Molecular Biological Characterisation of the Functional Microbial Communities in Anaerobic Digesters

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by

Saimon Malhotra

CONTENTS

Abbreviations

bp	base pair
BSA	bovine serum albumin
DNA	deoxyribonucleic acid
dH_2O	deionised water
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
BOD	biological oxygen demand
COD	chemical oxygen demand
TOC	total organic carbon

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
TGGE	temporal gradient gel 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

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Mum and Dad
...this is for you.

ABSTRACT

The microbial population in sludge taken from a domestic anaerobic digester was investigated by methods not requiring direct cultivation. Molecular techniques based on direct nucleic acid recovery were used to target key bacterial functional groups central to the anaerobic degradation of organic matter *i.e.* microbial groups involved in the critical processes of cellulolysis (clostridia), sulphate-reduction (sulphate-reducing bacteria) and methanogenesis (methanogens).

Archaeal (methanogen) 16S rRNA genes were PCR-amplified using DNA extracted from both raw and digesting sludge. Hybridisation using group-specific probes indicated the presence of large populations belonging to the orders *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales* and *Methanosarcinaceae*. In the latter group, members of the genus *Methanosaeta* (which produce methane from acetate only) were found to predominate.

The majority of the sulphate-reducing bacteria (SRB) are divided into six phylogenetic subgroups based on 16S rRNA sequence information. PCR amplification primers and confirmatory oligonucleotide probes were applied to detect the six genus-level subgroups, *Desulfotomaculum*; *Desulfobulbus*; *Desulfobacterium*; *Desulfobacter*; *Desulfococcus-Desulfonema-Desulfosarcina* and *Desulfovibrio-Desulfomicrobium*. Direct PCR amplification enabled the detection of *Desulfococcus* and *Desulfovibrio* groups in raw sludge, but only one dominant subgroup, the *Desulfococcus* group, was found in digesting sludge. All other groups except *Desulfobacterium* and *Desulfobacter* were detected in both sludge types via a more sensitive nested PCR approach, implying their presence in lower numbers than the dominant subgroups detected by direct PCR.

Primer sets specific for four clostridial groups (I, III, IV, XIVab) containing cellulolytic, proteolytic and mesophilic representatives were used for amplification of digester DNA. The results demonstrated the presence of all four clostridial groups in both raw and digesting sludge.

Temperature gradient gel electrophoresis (TGGE) of the amplified bacterial 16S rRNA genes showed the genetic diversity within each microbial group. The TGGE profiles also demonstrated differences in these groups between raw and digesting sludge samples suggesting that digester operation imposes a selective pressure on the incoming microbial population. TGGE profiles of clusters I, IV and XIV showed limited diversity within each group. However, cluster III clostridia revealed a much greater diversity in both raw and digesting sludge with relatively few bands common to both samples thus suggesting a population shift during digestion. TGGE profiling of SRB groups showed limited diversity with an overall trend of decrease in SRB diversity from raw to digesting sludge. An impressive archaeal diversity was observed in both raw and digesting sludge samples through TGGE. An equal number of discernable bands were present in both sludge types with a noticeable variation in the community structure between raw and digesting sludge. This suggests that the majority of the *Archaea* present in the digesting sludge were specifically selected for in the digester environment.

Sequencing and phylogenetic analysis of cloned clostridia and SRB 16S rDNA along with excised TGGE bands from archaeal profiles have shown them to cluster within the specific subgroups for which the PCR primers and oligonucleotide probes were designed. The majority of the sequences analysed were identified as being new as yet undescribed species.

CHAPTER ONE

1. Introduction

1.1. Overview

Anaerobic digestion is one of the oldest means of wastewater treatment and is exploited as a simple and effective biotechnological process to reduce pollution caused by organic wastes. The reaction commonly occurs in engineered systems known as digesters in which a complex feedstock is converted into a range of simpler compounds including methane, by microorganisms in the absence of oxygen (Hughes 1979). The complete anaerobic digestion of organic material requires a number of bacterial functional groups whose co-ordinated activity ensures process stability during anaerobic digestion. The information regarding these functional groups is still rather rudimentary and an appreciation of the microbiology is fundamental to an understanding of the factors that influence waste decomposition. This, in turn, is critical for the development and operation of controlled systems designed to optimise the treatment of waste and the production of biogas and digestate.

Due to the anaerobic nature of these organisms, their isolation is difficult. However, the application of molecular biological tools can provide information on the structure and diversity of the functional communities involved in environmental samples, that would not be possible using culture dependent methods.

1.2. Molecular Microbial Ecology

The study of microbial ecology provides information about the physiological diversity and community structure of bacteria in their natural habitats, and about their relationship with other biological and non-biological components.

In the past, detection and analysis of bacteria in the environment was performed mainly by methods based on their phenotypic characteristics and laboratory cultivation. Characterisation of naturally occurring populations of microorganisms using culture-based techniques has been recognised to misrepresent microbial biomass and has proved inadequate for the proper study of microbial ecology (Wagner *et al.*, 1993; Reeves *et al.*, 1995; Kampfer *et al.*, 1996). The biases of

cultivation-based approaches were first recognised by Winogradsky (Winogradsky *et al.*, 1965 cited in Ward *et al.*, 1992). These methods exerted a selective bias for particular groups of organisms and revealed that those that grow are those that are best adapted to the cultivation conditions and not necessarily the most metabolically active, or abundant, in the environment. This in turn has led to misconceptions about the ecological importance of certain species in natural environments.

Over the last decade, the introduction of molecular biological techniques to the study of microbial ecology has had a dramatic impact on our appreciation of microbial diversity and the complexity of natural ecosystems. The pioneering work of Carl Woese (Woese *et al.*, 1987) provided a molecular means, based on 16S rRNA sequence divergence, for describing microbial diversity in phylogenetic terms. Ribosomal RNAs are universally distributed and have the same biological role in all cellular organisms, thus it is possible to conduct comparative analyses between bacteria and higher organisms.

It was the recognition of the potential of applying molecular analysis to 16S rRNA genes recovered directly from the environment that provided a mechanism to explore diversity independent of culture based methods (Pace *et al.*, 1986). Since then, studies involving molecular techniques have highlighted the biases caused by culture-dependent methods. For example, *Nitrosomonas spp* were considered to be primarily responsible for ammonia oxidation in the environment supported by the relative ease with which members of this genus could be grown in culture. However, application of molecular biological techniques to the study of ammonia-oxidisers in environmental samples has revealed that it is *Nitrosospira spp.*, which do not grow readily in culture, that are possibly of more ecological significance than *Nitrosomonas spp.* (Hiorns *et al*, 1995; Kowalchuk and Stephen, 2001).

To circumvent the problem of cultivation, modern molecular ecology techniques are being increasingly used to directly study the microbial community inhabiting natural and engineered environments. In principle, nucleic acid probes can be designed to hybridise with a complementary target sequence and thus provide a complete description independent of the growth conditions and the media used (Ward *et al.*, 1992; Amann *et al.*, 1995; Raskin *at al.*, 1994a and 1994b). Application of these methods has led to a tremendous increase in knowledge of microbial ecology and whole groups of uncultivated and unculturable microorganisms that may be

ecologically significant are now known only from nucleic acid sequences (Godon and Giovannoni, 1996; Felske *et a*l., 1997; Godon *et al.*, 1997; LaPara *et al.*, 2000).

The application of nucleic acid-based techniques such as the polymerase chain reaction, oligonucleotide hybridisation, cloning, sequencing and more recent developments such as gradient gel electrophoresis are now commonplace in the study of microbial communities in natural environments. These approaches all provide useful and novel information but also have limitations, which need to be recognised and resolved. These limitations relate to the extraction of nucleic acids from environmental samples, biases, artefacts associated with enzymatic amplification of nucleic acids and analysis of PCR products using techniques such as gradient gel electrophoresis. These biases and limitations are discussed further in the following sections.

Despite these limitations, molecular-based methods have revolutionised our appreciation of microbial diversity in the ecosystem and will continue to do so. The potential exists to complement these techniques with other technologies to relate community structure to function and activity (Head *et. al.*, 1998) thus bringing us closer to unlocking the deeper mysteries of microbial ecology.

1.3. Wastewater Treatment Processes

Wastewater treatment is probably the largest and globally most important use of biotechnology. Treatment plants around the world treat domestic and industrial wastewater through a combination of physical, biological and chemical processes. In biological treatment processes, heterogeneous cultures of largely undefined microorganisms are used to convert organic pollutants into environmentally acceptable products. The composition of industrial and domestic wastewaters are often complex and variable, therefore the metabolism of their constituents requires a versatile and flexible combination of enzymes, which can only be provided by a mix consortia of bacteria.

Wastewater treatment is generally a multistep process employing both physical and biological treatment steps (Fig. 1.1).

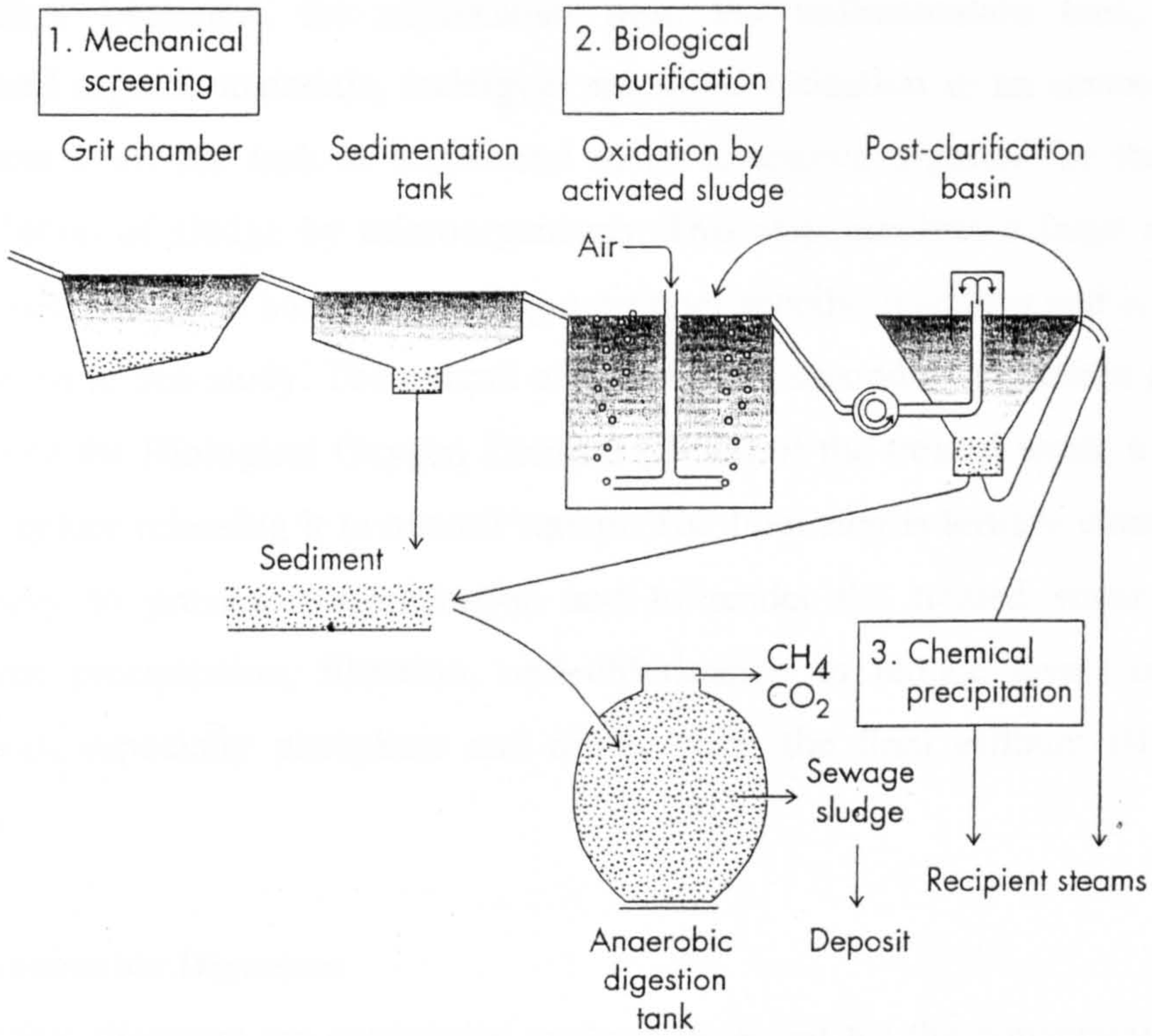


Figure 1.1. Flow of material through a wastewater treatment plant employing both biological and chemical purification. Taken from Schlegel (1986).

The stream of wastewater entering a treatment plant typically goes through a series of operations; primary, secondary and tertiary treatments (Brock *et al.*, 1994). The primary treatment consists of physical separations and involves the removal of large objects by passing the wastewater through a series of screens. The effluent is then left to settle in a sedimentation tank to allow suspended solids to sediment. In the secondary treatment, the supernatant from the sedimentation tank, containing dissolved organic materials, undergoes microbial oxidation in an aerated tank. The sediment from the tank is transferred to an anaerobic digester for the anaerobic degradation of sludge by microorganisms. This step involves a large spectrum of undefined anaerobic bacteria carrying out rather specific reactions and is the process of interest in this study. The overall effect of these secondary treatment processes is to reduce the Biological Oxygen Demand (BOD) of the treated waste to acceptable levels before releasing it to natural waters. The final step is tertiary treatment and is necessary to prevent eutrophication and to render the treated water potable. It involves precipitation, filtration, and chlorination to reduce levels of inorganic nutrients, especially phosphate and nitrate from the final effluent (Brock *et al.*, 1994).

1.4. Anaerobic Digesters

Anaerobic digesters are engineered systems designed for the conversion of organic wastes to methane. They are found commonly in municipal sewage treatment plants and are used to treat the particulate fraction (sludge) generated by primary settling of raw sewage and secondary settling of activated sludge.

Biologically-mediated anaerobic degradation occurs on the organic fraction of waste which is mainly polymeric consisting of undigested plant polymers in feces, paper, and microbial cells. The dry solid portion of most municipal sludges contains approximately 19% protein, 18 to 50% carbohydrate, 5% pectin, and 25 to 36% cellulose (Hunter and Heckelekian, 1965). Therefore, the hydrolysis of organic polymers is often the rate-limiting step in the anaerobic digestion of municipal sludge.

The two basic types of digester configuration are batch and continuous. Batch-type digesters are relatively simple and once the digestion is complete, the effluent is removed and the process is repeated. In a continuous digester, organic material is constantly or regularly fed into the digester. The material moves through the digester

either mechanically or by the force of the new feed pushing out digested material. Unlike batch-type digesters, continuous digestion produces biogas without the interruption of loading material and unloading effluent.

Biogas produced in anaerobic digesters consists of methane (50-80%), carbon dioxide (20-50%), and trace levels of other gases such as hydrogen, carbon monoxide, nitrogen, oxygen, and hydrogen sulphide (Day, 1995). The relative percentage of these gases in biogas depends on the feed material digested and the management of the process.

Mesophilic anaerobic digestion is usually achieved in a completely mixed reactor, designed to operate in the temperature range 25-37 °C with a retention time of between 15 and 35 days. As the basis of anaerobic treatment, a strict anaerobic environment is essential to the growth of the anaerobes. The upper limit of redox potential (Eh) range from -450 mV to –300 mV, which corresponds to an extremely low oxygen concentration (Day, 1995). Other environmental factors of primary importance in anaerobic processes are temperature, nutrient requirements, pH and toxic substances.

1.5. Anaerobic Digestion in sludge digesters

Anaerobic digestion in digesters follows the same biochemical route as other methanogenic ecosystems in nature, such as marine sediments, marshes, swamps and rice soils. The digestion process is a multistep process involving many different physiological groups of microorganisms whose coordinated activity leads to waste stabilisation (Fig. 1.2).

The microbial process commences with the hydrolysis of biological polymers (cellulose, hemicellulose, carbohydrate, fats and protein) by cellulolytic and other hydrolytic bacteria (e.g. clostridia) to soluble sugars, amino acids and long chain fatty acids. This activity results in the rapid production of CO_2 and the depletion of oxygen that allows fermentative acid forming bacteria to use these substrates to produce short chain fatty acids (e.g. acetate, butyrate, and propionate), alcohols, carbon dioxide and hydrogen. The most significant short chain fatty acid is acetate, which is an important substrate for methane generation. Acetate is produced directly in the primary fermentation step but, in addition, the H_2-producing acetogenic bacteria, *Syntrophobacter* and *Syntrophomonas*, convert propionate and short chain fatty acids to acetate, CO_2 and hydrogen, when H_2

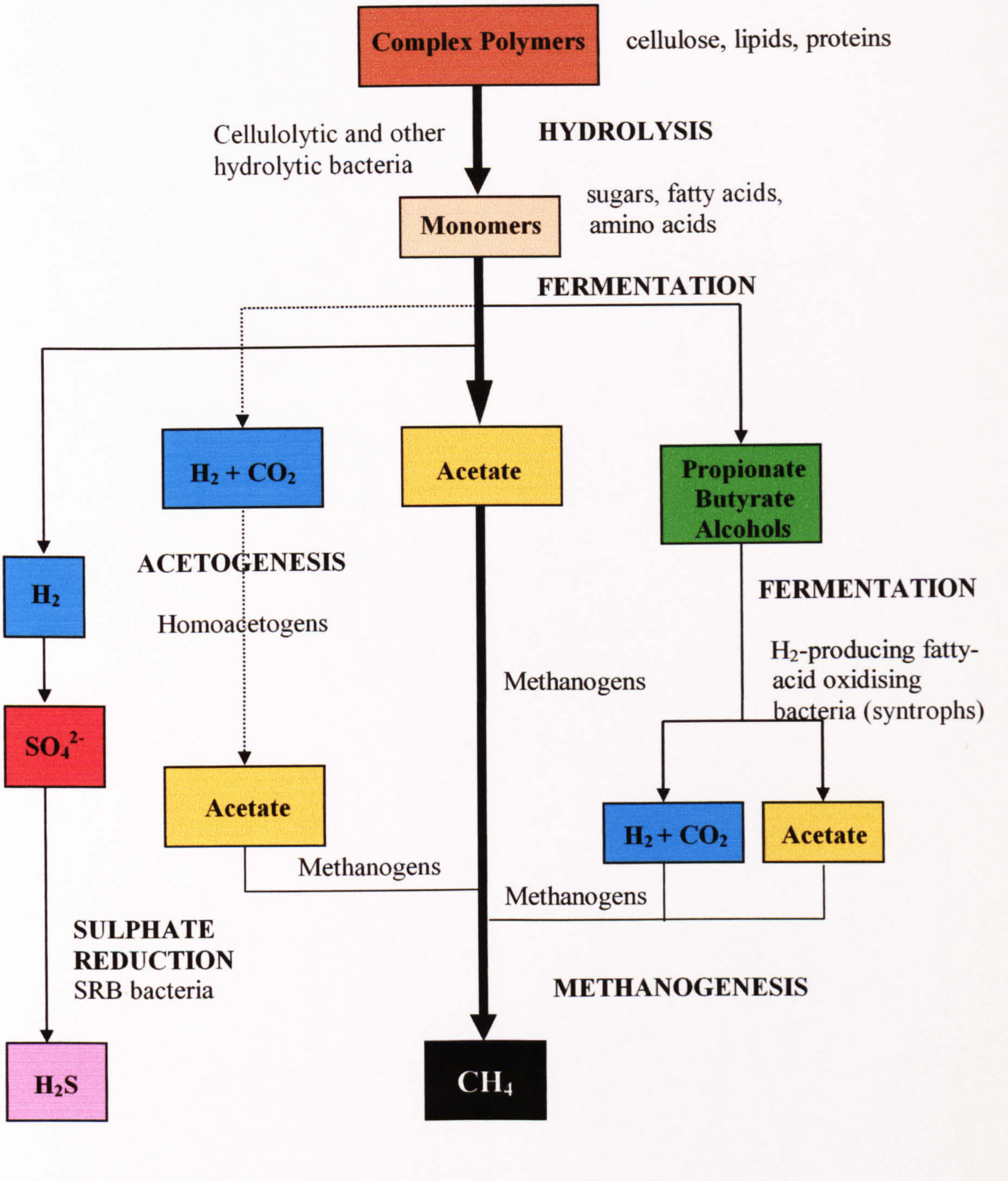


Figure 1.2. Overall process of anaerobic decomposition in anaerobic digesters showing the major metabolic stages in the conversion of complex organic wastes into methane and carbon dioxide. Modified from Brock *et al* (1994).

concentrations are favourably low (Brock *et al.*, 1994). Low hydrogen concentrations are biologically maintained by methanogens and sulphate-reducers which both require hydrogen to generate the end products of anaerobic degradation, methane or hydrogen sulphide, respectively. In anaerobic sludge digestion the preferred end product is methane from methanogenesis. In addition to acetate, H_2, and CO_2, substrates such as formate, methanol and methylated amines can also be used by methanogens.

Sulphate-reducing bacteria are able to compete with methanogens for available electrons in digesters with high sulphate concentrations. However, sulphate concentrations in sewage sludge are typically low in the range of 5-30 mg/l (Raskin *et al.*, 1995) and this should result in low sulphate-reduction activity in the digester. When sulphate levels are depleted, certain SRB populations can act as syntrophic bacteria and favour methanogenesis by providing their substrates hydrogen, formate and acetate (e.g. Jones *et al.*, 1984; Tasaki *et al.*, 1993; Wu *et al.*, 1991).

1.6. Analysis of Major Functional Groups

Trophic groups have been identified that drive the critical processes of cellulolysis, sulphate reduction and methanogenesis in anaerobic sludge digestion.

Cellulose and other polymeric material in sludge is likely to be degraded by hydrolytic fermentative bacteria related to the genera *Clostridium* and *Eubacterium*. *Clostridium* is heterogeneous displaying a wide range of phenotypic characteristics. Phylogenetic comparison of *Clostridium* and relatives based on 16S rRNA genes show this group to comprise deeply branching clusters containing a number of non-clostridial species (Collins *et al.*, 1994). Group specific amplification primers have been developed for four clostridial groups (Van Dyke *et al.*, 2002) whose members are believed to be involved in hydrolytic activity. These tools have been applied in landfill studies (Van Dyke *et al.*, 2002) and were used in this study for detection of these groups in sludge samples.

Sulphate reducing bacteria (SRB) play an important role in degradation when a high concentration of sulphate is present resulting in competition for H_2 with methanogens. Typically, sulphate concentrations in digester systems are kept to a minimum allowing methanogenesis to be the end reaction. In contrast, SRB populations are usually associated with sulphate-rich environments such as freshwater and marine sediments (Purdy *et al.*, 2001; Sahm *et al.* 1999). 16S rRNA phylogenetic studies have placed most of the SRB in six 'natural' groups (Devereux *et al.*, 1992; Daly *et al.*, 2000). A series of PCR amplification primers and confirmatory oligonucleotide probes have been designed and verified for recovery and identification of the SRB groups in landfill sites (Daly *et al.*, 2000). These molecular biological tools have been evaluated for use with anaerobic digester sludges.

The third group of interest are the methanogens. They are an important group in anaerobic digester microbiology as methanogenesis is the final step and the reaction of great significance in terms of digester activity and stability. Group-specific methanogen 16S rRNA probes have been described by Raskin *et al.* (1994a) and were used in this study to detect subgroups of methanogens in anaerobic sludge, after DNA was amplified with non-specific 16S rDNA archaeal primers.

The taxonomy, physiology and ecology of these groups of bacteria are discussed in the following sections.

1.7. *Clostridium*

1.7.1. Taxonomy

The genus *Clostridium* is a diverse group of anaerobic, Gram-positive, rod-shaped, endo-spore forming bacteria. The taxon is very heterogenous, comprising organisms with considerable variation in genome size (2.5 to 6.5Mb) and G+C content. In addition members of this group exhibit a wide range of phenotypic characteristics (Hippe *et al.*, 1992). Phylogenetic studies based on comparative 16S rRNA gene sequence analyses have allowed the division of this group into many deeply branching clusters that include non-clostridia species (Collins *et al.*, 1994). With over a hundred species, the study defined nineteen clusters. Some clostridial species were phylogenetically so distinct that they have either been assigned as new genera (e.g. *Filifactor*, *Caloramator*, *Moorella*, *Oxobacter*, *Oxalophagus*) or to existing genera (e.g. *Eubacterium*, *Sporohalobacter*, *Syntrophospora*, *Paenibacillus*, *Thermoanaerobacterium*) (Collins *et al.*, 1994).

With respect to this study only clusters I, III, IV, and XIVab are of interest since they include mesophilic cellulose-degrading stains. Cluster I is the largest of the clostridial groups and members of this group include saccharolytic and proteolytic species, as well as psychrophiles, mesophiles, and thermophiles. Cluster III contains both mesophiles and thermophiles and comprise only cellulose degrading strains. Cluster IV include representatives from the genera *Clostridium*, *Eubacterium*, and *Ruminococcus*. The cluster contains both mesophiles and thermophiles and comprise of cellulolytic and non-cellulolytic stains. The *Clostridium* cluster XIVab is another large and diverse group containing both cellulolytic and noncellulolytic members (Collins *et al.*, 1994).

1.7.2. Physiology and Ecology

Members of the genus *Clostridium* are anaerobic spore-formers and their resistant spores ensure wide dispersal and survival. In general, the genus is extremely metabolically diverse, comprising saccharolytic, proteolytic, lipolytic, and other species with sometimes broad and sometimes very narrow substrate specificities (Schink 1994). The saccharolytic species are able to ferment a wide range of substrates such as xylose, mannitol, glucose, fructose, lactose, starch, cellulose, pectin, and chitin (Schink 1994). As a result they produce a wide range of

fermentation products (butyrate, acetate, CO_2, ethanol, H_2, formate, succinate, and lactate (Fenchel and Finlay, 1995). Some species of *Clostridium* are known to possess nitrogenase activity and are able to fix molecular nitrogen (eg. *Clostridium papyrosolvens*; (Leschine, 1995). Others can serve as homoacetogens (eg. *Clostridium thermoaceticum*) and can grow heterotrophically using CO_2 or autotrophically using molecular hydrogen, to form acetate (Fenchel and Finlay, 1995). They are strict anaerobes and grow only at neutral or alkaline pH so their growth can be completely inhibited by the acid products of other fermenters (Fenchel and Finlay, 1995).

Due to their ability to use a wide range of substrates, it is no wonder that clostridia are found in many diverse anaerobic environments. These include the human gut (Suau *et al.*, 1999), the rumen (Tajima *et al.*, 1999), landfill (Van Dyke and McCarthy, 2002), anaerobic digesters (Godon *et al.*, 1997); sewage sludge (Patel *et al.*, 1980), compost (Petitdemange *et al.*, 1984), and rice fields (Weber *et al.*, 2001)

1.8. Sulphate-Reducing Bacteria

1.8.1. Taxonomy

Sulphate-reducing bacteria (SRB) are a morphologically and physiologically diverse group of anaerobic bacteria, which all share the ability to obtain energy from dissimilatory reduction of inorganic sulphate. They are involved in the terminal stages of anaerobic digestion and are able to utilise sulphate and other oxidised sulphur compounds as terminal electron acceptors, that are subsequently reduced to sulphide (either completely or incompletely) in the mineralisation of organic compounds (Gibson, 1990).

Analysis of 16S rRNA sequences has placed the SRB species into four distinct groups: Gram-negative mesophilic SRB; Gram-positive spore-forming SRB, thermophilic bacterial SRB; and thermophilic archaeal SRB (Castro *et al.*, 2000). The assignment of SRB based on rRNA analysis correlates well with traditional classification based on physiological and biochemical characteristics (Stackebrandt *et al.*, 1995; Castro *et al.*, 2000). The thermophilic bacterial and archaeal SRB, to date, consist of only a few members. The thermophilic bacterial SRB contain the

genera *Thermodesulfobacterium* and *Thermodesulfovibrio*, each containing two species. Not only do members of these two genera exhibit incomplete oxidation of acetate and utilise a limited number of electron donors, they are phylogenetically separate from other eubacterial genera (Widdel, 1992b). Archabacterial sulfate reducers of the genus *Archaeoglobus* (Achenbach-Richter *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).

Mesophilic, Gram-negative, non-sporeforming SRB, belong to the δ-subclass of the Proteobacteria, comprised of myxobacteria and bdellovibrios (Fowler *et al.*, 1986). Recently, this taxon has been expanded by addition of the genera *Pelobacter* (Stackebrandt *et al.*, 1989) and *Geobacter* (Lovely *et al.*, 1993). The well-characterised SRB genera include *Desulfobulbus*; *Desulfomicrobium*; *Desulfomonas*; *Desulfovibrio*; *Desulfobacter*; *Desulfobacterium*; *Desulfococcus*; *Desulfomonile*; *Desulfonema*; *Desulfosarcina*. Two recently described genera; *Desulfohalobium* and *Desulfonatronum* also fall firmly within this group (Castro *et al.*, 2000). The mesophilic Gram-negative SRB are the most widespread in nature and members of approximately half of the genera are able to oxidise organic substrates completely to CO_2 while the remainder can only oxidise organic incompletely to the level of acetate (Devereux *et al.*, 1989; Fauque, 1995).

Gram-positive spore-forming SRB is dominated by the genus *Desulfotomaculum* and exhibit complete and incomplete oxidising species. They are placed within the low GC Gram-positive bacteria such as *Bacillus* and *Clostridium* and include the only SRB known to form heat-resistant endospores. The genus *Desulfotomaculum* also contains moderately thermophilic species and dissimilatory sulfate reduction in environments with temperatures between 50-65 °C is mainly due to the spore-forming species (Widdel, 1992c).

Phylogenetic analysis by Daly *et al.* (2000) based on 16S rRNA sequence comparisons has confirmed the phylogeny of SRB previously described (Devereux *et al.*, 1989) and allowed the identification of six main 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). Diagnostic group-specific 16S rDNA-targeted PCR primers and internal 16S rDNA-targeted oligonucleotide probes have been designed

(Devereux *et al.*, 1992; Daly *et al.*, 2000) and used to study SRB populations in complex communities such as biofilms (Amann *et al.*, 1992), marine sediments (Devereux and Mundfrom, 1994), landfill (Daly *et al.*, 2000) and radioactive sites (Lockhart *et al.*, unpublished).

1.8.2. Physiology and Ecology

The metabolic diversity of SRB is not restricted to using sulphate as a terminal electron acceptor but they can utilise a wide range of electron acceptors in the consumption of organic compounds. These include other oxidised sulphur compounds e.g. thiosulfate (Jorgensen and Bak, 1991) and sulfite (Kramer and Cypioka, 1989), and also elemental sulphur (Lovely and Philips, 1994). 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 (Daalsgaard and Bak, 1994), metals such as iron and manganese (Coleman *et al.*, 1993; Lovely and Phillips, 1994), toxic heavy metals such as mercury (Choi *et al.*, 1994), uranium (Lovely and Philips, 1992) and chromium (Fude *et al.*, 1994) and even oxygen (Dilling and Cypionka, 1990).

SRB are also capable of utilising a diverse range of electron donors. The types of carbon sources utilised for the reduction of sulphate 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).

SRB are known to be ubiquitous and to play an important ecological role in diverse environments such as freshwater lake sediments (Sass *et al.*, 1997), marine sediments (Devereux and Mundfrom, 1994), oil production facilities (Rabus *et al.*, 1996), wastewater treatment facilities (Santegoeds *et al.*, 1998; Schramm *et al.*, 1999), the rumen (Tajima *et al.*, 1999), and rice paddy fields (Scheid and Stubner, 2001). 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.*, 1996; Purdy *et al.*, 2001; Sahm *et al.*, 1999). In particular, it has been estimated that sulphate-reduction

accounts for up to 50% of organic matter degradation in some marine sediments (Jorgensen, 1982) and aerobic wastewater biofilms (Kuhl and Jorgensen, 1992).

1.9. Methanogenic *Archaea*

1.9.1. Taxonomy

Methanogens belong to the *Archaea* (*Archaebacteria*), which also include the extreme halophiles, extreme thermophiles, and thermophilic sulfate reducers. Two major kingdoms of *Archaea* are identified, *Euryarchaeota* (methanogens, extreme halophiles and thermoacidophiles) and *Crenarchaeota* (extreme thermophiles) (Woese *et al.*, 1990). The possible existence of a third kingdom, the *Korarchaeota*, was raised with the identification of two uncultured thermophilic organisms, pJP27 and pJP27 (Barns *et al.*, 1996). Methanogens are extremely diverse exhibiting a G+C content range from 23 to 61 mol %, with bacillary, coccal, and spiral morphologies and a variety of cell envelope structures. However, they are unified by their ability and need to synthesize methane.

Methanogens are classified into five orders, which consist of 10 families and 26 genera that comprise over 50 species (Fig. 1.3) (Garcia *et al.*, 2000).

1.9.2. Physiology and Ecology

Despite the great phylogenetic diversity represented by the methanogens, as a group they can only use a limited number of substrates. This degree of substrate specialisation renders methanogens dependent on other organisms for there substrates.

Substrates for methane generation include H_2, CO_2, acetate, formate, methanol and methylamines; different genera/species vary in the type(s) of substrate utilised (Fig. 1.3). As a result, catabolic pathways of methanogens can be divided into three groups. The CO_2-reducing pathway is the most widespread catabolic reaction among the methanogens, and involves the reduction of CO_2 to CH_4 using H_2 as a source of electrons. This source of H_2 is catabolic product obtained from other bacteria (Whitman *et al.*, 1991; Boone *et al.*, 1993). Many of these H_2-using methanogens (hydrogenotrophs) can also use formate, a common fermentation product in anaerobic ecosystems, as an electron donor for the reduction of CO_2 to CH_4. In

addition, short chain alcohols (primary and secondary alcohols) can also serve as electron donors and are oxidised by a limited number of these methanogens for the CO_2 reduction to CH_4 (Widdel, 1986; Zellner and Winter, 1987).

The methylotrophic pathway exploits methyl groups of compounds such as methanol, trimethylamine, and dimethyl sulfide and reduces them to methane using H_2 as an electron donor (Boone *et al.*, 1993).

Of the many methanogenic genera, only two, *Methanosaeta* and *Methanosarcina*, are known to grow by the acetoclastic pathway, producing methane from acetate (Zinder, 1993). *Methanosaeta* spp. are solely acetoclastic, whilst *Methanosaricinia* spp. are also capable of growing using the other two pathways.

Methanogenic bacteria inhabit a variety of niches, and the type of species present is dependent on parameters such as temperature, pH, osmotic pressure and substrate concentration, all of which may differ from one habitat to another. Methanogens are found in habitats such as wastewater treatment facilities (Godon *et al.*, 1997; Plumb *et al*, 2001), the rumen (Tajima *et al.*, 1999), salt marsh sediments (Munson *et al*, 1997) and rice field soils (Lueders and Friedrich, 2000). The latter are estimated to contribute up to 25% to global methane emissions (Neue, 1993).

	Substrates utilised
ORDER I: *Methanobacteriales*	
FAMILY I: *Methanobacteriaceae*	
Genus I: *Methanobacterium*	H_2, CO_2 and formate
Genus II: *Methanobrevibacter*	H_2, CO_2 and formate
Genus III: *Methanosphera*	H_2, and methanol
Genus IV: *Methanothermobacter*	H_2, CO_2, and formate
FAMILY II: *Methanothermaceae*	
Genus I: *Methanothermus*	H_2 and CO_2
ORDER II: *Methanococcales*	
FAMILY I: *Methanococcaceae*	H_2 and formate
Genus I: *Methanococcus*	
Genus II: *Methanothermococcus*	
FAMILY II: *Methanocaldococcaceae*	
Genus I: *Methanocaldococcus*	
Genus II: *Methanoignis*	
ORDER III: *Methanomicrobiales*	
FAMILY I: *Methanomicrobiaceae*	H_2, CO_2, formate, secondary alcohols
Genus I: *Methanomicrobium*	
Genus II: *Methanogenium*	
Genus III: *Methanoculleus*	
Genus IV: *Methanolacinia*	
Genus V: *Methanofollis*	
Genus VI: *Methanoplanus*	
Genus VII: *Methanocalculus*	
FAMILY II: *Methanocorpusculaceae*	
Genus I: *Methanocorpusculum*	H_2, CO_2 and formate
FAMILY III: *Methanospirilliaceae*	
Genus I: *Methanospirillum*	H_2, CO_2 and formate
ORDER IV: *Methanosarcinales*	
FAMILY I: *Methanosarcinaceae*	
Genus I: *Methanosarcina*	H_2, CO_2, formate, acetate, methyl
Genus II: *Methanococcoides*	use only methanol and methylamines
Genus III: *Methanolobus*	
Genus IV: *Methanohalophilus*	
Genus V: *Methanosalsus*	
Genus VI: *Methanohalobium*	
FAMILY II: *Methanosaetaceae*	
Genus I: *Methanosaeta*	acetate only
ORDER V: *Methanopyrus*	
FAMILY I: *Methanopyraceae*	
Genus I: *Methanopyrus*	H_2, CO_2

Figure 1.3. Classification of methanogens and the substrates utilised, modified from Raskin *et al.*, 1994a.

1.10. Molecular Ecology of Anaerobic Digesters

Earlier studies based on isolation and culture techniques have provided an indication of the digester microbiology involved in anaerobic digestion processes (Kirsch 1969; Toerien 1970; Ueki *et al.*, 1978; Patel *et al.*, 1980; Sleat and Mah, 1985; Yang *et al.*, 1990; Wu *et al.*, 1992) Most recently reported was the isolation and characterisation of two glycerol fermenting clostridial strains from an anaerobic digester treating high lipid and protein content (Jarvis *et al.*, 1999). However, microbial populations in anaerobic ecosystems are highly interdependent and while only a few are culturable with ease, many are difficult to isolate and grow in pure culture. As a result, isolation studies provide a poor indication of the microbiology involved in anaerobic digester systems.

Over the last decade, the molecular biological characterisation of microbial populations in anaerobic digesters has provided a wealth of information into the microbial complexity of these anaerobic ecosystems. Molecular inventories based on 16S rDNA sequences have shown a huge diversity in the microbial community structure of anaerobic digesters (Ng *et al.*, 1994; Godon *et al.*, 1997; Godon *et al.*, 1997b; Sekiguchi *et al.*, 1998). None of these molecular inventories has proved exhaustive and a particular ecosystem contained more than 133 species (Godon *et al.*, 1997). In addition, the majority of the species identified were new species, some of them belonging to phylogenetic groups without any known function (Godon *et al.*, 1997; Sekiguchi *et al.*, 1998). Furthermore, a study by Delbes *et al.* (1998) used a combination of culture-based analysis with 16S rDNA identification of the isolates to compare to the 16S rDNA sequences directly retrieved previously by Godon *et al.* (1997). Recently, molecular biological techniques have been used to investigate the microbial structure of various wastewaters undergoing anaerobic digestion (Raskin *et al.*, 1995; Godon *et al.*, 1997; LaPara *et al.*, 2000; Plumb *et al.* 2001; Wu *et al.*, 2001; Pereira *et al.*, 2002) and has indicated the presence of specific populations.

Community dynamics in reactors over time have been assessed by Fernandez *et al.* (1999) and Zumstein *et al.* (2000) and have independently shown that digesters that appeared functionally stable with respect to environmental parameters were not stable in respect to the microbial community. In another study, the comparison of two ecosystems subjected to substrate loading shock suggests that the most stable microbial community structure was associated with greater functional instability (Fernandez *et al.*, 2000).

Characterisation of microbial communities in anaerobic digesters have been demonstrated using hybridisation probes to target populations of sulphate-reducing bacteria (Raskin *et al.*, 1995; 1996; Oude Elferink *et al.*, 1998), and methanogens (Raskin *et al.*, 1994b; 1995; 1996). In addition, immunological methods have been used to detect major groups of methanogens in samples from anaerobic reactors (Sorensen and Ahring, 1997). Other molecular techniques applied to assess microbial structure and diversity in digesters include restriction fragment length polymorphism (RFLP) (Fernandez *et al*, 2000), denaturing gradient gel electrophoresis (DGGE) (LaPara *et al.*, 2000), fluorescent *in situ* hybridization (FISH) (Plumb *et al.*, 2001), and single – strand conformation polymorphism (SSCP) (Delbes *et al.*, 2000; Zumstein *et al.*, 2000)

1.11. Molecular Biological Detection of Bacterial groups

1.11.1. Extraction of nucleic acids from environmental samples

An essential tool of molecular microbial ecology is the isolation of nucleic acids (DNA or RNA) from different habitats, with a purity suitable for subsequent techniques. There have been many published methods and protocols for extracting DNA from environmental samples (Orgam *et al*, 1987; Fuhrman *et al*, 1988; Steffan *et al*, 1988; Selenska and Klingmuller, 1991; Tsai and Olson, 1991; Bruce *et al*, 1992). Due to the microbial complexity of some environmental samples, standard extraction methods may not always deliver efficient extraction of DNA. It is for this reason that studies of natural communities are routinely performed by methods that have been optimised to suit either the microbial community of interest or the characteristics of the medium harbouring them (Leff *et al*, 1995).

A major problem presented by certain environmental samples is the presence of humic acids, phenolic compounds and chealating agents. These are co-extracted with the DNA and are able to inhibit the enzymatic action of PCR amplification (Tsai and Olson, 1992; Tebie and Vahjen, 1993). The removal of such inhibitory factors is necessary to provide a DNA template for PCR. Various studies have put forward protocols for the purification of extracted DNA. These include CsCl-gradient ultracentrifugation (Steffan *et al*, 1988), sephadex gel separation (Tsai and Olson, 1992), phenol chloroform extraction (Bruce *et al*, 1992) and by the addition of sequestering proteins and compounds, such as bovine serum album (BSA), polyvinylpolypyrrolidone (PVPP) and polyvinylpyrroline (PVP) (Young *et al*, 1993; McGregor *et al*, 1996).

In addition, combined DNA extraction and purification kits are now commercially available that can provide high yield, high quality DNA that is readily amplifiable via PCR.

1.11.2. Polymerase Chain Reaction

The use of PCR as a diagnostic tool allows sensitive detection of DNA sequences and its application has revealed a much higher diversity in natural microbial communities than the classical approach based on culture enrichment (Giovannoni *et al*, 1990; Amann *et al*, 1991; Godon *et al*., 1997).

The extracted DNA is subjected to PCR amplification using oligonucleotides (15 to 20 bases long) that are complementary to signature sequences at different sites on the rRNA molecule or the gene that encodes it (rDNA). This amplifies a region of the DNA targeting a particular group of interest. The technique is highly sensitive and specific for the target genes when used in conjunction with confirmatory oligonucleotide probing. The main disadvantage is that it is difficult to generate data that are quantitatively meaningful (Head *et al*, 1998).

The sensitivity and specificity can be improved by adopting a 'nested' approach to PCR, whereby initial amplification is carried out with a pair of primers with broad specificity. A second round of amplification is conducted on the product using primers with target sites internal to the first primer pair and of greater specificity. This approach has been successfully used to detect autotrophic ammonia-oxidising bacteria in diverse environments (Hiorns *et al*, 1995; Hastings *et al*., 1997) and SRB populations in landfill communities (Daly *et al*, 2000).

Single strand conformation polymorphism (SSCP) is a method based on PCR and has been applied to anaerobic sludge (Delbes *et al*., 2000; Zumstein *et al*., 2000). The method is based on the folded structure of a single stranded DNA determined by intramolecular interactions and nucleotide sequence. The DNA fragments can then be separated by differences in electrophoretic mobility related to the shape of the DNA. The bands can then be compared using a laser beam densiometer and their occurrence and intensity analysed statistically.

1.11.3. Limitations of PCR

Although much new and valuable knowledge can be gained from the use of PCR in studies of microbial ecology and diversity, there are important limitations that must be recognized when using this technique. A number of factors are known to bias amplification resulting in differential PCR and causing the preferential amplification of sequences that in turn may not reflect the true diversity of an environment.

Amplification may be hampered by suboptimal reaction conditions and the lack of primer specificity. The amplification of unknown and unrelated genes containing the same primers sites as the target gene can occur, especially when amplifying DNA from environmental samples serving as a rich gene pool source. To overcome this, the use of specific PCR primers coupled with a specific confirmatory oligonucleotide probe internal to the primer sites can be used to confirm the identity of the amplified product (Head *et al.*, 1998). In addition, primers annealing to sites that contain mismatches in the nucleotide sequence of the target region may occur at low stringency. However, the thermal stability of *Taq* polymerase allows PCR to be performed at elevated temperatures that can prevent mispriming thus increasing the probability of specifically amplifying the target gene (Steffan and Atlas, 1991).

Increasing the stringency of PCR can also be achieved by introducing a 'hot start' PCR, whereby primers and template DNA are completely denatured before PCR cycling begins, therefore preventing primer annealing at low temperatures.

