The Microbial Ecology of Acidic Environments

By

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Tables and Figures

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Declaration

I declare that this thesis has been composed by myself and has not been used in any previous application for a degree. All results have been obtained by myself under the supervision of Dr Paul Norris unless otherwise stated.

Summary

The microflora of two acidic environments was investigated using analysis of 16S rDNA amplified by the polymerase chain reaction (PCR) from environmental DNA. These environments had different chemical characteristics from most of the acidic environments studied by others. The first sample site, a coal spoil (Birch Coppice, Warwickshire), might have been expected to produce niches enriched in humic matter. The second, comprising geothermal vents on the Island of Vulcano, was unusual for natural acidic environments since it was saline. Three vent regions of different temperatures (30°C, 45°C and 80°C) were examined.

Prior to the 16S rDNA analysis of the sites, a brief investigation into selection of a suitable method of DNA extraction was carried out. A bead-beating method and a chemical lysis/freeze-thaw method were compared. With regard to clone types found via each method, there was little qualitative difference. DNA was extracted from the two sites and 16S rRNA genes were amplified by PCR. PCR products were ligated and competent *E. coli* cells were transformed to produce clone libraries. Restriction fragment length polymorphisms (RFLPs) were examined and representatives of each RFLP type were sequenced and analysed with reference to RNA gene sequence data bases.

The coal spoil clone library was dominated by sequences related to those from uncultured actinobacteria, particularly those found previously in peat bogs and various soils. Representatives of some well-known acidophiles were also found (e.g. *Leptospirillum* species). The clone bank from the saline, geothermal site DNA comprised sequences from acidophiles capable of growth at the respective temperatures of different samples. The lowest temperature samples produced sequences from a novel *Acidithiobacillus* species and also indicated a novel species probably related to *Thiobacillus prosperus* (which was isolated previously from Vulcano). A high temperature sample gave sequences from archaeal acidophiles, *Acidianus brierleyi* and, previously isolated from Vulcano, *Acidianus infernus* and *Thermoplasma volcanium*.

Where the clone banks revealed the presence of novel organisms, attempts were made to isolate and characterise them. The novel actinobacteria did not appear to grow in laboratory enrichment cultures. The novel *Acidithiobacillus* species and two novel *Thiobacillus prosperus*-like species were characterised.

Abbreviations

A Adenine

AMD Acid mine drainage

bp Base pairs

C Cytosine

CsCl Caesium chloride

CTAB Cetyltrimethylammonium bromide

Dig Digoxigenin

DGGE Denaturing Gradient Gel Electrophoresis

DIMA Dot-Immuno-Assays

DNA Deoxyribonucleic acid

dNTP Deoxynucleotide triphosphate

EDTA Ethylenediaminetetraacetic acid

EtBr Ethidium bromide

FISH Fluorescent *In Situ* Hybridisation

G Guanine

GCG Genetics Computer Group

KDa Kilodaltons

KSCN Potassium thiocyanate

lbs Pounds (pressure)

LB Luria Bertini broth

Me Metal

MeS Metal sulphide

MQ MilliQ water

Mt Metric ton

PAGE Polyacrylamide Gel Electrophoresis

PCR Polymerase Chain Reaction

PFGE Pulse Field Gel Electrophoresis

pM Picomole

PVP Polylvinylpyrrolidone

PVPP Polyvinylpolypyrrolidone

RDP Ribosomal Database Project

RFLP Restriction fragment length polymorphism

RNA Ribonucleic acid

rRNA Ribosomal RNA

S Svedberg unit

SDS Sodium dodecylsulphate

SSC NaCl-Na citrate

T Thymine

Taq *Thermus aquaticus*

TBE Tris-boric acid-EDTA

$T_{10}E_1$ Tris (10mM) EDTA (1mM)

$T_{50}E_1$ Tris (50mM) EDTA (1mM)

Temed Tetramethylethylenediamine

Tet Potassium tetrathionate

V/v Volume per volume

W/v Weight per volume

YE Yeast extract

CHAPTER 1 INTRODUCTION

1.1 Molecular Ecological Techniques In Microflora Analysis

The study of microbes in environmental samples using molecular biology techniques has been practised since the mid 1980s (Head *et al.*, 1998; Amann *et al.*, 1995), and their usage has changed the way microbiological studies have been performed.

Prior to the usage of molecular biological techniques, only bacteria that could be cultured were identified and characterised. The majority of bacterial cells visualised by microscopy do not form colonies, in fact only 0.1-10% of prokaryotes do, and therefore traditional plating techniques can only give a limited picture of *in situ* populations (Head *et al.*, 1998).

The advance of molecular biology has led to new insights into the identification of uncultivated microorganisms. The use of rRNA in the study of microbes in environmental samples has advanced the understanding of microbial ecology. rRNA is universal in organisms and its analysis allows inferences to be made about the relationships among organisms. Originally 5S rRNA was used in molecular biology studies; however, the information gained from 5S rRNA sequences is limited as the molecule is only 120 nucleotides long. 16S rRNA and 23S rRNA are approximately 1500 and 3000 nucleotides long, respectively, and have proved to be more useful. Large rRNA sequence databases have been established to allow ease of sequence comparison (Manz *et al.*, 1992).

Culture collections contain many inappropriately named strains, misidentified due to the incorrect interpretation of traditional culturing, morphological and biochemical data (Amann *et al.*, 1995). The study of rRNA sequences can allow the correction of these inaccuracies as the alignment and comparison of rRNA sequences can place organisms in their correct phylogenetic groups. Unculturable organisms can be identified and inferences might be possible in some cases with regard to their physiology by their positioning in phylogenetic groups. Steps might then be taken to create suitable conditions for their growth and isolation.

A molecular ecological approach can give valuable insights into community composition, spatial relationships and functions of populations. These insights may also relate to the understanding of symbiotic relationships and biogeochemical cycles (Amann *et al.*, 1995). For example this approach has been used in the study of methane cycling where methanotrophic and methanogenic organism population dynamics have been analysed through the use of oligonucleotide probes, PCR and other molecular biological tools. It is hoped that through the study of these organisms a way may be found to decrease the amount of methane released into the atmosphere and therefore reduce global warming. (McDonald *et al.*, 1995; Murrell *et al.*, 1998; McDonald *et al.*, 1999)

1.1.1 DNA extraction methods.

A number of methods have been devised to extract DNA from environmental samples and cell cultures. Different methods can give different results with regard to the lysis of organisms: some organisms lyse more efficiently than others, gram-positive organisms, for example, are notoriously harder to lyse than gram-negatives. The type of environment from which DNA is to be extracted can also affect the efficiencies of different methodologies. A number of papers have been written about DNA extraction methods, particularly extractions from soils and sediments. Many of these methods are adaptations from three protocols developed in the late 1980s and the early 1990s: Ogram *et al.* method (1987), Tsai and Olsen's method (1991) and Jacobsen and Rasmussen's method (1992).

DNA extraction methodologies use chemicals as a means of cell lysis or mechanical cell disruption, many protocols require a combination of both. Chemicals used in DNA extractions include detergents such as SDS (Ogram *et al.*, 1987) or sarkosyl, or enzymes such as lysozyme or proteinase K. Mechanical cell disruption can be achieved by freeze thawing (Tsai and Olsen, 1991), homogenisation in a blender (Steffan *et al.*, 1988) or bead-beating (Ogram *et al.*, 1987). Jacobsen and Rasmussen (1992) developed a method using cation exchange resin to separate bacteria from samples prior to lysis. These techniques are discussed in the following paragraphs.

The "Ogram *et al.* Method"

This method involves the direct lysis of the bacteria in the sample using the physical disruption method of bead beating. This is an efficient way of breaking up the cells but the DNA can be sheared in the process.

The "Tsai and Olsen Method"

The Tsai and Olsen method involves the use of chemicals (lysozyme, SDS and phenol) and freeze-thawing. The environmental sample is washed and pelleted in a sodium phosphate buffer and re-suspended in a lysis solution that contains sodium chloride and EDTA plus lysozyme, before incubation at 37°C. Following this incubation, a buffer containing sodium chloride and Tris-HCl is added plus SDS. The mixture undergoes freeze-thawing in dry ice-ethanol (-70°C) and a 65°C water bath, which releases DNA from the organism cells. After the freeze-thawing the sample is mixed with Tris-saturated phenol, vortexed and centrifuged. The aqueous phase is removed and added to a phenol-chloroform-isoamylalcohol mixture, vortexed and centrifuged. The resulting aqueous phase is mixed with cold isopropanol and placed in a freezer at –20°C overnight to precipitate the DNA. This method is relatively simple and quick compared to methods by Ogram, and the resulting DNA is less sheared than DNA extracted by bead-beating.

The "Jacobsen and Rasmussen method"

The Jacobsen and Rasmussen method separates the bacteria from the environmental sample using a cation exchange resin. This separation eliminates the possibility of DNA being contaminated by eukaryotic or extracellular DNA. However, as discussed in a paper by Steffan *et al.* (1988), separation of the bacteria from the sample (using a blender, a method developed by Holben *et al.*, 1988) can be very time consuming and the results are sometimes disappointing. The Jacobsen and Rasmussen method aims to make the bacterial separation a more viable method in terms of the amount of time spent on the method and the quality of results gained. The method is based on the fact that soil particles bind to the bacteria via cations. The cation exchange resin releases the bacteria as the soil preferentially binds to the resin

rather than the bacteria. The sample (soil) is put in a centrifuge bottle with Chelex 100, sodium-deoxycholate and polyethylene glycol 6000 and shaken. The soil particles are pelleted by centrifugation and the supernatant is filtered to remove the Chelex 100. The bacteria are harvested by centrifugation and lysed with lysozyme and SDS.

1.1.2 DNA purification

Generally, with samples such as soil, the extracted DNA is contaminated, often with humic substances. This contamination can render the DNA unusable in terms of restriction enzyme digestion, PCR and probing. It denatures biological molecules by bonding to N-substituted amines or oxidises to form quinones which covalently bond to DNA or proteins (Young *et al.*, 1993). Therefore the DNA must be purified prior to molecular analysis. Chemicals such as polyvinylpolypyrrolidone (PVPP), CsCl and hydroxyapatite have all been used to remove humic substances from DNA.

PVPP forms hydrogen bonds with phenolic compounds from the humic matter, forming PVPP-phenolics which precipitate and which can be removed by centrifugation. A number of papers describe the effective use of PVPP (Steffan *et al.*, 1988), or the soluble form polyvinylpyrrolidone (PVP) (Young *et al.*, 1993), to remove humic substances. Steffan *et al.* (1988), added the PVPP directly to the sample mixture prior to cell separation. Young *et al.* (1993) added PVP to agarose gels; when the DNA is electrophoresed in the gel the PVP separates the DNA from the humic matter.

Steffan *et al.* (1988) also mixed DNA with hydroxyapatite to remove the contaminants, others mixed the DNA with CsCl and EtBr and centrifuged to separate the DNA from impurities.

DNA can also be separated from humic substances by the use of Sepharose 4B (Jackson *et al.*, 1997), where the DNA is extracted using the high-salt, SDS based method of Zhou *et al.* (1996). The crude nucleic acids were placed in spin columns containing Sepharose 4B (made using syringes packed with the gel matrix) and centrifuged, the humic substances bound to the sepharose gel matrix and the DNA

was eluted from the column. More recently, commercial kits have been developed that use spin columns in this way, creating quick and easy methods for DNA purification. Other kits have been developed to remove the DNA from the agarose gel after it has been electrophoresed and separated from the impurities. A paper by Miller *et al.* (1999) provided a comparison of different methods of DNA extraction and concluded that all physical disruption methods shear the DNA in some way. They also concluded that bead-beating alongside phenol and SDS yields larger amounts of DNA than freeze-thawing with SDS and enzymatic pre-treatment, although when large sample sizes are involved some methods are not always practical.

1.1.3 The polymerase chain reaction (PCR)

The advent of the polymerase chain reaction, developed by Saiki *et al.* in 1988, has been one of the most important discoveries for molecular microbiology. It allows the selective amplification of specific genes from genomic DNA samples, the most useful genes for organism identification being the rRNA genes. Thus, in a sample containing a mixture of chromosomal DNA from different organisms, the rRNA genes can be amplified, cloned and sequences identified for the organism types present in the sample. Although this technique is not quantitative, it allows a quick assessment of the biodiversity of microbes in an environmental sample as organism types can be revealed that may otherwise remain undiscovered. From these sequences comparisons to other rRNA sequences can be made and phylogenetic trees can be created to give a visual representation of the evolutionary relationships between organisms or sequences. The high numbers of certain clone types might indicate organisms that were major components of *in situ* populations. However, PCR and the study of 16S rRNA sequences are only qualitative in the light of various analytical biases, including sample handling procedures and potentially differential cell lysis and gene amplification (Wintzingerode *et al.*, 1997).

PCR is not without its disadvantages, biasing can occur at all stages starting with the recovery of the DNA from the environmental samples. Qualitative recovery of DNA is almost impossible as without knowing the amount of total DNA present in the sample it is difficult to assess the efficiency of recovery (Head *et al.*, 1998).

PCR itself has many biases, some organisms' DNA may be more selected for during amplification than others and different organisms have different rRNA gene copy numbers thus the organisms with the most rRNA gene copy numbers will be most represented in amplification. Novel taxa could be discovered that may just be the creation of artefacts caused by misincorporation of DNA by some DNA polymerases. Chimeras can form, but these are easily identified if the organisms from which they are composed are already well known and distinct. However, problems could occur if the chimeras were formed from closely related novel organisms. Other factors that can bias the relative frequencies of genes in the PCR products of mixed template reactions include the G+C% Mol content of template DNA; the accessibility of the gene to primer hybridisation following denaturation; the efficiencies of the formation of primer-template hybrids and the efficiency of the extension step by the polymerase (Suzuki and Giovannoni, 1996).

However, PCR is an invaluable technique for assessing the biodiversity of a particular environment if numbers of each particular organism is not important. Many organism types have been discovered which would otherwise have been missed if it were not for the use of PCR. "The history of PCR is short but very successful. Enzymes, whole reaction kits and automatic thermocyclers are now commercially available. Within a few years, it has become a routine method and has found many exciting and beneficial applications" (Schleifer *et al.*, 1993 p. 493)

1.1.4 Nucleic acid probes

A nucleic acid probe is a fragment of single stranded nucleic acid that binds to complementary DNA or RNA. Nucleic acid probes can be isolated from the DNA of the target organisms or synthesised in the laboratory (Schleifer *et al.*, 1993). The use of nucleic acid probes, as a tool for diagnostic and detection purposes, is becoming one of the most valuable developments in molecular biology. Nucleic acid probes – usually oligonucleotide rather than polynucleotide – allow an in-depth study of the microbial ecology of environments and can be used in the analysis of population structure and dynamics in microbial ecosystems. Labelled nucleic acid probes directed against the intracellular rRNA of organisms, combined with techniques to retrieve rRNA sequence information directly from complex ecosystems, can be used

to monitor uncultivated bacteria (Zarda *et al.,* 1991). When using probes, the microbial ecologist should be able to take a phylogenetic census of any microbial niche using the proper inclusive set of specific taxonomically nested whole organism probes (Burggraf *et al.,* 1994).

Nucleic acid probes can be labelled and used in a variety of methods. They can be labelled with radioactive isotopes – a very sensitive technique that gives good results but carries usage and disposal problems. Alternatives to radioactive isotope labelling include biotin, fluorescent and enzymic labels. The labelling of a probe can be carried out directly or indirectly. For direct labelling the marker is covalently bound to the oligonucleotide, markers labelled in this way include radioactive, fluorescent or enzymic. When indirect labelling is carried out the unlabelled reporter group is attached to the probe and is detected by a labelled binding protein. Reporter groups include biotin (nucleic acids can be biotinylated and detected by the use of anti-biotin antibodies conjugated to a variety of enzymes of fluorescent labels), immunologically detected haptens (eg N-acetoxy-N-2-acetyl aminofluorene, used for the modification of cytosine residues) and dioxygenin (anti-digoxygenin antibodies are conjugated with chemiluminescence enzymes) (Schleifer *et al.,* 1993).

The targets for nucleic acid probes can vary from whole cell DNA to DNA fragments to 16S and 23S rRNA. With whole cell DNA probes the whole cells are blotted and lysed onto membrane filters and the DNA from reference strains (chromosomal probes) are hybridised. This is a quick and simple method but it lacks specificity and unspecific background hybridisations can occur.

In random DNA fragment probing, the DNA fragments are obtained by restriction enzyme endonuclease digestion and cross-hybridising fragments are removed. The remaining fragments are used to design the probe for probing the original DNA.

The most useful (and most frequent) types of nucleic acid probes used are rRNA gene probes. 16S and 23S rRNA have regions of highly conserved sequence interrupted by more variable sequence. rRNA can be present in high copy numbers; this increases the sensitivity of the probe. rRNA probes are extremely useful for

differentiating phylogenetic groups of organisms and can lead to the rapid classification of unknown organisms.

rRNA probes can again be used in a variety of methods, for example Southern (DNA) and Northern (RNA) hybridisations and dot-blot hybridisations. In dot-blot hybridisation the target nucleic acids are applied directly to a membrane, hybridised with a probe and after an incubation time at an optimised temperature, the excess probe is washed off and the blot is detected. With digoxigenin (DIG) labelled probes, the hybridised probes are bound with an antibody, which is in turn bound with a chemiluminescent agent; this greatly amplifies the signal given off from positive hybridisations. The blot is applied to X-ray film and when the film is developed the positive hybridisations can be identified. This method can be carried out to give qualitative results either by blotting the target nucleic acids against standardised nucleic acids, or by hybridising the target nucleic acid with a universal probe followed by detection and then removing the universal probe and re-hybridising with the specific probe. The amount of target nucleic acid blotted on the membrane can then be determined relative to the known standard, or the total amount of target present hybridised to the universal probe, by use of a densitometer.

These methods of nucleic acid probing require the extraction of the nucleic acids from the target organisms. This can be difficult as lysis and extraction methods are not efficient for all organism types, thus the organism types in the sample may be present in equal numbers but different lysing efficiencies may give different blotting results. An added problem can occur when samples come from environments such as soil where the nucleic acids can adsorb to the soil particles thus increasing the difficulty of obtaining a comprehensive blot result.

In situ hybridisation does not require the extraction of nucleic acids from the cells. *In situ* methods can range from the bacterial cells grown on filters to the *in situ* detection of specific nucleic acid sequences in cells. When detecting cells in their environment or culture using *in situ* techniques it has been found that fluorescently labelled probes give the best results. The fluorescently labelled rRNA targeting oligonucleotides enter cells permeated with specific chemicals and bind to the intracellular rRNA. This technique works most efficiently when the cells are growing

exponentially so the rRNA content of the cell is at its maximum. The cells can then be enumerated by counting the fluorescing cells using filters under a microscope. Probes targeting different sequences (different organisms) can be labelled with different fluorochromes so that a mixture of organism types can be detected in one hybridisation (Schleifer *et al.*, 1993). This technique is particularly useful in the analysis of mixed culture and bioreactor population dynamics.

Thus nucleic acid probes provide fast, sensitive and specific methods for identifying and enumerating microorganisms without the need for cultivation. However, improvements need to be made to increase cell permeability without causing cell lysis and to design more efficient and simple nucleic extraction methods.

Other molecular biological techniques include immunological methods: dot-immuno-assays (DIMA), Pulse Field Gel Electrophoresis (PFGE) and Denaturing Gradient Gel Electrophoresis (DGGE). DIMA is used for the determination of ore adhered microorganisms; in PFGE the total DNA of organisms is cleaved using restriction enzymes and the entire fragmented genome can be separated by PFGE giving macro restriction patterns which can be used in the identification of the organism. DGGE is a method in increasing use, DNA fragments of the same length are separated on the basis of their nucleotide sequence. PCR-amplified 16S rDNA fragments are electrophoresed in polyacrylamide gels containing a linearly increasing gradient of denaturants. Separation is based on the electrophoretic mobility of a partially melted DNA molecule, which is decreased, compared to that of a completely helical form of the molecule. Fragment melting proceeds in the melting domains – stretches of base pairs with identical melting temperatures. When the melting domain with the lowest melting temperature gets to its melting temperature in the DGGE gel, there is a transition of helical to partially melted molecule and migration down the gel stops. Thus different fragments of DNA will stop at different points down the gel and the microbial diversity of the environment from which the sample was taken can be observed (Muyzer *et al.*, 1993).

1.2 Previous analysis of acidic environments

The environments of particular interest in this study are acidic, mineral sulphide rich sites such as biomining heaps, acidic mine tailings heaps and sulphurous geothermal hot springs. Only a few acidic sites have been studied using molecular methods, (see Table 1.1). These areas are of biotechnological and environmental importance because of the nature of the microorganisms present. These microbes tend to be acidophilic and many exhibit some form of thermotolerance. Many can oxidise sulphur and iron, which for bioleaching purposes is crucial but in non-commercial bioleaching environments such properties can give rise to pollution. Acidification and the release of metal ions into soil and watercourses is detrimental to the flora and fauna of the surrounding area.

The study of these sites could lead to the discovery of novel organisms, which may prove useful to the biomining industry, and analysis of the biodiversity of these environments could provide information about the causative organisms and their roles in pollution.

Studies of three sites (Table 1.1) have shown that, in the mineral rich acidic environments of Mount Isa and Iron Mountain, the microbiology appears similar. The same sorts of organisms have been found both by 16S rDNA analysis and traditional culturing methods, there is little in the way of biodiversity and few novel species were present. However, at the Montserrat geothermal springs, some of the organisms found were of similar species to those of Mount Isa and Iron Mountain but other novel types were shown to be present as well.

Table 1.1 Acidic environments studied by 16S rDNA analysis

SITE	pH	Temp (°C)
Montserrat geothermal springs (Burton and Norris, 2000)	1.5-3.2	33-98
Iron Mountain California: disused mine workings (Edwards *et al.*, 1999)	0.5-2.5	20-50
Mount Isa, Queensland: ore heap run-off pool (Goebel and Stackebrandt, 1994)	1.5	ambient

Although these sample sites differed in their temperature ranges, the pH and general chemistry/mineralogy were very similar, thus similar organism types were found. At the Mt Isa mine, all the cloned sequences were similar to those of previously cultivated laboratory strains. At the Iron Mountain mine the cloned sequences were similar to laboratory strains plus some novel groups clustering with known acidophiles. With the Montserrat springs the cloned sequences were similar to known strains, but novel species and genera were also indicated. Other extreme non-acidic sites that have been studied using 16S rDNA analysis include neutral hot springs at Yellowstone National Park (Barns *et al.*, 1994; Hugenholtz *et al.*, 1998), alkaline salt lakes (Jones *et al.*, 1998) and deep-sea sites (Takami *et al.*, 1999); all of these sites indicated the existence of novel groups of extremophiles.

1.3 Bioleaching

Bioleaching is the leaching of metals from their ores, usually sulphides, into solution by the direct or indirect actions of bacteria (Goebel and Stackebrandt, 1994). It is used to pretreat refractory sulphide gold ores and to leach copper from chalcocite (CuS) ores. There are two main process designs for commercial bioleaching: these involve bioreactors or heaps of ore. Lower grade ores are leached in heaps, while reactors treat concentrates.

In heap bioleaching the ore is crushed, acidified, usually with sulphuric acid, and stacked on lined pads. The leach solution flows through the heap and the pregnant leaching solution passes to a solvent extraction/ electrowinning (SX/EW)

plant to recover the metal from the solution. (Schnell, 1997) In chalcocite leaching the stacked ore is irrigated with effluent from the SX/EW plant adding to the efficiency of the bioleaching process.

It was thought there were two ways in which the bacteria could leach the metal; denoted as the 'indirect' and 'direct' methods.

Indirect method – here the bacteria generate a lixiviant, usually ferric sulphate, in acidic solution. This oxidises the sulphide in the mineral. (Where Me is the metal to be leached).

$$MeS + Fe_2(SO_4)_3 \longrightarrow MeSO_4 + 2FeSO_4 + S$$

The bacteria may also oxidise the produced elemental sulphur to sulphuric acid (Bosecker, 1997).

$$2S + 3O_2 + 2H_2O \longrightarrow 2H_2SO_4$$

Direct method – here the bacteria are in intimate contact with the ore.

$$MeS + 2O_2 \longrightarrow MeSO_4$$

However, it has now been proposed that the bacteria in contact with the ore are in a micro-environment where the ferric sulphate is produced and active as in the indirect method (Sand *et al.*, 1995).

$$MS + 2Fe^{3+} \xrightarrow[\text{reaction}]{\text{Chemical}} M^{2+} + S + 2Fe^{2+}$$

$$2H^+ + 0.5O_2 + 2Fe^{2+} \xrightarrow[\text{reaction}]{\text{Bacterial}} 2Fe^{3+}\ H_2O$$

$$S + 1.5O_2 + H_2O \xrightarrow[\text{reaction}]{\text{Bacterial}} SO_4^{2+} + 2H^+$$

$$MS + 2O_2 \xrightarrow[\text{Bacterial reaction}]{\text{Chemical+}} M^{2+} + SO_4^{2-}$$

Bioleaching is used for economical and technical reasons, economically because it is a cost effective way of using waste ores that do not contain much of the metal and therefore normal smelting processes would be too expensive; or technically because the refractory nature of some gold ores, which are encapsulated in a pyrite

(FeS_2) or arsenopyrite (FeAsS) shell, means that the direct extraction with cyanide cannot be used. The use of a biological pre-treatment process can remove this shell.

Reasons for heap bioleaching refractory gold ores

1. The gold grade is too low for normal processes, even when the ore is concentrated it is too low to support the cost of a roaster.
2. The ore is normally considered as waste but it can be bioheap leached because of the low capital and operating costs.
3. The ore cannot be concentrated because of its mineralogy or is refractory to conventional treatment.

Reasons for heap bioleaching copper sulphide ores

1. The cost of construction of a smelter would be too great and the mine would not be able to support it economically.
2. Smelter charges (the charges imposed by toll smelters because of contaminants in the concentrate) would be too costly.
3. Transport costs to take the concentrate to a smelter would be too great because of the remoteness of the sites.
4. There is no need to build tailing ponds or effluent treatment plants as there is no aqueous discharge.
5. Only a relatively untrained workforce is needed thus sites can be built in remote places in undeveloped countries.
6. Overall bioleaching of copper is more cost effective than conventional methods of extraction and more environmentally friendly. (Brierley, 1997).

In copper bioleaching heaps, the usage of which is on the increase because of their economical and environmental advantages, acidophilic autotrophic bacteria are used to enhance the oxidation of copper sulphides and so solubilise the copper. These plants run with high concentrations of sulphate salts, such high concentrations are caused by sulphuric acid being continuously added and the leaching solution being recycled.

There are a number of commercial bioheaps around the world (Table 1.2). These are large scale, some copper sites processing 1,500- 2000 metric tons (mt) of chalcocite ore per day, producing 10-14000 mt Cu/year. The Newmont-Carlin plant processes 10,000mt refractory gold ore per day (Brierley, 1997).

Figure 1.1 is a schematic diagram depicting the theoretical variations occurring within a mineral leaching heap.

Table 1.2 Three examples of large scale commercial mineral leaching heaps

SITE AND ORE	TONNAGE	COMMISSIONED
Ivan-Zar, Chile. Chalcocite ore, 2.5% Cu	1500mt/day 12,000mt Cu/year	1994
Girilambone, Australia. Chalcocite ore, 2.5% Cu	2,000mt/day 14,000 mt Cu/year	1993
Newmont-Carlin, USA. Sulphidic gold ore 1g Au/mt	10,000mt/day	1995

Fig 1.1 Theoretical Variations in key conditions within a bioleaching heap (taken from Casas *et al.*, 1995)

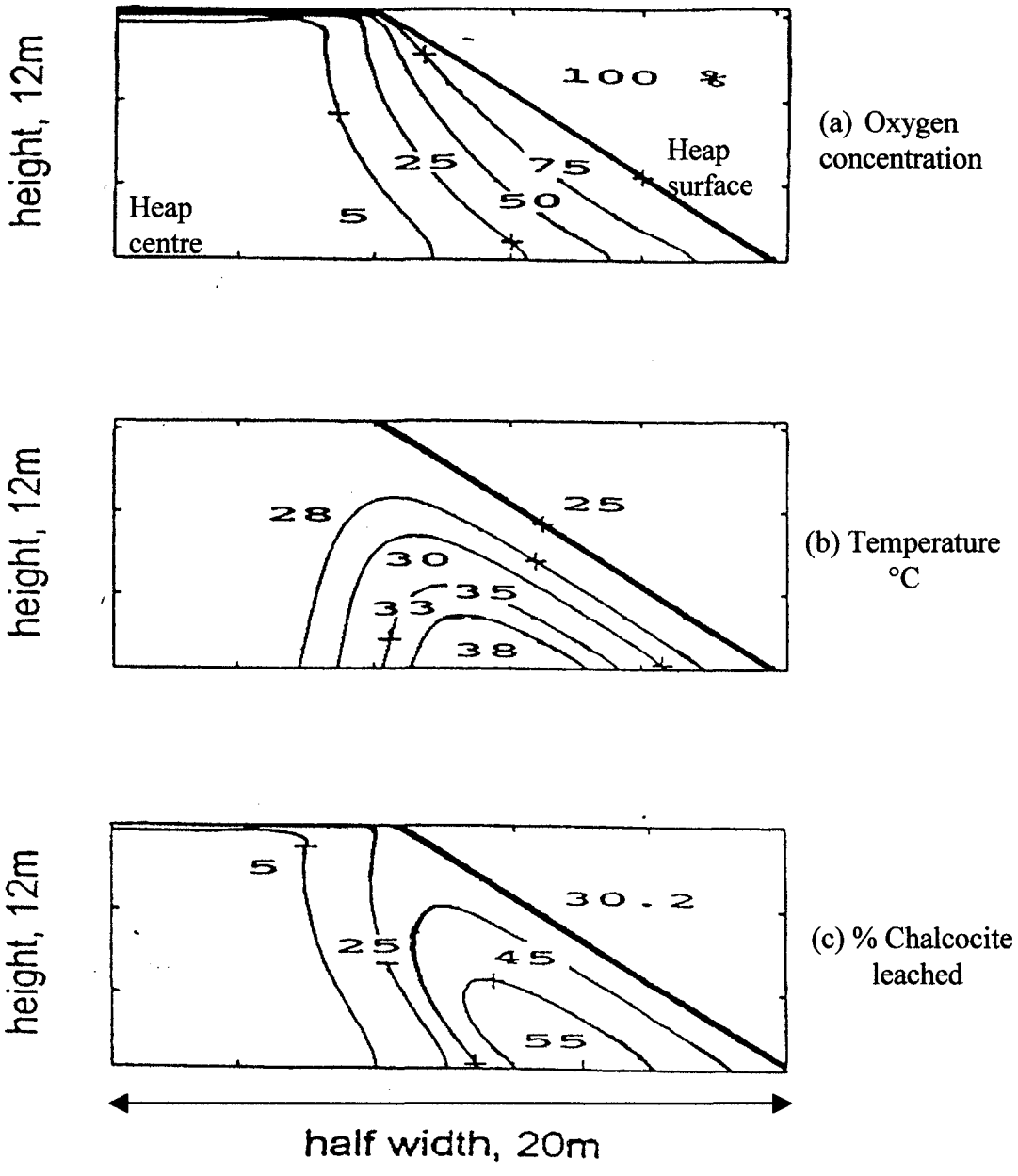


Figure 1.1 illustrates the heterogeneous nature of a heap leaching environment Contour plots are shown predicting (a) the oxygen concentration, (b) the temperature and (c) the percentage of chalcocite ore leached in one year for a dump 12m high and 20m half-width at the base.

It can be seen in (a) that the oxygen concentration is predicted to decrease with depth or distance from the edge, which corresponds with the temperature of the heap that is lowest where there is the least amount of oxygen and therefore least bacterial

oxidation activity. The highest temperature of this 'heap' is 38°C, in larger commercial heaps the temperature can be higher as there is more surrounding insulation. The heat originates from the exothermic bacterial oxidation of the mineral sulphides.

The percentage chalcocite conversion also corresponds to the oxygen concentration and temperature. The lowest value of chalcocite conversion is 5%, this is where the oxygen is lowest, thus the bacteria are not oxidising the ore. Conversely, the highest value of chalcocite conversion is where the temperature is highest, i.e., where maximum bacterial activity is taking place. If heaps have aeration pipes running beneath them, as many now do, the efficiency of bioleaching is much greater. Another point to note about these contour plots is the gradients of different conditions through the heap. This ensures various niches and so ensures a wide variety of microorganisms in such heaps.

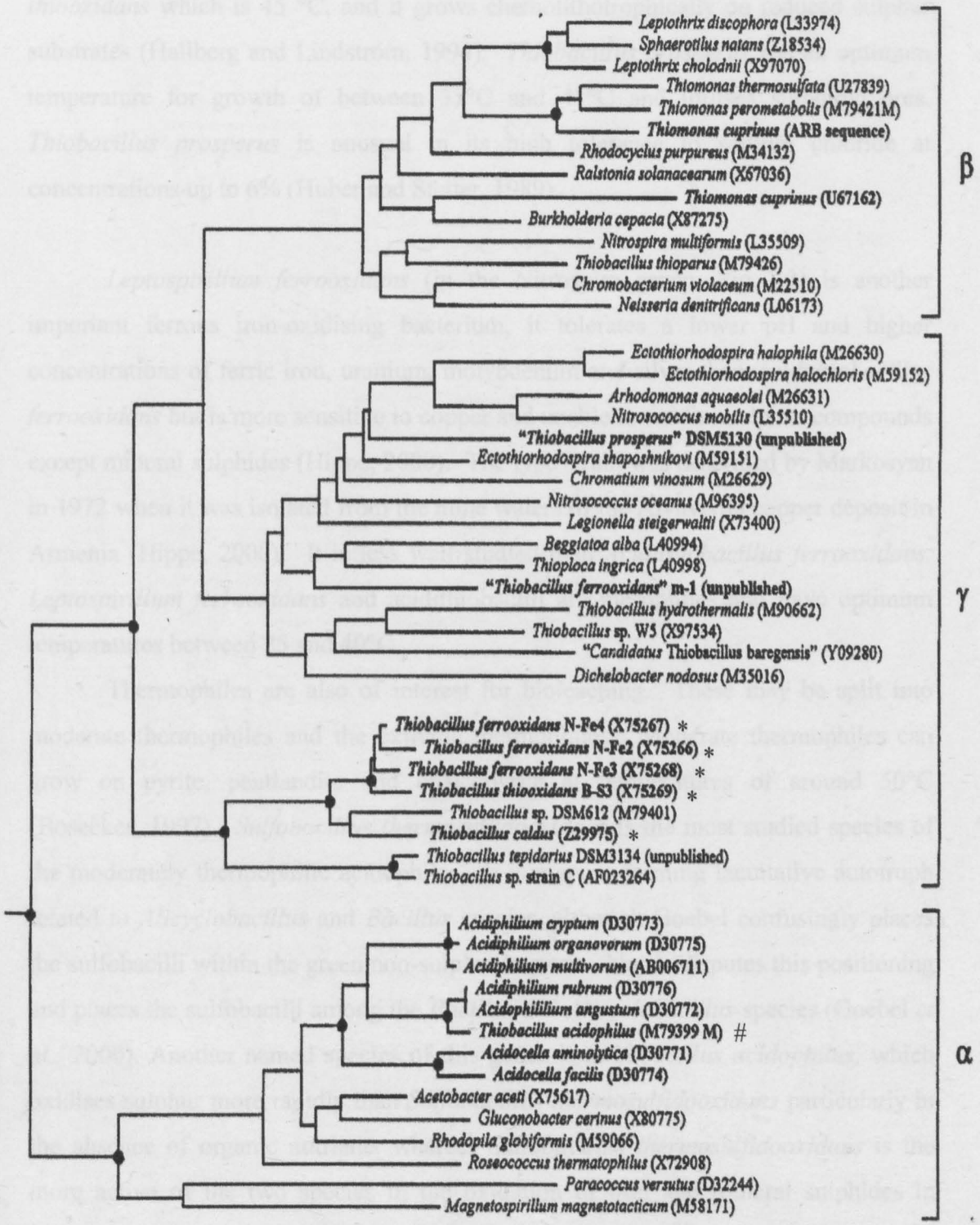
1.4 Bacteria involved in bioleaching

The environments studied in this project were chosen because of the increasing interest in iron- and sulphide- oxidising microorganisms and their ecological/environmental roles. The sulphur cycle is very geochemically important, one of the main components of the sulphur cycle is the release of sulphate into solution through the oxidation of sulphide minerals. Pyrite is the most common sulphidic compound in the earth's crust (Schrenk *et al.*, 1998) and chemolithotrophic microorganisms can enhance the dissolution of pyrite and thus are an important factor in acid mine drainage pollution. The study of such organisms is important to try and combat the problems caused by acid mine drainage, but such organisms may be also beneficial to industry in biomining operations as mentioned earlier. Acidic environments can occur from natural processes, however, since the industrial revolution many acidic environments are man-made. The majority of man-made acidic environments originate from the mining of metals and coal. When mining mineral sulphide ores, much of the waste is composed of iron sulphides as iron sulphides are often associated with other, more valuable metal ores. Iron sulphides are often present in coal deposits, thus coal-spoils often contain these polluting sulphides (Johnson, 1998).

There are a number of bacteria involved in bioleaching and they are dispersed widely phylogenetically (Lane *et al.*, 1992). Bioleaching bacteria need to be acidophilic due to the low pH of bioleaching environments. When bioleaching was first studied it was believed that the acidithiobacilli were the main bacteria involved. These are located in the Proteobacteria (Fig 1.2). They are non-spore forming rods that are facultatively anaerobic, obtaining energy from oxidizing reduced sulphur compounds. They were previously known as thiobacilli but the name was changed as more information obtained from 16S rRNA analyses and DNA-DNA hybridisation enabled a re-classification of some of the species to the new genus *Acidithiobacillus* (Kelly and Wood, 2000). Bacterial leaching is carried out in acidic environments at pH values between 1.5 and 3, thus *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* are particularly important as they tolerate these conditions well (Leduc and Ferroni, 1994). *Acidithiobacillus ferrooxidans* is an obligate chemolithotroph, gaining energy from ferrous iron as well as sulphur

compounds; it fixes carbon dioxide. It was first isolated from an acid mine drainage site in 1950 by Colmer and his co-workers and named as *Thiobacillus ferrooxidans* by Temple and Colmer in 1951.

Fig 1.2 Evolutionary distance tree of the Proteobacteria (Goebel *et al.*, 2000)



* denotes genus change from *Thiobacillus* to *Acidithiobacillus*

\# denotes name change from *Thiobacilus acidophilus* to *Acidiphilium acidophilus*

There are two other members of the Thiobacilli/Acidithiobacilli that are considered important acidophilic mineral oxidisers; these are *Thiobacillus prosperus* and *Acidithiobacillus caldus*. *Acidithiobacillus caldus* has a higher optimum temperature for growth than either *Acidithiobacillus ferrooxidans* or *Acidithiobacillus thiooxidans* which is 45 °C, and it grows chemolithotrophically on reduced sulphur substrates (Hallberg and Lindstrom, 1994). *Thiobacillus prosperus* has an optimum temperature for growth of between 37°C and 41°C and utilises sulphidic ores. *Thiobacillus prosperus* is unusual in its high tolerance to sodium chloride at concentrations up to 6% (Huber and Stetter, 1989).

Leptospirillum ferrooxidans (in the Nitrospira group, Fig 1.3) is another important ferrous iron-oxidising bacterium, it tolerates a lower pH and higher concentrations of ferric iron, uranium, molybdenum and silver than *Acidithiobacillus ferrooxidans* but is more sensitive to copper and unable to oxidise sulphur compounds except mineral sulphides (Hippe, 2000). The type strain was described by Markosyan in 1972 when it was isolated from the mine waters of the Alvaverda copper deposit in Armenia (Hippe, 2000). It is less well studied than *Acidithiobacillus ferrooxidans*. *Leptospirillum ferrooxidans* and acidithiobacilli are mesophiles and have optimum temperatures between 25 and 40°C.

Thermophiles are also of interest for bioleaching. These may be split into moderate thermophiles and the extreme thermophiles. Moderate thermophiles can grow on pyrite, pentlandite and chalcopyrite at temperatures of around 50°C (Bosecker, 1997). *Sulfobacillus thermosulfidooxidans* is the most studied species of the moderately thermophilic acidophiles. It is a spore-forming facultative autotroph related to *Alicyclobacillus* and *Bacillus* species, although Goebel confusingly places the sulfobacilli within the green non-sulphur bacteria. Norris disputes this positioning and places the sulfobacilli among the *Bacillus* and *Alicyclobacillus* species (Goebel *et al.*, 2000). Another named species of this genus is *Sulfobacillus acidophilus*, which oxidises sulphur more rapidly than *Sulfobacillus thermosulfidooxidans* particularly in the absence of organic nutrients whereas *Sulfobacillus thermosulfidooxidans* is the more active of the two species in the oxidation of iron and mineral sulphides in laboratory culture (Norris *et al.*, 1996). Moderate thermophiles can grow in mixed cultures with mesophiles or extreme thermophiles at their lower end and higher end of their temperature ranges respectively.

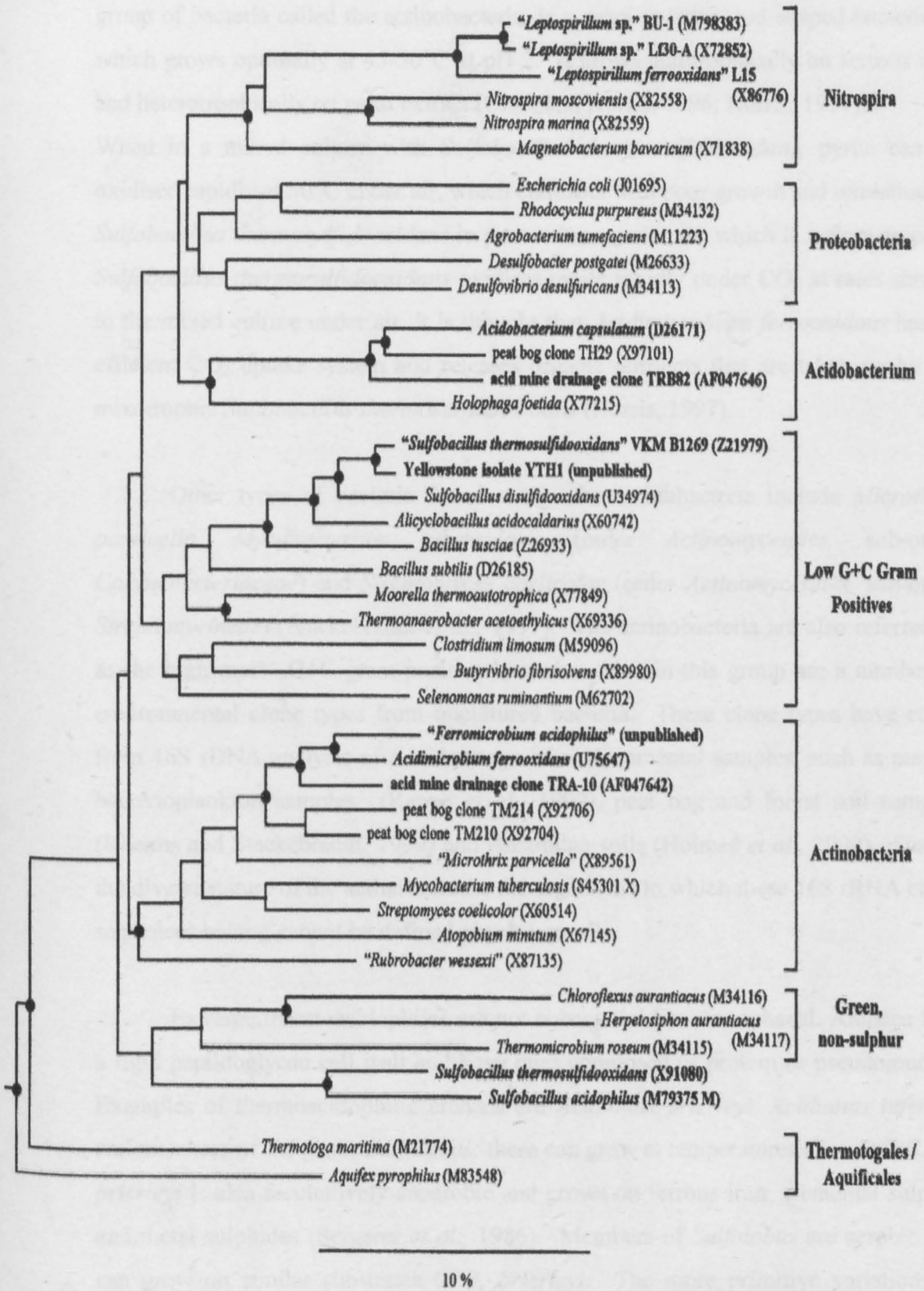


Fig 1.3 Evolutionary distance tree of the Eubacterial phyla, important bioleaching organisms are highlighted in bold (Goebel *et al.*, 2000)

Acidimicrobium ferrooxidans, from the order Acidimicrobiales, part of a diverse group of bacteria called the actinobacteria, is a gram-positive, rod-shaped bacterium, which grows optimally at 45-50°C at pH 2. It grows autotrophically on ferrous iron and heterotrophically on yeast extract (Clark and Norris, 1996; Norris, 1997).
When in a mixed culture with *Sulfobacillus thermosulfidooxidans,* pyrite can be oxidised rapidly at 50°C under air, which contrasts with poor growth and oxidation by *Sulfobacillus thermosulfidooxidans* in pure culture under air, which is a slow process. *Sulfobacillus thermosulfidooxidans* oxidises pyrite rapidly under CO_2 at rates similar to the mixed culture under air. It is thought that *Acidimicrobium ferrooxidans* has an efficient CO_2 uptake system and releases organic nutrients that are taken up by the mixotrophic *Sulfobacillus thermosulfidooxidans* (Norris, 1997).

Other types of bacteria found within the actinobacteria include *Microthrix parvicella, Mycobacterium tuberculosis* (order *Actinomycetales,* sub-order *Corynebacteriaceae*) and *Streptomyces coelicolor* (order *Actinomycetales,* sub-order *Streptomycineae*) (Stackebrandt *et al.,* 1997). The actinobacteria are also referred to as the high mol% G+C gram-positive bacteria. Also in this group are a number of environmental clone types from uncultured bacteria. These clone types have come from 16S rDNA analysis of a wide range of environmental samples, such as marine bacterioplankton samples (Rappe *et al.,* 1999), peat bog and forest soil samples (Rheims and Stackebrandt, 1999) and Australian soils (Holmes *et al.,* 2000). Due to the diverse nature of the actinobacteria, the organisms to which these 16S rRNA clone sequences belong cannot be defined physiologically.

Extreme thermoacidophiles are not eubacterial but are archaeal. Archaea lack a rigid peptidoglycan cell wall and have ones composed of protein or pseudomurein. Examples of thermoacidophilic archaea are *Acidianus brierleyi, Acidianus infernus* and members of the genus *Sulfolobus;* these can grow at temperatures above 60°C. *A. brierleyi* is also facultatively anaerobic and grows on ferrous iron, elemental sulphur and metal sulphides (Sergerer *et al.,* 1986). Members of *Sulfolobus* are aerobic and can grow on similar substrates to *A. brierleyi.* The more primitive variations of oxidation and reduction metabolism of the archaea have added new dimensions to the evolutionary ideas about lithotrophic metabolism. The evolutionary interest of

bioleaching organisms comes from the influence of chemolithotrophs on the shaping of our planet (Lane *et al.*, 1992).

The previously mentioned autotrophic bacteria and archaea are the main ones that have been studied in association with bioleaching. However, heterotrophic bacteria may also have importance in bioleaching processes and need to be studied further to establish their roles. Acidophilic heterotrophs that have been described include *Alicyclobacillus* species (gram-positive, low G+C group, Fig 1.3). These are moderately thermophilic organisms growing optimally at 45-65°C. The pH optimum of these organisms is higher than the autotrophic iron/sulphur oxidisers at 3.5-4 (Albuquerque *et al.*, 2000). Strains of alicyclobacilli have been isolated from solfatara soils within geothermal areas. Other major heterotrophs of potential significance are mesophiles of the genus *Acidiphilium* (α-Proteobacteria, Fig 1.2). Heterotrophic organisms do not usually gain energy from mineral oxidation, growth can occur from the utilisation of organic compounds released from the autotrophs or other organic materials. Heterotrophic organisms such as actinobacteria may aid the dissolution of the pyrite in the heap by preventing the inhibition of the growth of iron-oxidising bacteria by their own organic by-products. *Leptospirillum ferrooxidans* and *Acidithiobacillus ferrooxidans* are sensitive to organic compounds and heterotrophic bacteria metabolise these compounds thus preventing the inhibition of the autotrophic bacteria. Co-cultures of iron-oxidising bacteria and heterotrophic bacteria have been proven to have enhanced leaching capabilities compared to pure cultures of the iron oxidisers alone. Examples of such co-cultures are: *Leptospirillum ferrooxidans* and *Acidithiobacillus thiooxidans*/*Acidithiobacillus caldus*, this co-culture shows more efficient dissolution than *Leptospirillum ferrooxidans* alone, and *Sulfobacillus* species and *Acidimicrobium ferrooxidans* give rapid oxidation of ferrous iron without extra organic compounds or enhanced carbon dioxide levels (Johnson, 1998). See Figure 1.4 for a schematic overview of microbial interactions in an acidic, mineral-oxidising environment and Table 1.3 for a summary of organisms encountered in such environments.

