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A study of microbial communities and interactions in extremely acidic environments.

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A study of microbial communities and interactions in extremely acidic environments

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Abstract

Microbial communities and interactions in extremely acidic environments were studied in this project. Microbial biomass growing as biofilms ("acid streamers") in acidic metal-rich water was collected from a chalybeate spa and an abandoned pyrite mine in North Wales, U.K.. The microbial communities found in these materials and those in extremely acidic, metal-rich waters were investigated using a combination of cultivation-dependent and cultivation–independent techniques. The majority (>90%) of microbes in the samples were unculturable using the methods used. Culture-independent approaches revealed that the acid-streamer microbial community was predominantly bacterial and that the dominant members were previously uncharacterized β-proteobacteria. Significant variations in microbial communities were found between streamers from different locations; microbes detected included members of the classes *α-* and *γ-Proteobacteria, Acidobacteria Actinobacteria, Bacilli* and *Nitrospira*.

Sulfidogenesis at low pH (3.8-4.2) was demonstrated by acidophilic/acid-tolerant sulfate reducing bacteria (aSRB), *Desulfosporosinus*-isolate M1, and by a defined mixed-culture of M1 grown in mixed culture with an apparently obligately aerobic heterotrophic acidophile (*Acidocella*-isolate PFBC). The aSRB-isolate was found to be an incomplete oxidizer of glycerol (producing acetic acid) and was also able to use molecular hydrogen as electron donor. A sulfidogenic bioreactor, containing the defined mixed culture, was studied in detail. No acetic acid accumulation was recorded when glycerol was supplied as electron donor and the amount of sulfide produced indicated that glycerol was being completely oxidized to CO_2. A hypothesis was developed to explain the observed phenomena, involving interspecies transfer of hydrogen. Subsequent experimental data supported this hypothesis, and suggested a mechanism whereby sulfidogenesis may occur in extremely acidic environments.

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Abbreviation

%	percent
$\Delta G^{0'}$	change in free energy during a reaction
ul	microliter
ug	microgram
umoles	micromoles
A	adenine
AAS	atomic absorbance spectroscopy
acetyl-CoA	acetyl-coenzyme A
ALD	anoxic lime stone drain
ADP	adenosine diphosphate
AMD	acid mine drainage
AMP	adenosine monophosphate
APS	adenosine-5'-phosphosulfate
ARD	acid rock drainage
aSRB	acidophilic/acid-tolerant sulfate reducing bacteria
ATP	adenosine triphosphate
BChl	bacteriochlorophyll
BLAST	basic local alignment search tool
bp	base pair
C	cytosine
ca.	*circa*
°C	degrees Celsius
CFU	Colony forming unit
DABCO	1,4-diazabicyclo[2.2.2]octane
DAPI	4',6-diamidino-2-phenylindole
DGGE	denaturing gradient gel electrophoresis
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanine triphosphate
dTTP	deoxythymine triphosphate
dNTP	deoxynucleotide triphosphate
DO	dissolved oxygen
DOC	dissolved organic carbon
EDAX	energy dispersive analysis of X-ray
EDTA	Na_2ethylenediamnetetra-acetic acid
Eh	redox potential
Feo	iron overlay medium
FeSo	iron tetrathionate medium
FISH	fluorescent *in situ* hybridization
g	gram
g	gravitational acceleration (in ms^{-1})
G	guanine
HBS	"heterotrophic" basal salts
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
IPTG	isopropyl-beta-d-thiogalactopyranoside
KeV	kilo electron volt
KHP	anhydrous potassium hydrogen phthalate
L	liter
LB	Luria-Bertani
M	molar
mA	milliamps

mg	milligram
ml	milliliter
mM	millimolar
mmols	millimoles
MPN	most probable number
MW	molecular weight
mS/cm	Millisiemens per centimeter
MS	metal sulfide
mV	millivolt
µM	micromolar
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	national center for biotechnology information
ng	nanogram
nm	nanometer
nmols	nanomoles
no.	number
nt	nucleotides
ODP	oligonucleotide database nomenclature
OFN	oxygen free nitrogen
PAR	[4-(2-pyridylazo) resorcinol]
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PDCA	pyridine-2,6-dicarboxylic acid
*p*Ka	dissociation constant of an acid
*p*Ksp	solubility product of a sparingly soluble salt
PFA	paraformaldehyde
pm	picomoles
PP	pyrophosphate
psi	pounds per square inch
RFLP	restriction fragment length polymorphism
RISCs	reduced inorganic sulfur compounds
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate
SEM	scanning electron microscope
SRA	sulfate reducing archaea
SRB	sulfate reducing bacteria
SRP	sulfate reducing prokaryotes
T	thymine
TBE	tris-hydroxymethyl-methylamine
TE	trace elements
TFB	transformation buffer
TGGE	temperature gradient gel electrophoresis
Thioo	thiosulfate overlay medium
Tm	melting temperature
T-RF	terminal restriction fragment
T-RFLP	terminal restriction fragment length polymorphism
TSB	tryptone soya broth
U.K.	United Kingdom
U.S.A.	United States of America
UV	ultraviolet
V	volume
W	weight
YE	yeast extract
YEo	yeast extract overlay

Chapter 1 General Introduction

1.1 Origin and characteristics of acidic environments

Acidic environments are widely distributed on planet earth. The degree of acidity of these environments can vary from moderate (pH 6.0 to 3.0) to extreme (pH less than 3.0). Acidity may result from microbial activities, for example production of organic acids during fermentation and, in some cases, by aerobic microorganisms, and under aerobic oxidizing conditions due to nitrification and as a result of sulfuric acid production during the oxidation of elemental sulfur and sulfide minerals. Acid-impacted sites occur as a result of both natural (e.g. volcanic) and anthropogenic (e.g. mining) processes.

Some extremely acidic environments occur naturally at geothermal areas such as Yellowstone National Park (U.S.A.), Montserrat (West Indies), Krisuvik (Iceland) and volcanic lakes in New Zealand (Rawlings & Johnson 2003). Volcanic gas evolving from deep in the earth crust often contains large quantities of H_2S that reacts with SO_2 and produces elemental sulfur (Equation 1.1), which is further oxidized to sulfuric acid, as in Equation 1.2.

$$2H_2S + SO_2 \rightarrow 3S^0 + 2H_2O \quad (1.1)$$

$$S^0 + H_2O + 1.5O_2 \rightarrow SO_4^{2-} + 2H^+ \quad (1.2)$$

Mining activity is commonly associated with formation of acidic environments. There are various forms of sulfide minerals, e.g. pyrite (FeS_2), marcasite (FeS_2), pyrrhotite (Fe_7S_8-FeS), chalcopyrite ($CuFeS_2$), sphalerite (ZnS) and galena (PbS) (Southwood 1995). Commercially-important metals often occur as sulfide minerals. Sulfidic minerals of no commercial value, in particular pyrite and marcasite, are frequently found in ore and coal deposits, and waste materials may contain large amount of these minerals. Waste materials from froth flotation (tailings) are generally fine-grain deposits and are considered to be a greater danger to the environments because of their greater reactivity than waste rock (spoil) (Johnson 2003).

Once sulfide minerals are exposed to both air and water, the oxidation of these minerals occurs spontaneously. Sulfide minerals can be divided into acid-soluble and acid-insoluble varieties. Acid-soluble sulfides are oxidized

by ferric iron or by protons (Equation 1.3) producing H_2S^+ radicals that eventually form polythionates (Schippers & Sand 1999).

$$MS + Fe^{3+} + 2H^+ \rightarrow M^{2+} + Fe^{2+} + H_2S^{\cdot +} \quad (1.3)$$

In contrast, acid-insoluble sulfides, such as pyrite, are solubilized by the process illustrated in Equation 1.4, where the oxidizing agent is ferric iron (Schippers & Sand 1999).

$$FeS_2 + 6Fe(H_2O)_6^{3+} + 3H_2O \rightarrow Fe^{2+} + S_2O_3^{2-} + 6Fe(H_2O)_6^{2+} + 6H^+ \quad (1.4)$$

Thiosulfate, formed in Equation 1.4, is unstable at low pH and hydrolyzes to form various reduced inorganic sulfur compounds (RISCs) such as tetrathionate, and also elemental sulfur (Schippers & Sand 1999). RISCs and ferrous iron produced from iron sulfide oxidation can serve as energy sources for acidophilic iron- and sulfur-oxidizing prokaryotes. Oxidation of tetrathionate (as in Equation 1.5) generates further acidity:

$$S_4O_6^{2-} + 3.5O_2 + 3H_2O + 2H^+ \rightarrow 8H^+ + 4SO_4^{2-} \quad (1.5)$$

Oxidation of ferrous iron by iron-oxidizing microorganisms (Equation 1.6) produces ferric iron which leads to further oxidation of sulfides, and these (mostly) chemolithotrophic bacteria may therefore greatly accelerate the rates of metal sulfide oxidation.

$$2Fe^{2+} + 0.5O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O \quad (1.6)$$

The oxidation of sulfide minerals results in acidification of water draining from mines and mine wastes. The acidic waste wasters typically contain elevated concentrations of dissolved iron, sulfate, heavy metals and metalloids, and are generally referred to as acid mine drainage (AMD) or acid rock drainage (ARD). The acidity of AMD originates from both proton acidity (pH) and mineral acidity. Mine waters vary greatly in their chemistries, including pH. The lowest pH (-3.6) of any environmental water body was recorded in water droplets found deep within the Richmond Mine at Iron Mountain in the U.S.A. (Nordstrom et al. 2000). The most significant metals that give rise to mineral acidity, which is generated by metal hydrolysis, are aluminium, iron and manganese (Johnson 2000). As many cationic metals are considerably more soluble in acidic than in neutral waters, the acidity of AMD greatly influences the characteristics of chemical composition of the water. Examples of physico-chemical characteristics of AMD are shown in Table 1.1.

Table 1.1. Physico-cheminal characteristics of some AMD waters (adapted from Johnson & Hallberg 2003). Key (*) = Concentrations in mg/L; (-) = Data not available.

	Mynydd Parys, U.K.	King's mine Norway	Iron Mountain, U.S.A.	Rio Tinto, Spain
pH	2.5	2.8	0.5-1.0	2.2
Eh (mV)	+685	-	-	+450
Total dissolved Fe*	650	172	13000-19000	2300
Al*	70	23	1400-6700	-
Mn*	10	0.8	17-120	-
Cu*	40	15.8	120-650	109
Zn*	60	25	700-2600	225
Sulfate*	1550	668	20000-108000	10000

As the chemical composition of AMD depends on the nature of the source material, some AMD contains elements that are not listed in Table 1.1 including arsenic, barium, cadmium, molybdenum, nickel and lead. The characteristics of AMD greatly influence its surroundings, and allow development of unique ecosystems within and around AMD-impacted environments.

1.2 Physiology and ecology of acidophilic microorganisms

Acidophilic microorganisms grow optimally in acidic environments, and may be sub-divided into moderate (pH_{optima} 3.0 to 5.0) and extreme (pH_{optima} < 3.0) acidophiles. Most acidophilic life-forms are microorganisms and these are distributed within the *Bacteria*, *Archaea* and *Eukarya* domains (Hallberg & Johnson 2001).

1.2.1 Carbon metabolism of acidophilic prokaryotes

Acidophilic prokaryotes can be divided into three groups; autotrophs, mixotrophs and heterotrophs, according to their varying abilities to utilize organic and inorganic forms of carbon.

1.2.1.1 Autotrophic acidophiles

Extremely acidic environments are often oligotrophic and contain <20 mg dissolved organic carbon (DOC)/L (McGinness & Johnson 1993). Chemoautotrophic microorganisms that obtain energy from the oxidation of inorganic substances, including iron and sulfur, are the primary producers in these environments in the absence of sunlight, such as within deep mines (Johnson 1998). These prokaryotes synthesize ATP by oxidizing inorganic electron donors, usually coupled to the reduction of molecular oxygen, and fix CO_2 as carbon source. In order to fix carbon for cellular biosynthesis, chemoautotrophs use ATP and NADPH generated from the oxidation of their inorganic electron donors (though iron-oxidizers necessarily use ATP to synthesise NADPH, as the redox potential of the ferrous/ferric couple is too high to reduce $NADP^+$ directly); The pathway for CO_2 reduction is usually the Calvin cycle (the reductive pentose cycle; Madigan et al. 1997). The overall stoichiometry of this pathway is shown, in general terms, in Equation 1.7.

$$6CO_2 + 12NADPH + 18ATP \rightarrow C_6H_{12}O_6(PO_3H) + 12NADP^+ + 18ADP + 17P \quad (1.7)$$

In the Calvin cycle, 18 ATP and 12 NADPH molecules are used to synthesize 1 hexose molecule (fructose 6-phosphate) that can subsequently be used to synthesize storage polymers such as glycogen and starch (Madigan et al. 1997). To generate energy for biosynthesis, acidophilic chemolithotrophs

utilize various inorganic electron donors. Because of the nature of the environments in which acidophiles are found, ferrous iron, elemental sulfur and reduced sulfur compounds are generally abundant and important energy sources, whilst other potential inorganic electron donors (e.g. ammonium) are insignificant. Interestingly, some acidophiles (such as *Acidithiobacillus ferrooxidans*) have been shown to use hydrogen as an energy source (Drobner et al. 1990), though the importance of hydrogen in acidic environments is unknown. The oxidation of ferrous iron yields relatively little energy (significantly less than sulfur oxidation) and is also influenced by pH (Table 1.2).

Table 1.2. Free energy yields of ferrous iron (at varying pH values), elemental sulfur and thiosulfate oxidation (both at pH 7.0) (Kelly 1978).

Reaction	Energy yield
$S^0 + 1.5O_2 + H_2O \rightarrow 2H^+ + SO_4^{2-}$	-496 kJ reaction^{-1}
$S_2O_3^{2-} + H_2O + 2O_2 \rightarrow 2H^+ + 2SO_4^{2-}$	-936 kJ reaction^{-1}
$4FeSO_4 + O_2 + 2H_2SO_4 \rightarrow 2Fe_2(SO_4)_3 + 2H_2O$	pH 3.0 -25 kJ mol^{-1} $FeSO_4$
	pH 2.0 -30 kJ mol^{-1} $FeSO_4$
	pH 1.5 -33 kJ mol^{-1} $FeSO_4$

Due to the relatively high redox potential of the ferrous/ferric couple (+770 mV at pH 2.0, which is close to that of the O_2/H_2O couple, +820 mV), the only feasible electron acceptor for iron-oxidizing acidophiles is molecular oxygen. In contrast, some chemolithotrophs couple oxidation of RISCs to the reduction of ferric iron under anaerobic conditions, including *At. ferrooxidans* (Pronk et al. 1992; Equation 1.8).

$$S^0 + 6Fe^{3+} + 4H_2O \rightarrow SO_4^{2-} + 6Fe^{2+} + 8H^+ \quad (1.8)$$

This reaction provides free energy of –314 kJ·mol^{-1}. *At. ferrooxidans* is also capable of growth by coupling Fe^{3+} reduction to H_2 oxidation (Ohmura et al. 2002).

