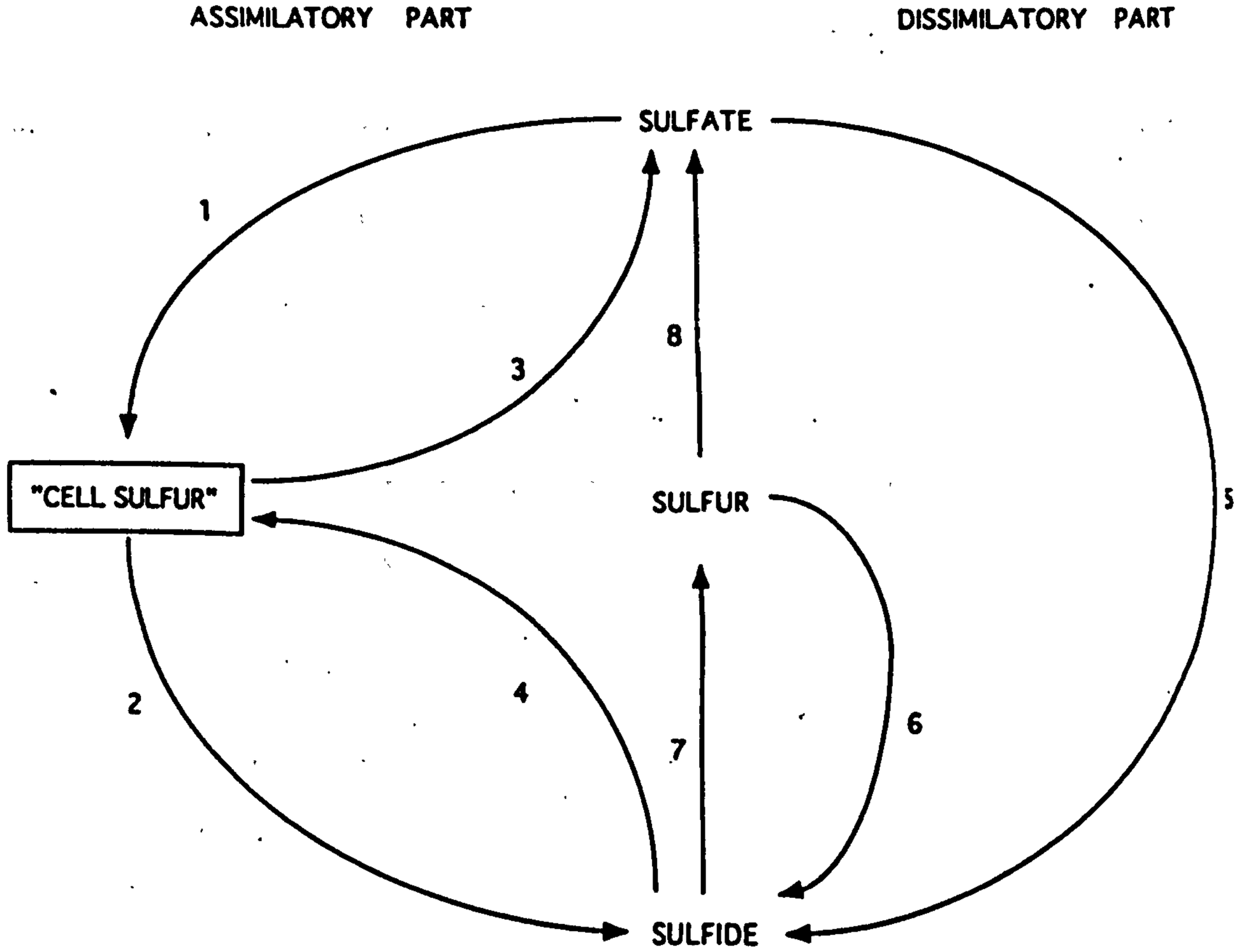


Figure 1.1. The Biological Sulfur Cycle.
"Cell Sulfur" includes sulfur bound in bacteria, fungi, animals and plants.
(1) Assimilatory sulfate reduction by bacteria, plants and fungi; (2) Death and decomposition by bacteria and fungi; (3) Sulfate excretion by animals; (4) Sulfide assimilation by bacteria (and some plants); (5) Dissimilatory sulfate reduction; (6) Dissimilatory elemental sulfur reduction; (7) Chemotrophic and phototrophic sulfide oxidation; (8) Chemotrophic and phototrophic sulfur oxidation.
(from Fauque, 1995).

Sulfate reduction can be divided into two distinct processes: assimilatory and dissimilatory sulfate reduction. Assimilatory sulfate reduction is purely a biosynthetic process in which the sulfide produced is incorporated into amino acids. Most bacteria, fungi and plants are capable of performing assimilatory sulfate reduction (Gibson, 1990). With dissimilatory sulfate reduction, however, virtually all the sulfide produced is excreted and may be converted outside the cell to H_2S or other sulfur compounds such as metal sulfides (Gibson, 1990).

The dissimilatory reduction of sulfur compounds is an essential step in the biological sulfur cycle (LeGall and Fauque, 1988; Fauque *et al*, 1991) and is carried out by a specialized group of anaerobic bacteria, the sulfate-reducing bacteria (SRB).

1.5. Sulfate-Reducing Bacteria

1.5.1. Taxonomy

The SRB can be described as a morphologically and physiologically diverse group of anaerobic bacteria that share the ability to utilize sulfate (or other oxidized sulfur compounds) as a terminal electron acceptor in the mineralization of organic compounds (Gibson, 1990; Odom and Singleton, 1993).

The dissimilatory SRB comprise more than 15 eubacterial genera and one archaebacterial genus (Widdel and Bak, 1992; Fauque, 1995). Well characterized eubacterial genera include: *Desulfovibrio* (Dsv); *Desulfobacter* (Dsb); *Desulfobulbus* (Dbb); *Desulfococcus* (Dcc); *Desulfosarcina* (Dss); *Desulfonema* (Dnm);

Desulfomicrobium (Dmb); *Desulfobacterium* (Dbm); *Desulfohalobium* (Dhb); *Desulfomonile* (Dmn); *Desulfotomaculum* (Dfm); *Thermodesulfobacterium* (Tdb) (Widdel, 1992a, 1992b; Widdel and Bak, 1992). The *Archaea* are represented by the genus *Archaeoglobus* (Stetter, 1992). However, members of novel genera are still being isolated and characterized from environmental samples e.g. *Desulfotalea gen. nov.* and *Desulfofrigus gen. nov.* isolated from marine arctic sediments (Sahm *et al*, 1999a).

Mesophilic, Gram-negative, non-sporeforming SRB are the most widespread in nature. Members of approximately half of the genera are able to oxidize organic substrates completely to CO_2, while the remainder can only oxidize organic compounds incompletely to the level of acetate (Devereux *et al*, 1989; Fauque, 1995). *Desulfovibrio* spp. and *Desulfobulbus* spp. are equally common in marine and freshwater environments. Species of *Desulfobacter*, *Desulfosarcina*, *Desulfonema*, *Desulfobacterium* and *Desulfohalobium* are mainly found in brackish or marine environments, while *Desulfomonile* spp. and *Desulfomicrobium* spp. have been primarily isolated from freshwater environments (Postgate 1984; Widdel and Bak, 1992; Fauque, 1995).

Complete Oxidation	Incomplete Oxidation
Desulfobacter	*Desulfobulbus*
Desulfococcus	*Desulfovibrio*
Desulfosarcina	*Desulfomicrobium*
Desulfonema	*Desulfohalobium*
Desulfomonile	

Table 1.1. Genera of Gram-negative SRB capable of complete or incomplete oxidation of organic substrates

Gram-positive, spore-forming SRB of the genus *Desulfotomaculum* include complete and incomplete oxidizing species. *Desulfotomaculum* spp. have been isolated from freshwater environments or other habitats with relatively low salt concentrations (Jones and Simon, 1984). The genus *Desulfotomaculum* also contains moderately thermophilic species and dissimilatory sulfate reduction in environments with temperatures between 50-65°C is mainly due to spore-forming species (Widdel, 1992a).

The genus *Thermodesulfobacterium* contains two species which are incomplete oxidizers and phylogenetically separate from other eubacterial genera (Widdel, 1992b).

Archaebacterial sulfate reducers of the genus *Archaeoglobus* (Achenbach-Richter *et al*, 1987; Stetter *et al*, 1987; Stetter, 1988) are only found in anaerobic environments with extremely high temperatures such as deep sea hydrothermal vents and volcanic hot springs (Stetter *et al*, 1987).

Comparative 16S rRNA sequence analyses have placed the mesophilic, Gram-negative species of SRB within the δ-subclass of the Proteobacteria (Oyaizu and Woese, 1985; Fowler *et al*, 1986). The δ-subclass contains the myxobacteria, bdellovibrios and SRB (Woese, 1987) and has recently been expanded to include the genus *Pelobacter* (Stackebrandt *et al*, 1989) and the iron-reducing bacterium *Geobacter metallireducens* (Lovley *et al*, 1993). Phylogenetic analysis based on 16S rRNA sequence comparisons has also divided the major SRB genera into a number of distinct lineages (Devereux *et al*, 1989) (see Chapter 3). Generally, classification of SRB by sequence analysis of 16S rRNA correlates well with traditional classification based on physiological and biochemical characteristics (Stackebrandt *et al*, 1995).

1.5.2. Biochemistry

All SRB share the ability to perform anaerobic oxidative phosphorylation with sulfate as a terminal electron acceptor (Fauque *et al*, 1991). In these reactions, sulfate is stoichiometrically reduced to sulfide according to the equation (Gibson, 1990):

$$2CH_2O + SO_4^{2-} \rightarrow 2HCO_3^- + H_2S$$

The initial step in the biochemical pathway of sulfate reduction is the transport of exogenous sulfate across the bacterial cell membrane into the cell (Cypionka, 1987, 1989). Sulfate dissimilation then proceeds by the action of ATP sulfurylase which combines sulfate with ATP to produce the highly activated molecule adenosine phosphosulfate (APS). The cytoplasmic enzyme APS reductase then rapidly converts APS to sulfite (SO_3^-) (Stille and Truper, 1984). There have been a number of different sulfite reductases reported in SRB (LeGall and Postgate, 1973). However, the most commonly recognized, particularly among members of the genus *Desulfovibrio*, are desulfoviridin (Lee and Peck, 1971) and desulforubidin (Lee *et al*, 1973), which share the common function of catalyzing the reduction of sulfite, via a series of intermediates, to sulfide which is then released from the cell (Gibson, 1990).

1.5.3. Physiology

Although reduction of sulfate is considered to be the classic role of SRB in the natural environment, recent studies have demonstrated that these bacteria exhibit metabolic diversity that includes nitrogen fixation (Postgate *et al*, 1988), metal methylation

(Compeau and Bartha, 1985; Choi *et al*, 1994) and disproportionation of oxidized sulfur compounds (Bak and Pfennig, 1987).

Once thought to be restricted primarily to using sulfate as a terminal electron acceptor, SRB have now been demonstrated to be capable of utilizing a wide range of electron acceptors in the consumption of organic compounds. These include other oxidized sulfur compounds e.g. thiosulfate (Bak and Cypionka, 1987; Jorgensen and Bak, 1991) and sulfite (Kramer and Cypionka, 1989), and also elemental sulfur (Lovley and Phillips, 1994b). Other electron acceptors associated with SRB include nitrate and nitrite, which are reduced to ammonia that can then serve as a nitrogen source for cell growth (Dannenburg *et al*, 1992; Daalsgaard and Bak, 1994), metals such as iron and manganese (Coleman *et al*, 1993; Lovley and Phillips, 1994b), toxic heavy metals such as mercury (Compeau and Bartha, 1985; Choi *et al*, 1994), uranium (Lovley and Phillips, 1992; Lovley *et al*, 1993) and chromium (Fude *et al*, 1994; Lovley and Phillips, 1994a), and even oxygen (Cypionka *et al*, 1985; Dilling and Cypionka, 1990; Dannenburg *et al*, 1992).

SRB are also capable of utilizing a diverse range of electron donors. The types of carbon sources utilized for the reduction of sulfate vary according to genus (Gibson, 1990). Preferred carbon sources are generally the products of fermentative bacteria such as volatile fatty acids (e.g. acetate, butyrate and propionate), C3 and C4 fatty acids (e.g. lactate, pyruvate, malate), alcohols (e.g. ethanol, propanol), and molecular H_2/CO_2 (Laanbroek and Pfennig, 1981; Widdel and Pfennig, 1981; Widdel, 1982; Gibson, 1990). However, close to 100 electron donors for sulfate reduction have now been described (Hansen, 1993), including hydrocarbons in crude oil (Reuter *et al*, 1994) which has

economic implications for the oil industry. The oxidized carbon compounds that can be produced as metabolic end products of sulfate-reducing activity are also used as electron donors by other SRB. For example, many SRB have an incomplete TCA cycle and therefore release acetate from the oxidation of higher fatty acids (e.g. butyrate, propionate, lactate). The acetate thus formed and released may then be further utilized by SRB capable of the oxidation of acetate to H_2 and CO_2. Thus, the end products of the metabolic activities of one group may frequently serve as carbon sources for another (Gibson, 1990).

1.5.4. Ecology

SRB are ubiquitous in the environment, and have been isolated or detected from almost every type of habitat on Earth. They are important in the anaerobic degradation of organic matter and have pivotal roles in the biogeochemical cycling of carbon and sulfur. Microbial sulfur transformations are closely linked with the carbon cycle in which sulfate reduction coupled with organic matter utilization is a major mineralization pathway in anaerobic environments (Hines *et al*, 1997).

Marine, estuarine and saltmarsh sediments as well as those of saline and hypersaline lakes are the most significant habitats of SRB in nature because of their high sulfate concentrations (Devereux and Mundfrom, 1994; Devereux *et al*, 1996a, 1996b; Rooney-Varga *et al*, 1997, 1998; Trimmer *et al*, 1997; Teske *et al*, 1998; Hines *et al*, 1999; Sahm *et al*, 1999b). Consequently, sulfate-reduction is thought to be responsible for up to 50% of organic matter degradation in these high sulfate environments (Jorgensen, 1982).

However, sulfate-reduction is also important in the decomposition of organic material in low sulfate environments such as soils and freshwater sediments (Jones and Simon, 1984; Bak and Pfennig, 1991a, 1991b; Sass *et al*, 1997; Li *et al*, 1999).

In addition to soils and sediments, SRB have been detected in a wide range of other habitats. These include microbial mats (Frund and Cohen, 1992; Risatti *et al*, 1994), anaerobic biofilms (Amann *et al*, 1992; Ramsing *et al*, 1993; Raskin *et al*, 1996), activated sludge (Manz *et al*, 1998; Schramm *et al*, 1999), human intestines (Gibson *et al*, 1988), rice paddy fields (Ouattara and Jacq, 1992; Wind and Conrad, 1995), and oil field production waters (Rosnes *et al*, 1991; Voordouw *et al*, 1992, 1996).

Although SRB are mainly found in anaerobic environments, the presence of SRB and significant rates of sulfate reduction have been reported in the oxic zones of sediments (Laanbroek and Pfennig, 1981; Battersby *et al*, 1985; Jorgensen and Bak, 1991; Sass *et al*, 1997), biofilms (Ramsing *et al*, 1993; Santegoeds *et al*, 1998) and microbial mats (Canfield and Des Marais, 1991; Frund and Cohen, 1992; Krekeler *et al*, 1997; Teske *et al*, 1998), and these observations have cast doubts upon the conventional wisdom that SRB are obligate anaerobes.

SRB have also been detected in more extreme environments. These include highly acidic environments (pH 2.5 - 4.5), such as acid mine drainage and acidic mine tailings (Gyure *et al*, 1990; Fortin *et al*, 1996), hypersaline lakes (Teske *et al*, 1998), deep sea hydrothermal vents (Cottrell and Cary, 1999) and permanently cold marine arctic sediments (Sahm *et al*, 1999a).

Environmental factors can often have a major influence on the occurrence and community structure of SRB populations. In marine environments, the sulfate

concentration is on average 28 mM (Goldhaber and Kaplan, 1974), whereas in freshwater environments, the sulfate concentration is usually much lower and ranges from about 0.01 - 0.2 mM (Ingvorsen *et al*, 1981). This can lead to differences in the physiology and structure of the respective SRB populations. For example, freshwater-adapted SRB appear to have a greater affinity for sulfate than their marine adapted counterparts (Smith and Klug, 1981; Ingvorsen *et al*, 1984; Dalsgaard and Bak, 1994), allowing them to continue to reduce sulfate at much lower sulfate concentrations.

The availability of certain electron donors can also influence SRB populations. Acetate is the predominant carbon source for sulfate reduction in marine and estuarine sediments (Sorensen *et al*, 1981; Winfrey and Ward, 1983; Christensen, 1984), thereby favouring completely oxidizing SRB capable of utilizing acetate. However, there can be pronounced differences in the relative importance of particular substrates in natural environments, dependent upon the input and nature of organic material, and these differences can have a major influence upon the community structure and relative importance of different SRB (Purdy *et al*, 1997).

In natural environments, the activities of SRB are interlinked with the activities of sulfur- and sulfide-oxidizing bacteria within a biological sulfur cycle known as a 'sulfuretum' (Fauque, 1995). In a natural ecosystem such as a marine coastal sediment, only *ca.* 10% of the sulfide produced is precipitated by metal ions (Jorgensen, 1977). The remainder is potentially available for oxidation by biological or chemical processes. Sulfide can be re-oxidized by purple or green sulfur bacteria, colourless sulfur bacteria or cyanobacteria. Similarly, if sulfide diffuses to aerobic regions, bacteria such as

Thiobacillus are able to oxidize sulfide or sulfur to sulfate again, thereby stimulating the activity of SRB. Thus a complete sulfur cycle or sulfuretum can develop (Gibson, 1990).

1.6. Economic and Environmental Importance of Sulfate Reduction

The primary metabolic end product of sulfate reduction is sulfide, which can be further converted to H_2S in the presence of external H^+ ions. The presence of SRB with a high metabolic activity is therefore easily detected by the characteristic odour of H_2S. Sulfide itself is extremely corrosive and binds rapidly to metals, while H_2S also has marked effects upon external conditions by virtue of being a strong reducing agent and able to suppress the growth of some aerobic organisms. Furthermore, H_2S is directly toxic to a large range of bacteria and higher organisms, including man (Gibson, 1990). In the oil industry, production of H_2S in oil field waters can cause great economic and environmental problems including the contamination of oil and gas with a concomitant increase in sulfur content, the corrosion of pipelines and other containments made of steel and the stabilization of undesirable oil-water emulsions, as well as the risk of poisoning from inhalation of H_2S gas (Odom, 1993). SRB can also cause problems for the waste industry where their growth and activity can inhibit methanogenesis in landfill sites and lead to the release of toxic H_2S into populated areas.

1.7. Landfill

1.7.1. Economic and Environmental Importance of Landfill

Land disposal of solid waste has been practiced for centuries (Senior, 1990) with the assumption that micro-organisms will degrade such wastes into more environmentally acceptable products. However, increased awareness of the environmental consequences of landfilling has led to a need for understanding the microbiological processes involved (Evans, 1991).

Landfills are the principal disposal alternative for municipal solid waste (MSW), as well as certain industrial wastes, water and wastewater treatment sludges, and agricultural residues (Barlaz, 1997). A significant amount of construction and demolition waste is also deposited in landfills (Suflita *et al*, 1992; Barker, 1998). More than 90% of the MSW produced annually in the United Kingdom and more than 70% in the United States is disposed of to landfill. This amounts to over 175 m tonnes of refuse buried annually in these two nations alone. There are more than 100,000 active and closed landfill sites in the United States (Suflita *et al*, 1992) and more than 3000 active landfill sites in the UK (Barker, 1998). Despite European Community directives aimed at reducing the organic fraction of landfilled wastes and increases in other waste alternatives (recycling, incineration and composting), economic considerations will ensure that landfills remain the most significant waste repository for the foreseeable future (Barlaz, 1997).

The main environmental implications of landfill concern the release of landfill gas and the migration of leachate to the groundwater. In the past, a landfill often represented little more than an open hole or marsh where waste was dumped. The refuse was often not

covered properly and there was little effort to control storm water runoff and downward migration of leachate into groundwater. However, there has been substantial evolution in landfill design and management, and landfill sites have now become highly engineered facilities designed to contain the refuse and separate it from the environment, capture leachate and control gas migration (Barlaz, 1997).

Increasing public concern over the build up of atmospheric gases contributing to global warming (the 'greenhouse effect') has led to increased interest into the release and effects of landfill gas. Landfill gas is comprised mainly of methane and carbon dioxide, both of which are considered to be greenhouse gases. Volume for volume methane is estimated to be 20 times more damaging than CO_2, and methane released by landfills has a significant impact on the accumulation of greenhouse gases in the atmosphere (Barlaz, 1991). In 1996 in the UK alone, total methane emissions amounted to 3.7m tonnes with landfill gas (46%) being by far the main source (Barker, 1998).

However, methane gas could be economically viable through its recovery and use as an alternative energy source and landfill gas recovery projects impact favourably on the global methane budget. In 1994, methane was recovered in commercial quantities from 119 landfill sites in the US and Canada (Barlaz, 1997). In addition to the benefits of methane recovery as an alternative energy source and for the reduction of atmospheric accumulation, there are other environmental implications of methane production. Toxicity of leachate is reduced with the onset of methanogenesis, which reduces the risk of groundwater contamination. Active decomposition also results in the settling of refuse which increases the volume of landfill space available (Barlaz, 1991).

The increased understanding of the microbiological processes involved in landfill decomposition and of the factors affecting the onset and rate of methanogenesis can only enhance these benefits and lead to more efficient, cost-effective and environmentally acceptable landfill waste disposal.

1.7.2. Anaerobic Degradation in Landfill

Municipal solid waste (MSW) is a heterogeneous mixture comprising organic waste material (e.g. paper, cardboard, food waste), plastics and inorganic waste fractions such as glass and metals (Evans, 1991). The organic fraction of MSW is subjected to biodegradation in landfill under anaerobic conditions. Typical composition of organic waste material is: 30-50% cellulose; 15-30% lignin; 10-12% hemicellulose; 4-5% protein; <1% soluble sugars and starch. Only lignin is recalcitrant to anaerobic degradation (Young and Frazer, 1987). Thus, landfills represent active anaerobic ecosystems that harness the co-ordinated activity of several trophic groups of bacteria in order to drive a complex series of biological and chemical reactions, the overall reaction being the decomposition of MSW to methane and carbon dioxide (Barlaz, 1997).

The microbial processes commence with the hydrolysis of biological polymers (cellulose, hemicellulose, carbohydrates, fats and proteins) by cellulolytic and other hydrolytic bacteria (e.g. *Clostridia*) to soluble sugars, amino acids, long chain fatty acids and glycerol. These are the substrates for fermentative bacteria which produce short chain fatty acids (e.g. acetate, butyrate and propionate), alcohols, carbon dioxide and hydrogen. Syntrophic H_2-producing fatty acid-oxidizing bacteria and acetogenic bacteria

can then further ferment fatty acids such as butyrate and propionate into acetate, CO_2 and H_2. The terminal step in this anaerobic decomposition process is methanogenesis. The most common methanogenic substrates being acetate, H_2 and CO_2. Other substrates include formate, methanol and methylated amines, with methane as the major end product formed (Barlaz, 1997) (Fig. 1.2, p.19).

It is important to remember that the stages in this degradation process are strongly interlinked so that degradation occurring at each stage is affected by activities within the others. For example, the oxidation of short chain fatty acids to acetate, CO_2 and H_2 by obligate H_2-producing acetogenic bacteria can only occur at very low H_2 concentrations (Zehnder, 1978). Thus, these bacteria function only in syntrophic association with H_2-scavengers such as methanogens or sulfate-reducers (Barlaz, 1997).

When MSW is placed in a landfill, anaerobic degradation as described above does not proceed immediately. Although all of the microbial groups required for decomposition of MSW to methane are present, a lag period is observed, ranging from months to years, while the proper growth conditions and microbiological system become established (Barlaz, 1997).

Decomposition of MSW can be described as occurring in four phases. Phase 1 is the aerobic phase, where soluble sugars serve as the main carbon source for microbial activity and oxygen is consumed. In the anaerobic acid phase (phase 2) hydrolysis of biological polymers commences and fatty acids begin to accumulate. The imbalance caused by this fermentative activity and lack of acetogenic and methanogenic activity results in a decrease in pH. However, as phase 2 proceeds, methanogenic populations begin to increase and methane can be detected in the landfill gas. Phase 3, the accelerated

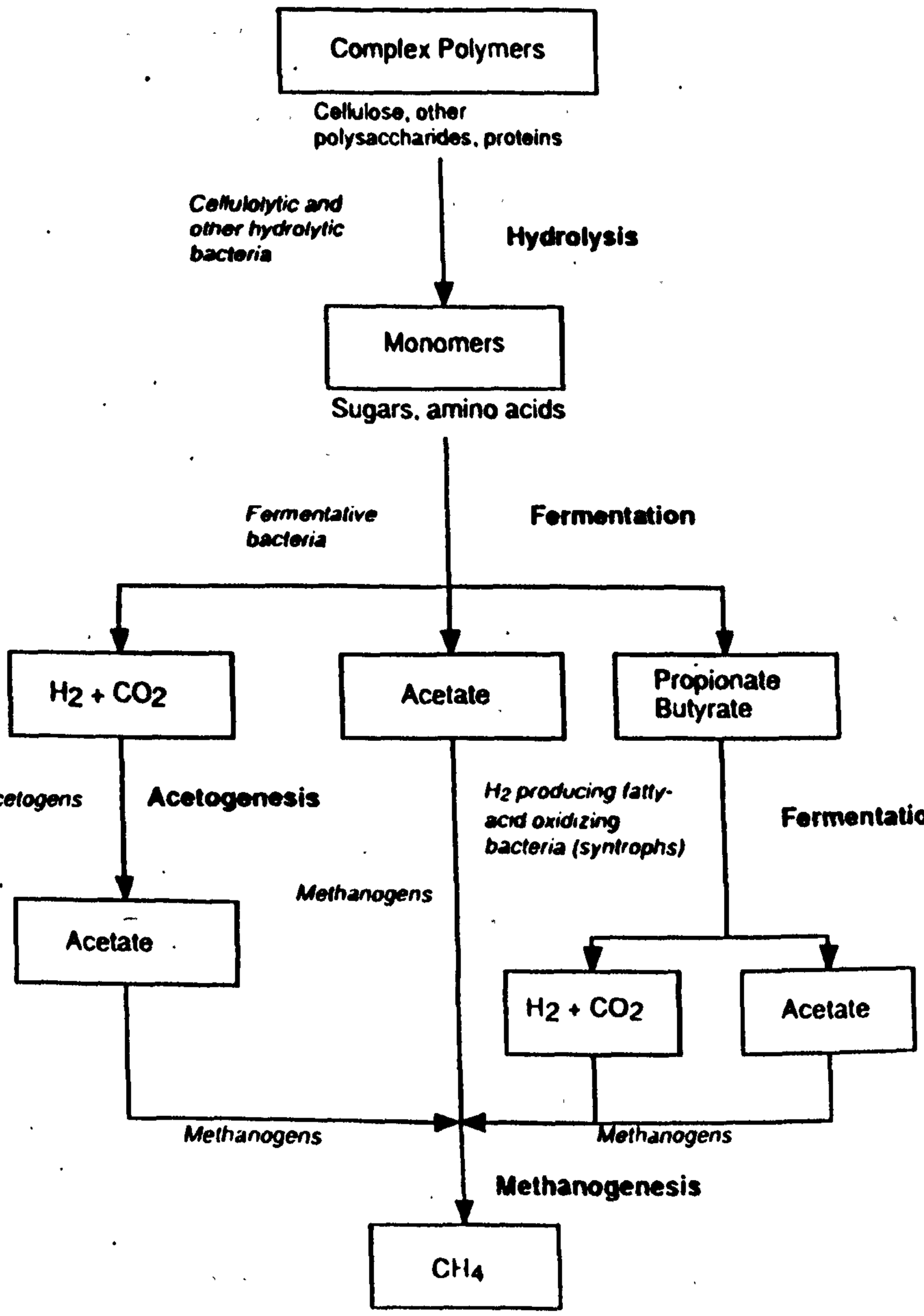


Figure 1.2. Pathway of the anaerobic degradation of organic matter. (from Brock and Madigan, 1991).

methane production phase results in a rapid increase in the rate of methanogenesis to a maximum value depending on conditions within the landfill. Characteristics of this phase include a 50-60% (v/v) methane concentration in the landfill gas, decreasing fatty acid concentrations due to consumption by methanogens, a resultant increase in pH and increases in the populations of cellulolytic, acetogenic and methanogenic bacteria. The final phase (phase 4) is termed the decelerated methane production phase. Fatty acids are depleted and the rate of methanogenesis decreases. The stabilization time for landfills, that is the time taken for methane production to reach a minimal steady-state phase and for the degradation of MSW to produce stable products unlikely to cause environmental pollution, can be anything from 15 to 30 years (Barlaz, 1997).

1.7.3. Factors Affecting Anaerobic Degradation in Landfill

Landfill sites are extremely heterogeneous environments as a consequence of the waste buried in them. This heterogeneity of waste, and the manner in which it is deposited (particularly the absence of mixing once emplaced), has led to the idea that a landfill can be considered to be composed of a series of isolated discrete niches or 'mini-environments'. The conditions in one niche may vary greatly from conditions in another. Microbial contact between niches is facilitated largely by the movement of moisture through the landfill, but may be limited. The extent of degradation and rate of methane production is therefore heavily influenced by environmental conditions both at the level of each niche and for the entire landfill (Evans, 1991).

It has been demonstrated that the actual methane recovery based on the biodegradable fraction of MSW is typically only 1-50% of that theoretically expected (Barlaz *et al*, 1990). This then suggests that conditions for methanogenesis are not optimal in landfill sites. There are a number of environmental factors including moisture content and flow, pH, particle size, inoculum addition, nutrient concentrations, temperature and availability of alternate electron acceptors that have been shown to influence the onset and rate of methane production (Gurijala and Suflita, 1993; Barlaz, 1997; Gurijala *et al*, 1997). The three variables that appear to be most critical in controlling landfill methanogenesis are moisture content, pH and sulfate reduction (Gurijala and Suflita, 1993; Gurijala *et al*, 1997). Landfill samples that were amended with at least an equivalent weight of water were shown to exhibit increased methane production, while samples containing less than 33% moisture did not produce methane at all (Barlaz *et al*, 1990; Gurijala and Suflita, 1993). In addition, high moisture content will promote the dissolution and mixing of soluble substrates and nutrients and will also provide a mechanism for microbial transport within the landfill (Barlaz, 1997). A pH of *ca.* 6.8-7.2 has been reported to be optimal for maximised methane production in municipal waste samples (Kasali *et al*, 1988), while Gurijala and Suflita (1993) demonstrated little or no methane production in landfill samples which fell outside this circumneutral pH range. The influence of sulfate reduction on landfill methanogenesis is discussed in section 1.7.4.

1.7.4. Sulfate Reduction in Landfill

Landfill sites have long been overlooked as important habitats for sulfate reduction and SRB due to the fact that methanogenesis predominates as the key terminal process of carbon mineralization in the absence of significant concentrations of sulfate. Thus, our knowledge of SRB occurrence and distribution in landfill is extremely limited. In landfill sites, the breakdown of waste material ultimately to methane is a complex process involving a series of microbially driven transformations that harness the co-ordinated activity of several bacterial consortia. While the key terminal process is methanogenesis, SRB can compete with methanogenic bacteria for available electron donors such as acetate and H_2 (Schonheit *et al*, 1982; Robinson and Tiedje, 1984) (Fig 1.3, p.23), and have the potential to inhibit the methanogenic decomposition of waste organic matter resulting in decreased methane production, increased production of H_2S and the phenomenon of 'souring' (Gurijala & Suflita, 1993; Harvey *et al*, 1997). Conventional wisdom suggests that the low availability of sulfate outside the marine environment will limit sulfate reduction and therefore SRB populations, but this may not be true of landfill sites. Exogenous sources of sulfate (e.g. gypsum from construction and demolition debris) have been thought to be responsible for sulfate levels as high as 80 mmol kg dry weight^{-1} waste material in particular landfill sites (Suflita *et al*, 1992; Gurijala & Suflita, 1993). These workers have also shown that cellulosic material can account for over 40% of the volume of a landfill site and act as a reservoir of sulfate that originates from other waste fractions. Consequently sulfate may be present in landfills in significant amounts. Inhibition of methanogenesis by sulfate has been observed in a range of environments (Oremland & Polcin, 1982; Beeman & Suflita, 1987; Raskin *et al*, 1996) and so could

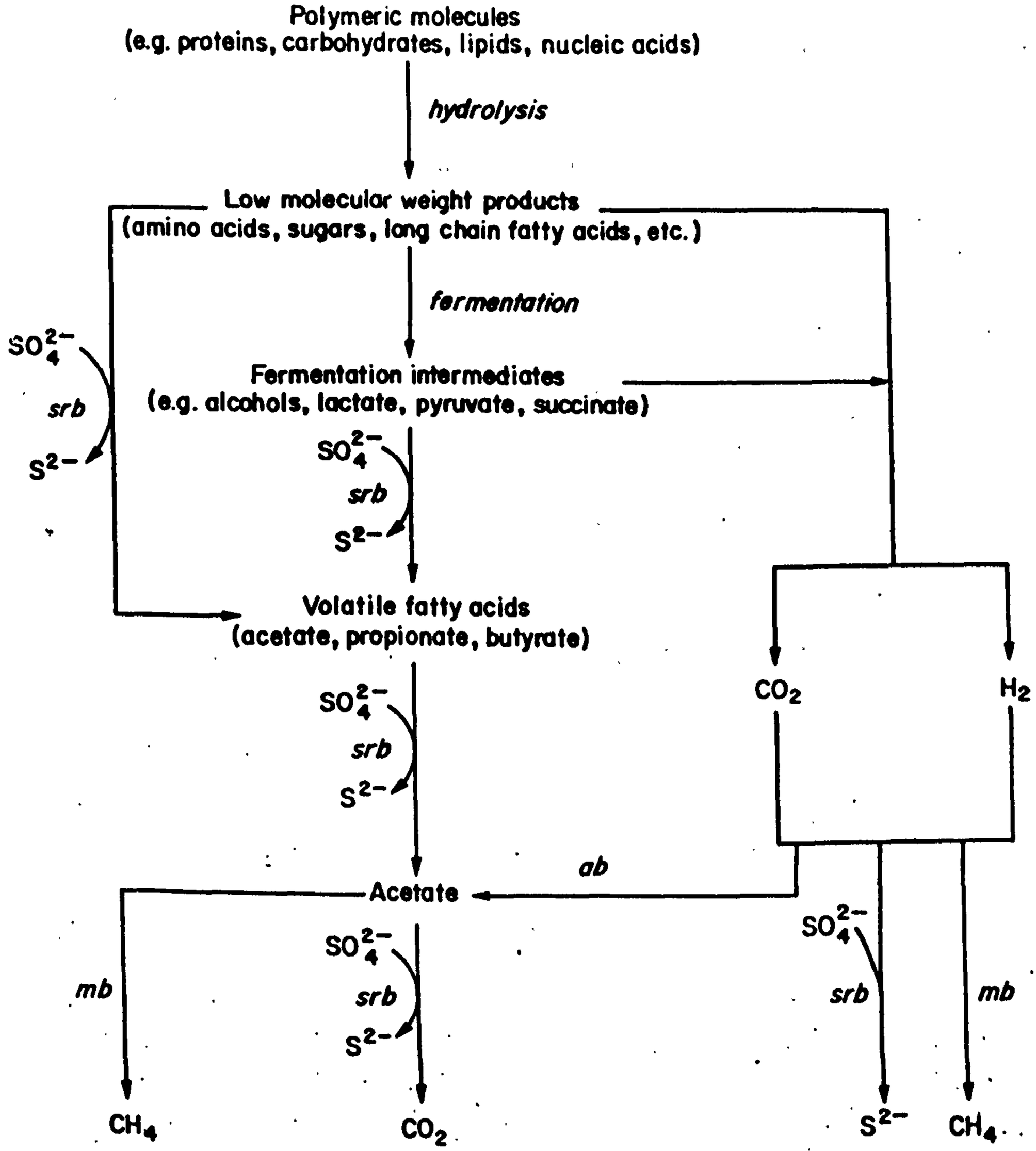


Figure 1.3. Pathway of the anaerobic degradation of organic matter, showing potential interactions of sulfate-reducing bacteria (srb = sulfate-reducing bacteria; mb = methanogenic bacteria; ab = acetogenic bacteria). (from Gibson, 1990).

clearly occur in landfill. Gurijala & Suflita (1993) showed that landfill samples amended with molybdate to inhibit sulfate reduction exhibited an increased rate of methanogenesis, while in samples amended with sulfate, methane production was inhibited relative to the sulfate-unamended control. The results obtained by these workers were analogous, suggesting that these effects may not necessarily be limited to selected landfill samples. The SRB are therefore one of a number of important functional bacterial groups whose structure and activity in landfill sites needs to be directly addressed.

1.8. Molecular Biological Detection of Sulfate-Reducing Bacteria

1.8.1. Extraction of nucleic acids from environmental samples

The starting point for all molecular-based analysis of microbial communities in natural environments is the extraction of nucleic acids from environmental samples. The DNA extracted must then be of sufficient quality to permit activity of the enzymes used in subsequent procedures.

The efficiency of the extraction technique and the purity of the extracted DNA both have implications for the study of microbial ecology. There are many published methods and protocols for extracting DNA from environmental samples (e.g. Ogram *et al*, 1987; Fuhrman *et al*, 1988; Steffan *et al*, 1988; Selenska and Klingmuller, 1991; Tsai and Olson, 1991; Bruce *et al*, 1992). However, environmental samples contain extremely complex mixtures of micro-organisms: *Eubacteria* and *Archaea*; Gram-positive and Gram-negative; vegetative cells and spores, and so standard protocols may not work

efficiently with every environmental sample, and extraction must therefore be optimised on an individual basis.

Humic acids, phenolic compounds and chelating agents that are co-extracted with the DNA can inhibit enzymatic manipulations of DNA, particularly PCR amplification (Tsai and Olson, 1992; Tebbe and Vahjen, 1993) and therefore must be removed. Any DNA not associated with humic material would be expected to offer a preferential template for PCR. Sephadex gel separation (Tsai and Olson, 1992), CsCl-gradient ultracentrifugation (Steffan *et al*, 1988), dialysis (Bruce *et al*, 1992), phenol/chloroform extraction (Bruce *et al*, 1992) and polyvinylpolypyrrolidone (PVPP) treatment (Steffan *et al*, 1988; Weller and Ward, 1989) have all been put forward as protocols for the purification of extracted DNA. However, there are now various commercially available one-step DNA extraction and purification kits that can provide high yield, high quality DNA that is readily amplifiable via PCR.

1.8.2. Polymerase Chain Reaction

The use of PCR as a diagnostic tool for the detection of bacterial populations in environmental samples is now commonplace. Specific PCR primers have been used to amplify fragments of rRNA genes in order to detect the presence of specific organisms or groups of organisms in a wide range of environmental samples (Bej *et al*, 1990; Erb and Wagner-Dobler, 1993; Hiorns *et al*, 1995; Hales *et al*, 1996). However, the use of PCR primers for the specific amplification and detection of SRB in the environment has not

been previously described. The phylogeny of the SRB, though, makes them amenable to the development of primers and probes based on 16S rRNA sequences (see Chapter 3). The main advantage of PCR is that it can be both highly specific and sensitive, and can be used to detect specific organisms in environmental samples without the need for prior cultivation. The main disadvantage is that it is difficult to generate data that are quantitatively meaningful (Head *et al*, 1998).

The sensitivity and specificity of PCR can be improved by adopting a 'nested' approach to PCR, whereby initial amplification is performed with a pair of broad specificity primers. A second round of amplification is then conducted on the product using primers of greater specificity with target sites internal to the first pair. This approach has been used successfully to detect low levels of ammonia-oxidizing bacteria in samples of lakewater (Hiorns *et al*, 1995).

1.8.2.1. Limitations of PCR

There are, however, some limitations to the use of PCR in studies of microbial ecology and diversity. One of the main problems with PCR is the amplification of unknown and unrelated genes that contain the same primer sites as the target genes. This is especially true when amplifying DNA from environmental samples. One approach to solving the problem of detecting unrelated organisms is the use of specific PCR primers coupled to confirmation of the identity of the amplified products by the use of a specific oligonucleotide probe. While a single oligonucleotide target sequence may be found in a

number of unrelated taxa, the probability that target sites for three specifically designed oligonucleotides are found in a non-target organism is much reduced (Head *et al*, 1998). In addition, at low stringency, primers may anneal to sites that contain mismatches in the nucleotide sequence of the target region. However, the thermal stability of *Taq* polymerase allows the PCR reaction to be performed at elevated temperatures that can prevent mispriming thus increasing the probability of specifically amplifying the target gene (Steffan and Atlas, 1991). Methods such as 'hot-start' PCR, whereby primers and template DNA are completely denatured before PCR cycling begins, therefore preventing primer annealing at low temperatures, can also be used to increase the stringency of PCR and minimise these problems.

PCR can also introduce biases that can affect the results of molecular biological measures of diversity through the preferential amplification of particular sequences. Potential sources for these biases include variation in nucleotide sequence at primer sites and the formation of secondary structures or hybrids which can inhibit primer extension (Wilson, 1997). In addition, different binding energies, resulting from primer degeneracy, that can influence the formation of primer-template hybrids, and the mole % G+C content of template DNA have also been reported to influence gene amplification by PCR. Reysenbach *et al* (1992) found that rRNA genes from thermophilic members of the *Archaea* could not be readily amplified by PCR due to the high mole % G+C content preventing efficient denaturation during thermal cycling.

Suzuki and Giovannoni (1996) demonstrated preferential amplification by PCR due to the reannealing of genes present in high concentrations inhibiting the formation of primer-template hybrids. This bias resulted in 1:1 mixtures of genes in the final products,

regardless of the initial proportions of the templates, and was strongly dependent on the number of cycles of replication. However, they concluded that, if using DNA extracted from environmental samples, this PCR-produced bias would be small, since it is unlikely that the amplification of any particular gene will produce products at a high enough concentration to reproduce the reannealing inhibition effect. Furthermore, as the amount of bias is dependent on the number of cycles, it can be minimized by keeping the number of cycles a low as possible.

A further problem in PCR-based studies of microbial ecology is the formation of recombinant or chimeric products (Liesack *et al*, 1991) in which fragments from two different sequences become fused during the amplification process. Inclusion of such chimeric products in phylogenetic analyses can cause significant errors as the sequence may not actually exist in a single continuous stretch of DNA within any organism. There are a number of computer programs that have been developed to help identify chimeric sequences such as CHECK_CHIMERA from the RDP (Maidak *et al*, 1997) and Chimeric Alignment (Komatsoulis and Waterman, 1997) which uses the GCG suite of programs (Wisconsin Package Version 10.0, Genetics Computer Group (GCG), Madison, Wisc., USA).

The choice of DNA polymerase can also influence the sequences obtained by PCR. The fidelity of PCR amplification can vary depending on the particular DNA polymerase used. Nucleotide misincorporation rates have been reported in the range of 0.000002% - 1.3% for different DNA polymerases (Head *et al*, 1998). Careful analysis of sequences and of secondary interactions should, however, normally identify discrepancies due to misincorporation of nucleotides during PCR (Head *et al*, 1998). Giovannoni (1991)

though, considered *Taq* incorporation errors to be insignificant in phylogenetic analysis when comparing rRNA sequence differences between species, so long as emphasis is not placed on discriminating species or clusters on the basis of one or two nucleotide changes.

1.8.3. Temporal Thermal Gradient Electrophoresis

Temporal thermal gradient electrophoresis (TTGE) is a variant of denaturing/temperature gradient gel electrophoresis (DGGE/TGGE) (Myers *et al*, 1985; Reisner *et al*, 1989) and like DGGE/TGGE can be used to directly determine the composition and genetic diversity of complex microbial populations. With TTGE, separation of specifically-amplified PCR products on the basis of sequence heterogeneity is facilitated by electrophoresis through a denaturing acrylamide gel that is subjected to increases in temperature over time. The addition of a GC-rich sequence (GC clamp) to the 5'-end of either the forward or reverse primer imparts melting stability to the PCR products in a denaturing gradient gel and improves the detection of individual sequences (Myers *et al*, 1985; Sheffield *et al*, 1989). The different melting characteristics caused by variation in sequence between PCR products of the same size causes their migration through the gel to halt at unique positions, forming discrete bands in the gel. Since each single band may represent a single microbial 'phylotype', analysis of banding patterns can be used as a measure of genetic diversity within particular microbial populations.

The use of gradient gel electrophoresis to investigate the genetic diversity of natural microbial communities was introduced to microbial molecular ecology by Muyzer *et al*

(1993) who demonstrated DGGE profiles of PCR-amplified 16S rDNA extracted from environmental samples. Subsequently, gradient gel electrophoresis has been used to investigate the composition and genetic diversity of complex microbial populations in a number of different environments including cyanobacteria in a hot spring microbial mat (Ferris *et al*, 1996; Ferris and Ward, 1997), actinomycetes in soils (Heuer *et al*, 1997), ammonia-oxidizing bacteria in coastal sand dunes (Kowalchuk *et al*, 1997) and sulfate-reducing bacteria in a marine fjord (Teske *et al*, 1996). Muyzer *et al* (1993) also demonstrated the sensitivity of this technique by showing that a specific band in a mixture of PCR products could be distinguished even when the target DNA comprised less than 1% of the total DNA in the mixture. This indicated that minority species in microbial populations would also be detected.

Gradient gel electrophoresis also offers the possibility of providing phylogenetic information on the microbial populations analyzed through the excision, reamplification and sequencing of individual bands (Ferris *et al*, 1996; Ferris and Ward, 1997; Teske *et al*, 1996; Kowalchuk *et al*, 1997).

Therefore, the presence, relative abundance and identity of different phylotypes in complex microbial communities can be discerned in a qualitative and perhaps semi-quantitative way using this technique.

1.8.3.1. Limitations of Gradient Gel Electrophoresis

However, limitations also exist in the use of gradient gel electrophoresis. There have been some concerns raised over PCR bias towards or against certain sequence types

(Reysenbach *et al*, 1992; Ferris *et al*, 1996) which could affect the appearance and intensity of bands on the gel and therefore discount any semi-quantitative assessment. However, Heuer and Smalla (1997) have shown that the intensity of bands in a TGGE analysis of soil microbial communities corresponded semi-quantitatively with the abundance of species and concluded that the bias of preferred amplification may be overestimated.

A further limitation is that the separation of fragments from highly diverse microbial communities can often be poor, though resolution can be improved by using a narrower gradient range, two-dimensional electrophoresis (Fischer and Lerman, 1979) or by employing group-specific PCR primers to narrow the target range.

There is always the potential that PCR products with different sequences can exhibit very similar melting characteristics and so co-migrate to the same position in the gel. Therefore, it is important to remember that single bands in the gel do not necessarily represent single phylotypes and that the number of bands generated by the gradient gel electrophoresis may underestimate the number of different phylotypes in the population analyzed. Conversely, some bacteria contain multiple rRNA operons of varying sequence and so individual species could potentially be responsible for multiple bands on the gel (Nubel *et al*, 1996).

The phylogenetic information gained from the sequencing of excised bands is also limited by the size of the fragment run on the gradient gel. Typically, only fragments up to 500 bp can be well separated. However, in this study, a novel method of screening clones derived from landfill PCR products by TTGE (van Dyke, Personal

communication) (section 2.12) has allowed sequencing of fragments up to 1150 bp to be performed.

1.9. Measurement of Bacterial Phylogenetic Relationships

1.9.1. The Ribosomal RNA (rRNA) Approach

A molecule whose sequence changes randomly in time can be considered a molecular chronometer. To be a useful chronometer a molecule must fulfill certain criteria: changes in its sequence must occur as randomly as possible; rates of change have to be commensurate with the spectrum of evolutionary distances being measured; the molecule must be large enough to provide an adequate amount of information (Woese, 1987). For these reasons, it is the larger rRNA molecules (16S and 23S in bacteria), and in particular the 16S rRNA molecule, which have proved to be the most useful and widely used molecular chronometers.

16S rRNA is ubiquitous in bacteria, exhibits a high degree of functional conservation, provides a large amount of information (*ca.* 1,500 nucleotides), is of a size that can be readily sequenced and different positions in the sequence change at very different rates, thereby allowing most phylogenetic relationships to be measured (Woese, 1987). In addition, the advent of PCR has also made it much easier to directly amplify and sequence the 16S rRNA gene (16S rDNA).

Extensive sequence analysis of rRNA molecules has enabled all cellular life to be assigned to one of three domains: *Bacteria*; *Archaea*; *Eukarya* (Woese, 1987). In the domain *Bacteria*, further comparative analysis of the 16S rRNA molecule has allowed

major lineages (divisions) to be defined, within which different phylogenetically distinct groups can be discerned.

The 16S rRNA molecule comprises highly conserved sequence domains interspersed with semi-conserved and hypervariable regions (Gutell *et al*, 1994; Van de Peer *et al*, 1996). It is these semi-conserved and hypervariable regions that provide phylogenetic resolution to species level and perhaps beyond. The secondary structure of the 16S rRNA molecule is presented in Fig. 1.4 (p.34).

1.9.2. Analysis of Sequence Data

The most commonly used form of comparative rRNA sequence analysis is the construction of phylogenetic trees. Combinations of multiple sequencing runs allows the production of a consensus sequence which minimizes the possibility of sequencing errors. Once ascertained, sequences are arranged into a database in order to extract phylogenetic data (Pace *et al*, 1986). Regions of conserved nucleotides that are dispersed throughout the primary structure facilitate the alignment of rDNA sequences. These conserved regions, once aligned, provide a framework for the alignment of more variable regions.

Regions of sequence that cannot be unambiguously aligned are normally not included in phylogenetic analyses (Head *et al*, 1998). Once the sequences have been aligned phylogenetic analyses can be undertaken. In the construction of phylogenetic trees, the ancestral start point of the tree may be unknown and thus the tree is described as 'unrooted'. A point of reference can be provided by the inclusion of sequence data from

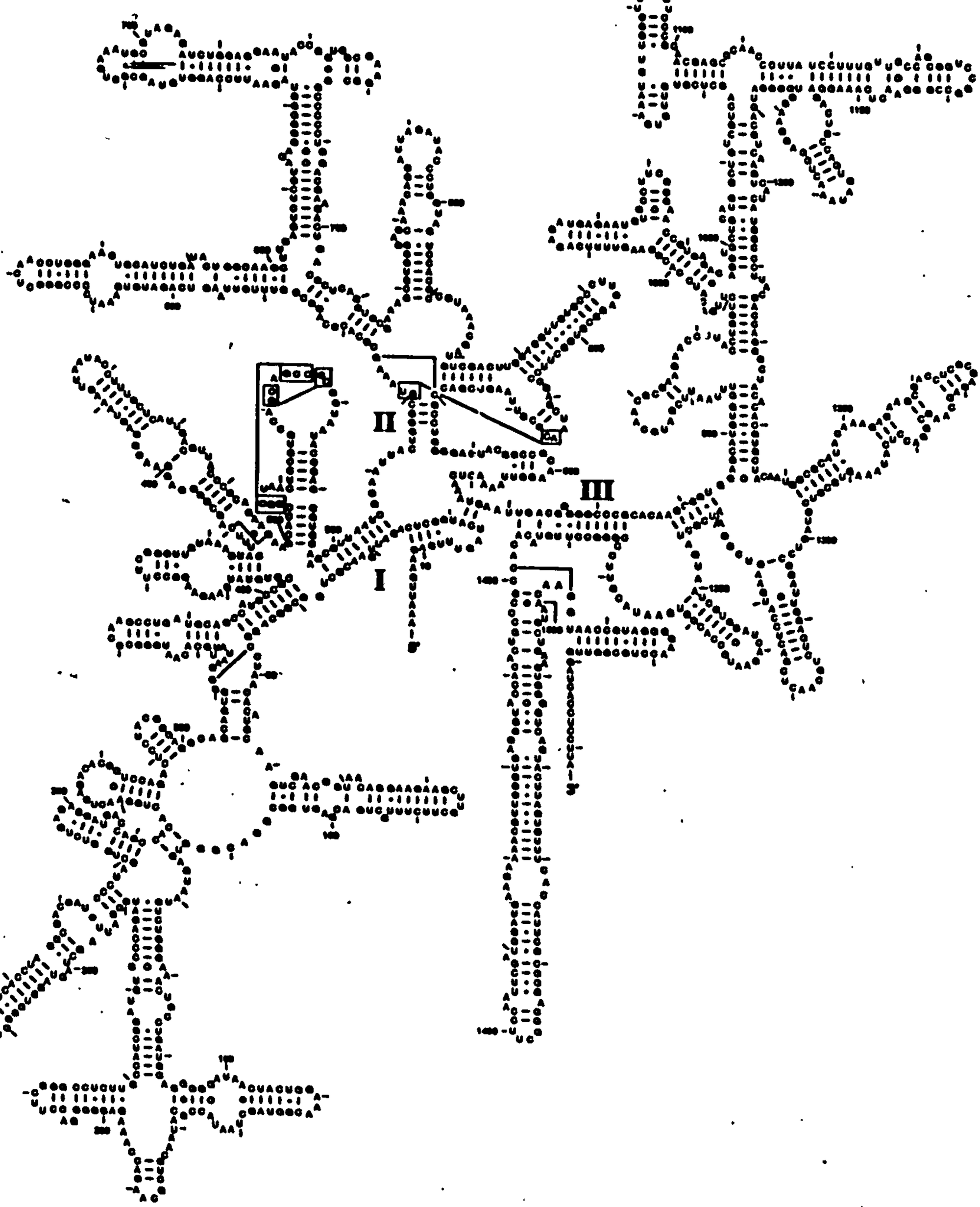


Figure 1.4. Secondary structure model of eubacterial (*E. coli*) 16S rRNA. (from Gutell *et al*, 1994).

a distant evolutionary branch known as an 'outgroup'. Two widely used approaches for inferring phylogenetic trees are employed in the work described in this thesis, distance matrix and maximum parsimony.

1.9.2.1. Distance Matrix Methods

Distance methods are conceptually the most simple. Pairwise comparisons of a set of aligned sequences are used to construct a distance matrix, usually using a model of base substitution to account for multiple substitutions at a single site (e.g. Jukes and Cantor, 1969). The evolutionary distances calculated can then be converted into an optimal tree topology by grouping the most closely related sequences. This method can underestimate the true evolutionary distances between sequences due to multiple events occurring at different rates (Woese, 1987).

1.9.2.2. Maximum Parsimony Analysis

Unlike distance matrix analysis, maximum parsimony does not reduce the differences between sequences to a single distance; it considers each nucleotide position independently. Each branch point in the tree is defined by specific changes postulated to have occurred in the evolution of some ancestral sequence. The assumption of maximum parsimony is that the correct tree is that which requires the smallest number of mutational changes to have occurred in that ancestral sequence i.e. the most parsimonious tree (Woese, 1987).

1.10. Aims of the Project

The main aims of this project were to evaluate the relative occurrence, distribution and diversity of SRB populations in landfill leachate by the application of molecular biological techniques.

This was to be achieved by the design and development of 16S rDNA-targeted PCR primers and oligonucleotide probes specific for subgroups within SRB that would facilitate the amplification and identification of SRB DNA extracted directly from leachate samples. The primers and probes were also to be designed for the study of SRB ecology in general.

PCR-based analyses such as gradient gel electrophoresis and DNA sequencing could then be undertaken to elicit information on the diversity of SRB populations in this environment, to verify SRB designates based on oligonucleotide probing and also to provide information on the phylogenetic relationships between extracted sequences.

CHAPTER 2. Materials and Methods.

2.1. Chemicals and Reagents

Chemicals and reagents used throughout this study were of Analar grade (or equivalent) and were obtained from Sigma (Sigma Chemical Company, Poole, Dorset), BDH (BDH Ltd., Gillingham, Dorset) or Fisons (Fisons Scientific Equipment, Loughborough), unless otherwise stated.

2.2. Bacterial strains

Type strains of bacteria were obtained from either the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM) or the National Collection of Industrial and Marine Bacteria (NCIMB). Details of the strains used as controls in this study are provided in Table 2.1.

ORGANISM	SOURCE[a]
Desulfotomaculum nigrificans (Group 1)	NCIMB 8395
Desulfobulbus propionicus (Group 2)	DSM 2032
Desulfobacterium autotrophicum (Group 3)	DSM 3382
Desulfobacter curvatus (Group 4)	DSM3379
Desulfosarcina variabilis (Group 5)	DSM 2060
Desulfovibrio desulfuricans (Group 6)	DSM 642
Zymomonas mobilis	NCIMB 8938
Clostridium aurantibutyricum	NCIMB 10659
Desulfobacterium vacuolatum	DSM 3385
Pelobacter carbinolicus	DSM 2380

Table 2.1. Bacterial strains used in this study.

[a]Abbreviations : NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland ; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

The non-SRB reference strains included are those identified in the Ribosomal Database Project (RDP) SSU_ rRNA database (Maidak *et al*, 1997) as containing 1 or 2 bp mismatches within the target regions of the oligonucleotide probes. They were therefore appropriate controls for evaluating the specificity of the SRB group-specific oligonucleotide probes.

2.3. Collection of environmental samples

Samples of fresh, pooled landfill leachates were collected by the landfill operators of seven conventional municipal landfill sites in the North West of England. Location of each landfill site and the year of sampling are provided in Table 2.2. Unfortunately, it was not possible to obtain any information on the physical or chemical characteristics of the landfill sites.