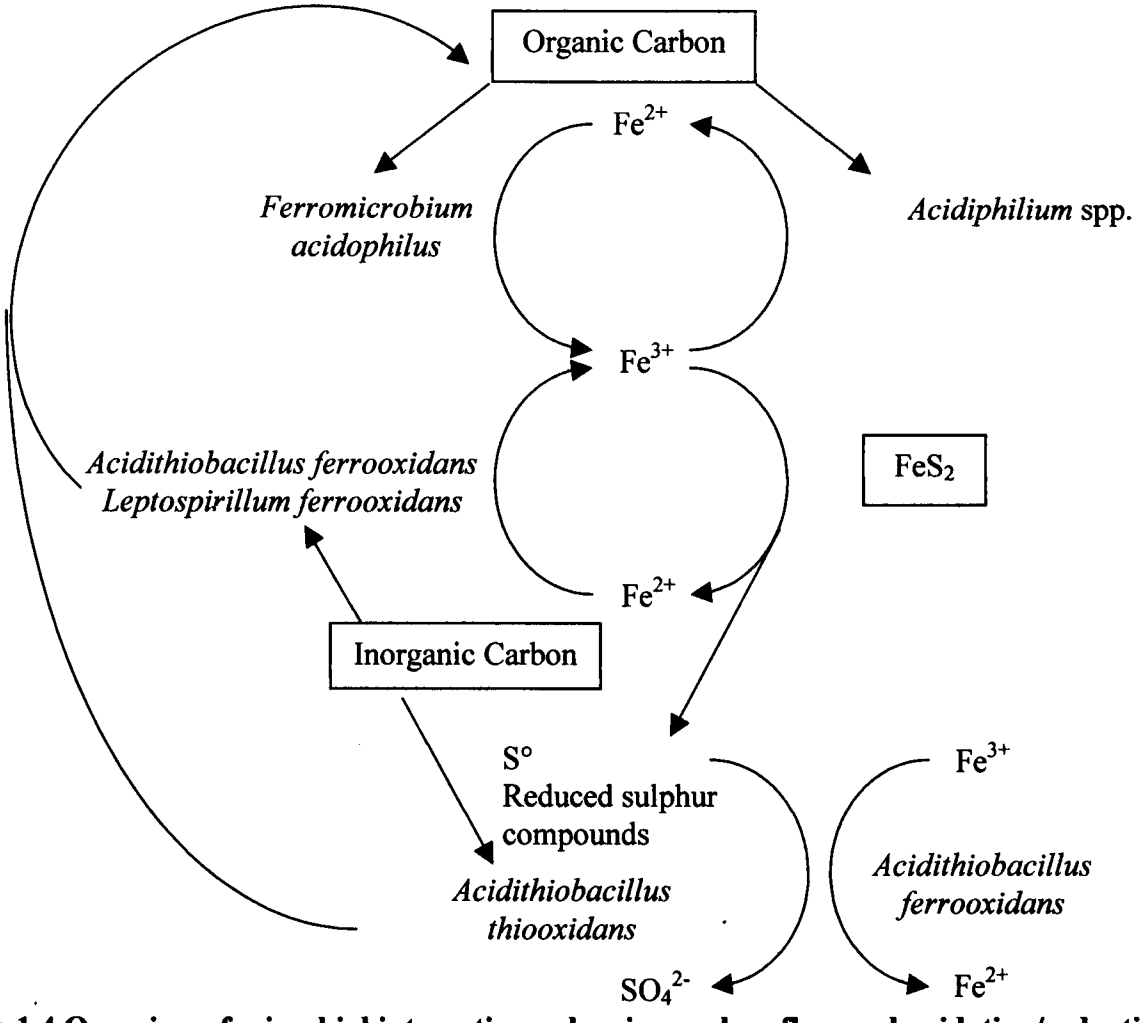


Fig 1.4 Overview of microbial interactions, showing carbon flow and oxidation/reduction of Fe and S in and acidic mineral-leaching environment. (Johnson, 1998)

Many of these bacteria have been found occurring naturally in places such as hot springs, volcanic areas and also waste dump sites such as coal-spoil heaps, these places are often acidic and of elevated temperatures. Here the bacteria can cause environmental damage due to the acid produced and the solubilisation of metals, which leach into the surrounding area where they are toxic to other living organisms.

Table 1.3 Summary of organisms encountered in acidic environments

Organism	Group	Environments isolated from	Substrate	Optimum growth temp °C
Acidithiobacillus ferrooxidans	γ Proteobacteria	Acid mine drainage, Coal spoil	Iron, sulphur and metal sulphides	30°C
Acidithiobacillus thiooxidans	γ Proteobacteria	Coal refuse piles	Reduced sulphur	30°C
Acidithiobacillus caldus	γ Proteobacteria	Coal refuse piles	Reduced sulphur	45°C
Thiobacillus prosperus	γ Proteobacteria	Solfataras	Iron, sulphur and metal sulphides	37°C
Leptospirillum ferrooxidans	γ Proteobacteria	Mine waters, Coal spoil	Iron	30-40°C
Sulfobacillus thermosulfidooxidans	Low G+C gram positive	Geothermal environments, Coal spoil	Reduced sulphur	45-50°C
Sulfobacillus acidophilus	Low G+C gram positive	Geothermal environments, Coal spoil	Reduced sulphur	45-50°C
Thermoplasma acidophilum	Euryarchaeaota	Coal refuse piles/solfataras	Heterotrophic	59°C
Thermoplasma volcanium	Euryarchaeaota	Solfataras	Heterotrophic	60°C
Sulfolobus spp.	Crenarchaeaota	Solfataras .	Sulphur, some are mixotrophic	70-87°C
Acidianus infernus	Crenarchaeaota	Solfataras	Sulphur, iron	90°C
Acidianus brierleyi	Crenarchaeaota	Solfataras	Sulphur, iron	70°C
Acidimicrobium ferrooxidans	Actinobacteria	Coal refuse piles	Mixotrophic iron oxidiser	45-50°C
Alicyclobacilli	Low G+C gram positive	Solfataras, Coal spoil	Heterotrophic	45-65°C
Acidiphilium spp.	α Proteobacteria	AMD, Coal spoil	Heterotrophic	25-40°C

1.5 Molecular biology and the biomining industry

The use of molecular ecological methods is becoming of interest in the biomining industry and is now being developed for monitoring the population dynamics of the organisms. Culturing methods for acidophiles, both mesophiles and thermophiles, can take several days by which time the population could have changed dramatically if growth conditions in a bioreactor or heap have been altered. A slight change in the composition of the mineral being fed into a reactor could alter the

numbers of organisms thereby upsetting the bioleaching process. Molecular methods could allow the swift and accurate monitoring of bioreactors so that any detrimental changes occurring to the microbial composition can be quickly recognised. Jerez (1997) highlighted the need for quick and simple techniques that could be employed at biomining plants to analyse and enumerate microbial populations. He discussed methods such as those mentioned previously, the use of probes in dot blot and Southern hybridisations, PCR and *in situ* fluorescent probing. He also included immunological methods: dot-immuno-assays (DIMA), Pulse Field Gel Electrophoresis (PFGE) and Denaturing Gradient Gel Electrophoresis (DGGE).

1.6 Project aims

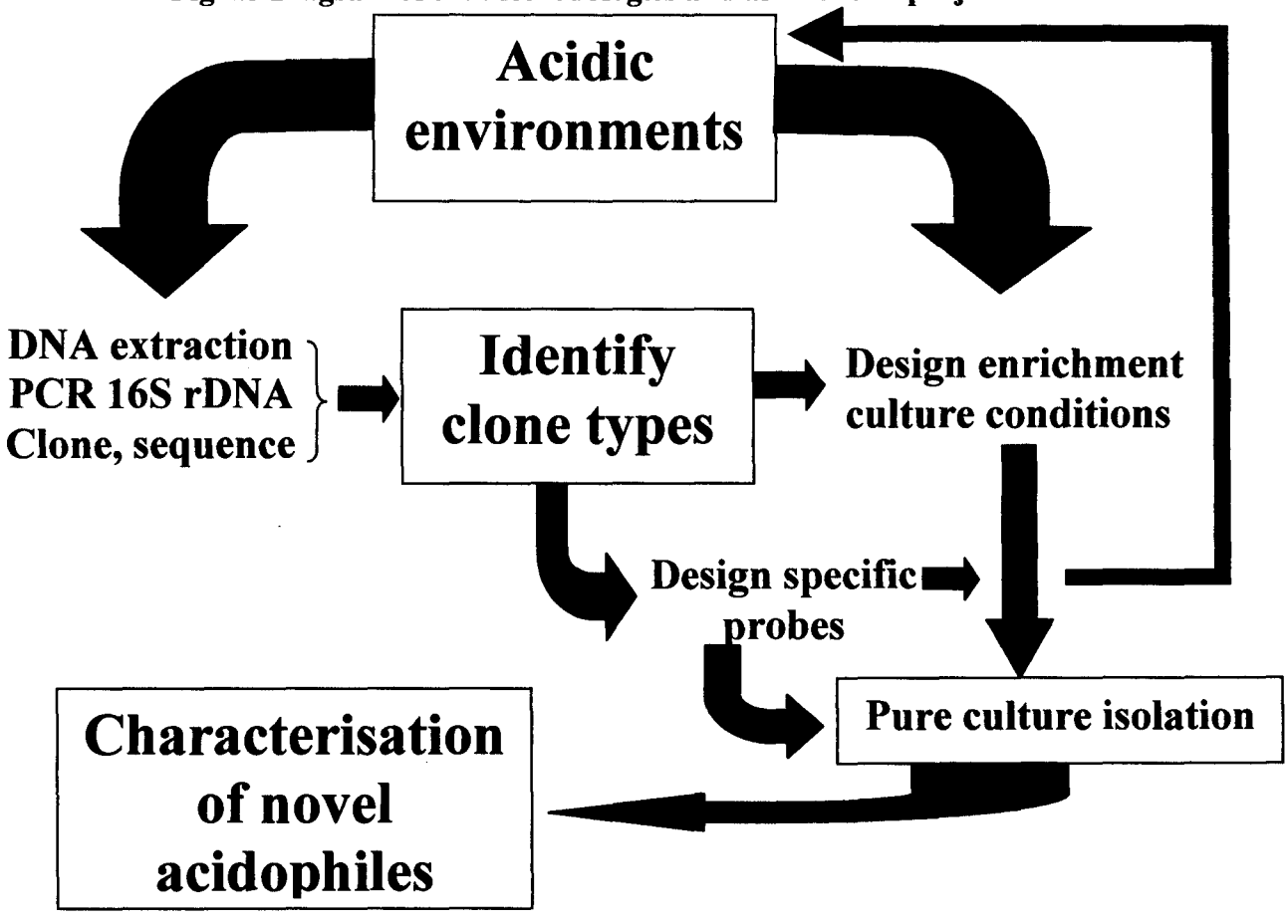
This project involved the 16S rDNA analysis of two acidic environments with different mineral compositions to the acidic sites in Table 1.2. These two sites were Birch Coppice, an organic matter and pyrite rich coal-spoil heap in north Warwickshire and geothermal vents on Vulcano Island, Italy, an acidic, iron rich, hot and saline site. These two sites were chosen with the expectancy of different biodiversities to the previously studied sites and perhaps of novel organisms in the indigenous populations.

From the acidic environments DNA was to be extracted and clone types identified. These identified clone types were intended to aid the design of enrichment culture conditions to isolate organisms from the acidic environments. The identified clone types should also have allowed the design of specific probes, which could have been used to probe the enrichment cultures and the DNA from the acidic environments. Used together, these methodologies should allow the characterisation of novel acidophiles. Figure 1.5 illustrates the methodologies involved in this project.

In summary the aims were:

1. To analyse the microflora of Birch Coppice coal-spoil heap and geothermal pools of Vulcano Island using 16S rDNA molecular analysis
2. To isolate and characterise novel organisms
3. To design and use labelled oligonucleotide probes to study bioreactor and microcosm microflora

Fig 1.5 Diagram of the methodologies and aims of this project



CHAPTER 2 MATERIALS AND METHODS

2.1 Sampling

2.1.1 The coal-spoil samples used in this study were taken from Birch Coppice colliery in north Warwickshire. A number of small samples were taken using 20ml sterile plastic universals; larger (10 kg) samples were taken using a sterile trowel to fill plastic autoclave biohazard bags. The samples were taken back to the laboratory and those not used immediately for DNA extractions or culturing were placed in a -80°C freezer until further use. Some of the larger sample was frozen, the remainder (8 kg approx) was placed in a large plastic box cleaned with ethanol, and this was used as a simple microcosm. More information is given about this sample site in Chapter 3 section 3.1

2.1.2 Geothermal hot pool samples were obtained from Vulcano Island, Italy. Samples were obtained from hot pools of differing conditions using 20ml plastic universals. More information is given about the samples obtained from Vulcano and site description in Chapter 4, section 4.1.

2.2 General chemicals and reagents.

All chemicals unless otherwise stated were obtained from the following manufacturers:

British Drug House (BDH) Ltd., Poole, Dorset,UK.
Sigma Chemical Co. Ltd., Poole, Dorset, UK.
Fisons Scientific Equipment Ltd., Loughborough, UK.
Lab M, Salford, UK.

2.3 Organism growth media

2.3.1 Organisms used

Sulfobacillus thermosulfidooxidans strain BC1, *Sulfobacillus acidophilus* strain NAL, *Leptospirillum ferrooxidans* DSMZ 2705, *Acidithiobacillus thiooxidans* ATCC 8085

Acidithiobacillus ferrooxidans DSMZ 583, *Acidithiobacillus caldus* DSMZ 8584 and *Alicyclobacillus* types 8a, 8c and K2.

2.3.2 Media

Table 2.1 Basal salts

Compound	Single strength liquid media	Double strength liquid media	Solid media
$MgSO_4.7H_2O$	0.4g/l	0.5g/l	0.5g/l
$(NH_4)_2SO_4$	0.2g/l	0.4g/l	0.4g/l
K_2HPO_4	0.1g/l	0.2g/l	-
KCl	0.05g/l	0.1g/l	-
K_2SO_4	-	-	0.1g/l
Phytagel	-	-	0.45%w/v

The media were adjusted to the appropriate pH with 5% (v/v) sulphuric acid (in distilled water) and were sterilized by autoclaving at 121°C for 15 min (15 lbs/sq inch) unless otherwise stated. More growth occurs on sulphide minerals than on ferrous sulphate therefore the primary limiting nutrients were increased with the use of the double strength medium.

Salts for serial dilutions.

	$g\ l^{-1}$
$MgSO_4.7H_2O$	0.5
$(NH_4)_2SO_4$	0.4

This was adjusted to the appropriate pH with 5% (v/v) H_2SO_4 (in distilled water)

Solid medium for growth of *Sulfobacillus* spp.

The following solutions were made and autoclaved. 250 ml of double the concentration of double strength salts medium (which contained K_2SO_4 at a final concentration of 0.2 g l^{-1} in place of K_2HPO_4 and KCl). Two and a quarter grams of phytagel was added to 250 ml dH_2O (when mixed with the salts the phytagel was at a final concentration of 0.45%) and allowed to dissolve with no shaking in order to prevent lumps forming. The solution was boiled prior to autoclaving.

The salts solutions were allowed to cool to 50°C and supplemented with ferrous sulphate, potassium tetrathionate (or yeast extract) and aluminium sulphate at the required concentration prior to mixing with the solidifying agent. For pour plates, 20 ml of the medium was poured into each petri dish. The plates were allowed to set at room temperature prior to spreading with 100 μl of a serial dilution of cells in exponential growth. The plates were allowed to dry, inverted and then incubated at 48°C in sealed autoclave bags for 5-14 days.

A similar medium was used for the growth of *Leptospirillum ferrooxidans,* in this case the potassium tetrathionate was omitted and the organism was grown at 30-37°C. For *Acidithiobacillus thiooxidans,* the ferrous iron was omitted.

2.3.3 Media supplements

Ferrous Sulphate.

1 M stock solutions of $FeSO_4.7H_2O$ (Analytical Research grade) were prepared. The pH was adjusted to 1.3 using H_2SO_4 and the solution was autoclaved at 115°C for 10 min (10lbs/sq inch). Ferrous sulphate was used at a final concentration of 50 mM in liquid media and 20 mM in solid media.

Yeast Extract.

1% (w/v) stock solutions of yeast extract were prepared and sterilized by autoclaving at 121°C for 15 min. Yeast extract was used at a final concentration of 0.02% (w/v) in liquid media and 0.01% (w/v) in solid media.

Potassium Tetrathionate.

3% stock solutions of $K_2S_4O_6$ (Fluka Chemicals Ltd., Gillingham Dorset) were prepared and sterilized by autoclaving at 115°C for 10 min. Potassium tetrathionate was used at a final concentration of 0.5 mM or 1mM as a reduced sulphur source where ferrous iron was the substrate for autotrophic growth.

Aluminium Sulphate.

100 mM stock solutions of $Al_2(SO_4)_3.16H_2O$ were prepared and sterilized by autoclaving at 115°C for 10 min. Aluminium sulphate was used at a final concentration of 2.0 mM.

The aluminium forms a positively charged hydroxide and attracts organic acids which are toxic to some of the organisms used in this project. Aluminium hydroxide or ferric hydroxides (both are trivalent metals) are used in drinking water treatment, though there it is to attract negatively charged bacteria and viruses, as well as organics, to a floc that can be filtered/settled out. Ferric iron works as well as aluminium but it is not used to avoid complications of iron being a substrate.

2.4 Cell growth and harvesting

2.4.1 Batch culture.

Conical flasks (250 ml) containing 100 ml total volume of medium (before addition of substrate and inoculum) were used to grow stock cultures. These were agitated in rotary shakers at 120 rpm at the appropriate temperature for optimal growth. Flask necks were plugged with foam bungs.

2.4.2 Cell Harvesting.

Cultures were harvested by centrifugation at 11500 rpm for 15 min in a fixed angle Beckman JA10 rotor or at 13000 rpm for 5 min in a bench top microcentrifuge at room temperature. Cell pellets were washed twice in acid water (pH 1.7) and either frozen rapidly in liquid nitrogen and stored at -80°C or were re-suspended in an appropriate volume of sterile distilled water or appropriate buffer and used immediately.

2.5 Buffers, reagents and solutions

2.5.1 General-purpose buffers and solutions all made with MQ H_2O

1M Tris pH 8

0.25M EDTA

10% w/v SDS

10M NaOH

3M sodium acetate pH 5.2

1M glucose

1M $MgCl_2$

$T_{10}E_1$ – 10mM Tris pH 8, 1mM EDTA

2.5.2 DNA extraction buffers and solutions

Extraction buffer - 0.5M Tris pH 8, 0.1M NaCl, 1mM Na citrate

Lysis buffer - 0.2M Tris pH 8, 0.1M NaCl, 4% w/v SDS

Phenol-chloroform-isoamylalcohol - ratios of 25:24:1 of phenol:chloroform:isoamylalcohol

BIO101 FastDNA®Kit (catalog No. 6540-400) for the Hybaid Ribolyser™ machine

2.5.3 DNA/RNA gel electrophoresis buffers and solutions

TBE(10X) – (per litre)100g Tris base, 5.5g Boric acid, 40ml 0.5M EDTA pH8

5X DNA loading buffer - 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol 15% w/v ficoll 400

Markers – Made according to manufacturers instructions (Gibco) with lambda DNA and DNA loading buffer. *Hin*d III for 1% agarose gels, *Pst*I for 2% agarose gels. Lambda DNA final concentration 50ng/µl, 6µl of marker was loaded in wells.

Ethidium bromide - 10mg/ml in MQ water

2.5.4 DNA clean-up solutions

Bio101 FastDNA kit - Binding matrix solution, SEWS-M, DES

Cetyltrimethylammonium bromide (CTAB)

Dilution-precipitation buffer – 50mM Tris-HCl, 10mM EDTA, 1% w/v CTAB

Wash solution – 0.4M NaCl

DNA salt dissolving solution – 2.5M ammonium acetate

Electroelution

TBE

Tris saturated butanol

Electro-elution BioRad model 422 Electro-eluter (catalog No. 165 2976/2977)
DNA elution buffer – Tris 40mM, glacial acetic acid 20mM, EDTA 1mM, SDS 0.1%.

2.5.5 SDS PAGE buffers and solutions

Acrylamide solution - 30g acrylamide, 0.8g Bis- acrylamide in 100ml H_2O

Upper Tris buffer - 0.5M Tris, 0.4% w/v SDS, pH 6.8 adjusted with HCl

Lower Tris - 1.5M Tris, 0.4% w/v SDS, pH 8.8 adjusted with HCl

10X Running buffer - 188g glycine, 30.2g Tris, 10g SDS in 1 litre H_2O

5X Sample buffer (reductase) – 4ml distilled water, 1ml 0.5M Tris-HCl pH 6.8, 0.8ml glycerol, 1.6ml SDS (10% w/v), 0.4ml β-mercaptoethanol, 0.2ml bromophenol blue (0.05%w/v)

Coomassie blue stain - 250ml ethanol, 200ml H_2O, 50ml acetic acid, 0.6g Coomassie R250

Destain - 150ml acetic acid, 250ml methanol, 1600ml H_2O.

2.5.6 Buffers and solution for plasmid extraction from *E. coli* (Sambrook *et al.*, 1989)

Solution I – 50mM glucose, 25mM Tris-HCl (pH 8.0), 10mM EDTA (pH 8.0)

Solution II - 0.2M NaOH, 1% w/v SDS

Solution III - 60ml 5M potassium acetate, 11.5ml glacial acetic acid, 28.5ml H_2O

2.5.7 Solutions and reagents for DNA/RNA dot blotting (Boehringer Mannheim dig kit protocol)

Most solutions and reagents were supplied with the kits, additionally required solutions were: -

20X SSC stock solution – 3M NaCl, 0.3M sodium citrate adjusted to pH 7.0 with 10M NaOH

10X SDS – w/v in MQ H2O

Maleic acid buffer – 0.1M maleic acid, 0.15M NaCl adjusted to pH 7.5 with solid NaOH

N-laurylsarcosine

Tween 20

Blocking solution – Blocking reagent (from kit) 10% w/v in maleic acid buffer

Hybridisation solution – 5X SSC, blocking reagent 1% w/v (from 10% stock), N-laurylsarcosine 0.1% w/v and SDS 0.02% w/v

Stringency washing solution (2X) - 2X SSC, 0.1% SDS

Stringency washing solution (0.1X) - 0.1X SSC, 0.1%SDS

Washing Buffer - Maleic acid buffer plus 0.3% v/v tween 20

Detection Buffer – 0.1M Tris-HCl, 0.1M NaCl, pH 9.5

Also used were Fuji Super RX X-ray film, Kodak Dektol developer and Kodak X-ray fixer.

2.5.8 Sequencing solutions and reagents

(i) Ethanol

(ii) Solution III from plasmid preps (2.5.6)

(iii) ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction mix

(iv) Primers at 3.2pM

2.6 DNA extraction and purification methods

2.6.1 Ribolyser™ following FastDNA kit protocol from BIO 101 (catalogue number 6540-400)

Half a gram of sample was put in a tube with lysing matrix, 1ml CLS-TC and 0.25g PVPP. This was homogenised in the Ribolyser™ for 30 seconds at a speed of 5.0m/s.

The debris was then pelleted in a microcentrifuge and 600µl of the supernatant was put into a new microcentrifuge tube. 600µl binding matrix was added to this, incubated at room temperature and microcentrifuged before re-suspension of the pellet in 500µl SEWS-M. The sample was again microcentrifuged and the DNA was eluted by re-suspending in 100µl DES and incubating followed by microcentrifuging.

2.6.2 Chemical lysis method (adapted from Barns *et al.*, 1994 and Tsai and Olsen, 1991)

Ten millilitres of extraction buffer with lysozyme (5 mg/ml extraction buffer) was added to 10ml sample and incubated for 1 hour at 37°C followed by the addition of 1mg/ml proteinase K and another incubation at 37°C for 30 minutes. Sixteen millilitres of lysis buffer was then added and mixed gently by inversion. Freeze-thaw cycles (dry ice + ethanol and 65°C water bath) were repeated three times followed by extraction with an equal volume of phenol and centrifugation at 11500 rpm for 10 minutes. A phenol-chloroform-isoamyl alcohol extraction was then carried out using the aqueous phase resulting from the phenol extraction and an equal volume of phenol-chloroform-isoamyl alcohol, this mixture was centrifuged at 11500 rpm 10 minutes.

The DNA was precipitated with an equal volume of propan-2-ol and centrifuged 15000 rpm at 4°C for 20 minutes. The pellet was re-suspended in 1ml of $T_{10}E_1$ and 0.1g PVPP was added. This was incubated for 1 hour 37°C followed by microfuging for 5 minutes at 13000 rpm and filtration of the supernatant.

The DNA in the supernatant was precipitated in 2 volumes of ethanol and 0.1 volumes of 3M sodium acetate at –80°C for 30 minutes or –20°C overnight and washed with cold 70% ethanol. The resulting DNA pellet was re-suspended in MQ water.

2.6.3 DNA extraction from pure cultures.

Grown cells were harvested, washed twice in acid water (pH 1.7 for cells grown at pH 1.7 or pH 3 for cells grown at pH 3), once in distilled water and once in 50 mM Tris, 1 mM EDTA (pH 8.5) and re-pelleted. The cell pellet was re-suspended in 0.5 ml of 50 mM Tris (pH 8.0) and lysed. 900 µl 0.25 M EDTA and 100 µl 10% (w/v) SDS were added to the cell lysate. The mixture was split into two Eppendorf tubes and an equal volume of $T_{10}E_1$ saturated phenol was added to each. The phases were separated by microcentrifugation at 13000 rpm for 10 min. The aqueous phases were transferred into sterile Eppendorf tubes containing equal volumes of phenol:chloroform:isoamylalcohol (24:24:1). The phases were separated by microcentrifugation at 13000 rpm for 5 min. The aqueous phases were transferred into sterile Eppendorf tubes and nucleic acids were precipitated from solution with equal volumes of isopropyl alcohol and microcentrifugation at 13000 rpm for 10 min. The resulting pellets were washed in 500 µl 70% (v/v) ethanol, air dried, re-suspended in an appropriate volume of sterile $T_{10}E_1$ buffer and pooled.

2.6.4 RNase treatment of DNA.

DNase-free RNase (Pharmacia Biotech) was used for the selective removal of RNA from both genomic DNA and plasmid DNA preparations. A stock solution of RNase A (10 mg ml^{-1}) in distilled water was prepared and heated in a boiling water bath for 15 min, cooled to room temperature and stored at -20°C. DNA samples were treated with RNase at a final concentration of 20 μg ml^{-1} with incubation at 37°C for 60 min.

2.6.5 DNA Purification

(i) Bio101 FastDNA kit solutions

The solution containing the crude DNA was made up to 0.6 ml with MQ water and added to 0.6 ml of Binding matrix solution, this was incubated for 5 minutes at room temperature.

The mixture was microcentrifuged for 1 minute and the supernatant was discarded. The pellet was resuspended in 0.5 ml SEWS-M and again microcentrifuged for 1 minute and the supernatant discarded.

DNA was eluted from the binding matrix by resuspending in 0.1ml DES followed by incubation at room temperature for 3 minutes. After centrifugation for 1 minute the supernatant was transferred to a new tube. This elution procedure was repeated a number of time to remove all of the DNA from the Binding matrix.

The DNA from each elution repeat was run on a 1% agarose gel and visualised with ethidium bromide on a transilluminator. All the DNA suspensions from each repeat were pooled and precipitated with 2 volumes of ethanol and 0.1 volume of sodium acetate.

(ii) Electroelution from gel slices

Agarose gel slices containing DNA bands were placed in dialysis tubing (diameter 8/32, Scientific Industries International) with some TBE buffer and placed in a mini gel electrophoresis tank and covered with TBE. A current was placed across the tubing causing the DNA to move out of the gel but remain in the tubing. The tubing and gel were briefly viewed using a UV transilluminator to check all of the DNA had been removed from the gel slices. The DNA was removed from the tubing and the tubing was rinsed with TBE.

The TBE containing the DNA was extracted with an equal volume of Tris saturated butanol a few times to remove the ethidium bromide and the DNA was precipitated using 2 volumes of ethanol and 0.1 volume of 3M sodium acetate.

A BioRad electroelution apparatus (Model 422 Electro-Eluter) was also used to obtain DNA from gel slices (manufacturers protocol).

(iii) Precipitation using Cetyltrimethylammonium bromide (CTAB) (Johnson, 1991)

An equal volume of CTAB dilution precipitation buffer was added to the solution containing crude DNA. The CTAB-nucleic acid salts were pelleted by microcentrifugation at 8000 rpm for 10 minutes and the precipitate was washed 2-3 times with 300µl of cold wash solution to remove any free CTAB.

The CTAB-nucleic acid pellet was dissolved in 100 µl salt dissolving solution and the DNA was precipitated with 2 volumes of 95% ethanol and microfuging for 10 minutes. The resulting DNA pellet was washed with cold 70% ethanol and re-suspended in MQ water.

(iv) Purification using Phenol-chloroform-isoamylalcohol

The crude DNA solution was made up to 0.5 ml with MQ water and an equal volume of phenol-chloroform-isoamylalcohol was added, inverted and microcentrifuged at 11000 rpm for 5 minutes. The aqueous phase was removed and added to an equal volume of chloroform-isoamylalcohol, inverted and again microcentrifuged for 5 minutes. The aqueous phase was removed and the DNA was precipitated from it with either an equal volume of propan-2-ol, or 2 volumes of ethanol + 0.1 volumes of sodium acetate and chilled at –80°C or –20°C. The resulting DNA pellet was washed with cold 70% ethanol and re-suspended in MQ water.

The resulting DNA from the extractions/purifications were visualised for quality on 1% agarose gels.

2.6.6 Determination of concentration of nucleic acids.

The concentration and purity of nucleic acid in aqueous solution was determined by measuring absorbance at 260 nm and 280 nm in a quartz cuvette of 1 cm path length against a distilled water blank in a Hewlett Packard 8453 spectrophotometer. The nucleic acid concentration was determined on the assumption that an A_{260} of 1.0 was equivalent to the following concentrations: oligonucleotides (20 $\mu g\ ml^{-1}$), RNA (40 $\mu g\ ml^{-1}$) and double-stranded DNA (50 $\mu g\ ml^{-1}$), (Sambrook *et al.*, 1989). The purity of nucleic acid samples was investigated by determining the

ratio of A_{260}/A_{280}. A ratio of >1.5:1 indicated relatively pure DNA with no contaminating proteins or polysaccharides.

The minigel method (Sambrook *et al.*, 1989), using Lambda DNA (60 ng μl^{-1} digested with *Hin*dIII) as the standard was used to estimate the concentration of plasmid DNA.

2.6.7 Agarose gel electrophoresis of DNA.

Agarose gels were made and run in 0.5 × TBE buffer. Routine analysis of plasmids, PCR products and genomic DNA samples used 1% (w/v) agarose gels. Ethidium bromide (EtBr) was added to the gel at a final concentration of 0.5 $\mu g\ ml^{-1}$. 50 ml of melted agarose was required for 'mini' gels (10 cm × 8 cm). Minigel tanks were supplied by Flowgen Instruments Ltd. Minigels were run at a constant current of 60 mA. DNA was visualized by placing the gel on a short wavelength UV trans-illuminator (260 nm). Gels were photographed using a Polaroid camera (CU5 Land Camera) and Polaroid 665 positive/negative film).

2.7 16S rDNA amplification by PCR and clone libraries

2.7.1 PCR conditions and reagents

The polymerase chain reaction (PCR) was used to amplify eubacterial 16S rRNA genes from genomic DNA of the environmental and bioreactor samples. The primers 27f and 1492r designed for the amplification of eubacterial 16S rRNA genes, were described by Lane (1991) (see table 3.1). Standard PCR amplification was performed in a total volume of 50 μl in 0.5 ml microcentrifuge tubes under a layer of DNase free mineral oil (Sigma) using a Perkin Elmer Cetus 480 thermal cycler. Each tube contained 1.5 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 100 μM of dNTPs, 10 mM bovine serum albumin, 50 pmol of each primer and 1 μl of either a 10^{-1}, 10^{-2}, 10^{-3} or 10^{-4} dilution of template DNA. The reaction mix underwent a "hot start" where the temperature was increased to 94°C for 5 min followed by a temperature decrease to 60°C for 1 min. At this stage, 2.5 units of *Taq* DNA Polymerase (Gibco BRL) was added and the reaction continued. 30-32 cycles of PCR amplification were performed as follows: 94°C for 1 min (denaturation), 60°C for 1

min (annealing) and 72°C for 1.5 min (extension). After completion of the cycles a final extension step of 15 min completed the PCR.

2.7.2 Cloning of PCR products.

Taq DNA polymerase was used in PCR amplification because it has no 3' to 5' exonuclease activity and therefore generated PCR products with a 3' adenine overhang on the end. PCR products were cloned using the TA cloning kit or the TOPO cloning kit (Invitrogen, San Diego) according to manufacturers instructions.

2.7.3 Ligation/transformation The TOPO TA cloning kit (Invitrogen) protocol (catalog No. K4500) using chemically competent cells and pCR®2.1 TOPO plasmid vector was followed.

2.7.4 Plasmid preps One-step 'miniprep' method for the isolation of plasmid DNA protocol (Chowdhury, 1991) and the alkaline lysis method (Sambrook *et al.*, 1989) were used.

2.7.5 Restriction endonuclease digestion of DNA.

Restriction endonucleases and buffer solutions were supplied by Gibco BRL. DNA was digested according to the manufacturers instructions.

Plasmids containing 16S rDNA PCR products were extracted from transformed competent cells and digested with restriction endonucleases. Restriction fragments were separated using agarose gel electrophoresis. Plasmids were initially digested with *Eco*R1 in reaction buffer 3 for at least 2 hours. Restriction fragments were run on a 1% (w/v) agarose gel to determine which plasmids contained products of the correct size. The plasmids that contained correct size inserts were digested with two different sets of restriction endonucleases: *Eco*R1/*Sau*3AI, and *Eco*R1/*Rsa*I in reaction buffer 4 for at least 2 hours. Restriction fragments were run on a 2% (w/v) agarose gel.

*Eco*RI, six base cutter at G*AATTC
*Rsa*I, four base cutter at GT*AC
*Sau*3AI, four base cutter at *GATC

2.7.6 Medium for culture and maintenance of *Escherichia coli.*

Luria Bertini broth (LB) was routinely used for the growth and maintenance of *Escherichia coli* strain INVαF' (Invitrogen, San Diego). The recipe for this medium is detailed in Sambrook *et al.* (1989). LB was solidified by the addition of Difco Bacto agar at 1% (w/v).

Cultures of *E. coli* were incubated at 37°C both on plates and in liquid culture. Liquid cultures (10 ml volume) were incubated in glass universals in an orbital shaker at 200 rpm. To ensure plasmid maintenance and strain purity, the appropriate antibiotic (100 μg ml^{-1} ampicillin) was added to the medium.

2.7.7 Long term storage of *E. coli* in glycerol.

Fresh overnight cultures of *E. coli* (175 μl) were added to 75 μl of glycerol:H_2O (1:1), mixed well and then frozen rapidly in a dry ice-ethanol bath. Glycerol stocks were stored at -80°C. To recover the strain, a loopful of glycerol stock was streaked onto solid LB medium and incubated at 37°C overnight. A single colony was used to inoculate LB medium.

2.8 Protein gels

2.8.1 SDS gel preparation

Separating gel (resolving gel)

The resolving gel was composed of 12 ml of Acrylamide (30%:0.8%), 9 ml of lower Tris, 13 ml of water, 200 µl of ammonium persulphate (APS) and 20µl of Temed.

Stacking gel

The stacking gel was composed of 1.6 ml of Acrylamide, 2.8 ml of upper Tris, 3.3 ml of water, 100 µl of APS and 11 µl of Temed.

2.8.2 Sample preparation

Cells were pelleted, washed in acid water followed by distilled water and resuspended in $T_{50}E_1$ with 1mg/ml lysozyme for 1 hour at 37°C. Lysis was completed by boiling for 3 mins in sample buffer reductase followed by microcentrifugation for 3 mins 13000 rpm.

SDS-PAGE was routinely carried out using 10% (w/v) SDS-polyacrylamide resolving gels with 5% (w/v) stacking gels according to the method of Laemmli (1970). Standard LKB (Bromma, Sweden) electrophoresis plates (20×20 cm), a vertical electrophoresis unit, and an LKB 2197 power unit were used. SDS-PAGE was performed at 35-40 mA for 3-4 hours. Low molecular weight protein markers were supplied by Pharmacia Inc (Uppsala, Sweden) and comprised:

Protein	kDa
phosphorylase b	94
bovine serum albumin	67
ovalbumin	43
carbonic anhydrase	30
soya bean trypsin inhibitor	20.1
alpha- lactalbumin	14

Gels were stained overnight in Coomassie blue stain. Gels were de-stained by frequent changes in destain.

2.9 RNA extractions

Cells were harvested by microcentrifugation at 7000rpm for 3-5min and the supernatant was discarded. Cells were resuspended in 100µl $T_{10}E_1$ plus lysozyme by vortexing. Three hundred and fifty microlitres of kit buffer RLT (Rneasy kit, Qiagen Ltd) was added and vortexed followed by microcentrifugation at maximum speed. 250µl of ethanol was added to the resulting supernatant and mixed by pipetting. This mixture was then transferred to a spin column in a collection tube and microcentrifuged at 10000rpm for 15 seconds; the flow through was discarded. Seven hundred microlitres of kit buffer RW1 was added to the spin column and microcentrifuged for 15 seconds, and the flow through was discarded. Five hundred microlitres of kit buffer RPE was added, microcentrifuged for 15 seconds and the flow through was discarded. Another 500µl of RPE was added and microcentrifuged

for 2 min. The spin column was transferred to a new collection tube and 30-50µl of RNase free water was pipetted into the tube. The column was microcentrifuged for 1 min at 10000 rpm to elute the RNA. The RNA was then visualised on an agarose gel.

2.10 Oligonucleotide probes

2.10.1 3′ labelling (Boehringer) as protocol

2.10.2 Probe design Sequences were aligned using the pileup program in GCG (2.14). Probes were then designed from suitable regions in the aligned sequences, these probes were synthesised by GibcoBRL Custom Primers. Primer stocks were diluted to 100 pml when used for DIG-labelling.

2.10.3 DNA/RNA dot blotting equipment – Hybaid oven, Hybaid tubes, X-ray film, dark room, X-ray film developer and fixer

2.10.4 Dot-blot hybridization (Boehringer Mannheim protocol).

Nucleic acids of known concentration were spotted onto nylon membranes (Hybond-N) and fixed with a UV "Stratalinker" (Stratagene). Nucleic acids were detected with 16S rRNA specific oligonucleotide probes designed to clone types in rDNA clone libraries (Tables 3.1 and 4.3) using the digoxigenin (DIG) nonradioactive nucleic acid labelling and detection system (Boehringer Mannheim). In summary, oligonucleotide DNA probes were enzymatically 3'end-labelled with terminal transferase by incorporation of a single digoxigenin-labelled dideoxyuridinetriphosphate (DIG-ddUTP) according to the manufacturers instructions. Membrane-bound nucleic acids were hybridized with 25 pmol of DIG-ddUTP-labelled probe in hybridization solution (5 × SSC, 1% (w/v) Blocking Reagent (Boehringer Mannheim GmbH), 0.1% (w/v) N-laurylsarkosine, 0.02% (w/v) SDS). Hybridization was performed overnight at 45°C in glass tubes (Hybaid) in a hybridization oven (Hybaid). Following hybridization, unbound oligonucleotides were removed by washing membranes 2 × 5 min at 45°C with 50 ml 2 × SSC, 0.1% (w/v) SDS and 2 × 5 min at 45°C with 50 ml 0.1 × SSC, 0.1% (w/v) SDS.

Following hybridization with target nucleic acids and stringency washes, DIG-labelled probes were detected (according to the manufacturers instructions) by an enzyme-linked immunoassay using an antibody-conjugate (anti-digoxigenin alkaline phosphatase conjugate, anti-DIG-AP). Anti-DIG-antibody conjugates were then visualized with the chemiluminescence substrate CSPD®. Enzymatic dephosphorylation of CSPD® by alkaline phosphatase leads to a light emission at a wavelength of 477 nm which was visualized on X-ray film.

2.11 Microscopy and photography

2.11.1: Phase Contrast Microscopy.

Routine light microscopy was carried out using a Kyoga-Unilux III (Tokyo) phase contrast microscope. Microbial cultures were examined using either 1000 X magnification under oil immersion or 400 X magnification.

2.11.2 PHOTOGRAPHY.

Agarose gels were photographed using a Polaroid camera (CU5 Land Camera, loaded with Polaroid 665 positive/negative film).

2.12 G+C contents of organism DNA

2.12.1 Large scale culture procedure

Organisms were grown in 20 L jars in the appropriate medium. These jars were placed on heater stirrers connected with feed-back temperature control thermometers in the vessels, and gassed with air or with 1% CO_2 in air as required (1L per min). The cells were harvested using a cross flow filter and centrifugation in Beckman JA-10 pots.

2.12.2 Large Scale DNA extractions for G+C content analysis

The cell pellets were washed in 0.12 M NaCl, 0.05 M EDTA pH 8 and repelleted at 10000 rpm for 10 minutes room temperature. The resulting pellet was re-suspended in 10ml Tris-sucrose pH 8 (0.05 M Tris pH 8, 25% sucrose w/v) to which 0.5 ml of 20 mg/ml lysozyme was added (final concentration 2 mg/ml) before

incubation at 37°C for 10 minutes. Fifteen millilitres 0.25 M EDTA was then added and incubated at 37°C for 50 min followed by the addition of Sarkosyl to a final concentration of 2% v/v.

An equal volume of phenol-chloroform-isoamylalcohol was added, the solution was vortexed and centrifuged at 10000 rpm for 10 min. The aqueous phase (about 30 ml) was removed and used for the next step.

Thirty grams of caesium chloride was added to the aqueous phase (1g/ml) and dissolved. One millilitre of ethidium bromide (from a 10 mg/ml stock) was added and the mixture was centrifuged at 15000 rpm for 15 min to remove any debris. The mixture was then placed in an ultra-centrifuge tube and centrifuged at 45000 rpm at 15°C for 16 hours. When ultracentrifugation had finished the fluorescent DNA band was removed with an 18-gauge needle. An equal volume of Tris-saturated butanol was added to the resulting pink DNA, inverted and centrifuged at 15000 rpm for 3 min at room temperature.

The lower aqueous phase was collected and the butanol extraction procedure was repeated until any trace of ethidium bromide had gone. The caesium chloride was removed by dialysis against $T_{10}E_1$ (5 litres, 24-48 hr, changed twice)

The DNA was precipitated by adding a third of the volume of 3M sodium acetate and two volumes of ethanol followed by inversion and chilling for at least 2 hours at –20°C followed by centrifugation at 18000 rpm for 15 min at 4°C. The DNA pellet was then washed in 70% ethanol, vacuum dried and resuspended in 1ml $T_{10}E_1$.

One microlitre of the DNA suspension was run on a 1% agarose gel and the DNA was also checked for quantity and purity by 260/280 ratio measurement with a spectrophotometer. The DNA was then diluted in 0.1X SSC to give an absorbance (OD) at 260 of 0.6.

Controls (ultra pure DNA standards) were also dissolved in 0.1X SSC and all of the DNA samples were dialysed together against 0.1X SSC for 16 hours at 8°C. DNA from novel organisms and DNA (Sigma) from *Clostridium perfringens* (26.5 mol % G + C), *Escherichia coli* (52 mol % G + C) and *Micrococcus luteus* (72 mol % G + C) was dialysed three times against 0.1X SSC.

To prepare the dialysis tubing it was boiled for 10 mins in 2% sodium bicarbonate and 1mM EDTA followed by a thorough rinse in distilled water. It was then boiled in distilled water for 10 min, allowed to cool and stored at 5°C. Before use it was rinsed with distilled water.

Harvesting of the cells and the DNA extraction was mainly carried out by technician Andrew Price.

2.12.3 DNA melting and G+C analysis (Owen and Hill, 1979)

Melting curve mid-points (T_m) were determined using a Hewlett Packard automated DNA melt testing system and 8452A spectrophotometer. Unknown base compositions were calculated from the formula mol % G + C = mol % G + C of X + 2.08(T_m - T_m of X) where X was the DNA of known base composition (Owen & Hill, 1979). Means of triplicate T_m determinations were used and the mol % G + C calculated for each novel organisms was the mean of the three values calculated with reference to the three DNA standards

2.13 16S rDNA sequencing

A number of primers, both eubacterial and archaeal were used in DNA sequencing of 16S rDNA clones. 500-750 ng plasmid DNA was mixed with 1µl 3.2pM primer and 4µl dye terminator. This was made up to 10µl with MQ water and placed in a thermal cycler. Cycle sequencing conditions were carried out using a Hybaid Touchdown thermal cycling system. 25 cycles of 30 seconds at 96°C, 15 seconds at 50°C and 4 min at 60°C were carried out as recommended by PE Biosystems for their dye terminator.

The DNA was precipitated by adding 1µl of acetic acid and 25µl of ethanol and left on ice for 15 min, followed by microcentrifuging for 15min, 13000 rpm at 4°C. The resulting pellet was washed in 70% ethanol and dried.

The sequencing products were resolved using Applied Biosystems (USA) models 373A and 377 Automated Sequencers.

2.14 Phylogenetic analysis of sequences

Cloned sequences were checked using the CHIMERA_CHECK version 2.7 of the RDP at the University of Illinois (Maidak *et al.*, 1994). Data bases were searched for similar sequences using the RDP (Olsen *et al.*, 1991; Maidak *et al.*, 1994) and BLAST

programmes in GenBank (Altschul *et al.*, 1997). Percentage identities of selected sequences were determined following alignments using the BESTFIT programme of the Genetics Computer Group (GCG) Sequence Analysis Software Package, Version 7.2 (University of Wisconsin, USA). Sequences were aligned using the PILEUP programme of the GCG Sequence Analysis Software Package, with minor manual modifications where appropriate. Phylogenetic analysis of 16S rRNA sequences used the PILEUP programme of GCG and a neighbour joining method (Jukes and Cantor, 1969) was used in the DNAdist, DNApars and FITCH programmes of PHYLIP version 3.57 (Felsenstein, 1995). Boostrap values were calculated using SEQBOOT and CONSENSE programmes. Trees were visualised in TreeView.

2.15 Culture conditions and growth measurements

2.15.1 Vessels For many of the experiments to determine growth rates the organisms were grown in water-jacketed vessels. The vessels had a capacity of about 800 ml but only 400 ml of medium was used in each vessel for the experiments.

For some of the growth experiments flasks were used, when small flasks are mentioned it is 250 ml flasks that are being referred to, usually with 50-100 ml of medium. Large flasks refers to 2L flasks with 500 ml medium.

2.15.2 Growth rate measurements Growth was estimated as optical density measured at 440 nm in a spectrophotometer.

Growth of tetrathionate oxidising strains was also estimated from substrate utilisation with residual tetrathionate measured by the cyanolysis method of Kelly *et al.* (1969).

Tetrathionate assay protocol:

1. 0.1M KCN
2. **Buffer** (i) 50 ml 0.2M NaH_2PO_4
   (ii) 39 ml 0.M NaOH
3. **Ferric nitrate reagent**

217.4 ml 70% perchloric acid

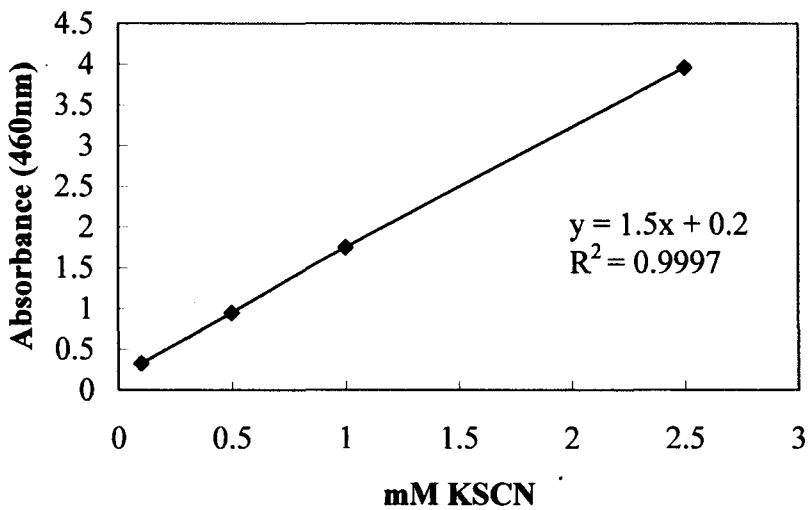
15 ml H_2O

303 g $Fe(NO)_3.9H_2O$

4. 1 mM KSCN standard

To a 25 ml volumetric flask 4 ml of buffer was added plus the sample or the standard (1ml 1mM KSCN ≡ 1mM tetrathionate), then 5ml KCN was added (to the samples only) and this was left for 10 minutes. After 10 minutes, 3 ml ferric nitrate reagent was added and mixed continuously. Following this the volume was made up to 25 ml with H_2O and the mixture was read at 460 nm immediately.
The readings from the spectrophotometer were measured against a calibration curve made using the KSCN standards (see Fig 2.1).