Several other factors might bias the relative frequencies of genes in PCR products of mixed-template reactions. The formation of secondary structures of the template can inhibit primer extension (Wilson, 1997), and different binding energies resulting from primer degeneracy, that can influence the formation of primer-template hybrid, are all plausible sources of bias in PCR.

The mol % G+C content of the primer site can also affect primer annealing efficiency. 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 mol % G+C content preventing efficient denaturation during thermal cycling. However this can be improved by the addition of denaturants such as acetamide (Reysenbach *et al.*, 1992) or dimethylosulfoxide (Baskaran *et al.*, 1996).

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 toward a 1:1 product formation ratio

irrespective of the initial template ratio, and was strongly dependant on the number of cycles of replication. However, they concluded that, if using DNA extracted from environmental samples, this PCR-product 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 dependant on the number of cycles, it can be minimized by keeping the number of cycles a low as possible.

Moreover, the misincorporation rate of *Taq* polymerase (Eckert and Kunkel, 1991) or the formations of chimeric molecules during PCR (Liesack *et al.*, 1991) were also reported to bias PCR product formation. The fidelity of PCR amplification can vary depending on the particular DNA polymerase used. Nucleotide misincorporation rates have been reported in the range of 0.00002% - 1.3% for different DNA polymerases (Head *et al.*, 1998). Careful analysis of sequences and of secondary interaction 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.

Chimeric molecules can be created in PCR from the recombination of two or more homologous DNA fragments to produce a genetic hybrid that may be interpreted as a novel sequence (Liesack *et al.*, 1991). It has been demonstrated that up to 30% of the products generated during coamplification of similar templates were chimeric (Wang and Wang, 1997). 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).

1.11.4. Temporal Gradient Gel Electrophoresis

To study population structure and dynamics, genetic fingerprinting techniques that provide a pattern or profile of genetic diversity are required. Temporal gradient gel electrophoresis (TGGE) is one such technique and is a variant of denaturing gradient gel electrophoresis (DGGE) (Myers *et al.*, 1985; Reisner *et al.*, 1989). Like DGGE, TGGE of 16S rDNA amplicons have been demonstrated to be suitable tools for the analysis of microbial communities because they permit the detection of species and changes in community structure quickly and economically (Muyzer and Smalla, 1998). In TGGE, DNA fragments of the same length but with different sequences can be separated based on the decreased electrophoretic mobility of a partially melted double-starnded DNA (dependent on number of covalent G-C bonds) molecule in polyacrylamide gels containing a linear gradient of DNA denaturants (eg. urea and formamide) 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 and allows improved detection of individual sequences in TGGE/DGGE (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.

Since its introduction to molecular ecology by Muyzer *et al.*, (1993), who demonstrated DGGE profiles of PCR-amplified 16S rDNA from environmental samples, the technique has been widely applied to investigate the composition and genetic diversity of complex microbial populations in a number of different environments. The technique enables the simultaneous analysis of multiple samples and allows the monitoring of complex microbial population dynamics over time. Muyzer *et al.* (1993) also demonstrated the sensitivity of this techniques by showing that a specific band in a mixture of PCR products could be distinguished even when the target DNA comprised less than 1% total DNA in the mixture. This indicated that minority species in microbial populations would also be detected.

In addition to providing a description of the community structure, TGGE/DGGE can also provide phylogenetic information on the microbial populations analysed through the excision, reamplification and sequencing of individual bands (Ferris and Ward,

1997; Kowalchuk *et al.*, 1997). Therefore, the presence, relative abundance and identity of different phylotypes in complex microbial communities can be discerned in a qualitative way using this technique.

The TGGE/DGGE technique has been applied to assess the microbial community structure in a range of environments (Whiteley and Bailey 2000; Van Dyke and McCarthy 2002; Teske *et al.*, 1996; Chan *et al.*, 2002).

In addition to TGGE/DGGE, other fingerprinting techniques include terminal restriction fragment length polymorphism (T-RFLP) (Liu *et al.*, 1997) and single–strand conformation polymorhism (SSCP) (Lee *et al.*, 1996).

1.11.5. Limitations of Gradient Gel Electrophoresis

As with PCR, the use of gradient gel electrophoresis is associated with limitations. A potential source of bias stems from the PCR and questions the quantitative meaningfulness of the banding pattern. PCR bias towards certain sequence types could influence the appearance and intensity of bands on the gel (Reysenbach *et al.*, 1992; Ferris *et al.*, 1996). However, it has been shown that the intensity of bands in a TGGE analysis of soil microbial communities corresponds semi-quantitatively with the abundance of species and concludes that the bias of preferred amplification may be overestimated (Heur and Smalla, 1997).

The separation of fragments from highly complex microbial populations can often be poor thus presenting interpretation difficulties, especially when large numbers of samples to be compared are involved. The resolution of such profiles can be improved by using narrower gradient range, two-dimensional electrophoresis (Fischer and Lerman, 1979) or by employing group-specific PCR primers to narrow the target range.

There is the possibility that two different sequences, sharing similar melting characteristics, may comigrate to the same position on a gel. In this case, single bands on the gel do not necessarily represent single phylotypes, and this could lead to an under estimation of diversity in the population analysed. Conversely, overestimates of the diversity can be caused by a number of factors. Some bacteria have more than one rRNA operon of varying sequence and could lead to individual species generating multiple bands (Nubel *et al.*, 1996). The formation of hetroduplex fragments (where two similar but different stands join together) can be problematic for the analysis of mixed bacterial populations, because it overestimates the real

number of community constituents (Myers *et al.*, 1989; Ferris *et al.*, 1997). However, Jensen and Straus (1993) reported that hetroduplex formation could be reduced by using a higher ionic strength, higher primer concentrations, and a lower annealing temperature as well as by decreasing the number of amplification cycles.

The phylogenetic information gained from the sequence of excised bands is also limited by the size of the fragment run on the gradient gel. Typically, only fragments up to 500bp can be well separated. This can be overcome by creating clone libraries that can be screened with gradient gel electrophoresis to find phylotypes that are representative of environmental microbial communities. In this way, increased sequence information can be obtained from the clones.

1.12. Measurement of Bacterial Phylogenetic Relationships

1.12.1. The rRNA Approach

The phylogenetic relationship between different organisms could be measured by comparing sequences from appropriate homologous biomacromolecules. The criteria adopted, and one that has found wide application for the inference of evolutionary relationships has been described comprehensively by Carl Woese (1987). Woese outlines that a molecule whose sequence changes randomly with time can serve as a universal molecular chronometer (Woese, 1987). Such a molecule needs to behave in a clock–like manner, whereby changes in sequences occur randomly and the rate of change must be commensurate with the spectrum of evolutionary distances being measured. In addition to this, the molecule has to be large enough to provide an adequate amount of information and be ubiquitous in all cellular life forms. It is for these reasons that larger rRNA molecules are targeted for determining the evolutionary relationships of all living things (Woese *et al.* 1990), and in particular the 16S rRNA molecules have proved to be the most useful and widely used molecular chronometers.

The 16S rRNA molecule comprises highly conserved sequence domains interspersed with semi-conserved and hypervariable regions (Gutell *et al.*, 1994). It is these semi-conserved and hypervariable regions that provide phylogenetic resolution to species level and perhaps beyond.

In addition, the advent of PCR has made it easier to directly amplify and sequence 16S rRNA to define phylogenetic relationships further enhancing the field of bacterial taxonomy.

1.12.2. Analysis of Sequence Data

To determine the identification of the unknown sequences retrieved from environmental samples, BLAST (Altshul *et al.*, 1990) and FASTA (Pearson and Lipman, 1998) searches are carried out that involve comparing against known DNA or protein sequences catalogued in databases. Sequence identities are inferred from the percentage of nucleotides/amino acids that are shared with known sequences.

To assess the quality, the sequences can be investigated with the RDP Chimera Check program (Maidak *et al.*, 1997) for the presence of chimeric sequences, which result when fragments from two different sequences become fused during the amplification process.

The construction of phylogenetic trees requires the comparative analysis of rRNA sequences. Each sequence retrieved undergoes a combination of multiple sequencing runs to form 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. From this a phylogenetic tree is generated that reflects the evolutionary relationship of the sequences. The tree is considered 'unrooted' if the ancestral start point is unknown, however, a point of reference can be provided by the inclusion of data from a distance evolutionary branch known as an outgroup. The two widely used approaches for inferring phylogenetic trees are distance matrix and maximum parsimony, both of which are employed in the work described in this thesis.

1.12.3. Distance Matrix Methods

Distance matrix methods are the simplest methods for analysing bacterial relationships. DNA sequences are aligned pairwise and base substitutions are used to construct a distance matrix. Distance methods use this matrix as the data from which branching order and branch length are computed. There are several algorithms to interpret base substitutions at a single site, the simplest being the Jukes and Cantor model (Jukes and Cantor, 1969), which assumes the probabilities of any nucleotide changing to any other nucleotide, are equal. However, this method can underestimate

the true evolutionary distances between sequences due to multiple events occurring at different rates (Woese, 1987).

1.12.4. Maximum Parsimony Analysis

Unlike distance methods, maximum parsimony does not reduce the differences between sequences to a single distance; it considers each nucleotide position independently thus offering more evolutionary information. These methods select evolutionary trees that assume the least number of substitutions has occurred; maximum parsimony, or that fit the observed data, maximum likelihood.

1.13. Aims of this Project

This project aimed, primarily to elucidate community structure and diversity of key bacterial functional groups involved in the critical processes of cellulolysis, sulphate-reduction and methanogenesis in the anaerobic degradation of municipal sludge. The anaerobic populations in digester environments are highly interdependent and due to their anaerobic nature are difficult to isolate and grow in pure culture. Isolation studies done previously by others give a poor indication of the microbiology residing in digester ecosystems. Here, molecular biological techniques were applied to target these key functional groups in a mesophilic anaerobic digester, in order to assess the difference in occurrence and diversity in sludge before and during digestion.

The study therefore has two aims:

1. to describe the structure of a normal functioning microbial community in an anaerobic sludge digester to serve as a benchmark for the investigation of perturbation effects, and ultimately the development of molecular biological tools for monitoring the process;

2. to describe how the functional communities change as anaerobic digestion proceeds i.e. is diversity maintained or does selection operate, and to what extent.

CHAPTER TWO

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

Non-target reference strains were also used in this study. For SRB, the non-target species used are those described by Daly *et al.*, (2000) as containing 1 or 2 bp mismatches within the oligonucleotide probe target region, identified from the Ribosomal Database Project (RDP) (Maidak *et al.*, 1997). Probe specificities for methanogen groups, under appropriate hybridization and wash conditions, have been demonstrated empirically by Raskin *et al.*, (1994a). In this study, for methanogen group oligonucleotide probing, reference strains not belonging to the probe target groups were used as negative controls.

Table 2.1. Bacterial strains used in this study

Reference Strains	Source*
Desulfotomaculum nigrificans (Group 1)[a]	NCIMB 8395
Desulfobulbus propionicus (Group 2)[a]	DSM 2032
Desulfobacterium autotrophicum (Group 3)[a]	DSM 3382
Desulfotobacter curvatus (Group 4) [a]	DSM 3379
Desufosarcina variabilis (Group 5)[a]	DSM 2060
Desulfovibrio desulfuricans (Group 6)[a]	DSM 642
Zymomonas mobilis	NCIMB 10659
Clostridium aurantibutyricum	NCIMB 10659
Desulfobacterium vacuolatum	DSM 3385
Pelobacter carbinolicus	DSM 2380
Clostridium sporogenes (Cluster I)[b]	LIV 91
Clostridium termitidis (cluster III)[b]	DSM 5398
Clostridium sporosphaeroides (cluster IV)[b]	NCIMB 10772
Clostridium celecrescens (cluster XIVab)[b]	NCIMB 12839
Methanobacterium espanolae	University of Liverpool
Methanobacterium bryantii	DSM 862
Methanococcus thermolithotrophicus	DSM 2095
Methanocorpusculum aggregans	University of Liverpool
Methanosaeta concilii	DSM 3013
Methanosarcina mazeii	University of Liverpool
Methanolobus tindariz	DSM 2278
Escherichia coli	NCIMB 10243

*Abbreviations: NCIMB, Nationnal Collection of Industrial and Marine Bacteria, Aberdeen, Scotland; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

[a] Sulphate-reducing bacteria group designations from Daly *et al.*, 2000

[b] Clostridial cluster designations from Collins *et al.*, 1994

2.3. Collection of Environmental Samples

The environmental samples used in this study were collected from the Exeter Municipal treatment plant that treats primarily domestic waste. Samples were obtained from the primary settling tanks holding the raw sludge that serves as the feed for the digester. The digesting sludge samples were taken from a mesophilic anaerobic digester at the site. Samples (20 litres) of each sludge type were collected, and 500ml sub-samples were forwarded to Liverpool for this study. The sludge samples were collected by staff at AstraZeneca Brixham Laboratories.

2.4. Extraction of DNA from sludge samples

The sludge samples were processed immediately upon receipt. Both the raw and digesting sludge were further concentrated by centrifuging 1.5ml aliquots of each sludge sample at 13,000g for 10 min. The supernatant was discarded and the pellets stored at –80°C until required. These concentrated sludge pellets were thawed on ice and resuspended in 200µl of sterile dH_2O. Genomic DNA was extracted from ribolysed sludge samples using the FAST DNA SPIN Kit for soil (Anachem Catalog no. 6560-200). A 978ml volume of 200mM sodium phosphate buffer (pH 8.0), 122ml of MT buffer and 200µl of the sludge preparation were added to a FastDNA tube containing a matrix designed to lyse most cell types. The mixture was shaken in the ribolyser for 30 s at 5.5 m/s and then centrifuged at 13,000g for 30 s. One ml of supernatant was removed and mixed with 250µl of protein precipitating solution (Anachem). This mixture was centrifuged at 13,000g for 5 min at room temperature. DNA purification was carried out by adding the sludge supernatant to a spin filter with 1 ml binding matrix. This tube was gently inverted five times, incubated for 5 min at room temperature, and then centrifuged for 30 s at 13,000g. For this step and all other purification steps, the eluate in the catch tube was discarded after centrifugation. The pellet in the spin filter was washed twice. Each wash was achieved by adding 500µl of salt-ethanol wash solution and then centrifuging for 30 s at 13,000g. The spin filter was then centrifuged for 1 min at 13,000g to dry the pellet. The DNA was eluted by transferring the spinfilter to a new catch tube, adding 100µl of DNA elution solution, gently flicking the tube five times, and then centrifuging for 1 min at 13,000g. To minimize DNA shearing, vortex mixing was avoided.

DNA was extracted from control strains (Table 2.1) by resuspending freeze dried cultures in 200µl sterile dH_2O and applying the FAST DNA SPIN Kit as described. DNA recovery, purity and yield were evaluated by agarose gel electrophoresis (section 2.7). DNA quantity from sludge samples was determined by comparing the visual intensity of ethidium bromide fluorescence of the extracted DNA to bands of the molecular marker of known concentrations. The quality of DNA was assessed by successful PCR amplification of extracted DNA.

2.5. Oligonucleotides

All oligonucleotides used in this study were commercially synthesized by MWG-Biotech AG (Ebersberg). On arrival, the oligonucleotides were resuspended in sterile dH_2O to give a final stock concentration of 100pmol. For PCR application, the oligonucleotides were diluted to give a final concentration of 10 pmol μl^{-1}. Details of oligonucleotides are provided in Tables 2.2 - 2.5.

2.6. PCR amplification

Total DNA extracts were serially diluted ten-fold to determine the optimal sample concentration for PCR amplification. In all cases, the PCR was performed with a DNA thermal cycler 480 (Perkin Elmer Cetus) in 50µl reaction mixtures. Each 50µl tube contained: 1µl each primer (10pmol μl^{-1}), 1µl dNTP (10mM each) (HT Biotech, Ltd), 3µl 10mM $MgCl_2$, 40µl dH_2O, 5µl 10 x PCR buffer (HT Biotech, Ltd), 0.1µl 10% BSA (Boehringer Mannheim), 1U Super*Taq* polymerase (HT Biotech, Ltd) and a 1-5µl aliquot of DNA template (equivalent to 50-100ng environmental DNA or 1ng control DNA).

PCR amplifications of 16S rDNA extracted from environmental samples and control strains were performed with universal PCR primers targeting *Bacteria* and *Archaea* as well as group-specific primers for SRB and clostridia (Tables 2.2 - 2.4). The PCR program, unless otherwise stated, involved 30 cycles consisting of 94°C 1 min, 1 min at appropriate annealing temperature, 72°C for 1 min, with a final elongation step of 10 min at 72°C after the 30 cycles. Reaction tubes were held at 0°C upon completion.

2.6.1. 'Hot-Start' PCR

All PCR amplifications in this study were performed using a 'hot-start' PCR protocol. This approach includes an initial denaturation step of 94°C prior to PCR cycling. In this way the DNA template and PCR primers are fully denatured and dissociated from one another in order to enhance the product yield for environmental samples. Each reaction was therefore heated to 94°C for 6 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 for addition of the enzyme. Each reaction was then overlaid with mineral oil prior to amplification.

2.6.2. 'Nested' PCR

Some bacterial groups in complex environmental samples are not detectable when PCR amplification is applied directly. 'Nested' PCR is a method which increases the sensitivity of PCR by employing two rounds of amplification with different primer sets, the second set internal to the first. In this study, templates produced by amplification with universal bacterial 16S rDNA PCR primers pA & pH' (Table 2.2) (Edwards *et al.*, 1989) were suitably diluted in dH_20 to prevent interference due to primers from the initial PCR reaction. Aliquots of these products were used in a second PCR reaction involving a pair of SRB or clostridial group-specific primers.

Bacterial amplification primers

Primer	Sequence 5' – 3'[a]	Specificity[b]	Annealing temp.	Product size	Reference
pA	AGA GTT TGA TCC TGG CTC AG	*Bacteria*	55°C	1500bp	Edwards *et al.*, 1989
pH'	AAG GAG GTG ATC CAG CCG CA				

***Clostridium* Clusters I, III, IV, XIVab**

Primer	Sequence 5' – 3'[a]	Specificity[b]	Annealing temp.	Product size	Reference
S-*-Chis-0150-a-S-23	AAA GGR AGA TTA ATA CCG CAT AA	*Clostridium*	65°C	820bp	Franks *et al.*, 1998
S-*-Cbot-0983-a-A-21	CAR GRG ATG TCA AGY CYA GGT	cluster I			Van Dyke & McCarthy 2002
S-*-Cther-0650-a-S-23	TCT TGA GTG YYG GAG AGG AAA GC	*Clostridium*	60°C	720bp	Van Dyke & McCarthy 2002
S-*-Cther-1352-a-A-19	GRC AGT ATD CTG ACC TRC C	cluster III			Van Dyke & McCarthy 2002
S-*-Clos-0561-a-S17	TTA CTG GGT GTA AAG GG	*Clostridium*	60°C	580bp	Van Dyke & McCarthy 2002
S-*-Clept-1129-a-A-17	TAG AGT GCT CTT GCG TA	cluster IV			Van Dyke & McCarthy 2002
S-*-Erec-0482-a-S-19	CGG TAC YTG ACT AAG AAG C	*Clostridium*	55°C	620bp	Franks *et al.*, 1998
S-*-Ccoc-1112-a-A-19	TGG CTA CTR DRV AYA RGG G	cluster XIVab			Van Dyke & McCarthy 2002

Table 2.2. Primer sequences used to target 16S rDNA of *Bacteria* and *Clostrdium* groups I, III, IV & XIVab.
[a]Ambiguities: R (G or A); V (G , C or A); D (A, G or T); Y (C or T).
[b]Clusters as defined by Collins *et al.* 1994.

Sulphate-Reducing Bacteria

Primer	Target site[a]	Sequence 5'- 3'[b]	Specificity	Annealing temp.	Product size	Reference
DFM140	140-158	TAG MCY GGG ATA ACR SYK G	Group 1	50°C	700bp	Daly *et al.*, 2000
DFM842	842-823	ATA CCC SCW WCW CCT AGC AC				
DBB121	121-142	CGC GTA GAT AAC CTG TCY TCA TG	Group 2	66°C	1120bp	Daly *et al.*, 2000
DBB1237	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	Daly *et al.*, 2000
DBM1006	1006-986	ATT CTC ARG ATG TCA AGT CTG				
DSB127	127-148	GAT AAT CTG CCT TCA AGC CTG G	Group 4	60°C	1150bp	Daly *et al.*, 2000
DSB1273	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	Daly *et al.*, 2000
DCC1165	1165-1144	GGG GCA GTA TCT TYA GAG TYC				
DSV230	230-248	GRG YCY GCG TYY CAT TAG C	Group 6	61°C	610bp	Daly *et al.*, 2000
DSV838	838-818	SYC CGR CAY CTA GYR TYC ATC				

Probe	Target site[a]	Sequence 5'- 3'[b]	Specificity	Hybridisation temp.	References
DFM228	228-242	GGG ACG CGG AYC CAT	Group 1	48°C	Daly *et al.*, 2000
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	Daly *et al.*, 2000
DCC868	868-885	CAG GCG GAT CAC TTA ATG	Group 5	46°C	Daly *et al.*, 2000
DSV687	687-702	TAC GGA TTT CAC TCC T	Group 6	45°C	Devereux *et al.*, 1992

Table 2.3. Primers and probes used in this study to target 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).

Methanogens

Primer	Target site[a]	Sequence 5'- 3'[b]	Specificity	Annealing temp.	Product size	Reference
1Af	1-20	TCY GGT TGA TCC TGC CRG AG	*Archaea*	60°C	1400bp	Munson *et al.*, 1997
1404r	1404-1387	CGG TGT GTG CAA GGR GC				Munson *et al.*, 1997

Probe	Target site[a]	Sequence 5'- 3'[b]	Specificity	Hybridisation temp.	Reference
MB1174	1195-1174	TAC CGT CGT CCA CTC CTT CCTC	*Methanobacteriales*	62°C	Raskin *et al.* 1994a
MC1109	128-1109	GCA ACA TAG GGC ACG GGT CT	*Methanococcales*	55°C	Raskin *et al.* 1994a
MG1200	1220-1200	CGG ATA ATT CGG GGC ATG CTG	*Methanomicrobiales*	53°C	Raskin *et al.* 1994a
MS1414	1434-1414	CTC ACC CAT ACC CAC TCG GG	*Methanosarcinaceae*	58°C	Raskin *et al.* 1994a
MS821	844-821	CGC CAT GCC TGA CAC CTA GCG AGC	*Methanosarcina*	60°C	Raskin *et al.* 1994a
MX825	847-825	TCG CAC CGT GGC CGA CAC CTA GC	*Methanosaeta*	59°C	Raskin *et al.* 1994a

Table 2.4. Primers and probes used in this study to target methanogen groups.
[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).

2.7. Agarose gel electrophoresis

The extracted DNA and PCR products were subjected to agarose gel electrophoresis for analysis. This involved electrophoresis through a 1% (w/v) agarose gel containing 5µl ethidium bromide (10mg ml^{-1}) in 1 x Tris acetate EDTA (pH 8.0) buffer (50 x Tris acetate EDTA: 2M Tris; 57.1ml l^{-1} glacial acetic acid; 0.05M EDTA; adjusted to pH 8.0).

Nucleic acid samples (5µl) were mixed with (1µl) 6 x loading buffer prior to loading onto the gel. Nucleic acids were separated by electrophoresis at a constant voltage of 75V for 1 h and visualised by UV illumination at 320nm. The gels were recorded and stored with a gel-reading program (Genetool, Syngene).

λDNA/*HindIII* and GeneRuler 100bp DNA Ladder Plus (MBI Fermentas) markers were included to enable estimation of the molecular weight and yield of the DNA extracted and amplified.

2.8. Southern transfer of DNA

PCR amplified products and DNA extracts were transferred from 1% (w/v) agarose gels to positively-charged membranes by Southern Blotting. Transfer was facilitated by capillary action using alkali transfer buffer (0.25M NaOH; 1.5M NaCl). Blotting was allowed to proceed for at least 4 h, after which time the DNA was then fixed to membranes by air drying for 30 min followed by UV crosslinking at 320nm for 6 min. If required, the membranes were wrapped in cling film and stored at 4°C.

2.9. Oligonucleotide probing

The membranes were prehybridised for approximately 2 h at the appropriate hybridization temperature (Tables 2.2 and 2.4) with 40 ml standard 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]) to prevent non-specific binding of the probe.

The labeled probes were diluted in 10 ml of hybridization solution to a final cocentration of 10 pmol ml^{-1} and membranes were incubated overnight at the hybridization temperature inside a thermostatically controlled oven (Hybaid). After hybridization, the membrane was washed twice at room temperature for 5 min with 30 ml of a solution containing 2 x SSC-0.1% (w/v) SDS and twice at hybridization

temperature, with 30 ml of a solution containing 0.1 x SSC-0.1% (w/v) SDS. The membrane was incubated for 30 min with blocking solution containing 1% (w/v) blocking reagent, 0.1 M maleic acid, and 0.15 M NaCl (pH 7.5). The DIG-labelled oligonucleotide probe was subsequently detected by an enzyme-linked immunoassay with 2μl of anti-DIG alkaline phosphatase conjugate in 10 ml of the blocking solution. After 1 h of incubation, the membrane was washed twice for 15 min with washing buffer (0.1 M maleic acid) 0.15 M NaCl, 0.3% (v/v) Tween 20) and equilibrated for 5 min with detection buffer (0.1 M Tris-HCl, 0.1 M NaCl [pH 9.5]). A subsequent enzyme-catalysed reaction with the chemiluminescent reagent CDP-Star (Boehringer Mannheim) allowed the detection of the hybrids with X-ray film (Kodak) at room temperature for 1-5 min.

2.10. Labelling of oligonucleotide probes

Synthetic oligonucleotide probes (100pmol) were labelled with digoxigenin at their 3' end by incorporation of a single digoxigenin (DIG)-labelled nucleotide, using the enzyme terminal transferase. The chemicals for this labelling reaction were obtained from Boehringer Mannheim Biochemicals, and the reaction was performed according to the manufacturer instructions. Probe labelling efficiency was also assessed according to the manufacturer's instructions.

2.11. Stripping and re-probing of membranes

In some cases, the DNA-containing nylon membranes were stripped and re-used with another probe. Removal of the probe from the membrane involved rinsing the membrane thoroughly in water for 1 min. The membranes were then incubated in alkaline probe stripping solution (0.2 M NaOH, 0.1% (w/v) SDS) at 37°C for 20 min. This was followed by a final step of rinsing the membrane in 2 x SSC. The stripped membrane was dried and stored at -20°C until required or used directly for hybridisation.

2.12. Temporal gradient gel electrophoresis

Sequence-specific separation of PCR products was performed by parallel TGGE using the D-Gene system (Bio-Rad, Inc) as specified by the manufacturer. Aliquots of PCR products amplified with appropriate GC primer sets (section 2.13.) 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 TGGE gels. TGGE gels comprised 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) deionised formamide).

Gels were allowed to polymerise for at least 1 h. A 5ml stacking gel, without denaturant, was added after polymerization. Products from amplification with the appropriate GC-clamped primer pair were electrophoresed in the gels with a 1.25 x Tris acetate EDTA (pH 8.0) buffer system. For each of the bacterial groups analysed, approximately 200 ng of each sludge product were run at the appropriate temperature range and voltage settings as outlined in Table 2.5, to give improved resolution of PCR products. After the runs, gels were removed from setup and stained for 20 min with 0.2 x conc. SYBR Green 1 nucleic acid stain (Flowgen) in 1.25 x Tris acetate EDTA (pH 8.0), after which they were inspected under UV illumination using a STORM optical scanner (Molecular Dynamics). Gel images were visualized and stored using ImageQuant software.

GC-PCR PRODUCTS	Temperature range °C	Ramp rate °C h^{-1}	Voltage V
Clostridia	43 – 51	0.5	80
SRB	51 – 56	0.4	75
Archaeal	43 – 51	0.5	80

Table 2.5. TGGE running conditions for clostridia, SRB, and archaeal GC-PCR products

2.13. GC-clamped PCR amplification

PCR products amplified from the two sludge types using group-specific primers (section 2.6) were diluted 100-fold and re-amplified with the appropriate TGGE primer sets to generate a PCR fragment of less than 500bp suitable for TGGE analysis. A 40 nucleotide GC rich sequence (GC-clamp) was incorporated at the 5' end of the forward primer of each TGGE primer set (Table 2.6) to facilitate separation of fragments. PCR reactions were performed under the same reaction conditions as described in section 2.6. The PCR program involved 25 cycles consisting of 94°C 1 min, 1 min at appropriate annealing temperature, 72°C for 1 min, with a final elongation step of 10 min at 72°C after the 25 cycles. Reaction tubes were held at 0°C upon completion.

2.14. Recovery of DNA from TGGE bands

DNA bands of interest in TGGE gels were cut out with a razor blade and eluted overnight at 37°C in 50µl of sterile distilled water. For sequencing, bands were re-amplified using the primer set without GC clamp and purified with QIAquick PCR purification kit (Qiagen, Ltd).

2.15. Cloning of PCR products

Prior to cloning, the amplified PCR products were purified with the Qiagen PCR purification kit (Qiagen). The purified amplicons were then ligated into the pGEM-T plasmid vector (Promega) and cloned into competent E.*coli* JM109 cells according to the manufacturer's instructions. Cells were plated out onto LB agar (10g l^{-1} tryptone, 5g l^{-1} yeast extract, 5g l^{-1} NaCl, adjusted to pH 7.0) containing 100µg ml^{-1} ampicillin with 0.5 mM IPTG and 40µg 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 100µg ml^{-1} ampicillin, 0.5 mM IPTG, 40µg ml^{-1} X-Gal and incubated overnight at 37°C. Clones were then stored at 4°C until required.

Primer sets for GC amplifications

Methanogen

Primer	Sequence 5'- 3'[a]	Specificity	Annealing temp.	Product size	Reference
1100Af (GC)	(GC-clamp)[b]-CAA CGA GCG AGA CCC A	*Archaea*	60°C	300bp	Munson *et al.*, 1997
1404r	CGG TGT GTG CAA GGR GC				Munson *et al.*, 1997

***Clostridium* clusters I, III, IV, and XIVab**

Primer	Sequence 5'- 3'[a]	Specificity	Annealing temp.	Product size	Reference
S-D-Bact-0907-a-S-20	AAA CTC AAA GGA ATT GAC GG	Cluster III	60°C	310bp	Lane *et al.*, 1985
S-D-Grps-1097-a-A-20	(GC-clamp)-ACA TAA GGG GCA TGA TGA TT				Fry *et al.*, 1997
S-D-Bact-0786-a-S-20	(GC-clamp)-GAT TAG ATA CCC TGG TAG TC	Cluster IV & XIV	62°C	300bp	Woese 1987
S-D-Bact-1060-a-A-21	TCA CGA CAC GAG CTG ACG ACA				Woese 1987
pC(gc)	(GC-clamp)-CTA CGG GAG GCA GCA GTG GG	Cluster I	68°C	235bp	Edwards *et al.*,1989
pD'	GTA TTA CCG CGG CTG CTG				Edwards *et al.*,1989

Sulphate-reducing bacteria

pC(gc)	(GC-clamp)-CTA CGG GAG GCA GCA GTG GG	SRB	68°C	235bp	Edwards *et al.*,1989
pD'	GTA TTA CCG CGG CTG CTG	Groups 1 to 6			Edwards *et al.*,1989

Table 2.6. GC-clamp primer sets used in this study for TGGE analysis of target groups.
[a]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).
[b]GC-clamp, CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC.

2.16. TGGE screening of clones

Clones stored at 4°C were subcultured into 10ml LB broth containing 100μg ml^{-1} ampicillin and incubated overnight at 37°C. 1ml of overnight culture was pelleted by centrifugation (13,000 x g, 5 min) and resuspended in 100μl sterile dH_20. The samples were then placed in a boiling water bath for 10 min to lyse the cells and release plasmid DNA. The cell lysates were used to screen the transformants by PCR using the appropriate GC primer sets followed by TGGE analysis as described in sections 2.12. and 2.13. TGGE profiles of amplified DNA from each bacterial group and sludge type were run alongside the clones derived from the amplification products in order to identify clones of interest.

2.17. Plasmid isolation

Clones that equated with bands on TGGE gels were subcultured into fresh LB broth containing 100μg ml^{-1} ampicillin and incubated overnight at 37°C. 2 ml of overnight culture was pelleted by centrifugation (13,000 x g, 5 min) and plasmid DNA containing the relevant insert 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.

2.18. DNA sequencing

Sequence analysis was carried out using purified plasmid DNA and sequencing primers complementary to the 16S rDNA sequences (Tables 2.2 - 2.4). Automated DNA sequencing was performed with the CEQ 2000XL DNA Analysis System (Beckman Coulter, USA) at the School of Biological Sciences sequencing facility, University of Liverpool.

2.19. Phylogenetic analysis

Excised bands and selected 16S rDNA clonal sequences were submitted to the BLASTN (Altschul *et al.*, 1990) database searches in order to identify the unknown sequences, by comparing them against the known DNA sequences in databases. These sequences were then aligned to homologous 16S rRNA sequences of closely related microorganisms obtained from the GenBank (Benson *et al.*, 1997), EMBL (Stoesser, 1997) and Ribosomal Database Project (Maidak *et al.*, 1997) databases. Sequence data were aligned with the CLUSTAL X package (Thompson *et al.*, 1997) and corrected by manual inspection on GDE. Only unambiguously aligned base positions were used in the analysis. Calculations of distance values were performed using Phylogeny Inference Programs (PHYLIP 3.4) (Felsenstein, 1993). Phenograms illustrated in this thesis were generated using the Jukes & Cantor (1969) correction in the DNADIST program from PHYLIP 3.4. Phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis was performed using SEQBOOT (PHYLIP 3.4) to evaluate the robustness of the inferred phenograms and CONSENSE (PHYLIP 3.4) was used to generate a consensus phenogram using the neighbour-joining method. The topologies of phenograms were corroborated by maximum parsimony analysis using PHYLIP 3.4.

CHAPTER THREE

3. Identification and detection of sulphate-reducer, methanogen and clostridial populations in sludge before and during anaerobic digestion in a municipal treatment plant.

3.1. Introduction

Molecular biological techniques based on rRNA analysis have demonstrated the ability to characterise microbial populations in natural and engineered environments without the need for prior cultivation and isolation (e.g. Amann, *et al.*, 1995; Pace *et al*, 1986; Godon *et al.*, 1997). The direct amplification of 16S rRNA genes from environmental samples by using the polymerase chain reaction has been proven to provide information on the structure of different bacterial communities, because it avoids the well-known biases of culture methods. Similarly, oligonucleotide hybridisation probes, designed from sequence data, have been used in determinative and quantitative studies of microbial populations within complex communities (e.g. Amann *et al.*, 1990; Weber *et al.*, 2001; Raskin *et al.*, 1994b).

In this chapter, a combination of PCR primers and oligonucleotide probes have been applied to detect and identify microbial populations involved in the processes of cellulolysis, sulphate-reduction and methanogenesis during anaerobic degradation of domestic waste and assess the comparative occurrence and distribution of these groups in sludge before and during digestion.

Sludge samples used for analysis were taken from a municipal treatment plant treating primarily domestic waste. Samples were obtained from the primary settling tank (raw sludge) that serves as the feed for the digester (Fig. 3.1). The digesting sludge samples were taken from a mesophilic anaerobic digester at the site (Fig. 3.2).

Figure 3.1. Primary settling tanks at Exeter treatment plant from which raw sludge was obtained

Figure 3.2. Mesophilic anaerobic digester at the Exeter treatment plant from which digesting sludge was obtained.

3.2. Results

3.2.1. Extraction of DNA from sludge samples

Genomic DNA was isolated from both raw and digesting sludge as described in the DNA extraction protocol in section 2.4. Equal volumes (5µl) of the isolated DNA were analysed on an ethidium bromide-stained agarose gel (Fig. 3.3) to assess DNA recovery, purity and yield. The yield and integrity of the DNA was observed to be good and reproducible. The concentration of DNA was estimated by comparing the visual intensity of ethidium bromide fluorescence to that of the DNA molecular marker. The quality of the DNA was determined on the success of PCR amplification.

3.2.2. Bacterial PCR amplification of 16S rDNA from sludge samples

Specific PCR amplification of 16S rDNA extracted from both sludge types was performed using primers pA & pH' (Edwards *et al.*, 1989) targeting the bacterial 16S rRNA gene. A single band corresponding to the expected size of amplified bacterial DNA (approximately 1.5 kb) was observed for both sludge DNA preparations on agarose gel electrophoresis (Fig. 3.4). All PCR reactions on sludge samples were performed in duplicate in this study unless otherwise stated.

These bacterial amplifications not only confirmed the presence of bacterial 16S rDNA in the sludge microbial community, but also demonstrated that the extracted DNA was sufficiently pure to undergo PCR amplification. In addition, the PCR products obtained here would serve as a DNA template of enriched eubacterial genes for any subsequent 'nested' PCR reactions that might be required. This is required if direct PCR amplifications fail to generate visible products from DNA extracts in a single step reaction using group-specific primers.

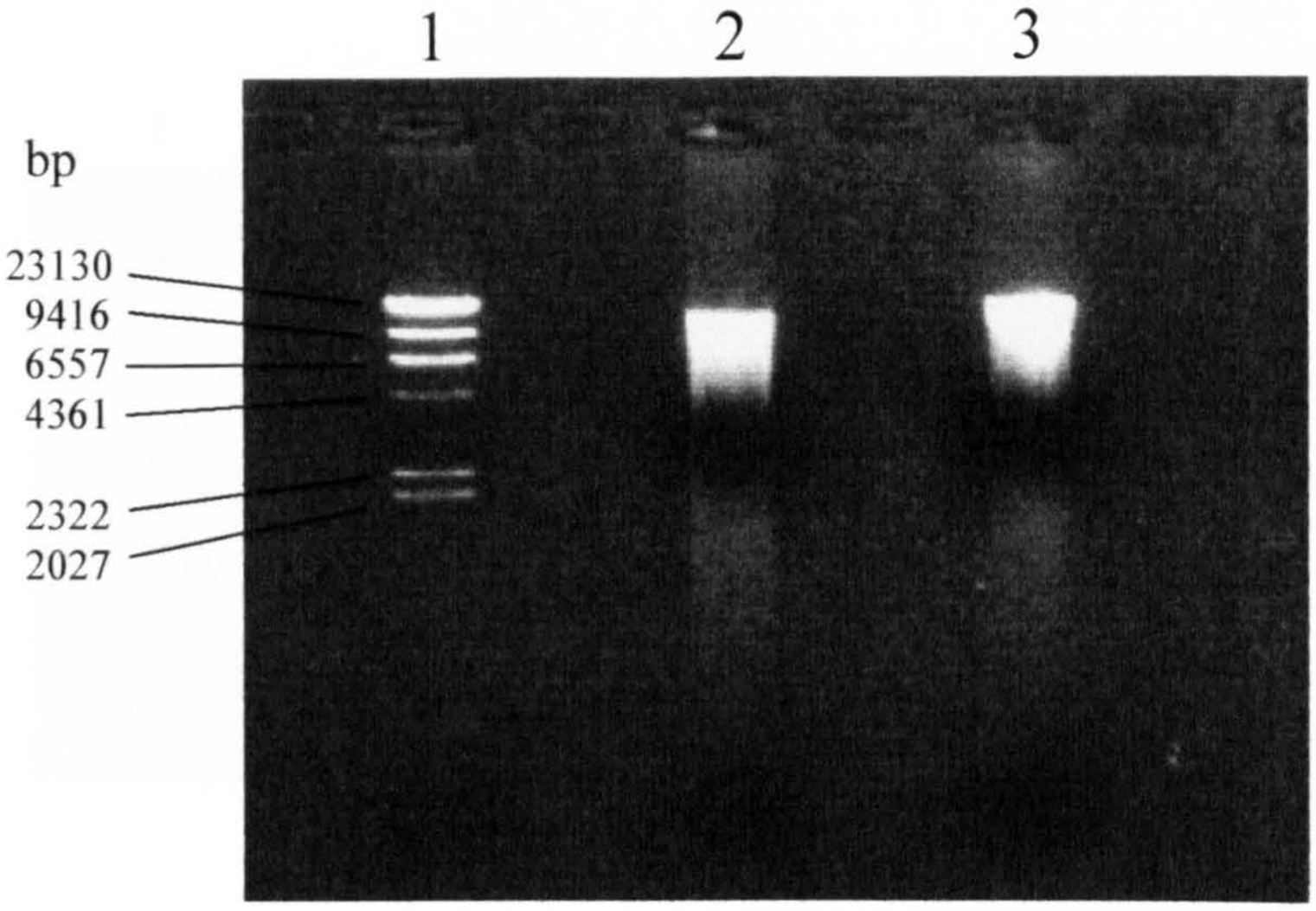


Figure 3.3. Agarose gel electrophoresis of DNA extracted from raw and digesting sludge using the Bio101 FastDNA Spin kit (Anachem).

Lane 1 – lambda DNA/HindIII (MBI Fermentas); Lane 2 – raw sludge;
Lane 3 – digesting sludge

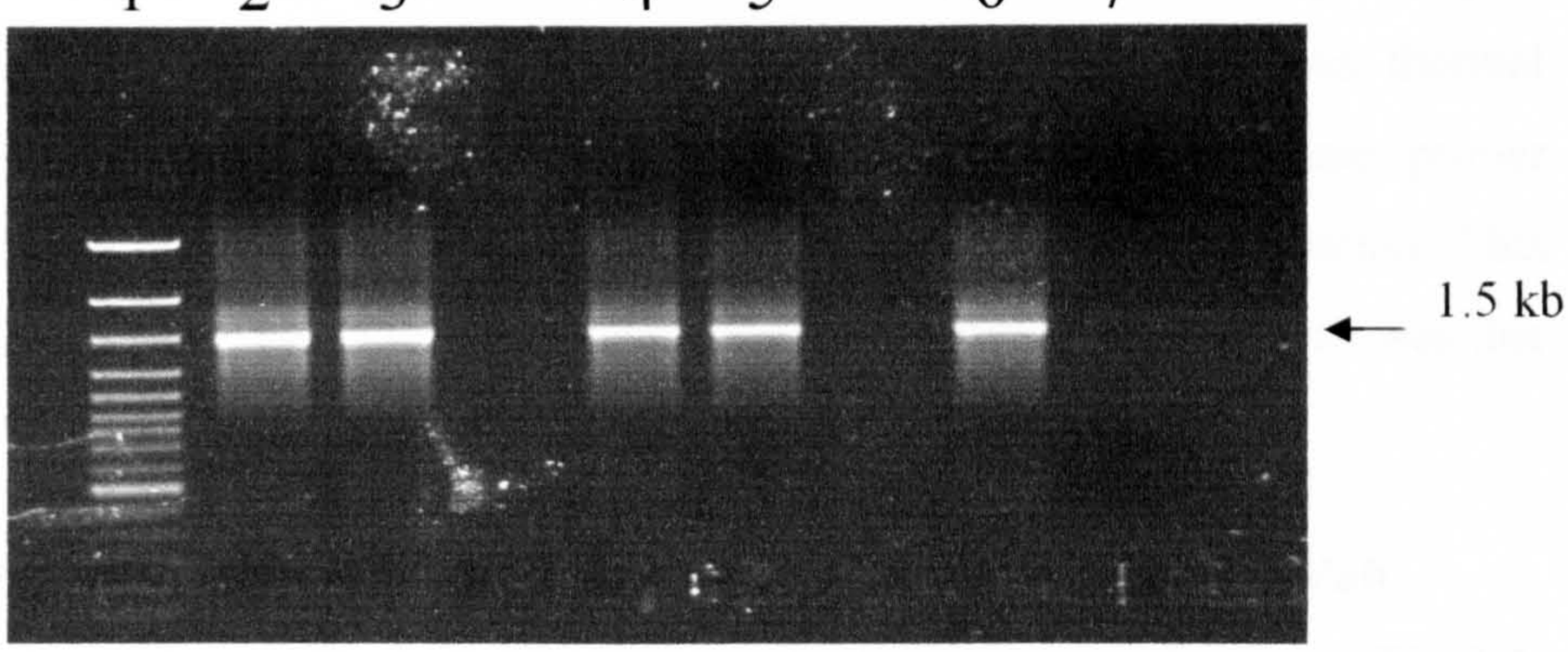


Figure 3.4. PCR amplification of 16S rDNA extracted from raw and digesting sludge using bacterial primers pA & pH' (Edwards *et al.*, 1989).