LANDFILL SITE	YEAR SAMPLED
Pilsworth, Bury, Lancashire	1997
Butchersfield, Warrington, Cheshire	1997 & 1998
Buff Quarry, St.Helens, Merseyside	1997
Risley, Warrington, Cheshire	1998
Chadderton, Oldham, Lancashire	1998
Holiday Moss, Rainhill, Merseyside	1998
West Leigh, Greater Manchester	1998

Table 2.2. Landfill sites sampled in this study

2.3.1. Preparation of environmental samples

The leachate samples were processed immediately upon receipt. Each 1 litre sample was concentrated by centrifugation (27,000 x *g*, 40 min) and the pellet resuspended in 20 ml 0.1M K_2HPO_4. Aliquots (1.5 ml) of this concentrated sample were centrifuged (22,000 x *g*, 5 min) and the pellets stored at -80°C until required.

2.4. Extraction of DNA

Pellets of concentrated leachate stored at -80°C were thawed on ice and resuspended in 200 µl sterile dH_2O to give a final 375-fold concentration of the leachate solids. DNA was extracted and purified from this concentrated leachate using the FastDNA SPIN kit (BIO101, Inc.) and a Ribolyser (Hybaid, Ltd.) according to the manufacturers' instructions. DNA was extracted from control strains (Table 2.1, p.37) by resuspending freeze-dried cultures in 200 µl sterile dH_2O and applying the BIO101 kit and Hybaid Ribolyser protocol described above. DNA recovery, purity and yield were evaluated by agarose gel electrophoresis (section 2.7).

2.5. Oligonucleotide synthesis

Oligonucleotides were synthesised at the School of Biological Sciences, University of Liverpool (ABI 392 oligonucleotide synthesiser) or commercially synthesised by Perkin Elmer (Perkin Elmer Biosystems, Warrington). Oligonucleotides were ethanol precipitated with 0.1 vol. 3 M sodium acetate and 3 vol. ice cold absolute ethanol and resuspended in sterile dH_2O prior to application. Details of oligonucleotides are provided in Tables 3.1 & 3.2 (p.54 & 56).

2.6. PCR amplification

PCR amplification of 16S rDNA extracted from control strains and from each landfill site was performed with eubacterial (Edwards *et al*, 1989) and SRB group-specific PCR primers (Table 3.1, p.54). Reactions were carried out as follows: 95°C for 1 min; annealing for 1 min; 72°C for 1 min, for 30 cycles, with a final extension step of 72°C for 10 min. Each reaction tube (100 µl) contained: 2 µl each primer (10 pmol μl^{-1}), 2 µl dNTP (10 mM each)(HT Biotech, Ltd.), 85 µl dH_2O, 10 µl 10xPCR buffer (HT Biotech, Ltd.), 0.2 µl 10% (w/v) BSA (Boehringer Mannheim), 1U Super*Taq* polymerase (HT Biotech, Ltd.) and DNA template (approximately 100-150 ng). Each reaction was overlaid with approximately 50-100 µl mineral oil prior to cycling. All PCR reactions were performed with a DNA thermal cycler 480 (Perkin-Elmer Cetus).

2.6.1. 'Hot-start' PCR

'Hot-start' PCR is a method by which the DNA template and PCR primers are fully denatured and dissociated from each other by heating to 95°C prior to PCR cycling. All PCR amplifications in this study were performed using a 'hot-start' PCR protocol. This was achieved using either of two methods. Firstly, each reaction, without Super*Taq* polymerase (HT Biotech, Ltd.), was heated at 95°C for 5 min to fully denature and dissociate the DNA template and PCR primers. The tubes were then cooled to 80°C and maintained at this temperature while the enzyme was added. Each reaction was then overlaid with mineral oil prior to cycling. Secondly, HotStar*Taq* (Qiagen, Ltd.), a commercially available *Taq* polymerase designed to facilitate 'hot-start' PCR was used. Each reaction, containing 1U HotStar*Taq* (Qiagen, Ltd.) and overlaid with mineral oil,

was heated at 95°C for 15 min to denature and dissociate template and primers and also to activate the enzyme. PCR cycling then commenced as described above.

2.6.2. 'Nested' PCR

'Nested' PCR as developed is a method which increases the sensitivity of PCR by employing two rounds of amplification with two different primer sets, the second set being internal to the first. 16S rDNA extracted from landfill leachate was first amplified with eubacterial primers pA & pH' (Edwards *et al*, 1989), then aliquots of these eubacterial amplification products were diluted 100-fold into fresh PCR reaction mixtures containing a pair of SRB group-specific primers (Table 3.1, p.54). PCR amplifications were performed as described above.

2.7. Agarose gel electrophoresis

Extracted DNA and PCR products were electrophoresed through a 1% (w/v) agarose gel in 1 x Tris acetate EDTA (pH 8.0) (50 x Tris acetate EDTA: 2 M Tris; 57.1 ml l^{-1} glacial acetic acid; 0.05 M EDTA; adjusted to pH 8.0) containing ethidium bromide (0.2 μg ml^{-1}). Electrophoresis was performed at a constant voltage of 100 V for 1 h and DNA was visualised by UV illumination at 320 nm. Markers λ DNA/*Hind*III and pBR322 DNA/*Alw*441/*Mva*1 (MBI Fermentas) were included to enable estimation of the molecular weight and yield of the DNA extracted and amplified.

2.8. Immobilisation of nucleic acids

2.8.1. Dot blotting of DNA using a vacuum manifold

DNA extracted from SRB and non-SRB control strains was diluted in 1 vol. denaturing solution (1 M NaOH; 3 M NaCl) and transferred to positively-charged nylon membrane (Boehringer Mannheim) using a dot blot apparatus (Minifold, Schleicher and Schuell) connected to a vacuum pump. DNA was then fixed to membranes by air drying for 1h and UV crosslinking at 320 nm for 3 min. If required, membranes were wrapped in cling film and stored at 4°C.

2.8.2. Southern transfer of DNA

PCR products amplified from landfill leachate with SRB group-specific primers were transferred to positively-charged nylon membrane (Boehringer Mannheim) by Southern blotting using alkali transfer buffer (0.25 M NaOH; 1.5 M NaCl). Transfer of DNA was allowed to proceed for at least 3 h. DNA was fixed to membranes by air drying and UV crosslinking as described above.

2.9. Oligonucleotide probing

DNA fixed to membranes was first incubated in 10-15 ml standard prehybridization solution (5xSSC; 0.1% (w/v) N-lauroyl sarcosine; 0.02% (w/v) SDS; 1% (w/v) blocking reagent [Boehringer Mannheim]) at the appropriate hybridization temperature (Table 3.2, p.56) for 1 h to prevent non-specific binding of the probe. 100 pmol of concentrated DIG-labelled probes were diluted in 10 ml prehybridization solution to a final

concentration of 10 pmol ml^{-1} and membranes were incubated overnight at hybridization temperature inside a thermostatically controlled oven (Hybaid). After hybridization, two 15 min high stringency washes (Maleic acid buffer + 0.3% (v/v) Tween 20) were performed at hybridization temperature. DIG-labelled DNA was then detected using the standard DIG luminescent detection procedure (Boehringer Mannheim) and membranes were exposed to X-ray film (Kodak) at room temperature for 1-5 min.

2.9.1. Labelling of oligonucleotide probes

Oligonucleotide probes were 3'-end labelled with non-radioactive Digoxigenin-11-ddUTP using terminal transferase (Boehringer Mannheim) according to the manufacturer's instructions. Probe labelling efficiency was assessed according to the manufacturer's instructions.

2.9.2. Solutions for oligonucleotide probing

Maleic acid buffer:

0.1 M Maleic acid; 0.15 M NaCl; adjusted to pH 7.5.

10 x Blocking reagent:

10% (w/v) blocking reagent (Boehringer Mannheim) dissolved in maleic acid buffer.

Standard Prehybridization/Hybridization solution:

5 x SSC (20 x SSC: 0.3 M Sodium citrate; 3 M NaCl; adjusted to pH 7.0); 0.1% (w/v) N-lauroyl sarcosine; 0.02% (w/v) SDS; 1% (w/v) blocking reagent (Boehringer Mannheim).

2.10. Temporal thermal gradient electrophoresis

PCR products amplified from landfill leachate with SRB group-specific primers were diluted 1000-fold and reamplified with eubacterial primers pC & pD' (Edwards *et al*, 1989) to generate a 235 bp PCR fragment encompassing the V3 region of the 16S rRNA gene suitable for TTGE analysis. A 40 bp GC-clamp was incorporated onto primer pC to facilitate separation of fragments. PCR reactions were performed using HotStar*Taq* (Qiagen, Ltd.) (section 2.6.1) with primer annealing at 68°C for 25 cycles. Aliquots of these PCR products (20-30 ng of pure culture product or 200 ng of landfill product) were diluted in 1 vol. 2 x loading buffer (0.08% (w/v) bromophenol blue; 0.08% (w/v) xylene cyanol FF; 10% (v/v) glycerol) and applied directly to the TTGE gels. TTGE gels were composed of 6% (w/v) polyacrylamide (37:1 acrylamide:bisacrylamide), 1.25 x Tris acetate EDTA (pH 8.0), 2.5% (v/v) 80% glycerol, 0.1% (w/v) ammonium persulfate, 0.1% (v/v) TEMED, and 50% denaturant (7 M urea, 20% (v/v) deionized formamide). Gels were allowed to polymerize for at least 1 h. A 5 ml stacking gel, without denaturant, was added after polymerization. TTGE was performed in 1.25 x Tris acetate EDTA (pH 8.0) at a constant voltage of 60 V for 16 h. The temperature range for separation of PCR products amplified from landfill leachate with SRB group-specific primers was 49.5°C-57.5°C with a gradient of 0.4°C h^{-1}. The D-GENE system (Bio-Rad,

Inc.) was used for all TTGE analyses according to the manufacturer's instructions. TTGE gels were stained with 0.2 x conc. SYBR Green 1 nucleic acid stain (Flowgen) in 1.25 x Tris acetate EDTA (pH 8.0) for 30 min and scanned under UV illumination using a STORM 860 optical scanner (Molecular Dynamics). Gel images were visualised and stored using ImageQuant software.

2.11. Cloning of PCR products

PCR products amplified from landfill leachate with SRB group-specific primers were ligated into the pGEM-T vector (Promega) and cloned into competent *E.coli* JM109 cells according to the manufacturer's instructions. Cells were plated out onto LB agar (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 5 g l^{-1} NaCl, adjusted to pH 7.0) containing 100 µg ml^{-1} ampicillin with 0.5 mM IPTG and 40 µg ml^{-1} X-Gal to facilitate blue/white screening. After overnight incubation at 37°C, white colonies containing vector + insert were subcultured onto fresh LB agar containing 100 µg ml^{-1} ampicillin, 0.5 mM IPTG, 40 µg ml^{-1} X-Gal and incubated overnight at 37°C. Clones were then stored at 4°C until required.

2.12. TTGE screening of clones

Clones stored at 4°C were subcultured into 10 ml LB broth containing 100 µg ml^{-1} ampicillin and incubated overnight at 37°C. 1 ml of overnight culture was pelleted by centrifugation (22,000 x *g*, 5 min) and resuspended in 100 µl sterile dH_2O. The samples were then placed in a boiling water bath for 10 min to break open the cells and release plasmid DNA. Cell debris was pelleted by centrifugation (22,000 x *g*, 1 min) and the

resultant supernatant (crude cell extract) was decanted. 1 µl aliquots of the crude cell extracts were subsequently used as DNA template in PCR reactions (section 2.6) with the relevant SRB group-specific primers to screen for the correct sized insert. PCR products from positive reactions were diluted 1000-fold and reamplified with primers pC(GC-clamp);pD'. Aliquots of these PCR products (20-30 ng) were then analysed by TTGE as described in section 2.10. TTGE profiles of the appropriate landfill site were run alongside to identify clones of interest.

2.13 Plasmid isolation

Bands of interest on the TTGE gels were noted and the relevant clones subcultured into fresh LB broth containing 100 µg ml^{-1} ampicillin and incubated overnight at 37°C. 3 ml of overnight culture was pelleted by centrifugation (22,000 x *g*, 5 min) and plasmid DNA containing the relevant insert was extracted and purified using the QIAprep Spin Miniprep kit (Qiagen, Ltd.) according to the manufacturer's instructions. DNA was precipitated with 1 vol. 13% PolyEthyleneGlycol (8000); 1.6 M NaCl and resuspended in 10 mM Tris.HCl (pH 8.5) prior to sequencing.

2.14. DNA sequencing

Automated DNA sequencing was performed with a laser fluorescence ABI 373S automated sequencer (Applied Biosystems) at the School of Biological Sciences sequencing facility, University of Liverpool.

2.15. Analysis of sequence data

Sequence data was analysed using the GCG suite of programs (Wisconsin Package Version 10.0, Genetics Computer Group (GCG), Madison, Wisc., USA) running on the UNIX computer system at the University of Liverpool. 16S rDNA sequences derived from landfill samples were aligned by eye in conjunction with reference sequences obtained from the GenBank (Benson *et al*, 1997), EMBL (Stoesser, 1997) and Ribosomal Database Project (Maidak *et al*, 1997) databases. Data analysis and manipulation was performed using the Genetic Data Environment (GDE) program running on the UNIX system at the University of Liverpool. The application of a 'mask' sequence allowed the alignment of unambiguous sequences for comparison. Calculations of distance values were performed using Phylogeny Inference Programs (PHYLIP 3.4) (Felsenstein, 1993). Phylogenetic trees were constructed from the calculated distance values using the neighbour-joining method of Jukes and Cantor (1969) and produced by the TREEVIEW program (PHYLIP 3.4). The robustness of the inferred phylogeny was determined by bootstrap analysis consisting of 100 resamplings of the data performed using SEQBOOT (PHYLIP 3.4) and a consensus phenogram was generated using the program CONSENSE (PHYLIP 3.4).

The topologies of the phylogenetic trees were corroborated by maximum parsimony analysis using PAUP 3.0 (Swofford, 1991). The SEQBOOT and CONSENSE programs (PHYLIP 3.4) performed bootstrap analysis of 100 data sets.

CHAPTER 3. Design and Evaluation of SRB Group-Specific 16S rDNA-Targeted PCR Primers and Oligonucleotide Probes.

3.1. Introduction

Molecular biological methods have been used to investigate microbial populations in a variety of different environments. The application of molecular biological methods to investigate the occurrence and distribution of bacteria in the environment has the advantage of providing direct information on community structure. Not only do culture-based methods only recover a fraction of the natural population, estimated at 0.1-10% of the bacteria that can be visualized using direct count methods (Head *et al*, 1998), but for many bacteria including SRB, the isolation of axenic cultures from environmental samples is not straightforward. Therefore, the development of molecular biological tools is of paramount importance in continuing investigations of microbial community structure and activity in the natural environment.

The rapidly expanding database of 16S rRNA sequences now contains several thousand sequences, and represents an invaluable resource. By comparison of the more variable regions of the 16S rRNA molecule, it is possible to design oligonucleotides of varying phylogenetic resolution. Hypervariable regions can be used to design genus- or species-specific primers or probes while regions of increasing conservation can be used to target more general assemblages of bacteria. Finally, highly conserved tracts common to all sequenced species can be used to design universal primers or probes (Amann *et al*, 1990). Detection of specific organisms, without cultivation, can be achieved by PCR alone, or combined with the use of diagnostic oligonucleotide probes (Head *et al*, 1998).

This chapter describes how regions of hypervariability in the 16S rRNA molecule were used to enable the design of group-specific PCR primers and oligonucleotide probes for the determinative amplification and hybridization of SRB in environmental samples.

3.2. Results

3.2.1. Construction of a Phylogenetic Tree and Identification of SRB Subgroups

A phylogenetic tree was constructed from aligned SRB 16S rRNA sequences obtained from the GenBank (Benson *et al*, 1997), EMBL (Stoesser, 1997) and Ribosomal Database Project (Maidak *et al*, 1997) databases using the neighbour-joining method of Jukes and Cantor (1969) and produced by the TREEVIEW program (section 2.15). The topology of the tree (Fig. 3.1, p.50) confirmed the phylogeny of SRB previously described by Devereux *et al* (1989) and allowed identification of six main lineages (subgroups) of SRB: Group 1-*Desulfotomaculum* (DFM); Group 2-*Desulfobulbus* (DBB); Group 3-*Desulfobacterium* (DBM); Group 4-*Desulfobacter* (DSB); Group 5-*Desulfococcus-Desulfonema-Desulfosarcina* (DCC-DNM-DSS); Group 6-*Desulfovibrio-Desulfomicrobium* (DSV-DMB) (Fig. 3.1, p.50). This provided the platform for the design of group-specific 16S rDNA-targeted PCR primers and internal 16S rDNA-targeted oligonucleotide probes that could be used as diagnostic tools to screen environmental samples for the presence of SRB.

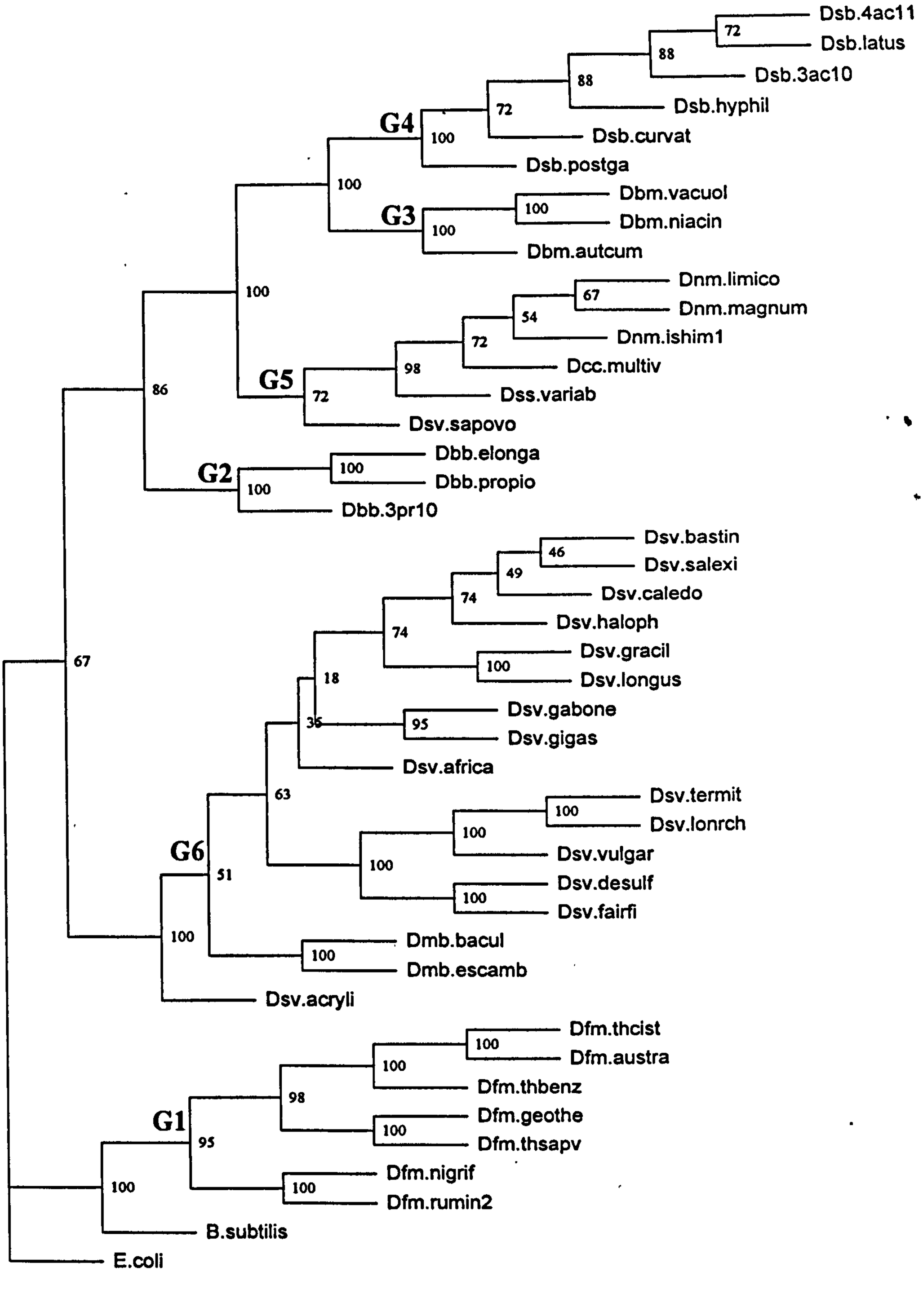


FIGURE 3.1. 16S rDNA bootstrap consensus tree showing the lineages of the six main subgroups of SRB. The tree was constructed using the neighbour-joining method of Jukes and Cantor (1969) and analysis was based on 1351 nucleotides. Bootstrap values (out of 100 trees) are shown adjacent to nodes. SRB subgroups marked G1-G6.

KEY FOR PHYLOGENETIC TREES:

E.coli	*Escherichia coli*
B.subtilis	*Bacillus subtilis*
Dfm.aerona	*Desulfotomaculum aeronauticum*
Dfm.austra	*Desulfotomaculum australicum*
Dfm.geothe	*Desulfotomaculum geothermicum*
Dfm.kuznet	*Desulfotomaculum kuznetsovii*
Dfm.nigrif	*Desulfotomaculum nigrificans*
Dfm.putei1	*Desulfotomaculum putei*
Dfm.rumin2	*Desulfotomaculum rumunis*
Dfm.thacet	*Desulfotomaculum thermoacetoxidans*
Dfm.thbenz	*Desulfotomaculum thermobenzoicum*
Dfm.thcist	*Desulfotomaculum thermocisternum*
Dfm.thsapv	*Desulfotomaculum thermosapovorans*
Dfm.reduce	*"Desulfotomaculum reducens"*
Dbb.3pr10	*Desulfobulbus* strain 3pr10
Dbb.elonga	*Desulfobulbus elongatus*
Dbb.propio	*Desulfobulbus propionicus*
Dbb.rhabdo	*Desulfobulbus rhabdoformis*
Dbm.autcum	*Desulfobacterium autotrophicum*
Dbm.niacin	*"Desulfobacterium niacini"*
Dbm.vacuol	*"Desulfobacterium vacuolatum"*
Dsb.3ac10	*Desulfobacter* strain 3ac10
Dsb.4ac11	*Desulfobacter* strain 4ac11
Dsb.curvat	*Desulfobacter curvatus*
Dsb.hyphil	*Desulfobacter hydrogenophilus*
Dsb.latus	*Desulfobacter latus*
Dsb.postga	*Desulfobacter postgatei*
Dsb.vibrio	*Desulfobacter vibrioformis*
Dsb.haloto	*Desulfobacter halotolerans*

Dcc.multiv	*Desulfococcus multivorans*
Dnm.ishim1	*Desulfonema ishimotoei*
Dnm.limico	*Desulfonema limicola*
Dnm.magnum	*Desulfonema magnum*
Dss.variab	*Desulfosarcina variabilis*
Dsv.sapovo	*"Desulfovibrio sapovorans"*
Dsv.acryli	*Desulfovibrio acrylicus*
Dsv.africa	*Desulfovibrio africanus*
Dsv.alvora	*Desulfovibrio alcoholovorans*
Dsv.amphil	*Desulfovibrio aminophilus*
Dsv.bastin	*"Desulfovibrio bastinii"*
Dsv.caledo	*"Desulfovibrio caledoniensis"*
Dsv.desulf	*Desulfovibrio desulfuricans*
Dsv.fairfi	*"Desulfovibrio fairfieldensis"*
Dsv.frvora	*Desulfovibrio fructosovorans*
Dsv.gabone	*Desulfovibrio gabonensis*
Dsv.gigas	*Desulfovibrio gigas*
Dsv.gracil	*"Desulfovibrio gracilis"*
Dsv.haloph	*Desulfovibrio halophilus*
Dsv.litora	*Desulfovibrio litoralis*
Dsv.longus	*Desulfovibrio longus*
Dsv.lonrch	*Desulfovibrio longreachensis*
Dsv.salexi	*Desulfovibrio salexigens*
Dsv.suldis	*Desulfovibrio sulfodismutans*
Dsv.termit	*Desulfovibrio termitidis*
Dsv.vulgar	*Desulfovibrio vulgaris*
Dmb.apsher	*Desulfomicrobium apsheronum*
Dmb.bacul	*Desulfomicrobium baculatum*
Dmb.escamb	*Desulfomicrobium escambiense*
Dmb.hypoge	*Desulfomicrobium hypogeium*
Dmb.norveg	*Desulfomicrobium norvegicum*

3.2.2. Design of SRB Group-Specific PCR Primers

16S rDNA -targeted PCR primers were designed from a collection of SRB 16S rRNA sequences obtained from the GenBank and EMBL databases and the RDP. *E.coli* and *B.subtilis* were used as reference points for the alignment of the SRB 16S rRNA sequences. Regions of variability between sequences representing each SRB subgroup and the reference sequences were located by eye. Potential candidates for PCR primers were compared to the aligned SSU_rRNA database of the RDP using the CHECK_PROBE utility (Maidak *et al*, 1997). The results of this cross-specificity check (Table 3.3, p.57) enabled the design of six 16S rDNA-targeted PCR primer pairs theoretically specific for each of the six main subgroups of SRB (Table 3.1, p.53).

3.2.3. Evaluation of SRB Group-Specific PCR Primers

The specificity of each group-specific primer pair was confirmed by amplifying DNA from target and non-target SRB strains (Table 2.1, p.37) with each of the six sets of primers (Table 3.1, p.53). The maximum annealing temperature for each primer pair was determined empirically and applied throughout (Table 3.1, p.53). All six primer pairs were specific for their target groups at the appropriate annealing temperatures and yielded PCR products of the expected size (Fig. 3.2, p.59). None of the primer sets amplified DNA from non-target SRB subgroups, with the exception of the DCC-DNM-DSS (Group 5) primers which gave amplification products of the expected size (860 bp) from DBM (Group 3) template DNA (Fig. 3.2, p.59), as predicted by the theoretical cross-specificity check between the two groups (Table 3.3, p.57).

PRIMER	TARGET SITE[a]	SEQUENCE 5'-3'[b]	SPECIFICITY	ANNEALING TEMP	EXPECTED SIZE PRODUCT
DFM140	140-158	TAG MCY GGG ATA ACR SYK G	Group 1	58°C	700bp
DFM842	842-823	ATA CCC SCW WCW CCT AGC AC			
DBB121[c]	121-142	CGC GTA GAT AAC CTG TCY TCA TG	Group 2	66°C	1120bp
DBB1237[c]	1237-1215	GTA GKA CGT GTG TAG CCC TGG TC			
DBM169	169-183	CTA ATR CCG GAT RAA GTC AG	Group 3	64°C	840bp
DBM1006	1006-986	ATT CTC ARG ATG TCA AGT CTG			
DSB127[c, d]	127-148	GAT AAT CTG CCT TCA AGC CTG G	Group 4	60°C	1150bp
DSB1273[c]	1273-1252	CYY YYY GCR RAG TCG STG CCC T			
DCC305	305-327	GAT CAG CCA CAC TGG RAC TGA CA	Group 5	65°C	860bp
DCC1165	1165-1144	GGG GCA GTA TCT TYA GAG TYC			
DSV230[c]	230-248	GRG YCY GCG TYY CAT TAG C	Group 6	61°C	610bp
DSV838	838-818	SYC CGR CAY CTA GYR TYC ATC			

Table 3.1. 16S rDNA-targeted PCR primer sequences specific for SRB subgroups.

[a]16S rDNA positions, *E.coli* numbering
[b]Ambiguities: R(G or A); Y(C or T); K(G or T); M(A or C); S(G or C); W(A or T)
[c]Primer sequences: DBB121; DBB1237; DSB127; DSB1273; DSV230 provided by Dr. Mark Munson, University of Essex (Personal Communication)
[d]Primer DSB127 derived from probe DSB129 described by Devereux *et al* (1992)

3.2.4. Design of SRB Group-Specific Oligonucleotide Probes

16S rDNA -targeted oligonucleotide probes were designed from the collection of SRB 16S rRNA sequences as described in section 3.2.2. Candidates for oligonucleotide probes were compared to the aligned SSU_rRNA database of the RDP using the CHECK_PROBE utility. The results of this cross-specificity check (Table 3.3, p.57) enabled the design of three group-specific 16S rDNA-targeted oligonucleotide probes to complement those already described by Devereux *et al* (1992) (Table 3.2, p.56).

3.2.5. Evaluation of SRB Group-Specific Oligonucleotide Probes

DIG-labelled oligonucleotide probes (section 2.9.1) were used in hybridization experiments (section 2.9) with DNA extracted from a range of SRB and non-SRB strains (Table 2.1, p.37). Probe EUB338 (Amann *et al*, 1990) that binds to 16S rDNA from all eubacteria was used as a control probe. Melting temperatures (Tm) for each probe were estimated and hybridization temperatures were determined empirically (Table 3.2, p.56). DNA extracts from target and non-target control strains (Table 2.1, p.37) were immobilised onto positively-charged nylon membrane by dot-blotting (section 2.8.1) and hybridized against each group-specific oligonucleotide probe as described in section 2.9. At these hybridization temperatures, target strains could be unambiguously and reproducibly discriminated from non-target strains that contained 1, 2 or 3 bp mismatches. Probe EUB338 gave strong positive signals for all of the strains (Fig. 3.3, p.60). A difference alignment of the group-specific oligonucleotide probe target regions

against 16S rRNA sequences from target and non-target species is presented in Table 3.4 (p.58)

PROBE	TARGET SITE[a]	SEQUENCE 5'-3'[b]	SPECIFICITY	HYB TEMP	REFERENCE
DFM228	228-242	GGG ACG CGG AYC CAT	Group 1	48°C	This study
DBB660	660-679	GAA TTC CAC TTT CCC CTC TG	Group 2	50°C	Devereux *et al* (1992)
DBM221	221-240	TGC GCG GAC TCA TCT TCA AA	Group 3	56°C	Devereux *et al* (1992)
DSB623	623-644	TGT TTC AAG TGC WCT TCC GGG G	Group 4	56°C	This study
DCC868	868-885	CAG GCG GAT CAC TTA ATG	Group 5	46°C	This study
DSV687	687-702	TAC GGA TTT CAC TCC T	Group 6	45°C	Devereux *et al* (1992)

Table 3.2 16S rDNA-targeted oligonucleotide probe sequences specific for SRB subgroups.

[a]16S rRNA positions, *E.coli* numbering
[b]Ambiguities: R(G or A); Y(C or T); K(G or T); M(A or C); S(G or C); W(A or T)

		PCR PRIMERS												PROBES					
		Group 1		Group 2		Group 3		Group 4		Group 5		Group 6		1	2	3	4	5	6
	SPECIES	DFM140	DFM842	DBB121	DBB1237	DBM169	DBM1006	DSB127	DSB1273	DCC305	DCC1165	DSV230	DSV838	DFM228	DBB660	DBM221	DSB623	DCC868	DSV687
Group 1	*Dfm.austra*	+	+									+		+					
	Dfm.thbenz	+	+									+		+					
	Dfm.geothe	+	+									+		+					
	Dfm.thacid	+	+									+		+					
	Dfm.nigrif	+	+											+					
	Dfm.rumin2	+	+											+					
Group 2	*Dbb.3pr10*			+	+										+				
	Dbb.elonga			+	+										+				
	Dbb.propio			+	+										+				
Group 3	*Dbm.autcum*					+	+			+	1bp					+			
	Dbm.niacin					+	+			+	1bp					+			
	Dbm.vacuol					+	+			+	1bp					+			
Group 4	*Dsb.3ac10*							+	+								+		
	Dsb.latus							+	+								+		
	Dsb.hyphil							+	+								+		
	Dsb.curvat							+	+								+		
	Dsb.postga							+	+								+		
Group 5	*Dcc.multiv*									+	+							+	
	Dnm.ishim1										+							+	
	Dnm.limico									1bp	1bp							+	
	Dnm.magnum									1bp	1bp							+	
	Dss.variab									+	+							+	
	Dsv.sapovo									+	+	+						+	
Group 6	*Dsv.acryli*											+							+
	Dsv.salexi											+	+						+
	Dsv.desulf											+	+						+
	Dsv.fairfi											+	+						+
	Dsv.lonrch											+	+						+
	Dsv.termit											+	+						+
	Dsv.vulgar											+	+						+
	Dsv.africa											+	+						+
	Dsv.gigas											+	+						
	Dsv.haloph											+	+						+
	Dsv.bastin											+	+						+
	Dmb.bacul											+	+						+
	Dmb.escamb											+	+						+

TABLE 3.3. Theoretical cross-specificity analysis of SRB group-specific PCR primers and oligonucleotide probes compared to SRB strains.
+ - sequence match in target region
1bp – 1bp mismatch in target region

PROBE	No. OF MISMATCHES	SPECIES	16S rRNA TARGET REGION[a]
DFM228			AUG GRU CCG CGU CCC
(Group 1)	0	*Dfm.nigrif* (G1)	AUG GAU CCG CGU CCC
	1	*Dsv.desulf* (G6)	AUG **A**GU CCG CGU CCC
	2	*Dss.variab* (G5)	AUG GG**C** CCG CGU **A**CC
	2	*Peb.carbin*	AUG **A**GU CCG CG**G** CCC
DBB660			CAG AGG GGA AAG UGG AAU UC
(Group 2)	0	*Dbb.propio* (G2)	CAG AGG GGA AAG UGG AAU UC
	2	*C.aurant*	**AG**G AGG GGA AAG UGG AAU UC
	3	*Peb.carbin*	**GG**G AG**A** GGA AAG UGG AAU UC
DBM221			UUU GAA GAU GAG UCC GCG CA
(Group 3)	0	*Dbm.autcum* (G3)	UUU GAA GAU GAG UCC GCG CA
	0	*Dbm.vacuol* (G3)	UUU GAA GAU GAG UCC GCG CA
	3	*Dss.variab* (G5)	UUU GAA GAU G**G**G **C**CC GCG **U**A
DSB623			CCC CGG AAG WGC ACU UGA AAC A
(Group 4)	0	*Dsb.curvat* (G4)	CCC CGG AAG UGC ACU UGA AAC A
	2	*Dbm.vacuol* (G3)	CCC CGG A**C**G UGC A**U**U UGA AAC A
	3	*Dbm.autcum* (G3)	CCC **U**GG AAG UGC A**U**U UGA AAC **U**
	3	*Dss.variab* (G5)	CCC CGG AAG UGC A**U**U UGA **U**AC **U**
DCC868			CAU UAA GUG AUC CGC CUG
(Group 5)	0	*Dss.variab* (G5)	CAU UAA GUG AUC CGC CUG
	1	*Zym.mobilis*	CAU UAA GU**U** AUC CGC CUG
	2	*Dsb.curvat* (G4)	CAU UAA GUG **UA**C CGC CUG
	2	*C.aurant*	CAU UAA GU**A** **U**UC CGC CUG
DSV687			AGG AGU GAA AUC CGU A
(Group 6)	0	*Dsv.desulf* (G6)	AGG AGU GAA AUC CGU A
	1	*Peb.carbin*	AGG **G**GU GAA AUC CGU A
	3	*Dfm.nigrif* (G1)	AG**C** **G**GU GAA AU**G** CGU A
	3	*Dss.variab* (G5)	AG**A** **G**GU GAA AU**U** CGU A

Table 3.4. Difference alignments of the 16S rRNA target regions for the SRB group-specific oligonucleotide probes. Sequences shown are for the respective target (0 mismatch) and non-target species (1-3 mismatches).

[a]Mismatches are underlined in bold type

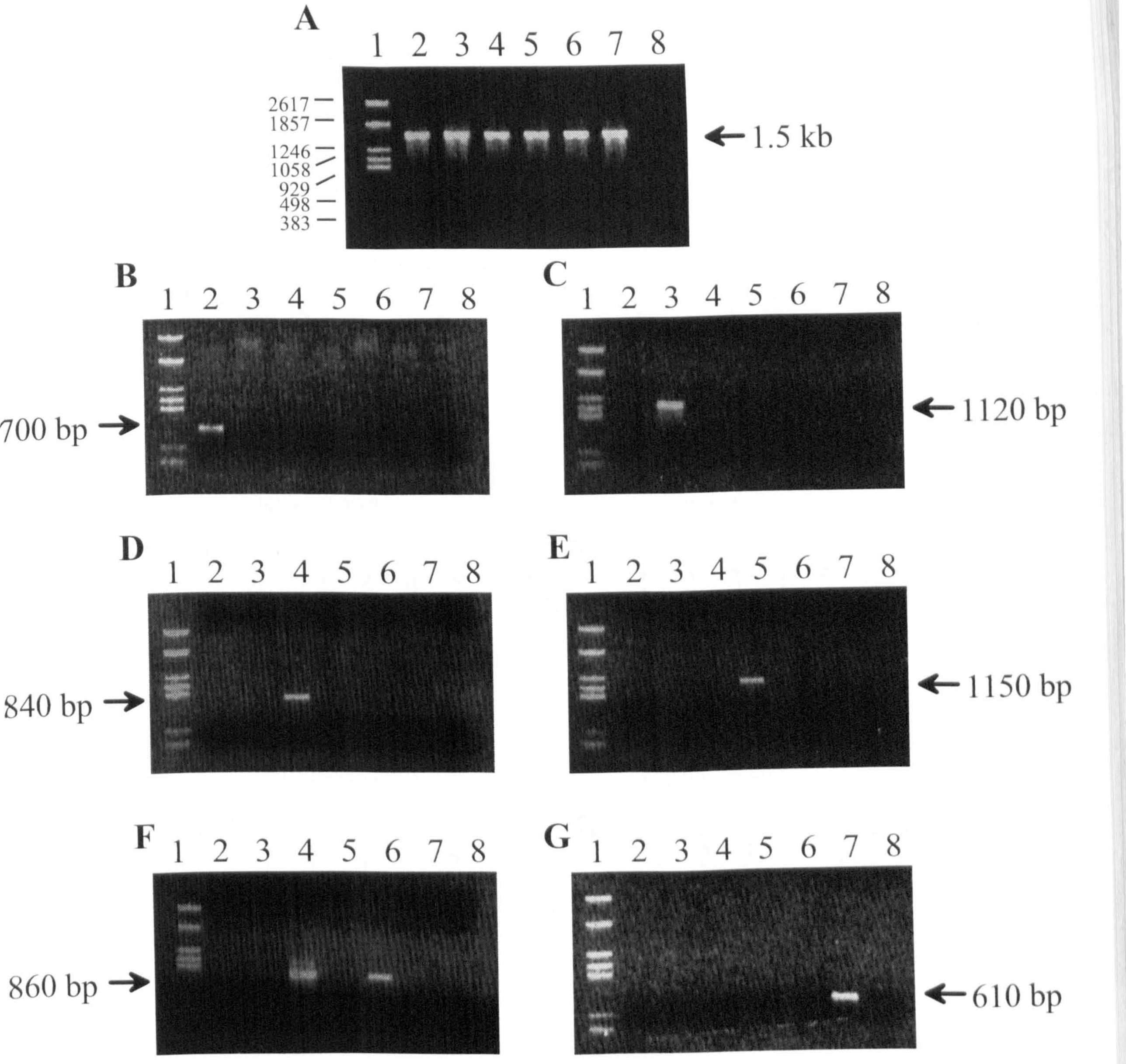


Figure 3.2. PCR amplifications of SRB 16S rDNA using eubacterial and group-specific primers. **A** - pA & pH' (Edwards *et al*, 1989), 55°C; **B** - DFM140 & DFM842 (Group 1), 58°C; **C** - DBB121 & DBB1237 (Group 2), 66°C; **D** - DBM169 & DBM1006 (Group 3), 64°C; **E** - DSB127 & DSB1273 (Group 4), 60°C; **F** - DCC305 & DCC1165 (Group 5), 65°C; **G** - DSV230 & DSV838 (Group 6), 61°C.

Lane 1 - pBR322 DNA/*Alw*441/*Mva*1 (MBI Fermentas);
Lane 2 - *Desulfotomaculum nigrificans*; Lane 3 - *Desulfobulbus propionicus*; Lane 4 - *Desulfobacterium autotrophicum*;
Lane 5 - *Desulfobacter curvatus*; Lane 6 - *Desulfosarcina variabilis*;
Lane 7 - *Desulfovibrio desulfuricans*; Lane 8 - PCR negative control

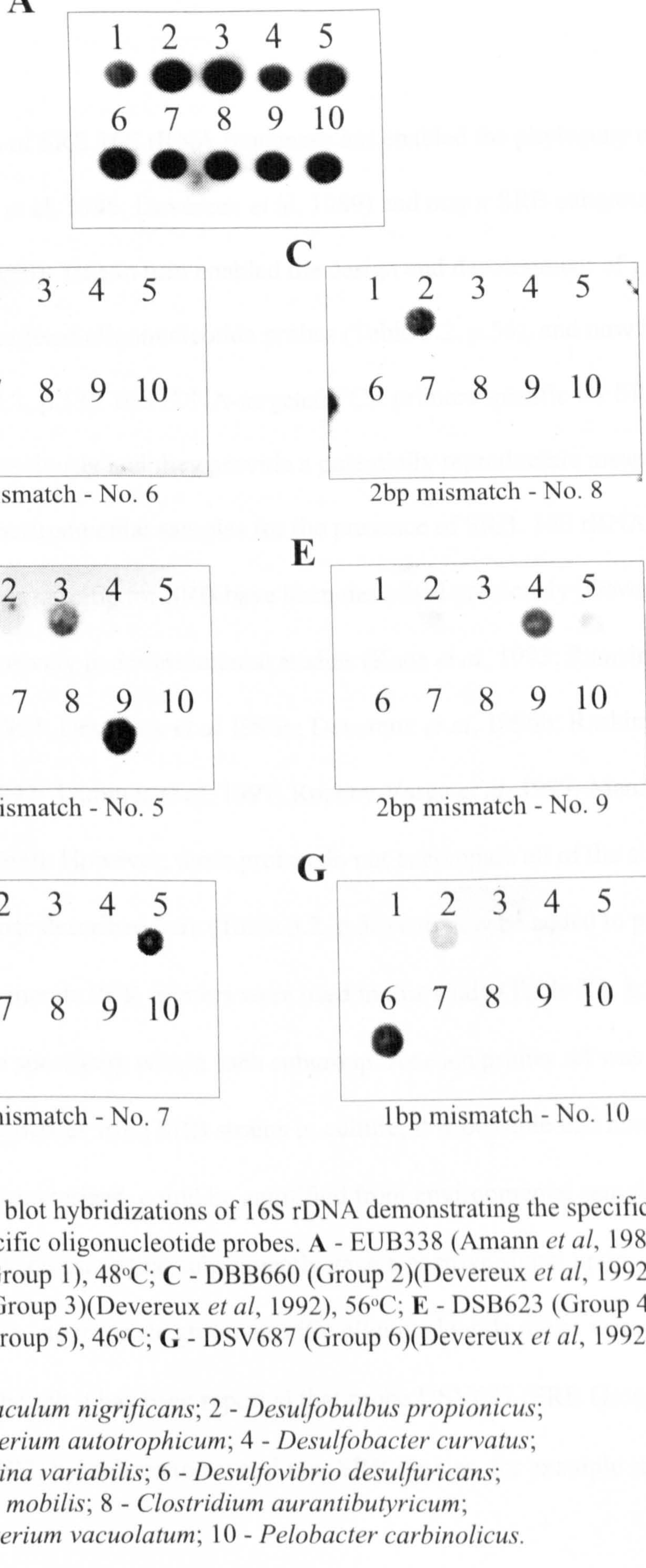


Figure 3.3. Dot blot hybridizations of 16S rDNA demonstrating the specificity of SRB group-specific oligonucleotide probes. **A** - EUB338 (Amann *et al*, 1989), 45°C; **B** - DFM228 (Group 1), 48°C; **C** - DBB660 (Group 2)(Devereux *et al*, 1992), 50°C; **D** - DBM221 (Group 3)(Devereux *et al*, 1992), 56°C; **E** - DSB623 (Group 4), 56°C; **F** - DCC868 (Group 5), 46°C; **G** - DSV687 (Group 6)(Devereux *et al*, 1992), 45°C.

1 - *Desulfotomaculum nigrificans*; 2 - *Desulfobulbus propionicus*;
3 - *Desulfobacterium autotrophicum*; 4 - *Desulfobacter curvatus*;
5 - *Desulfosarcina variabilis*; 6 - *Desulfovibrio desulfuricans*;
7 - *Zymomonas mobilis*; 8 - *Clostridium aurantibutyricum*;
9 - *Desulfobacterium vacuolatum*; 10 - *Pelobacter carbinolicus*.

3.3. Discussion

Comparative analysis of SRB 16S rRNA sequences has enabled the phylogeny of SRB to be described (Fowler *et* al, 1986; Devereux *et* al, 1989) and major SRB subgroups to be identified (Fig. 3.1, p.50). This in turn enabled the design and development of group-specific 16S rDNA-targeted oligonucleotide probes (Table 3.2, p.56), and now PCR primers also (Table 3.1, p.53). 16S rDNA-targeted PCR primers specific for SRB have not been described previously and they provide a potentially reproducible means of routinely screening environmental samples for the presence of SRB. 16S rRNA-targeted oligonucleotide probes specific for SRB have been described previously (Devereux *et al*, 1992) and used extensively in environmental studies (Kane *et al*, 1993; Ramsing *et al*, 1993; Risatti *et al*, 1994; Devereux *et al* 1996a; Devereux *et al*, 1996b; Raskin *et al*, 1996; Purdy *et al*, 1997; Trimmer *et al*, 1997; Rooney-Varga *et al*, 1997; Manz *et al*, 1998; Sahm *et al*, 1999). However, these probes do not encompass all of the six main SRB groups, and those described here (Table 3.2, p.56) can now be added to provide a complete suite. Degenerate PCR primers were used in this study (Table 3.1, p.53) in order to broaden the specificity within each subgroup. As each primer set was designed using 16S rRNA sequences from SRB strains in culture, it is possible that non-target species, as yet uncharacterized, could be amplified from environmental samples. Therefore, only PCR products that subsequently gave a positive signal upon hybridization with the appropriate group-specific oligonucleotide probe were recorded as SRB positives. Although it has been reported that probe DSV687 (SRB Group 6) (Devereux *et al*, 1992) hybridizes to several non-SRB species, for example some

members of the family *Geobacteriaceae* (Lonergan *et al*, 1996), the DSV (Group 6)-specific PCR primer sequences used in this study do not occur in these non-SRB species. Consequently, application of the DSV-DMB (Group 6)-specific primers described here with confirmation by hybridization to probe DSV687 provides firm evidence that these SRB are present. Theoretical cross-specificity analysis of the primers and probes designed for this thesis (sections 3.2.2 & 3.2.4) indicated that primer-probe combinations would provide highly specific molecular tools for unequivocal detection of each of the six SRB subgroups in environmental samples. This was confirmed experimentally by probing specifically amplified DNA from strains representing each of the six SRB subgroups alongside non-SRB strains with 1, 2 or 3 bp mismatches in the oligonucleotide probe target region, providing further confidence in the data on SRB subgroup detection in the landfill leachate samples.

CHAPTER 4. Detection and Identification of SRB 16S rDNA in Landfill Sites.

4.1. Introduction

Direct analysis of microbial community structure in natural environments, without the need for prior cultivation, is now possible through the amplification of specific DNA targets using the Polymerase Chain Reaction (Giovannoni *et al*, 1990; Schmidt *et al*, 1991; Amann *et al*, 1995). As molecular biological techniques, such as PCR, are applied directly to DNA extracted from environmental samples, they can provide information on the ecological importance of different bacterial communities *in situ*, thus eliminating the biases caused by cultivation.

The Polymerase Chain Reaction has been used to study several bacterial communities in environmental samples, for example ammonia-oxidizing bacteria (Hiorns *et al*, 1995), methanogenic bacteria (Hales *et al*, 1996), coliform bacteria (Bej *et al*, 1990) and hydrocarbon-degrading bacteria (Erb and Wagner-Dobler, 1993). However, there are no published studies concerning the direct extraction and amplification of specific SRB DNA targets from environmental samples using PCR. Coupled with specific oligonucleotide hybridization to further confirm the identity of specifically amplified DNA, PCR represents a powerful and highly specific means of investigating the community structure and ecological importance of SRB in natural environments.

Landfill sites are essentially bioreactors in which anaerobic bacterial communities mediate the mineralization and stabilization of organic matter (Barlaz, 1997). The SRB are important members of landfill communities because they have the potential to compete with methanogenic bacteria for available electron donors (Schonheit *et al*, 1982;

Robinson and Tiedje, 1984) resulting in decreased methane production and souring of the landfill site (Gurijala & Suflita, 1993; Harvey *et al*, 1997). The key issue in the control of microbially induced souring is the early detection of the SRB populations that are responsible (Harvey *et* al, 1997). Data on their occurrence and distribution could ultimately enable the development of detection protocols that can be used to monitor the microbiology of landfill sites in order to provide information for site management. For example, molecular biological methods could give SRB population profiles that provide an early warning of interference with methanogenesis by sulfate reduction.

This chapter describes the isolation and specific PCR amplification of SRB 16S rDNA from landfill leachate in order to provide the first insight into SRB occurrence and distribution in landfill sites.

4.2. Results

4.2.1. Extraction of DNA from landfill leachate samples

DNA was extracted from samples of landfill leachate as described in section 2.4. DNA recovery, purity and yield were indicated by agarose gel electrophoresis (section 2.7) (Fig. 4.1, p.65).

4.2.2. Eubacterial PCR amplification of 16S rDNA from landfill leachate

PCR amplification of 16S rDNA extracted from landfill leachate was performed with eubacterial-specific primers pA & pH' (Edwards *et al*, 1989). PCR reactions were

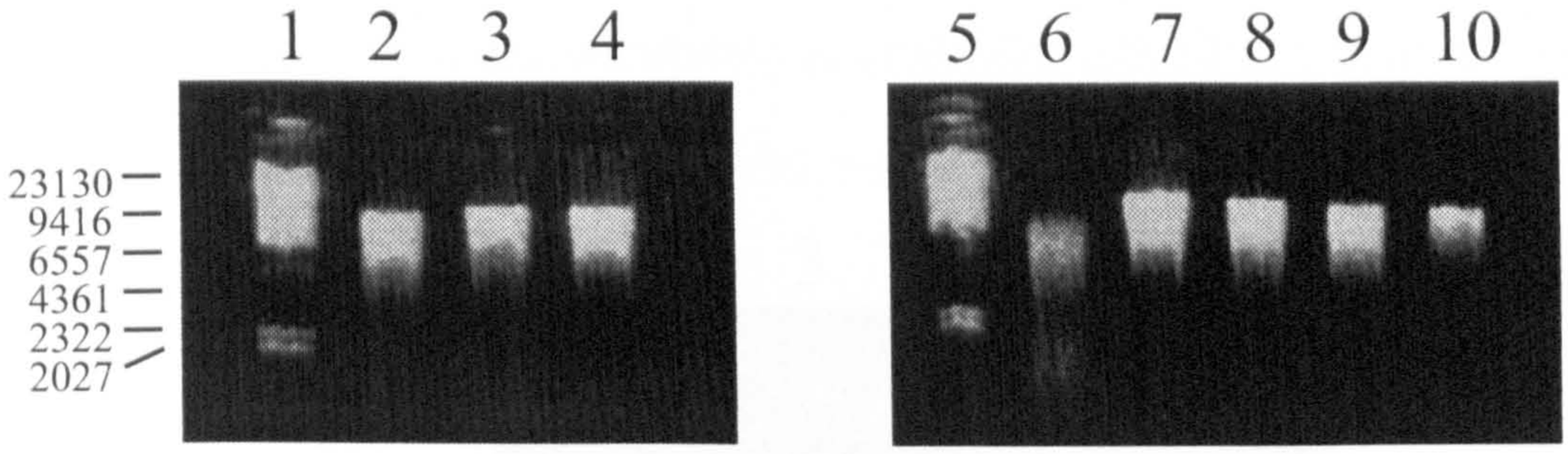


Figure 4.1. Agarose gel electrophoresis of DNA extracted from landfill leachate using the Bio101 FastDNA Spin kit (Bio101, Inc.).

Lane 1 - *lambda* DNA/*Hin*dIII (MBI Fermentas); Lane 2 - Pilsworth; Lane 3 - Butchersfield [97]; Lane 4 - Buff Quarry; Lane 5 - *lambda* DNA/*Hin*dIII (MBI Fermentas); Lane 6 - Risley; Lane 7 - Chadderton; Lane 8 - Holiday Moss; Lane 9 - West Leigh; Lane 10 - Butchersfield [98]

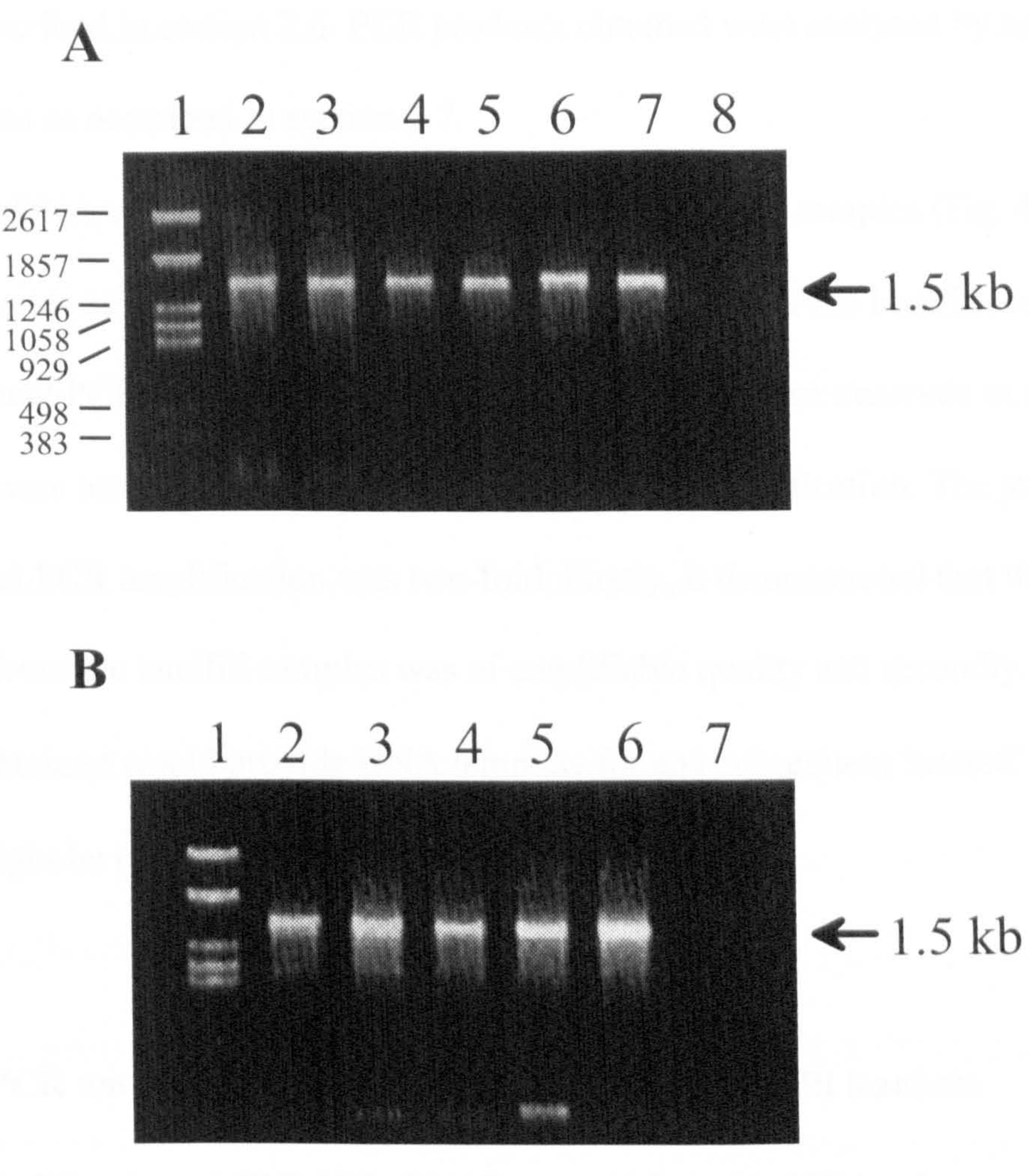


Figure 4.2. PCR amplification of 16S rDNA extracted from landfill leachate using eubacterial primers pA & pH' (Edwards *et al*, 1989).
Annealing Temp. 45°C.

A
Lane 1 - pBR322 DNA/*Alw*441/*Mva*1;
Lanes 2&3 - Pilsworth;
Lanes 4&5 - Butchersfield [97];
Lanes 6&7 - Buff Quarry;
Lane 8 - PCR negative control

B
Lane 1 - pBR322 DNA/*Alw*441/*Mva*1;
Lane 2 - Risley;
Lane 3 - Chadderton;
Lane 4 - Holiday Moss;
Lane 5 - West Leigh;
Lane 6 - Butchersfield [98];
Lane 7 - PCR negative control

carried out as described in section 2.6. PCR products obtained were analysed by agarose gel electrophoresis as described in section 2.7.

Eubacterial 16S rDNA was successfully amplified from all landfill samples (Fig. 4.2, p.66). Discrete bands of the expected size were obtained for each of the landfill leachate samples. Additional PCR bands smaller than expected size were also observed in some samples. These were assumed to be products of non-specific amplification. The purpose of this eubacterial PCR amplification was two-fold. Firstly, it demonstrated that the DNA extracted from the landfill samples was of amplifiable quality and secondly, the PCR products obtained would provide DNA template for any subsequent 'nested' PCR reactions that might be required (section 2.6.2).