1.2.1.2 Mixotrophic acidophiles

There are several acidophiles that can use organic compounds as carbon sources as well as fix CO_2 when organic carbon source is not available.

These are referred as mixotrophs, and examples are *Sulfobacillus* spp. (Wood & Kelly 1984; Norris & Barr 1985) and *Acidiphilium acidophilum* (Harrison 1983; Mason & Kelly 1988; Pronk et al. 1990a; Pronk et al. 1990b; Pronk et al. 1990c). *Sb. acidophilus* (strain ALV) was shown to grow faster when growing mixotrophically than when growing as an autotroph, and assimilated 81.5% of its carbon from glucose and 18.5% from CO_2 when grown mixotrophically in the presence of glucose (Wood & Kelly 1984).

1.2.1.3 Heterotrophic acidophiles

Obligatory heterotrophic acidophiles require organic carbon and do not fix CO_2. Most eukaryotic acidophiles, as well as some bacteria and archaea, fall into this category. Heterotrophs are often scavengers that rely on leakage and lysis products from other organisms including photoautotrophs and chemoautotrophs (Norris & Johnson 1998).

1.2.2 Temperature profiles of acidophilic microorganisms

Acidophilic microorganisms may also be sub-divided on the basis of their temperature optima. There are three groups of acidophilic prokaryotic microorganisms that have often been recognized; mesophiles (optimum growth temperatures 20-40°C), moderate thermophiles (optimum growth temperatures 40-60°C) and extreme thermophiles (optimum growth temperatures >60°C). The majority of mesophilic microorganisms are rod-shaped, Gram-negative eubacteria (e.g. *At. ferrooxidans* and *At. thiooxidans*) whereas the moderate thermophiles include Gram-negative, sulfur-oxidizing autotrophs (e.g. *At. caldus*), Gram-positive *Bacillus*-like facultative chemolithotrophs (e.g. *Sb. acidophilus* and *Sb. thermosulfidooxidans*), heterotrophs (e.g. *Alicyclobacillus* spp.) and two genera of archaea (*Thermoplasma* and *Picrophilus*) (Norris & Johnson 1998; Johnson 1998). Archaea are the only domain to be found to exist at extreme acidity and high temperatures (>60°C). Extremely thermophilic archaea include *Sulfolobus* spp., some of which are capable of sulfur oxidation, and *Acidianus* spp.. Although studies have indicated the presence of psychrotolerant acidophiles

in some environments (Berthelot et al. 1994), no psychrophilic acidophiles have been isolated to date.

1.2.3 Interactions between acidophilic microorganisms

In anthropogenic as well as natural environments, acidophilic microorganisms interact with each other. A variety of relationships have been described, including mutualism (an association of two organisms from which both benefit), synergism (an association of two or more organisms with complementary activities allowing them to grow when together but not in pure cultures), competition, ammensalism (a repression of one species by toxins produced by another) and predation (grazing of bacteria by acidophilic protozoa). These interactions among acidophiles can be studied in depth using biofilms and acid streamers. Johnson et al. (1992) investigated gelatinous acid streamers and found that the streamers consisted of a wide range of microorganisms including unicellular and filamentous bacteria, together with protozoa and rotifera that graze on other microorganisms. Other studies have been conducted by Bond et al. (2000a; 2000b) on submerged slimes and streamers in an acid mine drainage site at Iron Mountain. Phylogenetic analyses of 16S rRNA showed a diversity of mostly novel sequences (Bond et al. 2000a); the biofilms were composed mostly of extracellular polymers surrounding spirillum-shaped cells and small cocci. This study also showed that there might be oxygen-limited microenvironments within the slimes that supported growth of anaerobic and microaerophilic bacteria. These studies imply that studying microbial communities using molecular techniques may reveal additional interactions among acidophilic microorganisms to those noted previously.

1.2.4 Adaptation to acidic environments

One of the most important factors that influences microorganisms in all environments is the concentration of hydrogen ions. It affects the ionic state and thus the availability of inorganic ions and metabolites in the environment. For example, the solubility of CO_2 decreases at low pH but that of many metal ions, which are toxic to many organisms at high concentrations (such as Al^{3+}, Fe^{3+}, Mn^{2+}, Cu^{2+} and Zn^{2+}) increases (Langworthy 1978).

Besides the availability of CO_2 and toxic metals, acidophiles face various theoretical problems including the survival and functioning of biomolecules exposed to extreme acidity, the condition of the periplasm, maintenance of cytoplasmic pH, and the functioning of the chemiosmotic mechanism with regard to a large pH difference across the cytoplasmic membrane (Norris & Ingledew 1992).

Acidophiles are, in general, highly sensitive to small molecular weight organic acids, such as acetic acid, lactic acid and formic acid, that are less toxic to neutrophilic microorganisms (Norris & Ingledew 1992). The toxicity of organic acids is caused by organic acids becoming increasingly protonated (AH) as the solution pH declines, and they exist predominantly as protonated (undissociated) acids below their *p*Ka values. Undissociated acids are often lipophilic, and therefore membrane-permeable. Once they have entered the cells, organic acids dissociate ($A^- + H^+$) since the cytoplasmic pH of active microorganisms is around 6.5 (Norris & Ingledew 1992). Since the dissociated organic acids are charged, they are no longer membrane permeable hence organic acids accumulate in the cell. This leads to accumulation of organic acids and protons in the cell resulting in decrease of the trans-membrane pH gradient. Figure 1.1 shows a schematic diagram showing this phenomenon using an accumulation of acetic acid as an example. As suggested by the chemiosmotic hypothesis (Mitchell 1966), the proton concentration gradient and membrane potential generates proton-motive force for driving ATP synthesis and decreasing the pH gradient reduces the potential for energy generation. In addition, increase in hydrogen ion concentration reduces cytoplasmic pH, affecting the stability and function of macromolecules leading to malfunctioning of biological processes.

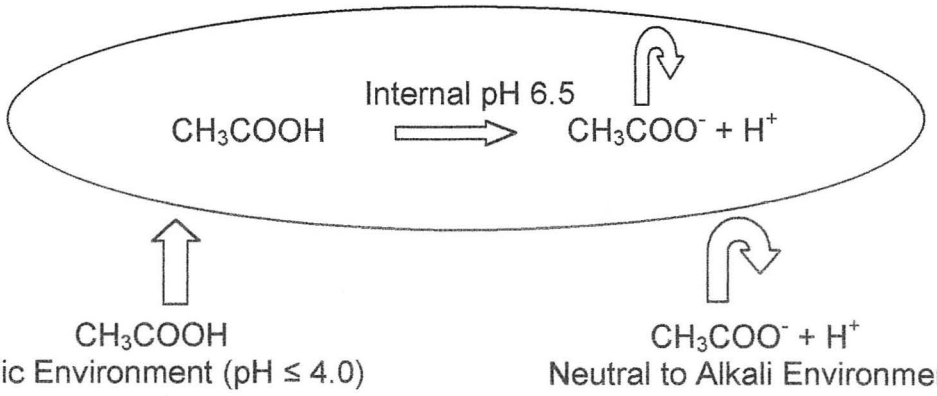


Figure 1.1. A schematic diagram showing the mechanism of acetic acid accumulation inside a prokaryotic cell in an acidic environment.

Acidophiles can also be sensitive to inorganic anions, except for sulfate which is relatively impermeable (Norris & Ingledew 1992). In an active acidophilic cell, the membrane potential ($\Delta\Psi$) is relatively small (+50 mV) but the value is much greater (+120 mV) in an inactive cell (Matin 1999). As ions that are permeable move across the membrane according to the membrane potential, anions enter and cations are excluded from the inactive cell where the membrane potential is high. This can result in the depletion of required cations, and anion poisoning of the cell. The exclusion of cations, however, benefits acidophiles and allow them to tolerate highly elevated concentrations of many cationic metals, such as copper and zinc (Norris & Ingledew 1992). In active cells, the membrane potential is maintained at low $\Delta\Psi$, which avoids anion accumulation and loss of cations. This is achieved by combination of proton diffusion potential, which is generated by influx of protons, and the Donnan potential, which is the osmotic pressure of protein solution caused by impermeable protein molecules (Matin 1999). The former has been suggested by Matin (1999) to be more significant phenomenon in active cells while the latter is the more important in inactive cells.

Although life forms face a number of survival problems in low pH environments, there is one theoretical advantage, which is the existence of a pH differential across the cell membrane that would facilitate the synthesis of ATP via proton influx. Unlike neutrophiles and alkaliphiles, acidophiles have a "ready made" pH gradient (of *ca.* 3-5 pH units) as the external proton concentration is higher than that of inside the cell. A study using *At. caldus*

confirmed that ATP could be generated from a pH gradient created across the membrane (Dopson 2002). However, in order to prevent generation of inhibitory membrane potential created from inward flow of protons, acidophiles need to balance the charge of protons by either a co-influx of anions or counter flow of cations. As high concentrations of anions are often toxic to acidophiles (Matin 1990), cation counter flow is likely to be used to balance the charge. In the case of *At. caldus*, K^+ was suggested to be used as a counter-ion (Dopson 2002).

In order to grow in acidic environments, acidophilic microorganisms have specific adaptations that differentiates them from neutrophiles. Madigan et al. (1997) suggested that the cytoplasmic membrane was the most critical structural component in maintaining the integrity of acidophilic microorganisms, as it needs to be resistant to high acidity and highly impermeable to protons. However, with few exceptions (such as the acid tolerance of the redox protein rusticyanin, which is located in the periplasm of the iron-/sulfur-oxidizer, *At. ferrooxidans*) (Walter et al. 1996) other mechanisms of adaptation to extremely acidic environment (e.g. of the cell walls of acidophiles) have yet to be fully investigated.

1.2.5 Acid streamers

One of the most remarkable forms of microbial life found in acidic waters is filamentous gelatinous growths that are often referred to as "acid streamers". These were first reported by Lackey in 1938. He found acid streamer growths in acid mine drainage streams in West Virginia and originally believed that the long, colorless or light brown structures were fungal. However, microscopic examination of the acid streamer revealed that they were "bacterial masses, presumably in a zoogloeal jelly". The descriptions of acid steamers vary significantly. Leathen (1952) reported they were composed of fibrous masses of sheath-like structures that did not contain any bacterial cells. Although Leathen reported the absence of bacterial cells in acid streamers, a report made by Temple and Koehler (1954) agreed with the observation made by Lackey stating that acid steamers consisted of non-filamentous, non-orientated bacteria surrounded by a tough slime. Some of

the acid streamers found within mines were reported to be encrusted with orange-brown ferric precipitates and those found outside the mines were green-colored as a result of colonization by phototrophic *Euglena* spp. (Temple & Koehler 1954).

Studies of acid steamers revealed that they are composed of wide range of microorganisms. Dugan et al. (1970) isolated a neutrophilic *Bacillus* sp. from acid streamers that produced large amount of extracellular slime in liquid media and suggested that the isolate played an important role within the acid streamer community.

A variety of neutrophilic bacteria were isolated from acid streamers growing in Cae Coch, an abandoned pyrite mine in North Wales, by Johnson et al. (1979) and some of these isolates produced exopolysaccharides. The pH of the site was between 2.10 and 2.45 and the temperature ranged from 7.9 and 8.4°C (McGinness & Johnson 1993). The streamer material found in the mine was described to be composed of bacteria embedded in a fibrous polysaccharide matrix. Some ciliates and rotifers were found to graze on streamer material, but no fungi or yeasts were observed in the streamer. The investigation reported that the bacterial component of the streamer was exclusively heterotrophic though the later studies showed acidophilic chemolithotrophic bacteria were also present in the streamer (Johnson et al. 1983; Johnson et al. 1992). The authors stated that acid streamers contained a mixed community of iron- and sulfur-oxidizing bacteria that were the primary producers in Cae Coch, and heterotrophic microorganisms that used cell exudates and lysates as their growth substrates. One of the microorganisms (isolate CCH7) isolated from this mine was an obligately acidophilic (pH range 2.0-4.4) heterotroph that oxidized ferrous iron (Johnson et al. 1992). This isolate was filamentous and formed streamer-like growths when grown in liquid media.

A culture-independent study was carried out on acid streamer-like communities, which was a 1 cm thick slime biofilm that developed on a slump of finely disseminated pyrite ore within an "extreme" acid mine drainage (pH

between 1.3 and 0.5 and temperate between 25-50°C) site at Iron Mountain in California, U.S.A. (Bond et al. 2000a). The clones obtained from a clone library constructed from 16S rRNA genes amplified from the biofilm were mostly novel and most of the clone sequences were closest to sequences of iron-oxidizing acidophiles; *Leptospirillum, Acidimicrobium, Thermoplasma* and *Ferroplasma*. The clone library study also detected sequences that belonged to the δ-*Proteobacteria* class, which contains sulfate-reducing bacteria. The biofilm community was also studied using fluorescent *in situ* hybridization (FISH) analysis and results showed that only one to three types of organisms dominated each biofilm sample (Bond et al. 2000b). The microorganisms found to dominate the slime samples from FISH analysis included *Ferroplasma, Leptospirillum, Sulfobacillus* and *Acidimicrobium*.

Another culture-independent study of acid streamers was carried out in the River Tinto, Spain (water pH 2.0, and temperature 21°C) by López-Archilla et al. (2004). These streamers were very large (up to 1.5 m long and 5 cm thick) and were attached to a small still dam in the river. From construction and analysis of clone libraries, these researchers showed that this particular streamer community was composed of completely different microorganisms from that of Iron Mountain. The community was dominated by γ-*Proteobacteria* (*Pseudomonas*) and α-*Proteobacteria* (*Sphingomonas*); minor components of the streamer microbial community included β-*Proteobacteria* (*Ralstonia*), *Bacilli* (*Bacillus*) and *Actinobacteria*.

From the limited number of studies carried out on acid streamers, it seems that their component microorganisms vary between materials found in environments with different physicochemical characteristics, such as pH and temperature.

1.3 Phylogeny of acidophiles

Advances in cultivation and molecular techniques have resulted in increased numbers of characterized genera of acidophiles. This section will describe currently known acidophilic prokaryotic microorganisms. Table 1.3 shows the list of generic abbreviations for acidophiles proposed by Hallberg and Johnson (2001). These abbreviations are used throughout this thesis.

Table 1.3. List of proposed generic abbreviations for acidophiles (adapted from Hallberg & Johnson 2001).