Lane 1 – GeneRuler 100bp DNA Ladder Plus;
Lane 2 & 3 – raw sludge;
Lane 4 & 5 – digesting sludge;
Lane 6 – *Escherichia coli*
Lane 7 – PCR blank

3.3. *Clostridium* populations in raw and digesting sludge

3.3.1. 'Direct' PCR amplification of clostridial groups I, III, IV and XIVab

'Direct' PCR amplification of 16S rDNA with *Clostridium* group-specific primers (Table 2.3) was performed as described in section 2.6. In each sludge type, products of the expected size were not generated for clostridial groups I, III, IV & XIVab (Fig. 3.5; Fig.3.6; Fig.3.7 and Fig.3.8 respectively; lanes 4, 5, 8, 9). Several attempts at 'direct' PCR amplification of indigenous 16S rDNA genes in the sludge samples were unsuccessful. Different parameters of the PCR protocol, such as thermal cycling times and temperatures, magnesium concentration, *Taq* polymerase, primer concentration and amount of DNA template, were modified without success. This strategy suggested that the failure to obtain detectable amplifiable products was due to insufficient template, and the 'nested' approach was therefore applied.

3.3.2. 'Nested' PCR amplification of clostridial groups I, III, IV & XIVab

Amplicons obtained from the initial bacterial (pA/pH) PCR amplifications (Fig 3.4) were diluted appropriately and used as a template DNA for the 'nested' PCR reaction with primers specific to target each of the four clostridial groups I, III, IV & XIVab. The PCR products obtained were subjected to agarose gel electrophoresis.

Cluster I: amplification products, observed as a single band corresponding to the expected size (0.82 kb), were obtained from both raw and digesting sludge using *Clostridium* cluster I primer pair (Fig. 3.5).

Cluster III: amplification products, observed as a single band corresponding to the expected size (0.72 kb) were obtained from both raw and digesting sludge using the *Clostridium* cluster III primer pair (Fig. 3.6).

Cluster IV: amplification products, observed as a single band corresponding to the expected size (0.58 kb), were obtained from both raw and digesting sludge using the *Clostridium* cluster IV primer pair (Fig. 3.7).

Cluster XIVab: amplification products, observed as a single band corresponding to the expected size (0.62 kb), were obtained from both raw and digesting sludge using the *Clostridium* cluster XIVab primer pair (Fig. 3.8).

All of the clostridial cluster groups (Collins *et al.*, 1994) that are known to contain cellulolytic bacteria could therefore be detected in sludge by 'nested' PCR, but not 'direct' PCR. The specificity of the primers is such (Van Dyke and McCarthy, 2002) that confirmation by oligonucleotide probe hybridisation was not required.

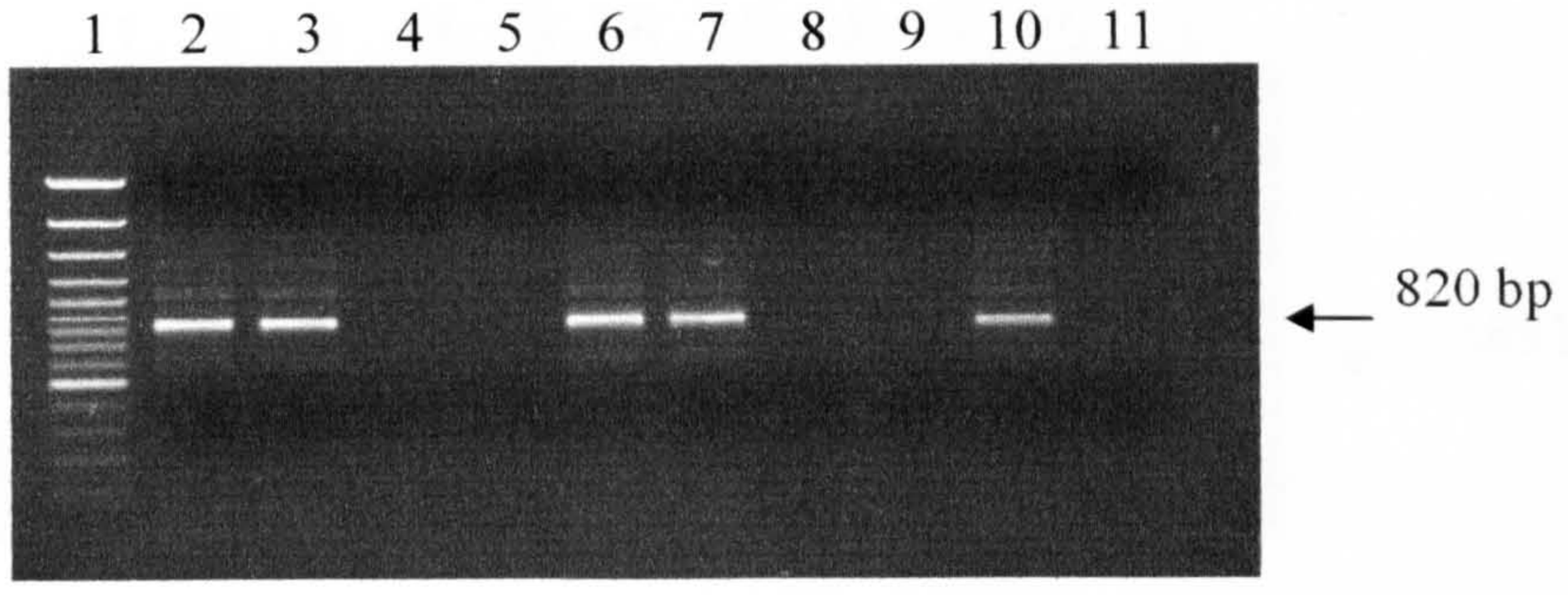


Figure 3.5. 'Direct' and 'nested' PCR amplification of 16S rDNA from DNA extracts of raw and digesting sludge using *Clostridium* cluster I primers.

Lane 1 – GeneRuler 100bp DNA Ladder Plus; Lane 2 & 3 – raw sludge 'nested' PCR;
Lane 4 & 5 – raw sludge 'direct' PCR; Lane 6 & 7 – digesting sludge 'nested' PCR;
Lane 8 & 9 – digesting sludge 'direct'; Lane 10 – *C. sporogenes* (+ve control);
Lane 11 – PCR blank control.

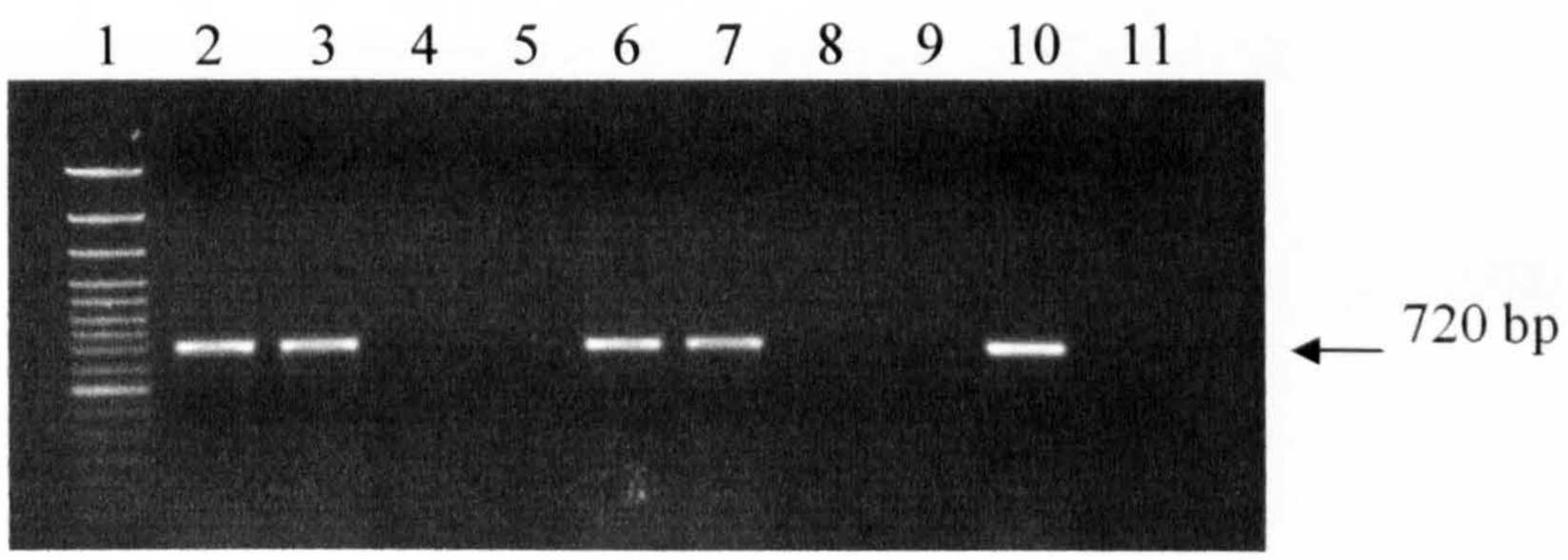


Figure 3.6. 'Direct' and 'nested' PCR amplification of 16S rDNA from DNA extracts of raw and digesting sludge using *Clostridium* cluster III primers.

Lane 1 – GeneRuler 100bp DNA Ladder Plus; Lane 2 & 3 – raw sludge 'nested' PCR;
Lane 4 & 5 – raw sludge 'direct' PCR; Lane 6 & 7 – digesting sludge 'nested' PCR;
Lane 8 & 9 – digesting sludge 'direct'; Lane 10 – *C. thermocellum* (+ve control);
Lane 11 – PCR blank control.

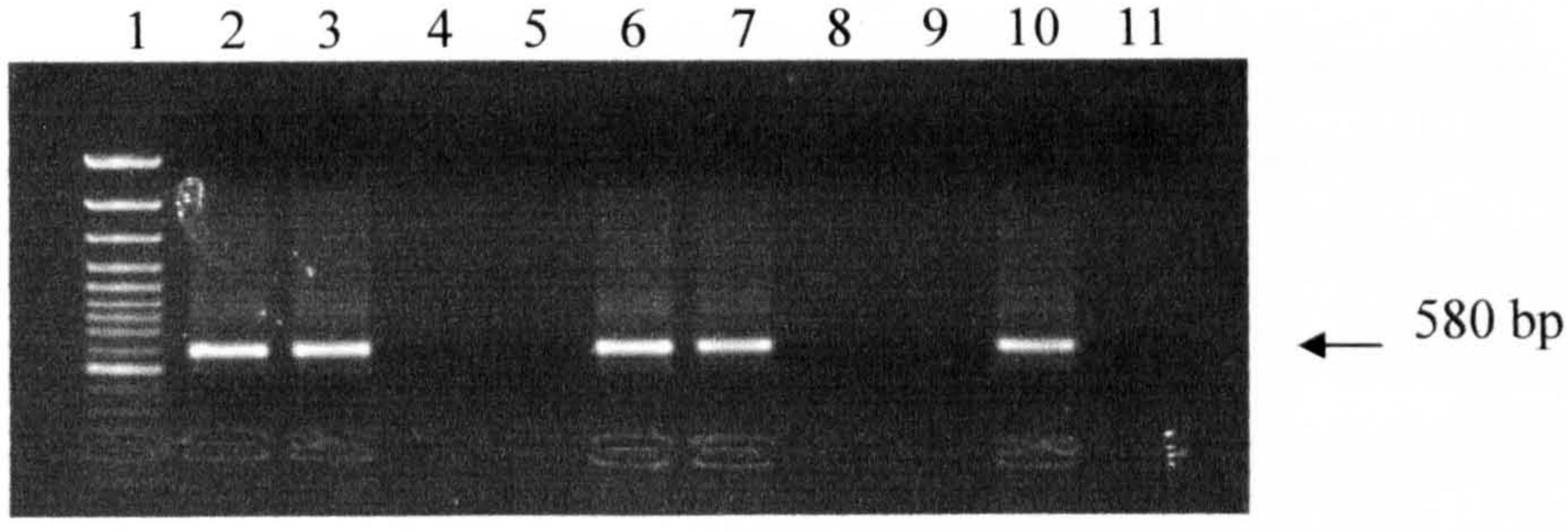


Figure 3.7. 'Direct' and 'nested' PCR amplification of 16S rDNA from DNA extracts of raw and digesting sludge using *Clostridium* cluster IV primers.

Lane 1 – GeneRuler 100bp DNA Ladder Plus; Lane 2 & 3 – raw sludge 'nested' PCR; Lane 4 & 5 – raw sludge 'direct' PCR; Lane 6 & 7 – digesting sludge 'nested' PCR; Lane 8 & 9 – digesting sludge 'direct'; Lane 10 – *C. sporosphaeroides* (+ve control); Lane 11 – PCR blank control.

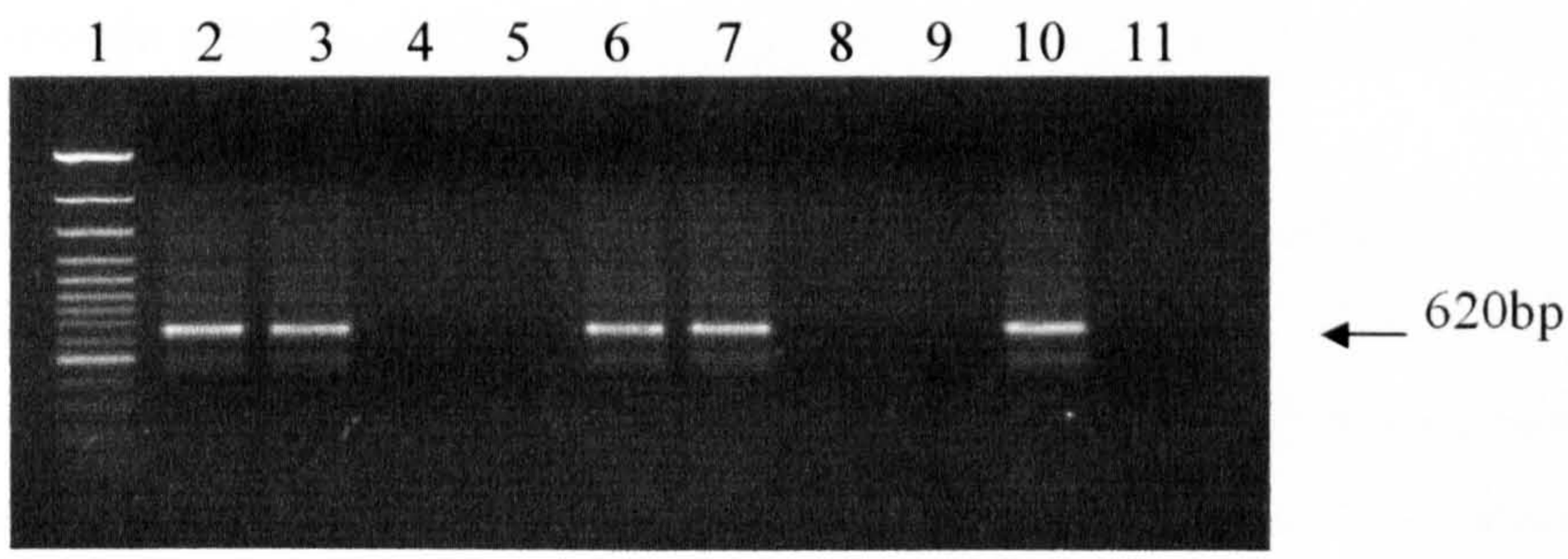


Figure 3.8. 'Direct' and 'nested' PCR amplification of 16S rDNA from DNA extracts of raw and digesting sludge using *Clostridium* cluster XIVab primers.

Lane 1 – GeneRuler 100bp DNA Ladder Plus; Lane 2 & 3 – raw sludge 'nested' PCR; Lane 4 & 5 – raw sludge 'direct' PCR; Lane 6 & 7 – digesting sludge 'nested' PCR; Lane 8 & 9 – digesting sludge 'direct'; Lane 10 – *C. celecrescens* (+ve control); Lane 11 – PCR blank control.

3.4. Sulphate-reducing bacteria (SRB) populations in raw and digesting sludge

3.4.1. 'Direct' PCR amplification of SRB groups from raw and digesting sludge

Sulphate-reducing bacterial 16S rDNA was directly amplified from raw and digesting sludge DNA extracts. PCR was performed with primers specific for each of the six main subgroups of SRB (Table 2.2) and carried out using the conditions described in section 2.6. The PCR products obtained were subjected to agarose gel electrophoresis and then transferred to positively-charged nylon membranes by Southern blotting (section 2.8). DNA fixed to membranes was then hybridised against the appropriate group-specific oligonucleotide probe (Table 2.2). Due to the degeneracy of the PCR primers, non-SRB DNA can also be amplified. However, the authenticity of amplified SRB 16S rDNA was confirmed by Southern blotting and therefore, only PCR products that subsequently gave a positive signal upon hybridisation were recorded as containing SRB DNA.

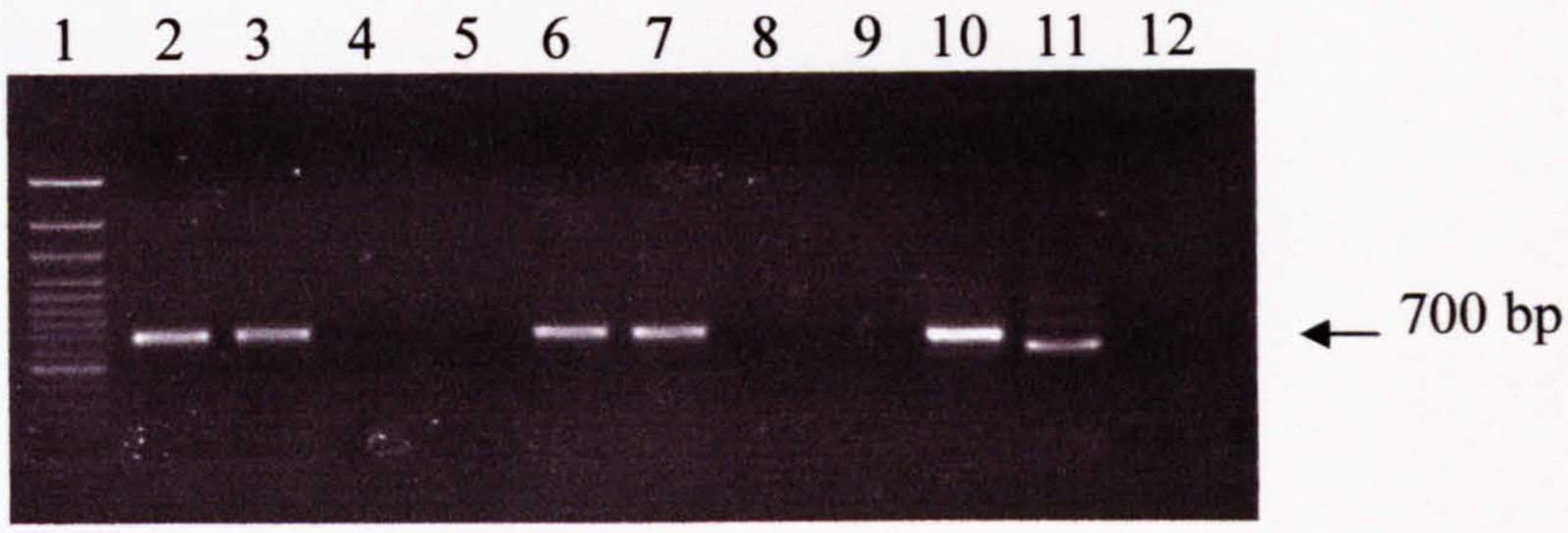
***Desulfotomaculum* (DFM Group 1)**: amplification products were not obtained from any of the two sludge types using the 'direct' PCR approach (Fig. 3.9.A; lanes 4, 5, 8, 9). The possibility that Group 1 SRB 16S rDNA was present at levels undetectable by ethidium bromide staining can be discounted because these lanes did not bind to the Group 1 probe DFM228 in Southern blots (Fig. 3.9.B).

***Desulfobulbus* (DBB Group 2)**: amplification products were not obtained from any of the two sludge types using the 'direct' PCR approach (Fig. 3.10.A; lanes 4, 5, 8, 9). The possibility that Group 2 SRB 16S rDNA was present at levels undetectable by ethidium bromide staining can be discounted because these lanes did not bind to the Group 2 probe DBB660 in Southern blots (Fig. 3.10.B).

***Deulfobacterium* (DBM Group 3)**: amplification products were not obtained from any of the two sludge types using the 'direct' PCR approach (Fig. 3.11.A; lanes 4, 5, 8, 9). The possibility that Group 3 SRB 16S rDNA was present at levels undetectable by ethidium bromide staining can be discounted because these lanes did not bind to the Group 3 probe DBM221 in Southern blots (Fig. 3.11.B).

***Desulfobacter* (DSB Group 4)**: amplification products were not obtained from any of the two sludge types using the 'direct' PCR approach (Fig. 3.12.A; lanes 4, 5, 8, 9). The possibility that Group 4 SRB 16S rDNA was present at levels undetectable by ethidium bromide staining can be discounted because these lanes did not bind to the Group 4 probe DSB623 in Southern blots (Fig. 3.12.B).

A



B

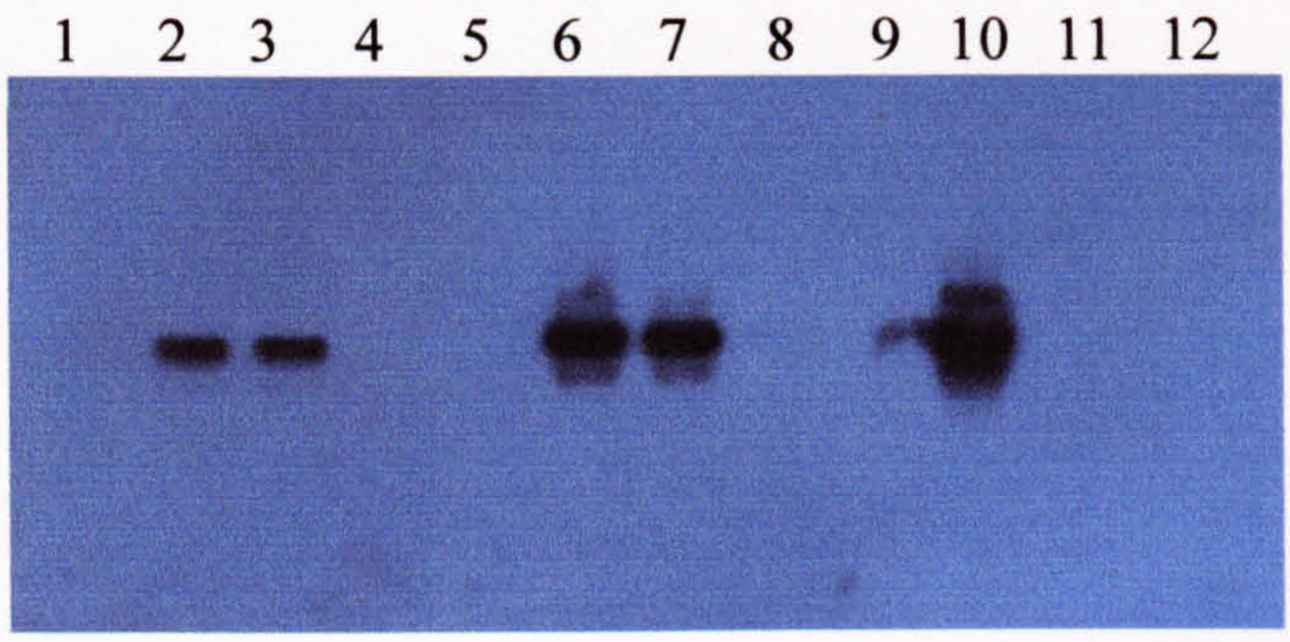
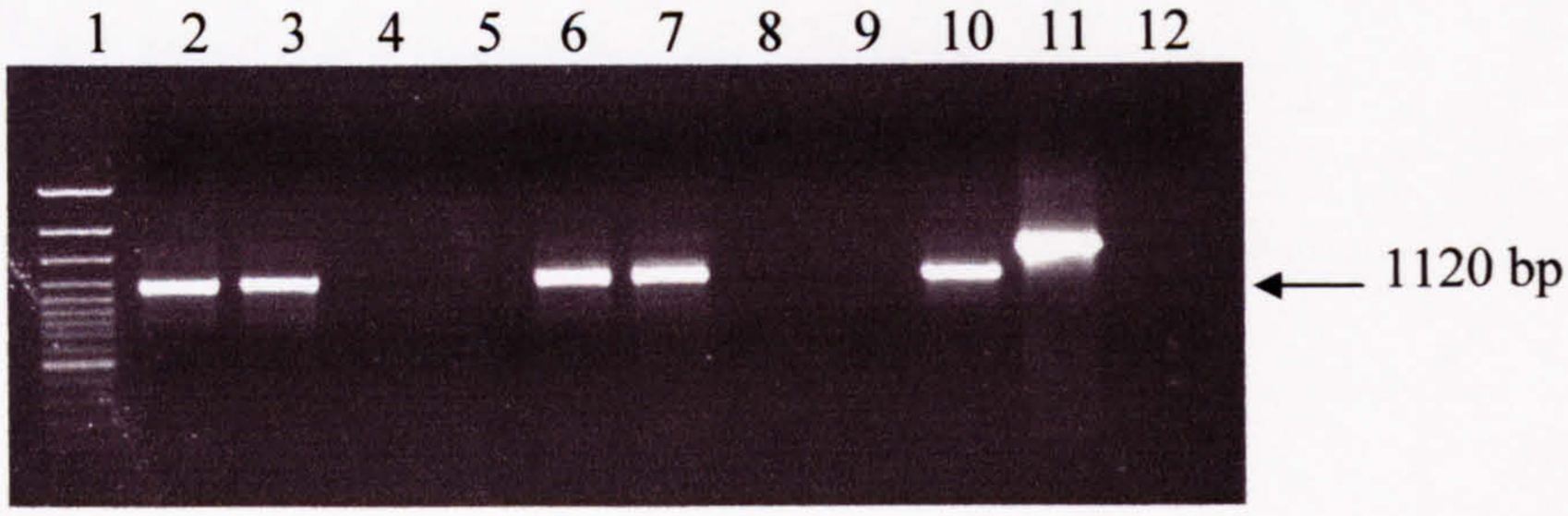


Figure 3.9. A - 'Direct' and 'nested' PCR amplification of SRB 16S rDNA extracted from raw and digesting sludge using primers DFM140 & DFM842 (Group 1);
B – Southern blot hybridised against probe DFM228 (Group 1).

Lane 1 – GeneRuler 100bp DNA Ladder Plus; Lane 2 & 3 – raw sludge 'nested';
Lane 4 & 5 – raw sludge 'direct'; Lane 6 & 7 – digesting sludge 'nested';
Lane 8 & 9 – digesting sludge 'direct'; Lane 10 – *Dfm. nigrificans*;
Lane 11 – *D. desulfuricans* (1 bp mismatch in probe region);
Lane 12 – PCR blank control.

A



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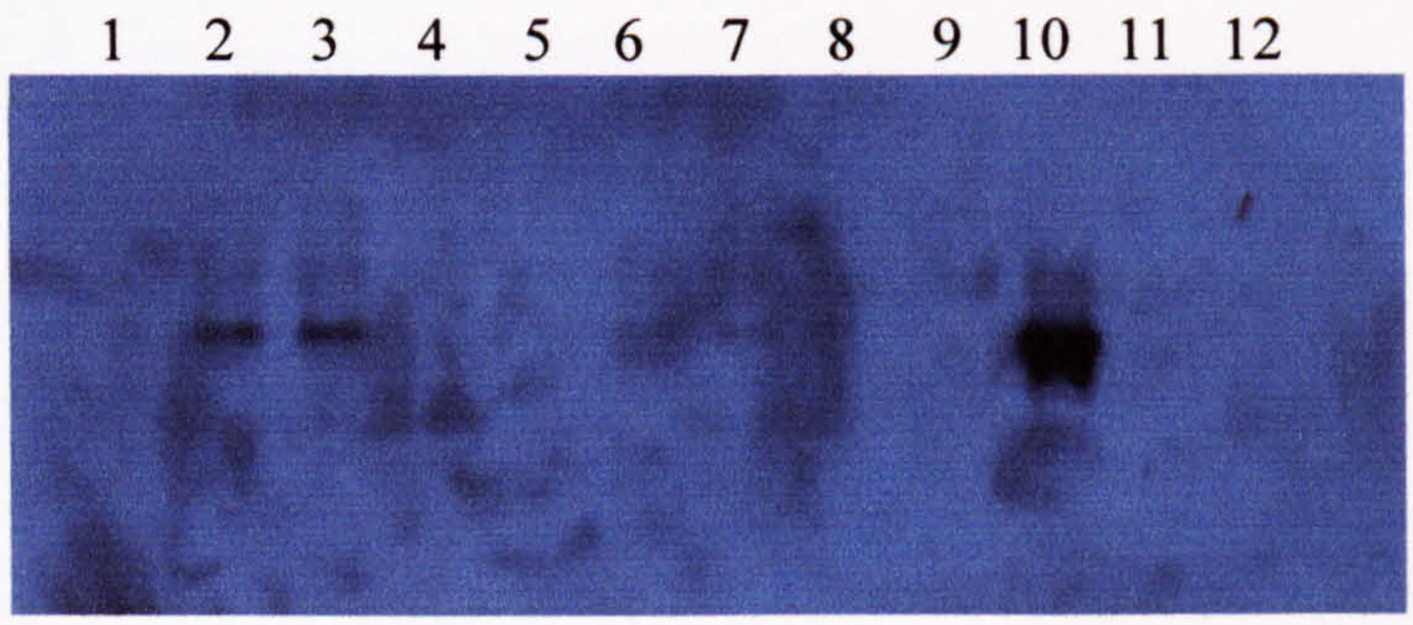
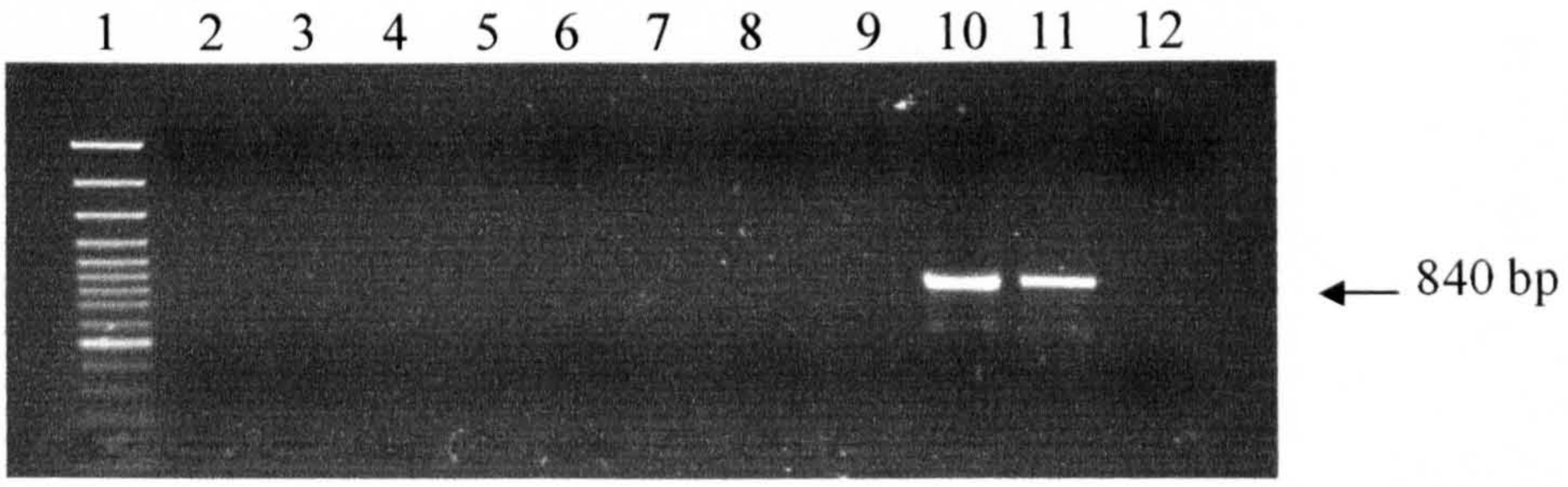


Figure 3.10. A 'Direct' and 'nested' PCR amplification of SRB 16S rDNA extracted from raw and digesting sludge using primers DBB121 & DBB1237 (Group 2);
B – Southern blot hybridised against probe DBB660 (Group 2).

Lane 1 – GeneRuler 100bp DNA Ladder Plus; Lane 2 & 3 – raw sludge 'nested';
Lane 4 & 5 – raw sludge 'direct'; Lane 6 & 7 – digesting sludge 'nested';
Lane 8 & 9 – digesting sludge 'direct'; Lane 10 – *Dbb. propionicus*;
Lane 11 – *C. aurantibutyricum* (2 bp mismatch in probe region);
Lane 12 – PCR blank control.

A



B

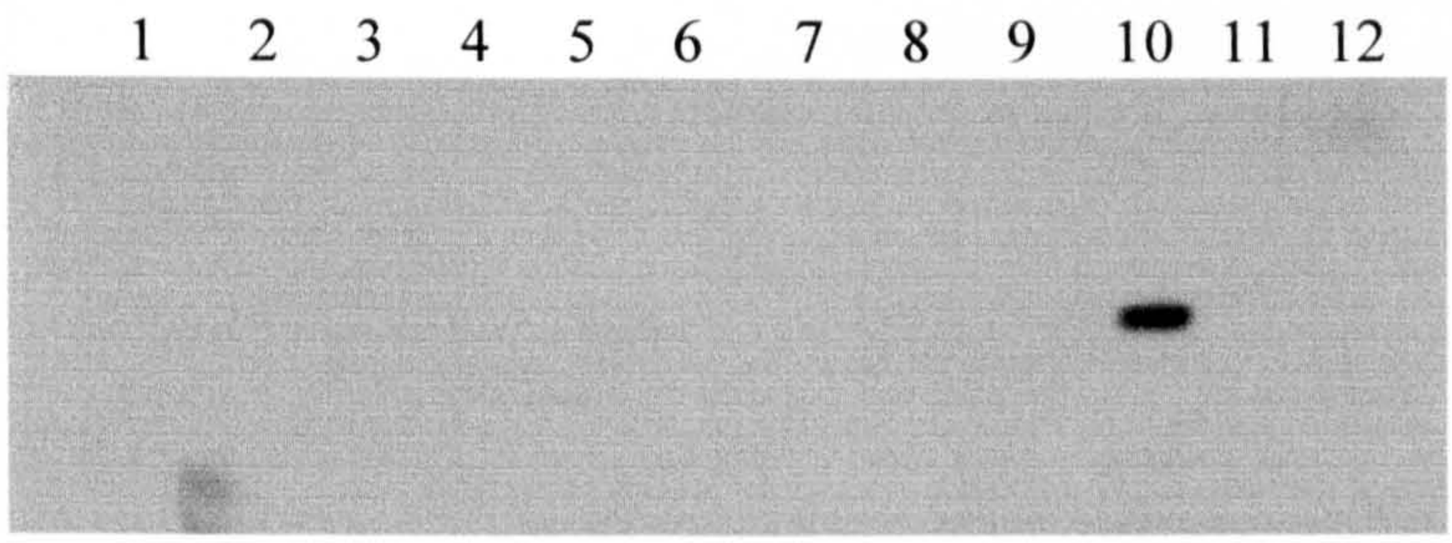
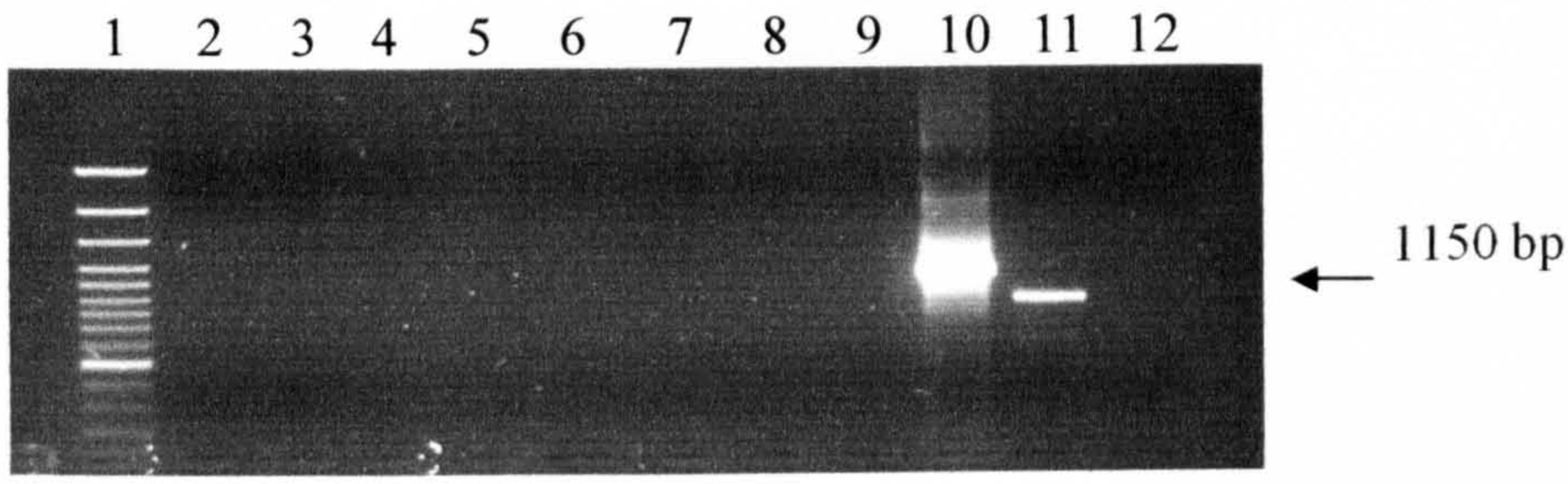


Figure 3.11. A 'Direct' and 'nested' PCR amplification of SRB 16S rDNA extracted from raw and digesting sludge using primers DBM169 & DBM1006 (Group 3);
B – Southern blot hybridised against probe DBM221 (Group 3).

Lane 1 – GeneRuler 100bp DNA Ladder Plus; Lane 2 & 3 – raw sludge 'nested';
Lane 4 & 5 – raw sludge 'direct'; Lane 6 & 7 – digesting sludge 'nested';
Lane 8 & 9 – digesting sludge 'direct'; Lane 10 – *Dbm. autotrophicum*;
Lane 11 – *D. variabilis* (3 bp mismatch in probe region);
Lane 12 – PCR blank control.

A



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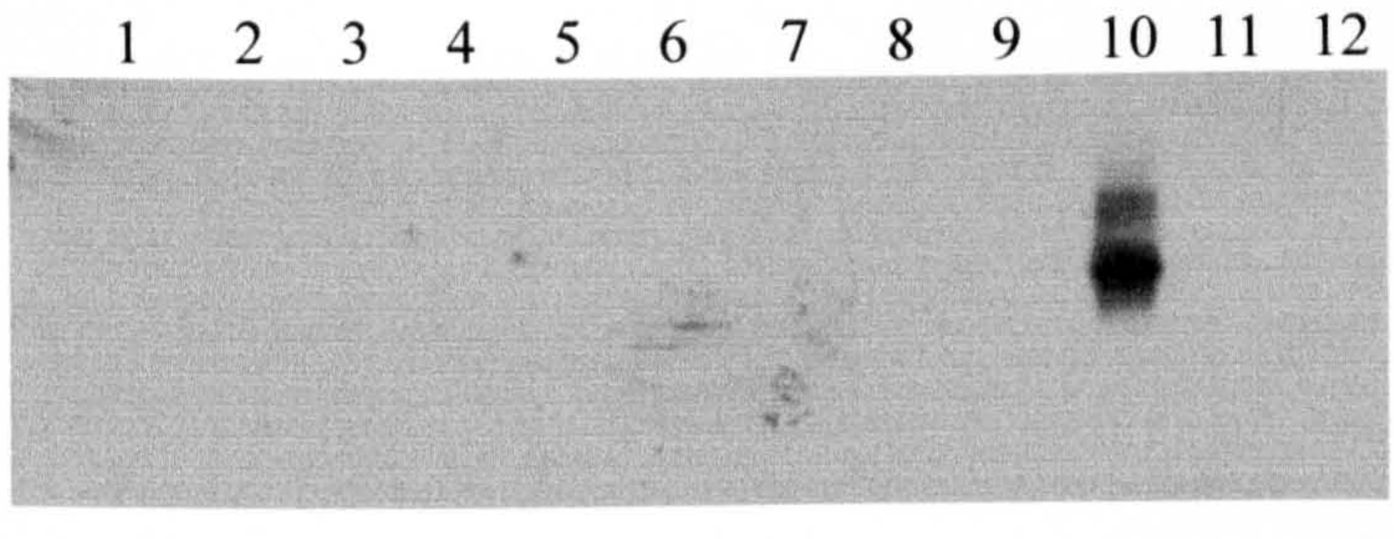


Figure 3.12.A 'Direct' and 'nested' PCR amplification of SRB 16S rDNA extracted from raw and digesting sludge using primers DSB127 & DSB1237 (Group 4);
B – Southern blot hybridised against probe DSB623 (Group 4).

Lane 1 – GeneRuler 100bp DNA Ladder Plus; Lane 2 & 3 – raw sludge 'nested';
Lane 4 & 5 – raw sludge 'direct'; Lane 6 & 7 – digesting sludge 'nested';
Lane 8 & 9 – digesting sludge 'direct'; Lane 10 – *Dsb. curvatus*;
Lane 11 – *D. vacuolatum* (2 bp mismatch in probe region);
Lane 12 – PCR blank control.

***Desulfococcus-Desulfonema-Desulforsarcina* (DCC-DNM-DSS Group 5):** Although no PCR products were visible of the expected size (0.86 kb) on the agarose gel, extremely faint signals could be discerned for both raw and digesting sludge types upon hybridisation against probe DCC868 (Fig. 3.13.A/B; lanes 4, 5, 8, 9). These signals, however faint, were recorded as a positive result since the 1bp negative control *Z. mobilis* gave no hybridisation signal under the conditions used.

***Desulfovibrio-Desulfomicrobium* (DSV-DMB Group 6):** Amplification products of the expected size (0.61 kb) were obtained from raw sludge only (Fig. 3.14.A; lanes 4 & 5). A discrete band was generated and gave a positive signal upon hybridisation against probe DSV687 (Fig. 3.14.B; lanes 4 & 5). However, no amplification products were obtained for digesting sludge and this was confirmed by a negative result from subsequent probing.

3.4.2. 'Nested' PCR amplification of SRB groups from raw and digesting sludge

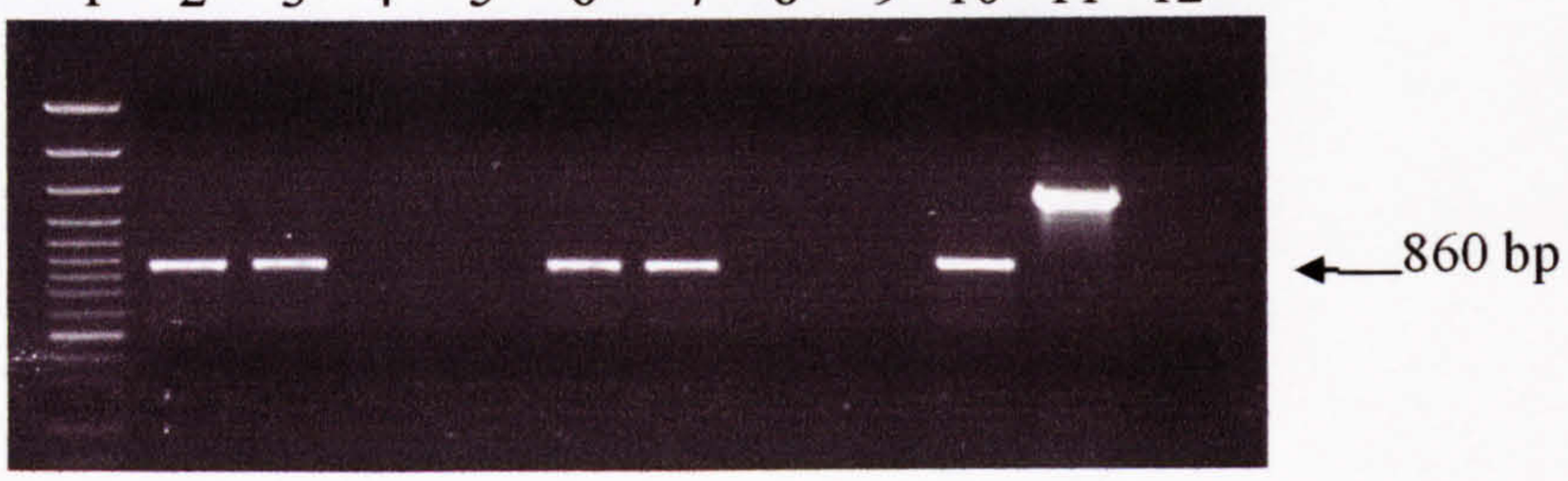
The eubacterial 16S rDNA PCR products obtained from both the raw and digesting sludge samples (section 3.2.2.) were appropriately diluted and used as DNA templates for 'nested' PCR amplification. PCR amplification of SRB 16S rDNA with primers specific for all six main subgroups (Table 2.2) were analysed on an agarose gel as before.