Fig 2.1 Calibration curve for Tetrathionate assay
(regression line not shown for the sake of clarity)



Growth of organisms on ferrous iron was monitored by assaying ferrous iron in solution using ceric sulphate titrations. The rate of ferrous iron oxidation has previously been demonstrated as a valid method of growth estimation for bacteria (Marsh and Norris, 1983). 0.5 ml culture aliquots were titrated against 0.05 M ceric sulphate in 5% (v/v) H_2SO_4 with 1, 10-phenanthroline-ferrous sulphate complex solution used as the end point indicator.

2.15.3 Cell counts The CellFacts particle size analyser (Microbial Systems Ltd., Coventry, UK) uses orifice electrical flow impedance to size and count cells in a suspension in real time (sample time lass than one minute). Patented technology also ensures that the orifice does not block in the presence of solids so that the instrument can be used in the presence of mineral sulphide particles, elemental sulphur or iron precipitates that can be found in growth media of acidophilic bacteria.

CHAPTER 3. COAL-SPOIL ENVIRONMENTAL rDNA ANALYSIS

3.1 Introduction

Coal-spoil and mine tailings sites are of environmental concern due to acidic run-off and the toxicity of metals leached from the sulphides they contain in the form of acid mine drainage. However, the organisms producing the acid and leaching the metals are of special interest to the mining industry because of their ability to withstand low pH and cause mineral sulphide oxidation. Acid mine drainage and mine tailings sites have been the focus for many projects. These sites are rich in mineral sulphides but lack organic matter so the organisms present at sites are often predictable, as sites of similar mineralogy usually have similar bacterial flora. Coal-spoil heaps, however, are also rich in organic matter which may create a difference in the bacterial flora present compared to non-coal acid mine drainage and mine tailings samples. Coal-spoil heaps have not been studied in depth using molecular ecology techniques. They have been sampled with regards to bacterium isolation and one of the first *Sulfobacillus* species was isolated from a coal-spoil heap – as mentioned in Chapter one. Other organisms that have been isolated from coal-spoil heaps include *Leptospirillum ferrooxidans, Acidithiobacillus ferrooxidans* and *Acidimicrobium ferrooxidans.* Organisms such as these are readily isolated from acidic mineral rich sites as they are easily cultured under the right conditions. However, some organisms are difficult to isolate and many types could be missed when standard culturing methods are used. This chapter reveals the results of 16S rDNA analysis of samples taken from Birch Coppice coal-spoil heap in North Warwickshire. The difficulty of obtaining novel organisms in culture is also shown which emphasises the value of combining molecular methods with more traditional techniques.

3.1.1 Previous 16S rDNA analysis with Birch Coppice samples (unpublished).

The results of a preliminary analysis carried out by N. P. Burton (University of Warwick) on a sample taken from an acidic iron rich pool found at Birch Coppice is shown in Figure 3.1. As can be seen in this figure, the clone library contained clone types with rRNA sequences most closely related to *Sulfobacillus thermosulfidooxidans, Acidimicrobium/Ferromicrobium* species and *Acidithiobacillus ferrooxidans.* The numbers in brackets show the percentage sequence identity of the clone sequence to the named organisms on the histogram and the number of bases over which the comparison was made.

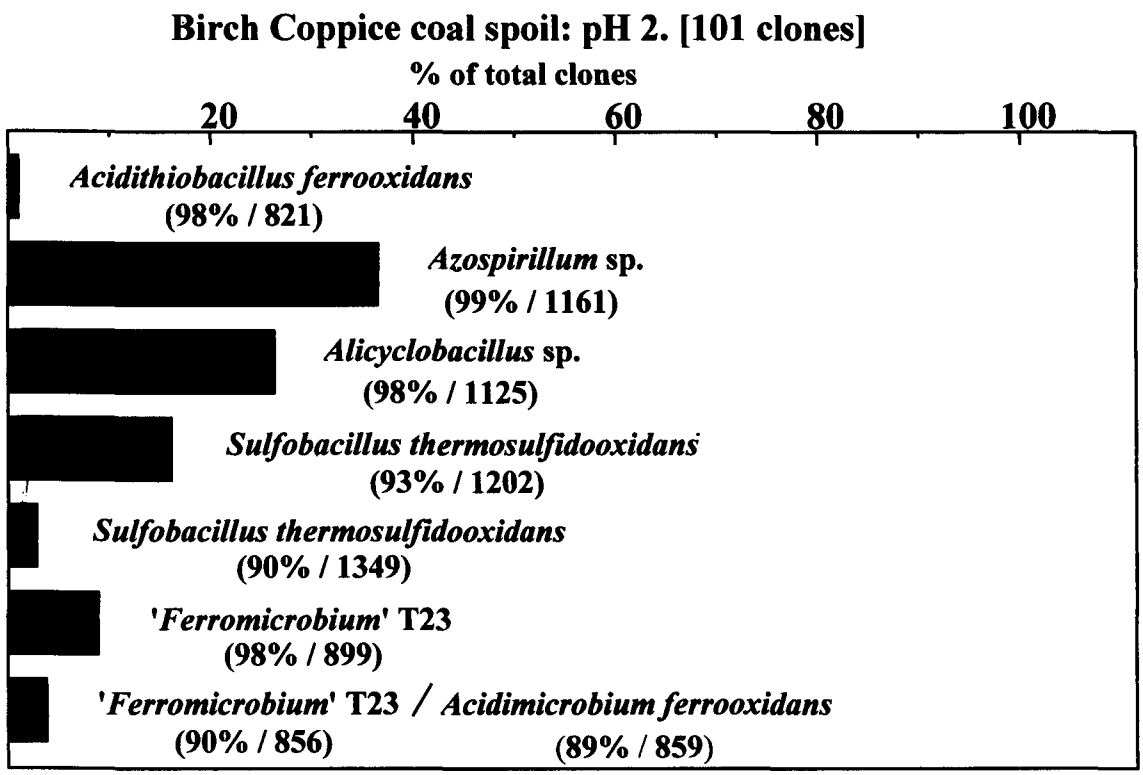


Fig 3.1 Environmental rDNA analysis of acidic pool sample (Dr N. Burton)

Most of these organisms are known to be present at such sites. However, the *Azospirillum* species found was unexpected as azospirilli are nitrogen-fixing organisms that do not tolerate acidity; it was presumed that this was a contaminant. This was later shown to be the case using DNA dot blots with probes designed to the sequences found in the clone library – the *Azospirillum* probe was negative with the Birch Coppice sample DNA.

Leptospirillum ferrooxidans clone types were not present in this clone library even though it is an organism that is readily isolated from coal-spoil heaps and has been previously isolated from Birch Coppice (Norris, unpublished). Only one method was used for extracting the DNA from the samples. This was a chemical lysis method adapted from Barns *et al.* (1994) and Tsai and Olsen (1991). It is thought that bias may occur with different methods of DNA extraction. For example, if some organisms did not lyse as well as others with the previously used extraction method at Birch Coppice, then some organism types may have been missed.

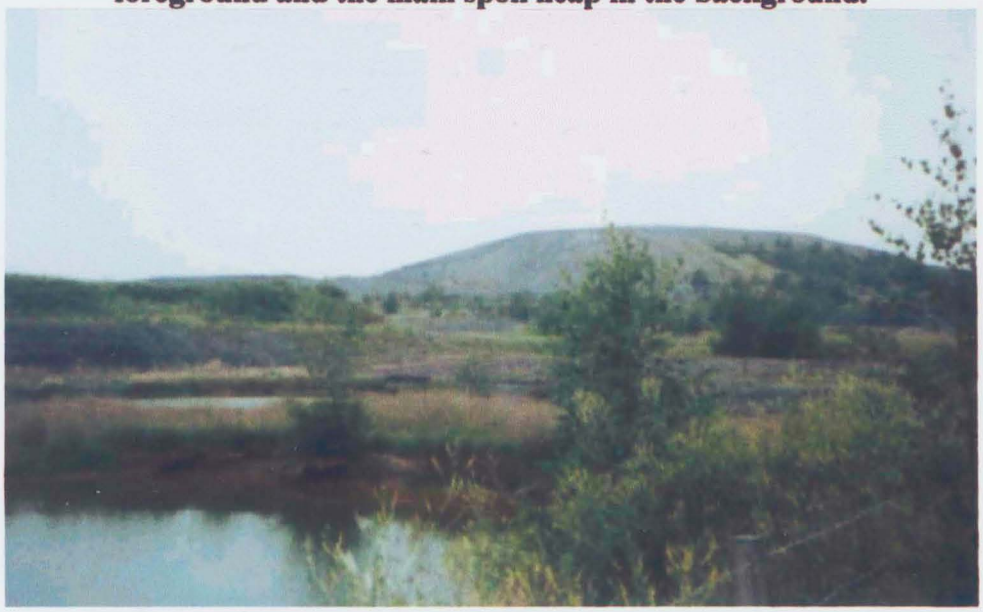
For this further investigation of Birch Coppice samples, two different DNA extraction methods were used to assess any obvious selectivity in relation to the methodology. The sequences obtained from the clone libraries were then used to design oligonucleotide probes to probe the coal-spoil DNA to avoid PCR biasing. The final part of this section of work attempted to isolate the novel organisms indicated by the clone library.

3.1.2 Sample site description

Mining at Birch Coppice colliery at Dordon, North Warwickshire has left approximately 9 million tonnes of coal spoil material. The site is very barren (Fig 3.2) as vegetation cannot survive due to the low pH of the waste in places, which is as low as pH 2. The acidity increases the solubility of the phytotoxic elements in the heap, a problem that is widespread in many pyrite rich tailings and waste heaps around the world (Schippers *et al.*, 2000). Some areas of spoil heaps at Birch Coppice used to be hot due to the exothermic reactions of mineral oxidation.

Iron is leached out of the heaps in acid run-off following pyrite oxidation, which results in visible deposition of red/brown hydrated ferric oxides in pools around the site. The acid is probably produced by the oxidation of the sulphides present in the heap by bacteria. The pH of the run off water has been so low that the spoil has been limed to neutralise acidity and allow release of higher quality water from the site.

Fig 3.2. (a) The abandoned Birch Coppice colliery site with a run-off pond in the foreground and the main spoil heap in the background.



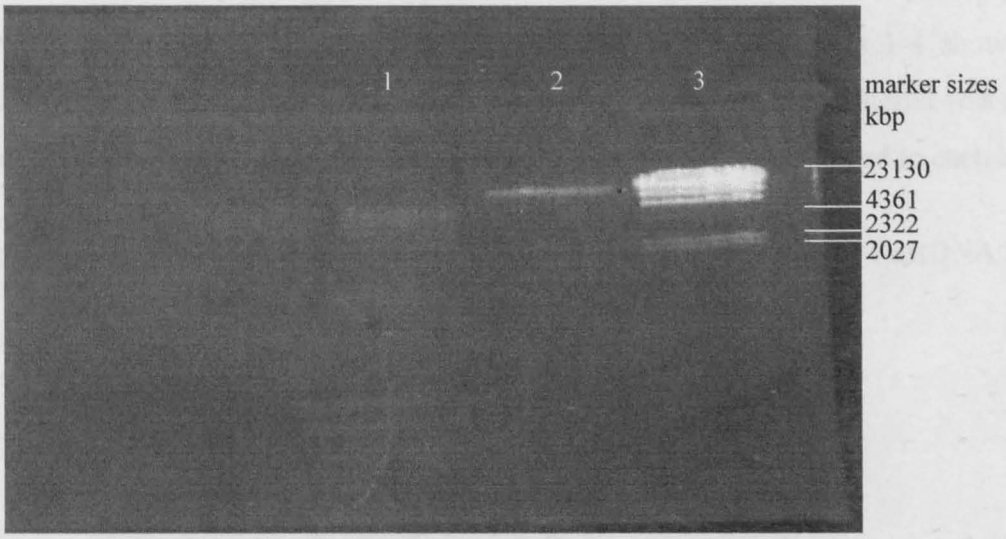
There is a run-off stream along the bottom of the heap where leached minerals collect and run down to the pools.

Fig 3.2 (b) The sampling site on the side of coal-spoil heap at Birch Coppice.

3.2 Environmental DNA extraction

DNA was extracted from the coal-spoil using adaptations of the chemical lysis methods of Barns *et al.,* (1994) and Tsai and Olsen (1991) and the Ribolyser method (Borneman *et al.,* 1996). More DNA was obtained with the Barns/Tsai and Olsen method and the DNA was of better quality than with the Ribolyser method where the resulting DNA was sheared. These results are illustrated in Figure 3.3. Lane 1 shows the DNA extracted using the Ribolyser method, lane 2 shows the DNA extracted using Barns/Tsai and Olsen method and lane 3 shows the lambda *Hin*dIII markers. The DNA was extracted from 0.5g coal-spoil sample using each method. 5µl of the resultant 20µl of DNA extracted via the Barns/Tsai and Olsen method and 20µl of the 100µl of DNA obtained from the Ribolyser method was loaded on the gel.

Fig 3.3 DNA extractions using the Barns/Tsai and Olsen method and the Ribolyser method.



Lane 1 shows DNA extracted by the Ribolyser methods, lane 2 shows DNA extracted by the Barns/Tsai and Olsen method and lane 3 shows the markers.

When the DNA was amplified by the polymerase chain reaction the PCR products appeared to differ little in quality and quantity. However, the DNA obtained from the Barns/Tsai and Olsen method was impure and needed PVPP treatment and dilution before any PCR products were obtained, whereas the DNA product from the Ribolyser extraction method was relatively clean and PCR products were obtained without the need for decontamination steps (results for PCR not shown). Therefore, if only enough DNA is required to perform PCR, then the Ribolyser method is a suitable

quick, and easy method and does not require the use of toxic chemicals such as phenol.

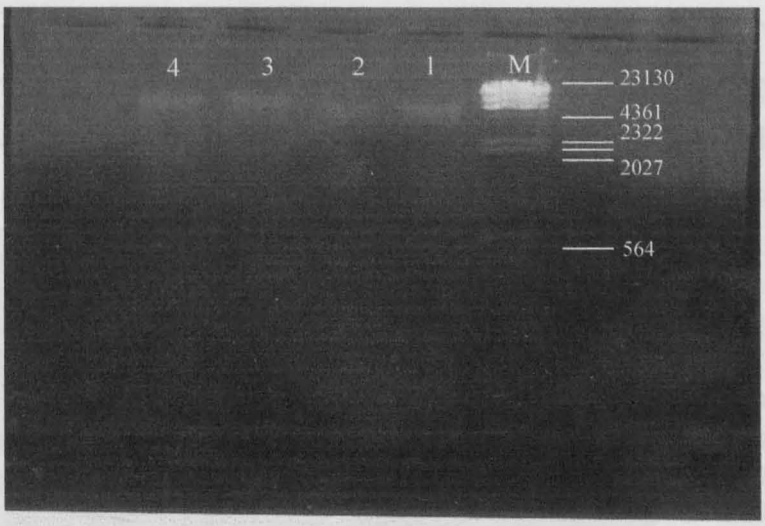
With regard to the Ribolyser method, it was also necessary to establish which lysing matrix combination was most efficient at extracting the DNA. Lysing matrix components consisted of ceramic cylinders, ceramic spheres and tiny garnet crystals. These could be used in different combinations to optimise yield and size of the resulting DNA.

There were four matrix combinations available:

1. ¼" sphere,
2. ¼" sphere + ¼" cylinder,
3. ¼" sphere + garnet matrix
4. ¼" sphere + garnet + ¼" cylinder

These item descriptions refer to the proprietary materials as given in the Fast DNA kit and have different efficiencies of DNA extraction depending on the type of sample. DNA was extracted using the different matrix combinations and examined by electrophoresis (Fig 3.4). M is the Lambda *Hin*d III marker; lanes 1-4 show the results of the DNA extracted using matrices 1-4. 0.5g of coal-spoil material was used in each extraction resulting in a 100µl DNA suspension; 20µl was loaded in each lane.

Fig 3.4. DNA extractions using the 4 different lysis matrices of the FastDNA kit.



M indicates the markers, lane 1 shows DNA extracted using lysing matrix combination 1, lane 2 shows DNA extracted using lysing matrix combination 2 and so on for lanes 3 and 4.

Matrix combination three (sphere +garnet) indicated the best quality of DNA out of all four matrix combinations. This matrix combination resulted in the least sheared, larger sized and most amount of DNA. Matrix combination two (sphere + cylinder) resulted in the lowest quality DNA. From these results it was difficult to relate DNA quality to matrix combination, however, it appears that combinations involving garnet give a better quality DNA than combinations involving the sphere, the cylinder or both.

3.3 Environmental rDNA analysis

3.3.1 Clone library analysis

DNA was extracted from two samples, designated A and B, obtained from the side of the heap at Birch Coppice using the Barns/Tsai methods of chemical lysis and the Ribolyser method of mechanical lysis with matrix combination three. The PCR was performed with extracted DNA and the PCR products were ligated and transformed using the TOPO TA cloning kit from Invitrogen. Plasmid preparations were carried out using the one step miniprep method (Chowdhury, 1991) and restriction digests were performed on the plasmids using *Eco*RI to check for correct insert size. Double digests were carried out using *Eco*RI/*Sau*3AI and *Eco*RI/*Rsa*I to look for different restriction fragment length polymorphisms (RFLPs).

Figure 3.5 shows examples of the different RFLP types obtained from the samples using *Rsa*I/*Eco*RI; M indicates Lambda *Pst*I markers. Over 170 clones were examined for each sample extracted with each method but not all had inserts. 128 clones from sample A and 85 clones from sample B using the Ribolyser method had inserts. 50 clones from sample A and 81 clones from sample B using the chemical method had inserts. Representatives of each RFLP type were sequenced.

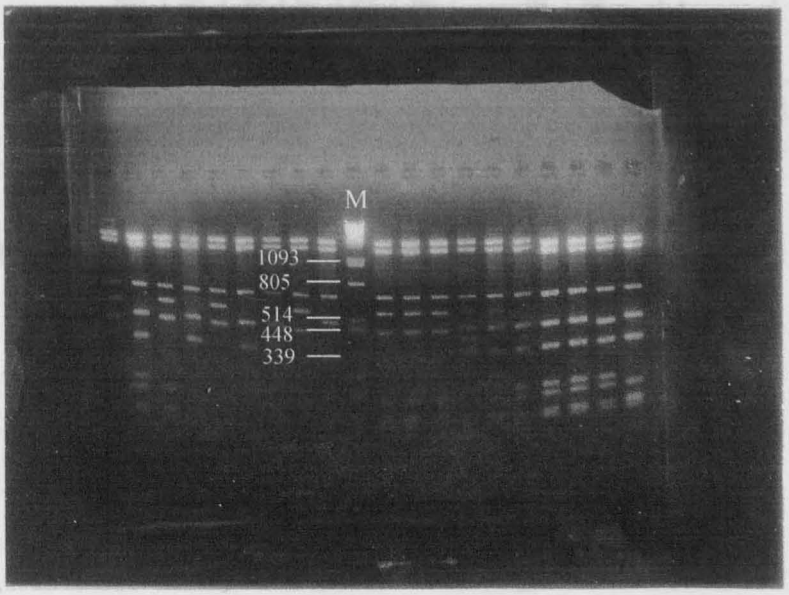


Fig 3.5 Representative RFLPs of clones containing Birch Coppice spoil environmental rDNA using *Rsa*I/*Eco*RI restriction enzymes. M indicates the *Pst*I markers.

The percentage of each clone type obtained from each sample with each extraction method is illustrated in Figure 3.6 and Figure 3.7 shows most related data-base sequences to the major clone types found. In Figure 3.6 A and B indicate the two different samples. Thus clone type 1 represent 32% of the clones found in sample A using the chemical method and represent 41% of the clone types obtained from sample A using the Ribolyser method, whereas clone type 1 represents 45% of the clone types found in the clone library of sample B using the Ribolyser method and so on. From this histogram it can be seen that qualitatively there was little or no apparent biasing regarding the DNA extraction method used. All clone types were found in both samples using both extraction methods. These results suggest that the samples represented a relatively homogeneous spoil tip area and that the DNA extraction method used was of no consequence with regards to clone library generation.

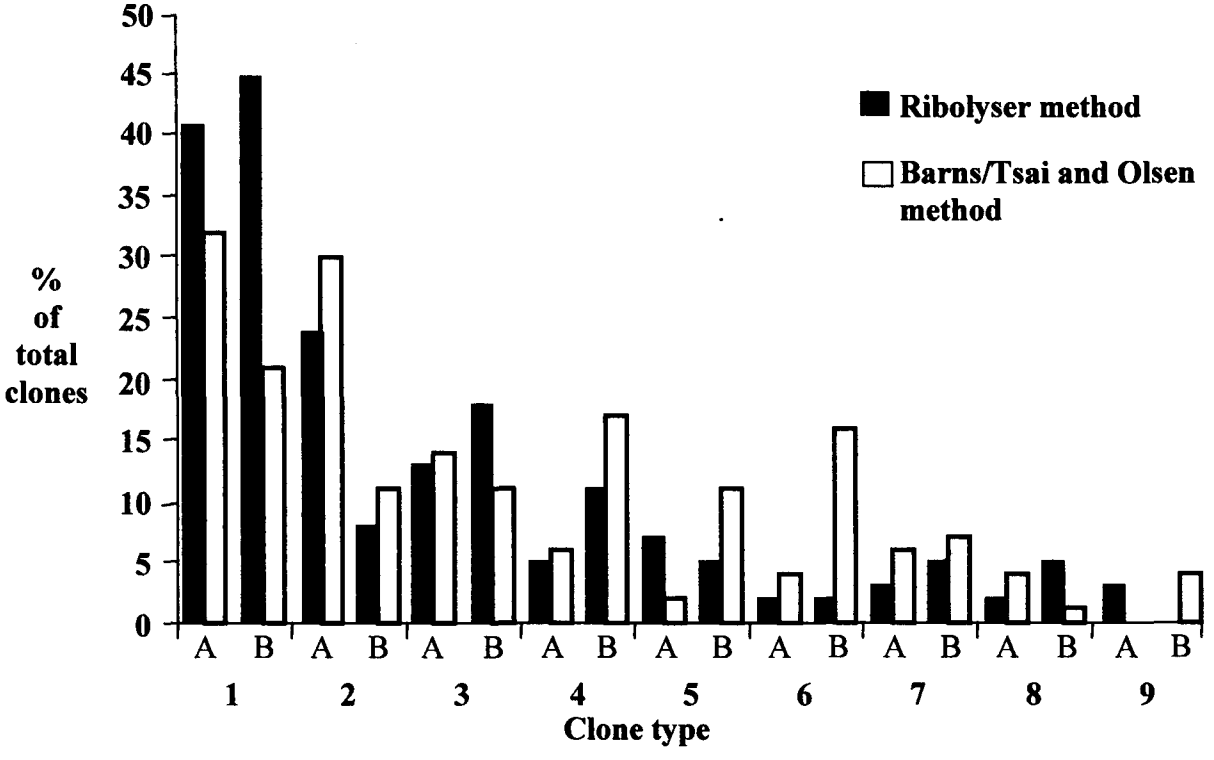


Fig 3.6 Percentages of each clone type found with both extraction methods in two samples A and B.

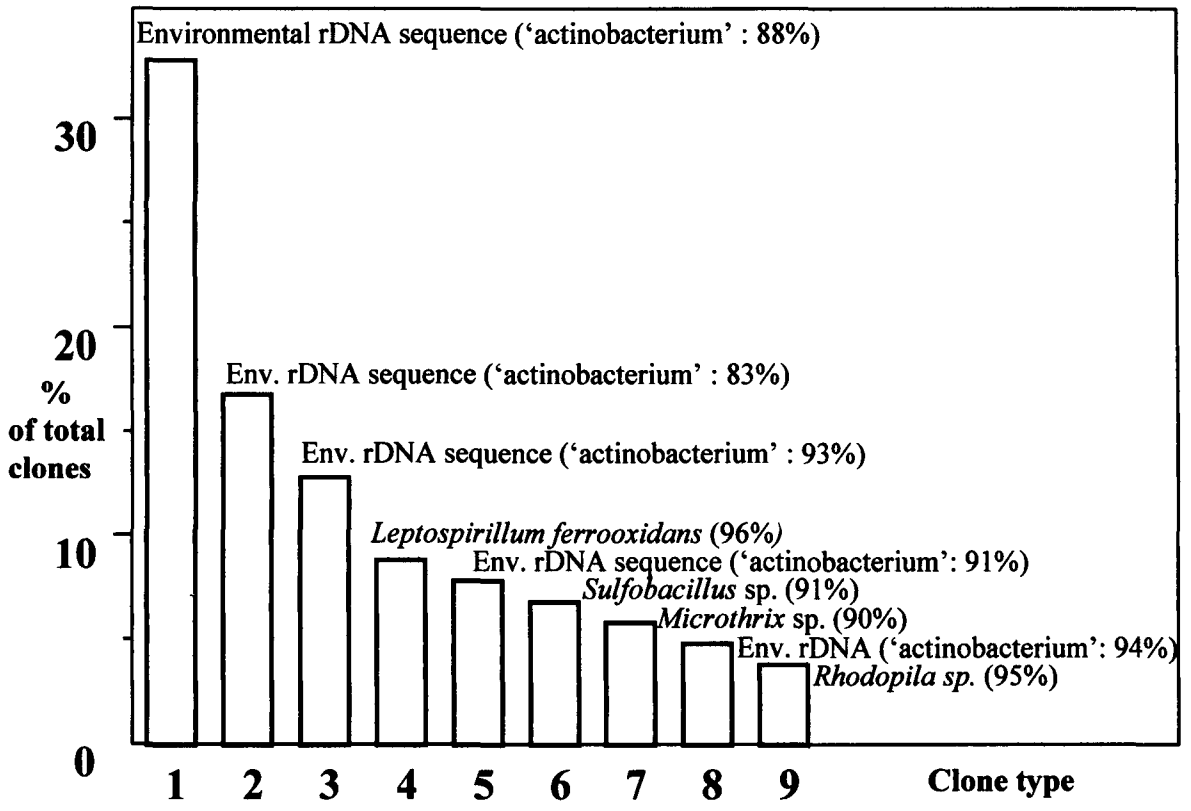
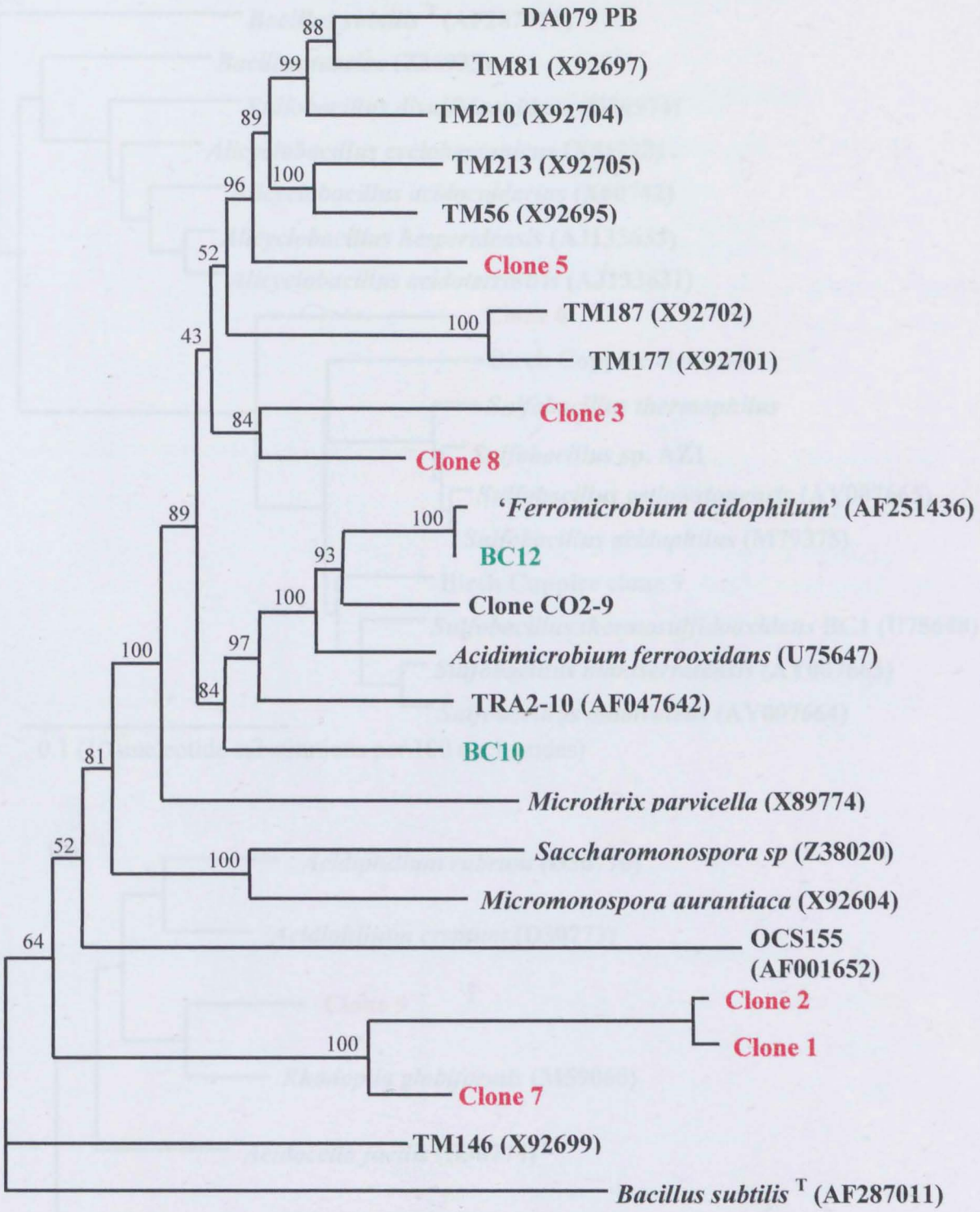


Fig 3.7 16S rDNA clone type analysis
The percentage of total clones are shown and percentage identity to database sequences are shown in brackets. All different clone types were sequenced fully to around 1460 bases.

The most numerous of the clone types had sequence identities closest to a group of bacteria called the actinobacteria. In this clone library six different actinobacteria were found with sequence similarities of the clone type to the data-base sequence ranging between 88% and 95%. Clone 1 had closest sequence identity to an unidentified eubacterium U62827, clone 2 to an uncultured landfill bacterium, AJ013608, clone 3 to Actinomyces species X92704, clone 5 to Actinomyces type X92705, clone 7 to *Microthrix parvicella* X89560 and clone 8 to Actinomyces type X92704. The other clone types found in the library had sequence similarities most closely related to *Leptospirillum ferrooxidans* X72852 (clone 4), *Sulfobacillus* sp. X91080 (clone 6) and *Rhodopila* sp. D86513 (clone 9). Many of these organisms are described in Chapter one.

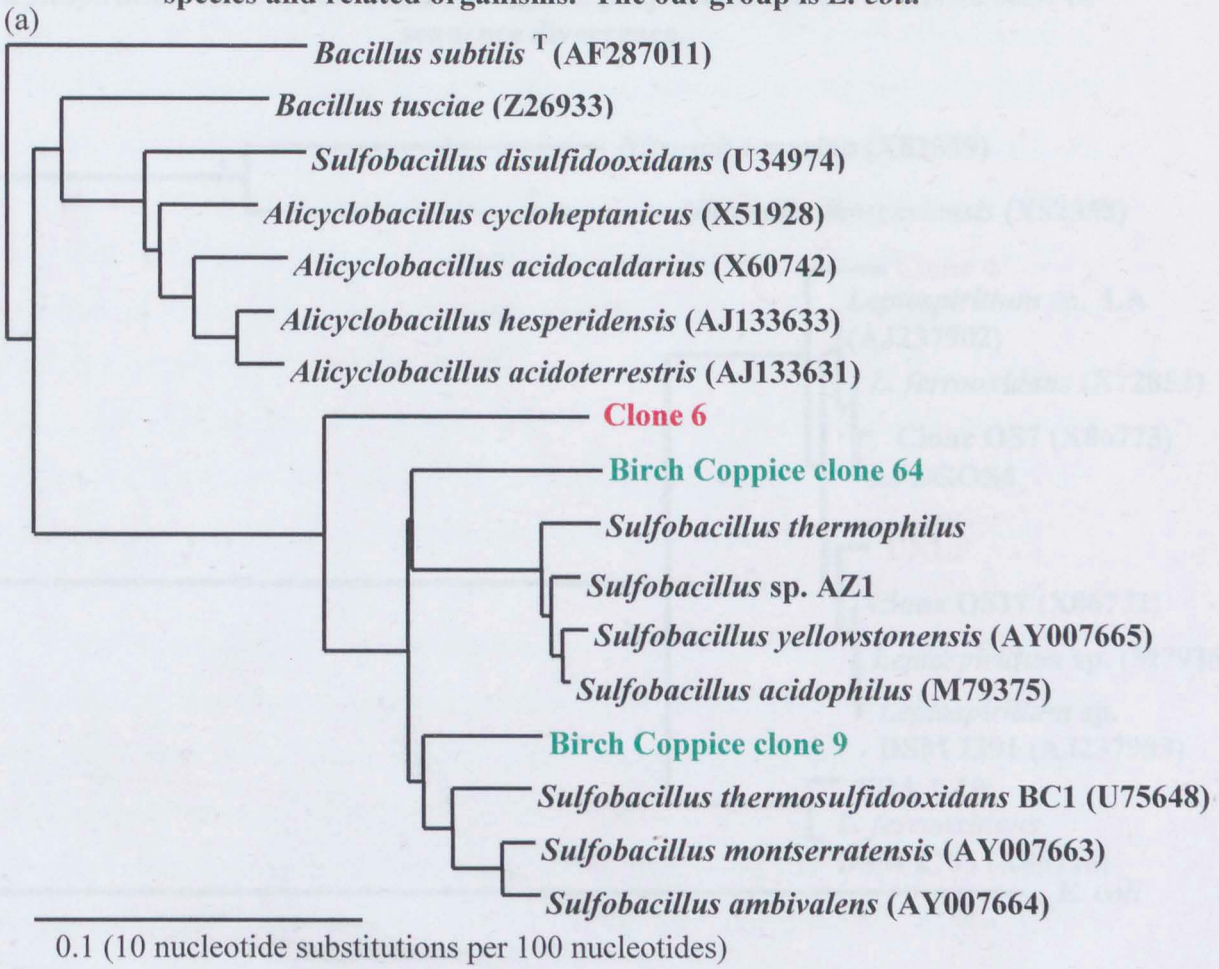
These clone sequences were aligned with their closest related sequences and positioned on phylogenetic distance trees (Figures 3.8-3.10) to gain more information about how they relate to other organisms.

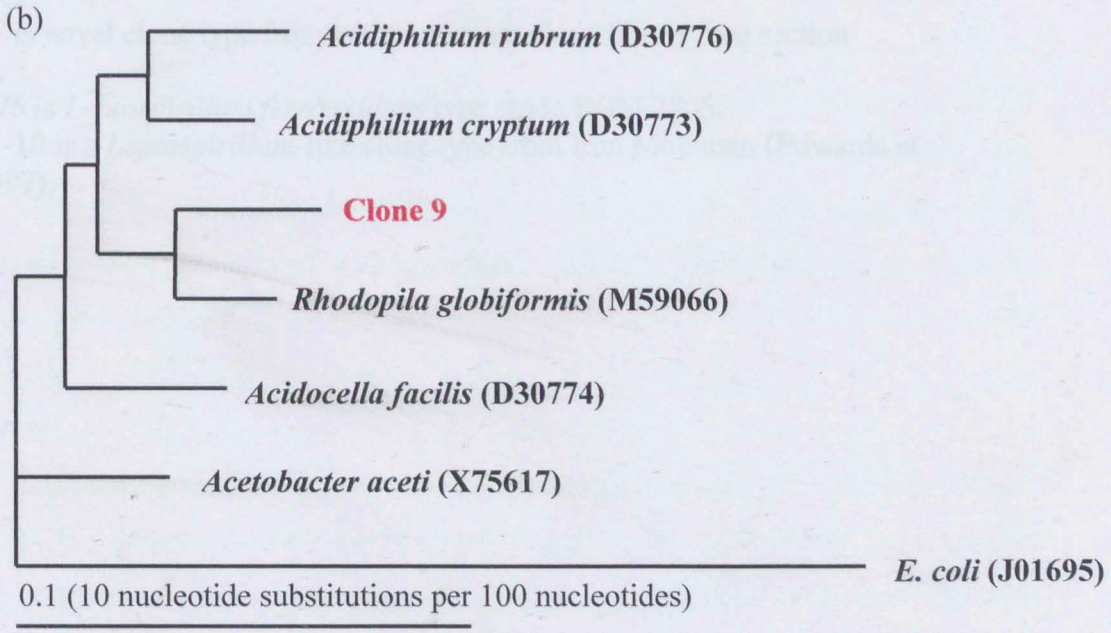
Fig 3.8 Unrooted phylogenetic distance tree of the actinobacteria showing the Birch Coppice clone types; *Bacillus subtilis* is the out-group. Bootstrapp values from 100 replicates are shown at branch nodes



Key:
TM = Grassland clones, DA = peat bog clones and OC = ocean clones
Birch Coppice actinobacterial clone types from this analysis
Birch Coppice actinobacterial clones types found by Nick Burton
GenBankAccession numbers are in brackets.

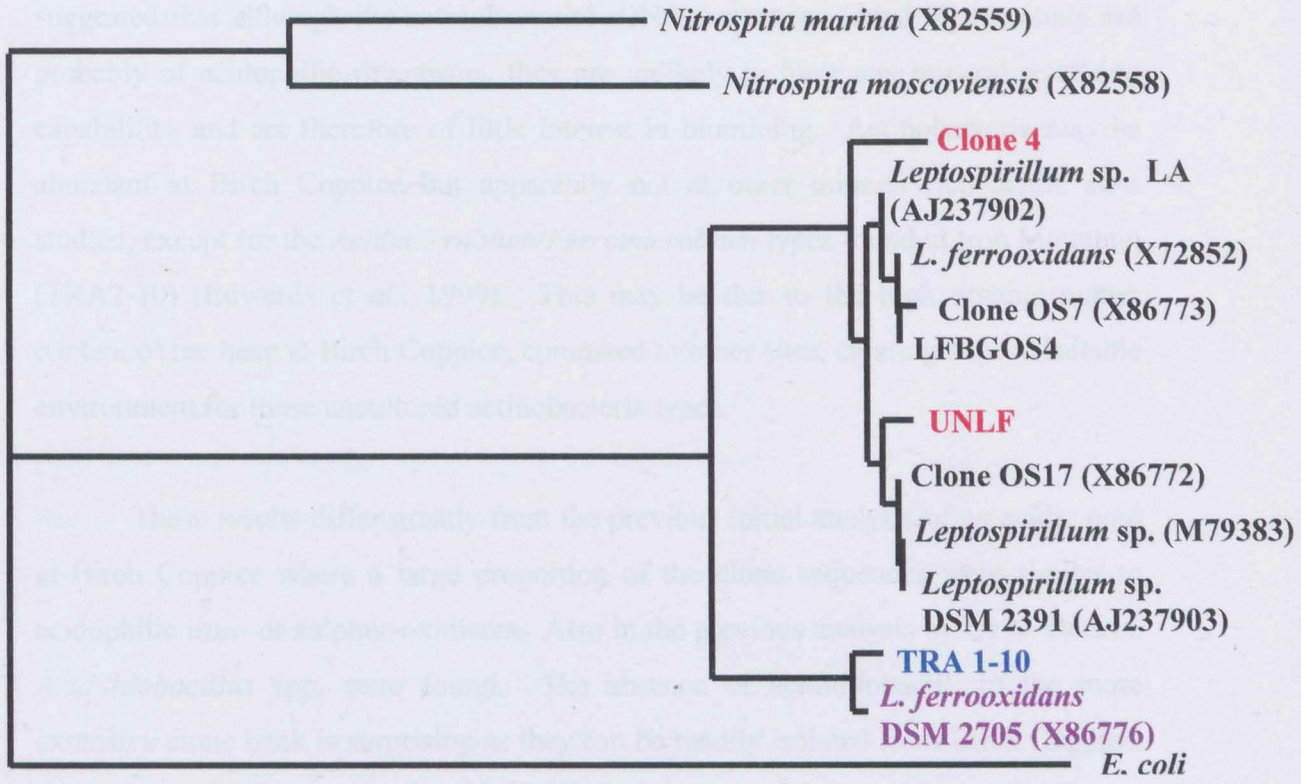
Fig 3.9 Unrooted distance trees of (a) position of clone 6 among the sulfobacilli and the alicyclobacilli, and (b) position of clone 9 in relation to *Acidiphilium* species and related organisms. The out-group is *E. coli*.





Key:
Birch Coppice clone types found in this analysis
Birch Coppice clone types found in previous analysis
GenBankAccession numbers are in brackets

Fig. 3.10 Unrooted phylogenetic distance tree of *Leptospirillum and Nitrospira* species. *E.coli* is the out-group. This tree is to demonstrate the positioning of the *Leptospirillum* clone types found during this project and therefore has no scale of sequence divergence.



Key:
Clone 4 is novel *Leptosprillum* type from clone bank of Birch Coppice samples.
UNLF is novel clone type from lab microcosm clone library (see section 3.3.2).
X86776 is *Leptospirillum ferrooxidans* type strain DSM 2705.
TRA1-10 is a *Leptospirillum*-like clone type from Iron Mountain (Edwards *et al.*, 1997).

The actinobacterial clone sequences found in the library form two main clusters - clones 1,2,7, and 3,8. These two groups are phylogenetically related to sequences from uncultured organisms of grassland (TM), peat bog (DA) and ocean samples (OC) (Rheims and Stackebrandt, 1999) with clone 5 on its own (Figure 3.8). This tree suggested that although the actinobacterial rDNA sequences found in this study are probably of acidophilic organisms, they are unlikely to have any mineral oxidising capabilities and are therefore of little interest in biomining. Actinobacteria may be abundant at Birch Coppice but apparently not at other mineral rich acidic sites studied, except for the *Acidimicrobium/Ferromicrobium* types found at Iron Mountain (TRA2-10) (Edwards *et al.,* 1999). This may be due to the high organic matter content of the heap at Birch Coppice, compared to other sites, creating a more suitable environment for these uncultured actinobacteria types.

These results differ greatly from the previous initial analysis of an acidic pool at Birch Coppice where a large proportion of the clone sequences were similar to acidophilic iron- or sulphur-oxidisers. Also in the previous analysis by Dr N. Burton, *Acidithiobacillus* spp. were found. The absence of acidithiobacilli in the more extensive clone bank is surprising as they can be readily isolated from Birch Coppice. However the preliminary analysis by Nick Burton was carried out on samples from a very acidic iron rich red pool that was specifically chosen with the view of finding acidophilic iron oxidisers.

BC12 and BC10 are two of the clone types found in the earlier study and are positioned within the *Acidimicrobiales* group. Another clone sequence on this tree, (CO2-9) came from the analysis of a mixed mineral sulphide oxidising reactor in the laboratory at the University of Warwick (Cleaver, 2001). Unfortunately, the organism to which this 16S rDNA sequence belongs could not be isolated in pure culture although several attempts were made.

The positions of clone 6 and clone 9 on phylogenetic trees are shown in Figure 3.9, and the position of clone 4 is shown in Figure 3.10. Clone 6 is not positioned among the sulfobacilli or the Alicyclobacilli but somewhere in between the two groups of organisms, making the determination of the type of organism to which

clone 6 belongs to difficult. Clone 9 has an obvious position among the α Proteobacteria, close to *Rhodopila globiformis.*

Clone 4 is positioned in among the leptospirilla but it does not appear to be closely related to the other *Leptospirillum* type 16S rDNA sequences. Clone UNLF from the microcosm clone library (see section 3.3.2) has greater sequence similarities with other *Leptospirillum* type 16S rDNA sequences than clone 4

3.3.2 Clone probe hybridisations

From the clone sequences oligonucleotide probes were designed and dig-labelled using the protocol described in chapter 2. Specific probes were designed for the *Rhodopila*-type - clone 9, the *Sulfobacillus* type – clone 6, the *Leptospirillum* type – clone 4 and the actinobacterium type clone 8. A group probe was designed for the cluster on the tree containing clones 1, 2 and 7 (Fig 3.8). A probe was designed for other actinobacteria clone types 5 and 3 but proved unspecific. *Acidithiobacillus* probes were also designed. An *Acidimicrobium* probe designed by Adam Cleaver at the University of Warwick and a general *Leptospirillum* genus probe designed by de Wulf Durand *et al.* (1997) were also used. The probe regions are shown in Figure 3.11 and the designed probes are shown in Table 3.1.

Figure 3.12 a-i are blots illustrating the probe specificities. (a) shows all clone rDNA hybridising with the R1492 probe, whereas the 1-2-7 probe only hybridises with the rDNA of the clones types to which it was designed. Probe –9- hybridises with the rDNA of clone type 9 and none of the other rDNA's, however, it is only assumed that this probe was specific to clone 9 as rDNA from closely related sequence clone types was not blotted. The THIO probe works with the rDNA from the clones made from *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thioooxidans* but not *Acidithiobacillus caldus.* The S-6- probe is specific to clone 6 rDNA and does not hybridise with other sulfobacilli, likewise the SULF probe does not react with clone 6 but it does hybridise with BC1 (*Sulfobacillus thermosulfidooxidans*) and NAL (*Sulfobacillus acidophilus*). Probe LF-4- is specific to the rDNA from clone 4 and none of the other Leptospirilla, 2705 is the type strain and CF12 is a leptospirilla isolated by B. Johnson. The RNA on this blot is from strain 2705 to demonstrate that R1492 hybridises with both RNA and rDNA.