Genus name		Proposed abbreviation	Phylogenetic division
Archaea	*Acidianus*	*Ad.*	Sulfolobales
	Acidilobus	*Al.*	Sulfolobales
	Ferroplasma	*Fp.*	Thermoplasmales
	Metallosphaera	*M.*	Sulfolobales
	Picrophilus	*P.*	Thermoplasmales
	Stygiolobus	*Sg.*	Sulfolobales
	Sulfolobus	*S.*	Sulfolobales
	Sulfurisphaera	*Ss.*	Sulfolobales
	Sulfurococcus	*Sc.*	Sulfolobales
	Thermoplasma	*Tp.*	Thermoplasmales
Bacteria	*Acidimicrobium*	*Am.*	*Actinobacteria*
	Acidiphilium	*A.*	*α-Proteobacteria*
	Acidisphaera	*As.*	*α-Proteobacteria*
	Acidithiobacillus	*At.*	*β/γ-Proteobacteria*
	Acidobacterium	*Ab.*	*Acidobacteria*
	Acidocella	*Ac.*	*α-Proteobacteria*
	Acidomonas	*Amn.*	*α-Proteobacteria*
	Alicyclobacillus	*Alb.*	*Bacilli*
	"Ferrimicrobium"	*Fm.*	*Actinobacteria*
	Hydrogenobacter	*H.*	*Aquificae*
	Leptospirillum	*L.*	*Nitrospira*
	Sulfobacillus	*Sb.*	*Bacilli*
	Thiomonas	*Tm.*	*β-Proteobacteria*

1.3.1 Eukarya

Eukaryotes found in acidic environments include acid-tolerant and acidophilic microorganisms. These include fungi, yeasts, protozoa, microalgae and rotifera. Descriptions of these acidophiles are included in reviews by Deneke (2000), Gross & Robbins (2000) and Gross (2000). Since acidophilic eukaryotes were not the focus of the present study, they are not described here.

1.3.2 Bacteria

In the domain *Bacteria*, acidophilic bacteria that have been characterized are found in eight classes; *Acidobacteria, Actinobacteria, Aquificae, Bacilli, Nitrospira, α-Proteobacteria, β-Proteobacteria* and *γ-Proteobacteria*.

1.3.2.1 Acidobacteria

Acidobacterium is currently the only acidophilic/acid-tolerant genus of this class. Some clones from environmental DNA libraries of samples taken from peat bogs, soil, freshwater and marine sediments, and wetlands have been identified as *Acidobacterium* (Barns et al. 1999). *Ab. capsulatum*, which is one of the few cultivated bacteria of the class, is motile rod-shaped bacterium and forms a capsule (Kishimoto et al. 1991). This mesophilic chemoorganotrophic bacterium grows between pH 3.0-6.0 and 20-37°C. The other distinct characteristics of the microorganism are formation of orange-colored colonies on solid media, and production of exopolymers.

1.3.2.2 Actinobacteria

The class *Actinobacteria* includes a physiologically diverse group of bacteria. Members of this group are characterized by a high G+C content of their chromosomal DNA (Stach 2003). There are two acidophilic species in this class; *Acidimicrobium ferrooxidans* and "*Ferrimicrobium acidiphilum*", both of which oxidize iron in the presence of yeast extract and reduce ferric iron under anaerobic conditions. *Am. ferrooxidans* is a moderate thermophile (Clark & Norris 1996) and *"Fm. acidiphilum"* is a mesophile (Johnson et al. 1995). The latter microorganism was found not to be able to fix CO_2 and is incapable of sulfur oxidation (Hallberg & Johnson 2001).

1.3.2.3 Aquificae

The only acidophile known in this group is *Hydrogenobacter acidophilus* which has been proposed to be renamed *Hydrogenobaculum acidophilum* (Stohr et al. 2001). This is a thermoacidophilic, obligately chemolithotrophic, aerobic, hydrogen-oxidizing Gram-negative bacterium originally isolated from a solfataric field (Shima & Suzuki 1993). It requires elemental sulfur for growth by hydrogen oxidation and the optimum pH for growth is between 3.0 and 4.0.

1.3.2.4 Bacilli

There are two groups of acidophiles in this group, *Sulfobacillus* and *Alicyclobacillus*.

Sulfobacillus spp. are mostly moderately thermophilic (typically growing between 40 and 60°C). They are iron-oxidizing acidophiles that have been isolated from hot springs, coal spoil heaps and AMD. *Sulfobacillus* spp. exhibit a variety of metabolic capabilities. They are mixotrophs that can grow using S^0, Fe^{2+}, pyrite or organic substrates as energy sources, and fix CO_2 in the absence of organic carbon (Wood & Kelly 1984; Norris & Barr 1985). *Sulfobacillus* spp. are facultative anaerobes that can grow on glycerol using Fe^{3+} as an alternative electron acceptor when oxygen is limiting (Bridge & Johnson 1998). *Sb. acidophilus* is capable of reducing ferric iron coupled with tetrathionate oxidation under anaerobic conditions (Bridge & Johnson 1998). *Sb. disulfidooxidans* is unusual microorganism among *Sulfobacillus* spp. as it is an aerobic mesophile which grows optimally at 35°C (Dufresne et al. 1996). It can grow by using elemental sulfur and pyrite as sole energy source.

Two mesophilic isolates (RIV14 and L15) belonging to the *Sulfobacillus* genus have been described (Bridge & Johnson 1998); the proposed names for these isolates are "*Sb. ambivalens*" and "*Sb. montserratensis*", respectively. The microorganisms were isolated from the Caribbean island of Montserrat, and are facultative anaerobes, like other *Sulfobacillus* spp.. Isolate-L15 can grow between 30-43°C (optimum 37°C) and pH 0.7-2.0

(optimum 1.5). It grows using variety of organic and inorganic electron donors, and can fix CO_2. It oxidizes ferrous iron, elemental sulfur and pyrite, and can also reduce ferric iron.

Another *Sulfobacillus* isolate, YTF1 (proposed name "*Sb. yellowstonensis*"), was isolated from the Frying Pan Hot Springs and the Sylvan Springs area of Yellowstone National Park (Johnson et al. 2001b). "*Sb. yellowstonensis*" is more thermotolerant than other *Sulfobacillus* spp. with a temperature range of 35-60°C (optimum 55°C). It grows between pH 1.7-3.0 (optimum 2.5) and oxidizes ferrous, elemental sulfur and pyrite (Johnson et al. 2001a). It can reduce ferric iron and is capable of fixing CO_2.

Alicyclobacillus spp. are thermoacidophilic, rod-shaped, Gram-positive microorganisms and have been found in geothermal sites, soil, organic compost, fruit, acidic beverages and heat-processed foods (Hiraishi et al. 1997; Goto et al. 2002; Matsubara et al. 2002). There are currently eight species recognized; *Alb. acidiphilus* (Matsubara et al. 2002), *Alb. sendaiensis* (Tsuruoka et al. 2003), *Alb. pomorum* (Goto et al. 2003), *Alb. hesperidum* (Albuquerque et al. 2000), *Alb. herbarius* (Goto et al. 2002), *Alb. acidocaldarius* (Darland & Brock 1971; Wisotzkey et al. 1992), *Alb cycloheptanicus* and *Alb. acidoterrestris* (Deinhard et al. 1987a; Wisotzkey et al. 1992). Unlike *Sulfobacillus* spp., *Alicyclobacillus* spp. are obligate heterotrophs and the major lipid component of the cell membranes is omega-alicyclic fatty acids (Wisotzkey et al. 1992). *Alb. acidiphilus,* was isolated from acidic beverage and was found to grow between 20 and 55°C (optimum 50°C) and between pH 2.5 and 5.5 (optimum 3.0) (Matsubara et al. 2002). *Alb. sendaiensis* was isolated from a soil sample in Aoba-yama park (Japan) and is slightly more thermophilic (40–65°C; optimum 55°C) than *Alb. acidiphilus* (Tsuruoka et al. 2003). *Alb. hesperidum* was isolated from solfataric soil at Furnas on the Island of Sao Miguel in the Azores (Albuquerque et al. 2000) and *Alb. herbarius* was isolated from a herbal tea made from the dried flowers of hibiscus (Goto et al. 2002). Unlike other members of *Alicyclobacillus*, *Alb. pomorum* does not contain ω -alicyclic fatty

acid which, besides being a diagnostic characteristic of this genus, also gave rise to the genus name. *Alb. acidocaldarius* (Darland & Brock 1971; Wisotzkey et al. 1992), *Alb cycloheptanicus* (Deinhard et al. 1987b; Wisotzkey et al. 1992) and *Alb. acidoterrestris* were all originally classified as *Bacillus* spp..

Alicyclobacillus isolate YTH1, isolated from the Sylvan Springs area of Yellowstone National Park (Johnson et al. 2001b), grows optimally at *ca.* 45°C and pH 2.0. It does not oxidize ferrous iron, elemental sulfur or pyrite, but can reduce ferric iron (Johnson et al. 2001a). A similar isolate (Y004), also from Yellowstone National Park, is incapable of ferrous iron or tetrathionate oxidation. Isolate Y004 can couple the oxidation of glucose to the reduction of ferric iron under anaerobic conditions, though only to a limited extent. The maximum temperature for growth of isolate Y004 was found to be 60°C and its lower pH limit for growth was 1.0 (Johnson et al. 2003).

There are two other groups of currently unclassified acidophiles that are related to the Gram-positive bacteria described above. Isolate GSM (proposed name "*Acidibacillus ferrivorum*") is more closely related to *Alb. cycloheptanicus* (93%; 16S rRNA gene sequence identity) than to *Sb. thermosulfidooxidans* (88%) and was isolated from mine spoil material from the Golden Sunlight mine at Montana, U.S.A. (Yahya 2000). It is a Gram-positive moderate thermophile that grows between 35-55°C (optimum temperature is 45°C) and grows optimally at pH 1.8 (Johnson et al. 2001a). This microorganism is a facultative anaerobe that can oxidize ferrous iron, elemental sulfur and pyrite, and can also reduce ferric iron. It grows both autotrophically and mixotrophically and produces high cell yields (more than 10^9 cells/ml) when grown on organic substrates. A microorganism which is closely related to isolate-GSM has also been isolated from Sydney bay in Australia (Holden et al. 2001). Isolates SLC1, SLC2 and SLC66 were isolated from sulfidic regoliths subjected to accelerated oxidation in humidity cell chambers and are low G+C Gram-positive bacteria (Johnson 1995). These bacteria form colonies with typical "fried egg" morphologies, with ferric iron

precipitates in the centers of colonies which have gelatinous white fringes. Isolate SLC1 grows between 10-45°C (optimum temperature 37°C) and pH 1.7-3.7 (optimum pH *ca.* 2.5) (Johnson et al. 2001a). It oxidizes ferrous iron and pyrite only in the presence of organic substrates and does not fix CO_2.

1.3.2.5 Nitrospira

Leptospirillum spp. are the only acidophiles in this class and there are currently three recognized species; *L. ferrooxidans* and *L. thermoferrooxidans* (Hippe 2000) and *L. ferriphilum* (Coram & Rawlings 2002). *Leptospirillum* spp. have been found to be the dominant microorganisms in many bioleaching plants especially at low pH (Norris et al. 1988), and considered to be the primary mineral-oxidizers in these situations. All *Leptospirillum* isolates grow exclusively by coupling ferrous iron oxidation and molecular oxygen reduction, and are small, Gram-negative, vibrioid or spiral-shaped bacteria (Coram & Rawlings 2002). *Leptospirillum* spp. are more acidophilic than *At. ferrooxidans* (Section 1.3.2.8), and can grow as low as pH 1.1 (optimum growth 1.3-2.0; Johnson 2001). *L. ferriphilum* is slightly more acidophilic than *L. ferrooxidans* and can grow optimally between pH 1.4 and 1.8. *L. ferriphilum* has a higher G+C mole percentage of its chromosomal DNA (55-58%, compared to 51-56% for *L. ferrooxidans;* Coram & Rawlings 2002). Unlike *L. ferrooxidans*, which is a mesophile, some strains of *L. ferriphilum* are thermo-tolerant (can grow at 45°C; Coram & Rawlings 2002) and *L. thermoferrooxidans* are moderate-thermophiles with the maximum growth temperature 55°C (Golovacheva 1993). There are some similarities between *L. ferriphilum* and *L. thermoferrooxidans,* including G+C content (about 56 %) and growth at higher temperature, and there is a possibility of the two being the same. However, since the only isolate of *L. thermoferrooxidans* has been lost before phylogenetic analyses were performed, this cannot be confirmed.

1.3.2.6 α-Proteobacteria

Acidophilic members of *α-Proteobacteria* class are mainly heterotrophs and include *Acidiphilium* spp., *Acidocella* spp., *Acidomonas methanolica* and *Acidisphaera rubrifaciens.*

Acidiphilium spp. (including *A. angustum*, *A. rubrum, A. multivorum, A. organovorum* and *A. cryptum*) are all obligate heterotrophs and the optimum pH ranges from 2.5 to 6.0 (the minimum pH range for growth is 1.9). All *Acidiphilium* spp. are capable of reducing ferric iron to ferrous iron in anaerobic conditions (Johnson & McGinness 1991). *A. acidophilum* is the only recognized mixotrophic species in the genus. It can fix CO_2 as well as use organic substrate as carbon source. It is also capable of chemolithotrophic growth on reduced inorganic sulfur compounds (Guay & Silver 1975).

Although *Acidiphilium* is not capable of photosynthetic growth, it produces bacteriochlorophyll (BChl) only under aerobic conditions (Hiraishi et al. 1998; Wakao et al. 1996). The photopigment of this species is a BChl *a,* which is chelated with zinc instead of magnesium, and it is stable under acidic condition (Kobayashi 1998). They also contain the *puf* operon which codes for the proteins of the photosynthetic reaction center and the core light-harvesting complex of the purple photosynthetic bacteria (Nagashima et al. 1997).

There are currently two classified species of *Acidocella*; *Ac. facilis* (Wichlacz et al. 1986) and *Ac. aminolytica* (Kishimoto et al. 1993). *Acidocella* is less acidophilic (grows between pH 2.5 and 6.0) and less tolerant to some metals and organic acids than *Acidiphilium*. *Acidocella* is motile and forms small flocs and chains (Kishimoto 1995). Another proposed species of this genus, "*Ac. aromatica*", was found to be able to grow on a variety of aromatic compounds such as benzoate, phenol and naphthalene (Hallberg et al. 1999). Although it is capable on growth using fructose and aliphatic acids (including acetic acid) as sole carbon and energy sources, "*Ac. aromatica*" is incapable of utilizing glucose and glycerol that are commonly used by other acidophilic heterotrophs (Gemmell & Knowles 2000).

Acidomonas methanolica shares many physiological characteristics with *Acidiphilium* including growth on methanol, and grows between pH 2.0 and

5.5 (Urakami 1989). The other acidophile currently belonging to *α-Proteobacteria* class is *Acidisphaera rubrifaciens* which was first isolated from acidic hot springs in Japan (Hiraishi 2000). This obligate aerobic chemo-organotrophic and facultative photo-organotrophic microorganism is non-motile and forms salmon-pink to red colored colonies on solid media. The pH range of the microorganism is 3.5-6.0 (optimum 4.5 to 5.0) and temperature range is 20-40°C (optimum 30-35°C).