***Desulfotomaculum* (DFM Group 1):** amplification products of the expected size (0.7 kb) were obtained from both raw and digesting sludge and confirmed positive upon hybridisation against probe DFM228 (Fig. 3.9.A & B; lanes 2, 3, 6, 7).

***Desulfobulbus* (DBB Group 2):** amplification products of the expected size (1.12 kb) were obtained from both raw and digesting sludge and confirmed positive upon hybridisation against probe DBB660 (Fig. 3.10.A & B; lanes 2, 3, 6, 7). A much weaker hybridisation signal was obtained for the product from digesting sludge in comparison to raw sludge, and this was recorded as a positive result since the 2bp mismatch control *C. aurantibutyricum* gave no hybridisation signal under the conditions used.

***Desulfobacterium* (DBM Group 3):** amplification products were not obtained from any of the two sludge types using the 'nested' PCR approach followed by hybridisation against probe DBM221 (Fig. 3.11.A & B; lanes 2, 3, 6, 7).

A



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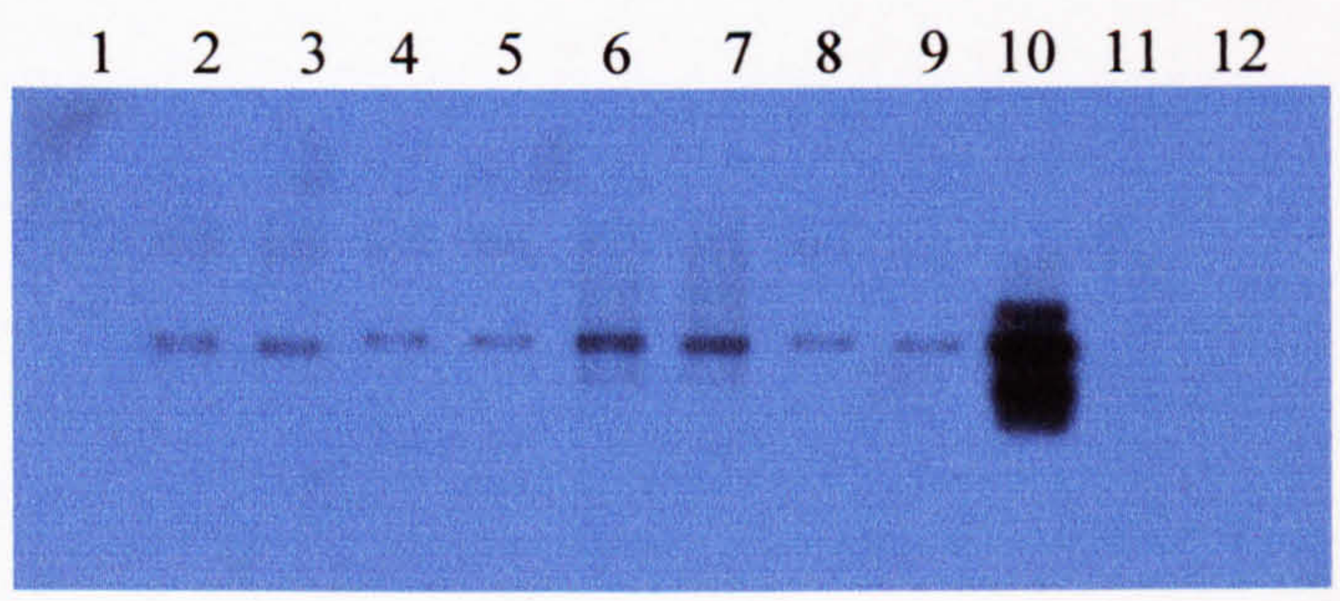
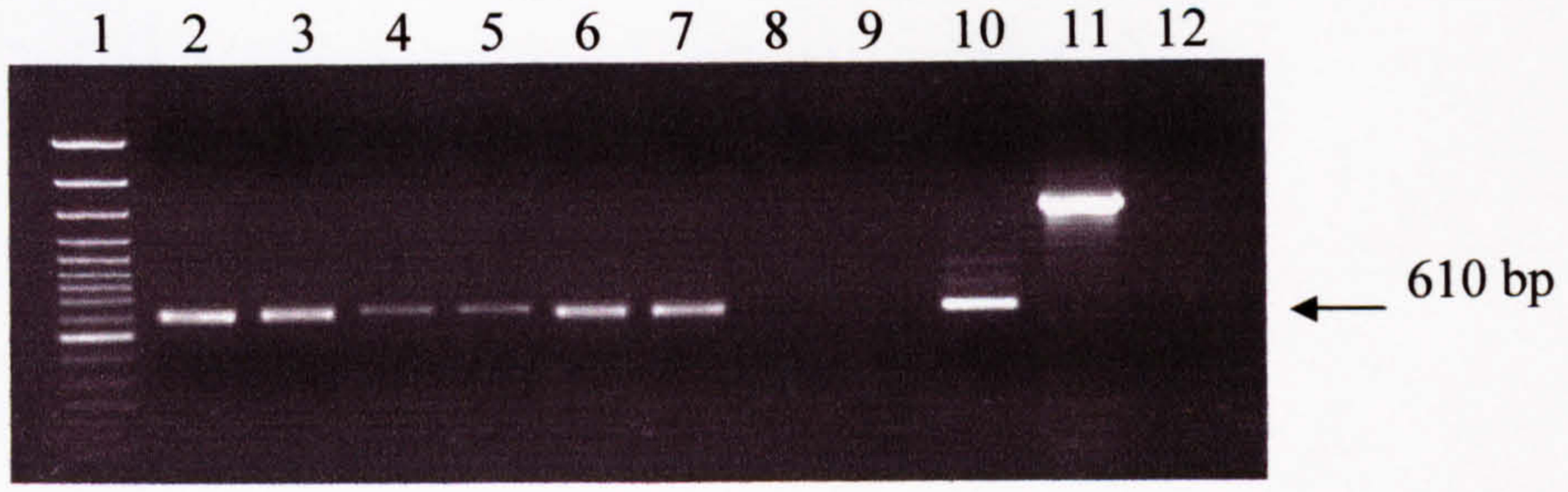


Figure 3.13.A -'Direct' and 'nested' PCR amplification of SRB 16S rDNA extracted from raw and digesting sludge using primers DCC305 & DCC1165 (Group 5);
B – Southern blot hybridised against probe DCC1165 (Group 5).

Lane 1 – GeneRuler 100bp DNA Ladder Plus; Lane 2 & 3 – raw sludge 'nested';
Lane 4 & 5 – raw sludge 'direct'; Lane 6 & 7 – digesting sludge 'nested';
Lane 8 & 9 – digesting sludge 'direct'; Lane 10 – *Dss. variabilis*;
Lane 11 – *Z. mobilis* (1 bp mismatch in probe region);
Lane 12 – PCR blank control.

A



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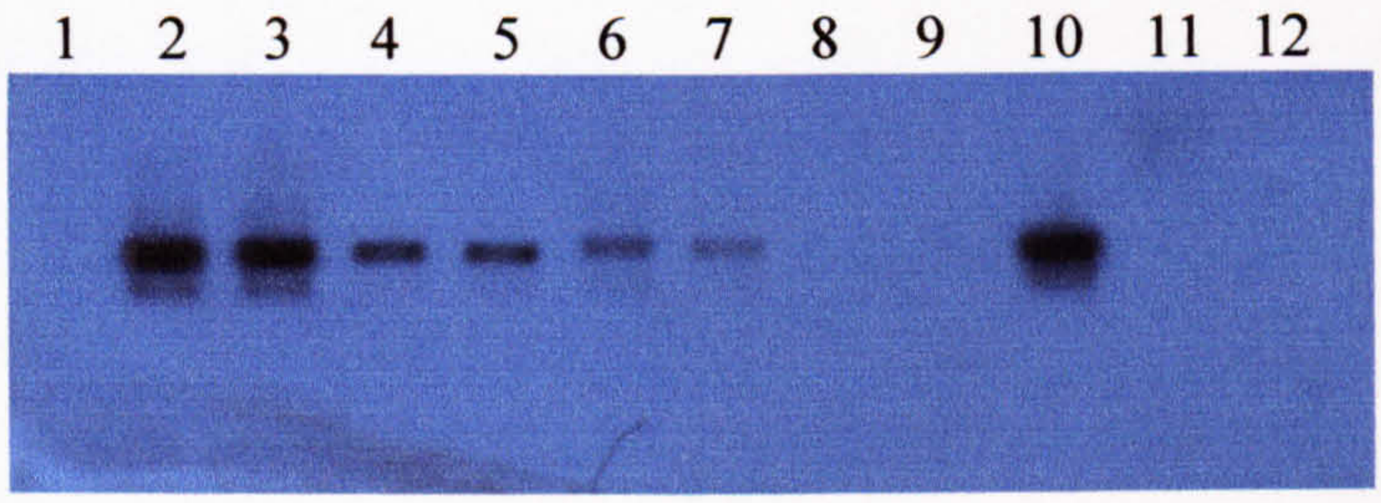


Figure 3.14.A -'Direct' and 'nested' PCR amplification of SRB 16S rDNA extracted from raw and digesting sludge using primers DSV230 & DSV838 (Group 6);
B – Southern blot hybridised against probe DSV687 (Group 6).

Lane 1 – GeneRuler 100bp DNA Ladder Plus; Lane 2 & 3 – raw sludge 'nested';
Lane 4 & 5 – raw sludge 'direct'; Lane 6 & 7 – digesting sludge 'nested';
Lane 8 & 9 – digesting sludge 'direct'; Lane 10 – *Dsv. desulfuricans*;
Lane 11 – *P. carbinolicus* (1 bp mismatch in probe region);
Lane 12 – PCR blank control.

***Desulfobacter* (DSB Group 4)**: amplification products were not obtained from any of the two sludge types using the 'nested' PCR approach followed by hybridisation against probe DSB623 (Fig. 3.12.A & B; lanes 2, 3, 6, 7).

***Desulfococcus-Desulfonema-Desulforsarcina* (DCC-DNM-DSS Group 5)**: amplification products of the expected size (0.86 kb) were obtained from both raw and digesting sludge and confirmed positive upon hybridisation against probe DCC868 (Fig. 3.13.A & B; lanes 2, 3, 6, 7).

***Desulfovibrio-Desulfomicrobium* (DSV-DMB Group 6)**: amplification products of the expected size (0.61 kb) were obtained from both raw and digesting sludge and shown to be positive upon hybridisation against probe DSV687 (Fig. 3.14. A & B; lanes 2, 3, 6, 7). A weaker hybridisation signal was obtained for digesting sludge in comparison to raw sludge despite similar intensities of the PCR products.

A summary of results for the 'direct' and 'nested' PCR amplification of 16S rDNA extracted from raw and digesting sludge using SRB group-specific primers and hybridisation against group-specific oligonucleotide probes is presented in Table 2.6

	Direct PCR		Nested PCR	
	Raw sludge	Digesting sludge	Raw sludge	Digesting sludge
SRB Group 1	-	-	+	+
SRB Group 2	-	-	+	+
SRB Group 3	-	-	-	-
SRB Group 4	-	-	-	-
SRB Group 5	+	+	+	+
SRB Group 6	+	-	+	+

Table 2.6. Summary of results for SRB group analysis.

+ : indicates a positive signal upon hybridisation against group-specific probe
- : indicates a negative signal upon hybridisation against group-specific probe

SRB groups 3 and 4 could not be detected in raw and digesting sludge. However, the other four groups (Groups 1, 2, 5 & 6) were present in both raw and digesting sludge. The detection of Group 5 SRB by 'direct' PCR suggests that they could predominate amongst the SRB. The 'direct' PCR results also suggest that Group 6 SRB decline during digestion.

3.5. Archaeal 16S rDNA amplification

The primers 5Af and 1404Ar (Munson *et al.*, 1997) were used to amplify a 1.4 kb region of 16S rDNA specific for *Archaea*. PCR reactions were carried out in quadruplets and products were obtained from both raw and digesting sludge samples. In comparison to digesting sludge, for the same amount of DNA template used, lower levels of archaeal DNA could be amplified from raw sludge (Fig. 3.15) as suggested by the visual intensity of the PCR product yield on the gel.

These products were then used to detect methanogenic groups in the two sludge samples using oligonucleotide hybridisation probes as devised by Raskin *et al.* (1994a). The specificity of these probes had previously been tested only against rRNA (Raskin *et al.*, 1994a), and it was therefore necessary to evaluate the probes in hybridisations against DNA. Equal concentrations of 16S rDNA amplified from sludge DNA and genomic DNA extracts of control methanogen strains were electrophoresed on an agarose gel (Fig. 3.16) and then transferred to a positively charged nylon membrane by Southern blotting. DNA fixed to the membrane was then hybridised against a number of oligonucleotide probes under the appropriate hybridisation conditions (Table 2.4) to target methanogen groups belonging to the orders *Methanobacteriales*, *Methanococcales*, and *Methanomicrobiales*. Probing was carried out as described in section 2.9 and after each hybridisation, the membrane was stripped of its probe and re-used for hybridisation with another probe as described in section 2.11. All probes used in this study, when used at the optimum temperature previously determined with RNA's by Raskin and co-workers (1994a), yielded strong signals with target DNA and undetectable signals with non-target DNA.

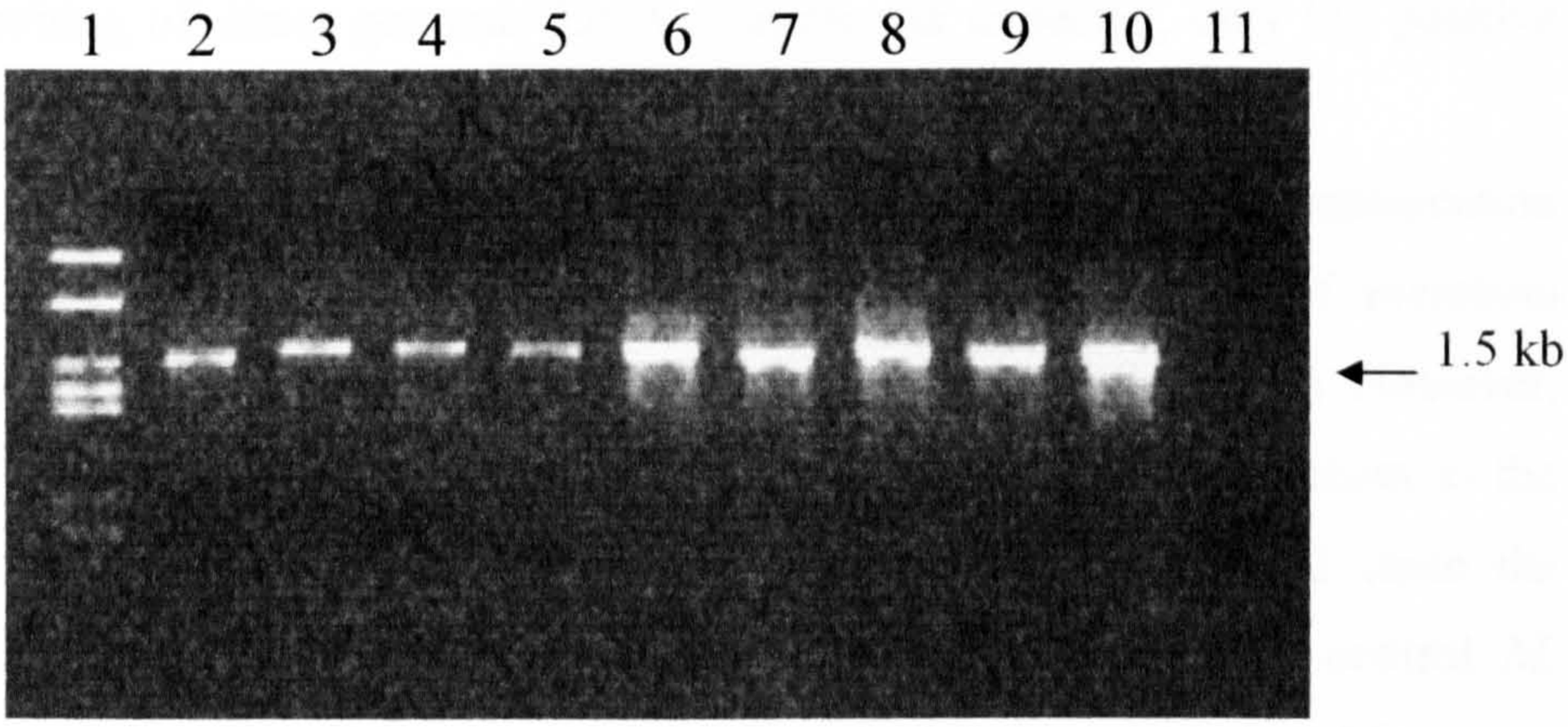


Figure 3.15. PCR amplification of archaeal 16S rDNA from raw and digesting sludge DNA using primers 5Af and 1404Ar (Munson *et al*., 1997).

Lane 1 –Marker 21, pBR322 DNA/*Alw*441/*Mva*1 (MBI Fermentas);
Lane 2, 3, 4 & 5 – raw sludge;
Lane 6, 7, 8 & 9 – digesting sludge;
Lane 10 – *Methanolobus tindarius*;
Lane 11 – PCR blank

Results of Southern hybridisation against each methanogenic probe are as follows:

***Methanobacteriales* (MB1174)**: hybridisation of archaeal 16S rDNA amplification products to the internal probe MB1174 confirmed the detection of members belonging to the order *Methanobacteriales* (except *Methanothermaceae*) in both raw and digesting sludge (Fig. 3.17). In addition, no hybridisation signals were obtained from the probing of direct genomic DNA extracts. As expected, only the positive control *M. bryantii* (lane 2) gave a positive signal with this probe.

***Methanococcales* (MC1109)**: hybridisation of archaeal 16S rDNA amplification products to the internal probe MC1109 confirmed the detection of members belonging to the order *Methanococcales* in both sludge types (Fig. 3.18). However, very weak hybridisation signals were obtained (lanes 7 & 8) in comparison to the positive control (lane 1). Despite this, a positive result was recorded since the negative controls showed no signal. As expected, only the positive control *M. thermolithotrophicus* (lane 1) gave a positive signal with this probe. In addition, no hybridisation signals were obtained from the probing of direct genomic DNA extracts.

***Methanomicrobiales* (MG1200)**: hybridisation of archaeal 16S rDNA amplification products to the internal probe MG1200 confirmed the detection of members belonging to the order *Methanomicrobiales* in both sludge types (Fig. 3.19). In contrast to raw sludge, a much stronger hybridisation signal was obtained for digesting sludge. However, genomic DNA extracts gave no signal upon hybridisation with probe MC1109. As expected, positive control *M. organophilium* (lane 3) showed strong hybridisation with this probe. Weaker band signals were observed in control lanes 1 and 2, probably due to non-specific binding of the probe, and therefore were viewed as negatives.

***Methanosarcinaceae* (MS1414)**: hybridisation of archaeal 16S rDNA amplification products against probe MS1414 confirmed the presence of members within this order belonging to the genera *Methanosarcina*, *Methanococcoides*, *Methanolobus*, and *Methanohalophilus*, in both sludge types (Fig. 3.20). The signal obtained from digesting sludge was weaker in comparison to the hybridisation signal obtained from raw sludge. However, genomic DNA extracts gave no signal upon hybridisation with probe MS1414. As expected, only the positive control *M. tindarius* (lane 4)

gave a positive signal with this probe. The extra band observed above this is the result of this control DNA not cleared from the gel well.

***Methanosarcina* (MS821)**: archaeal 16S rDNA amplification products were hybridised to the genus-specific probe MS821 in an attempt to demonstrate the presence of *Methanosarcina* in both sludge types (Fig. 3.21). Both sludge samples confirmed their detection upon hybridisation with probe MS821. Similar detection signals were observed to that of probe MS1414 with the signal obtained from digesting sludge being weaker in comparison to the hybridisation signal obtained from raw sludge. Again, genomic DNA extracts gave no signal upon hybridisation with probe MS821. As expected, only the positive control *M. mazei* (lane 5) gave a positive signal with this probe.

***Methanosaeta* (MX825)**: archaeal 16S rDNA amplification products were hybridised to the genus-specific probe MX825 in an attempt to demonstrate the presence of *Methanosaeta* in both sludge types. Both sludge samples confirmed their detection upon hybridisation with probe MX825 (Fig. 3.22). The signals obtained here differed in comparison to hybridisation signals obtained previously with probes MS1414 and MS821, which target members within the same order. Here, *Methanosaeta* were found to be dominant in digesting sludge as suggested by a much stronger hybridisation signal observed from the two samples. Again, genomic DNA extracts gave no signal upon hybridisation with probe MX825. As expected, positive control *M. concilii* (lane 6) showed strong hybridisation with this probe. Weaker band signals were observed in control lanes 1, 3, 4, and 5, probably due to non-specific binding of the probe, and therefore were viewed as negatives because there is little or now homology between the probe and these targets.

In all cases, methanogen probes applied directly to sludge extracts did not give positive hybridisation signals. These were only obtained with 16S rDNA amplified from sludge DNA.

Hybridisation responses of methanogen probes to archaebacterial DNA amplified from raw and digesting sludge along with genomic DNA extracts, target and nontarget methanogens.

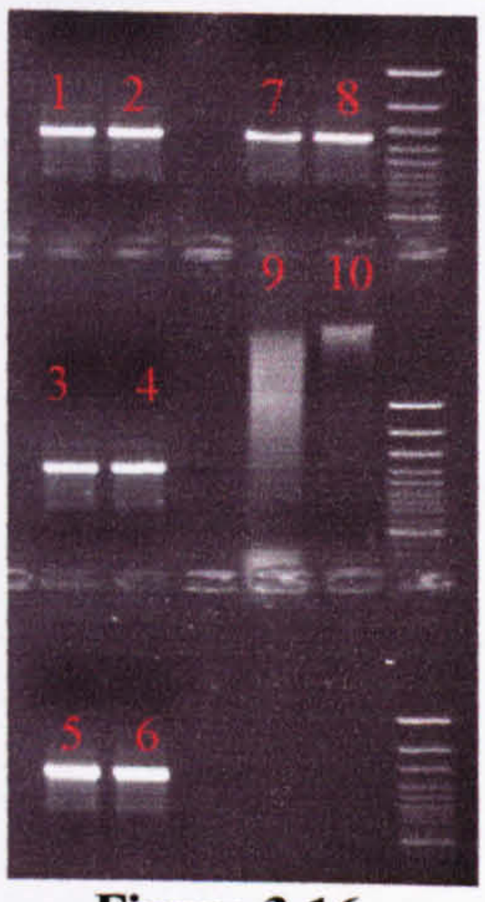


Figure 3.16

MB1174

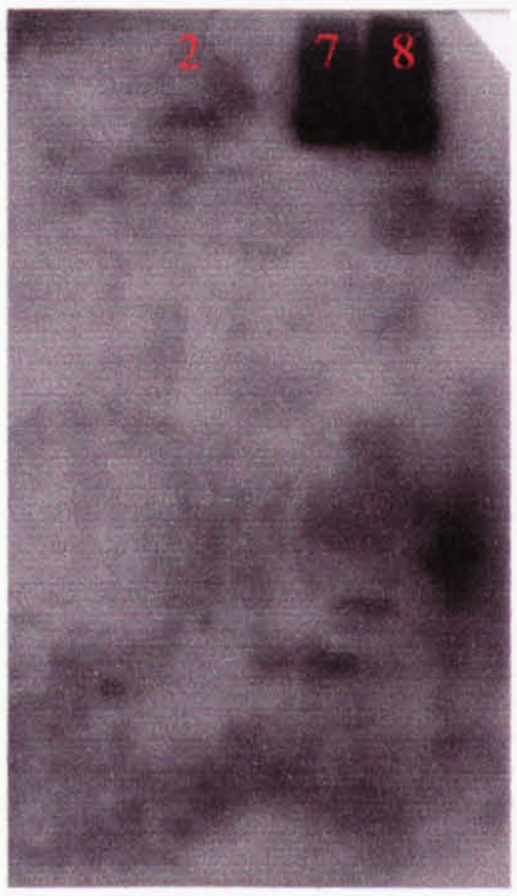


Figure 3.17

MC1109

Figure 3.18

MG1200

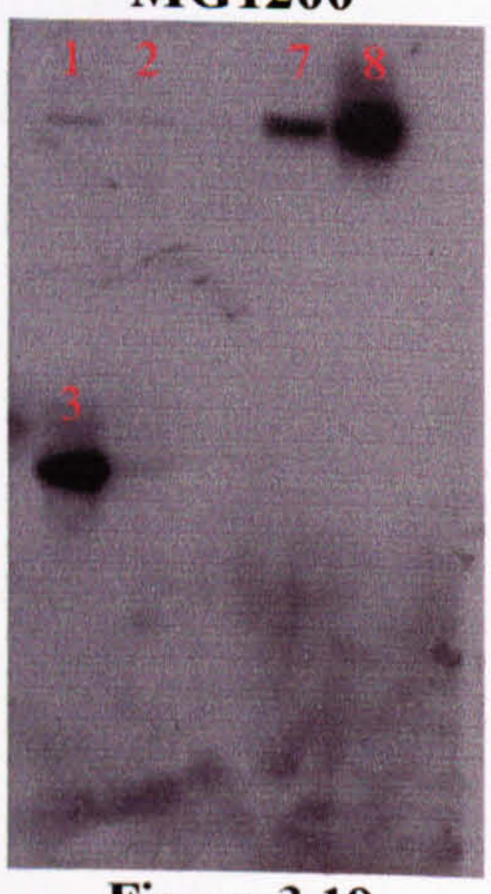


Figure 3.19

MS1414

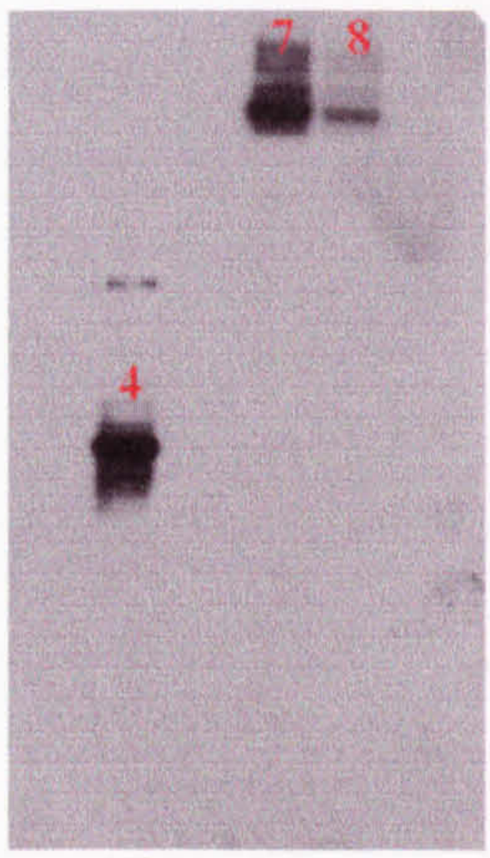


Figure 3.20

MS821

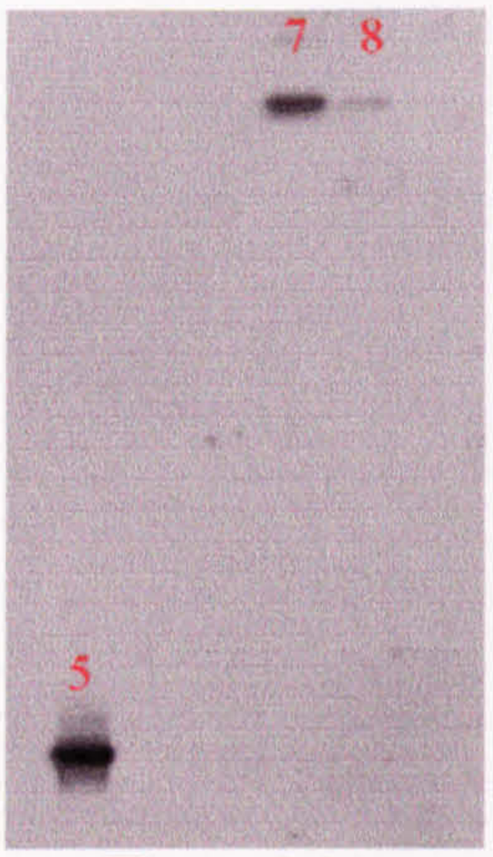


Figure 3.21

MX825

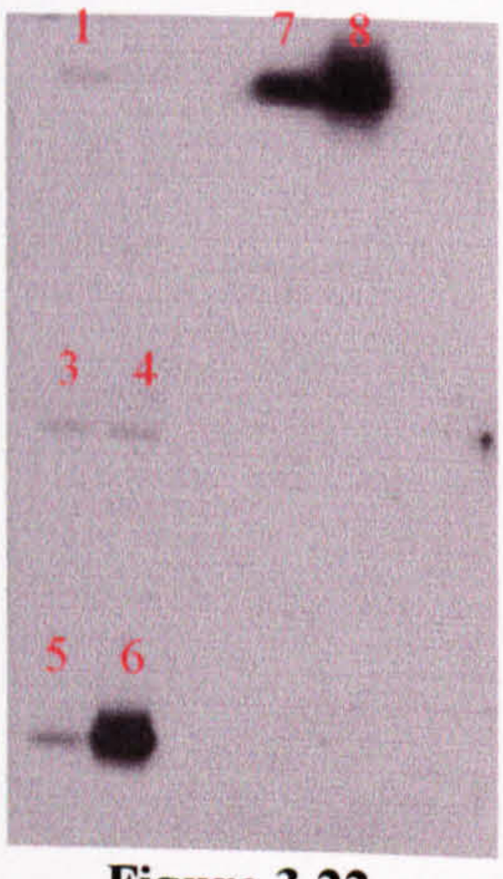


Figure 3.22

Lanes in Figure 3.16

1 – *Methanococcus thermolithotrophicus* (MC1109)
2 – *Methanobacterium bryant* (MB1174)
3 – *Methanogenium organophilium* (MG1200)
4 – *Methanolobus tindarius* (MS1414)
5 – *Methanosarcina mazei* (MS821)
6 – *Metanosaeta concilii* (MX825)
7 – raw sludge (archaeal 16S rDNA PCR product)
8 – digesting sludge (archaeal 16S rDNA PCR product)
9 – raw sludge (DNA extract)
10 – digesting sludge (DNA extract)

4. Discussion

The limitations associated with using culture-dependent methods for studying the diversity of complex microbial communities are well known. As a consequence, the application of small subunit rRNA molecular based methods to characterise the microflora residing in such environments has now become common practice in microbial ecology. With the advent of molecular techniques, it is only over the last decade that increasing attention has begun to be focussed towards wastewater treatment systems in particular aerobic (activated sludge) and anaerobic (anaerobic digesters) sludge processes. Although there is an increasing body of information now available in this area, studies published on anaerobic processes are minimal in comparison to aerobic processes and only fragmentary information is available about the bacterial groups involved.

Anaerobic digesters are engineered environments whose function relies on the interaction of diverse microbial groups for the treatment of sludge. The complete anaerobic degradation of relatively complex organic molecules to carbon dioxide and methane requires the concerted effort of many trophic groups of bacteria as addressed in Chapter 1 (Fig.1.1). Recently, a comprehensive study on the microbial diversity of an anaerobic digester was carried out by Godon *et al.*, (1997). They isolated DNA from a fluidised bed reactor, amplified a portion of the 16S rDNA with conserved primers, sequenced selected clones from libraries of amplified 16S rDNA fragments and compared the sequences with those in the public databases. They were able to describe the phylogeny of the microbial community structure of *Archaea*, *Procarya*, and *Eucarya* populations. In contrast to the approach by Godon *et al.*, (1997), this study makes use of molecular tools to target the functional sub-groups responsible for hydrolysis, sulphate-reduction and methanogenesis in sludge from a mesophilic anaerobic digester treating primarily domestic waste at Exeter, to serve as a model system to investigate the bacterial community present. In addition, this study was a comparative analysis of the population diversity regarding these functional groups between raw sludge, obtained from the settling tanks serving as the feed for anaerobic digesters, and sludge undergoing anaerobic digestion in the digester.

The availability of primer and probe sequences to detect groups of clostridia, sulphate-reducing bacteria and methanogens has provided new tools for studying these organisms in anaerobic environments (Daly *et al.*, 2000; Raskin *et al.*, 1994a; Raskin *et al.*, 1994b; Van Dyke and McCarthy, 2002). These molecular tools have

been developed on the basis of published nucleotide sequences and have shown to be reasonably specific for the groups they detect under the appropriate reaction conditions. For clostridia, the primers are highly specific for the clusters they target. General archaeal primers are used to target methanogen populations, and the identification of methanogen groups achieved by group-specific probing. The SRB groups are targeted by degenerate primers that are always supported by oligonucleotide hybridisation to confirm identity. The validity of these tools has been confirmed upon application against pure strains and 16S rRNA genes extracted from the environment (Daly *et al.*, 2000; Raskin *et al.*, 1994a; Raskin *et al.*, 1994b; Van Dyke and McCarthy, 2002). It is for this reason that without further verification they were applied directly for the analysis of these bacterial populations in this study. The DNA extraction protocol described here was applied successfully for the isolation of total nucleic acids from raw and digesting sludge samples. Agarose gel electrophoresis of the DNA isolated demonstrated the presence of intact genomic DNA without obvious signs of shearing (Fig. 3.3). The DNA was proven to be of amplifiable quality and was used to target group specific DNA segments from such a diverse group of microorganisms present in these sludge samples.

Clostridium. Since the dry-solid portion of most municipal sludge contains approximately 25 to 36% cellulose, 18 to 50% carbohydrate, 19% protein and 5% pectin (Hunter and Heckelekian, 1965), digestion of these components is critical in the reduction of the total contents and for the provision of carbon and energy to the other microorganisms. Bacteria belonging to the genera *Clostridium* and *Eubacterium* are involved in the degradation of cellulose and other polymeric materials and have often been isolated from sewage sludge and anaerobic digesters (Murray *et al.*, 1984; Palop *et al.*, 1989; Patel *et al.*, 1980; Sleat *et al.*, 1985; Yang *et al.* 1990; Jarvis *et al.* 1999).

In this study, four PCR primer pairs (Van Dyke and McCarthy, 2002) were used to target the members of four clusters within the *Clostridium* phylogeny, as established by Collins *et al* (1994). All known cellulolytic clostridia can be found in the four clusters, although only one (cluster III) contains strains that are all cellulolytic. These primer pairs have been authenticated for application to the analysis of environmental DNA preparations, but have only been used to analyse landfill (Van Dyke and McCarthy, 2002). This is the first time that the four sets of cluster-specific clostridial

16S rDNA primers have been applied to anaerobic digester DNA, and evidence for the presence of members of all four cluster groups was obtained. Their detection was only possible using a nested primer approach employing initially oligonucleotides pA and pH' (Edwards *et al.*, 1989) followed by a second round of amplification with group specific primers. The nested approach indicated that these groups of bacteria might be present in low numbers undetectable by a one-step PCR. Due to the severe lack of information regarding the concentration of clostridia in municipal sludge it is difficult to say whether the requirement for nested PCR is a reflection of population size or a feature of PCR efficiency. Van Dyke and McCarthy (2002) were able to detect clostridial 16S rDNA by direct PCR from landfill leachate, but Molyneux (personal communication) could only detect these clostridia by 'nested' PCR of soils to which anaerobically digested sludge had been applied.

However, most-probable number determination of cellulolytic bacteria in swine manure digesters showed that the cellulolytic and hemicellulolytic bacteria comprised less than 0.1% of the total digester population (Iannotti *et al.*, 1982). It has also been shown that culture dependent studies carried out by Chen (1987) and Ceki *et al.*, (1978) have indicated that sporeforming bacteria constitute less than 10% of the total bacterial population in anaerobic municipal sludges fermented at mesophilic temperatures. Since the clostridial groups targeted here are spore-formers, their detection in this pool of sporeforming bacteria is supportive with the findings of this study and justifies the application of a nested PCR for their detection. This could suggest that these clostridia in general are a minor component of the digester population, and that other clostridial clusters possibly predominate.

The PCR detection of all four clusters in both raw and digesting sludge shows the diversity of bacteria involved within this trophic group but demonstrates no observable selection imposed by the digester environment. However, this will be better assessed by investigating the diversity within each cluster through community profiling using the TGGE technique (see Chapter 4).

Since the hydrolysis and liquefaction of complex organic material drives the entire digestion process, their detection here at low levels could suggest that clostridia may not be the predominant cellulolytic bacteria in digester environments. In addition to bacterial activity, other microorganisms such as anaerobic fungi, and to a lesser extent protozoa, may contribute to the degradation of cellulose in digester environments. These organisms have been found in studies of rumen populations

(Erikson *et al.*, 1990; Leschine, 1995), but their presence has yet to be demonstrated in anaerobic digesters. It is also possible that gram-negative anaerobes belonging to the *Bacteriodes* and *Fibrobacter* are involved in cellulose hydrolysis in the digester since these are among the primary cellulose degrading bacteria in the rumen (Tajima *et al.*, 1999; Lin *et al.*, 1994; Lin and Stahl, 1995).

Sulphate-reducing bacteria. The comparison of direct and nested amplification provides some indication of the relative abundance of the different SRB subgroups. The results of the nested amplification protocol in particular, demonstrate that a diverse SRB community resides amongst the anaerobic digester microflora. The amplification primers vary in their specificity and degree of degeneracy, but the application of oligonucleotide probes to amplified products removes any uncertainty. When these primers are applied to complex environmental samples, it is possible that non-target species, as yet uncharacterised, could be amplified. It is for this reason that only PCR products that gave a positive signal upon hybridisation against the appropriate group-specific probe were recorded as positive. Thus it is the primer-probe combinations that are highly specific. In studies of landfill samples (Daly, 2000), follow up sequencing of DNA clones obtained in this way always confirmed its identity as SRB of the predicted subgroup and the same level of confidence is expected for SRB analysis in raw and digesting sludge samples. Thus, although it is possible that amplification products obtained with these primers may not be SRB, the previous application to landfill sites recorded only SRB DNA.

The results obtained using the 'direct' PCR amplification approach suggest that there would appear to be two subgroups dominant in raw sludge – *Desulfococcus-Desulfonema-Desulforsarcina* (Group 5) and *Desulfovibrio-Desulfomicrobium* (Group 6) – and only one subgroup dominant in digesting sludge, Group 5. No other groups were detected using this approach. The differences observed here suggest that selective pressure is applied to the digester SRB population. In comparison, the *Desulfococcus* group (Group 5) was also one of the groups detected in landfill sites by 'direct' PCR (Daly *et al.*, 2000). It is presumed that SRB groups detected by 'direct' PCR are present in higher numbers than those detected by 'nested' PCR, therefore the direct detection of Group 5 SRB in both digester sludge and landfill sites may suggest the importance of this group to waste stabilisation processes in anaerobic environments.

However, 'nested' PCR amplification revealed the presence of other subgroups not detected by 'direct' PCR. In addition to the subgroups already detected by 'direct' PCR, and confirmed by 'nested' PCR as expected, three other subgroups were detected. Nested amplification revealed the presence of SRB DNA belonging to the groups *Desulfotomaculum* (Group1) and *Desulfobulbus* (Group 2) in both raw and digesting sludge samples. The *Desulfovibrio* group (Group 6) was also detected in digesting sludge by 'nested' PCR, not previously detected by 'direct' PCR. Like the clostridial groups, the detection of greater genus level SRB diversity in sludge samples when a nested PCR is employed suggests that these groups are present in lower numbers than members of the dominant groups detectable by 'direct' PCR. In this case, the dual application of 'direct' and 'nested' can serve as a qualitative measure of the relative predominance of SRB groups in both sludge types. 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 real quantitative significance. It could also be argued that the requirement for a nested PCR to detect members of Group 1, Group 2 and Group 6 (digesting sludge) in these sludge samples could be due to the comparative efficiency of these primers in PCR reactions, rather than reflection of a relatively small population size. However, no significant differences in the performance of the primer pairs was noted when DNA extracted from pure cultures was PCR amplified alongside environmental samples yielding similar amounts of PCR product.

Amplification products belonging to the groups *Desulfobacterium* (Group 3) and *Desulfobacter* (Group 4) were never obtained from any sludge sample using either PCR approach. The absence of *Desulfobacterium*-like members in sludge samples is not surprising since most of the known species of the genus *Desulfobacterium* are associated with the marine environment (Postgate, 1984; Fauque, 1995), and nor were members of this group detected in a study of landfill leachates (Daly *et al.*, 2000). However, their detection has been reported in anaerobic digester sludge at very low levels by rRNA analysis involving total nucleic acid extraction followed by probing (Raskin *et al.* 1995). The same study also detected *Desulfobacter* spp (Group 4), but again at very low levels. This group of sulphate reducers primarily degrade acetate (Widdel, 1988) and their lack of detection here is probably down to successful competition from acetate-utilising methanogens (*Methanosaeta*), which are abundant in these sludge samples (see below). In competition studies with

sulphate limited anaerobic reactors, acetate seemed to be the least favoured substrate for sulphate reduction (Visser *et al.* 1993; Uberoi *et al*, 1995) which in turn supports the absence of the acetate utilising *Desulfobacter* group (Group 4). Generally, properly functioning municipal waste anaerobic digesters can be regarded as sulphate-limited.

The results obtained from the nested PCR suggest that several phylogenetic groups of SRB play a fundamental role in the anaerobic digester as four out of six main subgroups of SRB have been detected in these sludge samples. This correlates with investigations of SRB occurrence and distribution in studies of anaerobic sludge in which most of the main subgroups have been detected by oligonucleotide probing without the need for PCR amplification (Oude Elferink *et al.*, 1998; Santegoeds *et al.*, 1998; Raskin *et al.*, 1996; Santegoeds *et al.*, 1999; Raskin *et al.*, 1995, Okabe *et al.*, 1999). Although sulphate concentrations in the digesting sludge and sulphide production were not measured, the low levels of SRB and the high abundance of methanogens (described below) found here are indications of low sulphate levels present in digester sludge. It is for this reason that direct probing of the DNA extracted from raw and digesting sludge samples without PCR was not attempted. The anaerobic digester is operated to promote methanogenesis and should therefore select against SRB under normal conditions. However, the presence of SRB as demonstrated here, implies their importance to the degradation pathway of waste stabilisation. Recent studies have found large populations of SRB in sulphate-depleted environments (Wu *et al.*, 1992; Mancuso *et al*, 1990). Some sulphate reducers have fermentative or syntrophic capacities, and can grow in the absence of sulphate on compounds such as propionate, lactate, and ethanol. These fermentative and/or syntrophic sulphate reducers have been shown to play an important role in sulphate-depleted reactors (Wu *et al.*, 1992; Raskin *et al.*, 1995) by favouring methanogenesis through providing their substrates: hydrogen, formate and acetate (Jones *et al.*, 1984; Tasaki *et al.*, 1993; Wu *et al.*, 1991). It is important to establish which groups of SRB are present, and which persist and flourish during digestion. Once a pattern has been established, it should be possible to use SRB DNA digesting profiles to support the identification of abnormally functioning digesters, i.e. those digesters where there is potential for malfunction due to SRB out competing methanogens and interfering with overall methanogenesis, hence waste stabilisation.

Methanogens. Numerous studies have demonstrated that strictly anaerobic bacteria form the dominant population in digester environments, and that methanogens account for about 10% of the total microflora (Siebert *et al.*, 1967; Iannotti *et al.*, 1982).

Archaeal 16S rRNA genes were successfully PCR amplified using DNA extracted from both sludge types. In comparison to digesting sludge, lower levels of archaebacterial DNA could be amplified from raw sludge as suggested by the visual intensity of the PCR product yield on the gel (Fig. 3.15). This suggests that the archaeal population increases substantially during digestion, and this is of course in line with the high level of methanogens that characterise anaerobic sludge digesters.

The use of methanogen probes to characterise the microbial makeup of raw and digesting sludge is illustrated in Fig. 3.16 – 3.22. Both raw and digesting sludge demonstrated very similar methanogenic profiles. Hybridisation using methanogen group-specific probes (Raskin *et al.*, 1994a) has demonstrated the presence of *Methanobacteriales* (MB1174), *Methanococcales* (MC1109), *Methanomicrobiales* (MG1200), and relatives of the *Methanosarcinaceae* (MS1414), which include the genera *Methanosarcina* (MS821) and *Methanosaeta* (MX825). However, *Methanococcales* (MC1109) appeared to be present at insignificant levels as suggested by the relatively weak hybridisation signals observed in both raw and digesting sludge samples. This suggests that they play a minor role and are not overly important to the process of waste stabilisation in anaerobic digesters. In general, the *Methanococcales* have been isolated essentially from marine and coastal environments (Garcia *et al.*, 2000; Cytryn *et al.*, 2000; Huber *et al.*, 2002), hence their low level detection here is not surprising.