4.2.3. 'Direct' PCR amplification of SRB 16S rDNA from landfill leachate

'Direct' PCR amplification of SRB 16S rDNA extracted from landfill leachate was performed with primers specific for each of the six main subgroups of SRB (Table 3.1, p.53). PCR reactions were carried out as described in section 2.6. PCR products obtained were subjected to agarose gel electrophoresis (section 2.7) and then transferred to positively-charged nylon membrane by Southern blotting (section 2.8.2). DNA fixed to membranes was then hybridized (section 2.9) against the appropriate group-specific oligonucleotide probe (Table 3.2, p.56). The primers are degenerate and although they may amplify non-SRB DNA, the presence of SRB 16S rDNA was confirmed by Southern blotting. Therefore, only PCR products that subsequently gave a positive signal upon hybridization were recorded as SRB positives as described in section 3.3.

***Desulfotomaculum* (DFM Group 1)**: amplification products of the expected size (700 bp) were obtained from three of the seven landfill sites (Pilsworth, Buff Quarry & Chadderton) and shown to contain the target 16S rDNA by hybridization against probe DFM228 (Figs. 4.3 & 4.4, p.69 & 70). Faint bands smaller than the expected size were also obtained for some samples. These products did not hybridize to the DFM228 probe and are therefore not SRB 16S rDNA.

***Desulfobulbus* (DBB Group 2)**: amplification products were not obtained from any of the landfill sites using this 'direct' PCR approach (Figs. 4.5 & 4.6, p.71 & 72).

***Desulfobacterium* (DBM Group 3)**: amplification products were not obtained from any of the landfill sites using this 'direct' PCR approach (Figs. 4.7 & 4.8, p.73 & 74).

***Desulfobacter* (DSB Group 4)**: amplification products of the expected size (1150 bp) were obtained from two landfill sites (Pilsworth & Butchersfield [97 & 98]) and these hybridized to probe DSB623 (Figs. 4.9 & 4.10, p.75 & 76). Additional PCR products of approximately 100-150bp were obtained from the Pilsworth landfill site using the DSB (Group 4)-specific primers. However, these products did not give a positive signal upon hybridization against probe DSB623 and so were thought to be a product of non-specific amplification.

***Desulfococcus-Desulfonema-Desulfosarcina* (DCC-DNM-DSS Group 5)**:

amplification products of the expected size (860 bp) were obtained from four of the seven landfill sites (Pilsworth, Butchersfield [97], Chadderton & West Leigh), confirmed by hybridization against probe DCC868 (Figs. 4.11 & 4.12, p.78 & 79). Although PCR products of the expected size were obtained for Butchersfield [98], and no visible PCR products obtained for Holiday Moss, extremely faint signals could be discerned for these

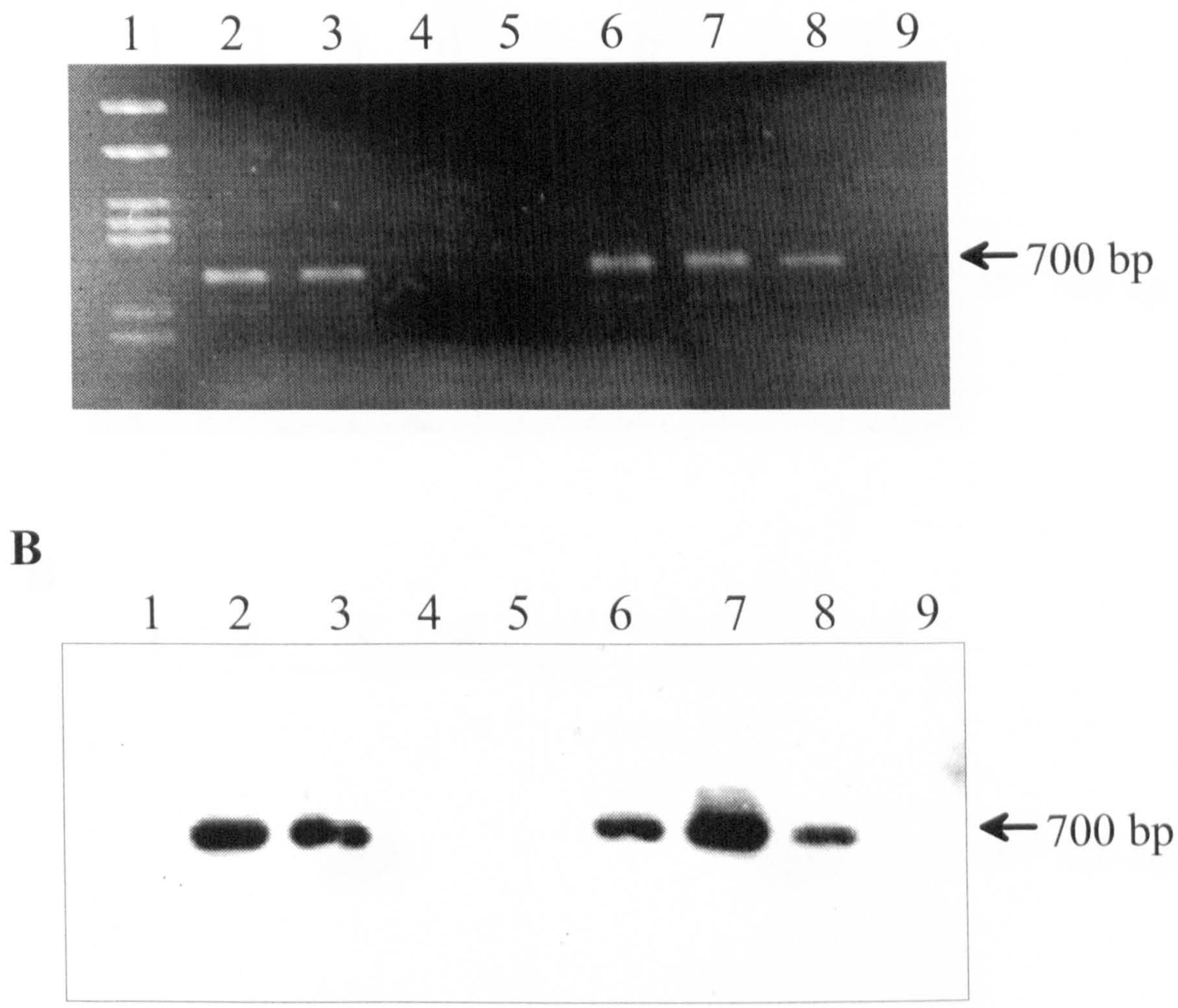
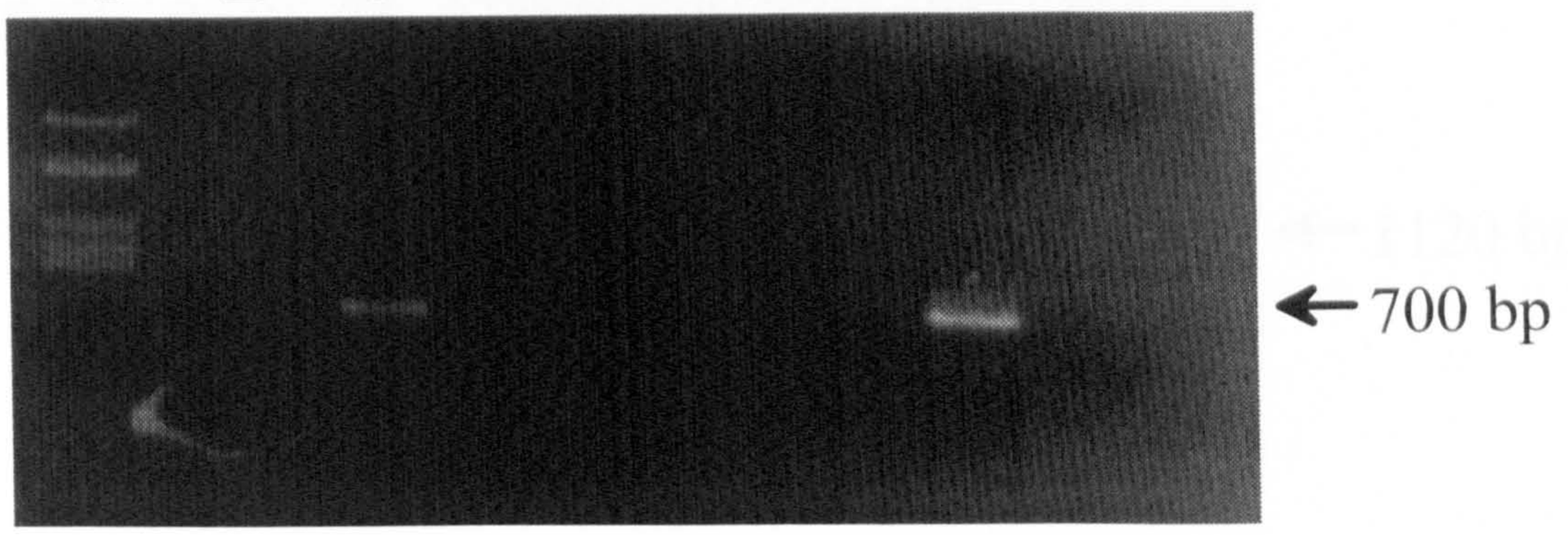


Figure 4.3. A - 'Direct' PCR amplification of SRB 16S rDNA extracted from landfill leachate using primers DFM140 & DFM842 (Group 1); **B** - Southern blot hybridized against probe DFM228 (Group 1).

Lane 1 - pBR322 DNA/*Alw*441/*Mva*1(MBI Fermentas); Lanes 2&3 - Pilsworth;
Lanes 4&5 - Butchersfield [97]; Lanes 6&7 - Buff Quarry;
Lane 8 - *Dfm. nigrificans*; Lane 9 - PCR negative control.

A



B

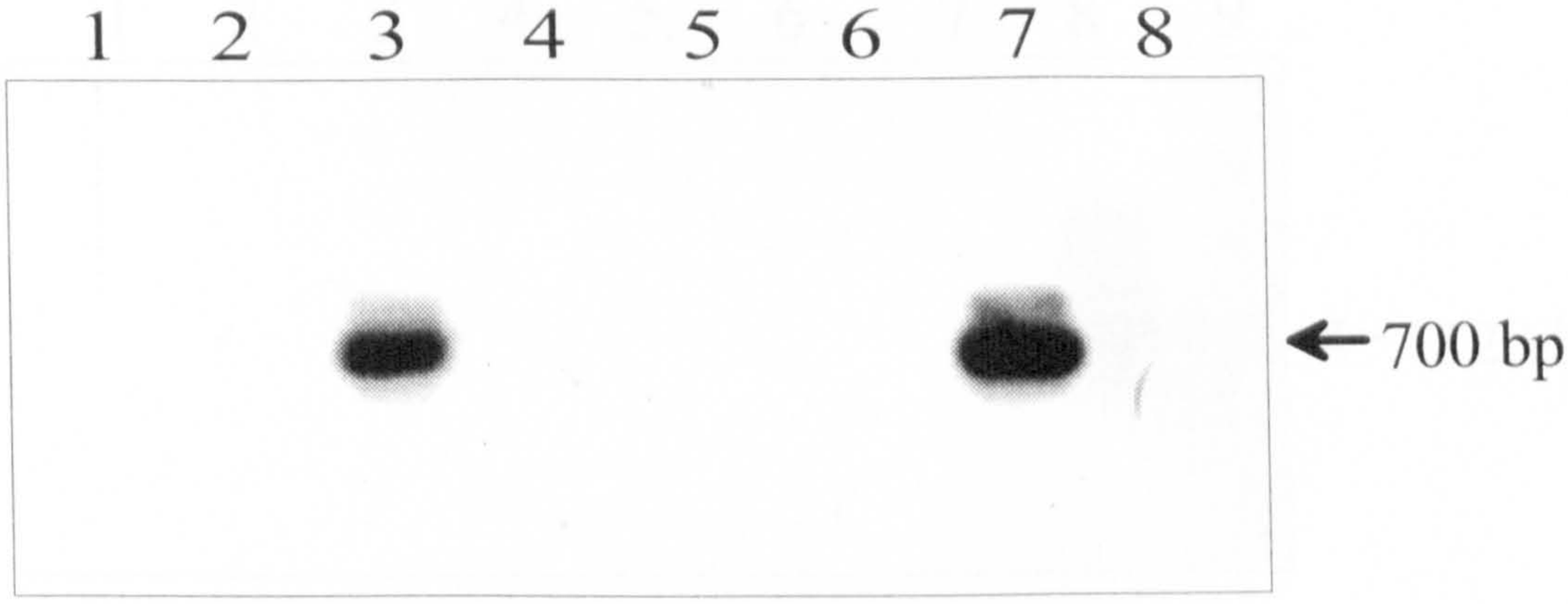


Figure 4.4. A - 'Direct' PCR amplification of SRB 16S rDNA extracted from landfill leachate using primers DFM140 & DFM842 (Group 1); **B -** Southern blot hybridized against probe DFM228 (Group 1).

Lane 1 - pBR322 DNA/*Alw*441/*Mva*1(MBI Fermentas); Lane 2 - Risley; Lane 3 - Chadderton; Lane 4 - Holiday Moss; Lane 5 - West Leigh; Lane 6 - Butchersfield [98]; Lane 7 - *Dfm. nigrificans*; Lane 8 - PCR negative control.

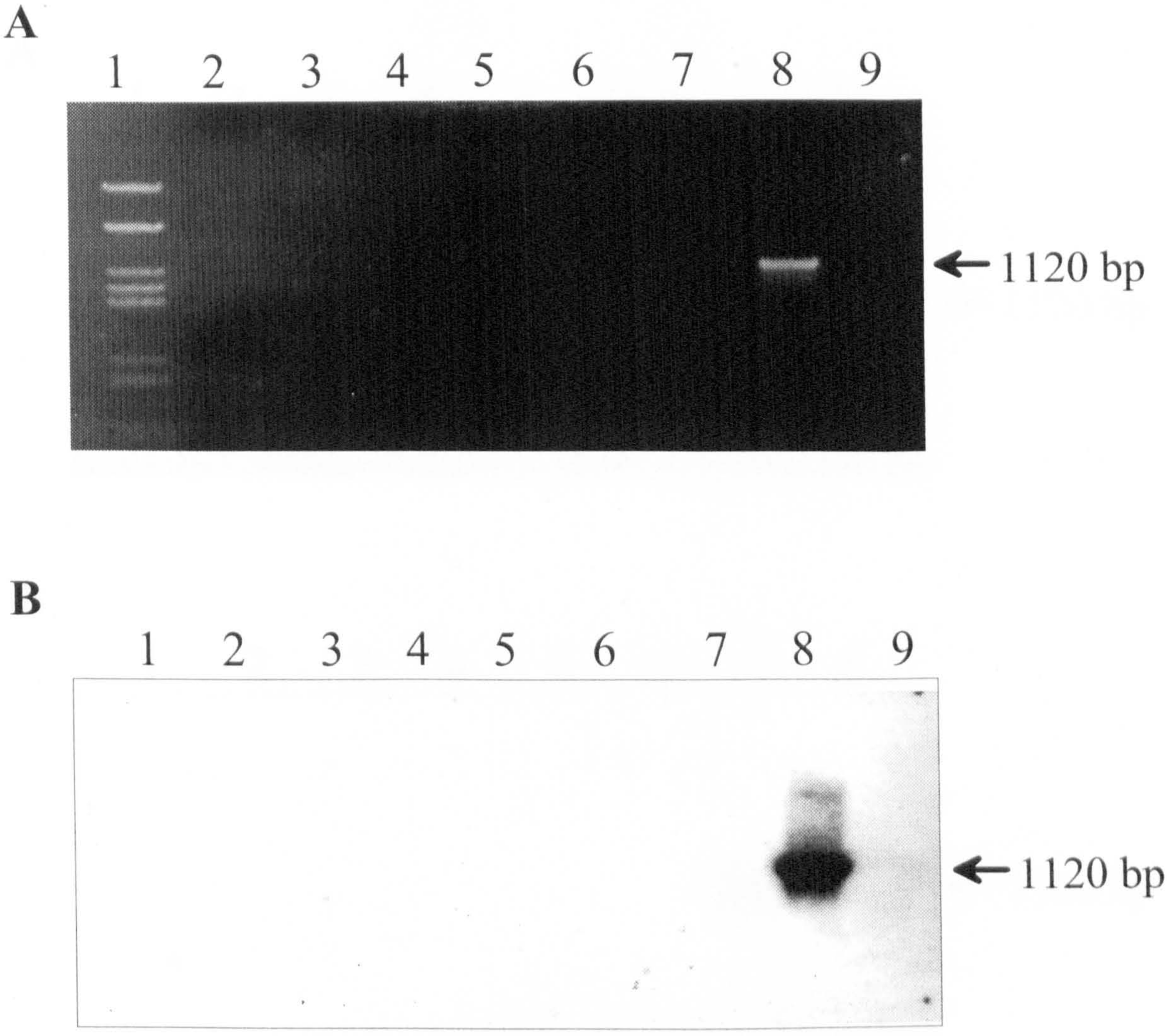


Figure 4.5. A - 'Direct' PCR amplification of SRB 16S rDNA extracted from landfill leachate using primers DBB121 & DBB1237 (Group 2); **B** - Southern blot hybridized against probe DBB660 (Group 2).

Lane 1 - pBR322 DNA/*Alw*441/*Mva*1(MBI Fermentas); Lanes 2&3 - Pilsworth; Lanes 4&5 - Butchersfield [97]; Lanes 6&7 - Buff Quarry;
Lane 8 - *Dbb. propionicus*; Lane 9 - PCR negative control.

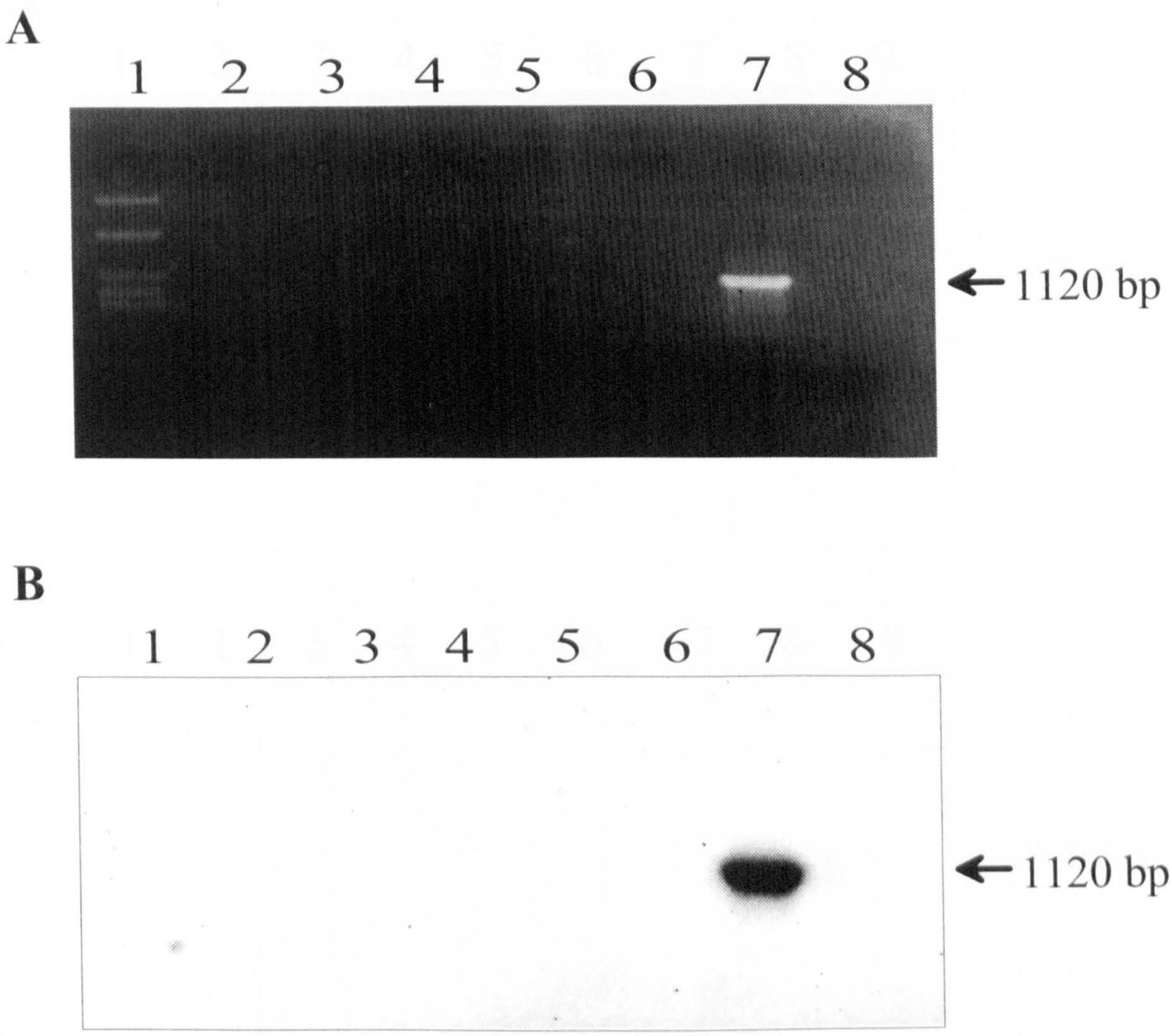


Figure 4.6. A - 'Direct' PCR amplification of SRB 16S rDNA extracted from landfill leachate using primers DBB121 & DBB1237 (Group 2); **B** - Southern blot hybridized against probe DBB660 (Group 2).

Lane 1 - pBR322 DNA/*Alw*441/*Mva*1(MBI Fermentas); Lane 2 - Risley;
Lane 3 - Chadderton; Lane 4 - Holiday Moss; Lane 5 - West Leigh;
Lane 6 - Butchersfield [98]; Lane 7 - *Dbb. propionicus*; Lane 8 - PCR negative control.

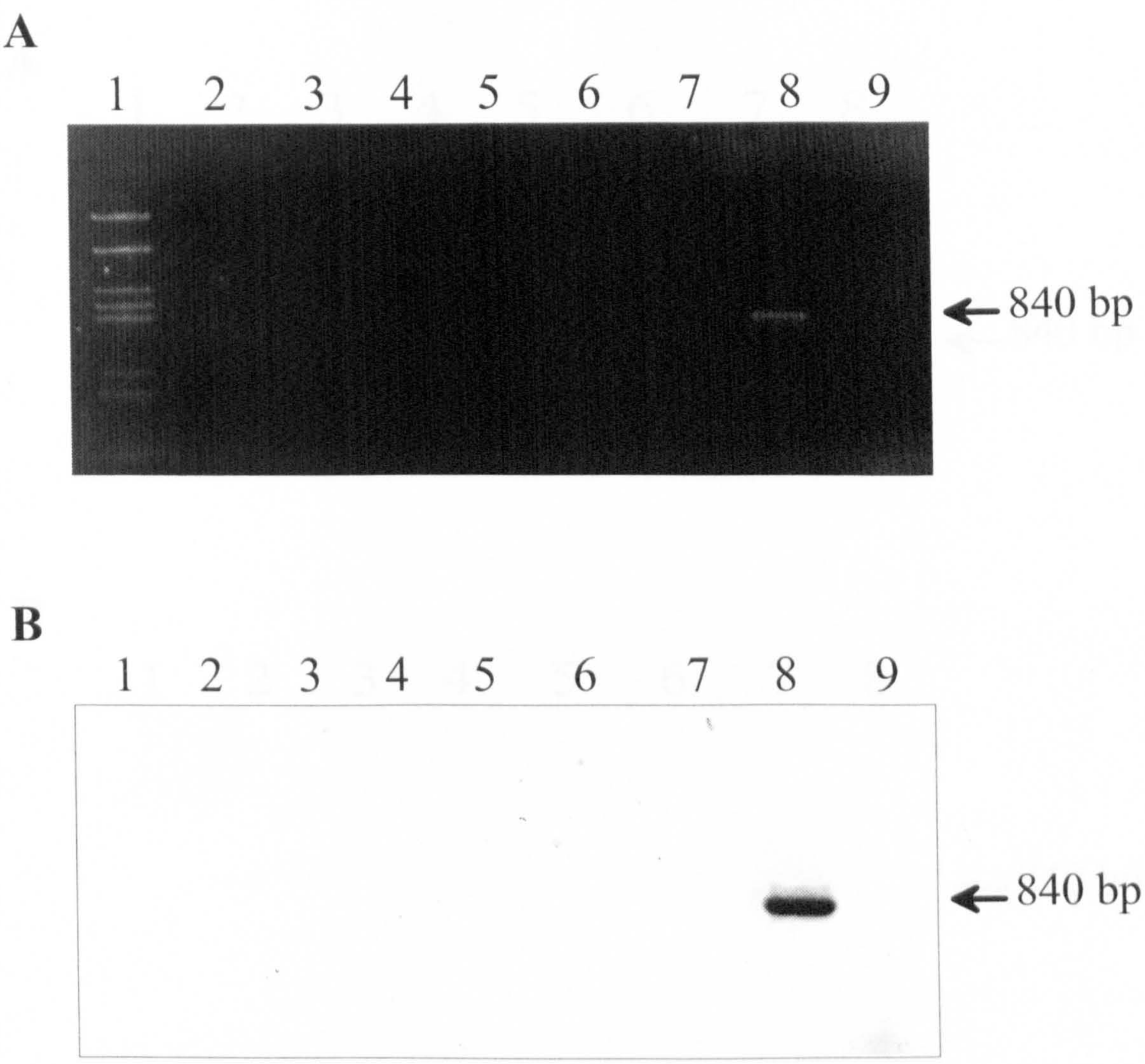


Figure 4.7. A - 'Direct' PCR amplification of SRB 16S rDNA extracted from landfill leachate using primers DBM169 & DBM1006 (Group 3); **B** - Southern blot hybridized against probe DBM221 (Group 3).

Lane 1 - pBR322 DNA/*Alw*441/*Mva*1(MBI Fermentas); Lanes 2&3 - Pilsworth;
Lanes 4&5 - Butchersfield [97]; Lanes 6&7 - Buff Quarry;
Lane 8 - *Dbm. autotrophicum*; Lane 9 - PCR negative control.

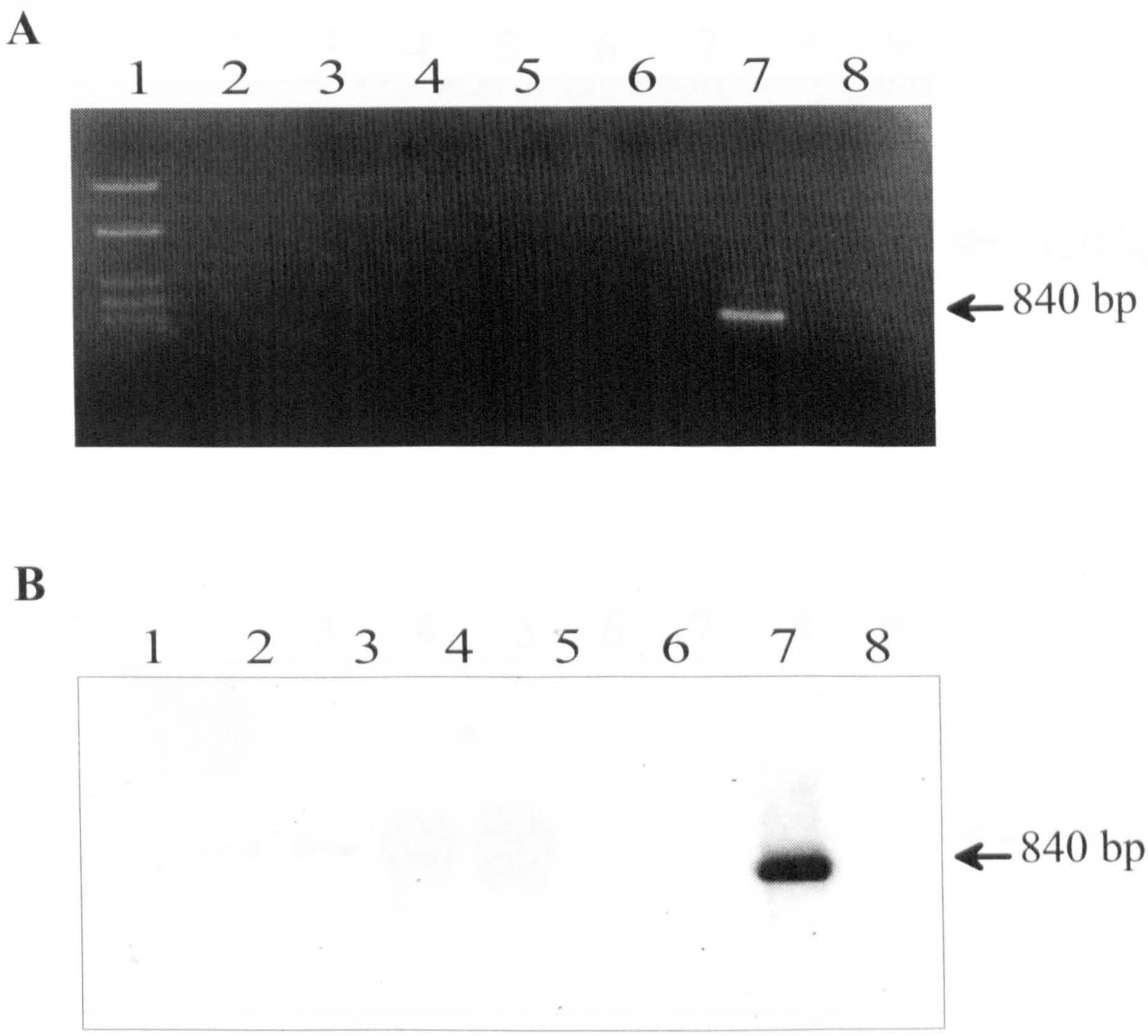


Figure 4.8. A - 'Direct' PCR amplification of SRB 16S rDNA extracted from landfill leachate using primers DBM169 & DBM1006 (Group 3); **B** - Southern blot hybridized against probe DBM221(Group 3).

Lane 1 - pBR322 DNA/*Alw*441/*Mva*1(MBI Fermentas); Lane 2 - Risley; Lane 3 - Chadderton; Lane 4 - Holiday Moss; Lane 5 - West Leigh; Lane 6 - Butchersfield [98]; Lane 7 - *Dbm. autotrophicum*; Lane 8 - PCR negative control.

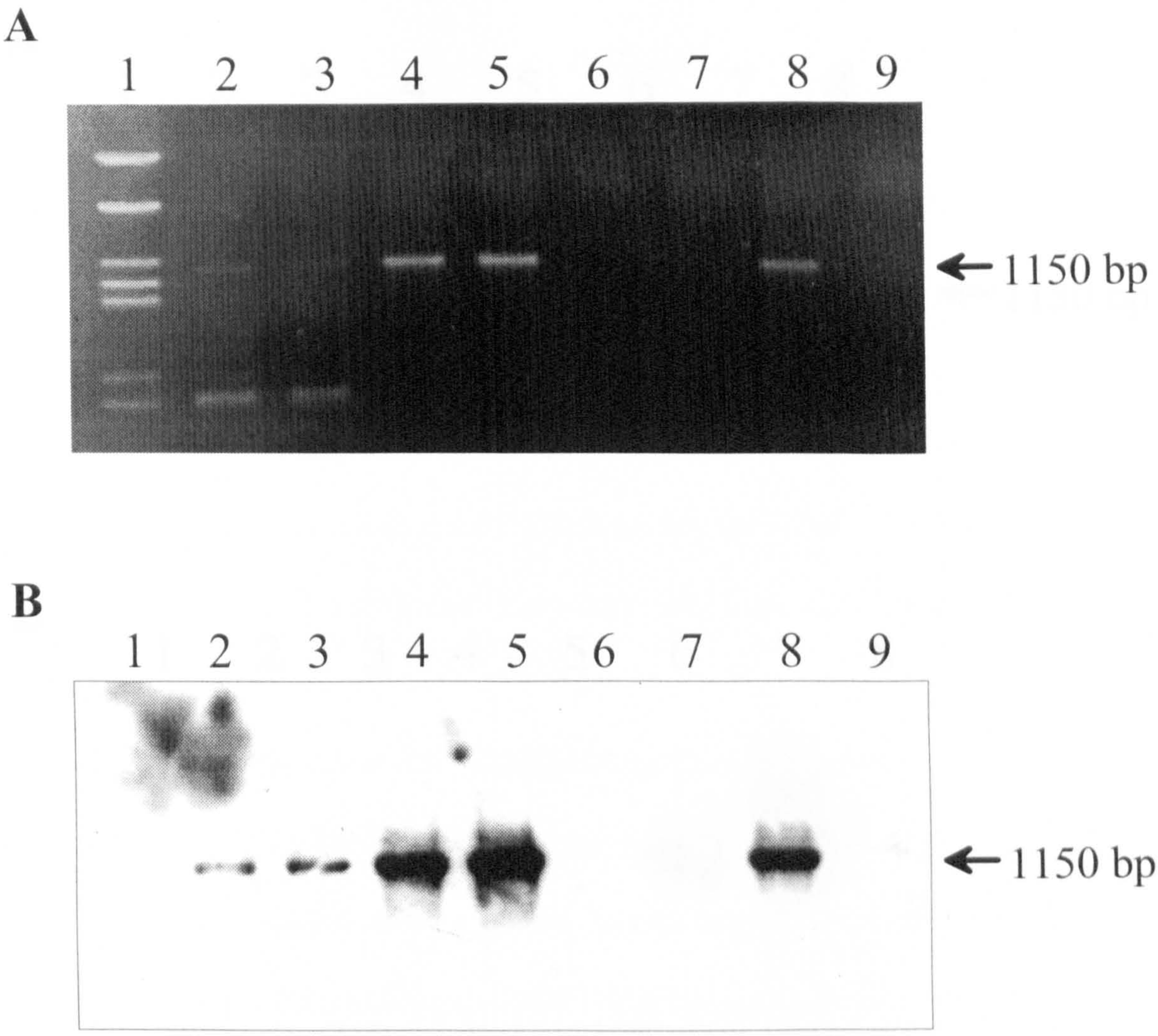


Figure 4.9. A - 'Direct' PCR amplification of SRB 16S rDNA extracted from landfill leachate using primers DSB127 & DSB1273 (Group 4); **B** - Southern blot hybridized against probe DSB623 (Group 4).

Lane 1 - pBR322 DNA/*Alw*441/*Mva*1(MBI Fermentas); Lanes 2&3 - Pilsworth; Lanes 4&5 - Butchersfield [97]; Lanes 6&7 - Buff Quarry;
Lane 8 - *Dsb. curvatus*; Lane 9 - PCR negative control.

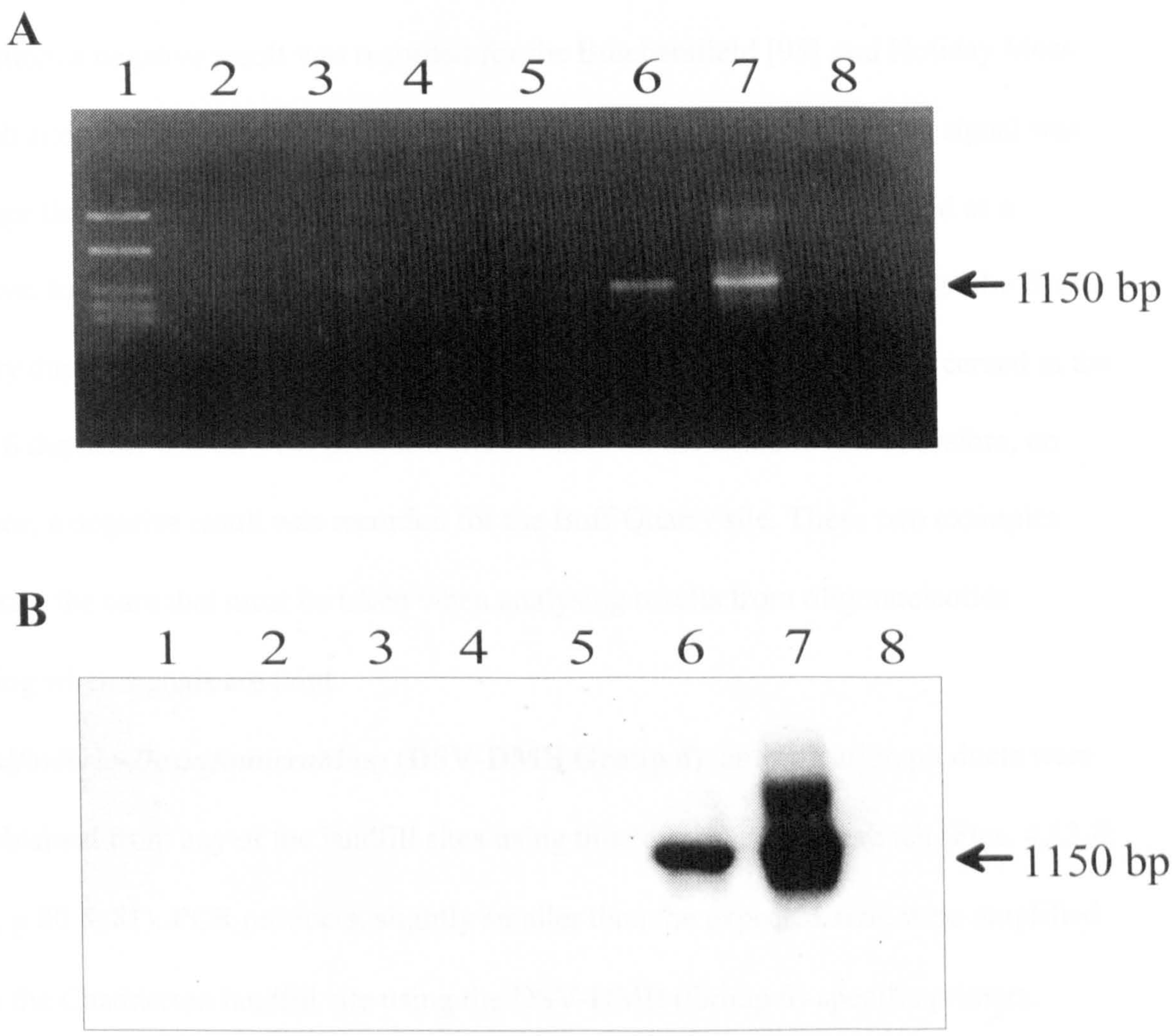


Figure 4.10. A - 'Direct' PCR amplification of SRB 16S rDNA extracted from landfill leachate using primers DSB127 & DSB1273 (Group 4); **B** - Southern blot hybridized against probe DSB623 (Group 4).

Lane 1 - pBR322 DNA/*Alw*441/*Mva*1(MBI Fermentas); Lane 2 - Risley; Lane 3 - Chadderton; Lane 4 - Holiday Moss; Lane 5 - West Leigh; Lane 6 - Butchersfield [98]; Lane 7 - *Dsb. curvatus*; Lane 8 - PCR negative control.

two landfill sites upon hybridization against probe DCC868. However, erring on the side of caution, a negative result was recorded for the Butchersfield [98] and Holiday Moss landfill sites. Chadderton also gave a faint signal upon hybridization but the signal was stronger than for either Holiday Moss or Butchersfield [98] and was recorded as a positive. In addition, a faint hybridization signal could be discerned for one of the Buff Quarry duplicates (Lane 7, Fig. 4.11, p.78). However, no signal could be discerned in the Lane 6 duplicate and no PCR products were visible on the agarose gel. Therefore, on balance, a negative result was recorded for the Buff Quarry site. These two examples illustrate the care that must be taken when analysing results from oligonucleotide probing when signals are faint.

Desulfovibrio-Desulfomicrobium **(DSV-DMB Group 6)**: amplification products were not obtained from any of the landfill sites using this 'direct' PCR approach (Figs. 4.13 & 4.14, p.80 & 81). PCR products, slightly smaller than the expected size, were amplified from the Chadderton landfill site using the DSV-DMB (Group 6)-specific primers. However, these products did not give a positive signal upon hybridization against probe DSV687 and therefore a negative result was recorded for the Chadderton landfill site.

A summary of results for the 'direct' PCR amplification of 16S rDNA extracted from landfill leachate using SRB group-specific primers and hybridization against group-specific oligonucleotide probes is presented in Table 4.1 (p.82).

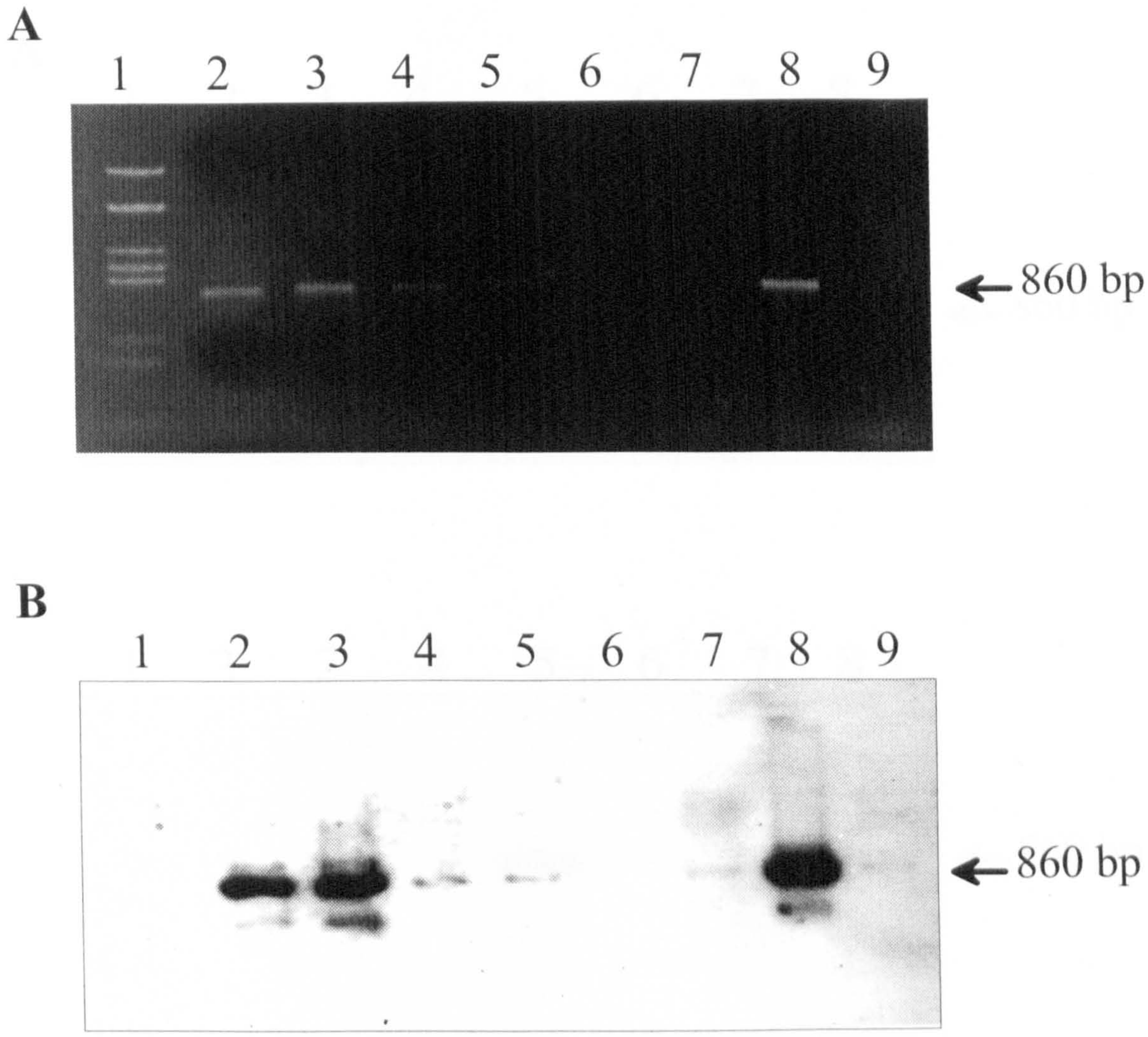


Figure 4.11. A - 'Direct' PCR amplification of SRB 16S rDNA extracted from landfill leachate using primers DCC305 & DCC1165 (Group 5); **B** - Southern blot hybridized against probe DCC868 (Group 5).

Lane 1 - pBR322 DNA/*Alw*441/*Mva*1(MBI Fermentas); Lanes 2&3 - Pilsworth; Lanes 4&5 - Butchersfield [97]; Lanes 6&7 - Buff Quarry;
Lane 8 - *Dss. variabilis*; Lane 9 - PCR negative control.

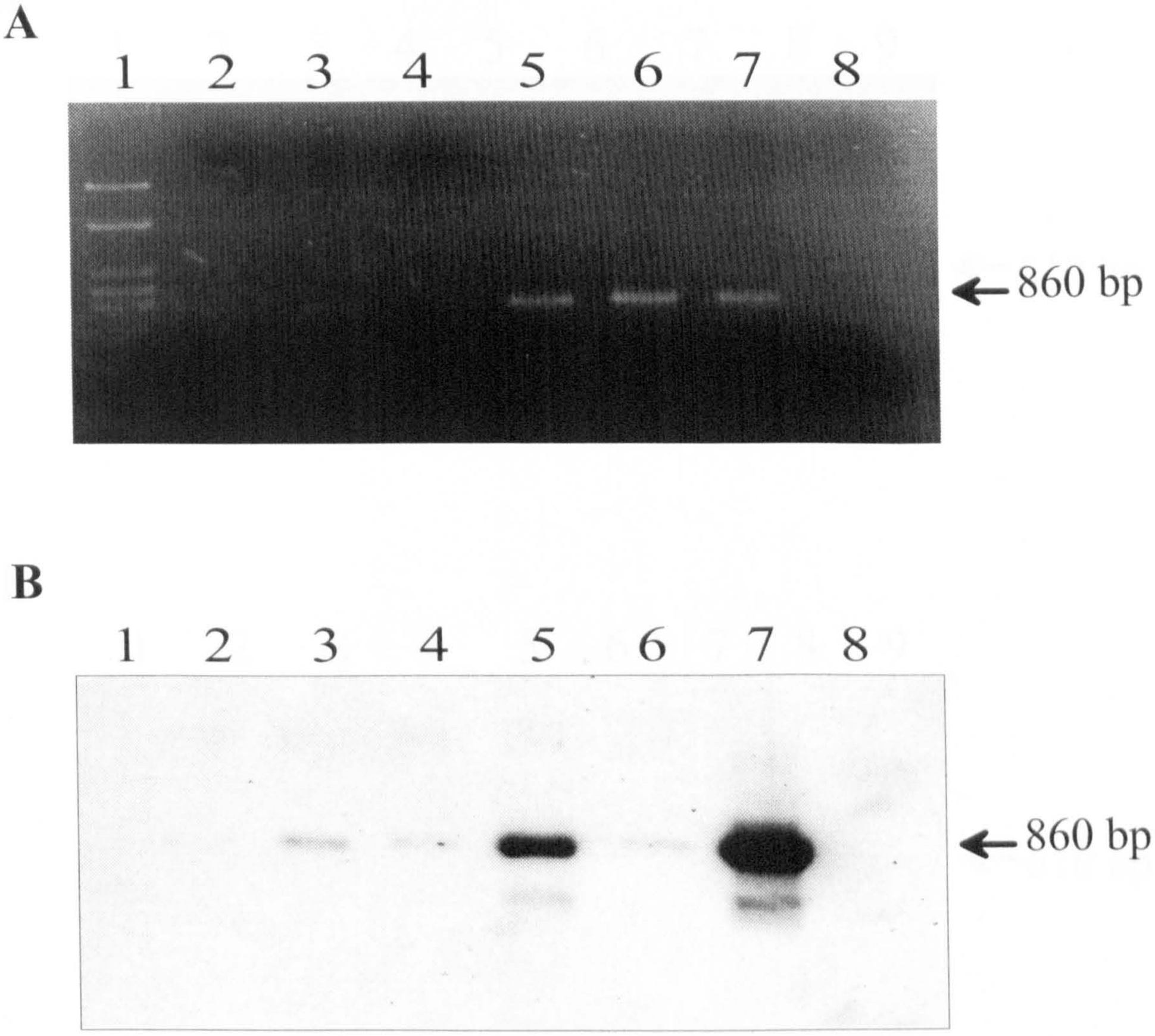


Figure 4.12. A - 'Direct' PCR amplification of SRB 16S rDNA extracted from landfill leachate using primers DCC305 & DCC1165 (Group 5); **B** - Southern blot hybridized against probe DCC868 (Group 5).

Lane 1 - pBR322 DNA/*Alw*441/*Mva*1(MBI Fermentas); Lane 2 - Risley; Lane 3 - Chadderton; Lane 4 - Holiday Moss; Lane 5 - West Leigh; Lane 6 - Butchersfield [98]; Lane 7 - *Dss. variabilis*; Lane 8 - PCR negative control.

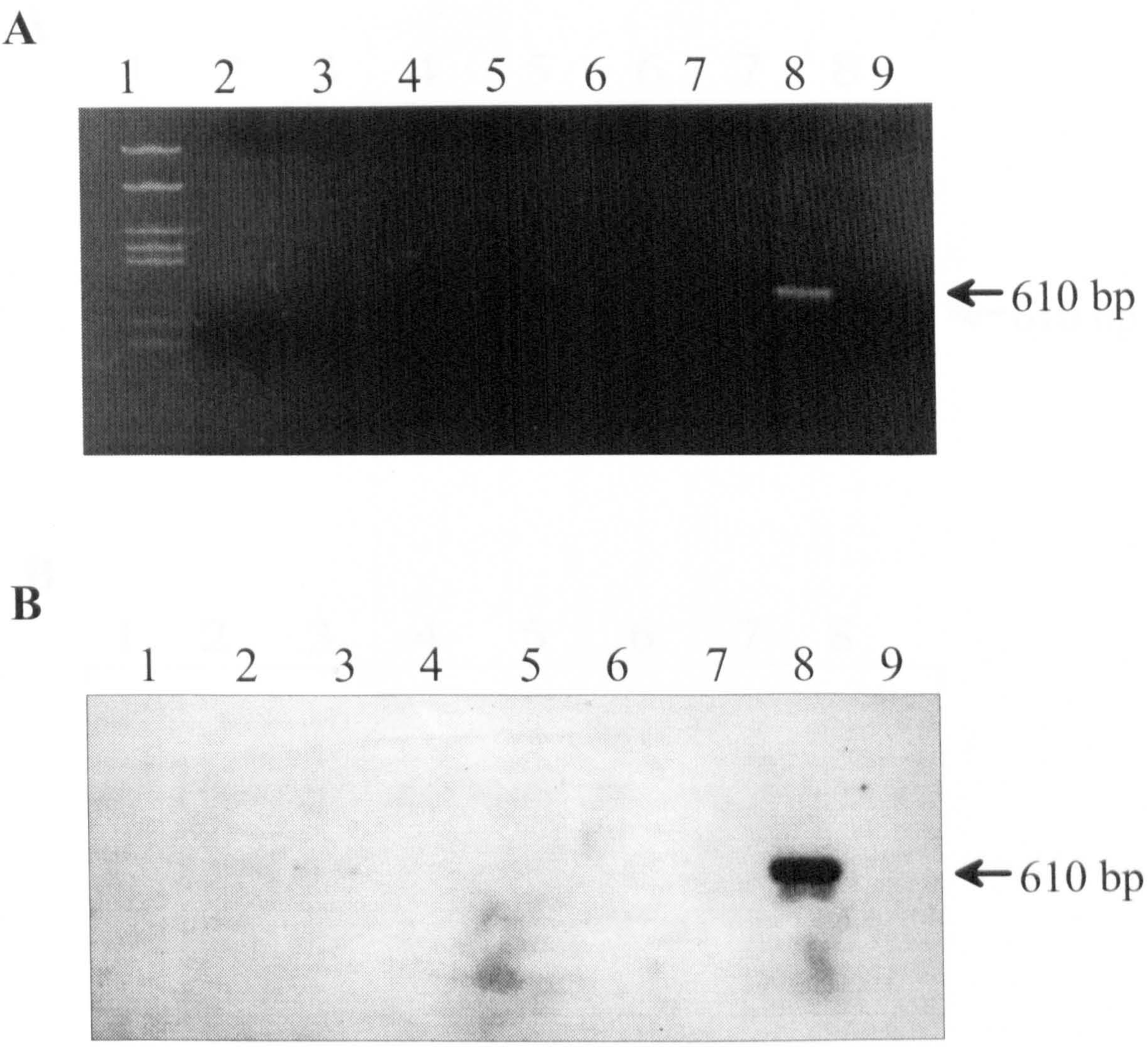
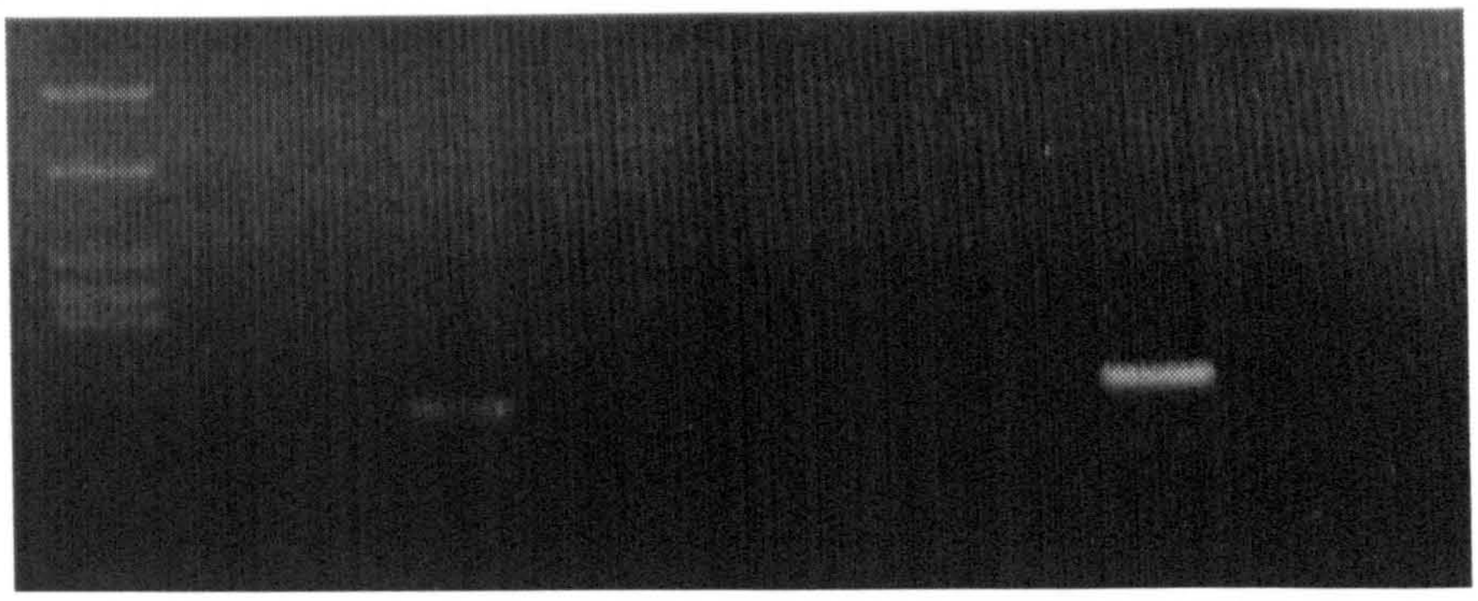


Figure 4.13. A - 'Direct' PCR amplification of SRB 16S rDNA extracted from landfill leachate using primers DSV230 & DSV838 (Group 6); **B** - Southern blot hybridized against probe DSV687 (Group 6).

Lane 1 - pBR322 DNA/*Alw*441/*Mva*1(MBI Fermentas); Lanes 2&3 - Pilsworth; Lanes 4&5 - Butchersfield [97]; Lanes 6&7 - Buff Quarry;
Lane 8 - *Dsv. desulfuricans*; Lane 9 - PCR negative control.

A



B

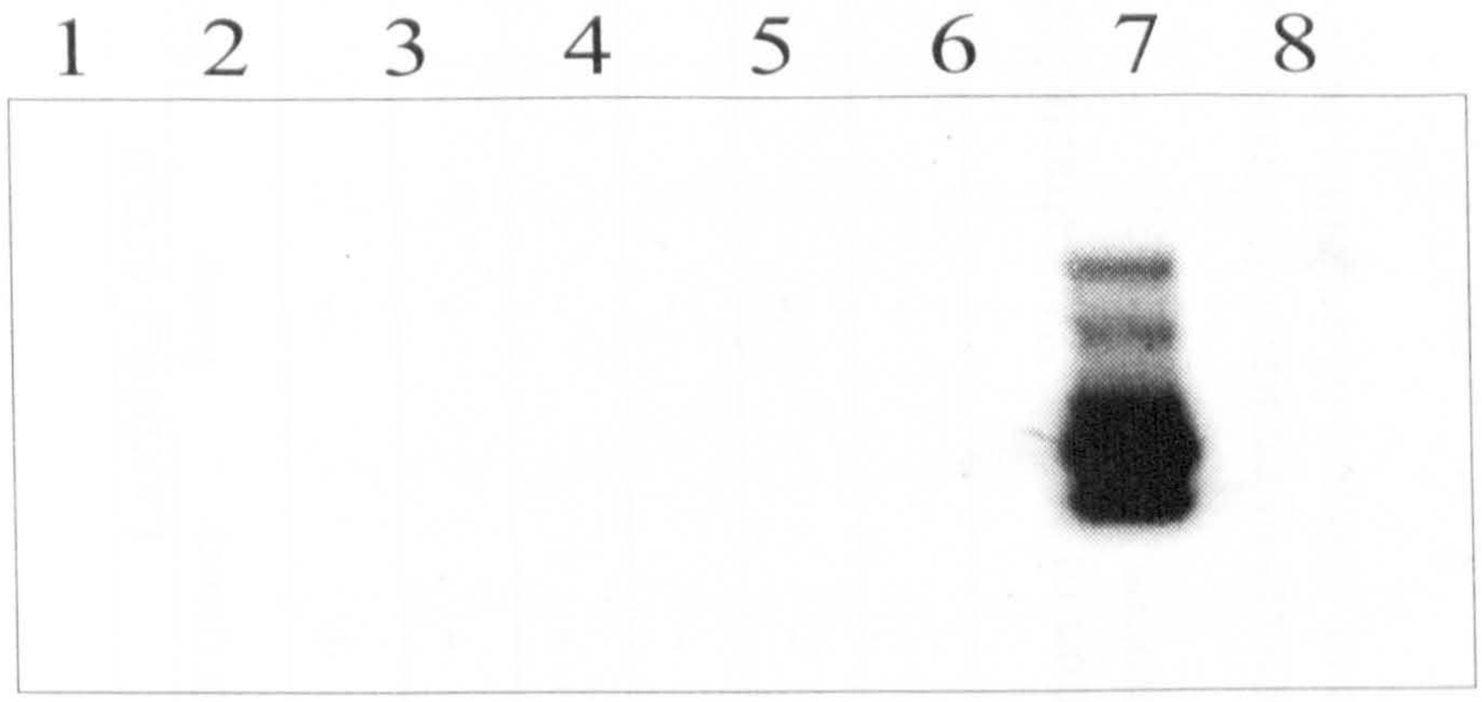


Figure 4.14. A - 'Direct' PCR amplification of SRB 16S rDNA extracted from landfill leachate using primers DSV230 & DSV838 (Group 6); **B** - Southern blot hybridized against probe DSV687 (Group 6).