1.3.2.7 β-Proteobacteria

Thiomonas cuprina (formerly *Thiobacillus cuprinus*), which was first isolated from solfatara fields in Iceland and a uranium mine in Germany, can grow heterotrophically using various organic compounds and autotrophically on elemental sulfur and reduced inorganic sulfur compounds (Huber & Stetter 1990). The pH range for growth of this bacterium is between 1.5 and 7.2 (optimum pH 3.0-4.0). The optimal temperature for growth is 30-36°C and it can grow between 20°C and 45°C (Huber & Stetter 1990). *Thiomonas cuprina* is unique among the genus as other species, *Tm. Intermedia, Tm. perometabolis* and *Tm. thermosulfata,* are not acidophilic. However, *Tm. thermosulfata* can grow between pH 4.3 and 7.8; when grown on thiosulfate, Shooner et al. (1996) noted that the pH of shake flask cultures fell from 7.0 to 2.5.

Thiomonas-like microorganisms have been isolated from ferruginous water draining a coal mine in south Wales and a passive AMD treatment plant in Cornwall (Hallberg & Johnson 2003). These isolates were found to be moderate acidophiles as they grew around pH 3.0 but not at pH 2.0. Unlike *Thiomonas* spp., these isolates can oxidize ferrous iron.

Another iron-oxidizing acidophilic β-proteobacterium, strain m-1, was originally considered to be a strain of *Thiobacillus ferrooxidans* (Harrison 1982). Unlike true *(Acidi)thiobacillus* spp., however, it is incapable of sulfur oxidation and it is phylogenetically unrelated to all other iron-oxidizing acidophiles (Hallberg & Johnson 2001).

1.3.2.8 γ-Proteobacteria

Acidophiles in the *γ-Proteobacteria* class includes members of the genus *Acidithiobacillus (At. ferrooxidans, At. thiooxidans, At. caldus* and *At. albertensis).* These are obligate acidophiles and can grow autotrophically using sulfur as an electron donor.

At. ferrooxidans is the most studied of all acidophilic microorganisms due to its previous perceived importance in acid mine drainage generation and bioleaching industry. It is an obligate acidophile and the pH range for growth is between 1.3 and 4.5 (optimum pH is 2.5). *At. ferrooxidans* is a Gram-negative, rod-shaped mesophile, and a facultative anaerobe. Studies on strains of *At. ferrooxidans* demonstrated that the members of this species are capable of growth by oxidizing Fe^{2+}, H_2S, S^0, SO_3^{2-}, thiosulfate, tetrathionate or H_2 (Friedrich 1998). Studies also showed that some strains can fix atmospheric nitrogen and oxidize UO_2 (Rawlings & Kusano 1994) or use formic acid as a carbon and energy source (Pronk et al. 1991). Although it had been considered to be an obligate aerobe, Pronk et al. (1992) found that it is capable of anaerobic elemental sulfur or formic acid metabolism coupled with ferric iron reduction. *At. ferrooxidans* is resistant to high concentrations of metallic and other ions including Zn^{2+}, Ni^{2+}, Cu^{2+}, Co^{2+} and Fe^{3+}. However, many strains are sensitive to silver and mercury, with the exception of some mercury resistant strains (Rawlings & Kusano 1994).

At. thiooxidans is a mesophilic, Gram-negative obligate lithotroph and can oxidize H_2S, S^0, SO_3^{2-}, thiosulfate and tetrathionate; strains are more motile than *At. ferrooxidans.* This microorganism can grow between pH 0.5 and 5.5 (optimum pH 2.0-3.0) and 28-30°C. Unlike *At. ferrooxidans,* it does not oxidize pyrite or ferrous iron. However, it can reduce ferric iron to ferrous iron with elemental sulfur in the absence of oxygen (Brock & Gustafson 1976), though this does not appear to support growth of the acidophile (Hallberg et al. 2001).

At. caldus is a small, Gram-negative, motile rod, and grows optimally between pH 2.0 and 2.5 (growth pH range 1.0-3.5). The growth temperature

range is between 32 and 52°C and the optimal temperature is 45°C. *At. caldus* has the highest G+C content of all acidophiles in this class (63.1-63.9 mol%). It is capable of chemolithoautotrophic growth on thiosulfate, tetrathionate, sulfide, elemental sulfur and molecular hydrogen (Hallberg & Lindström 1994). The bacterium can also grow mixotrophically with sulfur or tetrathionate and yeast extract or glucose.

At. albertensis was formally known as *Thiobacillus albertis* (Kelly & Wood 2000) and was first isolated from acidic soil adjacent to a sulfur stockpile in Alberta, Canada (Bryant et al. 1983). It is an obligate autotrophic sulfur oxidizer like *At. thiooxidans* with pH range of 2.0-4.5 (optimum between 3.5 and 4.0) and optimum growth temperature of 28-30°C. *At. albertensis* differs from *At. thiooxidans* in its G+C content (61.5 mol% compared to 50.0-52.0 mol% for *At. thiooxidans*). *At. albertensis* also has a glycocalyx, which is a viscous covering surrounding a cell, and a tuft of polar flagella (Bryant et al. 1983).

1.3.3 Archaea

Acidophilic archaea can be found in the phyla Euryarchaeota and Crenarchaeota.

1.3.3.1 The Euryarchaeota

Within the Euryarchaeota, acidophiles are present in three families; the *Thermoplasmaceae,* the *Picrophilaceae* and the *Ferroplasmaceae*. There is only one recognized genus in each of these families.

Thermoplasma have been found in moderately hot acidic solfatara fields and geothermal areas. Cells vary from spherical to filamentous, stain Gram-negative, and are generally motile. All current isolates are moderate thermophiles, which grow between 33 and 67°C, and have minimum pH for growth of 0.5, and grows optimally at *ca.* pH 2.0. *Thermoplasma* are obligate acidophiles and cells lyse at neutral pH (Reysenbach 2001). They are obligate heterotrophs and can grow aerobically, or in anaerobic conditions using elemental sulfur as a terminal electron acceptor. *Tp. acidophilum,*

which is one of the two characterized isolates, was first isolated from self-heating coal refuse piles in Southern Indiana and Western Pennsylvania (Darland et al. 1970). It grows between 45 and 63°C (optimum 59°C) and pH 0.5-4.0. The G+C content of DNA is 46 mol%. *Tp. acidophilum* has also been isolated from warm acidic solfatara fields and self-heating refuse piles (Darland et al. 1970). The other characterized species, *Tp. volcanium,* has been sub-divided into three "groups" (Segerer et al. 1988): group 1 was found in continental and submarine solfatara in Italy; group 2 was found in continental solfatara and tropical swamps in Java; group 3 was found in continental solfatara in Iceland and the U.S.A.. This species has a wider temperature range (33-67°C) than *Tp. acidophilum* and grows between pH 1.0 and 4.0. The G+C content is less than that of *Tp. acidophilum* (38 mol%; Segerer et al. 1988).

The archaeal genus *Picrophilus* currently comprises two distinct species, and represents the most acidophilic of all currently recognized life forms. *P. oshimae* was first isolated from hot (ca. 55°C) geothermal solfatara soils (pH <0.5) and hot springs in Hokkaido, Japan (Schleper et al. 1995). It is a moderate thermophile (range 47-60°C; optimum 60°C), hyperacidophilic (range, pH 0 and 3.5, optimum pH 0.7) and, in contrast to *Thermoplasma* spp., obligately aerobic. Cells are irregular cocci, about 1 µm in diameter and non-motile. *P. oshimae* grows heterotrophically with 0.1-0.5% (w/v) yeast extract and cannot grow on organic substrates in the absence of yeast extract. The G+C content of *P. oshimae* is slightly less than that of *Thermoplasma* (36 mol%). The other known *Picrophilus* sp., *P. torridus*, was isolated from the same location as *P. oshimae*. Besides having sufficient dissimilarity in their 16S rRNA genes to justify separate species, the two archaea were found to have significantly different growth rates (Schleper et al. 1996).

Ferroplasma acidiphilum belongs to the family *Ferroplasmaceae* and was first isolated from a bioleaching pilot plant (Golyshina et al. 2000). It is a mesophile (other members of Euryarchaeota are thermophilic) and the maximum temperature for growth is 45°C. The archaeon grows optimally at

pH 1.7 and lacks a cell wall (Golyshina et al. 2000). Unlike other acidophilic members of Euryarchaeota, *Fp. acidiphilum* is capable of oxidation of ferrous iron. In contrast to *Thermoplasma,* it cannot grow anaerobically or oxidize sulfur. There is a second species of genus *Ferroplasma, Fp. acidarmanus*; which was originally isolated from slime biofilm in Iron Mountain (U.S.A.) (Edwards et al. 2000). Like *Fp. acidiphilum*, it is capable of iron oxidation and can grow at pH 0. Although *Fp. acidiphilum* is not capable of anaerobic growth, *Fp. acidarmanus* is a facultative anaerobe, coupling chemoorganotrophic growth on yeast extract to the reduction of ferric iron (Dopson et al. 2004).

1.3.3.2 The Crenarchaeota

Characterized acidophilic archaea can be found in seven genera in the family *Sulfolobaceae* of the Crenarchaeota; *Acidianus*, *Acidilobus, Metallosphaera*, *Sulfolobus*, *Stygiolobus, Sulfurisphaera* and *Sulfurococcus.*

Acidianus spp. have been isolated from: acidic solfatara springs and mudholes at Solfatara Crater, Napoli in Italy and at Yellowstone National Park, U.S.A.; solfatara fields in Iceland and The Azores and Java in Indonesia; and geothermally heated acidic marine environments at the beach of Vulcano Island, Italy (Huber & Stetter 2001a). *Acidianus* can grow between 45-96°C and pH 1.0-6.0. It is a facultative anaerobe that can reduce elemental sulfur to sulfide using molecular hydrogen as electron donor. *Acidianus* is also capable of lithotrophic growth by elemental sulfur oxidation in aerobic conditions (Segerer et al. 1986). There are three characterized species of *Acidianus*: *Ad. infernus, Ad. ambivalens* and *Ad. brierleyi. Ad. infernus* is the most thermophilic of these, with an optimum growth temperature of 85-90°C (range 65-96°C) and is an obligate chemolithoautotroph which can oxidize elemental sulfur and molecular hydrogen (Segerer et al. 1986). Unlike other species of this genus, *Ad. ambivalens* cannot grow above pH 3.5 (optimum around 2.5) and its optimum temperature for growth is around 80°C (Zilling et al. 1986). *Ad. brierleyi* is the least thermophilic of the three species (temperature range 45-75°C; optimum around 70°C) and can grow between pH 1.0-6.0 (optimum pH 1.5-2.0;

Segerer et al. 1986). This is the only species that is capable of growing organotrophically on yeast extract, peptone, tryptone, Casamino acids and beef extract.

Acidilobus aceticus is an obligate anaerobic thermoacidophilic archaeon. It grows on complex organic substrates, such as starch, and addition of sulfur can stimulate its growth (Prokofeva et al. 2000).

Metallosphaera has been isolated from acidic solfatara springs and mud holes at Pisciarelli Solfatara, Napoli in Italy and hot deposits from heaps at the open cast mining area near Ronnenburg in Germany (Huber & Stetter 2001b). It is an obligate aerobe and is a facultative chemotroph which is capable of oxidizing molecular hydrogen, elemental sulfur and sulfide minerals (Huber et al.1989; Fuchs et al. 1996). It can also grow on complex organic substrates such as beef extract, peptone and yeast extract. The growth temperature ranges from 50 to 80°C, with an optimum of 75°C, and it grows between pH 1.0 and 4.5. There are three characterized species of this genus: *M. sedula, M. prunae* and *M. hakonensis*. *M. sedula* requires low oxygen concentrations (0.5%) to grow by molecular hydrogen oxidation, while *M. prunae* requires higher (8-12%) concentrations (Huber et al.1989; Fuchs et al. 1996). *M. prunae* possesses a fibrillar surface coat which is thought to be composed of carbohydrates. *M. hakonensis*, which was reclassified from *Sulfolobus hakonensis* based on its 16S rRNA gene sequence and genomic G+C content (Kurosawa et al. 2003), can grow between 50-80°C (optimum 70°C) and pH 1.0-4.0 (optimum 3.0; Takayanagi et al. 1996). *M. hakonensis* can be differentiated from other two species by its ability to oxidize FeS and tetrathionate (Kurosawa et al. 2003).

The largest number of known acidophilic archaea belongs to the genus *Sulfolobus*. These have been found in acidic continental solfatara fields including Yellowstone National Park (U.S.A.), New Mexico, Solfatara Crater and Pisciarelli Solfatara (Italy), Dominica republic, El Salvador, New Zealand, Iceland, Japan, the Azores and Sumatra. *Sulfolobus* was also isolated from hot deposits from heaps at the open cast mining area near Ronnenburg in

Germany (Huber & Stetter 2001c). *Sulfolobus* spp. are obligate aerobes and facultatively chemolithoautotrophs. Some species can oxidize sulfidic ores, sulfide, elemental sulfur, tetrathionate and ferrous iron. There are seven characterized species of this genus: *S. acidocaldarius, S. shibatae, S. solfataricus, S. yangmingensis, S. metallicus, S. islandicus* and *S. thuringiensis*. *S. acidocaldarius* can grow between 55-85°C (optimum 70-75°C) and pH 1.0-6.0 (optimum 2.0-3.0) (Brock et al. 1972). It can grow on glucose, sucrose, mannose, tryptophan and glutamate but not on galactose and lactose (Huber et al. 1987; Takayanagi et al. 1996). *S. shibatae* grows up to 85°C (optimum 81°C) and can on galactose, lactose, glucose and sucrose (Grogan et al. 1990). It is a facultative chemolithotroph and grows by oxidation of sulfur. *S. metallicus*, is considered to be the most important mineral-oxidizing microorganism above 60°C (Norris et al. 2000). It grows between 50-75°C (optimum 65°C) and pH 1.0-4.5. It is an obligate chemolithoautotroph which oxidizes sulfidic ores and elemental sulfur (Huber & Stetter 1991). *S. solfataricus* grows between 50-87°C (optimum 85°C) and pH 2.0-5.5 (optimum around pH 4.5; Zilling et al. 1980). It grows on yeast extract, tryptone, Casamino acids, galactose, lactose, glucose, sucrose, ribose, mannose, xylose and glutamic acid (Huber et al. 1987; Takayanagi et al. 1996). *S. yangmingensis* was isolated from Yang-Ming National Park, Taiwan and can grow between 65-95°C (optimum 80°C) and pH 2.0-6.0 (optimum 4.0; Jan et al. 1999). It can grow using sugars and amino acids as sole carbon sources.