On the basis of hybridisation signals, the results demonstrate population differences between raw and digesting sludge for the *Methanosarcina* (MS821) and *Methanosaeta* (MX825) groups. *Methanosarcina* spp. can use acetate and other substrates (H_2/CO_2, methanol and methylamines) and were detected at very low levels (weak band intensity) in the anaerobic digester (Fig. 3.21). This implies a decrease in the size of this population from raw to digesting sludge. On the other hand, *Methanosaeta* spp. (use acetate only) (Fig. 3.22) appear to predominate in digesting sludge implying an increase in their population during digestion. The dominance of *Methanosaeta* spp. in anaerobic digester sludge could be due to low acetate concentrations in the reactor as this group are often the dominant acetoclastic

methanogens in sludge. This is because low levels of acetate provide a competitive advantage for *Methanosaeta* spp. due to their higher affinity for acetate compared to *Methanosarcina* species (Jetten *et al.*, 1992). Unfortunately, this cannot be substantiated because acetate levels in the digester were not determined. However, 16S rRNA qualitative studies have previously shown the abundance of *Methanosaeta* spp. in anaerobic digesters containing low acetate concentrations (Oude Elferink *et al.*, 1998; Raskin *et al.*, 1994b; Raskin *et al.*, 1995).

This investigation has described the key microbial groups involved in the various stages of anaerobic digestion. The use of oligonucleotide primers and probes to specifically target groups of cellulolytic clostridia, sulphate-reducers and methanogens, is the first reported work in on profiling the anaerobic digester bacterial community. Although the approach has been largely targeted at providing evidence for the presence and absence of certain subgroups within these functional communities, comparisons of direct and nested PCR and signal intensities in Southern bots has enrolled some observations on relative predominance. The most significant is the predominance of *Methanosaeta* spp. over *Methanosarcinia* spp, both groups of acetate-utilising methanogens, as digestion occurs.

It is important to confirm that archaeal 16S rDNA contains methanogen DNA by Southern blotting. It is tempting to assume that in the methanogenic anaerobic environment, all *Archaea* are methanogens but in recent years analysis of DNA recovered directly from environmental samples is indicating the presence of large populations of uncultured *Archaea* of unknown physiology (Buckley *et al.*, 1998; GroβKopf *et al.*, 1998; Jurgens *et al.*, 2000).

The next step is to properly investigate the diversity of each of these bacterial groups. This is achieved by using techniques such as gradient gel electrophoresis and subsequent sequencing of 16S rDNA fragments for phylogenetic information. This will determine the community structure within sludge before and during digestion and demonstrate how the microbial population structure evolves during digestion.

4. Community structure and diversity of sulphate-reducers, methanogens and clostridial populations in anaerobic sludge determined by TGGE and sequencing analysis of cloned 16S rDNA fragments.

4.1. Introduction

Since their initial application to environmental 16S rDNA by Muyzer *et al.*, (1993), denaturing (DGGE) and later temperature gradient gel electrophoresis (TGGE) have become attractive techniques in molecular microbial ecology. They have been demonstrated to be suitable tools for the analysis of microbial communities because they permit the detection of species and changes in community structure quickly and economically on the basis of gene sequence variation (Muyzer and Smalla, 1998).

The banding pattern produced by TGGE corresponds to a fingerprint of the microbial community in that environment, with each band potentially representing a single bacterial phylotype. This can be interpreted as a measure of the genetic diversity of the bacterial community that is targeted by the primers used in the initial PCR amplification. In addition, multiple samples can be analysed by TGGE allowing a means for comparative analysis of different environments or population dynamics over time (Kowalchuk *et al.*, 1997, Ferris *et al.*, 1997).

In addition to providing information on the bacterial community structure within samples of interest, TGGE can also be applied to gain insight into the phylogenetic positions of the most prominent bacteria. Information about the individual members of the community can be obtained by hybridisation of the banding pattern with oligonucleotide probes or by excision and sequencing of individual bands (Ferris *et al.*, 1996, Heuer *et al.*, 1997, Teske *et al.*, 1996). Although the DNA fragments obtained through this way are small, typically up to 500bp, sequence information can be used to infer phylogenetic information. Alternatively, this can also be achieved by preparing and screening clone libraries on TGGE gels and sequencing clones corresponding to dominant bands in the TGGE pattern of environmental samples (Van Dyke and McCarthy, 2001).

This chapter describes the application of TGGE to PCR products amplified with group-specific primers to investigate differences in genetic diversity within populations of clostridia, SRB and *Archaea* (methanogens) before and during anaerobic digestion. The aim is to determine the stability of these functional group populations and to identify components that proliferate during digestion. Also, to determine whether diversity increases or decreases during digestion, and the extent of selection imposed on the incoming population. In addition, the sequencing of cloned 16S rDNA fragments allowed identification of community members whose phylogenetic affiliations were subsequently determined.

4.2. Results

4.2.1. TGGE analysis of *Clostridium* sub-groups using DNA amplified from raw and digesting sludge.

PCR amplified products obtained from each *Clostridium* sub-group using the nested PCR protocol were diluted appropriately and re-amplified using GC primer sets (Table 2.6). The amplification reaction mixtures were as described in section 2.13, with 5 ng of group-specific PCR product as a template and 25 amplification cycles at the appropriate annealing temperature. Products from amplification with the appropriate GC-clamped primer pair were separated using the Bio-Rad TGGE system as described in section 2.12. Approximately 200 ng of each sludge product were run at 80V from 43 to 51°C (0.5°C h^{-1}) for 16 hours. These running conditions were determined empirically and found to be optimal for producing banding patterns that gave a good separation of sequences for each of the four *Clostridium* sub-groups analysed.

The TGGE pattern for each of the four *Clostridium* sub-groups (Fig. 4.1) reveals the difference in bacterial genotypes between raw and digesting sludge, as shown by the different bands of 16S rDNA fragments. TGGE profiles for each *Clostridium* sub-group were repeated at least twice (as for all the analyses in this study), and the banding patterns shown to be reproducible.

The potential of TGGE for identifying populations was addressed by sequencing TGGE bands wherever possible. Several bands were excised from the TGGE gel for sequencing and are those that were identifiable under UV light after ethidium bromide staining. For the four clostridial-clusters (Collins *et al.*, 1994), a total of 8 bands were isolated, re-amplified by PCR and sequenced. The partial 16S rRNA gene sequences of each excised fragment were compared with those in the Genbank database to identify the most closely related database sequences. The results for each clostridial sub-group, revealed by Fig. 4.1, are as follows:

Cluster I: TGGE profiles obtained showed limited diversity with ≤ 3 dominant bands in both sludge types. The banding patterns obtained were very similar to one another, with the exception of one additional band observed in raw sludge suggesting that the organism represented by this sequence had not proliferated during digestion. Sequencing of bands ex1, ex2 and ex3 confirmed that they all belong to cluster I of

clostridia (Collins *et al.*, 1994). BLAST analysis of bands ex1 and ex3, common to both sludge profiles, respectively found them to have close identities to *C. magnum* (97%) and the uncultured gut bacterium clone p-406-03 (96%) (Leser *et al.*, 2002). The additional band (ex2) found in raw sludge, was closely related to an uncultured bacterium clone RSb16 (96%), previously isolated from an anoxic paddy soil (Weber *et al.*, 2001). This phylotype was not observed in the fingerprint of digesting sludge.

Cluster III: Unlike any of the other *Clostridium* sub-groups analysed, TGGE profiling revealed a greater diversity of cluster III clostridia in both raw and digesting sludge as shown by the total number of individual bands observed. Banding patterns generated from the two sludge types had approximately the same number of discernible bands (10 to 15) and were often distinct, with relatively few bands common to both samples (Fig. 4.1). This suggests a shift in population from raw to digesting sludge brought about by the prevailing conditions in the anaerobic digester environment. Bands that appeared to be dominant on the basis of staining intensity were excised, sequenced and confirmed as belonging to cluster III clostridia by BLAST searches. Bands ex4 and ex5 were present in raw sludge only, and gave closest related matches to *Acetivibrio cellulolyticus* (98%) and the uncultured bacterium clone IA-23 (98%) (Adrian *et al.*, unpublished), respectively. The dominant bands obtained from digesting sludge, ex6 and ex7, were shown to be closely related to the rumen isolated uncultured bacterium clone RC31 (97%) (Tajima *et al.*, 1999), and *Clostridium termitidis* (95%), respectively.

Cluster IV: This TGGE profile revealed a similar banding pattern for both sludge types with only subtle differences in band migration discernable. Like cluster I, this group showed limited diversity with a total of 3 or 4 unique discernible bands in both samples. A dominant band common to both profiles, ex8, was excised and sequenced. Sequencing results showed this phylotype to be closely related to the uncultured bacterium clone HB31 (95%), previously isolated from an anaerobic digester (Godon *et al.*, 1997), and grouping with cluster IV clostridia (Collins *et al.*, 1994).

Cluster XIVab: Like cluster IV, the amplified DNA from this group had limited sequence diversity and was similar in both raw and digesting sludge (Fig. 4.1). Excision and sequencing of these dominant bands was unsuccessful, yielding poor sequence data that could not be further analysed.

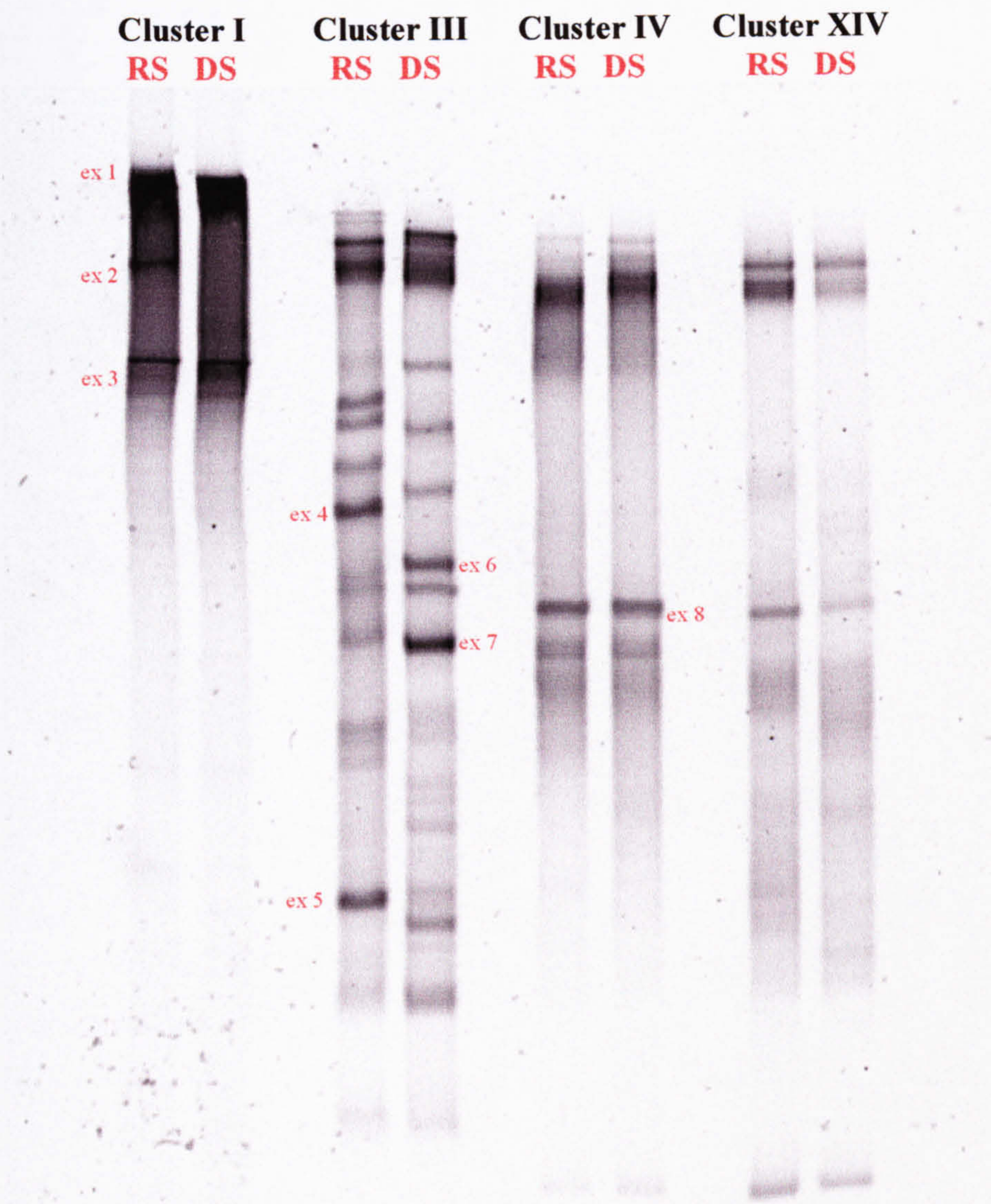


Figure 4.1. TGGE patterns of the 16S rDNA fragments of raw (RS) and digesting sludge (DS) samples for *Clostridium* clusters I, III, IV, and XIVab, as defined by Collins *et al* (1994). TGGE bands ex1 to ex8 were excised from the gels and sequenced.

4.2.2. Phylogenetic analysis of cloned *Clostridium* 16S rDNA fragments

While TGGE is useful for examining abundant populations it may not detect minor components of bacterial communities (Muyzer *et al.*, 1996). Therefore the number of bands generated by TGGE may not accurately reflect the number of sequences present in a mixture of PCR products. To gain more insight into the phylogenetic structure of the predominant and minor bacterial populations, 16S rDNA amplified with *Clostridium* sub-group specific primers was cloned into competent *E. coli* JM109 cells as described in section 2.15. Two clone libraries were constructed for each of the four-clostridial groups, one from raw sludge, and the other from digesting sludge. A total of 34 clones (17 from raw sludge, 17 from digesting sludge) were randomly chosen and screened for correct sized inserts by PCR and then analysed by TGGE. Clones that matched the position of bands of the original TGGE pattern were noted and sequenced. In addition, non-matching clones were also sequenced in order to further investigate digester bacterial diversity not represented in the original TGGE profiles of the sludge samples.

TGGE allowed the screening of clones to be achieved by grouping them according to their mobilities on the gel. Grouping clones in this way proved difficult on occasions, since some of the bands closely migrated to a group of bands all with similar but not identical migrations. This is sometimes observed when gels are not uniformly level and discrepancies within gels are formed during pouring and setting, thus manifesting in a wave-like profile of bands upon electrophoresis. In such cases, these clones were sequenced and if matched with sequences of the closely migrating bands, were grouped as such. This approach was used for all gels in this study to allow grouping of the clones with a strong degree of certainty.

Cluster I: Cloned PCR products amplified from raw and digesting sludge were analysed by TGGE (Figs. 4.2 & 4.3). Clones gIRS14, gIDS6 and gIDS13 did not yield readable sequence data, and thus were excluded from any further analysis.

Screening of clones according to their mobilities grouped them into six distinct groups for raw sludge (Table 4.1) and four distinct groups for digesting sludge (Table 4.2). When possible at least two representatives of each group were sequenced to confirm their identities, but only one representative was included in the phylogenetic analysis. The closest matches (and percentages of similarity) for the sequences analysed were determined by the BLAST program and resulted mainly in

matches with unknown and uncultured microorganisms assigned to cluster I clostridia from a wide diversity of environments. Although there are no exact 16S rDNA similarity limits for defining specific taxa such as genus and species, in general species definition requires sequence similarities greater than 98%. Only one group of sequences in digesting sludge (with nine representatives) can be identified as belonging to the uncultured bacterium clone p-406-o3 with 99% similarity, obtained from the pig gut (Leser *et al.*, 2002). The remaining sequences have a similarity value less than 98%; therefore to allocate these sequences with a reasonable degree of confidence to the corresponding taxa, phylogenetic analysis was performed to clarify their taxonomic position. It has been suggested that phylogenetic clustering of bacterial groups, rather than a specific similarity value, should be used as a guide for defining bacterial taxa (Paster *et al.*, 1991). The phylogenetic dendogram in Fig. 4.4 show the relationship of the RS and DS clones with clostridial cluster I type strain representatives and the uncultured sequences from the GenBank database. The bootstrap consensus trees generated by DNA distance and maximum parsimony analyses showed similar topologies and bootstrap values to one another, and therefore only one is shown.

In comparison to the original TGGE pattern, a greater diversity was observed by cloning of both raw and digesting sludge 16S rDNA. Sequencing data also suggests a change in the diversity from raw to digesting sludge in both number and phylotype. The majority of the sequences detected in raw sewage were most closely related and clustered with, *Clostridium scatlogenes* (clonal groups gIRS1, gIRS2 & gIRS9) and the rice paddy clone RSb16 (clonal group gIRS4) (Weber *et al.*, 2001). However, in the digesting sludge, many sequences (clonal groups gIDS1, gIDS3 & gIRS5) were closely associated and clustered with the gut bacterium clone p-406-o3 (Leser *et al.*, 2002). The next most prevalent group in digesting sludge, with four representatives, was related to *Clostridium quinii* (95%).

Clonal groups gIRS5 and gIDS3 matched the corresponding bands on the original TGGE profile, previously excised (ex3) and sequenced, and shared the closest sequence identity with the environmental clone p-406-o3. One clone (gIRS11) was distantly related to the *Clostridium quinii* cluster. The most similar affiliated sequence based on BLAST analysis was with that of a pig gut bacterial clone p-4936-6Wb (Leser *et al.*, 2002).

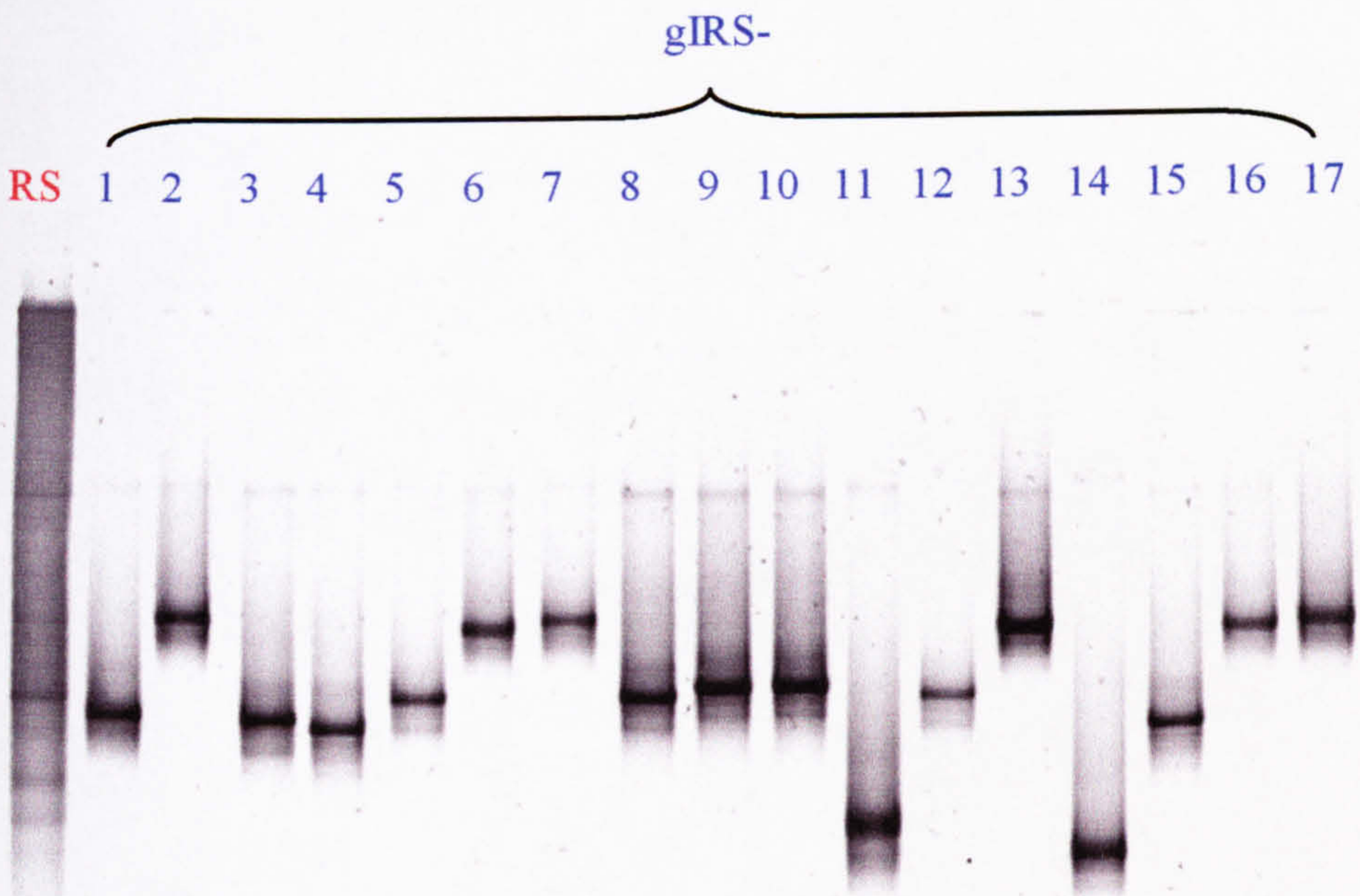


Figure 4.2. TGGE screening of raw sludge (RS) *Clostridium* cluster I 16S rDNA clones generated using 16S rDNA amplified with cluster I specific primers.

Table 4.1. Similarity values of *Clostridium* cluster I 16S rDNA sequences retrieved from raw sludge.

Clones	Closest Relative	Similarity	Sequence Length
gIRS1, gIRS3	Unidentified bacterium anoxSCC-41	94%	500bp
gIRS2, gIRS6, gIRS7 gIRS13, gIRS16, gIRS17	*Clostridium scatlogenes* strain FP	94%	499bp
gIRS4, gIRS15	Uncultured bacterium RSb16	96%	515bp
gIRS5, gIRS8, gIRS12	Uncultured bacterium p-406-o3	95%	499bp
gIRS9, gIRS10	*Clostridium scatlogenes* strain FP	97%	526bp
gIRS11	Uncultured bacterium clone p-4936-6Wb2	96%	562bp
gIRS14	Unreadable sequence		

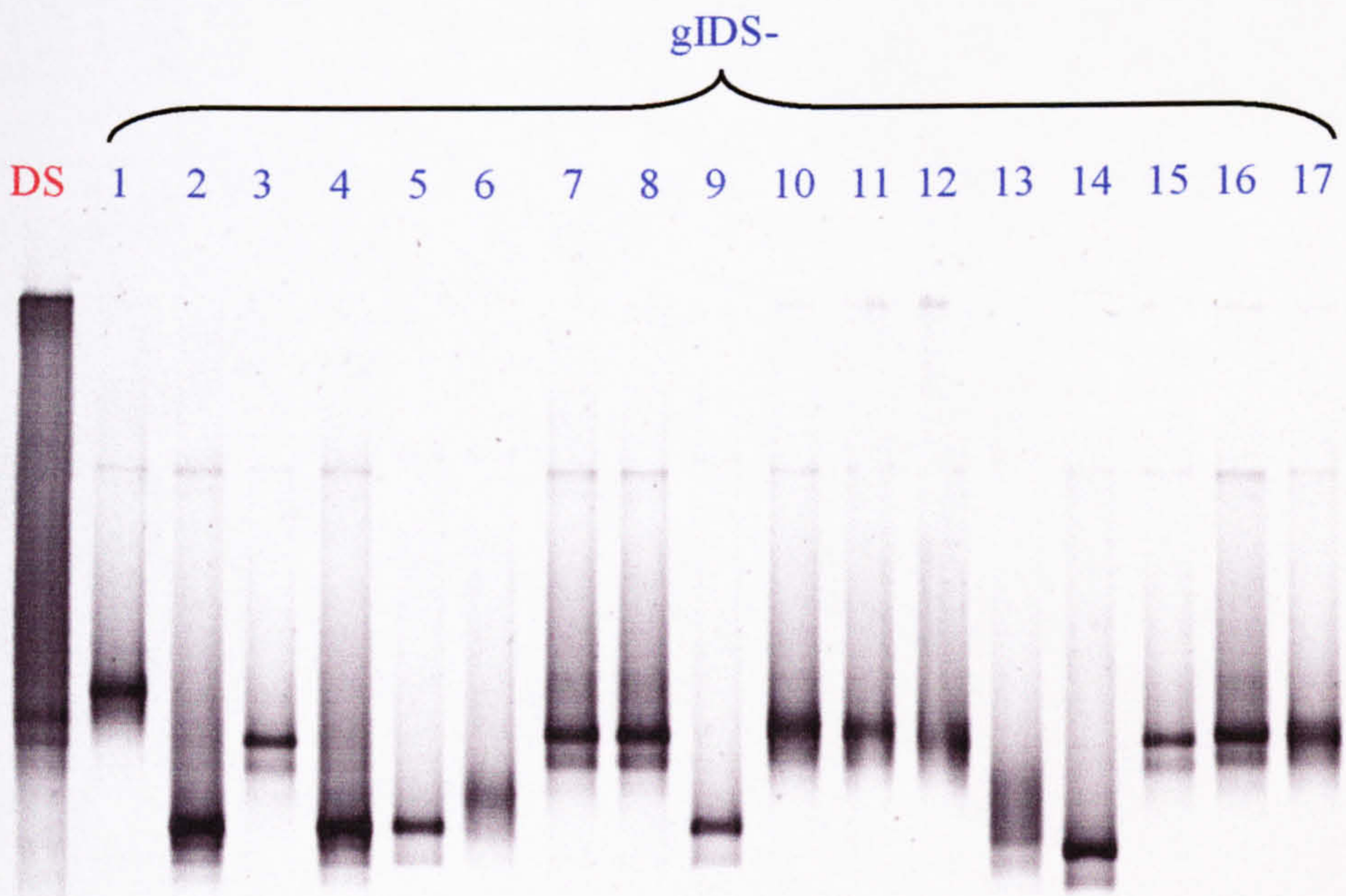


Figure 4.3. TGGE screening of digesting sludge (DS) *Clostridium* cluster I 16S rDNA clones generated using 16S rDNA amplified with cluster I specific primers.

Table 4.2. Similarity values of *Clostridium* cluster I 16S rDNA sequences retrieved from digesting sludge.

Clones	Closest Relative	Similarity	Sequence Length
gIDS1	Uncultured bacterium clone p-406-o3	94%	526bp
gIDS2, gIDS4, gIDS5 gIDS9,	*Clostridium quinii*	96%	516bp
gIDS3, gIDS7, gIDS8, gIDS10, gIDS11, gIDS12 gIDS15, gIDS16, gIDS17	Uncultured bacterium clone p-406-o3	99%	592bp
gIDS14	Uncultured rumen bacterium 4C0d-15	96%	477bp
gIDS6, gIDS13	Unreadable sequences		

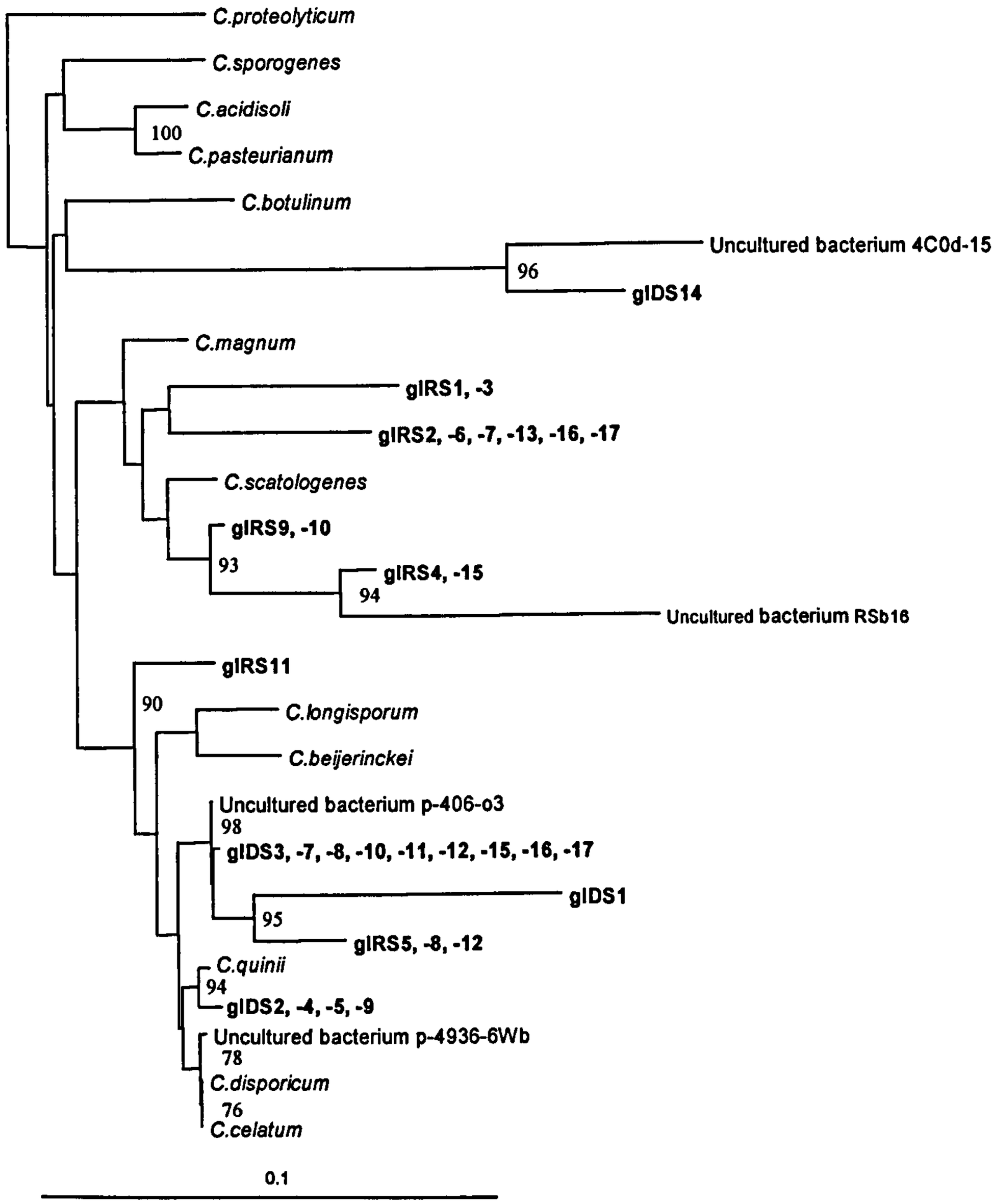


Figure 4.4. Phylogenetic tree generated from the alignment of *Clostridium* cluster I sequences with 16S rDNA cloned sequences derived from PCR products amplified from raw and digesting sludge. The tree was constructed using the neighbour-joining method of Jukes & Cantor (1969) and analysis was based on 578bp nucleotides. Bootstrap values above 70% are shown. The cluster II *C.proteolyticum* sequence served as the outgroup for rooting the tree. Bar shows estimated divergence in nucleotide sequences.

Cluster III: Cloned 16S rDNA amplified from raw and digesting sludge were analysed by TGGE (Figs. 4.5 & 4.6). Clones gIIIRS9, -15 and gIIIDS3, -10, -11, -14, & -16 yielded poor sequence data and were excluded from any further analysis. Screening of clones on the basis of mobility defined seven distinct groups in raw sludge and five in digesting sludge. The phylogenetic positions of cluster III RS and DS clones are shown in Fig. 4.7. The bootstrap consensus trees generated by DNA distance and maximum parsimony analysis showed similar topologies and bootstrap values to one another, and therefore only one is shown. Clones retrieved from raw sludge showed no matches with named cluster III *Clostridium* spp, however, BLAST searches (Tables 4.3 & 4.4) showed closest relatives to be unknown and uncultured microorganisms assigned to cluster III clostridia from a range of anaerobic environments. Three groups of clones (clonal groups gIIIDS6, gIIIDS7, gIIIDS8) retrieved from digesting sludge, were found to be closely related to and clustered with known cluster III clostridia. Only one of these clonal groups with two representatives, gIIIDS7, gIIIDS17, can be identified as belonging to *Clostridium termitidis* with 98% sequence similarity. The other two groups gIIIDS6 and gIIIDS8, with relatively low sequence identities (96%) are shown to be clearly related to, but distinct from, *Clostridium aldrichii* and *Acetivibrio cellulolyticus*, respectively. The phylogenetic placement of nine different clonal sequences (groups gIIIDS1, gIIIDS2, gIIIRS1, gIIIRS2, gIIIRS3, gIIIRS4, gIIIRS5, gIIIRS7 and gIIIRS11) formed two new closely related clusters with environmental sequences SHD-209 (Schloetelburg *et al.*, 2002), RC31 & RC4 (Tajima *et al.*, 1999), IA-23 (Adrian *et al.*, unpublished), and RA13CB (Afreider *et al.*, unpublished). These sequences showed 91-97% sequence identity to the sequences in the cluster.

Four groups containing gIIIRS1, gIIIRS3, gIIIRS4 and gIIIRS5 matched corresponding bands on the original RS profile (not existed) and only two groups, gIIIDS2 and gIIIDS7, matched bands on the original DS profile. The latter matched the corresponding excised bands on the DS profile and had similar sequences to each other (gIIIDS2 with ex6, gIIIDS7 with ex7).

Cluster IV: Cloned PCR products amplified from raw and digesting sludge were analysed by TGGE (Figs. 4.8 & 4.9). Clones gIVRS2, -9, -14, -17 and gIVDS6, -11, -12, & -15, yielded poor sequence data and were excluded from any further analysis. Screening of clones on the basis of mobility revealed a large number of clonal groups

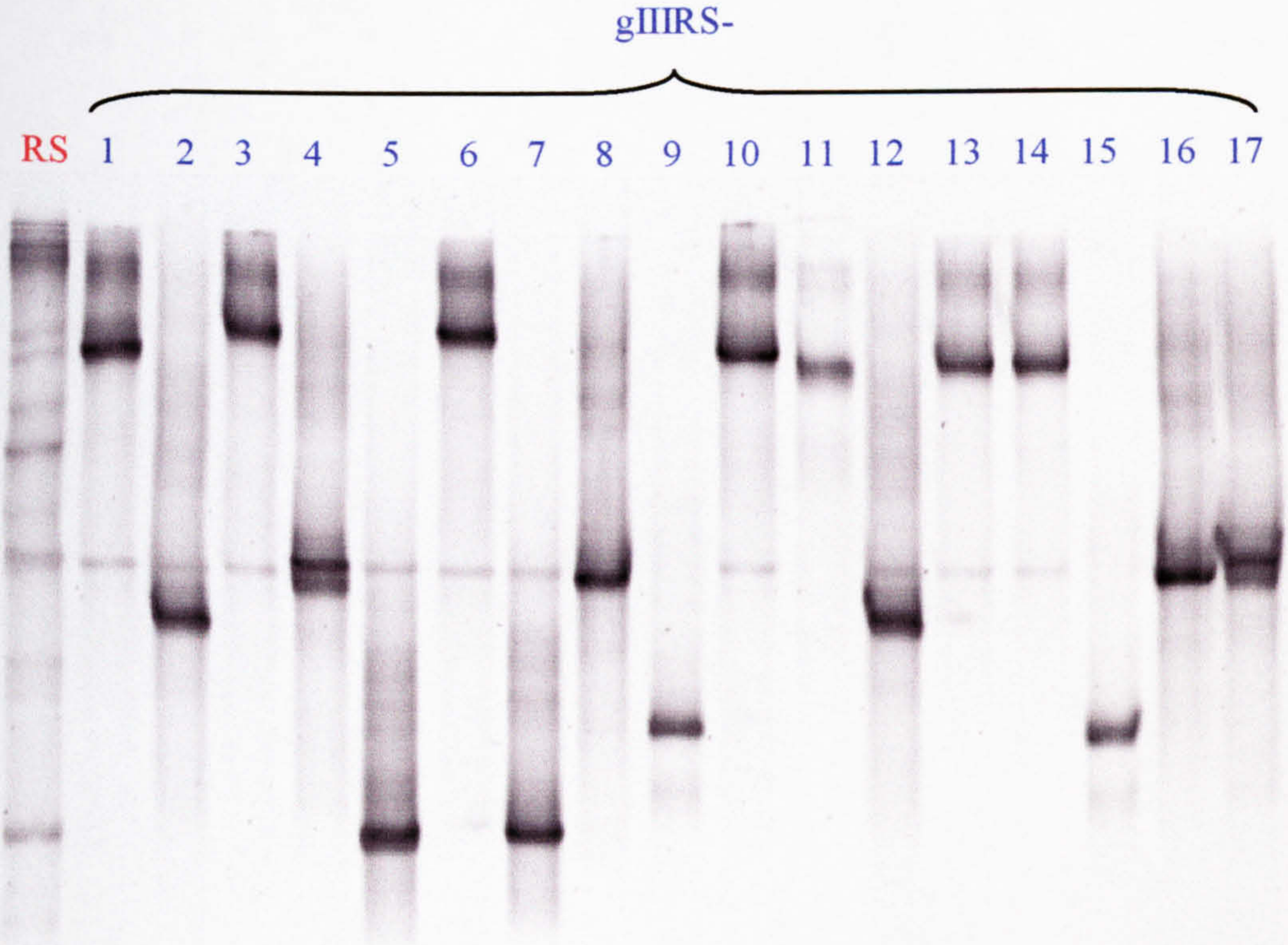


Figure 4.5. TGGE screening of raw sludge (RS) *Clostridium* cluster III 16S rDNA clones generated using 16S rDNA amplified with cluster III specific primers.

Table 4.3. Similarity values of *Clostridium* cluster III 16S rDNA sequences retrieved from raw sludge.

Clones	Closest Relative	Similarity	Sequence Length
gIIIRS1, gIIIRS10	Unidentified rumen bacterium RC31	94%	586bp
gIIIRS2, gIIIRS12	Uncultured bacterium clone RA13CB	95%	520bp
gIIIRS3, gIIIRS6	Unidentified rumen bacterium RC4	96%	515bp
gIIIRS4, gIIIRS8, gIIIRS16, gIIIRS17	Unidentified rumen bacterium RC31	93%	499bp
gIIIRS5	Uncultured bacterium clone IA-23	96%	520bp
gIIIRS7	Uncultured bacterium clone g3Br5	96%	504bp
gIIIRS11, gIIIRS13, gIIIRS14	Unidentified rumen bacterium RC4	93%	535bp
gIIIRS9, gIIIRS15	Unreadable sequence		

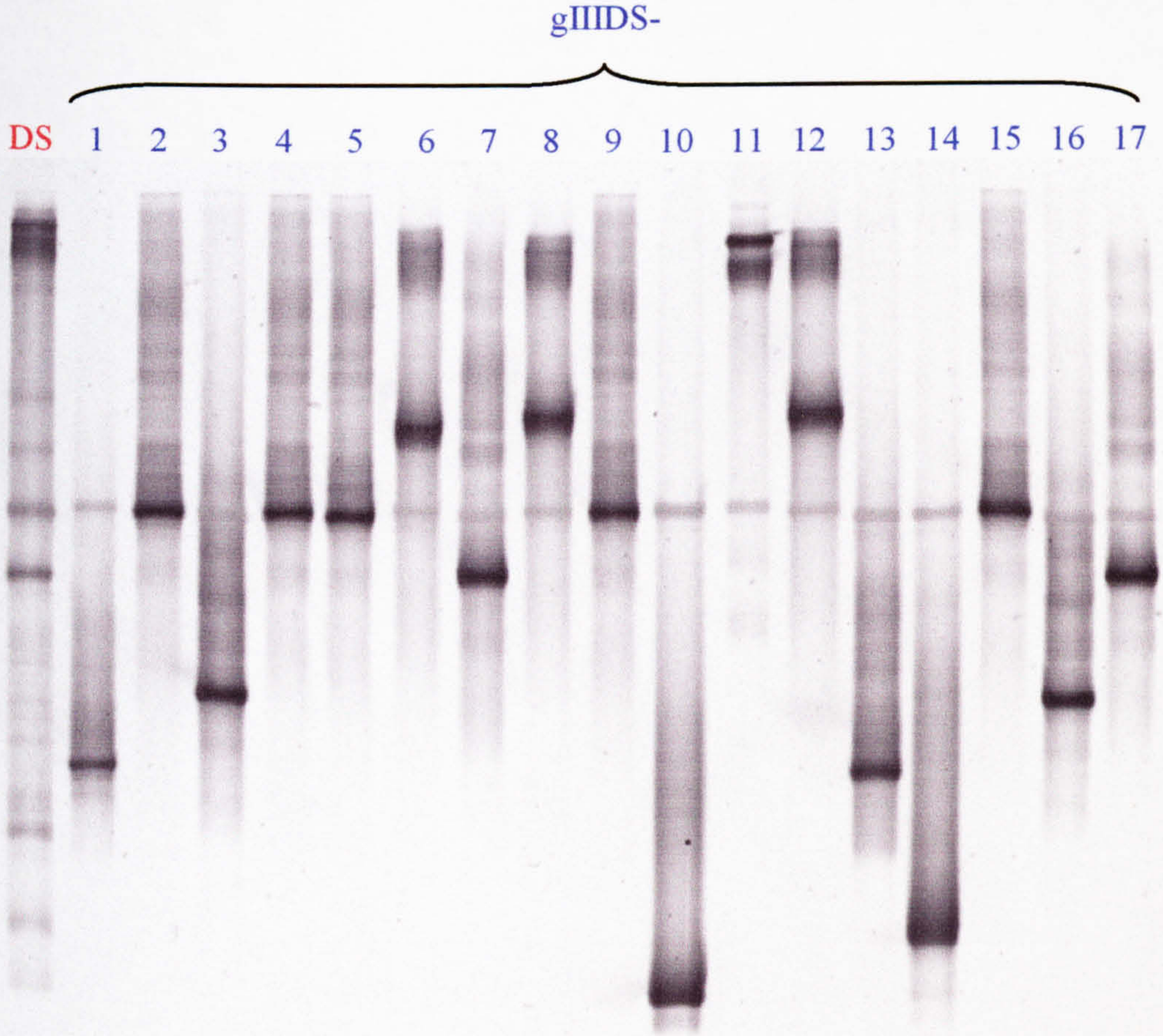


Figure 4.6. TGGE screening of digesting sludge (DS) *Clostridium* cluster III 16S rDNA clones generated using 16S rDNA amplified with cluster III specific primers.

Table 4.4. Similarity values of *Clostridium* cluster III 16S rDNA sequences retrieved from digesting sludge.