Lane 1 - pBR322 DNA/*Alw*441/*Mva*1(MBI Fermentas); Lane 2 - Risley; Lane 3 - Chadderton; Lane 4 - Holiday Moss; Lane 5 - West Leigh; Lane 6 - Butchersfield [98]; Lane 7 - *Dsv. desulfuricans*; Lane 8 - PCR negative control.

	LANDFILL SITES							
	Pilsworth	Butchersfield [97]	Buff Quarry	Risley	Chadderton	Holiday Moss	West Leigh	Butchersfield [98]
SRB Group 1 DFM	+	-	+	-	+	-	-	-
SRB Group 2 DBB	-	-	-	-	-	-	-	-
SRB Group 3 DBM	-	-	-	-	-	-	-	-
SRB Group 4 DSB	+	+	-	-	-	-	-	+
SRB Group 5 DCC-DNM-DSS	+	+	-	-	+	-	+	-
SRB Group 6 DSV-DMB	-	-	-	-	-	-	-	-

Table 4.1. Summary of results for 'direct' PCR amplification of 16S rDNA extracted from landfill leachate using SRB group-specific primers and hybridization against group-specific oligonucleotide probes.

+ : indicates a positive signal when amplification products were hybridized against the group-specific oligonucleotide probe
- : indicates a negative hybridization signal in the presence or absence of a visible band of PCR products on an agarose gel

4.2.4. 'Nested' PCR amplification of SRB 16S rDNA from landfill leachate

Eubacterial 16S rDNA PCR products obtained from landfill leachate (section 4.2.2.) were used as DNA templates for 'nested' PCR amplification (section 2.6.2) of SRB 16S rDNA with primers specific for all six main subgroups (Table 3.1, p.53). Analysis of PCR products was as described in section 4.2.3. Multiple bands of bigger than expected size seen in some of the agarose gels with 'nested' PCR is a phenomenon known as 'laddering' and is presumed to be a result of using a high concentration of template DNA containing primers used in the primary amplification.

***Desulfotomaculum* (DFM Group 1)**: amplification products of the expected size (700 bp) were obtained from all seven landfill sites, confirmed by hybridization against probe DFM228 (Figs. 4.15 & 4.16, p.84 & 85).

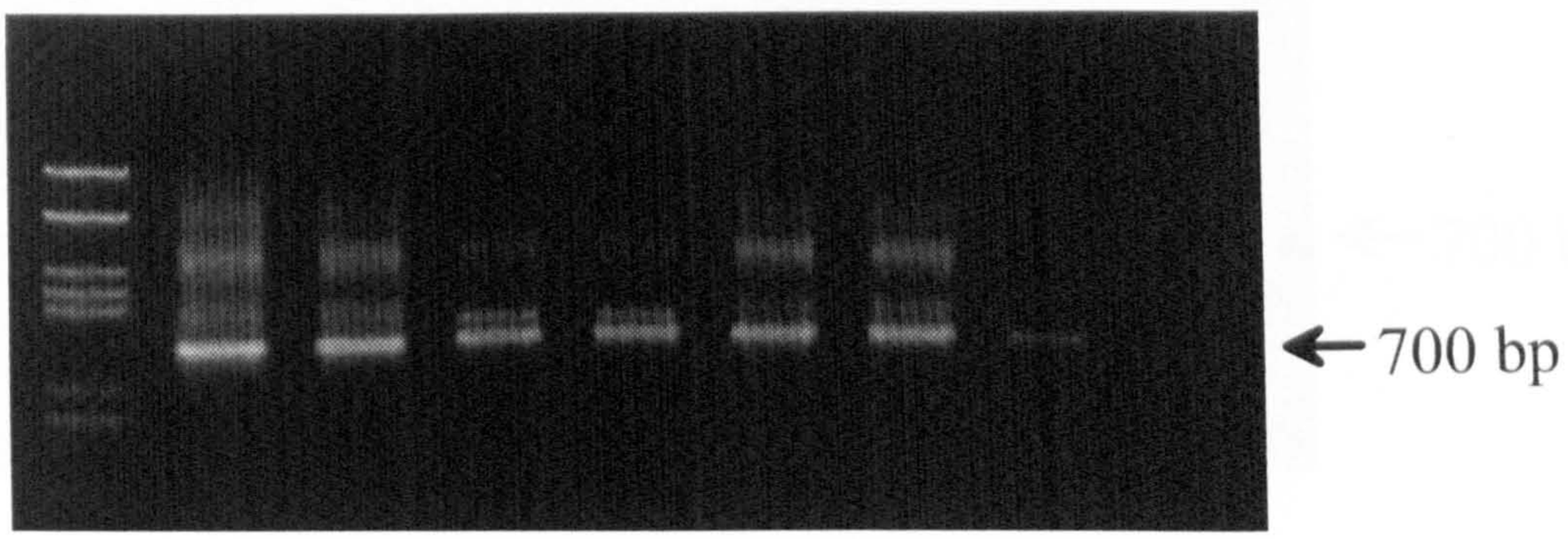
***Desulfobulbus* (DBB Group 2)**: amplification products of the expected size (1120 bp) were obtained from four landfill sites (Pilsworth, Butchersfield [97], Risley & West Leigh) and these hybridized with probe DBB660 (Figs. 4.17 & 4.18, p.86 & 87).

***Desulfobacterium* (DBM Group 3)**: amplification products were not obtained from any of the landfill sites using this 'nested' PCR approach (Figs. 4.19 & 4.20, p.88 & 89).

***Desulfobacter* (DSB Group 4)**: amplification products of the expected size (1150 bp) were obtained from four landfill sites (Pilsworth, Butchersfield [97 & 98], Buff Quarry & West Leigh) with hybridization against probe DSB623 (Figs. 4.21 & 4.22, p.90 & 91).

***Desulfococcus-Desulfonema-Desulfosarcina* (DCC-DNM-DSS Group 5)**: amplification products of the expected size (860 bp) were obtained from six out of seven landfill sites (all except Buff Quarry) confirmed by hybridization against probe DCC868

A



B

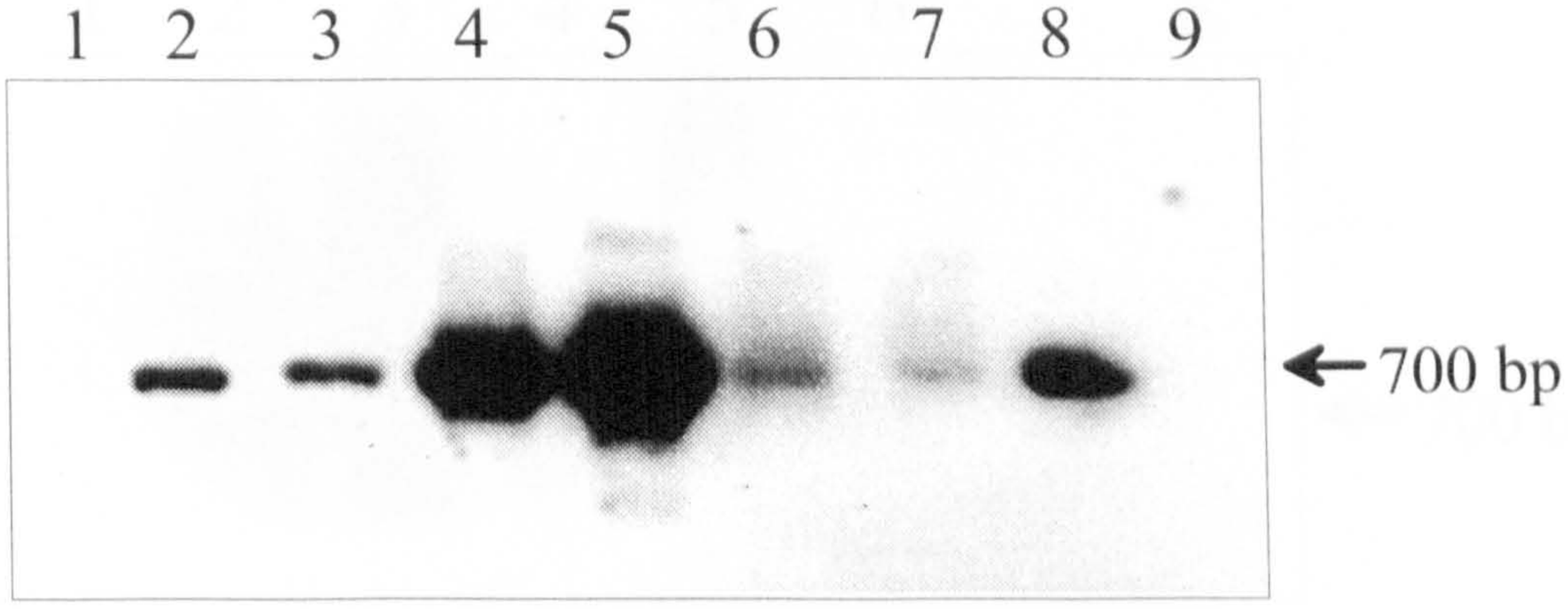


Figure 4.15. A - 'Nested' PCR amplification of SRB 16S rDNA extracted from landfill leachate using primers DFM140 & DFM842 (Group 1); **B** - Southern blot hybridized against probe DFM228 (Group 1).

Lane 1 - pBR322 DNA/*Alw*441/*Mva*1(MBI Fermentas); Lanes 2&3 - Pilsworth;
Lanes 4&5 - Butchersfield [97]; Lanes 6&7 - Buff Quarry;
Lane 8 - *Dfm. nigrificans*; Lane 9 - PCR negative control.

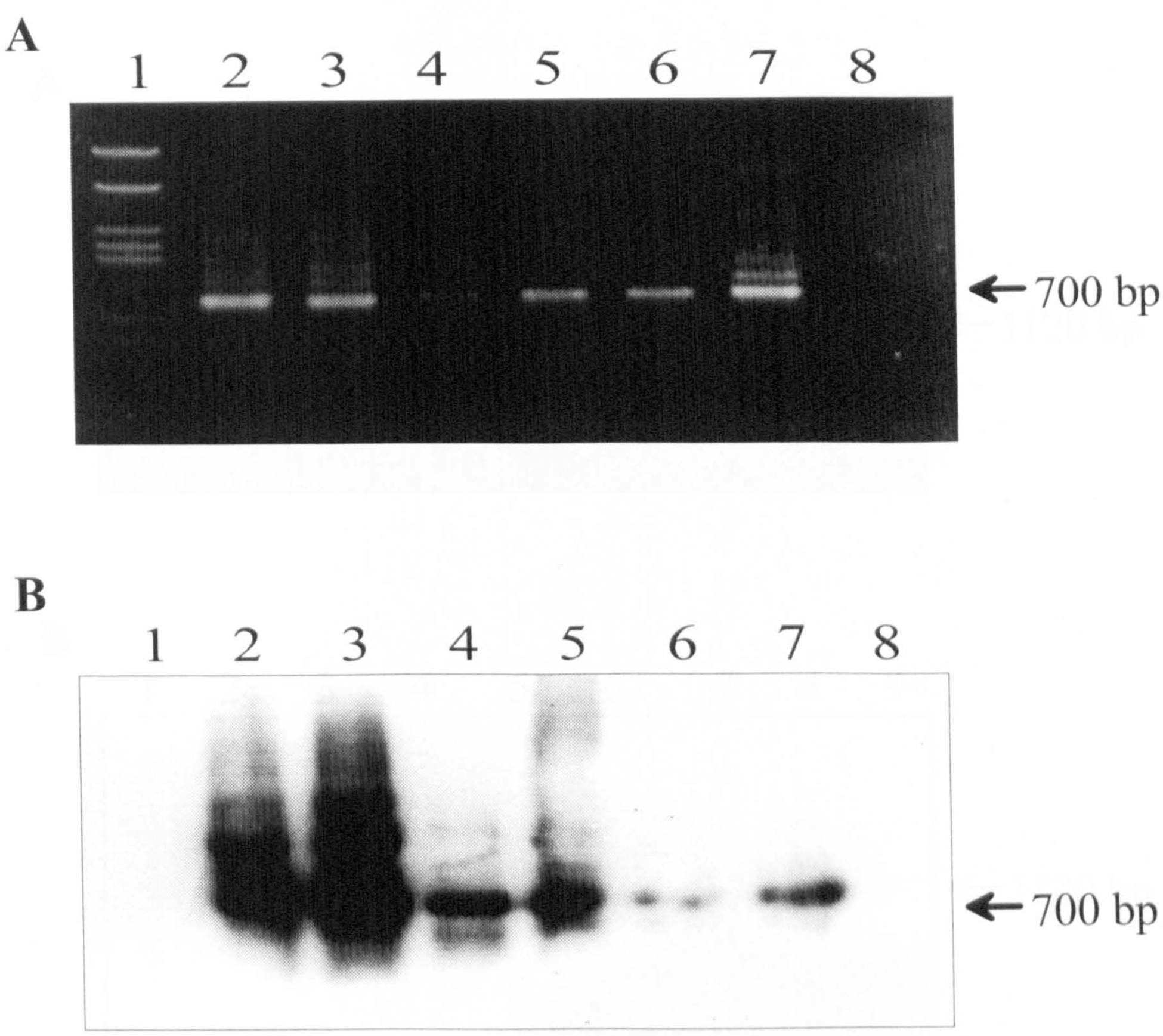
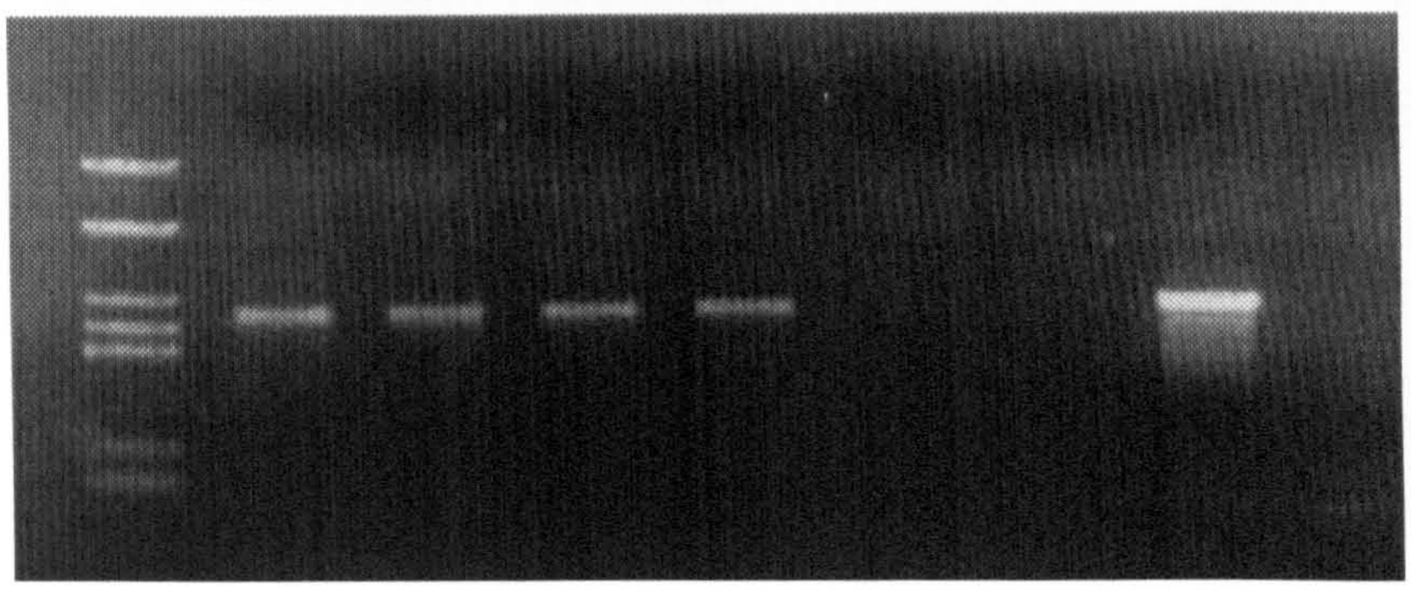


Figure 4.16. A - 'Nested' PCR amplification of SRB 16S rDNA extracted from landfill leachate using primers DFM140 & DFM842 (Group 1); **B** - Southern blot hybridized against probe DFM228 (Group 1).

Lane 1 - pBR322 DNA/*Alw*441/*Mva*1(MBI Fermentas); Lane 2 - Risley;
Lane 3 - Chadderton; Lane 4 - Holiday Moss; Lane 5 - West Leigh;
Lane 6 - Butchersfield [98]; Lane 7 - *Dfm. nigrificans*; Lane 8 - PCR negative control.

A



B

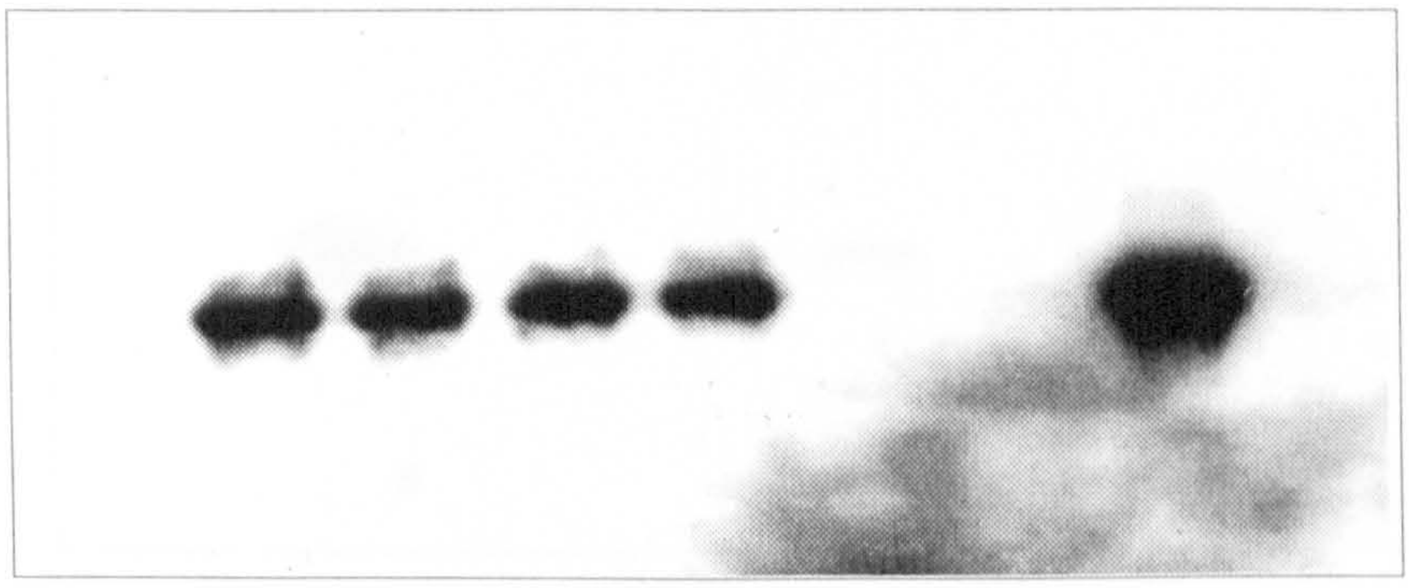


Figure 4.17. A - 'Nested' PCR amplification of SRB 16S rDNA extracted from landfill leachate using primers DBB121 & DBB1237 (Group 2); **B** - Southern blot hybridized against probe DBB660 (Group 2).

Lane 1 - pBR322 DNA/*Alw*441/*Mva*1(MBI Fermentas); Lanes 2&3 - Pilsworth; Lanes 4&5 - Butchersfield [97]; Lanes 6&7 - Buff Quarry;
Lane 8 - *Dbb. propionicus*; Lane 9 - PCR negative control.

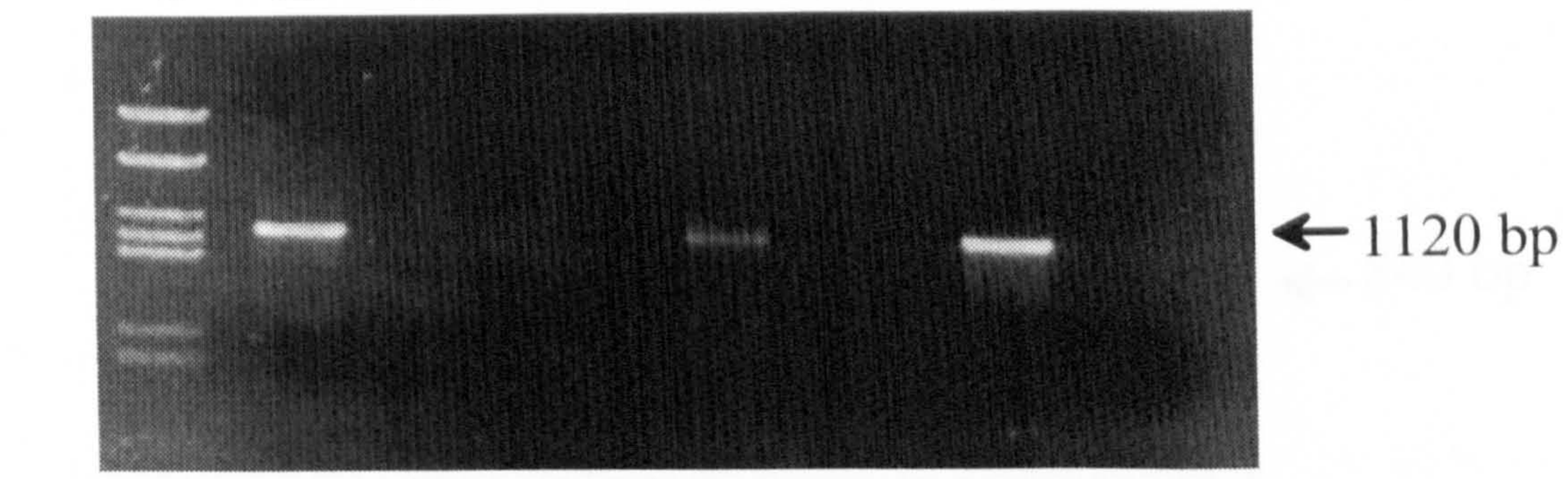


Figure 4.18. A - 'Nested' PCR amplification of SRB 16S rDNA extracted from landfill leachate using primers DBB121 & DBB1237 (Group 2); **B** - Southern blot hybridized against probe DBB660 (Group 2).

Lane 1 - pBR322 DNA/*Alw*441/*Mva*1(MBI Fermentas); Lane 2 - Risley;
Lane 3 - Chadderton; Lane 4 - Holiday Moss; Lane 5 - West Leigh;
Lane 6 - Butchersfield [98]; Lane 7 - *Dbb. propionicus*; Lane 8 - PCR negative control.

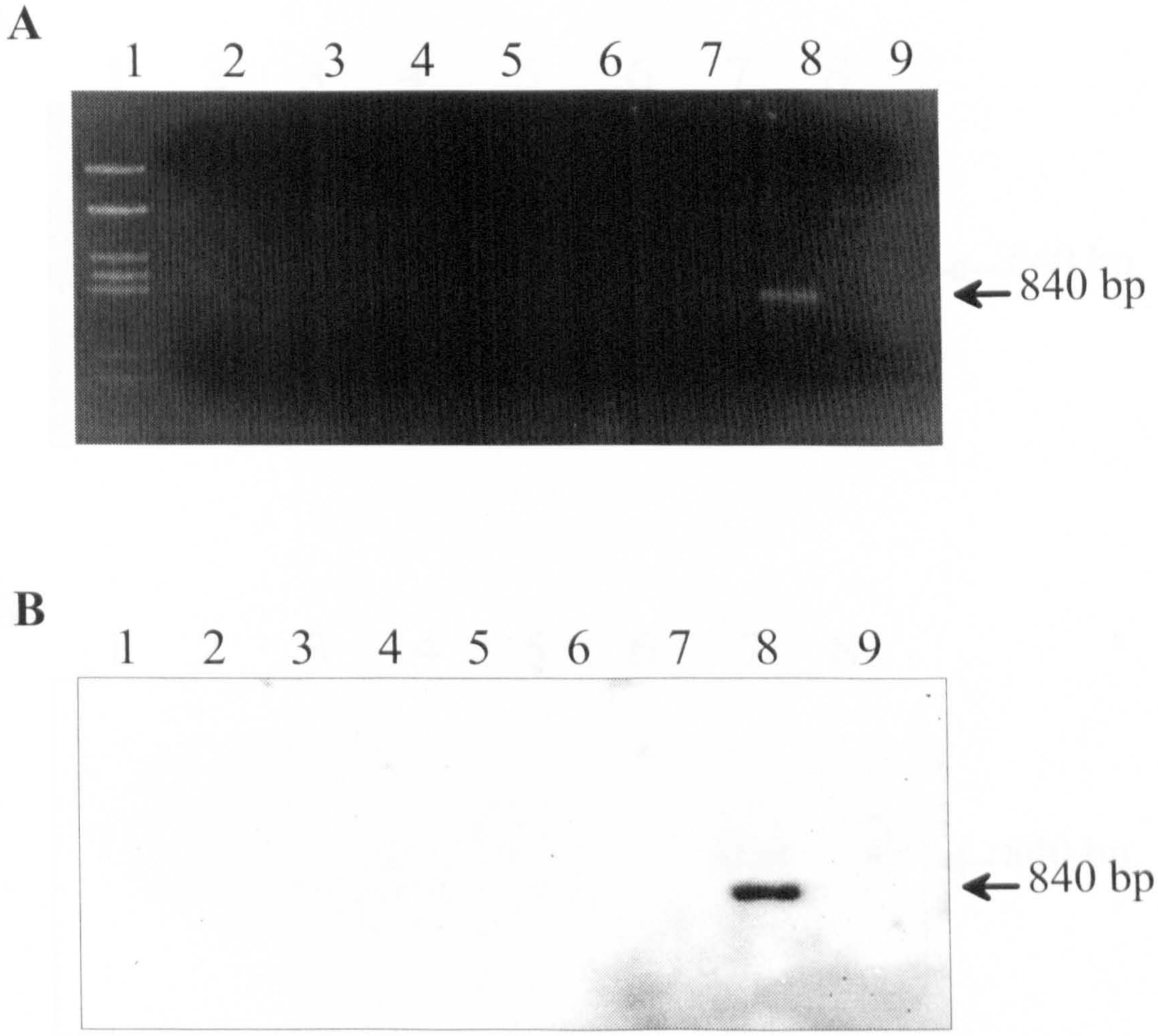


Figure 4.19. A - 'Nested' PCR amplification of SRB 16S rDNA extracted from landfill leachate using primers DBM169 & DBM1006 (Group 3); **B** - Southern blot hybridized against probe DBM221 (Group 3).

Lane 1 - pBR322 DNA/*Alw*441/*Mva*1(MBI Fermentas); Lanes 2&3 - Pilsworth;
Lanes 4&5 - Butchersfield [97]; Lanes 6&7 - Buff Quarry;
Lane 8 - *Dbm. autotrophicum*; Lane 9 - PCR negative control.

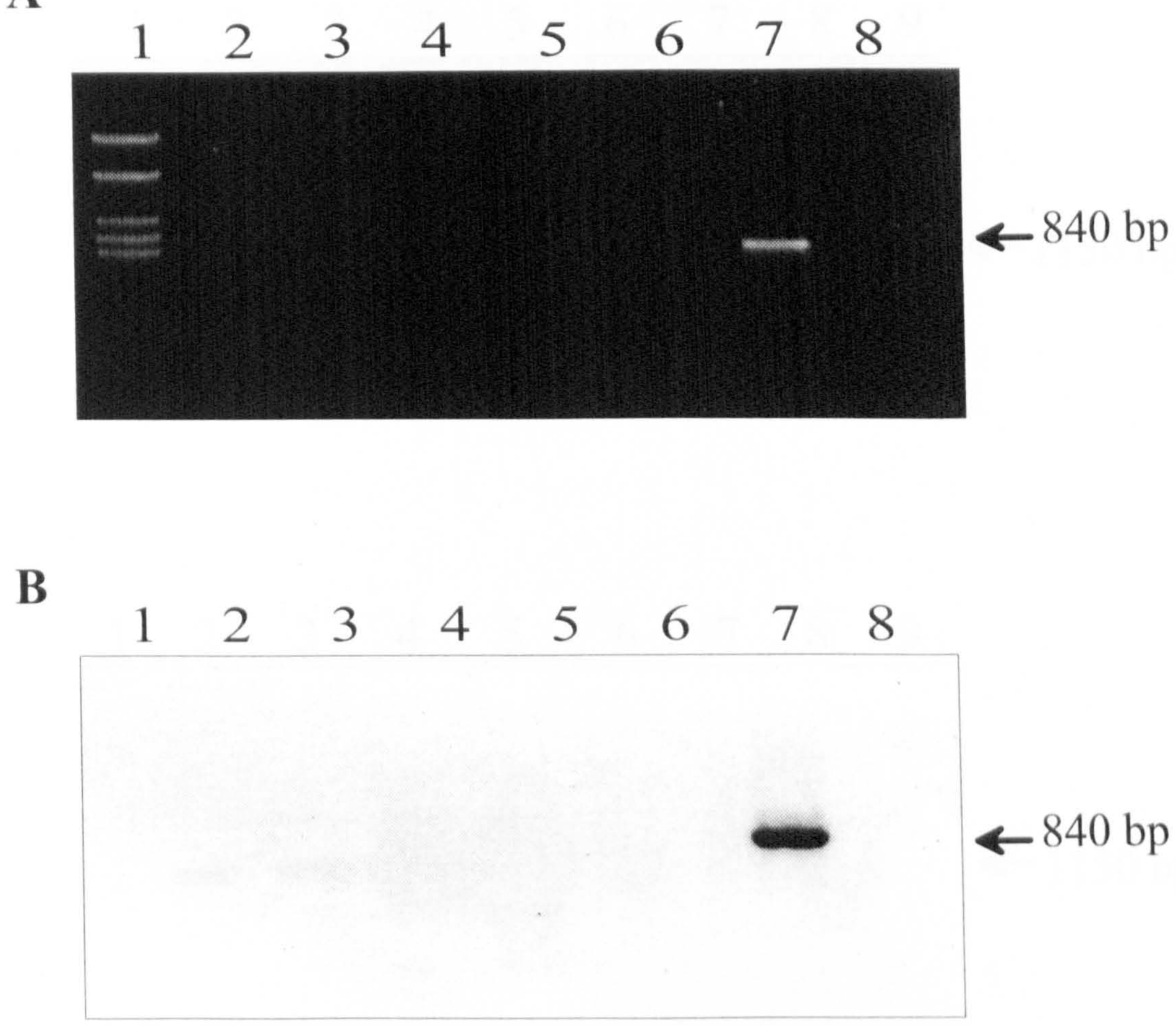
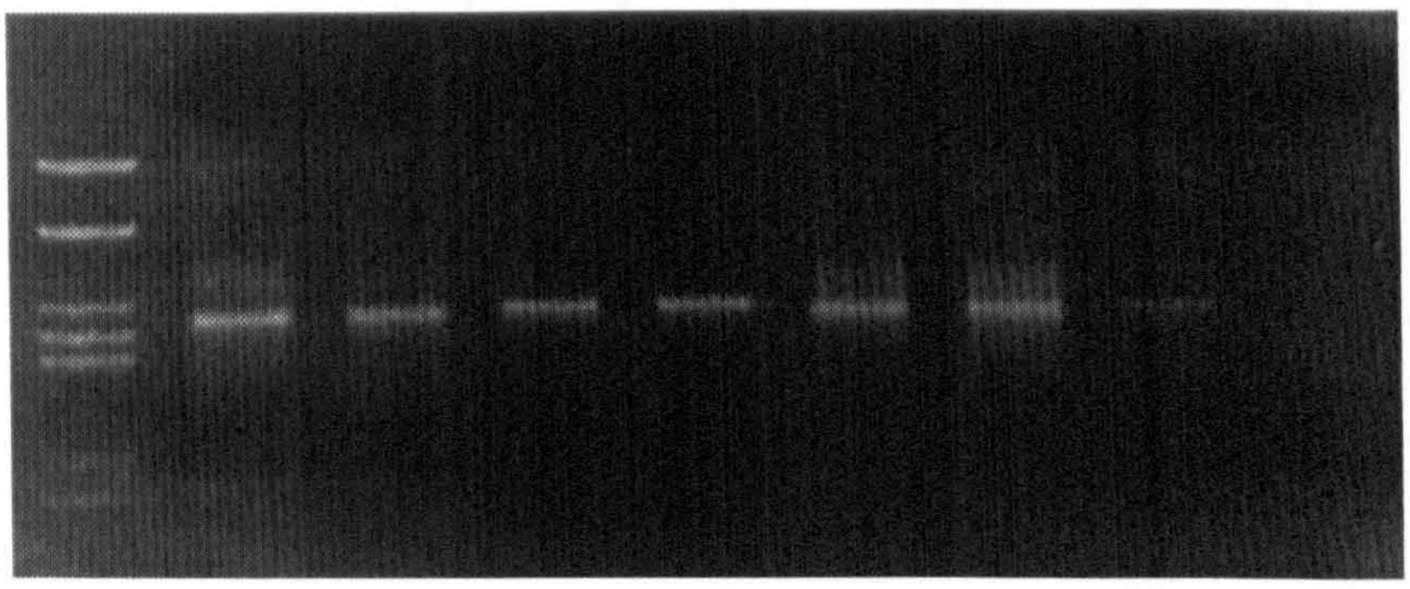


Figure 4.20. A - 'Nested' PCR amplification of SRB 16S rDNA extracted from landfill leachate using primers DBM169 & DBM1006 (Group 3); **B** - Southern blot hybridized against probe DBM221 (Group 3).

Lane 1 - pBR322 DNA/*Alw*441/*Mva*1(MBI Fermentas); Lane 2 - Risley;
Lane 3 - Chadderton; Lane 4 - Holiday Moss; Lane 5 - West Leigh;
Lane 6 - Butchersfield [98]; Lane 7 - *Dbm. autotrophicum*; Lane 8 - PCR negative control.

A



B

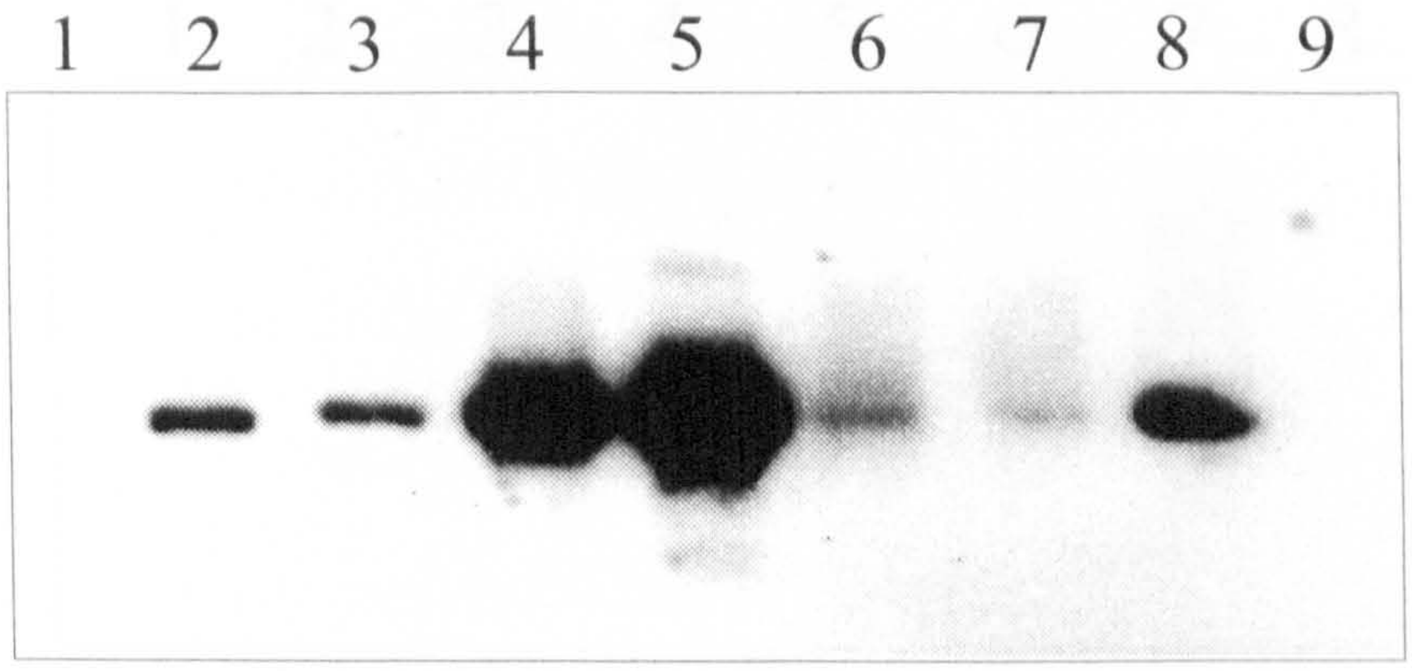


Figure 4.21. A - 'Nested' PCR amplification of SRB 16S rDNA extracted from landfill leachate using primers DSB127 & DSB1273 (Group 4); **B** - Southern blot hybridized against probe DSB623 (Group 4).

Lane 1 - pBR322 DNA/*Alw*441/*Mva*1(MBI Fermentas); Lanes 2&3 - Pilsworth;
Lanes 4&5 - Butchersfield [97]; Lanes 6&7 - Buff Quarry;
Lane 8 - *Dsb. curvatus*; Lane 9 - PCR negative control.

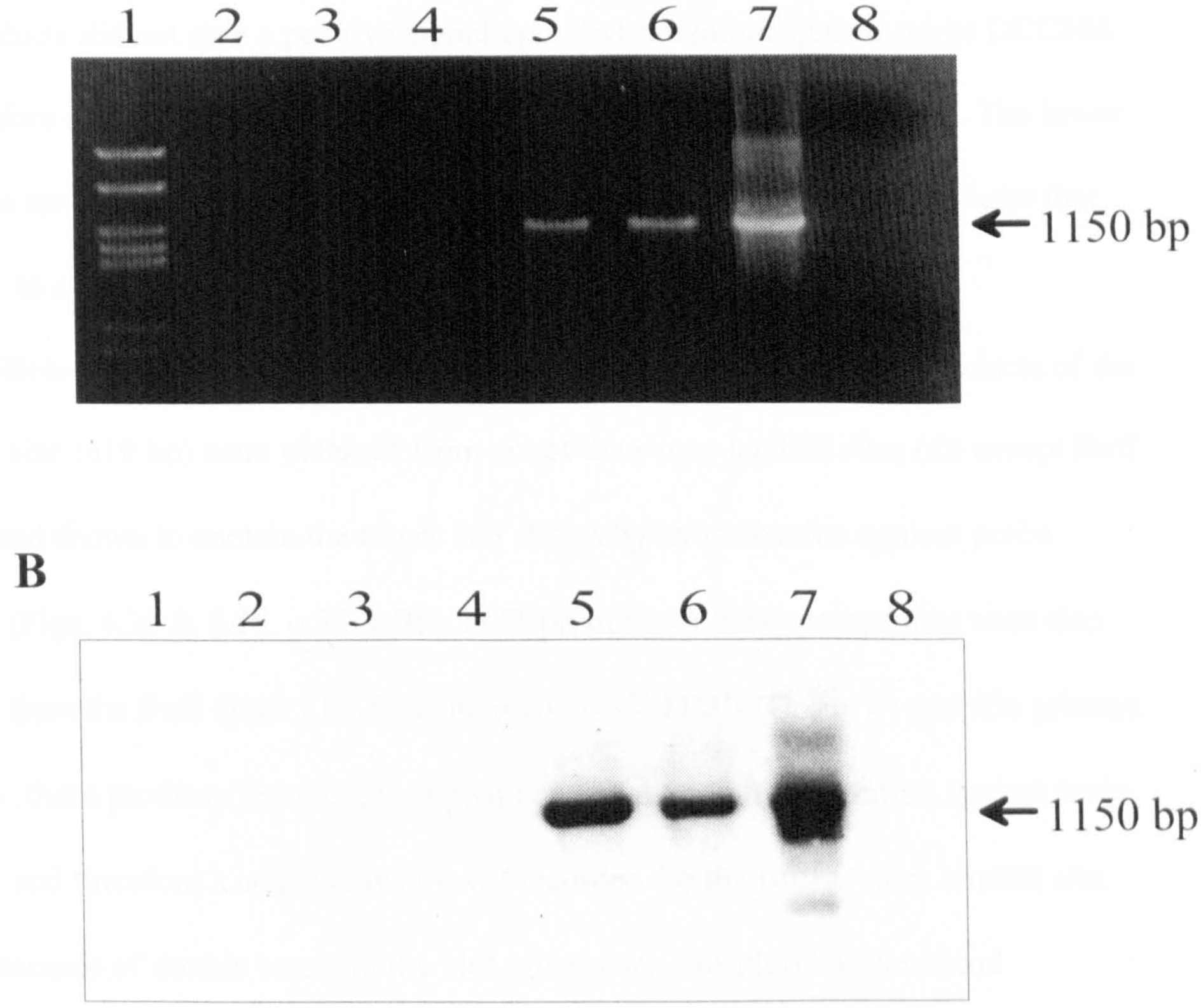


Figure 4.22. A - 'Nested' PCR amplification of SRB 16S rDNA extracted from landfill leachate using primers DSB127 & DSB1273 (Group 4); **B** - Southern blot hybridized against probe DSB623 (Group 4).

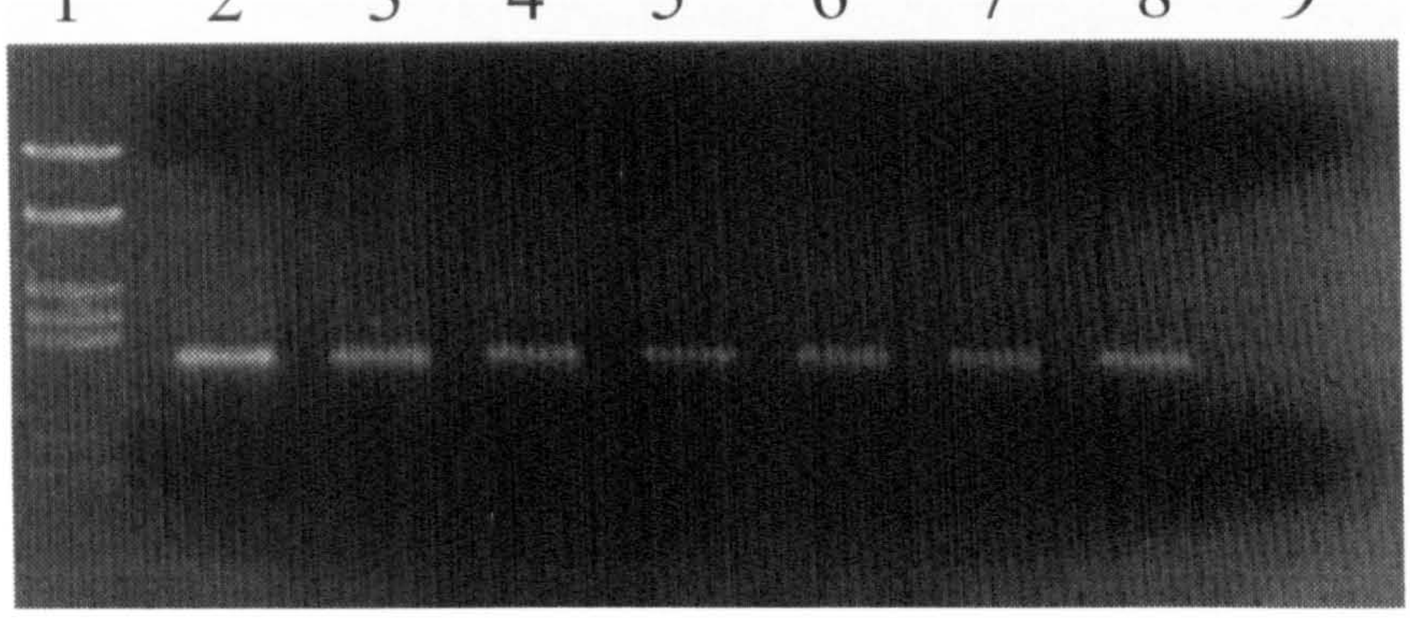
Lane 1 - pBR322 DNA/*Alw*441/*Mva*1(MBI Fermentas); Lane 2 - Risley; Lane 3 - Chadderton; Lane 4 - Holiday Moss; Lane 5 - West Leigh; Lane 6 - Butchersfield [98]; Lane 7 - *Dsb. curvatus*; Lane 8 - PCR negative control.

(Figs. 4.23 & 4.24, p.93 & 94). PCR products of the expected size were also obtained from Buff Quarry using the DCC-DNM-DSS (Group 5)-specific primers. However, these products did not give a positive signal upon hybridization against probe DCC868 and therefore a negative result was recorded for the Buff Quarry landfill site. The lower bands that can be seen on the Southern blot are most likely single strand products that can occur as a result of primer fatigue.

***Desulfovibrio-Desulfomicrobium* (DSV-DMB Group 6)**: amplification products of the expected size (610 bp) were obtained from six of the seven landfill sites (all except Buff Quarry) and shown to contain the target 16S rDNA by hybridization against probe DSV687 (Figs. 4.25 & 4.26, p.95 & 96). PCR products of the expected size were also obtained from the Buff Quarry landfill site using DSV-DMB (Group 6)-specific primers. However, these products did not give a positive signal upon hybridization against probe DSV687 and therefore a negative result was recorded for the Buff Quarry landfill site. The appearance of double bands in the blot images are a result of single strand amplification due to primer fatigue.

A summary of results for the 'nested' PCR amplification of 16S rDNA extracted from landfill leachate using SRB group-specific primers and hybridization against group-specific oligonucleotide probes is presented in Table 4.2 (p.97).

A



B

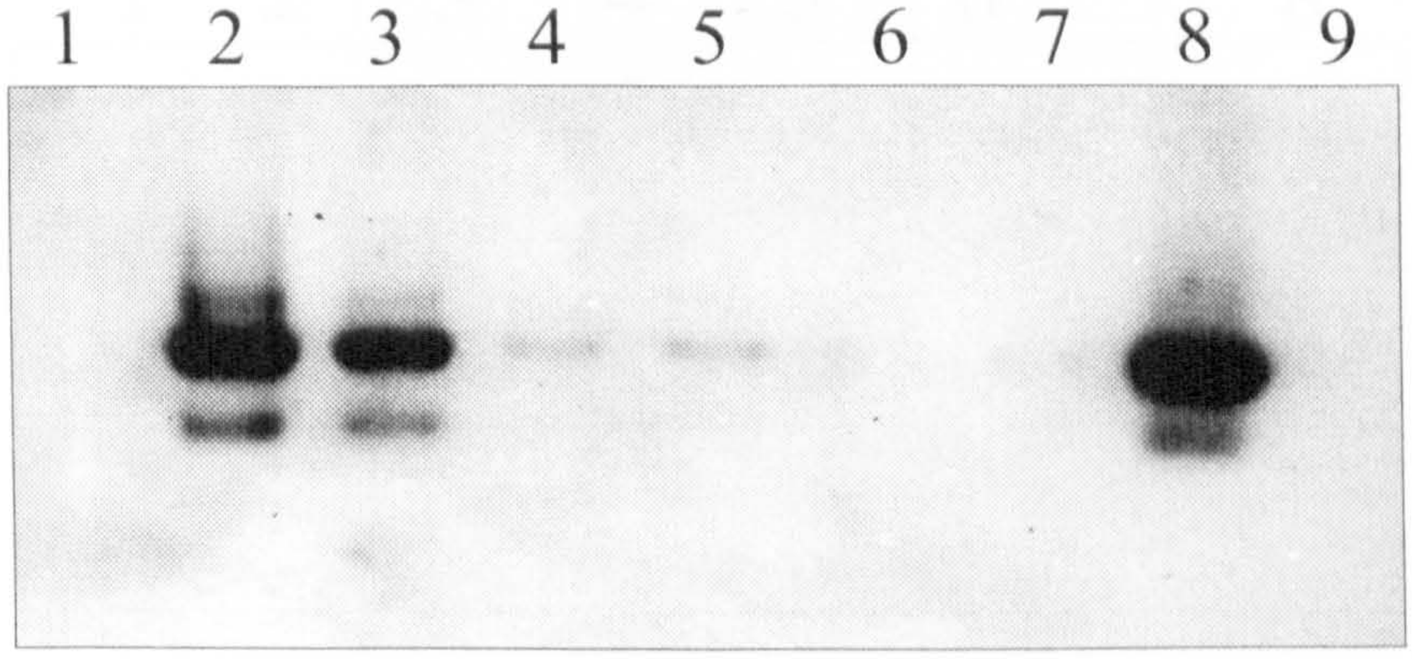


Figure 4.23. A - 'Nested' PCR amplification of SRB 16S rDNA extracted from landfill leachate using primers DCC305 & DCC1165 (Group 5): **B** - Southern blot hybridized against probe DCC868 (Group 5).

Lane 1 - pBR322 DNA/*Alw*441/*Mva*1(MBI Fermentas); Lanes 2&3 - Pilsworth; Lanes 4&5 - Butchersfield [97]; Lanes 6&7 - Buff Quarry;
Lane 8 - *Dss. variabilis*; Lane 9 - PCR negative control.

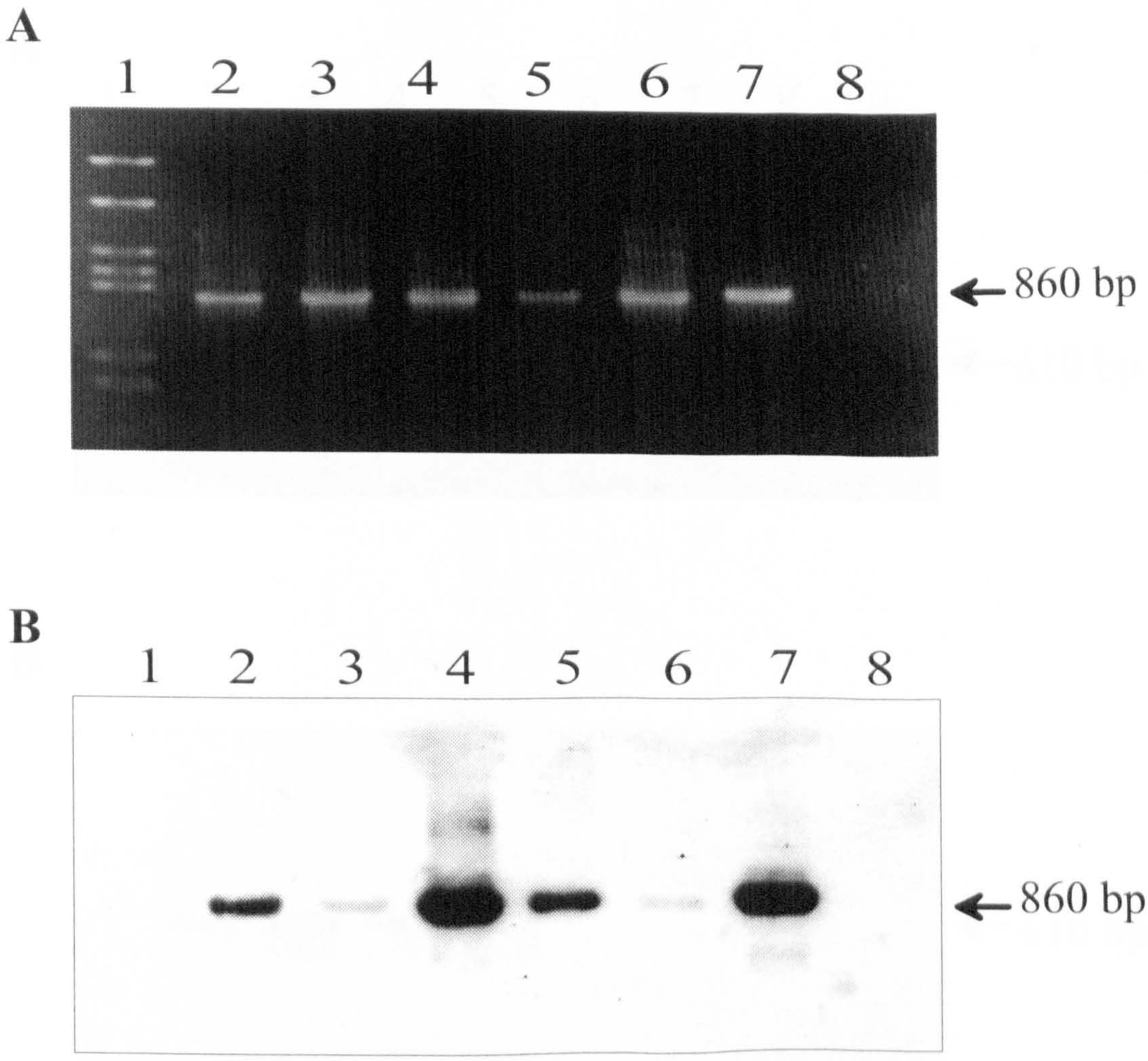
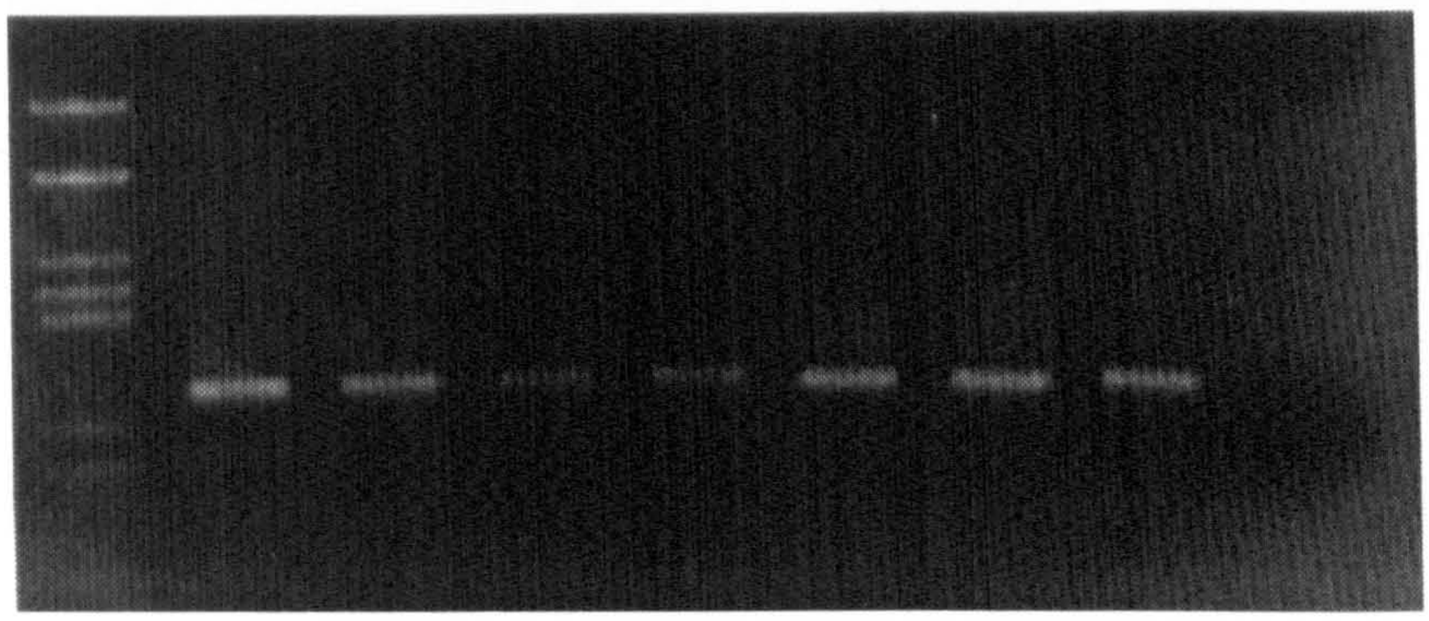


Figure 4.24. A - 'Nested' PCR amplification of SRB 16S rDNA extracted from landfill leachate using primers DCC305 & DCC1165 (Group 5); **B** - Southern blot hybridized against probe DCC868 (Group 5).