Stygiolobus azoricus is currently the only characterized species of the genus *Stygiolobus* and was isolated from hot acidic solfataric hot springs, mud and soil near Furnas and Ribeira Grande on Sao Miguel Island in Azores. It grows between 57-89°C (optimum around 80°C) and pH 1.0-5.5 (optimum 2.5-3.0) (Segerer et al. 1991). It is an obligate anaerobe and grows by molecular hydrogen oxidation coupled with elemental sulfur reduction, producing sulfide. *Sulfurisphaera ohwakuensis* is currently the only species of genus *Sulfurisphaera* and was isolated from hot acidic springs Hakone, Japan (Kurosawa et al. 1998). The temperature range for growth is between 63°C and 92°C (optimum 84°C) and grows between pH 1.0-5.0 (optimum

around pH 2.0). It is a facultative anaerobe that grows poorly by oxidation or reduction of elemental sulfur. It also grows aerobically on proteinaceous complex substrates, such as yeast extract and tryptone, but cannot grow on simple sugars and amino acids.

There are currently two characterized species in genus *Sulfurococcus*; *S. mirabilis* and *S. yellowstonensis*. Both species reproduce by binary fission and budding. They are strict aerobes that oxidize elemental sulfur under autotrophic and mixotrophic conditions. They also grow on variety of organic substrates including fructose, sucrose, glucose and yeast extract. *S. mirabilis* was isolated from the Crater of the Uzon volcano in Kamchatka, Russia (Golovacheva et al. 1987). It grows between 50-86°C (optimum 70-75°C) and pH 1.0-5.8 (optimum 2.0-2.6). *S. yellowstonensis* was isolated from Yellowstone National Park (U.S.A.) and can also oxidize ferrous iron and sulfide minerals (Karavaiko et al. 1994).

1.4 Sulfate reducing prokaryotes (SRP)

One of the AMD treatment systems which is being extensively researched, and that has also been employed in full-scale commercial operations, is the use of hydrogen sulfide gas generated by sulfate-reducing prokaryotes (SRP) to remove and recover dissolved metals from AMD. Virtually all SRP that have been isolated to date are bacteria rather than archaea. The phylogeny and major physiological characteristics of these microorganisms are described in this section.

1.4.1 Phylogeny of SRP

Sulfate reduction has been studied for many years as a process that is important in understanding ecosystem function, as well as environmental protection/remediation. Meyer first recognized in 1864 that the production of hydrogen sulfide in aquatic systems was due to the biologically-mediated reduction of sulfate. The first SRP to be isolated was *Spirillum desulfuricans* which grew on malate and aspartate as carbon/energy sources. Sulfate-reducing bacteria (SRB) had been thought to be a small, specialized group of bacteria that use only a limited spectrum of substrates, which are oxidized

incompletely, until Widdel and Pfenning (1977) discovered slowly-growing sulfate-reducers that could use a variety of organic substrates. Large numbers of sulfate-reducers have since been described. Sulfate-reducing bacteria (SRB) and archaea (SRA) utilize sulfate as terminal electron acceptor to oxidize organic acids, fatty acids, alcohols and H_2 (Madigan et al. 1997). Some SRB have also been found to be able to reduce various electron acceptors apart from sulfate, including sulfite, thiosulfate and elemental sulfur (Cypionka 2000). Classification of these prokaryotes was previously based on phenotypic characteristics, but modern classification is based on gene (e.g. 16S rRNA gene) analyses. Phylogeny of sulfate-reducers based on 16S rRNA genes was reviewed by Castro et al. (2000). On this basis, SRP can be divided into four groups; Gram-negative mesophilic SRB, Gram-positive spore-forming SRB, thermophilic SRB and thermophilic SRA. Some of the important characteristics of SRP are listed in Table 1.4.

1.4.1.1 Sulfate reducing bacteria

1.4.1.1.1 Gram-negative SRB

Gram-negative SRB belong to the δ-subdivision of the *Proteobacteria*; a large proportion of all known dissimilatory SRB isolates belong to this group (Widdel & Bak 1992). The typical habitats of these bacteria are the subsurface zones of aquatic environments, such as marine sediments, rice paddies and anaerobic digesters of sewage plants, sheep rumen and human intestines. Gram-negative SRB are found in the families; the Desulfovibrionaceae and the Desulfobacteriaceae, and in the genera *Thermodesulfobacterium* and *Thermodesulfovibrio.*

Table 1.4. Some important characteristics of representative SRP (Adapted from Castro et al. 2000).

	Cell shape	Motility	GC content of DNA (mol%)	Oxidation of organic substrates	Growth temper -ature (°C)
Gram-negative mesophilic SRB					
Desulfobulbus	Lemon to rod	-/+	59-60	Incomplete	25-40
Desulfomicrobium	Ovoid to rod	+/-	52-67	Incomplete	25-40
Desulfomonas	Rod	-	66	Incomplete	30-40
Desulfovibrio	Spiral to vibrioid	+	49-66	Incomplete	25-40
Desulfobacter	Oval to rod	+/-	44-46	Complete	20-33
Desulfobacterium	Oval to rod	+/-	41-52	Complete	20-35
Desulfococcus	Spherical or lemon	-/+	46-57	Complete	28-35
Desulfomonile	Rod	-	49	Complete	37
Desulfonema	Filaments	Gliding	35-42	Complete	28-32
Desulfosarcina	Oval rods or coccoid	+/-	51	Complete	33
Gram-positive spore-forming SRB					
Desulfotomaculum	Straight to curved rods	+	48-52	Incomplete / Complete	25-40 (most) 40-64 (some)
Desulfosporosinus	Straight to curved rods	+	45-46	Incomplete / Complete	30
Thermophilic SRB					
Thermodesulfobacterium	Vibrioid to rod	-/+	30-38	Incomplete	65-70
Thermophilic SRA					
Archaeoglobus	Coccoid	+/-	41-46	Incomplete	64-92

The Desulfovibrionaceae family is comprised of the genera *Desulfovibrio*, *Desulfomonas*, *Desulfohalobium*, *Desulfonatronum* and *Desulfomicrobium*. The most well studied genus of this group is *Desulfovibrio*. These SRB are usually curved, motile rods that oxidize lactate, pyruvate, ethanol, malate and fumarate incompletely, forming acetate; H_2 can also be used as an electron donor if acetate is supplied as a carbon source (Widdel & Bak 1992). The members of this genus have been found to form syntrophic relationships with H_2-utilizing microorganisms, such as methanogens, as described in Section 1.4.2.4. Another member of this family, *Desulfonatronum* spp., are obligately alkalophilic.

The Desulfobacteriaceae comprises the genera *Desulfobulbus*, *Desulfobacter*, *Desulfobacterium*, *Desulfococcus*, *Desulfosarcina*, *Desulfomonile*, *Desulfonema*, *Desulfobotulus*, *Desulfoarculus*, *Desulfobacula*, *Desulfospira*, *Desulfocella*, *Desulfobacca*, *Desulfacinum*, *Desulforhabdus*, *Desulfocapsa*, *Desulforhopalus* and *Desulfofustis*.

Desulfobulbus spp. ferment lactate in the absence of sulfate (Widdel & Bak 1992) and *Desulfobacter* spp. are the most effective acetate-oxidizers of all the Desulfobacteriaceae (Widdel & Bak 1992). *Desulfobacterium* spp. *Desulfococcus* spp. and *Desulfosarcina* spp. are metabolically versatile and can use fatty acids, alcohol, H_2, lactate and aromatic compounds as electron donors (Widdel & Bak 1992). *Desulfococcus* spp. can also oxidize various aromatic compounds (Peters & Rother 2004). *Desulfosarcina variabilis* is unique among the SRP as it can produce methane (Shcherbakova & Vainshtein 2000) and *Desulfomonile* spp. can completely oxidize acetate using thiosulfate as an electron acceptor. *Desulfonema* is the only gliding SRB that forms a multicellular filament (Widdel & Bak 1992) and *Desulfobacula toluolica* is a toluene degrader (Rabus et al. 1993). Two species in this family; *Desulfocapsa sulfoexigens* (Finster et al. 1998) and *Desulfocapsa thiozymogenes* (Janssen et al. 1996) are capable of sulfur disproportionation.

Two species of thermophilic Gram-negative SRB branch deeply in the *Bacteria* domain and have optimal growth temperatures between 65 and 70°C (Castro et al. 2000). *Thermodesulfobacterium commune* (Zeikus et al. 1983) and *Thermodesulfovibrio yellowstonii* (Henry et al. 1994) oxidize organic substrates incompletely to acetate, and use only a limited number of electron donors.

Other thermophilic Gram-negative SRB belong to the Desulfobacteriaceae. These are *Desulfacinum* spp., which can use sulfite and thiosulfate as electron acceptors (Rees et al. 1995; Sievert & Kuever 2000) and *Thermodesulforhabdus norvegicus* which is a sulfate- and sulfite-reducer (Beeder et al. 1995).

1.4.1.1.2 Gram-positive SRB

The Gram-positive SRB comprises two genera; *Desulfotomaculum* and *Desulfosporosinus* (Castro et al. 2002).

Desulfotomaculum spp. exhibit diverse physiologies, such as complete or incomplete oxidation of organic substrates and a range of temperature optima. Most have been isolated from habitats with low salt concentrations including soils, geothermal groundwater and thermophilic bioreactors (Fauque 1995). Some species of *Desulfotomaculum* are thermophilic and form heat-resistant endospores. *Desulfotomaculum* spp. can use various electron donors including acetate, acetone, aniline, catechol, ethanol, indole, nicotinate, phenol, stearate and succinate (Castro et al. 2000).

The genus *Desulfosporosinus* contains three recognized species; *Desulfosporosinus orientis* (formally known as *Desulfotomaculum orientis*), D. *meridiei, D. auripigmenti*, and two proposed species; "*D. idahoense*" and "*D. limneticum*". *D. orientis* can oxidize methoxy carbon and methanol to carbon dioxide, coupled to sulfate reduction, and can ferment methanol using CO_2 as an electron acceptor (Hanselmann et al. 1995). This bacterium is also capable of autotrophic growth on H_2 using CO_2 as a carbon source and SO_2 as an electron acceptor (Lee et al. 1994; Lee & Sublette 1994). *D. meridiei* was first isolated from groundwater contaminated with benzene, toluene, ethylbenzene and xylene (Robertson et al. 2001). Like *D. orientis, D. auripigmenti* was reclassified (originally a *Desulfotomaculum* sp.) and has physiological traits that distinguish it from both *D. orientis* and *D. meridiei*, including lack of motility and a smaller cell diameter (Stackebrandt et al. 2003). *D. auripigmenti* can also be differentiated from other species by its ability to use arsenate and thiosulfate, as well as sulfite and sulfate, as electron acceptors. It forms golden-colored colonies when grown on arsenate and sulfate, due to the production of As_2S_3 (Newman et al. 1997).

1.4.1.2 Sulfate reducing archaea (SRA)

Only one genus of sulfate-reducing archaea, *Archaeoglobus*, has so far been recognized. It belongs to the Euryarchaeota kingdom, along with

methanogens and extreme halophiles (Thaier & Kunow 1995). This archaeon has been isolated from highly saline thermal environments, such as hydrothermally-heated shallow marine sediments and abyssal hot sediments (Thauer & Kunow 1995). This genus currently comprises three species: *Archaeoglobus fulgidus, A. profundus* and *A. veneficus*.

1.4.2 Physiology and ecology of SRP

1.4.2.1 Dissimilatory sulfate reduction

In dissimilatory sulfate reduction, various organic compounds and, in some cases molecular hydrogen, may be used as electron donors. The reduction of sulfate to sulfide (an eight electron reduction) is achieved by several stages. In order to initiate sulfate reduction, sulfate has to be transferred across the cytoplasmic membrane as sulfate reduction takes place in the cytoplasm. This is an energy-consuming process if the sulfate concentration in the environment is low. The uptake of sulfate can be achieved by symporting protons (in freshwater environments) or sodium ions (in marine environments; Cypionka 1995). As sulfate is a highly stable anion, ATP is required to activate it, prior to reduction.

Sulfate is activated by reaction with ATP, forming adenosine-5'-phosphosulfate (APS) and releasing pyrophosphate (PP). The reaction is catalyzed by ATP sulfurylase (Peck 1962; Equations 1.9 and 1.10).

$SO_4^{2-} + ATP + 2H^+ \rightarrow APS + PP$ $\Delta G^{0'} = +46$ kJ/mol (1.9)

$PP + H_2O \rightarrow 2P$ $\Delta G^{0'} = -22$ kJ/mol (1.10)

Equation 1.9 shows the formation of APS. The pyrophosphate formed is hydrolyzed to phosphate by pyrophosphatase, thereby promoting the continued formation of APS (Akagi 1995). The activation of sulfate requires the energy equivalent of hydrolysis of two ATP molecules to ADP (Cypionka 1995). Other microorganisms that are unable to carry out dissimilatory sulfate reduction but can reduce sulfite to sulfide, are assumed to lack the APS system. Once APS is formed, it is directly reduced to bisulfite (HSO_3^{2-}) by APS reductase, releasing AMP (Equation 1.11).

$APS + H_2 \rightarrow HSO_3^- + AMP + H^+$ $\Delta G^{0'} = -69$ kJ/mol (1.11)

Electrons used to reduce APS (catalyzed by APS reductase) are obtained from the oxidation of H_2 by hydrogenase. Reactions 1.9, 1.10 and 1.11 are summarized in Equation 1.12, which shows the net transformation of sulfate to bisulfite:

$$SO_4^{2-} + 2ATP + H_2 + H^+ + H_2O \rightarrow HSO_3^- + 2ADP + 2P$$

$$\Delta G^{0'} = -45 \text{ kJ/mol} \quad (1.12)$$

Bisulfite is further reduced to form sulfide by dissimilatory bisulfite reductases (Fauque et al. 1991; LeGall & Fauque, 1988). In contrast to assimilatory sulfate reduction, sulfide is formed as the end product of dissimilatory sulfate reduction, and is excreted by SRP. Electrons from various donors, which are used for reduction of sulfate and sulfite, are transported via cytochrome c_3, as illustrated in Figure 1.2. Hydrogen, sourced directly from the environment or generated from organic electron donors is oxidized by periplasmic hydrogenase (Figure 1.2). Protons from the oxidation of hydrogen remain outside the cytoplasmic membrane, and electrons are transferred across the membrane and used in sulfate reduction. The proton motive force generated from this system can be used for ATP synthesis.