Clones	Closest Relative	Similarity	Sequence Length
gIIIDS1, gIIIDS13	Uncultured low G+C gram-positive bacterium SHD-209	96%	493bp
gIIIDS2, gIIIDS4 gIIIDS5, gIIIDS9, gIIIDS15	Uncultured rumen bacterium RC31	96%	522bp
gIIIDS6	*Clostridium aldrichii*	96%	497bp
gIIIDS7, gIIIDS17	*Clostridium termitidis*	98%	517bp
gIIIDS8, gIIIDS12	*Acetivibrio cellulolyticus*	96%	520bp
gIIIDS3, gIIIDS10 gIIIDS11, gIIIDS14, gIIIDS16	Unreadable sequence		

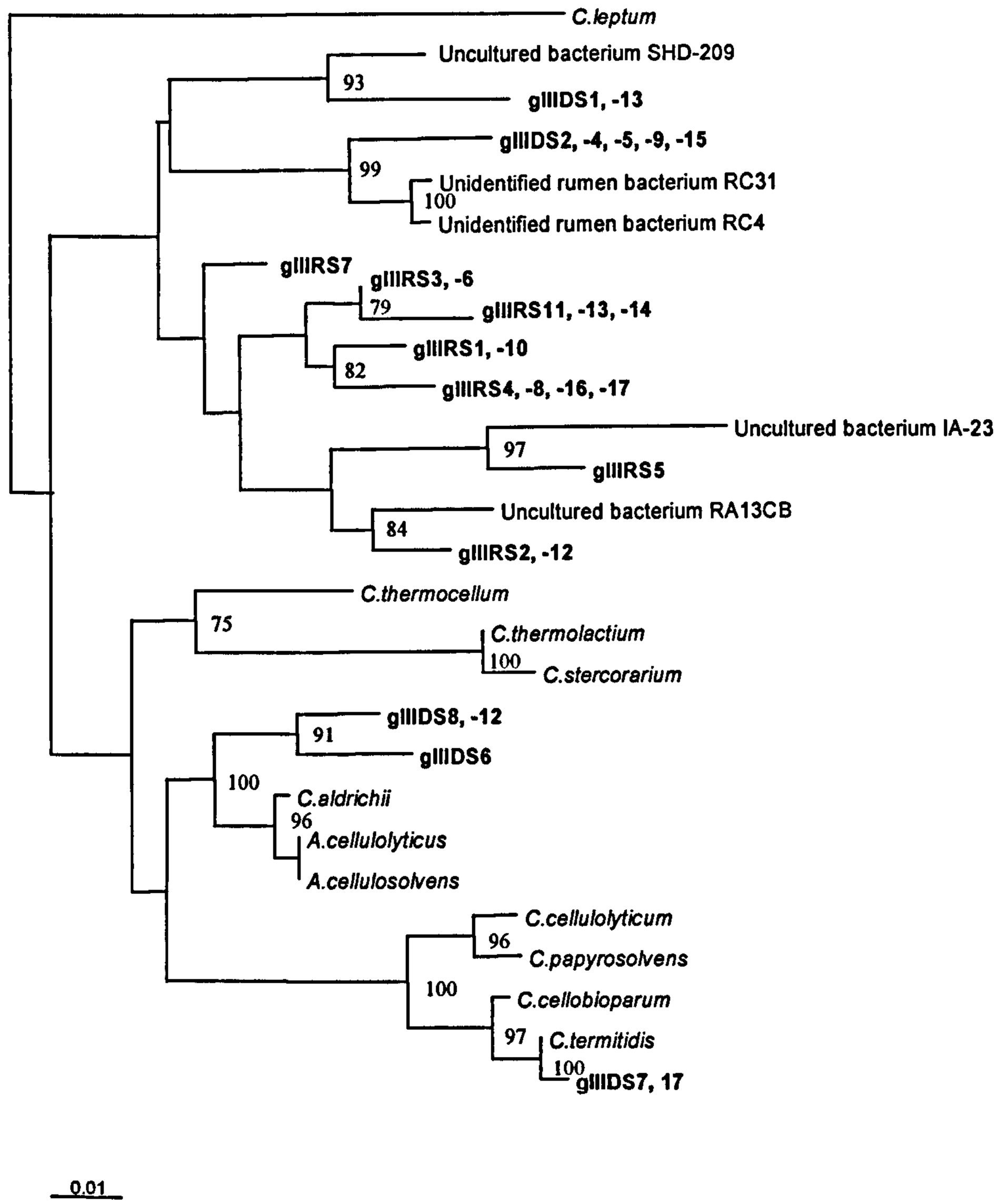


Figure 4.7. Phylogenetic tree generated from the alignment of *Clostridium* cluster III sequences with 16S rDNA cloned sequences derived from PCR products amplified from raw and digesting sludge. The tree was constructed using the neighbour-joining method of Jukes & Cantor (1969) and analysis was based on 566bp nucleotides. Bootstrap values above 70% are shown. The cluster IV *C.leptum* sequence served as the outgroup for rooting the tree. Bar shows estimated divergence in nucleotide sequences.

(seven from RS and eight from DS) suggesting the presence of a much greater diversity not observed on the original RS and DS TGGE profiles. As with the other clusters, sequencing followed by BLAST analysis of the bacterial clones resulted mainly in matches with uncultured environmental sequences assigned to cluster IV clostridia from a range of environments (Tables 4.5 & 4.6). The phylogenetic placement of cluster IV RS and DS clones are shown in Fig. 4.10. The bootstrap consensus trees generated by DNA distance and maximum parsimony analysis showed similar topologies and bootstrap values to one another, and therefore only one is shown.

Four clonal groups (gIVDS4, gIVDS5, gIVDS8, gIVRS4) formed a novel separate cluster that was not closely affiliated to any sequences in the database. The stability of this cluster was verified by a high bootstrap value of 100%. Another four groups (gIVDS2, gIVRS5, gIVRS6 and gIVRS12) formed part of a cluster containing only uncultured cluster IV sequences (HB31, BA18, CA26, and VadinHA42) retrieved from an anaerobic digester (Godon *et al.*, 1997). However, all the remaining clones are clustered with the closely related sequences identified by the BLAST searches.

Clonal groups gIVRS4 and gIVDS5 match the corresponding band (ex8) on the original TGGE profile. Sequencing results from these clones and the excised bands suggests that this phylotypes is closely related to the environmental sequence HB31 (Godon *et al.*, 1997).

Cluster XIV: Cloned PCR products amplified from raw and digesting sludge were analysed by TGGE (Figs. 4.11 & 4.12). Clones gXIVRS1, -5, -6, -7, & -12 and gXIVDS3, -5, -7, & -9 yielded poor sequence data, and thus were excluded from any further analysis. Screening clones according to their mobilities gave rise to six distinct groups in both sludge types, with majority of the clones closely related to uncultured environmental sequences assigned to cluster XIV clostridia (Tables 4.7 & 4.8). The phylogenetic positions of cluster XIV RS and DS clones are shown in Fig. 4.13. The bootstrap consensus trees generated by DNA distance and maximum parsimony analyses showed similar topologies and bootstrap values to one another, and therefore only one is shown.

Only one clonal group with three representatives (gXIVDS4, -6, & -17) can be identified as belonging to the fecal bacterium mpn-isolate group 18 with 98% sequence similarity (Mikkelsen *et al.*, unpublished). Apart from clones gXIVDS1, -

12, and gXIVRS14, the remaining clones form part of four separate clusters containing only uncultured environmental sequences obtained from the gastrointestinal tract of a pig (Leser *et al.*, 2002). Clones gXIVDS1 and gXIVDS12 are closely related to *Clostridium populeti* and *Clostridium indolis*, respectively. However, clone gXIVRS14 clusters with *Butyrivibrio fibrisolvens* and *Clostridium proteoclaticum* and is probably distantly related to these bacteria. Clone gXIVDS1 corresponds to a dominant band on the original DS profile and is closely related to *Clostridium populeti*.

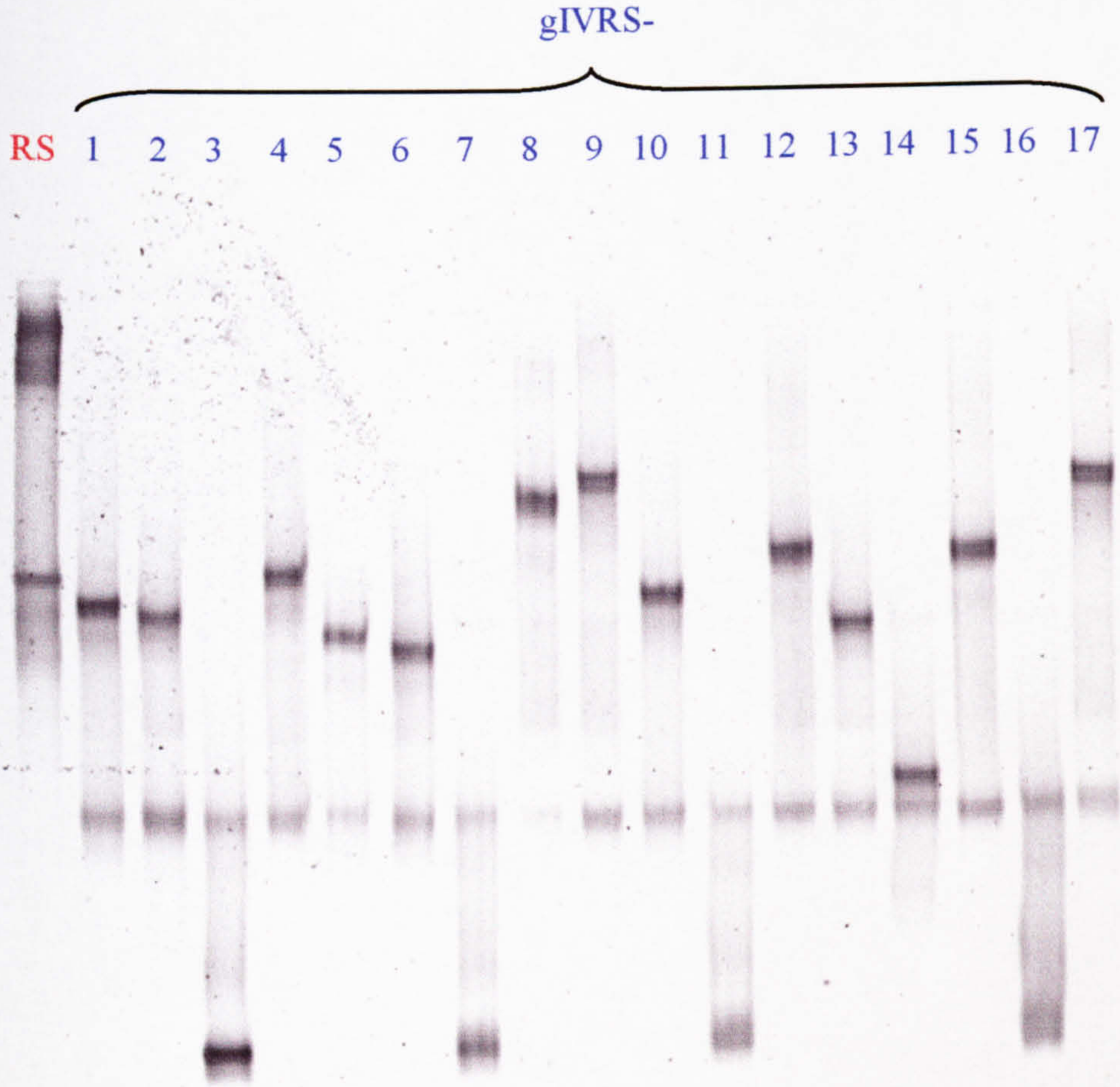


Figure 4.8. TGGE screening of raw sludge (RS) *Clostridium* cluster IV 16S rDNA clones generated using 16S rDNA amplified with cluster IV specific primers.

Table 4.5. Similarity values of *Clostridium* cluster IV 16S rDNA sequences retrieved from raw sludge.

Clones	Closest Relative	Similarity	Sequence Length
gIVRS1, gIVRS10	Uncultured bacterium clone p-5460-2Wb5	94%	528bp
gIVRS5, gIVRS13	Uncultured bacterium CA26	95%	475bp
gIVRS3, gIVRS7, gIVRS11, gIVRS16	*Clostridium virde*	95%	494bp
gIVRS4	Uncultured bacterium HB31	93%	495bp
gIVRS6	Uncultured bacterium BA18	95%	475bp
gIVRS8	Bacterium Irt-JG1-64	93%	513bp
gIVRS12, gIVRS15	Unidentified eubacterium clone VadinHA42	95%	456bp
gIVRS2, gIVRS9, gIVRS14, gIVRS17	Unreadable sequence		

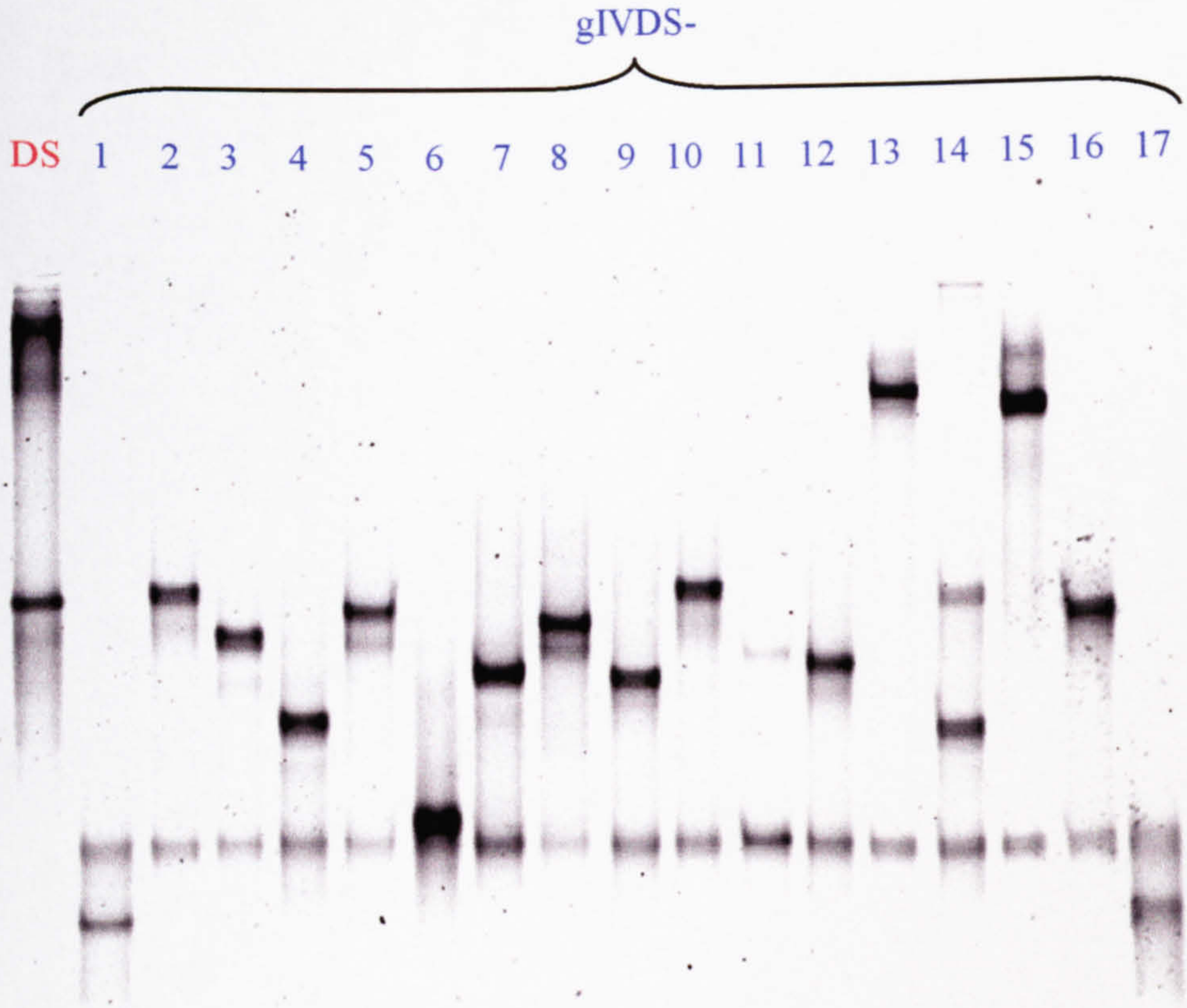


Figure 4.9. TGGE screening of digesting sludge (DS) *Clostridium* cluster IV 16S rDNA clones generated using 16S rDNA amplified with cluster IV specific primers.

Table 4.6. Similarity values of clostridia cluster IV 16S rDNA sequences retrieved from digesting sludge.

Clones	Closest Relative	Similarity	Sequence Length
gIVDS1, gIVDS17	*Clostridium virde*	96%	515bp
gIVDS2, gIVDS10	Uncultured bacterium BA18	97%	485bp
gIVDS3	Uncultured bacterium p-2031-s959	92%	473bp
gIVDS4, gIVDS14	Uncultured bacterium CA26	92%	481bp
gIVDS5, gIVDS16	Uncultured bacterium HB31	95%	495bp
gIVDS7, gIVDS9	*Clostridium orbiscindens* strain DSM 674	93%	477bp
gIVDS8	Uncultured bacterium BC09	91%	416bp
gIVDS13	*Ruminococcus flavefaciens* strain AR72	97%	468bp
gIVDS6, gIVDS11, gIVDS12, gIVDS15	Unreadable sequences		

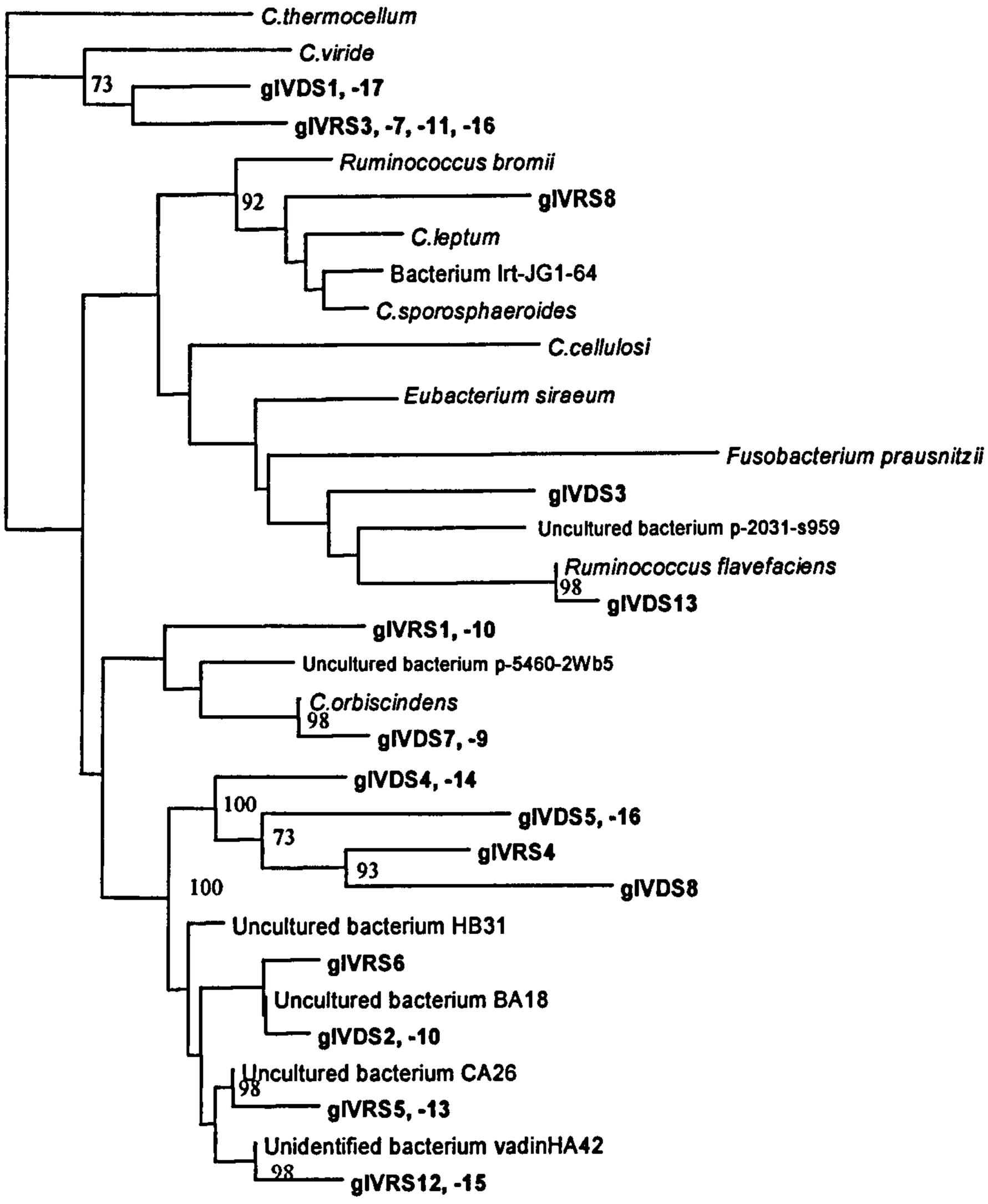


Figure 4.10. Phylogenetic tree generated from the alignment of *Clostridium* cluster IV sequences with 16S rDNA cloned sequences derived from PCR products amplified from raw and digesting sludge. The tree was constructed using the neighbour-joining method of Jukes & Cantor (1969) and analysis was based on 488bp nucleotides. The cluster III *C.thermocellum* sequence served as the outgroup for rooting the tree.Bootstrap values above 70% are shown.

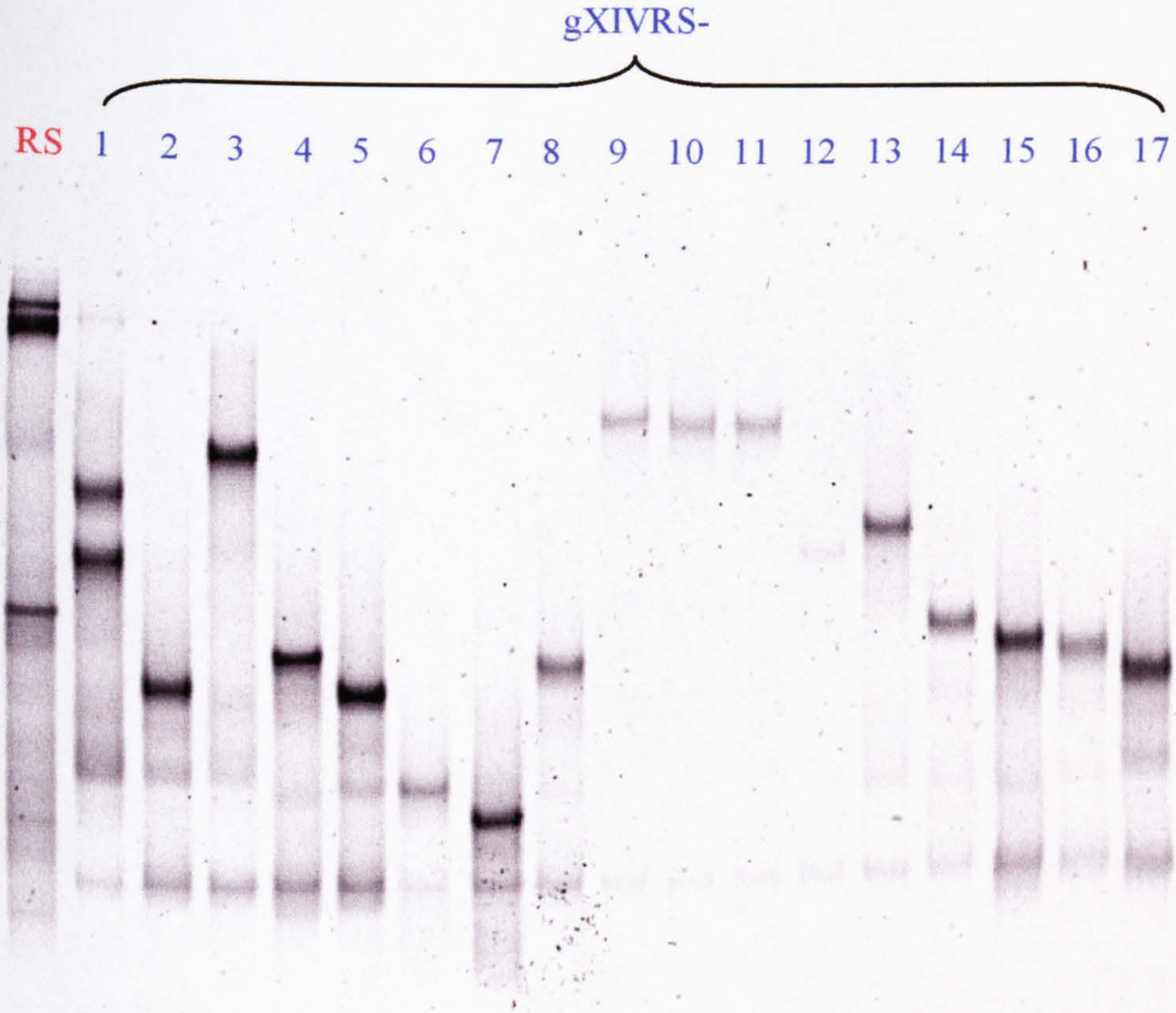


Figure 4.11. TGGE screening of raw sludge (RS) *Clostridium* cluster XIV 16S rDNA clones generated using 16S rDNA amplified with cluster XIV specific primers

Table 4.7. Similarity values of *Clostridium* cluster XIV 16S rDNA sequences retrieved from raw sludge.

Clones	Closest Relative	Similarity	Sequence Length
gXIVRS2, gXIVRS5, gXIVRS17	Uncultured bacterium clone p-969-s962-5	97%	582bp
gXIVRS3	Uncultured bacterium clone p-4162-6Wa5	93%	556bp
gXIVRS4, gXIVRS8, gXIVRS16	Uncultured bacterium clone p-619-a5	92%	546bp
gXIVRS9, gXIVRS10, gXIVRS11	Uncultured bacterium clone p-1028-a5	93%	538bp
gXIVRS13	Uncultured bacterium clone p-2195-s959-3	92%	559bp
gXIVRS14	*Clostridium proteoclasticum*	92%	524bp
gXIVRS1, gXIVRS5, gXIVRS6, gXIVRS7, gXIVRS12	Unreadable sequences		

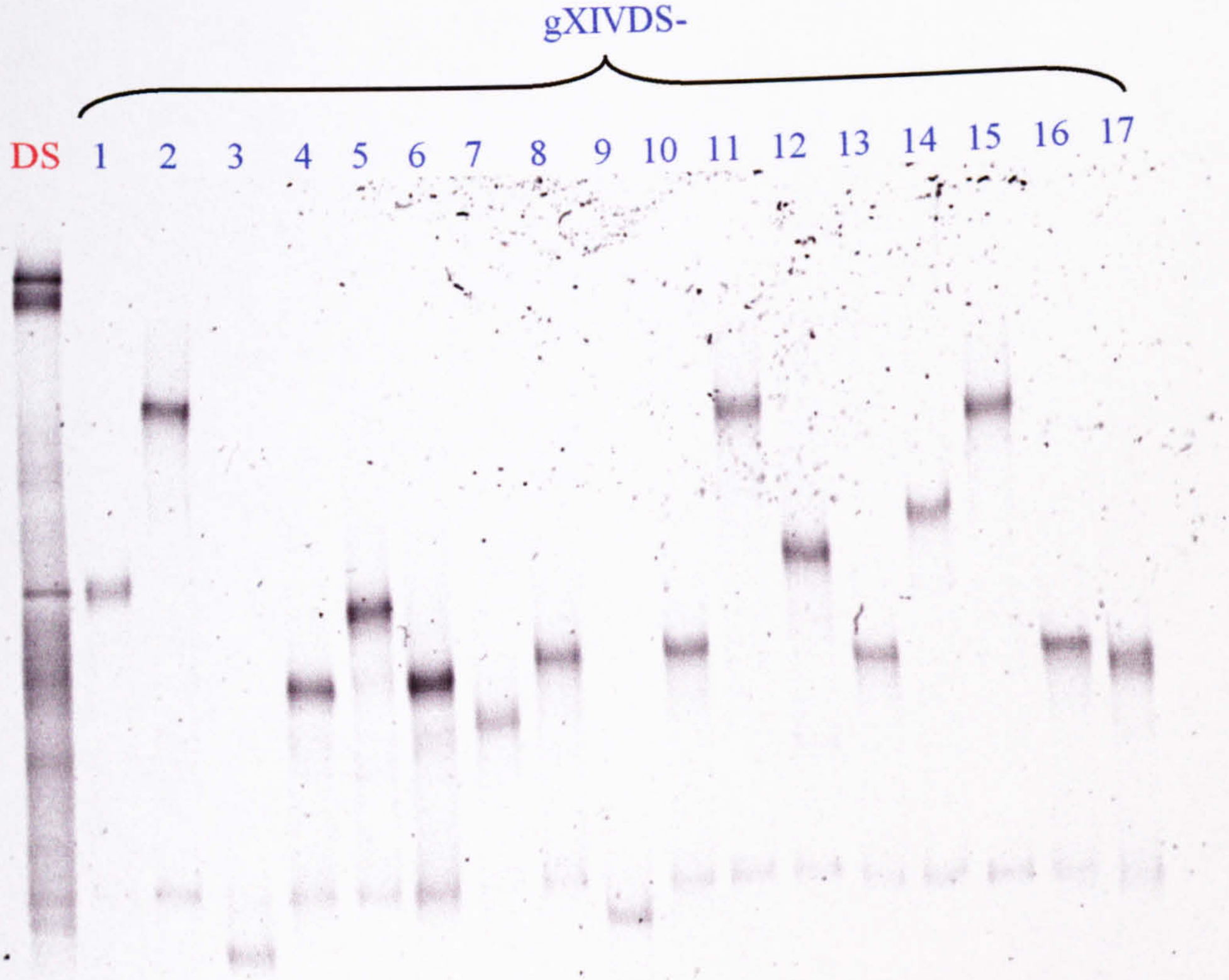


Figure 4.12. TGGE screening of digesting sludge (DS) *Clostridium* cluster XIV 16S rDNA clones generated using 16S rDNA amplified with cluster XIV specific primers

Table 4.8. Similarity values of *Clostridium* cluster XIV 16S rDNA sequences retrieved from digesting sludge.

Clones	Closest Relative	Similarity	Sequence Length
gXIVDS1	*Clostridium populeti*	97%	527bp
gXIVDS2, gXIVDS11, gXIVDS15	Uncultured bacterium clone p-4162-6Wa5	93%	556bp
gXIVDS4, gXIVDS6, gXIVDS17	Bacterium mpn-isolate group 18	98%	589bp
gXIVDS8, gXIVDS10, gXIVDS13, gXIVDS16	Uncultured bacterium clone p-393-o3	94%	559bp
gXIVDS12	*Clostridium indolis*	93%	559bp
gXIVDS14	Uncultured bacterium clone p-334-a3	92%	547bp
gXIVDS3, gXIVDS5, gXIVDS7, gXIVDS9	Unreadable sequences		

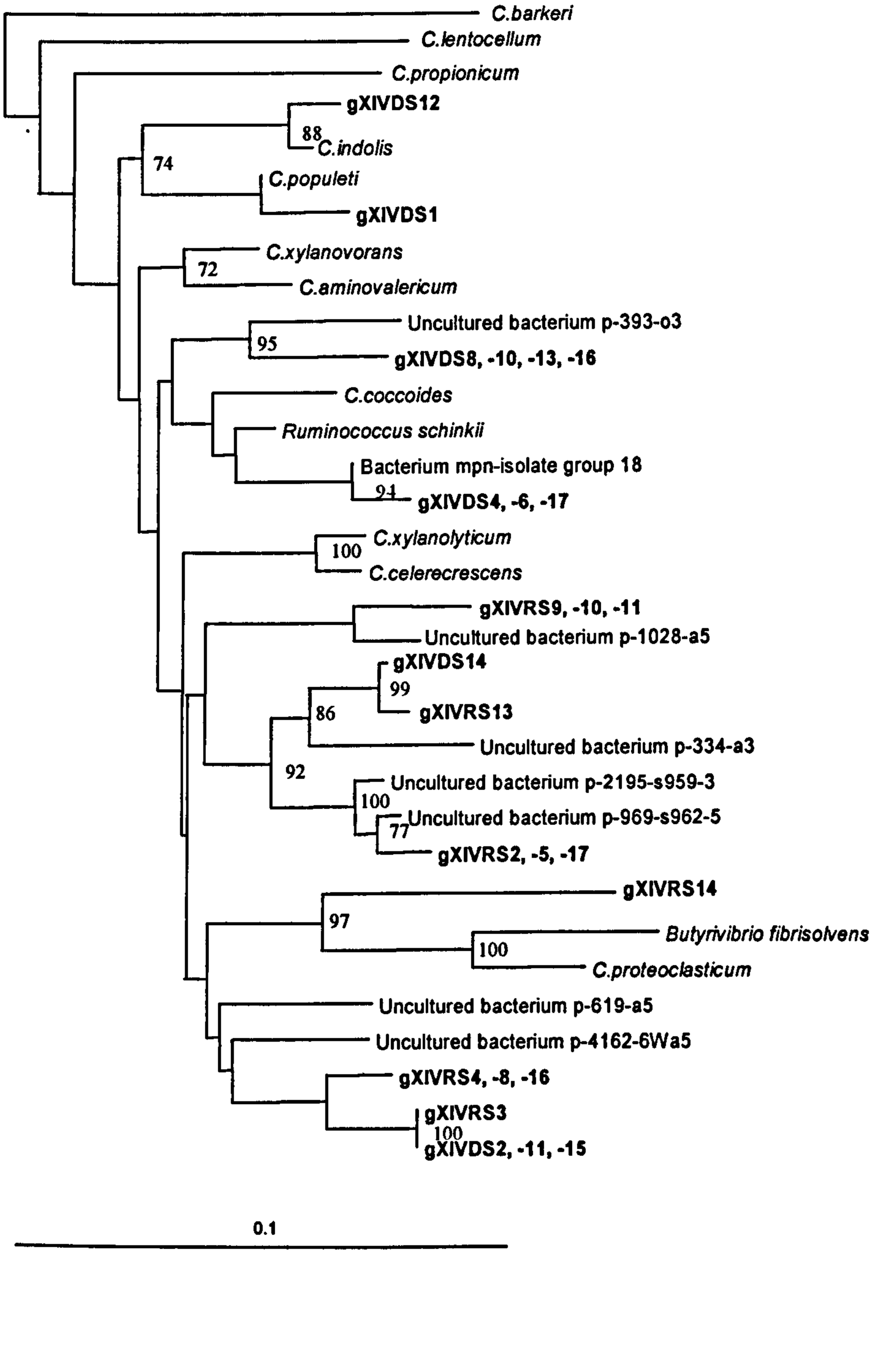


Figure 4.13. Phylogenetic tree generated from the alignment of *Clostridium* cluster XIV sequences with 16S rDNA cloned sequences derived from PCR products amplified from raw and digesting sludge. The tree was constructed using the neighbour-joining method of Jukes & Cantor (1969) and analysis was based on 578bp nucleotides. The cluster XV *C.barkeri* sequence served as the outgroup for rooting the tree. Bootstrap values above 70% are shown. Bar shows estimated divergence in nucleotide sequence.

4.3. TGGE analysis of SRB 16S rDNA amplified from raw and digesting sludge

PCR products amplified from raw and digesting sludge with SRB group-specific primers were diluted appropriately and re-amplified with eubacterial primers pC (GC-clamp); pD' (Edwards *et al.*, 1989) to generate fragments of 16S rRNA genes suitable for TGGE analysis. The amplification reaction mixtures were as described in section 2.13, with 5 ng of SRB group-specific PCR product as a template and 25 amplification cycles at the annealing temperature listed in Table 2.5. Approximately 200 ng of each amplification product were run at 75V from 51 °C to 56 °C (0.4 °Ch^{-1} gradient). These running conditions were empirically determined and were found to be optimal for producing good band separation for each of the SRB sub-groups. Only four of the six SRB sub-groups (SRB groups 1, 2, 5 & 6) were detected in sludge (Chapter 3) and are applied here to TGGE analysis to determine the degree of genetic diversity within each group (Fig. 4.14).

***Desulfotomaculum* (DFM Group 1)**: TGGE profiles showed the greatest diversity of all the SRB sub-groups analysed with approximately 5 to 6 discernable bands observed in both sludge types. The banding patterns were differentiated, with only 1 or 2 bands common to both samples.

***Desulfobulbus* (DBB Group 2)**: TGGE profiles showed a decrease in diversity from raw to digesting sludge. A total of six discernable bands (two dominant and four minor) were observed in raw sludge. In the digesting sludge profile, three dominant bands were observed that appeared not to co-migrate with any of the bands in the raw sludge profile.

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

TGGE profiles showed limited diversity in both raw and digesting sludge. A decrease in diversity was observed in the digesting sludge with the presence of only one strong band. Two dominant bands along with a few minor bands were observed in the raw sludge, and there was no evidence of co-migrating bands between raw and digesting sludge.

***Desulfovibrio-Desulfomicrobium* (DSV-DMB Group 6)**:

TGGE profiles revealed limited diversity with approximately the same number of discernable bands (3 to 4) in both sludge types. Despite the poor clarity of the profiles, it can be deduced that the banding patterns are very similar in both samples with the presence of an additional lower band in the DS profile that is not present in the RS profile.

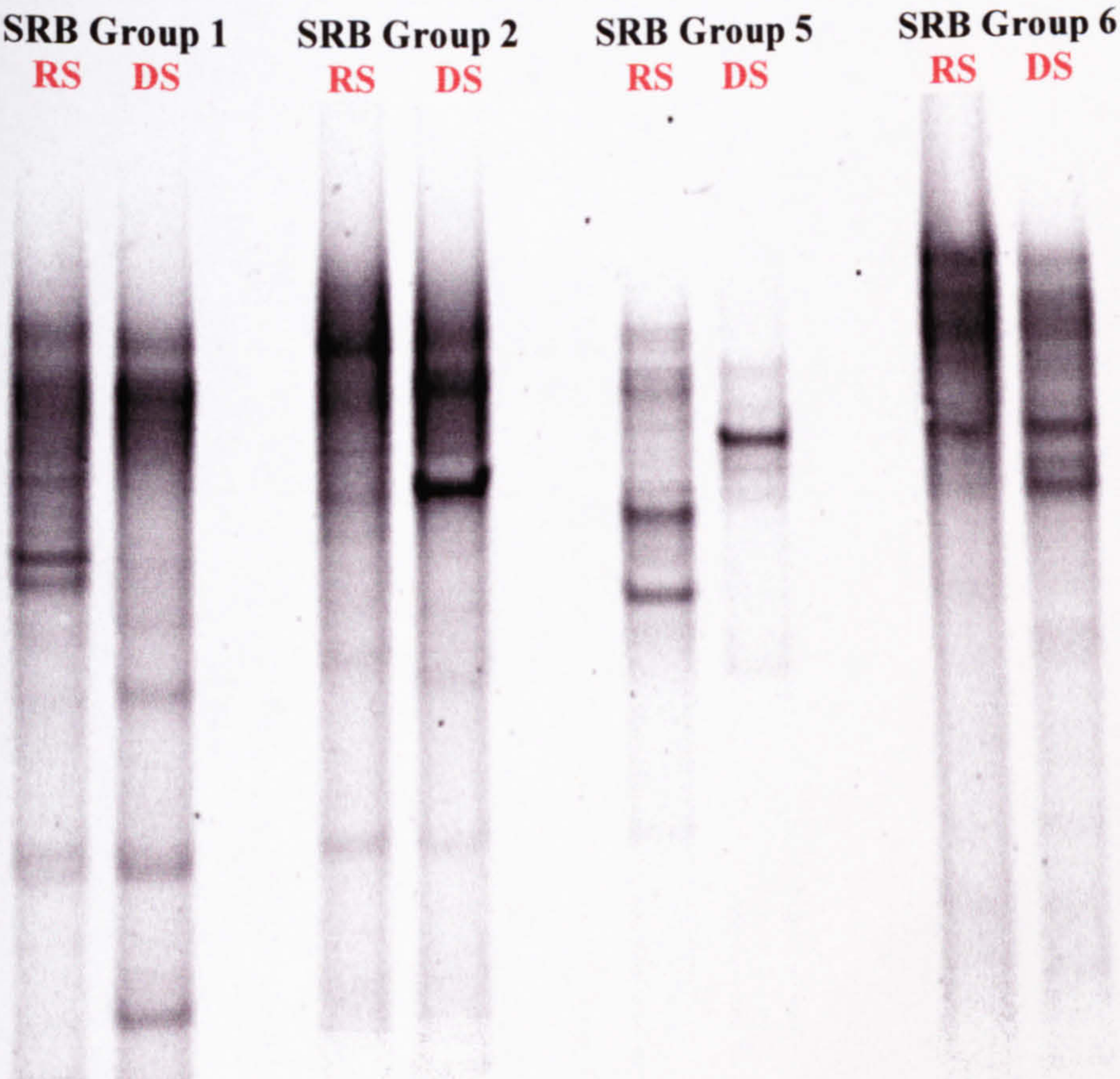


Figure 4.14. TGGE patterns of the 16S rDNA fragments of raw (RS) and digesting sludge (DS) samples for sulphate-reducing bacteria groups 1, 2, 5 & 6, as designated by Daly *et al* (2000).

4.3.1 Phylogenetic analysis of cloned SRB 16S rDNA fragments

PCR products amplified with SRB group-specific primers were cloned into competent *E. coli* cells as described in section 2.15. Two clone libraries were constructed for each of the SRB groups, one from raw sludge, and the other from digesting sludge. Since the primers targeting the SRB sub-groups are somewhat degenerate, the presence of non-SRB sequences amongst the clones is an issue. In order to screen the clones for SRB sequences, all clones obtained from each SRB sub-group were probed with their respective group-specific oligonucleotide probes.

Twenty white colonies were randomly picked from each SRB sub-group clone plate for raw and digesting sludge, and were incubated overnight at 37°C in 10 ml LB broth. 1ml of overnight culture was pelleted by centrifugation (13,000 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 lyse the cells and release plasmid DNA. The cell lysates were then PCR amplified with the appropriate group-specific primers, Southern-blotted onto positively charged nylon membranes (section 2.8) and hybridised with their respective group-specific probes in order to screen for SRB transformants. The data from this screening of clone libraries for SRB sequences are summarised in Table 4.9.

	RAW SLUDGE		DIGESTING SLUDGE	
	No. of randomly selected clones	Number of positive clones	No. of randomly selected clones	Number of positive clones
SRB Group 1	20	2	20	5
SRB Group 2	20	7	20	6
SRB Group 3	-	-	-	-
SRB Group 4	-	-	-	-
SRB Group 5	20	7	20	6
SRB Group 6	20	8	20	7

Table 4.9. Clones randomly selected from raw and digesting sludge for each SRB sub-group and identified by positive hybridisation signals with group specific probes.

Clones that gave a positive-signal upon hybridisation for each sub-group were analysed by TGGE. In addition to these clones that co-migrated with bands on the original RS and DS 16S rDNA profiles, clones that did not match were also sequenced. As before, the screening of clones by TGGE identified clonal groups with different melting characteristics. Whenever possible, at least two representatives of each group were sequenced to confirm there identities using the BLAST program, but only one representative was included in the phylogenetic analysis. The phylogenetic positions of SRB clones from both sludge types (RS and DS) are shown in Fig. 4.19. The bootstrap consensus trees generated by DNA distance and maximum parsimony analysis showed similar topologies and bootstrap values to one another, and only one is therefore shown.

***Desulfotomaculum* (DFM Group 1)**: Cloned PCR products that were positive upon hybridisation against probe DFM228 (Table 4.9) were analysed by TGGE (Fig. 4.15). Screening of clones on the basis of mobility defined two distinct groups in raw sludge and three distinct groups in digesting sludge (Table 4.10). However, a clonal group with one representative (DSdfm2) did not yield readable sequence information and was excluded from any further analysis. Only one of the clonal groups with two representatives, DSdfm1 and DSdfm5, can be identified as belonging to the anaerobic bacterium 'strain 7' (Letowski *et al.*, 2001) with 98% sequence similarity. The remaining sequences closely related to uncultured environmental sequence assigned to the *Desulfotomaculum* genus. Clones RSdfm1, RSdfm2, and DSdfm1, -5, formed part of a cluster containing only environmental sequences and grouped along with known members of Group 1 SRB. Clones DSdfm3, -4 formed a novel lineage within the subgroup (Fig. 4.19) and are distantly related to *Desulfotomaculum geothermicum* and *Desulfotomaculum thermosapovorans*.

***Desulfobulbus* (DBB Group 2)**: Cloned PCR products that were positive upon hybridisation against probe DBB660, were analysed by TGGE (Fig. 4.16). Screening of clones on the basis of mobility defined three distinct groups in raw sludge and two distinct groups in digesting sludge (Table 4.11). Clonal groups RSdbb1 (with five representatives) and DSdbb1 (with four representatives) were identified as almost identical to the uncultured sulphate-reducing bacterium clone 8B (99% similarity) from a wastewater biofilm (Ito *et al.*, 2002). The clonal groups RSdbb7 and DSdbb2 (with two representatives) share strong sequence similarities with *Desulfobulbus propionicus* lineage and are most closely related to an uncultured SRB clone 10B

with 96% similarities (Ito *et al.*, 2002). Clone RSdbb2 also groups in this cluster and is most closely related to an as-yet undescribed SRB clone R-PropA1 with 96% similarity. Clones obtained from RS and DS did not correspond to any of the bands in the original RS/DS TGGE profiles. This could be explained by the fact that the SRB primers are degenerate and the TGGE major bands may not be SRB.

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

Cloned PCR products that were positive upon hybridisation against probe DCC868, were analysed by TGGE (Fig. 4.17). Screening showed all clones to co-migrate at the same point in both raw and digesting sludge. Two clonal representatives (RSdcc2, RSdcc5 & DSdcc3, DSdcc6) sample were sequenced and BLAST analysis showed them to be identical (Table 4.12) – closely related to the unidentified sulfate-reducing bacterium clone DSB-DSb99-3 (96%), from a rice field (Scheid *et al.*, 2001). These fragments formed a cluster within the phylogenetic groupings of *Desulfonema* and *Desulfococcus*. Clones obtained from RS and DS did not correspond to any of the dominant bands in the original RS and DS profiles. However, these clones possibly match a weaker band observed in the RS profile, not obvious in the DS profile.

***Desulfovibrio-Desulfomicrobium* (DSV-DMB Group 6):**

Cloned PCR products that were positive upon hybridisation against probe DSV687, were analysed by TGGE (Fig. 4.18). Screening of clones according to their mobilities on the gel revealed three groups in RS and four groups in DS (Table 4.13). In general, BLAST searches resulted mainly in matches with known *Desulfovibrio* spp within this sub-group. Clonal groups RSdsv1 and DSdsv2 can be identified as *Desulfovibrio desulfuricans* with 98% similarity. The similar identities and migration patterns suggest that these two groups are probably the same phylotype, passed on from the raw feed to digestion. Clonal groups DSdsv1 and RSdsv4 are closely related to *Desulfovibrio mexicoense* (96%) and *Desulfovibrio termitidis* (96%), respectively. Two clones (DSdsv5 and 6) in digesting sludge were identified as the environmental sequence VadinHA40 (98%), retrieved from an anaerobic digester (Godon *et al.*, 1997). Clone DSdsv3 was distantly related to *Desulfovibrio fairfieldensis*.