Lane 1 - pBR322 DNA/*Alw*441/*Mva*1(MBI Fermentas); Lane 2 - Risley;
Lane 3 - Chadderton; Lane 4 - Holiday Moss; Lane 5 - West Leigh;
Lane 6 - Butchersfield [98]; Lane 7 - *Dss. variabilis*; Lane 8 - PCR negative control.

A



B

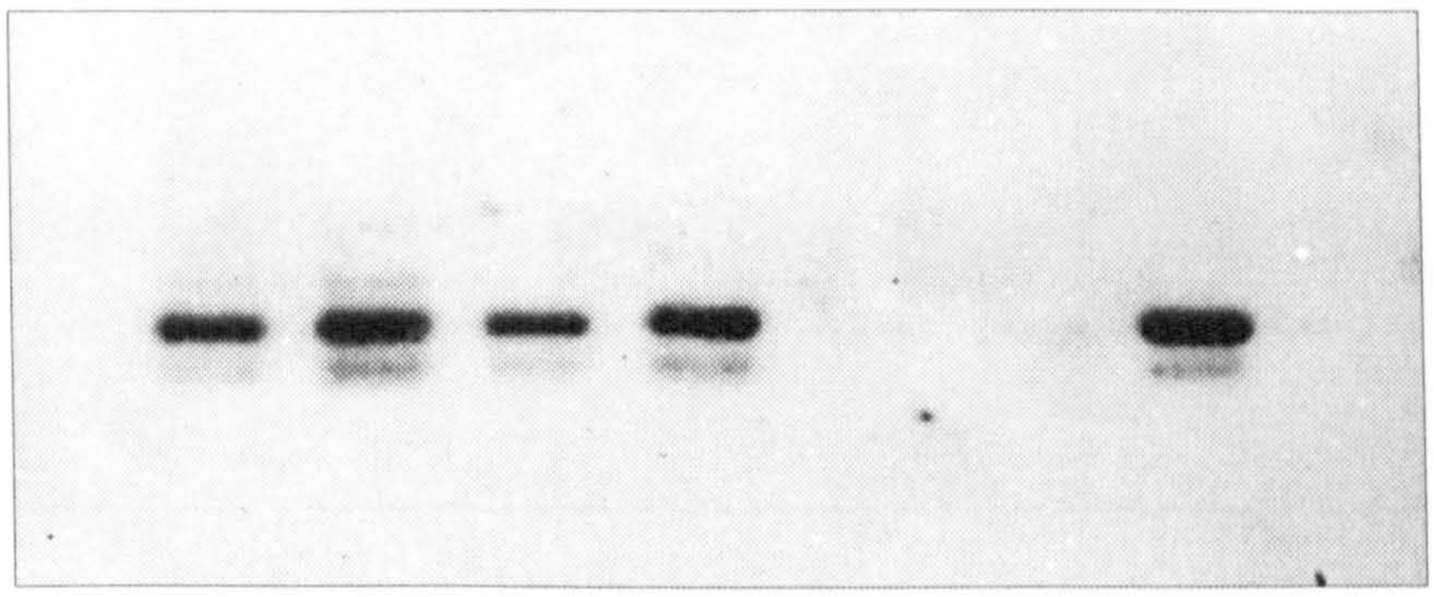
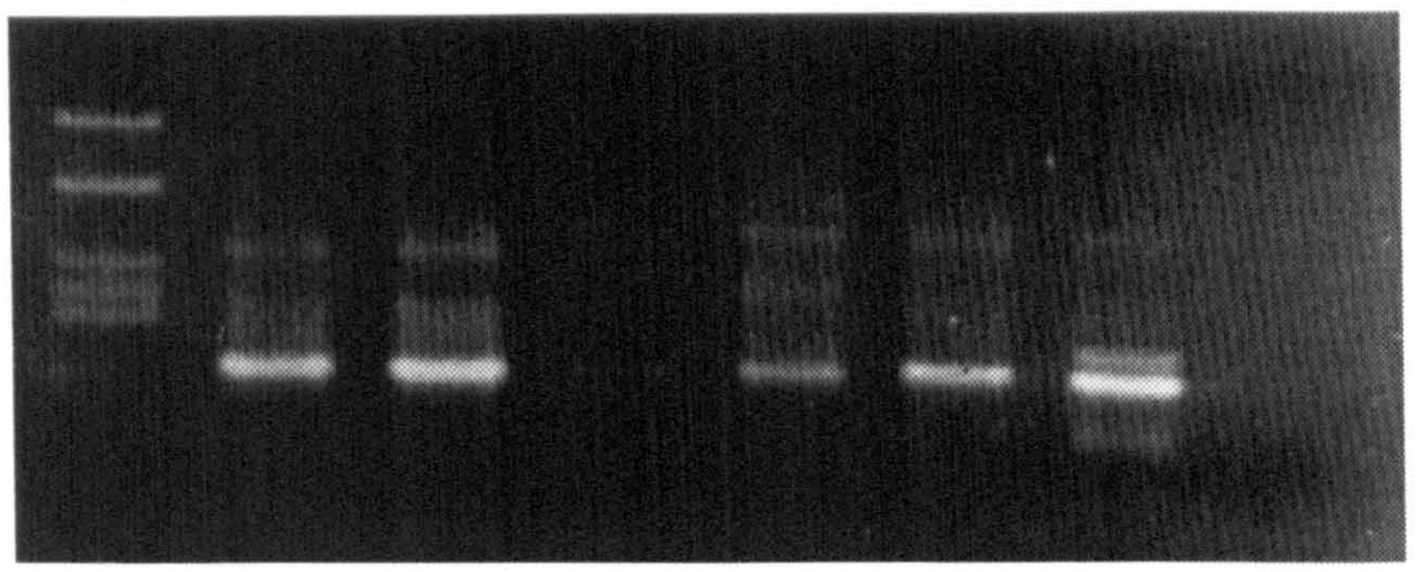


Figure 4.25. A - 'Nested' PCR amplification of SRB 16S rDNA extracted from landfill leachate using primers DSV230 & DSV838 (Group 6); **B** - Southern blot hybridized against probe DSV687 (Group 6).

Lane 1 - pBR322 DNA/*Alw*441/*Mva*1(MBI Fermentas); Lanes 2&3 - Pilsworth;
Lanes 4&5 - Butchersfield [97]; Lanes 6&7 - Buff Quarry;
Lane 8 - *Dsv. desulfuricans*; Lane 9 - PCR negative control.

A



B

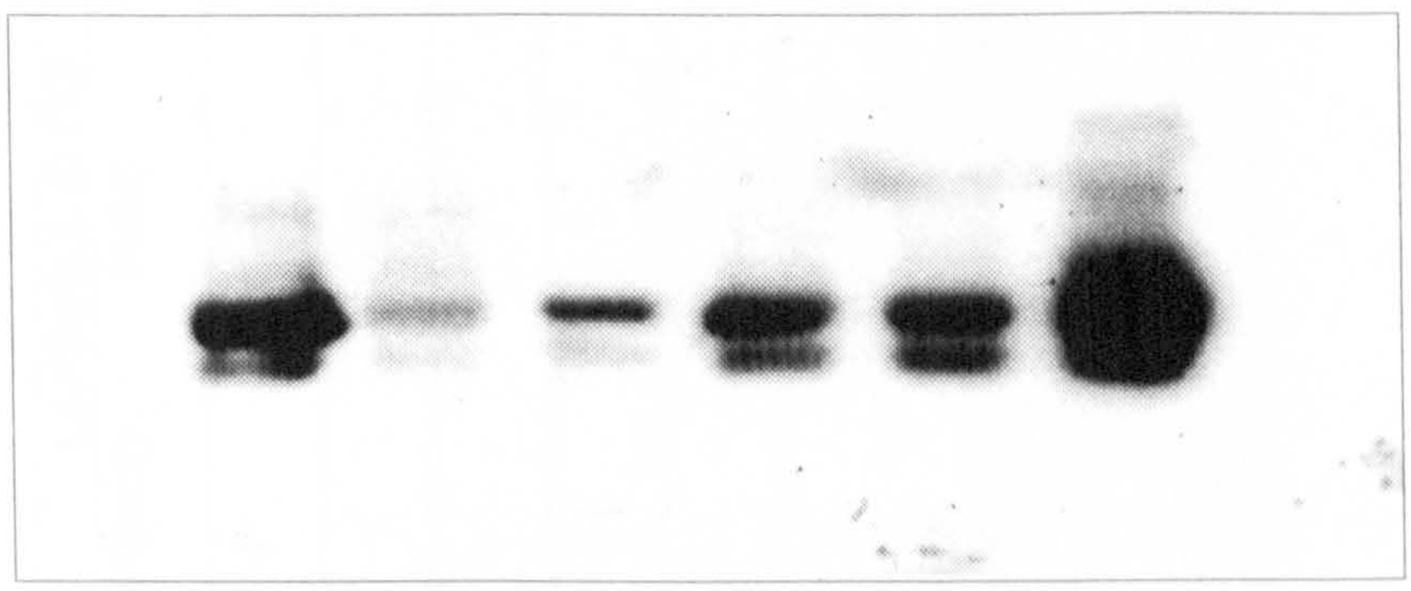


Figure 4.26. A - 'Nested' PCR amplification of SRB 16S rDNA extracted from landfill leachate using primers DSV230 & DSV838 (Group 6); **B** - Southern blot hybridized against probe DSV687 (Group 6).

Lane 1 - pBR322 DNA/*Alw*441/*Mva*1(MBI Fermentas); Lane 2 - Risley;
Lane 3 - Chadderton; Lane 4 - Holiday Moss; Lane 5 - West Leigh;
Lane 6 - Butchersfield [98]; Lane 7 - *Dsv. desulfuricans*; Lane 8 - PCR negative control.

	LANDFILL SITES							
	Pilsworth	Butchersfield [97]	Buff Quarry	Risley	Chadderton	Holiday Moss	West Leigh	Butchersfield [98]
SRB Group 1 DFM	+	+	+	+	+	+	+	+
SRB Group 2 DBB	+	+	-	+	-	-	+	-
SRB Group 3 DBM	-	-	-	-	-	-	-	-
SRB Group 4 DSB	+	+	+	-	-	-	+	+
SRB Group 5 DCC-DNM-DSS	+	+	-	+	+	+	+	+
SRB Group 6 DSV-DMB	+	+	-	+	+	+	+	+

Table 4.2. Summary of results for 'nested' PCR amplification of 16S rDNA extracted from landfill leachate using SRB group-specific primers and hybridization against group-specific oligonucleotide probes.

+ : indicates a positive signal when amplification products were hybridized against the group-specific oligonucleotide probe
- : indicates a negative hybridization signal in the presence or absence of a visible band of PCR products on an agarose gel

4.3 Discussion

The data showed that populations of SRB were detectable in landfill leachate by PCR amplification and probing, and that their occurrence would appear to be widespread.

SRB 16S rDNA was successfully amplified from five out of seven landfill sites using the 'direct' PCR approach and from all seven sites sampled using 'nested' PCR. This further demonstrates the ubiquity of SRB in the environment, and suggests that landfill sites can be regarded as habitats that contain SRB populations as a matter of routine and not just under certain circumstances.

The results obtained using the 'direct' PCR amplification approach suggest that there would appear to be one or two dominant subgroups of SRB in each of the landfill sites: *Desulfotomaculum* (Group 1) in Buff Quarry and Chadderton; *Desulfotomaculum* (Group 1) and *Desulfococcus-Desulfonema-Desulfosarcina* (Group 5) in Pilsworth; *Desulfobacter* (Group 4) in Butchersfield; *Desulfococcus-Desulfonema-Desulfosarcina* (Group 5) in West Leigh. Only in two landfill sites (Risley and Holiday Moss) were no SRB detected using this 'direct' PCR approach.

However, 'nested' PCR amplification revealed the presence of other subgroups not detected by the 'direct' PCR: *Desulfotomaculum* (Group 1) in Butchersfield, Risley, Holiday Moss and West Leigh; *Desulfobulbus* (Group 2) in Pilsworth, Butchersfield, Risley and West Leigh; *Desulfobacter* (Group 4) in Buff Quarry and West Leigh; *Desulfococcus-Desulfonema-Desulfosarcina* (Group 5) in Risley and Holiday Moss; *Desulfovibrio-Desulfomicrobium* (Group 6) in all except Buff Quarry.

It is presumed that SRB groups that can only be detected in landfill leachates when a second round of amplification is employed ('nested' PCR) are present in lower numbers than members of the dominant groups detectable by 'direct' PCR. Therefore, the dual application of 'direct' and 'nested' PCR can permit a rapid qualitative estimate of the relative predominance of SRB groups in landfill leachate. However, this is only a qualitative estimation of relative numbers based on detection through one round of PCR ('direct') compared to two rounds of PCR ('nested') and bears no statistical significance. It is possible that the requirement for 'nested' PCR to detect members of Group 2 (DBB) and Group 6 (DSV-DMB) in any leachate sample could be a feature of the PCR efficiency of these specific primers, rather than reflection of a relatively small population size. However, PCR amplifications of DNA extracted from pure cultures using all six group-specific primer sets (Fig. 3.2, p.59) yielded approximately equivalent amounts of PCR product, i.e. no significant differences in performance of the primer pairs was noted.

Desulfobacterium-like (Group 3) amplification products were never obtained from any of the landfill sites either by 'direct' or 'nested' PCR and this would appear to correlate with the association of most of the known species of the genus *Desulfobacterium* with the marine environment (Postgate, 1984; Fauque, 1995).

The apparent non-specific amplification of PCR products with the DCC-DNM-DSS (Group 5)- and DSV-DMB (Group 6)-specific primers suggests that these two primer sets are not specific for their target groups. However, it must be remembered that all of the primers described in this study are degenerate and based on a limited number of sequenced strains. Therefore, when applied to complex environmental samples, it is

possible that non-target species, as yet uncharacterized, could be amplified. It is also possible that the primer sequences are present in other DNA which may not be 16S rDNA or even bacterial DNA. It is for this reason that only PCR products that gave a positive signal upon hybridization against the appropriate group-specific oligonucleotide probe were recorded as positive results. Thus, it is the primer-probe combinations that are highly specific. It is also possible that the apparent non-specific amplification products were, in fact, from target species, as yet uncharacterized, that contained mismatches in the oligonucleotide probe target region. It would only require a 1 bp mismatch in the target region of both the DCC868 and DSV687 probes for a negative result to be obtained upon hybridization. The only way to resolve this issue would be to clone and sequence the PCR products, which would require time and effort beyond the scope of this study.

Nevertheless, the results obtained from the 'nested' PCR (Table 4.2, p.97) suggest that there is a high level of diversity in landfill as five out of the six main subgroups of SRB have been detected in these landfill sites. This correlates with investigations of SRB occurrence and distribution in other environments in which most of the main subgroups have been detected by oligonucleotide probing without the need for PCR amplification (Kane *et al*, 1993; Ramsing *et al*, 1993; Risatti *et al*, 1994; Devereux *et al* 1996a, 1996b; Raskin *et al*, 1996; Purdy *et al*, 1997; Trimmer *et al*, 1997; Rooney-Varga *et al*, 1997; Manz *et al*, 1998; Sahm *et al*, 1999b). This is the only study of SRB molecular ecology described to date in which DNA extracts have been amplified by specific PCR prior to confirmation by oligonucleotide hybridization. Direct probing of the DNA extracted from the landfill leachate samples without PCR was not attempted. However, it might be

predicted that due to the predominance of methanogenesis in landfill and the requirement for 'nested' PCR to amplify SRB DNA in most cases, detection of SRB from the leachate samples by direct probing would not have been successful. In landfill sites where there is a definite problem of sulfide production then direct detection of SRB without PCR may be possible. However, it was not possible to obtain samples from any landfill sites with sulfide problems, nor was it possible to obtain information on the characteristics of the landfill sites from which samples were obtained.

This apparent diversity of SRB, at least at the generic/suprageneric level, in landfill sites is not unexpected. The extremely high and varied organic carbon load together with long retention times encourages large and active populations of fermentative micro-organisms, which in turn produce various volatile fatty acids that serve as substrates for SRB. The scale of landfill sites and the extreme heterogeneity would promote microbial diversity. Also, as leachate results from the percolation of water through the site, high diversity would be expected even though SRB distribution could be non-uniform throughout the site. While it would be of interest to study SRB populations in solid landfill material, leachate is going to be the only practical sample material for routine analysis and SRB monitoring. Thus, the argument that SRB populations in leachate may be a poor representation of SRB population size and distribution in the landfill does not preclude its use as a practical source of useful information on landfill microbiology.

It is now well established that SRB and methanogens compete for fermentation products such as acetate and H_2 and that, in the presence of non-limiting levels of sulfate, SRB generally outcompete methanogenic bacteria (Oremland & Polcin, 1982; Beeman & Suflita, 1987; Raskin *et al*, 1996) with sulfate reduction being the key process of carbon

mineralization in these high sulfate environments. However, in landfill it is usually methanogenic bacteria that dominate with methanogenesis, not sulfate reduction, as the key terminal process of carbon mineralization. This therefore suggests that SRB populations in landfill be limited by the availability of sulfate, thereby allowing methanogenesis to dominate. However, the detection of SRB in these landfill sites suggests that the potential for sulfate reduction and the possible inhibition of methane production is present (Suflita *et al*, 1992; Gurijala and Suflita, 1993), and although there is no direct evidence that sulfate reduction is occurring in the landfill sites sampled here, it must be assumed that SRB populations detected could present a significant competitive threat to the methanogenic populations in the landfills should conditions begin to favour sulfate reduction over methanogenesis.

It is therefore important to be able to monitor SRB populations in landfill sites because their proliferation can potentially affect site performance via the inhibition of methanogenesis. This investigation provides the basis for using 16S rRNA-based methods to develop such a detection protocol for monitoring SRB in landfill and also provides the first insight into SRB community structure in landfill sites.

Furthermore, this investigation has described for the first time the use of PCR primers for the specific amplification of SRB 16S rDNA from environmental samples prior to oligonucleotide hybridization. This then provides the opportunity to investigate SRB diversity within environments by the analysis of specifically amplified PCR products using techniques such as gradient gel electrophoresis and subsequent sequencing of individual 16S rDNA fragments to provide phylogenetic information, as detailed in the next chapter.

CHAPTER 5. Genetic Diversity of SRB 16S rDNA Sequences Amplified from Landfill Leachate Determined by Temporal Thermal Gradient Electrophoresis and Sequencing of Cloned SRB 16S rDNA Fragments.

5.1. Introduction

The structure of natural microbial communities is often highly complex and can therefore be difficult to characterize. Temporal thermal gradient electrophoresis (and other forms of gradient gel electrophoresis) offer the potential to analyze the diversity of bacterial populations in environmental samples based on sequence variation of specifically-amplified PCR products. Other methods of investigating bacterial community structure such as the generation of clone libraries and subsequent sequencing of clones are both time-consuming and laborious. TTGE offers a more rapid and comprehensive approach to investigating genetic diversity within complex microbial populations, and can be used to screen large numbers of samples enabling better resolution of the distributions of bacterial community members. Subsequent cloning and sequencing of the DNA in resolved bands can then support the data obtained.

The application of gradient gel electrophoresis to specifically-amplified PCR products has been shown to facilitate investigations into microbial community structure of environmental samples from a number of habitats (Ferris *et al*, 1996; Teske *et al*, 1996; Ferris and Ward, 1997; Heuer *et al*, 1997; Kowalchuk *et al*, 1997), and has also been

used to infer phylogenetic affiliations of community members through the sequencing of cloned or excised fragments (Ferris *et al*, 1996; Teske *et al*, 1996).

Banding patterns generated by TTGE provide profiles of the amplifiable sequence types ('phylotypes') present in environmental samples and can be used as a measure of genetic diversity within distinct populations. In addition, the group-specific profile from one sample can be directly compared to the bacterial community pattern of a different sample so long as the same PCR fragment is analyzed, and can therefore be used to infer differences in the composition of microbial communities.

This chapter describes the application of TTGE to PCR products amplified with SRB group-specific primers to investigate genetic diversity within SRB subgroups detected in landfill samples. The sequencing of cloned SRB 16S rDNA fragments to provide phylogenetic information and to infer phylogenetic affiliations of SRB sequences in landfill is also described.

5.2. Results

5.2.1. TTGE analysis of SRB PCR products amplified from landfill leachate

PCR products amplified from landfill leachate with SRB group-specific primers were reamplified with eubacterial primers pC(GC-clamp);pD' (Edwards *et al*, 1989) to generate fragments encompassing the V3 region of the 16S rRNA gene suitable for TTGE analysis using the methods described in section 2.10. The V3 region was selected for TTGE analysis as it is a highly variable region which should provide sufficient sequence variation to obtain good separation of bands on the TTGE gel. Perpendicular

analysis of the melting behaviour of amplified 16S rDNA fragments is not possible using TTGE and so optimal conditions for TTGE were determined empirically. A temperature range of 49.5°C-57.5°C with a gradient of 0.4°C h^{-1} produced banding patterns that gave a good separation of sequences (section 2.10). Following the determination of the temperature gradient which would generate a good separation of sequences, TTGE analysis was then applied to PCR-amplified 16S rDNA from all samples.

***Desulfotomaculum* (DFM Group 1)**: TTGE profiles were obtained from both 'direct' and 'nested' *Desulfotomaculum* (DFM Group 1) PCR products. The profiles obtained from the 'direct' PCR products amplified from the Pilsworth, Buff Quarry and Chadderton landfill sites showed dissimilar banding patterns and only two individual bands were observed in each respective profile (Fig. 5.1, p.106). Banding patterns obtained from the 'nested' PCR products amplified from all seven landfill sites were also highly differentiated. However the increased number of bands in the profiles suggests that a greater diversity within the DFM subgroup is detected using 'nested' PCR (Fig. 5.2, p.107). Conversely, the lower band observed in the Buff Quarry 'direct' profile (Lane 2, Fig. 5.1, p.106) is not observed in the corresponding 'nested' profile (Lane 3, Fig. 5.2, p.107).

***Desulfobulbus* (DBB Group 2)**: PCR products were not obtained with 'direct' PCR using the DBB (Group 2)-specific primers. TTGE profiles were therefore obtained from 'nested' *Desulfobulbus* (DBB Group 2) PCR products amplified from the Pilsworth, Butchersfield [97], Risley and West Leigh landfill sites. Banding patterns generated from all four sites were observed to be similar to one another. Profiles obtained for the Pilsworth and Butchersfield [97] contained only two individual bands. Profiles obtained

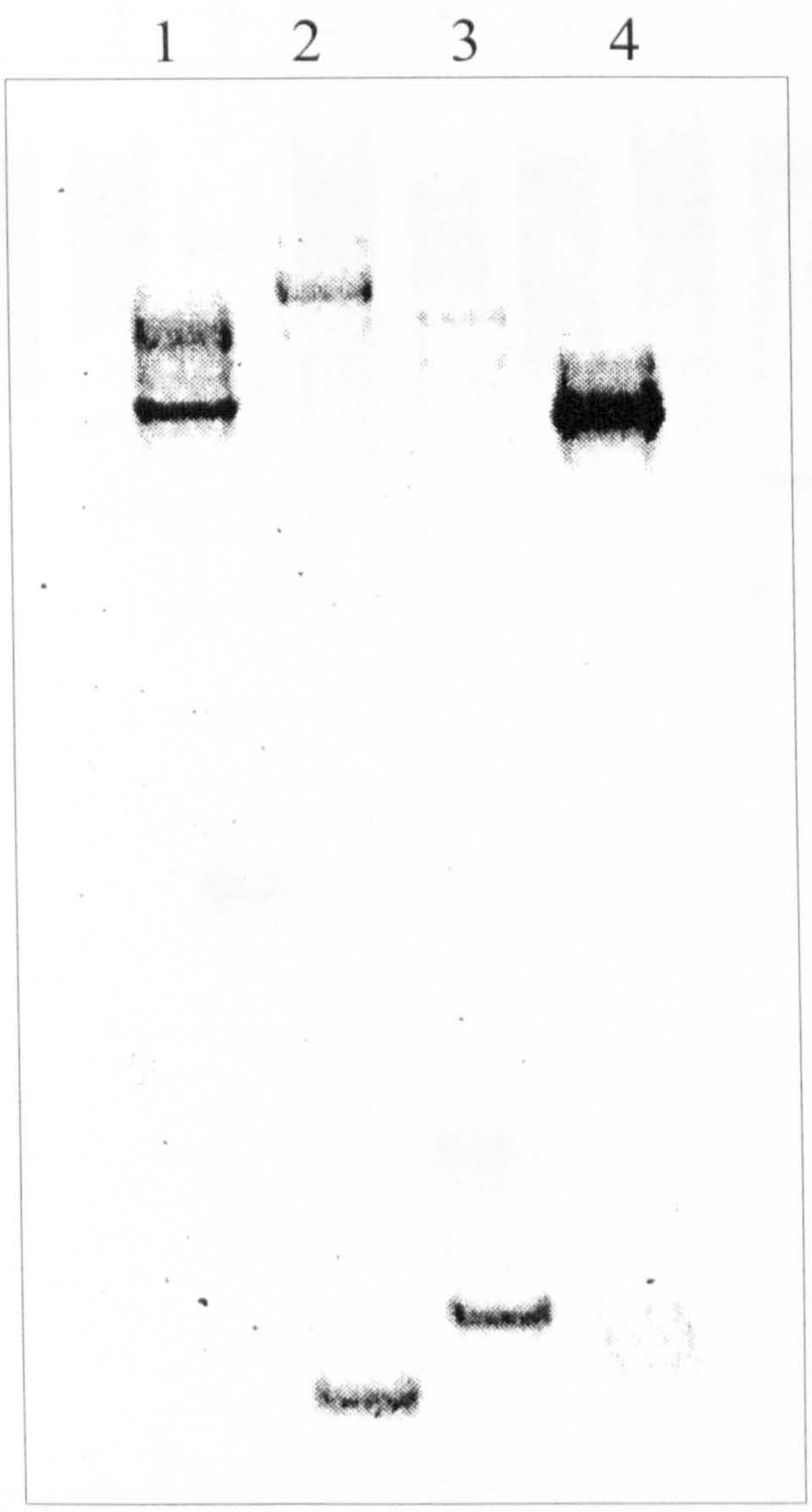


Figure 5.1. Temporal Thermal Gel Electrophoresis of DFM (Group 1) 'direct' PCR products amplified from landfill leachate.

Lane 1 - Pilsworth; Lane 2 - Buff Quarry; Lane 3 - Chadderton;
Lane 4 - *Dfm. nigrificans* (control)

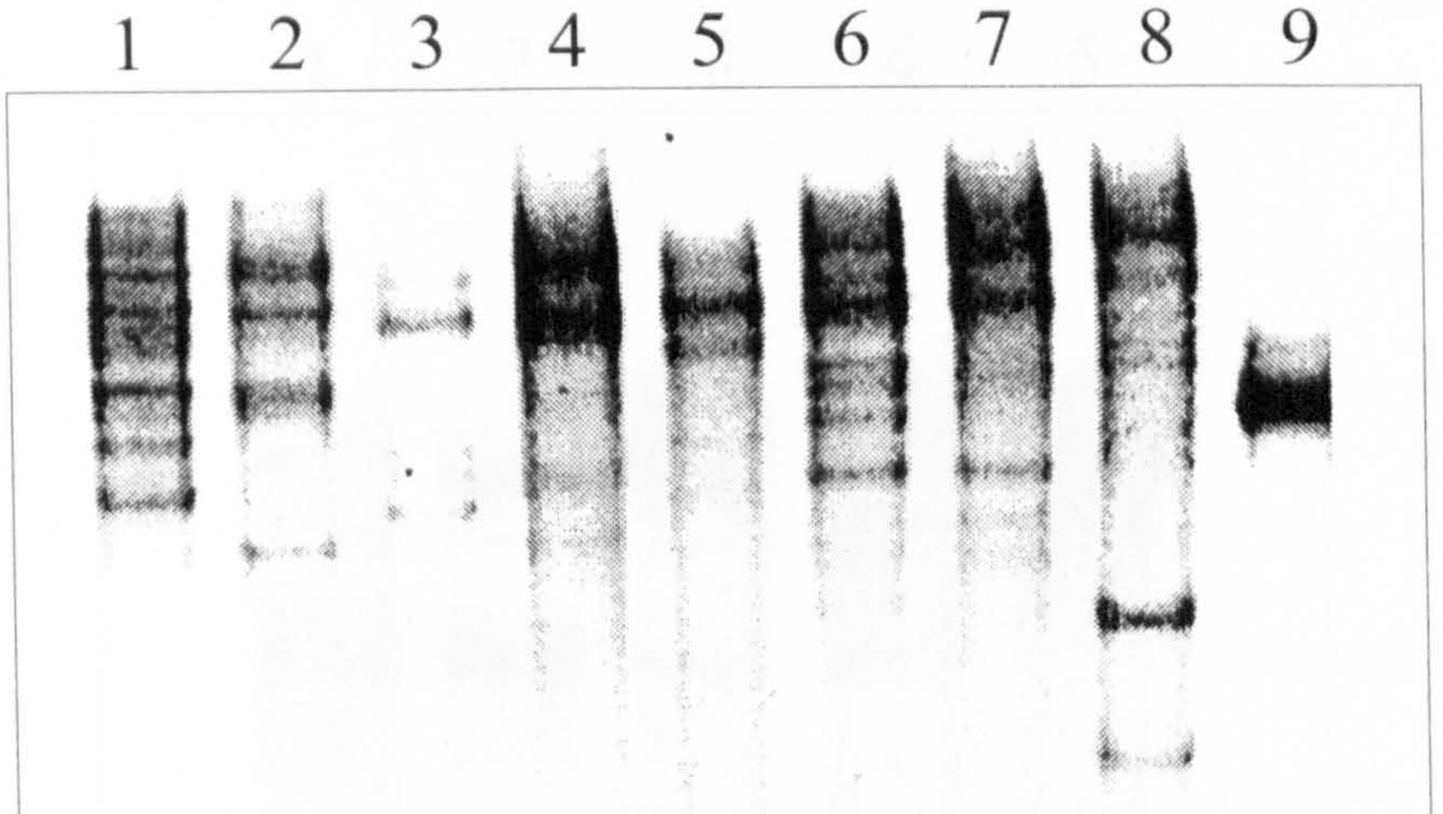


Figure 5.2. Temporal Thermal Gel Electrophoresis of DFM (Group 1) 'nested' PCR products amplified from landfill leachate

Lane 1 - Pilsworth; Lane 2 - Butchersfield [97]; Lane 3 - Buff Quarry;
Lane 4 - Risley; Lane 5 - Chadderton; Lane 6 - Holiday Moss;
Lane 7 - West Leigh; Lane 8 - Butchersfield [98]; Lane 9 - *Dfm. nigrificans* (control)

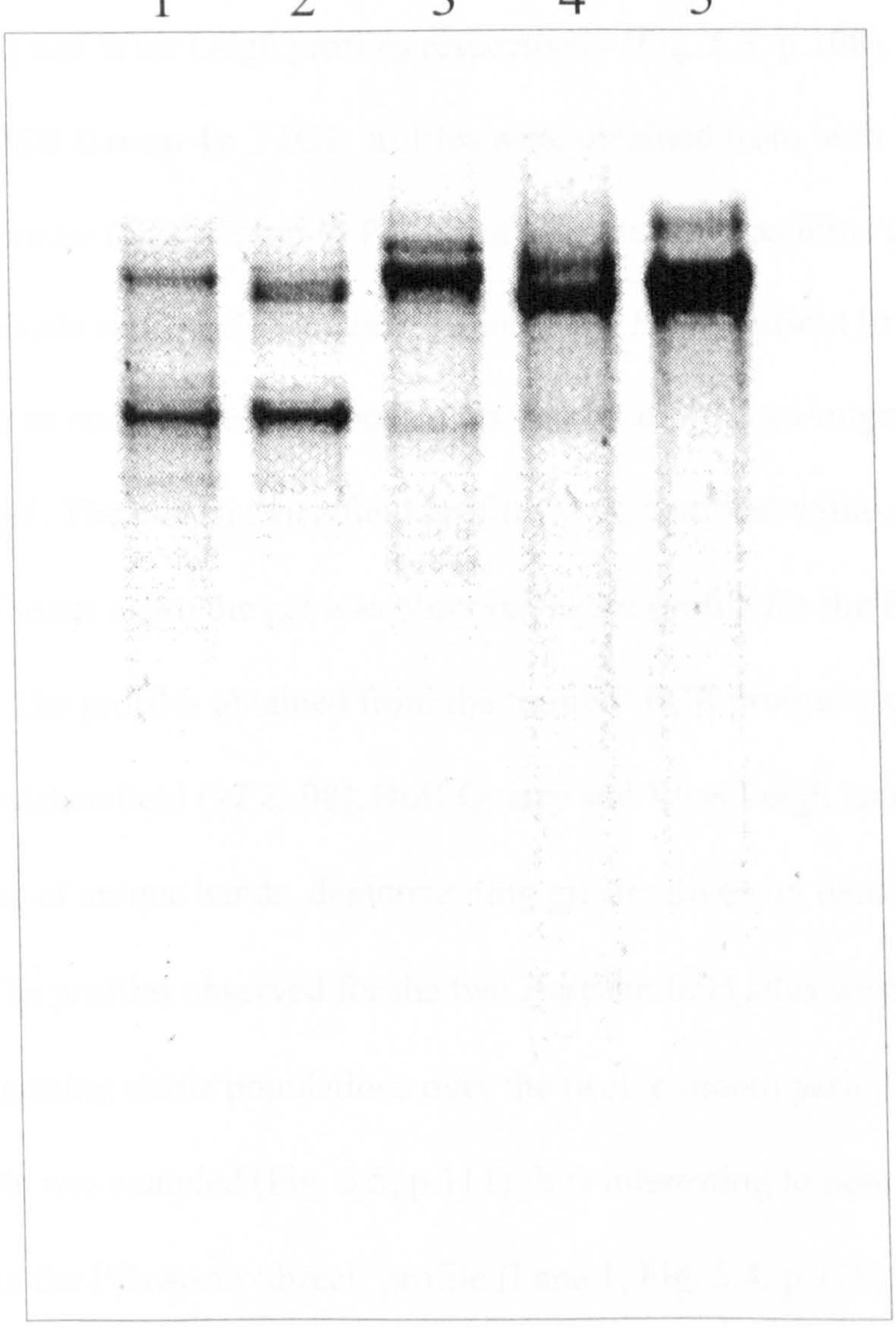


Figure 5.3. Temporal Thermal Gel Electrophoresis of DBB (Group 2) 'nested' PCR products amplified from landfill leachate.

Lane 1 - Pilsworth; Lane 2 - Butchersfield [97]; Lane 3 - Risley
Lane 4 - West Leigh; Lane 5 - *Dbb. propionicus* (control)

for the Risley and West Leigh landfill sites contained three bands each. The lower bands observed in all four profiles co-migrated to similar positions in the gel, whereas the main upper bands co-migrated to similar positions in the Pilsworth and Risley profiles, and the Butchersfield [97] and West Leigh profiles respectively (Fig. 5.3, p.108).

***Desulfobacter* (DSB Group 4)**: TTGE profiles were obtained from both 'direct' and 'nested' *Desulfobacter* (DSB Group 4) PCR products. Banding patterns generated from 'direct' PCR products amplified from the Pilsworth and Butchersfield [97 & 98] landfill sites were similar to one another and showed one common band co-migrating to similar positions in the gel. The two Butchersfield profiles were identical while one additional band migrating further down the gel was observed in the profile for the Pilsworth site (Fig 5.4, p.110). The profiles obtained from the 'nested' PCR products amplified from the Pilsworth, Butchersfield [97 & 98], Buff Quarry and West Leigh landfill sites showed a number of unique bands, demonstrating greater diversity detected through 'nested' PCR. The profiles observed for the two Butchersfield sites were again similar to one another suggesting stable populations over the twelve month period between the times that the site was sampled (Fig. 5.5, p.111). It is interesting to note that the upper band observed in the Pilsworth 'direct' profile (Lane 1, Fig. 5.4, p.110) is not observed in the Pilsworth 'nested' profile (Lane 1, Fig. 5.5, p.111).

***Desulfococcus-Desulfonema-Desulfosarcina* (DCC-DNM-DSS Group 5)**: TTGE profiles were obtained from both 'direct' and 'nested' *Desulfococcus-Desulfonema-Desulfosarcina* (DCC-DNM-DSS Group 5) PCR products. Banding patterns generated from 'direct' PCR products amplified from the Pilsworth and Chadderton landfill sites were observed to share a double band, although three other bands were observed in the

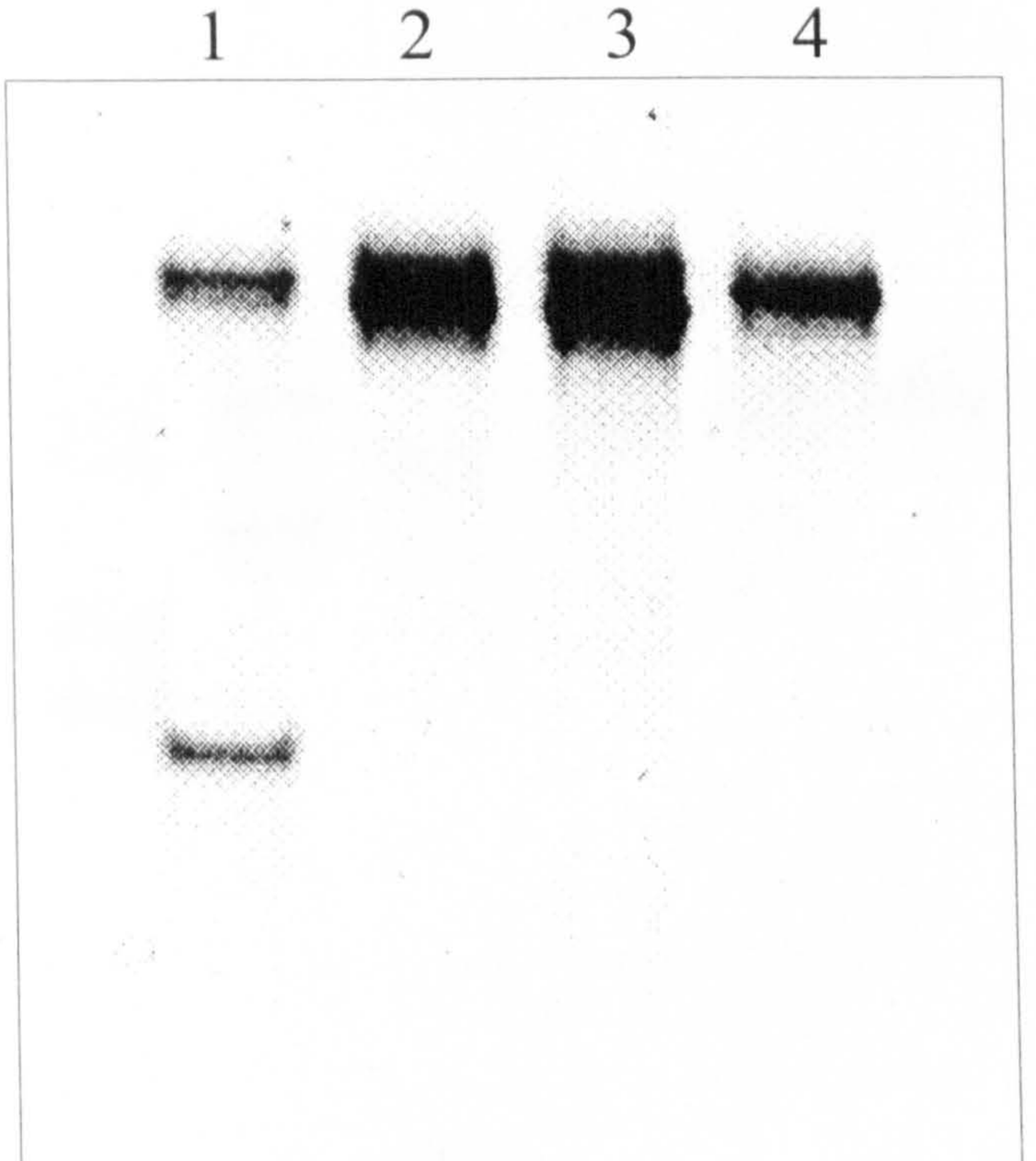


Figure 5.4. Temporal Thermal Gel Electrophoresis of DSB (Group 4) 'direct' PCR products amplified fron landfill leachate.

Lane 1 - Pilsworth; Lane 2 - Butchersfield [97]; Lane 3 - Butchersfield [98]; Lane 4 - *Dsb. curvatus* (control)

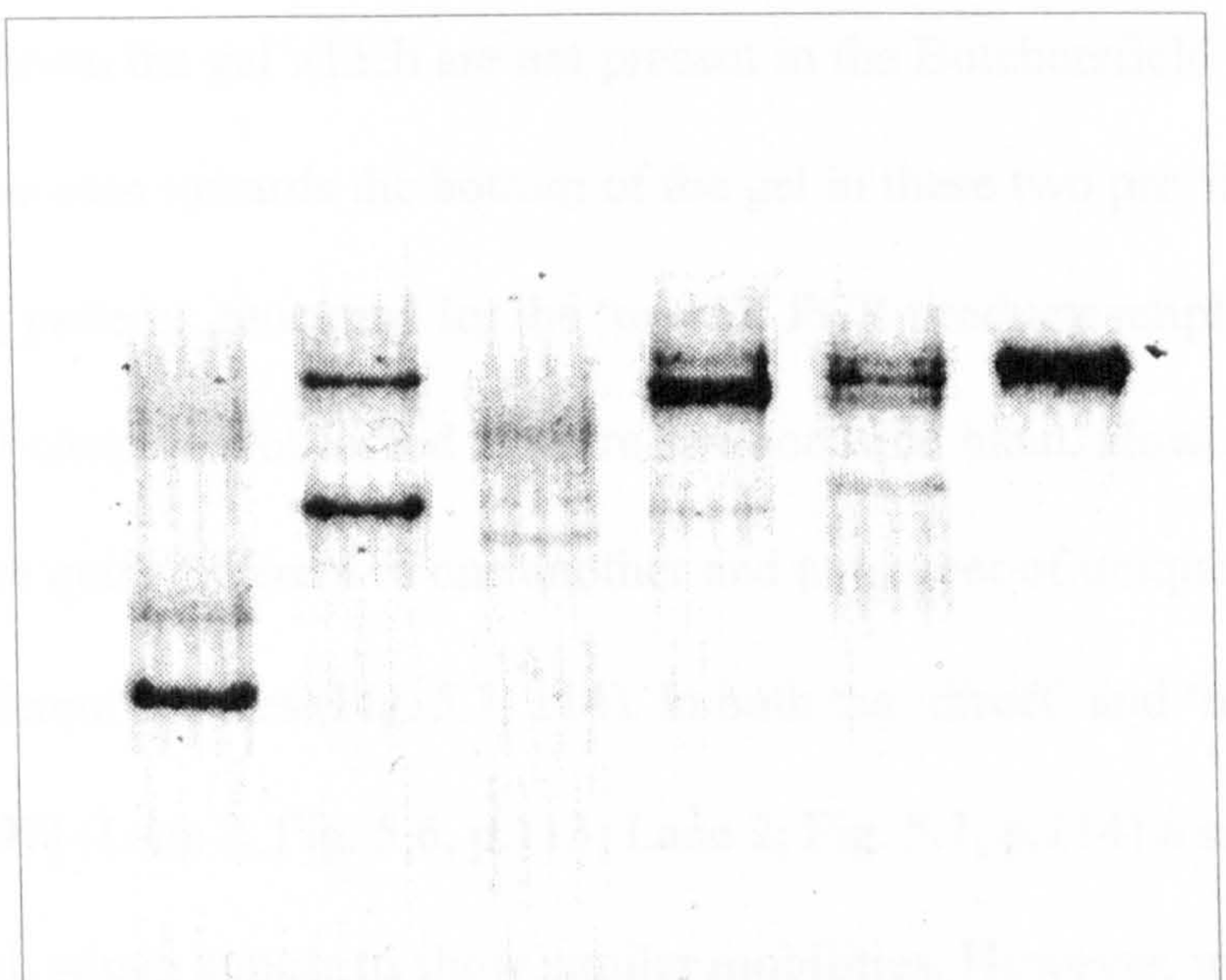


Figure 5.5. Temporal Thermal Gel Electrophoresis of DSB (Group 4) 'nested' PCR products amplified from landfill leachate.

Lane 1 - Pilsworth; Lane 2 - Butchersfield [97]; Lane 3 - Buff Quarry; Lane 4 - West Leigh; Lane 5 - Butchersfield [98]; Lane 6 - *Dsb. curvatus* (control)

Pilsworth profile and only one other band in the Chadderton profile. Banding patterns generated from Butchersfield [97] and West Leigh landfill sites show that they share the same major band. However, the West Leigh profile also contains two other bands slightly further down the gel which are not present in the Butchersfield profile. Faint bands can also be seen towards the bottom of the gel in these two profiles (Fig. 5.6, p.113). Banding patterns generated for the 'nested' PCR products amplified from six out of seven landfill sites were observed to share one common band. However, despite this they appear to be quite different to one another and a number of unique bands can be observed in different profiles (Fig. 5.7, 114). In both the 'direct' and 'nested' profiles for Butchersfield [97] (Lane 2, Fig. 5.6, p.113; Lane 2, Fig. 5.7, p.114) a single band is observed in each which appear to show similar mobilities. However, in comparison with the mobility of the control (*Dss. variabilis*) it can be seen that the 'nested' band has migrated slightly further down the gel than the 'direct' band, suggesting that the bands are of different sequence and that the 'direct' band is not amplified via the 'nested' PCR approach.

***Desulfovibrio-Desulfomicrobium* (DSV-DMB Group 6)**: No 'direct' PCR products were obtained for this subgroup. TTGE profiles were obtained from 'nested' *Desulfovibrio-Desulfomicrobium* (DSV-DMB Group 6) PCR products amplified from six out of seven landfill sites. Banding patterns showed all profiles to share one common band, while the profiles for the Risley, West Leigh and Butchersfield [98] sites seem to share a double band. Despite this, however, the profiles were quite dissimilar to one another and a number of unique bands could be observed (Fig. 5.8, p.115).

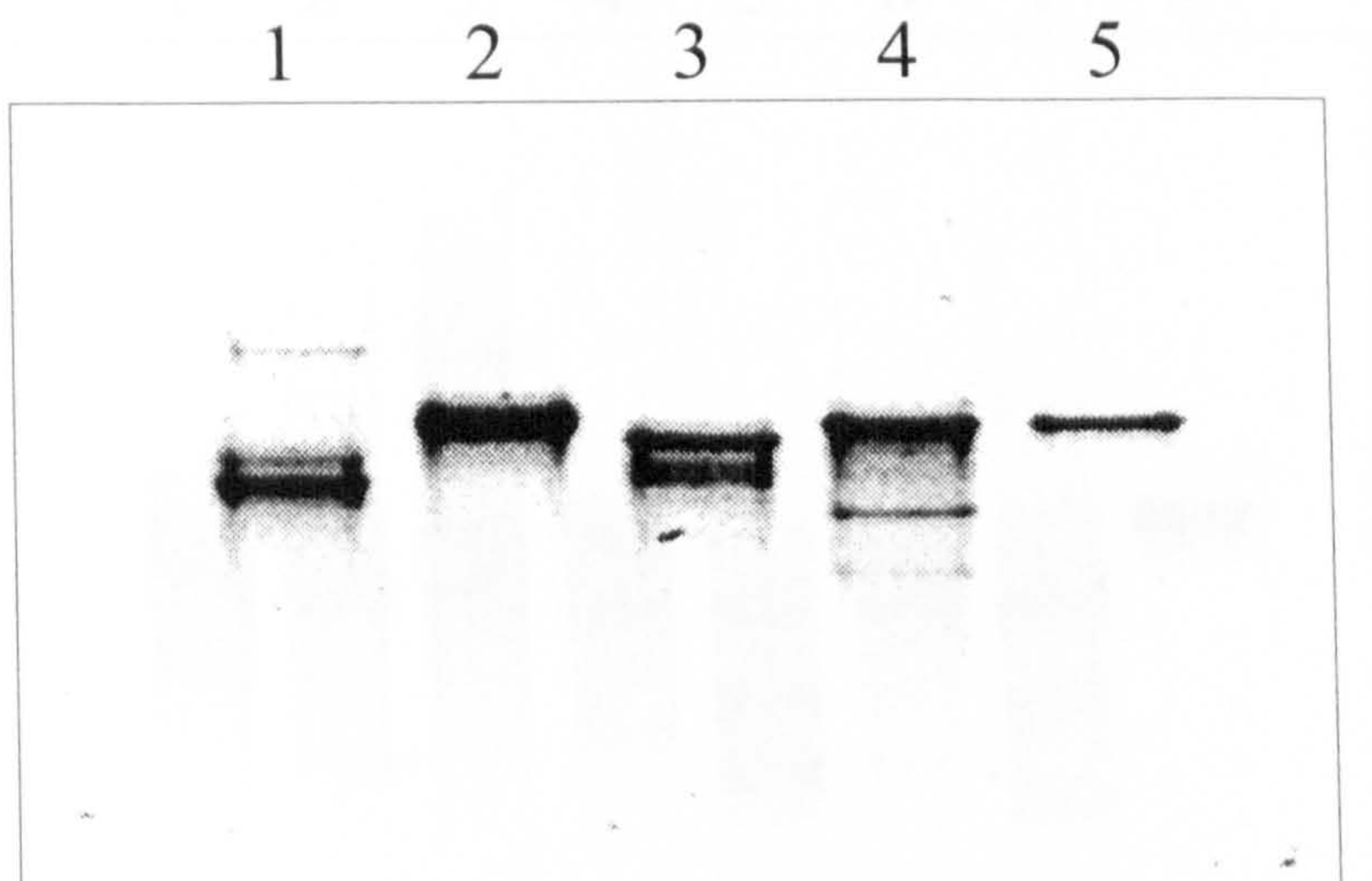


Figure 5.6. Temporal Thermal Gel Electrophoresis of DCC-DNM-DSS (Group 5) 'direct' PCR products amplified from landfill leachate.

Lane 1 - Pilsworth; Lane 2 - Butchersfield [97]; Lane 3 - Chadderton; Lane 4 - West Leigh; Lane 5 - *Dss. variabilis* (control)

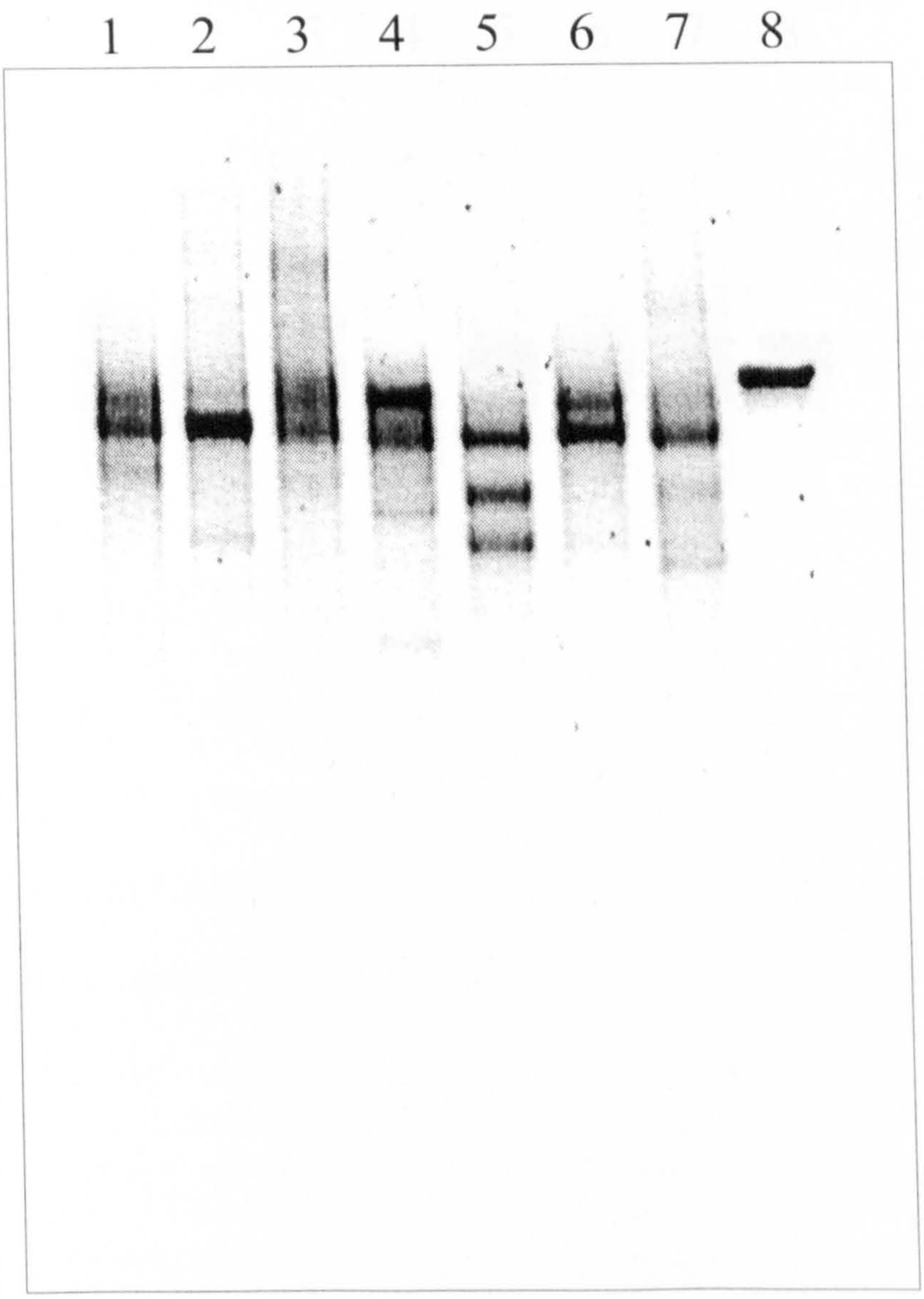


Figure 5.7. Temporal Thermal Gel Electrophoresis of DCC-DNM-DSS (Group 5) 'nested' PCR products amplified from landfill leachate.

Lane 1 - Pilsworth; Lane 2 - Butchersfield [97]; Lane 3 - Risley;
Lane 4 - Chadderton; Lane 5 - Holiday Moss; Lane 6 - West Leigh;
Lane 7 - Butchersfield [98]; Lane 8 - *Dss. variabilis* (control)

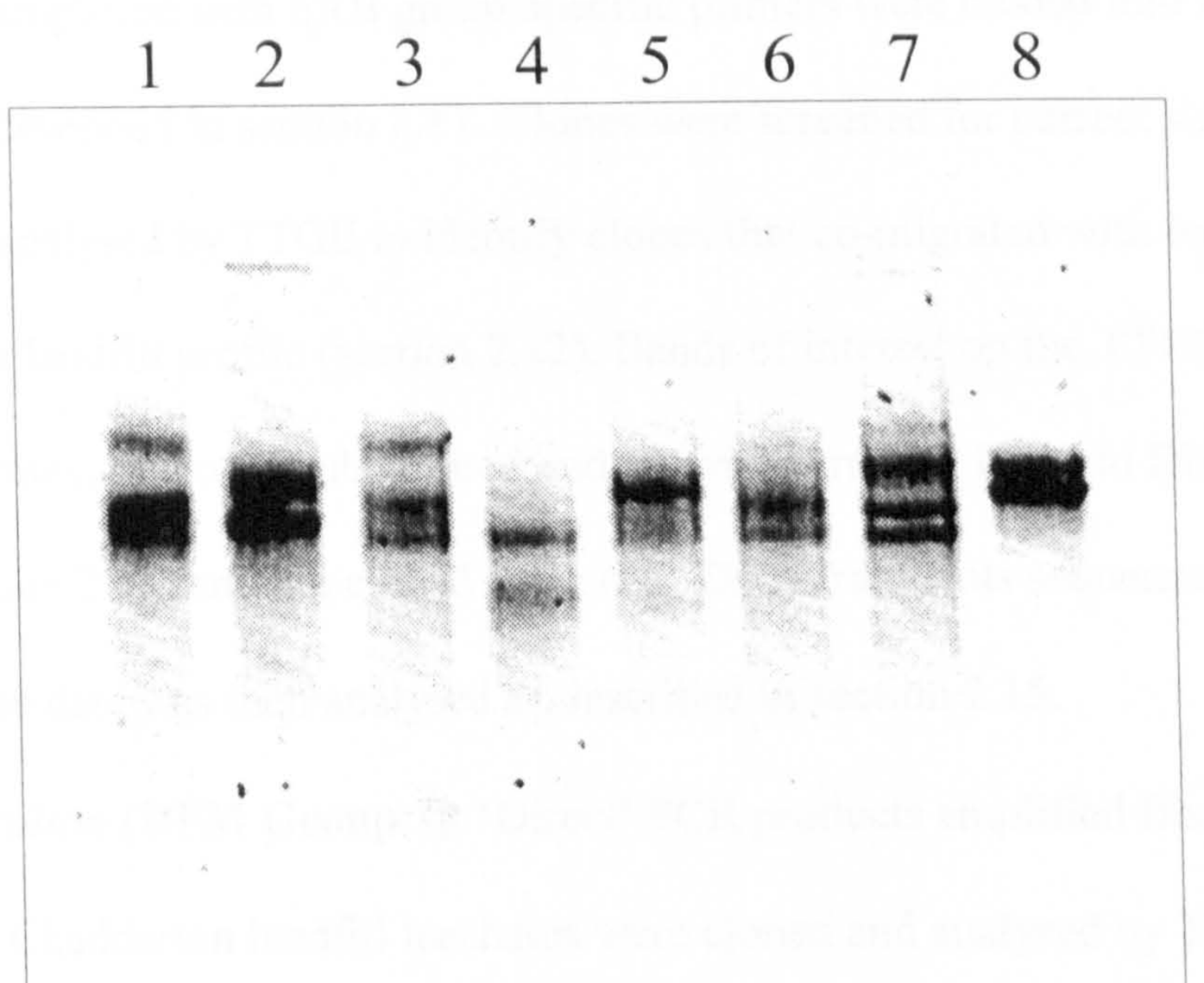


Figure 5.8. Temporal Thermal Gel Electrophoresis of DSV-DMB (Group 6) 'nested' PCR products amplified from landfill leachate.