Fig 3.11 Probe regions

(a) *Leptospirillum ferrooxidans* LF2705 (X86776) and clone 4 specific probes

```
                5'171                                          210 3'
TRA1            CAATACCGAA TATTGTCCGG GACCGTGAAG GGTTTCGGGG
X86776          CAATACCGAA TATTGTCCGG AGCCGTGAAG GGTTCCGGGG
LFBGOS4         CAATACCGAA TAGTATCCGG TTCCGTGAAG GGGGCCGGGG
X72852          CAATACCGAA TAGTATCCGG TTCCGTGAAG GGGGCCGGGG
X86773          CAATACCGAA TAGTATCCGG TTCCGTGAAG GGGGCCGGGG
AJ237903        CAATACCGAA TAGTATCCGG TTCCGTGAAG GGGACCGGGG
M79383          CAATACCGAA TAGTATCCGG TTCCGTGAAG GGGACCGGGG
X86772          CAATACCGAA TAGTATCCGG TTCCGTGAAG GGGACCGGGG
AJ237902        CAATACCGAA TAGTATCCGG TTCCGTGAAG GGGACCGGGG
CLONE 4         CAATACCGAA TAGAATCCGG TCCTGTGAAG GGGACCGGGG
UNLF            CAATACCGAA TAGAGTCCGG TTCCGTGAAG GGGACCGGGG
X82558          TAATACCGCA TA------CG ATTCCCGGAC TGCGGTTCGG
X82559          TAATACCCTA TA------CG CTATCATTTT TACGAAAAAG
E.coli          TAATACCGCA TAACGTC--- ---------- ----GCAAGA
```

(b) Actinobacteria clone 8, and 1-2-7 probes

```
                5'131                                          170 3'
CLONE 2         ACCAACCTCG AAGTTGGGAA TAGCTCTGCG AAAGCAGGGG
CLONE 1         ACCAACCTCG AAGTGGGGAA TAGCTCTGCG AAAGCAGGGG
CLONE 7         ACCAACCTCG AAGTTGGGAA TAGCTCTGCG AAAGCAGGGG
B.subt          ACCTGCCTGT AAGACTGGGA TAACTCCGGG AAACCGGGGC
TM 146          ACCTGCCTCG ATGACCGGGA CAACCCGAGG AAACTCGGGC
DA079           ACCTGCCCCG AAGACCGGGA CAACACCGGG AAACCGGTGC
CLONE 5         ACCTGCCCCG AAGACTGGGA TAACAGCGGG AAACCGCTGC
CLONE 8         ACCTACCCCG AAGTCTGGAA TAACACCGGG AAACCGATGC
CLONE 3         ACCTGCCCCG AAGTCTGGGA TAACAGTGGG AAACTGCTGC
BC12            ACCTACCCCA GAGCTTGGAA TAACACCGGG AAACCGGTGC

                5'171                                          210 3'
CLONE 2         TAATACCGAA TGTGGCCCGG CGCGGACATC CGCACCGGTC
CLONE 1         TAATACCGAA TGTGGCCCGG CGCGGACATC CGCACCGGTC
CLONE 7         TAATACCGAA TGTGGCCCGG CGCGGACATC CGCACCGGTC
B.subt          TAATACCGGA TGCT-TGTTT GAACCGCATG GTTCAAACAT
TM 146          TAATACCGGA TG-------- --------TG CCCGCAAGGG
DA079           TAATACCGGA TACCCCCACC TAATCGCATG ATCTGGTGAG
CLONE 5         TAATACCGGA TGCCCCCACC AGGTCGCATG GCCTGGCGAG
CLONE 8         TAATACTAGA TGCCCTGGCA GCATCGCATG GTGCAGCCAG
CLONE 3         TAATACCGGA TATTCGCGCT ATACTTACAT GAGGTGACGA
BC12            TAATACCGAA TACTCTCATT CGGCCGCATG ACTGAATGAG
```

(c) Clone 9 specific probe

```
            5'121                                          160 3'
A.aceti     AACGCGTAGG ATTCTATCCA TGGGTGGGGG ATAACTCCGG
A.facilis   AACACGTAGG AATCTATCCC AGGGTGGGGG ACAACAGCGG
A.cryptum   AACGCGTAGG AATCTATCCT TGGGTGGGGG ACAACCGTGG
A.rubrum    AACGCGTAGG AATCTATCCT TGGATGGGGG ACAACCGTGG
R.globif    AACGCGTAGG CATCTATCCC TGGGTGGGGG ACAACCGTGG
CLONE 9     AACGCGTAGG TATCTGTCTC CGGGTGGGGG ATAACCGCGG
E.coli      AATGTCTGGG AAACTGCCTG ATGGAGGGGG ATAACTACTG
```

(d) General *Sulfobacillus* probe and clone 6 specific probe

```
            5'641                                          680 3'
S.TH(WAR)   TCACCCGGAG GAGG-GCGGC TAAACGGTCG CGCTAGAGGG
X91080      TCACCCGGAG GAGG-GCGGC TAAACGGTCG CGCTAGAGGG
6B          TCACCCGACG GAGG-GCGGC TAAACGGCGC GGCTCGAGGG
NAL         TCACCCGACG GAGG-GCGGC TAAACGGCGC AGCTCGAGGG
CLONE 6     TCACCCGGGG GAGGCGGCGG CAAACGGCCG GGCTGGAGGG
E.coli      TCAACCTGGG AACTGCATCT GATACTGGCA AGCTTGAGTC

            5'1051                                        1090 3'
S.TH(WAR)   CTTCGGGGAG CGAG---CGC AGGT-CTCAT TGGTTGTCGT
X91080      CTTCGGGGAG CGAG---CGC AGGTGCTGCA TGGTTGTCGT
6B          GCTTCGGCAG AGCGGTCGTC AGGTGCTGCA TGGTTGTCGT
NAL         GCTTCGGCAG AGCGGTCGTC AGGTGCTGCA TGGTTGTCGT
CLONE 6     CTTCGGGG-G ACGGCGGCCC AGGTGCTGCA TGGTTGTCGT
E.coli      GCCTTCGGGA ACCGTGAGAC AGGTGCTGCA TGGCTGTCGT
```

(e) *Acidithiobacillus ferrooxidans* and *A. thiooxidans* probe

```
            5'821                                          860 3'
A.ferro     AGATGTTTGG TACCTAGCG- TACTGAGTGT CGTAGCTAAC
A.thio      AGATGTTTGG TGCCAAGCG- TACTGAGTGT CGTAGCTAAC
A.caldus    GGATGTTTGG CGAATTAGG- TGCTGAGTGT CGTAGCTAAC
B. subt     AAGTGTTAGG GGGTTTCCGC CCCTTAGTGC TGCAGCTAAC
```

Table 3.1 Summary of Probes Used

Primer/probe	Sequence 5'-3'	Base position
R1492	TAC GGY TAC CTT ACG TGC TGA A	R1492
F27	AGA GTT TGA TCK TGG CTC AC	F27
LF679	AAA TTC CGC TTC CCT CTC	R679
LF-4-	TTC ACA GGA CCG GAT TCT AT	R181
LF2705	ACG GCT CCG GAC AAT ATT CG	R176
-8-	GGC TGC ACC ATG CGA TGC TGC	R189
-9-	CCC CAC CCG GAG ACA GAT ACC T	R128
1-2-7	GCA GAG CTA TTC CCA ACT TCG AGG T	R134
ACID	CGA TCC TCT ACC GGA CTC	R654
SULF	CGT TTA GCC GCC CTC C	R649
S-6-	GCA CCT GGG CCG CCG TCC CC	R1055
THIO	CAG TAC GCT TGG CAC CAA A	R826
MC	CCT GTG TCT TGG CTC CCG AA	-

In Table 3.1, the base position refers to the base number at the start of the probe sequences.

R1492 and F27 are PCR primers designed by Lane (1991). R1492 was also dig-labelled and used in the blotting experiments to show that the DNA blotted was probeable.

ACID is a general *Acidimicrobium* spp. probe and SULF is a general *Sulfobacillus* spp. probe, both were designed by Cleaver (2001).

LF679 is a general *Leptospirillum* spp. probe from a paper by de Wulf Durand *et al.* (1997).

LF-4- is a specific probe designed for clone type 4 and LF2705 is a specific probe designed for *Leptospirillum ferrooxidans* strain DSM 2705 (accession X86776) from *Leptospirillum* spp. sequence alignments.

-8- is a specific probe designed for actinobacterium clone 8, 1-2-7 is a 'cluster specific' probe designed for clones 1, 2 and 7, which cluster together on the Birch Coppice actinobacterial phylogenetic tree.

-9- is a specific probe designed for *Rhodopila*-like clone type 9 and S-6- is a specific probe designed for *Sulfobacillus* – like clone type 6.

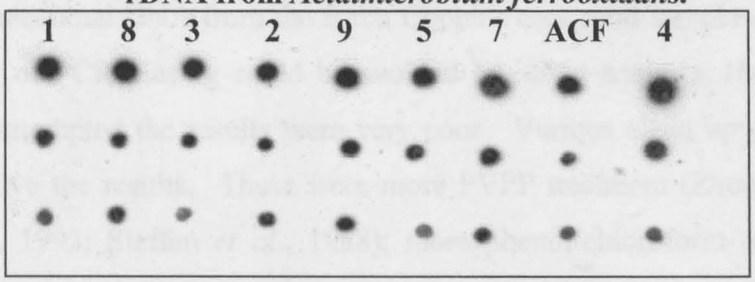
THIO is a probe that hybridises with *Acidithiobacillus thiooxidans* and *Acidithiobacillus ferrooxidans.*

MC is a *Methylococcus* spp. general probe designed by Bourne (2000).

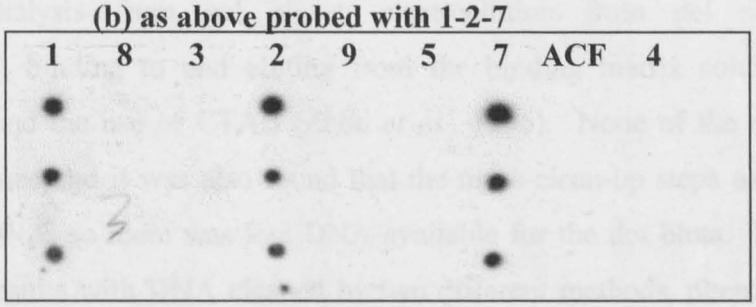
Probe specificity was tested using rDNA from the clone library and from organisms grown in the laboratory. Examples of these specificity-testing blots are shown in Figure3.12.

Fig 3.12 Specificity blots

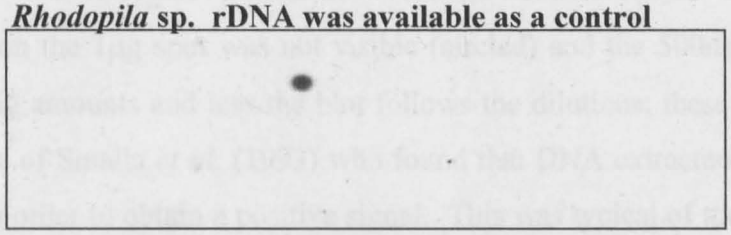
(a) Actinobacteria rDNA from clone library probed with R1492. ACF is rDNA from *Acidimicrobium ferrooxidans*.



(b) as above probed with 1-2-7

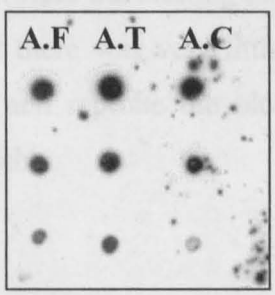


(c) as above probed with –9-, unfortunately no *Rhodopila* sp. rDNA was available as a control

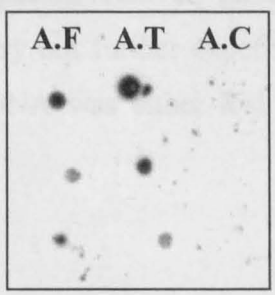


***Acidithiobacillus* spp. rDNA probed with (d) R1492 and (e) THIO (*A.ferrooxidans* (A.F), *A.thiooxidans* (A.T) and *A.caldus* (A.C))**

(d)

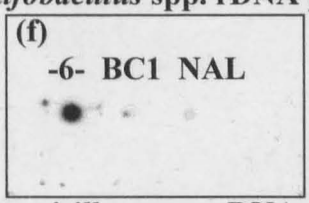


(e)

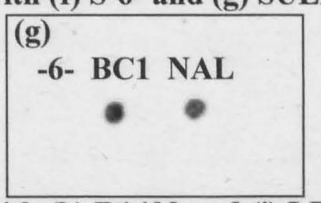


***Sulfobacillus* spp. rDNA probed with (f) S-6- and (g) SULF**

(f)

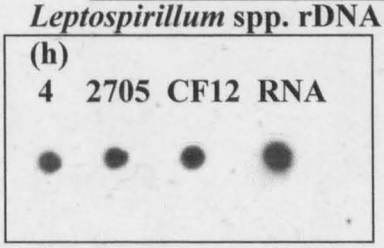


(g)

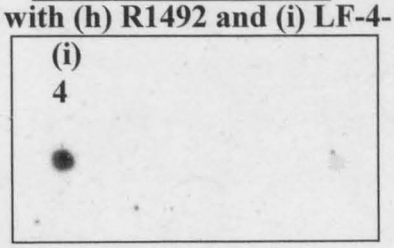


***Leptospirillum* spp. rDNA probed with (h) R1492 and (i) LF-4-**

(h)



(i)



It was hoped that using these labelled probes, DNA dot blots could be carried out with chromosomal DNA from the Birch Coppice coal spoil samples. In this way, any problems of PCR biasing could be avoided by direct analysis. However, when blotting was attempted the results were very poor. Various clean up methods were tried to improve the results. These were more PVPP treatment (Zhou *et al.*, 1996; Young *et al.*, 1993; Steffan *et al.*, 1988); more phenol-chloroform-isoamylalcohol treatment; dialysis from gel slices; electroelution from gel slices (Biorad electroeluter); binding to and eluting from the binding matrix solution from the BIO101 kit and the use of CTAB (Zhou *et al.*, 1996). None of the methods made much difference and it was also found that the more clean-up steps used the greater the loss of DNA, so there was less DNA available for the dot blots. Figure 3.13(a) shows blot results with DNA cleaned by two different methods, phenol-chloroform-isoamylalcohol and BIO101 solutions. The phenol-chloroform-isoamylalcohol cleaned DNA on the 1μg spot was not visible (circled) and the 500ng spot was not clear. At 200ng amounts and less the blot follows the dilutions; these findings were similar to those of Smalla *et al.* (1993) who found that DNA extracted from soil had to be diluted in order to obtain a positive signal. This was typical of the results of the dot blots after the use of most of the clean up methods with the exception of using the BIO101 solutions. Here the blotting results appeared relatively good but the DNA loss was so great that there was very little to carry out further experiments. It was also difficult to strip and reprobe the blots as DNA was either lost from the blots or rendered unprobeable.

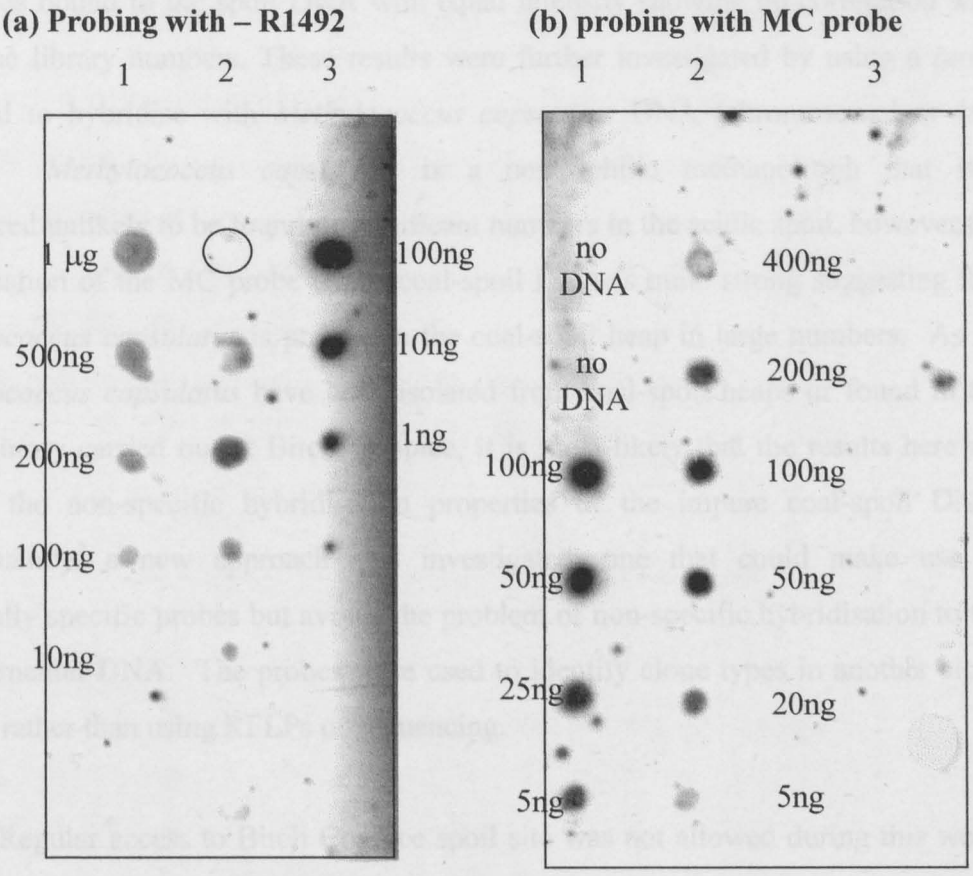


Fig 3.13 (a) Coal-spoil DNA blotting after two clean up methods and (b) Blot of *Methylococcus* DNA (lane 1), coal DNA (lane 2) and clone 4 rDNA as a negative control (lane 3) probed with a *Methylococcus* probe.

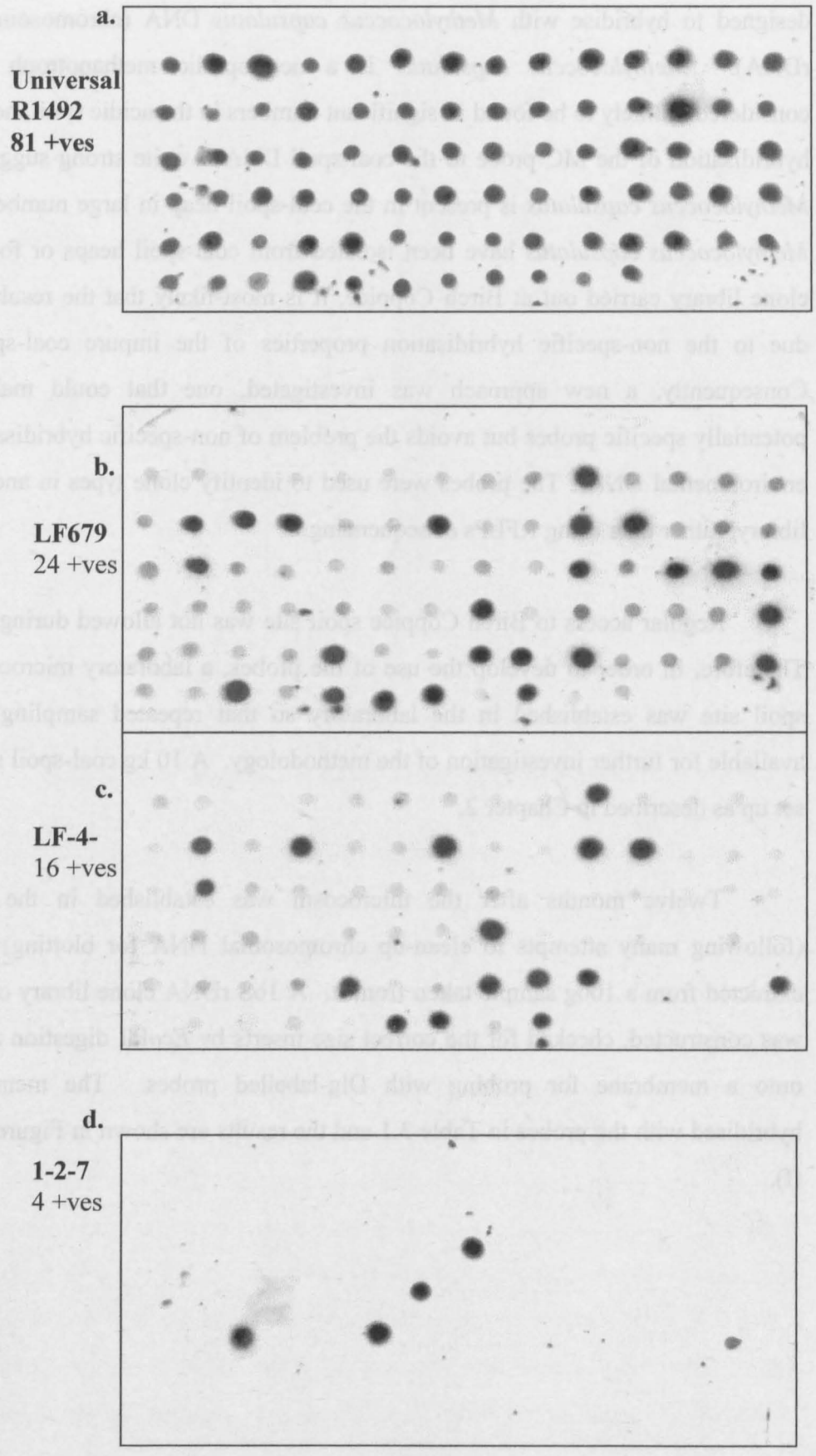
Figure 3.13 (a) shows coal-spoil DNA after being cleaned using BIO101 solutions (lane 1) and phenol (lane 2). In lane 3, clone 4 rDNA used as a positive control. The BIO101 solutions cleaning gave a satisfactory result but not enough DNA was recovered to carry out further required blotting experiments. The top spot (1µg) of the phenol purified DNA is not visible and the 500ng spot is not clear. Figure 3.13 (b) shows hybridisation of a general *Methylococcus* probe with *Methylococcus* sp. DNA and coal-spoil DNA with clone 4 (*Leptospirillum*) rDNA as a negative control. The probe bound unspecifically to the coal DNA, which also occurred with other probes designed to sequences of other organisms not thought to be found in coal spoil heaps. When chromosomal DNA blots were carried out with probes shown in Table 3.1, hybridisation of the probe to the DNA occurred even though the clone library indicated it should not. For example, the THIO probe gave positive results with spoil DNA dot blots but the clone library was negative for any *Acidithiobacillus*

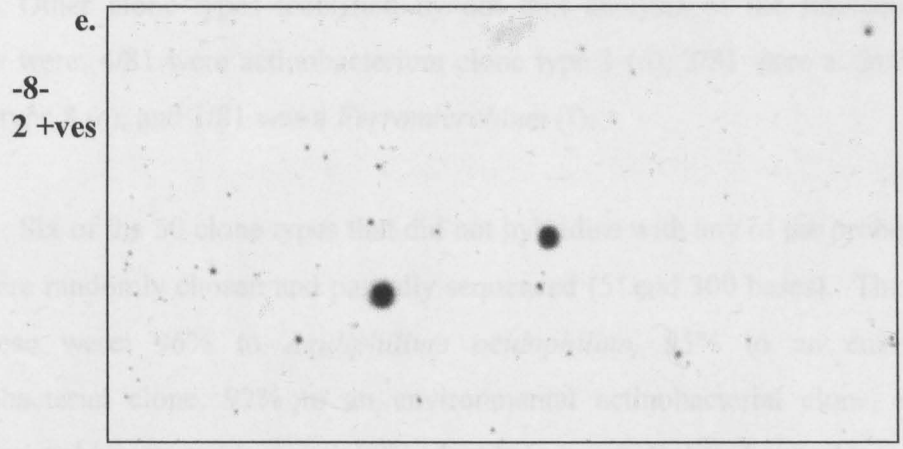
species, although this could indicate biasing occurring during PCR or cloning. Also, all probes bound to the spoil DNA with equal intensity showing no correlation with the clone library numbers. These results were further investigated by using a probe designed to hybridise with *Methylococcus capsulatus* DNA (chromosomal or 16S rDNA). *Methylococcus capsulatus* is a neutrophilic methanotroph that was considered unlikely to be found in significant numbers in the acidic spoil, however the hybridisation of the MC probe to the coal-spoil DNA is quite strong suggesting that *Methylococcus capsulatus* is present in the coal-spoil heap in large numbers. As no *Methylococcus capsulatus* have been isolated from coal-spoil heaps or found in the clone library carried out at Birch Coppice, it is most-likely that the results here are due to the non-specific hybridisation properties of the impure coal-spoil DNA. Consequently, a new approach was investigated, one that could make use of potentially specific probes but avoids the problem of non-specific hybridisation to the environmental DNA. The probes were used to identify clone types in another clone library, rather than using RFLPs or sequencing.

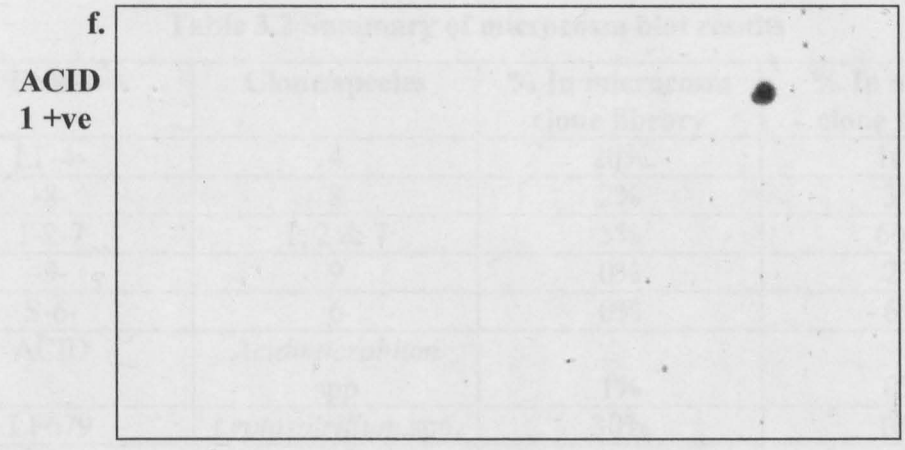
Regular access to Birch Coppice spoil site was not allowed during this work. Therefore, in order to develop the use of the probes, a laboratory microcosm of the spoil site was established in the laboratory so that repeated sampling would be available for further investigation of the methodology. A 10 kg coal-spoil sample was set up as described in Chapter 2.

Twelve months after the microcosm was established in the laboratory (following many attempts to clean-up chromosomal DNA for blotting) DNA was extracted from a 100g sample taken from it. A 16S rDNA clone library of 81 clones was constructed, checked for the correct size inserts by *Eco*R1 digestion and spotted onto a membrane for probing with Dig-labelled probes. The membrane was hybridised with the probes in Table 3.1 and the results are shown in Figure 3.14 (a) to (f).

Fig 3.14 Results of microcosm clone library rDNA dot blots hybridised with the previously designed probes LF679, LF-4-, 1-2-7, -8-, ACID and R1492.







It can be seen from the DNA dot blotting results in Figure 3.14 (a) with the 1492 probe that all 81 clones were positive and in Figure 3.14 (b) that 24 out of the 81 clones were positive with the general *Leptospirillum* probe 679. 16 of these 24 clones were positive with a specific probe designed for the novel *Leptospirillum* found in the clone bank, Figure 3.14 (c). Seven of these 24 were of another, possibly novel, *Leptospirillum* type, indicated by sequencing the first 800bp. One of these 24 clones was found to be a chimera, the first 300bp of the 5' terminus was from *Ferromicrobium*, the remaining 1100bp was from *Leptospirillum,* hence working with the general 679 *Leptospirillum* probe, which was designed to a region at around 680 nucleotides into the sequence but not with the *Acidimicrobium* probe designed at the 654 nucleotide region. These *Leptospirillum* sequences were put on a phylogenetic tree with other known leptospirilla sequences (Figure 3.10, Section 3.3.1), the novel type found in this microcosm clone library is called UNLF.

Other clone types identified by dot blot analysis of the microcosm clone library were: 4/81 were actinobacterium clone type 1 (d), 2/81 were actinobacterium clone type 8 (e), and 1/81 was a *Ferromicrobium* (f).

Six of the 50 clone types that did not hybridise with any of the probes in Table 3.1 were randomly chosen and partially sequenced (5′ end 300 bases). The identities of these were: 96% to *Acidiphilium acidophilum,* 95% to an environmental actinobacterial clone, 92% to an environmental actinobacterial clone, 91% to a *Ferromicrobium* sp and two with 90% identity to an unidentified eubacteria.

Table 3.2 Summary of microcosm blot results

Probe	Clone/species	% In microcosm clone library	% In original clone library
LF-4-	4	20%	10%
-8-	8	2%	3%
1-2-7	1, 2 & 7	5%	60%
-9-	9	0%	2%
S-6-	6	0%	6%
ACID	*Acidimicrobium* spp	1%	0%
LF679	*Leptospirillum* spp.	30%	10%
SULF	*Sulfobacillus* spp.	0%	0%
THIO	*Acidithiobacillus ferrooxidans* and *A. thiooxidans*	0%	0%

3.4 Novel organism isolation

Attempts were made to isolate some of the organisms indicated by the clone banks. The organisms were the *Leptospirillum* (Clone 4) from the original clone bank, the novel *Leptospirillum* type from the microcosm clone library; the *Sulfobacillus* like organism; and the actinobacteria.

3.4.1 Actinobacteria isolation

Little is known about the culturing conditions of the uncultured organisms found in the clone bank investigations, and it was therefore difficult to know what substrates and temperatures should be used. It was assumed that these uncultured organisms are acidophilic heterotrophs, therefore an acidic medium (pH 3) with different organic substrates was used for isolation at temperatures of 30°C and 45°C. The organic substrates used were cellulose, crab shell chitin and glucose. Serial subcultures in liquid media and plating on solid media were performed, as in Chapter 2. Growth in these flasks is noted in Table 3.3

A number of morphologically different colonies formed on the plates, but when isolated in pure culture and analysed by SDS PAGE against other known acidophilic heterotrophs, it was shown that an *Alicyclobacillus* species had been isolated.

Table 3.3 Growth in flasks 12 days after inoculation with 50g of coal sample

Substrate	**30°C**	**45°C**
Chitin	No growth	Growth
Cellulose	Few cells	Growth
Yeast Extract	Few cells	Growth
Coal pH 3	Few cells	Growth
Coal pH 1.7	No growth	Growth

Samples from these flasks were serially diluted and 100 µl from each dilution was plated on phytagel plates containing basal salts and chitin or cellulose as a substrate. Plates were incubated at 30°C and 45°C. After seven days of growth on the plates, colonies were picked and inoculated into 10 ml of medium containing glucose as a substrate. Colonies were either white or opaque and varied in size with the opaque colonies generally being larger than the white colonies. All the bacteria were 2-4µm long bacilli in shape except one which formed a filamentous colony and the

bacteria were shaped like long thin strands. The flasks were labelled 1-10 corresponding to the order in which the colonies were picked. No growth occurred on the cellulose plates incubated at 30°C.

Samples from the flasks containing glucose were serially diluted and plated out on phytagel plates containing basal salts plus glucose. Plates spread with samples from flasks 1-8 were incubated at 45°C, and a sample from flask 10 was incubated at 30°C. After five days incubation plates spread with samples 1-6 and 8 had colonies and 7 and 10 had no growth. As no growth had occurred on the 30°C cellulose plates, samples were taken from the original flask containing cellulose at 30°C and plated on glucose, growth occurred on these plates. The colonies on the plates incubated at 45°C were round, slightly domed, smooth and opaque or white, all were bacilli, some formed spores and some were motile. Some of the colonies from the plates incubated at 30°C were coloured blue or red, others were white or cream in colour, again all were bacilli with many being motile. The colonies from the 45°C glucose plates were cultured in flasks for SDS PAGE and run against *Alicyclobacillus* species. 30°C colonies were not cultured for protein gels as it was assumed from their colony morphology that they were not actinobacteria and therefore were of no interest. The 30°C organisms were probably *Acidiphilium* spp., and as there were no acidiphilia in the laboratory to run against the 30°C organisms on SDS protein gels no further analysis was carried out. The organisms that formed red colonies could have been a *Rhodopila* type, but again no further analysis was performed.

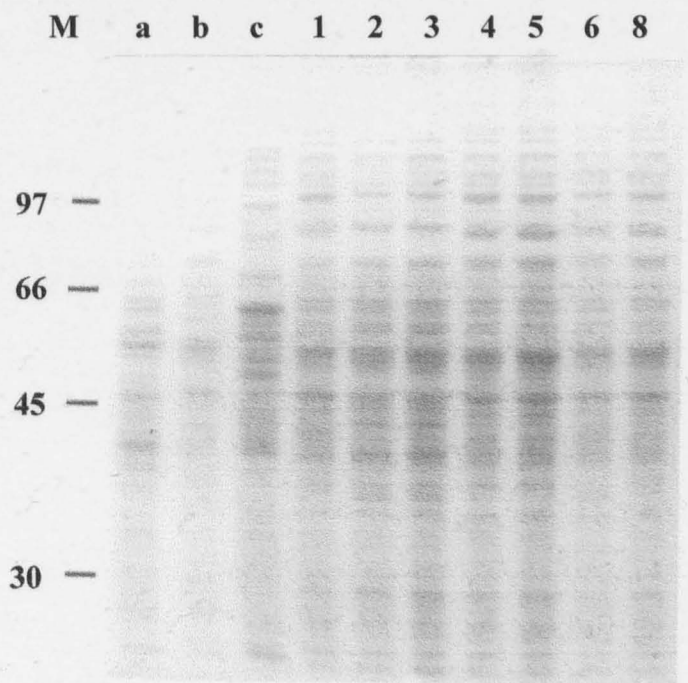


Figure 3.15 SDS-PAGE gel comparing the protein banding patterns of organisms isolated from Birch Coppice 45°C enrichment cultures (1-8) with *Alicyclobacillus* type organisms (a-c).

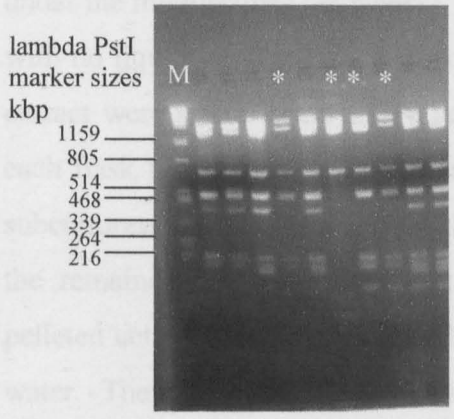
In Figure 5.15, a is organism 8c and b is organism 8a, both were isolated from site 8 at Montserrat (Atkinson *et al.,* 2000), c is organism K2 isolated from Kingsbury coal-spoil heap (Norris, unpublished work). All are *Alicyclobacillus* like organisms. There were two variants among unknown organisms 1-8, but all were 8a type. 8a has an optimum temperature range of 37-62°C and a pH range of 2-2.5, culturing conditions similar to those of the unknown organisms grown at 45°C at pH 3. Unknowns 2 and 3 have a slightly different pattern to 1 and 4-8.

3.4.2 *Sulfobacillus* isolation

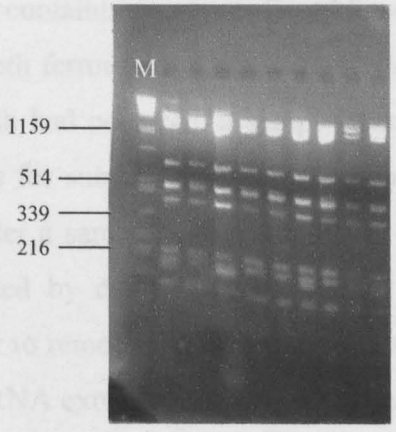
Cultures were set up in basal double salts medium (pH 1.7) plus ferrous sulphate (25mM final concentration) and yeast extract (0.01w/v % final concentration) and also on pyrite (1% w/v pH 2, double salts) and shaken at 47°C. At the third subculture the cultures were plated out on basal double salts medium plus yeast extract and iron sulphate. These were incubated at 47°C until individual colonies were visible. Single colonies were then picked and put into small flasks containing 10ml of medium until growth occurred then sub-cultured into 100ml of medium. The cultures were then grown and sub-cultured again for DNA extraction and protein gels. The organisms isolated were revealed as *Sulfobacillus thermosulfidooxidans* and *Sulfobacillus acidophilus* strains and not a novel *Sulfobacillus* sp. as was indicated by the coal-spoil clone bank from sequence data. This was shown by the RFLPs produced when the rDNA clones from these clone libraries were digested with *Eco*RI and *Rsa*I restriction enzymes and compared to restriction enzyme digestions of clones of *Sulfobacillus thermosulfidooxidans* strain BC1 and *Sulfobacillus acidophilus* strain NAL. Fig 3.16 shows the comparison of RFLPs of the unknown clones to BC1 and NAL clones. Protein gel results are not shown, but supported the RFLP results.

Fig 3.16 RFLPs of known *Sulfobacillus thermosulfidooxidans* (BC1) and *Sulfobacillus acidophilus* NAL) types and Sulfobacilli isolated from Birch Coppice samples.

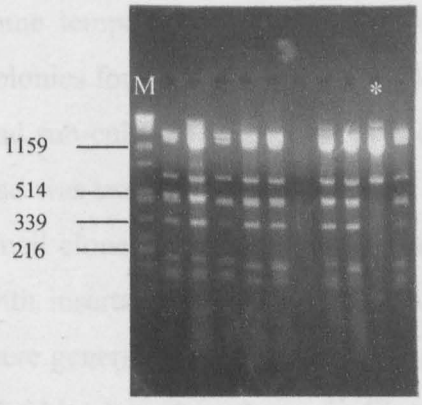
(a) *Eco*RI + *Rsa*I digest of plasmid preps of a NAL culture (not a pure culture).



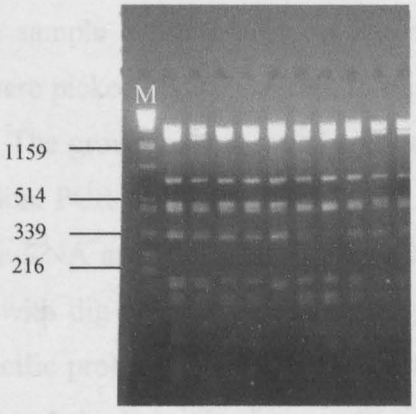
(b) *Eco*RI + *Rsa*I digests of unknown organism culture from enrichments - NAL like.



(c) *Eco*RI + *Rsa*I digests of a BC1 culture.



(d) *Eco*RI + *Rsa*I digest of unknown organisms from enrichment – BC1 like.



M indicates the *Pst*I marker.
*** indicates clone with wrong size insert**

It can be seen that the RFLPs in Figure 3.16 (b) are the same pattern as those in 3.16 (a), and those in (d) are the same as those in (c) indicating that the unknown organisms in these enrichment cultures are BC1 and NAL types.

3.4.3 *Leptospirillum* isolation

Flasks were set up containing pH 1.7 basal salts medium with 25mM ferrous sulphate and incubated at room temperature, 30°C and 37°C. They were inoculated with runoff from the lab coal-spoil microcosm and incubated until cells were visible under the microscope (1-2 weeks). A flask containing just runoff and ferrous sulphate with no liquid medium and a flask with both ferrous sulphate and 0.01% (w/v) yeast extract were set up at 30°C. When growth had occurred, a sample was taken from each flask for inoculation into fresh flasks for subculturing; this was done for three subcultures. At the second subculture, after a sample was removed for inoculation, the remainder of each flask was harvested by centrifugation at 9500 rpm. The pelleted cells were washed in pH 1.7 water to remove the iron and then with distilled water. The harvested cells were used for RNA extraction; this was also carried out at the first subculture of the flask with runoff/no added liquid media.

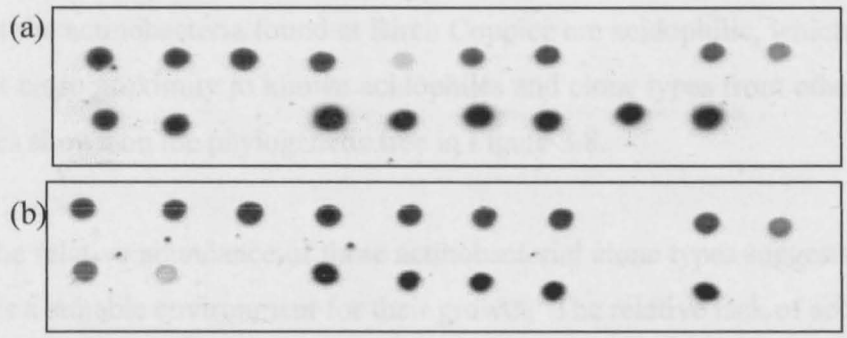
At the third subculture, the cultures were plated on phytagel plates containing 25mM ferrous sulphate and aluminium sulphate. The plates were incubated at the same temperatures as the flask that the sample came from, and left until visible colonies formed (2-3 weeks). Colonies were picked and transferred to liquid medium and sub-cultured when growth occurred. The grown culture was harvested as before and was used for DNA and RNA extraction. PCR was performed on the DNA and a small clone library was constructed. The RNA and the clone library rDNA (clones with inserts) were used for dot blotting with dig-labelled probes. The probes used were general *Leptospirillum* 679, and specific probes to *Leptospirillum* -4- and 2705. The blot layout is shown in Figure 3.17 and the hybridisation results are shown in Figure 3.18 (a) and (b).

Fig 3.17 layout of *Leptospirillum* blot

CL	CL	CL	CL	CL	CL	CL	CL	CL	CL
CL	CL		RNA1	RNA2	RNA3	RNA4	RNA5	RNA6	

CL = clone types from clone library of final culture
RNA1 = RNA from first subculture of run-off flask
RNA2 = RNA from second subculture of run-off flask
RNA3 = RNA from second subculture Fe 30°C flask
RNA4 = RNA from " " Fe/YE 30°C flask
RNA5 = RNA from " " Fe 37°C flask
RNA6 = RNA from " " Fe room temp flask

Fig 3.18 (a) Blot hybridised with general *Leptospirillum* probe LF679 and (b) blot probed with LF2705



From Fig 3.18 it can be seen that the LF679 probe worked with all but one of the DNA/RNA spots; this clone must not have been a *Leptospirillum* type. All of the DNA/RNA spots worked with the 2705 probe except the one that did not work with the 679 probe, which was expected, and RNA5, the RNA from the 37°C flask. There was not enough time to carry out RT PCR on this RNA to clone it and identify it, however it was not positive when probed with the LF -4- probe so it was not from the novel *Leptospirillum* type clone 4.

3.5 Discussion

As mentioned in chapter one, the Actinobacteria class is large and contains a diverse range of bacterial types such as *Microthrix parvicella* to *Acidimicrobium ferrooxidans*. Present in this class are a number of clone types of organisms that have yet to be isolated. These actinobacterial clone types have been found in many different environments, such as Australian arid soils (Holmes *et al.*, 2000), New Zealand geothermal soils, Finland soil, peat bogs, paddy fields and marine environments (Rheims *et al.*, 1996). *Microthrix parvicella* has been found in sewage plants, (Rheims *et al.*, 1996), so the ubiquitous nature of this class is fully demonstrated. However, because the organisms of these clone types have never been isolated in pure culture it is difficult to accurately discuss potential physiologies. It is likely that the actinobacteria found at Birch Coppice are acidophilic, which is inferred from their close proximity to known acidophiles and clone types from other relatively acidic sites shown on the phylogenetic tree in Figure 3.8.

The relative abundance of these actinobacterial clone types suggests that Birch Coppice is a suitable environment for their growth. The relative lack of actinobacteria in the acidic pool sampled by Dr N. Burton suggests that they either could not tolerate such a low pH or that they were out competed by the true acidophiles. The failure to isolate one of these actinobacteria in pure culture highlights the importance of molecular techniques over traditional culturing methods and the need to improve culture techniques. The 16S rDNA analysis has shown the presence of microorganisms that would otherwise have remained overlooked. However, 16S rDNA analysis as a tool for the study of microbial diversity cannot substitute for the need for isolation in pure culture to determine physiological characteristics. Molecular analysis can be used as a step to show what could be in the environment and perhaps indicate what organism isolation conditions need to be used to try to obtain the novel organisms in a pure culture. The isolation of the actinobacteria was shown not to be straight forward and it is likely that unique conditions need to be set up to obtain them in culture. This is probably why very few of the organisms of these actinobacterial clone type sequences, found in the database searches, have been isolated.

The actinobacterial clone types found at Birch Coppice are positioned throughout the other environmental actinobacteria clone types on the phylogenetic tree (Fig 3.8). However, they cluster together, forming distinct groups apart from the other clone types and organisms on the tree, so there is difficulty in inferring what their physiological relationship is with the other actinobacterial types. The cluster containing clones 1, 2 and 7 forms a distinct group from clones 3, 5 and 8. The closest identities to 3, 5 and 8 from Blast searches were all named as Actinomyces types with accession numbers X92704 or X92705, whereas the closest identity to clone 1 was an uncultured eubacterium. Clone 2 was an uncultured landfill bacterium and clone 7 was *Microthrix parvicella,* although on the tree, this cluster is also distinct from *Microthrix parvicella.* It is possible that clones 1, 2 and 7 may be physiologically similar to each other but different from clones 3, 5 and 8, whose physiologies may be similar to the environmental actinobacterial clones found by Rheims *et al.* (1996). Birch Coppice had actinobacteria, although widespread, are not usually encountered in acidic sulphide rich environments, with the exception of *Acidimicrobium ferrooxidans,* a common actinobacterial organism found in AMD sites and *"Ferrimicrobium"*- like Actinobacteria have also been reported to be widespread in acidic metal-rich environments (Johnson and Roberto, 1997). More recently Slime clone BA84, found in samples taken from slimes found within a mine (Bond and Banfield, 2001) is positioned close to clone type TM210 on the actinobacteria phylogenetic tree in this chapter (Fig 3.8) and has a sequence identity of 92% to clone types 5 and 3 and interestingly has a sequence identity of 94% to clone 8 found in the Birch Coppice analysis.

Leptospirillum ferrooxidans is a well-studied iron-oxidizing acidophilic gram-negative bacterium and, with clone 4's sequence identity to *Leptospirillum ferrooxidans* of 96%, it is possible that a novel *Leptospirillum* sp. was found at this site. The isolation of the novel leptospirilla was also difficult and it is possible that the conditions used to isolate the known strain 2705 were unsuitable for the novel types indicated by the clone banks. It is possible that a component found in the coal-spoil heap was required in the growth media to enable the novel types to grow, or perhaps they are extremely slow growers with a doubling time of days in the spoil-heap and were out competed by the 2705 strain in culture. The phylogenetic distance tree of the leptospirilla is based on 770 nucleotides and it shows the positions of the

novel leptospirilla found in the spoil sample and the microcosm. Both of these novel types are distinct from each other and also separate from their nearest sequences on the tree. It can be seen from the tree that there are two main groups of *Leptospirillum* spp., one containing the clone type TRA1-10, found by Edwards *et al* (1999), and strain DSM 2705 (X86776) with the other group containing the unknowns found at Birch Coppice and other clones/strains. It appears that clone 4 is not as closely related to the other leptospirilla as UNLF, as it branches separately from the other leptospirilla. New leptospirilla have been indicated via 16S rDNA analysis by other research groups. Recently Bond and Banfield (2001) reported novel *Leptospirillum* 16S rDNA found, via molecular analysis, in an AMD environment. This novel type, like the novel types indicated in this project, was difficult to cultivate and the paper suggests that previously uncultured species like these have been overlooked in previous studies even though the novel type reported was shown to be a major component of the microbial flora in the environment studied using 16s molecular techniques. Genbank Blast searches have indicated that the novel types found in this project have sequence identities of 92-96% to the novel *Leptospirillum* spp. found by Bond and Banfield (2001).

Sulfobacillus thermosulfidooxidans is a well characterised iron- and sulphur-oxidizing gram positive spore forming organism, but the clone sequence in the Birch Coppice coal-spoil clone library has only 91% identity to *S. thermosulfidooxidans*. It is therefore thought that the organism to which clone 6 has the 16S rDNA sequence may not be a *Sulfobacillus* spp. The novel *Sulfobacillus* type from the clone bank was a different case to the novel leptospirilla types. The novel leptospirilla had high sequence identities to known *Leptospirillum* species, whereas the novel *Sulfobacillus* type was not closely related to the sulfobacilli, as shown in the specificity blots (Fig 3.12) where the SULF probe did not hybridise with rDNA from clone 6. This is shown on the phylogenetic tree of the sulfobacilli and the alicyclobacilli (Fig 3.9). As can be seen, clone 6 is positioned on a separate branch in between the two genera, indicating that it is more closely related to the sulfobacilli than the alicyclobacilli, but not closely related to either. This lack of similarity meant that finding the right culturing conditions was difficult as the type of organism was unknown. This was shown by the results of the attempted isolation of this organism, where a known strain

of *Sulfobacillus* was isolated instead of the novel type, using the conditions usually associated with the isolation of *Sulfobacillus* spp..

If further work was to be carried out on the isolation of the novel organism types indicated by the clone banks, many more different culture conditions need to be investigated and reliance should not be placed entirely on known media types.

Clone 9 was positioned close to *Rhodopila globiformis* among the *Acidiphilium* and so it is possible that is has some form of acid tolerance and may well be an acidophilic heterotroph.

The DNA extraction methods used in this investigation gave similar results; DNA was extracted from both gram-negative organisms and gram-positive spore-forming bacteria. Both of the methods were quite harsh in the treatment of the samples, the lysozyme, freeze thawing and phenol extraction treatments of the Barns/Tsai and Olsen methods ensure that the majority of bacterial cells will lyse, as does the vigorous bead beating of the Ribolyser.

Oligonucleotide probing could not be put to its intended use due to the chemical composition of the sample. Both CTAB and PVPP are used in DNA extractions to remove humic matter from soil samples. Methods involving these chemicals were used in this investigation to try and improve the chromosomal DNA blotting results but these attempts proved fruitless.

RNA dot blotting is a method that could be used to overcome the problems that occurred with the chromosomal DNA dot blotting. RNA can be extracted from samples easily and can also be stripped and re-probed from blots. However, attempts to extract RNA from the coal produced little or no RNA, which could be due to relatively few RNA molecules being produced by the organisms growing slowly *in situ.*

Analysis of clone libraries by restriction fragment length polymorphisms can give a good indication of the number of different clone types. However, differentiating between similar RFLPs can be laborious and time-consuming and a keen eye is needed. The use of the oligonucleotide probes in the identification of the clone types in the microcosm clone library proved a useful and less intensive method.

The results were easily definable although the method is more time consuming than that of restriction enzyme digestion. Here it could be seen at a glance which of the clone types from the previous clone library were present in the microcosm. Three of the nine clone types from the original clone library were present in the microcosm, these were the novel *Leptospirillum* type (clone 4), clones 1, 2 & 7, although as the probe binds to all three not all three are necessarily present, and clone 8. A probe was not designed for clones 3 and 5 so their presence in the library was unknown and there was a negative result when the library was probed with the S-6- probe for clone 6. In addition to these clone types there were others present that were indicated by the blotting experiment, these were another *Leptospirillum* type and an *Acidimicrobium* type. It is possible that the warmer laboratory temperatures may have stimulated the growth of these organisms and enabled their detection by clone library analysis, whereas they may be present in too few numbers in their natural environment. No acidithiobacilli were detected in the microcosm clone library, as in the original clone library.