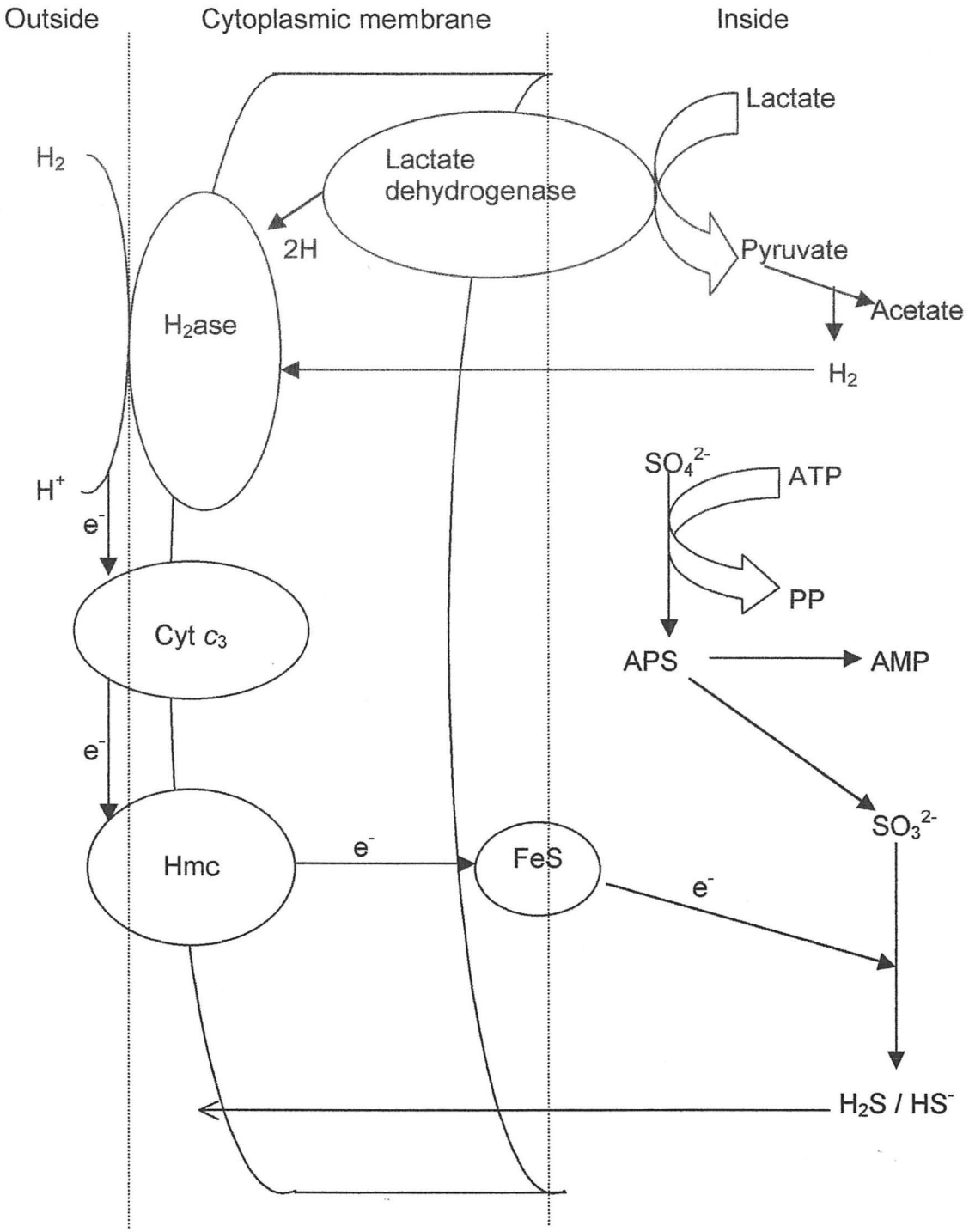


Figure 1.2. Electron transport and generation of a proton motive force in SRB (adapted from Madigan et al. 1997). Key: (H_2ase) Hydrogenase; (Cytc_3) Cytochrome c_3; (Hmc) cytochrome complex.

Some SRP can grow using molecular hydrogen as electron donor (Equation 1.13), and may grow as chemolithotrophs, using CO_2 as carbon source, whilst other hydrogen-oxidizing SRP require an organic carbon source, such as acetate (Widdel & Hansen 1992).

$$4H_2 + SO_4^{2-} + 2H^+ \rightarrow 4H_2O + H_2S \qquad (1.13)$$

In this reaction, molecular hydrogen is separated into protons and electrons by periplasmic or cytoplasmic hydrogenases. The electrons released from the reaction are used for sulfate reduction, and protons are used to generate ATP by creating a proton motive force (Widdel & Hansen 1992). Protons in the cytoplasm are then used to form sulfide during sulfate reduction. They can also be excreted from the cell to be used to symport sulfate into the cytoplasm.

As a physiological group, SRP are capable of using a variety of organic compounds including alcohols, organic acids, aromatic compounds and fatty acids. Alcohols, for example ethanol, are converted to acetyl-CoA via acetaldehyde by unknown electron or hydrogen carriers (Widdel & Hansen 1992). Oxidation of one of widely utilized organic acid, lactate, to pyruvate is mainly mediated by membrane-bound lactate dehydrogenases. Pyruvate produced from lactate oxidation is decarboxylated to acetyl-CoA (Ogata & Yagi 1986). These reactions are summarized in Figure 1.3.

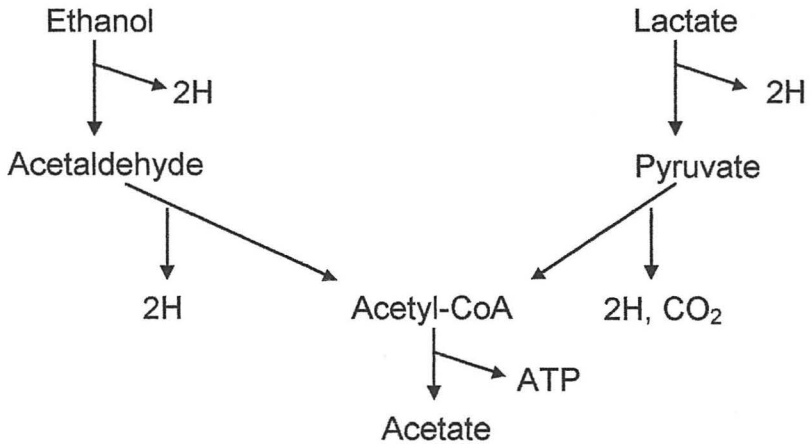


Figure. 1.3. Schematic diagram showing ethanol and lactate oxidation by SRP.

SRP that use organic compounds as electron donors can be divided into complete- or incomplete-oxidizers. The former produce only CO_2 as an end product whilst the latter produce and excrete various organic compounds including acetate, propionate (from *n*-propanol), butyrate (from *n*-butanol) or isobutyrate (from isobutanol; Widdel & Hansen 1992). The incomplete oxidation of organic substrates is due to the lack of an acetyl-CoA oxidation mechanism. Some complete-oxidizing SRP, however, excrete acetate when grown on organic substrates and only utilize this excreted acetate when the original substrate is depleted (Widdel & Hansen 1992). This phenomenon is a consequence of the rate of acetyl-CoA formation process being faster than its terminal oxidation, and the excess acetyl-CoA is converted to acetate, and excreted. The oxidation of acetate to CO_2 in SRP has been shown to be carried out via a modified citric acid cycle, or the carbon monoxide/C1-pathway. In the modified citric acid cycle, acetate is activated to acetyl-CoA and ATP is generated from conversion of acetyl-CoA to citrate (Madigan et al. 1997). The more commonly used acetate-oxidizing pathway is the carbon monoxide/C1 pathway which is the reverse system of acetyl-CoA pathway (Widdel & Hansen 1992). In this pathway, acetate is oxidized to CO_2, releasing H_2.

1.4.2.2 Elemental sulfur reduction and disproportionation

In addition to sulfate-reducing prokaryotes discussed above, some microorganisms can reduce elemental sulfur to sulfide but not sulfate to sulfide, and are referred to as dissimilatory sulfur-reducing bacteria (Madigan et al. 1997). This group of bacteria includes *Desulfuromonas, Desulfurella* and *Campylobacter.*

Another sulfide-generating process is called disproportionation. This is a process by which an element or compound in an intermediate oxidation state is converted to substances of higher and lower oxidation states (Jackson & McInerney 2000). This process does not require an extraneous electron acceptor as both atoms/molecules that donate and accept electrons are in the same molecule. Disproportionation of reduced inorganic sulfur compounds (RISCs) by SRB, such as *Desulfovibrio sulfodismutans,* isolated

from freshwater, brackish water or marine sediments was first discovered by Bak and Cypionka in 1987. An example of RISCs disproportionation, thiosulfate disproportionation by *Desulfovibria desulfuricans*, is described in Equations 1.14, 1.15 and 1.16 (Cypionka et al. 1998).

$$S_2O_3^{2-} \rightarrow S^0 + SO_3^{2-} \quad (1.14)$$

$$SO_3^{2-} + 0.5H^+ \rightarrow 0.25H_2S + 0.75SO_4^{2-} \quad (1.15)$$

$$S^0 + H_2O \rightarrow 0.75H_2S + 0.25SO_4^{2-} + 0.5H^+ \quad (1.16)$$

In Equation 1.14, thiosulfate is transformed into intermediates, i.e. S^0 and SO_3^{2-}, and these are disproportionated to sulfide (1.15) and sulfate (1.16). The net reaction of Equations 1.14, 1.15 and 1.16 is described as Equation 1.17.

$$S_2O_3^{2-} + H_2O \rightarrow H_2S + SO_4^{2-} \quad (1.17)$$

The overall free energy release is -21.9 kJ/mol thiosulfate.

Disproportionation of RISCs in an anaerobic condition has been mainly studied with the Gram-negative SRB in the *δ-Proteobacteria* and they were thought to be the only microorganisms that were capable of carrying out this metabolism. However, Gram-positive thermophilic SRB *Desulfotomaculum thermobenzoicum* (Jackson & McInerney 2000) were also been found to carry out thiosulfate disproportionation.

1.4.2.3 Interactions of SRP with other microorganisms

Like many other microorganisms in their natural environments, SRP interact with other indigenous species. Amongst the more important of these interactions are competition (e.g. for organic substrates) and syntrophy. Syntrophic interactions seem particularly important in the case of SRP. In a syntrophic relationship, the degradation of a substrate by one species is made thermodynamically possible through the removal of an end product by another species (Schink 1997). Hydrogen is a common end product of fermentative metabolism, and the oxidation of this gas, coupled to sulfate-reduction by SRP, may result in a change in the overall free energy (ΔG) thereby allowing an otherwise thermodynamically unfeasible reaction to proceed (Jackson & McInerney 2002). Interactions of this type can be

observed in a wide range of anaerobic environments, including sewage digesters, municipal landfills, hydrocarbon-contaminated soils, freshwater sediments and waterlogged soils.

An example of syntrophy was described by Jackson et al. (1999), involving *Syntrophus aciditrophicus* and *Desulfovibrio* strain G11. The oxidation of benzoate by *Syntrophus aciditrophicus* was shown to be only made possible when hydrogen was removed by hydrogen-utilizing *Desulfovibrio* strain G11, as benzoate degradation reaction is energetically unfavorable (Equation 1.18).

$$Benzoate^- + 7H_2O \rightarrow 3Acetate^- + HCO_3^- + 3H^+ + 3H_2$$

$$\Delta G^{0'} = +71 \text{ kJ/mol benzoate} \qquad (1.18)$$

However, in the presence of H_2-utilizing SRB, benzoate degradation becomes energetically favorable, as shown in the Equation 1.19.

$$4Benzoate^- + 3SO_4^{2-} + 16H_2O \rightarrow 12Acetate^- + 4HCO_3^- + 3HS^- + 9H^+$$

$$\Delta G^{0'} = -43 \text{ kJ/mol benzoate} \qquad (1.19)$$

Symbiotic associations between SRB and archaea have also been observed in microbial aggregates found in the crest of the southern Hydrate Ridge. Fluorescent *in situ* analysis of these aggregates revealed methanogenic archaea surrounded by SRB, related to *Desulfosarcina variabilis* (Boetius et al. 2000). In this consortium, the microbial population is assumed to be sustained by anaerobic oxidation of methane and sulfidogenensis, as described in Equation 1.20.

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O \qquad (1.20)$$

Boetius et al. (2000) proposed that reverse methanogenesis by methanogenic archaea is supported by effective removal of the end products, such as H_2 and acetate, by SRB.

1.5 Acid mine drainage (AMD)

1.5.1 Nature and characteristics of AMD

Acid mine drainage (AMD) is generated by oxidation of sulfur and/or sulfide minerals, and often results in the production of sulfuric acid. As AMD also frequently contains elevated concentrations of heavy metals (some of which are highly toxic), it can cause significant damage to adjacent and downstream environments. In many disused mine sites, such as Cae Coch in north Wales (U.K.), AMD is constantly released into the environment and has major impact on surrounding ecosystems (Banks et al. 1997). In many active mine sites, AMD produced from mining activities is contained in reservoirs to control the discharge. Although the release of AMD is monitored and regulated, accidental release may occur, and this can have devastating consequences. One of the incidents that illustrates the risk of uncontrolled discharge of AMD was the collapse of a tailings dam at the Los Frailes mine in Spain. This incident lead to the release of 5-7 x 10^6 m^3 acid sludge and water into the river Guadiamar (van Geen & Chase 1998). The discharge of AMD resulted in high input of Ag, As, Cd, Cu, Fe, Pb and Zn into the river (Achterberg et al. 1999) and had a devastating, though short-term, effect on aquatic life. This event highlights the hazard of AMD, the importance (where possible) of preventing AMD formation, and also the need to remediate AMD. There are various ways in which the formation of AMD might be prevented, and also a number of alternative strategies for remediating AMD, as summarized in Table (1.5).

Table 1.5. List of available AMD prevention and treatment options (Johnson & Hallberg 2002).

Prevention	➢ Flooding / sealing of underground mines ➢ Underwater storage of mine tailings ➢ Land-based storage in sealed waste heaps ➢ Coating technologies ➢ Application of anionic surfactants	
Treatment	Non-biological	➢ Active systems: Aeration/Lime addition ➢ Passive systems: Anoxic limestone drains
	Biological	➢ Active systems: Off-line sulfidogenic bioreactors/ Accelerated iron reduction
		➢ Passive systems: Aerobic wetlands/ Compost reactors/wetlands

1.5.2 Prevention of AMD formation

AMD production may be prevented or minimized by preventing contact of sulfidic minerals with either air or water, since these minerals are stable in dry or anoxic situations. This can be achieved by covering and sealing waste sulfidic heaps with dry (soil, plastic or clay) covers, or keeping sulfidic tailings under water preventing them to come in contact with air (Kuyucak 2002). Sulfide minerals such as pyrite can also be coated with phosphate to reduce their rate of oxidation (Nyavor & Egiebor 1995). Chemicals, such as anionic surfactants that are toxic to iron-oxidizing microorganisms that play key roles in AMD genesis, have been used to control AMD production (Clark 1995).

1.5.3 Treatment of AMD

Although preventative methods can be a practical approach especially in working mines, treatment of AMD is often unavoidable process in both working and disused mines. Treatment systems can be divided into non-biological and biological, as described below.