Lane 1 - Pilsworth; Lane 2 - Butchersfield [97]; Lane 3 - Risley;
Lane 4 - Chadderton; Lane 5 - Holiday Moss; Lane 6 - West Leigh;
Lane 7 - Butchersfield [98]; Lane 8 - *Dsv. desulfuricans* (control)

5.2.2. Sequence analysis of cloned SRB DNA fragments

PCR products amplified with SRB group-specific primers were cloned into competent *E.coli* cells as described in section 2.11. Clones were screened for correct sized inserts by PCR and then analysed by TTGE to identify clones that co-migrated with bands observed in the landfill profile (section 2.12). Bands of interest on the TTGE gels were noted and the relevant clones subcultured and grown overnight. Plasmid DNA was extracted (section 2.13) and the cloned SRB 16S rDNA fragments sequenced (section 2.14). Sequence data was then analysed as described in section 2.15.

***Desulfotomaculum* (DFM Group 1)**: 'Direct' PCR products amplified from the Pilsworth and Chadderton landfill leachates were cloned and analysed by TTGE (Figs. 5.9 & 5.10, p.117 & 118). Clones 'dfmp4'; 'dfmp6'; 'dfmp7' (from Pilsworth) and 'dfmc7'; 'dfmc8' (from Chadderton) were sequenced. Bootstrap consensus trees generated by DNA distance and maximum parsimony analysis showed similar topologies and bootstrap values to one another (Figs 5.11 & 5.12, p.119 & 120). The branching order of the trees demonstrates that the sequenced 16S rDNA fragments amplified from each landfill site respectively by the DFM (Group 1)-specific PCR primers segregate together in different clusters within the DFM group, forming novel lineages and are related to known DFM (Group 1) members.

***Desulfobulbus* (DBB Group 2)**: Cloned PCR products amplified from the Pilsworth and Risley landfill site leachates were analysed by TTGE (Figs. 5.13 & 5.14, p.121 & 122). Clones 'dbbp1'; 'dbbp2'; 'dbbp4' (from Pilsworth) and 'dbbr1'; 'dbbr2' (from Risley) were sequenced. DNA distance and maximum parsimony analysis produced trees with

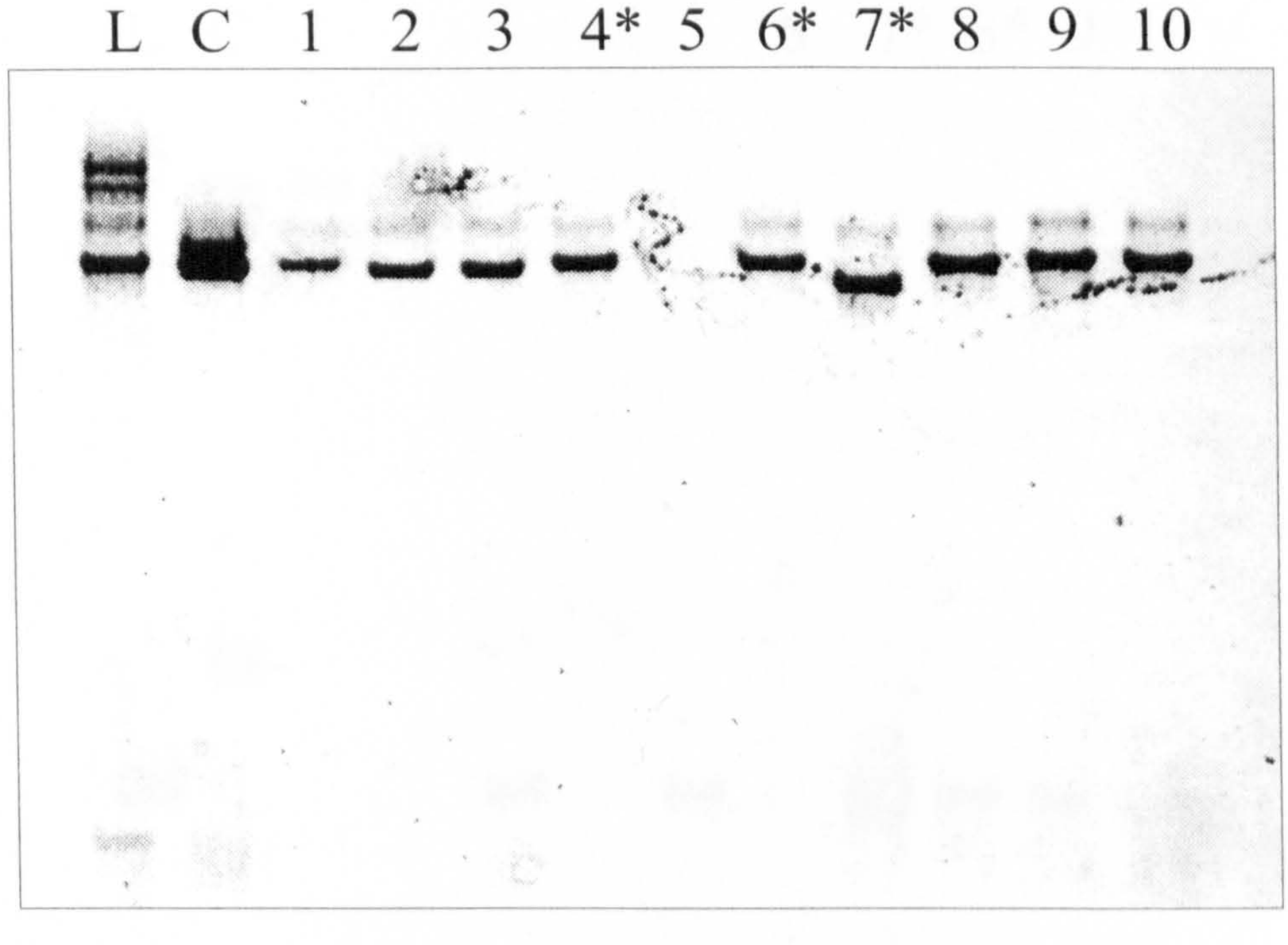


Figure 5.9. Temporal Thermal Gel Electrophoresis screening of landfill 16S rDNA clones generated using DFM (Group 1) 'direct' PCR products from Pilsworth landfill site.

L - Pilsworth landfill; C - *Dfm. nigrificans* (control); Lanes 1-10 - Pilsworth landfill clones (dfmp1-dfmp10).
* - clones selected for sequence analysis

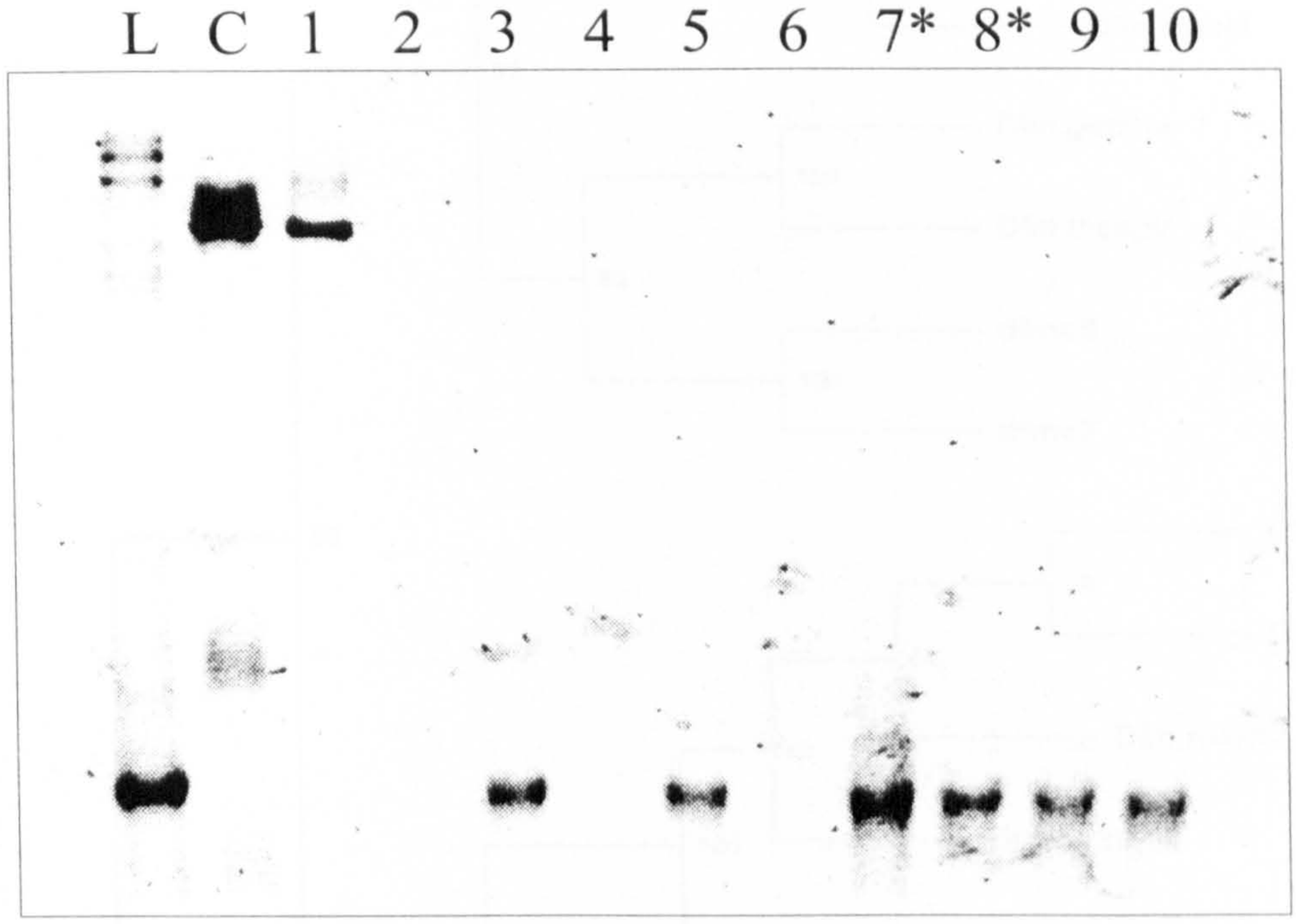


Figure 5.10. Temporal Thermal Gel Electrophoresis screening of landfill 16S rDNA clones generated using DFM (Group 1) 'direct' PCR products from Chadderton landfill site.

L - Chadderton landfill; C - *Dfm. nigrificans* (control); Lanes 1-10 - Chadderton landfill clones (dfmc1-dfmc10).
* - clones selected for sequence analysis

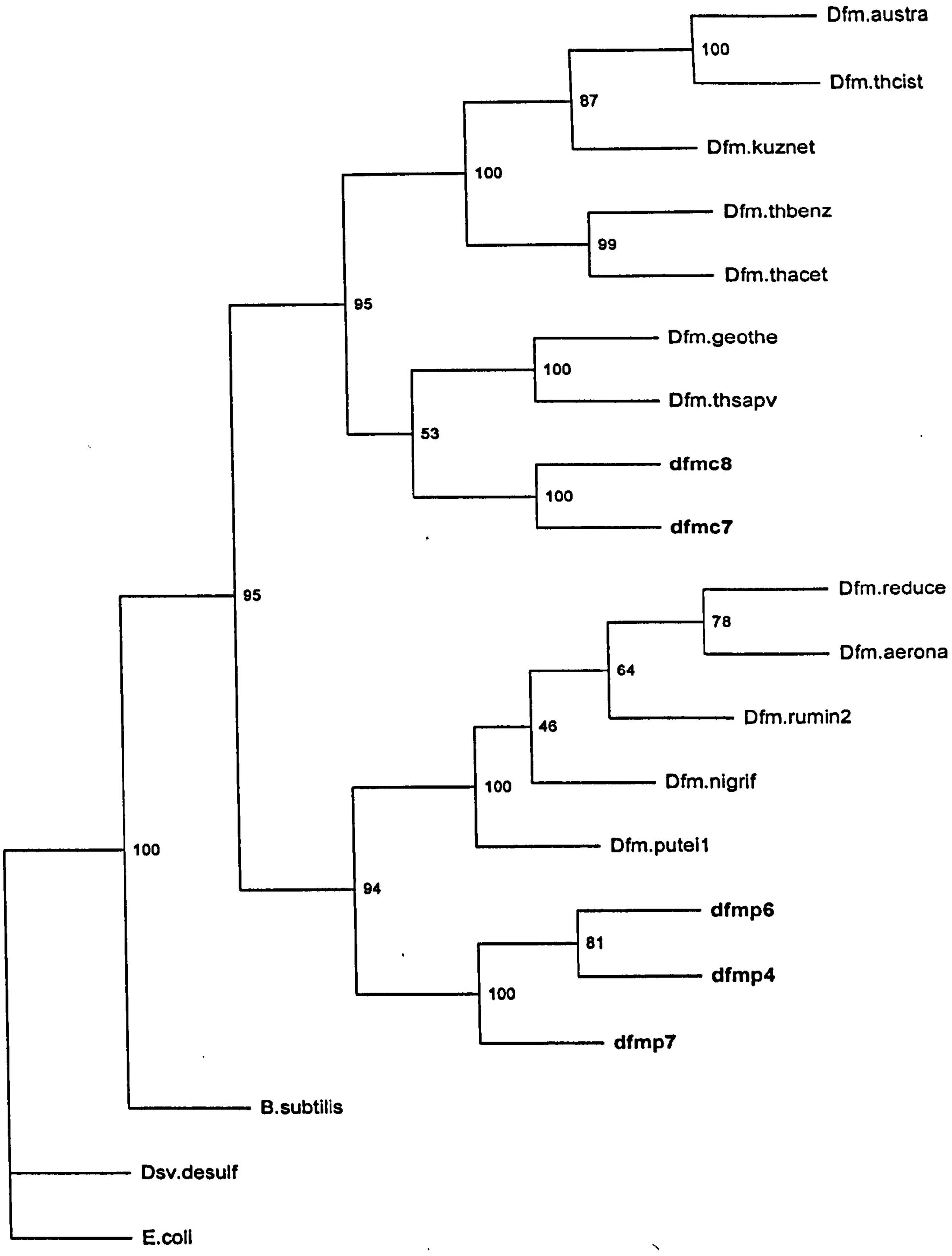


Figure 5.11. 16S rDNA bootstrap consensus tree of *Desulfotomaculum* (DFM Group 1) members and DFM (Group 1) clones derived from PCR products amplified from the Pilsworth and Chadderton landfill sites.
The tree was constructed using the neighbour-joining method of Jukes and Cantor (1969) and analysis was based on 625 nucleotides. Bootstrap values (out of 100 trees) are shown adjacent to nodes.

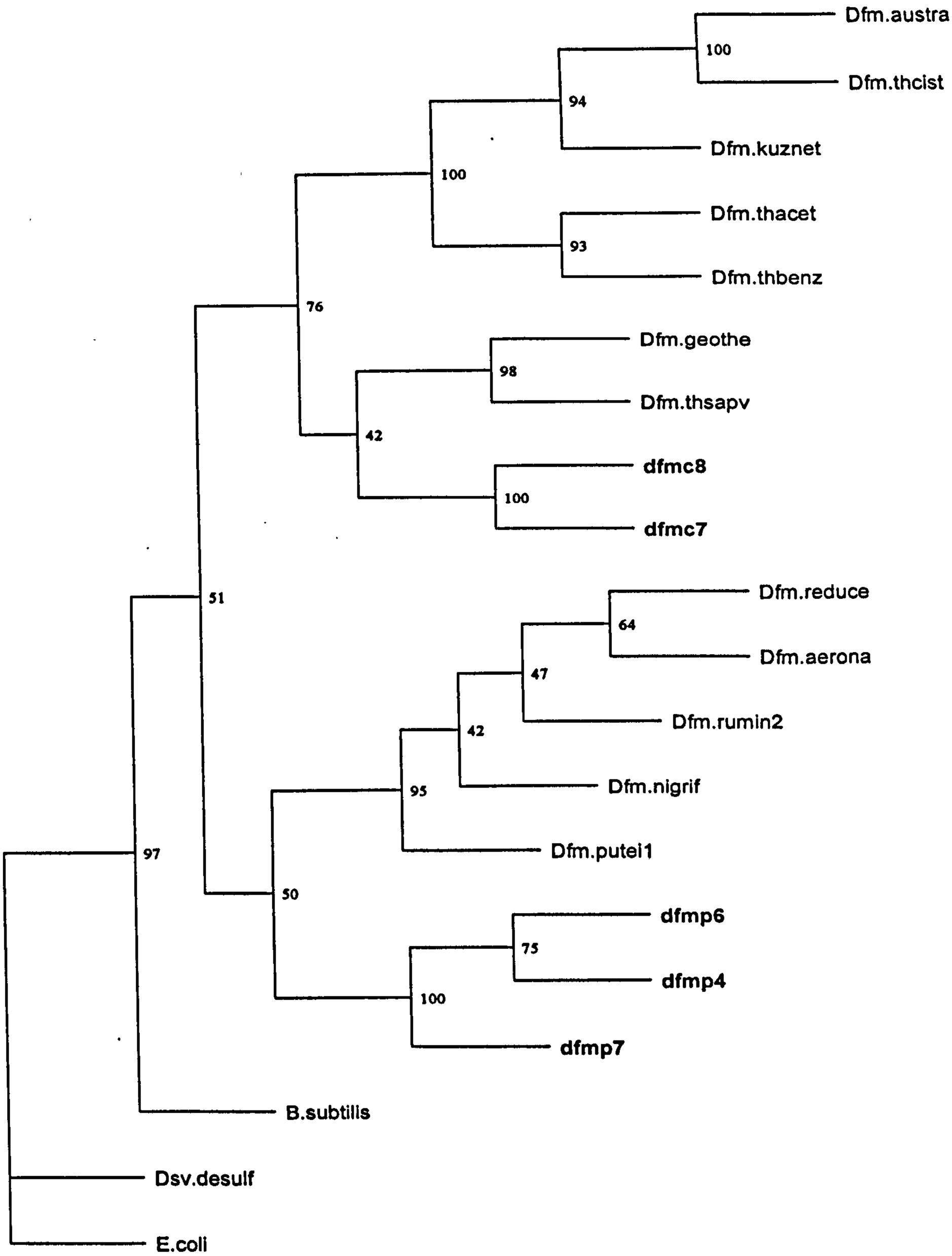


Figure 5.12. 16S rDNA bootstrap consensus tree of *Desulfotomaculum* (DFM Group 1) members and DFM (Group 1) clones derived from PCR products amplified from the Pilsworth and Chadderton landfill sites.
The tree was constructed using a maximum parsimony method (Swofford, 1991) and analysis was based on 625 nucleotides. Bootstrap values (out of 100 trees) are shown adjacent to nodes.

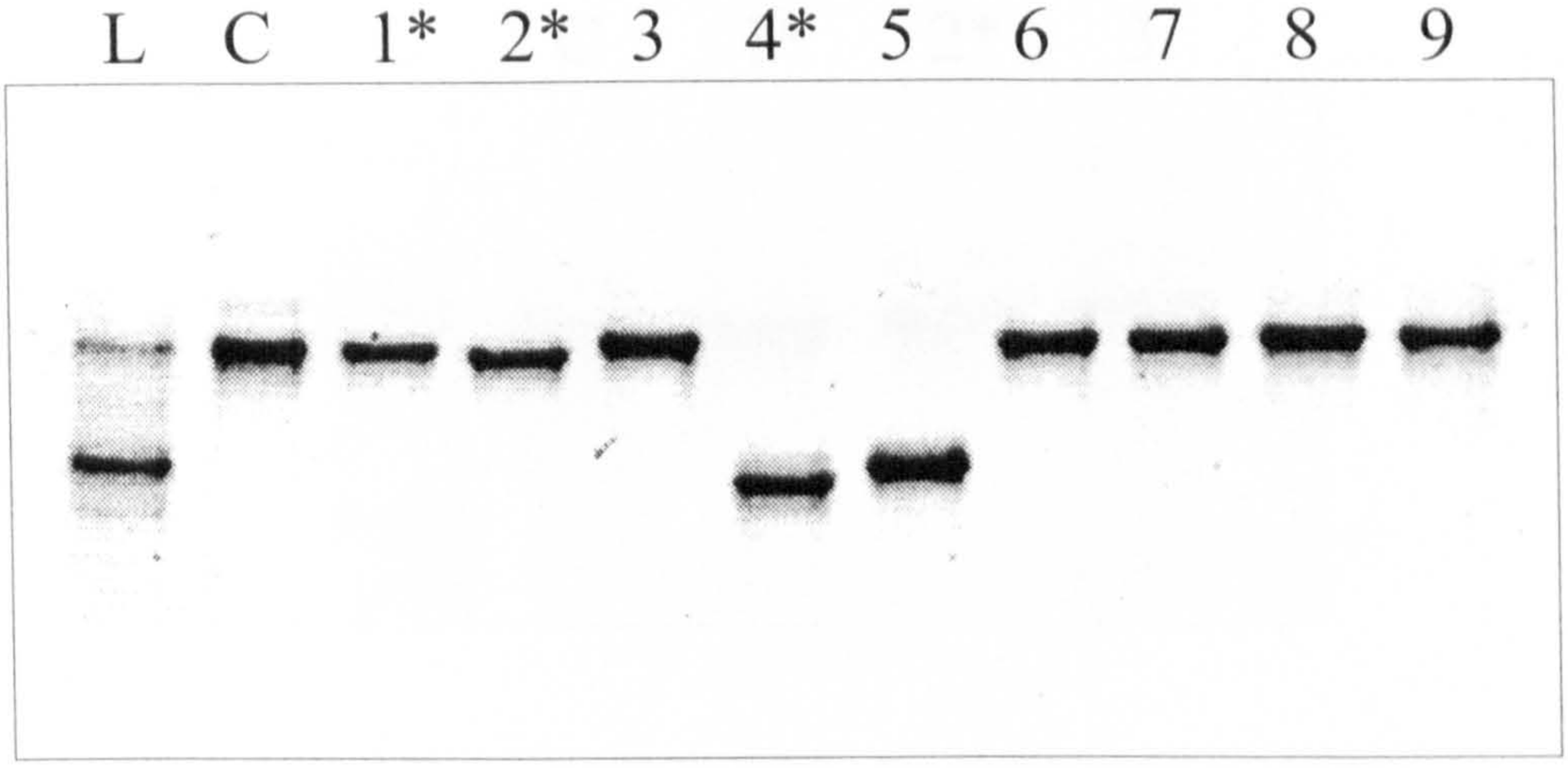


Figure 5.13. Temporal Thermal Gel Electrophoresis screening of landfill 16S rDNA clones generated using DBB (Group 2) 'nested' PCR products from Pilsworth landfill site.

L - Pilsworth landfill; C - *Dbb. propionicus* (control); Lanes 1-9 - Pilsworth landfill clones (dbbp1-dbbp9).
* - clones selected for sequence analysis

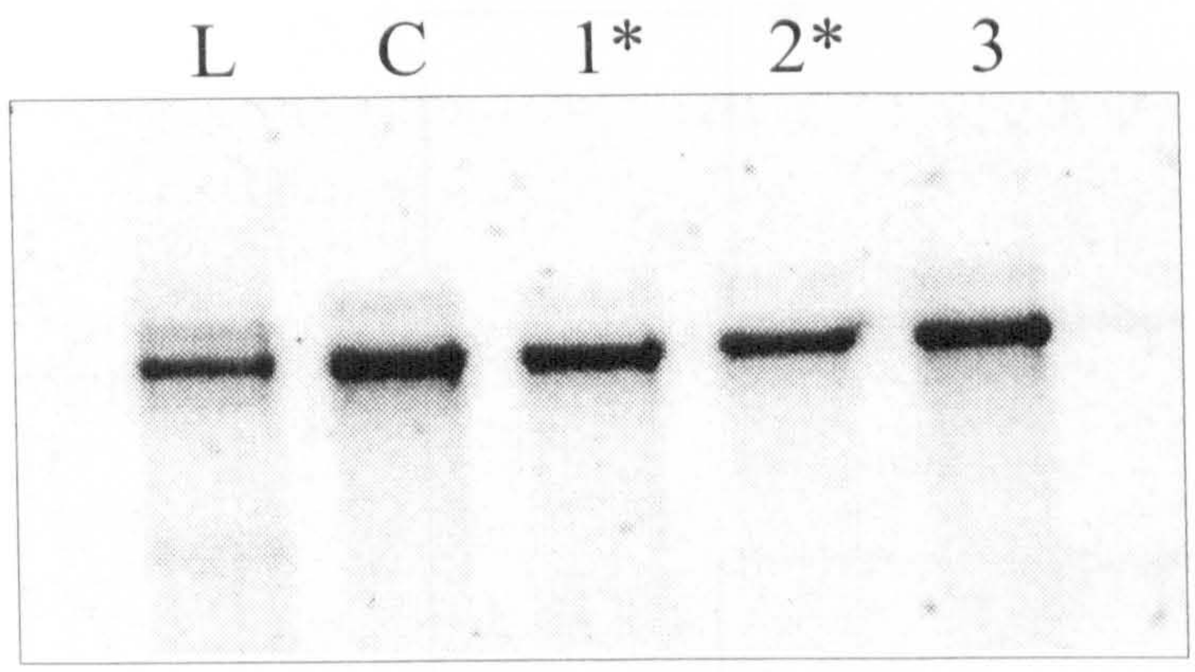


Figure 5.14. Temporal Thermal Gel Electrophoresis screening of landfill 16S rDNA clones generated using DBB (Group 2) 'nested' PCR products from Risley landfill site.

L - Risley landfill; C - *Dbb. propionicus* (control); Lanes 1-3 - Risley landfill clones (dbbr1-dbbr3).
* - clones selected for sequence analysis

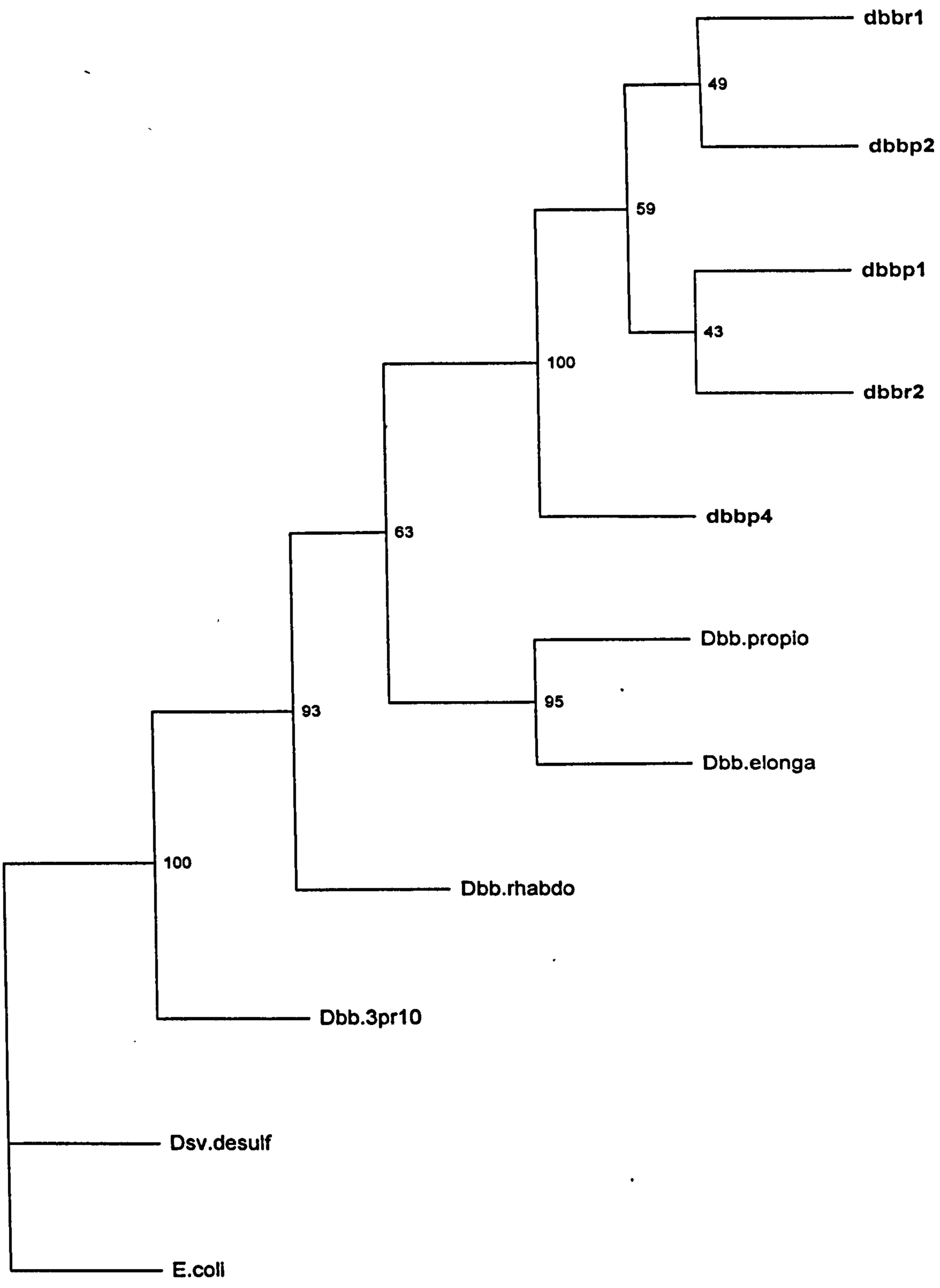


Figure 5.15. 16S rDNA bootstrap consensus tree of *Desulfobulbus* (DBB Group 2) members and DBB (Group 2) clones derived from PCR products amplified from the Pilsworth and Risley landfill sites.
The tree was constructed using the neighbour-joining method of Jukes and Cantor (1969) and analysis was based on 1022 nucleotides. Bootstrap values (out of 100 trees) are shown adjacent to nodes.

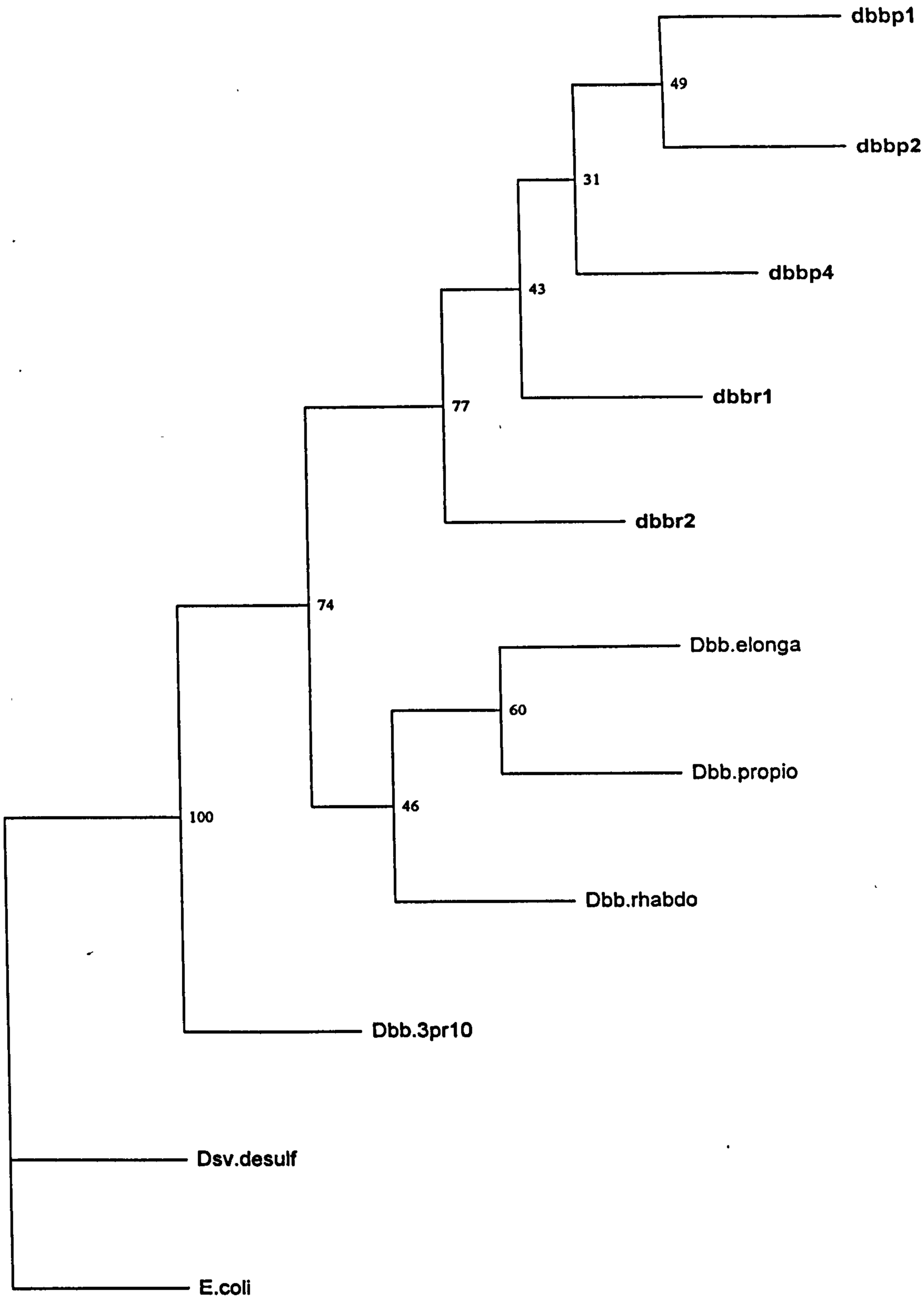


Figure 5.16. 16S rDNA bootstrap consensus tree of *Desulfobulbus* (DBB Group 2) members and DBB (Group 2) clones derived from PCR products amplified from the Pilsworth and Risley landfill sites.
The tree was constructed using a maximum parsimony method (Swofford, 1991) and analysis was based on 1022 nucleotides. Bootstrap values (out of 100 trees) are shown adjacent to nodes.

similar topologies and bootstrap values to one another (Figs. 5.15 & 5.16, p.123 & 124). The sequenced fragments formed a coherent cluster some distance away from the other DBB (Group 2) known members. However, FastA analysis of the cloned sequences showed that their nearest known relative was *Desulfobulbus elongatus* (*ca.* 91%-95% sequence similarity over 1140 bp) (Table 5.1, p.138), demonstrating that the sequences form a novel lineage within the DBB subgroup.

Desulfobacter **(DSB Group 4)**: 'Direct' PCR products amplified from the Butchersfield [98] landfill site were cloned and analysed by TTGE (Fig. 5.17, p.126). Clones 'dsbb1' and 'dsbb2' were sequenced. Bootstrap consensus trees generated by DNA distance and maximum parsimony analysis showed similar topologies and bootstrap values to one another (Figs. 5.18 & 5.19, p.127 & 128). The two fragments sequenced cluster within the DSB subgroup showing strong homology to the 16S rDNA sequence of *Desulfobacter postgatei* (clone 'dsbb1' showing 98.2% sequence similarity over 1167 bp) (Table 5.1, p.138).

Desulfococcus-Desulfonema-Desulfosarcina **(DCC-DNM-DSS Group 5)**: 'Direct' PCR products amplified from the Pilsworth and West Leigh landfill sites were cloned and screened by TTGE (Figs. 5.20 & 5.21, p.129 & 130). Clones 'dccp2'; 'dccp4'; 'dccp9' (from Pilsworth) and 'dccw2'; 'dccw3' (from West Leigh) were sequenced. DNA distance and maximum parsimony analysis produced trees with similar topologies and bootstrap values to one another (Figs. 5.22 & 5.23, p.131 & 132). Clone 'dccp4' was closely related to *Desulfococcus multivorans* (96.1% over 863 bp) (Table 5.1, p.138), while clones 'dccp2', 'dccp4' and 'dccw2', 'dccw3' respectively segregated together in different clusters forming novel lineages within the subgroup.

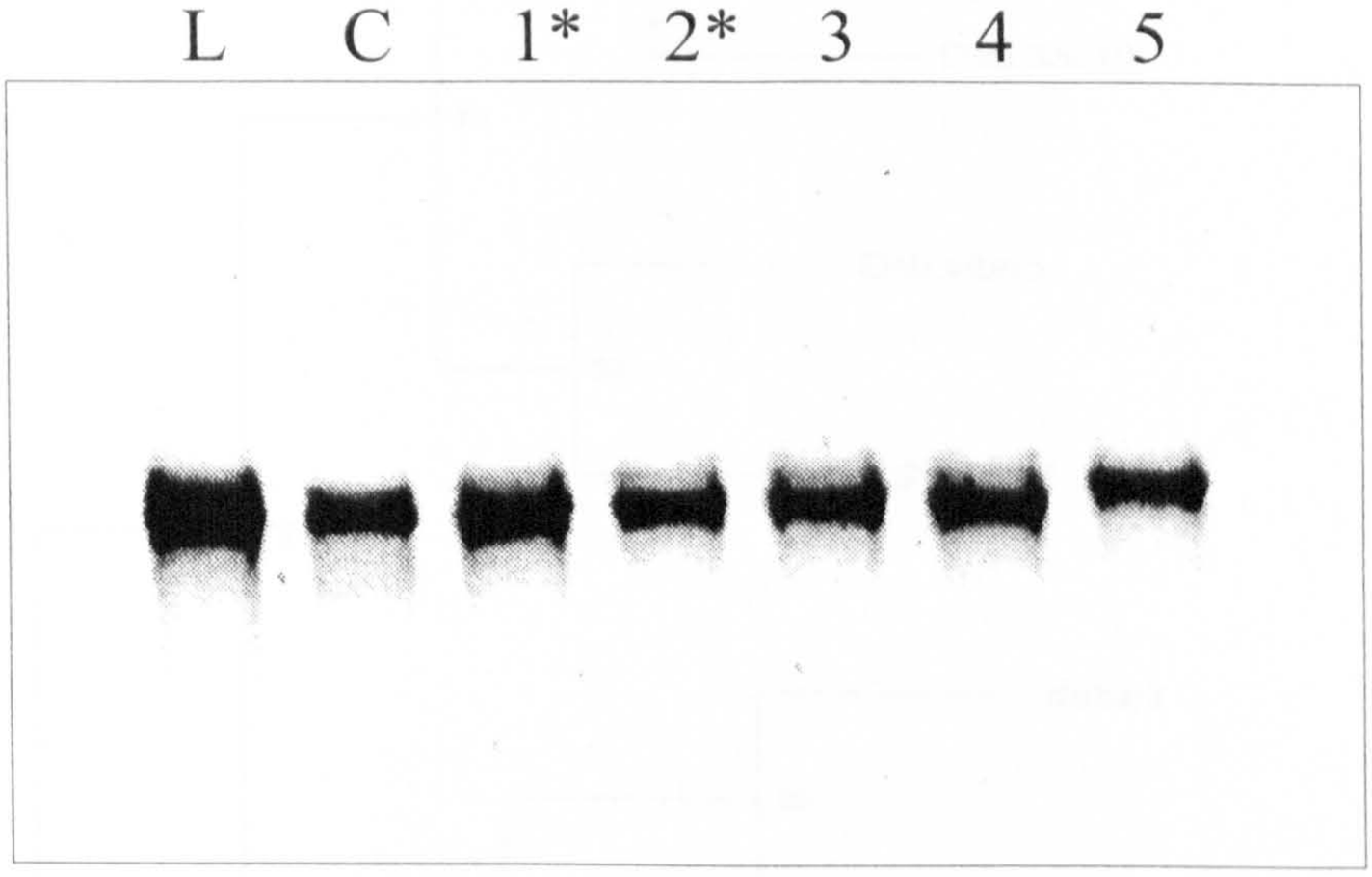


Figure 5.17. Temporal Thermal Gel Electrophoresis screening of landfill 16S rDNA clones generated using DSB (Group 4) 'direct' PCR products from Butchersfield [98] landfill site.

L - Butchersfield [98] landfill; C - *Dsb. curvatus* (control);
Lanes 1-5 - Butchersfield [98] landfill clones (dsbb1-dsbb5).
* - clones selected for sequence analysis

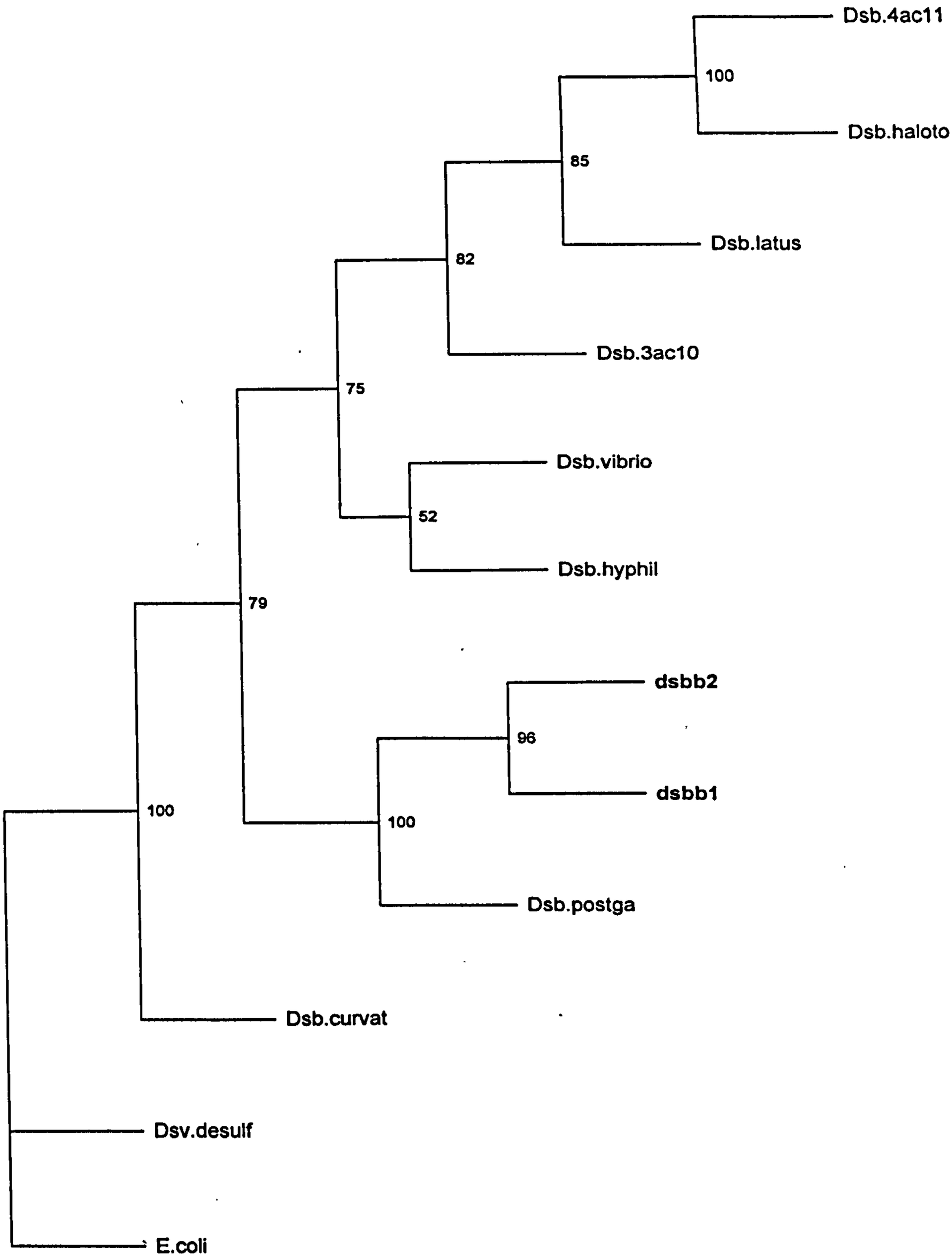


Figure 5.18. 16S rDNA bootstrap consensus tree of *Desulfobacter* (DSB Group 4) members and DSB (Group 4) clones derived from PCR products amplified from the Butchersfield [98] landfill site.
The tree was constructed using the neighbour-joining method of Jukes and Cantor (1969) and analysis was based on 811 nucleotides. Bootstrap values (out of 100 trees) are shown adjacent to nodes.

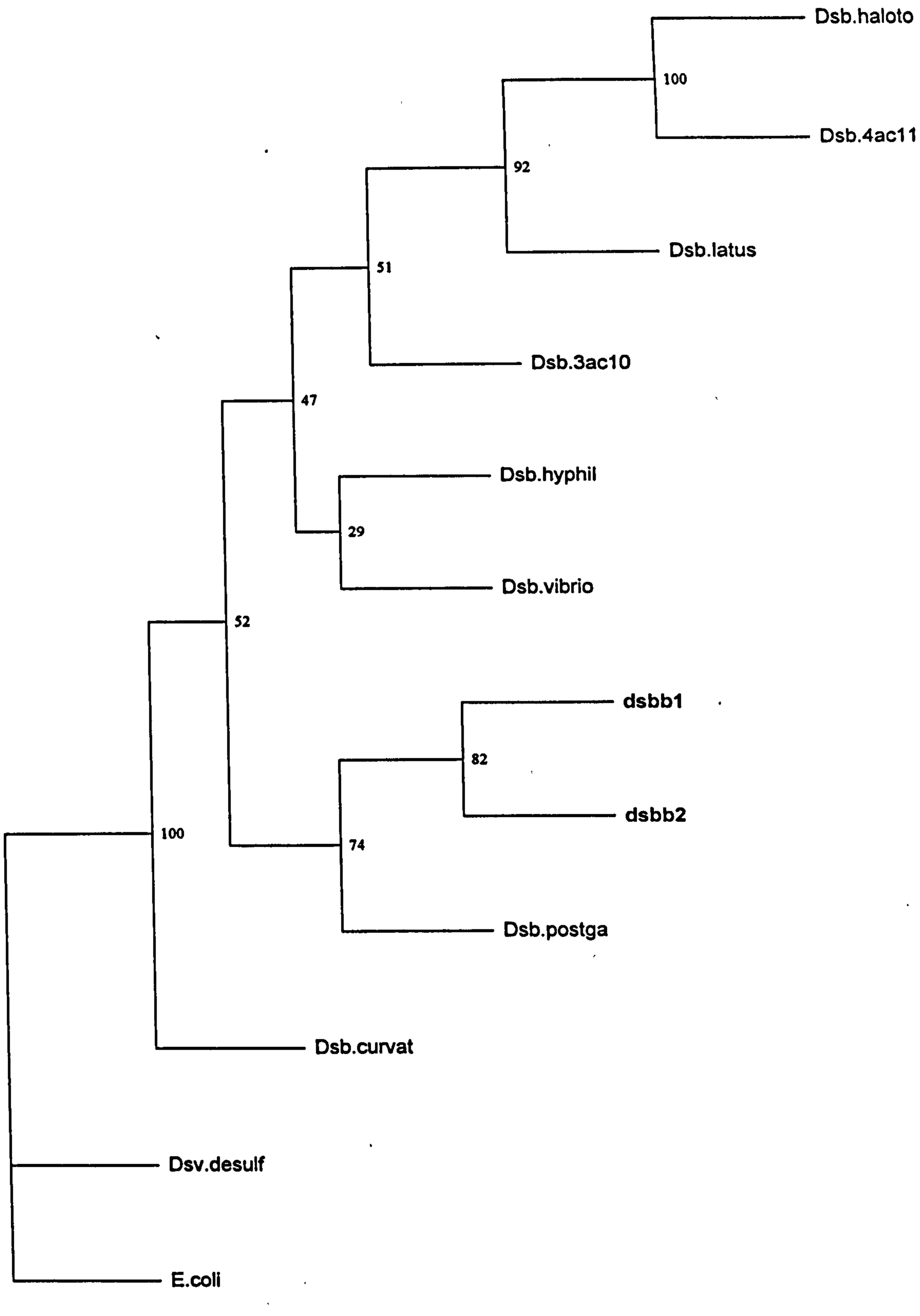


Figure 5.19. 16S rDNA bootstrap consensus tree of *Desulfobacter* (DSB Group 4) members and DSB (Group 4) clones derived from PCR products amplified from the Butchersfield [98] landfill site.
The tree was constructed using a maximum parsimony method (Swofford, 1991) and analysis was based on 811 nucleotides. Bootstrap values (out of 100 trees) are shown adjacent to nodes.

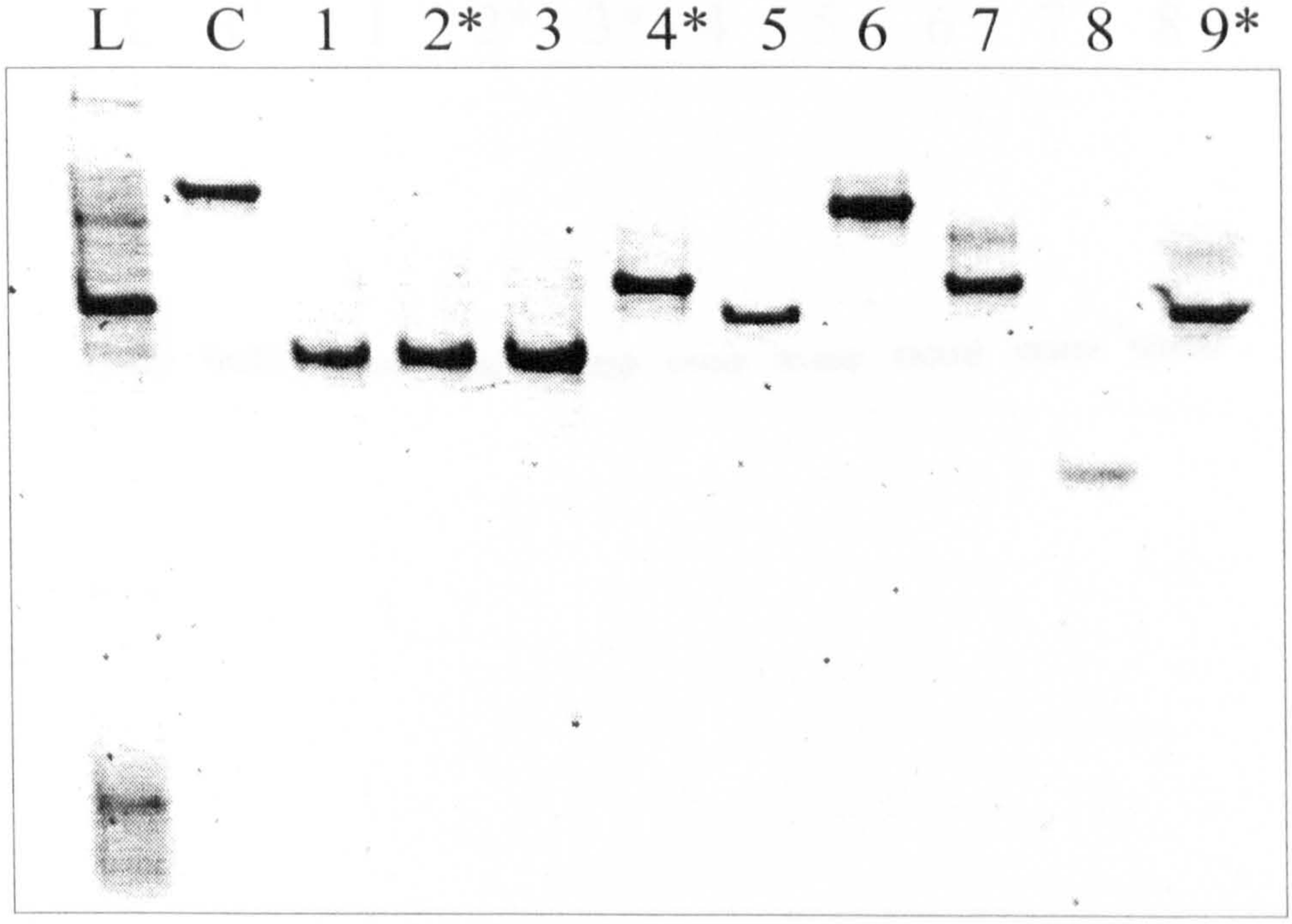


Figure 5.20. Temporal Thermal Gel Electrophoresis screening of landfill 16S rDNA clones generated using DCC-DNM-DSS (Group 5) 'direct' PCR products from Pilsworth landfill site.

L - Pilsworth landfill; C - *Dss. variabilis* (control); Lanes 1-9 - Pilsworth landfill clones (dccp1-dccp9).
* - clones selected for sequence analysis

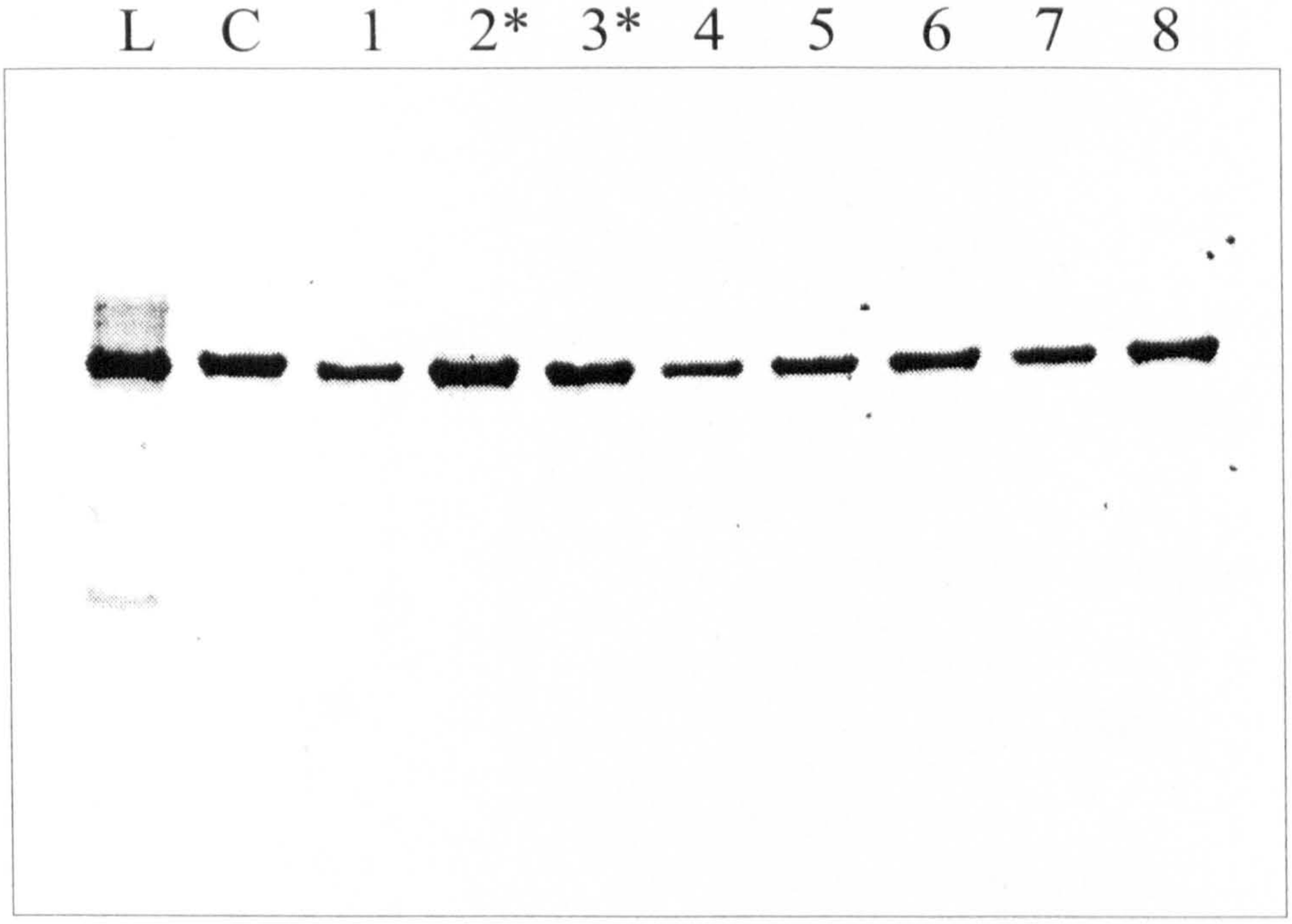


Figure 5.21. Temporal Thermal Gel Electrophoresis screening of landfill 16S rDNA clones generated using DCC-DNM-DSS (Group 5) 'direct' PCR products from West Leigh landfill site.