The clone types found in this analysis of the Birch Coppice coal-spoil sample have similarities to types found at other mineral rich acidic sites, as mentioned previously, both *Leptospirillum ferrooxidans* and *Sulfobacillus* spp. are often found in such sites and are indeed used in the biological processing of metal rich sulphidic ores. The organic rich nature of this spoil site has allowed the proliferation of novel, presumably heterotrophic organisms, yet to be isolated, and until their isolation very little will be understood about the role of these actinobacteria in nature.

CHAPTER 4 VULCANO 16S rDNA RESULTS

4.1 Introduction

Many of novel organisms have been discovered in geothermal areas. The diverse mineralogy and temperatures of these areas, both terrestrial and marine, have given rise to a diverse range of bacteria and archaea. Hydrothermal vents may appear to be unsuitable environments to sustain life. However, such places support microorganisms that have adapted to flourish in the chemical disequilibria caused by the mixing of hot reduced vents with the cold oxidised seawater (Gugliandolo *et al.*, 1999).

Marine hydrothermal vents, particularly deep-sea vents, have been the sites of discovery of potentially useful organisms for biotechnology. On land, the geothermal areas of Yellowstone National Park in the USA, Iceland, the Mediterranean and the Caribbean have all been analysed, which has led to the isolation of new organisms, some of which have been used in the biomining industry such as *Sulfobacillus thermosulfidooxidans*.

Yellowstone National Park has been extensively studied and is a particularly diverse site with regards to its mineralogy. It has hot, neutral-alkaline and carbonate rich parts and sulphurous acidic areas. The Caribbean island of Montserrat, mentioned previously in chapter one, was one site of sampling for molecular analysis and organism isolation. Here the 16S rDNA analysis revealed a number of novel organism types.

Vulcano has been sampled before and it was here that *Thiobacillus prosperus* and *Thermoplasma volcanium* were discovered, and although they were present in this saline site they are not true halophiles. Halotolerant organisms from Vulcano were available in the laboratory at the University of Warwick from previous sampling in 1998 (P. R. Norris, personal communication), but had not been characterised. The earlier samples from Vulcano Island were not analysed by molecular methods and it is possible that other novel haloacidophiles were missed by the traditional isolation methods. This and the following chapter reveal the results of 16S rDNA analysis of samples from three different temperature pools and vents from Vulcano and the characterisation experiments carried out on the organisms that had been previously isolated.

Sample site description

Vulcano Island is part of the volcanic Aeolian Island group off the north coast of Sicily, it is the second largest island of this chain and has the volcano of Gran Cratere or Fossa Vecchio, which is still active. According to classical mythology, the forges of Vulcan, the god of fire, were on one of the volcanoes found here, hence the name Vulcano. There are a number of hydrothermal vents at Vulcano, both on the island and in its coastal waters, the sampling sites were on one of the beaches. Figure 4.1 shows a map of the Aeolian islands showing the location of Vulcano.

Fig 4.1 Map of the Aeolian island showing the location of Vulcano north of Sicily

The sample sites on the Island that were of interest consisted of three geothermal pools or vents that were on the beach: a large pool at 35°C, water saturated volcanic sand at 45°C and a vent at 75°C, all were a few metres apart from each other. Table 4.1 and Figure 4.2 provide details of the sites.

Fig 4.2 Photographs of sample sites on the beach at Vulcano.

(a) Shallow 35°C pool with 75°C vent within it.

(b) 45°C water saturated volcanic sand on the beach with another 75°C vent on the left.

Table 4.1 Characteristics of samples

Sample site	temp (°C)	pH	Ferrous iron (mg/l)
Large shallow pool adjacent to beach	35	2.5	>500
Volcanic sand/gravel (water-saturated)	45	3.5	10
Hot water-filled vent	75	3	>500

The large shallow pool showed no apparent thermal activity except at one corner where there were gasses bubbling through the sand and a few small vents. The bulk of the pool was not heated, but it was still a few degrees higher than the air temperature at the time of sampling. The 45°C sample site consisted of hard-packed volcanic sand and gravel, the water saturating it was at a depth of 1cm (approximately) and there was continuous gas venting; under the surface of the sand and gravel the temperature was higher at 55-60°C. The hottest vent was actually inside the large shallow pool and contained a dark cloudy fluid. There was vigorous gas bubbling within this vent but it did not overflow; temperatures of around 75°C were found at the surface of this vent. The dissolved iron and pH of the of the sites were measured, indicating that the sites were iron rich and acidic, as previous reports have shown (Dando *et al.*, 1999).

4.2 Environmental rDNA analysis

Samples were taken back to the laboratory and placed in the -80°C freezer as soon as possible, although they were not stored on ice for the journey. Some samples were used as inocula for enrichment cultures and so were not frozen.

The samples were subjected to DNA extraction following the adapted Barns *et al.* (1994) and Tsai and Olsen (1991) methods as used for the coal-spoil work. The DNA underwent PCR with an R1492 universal primer and F27 eubacterial primer, or with

R1492 and F25 archaeal primers to amplify 16S rDNA. Products from the PCR reactions were only found with the low temperature sample DNA amplified with the R1492 + F27 set, indicating the lack of archaea at this sample site. The high temperature sample DNA only had a product with the R1492 + F25 primer set, thus indicating the presence of archaea only, and the 45°C sample DNA was amplified by both sets of primers. The PCR products were ligated into TOPO PCR 2.1 vectors and transformations were carried out using the TOPO TA cloning kit from Invitrogen. RFLPs of clone types were then studied from digests with E*co*R1 + S*au*3A1 and E*co*R1 + R*sa*1, see Figure 4.3.

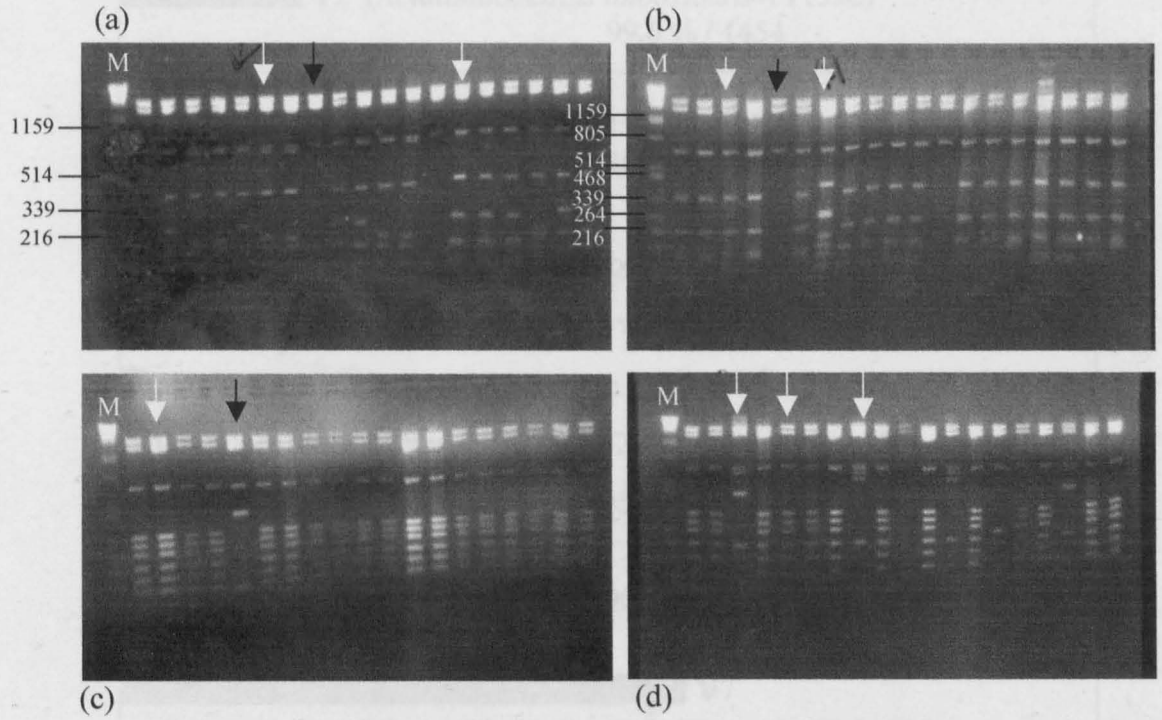


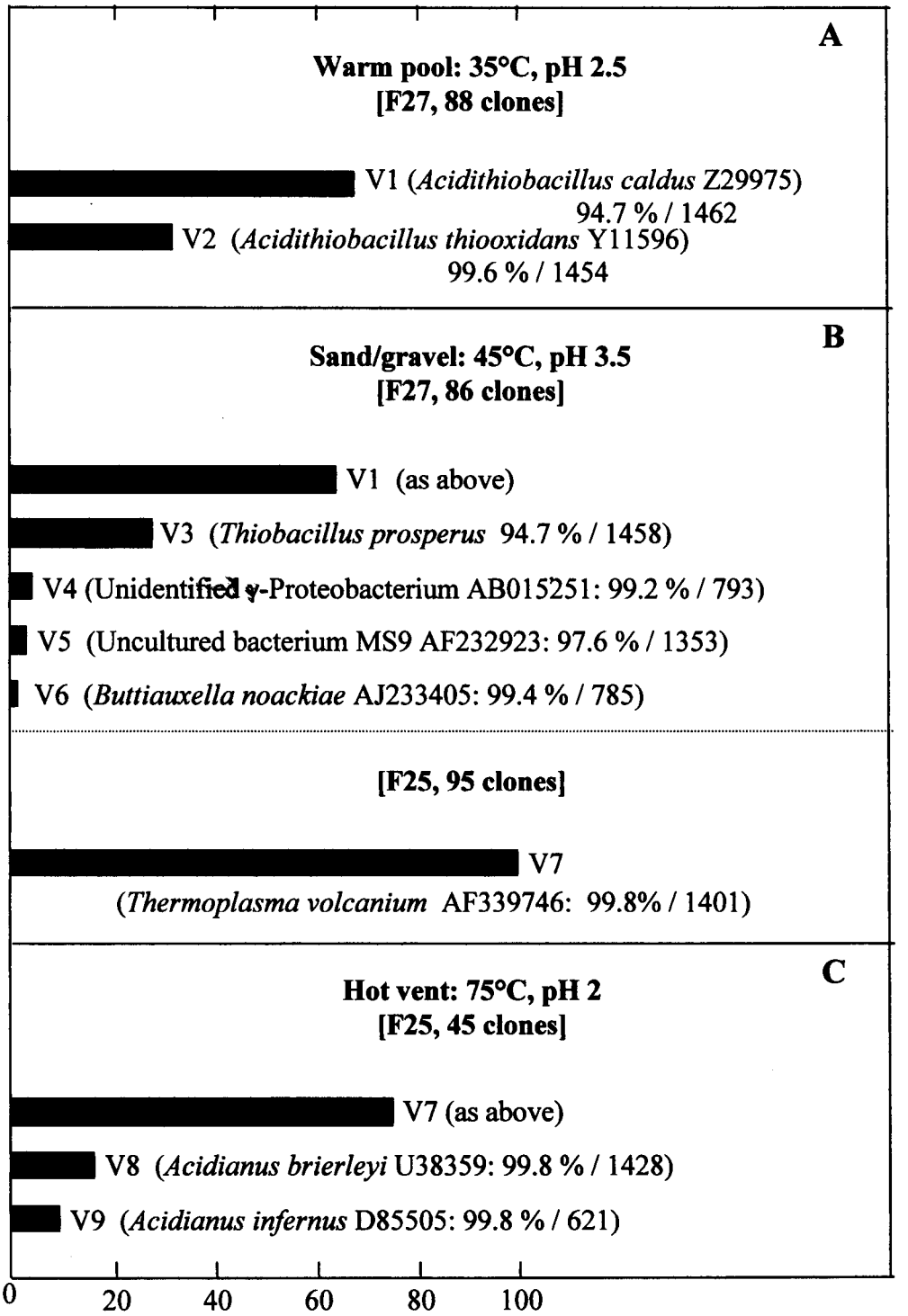
Fig 4.3 Examples of E*co*RI+R*sa*I restriction digests of minipreps from Vulcano sample clone libraries (a) 30°C pool eubacterial primers (b) 50°C pool eubacterial primers; (c) 50°C pool archaeal primers (d) 75°C pool archaeal primers.

White arrows indicate the different RFLP patterns; the black arrows indicate clones without inserts or with inserts of the wrong size. The 45°C sample clone library had more than two RFLP types but these are not shown. M indicates the Lambda P*st* marker with sizes of bands indicated (kbp).

Representatives of each clone types were fully sequenced, and several examples of each clone type were partially sequenced to about 700 bases. Figure 4.4

shows the percentages of each clone type found and their identifications as found through GenBank Blast searches.

Fig 4.4 Histogram of clone library results



4.2.1 35°C shallow pool

The RFLP analysis and 16S sequencing revealed only two clone types named here as V1 and V2. Clone V1 has a sequence identity closest to *Acidithiobacillus caldus,* but at only 95% similarity the V1 16S rDNA sequence indicates a novel species. Clone type V1 was also found at the 45°C site, suggesting a broad temperature range for growth or survival. V2 also has a 16S rDNA sequence identity closest to that of another *Acidithiobacillus* species, *A. thiooxidans*. *A. thiooxidans* is a mesophilic sulphur-oxidiser not known for its ability to tolerate high concentrations of sodium chloride. A strain corresponding to V1 was isolated after enrichment culture with sulphur or tetrathionate as a substrate by Dr Paul Norris after a previous visit to Vulcano. It was found to be a salt tolerant, slightly thermotolerant mesophile that differed from *Acidithiobacillus caldus* because of this salt tolerance and optimum temperature for growth at 40°C. Strain V1 characterisation experiments have been carried out and are described in Chapter 5. Strain V2 was also isolated after enrichment on sulphur but has not been further studied.

The initial RFLP analysis showed four clone types but after sequencing it was revealed that two of these clone types were V1/V2 chimeras.

4.2.2 45°C puddle

As mentioned above, clone type V1 was also found in the 45°C sample. This would be consistent with the temperature optimum of 39-42°C for the isolated organism with the same 16S sequence, although 42°C would be at the lower end of the temperature range found on the surface of the sediments at the sample site.

Clone type V3 had a sequence identity of 94% to *Thiobacillus prosperus*, which was discovered in the same area previously (Huber and Stetter, 1989). *T. prosperus* is an iron-oxidizing salt-tolerant acidophile, thus with a sequence identity of only 94%, V3 could be one of the other strains noted by Huber and Stetter but not characterised. Its sequence identity was closest to *T. prosperus* than to other phylogenetically related bacteria, alkaliphilic sulphur-oxidisers such as *Alkalispirillum mobilis* and *Thioalcalovibrio denitrificans,* which had 93% sequence identity and a sulphur oxidising strain (OAII2) from a shallow water hydrothermal vent.

Two organisms (V10 and V11) also with 94% identity to V3 and to *T. prosperus* have been previously isolated from ferrous iron enrichments of Vulcano samples (Norris, unpublished) but these were not the source organism of the clone V3 RNA sequence. Perhaps V3 was out-competed by the two isolates here, referred to as V10 and V11 (see section 5.3), or by *Sulfobacillus thermosulfidooxidans*, which was also isolated from Vulcano samples but was inhibited by concentrations of NaCl above 2% (P. Norris, personal communication). V10, V11 and *S. thermosulfidooxidans* were not found in this 16S clone library of the Vulcano samples. Characterisation experiments were carried out for V10 and V11 and the results are described in Chapter 5.

Clone type V4 had a sequence identity of 99% to an unidentified γ-Proteobacterium isolated from deep-sea sediments and close identities to several *Pseudomonas* species, but not to any organisms that exhibit acidophily or thermophily. V6 had a sequence identity of 99% to several *Buttiauxella* species; these are members of the *Enterobacteriaceae* and have been isolated from a number of different environments including soil, water, slugs and snails (Muller *et al.*, 1996). V6 did not have any sequence similarities to thermoacidophilic organisms, so it is possible that it was not indigenous to the sample niche.

The sequence of clone type V5 was closely related (97% identity, Fig 4.4) to one that was amplified from a hot, acidic pool on the Caribbean island of Montserrat (Burton and Norris, 2000). The next nearest relatives were of the *Thermosipho* and *Fervidobacterium* genera.

A single clone type comprised the clone bank established from the 45°C sample site using the F25 forward archaeal primer. It had a 16S rDNA sequence identity of 98% to *Thermoplasma volcanium*, an organism previously isolated from Vulcano, Iceland and Yellowstone National Park, USA – all geothermal environments (Sergerer *et al.*, 1988). However *T. volcanium* was not found in any of the enrichment cultures, as the correct conditions were not used for its isolation. V7 was also found in the 75°C sample site.

4.2.3 75°C vent

No bacterial 16S rRNA genes were amplified from the hot sample. Clone type V7 was again found at this sample site although 75°C is higher than *T. volcanium*'s maximum growth temperature of 67°C (Sergerer *et al.*, 1988). Occurrence of V7 in this sample site may be due the organism being released into the hot fluid from the cooler vent walls. Clone type V8 had a sequence identity of 99% to *Acidianus brierleyi* and V9 had 100% sequence identity to *Acidianus infernus*. The first V8 clone type sequenced was chimeric, with the majority of the sequence being *Acidianus brierleyi* at the 5′ end but the 3′ terminus was an *Acidianus infernus* sequence. More V8 clone types were sequenced and all were genuine with the full 16S sequence corresponding to *Acidianus brierleyi* even though all the RFLPs for V8, whether chimeras or not, were the same.

4.2.4 Enrichment cultures

Enrichment cultures of the 75°C sample were set up using sulphur and pyrite (separately) as substrates and incubated at 70°C. Good growth was not obtained on either of the substrates where sodium chloride was added at seawater concentration, but without the sodium chloride, growth could be maintained on pyrite and sulphur. 16S rDNA clone banks were made from cells lysed from the second serial subculture with each of these substrates. Two clone types were found by RFLP analysis and representatives were sequenced. One clone type corresponded to *Sulfolobus metallicus* with no other close matches in the database (see Table 4.2). The other clone had sequence closely related to *Acidianus brierleyi*, with one nucleotide difference from clone type V8 over the 5′-terminal 548 nucleotides. The main differences between the *A. brierleyi* sequences obtained from Vulcano and the database *A. brierleyi* sequences were contained in a region between helical stalks with -CCAAAA- instead of -TTAAAG- respectively (nucleotides 193-198 in *E. coli* nomenclature).

The selection of V8 over V9 in the enrichment culture is reflected in the optimum temperatures for growth, *A. brierleyi* has an optimum temperature of 70°C and *A. infernus* of about 90°C (Sergerer *et al.*, 1986). *S. metallicus* has a faster growth rate on pyrite than *A. brierleyi* at 70°C, hence its dominance on this substrate.

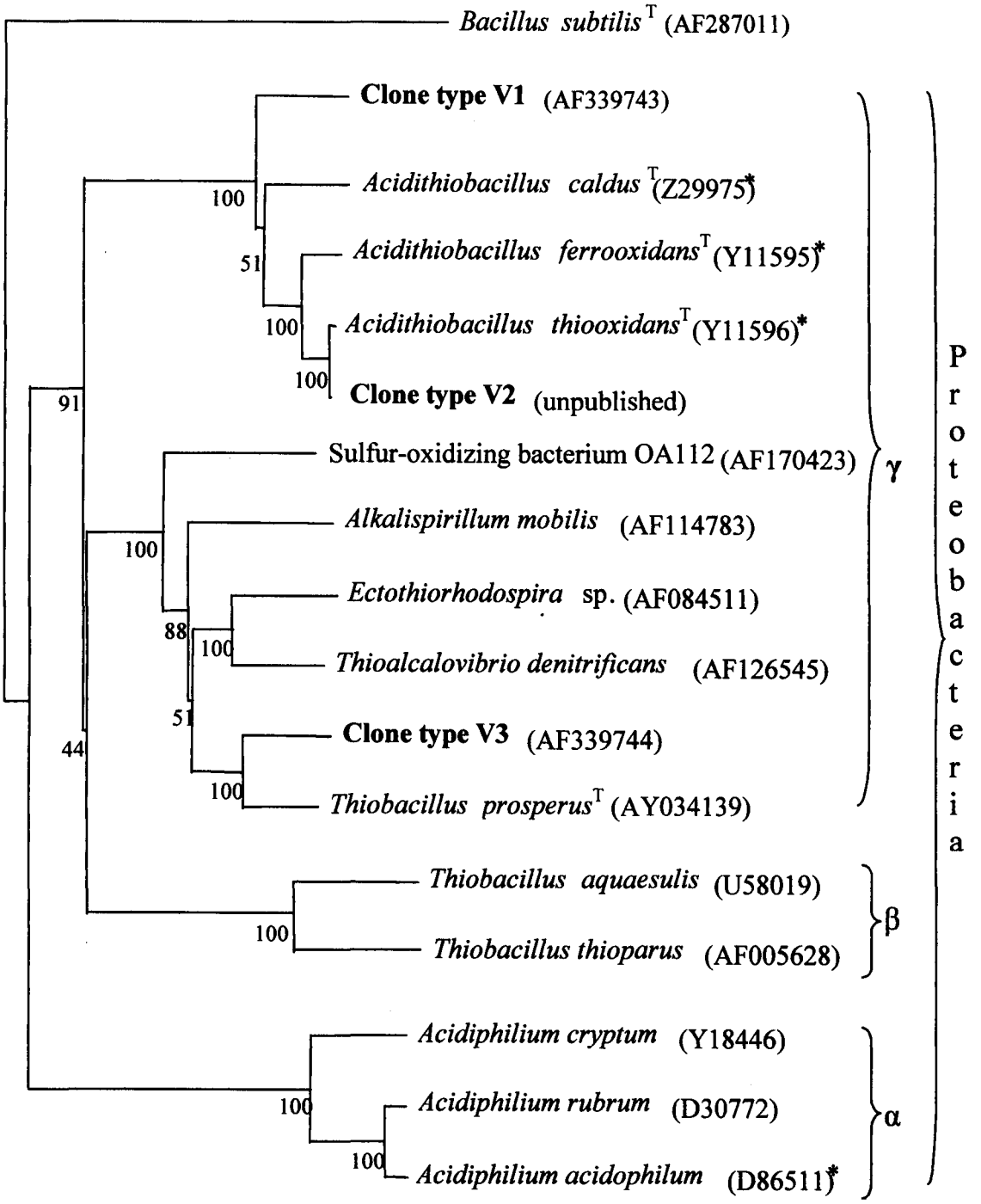
S. metallicus was not found in the clone bank of the Vulcano sample, however, it could have been in such few numbers that it was not detected.

Table 4.2. Identity of clone types from 16S rDNA clone libraries of enrichment cultures of acidophiles at 70°C; the sequences were compared over 548 nucleotides at the 5′ - terminus.

Substrate	cloned sequences	Corresponding species	16S rDNA sequence identity
sulphur	23	*S. metallicus* (13)	99.7%
		A. brierleyi (10)	99.4%
pyrite	29	*S. metallicus* (28)	as above
		A. brierleyi (1)	

The 16S rRNA gene sequences from the analysis of these clone libraries were identified using the GenBank database BLAST searches (Altschul *et al.*, 1997), aligned using the PILEUP program of GCG (Genetics Computer Group, University of Wisconsin). Phylogenetic trees were created using the DNAdist (Jukes and Cantor) and FITCH programs in PHYLIP, version 3.57 (Felsenstein, 1995) and bootstrap values were calculated using the SEQBOOT and CONSENSE programs. The trees are shown in Figure 4.5.

Fig 4.5 (a) Unrooted phylogenetic tree of the Proteobacteria. The clone types from the Vulcano clone library are highlighted in bold. The out group is *Bacillus subtilis.*



The asterisks indicate organisms that were originally known as *Thiobacillus* when deposited in the database.
The bootstrap values are at the branch nodes and are based on 100 re-samplings.

Fig 4.5 (b) Unrooted phylogenetic tree of the Euryarchaeaota showing the position of clone type V7. *Bacillus subtilis* and some of the Crenarchaeaota are the out-groups.

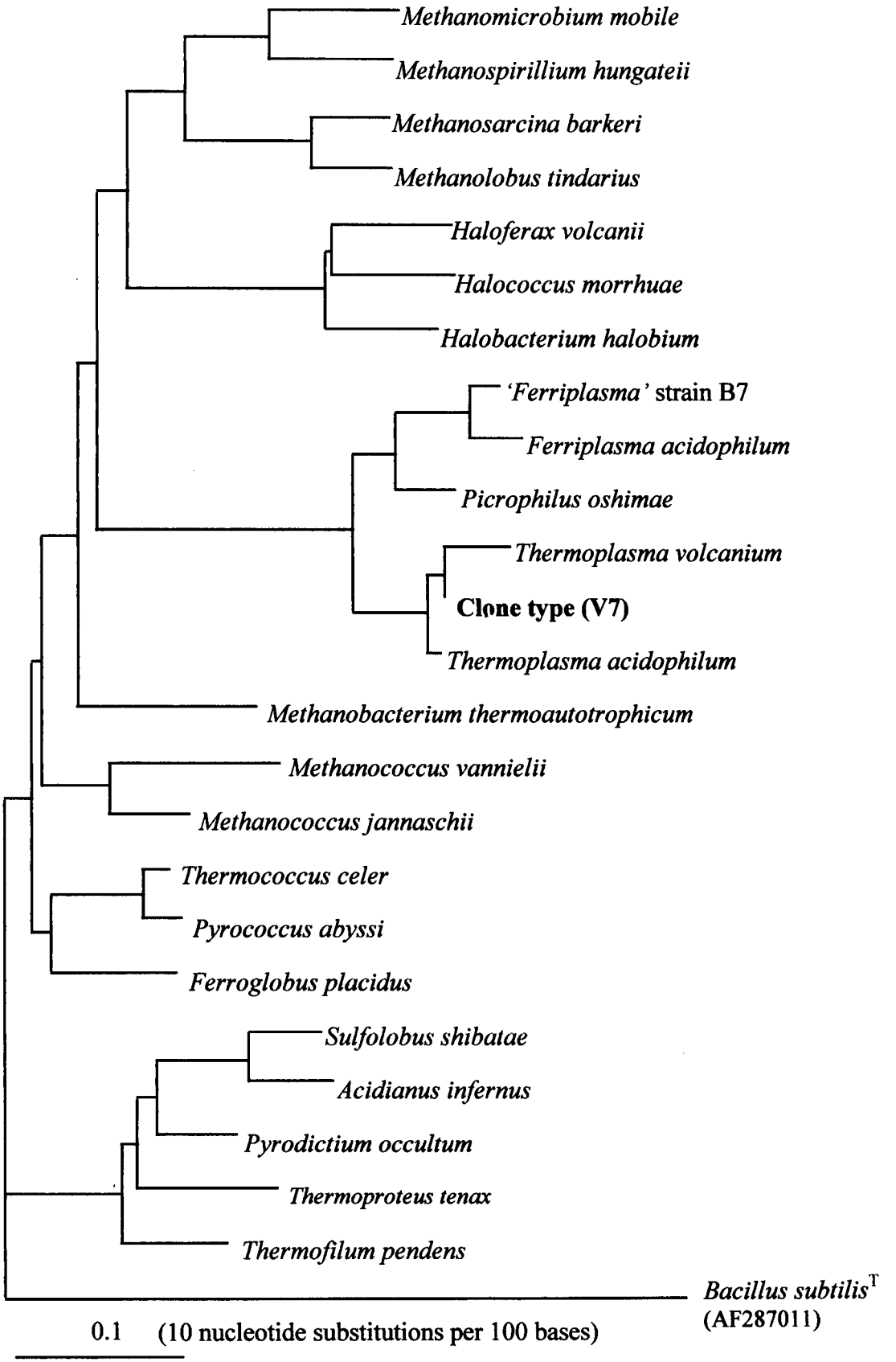
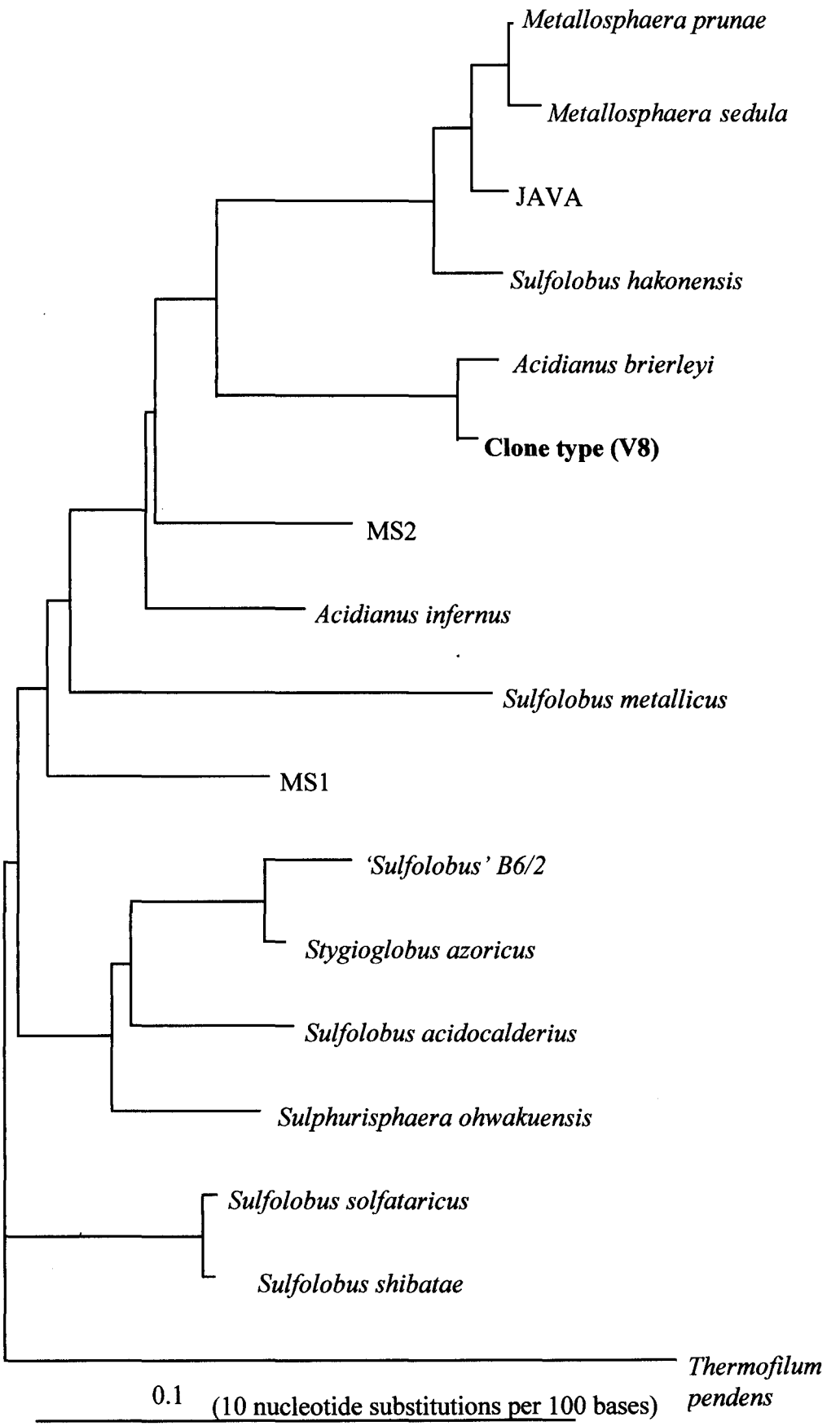


Fig 4.5 (c) Unrooted phylogenetic distance tree of the Crenarchaeaota showing the position of clone typeV8. The out-group is a member of the Euryarchaeaota *Thermofilum pendens.*



V2 trees very close to a clone type found in a saline bioreactor that had been inoculated with samples from Vulcano (more about the reactors is discussed in section 4.3). Clone type V3 is positioned between the marine bacteria *Alkalispirillum mobilis* and *Thioalcalovibrio denitrificans.* Clone type V7 is very similar to *Thermoplasma volcanium.* Clone type V8 also branches separately from its closest sequence identity, *Acidianus brierleyi,* although they are only different by a few bases. Clone type V9 is not shown on the tree as it has 100% sequence identity to *Acidianus infernus,* which is shown on the tree. MS1 and MS2 are clone types found at Montserrat (Burton and Norris, 2000). The accession numbers and bootstrap values are not shown in Figures 4.5 (b) and (c), as they are only simple trees to give an idea of the positioning of the clone types. The bootstrap values in Figure 4.5 (a) are important to gain an understanding of the relationships between the clone types and their closest sequence identities. Most of the bootstrap values are high indicating that those branches are showing the true representation of the sequence relationships.

4.3 Oligonucleotide probing of Vulcano cultures in bioreactors

From aligned sequences, oligonucleotide probes were designed to clone types V1, V2, V10 and V11. These were used to probe clone libraries of rDNA extracted from reactors inoculated with Vulcano samples (see Fig 4.6 for the probe regions and Table 4.3 for the probe designs). One culture contained 3% NaCl and was set up in December 1998 (by P. R. Norris). The other culture contained 6% NaCl and was set up in 1999. Both had pyrite as substrate at 35°C and were routinely serially subcultured every 7-14 days. The microflora of these reactors were examined via clone libraries of DNA extracted from them at 3-8 monthly intervals. Initially, the clone libraries were analysed via RFLPs, but once specific probes were designed, DIG-labelled and shown to work with the 16S rRNA of the desired organisms, the libraries were analysed via DNA dot blotting of the clones rDNA, which proved to be less work than restriction digests. Any clones that gave unusual or non-definable results were analysed via sequencing. Representatives of each positive clone type were also sequenced to recheck the probes specificity in practice.

Table 4.3 Probe designs for Vulcano 16S rRNA sequences

Probe name	Base position	Probe sequence
-V1-	82R	CCA CCA GGT GCA AGC AC
-V2-	457R	AA CAG CAA GTG ATA TTA GCA CTC A
Vx/THIOX	458R	CAG CAG GCG GTA TTA GCA CCC
V10	575F	CGT GCG TAG GTG GTT GGG T
	575R	ACC CAA CCA CCT ACG CAC G
V11	575F	CGT GTG TAG GCG GTT TAG T
	575R	ACT AAA CCG CCT ACA CAC G

R indicates a reverse (RNA/DNA) probe.
F indicates a forward (DNA) probe.
Vx is a novel clone type found in the reactors.

Fig 4.6 Probe regions from pileups (*A. thioox* = *A. thiooxidans; T. pros* = *T. prosperus)*

(a) V1

```
          5'70                                              109 3'
V1        CAGCA----C GGGTGCTTGC ACCTGGTGGC GAGTGGCGGA
A.caldus  CAGCA----- -GGTCCTTCG GGATGCTGGC GAGTGGCGGA
Vx        TAACA----- -GGTC--TTC GGATGCTGAC GAGTGGCGGA
V2        TAACA----- -GGTC--TTC GGATGCTGAC GAGTGGCGGA
A.thiox   TAACA----- -GGTC--TTC GGATGCGTAC GAGTGGCGGA
V10       TAGCA----G GCCCTT--CG GGGTGCTGAC GAGTGGCGGA
V11       TAACA----G ATCCTT--CG GGATGCTGAC GAGTGGCGGA
T. pros   TAACA----G GCCCTT--CG GGGTGCTGAC GAGTGGCGGA
```

(b) V2 and Vx

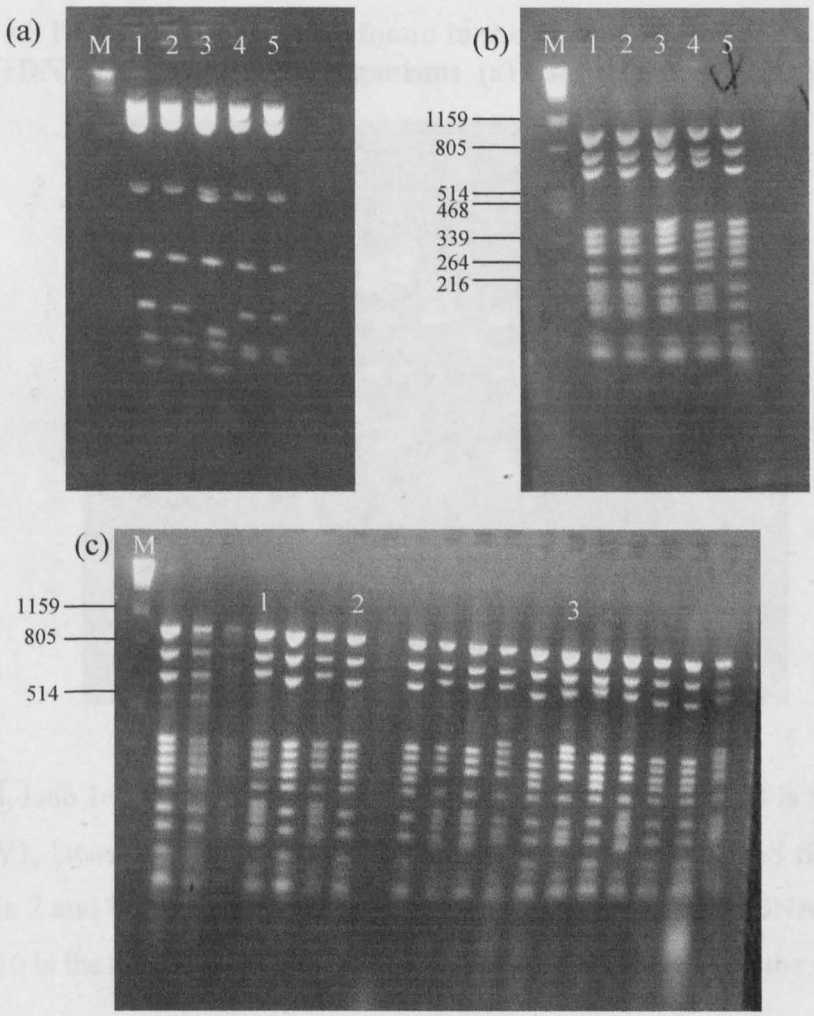
```
          5'450                                             489 3'
V1        GAAAAGGTGA TCGCTAATAT CGGTTACTGT TGACGTGAAC
A.caldus  GAAAAGGCGG ATCCGAATAC GGTCTGCTAT TGACGTGAAC
Vx        GAAAAGGTGG GTGCTAATAT CGCCTGCTGT TGACGTGAAT
V2        GAAAAGGTGA GTGCTAATAT CACTTGCTGT TGACGTGAAT
A.thiox   GAAAAGGTGG GTGCTAATAT CGCCTGCTGT TGACGTGAAT
V10       GAAAAGCTTA AGGTTAATAC CCTTGAGTCT TGACGTTACC
V11       GAAAAGCCTA GGGTTAATAC CCTTGGGTCT TGACGTTACC
T.pros    GAAAAGCCTA AGGTTAATAC CCTTGAGTCT TGACGTGAAC
```

(c) V10 and V11

```
          5'560                                             599 3'
V1        TCACTGGGCG TAAAGGGCGC GTAGGCGGTT GGTTACGTCT
A.caldus  TTACTGGGCG TAAAGGGCGC GTAGGCGGTG GGTTACGTCT
Vx        TCACTGGGCG TAAAGGGTGC GTAGGCGGTG CATTAGGTCT
V2        TCACTGGGCG TAAAGGGTGC GTAGGCGGTG CATTAGGTCT
A.thiox   TCACTGGGCG TAAAGGGTGC GTAGGCGGTG CATTAGGTCT
V10       TTACTGGGCG TAAAGCGTGC GTAGGTGGTT GGGTAAGTCA
V11       TTACTGGGCG TAAAGCGTGT GTAGGCGGTT TAGTAAGTCA
T.pros    TTACTGGGCG TAAAGCGTGC GTAGGCGGCT GGGTAAGTCA
```

When sequenced, representative clone types from the reactors had sequences identical to the 16S rRNA sequences of the organisms, except the V2-like reactor clone. This clone was very similar (99% sequence identity), but not identical, to the V2 16S rRNA sequence, which is reflected in their RFLPs. This can be seen in Figure 4.7.

Fig 4.7 RFLPs of clones of organisms from Vulcano run on a 2% agarose gel

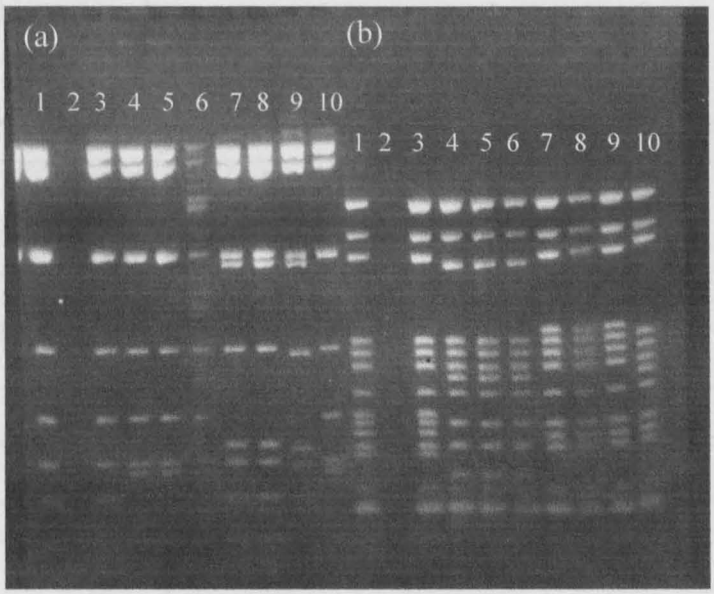


(a) R*sa*1+E*co*R1 and (b) S*au*3A1+E*co*R1 digests of 16S rDNA clones of 1,V1; 2,V1; 3,V2; 4,V10; 5,V11. (c) S*au*3A1+E*co*R1 digests of clones from clone library of 3% reactor, 1, V1 like clone; 2, V11 like clone; 3, V2 like clone.

Note that the R*sa*1+E*co*R1 RFLPs for V10 and V11 are the same, thus for the clone library work on the reactors, S*au*3A1+E*co*R1 digests were used to distinguish RFLP types. V10 was not found in the reactors. M indicates the Lambda P*st* markers, with indicated fragment sizes (kbp)

Figure 4.8 shows the RFLPs of clone types found in the reactors against the RFLPs of 16s rDNA clones of organisms previously isolated from Vulcano, demonstrating that the microflora in the reactors were Vulcano isolates V1 and V11. V10 and V2 were not present in the reactor but a clone type with a sequence identity very close to that of strainV2 was present, their RFLPs are almost identical except for a shift in the banding pattern.

Fig 4.8 RFLP's of clone types found in the reactor run on a 2% gel against 16S rDNA clones of known organisms. (a) R*sa*I+E*co*RI (b) S*au*3A1+E*co*RI,



In Fig 4.8, lane 1 is a V1-like clone type, lane 2 has no DNA, lane 3 is the 16s rDNA clone of V1, lanes 4 and 5 are V11 like clone types, lane 6 is the 16S rDNA clone of V11, lanes 7 and 8 areV2 -like clone types (Vx), lane 9 is the16S rDNA clone of V2, and lane 10 is the16S rDNA clone of V10. Markers were not run on the gel.

Different probes were designed to distinguish the two clone types. The Vx rDNA hybridises with a probe designed for *Acidithiobacillus thiooxidans,* whereas the 16S rDNA clone of V2 does not. The Vx is probably not *Acidithiobacillus thiooxidans* because (a) the 16S rRNA sequence is different and (b) its physiology must be different as *T. thiooxidans* would not be able to withstand such high levels of sodium chloride, though it could be an *A. thiooxidans* strain with salt tolerance.

Attempts were made to isolate the Vx organism from the reactor but growth on phytagel plates containing potassium tetrathionate did not occur, experiments were not carried out with alternative substrates such as thiosulphate. Table 4.4 is a summary of the clone types and their numbers found in the clone libraries of the 3% and the 6% NaCl reactors over time, a histogram of these results is shown in Figure 4.9. In the final clone library of the 3% reactor it can be seen that V1 and V11 are almost equal in population size indicating some stability of the population dynamics. Vx is out competed by V1 and V11, perhaps due to a slower growth rate. This cannot be fully understood as growth rate experiments were not carried out on Vx because it was not isolated in pure culture.

Table 4.4 Clone type numbers found in the 3% and 6% salt reactors over time.

Date (months from start)	No. clones analysed (salt %)	V1-like clones (%)	V2-like clones (%) (Vx)	V11-like clones (%)	unknowns (%)
5	90 (3)	2	35	62	
12	60 (3)	25	.32	43	
15	62 (3)	60	15	25	
16 (5)#	76 (6) *	0	1	95	4
19 (8)#	79 (3) *	43	4	46	7
	64 (6) *	0	8	89	3

Notes:
* Indicates analysis of clone libraries by rDNA dot blotting rather than by RFLPs.
# Numbers in brackets show the number of months after samples from the 3% reactor were inoculated into the 6% salt reactor.

The 4% of unknowns for the 6% reactor were found to be related to Proteobacterium B6 after analysis by sequencing. The unknowns for the 3% reactor at 19 months were found to be V1/V11 or V1/Vx chimeras. The 6% reactor unknowns for this date were related to *Staphylococcus epidermidis.* Figure 4.9 illustrates the 3% reactor results in the form of a histogram.

The analysis of these saline bioleaching reactors showed the higher salt tolerance of V11 compared to V1 and Vx, although both V1 and Vx exhibited some salt tolerance. It is interesting to note that V1 disappeared from the 6% salt reactor whereas Vx remains in it although at very low numbers. This may indicate that Vx

has a higher salt tolerance than V1 but is unable to grow as fast as V11. The tolerance of Vx to high salt concentrations is surprising as it has only one nucleotide difference in 16s rDNA from *Acidithiobacillus thiooxidans*, an organism not noted for its high salt tolerance.

Figure 4.9 Profile of bacterial types present in the 3% salt reactor over time.

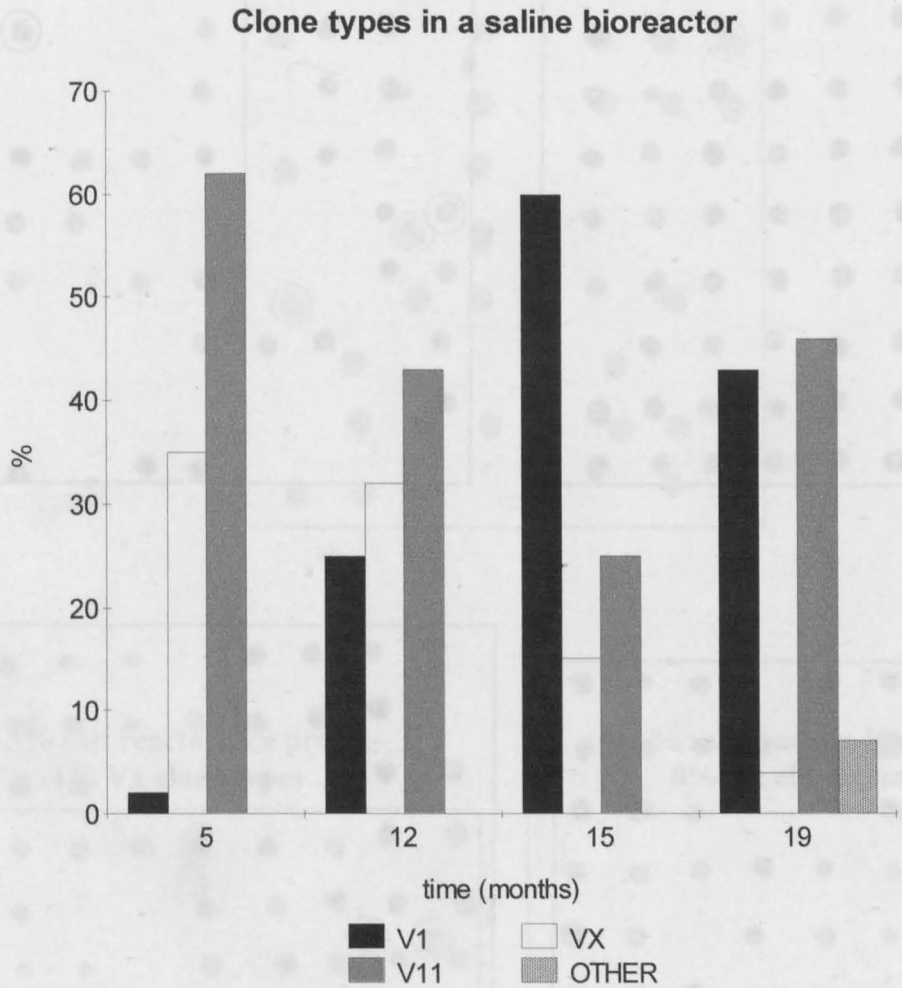
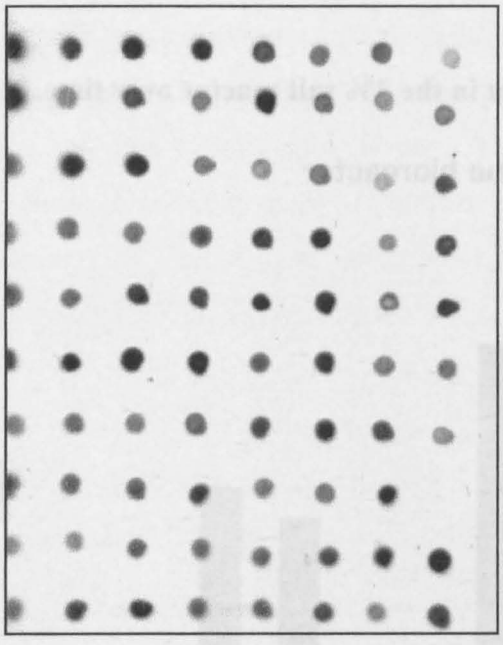


Figure 4.9 clearly shows the decrease of Vx in the 3% reactor and the increase of V1. V11 initially decreases in numbers but the final clone library shows that it is in almost equal numbers to V1 (if PCR biasing is not assumed). A profile is not shown of the 6% reactor results as only two clone libraries were carried out.