1.5.3.1 Non-biological treatment

In non-biological active systems, neutralization of effluents is achieved by adding alkali-generating chemicals such as lime (CaO) or limestone ($CaCO_3$). When lime is dissolved in water (Equation 1.21), it becomes calcium hydroxide and alkalinity is generated when calcium hydroxide comes contact with acidic effluents as shown in Equation 1.22.

$$CaO + H_2O \rightarrow Ca(OH)_2 \quad (1.21)$$

$$Ca(OH)_2 + 2H^+ + SO_4^{2-} \rightarrow CaSO_4 + 2\,H_2O \quad (1.22)$$

As shown in Equation 1.22, gypsum ($CaSO_4$) is produced. As a result of raised pH, many dissolved metals precipitate out of the effluent and the precipitated metals (along with the gypsum) can be collected as a bulky sludge. Another way to add alkalinity to AMD, but which avoids oxidizing the dissolved iron present, is to use an anoxic limestone drains (ALD). In this system, AMD passes through limestone beds that are kept anaerobic by coating with clay and plastic liners. When operated ideally, this system can increase pH to near neutrality. ALDs are not suitable, however, for treating

aluminium-rich AMD, as this results in the accumulation and blocking of drains by gelatinous aluminium hydroxides (Gazea et al. 1996).

The main problem in using liming materials to remediate AMD is that it results in an end product that is both bulky and requires disposal. Also in the case of drainage from metals mines, the sludge produced will contain a variety of heavy metals (as their hydroxides and carbonates) other than iron. This further complicates the disposal issue, in terms of the perceived hazard of the waste.

1.5.3.2 Biological treatment

As an alternative to non-biological AMD treatment systems, various biological options have been investigated and developed. As shown in Table 1.5, biological systems can be divided into to two categories: active and passive treatment.

1.5.3.2.1 Active biological treatment

Examples of active biological AMD treatment systems are use of accelerated iron oxidation by immobilized biomass and of off-line sulfidogenic bioreactors. The former approach uses immobilized biomass of iron-oxidizing bacteria to convert ferrous iron to ferric; the oxidized species is highly insoluble at pH >2.5, and spontaneously hydrolyzes and precipitates.

In an off-line sulfidogenic bioreactor system, hydrogen sulfide produced by SRB in a bioreactor is used as a source of alkalinity, and also to precipitate many of the dissolved metals present in AMD (Tabak et al. 2003). As SRB are sensitive to acidic water and high concentrations of dissolved metals, direct contact with AMD is inhibitory to biogenic hydrogen sulfide production (Poulson et al. 1997). Therefore it is necessary to isolate the sulfidogenic bioreactor and the metal precipitation tanks in these systems.

A study using bench-scale sulfidogenic bioreactors, where biogenic sulfide gas was used to selectively remove dissolved metals in AMD, showed

successful precipitation of Cd, Co, Cu, Fe, Mn, Ni and Zn (Tabak et al. 2003). In this system, AMD containing dissolved metals was treated in several stages and the pH was altered at each stage to selectively precipitate metals. Selective separation of metals can also be achieved by altering the redox potential of a solution in a precipitation container by regulating the flow of sulfide-containing solution (Pott & Mattiasson 2004). The selectively precipitated metal sulfides are more readily recycled than a mixture of metal sulfides. In order to make the system more cost-effective, hydrogen may be used as an electron donor rather than ethanol or acetate (Tabak & Govind 2003). An example of such a system in operation is a water treatment plant at the Budelco zinc refinery in the Netherlands. This refinery has caused considerable heavy metals and sulfate contamination of the groundwater below the site, and a commercial-scale off-line biological water treatment plant to remediate this was first installed in 1992. The initial system used ethanol as electron donor for the SRB, but more recently (1998) a new system using H_2/CO_2 derived from natural gas has been installed. The excess sulfide gas produced by the sulfidogenic reactor is biologically oxidized to elemental sulfur in an aerobic reactor (Boonstra et al. 1999) to prevent re-generation of sulfuric acid.

Apart from off-line systems, fluidized-bed reactors can be used to treat AMD. With these, biomass is retained on an inert carrier material in the reactor, and mass transfer of substrates and sulfide gas is superior to packed-bed reactors (Kaksonen et al. 2003a). Unlike off-line bioreactors, dissolved metal-containing water is directly pumped into the fluidized-bed reactors. The system was shown to remove soluble Zn and Fe by precipitation as metal sulfides, and also raised the pH from 2.5 to 7.9 by alkalinity generated by sulfidogenesis (Kaksonen et al. 2003b).

In most sulfidogenic reactors, mixed cultures of SRB and other prokaryotes are used as inocula. The use of a mixed population is particularly important if complex organic compounds or mixtures of organic substrates are used as

electron donors. Organic compounds such as lactate can be incompletely oxidized to acetate, and other SRB that are capable of acetate oxidation can (in theory) carry out further sulfidogenesis, thereby maximizing the efficiency. This is also important in order to minimize the amount of organic compounds contained in discharged effluents. Microorganisms, other than SRB, that have been detected in sulfidogenic reactors include bacteria related to *Geobacter*, *Magnetobacterium* and *Spirochaeta* (Kaksonen et al. 2004). Methanogens have also been found in these systems (Visser et al. 1996). However, since methanogens (and some other anaerobes) are known to compete with SRB for acetate and H_2, this results in reduced efficiency of the sulfidogenic reactor, compared to situations where complete substrate oxidation is carried out by SRB alone.

1.5.3.2.2 Passive biological treatment

Passive biological treatment of AMD involves either the use of (usually constructed) aerobic wetlands and/or compost reactor/wetlands. Passive treatment systems take advantage of natural biological processes to remediate AMD and are often the preferred choice for long-term AMD treatment projects due to their low maintenance costs (Gazea et al. 1996). Aerobic wetlands are used to treat "AMD" that is net alkaline, due to bicarbonate alkalinity naturally present or added *via* ALDs. Iron oxidation and precipitation occurs spontaneously at pH >4, though neutrophilic and moderately acidophilic iron-oxidizing bacteria present in these wetlands also appear to contribute to net iron oxidation (Hallberg & Johnson 2003). Aerobic wetlands can remove iron, and also other metals and metalloids (such as arsenic) that co-precipitate with ferric iron colloids.

Another passive biological treatment option are compost bioreactors/wetlands. In these systems, anaerobic reactions are used to generate alkalinity, and metals are removed by adsorption onto organic matter, and by precipitation reactions (chiefly as carbonates and sulfides). AMD is constrained to flow through anaerobic, organic-rich compost layers (to which limestone may be added to provide a further source of alkalinity)

using hydraulic pressure supplied by gravity (necessary for these to qualify as passive systems). Alkalinity is generated by a number of dissimilatory reductive processes, most notably microbial iron- (Equation 1.23) and sulfate-reduction (Equation 1.24). Alkalinity can also be generated as a result of methanogenesis (Equation 1.25).

$$Fe(OH)_3 + e^- \rightarrow Fe^{2+} + 3OH^- \quad (1.23)$$

$$SO_4^{2-} + 9H^+ + 8e^- \rightarrow HS^- + 4H_2O \quad (1.24)$$

$$CO_2 + 2\,H_2 + 4H^+ + 4e^- \rightarrow CH_4 + 2\,H_2O \quad (1.25)$$

Although the advantages in using passive biological AMD treatment system are described above, several problems associated with passive biological AMD treatment have been identified (Johnson & Hallberg 2002). The problems include production of excess sulfide, which results in discharge of gaseous sulfide and other reduced inorganic sulfur compounds, and oxidation of sulfide and ferrous iron, which generates acidity. In order to prevent such problems, more research should be carried out to understand the complex mechanism involved in passive biological treatment system.

1.6 Methods used for studying acidophiles

Prior to the development of biomolecular techniques, identification and classification of microorganisms depended exclusively on culture-dependent methods, including the isolation of pure cultures and defined co-cultures followed by multiple physiological and biochemical tests (Amann et al. 1995). Improvements in cultivation techniques have resulted in the isolation and characterization of many novel acidophiles and have contributed to the appreciation of the biodiversity of these microorganisms (Hallberg & Johnson 2001). However, it has been apparent for many years that culture-dependent techniques are not capable of targeting all microorganisms in acidic, as in other, environments. Enumeration of viable microorganisms from environmental samples has traditionally been carried out using plating (solid media) count and most-probable-number (MPN; liquid media) techniques. However, total counts (e.g. from direct microscopy) of microorganisms in environmental samples generally greatly exceed plate- or MPN-counts, the

so-called "great plate count anomaly" (Staley & Konopka 1985). This phenomenon is caused by the fact that a large proportion of microorganisms is unculturable using currently-available approaches. To overcome this problem, culture-independent techniques that do not rely on microorganisms being isolated, have been developed. In this section, culture-dependent and culture-independent techniques, as applied to the study of acidophilic microorganisms, are described.

1.6.1 Cultivation-dependent methodologies

Culture-dependent techniques have been used for a number of years for the study of acidophilic microorganisms. Enumeration, plate isolation and enrichment culture techniques are described in this section.

1.6.1.1 Enumeration of acidopiles

There are three commonly-used enumeration techniques: direct counts, most probable number counts and plate counts.

Direct counts may be carried out using a (phase-contrast) microscope and bacteria counting chambers (e.g. Thoma chambers). Although this is a simple and fast method to enumerate microorganisms, it generally requires relatively large numbers of microorganisms (> *ca.* 10^6 cells/ml), and is subject to errors if the sample solution contains small non-biological materials. For samples containing fewer microorganisms, cells can be concentrated by fixing onto membrane filters, and stained with e.g. DNA-binding dyes (such as DAPI), and counted using a (fluorescence) microscope. A limitation of enumerating populations in this way is that different physiological groups of microorganisms cannot be differentiated (e.g. autotrophic and heterotrophic acidophilic bacteria).

Most probable number counts involve making a series of dilutions (in multiple replicas) of samples, incubating cultures and testing for positive or negative growth. A problem associated with this technique is that some microorganisms may not be able to grow in the medium used. The plate count technique is a commonly used method for enumerating viable

microorganisms. This method can be very sensitive, allowing enumeration of <10 cells/ml sample. Plate counts also allow preliminary differentiation and (in some cases) identification of microorganisms based on their colony characteristics. However, as with the MPN technique, a major disadvantage of the plate count method is that not all of the indigenous microorganisms in an environmental sample are likely to grow on the solid media used.

1.6.1.2 Plate isolation

The plate isolation technique is commonly used to obtain pure cultures of microorganisms from environmental samples. In this technique, a liquid sample (or aqueous suspension) is streaked or spread onto a gelled medium, and single colonies are selected and re-streaked (several times) to obtain a pure isolate. However, attempts to isolate acidophiles using this approach met with little success for many years, and researchers reverted to serial dilution in liquid media to secure pure cultures. The latter approach is prone to significant error, however, in the case of acidophiles. For example, numbers of acidophilic heterotrophs often exceed those of iron-oxidizing autotrophs, even in "inorganic" ferrous iron medium (Johnson & Kelso 1983). Problems with growing acidophiles on solid media generally derive from impurities in the gelling agent use (e.g. bacteriological agar) and from on-going hydrolysis of the gelling agent during plate incubation. Agar is a polysaccharide and hydrolyzes under acidic conditions, releasing small molecular weight organic compounds, including pyruvic acid, which are toxic to acidophiles (Johnson 1995). Many acidophiles are sensitive to organic acids in general, due to the fact that many of these exist in their undissociated (protonated) states at the pH range in which acidophiles are active (Section 1.2.4). Washing of agar(ose) with dilute acid can remove much of the more easily hydrolysed components of the polymer without damaging the polymer itself, but there remains the problem of hydrolysis of the gelling agent during incubation. To eliminate both problems, the "overlay" medium was designed (Johnson & McGinness 1991; Johnson 1995). In this, heterotrophic acidophiles (*Acidiphilium* SJH or *Acidocella* WJB-3) are used to inoculate an underlayer of a solid medium which is then covered with a sterile overlayer. The heterotrophs in the lower layer continuously remove

potentially toxic small molecular weight organic compounds in the solid media, allowing more sensitive acidophiles, such as the iron-and sulfur-oxidizers, to grow. Figure 1.4 shows the general design of an overlay medium.

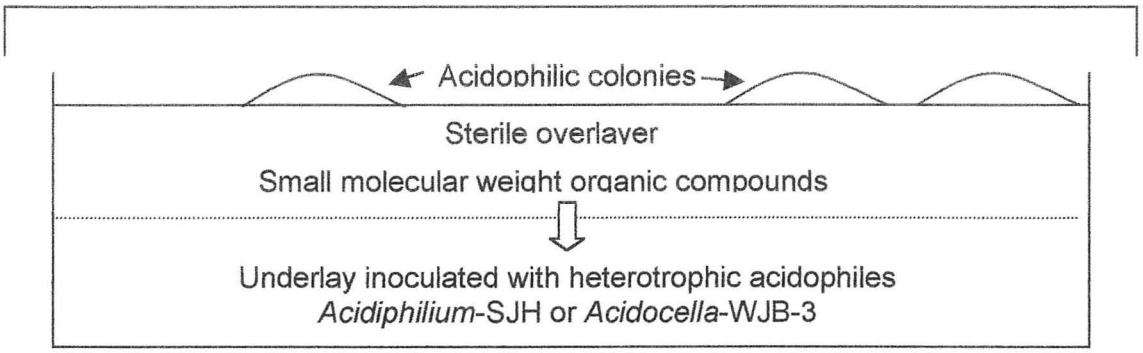


Figure 1.4. Diagram showing overlay medium used for acidophiles.

By varying the design and composition of the overlay technique, different media that specifically target certain acidophiles have been developed; Table 1.6 lists these media and their target microorganisms. With "iron overlay", "iron-tetrathionate overlay" and "iron thiosulfate overlay" media, the lower layer is inoculated with *Acidiphilium* SJH (Johnson & McGinness 1991), while the under-layers of "yeast extract 3" and "yeast extract 4" media are inoculated with *Acidocella* WJB-3 (Hallberg et al. 1999).

Table 1.6. List of overlay media used to isolate acidophilic prokaryotes.

Medium	Energy source	pH	Target
"Iron overlay"	Fe^{2+}/TSB*	2.6	Extremely acidophilic iron-oxidizers and heterotrophs
"Iron-tetrathionate overlay"	Fe^{2+}/tetrathionate /TSB*	2.6	Extremely acidophilic Iron oxidizers, sulfur oxidizers and heterotrophs
"Iron thiosulfate overlay"	Fe^{2+}/thiosulfate/ TSB*	4	Moderately acidophilic iron oxidizers, sulfur oxidizers and heterotrophs
"Yeast extract 3 overlay"	Yeast extract	3	Extremely acidophilic heterotrophs.
"Yeast extract 4 overlay"	Yeast extract	4	Moderately acidophilic heterotrophs.