L - West Leigh landfill; C - *Dss. variabilis*; Lanes 1-8 - West Leigh landfill clones (dccw1-dccw8).
* - clones selected for sequence analysis

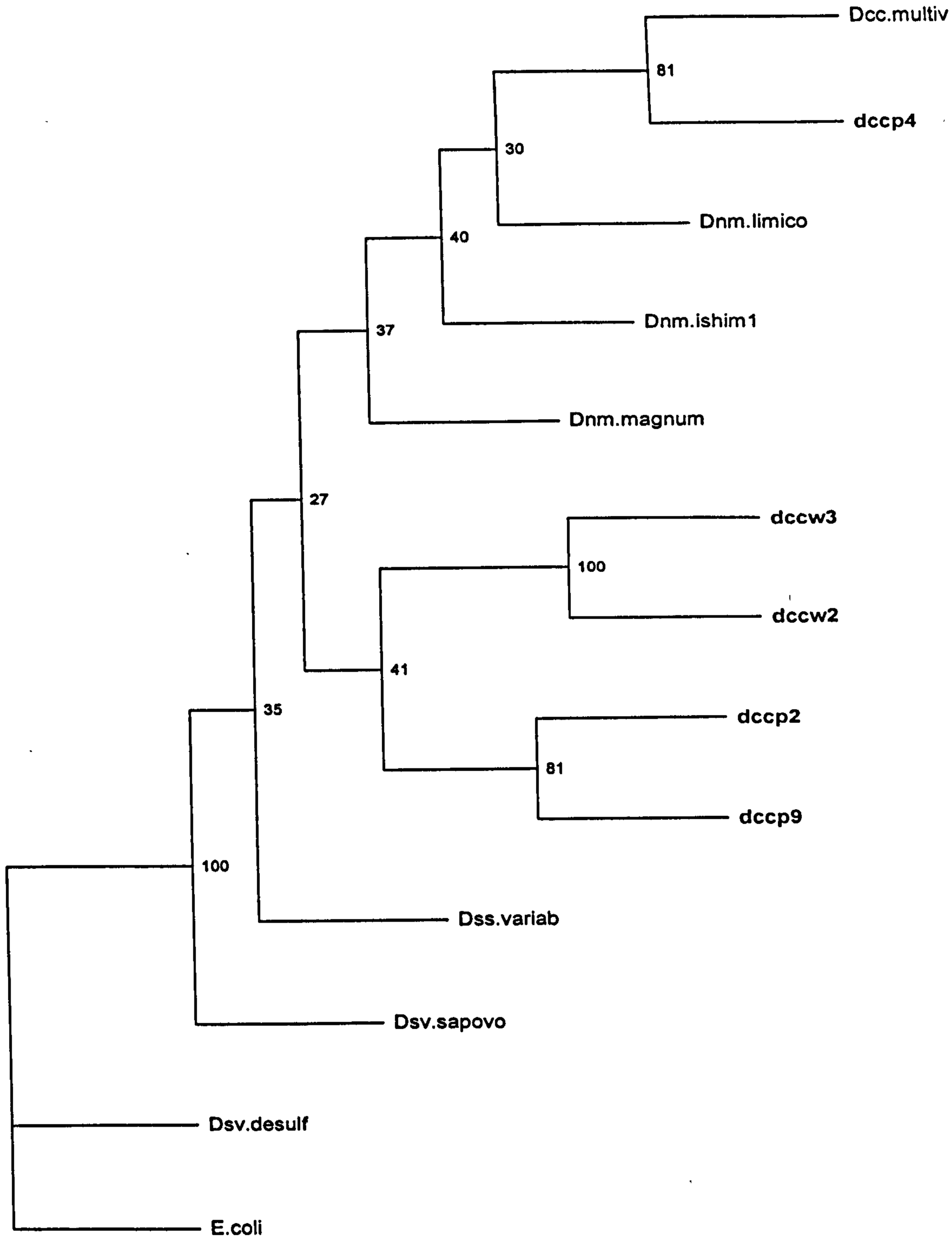


Figure 5.22. 16S rDNA bootstrap consensus tree of *Desulfococcus-Desulfonema-Desulfosarcina* (DCC-DNM-DSS Group 5) members and DCC-DNM-DSS (Group 5) clones derived from PCR products amplified from the Pilsworth and West Leigh landfill sites.
The tree was constructed using the neighbour-joining method of Jukes and Cantor (1969) and analysis was based on 850 nucleotides. Bootstrap values (out of 100 trees) are shown adjacent to nodes.

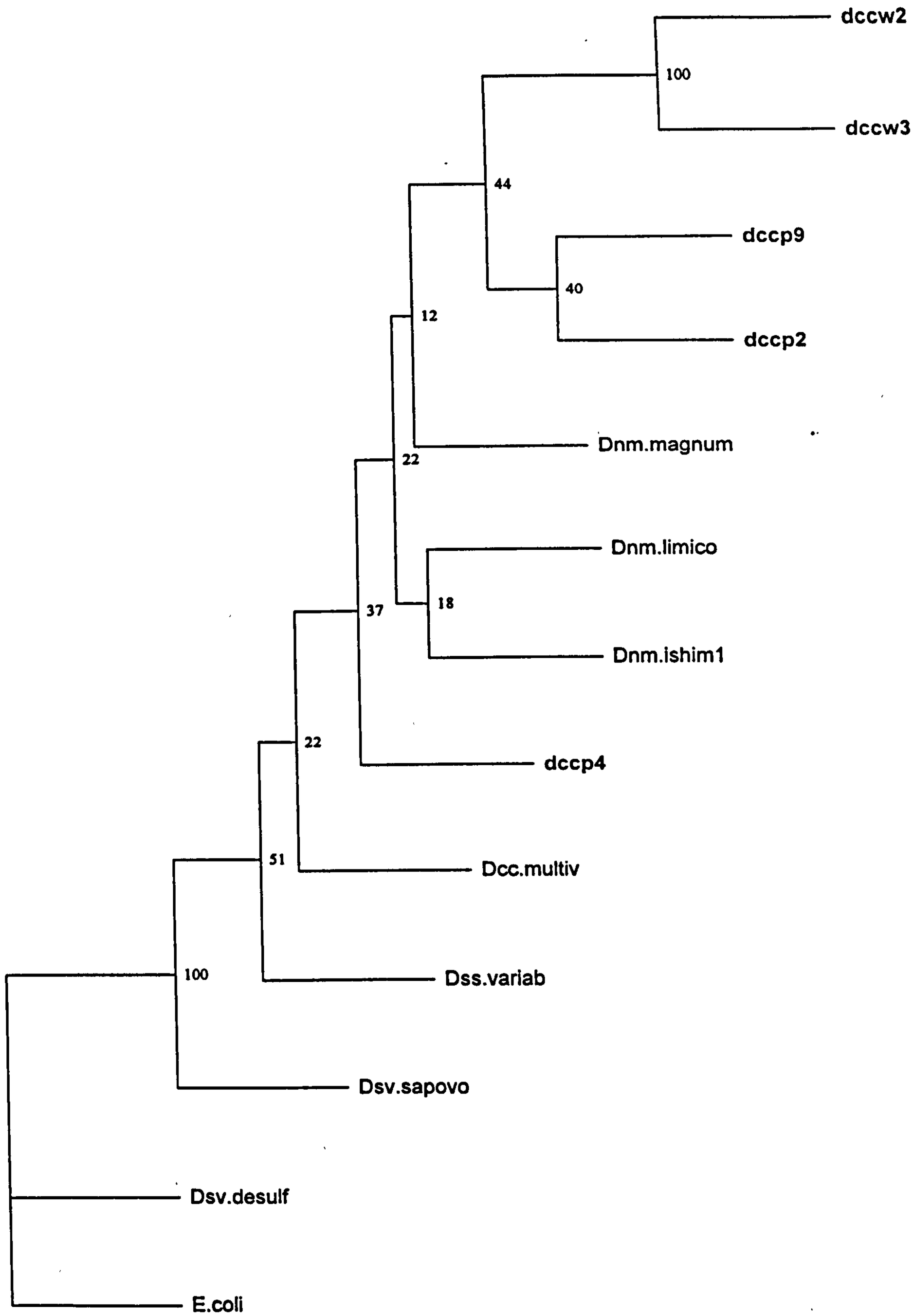


Figure 5.23. 16S rDNA bootstrap consensus tree of *Desulfococcus-Desulfonema-Desulfosarcina* (DCC-DNM-DSS Group 5) members and DCC-DNM-DSS (Group 5) clones derived from PCR products amplified from the Pilsworth and West Leigh landfill sites.
The tree was constructed using a maximum parsimony method (Swofford, 1991) and analysis was based on 850 nucleotides. Bootstrap values (out of 100 trees) are shown adjacent to nodes.

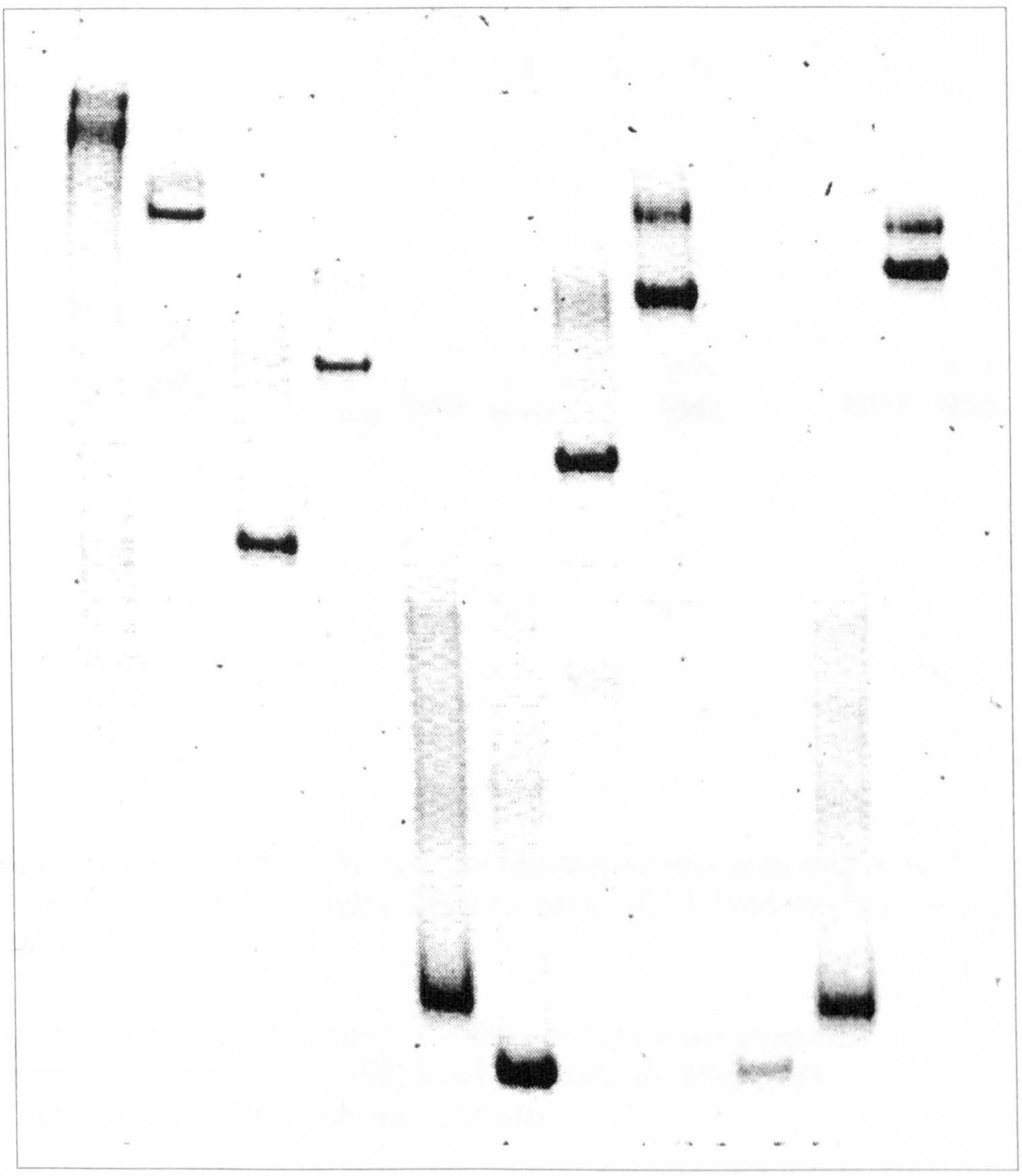


Figure 5.24. Temporal Thermal Gel Electrophoresis screening of landfill 16S rDNA clones generated using DSV-DMB (Group 6) 'nested' PCR products from Risley landfill site.

L - Risley landfill; C - *Dsv. desulfuricans* (control); Lanes 1-9 - Risley landfill clones (dsvr1-dsvr9).
* - clones selected for sequence analysis

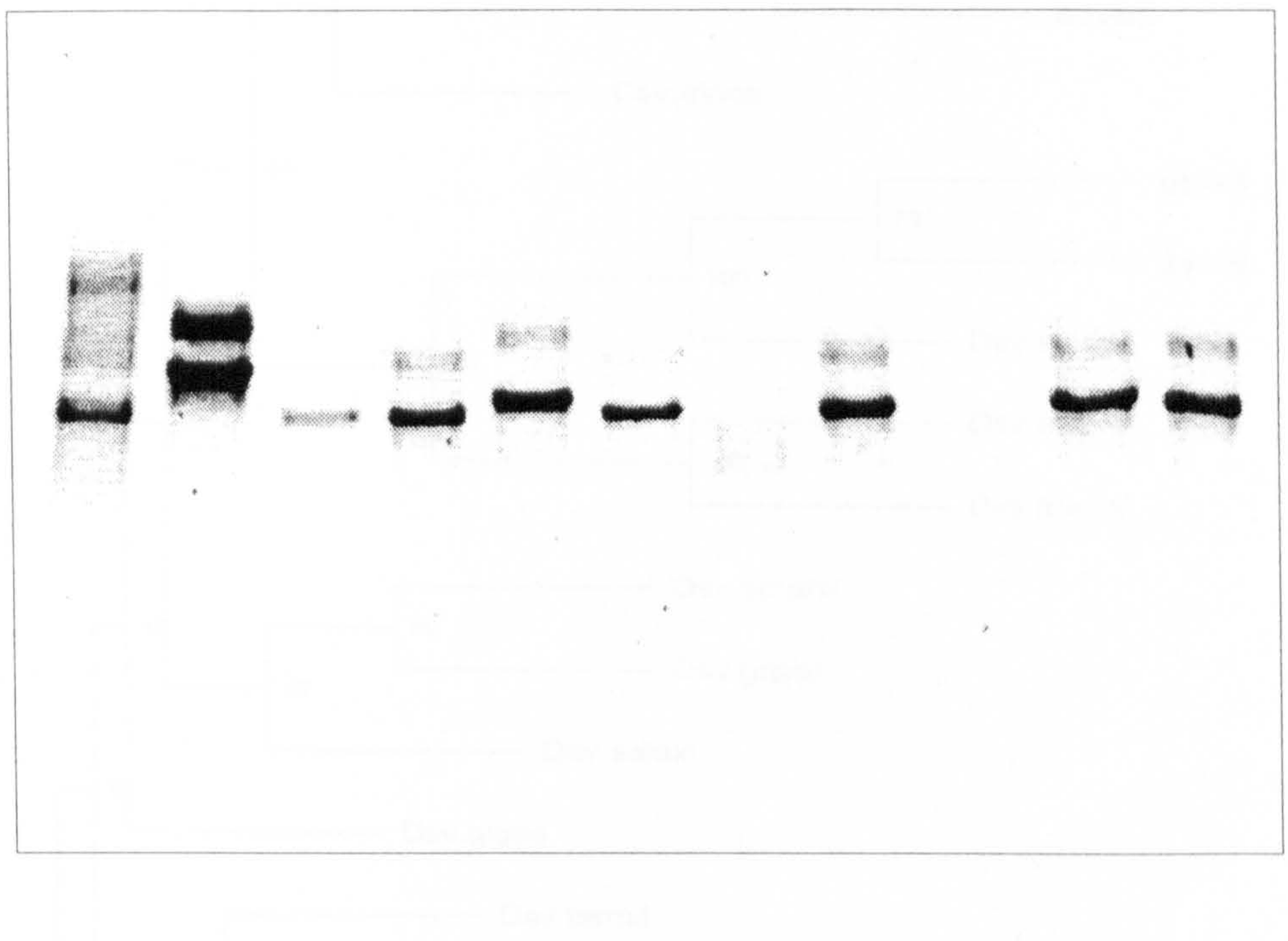


Figure 5.25. Temporal Thermal Gel Electrophoresis screening of landfill clones generated using DSV-DMB (Group 6) 'nested' PCR products from Butchersfield [98] landfill site.

L - Butchersfield [98] landfill; C - *Dsv. desulfuricans* (control);
Lanes 1-9 - Butchersfield [98] landfill clones (dsvb1-dsvb9).
* - clones selected for sequence analysis

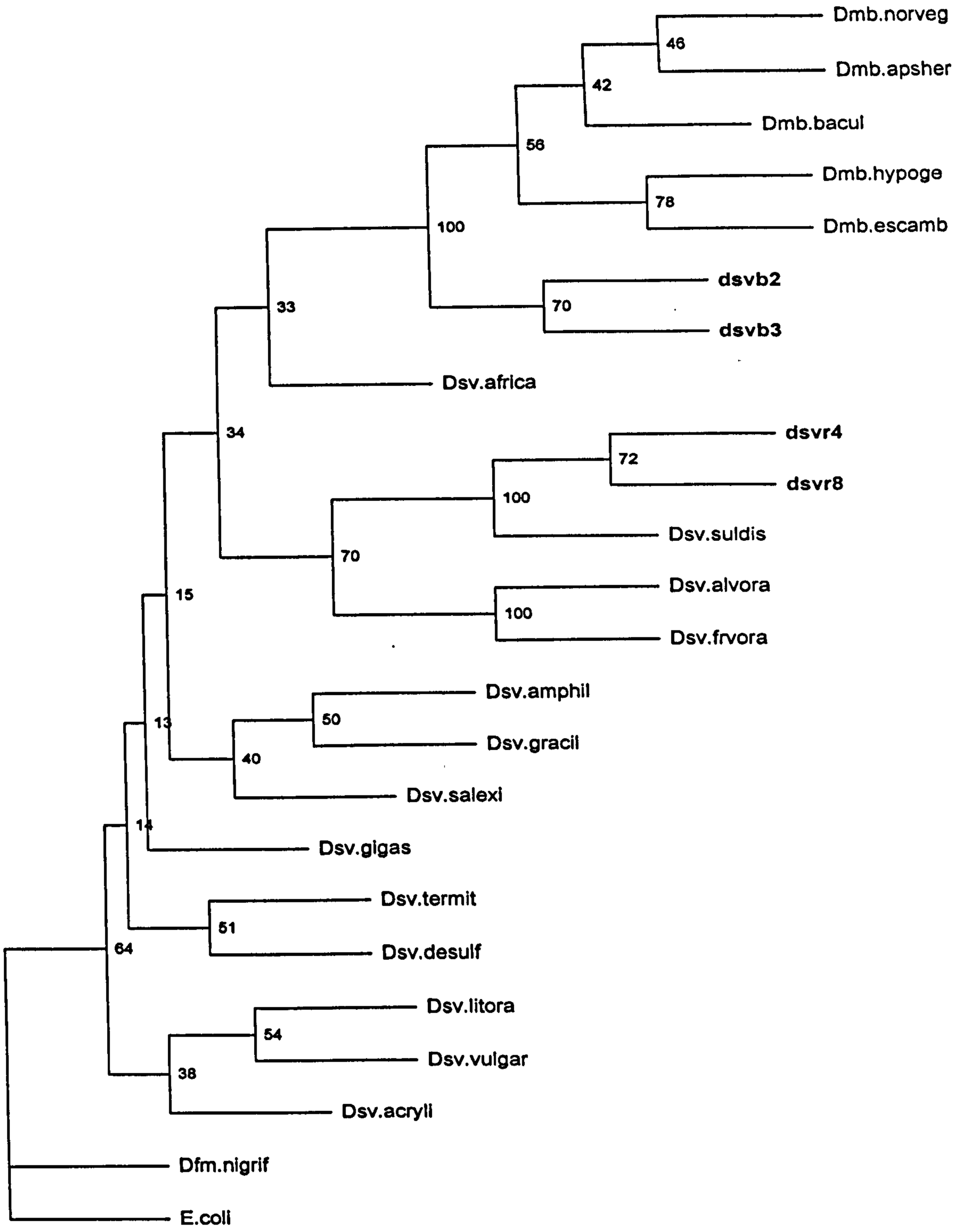


Figure 5.26. 16S rDNA bootstrap consensus tree of *Desulfovibrio-Desulfomicrobium* (DSV-DMB Group 6) members and DSV-DMB (Group 6) clones derived from PCR products amplified from the Risley and Butchersfield [98] landfill sites.
The tree was constructed using the neighbour-joining method of Jukes and Cantor (1969) and analysis was based on 585 nucleotides. Bootstrap values (out of 100 trees) are shown adjacent to nodes.

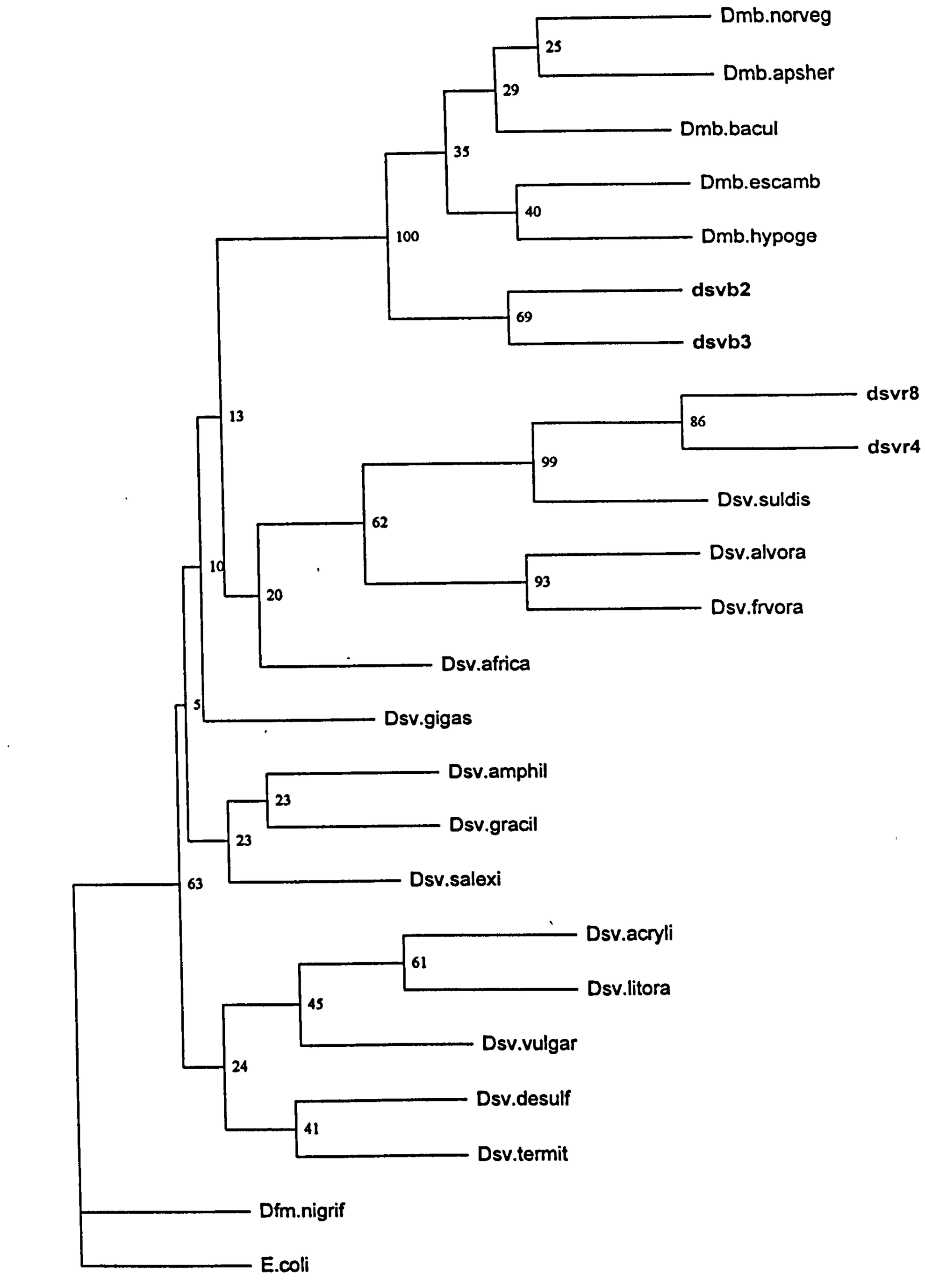


Figure 5.27. 16S rDNA bootstrap consensus tree of *Desulfovibrio-Desulfomicrobium* (DSV-DMB Group 6) members and DSV-DMB (Group 6) clones derived from PCR products amplified from the Risley and Butchersfield [98] landfill sites.
The tree was constructed using a maximum parsimony method (Swofford, 1991) and analysis was based on 585 nucleotides. Bootstrap values (out of 100 trees) are shown adjacent to nodes.

***Desulfovibrio-Desulfomicrobium* (DSV-DMB Group 6)**: Cloned *Desulfovibrio-Desulfomicrobium* (DSV-DMB Group 6) PCR products amplified from the Risley and Butchersfield [98] landfill sites were analysed by TTGE (Figs. 5.24 & 5.25, p.133 & 134). Clones obtained from the Risley landfill site did not correspond to any of the bands in the original landfill TTGE profile. The reason for this is not known, however they may result from sequences present in the original PCR products that have not been visible on the TTGE gel. Clones 'dsvr4'; 'dsvr8' (from Risley) and 'dsvb2'; 'dsvb3' (from Butchersfield [98]) were sequenced. Bootstrap consensus trees generated by DNA distance and maximum parsimony analysis showed similar topologies and bootstrap values to one another (Figs. 5.26 & 5.27, p.135 & 136). The sequences obtained from both landfill sites clustered within the DSV-DMB subgroup, although interestingly the sequences amplified from the Risley landfill site cluster within the *Desulfovibrio* assemblage while the sequences from the Butchersfield [98] landfill site cluster with the *Desulfomicrobium* group.

Sequence similarity values between the sequenced clones and their closest relatives are presented in Table 5.1 (p.138).

CLONE	CLOSEST RELATIVE	SEQUENCE SIMILARITY
'dfmp4'	*Desulfotomaculum aeronauticum*	88.2% over 687 bp
'dfmp6'	*Desulfotomaculum geothermicum*	87.5% over 686 bp
'dfmp7'	*Desulfotomaculum aeronauticum*	88.3% over 686 bp
'dfmc7'	*Desulfotomaculum geothermicum*	88.3% over 685 bp
'dfmc8'	*Desulfotomaculum geothermicum*	88.6% over 684 bp
'dbbp1'	*Desulfobulbus elongatus*	97.8% over 509 bp
'dbbp2'	*Desulfobulbus elongatus*	93.3% over 1142 bp
'dbbp4'	*Desulfobulbus elongatus*	91.2% over 1144 bp
'dbbr1'	*Desulfobulbus elongatus*	94.7% over 1142 bp
'dbbr2'	*Desulfobulbus elongatus*	98.3% over 466 bp
'dsbb1'	*Desulfobacter postgatei*	98.2% over 1167 bp
'dsbb2'	*Desulfobacter postgatei*	98.4% over 442 bp
'dccp2'	*Desulfosarcina variabilis*	93.5% over 863 bp
'dccp4'	*Desulfococcus multivorans*	96.1% over 863 bp
'dccp9'	*Desulfosarcina variabilis*	92.9% over 865 bp
'dccw2'	*Desulfonema magnum*	89.5% over 865 bp
'dccw3'	*Desulfonema magnum*	91.1% over 864 bp
'dsvr4'	*Desulfovibrio burkinabensis*	91.5% over 615 bp
'dsvr8'	*Desulfovibrio burkinabensis*	89.7% over 614 bp
'dsvb2'	*Desulfomicrobium apsheronum*	96.3% over 614 bp
'dsvb3'	*Desulfomicrobium apsheronum*	95.0% over 614 bp

Table 5.1. Sequence similarity values between cloned sequences amplified from landfill leachate and their closest relatives.

5.3. Discussion

The application of TTGE to separate specifically-amplified PCR products was successful in generating profiles of banding patterns that could be used as a measure of the genetic diversity within SRB subgroups present in landfill. It is possible to infer some level of diversity by counting the number of individual bands in each profile, as each band may represent a single microbial 'phylotype' within a particular microbial population.

The low numbers of individual bands observed in the profiles obtained for the five SRB subgroups detected in these landfill sites would suggest that the genetic diversity within each respective subgroup in particular landfill sites is limited. Banding patterns generated showed numbers of individual bands ranging from one band to no more than five bands in the profiles obtained. However, it is possible that other bands are present but at frequencies too low to be observed by visual examination of the TTGE gels. This would certainly seem to be the case for the DSV-DMB (Group 6) Risley profile (Fig. 5.24, p.133) where the clones generated from the Risley PCR products did not correspond to any of the bands observed in the landfill TTGE profile.

This result contrasts with a study by Rooney-Varga *et al* (1998) which investigated SRB diversity in a salt marsh sediment by sequencing enrichment cultures and environmental clones. From the sequence data obtained, they concluded there to be a high level of diversity of SRB inhabiting the salt marsh. However, salt marshes are a major habitat for SRB and sulfate reduction rates in these environments are one of the highest of any natural system (Howarth, 1993). Therefore, a high level of diversity in this environment would be expected. Landfill sites, though, are predominantly methanogenic in nature and

SRB may not be present in these environments in significant populations. Thus, you would not expect to find the same level of diversity within a landfill as in a salt marsh. The observation of a greater number of bands in most of the 'nested' profiles in comparison with profiles obtained with 'direct' PCR products would suggest that, overall, the application of 'nested' PCR does seem to reveal a greater diversity of sequences than 'direct' PCR. This would therefore suggest that genetic diversity within specific populations would be underestimated if 'nested' PCR were not to be applied in investigations of microbial ecology employing PCR.

However, there is also some evidence that the eubacterial primers pA & pH' (Edwards *et al*, 1989) used in this study to generate the 'nested' PCR products are biased against certain sequence types. The observation of bands in 'direct' profiles that were not subsequently observed in the corresponding 'nested' profiles (e.g. DFM (Group 1) Buff Quarry [Figs 5.1 & 5.2, p.106 & 107]; DSB (Group 4) Pilsworth [Figs. 5.4 & 5.5, p.110 & 111]; DCC-DNM-DSS (Group 5) Butchersfield [97] [Figs. 5.6 & 5.7, p.113 & 114]) suggests that the primary amplification using primers pA & pH' selects against these sequence types. This is possibly due to differences in primer annealing efficiency resulting in preferential amplification of certain sequence types over others or to mismatches in the primer target regions resulting in non-amplification. Although these primers are designed to be 'universal' eubacterial primers it is very doubtful that they will target all eubacterial sequences in a given environmental sample.

Although band numbers observed in profiles suggested low diversity within SRB subgroups present in particular landfill sites, this is probably an underestimation of the true level of the genetic diversity within the SRB subgroups. The number of bands

generated by TTGE may not accurately reflect the number of different sequence types in a mixture of PCR products, as heterogeneous sequences can exhibit equivalent mobilities depending on the conditions applied during electrophoresis. This means that individual bands on a TTGE gel may be comprised of two or more different sequence types ('phylotypes') that have co-migrated to the same point in the gel, thereby causing the diversity within the group to be underestimated.

Conversely, the presence of multiple heterogeneous rRNA operons in individual species (Nubel *et al*, 1996) could conceivably cause genetic diversity within mixed populations to be overestimated. However, this is thought unlikely in this case as the SRB are not known to contain multiple heterogeneous rRNA operons. This is demonstrated in the TTGE profiles by the production of a single band in all the controls run using 16S rDNA PCR products amplified from pure cultures of known SRB (Figs. 5.1-5.8, p.106-108, 110-111, 113-115). It is not known why a double band is observed for *Dsv. desulfuricans* in Fig. 5.25 (p.134), however it is thought that it may be a product of DNA degradation.

The true genetic diversity within the SRB subgroups may also be underestimated due to limitations in PCR. Banding patterns produced by TTGE from amplified PCR products represent the major constituents of the analyzed community. Unknown members of SRB subgroups may escape PCR targeting because their nucleotide sequences fall outside the specificity of the designed primers. If this is the case, additional sequence data obtained from new environmental isolates should be able to help in the design of more encompassing PCR primers.

As discussed in section 4.3, the possibility exists that unknown non-SRB sequences may be amplified from environmental samples by the SRB group-specific primers. These

sequences, forming bands in TTGE profiles, could lead to inaccuracies in measurements of genetic diversity within SRB subgroups. Ideally, DNA bands separated by TTGE gels should be oligonucleotide probed to confirm the identity of the amplified bands. Unfortunately, this was not possible in this case as the oligonucleotides specific for SRB described here and by Devereux *et al* (1992) do not target within the region of the 16S rRNA gene used for TTGE analysis. In this study, identification of DNA bands observed in TTGE profiles was confirmed by DNA sequencing.

One of the main advantages of TTGE is that profiles obtained from different samples, in this case different landfill sites, can be directly compared and differences in populations readily observed.

Thus, the observation of different bands in different profiles (e.g. Figs. 5.1, p.106; 5.2, p.107; 5.5, p.111) demonstrates differences in SRB community structure and shows that there are distinct populations of SRB in different landfill sites. The numbers of unique bands seen in TTGE profiles also suggests that there is a great deal of variation of SRB sequence types detected in these landfill sites. This has implications for the management of landfill sites as the differences in SRB populations are almost certainly a reflection of different environmental conditions within each site. Therefore, it would seem appropriate to suggest that any landfill management strategies aimed at keeping SRB populations under control be based upon information obtained directly from that site.

There are also some similarities in banding patterns between profiles from different landfill sites (e.g. Figs. 5.3, p.108; 5.4, p.110; 5.6, p.113; 5.8, p.115) which may suggest that there are related populations present in different sites. In addition, the observation of co-migrating bands in a few of the Butchersfield [97] and [98] profiles (Figs. 5.4, p.110;

5.5, p.111; 5.7, p.114) may also suggest population stability for particular SRB subgroups over the twelve month period between the times when the site was sampled. However, these observations cannot be reliably inferred on the basis of TTGE alone because, as discussed earlier, different sequences can exhibit similar melting characteristics. Therefore, the identification of bands must be verified by sequence analysis. Only differences between populations from different samples can be discerned by TTGE without sequence analysis.

There have been few other studies that have used gradient gel electrophoresis to investigate SRB ecology in environmental samples. Those that there are have used 'universal' or SRB semi-selective primers to amplify PCR products and have then relied upon oligonucleotide probing to identify SRB populations. The work described in this thesis is therefore the first to apply gradient gel electrophoresis to PCR products that have been amplified using primers specific for SRB and is the first to investigate genetic diversity within specific SRB subgroups. Teske *et al* (1996) investigated sulfate-reducing populations of a stratified marine water column by DGGE. Profiles of 16S rDNA- and rRNA-derived PCR products were compared to investigate differences between the presence and expression of particular SRB 16S rRNA genes. DGGE gels were membrane blotted and hybridized against probes designed by Devereux *et al* (1992) to identify SRB populations. In addition, the sequence of a resolved band excised from the DGGE gels formed a new, distinct phylogenetic lineage within the δ-subdivision which did not correspond to any known SRB sequences or to any sequences obtained from MPN cultures derived from the same environment. These workers thus concluded that the sequence would probably not have been identified by any other molecular method.

DGGE has also been used to investigate SRB populations within the DCC-DNM-DSS assemblage in a hypersaline microbial mat (Teske *et al*, 1998) and to investigate the temporal distribution of SRB populations during the development of a bacterial biofilm (Santegoeds *et al*, 1998). In both cases, SRB populations were identified through hybridization against SRB-specific oligonucleotides probes designed by Devereux *et al* (1992).

The application of gradient gel electrophoresis, however, is more widespread in studies of microbial molecular ecology in general and is fast becoming a major tool with which to investigate bacterial populations in environmental samples. Since Muyzer *et al* (1993) demonstrated a DGGE profile derived from PCR products amplified from a bacterial biofilm, gradient gel electrophoresis has been used to characterize cyanobacterial populations inhabiting a hot spring microbial mat (Ferris *et al*, 1996; Ferris and Ward, 1997), to investigate diversity within actinomycete populations present in soil (Heuer *et al*, 1997) and to study ammonia-oxidizing bacteria from coastal sand dunes (Kowalchuk *et al*, 1997). Furthermore, Heuer *et al* (1997) employed TGGE alongside DGGE and reported no significant differences in results obtained from either technique.

Phylogenetic analysis of 16S rRNA gene sequences from the amplified and cloned SRB fragments revealed that they were all from members of the δ-subclass of the Proteobacteria and were related to other known SRB within each subgroup. As well as providing information on the phylogenetic affiliations of the amplified fragments, the sequence analysis also demonstrated the ability of the designed PCR primers and oligonucleotide probes to specifically amplify and identify sequences that cluster within each respective subgroup from environmental samples. Although only 21 clones were

sequenced in total, the results obtained nonetheless validate the use of these primers and probes in investigations of SRB occurrence and distribution in the natural environment. None of the cloned fragments analysed was 100% homologous to any of the published sequences from known SRB. FastA searches of the GenBank and EMBL databases showed the amplified sequences to share 87.5%-98.4% similarity to the 16S rRNA gene sequences of their respective closest relatives (Table 5.1, p.138), with the topology of the bootstrap consensus trees showing the amplified sequences to form novel lineages within the subgroups. The amplification of novel 'phylotypes' from the leachate samples suggests that there could be as yet undescribed species of SRB present in landfill and that the limited number of published sequences from known SRB do not represent the SRB populations present in these landfill sites.

The phylogenetic affiliations of the amplified sequences can also be used to infer potential physiological capabilities of the bacterial populations. This is especially true with SRB where phylogenetic affiliations tend to correlate with physiological traits. Rooney-Varga *et al* (1998) found that the physiological characteristics of sequenced isolates enriched from a salt marsh sediment corresponded well with those of their closest relatives. It is therefore interesting to note the high sequence similarity between clone 'dsbb1' and *Desulfobacter postgatei* (98.2% over 1167 bp) (Table 5.1, p.138), when you consider that the main habitats for *Desulfobacter postgatei* are marine or brackish sediments (Fauque, 1995) and that this species has a requirement for NaCl in enrichment culture (Gibson, 1990; Stackebrandt *et al*, 1995). This result may therefore suggest that a related population to *Desulfobacter postgatei* has adapted to survive in a terrestrial environment or that the Butchersfield landfill site contains a relatively high

NaCl concentration. Furthermore, the main electron donor for *Desulfobacter postgatei* is acetate (Gibson, 1990; Stackebrandt *et al*, 1995) and therefore it is possible to infer that the species from which the amplified sequence was derived may also be capable of the complete oxidation of acetate to CO_2.

It is not known whether or not the SRB group-specific primers employed in this study targeted a significant proportion of the SRB populations in the landfill samples. However, although band numbers in individual TTGE profiles were low, the amplification of novel SRB 'phylotypes', as suggested by sequence analysis of cloned SRB fragments, does indicate that the PCR primers are not limited to detecting the known SRB sequences from which they were designed.

Bands observed to migrate to different positions in TTGE profiles from different landfill sites (e.g. clones 'dfmp4'; 'dfmc7' and 'dsvr4'; 'dsvb2') were confirmed to be of varying sequence (Figs. 5.11 & 5.26, p.119 & 135), while bands amplified using the DBB (Group 2)-specific primers from the Pilsworth and Risley landfill sites that were observed to co-migrate to similar positions (clones 'dbbp1'; 'dbbr1') were shown by sequence analysis to exhibit strong homology to one another (Fig. 5.15, p.123) suggesting related populations of *Desulfobulbus* in these two landfill sites.

The results obtained therefore validate the PCR/TTGE approach to the study of SRB ecology in environmental samples that has been described in this thesis.

By using TTGE coupled with sequence analysis of cloned 16S rDNA fragments, differences in SRB community structure could be detected between landfill sites that may correspond to important environmental factors such as moisture content, pH, availability of electron donors and concentration of sulfate. Such data not only provide

insights into the diversity and distribution of this ecologically important group of micro-organisms but can also help to generate testable hypotheses regarding potential physiological differences between 16S rDNA sequence clusters and their response to changing environmental conditions.

CHAPTER 6. General Discussion

Molecular biological approaches offer many advantages to the study of microbial community structure in the natural environment. Nucleic acid-based methods such as the polymerase chain reaction have enabled bacterial populations to be detected in environmental samples without the need for cultivation. Sulfate-reducing bacteria (SRB) are difficult to grow in pure culture and so are ideal as targets for molecular biological analysis.

The SRB are a diverse group of ecologically important bacteria that have been studied in a variety of different environments both by traditional microbiological and molecular biological methods. The application of molecular techniques has led to many insights into SRB occurrence, activity and community structure and also affords the possibility of directly studying the ecological significance of these bacteria in environmental samples.

SRB are ubiquitous in the environment and play an important role in the anaerobic degradation of organic matter. In high sulfate environments, for example marine sediments, sulfate reduction is thought to be responsible for up to 50% of organic matter degradation (Jorgensen, 1982). However, even in low sulfate environments, such as freshwater sediments, sulfate reduction can be a significant factor in anaerobic decomposition (Jones and Simon, 1984; Bak and Pfennig, 1991a, 1991b; Sass *et al*, 1997; Li *et al*, 1999).

Landfill sites have not been considered to be important habitats for SRB due to low sulfate levels within landfill sites leading to the predominance of methanogenesis as the key terminal process of organic matter degradation. However, exogenous sources

of sulfate (e.g. gypsum from construction and demolition waste) can increase sulfate levels within landfill sites significantly (Suflita *et al*, 1992; Gurijala and Suflita, 1993) which could potentially lead to increased growth of SRB and a subsequent increase in sulfate-reducing activity.

SRB can directly compete with methanogenic bacteria for electron donors such as acetate and H_2 (Schonheit *et al*, 1982; Robinson and Tiedje, 1984) and the presence of sulfate has been shown to inhibit methanogenic activity in a number of environments (Oremland and Polcin, 1982; Beeman and Suflita, 1987; Raskin *et al*, 1996). In addition, sulfate-reduction has been postulated to be one of the most important factors in the inhibition of methanogenesis in landfill sites (Suflita *et al*, 1992; Gurijala and Suflita, 1993).

Thus, the development of molecular biological tools to enable the characterization of SRB populations present in landfill sites is of prime importance in the ultimate development of landfill management strategies to keep SRB populations and sulfate-reducing activity under control.

The use of PCR to specifically amplify nucleic acids from environmental samples is now commonplace in the study of microbial ecology. PCR primers designed to target both conserved and variable tracts of rRNA genes have been used to detect bacterial populations in a range of environments (Bej *et al*, 1990; Erb and Wagner-Dobler, 1993; Hiorns *et al*, 1995; Hales *et al*, 1996). However, PCR primers designed for the specific amplification and detection of SRB 16S rDNA sequences in environmental samples have not been previously described.

Consequently, this is the first study to directly extract and amplify specific SRB 16S rDNA targets from environmental samples using PCR. In addition, this is the first

study to directly investigate SRB occurrence and diversity in landfill sites using molecular biological methods.

The phylogeny of the SRB as described by Devereux *et al* (1989) lends itself to the development of primers and probes based on 16S rDNA sequences. The construction of a phylogenetic tree (Fig. 3.1, p.50) allowed identification of six main clusters or subgroups of SRB, which in turn enabled the design and development of 16S rDNA-targeted PCR primers (Table 3.1, p.53) and oligonucleotide probes (Table 3.2, p.56) specific for each of the six main subgroups.

Comparative analysis of the designed primers and probe sequences against the SSU_rRNA database of the Ribosomal Database Project suggested that, when used in combination, the primers and probes would provide highly-specific molecular tools with which to investigate SRB populations in environmental samples.

This theoretical cross-specificity analysis was confirmed by subsequent amplification and probing of 16S rDNA from SRB strains representing each of the six subgroups alongside non-SRB strains with 1, 2 or 3 bp mismatches in the oligonucleotide probe target region.

When amplifying bacterial sequences from environmental samples by PCR, there is always the possibility of amplifying non-target sequences that are not represented in any of the databases. Degenerate primers, as used in this study to broaden the specificity within each subgroup, also increase the possibility of amplifying non-target sequences. Therefore, PCR products amplified using the SRB group-specific primers were only recorded as SRB-like positives if they subsequently gave a positive signal upon hybridization with the appropriate oligonucleotide probe. Thus, it is the

primer-probe combinations that are highly specific, not necessarily the primers and probes individually.

Recent updates of the GenBank and RDP databases have reaffirmed that the specificities of the primers and probes holds true even though more sequences have been deposited since their initial design.

The amplification of PCR products from samples of landfill leachate and confirmation of identity by oligonucleotide probing demonstrates the usefulness of these molecular tools for investigating SRB occurrence in environmental samples. Five out of six sets of primers were successful in amplifying positive PCR products from the leachate samples. Only the DBM (Group 3)-specific primers did not amplify from the leachate, although this is probably due to the lack of DBM (Group 3) DNA in the landfill, as members of this subgroup are found predominantly in marine environments. It would be interesting to see if these primers could be used successfully on marine sediment samples.

Phylogenetic analysis of cloned and sequenced PCR products has shown amplified sequences to cluster within the specific subgroups for which the PCR primers and oligonucleotide probes were designed. Even though only 21 clones were sequenced, this therefore validates the use of these primers and probes in environmental studies of SRB occurrence and demonstrates their ability to specifically amplify and detect populations of SRB in environmental samples.

The presence of detectable populations of SRB in landfill was confirmed by PCR amplification and oligonucleotide hybridization using the designed primers and probes. SRB 16S rDNA was detected by either 'direct' or 'nested' amplification from

all seven landfill sites sampled suggesting that landfill sites can be regarded as habitats for SRB as a matter of routine.

The assumption that bacterial populations that can be detected using 'direct' PCR are present in relatively high numbers compared to those populations that require 'nested' PCR for detection allows tentative estimations of the relative predominance of bacterial populations in environmental samples. Thus, the three subgroups detected by 'direct' PCR from the landfill sites: DFM (Group 1); DSB (Group 4); DCC-DNM-DSS (Group 5), would appear to be the predominant SRB present in these landfill sites. However, this is only a qualitative estimation of relative numbers based on detection through one round of PCR ('direct') compared to two rounds of PCR ('nested') and has no statistical significance.

Other studies that have measured the relative abundance of SRB subgroups by hybridization of oligonucleotide probes to RNA extracted from environmental samples have suggested that there is no definitive pattern to the predominance of SRB subgroups in the natural environment. *Desulfovibrio* spp. have been postulated to be the predominant SRB in both a freshwater river sediment (Trimmer *et al*, 1997) and an estuarine sediment (Devereux *et al*, 1996a, 1996b), *Desulfobulbus* spp. have been found to be dominant in a freshwater lake sediment (Li *et al*, 1999) and a salt marsh sediment (Devereux *et al*, 1996a, 1996b), while *Desulfobacter* spp. were reported dominant in the rhizosphere of marsh grass (Hines *et al*, 1999) and *Desulfobacterium* spp. in a coastal marine sediment (Sahm *et al*, 1999b).

'Nested' PCR, however, does reveal the presence of subgroups in the landfill samples that were not detected by 'direct' PCR. This also suggests a high level of diversity in landfill, at least at the generic level, which correlates with studies of SRB occurrence

and distribution in other environments (Kane *et al*, 1993; Ramsing *et al*, 1993; Risatti *et al*, 1994; Devereux *et al* 1996a, 1996b; Raskin *et al*, 1996; Purdy *et al*, 1997; Trimmer *et al*, 1997; Rooney-Varga *et al*, 1997; Manz *et al*, 1998; Sahm *et al*, 1999b). The detection of microbial populations through 'nested' PCR only further suggests that microbial diversity may be underestimated if 'nested' PCR is not applied in studies of molecular ecology in general.

The ability of SRB to compete with methanogenic bacteria for substrates such as acetate and H_2 makes them a potential threat to the efficient management of landfill sites where methane is the desired end-product of waste degradation. The presence of SRB in these landfill sites, determined by PCR amplification, suggests that the potential for sulfate-reduction and possible inhibition of methanogenesis is present. Furthermore, should conditions in the landfill sites begin to favour sulfate-reduction over methanogenesis, for example if sulfate concentrations were to increase, then these SRB populations may well present a significant competitive threat to methane production, leading to increased production of H_2S and the 'souring' of the landfill site. Molecular detection of SRB in landfill sites could provide early warnings of population shifts that lead to the souring of landfills.

Therefore, the ability to detect SRB populations in landfill samples through routine analysis is vitally important for the development of protocols that can be used to monitor the levels of SRB populations that could have a detrimental effect on landfill efficiency.

Genetic diversity within the SRB subgroups amplified from the landfill samples was investigated by temporal thermal gel electrophoresis. TTGE is a technique that offers the potential to analyze bacterial community structure within specific populations

through the electrophoretic separation of PCR products on the basis of sequence melting characteristics.

Banding patterns generated through the separation of PCR products provide profiles of the amplifiable sequence types present in environmental samples, with the numbers of individual bands in profiles being used as a measure of genetic diversity within specific populations. This technique also provides the opportunity to compare bacterial community patterns and to infer differences in the composition of microbial communities from different samples. Furthermore, phylogenetic analysis of community members can be performed through the sequencing of cloned or excised fragments. The analysis of specifically-amplified PCR products by gradient gel electrophoresis has been shown to facilitate investigations into microbial diversity and community structure in a range of environments (Ferris *et al*, 1996; Teske *et al*, 1996, 1998; Ferris and Ward, 1997; Heuer *et al*, 1997; Kowalchuk *et al*, 1997; Santegoeds *et al*, 1998;) and the sequencing of cloned or excised fragments has been used to infer phylogenetic affiliations of community members (Ferris *et al*, 1996; Teske *et al*, 1996, 1998).

TTGE analysis of PCR products amplified from landfill leachate using the SRB group-specific primers was successful in generating profiles of banding patterns that could be used as a measure of the genetic diversity within the SRB subgroups. The results obtained show low numbers of individual bands in profiles (<5) obtained for the SRB subgroups present in landfill, which suggests limited genetic diversity within each respective population in particular landfill sites. It is possible that other bands are present but at frequencies too low to be observed by visual examination of the TTGE gels.

In contrast, a study by Rooney-Varga *et al* (1998) concluded that the genetic diversity of SRB populations within a salt marsh sediment to be of a high level. However, whereas salt marshes are one of the main habitats for SRB, landfill sites are predominantly methanogenic, and therefore the same level of diversity would not be expected in these two distinct environments.

However, co-migration of different sequence types, PCR biases and limitations in primer design allowing unknown members of SRB subgroups to escape PCR detection can all cause genetic diversity within populations to be underestimated. Therefore, it is possible that the low genetic diversity, suggested by the results presented in this study, is not an accurate reflection of the true genetic diversity within the SRB subgroups present in the landfill samples. Nevertheless, the profiles obtained do demonstrate that TTGE of specifically-amplified 16S rDNA fragments is a viable technique for the analysis of genetic diversity within SRB populations from environmental samples.

The application of 'nested' PCR to amplify SRB sequences from the landfill leachates does seem to reveal a greater diversity within specific populations than the 'direct' PCR approach. However, there is also evidence that the 'universal' eubacterial primers used in this study to facilitate 'nested' PCR are selectively biased against certain sequence types. The observation of bands in 'direct' profiles that were not subsequently observed in the corresponding 'nested' profiles suggests that the primers are not targeting these sequences in the primary PCR amplification. Differences in SRB community structure in different landfill sites could be readily deduced through differences observed in banding patterns, thus demonstrating phylogenetically distinct populations of SRB in different landfill sites. The numbers

of unique bands seen in TTGE profiles also suggests that there is a great deal of variation of SRB sequence types detected by PCR between these landfill sites. The differences in SRB community structure observed for different landfill sites, in terms of distinct bands in TTGE profiles and different SRB populations detected by PCR, are almost certainly a reflection of different environmental conditions within each site. This has implications for landfill management as this suggests that strategies for the control of SRB should be based upon information obtained directly from the particular landfill site.

However, it was not possible to obtain any information on the physical or chemical characteristics of the landfill sites sampled in order to determine any significant differences between them.

Similarities observed in some profiles from different landfill sites indicated that there might be similar populations of SRB present in different sites. In addition, the co-migration of bands seen in the Butchersfield [97 & 98] profiles for *Desulfobacter* (DSB Group 4) (Figs. 5.4 & 5.5, p.110 & 111) and *Desulfococcus-Desulfonema-Desulfosarcina* (DCC-DNM-DSS Group 5) (Fig. 5.7, p.114) suggests that these populations were stable over the twelve months between the times when the site was sampled. These observations, though, cannot be inferred on the basis of TTGE alone. However, sequence analysis of co-migrating bands amplified from the Pilsworth and Risley landfill sites using the DBB (Group 2)-specific primers (Fig. 5.3, p.108) showed strong homology suggesting that these populations are related.

Screening of clones generated from specifically-amplified PCR products by TTGE to identify sequences of interest rather than excising bands directly from the TTGE gels was performed in order to increase the amount of sequence information available.

Fragments run on the TTGE gels were only *ca.* 250 bp in length including the 40 bp GC-clamp. By cloning PCR products, screening by TTGE and then sequencing from the original clone, fragments up to *ca.* 1150 bp were sequenced in this study, providing up to five times the amount of sequence information available through the sequencing of excised bands.

Sequence analysis of the cloned SRB 16S rDNA fragments revealed they were all members of the δ-subclass of the Proteobacteria and were related to known SRB within each respective subgroup. Construction of phylogenetic trees showed the sequences to form novel lineages within subgroups suggesting that undescribed species of SRB are present in these landfill sites. In some cases (e.g. DBB [Group 2]) the cloned sequences form coherent clusters some distance away from the other SRB in the group (Figs. 5.15 & 5.16, p.123 & 124), while in others (e.g. DFM [Group 1]; DCC-DNM-DSS [Group 5]; DSV-DMB [Group 6]) the sequences segregate together in different clusters within the group (Figs. 5.11 & 5.12, p.119 & 120; 5.22 & 5.23, p.131 & 132; 5.26 & 5.27, p.135 & 136). In the case of the DSB (Group 4) sequences, the clones were very closely related to a known SRB, *Desulfobacter postgatei* (Figs. 518 & 5.19, p.127 & 128). However, it would take the sequencing of many more clones than those described in this thesis to provide meaningful phylogenies and to reveal whether or not these novel sequences and clusters may be new centres of variation that could represent new species of SRB. Sequencing of PCR products amplified from a wide range of environments including other landfill sites could also provide evidence to suggest whether or not there are SRB phylotypes that are uniquely or predominantly associated with landfill sites.

The amplification of novel sequences from the landfill samples also shows that the group-specific primers are not limited to detecting the known SRB sequences from which they were designed.

The phylogeny of the SRB tends to correlate well with their physiological classification. Rooney-Varga *et al* (1998) concluded that the physiological capabilities of sequenced isolates from a salt marsh sediment did correspond to those of their closest relatives. Therefore, insights into the diversity and distribution of SRB in natural environments through TTGE analysis and sequencing of amplified fragments can provide the basis for further studies regarding SRB 16S rDNA sequence clusters and their physiological traits in relation to environmental conditions. Such studies could have a significant impact upon landfill management as environmental conditions can vary greatly both within and between landfill sites.

It is also possible to infer potential physiological capabilities of species present in environmental samples based on phylogenetic analysis. The high sequence similarity between clone 'dsbb1' and *Desulfobacter postgatei* (98.2% over 1167 bp) (Table 5.1, p.138) therefore suggests that the species from which the sequence was amplified may have similar physiological traits to *Desulfobacter postgatei*. This would further suggest that, as *Desulfobacter postgatei* is a predominantly marine micro-organism (Fauque, 1995), this related population has either adapted to survive in a terrestrial environment or that the Butchersfield landfill site contains a relatively high NaCl concentration. The amplification of sequences from the Butchersfield landfill site that are related to *Desulfomicrobium* spp. (Figs. 5.26 & 5.27, p.135 & 136), which have been primarily isolated from freshwater environments (Fauque, 1995), would suggest, however, that the levels of NaCl in this landfill site are not relatively high.

Further work into the characterization of SRB populations in landfill sites should include the use of rRNA as template for RT-PCR and TTGE analysis instead of rDNA as described here. As RNA is transient in nature this would allow targeting of metabolically-active populations of bacteria. TTGE profiles generated from rDNA and rRNA could then be directly compared to specifically identify metabolically-active populations of SRB. In addition, as rRNA is more abundant than rDNA, the application of RT-PCR might reveal there to be more diversity within the SRB subgroups than previously realised.

The use of rRNA, however, would not provide a direct link to sulfate-reducing activity within the landfill sites. For molecular biological techniques to provide information on actual sulfate reduction, genes encoding for enzymes required for sulfate respiration would have to be targeted. PCR primers designed to amplify the dissimilatory sulfite reductase gene of several known SRB have been described (Karkhoff-Schweizer, 1995; Wagner *et al*, 1998) and have been used to investigate the occurrence and diversity of sulfite reductase genes in SRB from a hypersaline microbial mat (Minz *et al*, 1999) and in bacteria associated with worms from a deep-sea hydrothermal vent (Cottrell and Cary, 1999). These PCR primers thus provide the opportunity of amplifying isolated mRNA to identify populations of SRB that are actively reducing sulfate.

It would have been interesting to obtain samples of leachate from landfill sites with problems of sulfide production to investigate any associated proliferation or increase in diversity of SRB within these sites compared to sites without sulfide problems. Unfortunately this was not possible as samples could not be obtained from any landfill sites with sulfide problems.

It would also be of interest to use solid landfill material, as opposed to leachate, to investigate occurrence and distribution of SRB. This would allow the detection of SRB populations attached to solid surfaces which may not be washed off into the leachate. Again, results from PCR and TTGE analysis could be directly compared to identify any differences in the SRB populations detected from solid material and leachate. This would give a good indication of the merits of using leachate as a practical source of information on landfill microbiology. In addition, the use of solid landfill material might also provide evidence of SRB 'hotspots' which may occur if sulfate is unevenly distributed and concentrated in discrete areas.