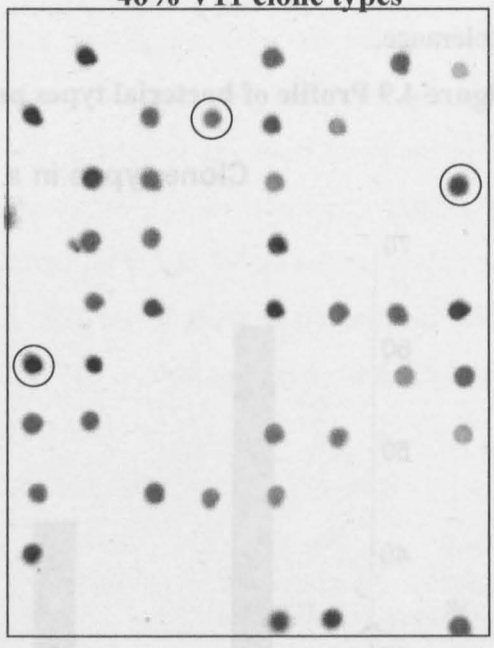
rDNA dot blots were carried out on the saline reactors using the probes in Table 4.3, the results are shown in Figure 4.10. The rDNA from 80 clones of the 3% reactor clone library were blotted on one membrane and 67 rDNA clones of the 6% reactor on the other. Any gaps on the membranes are a result of no rDNA being blotted or lack of insert.

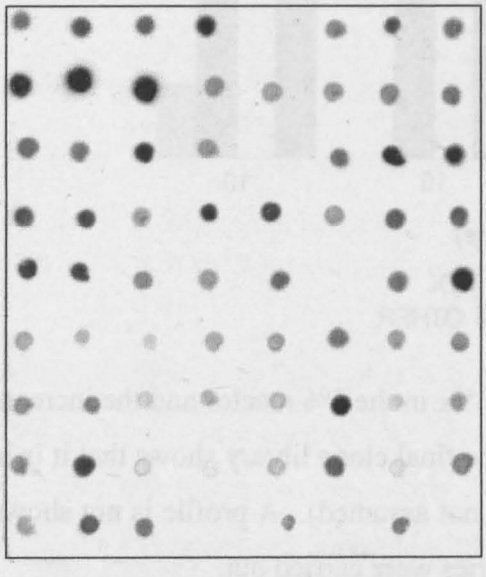
Fig 4.10 Examples of the reactor clone library rDNA dot blots, the probes used are in brackets, the circled spots are chimeric.

(a) 3% salt reactor R1492 probe: control

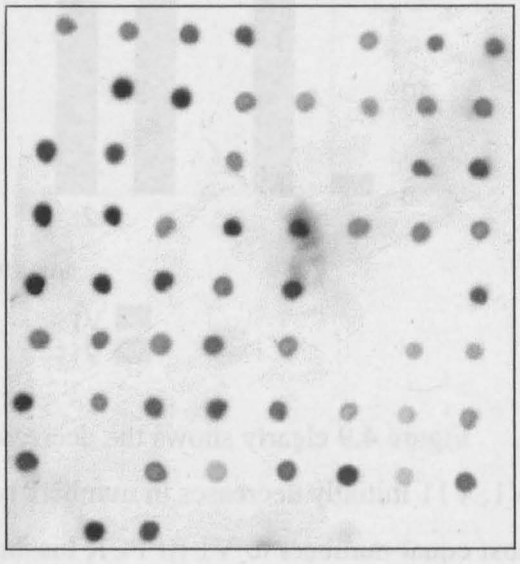


(b) 3% salt reactor: V11 probe - 46% V11 clone types



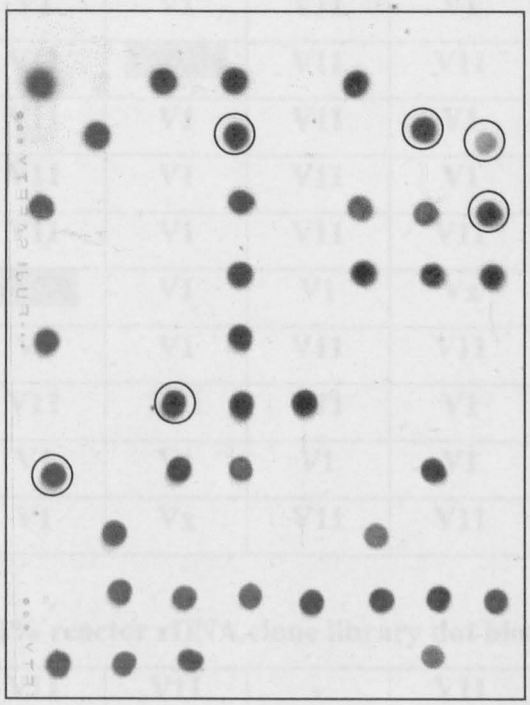


(c) 6% salt reactor R1492 probe: control

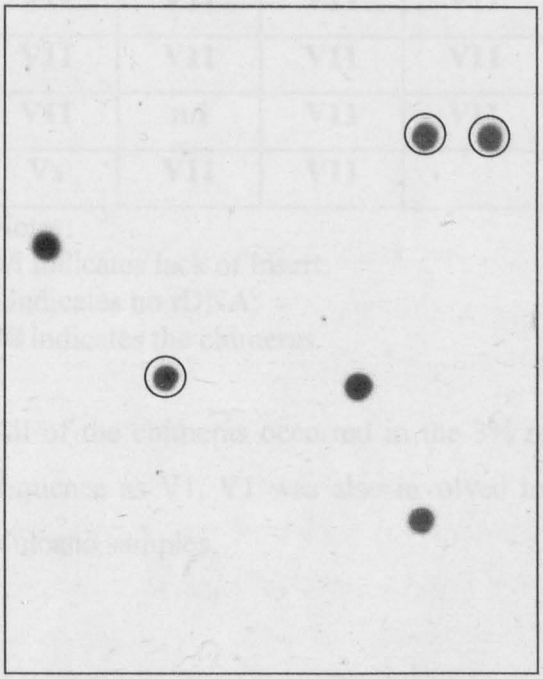


(d) 6% salt reactor: V11 probe – 89% V11 clone types

(e) 3% salt reactor: V1 probe – 43% V1 clone types



(f) 3% salt reactor: Vx probe – 4% Vx clone types



(g) 6% salt reactor: Vx probe - 8% Vx clone types

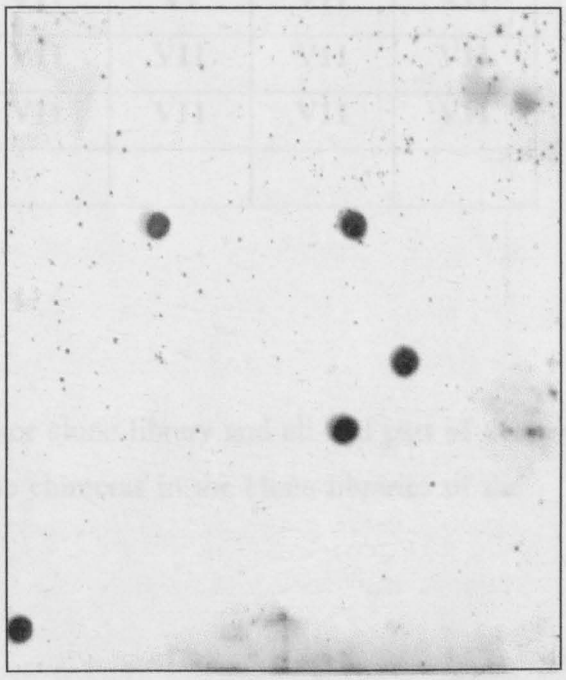


Fig 4.11 Summary of reactor rDNA clone library blots

(a) 3% reactor rDNA clone library blots

V1	V11	V1	V1	V11	V1	V11	V11
V11	V1	V11	V1/V11	V11	V11	V1/Vx	V1/Vx
V1	V11	V11	V1	V11	V1	V1	V1/V11
Vx	V11	V11	V1	V11	V1	V1	V1
V1	V11	V11	V1	V11	V11	V11	V11
V11	V11	V1/Vx	V1	V1	Vx	V11	V11
V1/V11	V11	V1	V1	V11	V11	V1	V11
V11	V1	V11	V11	V11	V1	Vx	-
V11	V1	V1	V1	V1	V1	V1	V1
V1	V1	V1	Vx	V11	V11	V1	V11

(b) 6% reactor rDNA clone library dot blots

V11	V11	V11	V11	-	V11	V11	V11
n/i	V11	V11	V11	V11	V11	V11	V11
V11	V11	Vx	V11	-	Vx	V11	V11
V11	V11	V11	V11	V11	V11	V11	V11
V11	V11	V11	V11	V11	-	Vx	V11
V11	V11	V11	V11	V11	Vx	V11	V11
V11	V11	V11	V11	V11	V11	V11	V11
V11	n/i	V11	V11	V11	V11	V11	V11
Vx	V11	V11					

Notes:
n/i indicates lack of insert;
- indicates no rDNA;
indicates the chimeras.

All of the chimeras occurred in the 3% reactor clone library and all had part of the sequence as V1, V1 was also involved in the chimeras in the clone libraries of the Vulcano samples.

4.4 Discussion

This analysis has confirmed some of the expected microbial diversity in a salt-rich, acidic environment. The prevalence of certain clone types might have indicated organisms that were major components of *in situ* populations but the results are only qualitative in the absence of more extensive sampling and in the light of various analytical biases, including sample handling procedures and potentially differential cell lysis and gene amplification (Wintzingerode *et al.*, 1997).

A great diversity of microorganisms, including many novel phylotypes, was revealed by rRNA-based analysis of samples from hot springs with pH values too high for proliferation of acidophiles (Barns *et al.*, 1994; Hugenholtz *et al.*, 1998). A similar analysis of strongly acidic, non-saline, hot springs on the island of Montserrat also indicated some novel phylotypes (Burton and Norris, 2000). The diversity appeared to be less in the acid springs, but the acidophile populations might have been dominated by relatively few strains so that minority components were not represented in small clone banks of environmental rDNA. Similarly, little diversity was revealed in environmental rDNA clone banks from acidic Vulcano samples and only three clone types (V1, V3 and V5) did not correspond closely to described species.

At shallow water vents in the Aegean sea, *Thiomicrospira* species were found to be significant members of communities but decreased in numbers along a gradient of increasing acidity (pH 7 to pH 5) towards vent centres (Brinkhoff *et al.*, 1999). The acidity of the niches sampled at Vulcano would be expected to select for *Acidithiobacillus* species, rather than *Thiomicrospira*, and the prevalence of these was indicated. The novel, salt tolerant species that corresponded clone type V1 might well have been isolated previously from Vulcano without being fully characterized (Gugliandolo and Maugeri, 1993). In contrast, *Acidithiobacillus caldus* with less salt tolerance did not feature in clone banks and was only readily isolated by enrichment culture in the absence of salt. Other acidophiles that lacked genuine salt tolerance such as *Sulfobacillus thermosulfidooxidans* also dominated enrichment cultures without salt but were not represented among the environmental rDNA clones. However, *Sulfobacillus* species were also readily found in enrichment cultures from a freshwater geothermal site but again were not represented in rDNA clone banks

(Burton and Norris, 2000). Any influence of sporulation by these organisms as a factor in their apparently easier revelation by enrichment culture than by direct rDNA extraction is unknown.

The archaea that were represented in the clone banks (*Thermoplasma volcanium*, *Acidianus infernus*, *Acidianus brierleyi*) have shown optimum growth in the absence of salt or at least at concentrations below that of seawater, but it is possible that they have slightly more salt tolerance than other acidophiles of similar physiology. For example, *Thermoplasma volcanium* grows with 2% w/v salt and some strains grow slowly with 4% w/v salt (Segerer *et al.*, 1988) whereas the related *Picrophilus* species are inhibited by about 1% w/v NaCl (Schleper *et al.*, 1995). Some isolates of *Acidianus infernus* show a broad tolerance of salt with growth between 0.1 and 4% w/v, which was independent of their marine or freshwater origins (Segerer *et al.*, 1986). The absence of *Sulfolobus metallicus* genes among the environmental rDNA clones might reflect the temperature of the hottest sample rather than its salinity. *Sulfolobus metallicus* grows optimally with less than 0.75% w/v salt but has some tolerance to 3% (Huber and Stetter, 1991), which does not distinguish it greatly from *Acidianus brierleyi* or *Acidianus infernus*. As noted above, the *Acidianus* species grow at higher temperatures than *Sulfolobus metallicus*. However, unknown factors could influence the natural populations since *Thermoplasma volcanium* has a lower maximum temperature for growth than *Sulfolobus metallicus* but rDNA from the *Thermoplasma* rather than from the *Sulfolobus* was amplified from the hot sample. There was no evidence for novel species of salt tolerant thermoacidophilic archaea from the environmental gene analysis or from enrichment cultures.

CHAPTER 5 CHARACTERISATION OF NOVEL ORGANISMS ISOLATED FROM VULCANO.

5.1 Introduction

Microorganisms grow best when an outwardly directed turgor pressure and a near neutral cytoplasmic pH is maintained (Roe *et al.*, 1998). Accumulation of anions in the cell can affect the cells turgor pressure. Acidophiles do not generally tolerate anions such as chloride very well, this is due to the difference in pH between the acidic environment and the near neutral pH of the cell cytoplasm causing an influx of anions.

The movement of ions across a cell membrane can be driven by the proton motive force. The proton motive force (Δp) is composed of the membrane potential ($\Delta\Psi$) and the transmembrane pH difference (ΔpH), ie, $\Delta p = \Delta\Psi - 60\ \Delta pH$ where 60 is derived from RT/F at 37°C (R is the gas constant, T is the absolute temperature and F is the Faraday constant). The proton motive force reflects the energy status of the cell and is variable as is the membrane potential, which has to vary in response to the energy state of the cell (Alexander *et al.*,1987). For example, if the environmental pH was pH 2 and the cell cytoplasm potential was pH 6 then the ΔpH would be 4, thus if the cell was de-energised and Δp was zero then 0mV = 240mV – 240mV (at 37°C). The outside of the cell is less negative than the inside so the membrane potential is +240mV. If the environment is high in chloride then the chloride is taken into the cell, this influx of the chloride causes a collapse of the membrane potential.

Some acidophilic organisms have developed methods of counteracting this membrane potential collapse generated by the chloride ions. These halotolerant acidophiles are not common as there are few saline acidic environments. Such organisms, especially iron or sulphur oxidisers, would be of benefit to the biomining industry as saline water could be used for bioleaching in remote places where there is not a fresh water supply.

Vulcano island has a rare, readily accessible source of saline, acidic water. As mentioned in Chapter four the island has some hydrothermal vents that contain high

levels of chloride and iron as well as a low pH. Samples from these vents were collected in 1998 (P. Norris, personal communication) and a number of organisms were isolated. These were established to have salt tolerance but no defining experiments were done to find the optimum conditions for their growth. This chapter describes experiments carried out to characterise some of these organisms.

5.2 Growth of strain V1

One of the organisms isolated by Dr P. Norris in 1998 from Vulcano had the same 16S rDNA sequence as V1 clone type sequence from the clone library (see Chapter 4, section 4.2). This organism, designated strain V1, had already been shown to be an acidophilic sulphur-oxidiser by Dr P. Norris. Its chloride tolerance, temperature optimum and growth kinetics were investigated.

5.2.1 Temperature Optimum

Five vessels containing 370 ml double strength basal salts medium (see Chapter two, Table 2.1) were set up at five different temperatures (28, 32.5, 37 41 and 44°C) with 5 mM potassium tetrathionate as a substrate. 10ml inoculum of strain V1 was used. Growth was measured by taking 1ml samples at time intervals of approximately one hour and measuring absorbance at a wavelength of 440 nm Fig 5.1).

It can be seen from Figure 5.2 that the optimum growth of the organism was achieved between 37°C and 41°C, with a doubling time of 3.2 hours.

Fig 5.1 Growth of V1 at various temperatures

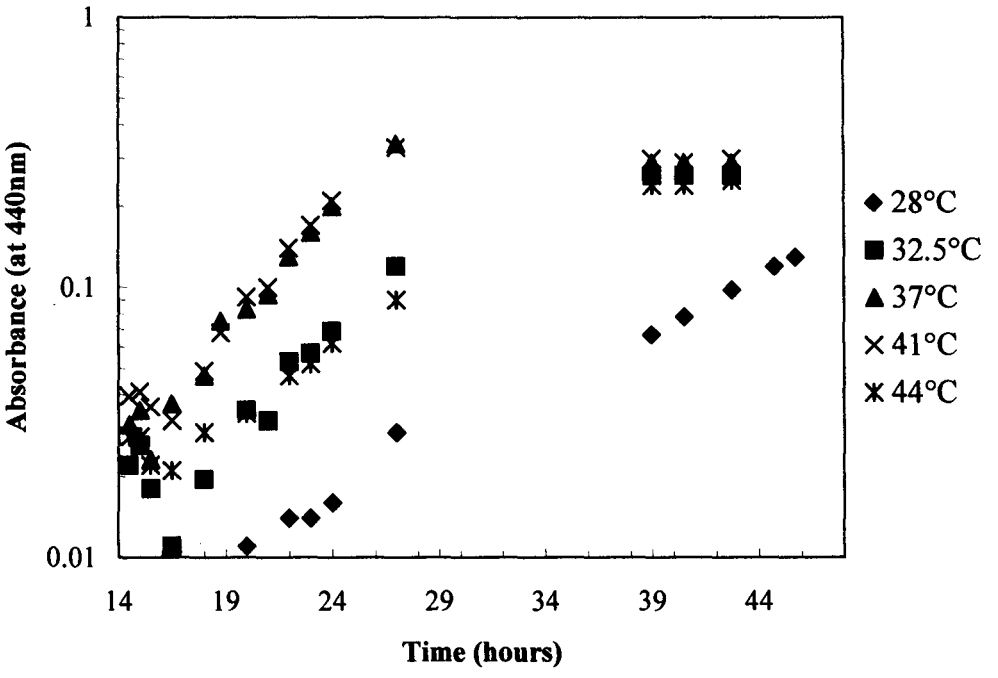
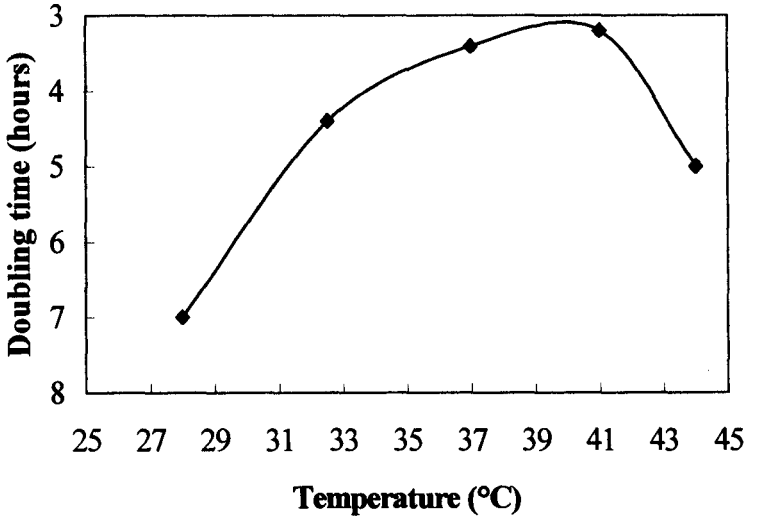


Fig 5.2 Effect of temperature on growth of strain V1

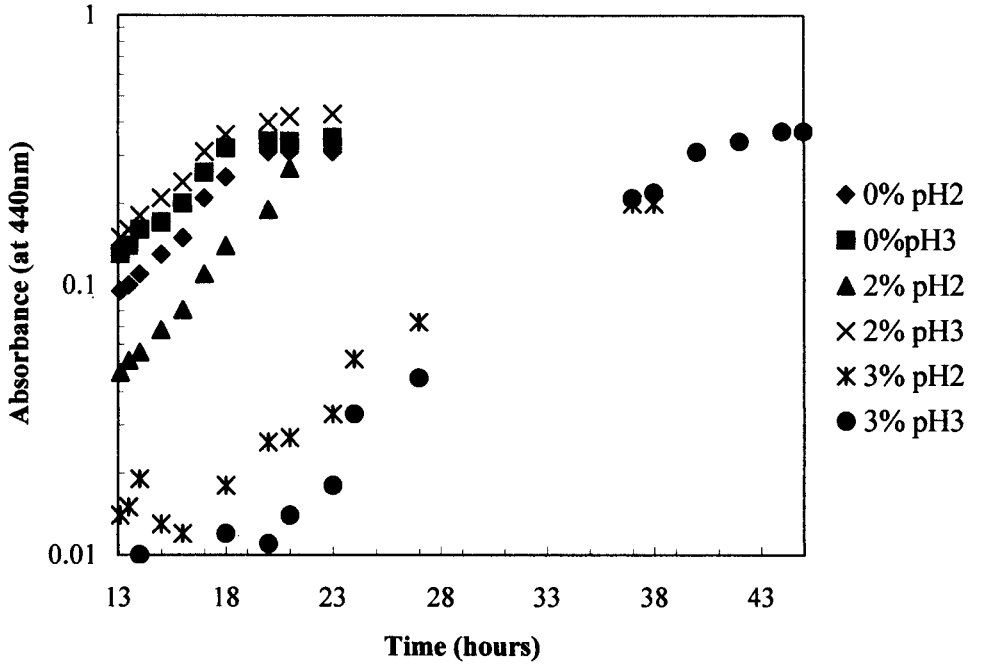


5.2.2 Chloride tolerance

It was necessary to ensure that any effects of NaCl on growth could be attributed to the chloride ions and not to sodium ions. This was done by setting up five vessels at 39°C containing 370 ml of double strength liquid medium. Two of the media contained 3% w/v sodium chloride (516 mM), two had 3.65% w/v sodium sulphate (250 mM) (all four had equal concentrations of sodium) and one medium had no sodium salts. Twenty millilitres of inoculum and 5mM tetrathionate was used in all cases. The two cultures with the sodium sulphate and no sodium salts both grew well indicating that there was not a sodium requirement and sodium was not detrimental to V1 growth at the concentrations used.

The next experiment was to determine the effect of pH at different salt concentrations. Six cultures were set up at 39°C, the different conditions were: pH 2.0, 0% NaCl; pH 3.0 0% NaCl; pH 2.0, 2% NaCl (344 mM); pH 3.0, 2% NaCl pH 2.0, 3% w/v NaCl (516 mM) and pH 3.0 3% NaCl. 10 ml inoculum and 5mM tetrathionate were used in all cases. Figure 5.3 shows the semi logarithmic graphs of the growth of V1 at these six different conditions. At pH 2.0 both the 0% and the 2% NaCl cultures, V1 had the fastest doubling time which was calculated to be 3.25 hours, but V1 at 0% pH 2.0 left the lag phase of growth earlier than the 2% at pH 2.0 (shown by its higher absorbance reading at 13 hours of growth). At pH 3.0 with these NaCl concentrations V1 had a doubling time of 3.75 hours. The lag phase was very similar for V1 at both concentrations, and shorter than at pH 2.0 indicating that the higher pH, salt concentration was not so crucial. At pH 3.0, V1 with 3% NaCl had a faster doubling time than at pH 2.0, 5.5 hours compared to the pH 2.0 vessel where V1 had a doubling time of 7 hours. However, although the growth rate was faster at the lower pH, V1 was slower to start growing compared to the higher pH.

Fig 5.3 Effect of NaCl on growth of strain V1 at different pH values



Experiments were carried out to compare the chloride tolerance of V1 to that of *Acidithiobacillus caldus,* its closest known relative (see Chapter 4). Each organism was inoculated into flasks with different nitrate or chloride concentrations. The flasks contained 0.1% (11.7 mM), 0.25% (29.25 mM), 0.5% (58.5 mM), 0.75% (87.75 mM) 1.0% (117 mM) and 1.25% (146.25 mM) sodium nitrate. Nitrate is a well-documented inhibitor of bacterial cell growth (Suzuki *et al.,* 1999) and it was used in this experiment to see if V1 exhibited nitrate tolerance as well as chloride tolerance, if so the organism may have some mechanism to allow it tolerance of anions in general, if not V1 may have a specific chloride resistance mechanism.

One millilitre of *Acidithiobacillus caldus* was inoculated from a growing culture, into flasks containing 0.1% (17.2 mM), 0.25% (43 mM), 0.5% (86 mM), 0.75% (129 mM), 1% (172 mM) and 1.25% (215 mM) NaCl. 1ml V1 was inoculated into flasks containing 0.5%, 1%, 1.5% (258 mM), 2%, 2.5% (430 mM), 3% and 3.5% (602 mM) NaCl – a wider NaCl range due to its expected greater chloride tolerance than *A. caldus.* Each organism was also inoculated into flasks with neither NaCl nor sodium nitrate, all flasks contained 5mM tetrathionate. The results of this experiment are shown in Figure 5.4 a-d.

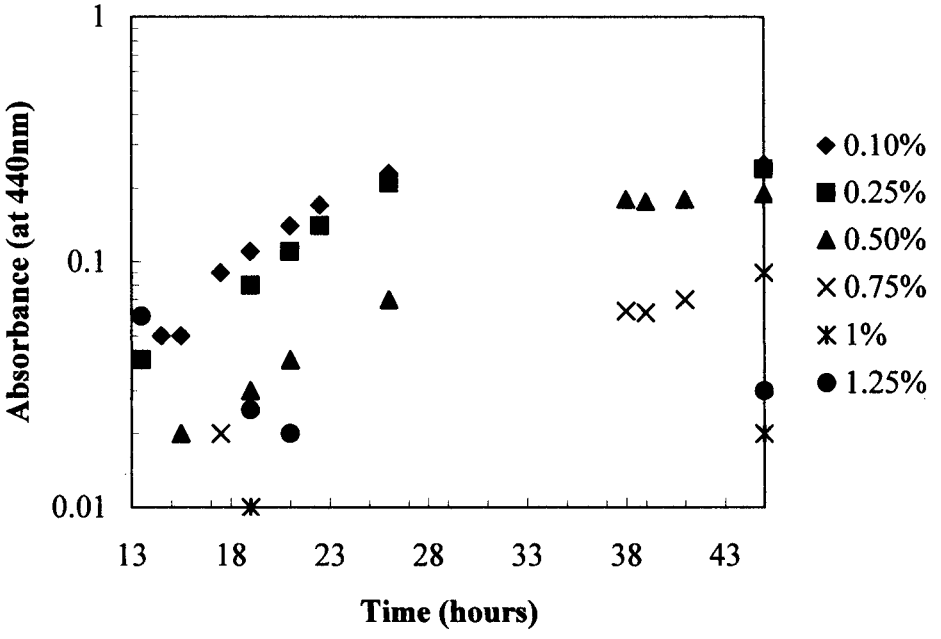
Problems occurred during this experiment as the tetrathionate formed a polysulphide and sulphur which precipitated in the *A. caldus* cultures.

However, *A. caldus* grew with 0.5% (w/v) NaCl with a doubling time of 4.75 hours this is quite slow compared to a normal doubling time of around 3 hours. Thus the organism was inhibited by NaCl but could still grow very slowly at 1.25% (215 mM) although precipitation caused problems with growth measurements. The growth of V1 in media containing NaCl, Figure 5.4(d), showed that it was able to grow well at 2.5% (430 mM) NaCl with a doubling time of 4 hours. This was not an optimum concentration as it grows faster at NaCl concentrations of 0%-1.5% with a doubling time of 3 hours. The flasks were grown at 39°C, the temperature considered to be optimum for the growth of the organism. Growth still occurred at 3% (516 mM) NaCl, with a doubling time of 6.75hours and at 3.5% (602 mM) NaCl, doubling time of 10 hours. All doubling time calculations were taken from the mid-exponential growth phase where possible, but this proved difficult at times as some of the mid-exponential growth phase was missed.

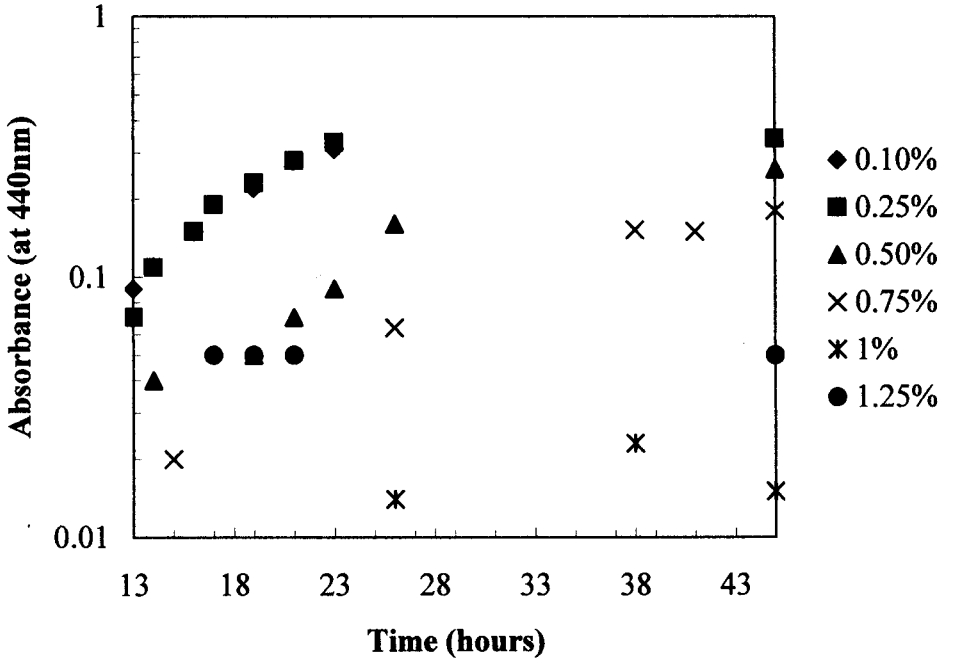
The growth of strain V1 was only inhibited by concentrations of NaCl above 2.5-3%, whereas growth of strain V1 in media containing even low concentrations of nitrate was poor. The results for V1 and *A. caldus* are very similar with both organisms being inhibited by the same concentrations of nitrate (Figs 5.4(a) (*A. caldus*) and (b) (V1)). At nitrate concentrations of 0.1% (11.7 mM) and 0.25% (29.25 mM) *A. caldus* had a doubling time of 4.5 hours, at 0.5% (58.5 mM) it had a doubling time of 6 hours and at 0.75% (87.75mM) a doubling time of 11 hours. V1 had doubling times of 4.25 hours at 0.1 and 0.25% nitrate, 4.5hours at 0.5% and 10 hours at 0.75%, little growth occurred above 0.75%. Figure 5.5 shows the doubling times and final absorbance measurements of each organism against sodium nitrate/sodium chloride concentration.

Fig 5.4

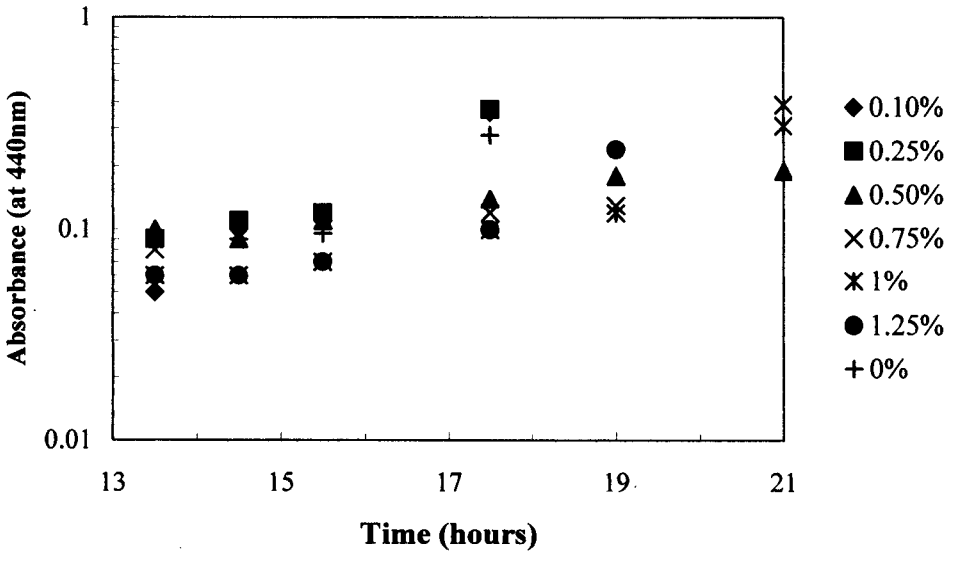
(a) Effect of $NaNO_3$ on growth of *A.caldus*



(b) Effect of $NaNO_3$ on growth of strainV1



(c) Effect of NaCl on growth of *A. Caldus*



(d) Effect of NaCl on growth of strain V1

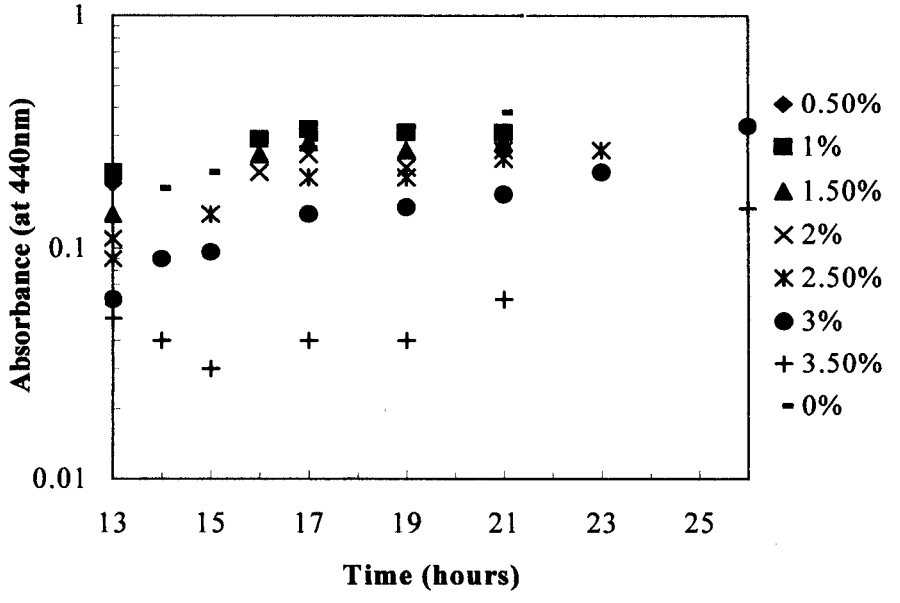
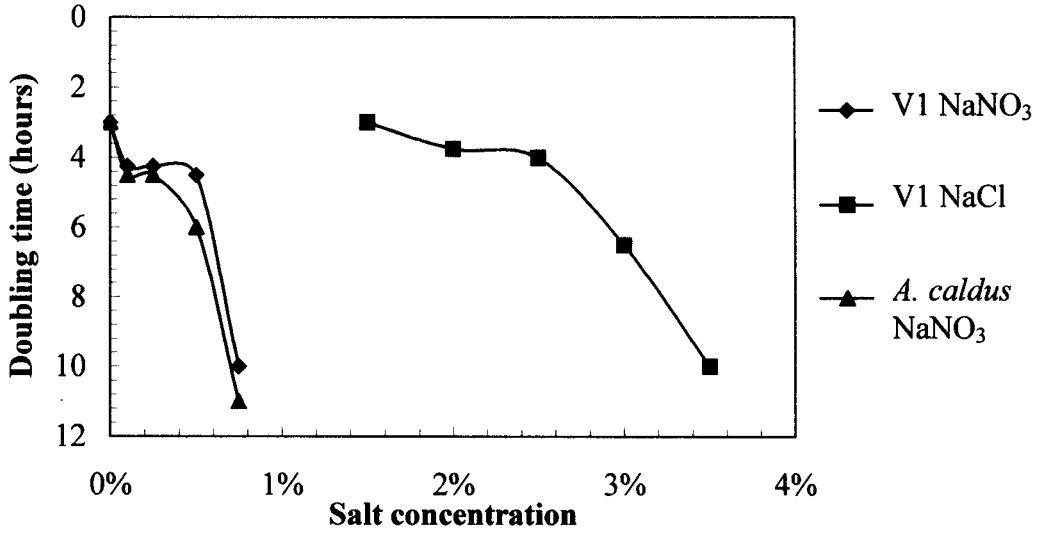


Fig 5.5 Effect of $NaNO_3$ and NaCl on the growth of strain V1 and *A.caldus*.



Note: There are no *A. caldus* NaCl doubling time data as accurate measurements could not be taken due to precipitation in the flasks which was possibly due to contamination of the potassium tetrathionate.

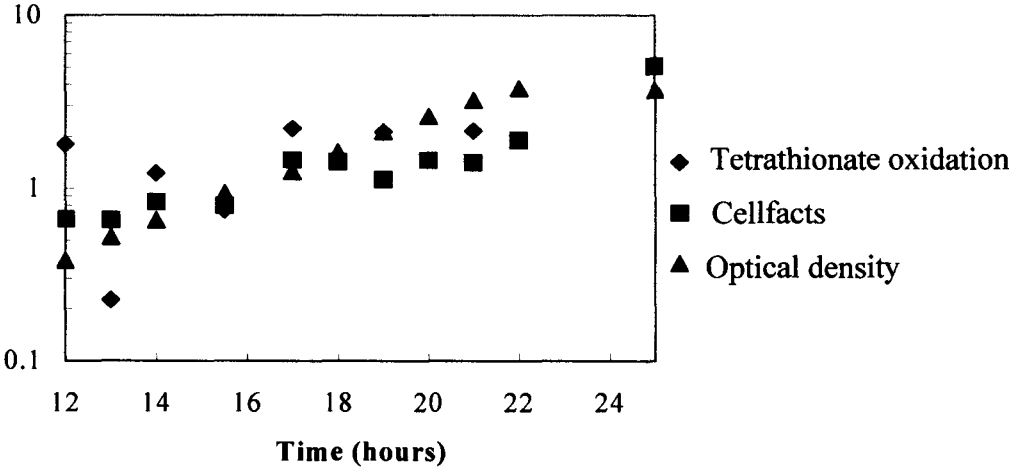
5.2.3 Final growth measurements for strain V1

The growth characteristics of strain V1 were determined by measurements of optical density, cell numbers and substrate utilisation. Three 2 litre flasks were set up with 500ml double strength medium plus 5 mM tetrathionate, containing either 0% NaCl, or 2% NaCl (both inoculated with 10ml V1), and a control flask containing the medium but no inoculum. A vessel was also set up containing 2% NaCl and inoculated with V1 to investigate whether the type of container used for the experiment affected the growth of V1. Figure 5.6 shows these results.

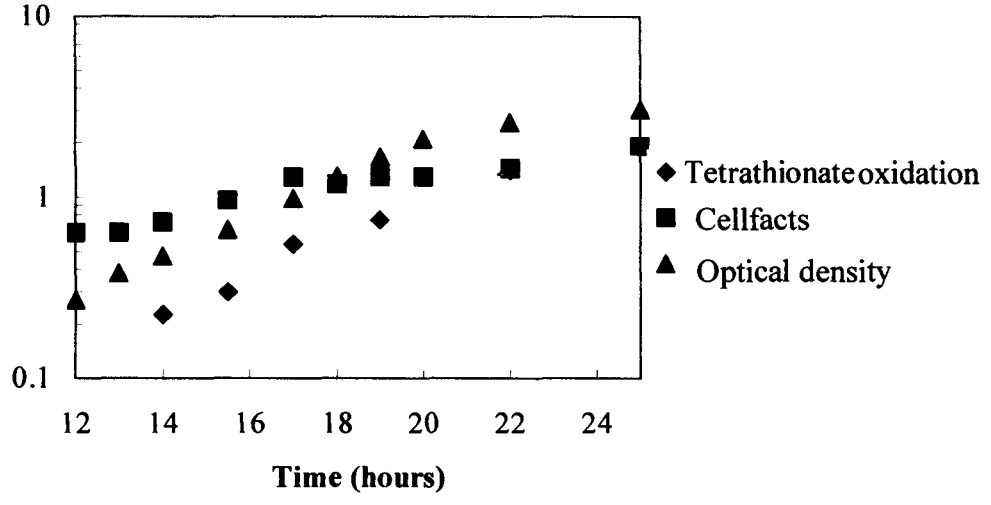
Strain V1 at 0% NaCl had a doubling time of 3.0 hours with all three measurements. With 2% NaCl the optical density readings and the tetrathionate assay, a doubling time of 3.0 hours was measured. A doubling time of 4.0 hours was calculated via cell counts measurements. Strain V1 in the vessel had a doubling time of 4.25 hours from the optical readings and the Cellfacts readings, a doubling time could not be determined from the tetrathionate assay. From these results it was observed that the optical density readings were most reliable and both the Cellfacts and the tetrathionate readings were not as reliable. The doubling time calculations for the tetrathionate results could only be made from the initial growth of V1. However the flask results are in keeping with the rest of the V1 experiment results with V1 having a fastest doubling time of 3 hours at pH 3.0, with 1.5-2% NaCl at 39°C.

Figure 5.6 Strain V1 growth measurements

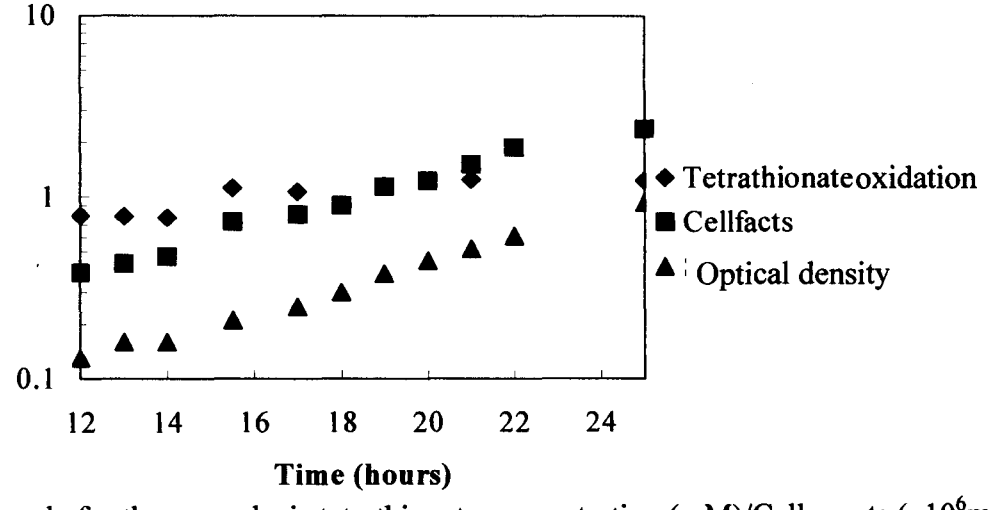
(a) Growth of strain V1 in 0% NaCl



(b) Growth of strain V1 in 2% NaCl



(c) Growth of strain V1 in vessel



The y axis scale for these graphs is tetrathionate concentration (mM)/Cellcounts ($x10^6 ml^{-1}$) /absorbance at 440nm

Table 5.1 Summary of doubling times for V1 growth experiments

Temp°C	**28**	**33**	**37**	**39**	**41**	**44**	
V1 (td, hrs)	8	4.5	3.5	3	3.5	4.5	
$NaNO_3$(%)	**0.1**	**0.25**	**0.5**	**0.75**	**1.0**	**1.25**	
V1 (td, hrs at 39°C)	4.25	4.25	4.5	10	-	-	
NaCl (%)	**0.5**	**1.0**	**1.5**	**2.0**	**2.5**	**3.0**	**3.5**
V1 (td, hrs at 39°C)	--	3	3	3.75	4	7	10

Note:
- indicates little or no growth,
-- indicates over-grown before measurements could be taken
The doubling time of V1 in medium containing no NaCl is 3 hours.

5.3 Characterisation of strains V10 and V11

Strains V10 and V11 were isolated from Vulcano but were not present in the 16S rDNA clone libraries of samples taken from the site. Both of these organisms oxidise iron and pyrite. The extent of their chloride tolerance and temperature range had not been established. This section investigates and compares the growth characteristics of these organisms.

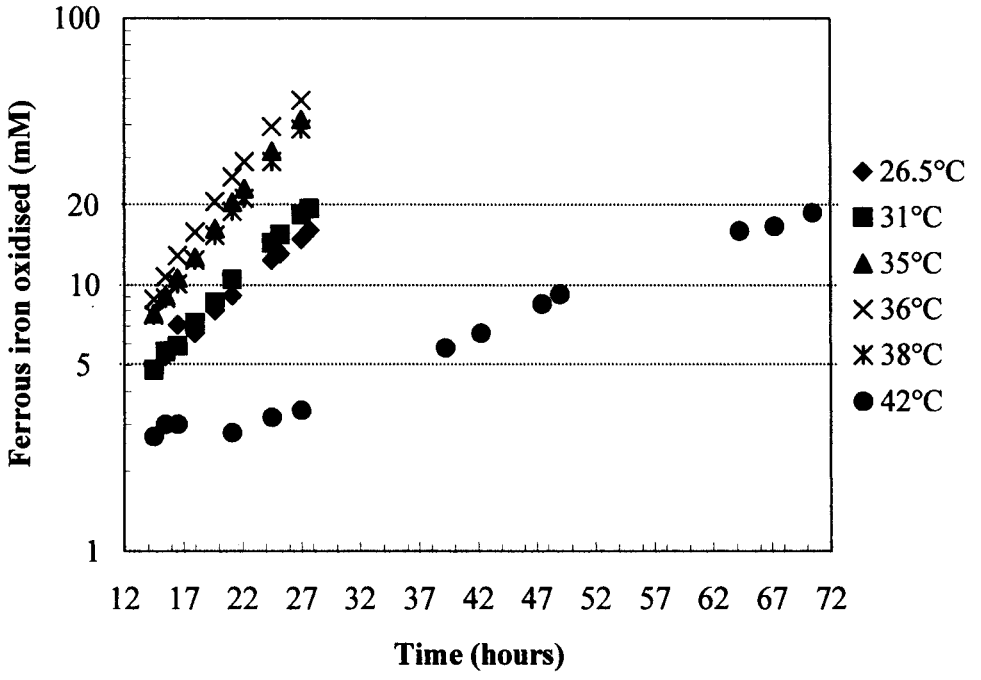
5.3.1 Temperature optima

Six vessels for each organism were set up containing 360 ml single strength basal salts medium, 2.5% NaCl, 2mM potassium tetrathionate and 50mM ferrous sulphate. These vessels were inoculated with 20 ml of either V10 or V11 and operated at 26.5°C, 31.5°C, 35.5°C, 36.5°C, 38.5°C and 42.5°C for V11 and 26.5°C, 31°C, 35°C, 36°C, 38°C and 42°C for V10. Growth was measured by using ceric sulphate titrations to measure residual ferrous iron.

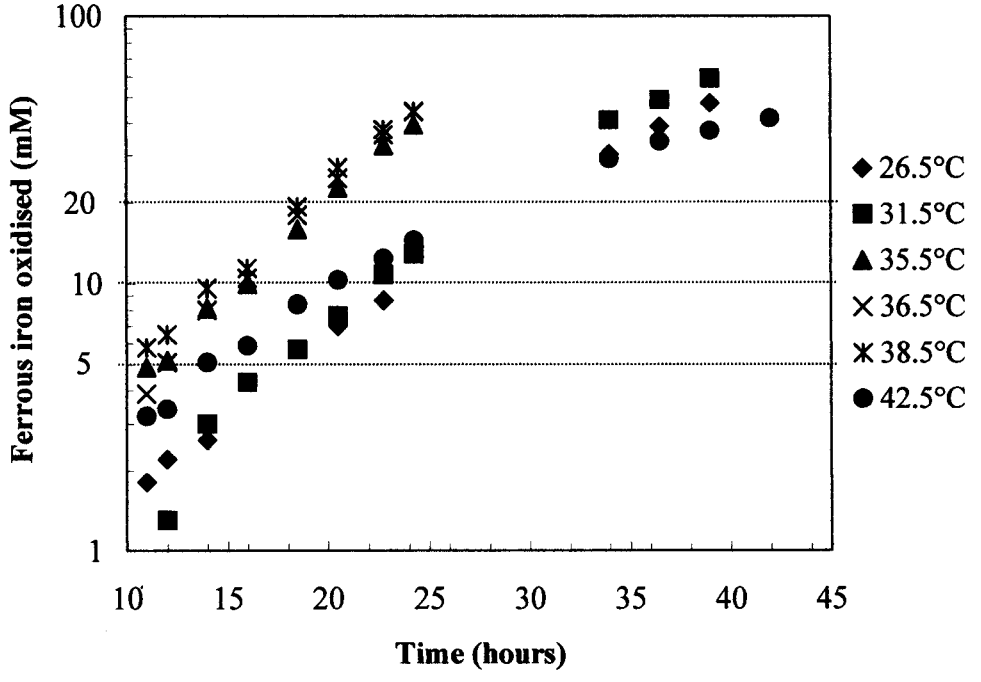
Figure 5.7 shows the growth of each organism at the different temperatures. Both organisms grew well at temperatures between 35 and 38°C. The growth at 26.5 and 31/31.5°C was very similar with a longer lag and a slower doubling time than at temperatures 35-38°C. At 42°C the growth of V10 was very poor and slower than at the other temperatures. At 42.5°C, V11 initially grew better than at 26.5 or 31.5°C but as the experiment progressed it can be seen that growth at 42.5°C was overall slower than at the other temperatures.