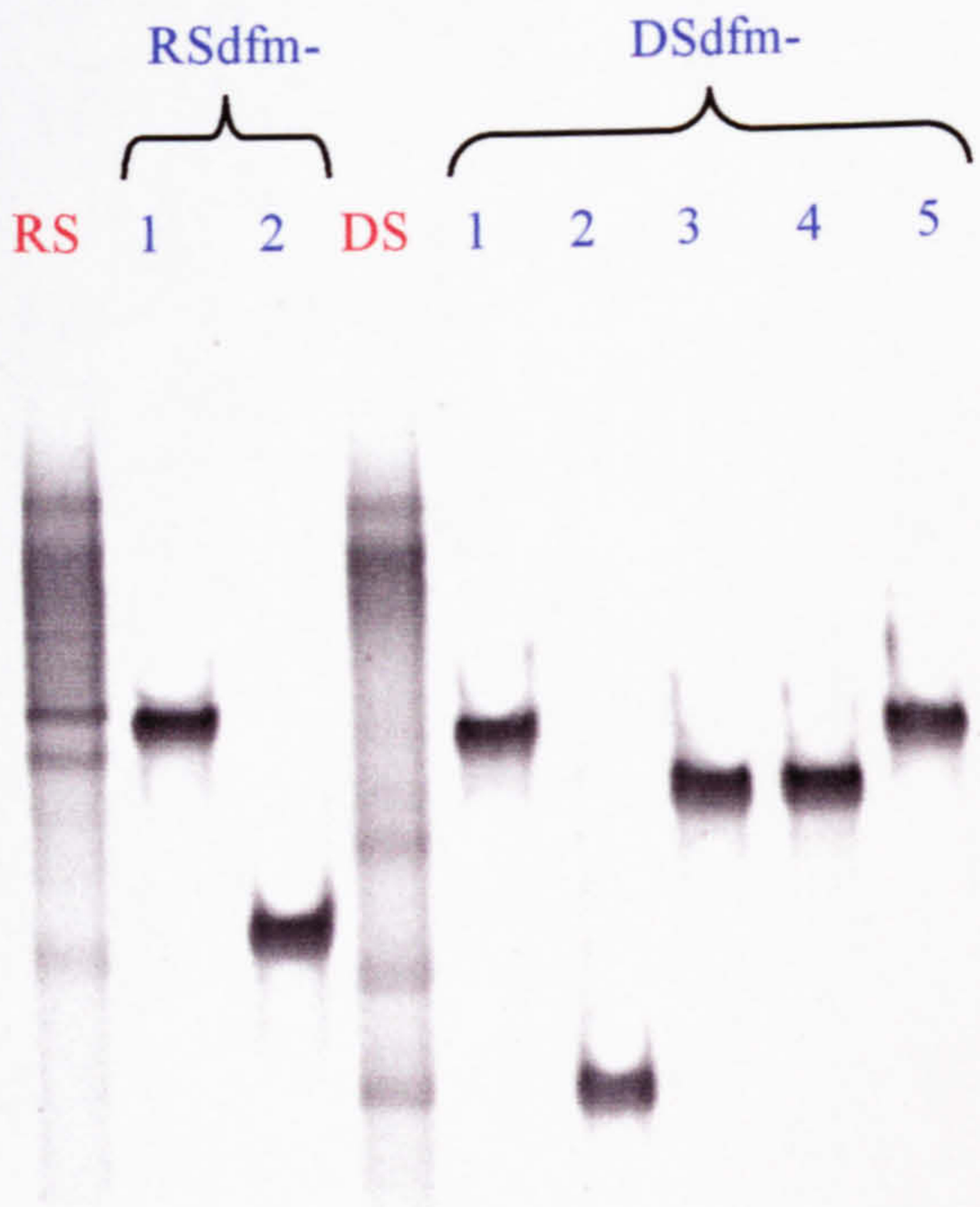


Figure 4.15. TGGE screening of raw (RS) and digesting sludge (DS) DFM 16S rDNA clones generated using SRB Group 1.

Table 4.10. Similarity values of SRB Group 1 16S rDNA sequences retrieved from raw and digesting sludge.

Clones	Closest Relative	Similarity	Sequence Length
RSdfm1	*Desulfotomaculum* sp. DEM-Kme99-2	96%	584bp
RSdfm2	Uncultured eubacterium WCHB1-20	95%	593bp
DSdfm1, DSdfm5	Anaerobic bacterium 'strain 7'	98%	594bp
DSdfm3, DSdfm4	Uncultured low G+C Gram- positive bacterium clone 36-20	95%	562bp
DSdfm2	Unreadable sequence		

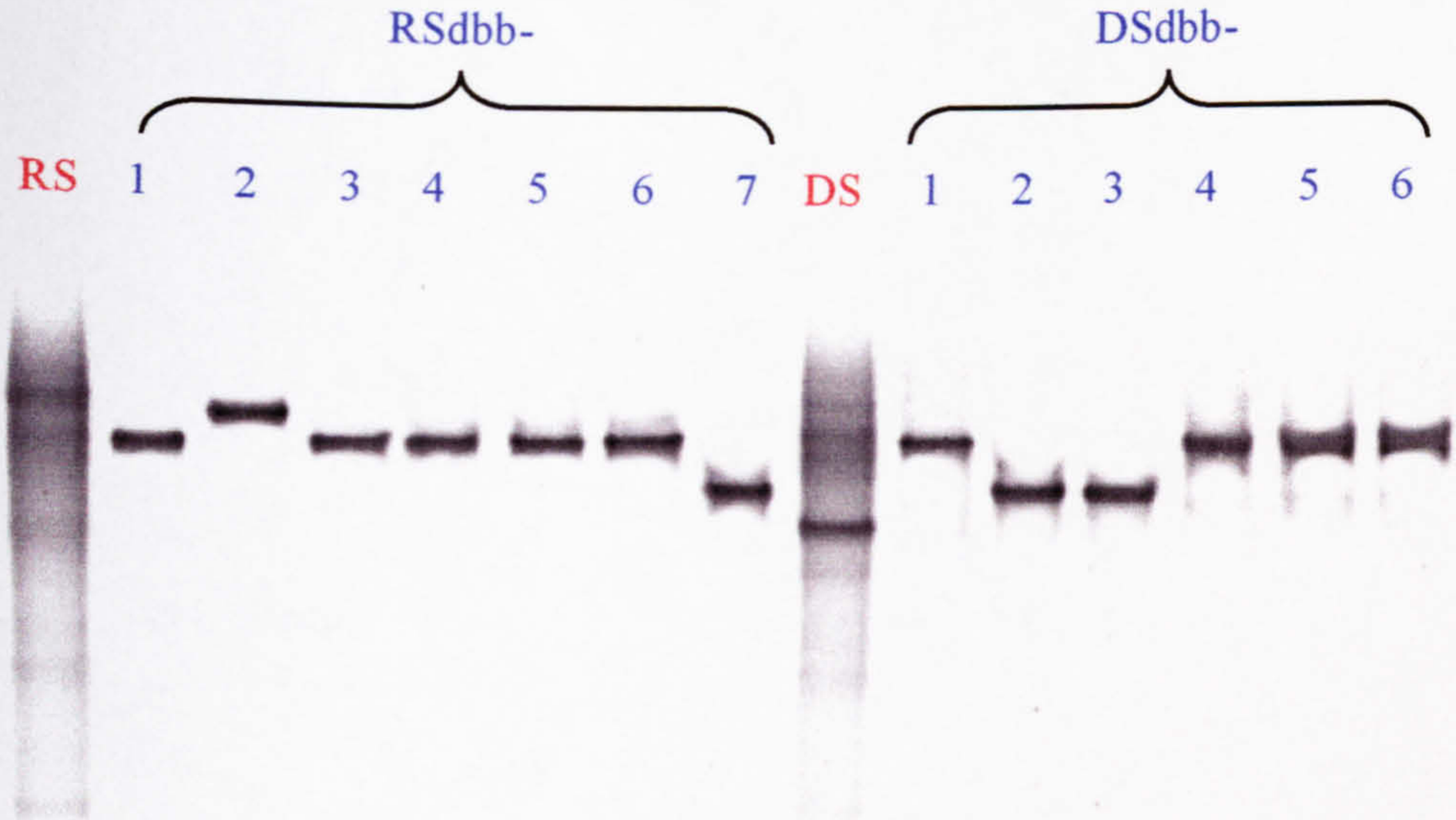


Figure 4.16. TGGE screening of raw (RS) and digesting sludge (DS) DBB 16S rDNA clones generated using SRB Group 2 amplification products.

Table 4.11 Similarity values of SRB Group 2 16S rDNA sequences retrieved from raw and digesting sludge.

Clones	Closest Relative	Similarity	Sequence Length
RSdbb1, RSdbb3, RSdbb4, RSdbb5, RSdbb6	Uncultured sulphate-reducing bacterium clone 8B	99%	665bp
RSdbb2	Sulphate-reducing bacterium R-PropA1	96%	665bp
RSdbb7	Uncultured sulphate-reducing bacterium clone 10B	96%	598bp
DSdbb1, DSdbb4, DSdbb5, DSdbb6	Uncultured sulphate-reducing bacterium clone 8B	99%	723bp
DSdbb2, DSdbb3	Uncultured sulphate-reducing bacterium clone 10B	96%	584bp

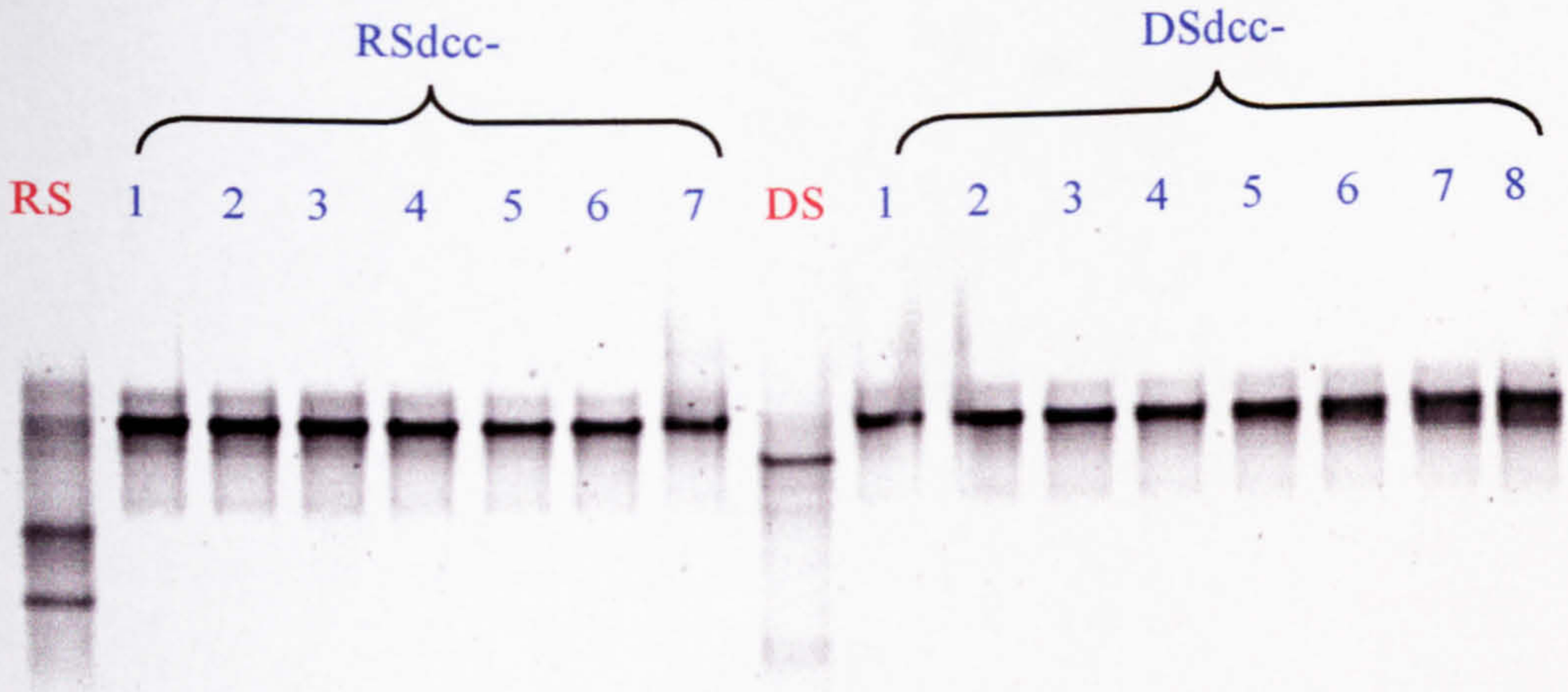


Figure 4.17. TGGE screening of raw (RS) and digesting sludge (DS) DCC 16S rDNA clones generated using SRB Group 5 amplification products.

Table 4.12 Similarity values of SRB Group 5 16S rDNA sequences retrieved from raw and digesting sludge.

Clones	Closest Relative	Similarity	Sequence Length
RSdcc2, RSdcc5	Unidentified sulphate-reducing bacterium DSB-DSb-99-3	96%	592bp
DSdcc3, DSdcc6	Unidentified sulphate-reducing bacterium DSB-DSb-99-3	96%	587bp

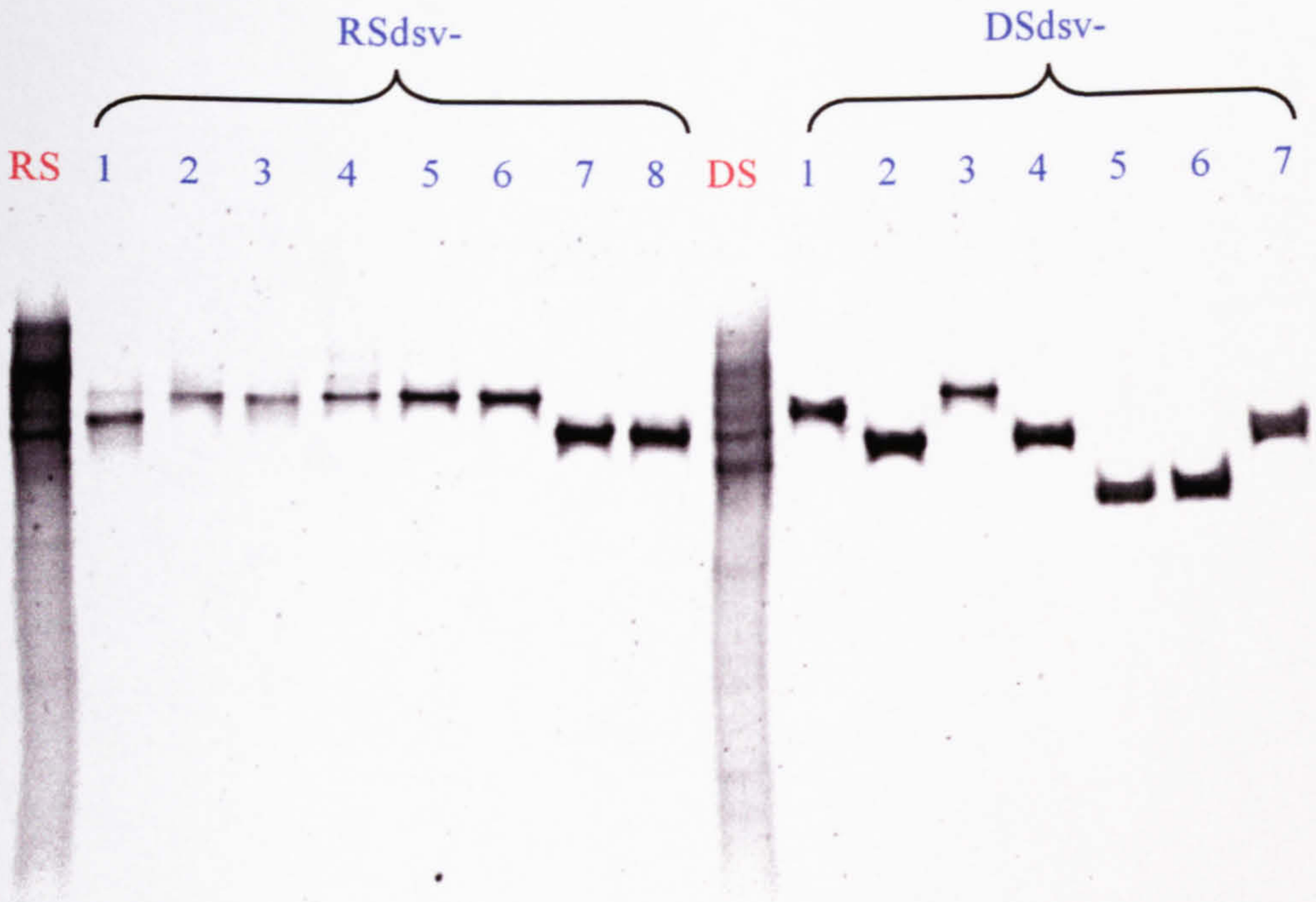


Figure 4.18. TGGE screening of raw (RS) and digesting sludge (DS) DSV 16S rDNA clones generated using SRB Group 6 amplification products.

Table 4.13. Similarity values of SRB Group 6 16S rDNA sequences retrieved from raw and digesting sludge.

Clones	Closest Relative	Similarity	Sequence Length
RSdsv1, RSdsv7, RSdsv8	*Desulfovibrio desulfuricans*	98%	540bp
RSdsv2, RSdsv3	*Desulfovibrio desulfuricans*	97%	544bp
RSdsv4, RSdsv5, RSdsv6	*Desulfovibrio termitidis*	96%	489bp
DSdsv1	*Desulfovibrio mexicoense*	96%	454bp
DSdsv2, DSdsv4, DSdsv7	*Desulfovibrio desulfuricans*	98%	555bp
DSdsv3	*Desulfovibrio fairfieldensis*	88%	465bp
DSdsv5, DSdsv6	Unidentified eubacterium clone vadinHA40	98%	528bp

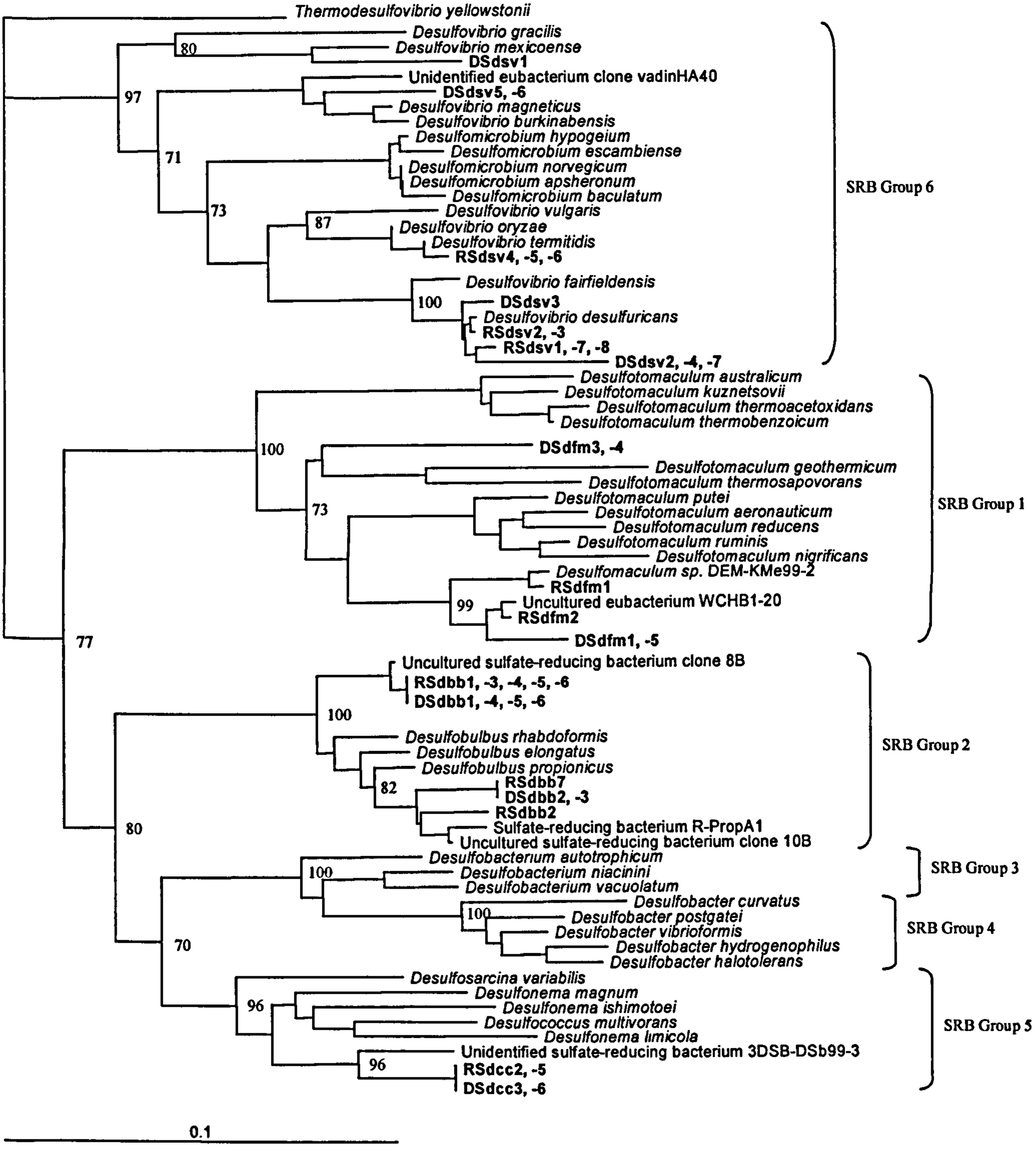


Figure 4.19. Phylogenetic tree generated from the alignment of the six SRB subgroups with 16S rDNA cloned sequences derived from PCR products amplified from raw and digesting sludge. The tree was constructed using the neighbour-joining method of Jukes & Cantor (1969) and analysis was based on 403bp nucleotides. Bootstrap values above 70% are shown. The *Thermodesulfovibrio yellowstonii* sequence served as the outgroup for rooting the tree. Bar shows estimated divergence in nucleotide sequences.

4.4. TGGE and sequence analysis of the Archaeal (methanogen) diversity in 16S rDNA amplified from raw and digesting sludge.

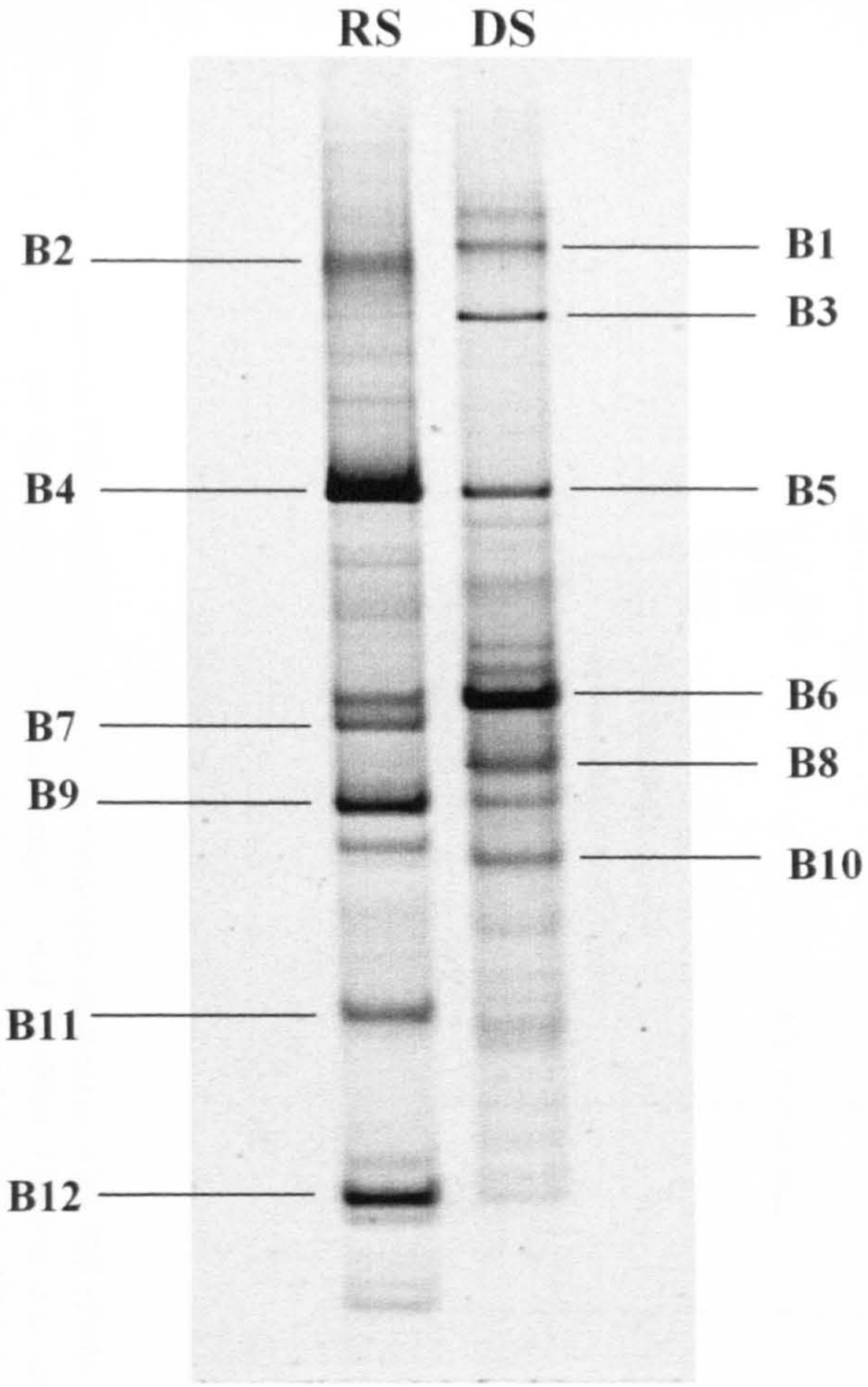
The archeal diversity of the digester ecosystem was determined by TGGE for both raw and digesting sludge. Archaeal PCR amplified products previously obtained using primers 1Af and 1404r (Chapter 3) were diluted appropriately and re-amplified using the GC primer pair 1100Af and 1404r(GC) to give a 304 bp fragment. The amplification reaction mixtures were as described in section 2.6, with 5 ng of archaeal-specific PCR product as a template and 25 amplification cycles at the annealing temperature listed in Table 2.5. Products from amplification with the GC primer pair were separated using the Bio-Rad TGGE system as described in section 2.12. Approximately 200 ng of products from each sludge were run at 80 V from 43 to 51°C (0.5°C h^{-1}). These running conditions were determined empirically and are optimal for producing well-separated bands for this group. The major bands in the TGGE gel were excised, reamplified and sequenced. The majority of the bands yielded quality sequence data, with the exception of two (B8 and B12) probably resulting from the co-migration of different sequences. Only data from bands that gave clear sequences were used for comparative sequence analysis.

An impressive archaeal diversity was observed in both raw and digesting sludge from the Exeter treatment plant (Fig. 4.20). In general, an equal number of discernable bands were present in both sludge types (ca.12 – 15) with a noticeable variation in the community structure between raw and digesting sludge. This suggests that the majority of the *Archaea* present in the digesting sludge were specifically selected for in the digester environment. TGGE profiles showed three obvious dominant bands, B4, B9 and B12, detected in the raw samples at very high relative intensity. The prominence of these bands decreased in the digesting sludge. However, bands B3 and B6 present in the raw sludge were apparently strongly selected within the digester.

Ten sequences in total were recovered from the two profiles and compared with available database sequences using BLAST. To investigate the relationship between microorganisms deduced from the sequence determined and reference strains, phylogenetic trees were constructed using DNA distance and maximum parsimony. Both trees showed similar topologies and bootstrap values to one another, and only one is therefore shown (Fig. 4.21). According to these analyses, all bands were confirmed as members of the domain *Archaea* and were most closely related to

methanogenic members of the *Euryarchaeota*. These sequences fell within two major phylogenetic groups, the *Methanosarcinales* and *Methanomicrobiales*.

Such is the shift in population, that only four bands were found to be common to both samples (B3, B4/B5, B6 and B9). Bands B4 and B5 ran to the same point on the gel and thus were sequenced to confirm that these similar migrating bands shared the same identity. All sequences except B3, B10 and B11, were most closely related to the uncultured archaeon clones 120A-4 and 61-2, from municipal wastewater sludge (Williams *et al.*, unpublished), and the TCB degrading clone SJD-114 (von Wintzingerode *et al.*, 1999), though the sequence identities were relatively low (91% - 96%). These sequences were shown to cluster within the *Methanosaeta* genus, implying the importance of these acetate-utilising organisms in digester function. The remaining sequences were closely related to *Methanospirilum hungatei* (B10, B11), whereas B3 was distantly related to the order *Methanomicrobiales*.



Band	Closest Relative	Similarity	Sequence length
B1	Uncultured archaeon 120A-4	91%	244bp
	Uncultured archaeon 61-2	91%	244bp
	Uncultured archaeon SJD-114	91%	244bp
B2	Uncultured archaeon 120A-4	94%	241bp
	Uncultured archaeon 61-2	94%	241bp
	Uncultured archaeon SJD-114	94%	241bp
B3	Uncultured archaeon clone LCD	93%	218bp
	Uncultured archaeon TA03	92%	218bp
	Methanocelleus sp. clone A3	92%	218bp
B4	Uncultured archaeon 120A-4	96%	246bp
	Uncultured archaeon 61-2	96%	246bp
	Uncultured archaeon SJD-114	96%	246bp
B5	Uncultured archaeon 120A-4	95%	287bp
	Uncultured archaeon 61-2	95%	287bp
	Uncultured archaeon SJD-114	95%	287bp
B6	Uncultured archaeon 120A-4	94%	253bp
	Uncultured archaeon 61-2	94%	253bp
	Uncultured archaeon SJD-114	94%	253bp
B7	Uncultured archaeon 120A-4	93%	242bp
	Uncultured archaeon 61-2	93%	242bp
	Uncultured archaeon SJD-114	93%	242bp
B9	Uncultured archaeon 120A-4	92%	214bp
	Uncultured archaeon 61-2	92%	214bp
	Uncultured archaeon SJD-114	92%	214bp
B10	*Methanospirillum hungatei*	94%	258bp
	Uncultured archaeon TA03	92%	264bp
	Uncultured archaeon clone GW70	92%	264bp
B11	Uncultured archaeon WCHA2-08	95%	241bp
	Uncultured archaeon clone LCD	95%	240bp
	Methanoculleus palmaeoli	95%	240bp
B8 & B12	poor sequence data		

Figure 4.20. Methanogenic archaeal diversity within raw (RS) and digesting sludge (DS) showing closest identity of bands from the TGGE gels of amplified 16S rDNA.

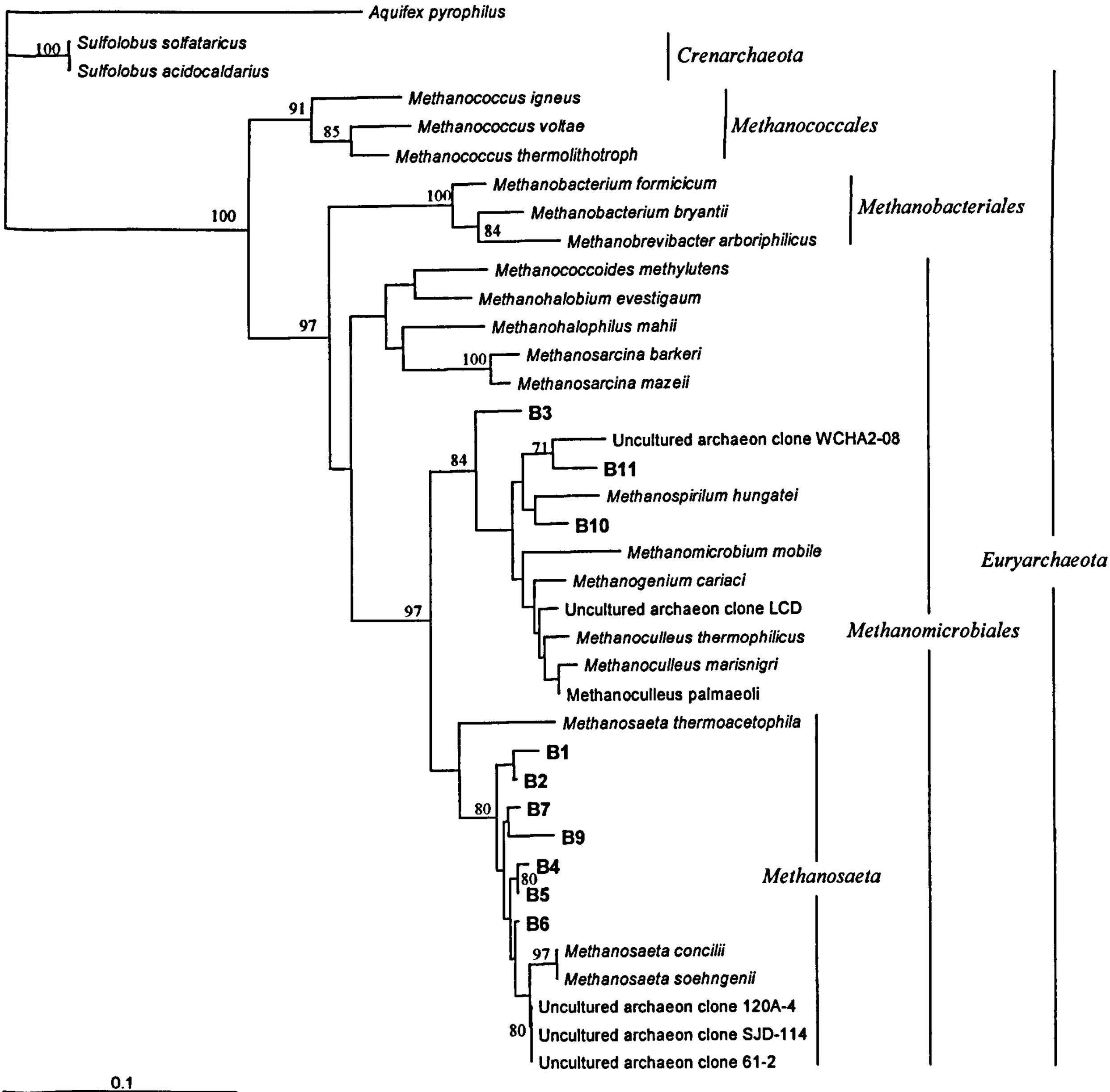


Figure 4.21. Phylogenetic tree generated from the alignment of methanogen sequences with 16S rDNA sequences recovered from bands B1 to B10 excised from raw and digesting sludge TGGE profiles. The tree was constructed using the neighbour-joining method of Jukes & Cantor (1969) and analysis was based on 268 nucleotides. Bootstrap values above 70% are shown. The *Aquifex pyrophilus* sequence served as the outgroup for rooting the tree. Bar shows estimated divergence in nucleotide sequences.

4.5. Discussion

It has been demonstrated here that the application of temporal gradient gel electrophoresis to wastewater sludge is a useful technique to reveal sequence diversity by generating population-specific fingerprints. The technique allows a relatively easy and quick comparison of profiles from related microbial assemblages and has now been widely used in many ecological studies (Muyzer and Smalla, 1998; LaPara *et al.*, 2000; Satokari *et al.*, 2001; Smalla *et al.*, 2001; Randazzo *et al.*, 2002). The microbial diversity of sub-groups of the major functional bacteria detected in chapter three was determined by the PCR-TGGE approach. The TGGE analysis performed on samples of raw and digesting sludge showed differences in population structure and composition within the digester in comparison to the feed. These changes in population are a direct consequence of the prevailing physio-chemical conditions in the digester, which imposes selection on the incoming microbial population.

An advantage of TGGE is that selected bands can be sequenced, and thus, the presence of a particular phylotype can be monitored in the environmental samples studied. However, due to the complexity of some TGGE-profiles obtained in this study, contamination-free excision of single TGGE bands was difficult. Therefore, only those bands that appeared distinct from others were excised and sequenced. Although TGGE is a convenient tool in this study for analysing community shifts, like most PCR-based molecular methods, it is not without limitations (see section 1.11.5, Chapter 1). One drawback is that only the most abundant bacteria within the population are detected, and it has been suggested that the empirical threshold for detection is approximately 1% of the total culturable population (Heur and Smalla, 1997). Moreover, only relatively short rDNA fragments can be separated within the gel, sequencing of which is sufficient to determine broad phylogenetic affiliations but inadequate to perform a precise phylogenetic analysis. Therefore, cloning of PCR products was performed to further assess the diversity in more detail. Clone libraries were only constructed for the sub-groups of SRB and clostridia. The partial sequences of the selected clones were analysed by the CHECK-CHIMERA program and no chimeric artefacts were detected. However, this cloning approach was not undertaken for *Archaea* since the banding pattern generated showed good separation of bands allowing sequence information to be determined by excision.

In this study, the 'nested' PCR products from Chapter 3 were chosen to facilitate the analysis of the 16S rDNA gene fragments of the different bacterial sub-groups by TGGE. TGGE analysis was attempted using the 'direct' PCR amplified 16S rDNA from SRB and clostridial subgroups but failed to generate any banding profile (data not shown), and thus were not used for further analysis. Previous work done on SRB in landfill by Daly (2000) has shown that the application of 'nested' PCR to TGGE reveals a greater diversity of sequences than 'direct' PCR. This would therefore suggest that the genetic diversity within specific populations would be underestimated if 'nested' PCR were not applied in investigations of microbial ecology employing PCR. However, the use of nested PCR should be evaluated with caution since a possible disadvantage of applying two or more successive PCR reactions is the introduction of an even greater bias due to preferential amplification (Suzuki and Giovannoni, 1996). According to some studies this bias of preferential amplification may be overestimated (Heur *et al.*, 1997; Heur and Smalla, 1997).

There is the possibility that individual bands in a TGGE gel may comprise two or more different sequence types that have co-migrated to the same point in the gel, thereby causing the diversity within the group to be underestimated. Conversely, one organism may produce more than one TGGE band because of multiple, heterogenous rRNA operons (Nubel *et al.*, 1996). It is also important to realise that when group-specific primers and probes based on cultured bacteria are used, only populations of known strains and their close relatives will be detected. Unknown members of the bacterial groups targeted may therefore escape PCR detection because their nucleotide falls 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.

Clostridium community structure

The four *Clostridium* clusters investigated here contain polysaccharide-degrading strains. The various steps in the hydrolysis of complex polymers should be achieved involving members of these clostridial groups. Here, the TGGE analysis of PCR products obtained with *Clostridium* group-specific primers generated profiles of banding patterns that could be used as a measure of genetic diversity within these *Clostridium* sub-groups in anaerobic sludge. The low number of individual bands observed in clusters I, IV and XIVab (Fig. 4.1) suggest that the genetic diversity

within each of these respective subgroups is relatively low. Similar banding profiles were observed in both raw and digesting sludge samples suggesting that these abundant phylotypes persist in the digestion process.

The cluster III profile shows a major shift in population from raw to digesting sludge, with only a few bands common to both sludges. Cluster III comprises only cellulolytic representatives (Collins *et al.*, 1994; Van Dyke and McCarthy, 2002), and not only do the TGGE profiles demonstrate selection, but suggest that this clostridia group is an active component of the digester population. It is not surprising that cluster III predominate since the cellulose content in most sewage sludge digesters is above 25% of the dry-solid portion (Hunter and Heckelekian, 1965). Cellulolytic members belonging to cluster III have been isolated from sewage sludge (Patel *et al.*, 1980), estuarine sediments (Madden *et al.*, 1982), and compost (Petitdemange *et al.*, 1984). In addition, molecular ecological studies have also shown their detection in cellulose-rich environments such as rice fields, landfill sites, rumen, and anaerobic digesters (Weber *et al.*, 2001; Van Dyke and McCarthy, 2002; Tajima *et al.*, 1999; Godon *et al.*, 1997). In comparison, PCR-TGGE studies have demonstrated cluster III and cluster IV to be the predominant groups in landfill sites with only one or two dominant phylotypes (Van Dyke and McCarthy, 2002).

Cluster I is the largest of the clostridial groups and consists of primarily saccharolytic and proteolytic strains. They have been isolated and characterised from the contents of a pilot scale, anaerobic digester treating waste with a high protein and lipid content (Jarvis *et al.*, 1999). During digestion, the predominant clonal sequence belonging to cluster I was closely related to the gut clone p406-o3 (99%), with over half the clones screened co-migrating with band ex3 in the original DS profile. The identity of band ex1 was closely related to *Clostridium magnum*, which is able to perform homoacetogenic fermentation and ferment carbohydrates to various fatty acids (Schink, 1984). Due to the high sequence similarity of the excised band (ex1 97%), the bacteria represented may operate similar metabolic pathways.

Clusters IV and XIV contain cellulolytic and noncellulolytic members and are shown to be present in anaerobic digester environments (Jarvis *et al.*, 1999; Godon *et al.*, 1997; Palop *et al.*, 1989). Many of the clonal sequences obtained for cluster IV in digesting sludge show close affiliation to environmental sequences previously obtained from a digester ecosystem (Godon *et al.*, 1997).

Phylogenetic analysis of 16S rRNA genes sequences from the amplified and cloned clostridial fragments revealed that they all belonged to their respective *Clostridium* clusters. As well as providing information on the phylogenetic affiliations of the amplified fragments, the sequence analysis also confirmed the specificity of the primers to specifically amplify and identify sequences that cluster within each respective subgroup. Since the analysis of clones can be laborious and time-consuming, only seventeen clones were randomly screened for each subgroup from both sludge types, partly because this is the maximum number that can be analysed on one TGGE gel. By comparing the single clone bands with those of the community profiles, the presumptive identities of some of the bands in the community profile were obtained. This method provides only circumstantial evidence of identity compared to sequencing of excised bands. However, those bands that were excised (ex1 to ex8) shared similar identity to the co-migrating clones and therefore in this report, the overall approach has credibility.

Clones were grouped according to their unique banding pattern and a much greater diversity was observed in this way for all four *Clostridium* sub-groups. Phylogenetic analysis of clones from all four clusters revealed that the majority of clones were closely related to as-yet-uncultured bacterial species in the digester to those found in anaerobic environments such as the rumen, rice paddy soils, and the gastrointestinal tract. This emphasises how poorly represented anaerobic bacteria are in laboratory collections of pure culture.

By screening seventeen clones, the actual diversity in the digester was only partially covered. This value was further lowered by the poor sequence data obtained from some of the screened clones, which accounted for up to a quarter of the clones analysed in some cases. However, it is not necessary to count every species in a community to estimate the number of different taxa within. The percentage coverage of a clone library can be calculated by applying mathematical modelling thus providing some indication of population type and abundance (Curtis *et al.*, 2001; Mullins *et al.*, 1995). Nevertheless, the collective information obtained from TGGE and cloning provides a glimpse of the difference in community structure and composition before and during digestion.

SRB community structure

TGGE banding profiles demonstrated differences in SRB community structure between the two sludge types with the overall trend of decreased SRB diversity in the digesting sludge for most of the subgroups. The *Desulfotomaculum* group (Group 1) showed the greatest diversity in both sludge types with the greatest number of individual bands (≤ 6). However, the banding pattern generated was not the same for both sludges and showed very few co-migrating bands. In all other groups, a low number of individual bands were observed suggesting limited SRB diversity within these groups. The *Desulfobulbus* group (Group 2) and the *Desulfococcus* group (Group 5) showed an overall decrease in diversity from raw to digesting sludge, whilst the *Desulfovibrio* group (Group 6) was indifferent. In nearly all cases there was very little or no evidence of co-migrating bands between raw and digesting sludge. In contrast to these results, a study by Rooney-Varga *et al.*, (1998) demonstrated by sequencing enrichment cultures and environmental clones, that a high level of SRB diversity inhabited a salt marsh sediment. Salt marshes are a major habitat for SRB and sulfate reduction rates in these environments are one of the highest of any natural systems (Howarth, 1993). Therefore, a high level of diversity in this environment would be expected.

Although, the results presented here show a decrease in diversity at a generic level, it is also possible that there could be an increase in species diversity. A decrease in SRB diversity is expected otherwise the normal functioning of biological processes in the digester may become upset and result in process failure if the concentrations of sulphides are high. The anaerobic digester is operated to promote methanogenesis and stabilisation should select against SRB under normal conditions. SRB are one of a number of functional groups whose population structure and activity in the digester may provide an indicator of digester function and possibly a predictor of process failure. So, a diverse community of SRB enters the digester, the operation of which imposes a selective pressure so that SRB diversity decreases.