Nevertheless, this study has designed and developed 16S rDNA-targeted PCR primers and oligonucleotide probes for the specific detection of SRB subgroups in environmental samples and has demonstrated their ability and usefulness for investigating SRB occurrence in landfill using leachate as a sample source. Populations of SRB were shown to be detectable in landfill through PCR and the application of TTGE to specifically-amplified PCR products was successful in providing information on the genetic diversity within SRB populations. Furthermore, phylogenetic analysis of cloned SRB 16S rDNA fragments validated the TTGE profiles and demonstrated the amplification of novel SRB sequences suggesting the presence of unknown populations of SRB in landfill.

CHAPTER 7. References

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APPENDIX 1. Nucleotide Sequences of Cloned SRB 16S rDNA Fragments Amplified from Landfill Leachate.

```
#dfmp4 (140-842)
T-AGAC-T-GGGAT-AACGG-CG-GGAAA-CTGGT-G-CTAATACCGGAT-AAG-CTCAA
--C-TG-GGC---ACAT----GCT--TGG-AT---------GAG-GAAA-GGA-------
---------CAAA---------------TCCG--CAT---TAAG--ATGGATCCGCGT-C
CC-ATTA-G-CTA-G---TTGG-CG-GTGTAAC-GG-ACCACC-AAGGC-AA-CGA-TG-
GGTAGCCGG-CCT-G-AGAGGGT---------------------GGACGG-CCACACTGG-
AACTGA-GA-CACGGTCCAGA-CTCCTACG-GGAGGC-AGCA-GT-GGGGAATCTTCCGC
AAT-GGGC-GAAA-GCC-TGACGGA-GCAACGCCGC-GTG-A-ATGATGAA--GGCC--T
T-CG--GG-TTGTAAA---------------------ATTCTGTC-TT-CAGG-GAA--GA
-A-------------------------AAAAAA------------T-GA-CGGT-A-CCT
-G-AGG-AGGAAG--C-CCC-GGCTAAATAC-GTGCCAGCAGCCGCGGTAAT-ACGTAG-
GGGGC-GAGCGTTGTCC-GGAATTACTGGGCGTAAAGGGC-GCGTAG-GCG-GTTT-GTT
-AAGTCAG-AGGTG-AAAAC-TATGGGC-TCAA-CCC-ATA-GCC--TGCCTTTGA-AAC
-TGGGA-GACTT-GAGGA-----CAGGA-GAGGGGAG-TGGAATTCCCAGTGTAGCG-GT
GAAA-TGCGT-AGAT-ATT-GGGAGG-AACA-CCAG--T-GGC-GAAGGCGG--CTTTCT
G-------------------------------GCCTGTAACTGACG-CTGAG-G-CG-CG
AAA-GC-GTGGG-GAGCGAACG-GGATTAGATAC-CCCGGTA-GTC-CACGCCGTAAAC-
GATGGGTG-CT-AGGAGTTGCGG-GT-----AT
```

Nucleotide sequence of cloned DFM (Group 1) 16S rDNA fragment 'dfmp4' (16S rDNA positions, *E. coli* numbering).

```
#dfmp6 (140-842)
T-AGAC-C-GGGAT-AACAG-CG-GGAAA-CTGGT-G-CTAATACCGGAT-AAG-CTCAT
--T-GG-GGC---ACAT----GCT--TTG-AT----------GAG-GAAA-GGA-------
---------GAAA---------------TCCG--CTT---TAAG--ATGGATCCGCGT-C
CC-ATTA-G-CTA-G---TTGG-CG-GTGTAAC-GG-ACCACC-AAGGC-AT-CGA-TG-
GGTAGCCGG-CCT-G-AGAGGGT---------------------GGACGG-CCACACTGG-
AACTGA-GA-CACGGTCCAAA-CTCCTACG-GGAGGC-AGCA-GT-GGGGAATCTTCCGC
AAT-GGGC-GAAA-GCC-TGACGGA-GCAACGCCGC-GTG-A-ATGATGAA--GGCC--T
T-CG--GG-TTGTAAA---------------------ATTCTGTC-TT-CAGG-GAA--AA
-A-------------------------AAAAAA------------T-GA-CGGT-A-CCT
-G-AGG-AGGAAG--C-CCC-GGCTAACTAC-GTGCCAGCAGCCGCGGTAAT-ACGTAG-
GGGGC-GAGCGTTGTCC-GGAATTACTGGGCGTAAAGGGC-GCGTAG-GCG-GTTT-CTT
-AAGTCAG-AGGTG-AAAAC-TATGGGC-TCAA-CCC-ATA-GCC--TGCCTTTGA-AAC
-TGGGA-GACTT-GAGGA-----CAGGA-GAGGGGAG-TGGAATTCCCAGTGTAGCG-GT
GAAA-TGCGT-AGAT-ATT-GGGAGG-AACA-CCAG--T-GGC-GAAGGCGG--CTCTCT
G-------------------------------GCCTGTAACTGACG-CTGAG-G-CG-CG
AAA-GC-GTGGG-GAGCGAACG-GGATTAGATAC-CCCGGTA-GTC-CACGCCGTAAAC-
GATGGGTG-CT-AGGTGATGGGG-GT-----AT
```

Nucleotide sequence of cloned DFM (Group 1) 16S rDNA fragment 'dfmp6' (16S rDNA positions, *E. coli* numbering).

#dfmp7 (140-842)
T-ACAC-C-GGGAT-AACRC-CT-GGAAA-CTGGT-G-CTAATACCGGAT-ACG-CTCGG
--A-TG-GAC---ACAT----GTT--CGT-AC---------GAG-GAAA-GGAG------
---------CAA----------------TCCG--CTT---TAAG--GTGGATCCGCGT-C
CC-ATTA-G-CTA-G---TTGG-GG-GTGTAAC-GG-ACCACC-AAGGC-AA-CGA-TG-
GGTAGCCGG-CCT-G-AGAGGGT--------------------GGACGG-CCACACTGG-
AACTGA-GA-CACGGTCCAGA-CTCCTACG-GGAGGC-AGCA-GT-GGGGAATATTCCGC
AAT-GGGC-GAAA-GCC-TGACGGA-GCAACGCCGC-GTG-A-ATGATGAA--GGCC--T
T-CG--GG-TTGTAAA----------------------ATTCTGTY-TT-CAGG-GAA--GA
-A----------ACAAA----------------------------T-GA-CGGT-A-CCT
-G-AGG-AGGAAG--C-CCC-GGCTAAMTAC-GTGCCAGCAGCCGCGGTAAA-ACGTAG-
GGGGC-GAGCGTTGTCC-GGAATTACTGGGCGTAAAGGGC-GCGTAG-GCG-GTTT-TTT
-AAGTCAG-AGGTG-AAAAC-TATGGGC-TCAA-CCC-ATA-GCC--TGCCTTTGA-AAC
-TGGGA-GACTT-GAGTG-----CAGGA-GAGGGGAG-TGGAATTCCCAGTGTAGCG-GT
GAAA-TGCGT-AGAT-ATT-GGGAGG-AACA-CCAG--T-GGC-GAAGGCGG--CTTTTT
G----------------------------------GCCTGTAACTGACG-CTGAG-G-CG-CG
AAA-GC-GTAGG-GAGCGAACG-GGATTAGATAC-CCCGGTA-GTC-CACGCCGTAAAC-
GATGGGTG-CT-AGGTGTAGCGG-GT-----AT

Nucleotide sequence of cloned DFM (Group 1) 16S rDNA fragment 'dfmp7' (16S rDNA positions, *E. coli* numbering).

#dfmc7 (140-842)
T-AGAC-C-GGGAT-AACAG-CG-GGAAA-CCGGT-G-CTAAAACCGGAT-ACG-CTCTT
--T-GG----------AG-GAAA-AAGTGCC---
-TT------TAAGGC----------GCTG--CTT---TTGG--ATGGGTCCGCGT-C
CC-ATTA-G-CTA-G---TTGG-TG-CGGTAAC-GG-CGCACC-AAGGC-GA-CGA-TG-
GGTAGCCGG-CCT-G-AGAGGGT----------GAGCGG-CCACACTGG-
GACTGA-GA-CACGGCCCAGA-CTCCTACG-GGAGGC-AGCA-GT-GGGGAATCTTCCGC
AAT-GGGC-GAAA-GCC-TGACGGA-GCAATGCCGC-GTG-A-GCGAAGAA--GGCC--T
T-CG--GG-TCGTAAA----------GCTCTGTC-CA-GGGG-GAA--GA
-A----------CAAA----------T-GA-CGGT-A-CCC
-C-TGC-AGGGAG--C-CCC-GGCTAAATAC-GTGCCAGCAGCCGCGGTAAC-ACGTAG-
GGGGC-AAGCGTTGTCC-GGAATTACTGGGCGTAAAGCGC-GCGTAG-GCG-GCCT-TAA
-AAGTCAG-AGGTG-AAAAC-CGGCAGC-TCAA-CTG-CAG-GCC--TGCCTCTGA-AAC
-TTTAA-GGCTT-GAGGA-----CAGGA-GAGGGGAG-TGGAATTCCCAGTGTAGCG-GT
GAAA-TGCGT-AGAT-ATT-GGGAGG-AACA-CCGG--T-GGC-GAAGGCGG--CTCCCT
G----------GCCTGTAACTGACG-CTGAG-G-CG-CG
AAA-GC-GTGGG-GATCAAACA-GGATTAGATAC-CCTGGTA-GTC-CACGCCGTAAAC-
GATGGGTG-CT-AGGTGTTGGGG-GT-----AT

Nucleotide sequence of cloned DFM (Group 1) 16S rDNA fragment 'dfmc7' (16S rDNA positions, *E. coli* numbering).

#dfmc8 (140-842)
T-AGAC-C-GGGAT-AACAG-CG-GGAAA-CCGGT-G-CTAAAACCGGAT-ACG-CTCTT
--T-GG----------AG-GAAA-AAGTGCC---
-TT-------TAAGGC----------GCTG--CTT---TTGG--ATGGGTCCGCGT-C
CC-ATTA-G-CTA-G---TTGG-TG-CGGTAAC-GG-CGCACC-AAGGC-GA-CGA-TG-
GGTAGCCGG-CCT-G-AGAGGGT----------GAGCGG-CCACACTGG-
GACTGA-GA-CACGGCCCAGA-CTCCTACG-GGAGGC-AGCA-GT-GGGGAATCTTCCGC
AAT-GGGC-GAAA-GCC-TGACGGA-GCAATGCCGC-GTG-A-GCGAAGAA--GGCC--T
T-CG--GG-TCGTAAA----------GCTCTGTC-CA-GGGG-GAA--GA
-A----------CAAA----------T-GA-CGGT-A-CCC
-C-TGC-AGGGAG--C-CCC-GGCTAAATAC-GTGCCAGCAGCCGCGGTAAC-ACGTAG-
GGGGC-AAGCGTTGTCC-GGAATTACTGGGCGTAAAGCGC-GCGTAG-GCG-GCCT-TAA
-AAGTCAG-AGGTG-AAAAC-CGGCAGC-TCAA-CTG-CAG-GCC--TGCCTCTGA-AAC
-TTTAA-GGCTT-GAGGA-----CAGGA-GAGGGGAG-TGGAATTCCCAGTGTASCG-GT
GAAA-TGCGT-AGAT-ATT-GGGAGG-AACA-CCGG--T-GGC-GAAGGCGG--CTCCCT
G----------GCCTGTAACTGACG-CTGAG-G-CG-CG
AAA-GC-GTGGG-GATCAAACA-GGATTAGATAC-CCTGGTA-GTC-CACGCCGTAAAC-
GATGGGTG-CT-AGGTGTTGGGG-GT-----AT

Nucleotide sequence of cloned DFM (Group 1) 16S rDNA fragment 'dfmc8' (16S rDNA positions, *E. coli* numbering).

#dbbp1 (121-1237)

```
C-GC-GTAAATAA--CCT-GC-CTTC---A-TGTC-T-GGAAT-AATAC-AC-CGAAA-G
GGGT-A-CTAATACCGGAT-ACA-CTTGC--T-TT--GT---ATAA----GT---AGA-G
T---------AAG-CAAA-GGTGGC-CTC-T-------GATT-------TA-A-GCTACT
G--CAT---GTTG--AGGGGTCTGCGT-ACC-ATTA-C-CTA-G---TAGG-TG-GGGTA
AT-GG-CCTACC-TAGGC-TA-CGA-TG-GTTAGCGGG-TCT-G-AAAGGAT--------
------------GATCCG-CCACACTGG-CACTGG-AA-CACGGGCCAnA-CTCCTACG-
GGAGGC-AGCA-GT-GAGGAATATTGCGCAAT-GGGG-GAAA-CCC-TGACGCA-GCGAC
CCCGC-GTG-A-GTGAGGAA--GGCC--TT-CC--GG-TCCTAAA--------------
-----GCTCTGTC-AA-GAGG-GAR--GA-AATGCG--TAATGGT-----T-AATA----
-CCTGTTA-TGT-TT-GA-CCGT-A-CCT-C-TAA-AGGAAG--C-ACC-GGCTAACTCC
-GTGCCACCAGCCGCGGTAAT-ACGGAG-GGTGC-AAGCGTTGTTC-GGAATCACAGGGC
GTAAACGGC-GCGCAG-GCG-GNTA-GGT-AAGTCAG-ATNTC-AANNC-CCACGGC-TT
AA-GCA-TGG-AGT--TGCATATGC-AAC-TGACA-NACTC-GAGTA-----CCACA-GG
GGAAAG-TGGAATTCCCGGTGT-------------------------------------
------------------------TTTCTG-------------------------------
-GCTGAGTACTGACG-CTGAG-G-CG-CGAAA-GC-GTGGG-GAGCAAACA-GGATTAGA
TAC-CCTGGTA-GTC-CACGCCGTAAAC-GATGTCAA-CT-AGATGTAGGGG-GT-----
-GTTGAT-----------------------------------CCCCTCTG-TGTCGCA-GCTA
ACGCATTAA-GTTGACCGCCT-G-GGGAGTACGGTC-GCAA-GATT-AAAACTC-AAA-G
GAATTG-ACGGGGG-CCC--GC-A--CA-AGCGGTGGAGTA-T-GT-GGT-TTAATT-CG
ATGCAACGCGAAGAA-CCTTA-CCTGGTCTTGACA-TC----------C---CGGG--
--AATCC-CTTT-GAAA-CTT-A-GGAG---TG-------(--CTTCATT----------
------------------------------------------------------------
----AG--AAG--G---------AG---CCCGG-AGA-------------------------
---------------------------CA-GGT-GCTGCATGGCTGTCGTCA-GCT---CG
-TGTC-GTGAGATGTTGGG-TTAA-GTCCCGCAA-CGAGC-GCAACCCTTG-CC--TTTA
G-TTGC-CAG-CAG-------------------------------TTCGG-----------
------------------------------------------------------------
------------------------------------------------------------
------------------------------------------------------------
------------------------------------------------------------
------------------------------------------------------------
-----------------------------------------CTGGG-CA-CT-C-T-A
AA-GG-GACTGCCGGTGT-CAA-ACCG--GAGG-A-AGG-TGGGGATG-ACG-TC-AAGT
CCTCAT-GGC-CTTTATG-AC-CA-GGGCT-ACA-CACGTA--CTAC
```

Nucleotide sequence of cloned DBB (Group 2) 16S rDNA fragment 'dbbp1' (16S rDNA positions, *E. coli* numbering).

```
#dbbp2 (121-1237)
C-GC-GTATATAA--CCT-GC-CTTC---A-TGTC-T-GGAAT-AATAC-AC-CGAAA-G
GGGT-A-CTAATACCGGAT-ACA-CTTGC--T-TT--GT---ATAA----TT---ATA-G
T----------AAG-CAAA-GGTGGC-CTC-T-------GATT-------TA-A-GCTACT
G--CAT---GTTG--AGGGGTCTGCGT-ACC-ATTA-C-CTA-G---TAGG-TG-GGGTA
AT-GG-CCTACC-TAGGC-TA-CGA-TG-GTTAGCGGG-TTT-G-AAAGGAT--------
------------GATCCG-CCACACTGG-CACTGG-AA-CACGGGCCAGA-CTCCTACG-
GGAGGC-AGCA-GT-GAGGAATATTGCGCAAT-GGGG-GAAA-CCC-TGACGCA-GCGAC
GCCGC-GTG-A-GTGAGGAA--GGCC--TT-CG--GG-TCGTAAA--------------
-----GCTCTGTC-AA-GAAG-AAA--GA-AATGCG--TAATGGT-----T-AATA----
-CCTGTTA-TGT-TT-GA-CGGT-A-CCT-C-TAA-AGGAAG--C-ACC-GGCTAACTCC
-GTGCCACCAGCCGCGGTAAT-ACGGGA-GGTGC-AAGCGTTGTTC-GGAATCACTGGGC
GTAAAGGGC-GCCCAG-GCG-GTTT-GGT-AAGTCAG-AAGTC-AAAGC-CCACCGC-TT
AA-CCG-TGG-AAG--TGCATTTGA-AAC-TGCCA-GACTT-GAGCA-----CCAGA-AG
GGAAAC-TGGAATTCCCGGTGTATAA-GTGAAA-TTCGT-ANAT-ATC-GGGAAG-AATN
-CCGG--T-GGC-GAATGCGA--CTTTCTG-----------------------------
-GCTGAATACTGACG-CTGAG-G-CG-CGAAA-GC-GGTGG-GAGCAAACA-GGATTAGA
TAC-CCTGGTA-GTC-CACGCCGTAAAC-GATGTCAA-NT-AGATGTAGGGG-GN-----
-GTTGAT-----------------------------------CCCCTCTG-TGTGGCA-GCTA
ACGCATTAA-GTTGACCGCCT-G-GGGAGTACGGTC-GCAA-GATT-AAAATTC-AAA-G
GAATTG-ACGGGGG-CCC--GC-A--CA-AGCGGTGGAGTA-T-GT-GGT-TTAATT-CG
ATGCAACGCGAAGAA-CCTTA-CCTGGTTTTGACA-TC------------C---CGGG--
--AATCC-TTTG-GAAA-ATT-A-GGAG---TG--------C--CTTCATT----------
--------------------------------------------------------------
----AG--AAG--G---------AG---CCCGG-AGA-----------------------
---------------------------CA-GGT-GCTGCATGGCTGTCGTCA-GCT---CG
-TGTC-GTGAGAWGTTGGG-TTAA-GTCCCGCAA-CGAGC-GCAACCCTTG-CC--TTTA
G-TTGC-CAG-CAG-------------------------------TTCGG-----------
--------------------------------------------------------------
--------------------------------------------------------------
--------------------------------------------------------------
--------------------------------------------------------------
--------------------------------------------------------------
-----------------------------------------NTGGG-CA-NT-N-T-A
AA-GG-GANTGCCGGTGT-CAA-ACCG--GAGG-A-AGG-TGGGGATG-ACG-TC-AAGT
CCTCAT-GGC-CTTTATG-AC-CA-GGGCT-ACA-CACGTA--ATAC
```

Nucleotide sequence of cloned DBB (Group 2) 16S rDNA fragment 'dbbp2' (16S rDNA positions, *E. coli* numbering).

```
#dbbp4 (121-1237)
C-GC-GTAAATAA--CCT-GC-CTTC---A-TGTC-T-GGAAT-AATAC-AC-CGAAA-G
GGGT-A-CTAATACCGGAT-ACA-CTTGC--T-TT--AT---ATAA----GT---AGA-G
T---------AAG-CAAA-GGTGGC-CTC-T-------GATA-------TA-A-GCTACT
G--CAT---GTTG--AGGGGTCTGCGT-ACC-ATTA-G-CTA-G---TAGG-TG-GGGTA
AT-GG-CCTACC-TAGGC-TA-CGA-TG-GTTAGCGGG-TCT-G-AAAGGAT--------
------------GATCCG-CCACACTGG-CACTGG-AA-CACGGGCCATA-CTCCTACG-
GGAGGC-AGCA-CT-GAGGAATATTGCGCAAT-GGGG-GAAA-CCC-TGACGCA-GCCAC
GCCGC-GTG-A-GTGAGGAA--GGCC--TT-CG--GG-TCCTATA--------------
-----TCTCTGTC-AG-AGGG-AAA--GA-AATGCC--TAATGGT-----T-AATA----
-CCCGTTA-TGT-TT-GA-CCGT-A-CCT-C-TAA-AGGAAG--C-ACC-GGCTAACTCC
-GTGCCACCAGCCGCGGTAAT-ACGGAG-GGTGC-AAGCGTTGTTC-GGAATCACAGGGC
GTAAAGGGC-GCGCAT-GCG-GTTT-GGT-AAGTCAG-ATGTG-AAAGC-CCACGGC-TT
AA-CCG-TGG-AAG--TGCATTTGA-AAC-TGCCA-GACTT-GAGTA-----CCAGA-GG
GGAAAG-TGGAATTCCCGGTGTAGAG-GTGAAA-TTTGT-AGAT-ATC-GGGGAG-AATA
-CCGG--T-GGC-GAAGGCGA--CTTTTTT-----------------------------
-GGTGAGTACTGACG-CTGAG-G-CG-CGAAA-GC-GTGGG-GAGCAAACA-GGATTAGA
TAC-CCTGGTA-GTC-CACGCCGTAAAC-GATGTCAA-CT-AGATGTAGGGG-GT-----
-GTTGAT-----------------------------------CCCCTCTG-TGTCGCA-GATA
ATGCATTAA-GTNGACCGCCT-G-GGGAGTACGGTC-GCAA-GATT-AAAATTC-AAA-G
GAATTG-ACGGGGG-CCC--GC-A--CA-AGCGGTGGAGTA-T-GT-GGT-TTAATT-CG
ATGCAACGGGAAGAA-CCTTA-CCTGGTNTTGACA-TG-------------T---CAAG--
--AAGCN-TTTG-AGAG-ATG-C-GGAG---TG-----------CC--TT----------
-------------------------------------------------------------
----CG--GG------------AA---CTTGA-ACA-------------------------
---------------------------CA-GGT-GCTGCATGGCTGTCTTCA-GCT---CG
-TGTC-GTGAGATGTTGGG-TTAA-GTCCCGCAA-CGAGC-GCAACCCTTG-CC--CTTA
G-TTGC-CAT-CAT-------------------------------TTAG-------------
-------------------------------------------------------------
-------------------------------------------------------------
-------------------------------------------------------------
-------------------------------------------------------------
-------------------------------------------------------------
-----------------------------------------------TTGGG-CA-CT-T-T-A
AG-GG-GACCTCCGGTGA-CAA-ACCG--GAGG-A-AGG-TGGGGATG-ACG-TC-AAGT
CATCAT-GGC-CCTTATG-AC-CA-GGGCT-ACA-CACGTA--CTAC
```

Nucleotide sequence of cloned DBB (Group 2) 16S rDNA fragment 'dbbp4' (16S rDNA positions, *E. coli* numbering).

```
#dbbr1 (121-1237)
C-GC-GTAAATAA--CCT-GC-CTTC---A-TGTC-T-GGAAT-AATAC-AC-CGAGA-G
GGGT-A-CTAATACCGGAT-ACA-CTTGC--T-TT--AT---ATAA----GT---AGA-G
T---------AAG-CAAA-GGTGGC-CTC-T-------GATA-------TA-A-GCTACT
G--CAT---GTTG--AGGGGTCTGCGT-ACC-ATTA-G-CTA-G---TAGG-TG-GGGTA
AT-GG-CCTACC-TAGGC-TA-CGA-TG-GTTAGCGGG-TCT-G-AAAGGAT---------
------------GATCCG-CCACACTGG-CACTGG-AA-CACGGGCCAGA-CTCCTACG-
GGAGGC-AGCA-GT-GAGGAATATTGCGCAAT-GGGG-GAAA-CCC-TGACNCA-GCGAC
GCCGC-GTG-A-GTGAGGAA--GGCC--TT-CG--GG-TCGTAAA---------------
-----GCTCTGTC-AA-GAGG-AAA--GA-AATGCG--TAATGGT-----T-AATA----
-CCTGTTA-TGT-TT-GA-CGGT-A-CCT-C-TAA-AGGAAG--C-ACC-GGCTAACTCC
-GTGCCAGCAGCCGCGGTAAT-ACGGAN-GGTGC-AAGCGTTGTTC-GGAATCACTGGGC
GTAAAGGGC-GCGCAA-GCG-GTTT-GGT-AAGTCAG-ATGTG-AAAGC-CCACGGC-TT
AA-CCG-KGG-AAR--TGCATTGGA-AAC-TGCCA-NACTT-GAGTA-----CCAGA-RG
GGAAAG-TGGAATTCCCGGTGTAGAA-GTGAAA-TTCGT-AGAT-ATC-GGGARG-AATA
-CCGG--T-GGC-GAAGGCGA--CTTTCTG-------------------------------
-GCTGARTACTGACG-CTGAG-G-CG-CGAAA-GC-GTGGG-GAGCAAACA-GGATTAGA
TAC-CCTGGTA-GTC-CACGCCGTAAAC-GATGTCAA-TA-AGATGTAGGGG-GT-----
-GTTGAT-------------------------------CCCCTCTG-TGTCGCA-GCTA
ACGCATTAA-GTTGACCGCCT-G-GGGAGTACGGTT-GCAA-GATT-AAAACTC-AAA-G
GAATTG-ACGGGGG-CCC--GC-A--CA-AGCGGTGGAGTA-T-GT-GGT-TTAATT-CG
ATGCAACGCGAAGAA-CCTTA-CCTGGTTTTGACA-TC------------C---CGGG--
--AATCC-TTTG-GAAA-ATT-A-GGAG---TG-------C----C--TT----------
-----------------------------------------------------------
----CATTAGAAGG---------AG---CCCGG-AGA-----------------------
---------------------------CA-GGT-GCTGCATGGCTGTCGTCA-GCT---CG
-TGTC-GTGAGATGTTGGG-TTAA-GTCCCGCAA-CGAGC-GCAACCCTTG-CC--TTTA
G-TTGC-CAG-CAG-------------------------------TTCGG-----------
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-----------------------------------------------------------
-----------------------------------------------------------
-----------------------------------------------------------
-------------------------------------------CTGGG-CA-CT-C-T-A
AA-GG-GACTGCCGGTGT-CAA-ACCG--GAGG-A-AGG-TGGGGATG-ACG-TC-AAGT
CCTCAT-GGC-CTTTATG-AC-CA-GGGCT-ACA-CACGTA--CTAC
```

Nucleotide sequence of cloned DBB (Group 2) 16S rDNA fragment 'dbbr1' (16S rDNA positions, *E. coli* numbering).

```
#dbbr2 (121-1237)
C-GC-GTAGATAA--CCT-GC-CTTC---A-TGTC-T-GGAAT-AATAC-AC-CGAGA-G
GGGT-A-CTAATACCGGAT-ACA-CTTGC--T-TT--AT---ATAA----GT---AGA-G
T----------AAG-CAAA-GGTGGC-CTC-T--------GATA--------TA-A-GCTACT
G--CAT---GTTG--AGGGGTCTGCGT-ACC-ATTA-G-CTA-G---TAGG-TG-GGGTA
AT-GG-CCTACC-TAGGC-TA-CGA-TG-GTTAGCGGG-TCT-G-AGAGGAT---------
------------GATCCG-CCACACTGG-CACTGG-AA-CACGGGCCAGA-CTCCTACG-
GGAGGC-AGCA-GT-GAGGAATATTGCGCAAT-GGGG-GAAA-CCC-TGACGCA-GCGAC
GCCGC-GTG-A-GTGAGGAA--GGCC--TT-CG--GG-TCGTAAA---------------
-----GCTCTGTC-AA-GAGG-AAA--GA-AATGCG--TAATGGT-----T-AATA----
-CCTGTTA-TGT-TT-GA-CGGT-A-CCT-C-TAA-AGGAAG--C-ACC-GGCTAACTCC
-GTGCCAGCAGCCGCGGTAAT-ACGGAG-GGTGC-AAGCGTTGTTC-GGAATCACTGGGC
GTAAAGGGC-GCGCAG-GCG-GTTT-GGT-AAGTCAG-ATGTG-AAAGC-CCACGGC-TT
AA-CGC-TGG-AGT--TGCATATGA-AAC-TGCCA-AACTT-GAGTA-----CCAGA-GG
GGAAAG-TGGAATTCCCGGTGT-------------------------------------
-----------------------------------------CAAACA-GGATTAGA
TAC-CCTGGTA-GTC-CACGCCGTAAAC-GATGTCAA-CT-AGATGTAGGGG-GT------
-GTTGAT-----------------------------------CCCCTCTG-TGTCGCA-GnTA
ACGCATTAA-GTTGACCGCCT-G-GGGAGTACGGTC-GCAA-GATT-AAAACTC-AAA-G
GAATTG-ACGGGGG-CCC--GC-A--CA-AGCGGTGGAGTA-T-GT-GGT-TTAATT-CG
ATGCAACGCGAAGAA-CCTTA-CCTGGTCTTGACA-TC------------C---CGGG--
--AATCC-TTTG-GAAA-CTT-A-GGAG---TG--------C----C--TT----------
-----------------------------------------------------------
----CATTAGAAGG---------AG---CCCGG-AGA-----------------------
-------------------------CA-GGT-GCTGCATGGCTGTCGTCA-GCT---CG
-TGTC-GTGAGATGTTGGG-TTAA-GTCCCGCAA-CGAGC-GCAACCCTTG-CC--TTTA
G-TTGC-CAG-CAG-------------------------TTCGG-------------
-----------------------------------------------------------
-----------------------------------------------------------
-----------------------------------------------------------
-----------------------------------------------------------
-----------------------------------------------------------
-----------------------------------------CTGGG-CA-CT-C-T-A
AA-GG-GACTGCCGGTGT-CAA-ACCG--GAGG-A-AGG-TGGGGATG-ACG-TC-AAGT
CCTCAT-GGC-CTTTATG-AC-CA-GGGCT-ACA-CACGTA--CTAC
```

Nucleotide sequence of cloned DBB (Group 2) 16S rDNA fragment 'dbbr2' (16S rDNA positions, *E. coli* numbering).

#dsbb1 (127-1273)
GATAA--TCT-GC-CTTC---A-AGCC-T-GGGAT-AACTA-TT-CGAAA-GGGTA-G-C
TAATACCGGAT-AAA-GTCGA--T-TT--AC---ACAA----GT---AGA-TT-------
--GAT-GAAA-GATTGC-CTC-TT-------CTTG--------AA-A-GCAATTG--TTT--
-GGAG--ATGAGTTTGCGT-ACC-ATTA-G-CTT-G---TTGG-TG-GGGTAAA-GG-CC
TACC-AAGGC-AA-CGA-TG-GTTAGCTGG-TCT-G-AGAGGAT----------------
-----GATCAG-CCACACTGG-AACTGG-AA-CACGGTCCAGA-CTCCTACG-GGAGGC-A
GCA-GT-GAGGAATTTTGCGCAAT-GGGG-GCAA-CCC-TGACGCA-GCAACGCCGC-GT
G-A-GTGAAGAA--GGCC--TT-TG--GG-TCGTAAA----------------------GCT
CTGTC-AA-CAGG-GAA--GA-AGTTAC--AATTGTT-----T-AACA-----GATGGTT
-GTA-TT-GA-CGGT-A-CCT-G-TGG-AGGAAG--C-GCC-GGCTAACTCC-GTGCCAG
CAGCCGCGGTAAC-ACGGGG-GGCGC-AA-CGTTATTC-GGAATTATTGGGCGTAAAGGG
C-GCGCAG-GCG-GTCT-TGT-CCGTCAG--TGTG-AAAGC-TCGGGGC-TCAA-CCC-C
GG-AAG--TGCACTTGA-AAC-AGCAA-GACTT-GAATA-----CGGGA-GAGGAAAG-C
GGAATTCCTGGTGTAGAG-GTGAAA-TTCGT-AGAT-ATC-AGGAGG-AACA-CCGA--T
-GGC-GAAGGCAG--CTTTCTG-------------------------------GACCGAT
ATTGACG-CTGAG-G-CG-CGAAG-GC-GTGGG-TAGCAAACG-GGATTAGATAC-CCCG
GTA-GTC-CACGCAGTAAAC-GTTGTACA-CT-CGGTGTAGCGG-GT------ATTAAA-
-----------------------------ACCTGCTG-TGCCCAA-G-TAACGCATTA
A-GTGTACCGCCT-G-GGAAGTACGGTC-GCAA-GACT-AAAACTC-AAA-GGAATTG-A
CGGGGG-CC---GC-A--CA-AGCGGTGGAGCA-T-GT-GGT-TTAATT-CGACGCAACG
CGAAGAA-CCTTA-CCTGGGTTTGACA-TC------------C---TGTG----AATAT-
CCCG-TAAT-TGG-G-ATAG---TG-----------CC--TT-----------------
---CG--
GG------------AG---CACAG-AGA-----------------------------------
------------------CA-GGT-GCTGCATGGCTGTCGTCA-GCT---CG-TGTC-GT
GAGATGTTTGG-TTAA-GTCCAGCAA-CGAGC-GCAACCCTTA-TC--GTTAG-TTGC-C
AG-CAT-------------------------------TTAAAG---------------------
----

-----------------------------------ATGGG-AA-CT-C-T-AAC-GA-GA
CTGCCCGGGT-CAA-CCGG--GAGG-A-AGG-TGGGGATG-ACG-TC-AAGTCCTCAT-G
GC-CCCTATA-TC-CA-GGGCT-ACA-CACGTG--CTACAATGGTAGGTA--CAAAGGGC
-A-GC-GA-CTT-CG-CGGGGGG

Nucleotide sequence of cloned DSB (Group 4) 16S rDNA fragment 'dsbb1' (16S rDNA positions, *E. coli* numbering).

```
#dsbb2 (127-1273)
GATAA--TCT-GC-CTTC---A-AGCC-T-GGGAT-AACTA-TT-CGAAA-GGGTA-G-C
TAATACCGGAT-AAA-GTCGA--T-TT--AC---ACAA----GT---AGA-TT-------
--GAT-GAAA-GATTGC-CTC-TT------CTTG-------AA-A-GCAATTG--TTT--
-GGAG--ATGAGTTTGCGT-ACC-ATTA-G-CTT-G---TTGG-TG-GGGTAAA-GG-CC
TACC-AAGGC-AA-CGA-TG-GTTAGCTGG-TCT-G-AGAGGAT---------------
----GATCAG-CCACACTGG-AACTGG-AA-CACGGTCCAGA-CTCCTACG-GGAGGC-A
GCA-GT-GAGGAATTTTGCGCAAT-GGGG-GCAA-CCC-TGACGCA-GCAACGCCGC-GT
G-A-GTGAAGAA--GGCC--TT-TG--GG-TCGTAAA--------------------GCT
CTGTC-AA-CAGG-GAA--GA-AGTTAC--AATTGTT-----T-AACA-----GATGGTT
-GTA-TT-GA-CGGT-A-CCT-G-TGG-AGGAAG--C-GCC-GGCTAACTCC-GTGCCAG
CAGCCGCGGTAAC-ACGGGG-GGCGC-AA----------------------------
----------------------------------------------------------
----------------------------------------------------------
----------------------------------------------------------
----------------------------------------------------------
----------------------------------------------------------
----------------------------------------------------------
----------------------------------ATTA
A-GTGTACCGCCT-G-GGAAGTACGGTC-GCAA-GACT-AAAACTC-AAA-GGAATTG-A
CGGGGG-CCC--GC-A--CA-AGCGGTGGAGCA-T-GT-GGT-TTAATT-CGACGCAACG
CGAAGAA-CCTTA-CCTGGGTTTGACA-TC----------C---TGTG----AATAT-
CCCG-TAAT-TGG-G-ATAG---TG----------CC--TT------------------
----------------------------------------------------CG--
GG----------AG---CACAG-AGA---------------------------------
------------------CA-GGT-GCTGCATGGCTGTCGTCA-GCT---CG-TGTC-GT
GAGATGTTTGG-TTAA-GTCCAGCAA-CGAGC-GCAACCCTTA-TC--GTTAG-TTGC-C
AG-CAT----------------------------TTAAAG----------------------
----------------------------------------------------------
----------------------------------------------------------
----------------------------------------------------------
----------------------------------------------------------
----------------------------------ATGGG-AA-CT-C-T-AAC-GA-GA
CTGCCCGGGT-CAA-CCGG--GAGG-A-AGG-TGGGGATG-ACG-TC-AAGTCCTCAT-G
GC-CCTTATA-TC-CA-GGGCT-ACA-CACGTG--CTACAATGGTAGGTA--CAAAGGGC
-A-CC-GA-CTT-CG-CGGGGGG
```

Nucleotide sequence of cloned DSB (Group 4) 16S rDNA fragment 'dsbb2' (16S rDNA positions, *E. coli* numbering).

#dccp2 (305-1165)

GATCAG-CCACACTGG-GACTGA-CA-CACGGTCCAGA-CTCCTACG-GGAGGC-AGCA-
GT-GAGGAATTTTGCGCAAT-GGGC-GAAA-GCC-TGACGCA-GCAACGCCGC-GTG-A-
GTGATGAA--GGCC--TT-CG--GG-TCGTAAA----------GCTCTGT
C-AA-GTGG-GAA--GA-ACCTGC--AGGAGGT-----A-AATA-----CCCTTTT-GC-
ACT-GA-CGGT-A-CCA-C-TGA-AGGAAG--C-ACC-GGCTAACTCC-GTGCCAGCAGC
CGCGGTAAT-ACGGGG-GGTGC-AAGCGTTATTC-GGATTTATTGGGCGTAAAGGGC-GC
GTAG-GCG-GCCT-GTT-AAGTCAS-ATGTG-AAAGC-CCGGGGN-TCAA-CTC-CGG-A
AG--TGCATTTGA-AAC-TAGCA-GGCTT-GAGTA-----TGGGA-GAGGGAAG-TGGAA
TTCCTGGTGTAGAG-GTGAAA-TTCGT-AGAT-ATC-AGGAGG-AACA-CCGG--T-GGC
-GAAGGCGG--TTTCCTG----------GACCAATACTG
ACG-CTGAG-G-CG-CGAAG-GC-GTGGG-GAGCAAACA-GGATTAGATAC-CCTGGTA-
GTC-CACGCAGTAAAC-GGTGATCA-CT-AGGTGTAGCGG-G-------TATTGA-----
----------CCCCTGCTG-TGCCGCA-GCTAACGCATTAA-GT
GATCCGCCT-G-GGGAGTACGATC-GCAA-GATT-AAAACTC-AAA-GGAATTG-ACGGG
GG-CCC--GC-A--CA-AGCGGTGGAGCA-T-GT-GGT-TTAATT-TGACGCAACGCGCA
GAA-CCTTA-CCTGGATTTGACA-TC----------T---GTGG-----AATT-TTGT
-TGAA-AGA-C-GAAAG--TG-------C---CC--TT----------
----------CA--GG--
-G--------AG---CCGCA-AGA----------
----------CA-GGT-GCTGCATGGCTGTCGTCA-GCT---CG-TGTC-GTGAGA
TGTTGGG-TTAA-GTCCCGCAA-CGAGC-GCAACCCTTG-TC--TTTAG-TTAC-CAG-C
AT----------TAAGT----------

----------TGGG-GA-CT-C-T-AAA-GA-TACTGC
CCC

Nucleotide sequence of cloned DCC-DNM-DSS (Group 5) 16S rDNA fragment 'dccp2'
(16S rDNA positions, *E. coli* numbering).

#dccp4 (305-1165)
GATCAG-CCACACTGG-GACTGA-CA-CACGGTCCAGA-CTCCTACG-GGAGGC-AGCA-
GT-GAGGAATTTTGCGCAAT-GGGG-GGAA-CCC-TGACGCA-GCAACGCCGC-GTG-A-
GTGAAGAA--GGCC--TT-CG--GG-TCGTAAA----------GCTCTGT
C-GA-GTGG-GAA--GA-ACCTTC--GTATGTT-----G-AATA-----TACATAC-GG-
ACT-GA-CGGT-A-CCA-C-AGA-AGGAAG--C-ACC-GGCTAACTCC-GTGCCAGCAGC
CGCGGTAAT-ACGGAG-GGTGC-AAGCGTTATTC-GGAATTATTGGGCGTAAAGAGC-GC
GTAG-ACG-GCTT-TGC-AAGTCAG-GTGTG-AAATC-CCGGGGC-TCAA-CCC-CGG-A
AG--AGCATTTGA-TAC-TGTGG-AGCTT-GAGTA-----TGGGA-GAGGGAAG-TGGAA
TTCCTGGTGTAGCG-GTGAAA-TGCGT-AGAT-ATC-AGGAGG-AACA-CCGG--T-GGC
-GAAGGCGG--CTTCCTG----------GACCAATACTG
ACG-CTGAA-G-CG-CCAAA-GC-GTGGG-GAGCAAACA-GGATTAGATAC-CCTGGTA-
GTC-CACGCAGTAAAC-GTTGATCA-CT-AGGTGTAGCGG-G--------TATTGA-----
----------CCCCTGCTG-CGCCGGA-GTTAACGCATTAA-GT
GATCCGCCT-G-GGGAGTACGATC-GCAA-GATT-AAAACTC-AAA-GGAATTG-ACGGG
GG-CCC--GC-A--CA-AGCGGTGGAGTA-T-GT-GGT-TTAATT-TGACGCAACGCGAA
GAA-CCTTA-CCTGGATTTGACA-TC----------C---GCGG-----GATT-CTTA
-TGAA-AAT-A-GGGAG--TG--------C---CC--TT----------
----------CG--GG--
-G--------AG---CCGCG-AGA----------
----------CA-GGT-GCTGCATGGCTGTCGTCA-GCT---CG-TGTC-GTGAGA
TGTTGGG-TTAA-GTCCCGCAA-CGAGC-GCAACCCCTG-TC--TTCAG-TTAC-CAT-C
AT----------TAAGT----------

----------TGGG-GA-CT-C-T-GAA-GA-TACTGC
CCC

Nucleotide sequence of cloned DCC-DNM-DSS (Group 5) 16S rDNA fragment 'dccp4'
(16S rDNA positions, *E. coli* numbering).

#dccp9 (305-1165)

GATCAG-CCACACTGG-GACTGA-CA-CACGGTCCAGA-CTCCTACG-GGAGGC-AGCA-
GT-GAGGAATTTTGCGCAAT-GGGG-GAAA-CCC-TGACGCA-GCAACGCCGC-GTG-A-
GTGATGAA--GGCC--TT-CG--GG-TCGTAAA----------GCTCTGT
C-AA-GTGG-GAA--GA-ACCTGC--AGAAAGC-----T-AACA-----TCTTTTT-GC-
ACT-GA-CGGT-A-CCA-C-TGA-AGGAAG--C-ACC-GGCTAACTCC-GTGCCAGCAGC
CGCGGTAAT-ACGGAG-GGTGC-AAGCGTTATTC-GGAATTATTGGGCGTAAAGGGC-GC
GTAG-GCG-GCCG-TTT-AAGTCAG-GTGTG-AAAGC-CCGGGGC-TTAA-CCC-CGG-A
AG--TGCATTTGA-TAC-TGAGC-GGCTT-GAGTA-----TGGGA-GAGGGAG-TGGAA
TTCCTGGTGTAGAG-GTGAAA-TTCGC-AGAT-ATC-AGGAGG-AACA-CCGG--T-GGC
-GAAGGCGA--YTTCCTG----------GACCAATACTG
ACG-CTGAG-G-CG-CGAAG-GC-GTGGG-GATCAAACA-GGATTAGATAC-CCTGGTA-
GTC-CACGCAGTAAAC-GGTGATCA-CT-AGGTGTAGCGG-G-------TATGAC-----
----------CCCTGCTG-TGCCGCA-GCTAACGCATTAA-GT
GATCCGCCT-G-GGGAGTACGATC-GCAA-GGTT-AAAACTC-AAA-GGAATTG-ACGGG
GG-CCC--GC-A--CA-AGCGGTGGAGCA-T-GT-GGT-TTAATT-TGACGCAACGCGAA
GAA-CCTTA-CCTGGGCTTGACA-TT----------T---GCGG-----AATT-TCTA
-TGAA-AGT-A-GGAAG--TG-------C---CC--TT----------
----------CG--GG--
-G--------AG---CCGCA-AGA----------
----------CA-GGT-GCTGCATGGCTGTCGTCA-GCT---CG-TGTC-GTGAGA
TGTTGGG-TTAA-GTCCCGCAA-CGAGC-GCAACCCCTA-TC--TTTAG-TTAC-CAT-C
AT----------TCAGT----------

----------TGGG-GA-CT-C-T-AAA-GA-TACTGC
CCC

Nucleotide sequence of cloned DCC-DNM-DSS (Group 5) 16S rDNA fragment 'dccp9'
(16S rDNA positions, *E. coli* numbering).

#dccw2 (305-1165)
GATCAG-CCACACTGG-GACTGA-CA-CACGGTCCATA-GTCCTACG-GGATGC-AGCA-
GT-CAGGAATTTTGCGCAAT-GGGG-GAAA-CCC-TGACGCA-GCAACGCCGC-GTG-A-
ATGATGAA--GGCC--TT-TG--GG-TCATAAA----------ACTCTGT
C-AT-CTGG-AAT--GA-AGTTAT--GGAGGGT-----T-AATA-----CCCCTTT-AT-
ATT-GA-CGGT-T-CCT-G-CAA-AGGAAG--C-ACC-GGCTAACTCC-GTGCCAGCATC
CGCGGTAAC-ACGGAC-GGTGC-AACCGTTATT--GGAATTATTGGGCGTAAAGGGC-GC
GTAG-GCG-GCCG-ATC-AGGTCAG-ATGTG-AAAGC-CCGGGGC-TTAA-CCC-CGG-A
AG--TGCATTTGA-AAC-CGGTT-GGCTT-GAGTA-----TGGGA-GAGGAGAG-CGGAA
TTCCTGGTGTAGAG-GTGAAA-TTTGT-AGAT-ATC-AGGAGG-AACA-CCGG--T-GGC
-GAAGGCGG--CTCTTTG----------GACCAATACTG
ACG-CTGAG-G-CG-CGAAG-GC-GTGGG-TAGCAAACA-GGATTAGATAC-CCTGGTA-
GTC-CACGCAGTAAAC-GTTGTTCA-CT-AGGTGTAGTGG-G-------TATTGA-----
----------CCCCTACTG-TGCCGCA-GCTAACGCATTAA-GT
GAACCGCCT-G-GGAAGTACGGTC-GCAA-GATT-AAAACTC-AAA-GGAATTG-ACGGG
GG-CCC--GC-A--CA-AGCGGTGGAGCA-T-GT-GGT-TTAATT-TGACGCAACGCGGA
GAA-CCTTA-CCTGGGTTTGACA-TT----------T---CGGG-----AAT--CTTA
-TGAA-AAT-A-GAGAG--TG-------C---CC--TT----------
----------CG--GG--
-G--------AG---CCCGA-AGA----------
----------CA-GGT-GCTGCATGGCTGTCGTCA-GCT---CG-TGTC-GTGAGA
TGTTGGG-TTAA-GTCCCGCAA-CGAGC-GCAACCCTTA-TT--TTCAG-TTAC-CAG-C
GA----------TTCGGT----------

----------CGGG-GA-CT-C-T-GAA-GA-TACTGC
CCC

Nucleotide sequence of cloned DCC-DNM-DSS (Group 5) 16S rDNA fragment 'dccw2'
(16S rDNA positions, *E. coli* numbering).

#dccw3 (305-1165)
GATCAG-CCACACTGG-GACTGA-CA-CACGGTCCAGA-CTCCTACG-GGAGGC-AGCA-
GT-GAGGAATTTTGCGCAAT-GGGG-GCAA-CCC-TGACGCA-GCAACGCCGC-GTG-A-
GTGATGAA--GGCC--TT-TG--GG-TCATAAA----------ACTCTGT
C-AG-CAGG-AAA--GA-AGTTAT--RGAGGGT-----T-AATA-----CCCCTTT-AT-
ATT-GA-CGGT-A-CCT-G-CAA-AGGAAG--C-ACC-GGCTAACTCC-GTGCCAGCAGC
CGCGGTAAC-ACGGAG-GGTGC-AAGCGTTATTC-GGAATTATTGGGCGTAAAGGGC-GC
GTAG-GCG-GCCG-ATC-AGGTCAG-ATGTG-AAAGC-CCGGGGC-TTAA-CCC-CGG-A
AG--TGCATTTGA-AAC-CGGTT-GGCTT-GAGTA-----TGGGA-GAGGARAG-CGGAA
TTCCTGGTGTAGAG-GTGAAA-TTCGT-AGAT-ATC-AGGAGG-AACA-CCGG--T-GGC
-GAAGGCGG--CTCTCTG----------GACCAATACTG
ACG-CTGAG-G-CG-CGAAG-GC-GTGGG-TAGCAAACA-GGATTAGATAC-CCTGGTA-
GTC-CACGCAGTAAAC-GTTGTTCA-CT-AGGTGTAGTGG-G-------TATTGA-----
----------CCCCTACTG-TGCCGCA-GCTAACGCATTAA-GT
GAACCGCCT-G-GGAAGTACGGTC-GCAA-GATT-AAAACTC-AAA-GGAATTG-ACGGG
GG-CCC--GC-A--CA-AGCGGTGGAGCA-T-GT-GGT-TTAATT-TGACGCAACGCGGA
GAA-CCTTA-CCTGGGTTTGACA-TC----------T---CGGG-----AAT--CTTA
-TGAA-AAT-A-GAGAG--TG-------C---CC--TT----------
----------CG--GG--
-G--------AG---CCCGA-AGA----------
----------CA-GGT-GCTGCATGGCTGTCGTCA-GCT---CG-TGTC-GTGAGA
TGTTGGG-TTAA-GTCCCGCAA-CGAGC-GCAACCCTTA-TC--TTCAG-TTAC-CAG-C
GA----------TTCGGT----------

----------CGGG-GA-CT-C-T-GAA-GA-TACTGC
CCC

Nucleotide sequence of cloned DCC-DNM-DSS (Group 5) 16S rDNA fragment 'dccw3'
(16S rDNA positions, *E. coli* numbering).

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#dsvr4 (230-838)
GAGCCTGCGT-CCC-ATTA-G-CTA-G---TTGG-CG-GGGTAAC-GG-CCCACC-AAGG
C-AA-CGA-TG-GGTAGCTGG-TCT-G-AGAGGAT----------GATCA
G-CCACACTGG-GAATGR-AA-CACGGCCCAGA-CTCCTACG-GGAGGC-AGCA-GT-GG
GGAATATTGCGCGAT-GGGG-GAAA-CCC-TGACGCA-GCGACGCCGT-GTG-A-GGGAA
GAA--GGCC--TT-CG--GG-TCGTAAA----------CCTCTGTC-GG-
GAGG-GAA--GA-ACCGCC--AGGTTTC-----G-AACAG----ASACCT---GGCCT-G
G-CGGT-A-CCT-Y-TAR-AGGAAG--C-GCC-GGCTAACTCC-GTGCCAGCAGCCGCGG
TAAT-ACGGAG-GGCGC-GAGCGTTAATC-GGAATCACTGGGCGTAAAGCGC-ACGTAG-
GCG-GCGA-RAT-AAGTCGG-GCGTG-AAAGC-CCTCGGC-CCAA-CCG-AGG-AAT--T
GCGTTCGA-TAC-TGTTT-GGCTT-GAAGTC----CTGGA-GAGGGTGG-CGGAATTCCG
GGTGTAGGA-GTGAAA-TCCGT-AGAT-ATC-SGGAGG-AACA-CCGG--T-GGC-GAAG
GCGG--CCACCTG----------GACAGGTACTGACG-C
TGAG-G-TG-CGAAA-GC-GTGGG-GAGCAAACA-GGATTAGATAC-CCTGGTA-GTC-C
ACGCTGTAAAC-GATGGATA-CT-AGGTGTCGGGG
```

Nucleotide sequence of cloned DSV-DMB (Group 6) 16S rDNA fragment 'dsvr4' (16S rDNA positions, *E. coli* numbering).

#dsvr8 (230-838)
GAGTCTACTT-CCC-AGTA-G-CTA-C---TTGG-TG-GGGTCCC-GG-CCCACC-AAGG
C-NA-CGA-TG-GGTACCTGG-TCT-G-AAAGGAT----------GATCN
N-CCACACTGG-GTACTG-AA-CACGGCCCAGA-CTCCTACG-GGAGGC-AGCA-GT-GG
GGAATATTGCGCAAT-GGGG-GAAA-CCC-TGACGCA-ACGACTCCGT-GTG-A-GGGAA
GAA--GGCC--TT-CG--GG-TCCTAAA----------CCTCTGTC-GG-
GAGG-GAA--GA-ACCGCC--AnGTTTC-----N-AACAA----AAACCT---GGCCT-G
A-CGGT-A-CCT-C-TAN-AGGAAG--C-GCC-GGCTAACTCC-GTGCCAGCAGCCGCGG
TAAT-ACGGAG-GGCGC-GAGCGTTAATC-GGAATCACTGGGCGTAAAGCGC-ACGTAG-
GCG-GCGW-RAT-AAGTCGG-GCGTG-AAASC-CCTCGGC-CCAA-CCG-AGG-AAT--T
GCGTTCGA-TAC-TGTTT-GGCTT-GAGTC-----CTGGA-GAGGGTGG-CGGAATTCCG
GGTGTAGGA-GTGAAA-TCCGT-AGAT-ATC-CGGAGG-AACA-CCGG--T-GGC-GAAG
GCGG--CCACCTG----------GACAGGTACTGACG-C
TGAG-G-TG-CGAAA-GC-GTGGG-GAGCAAACA-GGATTAGATAC-CCTGGTA-GTC-C
ACGCTGTAAAC-GATGGACG-CT-AGATGCCGGGC

Nucleotide sequence of cloned DSV-DMB (Group 6) 16S rDNA fragment 'dsvr8' (16S rDNA positions, *E. coli* numbering).

#dsvb2 (230-838)
GAGCCTGCGT-CCC-ATTA-G-CTA-G---TTGG-TA-TGGTAAY-GG-CCTACC-NANG
C-AA-CGA-TG-AGTANCTGG-TCC-G-AGAGGAT----------GATCN
A-CCACGCTGG-AACTGA-AA-CACGGTCCAGA-CTCCTACG-GGAGGC-AGCA-GT-GG
GGAATATTGCsCAAT-GGGC-GAAA-GCC-TGACGCA-GCAACGCCGT-GTG-A-GGGAT
GAA--GGCT--TT-CG--GG-TCGTAAA----------CCTCTGTC-GG-
AAGG-GAA--GA-ACGGGC--ATTGGTT-----T-AATAG----GCCTTT--GTT-TT-G
A-CGGT-A-CCT-T-TAG-AGGAAG--C-ACC-GGCTAACTCC-GTGCCAGCAGCCGCGG
TAAT-ACGGAG-GGTGC-AAGCGTTATTC-GGAATTACTGGGCGTAAAGCGC-ACGTAG-
GCG-GCCT-TGT-AAGTCAG-GGGTG-AAATC-CCCACGG-TCAA-CCG-TGG-AAC--T
GCCTTTGA-AAC-TGCAG-GGCTT-GAATC-----CTGGA-GAGGGTGG-CGGAATTCCT
GGTGTAGGA-GTGAAA-TCCGT-AGAT-ATC-AGGAGG-AACA-CCGG--T-GGC-GAAG
GCGG--CCACCTG----------GACAGGTATTGACG-C
TGAG-G-TG-CGAAA-GT-GTGGG-GAGCAAACA-GGATTAGATAC-CCTGGTA-GTC-C
ACACCGTAAAC-GATGGATA-CT-AGGTGTCGGGG

Nucleotide sequence of cloned DSV-DMB (Group 6) 16S rDNA fragment 'dsvb2' (16S rDNA positions, *E. coli* numbering).

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#dsvb3 (230-838)
GAGCCTGCGT-CCC-ATTA-C-CTA-N---TTGG-TA-GGGTNTT-GG-CCTACC-CCGG
C-AA-CGA-TG-AGTANCTGG-TCT-G-AAAGGAT--------------------GATCA
C-CCACGCTGG-GAACTN-AA-CACGGTCCAGA-CTCCTACG-GGAGGC-AGCA-GT-GG
GGAATATTGCGCAAT-GGGC-GAAA-GCC-TGACGCA-GCAACGCCGT-GTG-A-GGGAT
GAA--GGCT--TT-CG--GG-TCGTAAA--------------------CCTCTGTC-GG-
AAGG-GAA--GA-ACGGGC--WTTGGTC-----T-AATAG----GCCTTT--GTT-TT-G
A-CGGT-A-CCT-T-TAG-AGGAAG--C-ACC-GGCTAACTCC-GTGCCAGCAGCCGCGG
TAAT-ACGGAG-GGTGC-AAGCGTTATTC-GGAATTACTGGGCGTAAAGCGC-ACGTAG-
GCC-GCTT-TGT-AAGTCAG-GGGTG-AAATC-CCACGGC-TCAA-CCG-TGG-AAC--T
GCCTTTGA-AAC-TGCAG-AGCTT-GAATC-----CTGGA-GAGGGTGG-CGGAATTCCT
GGTGCAGGA-GTGAAA-TCCGT-AGAT-ATC-AGGAGG-AACA-CCGG--T-GGC-GAAG
GCGG--CCACCTG--------------------------------GACAGGTATTGACG-C
TGAG-G-TG-CGAAA-GT-GTGGG-GAGCAAACA-GGATTAGATAC-CCTGGTA-GTC-C
ACACCGTAAAC-GATGGACA-CT-AGATGCCGGGG
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Nucleotide sequence of cloned DSV-DMB (Group 6) 16S rDNA fragment 'dsvb3' (16S rDNA positions, *E. coli* numbering).