Fig 5.7

(a) Effect of Temperature on strain V10



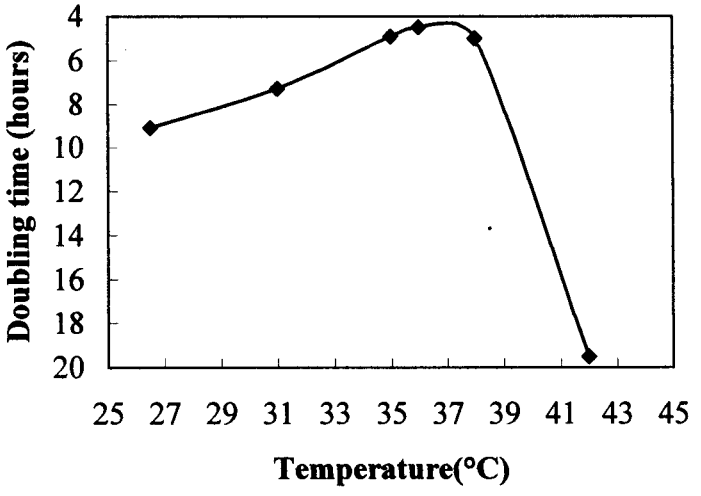
(b) Effect of temperature on strain V11



Note:
The dotted lines on the graphs show where 5mM, 10mM and 20mM of iron oxidised is. It is between these lines that doubling times are calculated.

Figure 5.8 shows the doubling times for each organism at the different temperatures. The optimum temperature for both of the organisms was at about 35°C – 37°C, with V10 having an overall slower growth rate than V11. For further experiments a temperature of 35°C was used. Two doubling times were taken for each organism, these were from growth between 5 and 10mM iron oxidised and 10 – 20mM iron oxidised, the 10 – 20mM range growth rate is slower of the two ranges and is the one used in calculations. However the 5 – 10mM doubling time for V10 was considerably slower than the doubling time over the same range for V11, the doubling times being 13 hours and 7.5 hours respectively.

Fig 5.8 (a) V10 doubling times at different temperatures (10-20mM)



(b) V11 doubling times at different temperatures (10-20mM)

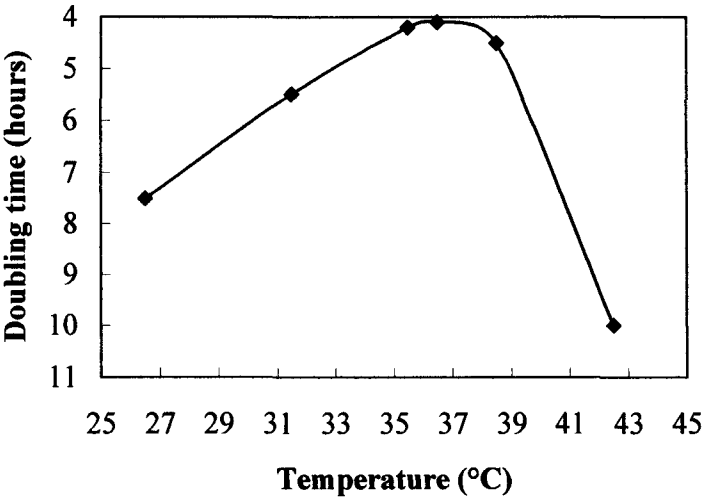


Table 5.2 Doubling times at six different temperatures for

(a) V11

Temp(°C)	5-10mM Fe	10-20mM Fe
26.5	5.9	7.5
31.5	5.0	5.5
35.5	4.1	4.2
36.5	3.7	4.1
38	4.0	4.5
42.5	7.4	10

(b) V10

Temp(°C)	5-10mM Fe	10-20mM Fe
26.5	7.2	9.1
31	5.9	7.3
35	4.4	4.9
36	4.3	4.5
38	4.4	5.0
42	13	19.5

5-10mM and 10-20mM are the amounts of iron oxidised over which the doubling times were calculated.

5.3.2 Effect of tetrathionate on growth on ferrous iron

Experiments were then set up to investigate the effect of different tetrathionate concentrations on each of the organisms.

Four flasks were set up at 35°C with single strength medium plus 50 mM iron sulphate and 2% (w/v) NaCl. These were shaken at 150 rpm. A different tetrathionate concentration was added to each flask, these were: 0.1mM, 0.5mM, 1mM and 1mM + 0.01% (w/v) yeast extract. The flasks were inoculated with 5ml of V10 from a culture containing 1 mM tetrathionate.

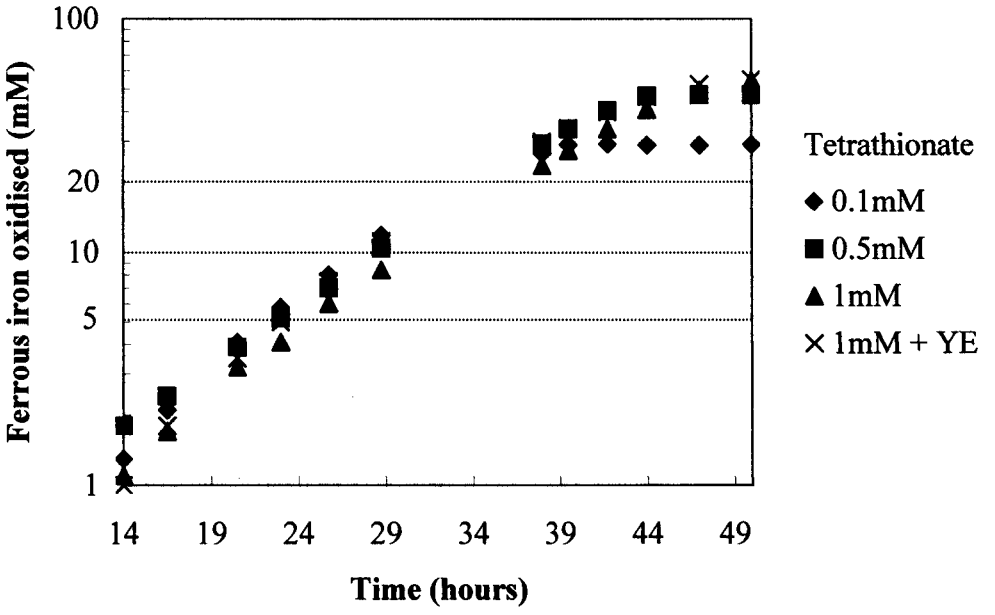
Figure 5.9(a) shows the results of V10 growth from this experiment. These results show that the addition of yeast extract to the medium had very little effect. The concentration of tetrathionate affected the yield of the organism rather than the growth rate, which is shown in Figure 5.9 (a). V10 supplemented with 0.1mM tetrathionate has a lower final substrate oxidation measurement than V10 supplemented with higher concentrations of tetrathionate. The average doubling time for V10 in this experiment was 5.8 hours.

An experiment was performed to investigate the effect of tetrathionate and yeast extract on the growth of V11. The conditions were the same as for V10 (35°C, 50mM iron sulphate, 150 rpm), but the tetrathionate, yeast extract and inoculum size was varied to see if there was any effect on the growth rate: 1mM tetrathionate, 0.01% (w/v) yeast extract, 2ml inoculum; 1mM tetrathionate, 0.01% yeast extract, 5ml inoculum; 0.01% yeast extract, 5ml inoculum; 1mM tetrathionate, 5ml inoculum. The yeast extract only flask was inoculated with a tetrathionate free inoculum obtained after several subcultures without the addition of tetrathionate, and the tetrathionate only flask was obtained after several subcultures without the addition of yeast extract.

Figure 5.9(b) shows the growth results of this experiment. The doubling time of V11 with both tetrathionate and yeast extract as well as iron sulphate as substrates was 4 hours, with yeast extract and iron sulphate as a substrates the doubling time was 5.3 hours and with tetrathionate and iron sulphate the doubling time was 10 – 40 hours depending, on where on the growth curve the doubling time was measured see (Table 5.3.)

Fig 5.9

(a) Effect of Tetrathionate on the growth of strain V10



(b) Effect of yeast extract, tetrathionate and inoculum size on the growth of strain V11

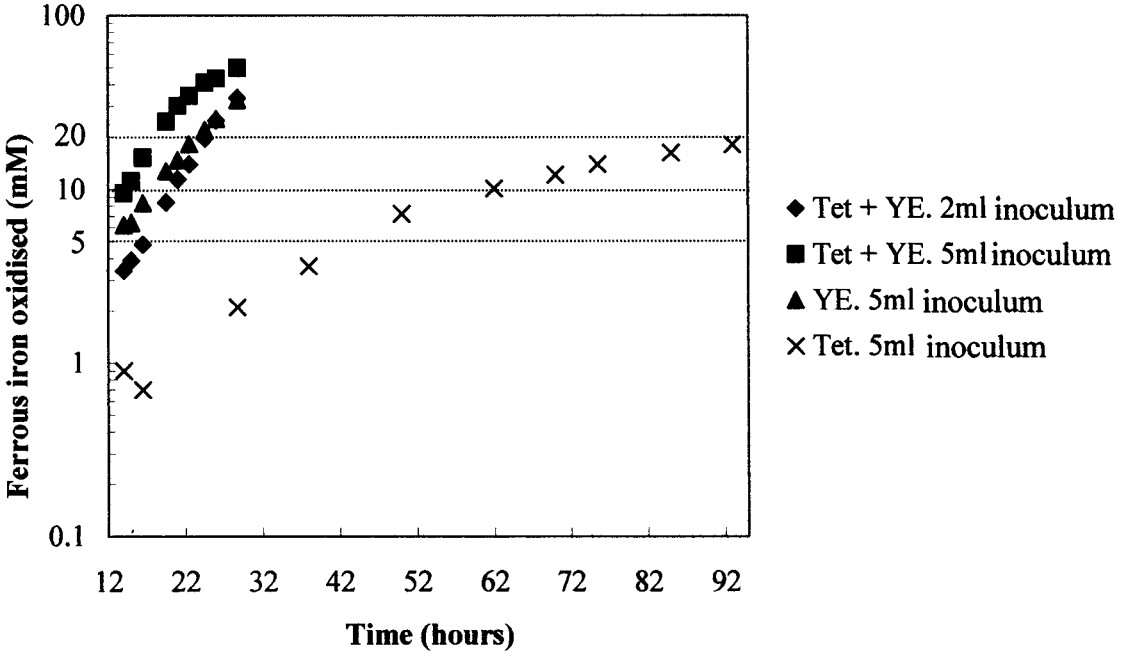


Table 5.3 Effect of growth supplements on the doubling times for ferrous iron oxidation

	V10 td (hours)	V11 td (hours)
Ferrous iron (50mM)	ng	ng
Ferrous iron (50mM) and tetrathionate (1mM)	5.7	15-40
Ferrous iron (50mM)+ tet (1mM) + YE (0.01%)	5.7	4.0
Fe 50mM + YE (0.01%)	ng	5.3

ng: no growth due to the requirements of tetrathionate (strain V10) or yeast extract (strain V11)

5.3.3 Effect of Sodium Chloride on growth of strains V10 and V11

It was necessary to determine the sodium chloride concentrations needed for optimal growth of V10 and V11 and also the range that is tolerated by these organisms.

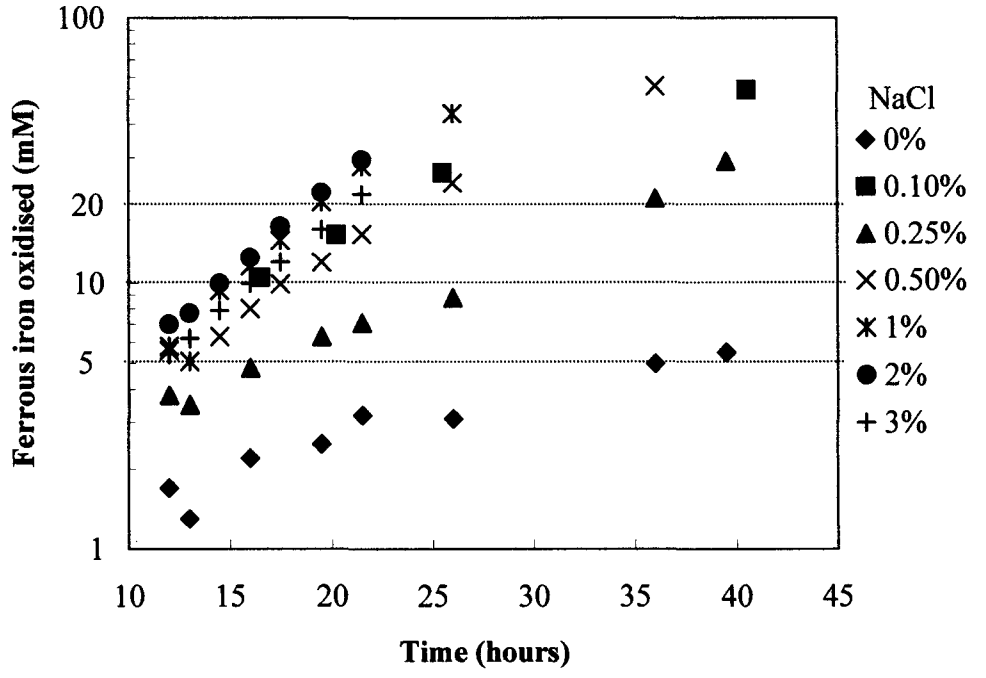
For this experiment a range of sodium chloride and sodium nitrate concentrations were used in the medium, these were for NaCl (w/v): 0%, 0.5%, 1%, 2%, 3%, 4% (688 mM), 6% (1032 mM) and 8% (1376 mM), and for $NaNO_3$ (w/v) : 0.5%, 1%, 1.5% (176 mM), 2% (234mM), 3% (351mM). 1mM tetrathionate and 50mM iron sulphate were added as substrates with 5ml inoculum added to each 100ml of medium. The growth results are shown in Figure 5.10(a).

However, the range of sodium chloride and sodium nitrate concentrations were not entirely suitable for V10 so a new experiment with a slightly different salt range was set up (Fig 5.10(b-e)). The new NaCl range for V10 was: 0% (with inoculum from a 0% NaCl stock), 0% (with NaCl carry-over from inoculum, 0.1%), 0.25%, 0.5%, 1%, 2%, 3%, and for $NaNO_3$ range: 0.1%, 0.25%, 0.5%. An additional flask containing 1% sodium sulphate was set up to assess any sodium requirement for V10. Iron, tetrathionate and temperature were as before. A comparison of the graphs in Figure 5.10 shows that V10 grew well with NaCl concentrations between 0.5 and 3% with the fastest growth occurring with 1-2% NaCl. There appeared to be better growth with 3% than 0.5%, but growth with 4% NaCl was quite slow. Growth with 0% NaCl was very poor as was growth in 6-8% NaCl where it appeared that V10 was strongly inhibited. V11, (Fig 5.10 (d)) on the other hand grew well in salt concentrations of 0.5%-4% NaCl, with good growth in 6% and 0.25% NaCl. Growth in 8% NaCl was slower but V11 was still able to oxidise nearly the same amount of iron as with 6% and 0.25% NaCl. Growth at 0% NaCl was very poor, starting much later than at the other concentrations and less than half of the iron was oxidised. V10 was also more affected by nitrate than V11, Figure 5.10 (c) and (e). V10 growth only occurred at nitrate concentrations up to 0.25% (w/v) whereas V11 grew in nitrate concentrations up to 1.5% (w/v).

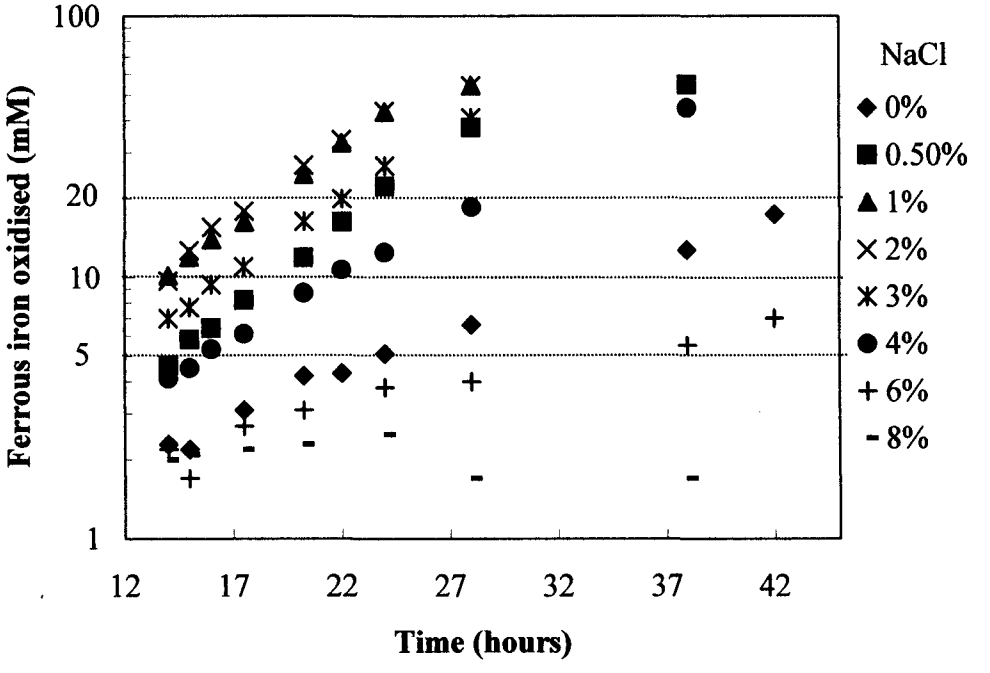
The doubling times at these conditions for each organism are shown in Figure 5.11 a-d. These results show that although V10 and V11 have a similar optimum NaCl concentration of around 1-2% V11 had a higher tolerance of NaCl than V10. Both organisms could not be maintained without NaCl. Table 5.4 summarises the doubling times.

Fig 5.10

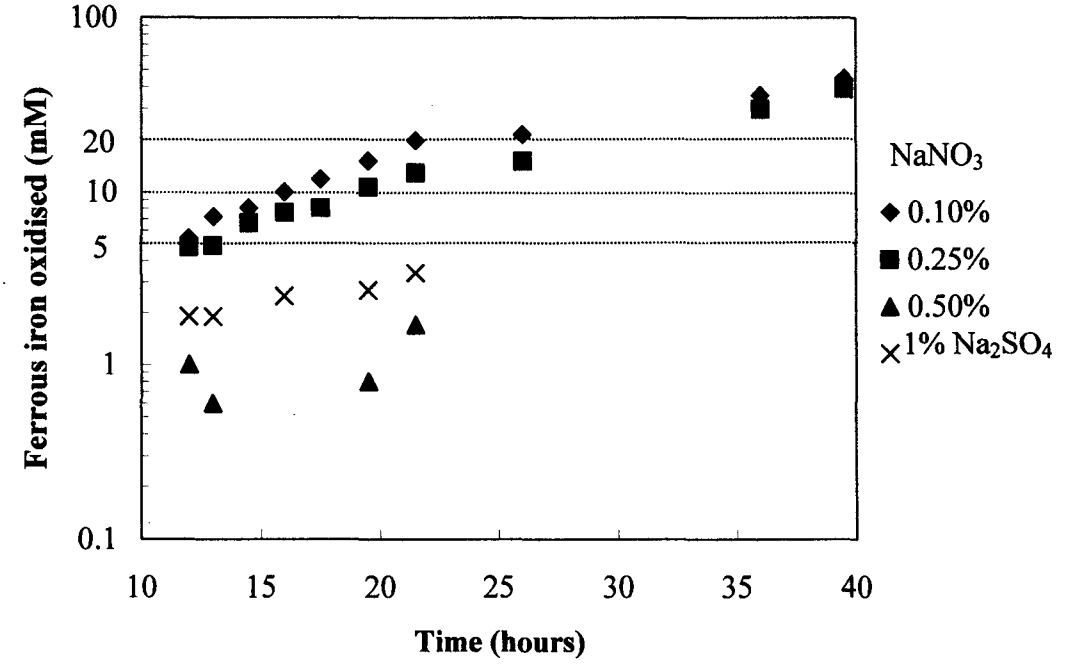
(a) Effect of NaCl on growth of strain V10 (1)



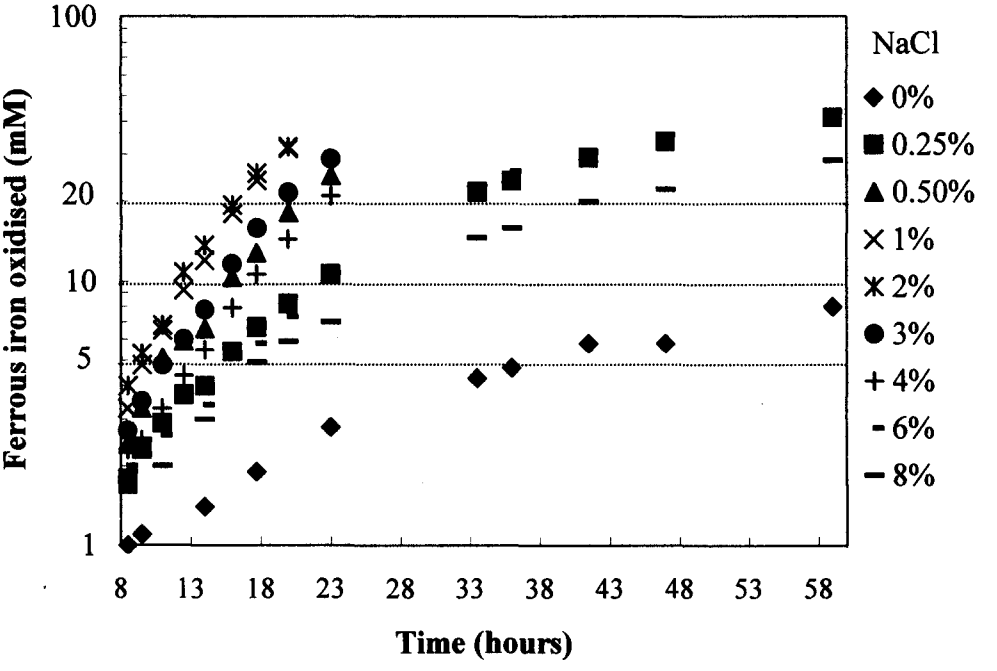
(b) Effect of NaCl on growth of strain V10 (2)



(c) Effect of $NaNO_3$ on growth of strain V10



(d) Effect of NaCl on growth of strain V11



(e) Effect of $NaNO_3$ on growth of strain V11

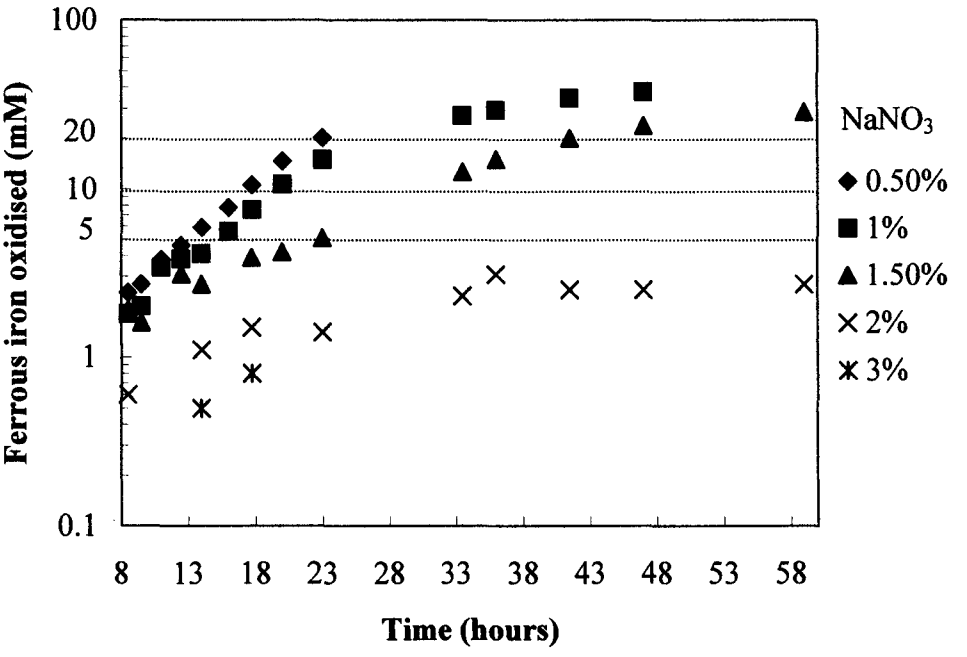
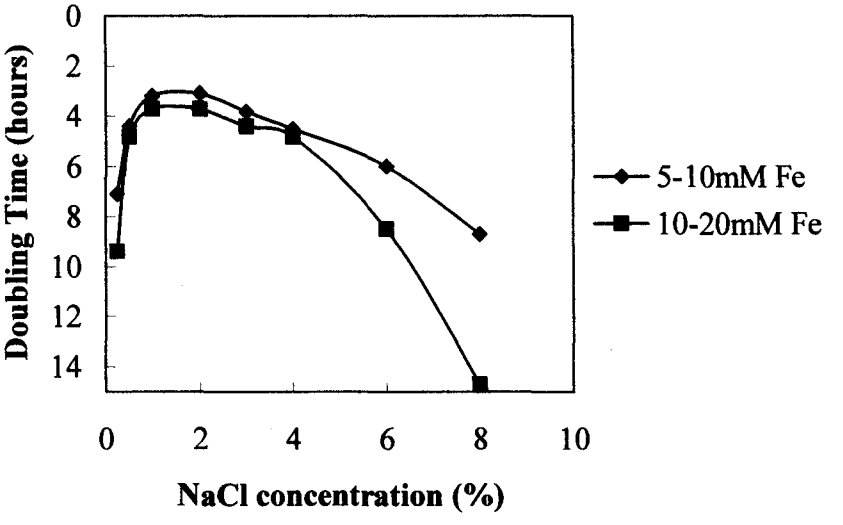
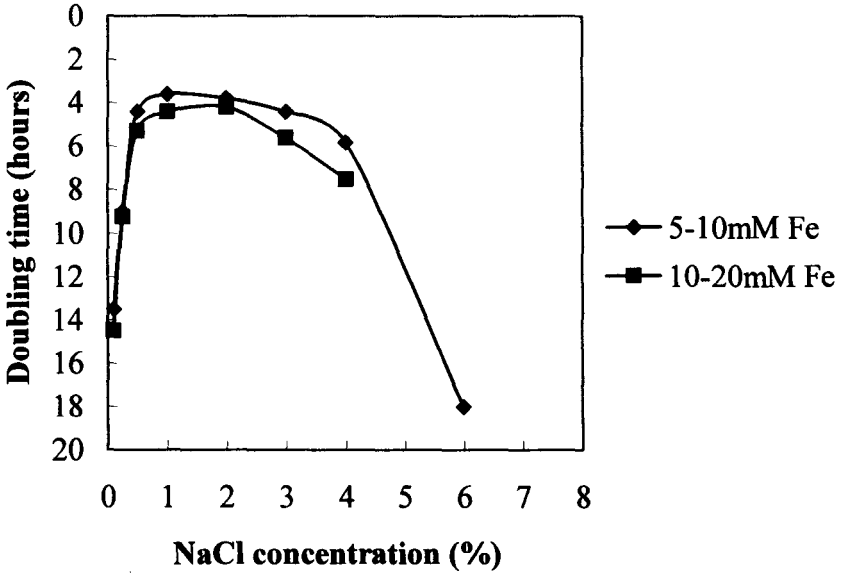


Fig 5.11

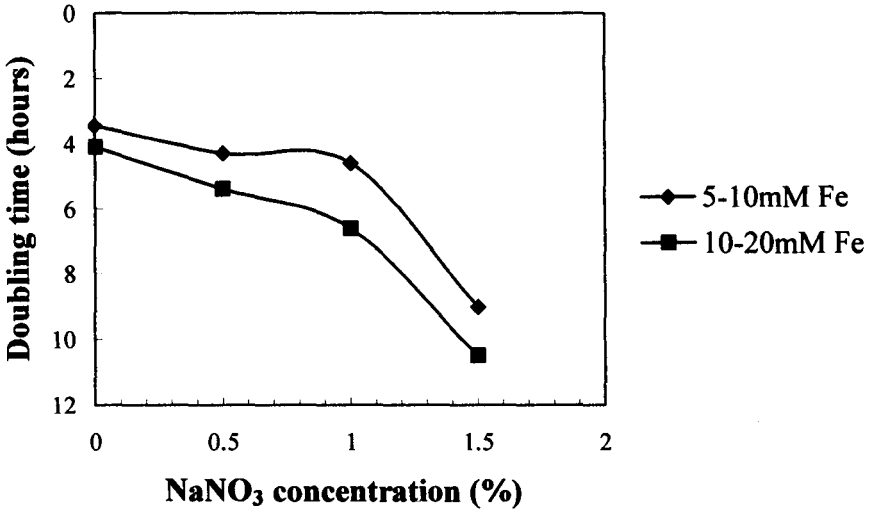
(a) Effect of NaCl on the growth of strain V11



(b) Effect of NaCl on the growth of strain V10



(c) Effect of $NaNO_3$ on the growth of strain V11



(d) Effect of $NaNO_3$ on the growth of strainV10

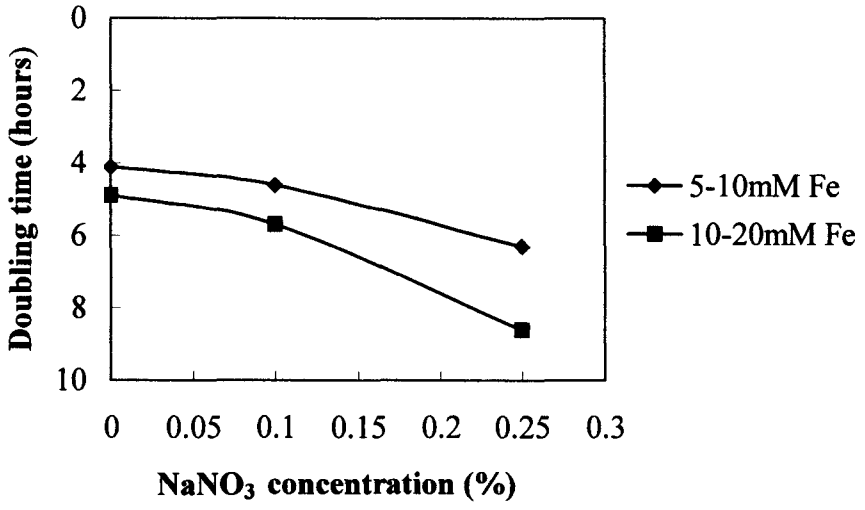


Table 5.4 Doubling times of V10 and V11 grown in different NaCl concentrations

(a) V11

NaCl (%)	5-10mM Fe td (hrs)	10-20mM Fe td (hrs)
0.1	-	-
0.25	7.1	9.4
0.5	4.4	4.8
1	3.2	3.7
2	3.1	3.7
3	3.8	4.4
4	4.5	4.8
6	6.0	8.5
8	8.7	14.7

(b) V10

5-10mM Fe td (hrs)	10-20mM Fe td (hrs)
13.5	14.5
9.0	9.25
4.4	5.3
3.6	4.4
3.8	4.2
4.4	5.6
5.8	7.5
18	-
-	-

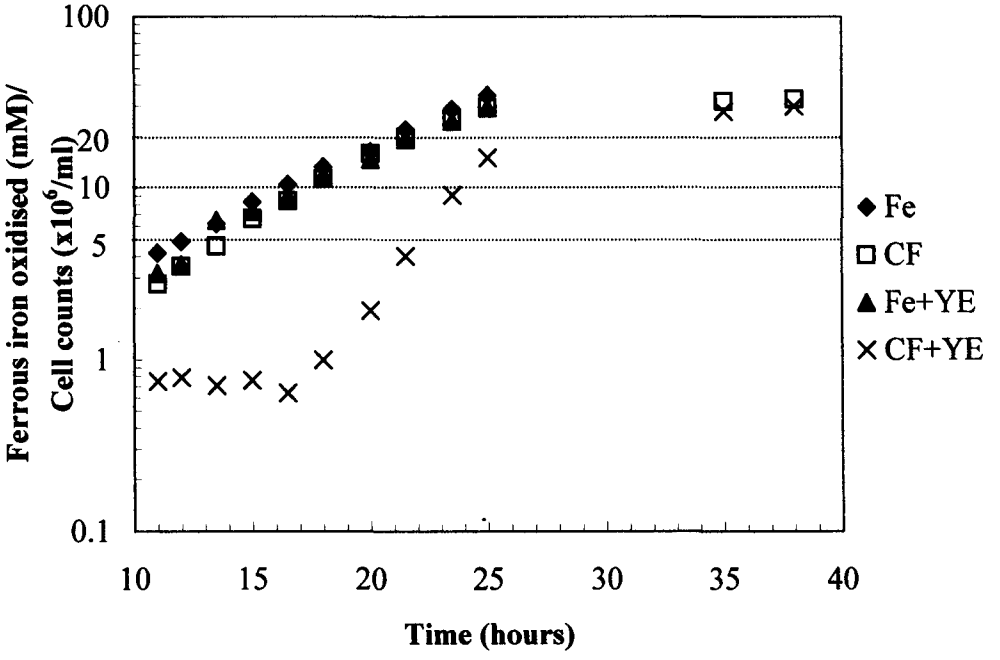
An experiment was then performed to see if yeast extract decreased the toxicity effect of nitrate on V10 to equal V11 grown with yeast extract. Here seven flasks were set up all containing single strength medium with 2.5% (w/v) (430 mM) NaCl. Two flasks had no sodium nitrate added; one of these had yeast extract added to a final concentration of 0.01%; two had 0.25% nitrate added; one had yeast extract added; two had 0.5% nitrate; one had yeast extract added and a final flask contained 1% nitrate plus yeast extract. As well as these small flasks, two large flasks (2L) containing 500ml of salts medium, plus 2.5% NaCl, 1mM tetrathionate, 50mM iron and 25ml of V10 from a grown culture, were set up to find out if large flasks would be more suitable for final growth rate measurements than small flasks. One of these large flasks contained 0.01% yeast extract. The growth in the small flasks was measured by substrate oxidation whereas growth in the large flasks was measured by substrate oxidation and cell counts. The only small flasks that showed growth of V10 were both of the ones with no nitrate added and the 0.25% nitrate flask with no yeast extract added. It was concluded that yeast extract could have increased the toxicity of nitrate to V10. The results from cultures in the large flasks were surprising. The substrate oxidation measurements were identical suggesting exactly the same growth rate for both cultures. The cell counts of the culture that did not contain yeast extract also indicated the same growth rate as the substrate oxidation measurements. However, the cell counts of the culture containing the yeast extract were very unusual. The readings showed few or no cells present in suspension for the first part of the growth curve followed by a dramatic increase in cells. The yeast extract appeared to cause V10 to form a biofilm on the inside of the flask so the cells were not in suspension and therefore not measured by Cellfacts. The iron was still oxidised by cells at the normal rate so the substrate oxidation measurements did not show this biofilm effect. The cells were suddenly released into the medium causing the dramatic rise in the cell counts. These results are shown in Figure 5.12.

5.3.4 Final growth rate measurements

A final growth experiment was set up to provide a full description of V10 and V11 growth with measurements taken for substrate oxidation measurements and cell counts. Two large flasks contained 500 ml of salts medium plus 2.5% NaCl, 1mM tetrathionate and 50mM ferrous sulphate. One had yeast extract added, both had 25ml

of inoculum. A third flask was set up with medium containing NaCl, ferrous sulphate and tetrathionate but no inoculum. The results are shown in Figure 5.13 (a) and (b).

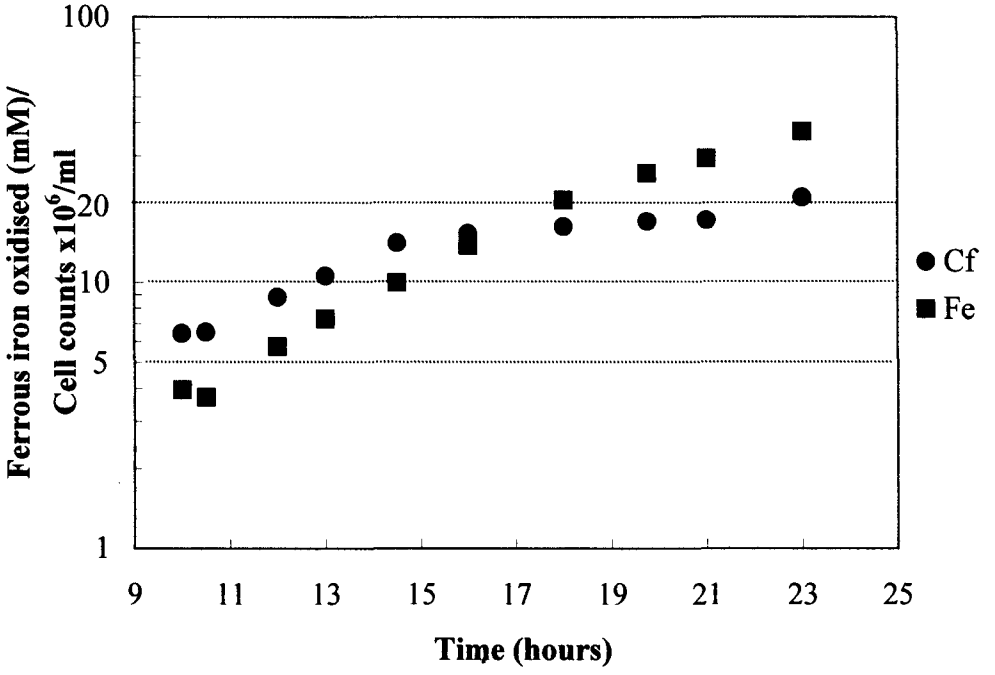
Fig 5.12
Effect of yeast extract on cell counts of strain V10



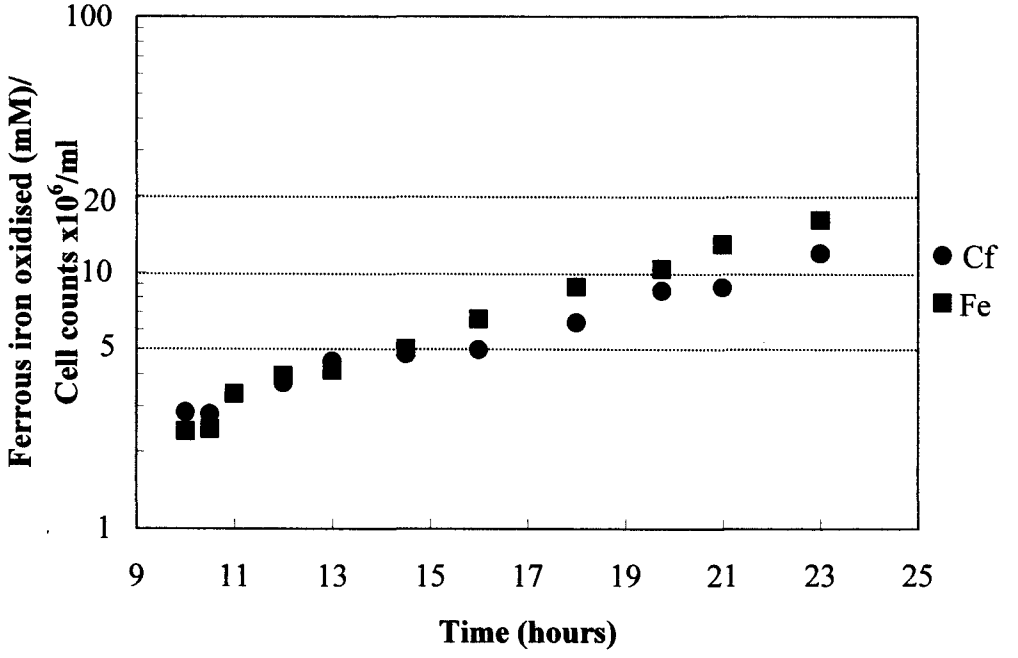
Fe: growth measured by ceric sulphate titrations, no YE added
CF: growth measured by cellfacts, no YE added
Fe+YE: as above but with YE added
CF+YE: as above but with YE added

Fig 5.13 Growth measurements of strains V10 and V11

(a) Growth curve for strain V11 plus yeast extract



(b) Growth curve for strain V10



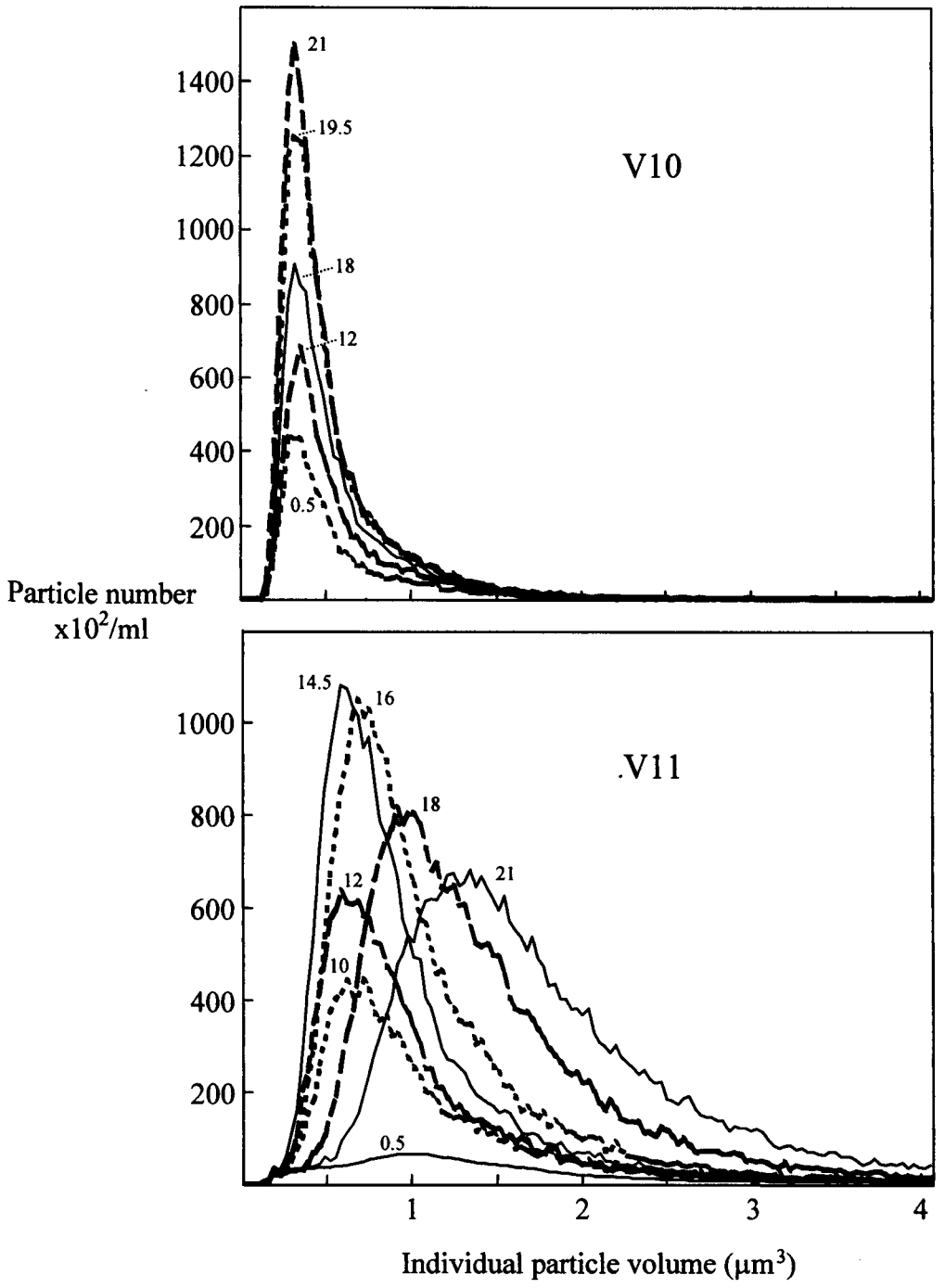


Figure 5.14 Comparison of V10 and V11 cell volumes over time by use of Cellfacts

Figure 5.14 shows charts generated by Cellfacts depicting the cell numbers and cell volume of V10 and V11 over the time course of the experiment. Cellfacts measures electrical flow impedence to count cells in a suspension and calculate their size (Chapter two, section 2.15). Cellfacts enables the user to differentiate cells in a mixed culture due to differences in cell volume. In figure 5.14 it can be seen that V10 and V11 exhibit different cell volumes over the time of the growth experiment. When V11 was inoculated into fresh medium from a grown stock culture its cells have a

large volume (0.5hours on the chart). As it grew in the fresh medium the cell volume decreased until after 14.5 hours when the cell volume started to increase again. This increase was due to the cells not separating after cell division when the substrate levels were depleted. V10, on the other hand, separated properly after cell division.

Table 5.5 Summary of growth characteristics of V1, V10 and V11.

	V1	V10	V11
Temperature range	(28) 30-45°C	(26) 33-38°C (42)	(26) 31-39°C (43)
Temperature optimum	39°C	35-36°C	35-36°C
Salt range	0-3% (3.5)	(0.1) 0.5-3% (4)	(0.25) 0.5-6% (8)
Salt optimum	0-2%	1-2.5%	1-2.5%
Iron oxidation	No	Yes	Yes
Doubling time	3 hours	5-10mM-3.7 hours 10-20mM-5.4hours	5-10mM-3.0hours 10-20mM-3.5hours

Note:
Numbers in brackets are the limits of the conditions at which the organisms will grow ie they will grow but very slowly.

V10 and V11 were grown, harvested and whole cell extracts were run on a 10% SDS-PAGE gel to compare electrophoretic profiles (see Fig 5.15). They showed some similarities but many significant differences, consistent with a different species status. Figure 5.16 shows the positioning of V1, V10 and V11 on phylogenetic trees among their closest related species.

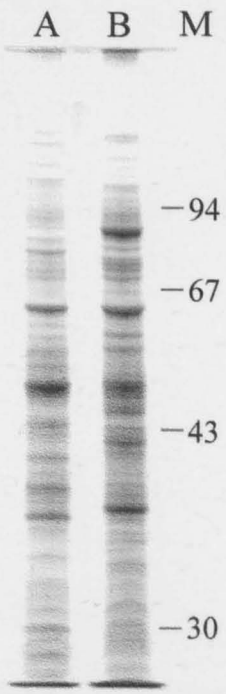


Fig 5.15 SDS-PAGE gel comparing the protein banding patterns of A, V10 and B, V11. The sizes and positions of molecular mass markers are shown (kDa).