Due to the degenerate nature of the SRB group-specific primers, it is difficult to comment weather or not the genetic diversity in raw and digesting sludge TGGE profiles is a meaningful reflection of the SRB population present. 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 TGGE profiles, could lead to inaccuracies in measurements of genetic diversity

within SRB subgroups. Ideally, DNA bands separated by TGGE 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 the SRB described by Daly (2000) and Devereux *et al.*, (1992) do not target within the region of the 16S rRNA gene used for TGGE analysis. However, attempts were made to excise bands but proved fruitless yielding poor sequence data.

The identification of SRB-related DNA bands observed in TGGE profiles was investigated by sequencing of co-migrating clones. Only those clones that gave a positive signal upon hybridisation with respective group-specific probes were screened on a TGGE gel alongside the environmental profiles. This approach revealed limited sequence diversity within each SRB subgroup in both raw and digesting sludge. Clone sequences from SRB groups 1, 2 and 5 were phylogenetically related to yet-as-uncultured bacterial species retrieved from other anaerobic environments. Only one unique banding pattern was observed for group 5 suggesting limited sequence variation in this subgroup. Mainly all SRB Group 6 cloned fragments were phylogenetically related to a known SRB group 6 member, the *Desulfovibrio* genera.

Methanogenic archaeal community structure

The work described in chapter three detected methanogenic archaea in both raw and digesting sludge samples by PCR amplification and oligonucleotide probing. Here, the archaeal diversity of the digester ecosystem was further assessed by TGGE analysis and compared with that of the raw sludge that serves as the feed source. In the TGGE fingerprint pattern, bands (B4/B5, B6, B9) were observed at the same gel positions for both raw and digesting samples. The bands at the same position of the fingerprint were likely to have originated from the same phylotype, which was supported by the identical band sequences retrieved from the raw sludge (B4) and the corresponding band in the digesting sludge (B5), both sharing high sequence similarity. This suggested that the archaeal community structures within raw and digesting sludge samples were comparable. However, on comparison of the two samples, a difference in banding pattern was clearly observed suggesting selection of certain methanogenic phylotypes under anaerobic digester conditions.

The TGGE bands excised and sequenced corresponded to methanogenic archaea from the *Euryarchaeota* and were observed in both raw and digesting sludge, which

suggests that the occurrence of methanogenic archaea is widespread and not just confined to the anaerobic sludge digester. *Archaea* have been detected in aerobic wastewater treatment processes essentially activated sludge plants. Anoxic microenvironments in activated sludge flocs (Schramm *et al.*, 1999) have been shown to harbour active methanogen populations (Gray *et al.*, 2002). Gray and co-workers detected 16S rRNA sequences related to the *Methanosarcinales*, *Methanomicrobiales* and *Methanobacterilaes*, and discovered that these populations, although active, play a minor role in carbon turnover in activated sludge due to the low rates of methanogenesis measured.

Nucleotide sequences related to *Methanosaeta*, a genus of acetoclastic methanogens, were found to be a major archaeal group using TGGE here. This is not surprising since their numerical dominance over other methanogens in anaerobic reactors has been reported previously (Ficker *et al.*, 1999; Merkel *et al.*, 1999; Sekiguchi *et al.*, 1999). Bands B1, B2, B4, B5, B6, B7 and B9, formed part of a cluster closely related to *Methanosaeta concilii*, *Methanosaeta soehngenii* and clones derived from environmental samples including municipal wastewater sludge (Williams *et al.*, 2001) and a trichlorobenzene-transforming microbial consortium (von Wintzingerode *et al.*, 1999). This demonstrates the capability of TGGE analysis to resolve DNA fragments with high sequence identities, and to provide information on the occurrence and distribution of closely related phylotypes. Band B6 had a higher relative intensity to its corresponding band in raw sludge, and to any other band in the digesting sludge profile. This may suggest the importance of this *Methanosaeta*-related phylotype in anaerobic methanogenic communities to metabolise acetate into carbon dioxide and methane. Such assumptions need to be treated with caution since PCR amplification is not quantitative, as preferential amplification can occur (Reysenbach *et al.*, 1992; Suzuki *et al.*, 1996). Therefore, band intensities cannot be extrapolated to indicate the abundance of a particular bacterial population, but they do provide information that is indicative. In addition, the phylotypes (B3, B10, B11) closely related to the genera *Methanosprilium*, *Methanoculleus*, and *Methanogenium* were retrieved and found in both sludge types. These species utilise hydrogen and formate for methanogenesis and are commonly found in anaerobic reactors.

Although the characterisation of archaeal communities detailed here are by no means a comprehensive report and sequence data was not obtained for every band, on balance the results indicate the predominant methanogen groups present and

demonstrates changes in population from raw to digesting sludge. For a more thorough analysis of methanogenic community structure in these sludge samples, cloning and sequencing of archaeal 16S rDNA fragments would need to be applied.

By using TGGE coupled with sequence analysis of cloned 16S rDNA fragments, differences in the community structure of members of *Clostridium*, sulphate-reducers and the methanogenic *Archaea* have been shown between raw and digesting sludge. To my knowledge, there have been no published data highlighting the differences on the composition of the microflora before and during digestion. Furthermore, this study targets key groups directly with the application of specific primers and probes. The potential of such tools and techniques to monitor key populations and their response to perturbations is therefore demonstrated.

CHAPTER FIVE

5. Archaeal population changes during anaerobic digestion in a lab-configuration reactor inoculated with primary raw sludge.

5.1. Introduction

In the previous chapter, TGGE has provided a comparative means to assess the genetic diversity of microbial groups before and during digestion based on analysis of samples from a full-scale commercial anaerobic digester.

However, the potential of this technique to study microbial communities over longer time periods has been demonstrated for a number of environments (Ito *et al.*, 2002; Smit *et al.*, 2001). This allows many samples to be taken at different time intervals and are simultaneously analysed by TGGE providing a relatively easy and quick comparison of microbial profiles over time for a given ecosystem. Community analysis involving multiple samples is best achieved using TGGE since the cloning approach is relatively time-consuming and labour intensive. The TGGE technique can be used to monitor shifts in microbial populations that occur in response to environmental perturbations (Atlas *et al.*, 1991; Ferris *et al.*, 1997; Ferris and Ward, 1997; Eichner *et al.*, 1999). The chapter employs TGGE to monitor the archaeal diversity over time in an operational small-scale lab based bioreactor.

5.2. Bioreactor set up and operation

An anaerobic, bench-top, continuously stirred fermentor with a six litre working volume was set up and operated at the Brixham Environmental Laboratory (Fig. 5.1). The bioreactor used in this study was supplied by Adaptive Biosystems Limited (U.K.). The bioreactor was fed with six litres of raw sludge obtained from the settling tanks at the Exeter treatment plant. This is the same source of raw sludge as analysed in chapters 2 and 3. Sludge digestion in the bioreactor was allowed to proceed by creating the same operating conditions used in the full-scale digester at the Exeter treatment plant. Monitoring and maintaining these conditions (pH, temperature, O_2 levels) was achieved by measurements collected from internal probes in the bioreactor and fed to a computer system. The bioreactor was operated under mesophilic conditions (35 °C) with the pH maintained between 6.8 – 7.2. The pH was maintained by the addition of acid or alkali. Any dissolved oxygen was removed from the vessel by bubbling the stirred media extensively with oxygen free nitrogen. Subsequently, the sludge was aerated in an oxygen-free atmosphere under a constant pressure of 100% nitrogen.

The reactor was in operation for a period of one month (32 days in total), during which time, sampling was carried out every fourth day. Samples (80 ml) were taken from the fermentor and stored in 50 % ethanol at –20 °C for molecular biological analysis.

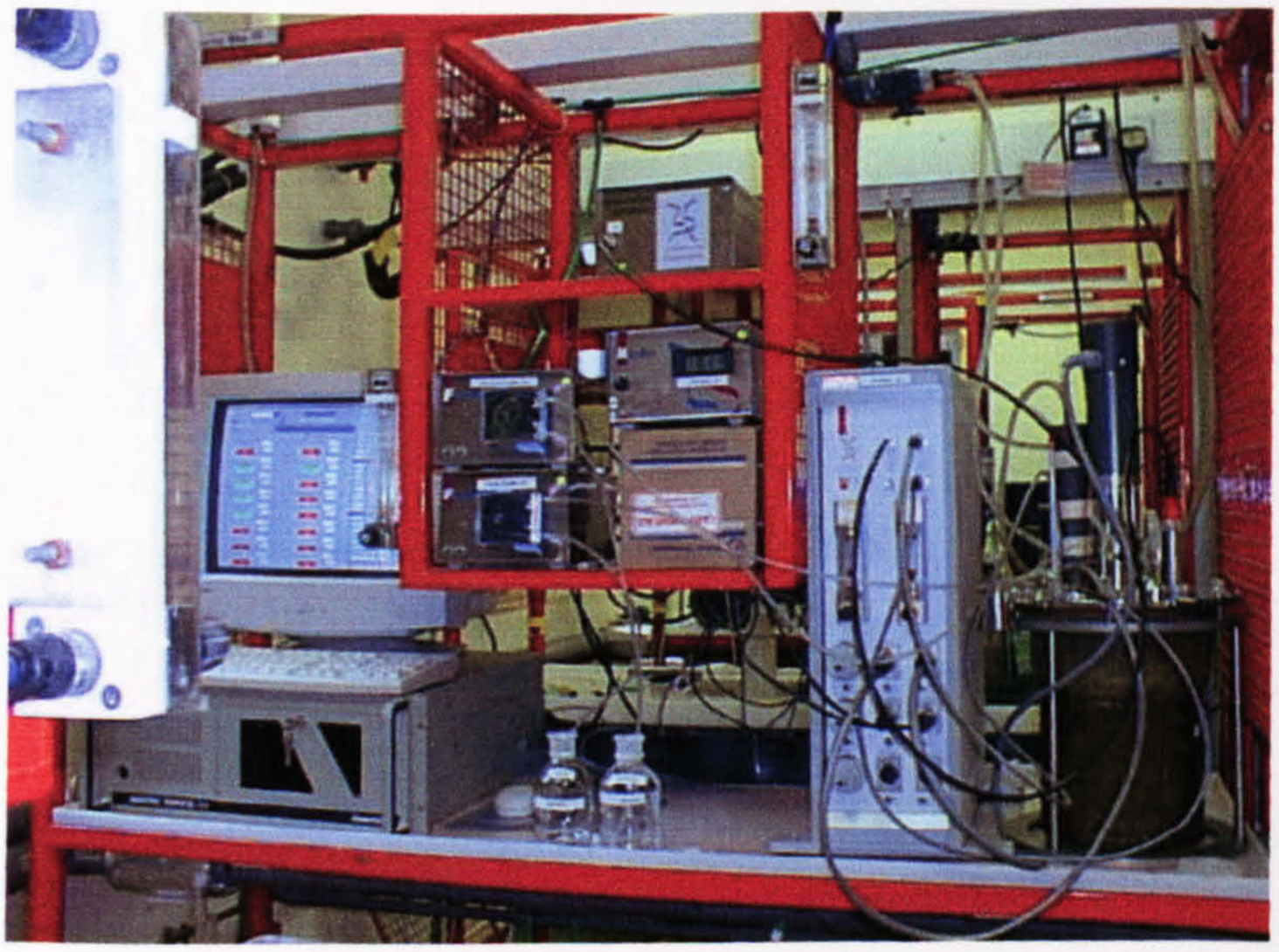


Figure 5.1. Lab scale bioreactor set up at the Brixham Environmental Laboratory

5.3. Molecular biological analysis of samples

Due to time constraints, only the Archaeal population dynamics were studied. A total of nine 80 ml samples were taken from the reactor for analysis. This included the raw sludge for inoculation (t=0) and eight subsequent samples taken every fourth day during operation (t =4, t =8, t =12, t =16, t =20, t =24, t =28, and t =32). As described in chapters two and three, DNA extracted from each of these sludge samples was subjected to PCR amplification using archaeal-specific primers (1Af and 1404r) under the appropriate PCR reaction conditions. Agarose gel electrophoresis of these PCR amplified products confirmed the detection of Archaeal DNA in all nine samples (Fig. 5.2).

For community analysis, Archaeal PCR products obtained using primers 1Af and 1404r were diluted appropriately and reamplified using the GC primer pair 1100Af and 1404r(GC). Products from amplification with the GC primer pair were separated using the Bio-Rad TGGE system at a running voltage of 80V, and temperature range 43 to 51°C (0.5°C h^{-1}). Repeated TGGE runs of the same PCR product produced similar banding profiles (data not shown). Running the samples in parallel on one temperature gradient gel enable the easy comparison of the banding patterns generated. From the fingerprints obtained, up to nine clearly visible bands were observed (Fig. 5.3). The bands in the profile represent most of the dominant microbial populations in the community, and their appearance and disappearance reflect important changes in the microbial community structure over time. In order to identify the species corresponding to certain bands in the TGGE profile, bands were excised from the acrlyamide gel, re-amplified and sequenced.

On the whole, the resulting banding pattern across the 32 day period was largely similar with only minor differences observed. A population shift with a minor increase in the number of visible bands was observed between t=12 and t=16. The appearance of band 7 at t=16, not observed previously in samples, was present up to t=28 and sequence analysis showed it to be closely related to *Methanospirilium hungatei* (93%) over 242 bp. Bands 2, 3, 4, 5 and 6 were present at t=0 and remained throughout digestion, with the exception of band 2 which disappeared between t=24 and t=28. However, band 8 only appeared during digestion and was not observed in the inoculating sludge. Another example of reactor selection is band 1, which appears at t =16 and disappears at t = 32.

The intensity of bands 4 and 6 increased at the start of digestion but gradually decreased as digestion proceeded. These sequences were closely related to the uncultured archaeon clone 120A-4 (similarity > 90% over 240 bp), previously isolated from municipal wastewater sludge (Williams *et al.*, Unpublished). Band 3 was present throughout all samples with the same visual intensity and was also closely related to clone 120A-4 (94% similarity over 244 bp). It is tempting to interpret band intensity in relation to species abundance but this must be approached with caution, because of the quantitative limitations of DNA extraction and PCR amplification (Suzuki *et al.*, 1996; Polz *et al.*, 1998). However, the intensity of an individual band can serve as an indicative measure for the relative abundance of this sequence in the population (Muyzer *et al.*, 1993).

Noteworthy is the presence of band 9, observed only in t=24 but no other samples. Attempts were made to excise and sequence this band, as with bands 1, 2, 5, and 8, but the sequence data obtained was poor.

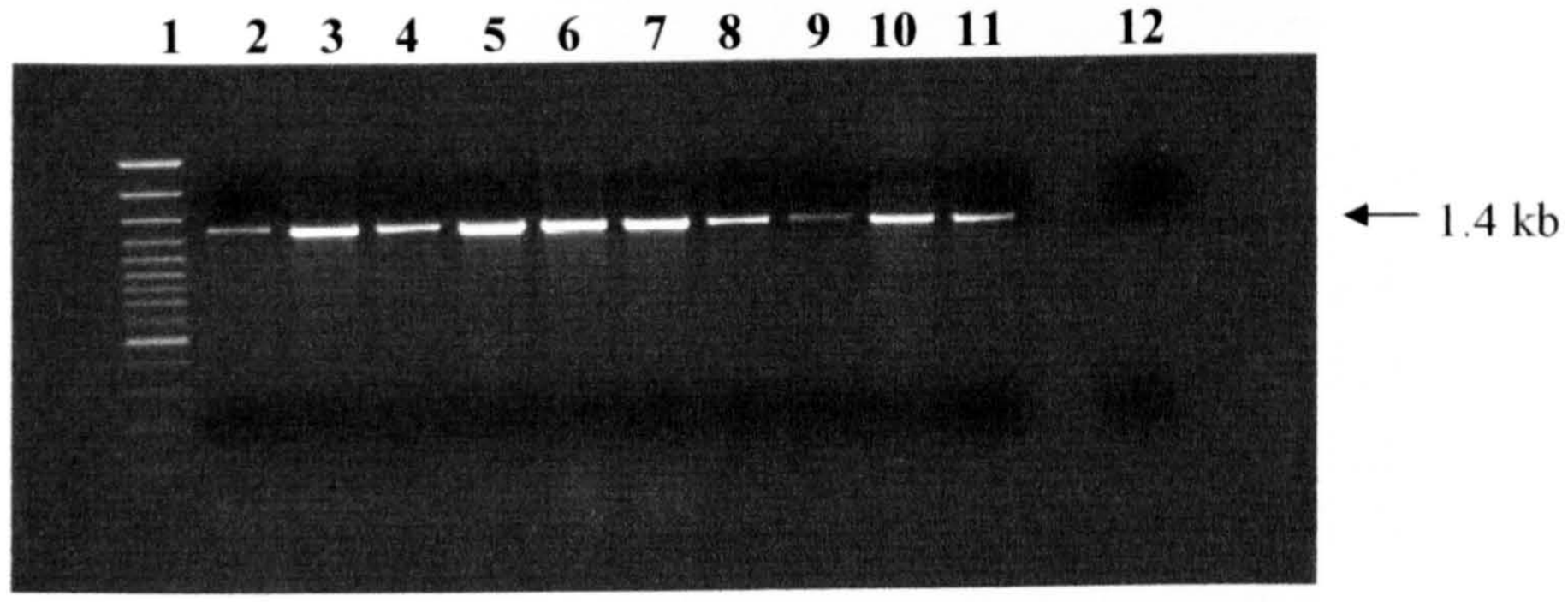


Figure 5.2. PCR amplification of archaeal 16S rDNA from reactor samples t = 0 to t = 32 using primers 1Af and 1404Ar (Munson *et al.*, 1997). **t** is the time in days on which sludge samples were taken from the reactor.

Lane 1 - Marker 21, pBR322 DNA/*Alw*441/*Mva*1 (MBI Fermentas);
Lane 2 – t = 0
Lane 3 – t = 4
Lane 4 – t = 8
Lane 5 – t = 12
Lane 6 – t = 16
Lane 7 – t = 20
Lane 8 – t = 24
Lane 9 – t = 28
Lane 10 – t = 32
Lane 11 – *Methanolobus tindarius* (positive control)
Lane 12 – PCR blank

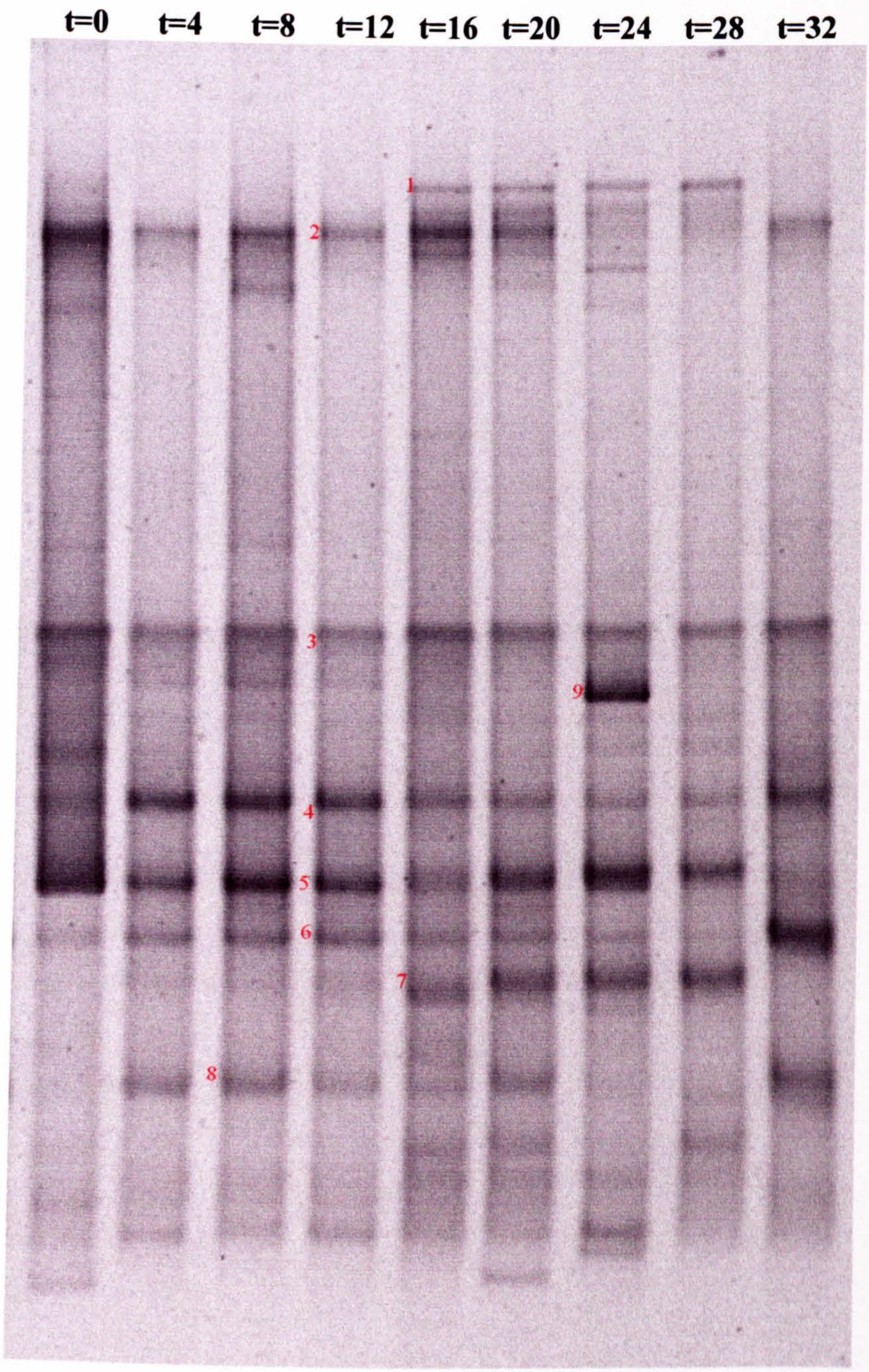


Figure 5.3. TGGE patterns of the *Archaeal* 16S rDNA fragments amplified from reactor samples. Dominant TGGE bands clearly visible are those designated 1 to 9. **t** is the time in days on which sludge samples were taken from the reactor.

5.4. Discussion

The study described here demonstrates that PCR-TGGE is a potentially powerful tool for monitoring microbial populations in anaerobic digesters. It is unfortunate that other anaerobic bacterial populations could not be maintained in the same way, and is clearly a priority for future work. Hopefully, the establishment of stable bacterial signatures for lab scale anaerobic reactors will provide a benchmark for determining the effect of perturbations, particularly the introduction of waste streams containing xenobiotics.

A laboratory scale batch-type bioreactor was set up by loading the vessel with raw sewage and allowing it to digest. Sludge samples were taken every fourth day and changes in Archaeal community structure was visualised by the TGGE banding profiles. The DNA-based fingerprints indicated that there are several dominant phylotypes that are relatively stable over the 32-days of digestion. The stability of these phylotypes is a characteristic of this digester, thus suggesting that each digester-type may have its typical set of dominant phylotypes. However, this tenuous conclusion would have to be substantiated by applying the same approach to other digester types treating various wastes.

Since time was a severe limiting factor in this study, digestion was only allowed to proceed for 32 days. This given time may not be enough for the microbial population to fully develop for optimum digestion. However, most digesters have a sludge retention time of between 15 and 35 days.

The sequencing of certain bands (bands 3, 4 & 6) from the digestion phase identified sequences belonging to the *Methanosaeta* group, closely related to the uncultured archaeon clone 120A-4 (Williams *et al.*, unpublished). This supports the sequence data obtained from the digestion profile in the previous chapter (section 4.4, Chapter 4) where bands were also found to be closely related to clone 120A-4. This suggests that the lab-scale bioreactor undergoing digestion can mimic a real scale reactor if physiochemical parameters are maintained throughout.

Methanogenic bacterial population dynamics during the start-up of anaerobic digesters has also been investigated using both molecular probe and fingerprinting methods (Griffin *et al.*, 1998; Leclerc *et al.*, 2001). In both cases, *Methanosaeta* species, which were the most abundant methanogens in the inoculum, were replaced by *Methanosarcina* species. This is because *Methanosaeta* spp. have a low threshold for acetate and therefore for have a competitive advantage over *Methanosarcina* spp.

at low acetate concentrations. However, if acetate accumulates to higher levels, *Methanosarcina* spp generally dominate (Zinder 1993). This shows some agreement with this study as the intensity of bands 4 and 6, identified as belonging to *Methanosaeta*, decreases as digestion proceeded. In order to determine if *Methanosarcina* species flourish as digestion occurs sequence information from other visible bands latter in digestion need to be analysed. The introduction and stability of band 7 from t=16 onwards may be a possible contender as a *Methanosarcina* spp. In addition, changes in acetate levels in the digester as digestion proceeds would provide some indication of methanogenic activity.

Although pH, temperature and oxygen levels were controlled, no other physiochemical measurements were determined (e.g. BOD, COD, CH_4 production rate, total solids and volatile solids) therefore additional information on digester stability and methanogen activity cannot be deduced.

Archaeal and bacterial community dynamics have been investigated in a methanogenic reactor fed with glucose by comparing ARDRA pattern frequencies during the course of reactor operation (Fernandez *et al.*, 1999). Their results showed that during a two-years long period, digesters that appeared to be functionally stable in respect to environmental parameters were not stable in respect to the microbial community. The dominant microorganisms detected by ARDRA changed continuously.

The significance of this small study is to demonstrate that this technique will be useful for monitoring changes in the microbial populations of digesters, which are undergoing stress from environmental perturbations. This can be in the form of toxic chemicals and xenobiotics in the feed as well as changes in operating conditions (e.g. pH, temperature, etc.). An understanding of the relationship between reactor performance and microbial community dynamics is necessary in order to enhance bioreactor predictability and reliability. In addition, the development and application of genus- and group-specific PCR with TGGE is valuable to study targeted microbial populations in the complex digester ecosystem.

CHAPTER SIX

6. General Discussion

Anaerobic processes involved in wastewater treatment have been long recognised as a key factor in the treatment of biodegradable wastes, but this is not reflected in the knowledge of the underlying microbiology, often viewed as a 'black box' process. An appreciation of the structure and function of complex microbial communities that drive the anaerobic digestion process would be a good starting point in achieving digester optimisation with respect to design, operation and control (Amann *et al.*, 1998). Early attempts to gain information on the microbiology of anaerobic sludge digesters were achieved with classical techniques. This involved isolation studies involving traditional culture-based techniques applied to human feces (Betian *et al.*, 1977; Moore *et al.*, 1974) sewage sludges (Patel *et al.*, 1980; Murray *et al.*, 1984) and anaerobic digesters (Palop *et al.*, 1989; Yang *et al.*1990). The former two serve as the feed source for many municipal digesters, and thus harbour microorganisms that may be directly involved in the digestion process. However, a detailed analysis of the microbial composition and structure has not been possible using this approach, and this in turn reflects the difficulty in studying the microbiology of anaerobic digesters.

It is with the advent of molecular biological techniques that over the last decade increased attention has been focussed in this area and with it has unleashed a wealth of information on digester microbiology involved in the treatment of various wastewaters (Plumb *et al.*, 2001; Sekiguchi *et al.*, 1998; Lapara, *et al.*, 2000; Raskin *et al.*, 1995; Godon *et al.*, 1997; Wu *et al.*, 2001; Ng *et al.*, 1994).

In this thesis, the community structure of three functional bacterial groups involved in the anaerobic degradation of organic matter was assessed by molecular analyses. These included four clostridial groups (groups I, III, IV, and XIVab; Collins *et al.*, 1994) involved in the hydrolysis of cellulose and other polymeric materials, and the two major groups involved in the terminal stages of anaerobic digestion, the sulphate-reducing bacteria and the methanogens. More specifically, this study was intended to compare the differences in community structure of these functional groups before (raw sludge) and during digestion (digesting sludge) so that selection of community members during digestion could be monitored. Unlike published culture-independent studies of digester microbiology which identify the bacterial

community structure by setting up clone libraries using general conserved primers (Godon *et al.*, 1997; Sekiguchi *et al.*, 1998), this study makes use of molecular tools specific to target the subgroups within these functional groups investigated. In addition to detection of these groups by means of PCR and oligonucleotide probing (where applicable), TGGE assessment of genetic diversity coupled with sequence analysis of clones and excised bands allowed phylogenetic analysis of the targeted groups.

The presence of clostridial cluster I, III, IV and XIVab 16S rDNA was demonstrated in both raw and digesting sludges. The detection of these groups was only possible using a 'nested' PCR protocol, suggesting their presence at low levels in anaerobic sludge.

From the six SRB groups targeted, all but two groups – *Desulfobacterium* (Group 3) and *Desulfobacter* (Group 4) were detected. The absence of *Desulfobacterium*-like members in sludge samples is not surprising since most of the known species of this genus are associated with the marine environment (Postgate, 1984; Fauque, 1995), and nor were members of this group detected in a study of landfill leachates (Daly *et al.*, 2000). However, their detection has been reported in anaerobic digester sludge at very low levels by rRNA analysis involving total nucleic acid extraction followed by probing (Raskin *et al.*, 1995). The same study also detected *Desulfobacter* spp (Group 4), but again at very low levels. This group of sulphate reducers primarily degrade acetate and their lack of detection here is probably down to successful competition from acetate-utilising methanogens (*Methanosaeta*). The dominant SRB subgroup during digestion was shown to be the *Desulfococcus* group (Group 5) since this was the only SRB group detected by 'direct' PCR. However, 'nested' PCR revealed the presence of SRB subgroups not initially detected by 'direct' PCR thus suggesting their presence at low levels.

Methanogenic populations belonging to the *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, and relatives of the *Methanosarcinaceae*, which include the genera *Methanosarcina* and *Methanosaeta*, were detected in both raw and digesting sludges. The most significant is the predominance of *Methanosaeta* spp. over *Methanosarcinia* spp as digestion occurs. However, 16S rRNA qualitative studies have previously shown the abundance of *Methanosaeta* spp. in anaerobic digesters containing low acetate concentrations (Oude Elfernik *et al.*, 1998; Raskin *et al.*, 1994b; Raskin *et al.*, 1995).

The genetic diversity within all these groups amplified from raw and digesting sludge samples was investigated by temporal gradient gel electrophoresis. TGGE is a technique that offers the potential to analyse 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 members in profiles being a measure of genetic diversity within specific populations. Comparison of diversity between raw and digesting sludges demonstrates differences in population structure suggesting selection imposed during digestion with respect to the incoming population. Differences in the banding patterns generated for raw and digesting sludges clearly showed selection imposed on populations of SRB, methanogens and clostridia, in particular cluster III (members of which degrade cellulose only).

Screening of clones generated from specifically-amplified PCR products by TGGE to identify sequences of interest was performed in order to increase the amount of sequence information available. Sequence analysis of cloned clostridia and SRB 16S rDNA fragments followed by phylogenetic analysis have shown them to cluster within the specific subgroups for which the PCR primers and oligonucleotide probes were designed. For methanogens, selected bands were excised and sequenced and phylogenetically confirmed to be related to methanogenic members.

Although this study proved far from exhaustive, the majority of the sequences analysed were identified as being new as yet undescribed species and this correlates well with the findings of other digester studies (Godon *et al.*, 1997; Sekiguchi *et al.*, 1998; Fernandez *et al.*, 1999, 2000).

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

The use of rRNA, however, would not provide a direct link to sulphate-reducing or methanogenic activity. Functional gene targets such as the *mcrA* gene and the sulfite reductase gene would have to be targeted to provide information on actual methanogenesis and sulphate reduction, respectively.

7. References

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APPENDIX

Aligned nucleotide sequences of cloned 16S rDNA fragments from raw and digesting sludge.

Clostridium cluster I clones 578bp

gIRS4	AAAGGATTTA	TATC-GCTTT	GAGATGGGCC	C-GCGGCGCA	TTAGCTAGTT
gIDS2	AAAGGATGTA	TATC-GCTTT	GAGATGGGCC	C-GCGGCGCA	TTAGCTAGTT
gIRS1	AAAGGATGTA	ATCC-GCTGT	AAGATGGGCC	C-GCGGCGCA	TTAGCTAGTT
gIRS2	AAAGGATGAA	ATCC-GCTAT	GAGATGGACC	C-GCGGCGCA	TTAGCTAGTT
gIDS3	AAAGGAGGCA	ATACCGCTAT	GAGATGGGCC	C-GCGGCGCA	TTAGCTAGTT
gIDS1	AAAGGAGGCA	ATACCGCTAT	GAGATGGGCC	C-GCGGCGCA	TTAGCTAGTT
gIRS5	AAAGGAGGCA	AT-CCGCTAT	GAGATGGGCC	C-GCGGCGCA	TTAGCTAGTT
gIDS14	AAAGGAGGCA	ATACCGCTAT	GAGATGGGCC	C-GCGGCGCA	TTAGCTAGTT
gIRS9	AAAGGATGTA	TT-C-GCTTT	GAGATGGGCC	C-GCGGCGCA	TTAGCTAGTT
gIRS11	AAAGGATGTA	TTGC-GCTTT	GAGATGGGCC	C-GCGGCGCA	TTAGCTAGTT

GGTGGGGTAA	CGGCTCACCA	AGGCAACGAT	GCGTAGCCGA	CCTGAGAGGG
GGTGAGGTAA	CGGCTCACCA	AGGCAACGAT	GCGTAGCCGA	CCTGAGAGGG
GGTGAGGTAA	CGGCTCACCA	AGGCGACGAT	GCCTAGCCGA	CCTGAGAGGG
GGTGAGGTAA	CGGCCCACCA	AGGCGACGAT	GCGTAGCCGA	CCTGAGAGGG
GGTGAGGTAA	CGGCTCACCA	AGGCGACGAT	GCGTAGCCGA	CCTGAGAGGG
GGTGAGGTAA	CGGCTCACCA	AGGCGACGAT	GCGTAGCCGA	CCTGAGAGGG
GGTGAGGTAA	CGGCTCACCA	AGGCGACGAT	GTGTAGCCGA	CCTGAGAGGG
GGTGAGGTAA	CGGCTCACCA	AGGCGACGAT	GCGTAGCCGA	CCTGAGAGGG
GGTGAGGTAA	CGGCTCACCA	AGGCAACGAT	GCGTAGCCGA	CCTGAGAGGG
GGTGAGGTAA	CGGCTCACCA	AGGCAACGAT	GCGTAGCCGA	CCTGAGAGGG

TGATCGGCCA	CATTGGAACT	GAGACACGGT	CCAGACTCCT	ACGGGAGGCA
TGATCGGCCA	CATTGGAACT	GAGACACGGT	CCAGACTCCT	ACGGGAGGCA
TGATCGGCCA	CATTGGAACT	GAGAAACGGT	CCAGACTCCT	ACGGGAGGCA
TGATCGGCCA	CATTGGAACT	GAGATACGGT	CCAGACTCCT	ACGGGAGGCA
TGATCGGCCA	CATTGGGACT	GAGACACGGC	CCAGACTCCT	ACGGGAGGCA
TGATCGGCCA	CATTGGGACT	GAGACACGGC	CCAGACTCCT	ACGGGANGCA
TGATCGGCCA	CATTGGGACT	GAGACACGGC	CCAGACTCCT	ACGGGAGGCA
TGATCGGCCA	CATTGGGACT	GAGATACGGT	CCAGACTCCT	ACGGGAGGCA
TGATCGGCCA	CATTGGAACT	GAGACACGGT	CCAGACTCCT	ACGGGAGGCA
TGATCGGCCA	CATTGGAACT	GAGACACGGT	CCAGACTCCT	ACGGGAGGCA

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GCAGTGGGGA	ATATTGCACA	ATGGGCGAAA	GCCTGATGCA	TCAACGCCGC
GCAGTGGAGA	ATGTTGCACA	ATGGGCGAAA	GCCTGATGCA	GCAACGCCGC
GCAGTGGGGA	ATATTGCACA	ATGGGCGAAA	GCCTGATGCA	GCAACGCCGC
GCAGTGGGGA	ATATTGCACA	ATGGGGGAAA	CCCTGATGCA	GCAACGCCGC
GCAGTGGGGA	ATATTGCACA	ATGGGGGAAA	CCC-GATGCA	TCAACGCCGC
GCAGTGGGGA	ATATTGCACA	ATGGGGGAAA	CCC-GATGTA	GCAACGCCGC
GCAGTGGGGA	ATATTGGGCA	ATGGGCGCAA	GCCTGACCCA	GCAACGCCGC
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GCAGTGGGGA	ATATTGCACA	ATGGGCGAAA	GCCTGATGCA	GCAACGCCGC

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GTGAGTGATG	AAGGCCTTCG	GGTTGTAAAG	CTCTGTCTTT	GGGGACGATA
GTGAGTGATG	ACGGCCTTCG	GGTTGTAAAG	CTCTGTCTTC	AGGGACGATA
GTGAGTGATG	ACGGCCTTCG	GGTTGTAAAG	CTCTGTCTCC	AGGGACGATA
GTGAGTGATG	ACGACCT-CG	GGTTGTAAAG	CTCTGTCTTC	GGGGACGATA
GTGAGGGAAG	AAGGTCTTCG	GATTGTAAAC	CTCTGTCCTT	GGGAAAAGTA
GTGAGTGATG	AAGGCCTTCG	GGTTGTAAAG	CTCTGTCTTT	GGGGACGATA
GTGAGTGATG	AATGCCTTCG	GGTTGTAAAG	CTCTGTCTTC	AGGGACGATA

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ATGACGGTAC	CCAAGGAGGA	AGCCACGGCT	AACTACGTGC	CAGCAGCCGC
ATGACGGTAC	CAGAAGAAGA	AGCCACGGCT	AACTACGTGC	CAGCAGCCGC
ATGACGGTAC	CCAAGGAGGA	AGCCACGGCT	AACTACGTGC	CAGCAGCCGC

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ATGACTGTAC CTGAGGACGA AGCCACTGCT AACTACGTGC C-GCAGCCGC
ATGACTGTAC CTGAGGAGGA AGCCACGGCT AACTACGTGC CAGCAGCCGC
AGGATGGTAG CCAAGGAGGA AGCTCCGGCT AACTACGTGC CAGCAGCCGC
ATGACGGTAC CCAAGGAGGA AGCCACGGCT AACTACGTGC CAGCAGCCGC
ATGACGGTAC CTGAGGAGGA AGCCACGGCT AACTACGTGC CAGCAGCCGC

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GGTAATACGT ATGTG--TTT ATCGTCGTCC GGATCTACTG TGCGTAAACG
GGTAATACGT ATGTGGCG-- AGCGTTGTCC CGAATTACGG GGCGTAAANG
GGTAATACGT ATGTNTTTTT AGCGTTGTCC GGATTTACTG GGCGTAAAGG
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TAGCCA--CA --GGAT-AGA TACCCGGG
GGAGCAA-CA --GGATTAGA TACCCTGG
AGAGC-AACA --GGATCATA TATCCTGG
G-AGCAA-CA --GGAT---A CATGCTGG
T-ANCAA-CA --GGAT-AGA TACCCTGG
GTAGCAA-CA --GGAT-AGA TANCCTGG
GGAGCAA-CA --GGATTAGA TACCCTGG
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Clostridium cluster III clones 566bp

gIIIRS7	GTGGCGAAAG	CGGCTTTCTG	GACGACAACT	GACAC-GAGG	CGCGAAAGCC
gIIIRS11	GTGGCGAAGG	CGGCTTTCTG	GACTGTAACT	GACACTGAGG	CACGAAAGCG
gIIIRS1	GTGGCGAAGG	CGGCTTTCTG	GACTGTAACT	GGCGC-GAGG	CATGAAAGCG
gIIIRS3	GTGGCGAAGG	CGGCTTTCTG	GACTGTAACT	GACGC-GAGG	CATGAAAGCG
gIIIRS5	GTGGCGAAGG	CGGCTTTCTG	GACTGTAACT	GACACAGAGG	CACGAAAGCG
gIIIRS2	GTGGCGAAGG	CGGCTTTCCG	GACTGTAACT	GACACTGAGG	CACGAAAGCG
gIIIRS4	GTGGCGAAGG	CGGCTTTCTG	GACTGTAACT	GACACTGAGG	CACGAAAGCG
gIIIDS2	GTGGCGAAGG	CGACTATCTG	GACAGTAACT	GACGCTGAGG	CGCGAAAGCG
gIIIDS1	GGGGCGAAGG	CGGCGTTCTG	GACGAAAACT	GACACTGAGG	CGCGAAAGCG
gIIIDS8	GTGGCGAAGG	CGGCTTTCTG	GACAGTAACT	GACGCTGAGG	CGCGAAAGCG
gIIIDS7	GTGGCGAAGG	CGGCTTTCTG	GACGATAACT	GACGCTGAGG	CGCGAAAGCG
gIIIDS6	GTGGCGAAGG	CGGCTTTCTG	GAC-GTAACT	GACGCTGAGG	CGCGAAAGCG

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CGCGAAGAAC CTTACCAGGG CTTGACATA- CCTCTGACGT ATTNTTTTTA

GATA---TGG CTTCCCTTCG -G-GGCAAGG AG--ACAGGT GGTGCATGGT
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GATA---TAG TTTCCCTTCG -G-GGCAGAG AG--ACAGGT GGTGCATGGT
GATA---TAG ATTCCCTTCG -G-GGCAGAG AG--ACAGGT GGTGCATGGT
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GATA---TAG TTTCCCTTCG -G-GGCAGAG AG--ACAGGT GGTGCATGGT
GATT---AAG CTTCCCTTCG -G-GGCAAGG AG--ACAGGT GGTGCATGGT
GATA---GAG TCTCCCTTCG -G-GGCAGAG AT--ACAGGT GGTGCATGGT
AATA---CAG CTTCCCTTCG -G-GGCAGAG TG--ACAGGT GGTGCATGGT
GATA---GTT ATTTCCTTCG -GGA-CAGAG GA-TACGGGT GGTGCATGGT
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GATA---GTT ATTTCCTTCG -GGGGCAGAG GA-TACAGGT GGTGCATGGT

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Clostridium cluster IV clones 488bp

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gIVDS3      GAAACTGCAG AACTTGAGTA CTGGAGAGGG TAGTGGAATT CCTAGTGTAG
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Sulphate-Reducing Bacteria clones 14 403bp

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Archaeal (methanogen) clones 268bp

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Accession numbers of the environmental sequences obtained from the public databases and listed in this study.

AJ387885	Unidentified bacterium anoxSCC-41
AJ289212	Uncultured bacterium RSb16
AF371835	Uncultured bacterium p-406-o3
AF371932	Uncultured bacterium clone p-4936-6Wb2
AB034028	Uncultured rumen bacterium 4C0d-15
AF001733	Unidentified rumen bacterium RC31
AF407407	Uncultured bacterium clone RA13CB
AF001697	Unidentified rumen bacterium RC4
AJ488074	Uncultured bacterium clone IA-23
AF401540	Uncultured bacterium clone g3Br5
AJ278163	Uncultured low G+C gram-positive bacterium SHD-209
AF371948	Uncultured bacterium clone p-5460-2Wb5
AF129862	Uncultured bacterium CA26
AF129863	Uncultured bacterium HB31
AF129861	Uncultured bacterium BA18
AJ295666	Bacterium Irt-JG1-64
UEU81762	Unidentified eubacterium clone VadinHA42
AF371748	Uncultured bacterium p-2031-s959
AF129864	Uncultured bacterium BC09
AF371610	Uncultured bacterium clone p-969-s962-5
AF371579	Uncultured bacterium clone p-4162-6Wa5
AF371578	Uncultured bacterium clone p-619-a5
AF371572	Uncultured bacterium clone p-1028-a5
AF371572	Uncultured bacterium clone p-2195-s959-3
AF357566	Bacterium mpn-isolate group 18
AF371587	Uncultured bacterium clone p-393-o3
AF371609	Uncultured bacterium clone p-334-a3
AJ276565	*Desulfotomaculum* sp. DEM-Kme99-2
AF050587	Uncultured eubacterium WCHB1-20
AF072863	Anaerobic bacterium 'strain 7'
AF351221	Uncultured low G+C Gram- positive bacterium clone 36-20
AB069772	Uncultured sulfate-reducing bacterium clone 8B
AJ012591	Sulfate-reducing bacterium R-PropA1
AB069773	Uncultured sulfate-reducing bacterium clone 10B
AJ300509	Unidentified sulfate-reducing bacterium DSB-DSb-99-3
UEU81725	Unidentified eubacterium clone vadinHA40
AF424772	Uncultured archaeon 120A-4
AF424767	Uncultured archaeon 61-2
AJ009515	Uncultured archaeon SJD-114
AB084243	Uncultured archaeon clone LCD
AF229776	Uncultured archaeon TA03
AJ133793	*Methanocelleus* sp. clone A3
AY062227	Uncultured archaeon clone GW70