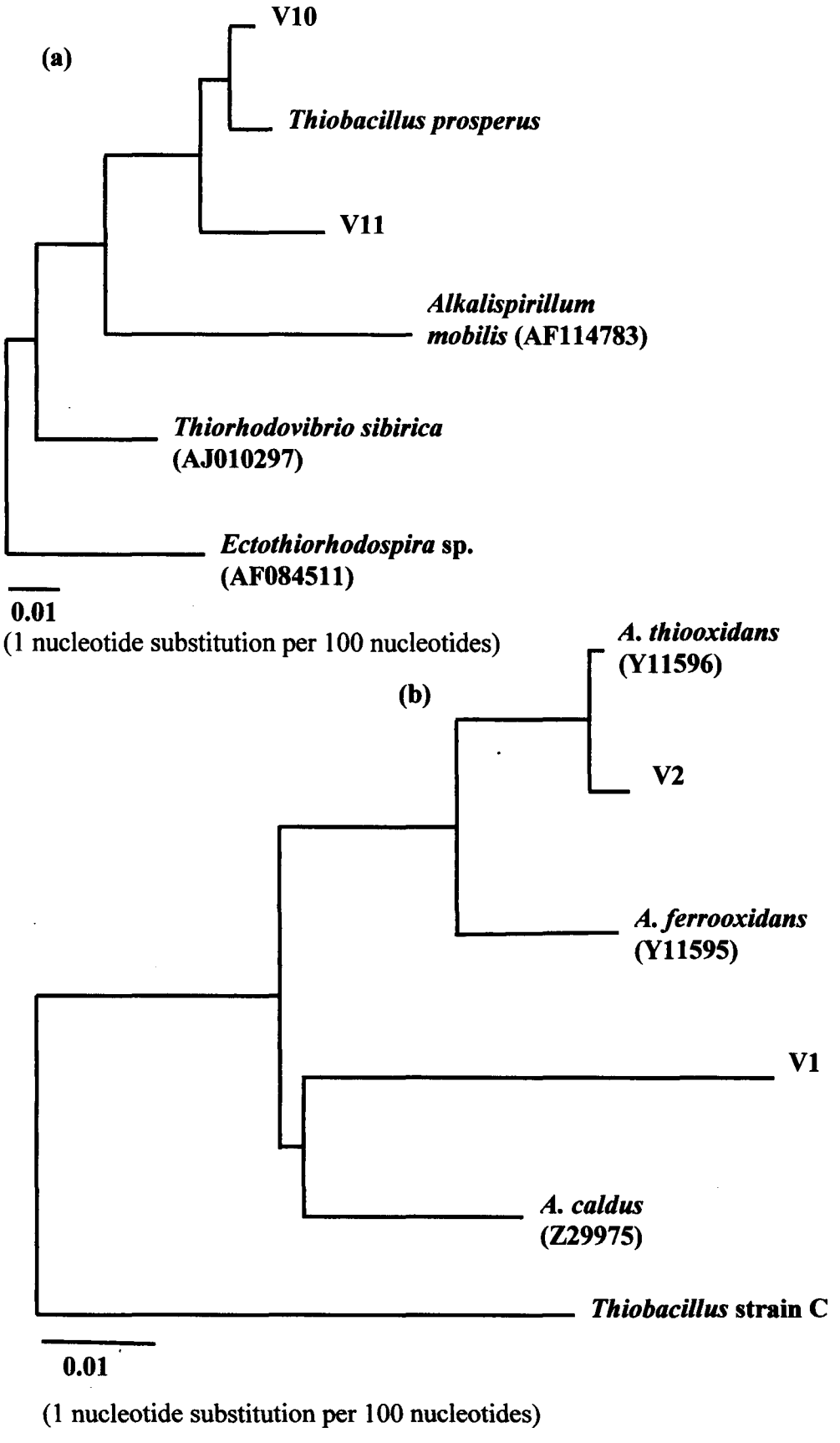


Figure 5.16 Simple unrooted phylogenetic distance trees of the positions of strains (a) V10 and V11 and (b) V1 and V2 among their nearest related sequences.

5.4 Discussion

Strain V1 exhibited some thermotolerance with growth at a wide range of temperatures (30 – 45°C) but with a lower maximum for growth than its nearest known relative, *Acidithiobacillus caldus* which can grow up to 55°C. It has a relatively high sodium chloride tolerance compared to other known acidophiles but it does not need chloride for growth as it grows well in media with no sodium chloride added. It may be considered that V1 is a terrestrial organism that has evolved to inhabit volcanic saline areas, it is not a true halophile due to its ability to grow at 0% sodium chloride, but it is deemed halotolerant as it can grow at sodium chloride concentrations of 3.5% (w/v).

Strains V10 and V11, now provisionally named *Acidihalobacter aeolicus,* and *Acidihalobacter ferrooxidans* respectively, appear to be marine organisms.

Strains V10 and V11 exhibit some similar characteristics, they have the same optimum temperature and both require tetrathionate and iron for growth (iron requirement results not shown). However, V11 has a wider range of salt tolerance than V10 both with chloride and nitrate salts and it also can grow over a greater range of temperature. Strains V10 and V11 also have different SDS-PAGE protein profiles and phylogenetic tree positions. Strain V1 has a greater temperature range than either V10 or V11, with a higher temperature optimum. Strain V1 also has a similar salt tolerance range to strain V10 as it can tolerate sodium chloride concentrations of 0-3.5% and strain V10 has the range 0.1%-4%, but strains V10 and V11 do not grow without sodium chloride demonstrating that they are obligate halophiles. Strain V1 was present in the clone libraries of two of the vents found at Vulcano, showing that it was abundant at the sample sites. Strains V10 and V11 were not found in any of the clone libraries, although clone types with similar sequence identities were, perhaps this indicates that they are present in low numbers relative to V1. Strains V10 and V11 are probably more likely to be found in the vents just off the shore rather than the vents on the beach as their growth conditions are more suited to the stable temperatures of the sea than the changeable conditions of the beach.

The tetrathionate supplementation experiments showed that yeast extract was more important to the growth of V11 than tetrathionate; it could still grow well with

the presence of yeast extract when tetrathionate was omitted from the medium as the reduced sulphur requirement for biosynthesis can be met by tetrathonate or yeast extract, but when tetrathionate but not yeast extract is added to the medium the growth of V11 is very slow. V10, on the other hand, has no such requirement as yeast extract does not affect its growth rate in any way apart from its role in biofilm formation. The formation of a biofilm in its natural environment could be advantageous as it could allow the organism to attach to the walls of the hydrothermal vent, gaining nutrients and energy from the mixing of hot reduced vent fluid with cold oxidised sea-water (Gugliandolo *et al.,* 1999).

Overall strain V1 demonstrated the fastest doubling time of these three organisms. This was reflected in the reactor flora demonstrated in chapter four. Strain V10 was not present in either the 3% NaCl reactor nor the 6% reactor, which could be due to its slower growth rate than V1 or V11, and therefore it being out-competed. An alternative explanation, is that V10 was not present in the reactor clone libraries as it was not in free suspension in the reactor, rather it had mostly formed a film on the solid surfaces.

Strains V1 and V10 were not present in the 6% NaCl reactor as the NaCl concentration was too high for these organisms. V11 was present in the 6% reactor and was the major component of the microbial flora. Also present was the unknown organism Vx, this organism was not obtained in pure culture so it is not known to what extent it is tolerant of NaCl. Vx was an unexpected find in the 6% reactor as it has a very high sequence identity to *Acidithiobacillus thiooxidans,* an organism not noted for its halotolerance.

It is not known whether the chloride tolerance of these organisms is due to the ability of the bacteria to exclude chloride or their possession of chloride efflux pumps. V11 does not grow without the presence of chloride ions thus this obligate halophile must need the chloride for some unknown function.

Experiments using fluorescently labelled chloride ions could be carried out to determine the intracellular concentrations, to investigate whether the chloride ions that enter the cell are pumped out or excluded from the cell entirely.

The permeability of nitrate ions into an acidophile is higher than that of chloride so much lower concentrations are needed to inhibit cell growth, as shown by Suzuki *et al.* (1999) with the effects of anions on *Acidithiobacillus thiooxidans* and Alexander *et al.* (1987) with *Acidithiobacillus ferrooxidans.* Alexander *et al.* (1987) also noted that if the cells were added to the medium containing the anion prior to the addition of the substrate needed for growth then the cell inhibition by the anion is greater than if the anion is added later. This result means that the chloride resistance in acidophiles is energy dependent. Suzuki *et al.* (1999) also showed that different cations also affected the amount of inhibition caused by anions, for example potassium chloride was less inhibitory than sodium chloride and lithium chloride is more inhibitory still. The theory behind this phenomenon is that potassium can enter as a counterion with the permeant anion (eg. chloride) therefore preventing the loss of the membrane potential but sodium is less effective as a counterion and lithium is ineffective. A small amount of certain anion such as sulphate added to the medium can actually promote cell growth, organisms without known salt tolerance actually grow a little faster with a small amount of anion added than with none (Suzuki *et al.*, 1999).

Very few halotolerant acidophiles have been isolated. *Thiobacillus prosperus* was isolated from Vulcano by Huber and Stetter (1989). It was the first organism of its kind to be isolated and is described in chapter four but there is little published about other halotolerant acidophiles. Much has been researched about marine microorganisms but these types were isolated from non-acidic environments and so do not have the energetic problems that acidophiles face. With regards to measurements taken for the growth rate, cell counts do not seem to give as reliable growth curves if there is cell attachment. Cell facts does, however, show when cells are stressed or lysing due to the changes in the cell size readings . Also, if there is a mixture of organisms eg V10 and V11, in the flasks, cell counts can show shifts in the microbial population between one organism and the other as they have different cell sizes and shapes that are clearly shown on the cell counts charts.

CHAPTER 6 GENERAL DISCUSSION AND CONCLUSIONS

This examination of acidic environments used a molecular ecological approach based on analysis of 16S rRNA genes amplified by PCR directly from environmental DNA. Although the PCR amplification would not be quantitative, it was expected to be a sensitive and specific route for detection of microorganisms in the environmental samples and thus to provide a good representation of the microbial flora (Kuske *et al.*, 1998).

This project has described the analysis of two chemically-distinct environments that are linked by their acidic nature. The environments studied in this project were chosen because of the increasing interest in iron- and sulphide-oxidising microorganisms with regard to their ecological/environmental roles and their biotechnological potential in the mining industry.

Via RNA gene sequence identification and phylogenetic analysis, characteristics of some of the principal organisms in the environments were inferred and suitable protocols for their isolation suggested or attempted. Thus, traditional methods of enrichment culture and strain characterisation were used in some cases to extend the molecular ecological work. Use of oligonucleotide probing of DNA extracted from a laboratory coal spoil microcosm and from saline, mineral bioleaching reactors combined the traditional and more recent molecular methodologies.

The combination of the two types of approach to microbiology is essential to further our understanding of the microbial diversity of habitats. 16S rDNA analysis provides valuable insights of the types of organisms that are likely to be found in the environment, but this type of analysis can only provide an overview of the organisms in the ecosystem. Traditional microbiology – culturing and characterisation provide information that 16S rDNA analysis cannot reveal, while the latter may reveal those organisms that cannot yet be cultured in the laboratory. Improving the understanding of the microbial population dynamics in extreme environments could lead to more efficient bioleaching processes and the study of extreme organisms may be useful in

the investigation of extraterrestrial life, or the origins of life on this planet (Bond and Banfield, 2001).

The oligonucleotide probes were not put to their full intended use. It was hoped that the probing experiments could be used as a quick and more reliable alternative to PCR and clone libraries for identifying the presence of certain organisms in samples. It has been shown that the direct extraction of chromosomal DNA from environmental samples, for example, soil/coal-spoil/sediments usually results in the coextraction of contaminants, such as humic substances. These humic substances can interfere with molecular biological analysis of the DNA, transformations, restriction digests and amplification with Taq polymerase can all be affected (Tebbe and Vahjen, 1993). Other DNA purification methods could have been used to produce DNA suitable for probing, such as hydroxyapatite and sepharose spin columns, but these can lead to a large loss of DNA as with other methods, which would preclude blotting experiments unless large samples were extracted. A number of factors can affect PCR of environmental DNA such as the purity and concentration of the DNA template, its quality and the amount of background DNA present in the sample (Kuske *et al.*, 1998). Reports that have discussed DNA extraction and purification methods (see Chapter one) dealt with soil and sediments that contained a lot of bacteria thus a large amount of DNA/RNA could be recovered. These papers were also focussing on purifying DNA for PCR and restriction digests, techniques, which do not need highly concentrated DNA for successful work. As little DNA was needed for PCR, purification of the chromosomal coal-spoil DNA was not a problem as PVPP treatment and dilutions of the DNA suspension were enough to allow successful PCR. Again, chromosomal DNA dot blots with DNA extracted from the Vulcano samples could not be carried out as very little DNA was extracted from the limited samples available.

There were a number of chimeras found in the Vulcano clone library, the reactor clone library and the coal spoil microcosm clone library. Chimeras form when fragments of two different sequences become fused during the amplification process. They were easily recognisable in the reactor and microcosm clone libraries as two different probes hybridised with the same spot of rDNA on blots. The V1/V2

chimaeras in the Vulcano clone libraries were also easily recognisable through comparing the sequences against both V1 and V2 sequences. Chimeras of the actinobacterial sequences in the Birch Coppice clone library would be much more difficult to identify as there are many different unknown actinobacterial clone types present in the database and it would be hard to recognise whether an actinobacterial clone type found in the Birch Coppice clone library was actually a chimera or a new clone type.

This project has indicated that the microbial ecology of acidic environments can differ greatly where chemical differences such as concentrations of salt or organic matter can probably select for different organisms. However, the levels or types of organic matter in the coal spoil were not investigated in this project. As well as clone types with sequence identities closest to the types of acidophiles generally found in mineral leaching heaps, such as *Leptospirillum ferrooxidans*, novel species were indicated and predicted to be adapted to the specific characteristics of the coal spoil and the geothermal vent sites.

At least superficial similarities can be envisaged between the Birch Coppice coal spoil and peat bogs where actinobacterial sequences have previously been found. The specific phylogenetic clusters of actinobacteria indicated in this work may represent even more acid-tolerant types than those previously indicated. These organisms could enhance the microbial oxidation and dissolution of pyrite in the spoil heap by reducing inhibition of iron-oxidising bacteria by their own organic by-products. Further work is necessary to isolate the actinobacterial types and the novel *Leptospirillum* type indicated in the clone libraries.

The Vulcano samples revealed organisms very similar to known bioleaching organisms and novel types. *Acidianus* species are well known acid tolerant extremophiles and are commonly found in natural high temperature acidic environments, their tolerance to salt has not been fully investigated but they must be able to withstand a certain level of sodium chloride to be found at this site. Clone types V1 and V2 had sequence identities closest to the acidithiobacilli species *Acidithiobacillus caldus* and *A. thiooxidans*. The potential of these novel types for a

useful roles in industrial bioleaching heaps is clear, particularly where only saline water is available. Strains V10 and V11, potentially the most useful strains in this context, had sequence identities closest to *Thiobacillus prosperus*, an organism originally found at Vulcano.

The project has provided sufficient characterisation of strains V10 and V11 for classification of the organisms (in conjunction with their 16S rRNA gene sequencing that was carried out separately by P.R. Norris and N. Foulis). The strains superficially resembled *Thiobacillus prosperus*, their closest relative, but this work has indicated significant differences from *T. prosperus* (which, unlike them, does not have an obligate salt requirement) that allow their separate species designation from *T. prosperus* and from each other. These included:

- different growth responses to reduced sulphur requirement factors (tetrathionate or yeast extract)
- differences in electrophoretic protein profiles
- different cell volume profiles during growth on ferrous iron
- slightly different tolerances of chloride and nitrate
- differences in 16S rRNA gene sequences (work of others, as above).

Further differences may be expected with examination of the sulphur metabolism of these strains. Strains V10 and V11 were deposited with the German Microorganism and Cell Culture Collection under the names:

Acidihalobacter aeolicus (V10) DSMZ 14174
Acidhalobacter ferrooxidans (V11) DSMZ 14175.

It is recommended that *Thiobacillus prosperus* should be reassigned to this proposed novel genus as *Acidihalobacter prosperus*. Technically, *T. prosperus* cannot remain in the redefined *Thiobacillus* genus (Kelly and Wood, 2000).

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APPENDIX

CLONE SEQUENCES

A. Birch Coppice Clone sequences (all approximately 1460 bases)

Clone 1 (Actinobacterium)

GGAGGATTAACGCTGGCGGCGTGCCTGACACATGCAAGTCGAACGAGGTCGCAGACC
TAGTGGCGGACGGGTGAGTAACACGTGACCAACCAACCTCGAAGTTGGGAATAGCTC
TGCGAAAGCAGGGGTAATACCGAATGTGGCCCGGCGCGGACATCCGCACCGGTCTAA
AGATTTATCGCTTCGAGACGGGGTCGCGGCCTATTAGCTTGTTGGTGGGGTAACGGC
CTACCAAGGCGATGATGGGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGGACTG
AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGAGGAATCTTCCGCAATGGGCGA
AAGCCTGACGGAGCAACGCCGCGTGTGGGATGAAGACTCTAGGGTTGTAAACCACTG
TCGGAGGGGACGATCGTGACGGTACCCTCCAAGAAAGCCCCGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGGGGCGAGCGTTATCCGGATTTACTGGGCGTAAAGCG
CGTTAAGGCGGATGGCCAGTTGGCGGTGAAATTTCGGGGCTCAACCCCGAAACTGCG
CCAAACTGGTCGTCTAGAGTATGGGAAGGAGAAGGTGGAATTCCGGGTGTAGTAGTG
AAATGCGTAGATATCCGGAGGAACACCAGCGGCGAAGGCGGCCTTCTGGCCCATAAC
TGACGCTGTACGCGAAAGCGTGGGGAGCGAACGGGATTAGATACCCCGGTAGTCCAC
GCCCTAAACTTTGTGCACTAGGTGTTGGGTGTATTGACGCGCTCAGCGCCGTAGCTA
ACGCATTAAGTGCACCGCCTGGGGACTACGGCCGCAAGGTTAAAACTCAAAGGAATT
GACGGGGGCCCGCACAAGCAGCGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAA
CCTTACCTGGGCTTGACATGCCGGTGAAACTCCTGGAGACAGGAGCCCCCGCAAGGA
CGCCGGCACAGGTGGTGCATGGCTGTCGTCAGCTCGTGCCGTGAGGTGTCGGGTTAA
GTCCTGCAACGAGCGCAACCCCTATGGTTAGTTGAATTTCTCTAGCCAGACTGCTGG
GAGAAACCCAGAGGAAGGTGGGGATGAGGTCAAGTCAGCATGCCCTTTATGTCCAGG
GCTACACACATGCTACAATGGGCGGTATCAACGGGCTGCGACCTCGCGAGAGGAAGC
GAATCCCTTAAAGCCGTCCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAG
TCGGAGTTGCTAGTAACCGCAGGTCAGCATACTGCGGTGAATATGTTCTCGGGCCTT
GTACGCACCGCCCGTCAAACCACCCGAGTCTGGTGCACCCGAAGTCGCTTTGCTCAA
ACCGCAAGGACAGCGGTGCCGAAGGTGTGCCTGGCGAGGGGGGT

Clone 2 (Actinobacterium)

GGATTAACGCTGGCGGCGTGCCTGACACATGCAAGTCGAACGAGGTCGCAAGACCTA
GTGGCGGACGGGTGAGTAACACGTGACCAACCAACCTCGAAGTTGGGAATAGCTCTG
CGAAAGCAGGGGTAATACCGAATGTGGCCCGGCGCGGACATCCGCACCGGTCTAAAG
ATTTATCGCTTCGAGACGGGGTCGCGGCCTATTAGCTTGTTGGTGGGGTAACGGCCT
ACCAAGGCGATGATGGGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTGAGGAATCTTCCGCAATGGGCGAAA
GCCTGACGGAGCAACGCCGCGTGGGGATGAAGACTCTAGGGTTGTAAACCACTGTCG
GAGGGGACGATCGTGACGGTACCCTCCAAGAAAGCCCCGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGGGCGAGCGTTATCCGGATTTACTGGGCGTAAAGCGCGTT
AAGGCGGATGGCCAAGTTGGCGGTGAAATTTCGGGGCTCAACCCCGAAACTGCCGCC
AAAACTGGTCGTCTAGAGTATGGGAGAGGAAGGTGGAATTCCGGGTGTAGTAGTGAA
ATGCGTAGATATCCGGAGGAACACCAGCGGCGAAGGCGGCCTTCTGGCCCATAACTG
ACGCTGTAGCGCGAAAGCGTGGGGAGCGAACGGGATTAGATACCCCGGTAGTCCACG
CCCTAAACTTTGTGCACTAGGTGTTGGGTGTATTGACGCGCTCAGCGCCGTAGCTAA
CGCATTAAATGCACCGCCTGGGGACTACGGCCGCAAGGTTAAAACTCAAAGGAATTG
ACGGGGGCCCGCACAAGCAGCGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAAC
CTTACCTGGGCTTGACATGCCGGTGAAACTCCTGGAGACAGGAGCCCCCGCAAGGGC
GCCGGCACAGGTGGTGCATGGCTGTCGTCAGCTCGTGCCGTGAGGTGTTGGGTTAAG
TCCCGCAACGAGCGCAACCCCTATGGTTAGTTGAATTTCTCTAGCCAGACTGCTGGG
AGAAACCCAAAGGAAGGTGGGGATGAGGTCAAGTCAGCATGCCCTTTATGTCCAGGG
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ATCCCTTTAAGCCGTCCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGTC
GGAGTTGCTAGTAACCGCAGGTCAGCATACTGCGGTGAATATGTTCTCGGGCCTTGT
ACACACCGCCCGTCAAACCACCCGAGTCTGGTGCACCCGAAGTCGCTTTGCTGAACC
GCCAGGGCTGCGGTGCCGAAGGTGTGCCTGGCGAGGGGGGT

Clone 3 (Actinobacterium)

GGACGAACGCTGGCGGCGTGCTTAATACATGCAAGTCNAACGGAATCTAGGCGGTGG
TAACACTGGCGAAATTTAGTGGCGAACGGTTGCGTAAGCACGTGAGCAACCTGCCCC
GAAGTCTGGGATAACAGTGGGAAACTGCTGCTAATACCGGATATTCGCGCTATACTT
ACATGAGGTGACGAGGAAAGCGCTTCGCTTCGGGAGGGGCTCGCGGCCTATCAGCTT
GTTGGTGAGGTAACGGCTCACCAAGGCAACGACGGGCAGCTGGTCTGAGAGGACGAT
CAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAA
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TAGGGTTGTAAACTCCTTTCAGTGGGAACGAAATTGACGGTACCCACAAAAGAAGCT
CCGGCCAACTACGTGCCAGCAGCCGCGGTGATACGTAGGGAGCGAGCGTTGTCCGGA
TTCATTGGGCGTAAAGAGCTCGTAGGCGGTTTAGTAAGTCGGGTGTGAAACCTCCAG
GCTCAACCTGGAGACGCCACCTGATACTGCTATGACTTGAGTCTGGTAGGGGACCAT
GGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCAGTAGCGAA
GGCGGTGGTCTGGGCCAGTACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGG
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CGACGGCTTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACCGGCC
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TTCGAAGCAACGCGAAGAACCTTACCTGGGCTTGACATGTAGGTTAAGGCGTGGAGA
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GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTATGTTGCCAGCGGG
TAATGCCGGGGACTCGTAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGA
GGTCAAGTCATCATGCCCCTTACGTCCAGGGCTGCACACATGCTACAATGGCCGGTA
CAAAGGGTTGCTATCCCGCGAGGGTGAGCCAATCCCAAAAAGCCGGTCTCAGTTCGG
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ATTGCCGGGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAACCACGAAA
GTCGGCAACACCCGAAGCCGGTGACCTAACCATTTGGAAGGAGCCGTCGAAGGTGGG
GTCGGTGATTGGGGTTAGTCGTAAC

Clone 4 (*Leptospirillum*)

AACGAACGCTGGCGGCGTGCCTAACACATGCAAGTCCAACGTGAAAGGGGAGCAATC
CCCCGGTAGGGTGGCAAACGGGTGAGTAATACATGGGTGATCTACCCTGGAGATGGG
GATATCCCTCCGAAAGGGGGGGCAATACCGAATAGAATCCGGTCCTGTGAAGGGGAC
CGGGGAAACGGAGGCCTCTGGAACAAGCTTCCGCTCCTGGATGAGCCCATGGCCCAT
CAGCTAGTTGGTAGGGTAAAGGCCTACCAAGGCGACGACGGGTAGCTGGTCTGAGAG
GACAACCAGCCACACTGGCACTGAGACACGGGCCAGACTCCTACGGGAGGCAGCAGT
GAGGAATATTGCGCAATGGGGGAAACCCTGACGCAGCAACGCCGCGTGTGGGAAGAA
GGCTTTCGGGTTGTAAACCACTTTTGCCCGGGACGAAAAGGGGCCCAATAATACGGG
GTCCCGATGACGGTACCGGGAGAATAAGCCACGGCTAACTCTGTGCCAGCAGCCGCG
GTAAGACAGAGGTGGCAAGCGTTGTTCGGAATTACTGGGCGTAAAGAGTCTGTAGGT
GGTTTGTCAAGTCTTTGGTGAAAGGCCGTGGCTTAACCATGGGAATGCCAAAGAGAC
TGGCAGACTGGAGGATGGGAGAGGGAAGCGGAATTTCTGGTGTAGCGGTGAAATGCG
TAGATATCAGAAGGAAGGCCGGTGGCGAAGGCGGCTTCCTGGAACATTCCTGACACT
GAGAGACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTA
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AAGTACCCCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGG
GCCCGCACAAGCGGTGGTGCATGTGGTTTAATTCGACGCAACGCGAAGAACCTTACC
TAGGTTTGACATGCCGCGAGTAGGGAACCGAAAGGGGACCGACCGGTTCAGTCCGGA
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TCCCGCAACGAGCGCAACCCTCGCCCTTTGTTGCCATCGGGTAAAGCCGGGCACTCT
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CCCTTATGTCTAGGGCAACACACGTGCAACAATGGCCGGTACAGACGGAGGCAAAGC
CGAGAGGTGGAGCAAACCCGAGAAAGCCGGTCTCAGTTCGGATTGAGGTCTGCAACT
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GTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAAAGTCTGTTGTACCTGAAG
TCGGTGCCCCACCCCGGAAACGGAGGGAGCCGCCCAGGTATGGCCGGTAATTGGGCC

Clone 5 (Actinobacterium)

ACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGAGGTTCAACCAGTAGCA
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AGACTGGGATAACAGCGGGAAACCGCTGCTAATACCGGATGCCCCCACCAGGTCGCA
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TGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCTGGTCTGAGAGGACGATCA
GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
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GGTTGTAAACCTCTTTCGAGCAGGGACGATAATGACGGTACCTGCAGAAGAAGCTCC
GGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGAGCGAGCGTTGTCCGGATT
CATTGGGCGTAAAGAGCTCGTAGGCGGCTTGGTAAGTCGGATGTGAAACCTCCAGGC
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AACTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAAGAACACCGGTGGCGAAGG
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GGGTTCCGTGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAG
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TACGGTGTCCGAAAGGGTCCGCGATAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCG
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CARCGCCGKGGTGGNTACGTTGCCGGGCCTTGTTCACACCGCCCGTCGCCCCACCAA
GGTCGGTGGCCCCCCCCGCCGCCGGTGGCCCAGCCGTTTATTTGGAGGGAACCGTCG
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CTGCAGATATC

Clone 6 (*Sulfobacillus*)

AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATGCATGCAAGTCG
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AACCTGCCCCGGGGCGGGCACTAGCGCTTCGAAAGGAGCGGTAATGGCCCATACGGC
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CGGTAAGACGTAGGGGGCGAGCGTTGTCCGGAATGACTGGGCGTAAAGGGCGTGTAG
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CCGAAGCGAATCCCCAAACGGCGTCGTAGTTCGGATCGCAGGCTGCAACTCGCCTGC
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GGCCTTGTACACACCGCCCGTCACACCNCGAAAGCCGCTCACACCCGAAGCCGGCGG
GGACGCCGTCGACGGTGGGGGGGGTAATTGNGGTCNAGTNGTAACAAGGTAAAAGTA
AAANGCGAATTCCTGCAGATATCC

Clone 7 (Actinobacterium)

ATCTGAGAATTCGNCCTAAGAGTTTGATCCTGGCTCAGGATTAACGCTGGCGGCGTG
CCTGACACATGCAAGTCGAACGAGGTCGCAAGGACCTAGCGGCGGACGGGTGAGTAA
CACGTGACCAACCAACCTCGAAGTTGGGAATAGCTCTGCGAAAGCAGGGGTAATACC
GAATGTGGCCCGGCGCGGACATCCGCACCGGTCTAAAGATTTATCGCTTCGAGACGG
GGTCGCGGCCTATTAGCTTGTTGGTGGGGTAACGGCCTACCAAGGCGATGATGGGTA
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CGCAGGAAAAGCCGCAGAGATGCGGTGTCCGCAAGGGCCTGCGATAGGTGGTGCATG
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GAGGGAGCTGTCGAAGGTGGGATCGGTGATTGGGGTGAAGTCGTAACAAGGTAGCCG
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Clone 8 (Actinobacterium)

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GAGGGAGCCGTCGAAAGGTGGGGTTGGTGATTGGGGTG

Clone 9 (*Rhodopila*)

AGAGTTTGATCCTGGCTCAGAGCGAACGCTGGCGGCACGCTCTGAACACATGCAAGT
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GTCTCCGGGTGGGGGATAACCGCGGGAAACTACGGCTAATACCGCATGACACCTGAG
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TGCAACTCGGGTGCATGAAGGTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGG
TGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTTCGA
CCTTAAGCCGGTGCGCCAACCTGGCAACAGGAGGCAGCCGACCACGGTCGGGTTANC
GACTGNGGCAAGTCGTAACAAGG

B. Vulcano Clone Sequences

V1 (*Acidithiobacillus* 1462 bases) Accession number (AF339743)

ATTGAACGCTGGCGGCATGCCTAACACATGCAAGTCGAACGGCAGCACGGGTGCTTG
CACCTGGTGGCGAGTGGCGGACGGGTGAGTAATGCGTAGGAATCTGTCCAATAGTTT
GGGACAACCCAGGGAAACTTGGGCTAATACCGGATACGTCCTGAGGGAGAAAGCGGG
GGATCTTTGGACCTCGTGCTATTGGAGGGGCCTACGTTCGATTAGCTAGTTGGCAGG
GTAAGGGCCTACCAAGGCGACGATCGATAGCTGGTCTGAGAGGACGATCAGCCACAC
TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTTCGCA
ATGGGGGCAACCCTGACGAAGCAATGCCGCGTGAATGAAGAAGGCCTTCGGGTTGTA
AAGTTCTTTCGTGGGAGACGAAAAGGTGATCGCTAATATCGGTTACTGTTGACGTGA
ACCCAAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTG
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TGGGAGAGGGTGATGGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGA
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GCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
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CCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCTTTATGTCCAGGGCTACAC
ACGTGCTACAATGGCGCGTACAGAGGGAAGCGAGACCGCGAGGTGGAGCAGACCCCA
GAAAGCGCGCCGTAGTTCGGATTGCAGTCTGCAACTCGACTGCATGAAGTCGGAATC
GCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACAC
CGCCCGTCACACCATGGGAGTGGATTGTACCAGAAGCAGCTAGCCTAACCTTCGGGG
GGGCGGTTACCACGGTATGGTTCATGACTGGGGTG

V2 (*Acidithiobacillus* 1454 bases)

ATTGAACGCTGGCGGCATGCCTAACACATGCAAGTCGAACGGTAACAGGTCTTCGGA
TGCTGACGAGTGGCGGACGGGTGAGTAAAGCGTAGGAATCTGTCTTTGAGTGGGGGA
CAACCCAGGGAAACTTGGGCTAATACCGCATAAGCCCTGAGGGGGAAAGCGGGGGAT
CTTCGGACCTCGCGCTGGAAGAGGAGCCTACGTCTGATTAGCTAGTTGGTAGGGTAA
AGGCCTACCAAGGCGACGATCGGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGG
ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTTCGCAATGG
GGGCAACCCTGACGAGGCAATGCCGCGTGAATGAAGAAGGCCTTCGGGTTGTAAAGT
TCTTTCGTGGAGGACGAAAAGGTGAGTGCTAATATCACTTGCTGTTGACGTGAATCC
AAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGGGGGTGCAAG
CGTTAATCGGAATCACTGGGCGTAAAGGGTGCGTAGGCGGTGCATTAGGTCTGTCGT
GAAATCCCCGGGCTCAACCTGGGAATGGCGGTGGAAACCGGTGTACTAGAGTATGGG
AGAGGGTGGTGGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAACAT
CAGTGGCGAAGGCGGCCACCTGGCCCAATACTGACGCTGAGGCACGAAAGCGTGGGG
AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGAATACTAGATGT
TTGGTGCCACGCGTACTGAGTGTCGTAGCTAACGCGATAAGTATTCCGCCTGGGAAG
TACGGCCGCAAGGTTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAG
CATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGGCTTGACATGTCTGGA
ATCCTGCAGAGATGCGGGAGTGCCCTTCGGGGAATCAGAACACAGGTGCTGCATGGC
TGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT
GTCCTTAGTTGCCAGCGGTTCGGCCGGGCACTCTAGGGAGACTGCCGGTGACAAACC
GGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCTTTATGTCCAGGGCTACACAC
GTGCTACAATGGCGCGTACAGAGGGAAGCCAAGCCGCGAGGTGGAGCAGACCCCAGA
AAGCGCGTCGTAGTTCGGATTGCAGTCTGCAACTCGACTGCATGAAGTCGGAATCGC
TAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCG
CCCGTCACACCATGGGAGTGGATTGTACCAGAAGCCGTTAGCCTAACCTTCGGGAGG
GCGATGACCACGGTATGGTTCATGACTGGGGTG·

V3 (*Thiobacillus prosperus* 1458 bases) Accession Number AF339744

ACGCTGGCGGCATGCCTAACACATGCAAGTCGAACGGTAACAGGAGCAGCTTGCTGC
TTGCTGACGAGTGGCGGACGGGTGAGTAACGCGTGGGAATCTGCCCAGTAGTGGGGG
ATAGCCCGGAGAAATCCGGATTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGA
TCTTCGGACCTTGCGCTATTGGATGAGCCCGCGTCTGATTAGCTAGTTGGTGGGGTA
ATGGCCTACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGG
AACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATG
GGGGAAACCCTGATCCAGCAATGCCGCGTGCGTGAAGAAGGCCTGCGGGTTGTAAAG
CACTTTCAGTAGCGAAGAAAAGCTCATGGCTAATACCCATGGGTCTTGACGTTAGCT
ACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCCCGGTAATACGGAGGGTGCGA
GCGTTAATCGGAATTACTGGGCGTAAAGCGTGTGTAGGCGGTTTGGTAAGTCAGATG
TGAAGGCCCCGGGCTTAACCTGGGAATTGTATCTGATACTGCTAGACTAGAGTTTGG
TAGAGGGCAGTGGAATTCCCGGTGTAGCGGTGAAATGCATAGATATCGGGAGGAACA
CCAGTGGCGAAGGCGACTGCCTGGCCCAAAACTGACGCTGAGACACGAAAGCGTGGG
GAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGAACTAGCCG
TTGGGGGTATTTACACCTTTAGTGGCGAAGCTAACGCGTTAAGTTCTCCGCCTGGGG
AGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGG
AGCATGTGGTTTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATCCACG
GAAGGCGCCAGAGATGGTGCTGTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGC
TGTCGTCAGCTCGCGTCGAGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT
GTCCCTAGTTGCCAGCGAATCAGTCGGGAACTCTAGGGAGACTGCCGGTGACAAACC
GGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTATGGGTAGGGCTACACAC
GTGCTACAATGGTCGGTACAGAGGGTTGCCAACCCGCGAGGGGGAGCTAATCCCACA
AAGCCGATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGATTCGC
TAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACC
GCCCGTCACACCATGGGAGTTGGCTGCACCAGAAGCCGGTAGTCTGACNTTCGGNAG
NNCGCCGTCCACGGTGTGGTCGATGACTGTTTTG

V4 (Unidentified Gamma proteobacterium, 793 bases)

ATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAGAGAGGAGCTTGC
TCCTCTTGAGAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGG
GATAACGTTCGGAAACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGG
ACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGT
AATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTG
GAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAAGTGGGGAATATTTGACAA
TGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAA
AGCACTTTAAGTTGGGAGGAAGGGCGGTAAATTAATACTTTGCTGTTTTGACGTTAC
CGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGC
AAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGA
TGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACTGACTAGAGTAT
GGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAA
CACCAGTGGCGAAGGCGACCACCTGGACTAATACTGACACTGAGGTGCGAAAGCGTG
GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCGTAAACGATGTCA

V5 (uncultured proteobacterium 1353 bases)

GACAAACGCTGGCGGCGTGCCTAACACATGCAAGTCGAACGACAGGCGAGGAATCCG
CAAGGAGGAATCGTGCTGAGTGGCGAACGGGTGAGTAATGCATGGGGACATACCCTC
GAGAGTGGGATAGCGTTCCGAAAGGGACGGTAATACCACGTACGCTCGGGAGAGGAA
AGGTGCAACAGCACCGCTCGAGGAGTGACTCATGGCCCATCAGCTAGTTGGTGAGGT
AAAGGCCCACCAAGGCAATGACGGGTAGCTGGTCTGAGAGGATGGTCAGCCACACTG
GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTTCCCAAT
GGGCGAAAGCCTGAGGGAGCGACGCCGCGTGGAGGAAGGAGTCTTTCGAGATGTAAA
CTCCTGTTGTAAGGGAGCAAGGGTACATGGAGTGGAAAGTCATGTACTCGGATAGTA
CCTTACGAGGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGG
CGAGCGTTGTCCGGAGTTACTGGGCGTAAAGGGTGCGTAGGTGGCCGATAAAGTCAA
GTGTGAAATTCCACGGCTCAACTGTGGGAGTGCGTTTGAAACTAATCGGCTTGAGGT
CAGGTCAGGAAGATGGAATTGTCAGTGTAGGGGTGAAATCCGTAGATATTGGCAAGA
ACGCCAGTGGTGAAGACGGTCTTCTGGGCTGATACTGACACTGAGGCACGAAAGCTA
GGGGAGCGAACGGGATTAGATACCCCGGTAGTCCTAGCCGTAAACGATGTTCACTGA
GTATGGGAGGCATTAGCCATCCGTGCTGAAGCAAACGCGTTAAGTGAACCGCCTGGG
GAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTG
GAGCGTTTGGTTTAATTCGACGCAACACGAAGAACCTTACCAAGGCTTGACATACAG
GTGGTAGTGAGACGAAAGTGGAACGACCCCCGCAAGGGGGAGCCTGTACAGGTGGTG
CATGGCTGTCGTCAGCTCGTGCCGTGAGGTGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCCTGTGGTTAGTTGCCAGCAGTTCGGCTGGGCACTCTAATCAGACTGCCGGAGA
AGATCCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCTTTATGCCTTGGGCG
ACACACGCGCTACAATGGCATGCACAGACAGGAGCGAAGCCGTGAGGCGGAGCGAAT
CTGAGAAAACATGCCTCAGTACAGATTGCAGGCTGAAACCCGCCTGCATGAAGTCGG
AATCGCTAGTAATCGCGGATCAGCCATGCCGCGGTGAATACGTTCCCGGGCCTTGTA
CACACCGCCCGTCAAACCACCCGAGTTGAGGATACCAGAAGTCATTGGTCCAACCGC
AAGGAGGAAGATGCCGAAGGTAGACTCAGTGAGGGGGGTT

V6 (*Buttiauxella* 785 bases)

ATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAGCACAGGAGAGCT
TGCTCTCCGGGTGACGAGCGGCAGACGGGTGAGTAATGTCTGGGAAACTGCCTGATG
GAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCTTCGGACCAAAG
AGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGG
TGAGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCC
ACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG
CACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGT
TGTAAAGTACTTTCAGCGAGGAGGAAGGCATTGTGGTTAATAACCGCAGTGATTGAC
GTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG
GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAG
TCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAG
AGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAAAGATCTGG
AGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAA
GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG

V7 (*Thermoplasma* 1401 bases)

TAAGCCATGCAAGTCACGGGGCCGTAAGGCACCGGCGAACAGCTCAGTAACACGTGG
ATAATTTACCCTCAGGCGGGGTATAACCTCGGGAAACTGAGGCTAATCCCCCATAGT
CATTACAAACTGGAACGTTGTAATGATGAAAGCTCCGGCGCCTGAGGATAAGTCTGC
GGCCTATCAGGTAGTAGGTGGTGTAAAGGACCACCTAGCCTAAGACGGGTACGGGCC
CTGAAAGGGGGAGCCCGGAGATGGACTCTGAGACAACAGTCCAGGCCCTACGGGGCG
CAGCAGGCGCGAAAACTGTGCAATGCGCGAAAGCGCGACACGGGGAACCTGAGTGCC
TTGACTTTTCGTCAAGGCTTTTCTGATGCCTAAAAAGCATCAGGAATAAGGGCTGGG
CAAGACGGGTGCCAGCCGCCGCGGTAACACCCGCAGCTCGAGTGGTGATCACTTTTA
TTGAGTCTAAAGCGTCCGTAACCGGTCTTATAAATCTTCAGATAAATTCTCCCGCTT
AACGGAAGAACTTCTGAAGAGACTGTAAGACTTGGGACCGGGTGAGGTTGAATGTAC
TTTCAGGGTAGGGGTAAAATCCTGTAATCCTGAAAGGACGACCGGTGGCGAAAGCGT
TCAACTAGAACGGATCCGACGGTGAGGGACGAAGGCTAGGGGAGCAAACCGGATTAG
ATACCCGGGTAGTCCTAGCTGTAAACGCTGCCCACTTGGTGTTGCTTCTCCGTTGAG
GGGGAGCAGTGCCGTAGCGAAGGTGTTAAGTGGGTCACTTGGGGAGTACGGCCGCAA
GGCTGAAACTTAAAGGAATTGGCGGGGGAGCACCGCAACGGGAGGAGCGTGCGGTTT
AATTGGATTCAACGCCGGAAAACTCACCGGGAGCGACCTTCGGATGAGGGTCAACCT
GACGAATTTACCCGATAGAAGGAGAGGTGGTGCATGGCCGTCGTCAGCTCGTACCGT
AGGGCGTTCACTTAAGTGTGATAACGAGCAAGACCCCCATCTCTAATTGCTAAGCTT
TCTTAGCGGGAAGCTGCACTTTAGAGGGACCGCCAGCGCTAAGCTGGAGGAAGGAGG
GGTCGACGGCAGGTCAGTACGCCCCGAATCTCCCGGGCTACACGCGCGCTACAAAGG
ACGGGACAATGAGTTGCAACCTCGAAAGGGGAAGCTAACCTCGAAACCCGTTCGTAG
TCAGGACTGAGGGCTGTAACTCGCCCTCACGAATGTGGATTCCGTAGTAATCGTAGG
TCAACAGCCTACGGTGAATATGCCCCTGCTCCTTGCACACACCGCCCGTCAAACCAT
CCGAGCTGGTGTTGGATGAGGGTCTGTCCATTGGATGGATTCGAATCTGATGTCAGT
GAGGAGGGTT

V8 (*Acidianus Brierleyi* 1428 bases)

ACCAGATCGCTATGGGGATAGGGCTAAGCCATGGGAGTCGTACGCTCTCGGTAAGAG
GGCGTGGCGGACGGCTGAGTAACACGTGGTCAACCTAACCTCGGGACTTGGATACCT
CCGGGAAACTGGAGCTAATCCAAGATAGGCAAAGGAATCTGGAACGATCCTTTGCCC
AAAAGCCTCTAGGCTAATACTGTCTAGGGGTGCCCGAGGATGGGACTGCGGCCCATC
AGGCTGTTGGTGGGGTAATGGCCCACCAAACCGATAACGGGTAGGGGCCGTGGGAGC
GGGAGCCCCCAGTTGGGCACTGAGACAATGGCCCAGGCCCTACGGGGCGCACCAGGC
GCGAAACGTCCCCAATGCGGGAAACCGTGAGGGCGCTATCCCCAGTGCCTCCGATAG
GAGGCTTTTCCCCACTTTAGAACGGTGGGGGAATAAGCGGGGGGCAAGGCTGGTGTC
AGCCGCCGCGGTAATACCAGCCCCGCGAGTGATCTGGACGTTTATTGGGCTTGAAGC
GCCCGTAGCCGGCCCATAAAGTCACTGTTTAAAGACCCGGGCTCAACCCGGGAAAGG
GCAGTGATACTTATGGGCTAGGGGGCGGGAAAGGTCGGAGGTACTCCCGGAGTAGGG
GCGAAATCCGTAGATCCCGGGAGGACCACCAGTGGCGAAAGCGTCCGGCTAGAACGC
GCCCGACGGTGAGGGGCGAAAGCCGGGGCAGCAAAAGGGATTAGATACCCCTGTAGT
CCCGGCTGTAAACGATGCAGGCTAGGTGTCGCGTGGGTCTAGAGCCCGCGCGGTGCC
GCAGGGAAACTGGTAAGCCCGCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTAA
AGGAATTGGCGGGGGAGCACCACAAGGGGTGGAACCTGCGGCTCAATTGGAGTCAAC
GCCTGGAATCTTACCGGAGGAGACCGCAGTGTGACGGTCAGGCTAATGACCTTACCT
GACTCGCGGAGAGGAGGTGCATGGCCGTCGCCAGCTCGTGTTGTGAAATGTCCGGTT
AAGTCCGGCAACGAGCGAGACCCCCACCTCTAATTGGCATTCTCTCCCCCGGGAGGG
ACCCACATTAGAGGGACTGCCGTCGTTAAGACGGAGGAAGGAGGGGGCCACGGCAGG
TCAGCATGCCCCGAAACCCCCGGGCCGCACGCGGGTTACAATGGCAGGGACAGCGGG
ATTCCGACCCCGAAAGGGGGAGGTAATCCCTCAAACCCTGCCTCAGTTGGGATCGAG
GGCTGAAACTCGCCCTCGTGAACGAGGAATCCCTA

V9 (*Acidianus infernus* 621 bases)

ACCCGACCGCTATGGGGGTAGGGCTAAGCCATGGGAGTCGTACGCCCTCGGGTAAGA
GGGCGTGGCGGACGGCTGAGTAACACGTGGCTAACCTACCCTCGGGACCCGGATAAC
TCCGGGAAACTGGAGCTAATCCGGGACAGGCGAAGGGTACTGGAACGTCCCTTCGCC
TAAAGGGGCATGGGCTATTTCCCGCTCATGCCCGCCCGAGGATGGGGCTGCGGCCCA
TCAGGCTGTTGGCGGGGTAACGGCCCGCCAAACCGATAACGGGTAGGGGCCGTGAGA
GCGGGAGCCCCCAGTTGGGCACTGAGACAAGGGCCCAGGCCCTACGGGGCGCACCAG
GCGCGAAACGTCCCCAATGCGGGAAACCGTGAGGGCGCTACCCCCAGTGCTCCCGAA
AGGGAGCTTTTCCCCGCTTTAAAACGGCGGGGGAATAAGCGGGGGGCAAGACTGGTG
TCAGCCGCCGCGGTAATACCAGCCCCGCGAGTGGTCGGGACGCTTACTGGGCTTAAA
GCGCCCGTAGCCGGCCCTGCAAGTCACTGCTTAAAGACCCGGGCTCAACCCGGGGAA
GGGCAGTGATACTGCAGGGCTAGGGGGCGGGAGAGGTCGGAGGTACTCCCG

VX (reactor clone 1440 bases)

ATTGAACGCTGGCGGCATGCCTAACACATGCAAGTCGAACGGTAACAGGTCTTCGGA
TGCTGACGAGTGGCGGACGGGTGAGTAAAGCGTAGGAATCTGTCTTTGAGTGGGGGA
CAACCCAGGGAAACTTGGGCTAATACCGCATAAGCCCTGAGGGGGAAAGCGGGGGAT
CTTCGGACCTCGCGCTGGAAGAGGAGCCTACGTCTGATTAGCTAGTTGGTAGGGTAA
AGGCCTACCAAGGCGACGATCGGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGG
ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTTCGCAATGG
GGGCAACCCTGACGAAGCAATGCCGCGTGAATGAAGGAAGCCTTCGGGTTGTAAAGT
TCTTTCGTGGAGGACGAAAAGGTGGGTGCTAATATCGCCTGCTGTTGACGTGAATCC
AAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGGGGGTGCAAG
CGTTAATCGGAATCACTGGGCGTAAAGGGTGCGTAGGCGGTGCATTAGGTCTGTCGT
GAAATCCCCGGGCTCAACCTGGGAATGGCGGTGGAAACCGGTGTACTAGAGTATGGG
AGAGGGTGGTGGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAACAT
CAGTGGCGAAGGCGGCCACCTGGCCCAATACTGACGCTGAGGCACGAAAGCGTGGGG
AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGAATACTAGATGT
TTGGTGCCACGCGTACTGAGTGTCGTAGCTAACGCGATAAGTATTCCGCCTGGGAAG
TACGGCCGCAAGGTTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAG
CATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGGCTTGACATGTCTGGA
ATCCTGCAGAGATGCGGGAGTGCCCTTCGGGGAATCAGAACACAGGTGCTGCATGGC
TGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCGCAACGAGCGCAACCCTTG
TCCTTAGTTGCCAGCGGTTCGGCCGGGCACTCTAGGGAGACTGCCGGTGACAAACCG
GAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCTTTATGTCCAGGGCTACACACG
TGCTACAATGGCGCGTACAGAGGGAAGCCAAGCCGCGAGGTGGAGCAGACCCCAGAA
AGCGCGTCGTAGTTCGGATTGCAGTCTGCAACTCGACTGCATGAAGTCGGAATCGCT
AGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGC
CCGTCACACCATGGGAGTGGATTGTACCAGAAGCCGTTAGCCTAACCTTCGGGAGGG
CGATGACCACGGTATGGTTCATGACTGGGGTG