TSB* = tryptone soya broth

1.6.1.3 Enrichment cultures

To isolate target acidophiles from environmental samples, enrichment culture techniques can be used in tandem with plate isolation. Using this approach it is possible to enrich selectively for a target microorganism(s), which might be present in relatively low numbers in an environmental sample. To do this, samples are inoculated into liquid media designed to promote the growth of the target microorganisms and cultures are incubated for periods of up to several weeks. The enrichment culture is then streaked or spread onto plates to obtain colonies of the microorganisms of interest. Some examples of enrichment media and their target acidophiles are listed in Table 1.7.

Table 1.7. Enrichment media and their target acidophiles. *the medium designation refers to the major source(s) in the liquid media; **as listed in Table 1.6.

Enrichment medium*	Overlay plate**	Target acidophiles
"$FeSO_4$"	"Iron "	*At. ferrooxidans*
"Fe^{2+}/pyrite"	"Iron"	*Leptospirillum* spp.
"S^0"	"Iron-tetrathionate"	*At. thiooxidans*
"Fe^{2+}/yeast extract"	"Iron"	*Ferrimicrobium* spp.
"Fe^{2+}/yeast extract"	"Ironthiosulfate"	*Sulfobacillus* spp.
"Yeast extract"	"Yeast extract 3"	*Acidiphilium* and *Acidocella*
"Yeast extract"	"Yeast extract 4"	*Acidobacterium* and *Acidisphaera*

1.6.2 Cultivation-independent methods

In order to study microorganisms, including acidophiles, that cannot be cultivated by the available culture techniques, cultivation-independent approaches have been developed. This section will describe cultivation-independent techniques, including immunological techniques and nucleic acid-based techniques.

1.6.2.1 Immunological techniques

Immunoassays have been widely used to enumerate acidophilic microorganisms (Muyzer et al. 1987). In this technique, acidophiles are fixed onto nitrocellulose membranes prior to the treatment using antibodies. The primary antibody, which is designed specific to target microorganisms of

interest, is used to react with antigens of the microorganisms and then the secondary antibody, specific to the primary antibody, is applied. The enzyme that reacts with a colored substance can be attached to the secondary antibody in order to detect target microorganisms. This technique can be used not only for enumeration, but also for monitoring physiological states of acidophilic microorganisms.

1.6.2.2 Nucleic acid-based techniques

Methods for characterizing the microbiology of environmental samples using rRNA genes were developed in the 1980s (Amann 1995). These techniques are often based on the extraction and amplification of 16S rRNA genes. These are extracted from environmental samples and amplified by the polymerase chain reaction (PCR), using general or specific primers. The PCR products can be analyzed using various methods, as described below and summarized in Figure 1.5.

1.6.2.2.1 Terminal restriction fragment length polymorphism (T-RFLP) analysis

In this method, terminal restriction fragments from a PCR-amplified marker are differentiated according to their size (Marsh 1999). Fluorescently-labeled primers, which can be designed to target microorganisms, are used to amplify environmental DNA, and the products are digested with various restriction enzymes. The digested PCR products are purified to remove unincorporated primers, and the fragments of different sizes are separated using capillary or gel electrophoreses. The fluorescence is measured and the fragment sizes are determined using size standards. Fragments are indicated in the form of fluorescent peaks, with each peak representing one or more microorganism. The fragments obtained from environmental samples can be compared to the values available in a database for identification.

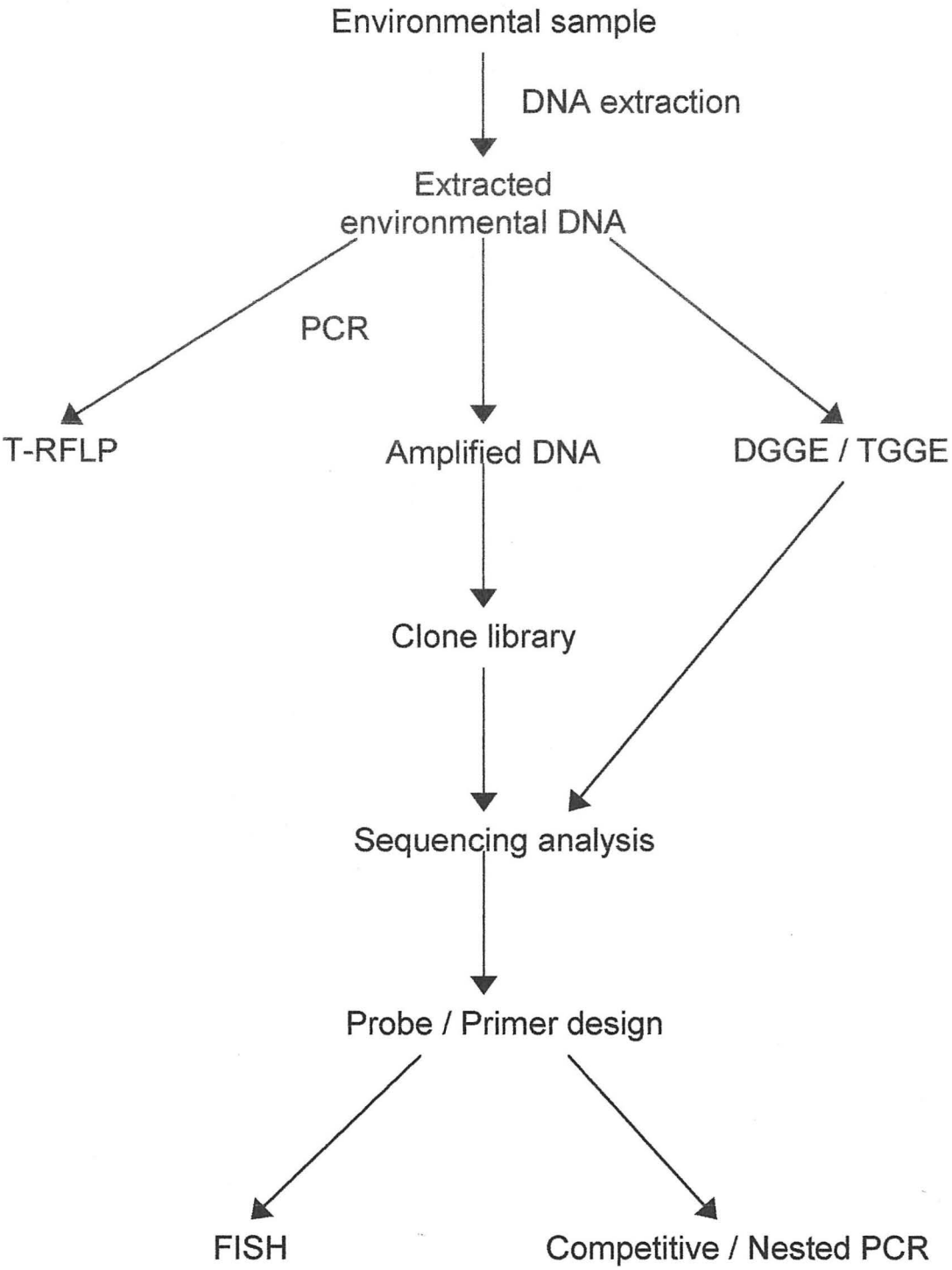


Figure 1.5. Schematic diagram showing nucleic acid-based techniques for studying microbial populations without the need for cultivation.

1.6.2.2.2 Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE)

In this technique, environmental DNA is amplified using a primer containing a "G+C clamp", which is rich in guanine and cytosine residues. The G+C clamp, incorporated in the amplified DNA fragments, acts as a high melting point domain and prevents the two DNA strands from dissociating into single strands (Muyzer & Smalla 1998). The sample is run on a polyacrylamide gel and DNA fragments of different sequences are separated on the basis of their electrophoretic mobilities. The separation can be achieved by varying the concentration of denaturant (a mixture of urea and formamide; DGGE) or *via* a temperature gradient (TGGE) of the gel. The melting of DNA fragments proceeds as "melting domains", which contain stretches of base-pairs with identical melting temperatures. The melting of DNA fragments at particular denaturant concentration or temperature halts their migration in the gel due to a transition of a helical to a partially-melted molecule (Muyzer & Smalla 1998). The variation in the melting temperature results from sequence differences, and therefore this technique enables separation of individual DNA fragments. The gel pattern can be used to show population diversity, and to identify bands if migration patterns of known microorganisms are available. The bands can also be excised from the gel to extract DNA for sequence analyses.

1.6.2.2.3 Cloning

The PCR products obtained from environmental samples can be used to construct a clone library. Individual 16S rRNA (or other) genes are ligated into a vector plasmid and the plasmid is incorporated into a host bacterium (usually *Escherichia coli*) in which multiple copies of the plasmids are made. The plasmids containing the inserted genes are then extracted and screened using techniques such as restriction fragment length polymorphism (RFLP). Cloned genes can also be sequenced and compared with known sequences in databases. The clone sequences obtained can be used to design primers and gene probes to target specific microorganisms.

1.6.2.2.4 Nested and competitive polymerase chain reactions

Primers designed to target desired microorganisms can be used in nested and competitive PCR to detect microorganisms in environmental samples. In nested PCR, primers specific to a certain group of microorganisms, for example Gram-positive bacteria- or *Proteobacteria*-specific primers, are used to amplify PCR products obtained by using more universal primers, such as domain-specific primers (Amann et al. 1995). This technique is very sensitive and can detect as few as 50 cells of the target organism in an environmental sample. Competitive PCR involves co-amplification of target DNA and an internal standard, or competitor DNA, which is similar but distinguishable from the target DNA (Phillips et al. 2000). This method may be used for quantification of a target microorganism in an environmental sample (Kondo et al. 2004).

1.6.2.3 Fluorescent in situ *hybridization (FISH)*

Nucleic acid probes can be designed when clone or TGGE/DGGE band sequences are available. The principal steps for the designing of probes are: (i) the alignment of rRNA (gene) sequences; (ii) the identification of unique sequences; (iii) synthesis of an oligonucleotide probe; (iv) labeling of the probe; (v) the experimental evaluation and optimization of the probe specificities and assay sensitivities (Amann et al. 1995). Accurate enumeration of active microorganisms in an environmental sample can be achieved by FISH as this technique is not subject to various biases encountered with PCR-based techniques (Wintzingerode et al. 1997). In FISH analysis, fluorescently-labeled oligonucleotide probes targeting rRNA are used to target active cells in a sample and the probes can be made universal or specific to target desired microorganisms. The number of the target cells can be enumerated proportional to total active cells in the sample. DNA-binding 4', 6-diamidino-2-phenylindole (DAPI) can also be used as a general stain to obtain total cell counts.

The most frequently encountered problem in FISH analysis is the low signal intensity. This is caused by low copy numbers of rRNA in inactive cells or difficulty in accessing the target site. The latter is caused by the three-dimensional structure of rRNA, and probe accessibility has been investigated in detail (Fuchs et al. 1998; Behrens et al. 2003). In order to target the region of rRNA that has poor accessibility, unlabeled helper oligonucleotides that target both sides or opposite of the probe site can be used (Fuchs et al. 2000). Since FISH can reveal the proportion of target microorganisms in environmental samples to an accurate degree, the technique has been used widely. FISH is particularly important to the study of biofilms as the technique allows enumerating the target as well as indicating the position of target cells within the biofilm. Examples of the application of this technique include a study of the spatial distribution of SRB in wastewater biofilms (Okabe et al. 1999) and investigation of submerged slimes and streamers in acid mine drainage site at Iron Mountain (Bond et al. 2000b).

1.7 Scope of the current project

This project had the following major aims; (i) to obtain a better understanding of microbial communities found in extremely acidic environments and (ii) to study sulfidogenesis at low pH.

The project structure can be sub-divided as follows:

1. Investigation of "acid streamer" microbial communities found in Trefriw spa (a chalybeate spa located in North Wales, U.K.) using cultivation-dependent and culture–independent techniques.
2. Investigation of microbial communities found within an abandoned pyrite mine (Cae Coch; North Wales, U.K.) using cultivation-dependent and cultivation–independent techniques.
3. Characterization of a novel acidophilic/acid-tolerant SRB isolates, grown in pure and mixed cultures.

In addition, a study was carried out on anaerobic metabolism by a novel thermo-acidophile that was isolated during the course of the study.

Chapter 2 Materials and Methods

The materials and methods described in this chapter are those used routinely throughout the project. Any modifications to these and specific procedures used for individual experiments are described in detail in respective chapters. All chemicals used in the research were supplied by Sigma-Aldrich (U.S.A.), Merck-BDH (U.S.A.) or Fisher Scientific (U.S.A.), unless stated otherwise.

2.1 Microorganisms

The microorganisms used in this study are listed in Tables 2.1 and 2.2. The codes in brackets are NCBI accession numbers.

Table 2.1. List of acidophilic/acid-tolerant sulfate reducing bacteria (aSRB) used in this study.

Isolate code	Nearest relative	Homology (%)	Reference
M1	*Desulfosporosinus orientis* (AJ493052)	94	Sen 2001
P1	*D. orientis*	95	Sen 2001
CC1	*D. orientis*	93	Sen 2001
PFB	*D. orientis*	95	Sen 2001

Table 2.2. List of acidophilic bacteria used in this study.

Isolate code	Nearest relative	Homology (%)	Reference
WJB-3 (AF253412) "*Acidocella aromatica*"	*Acidocella aminolytica*	97	Hallberg et al. 1999
PFBC	*"Acidocella aromatica"*	99	This study
SJH (AY040740)	*Acidiphilium cryptum*	99	Hallberg & Johnson 2001
Y008 (AY140238)	*Acidisphaera rubrifaciens*	93	Okibe 2002

2.2 Microbiological techniques

Isolation and maintenance of microorganisms were carried out using appropriate media and incubation conditions as described below.

2.2.1 Media and culture conditions

Both solid and liquid media were prepared using various basal salts formulations (designated "autotrophic", "heterotrophic" and "modified heterotrophic") and trace elements. These are listed in Tables 2.3 and 2.4. Basal salts were prepared as 50X concentrates, and the trace elements mix as a 1000X concentrate.

Table 2.3. List of the components of the different basal salts formulations used. The numbers in brackets are concentrations (in g/L) in the final media.

"Autotrophic" basal salts		"Heterotrophic" basal salts		"Modified heterotrophic" basal salts	
$(NH_4)_2SO_4$	(0.15)	$(NH_4)_2SO_4$	(0.45)	$(NH_4)_2SO_4$	(1.25)
KCl	(0.05)	KCl	(0.05)	$MgSO_4 \cdot H_2O$	(0.5)
$MgSO_4 \cdot 7H_2O$	(0.5)	$MgSO_4 \cdot 7H_2O$	(0.5)		
KH_2PO_4	(0.05)	KH_2PO_4	(0.05)		
$Ca(NO_3)_2 \cdot 4H_2O$	0.014)	$Ca(NO_3)_2 \cdot 4H_2O$	(0.014)		

Table 2.4. List of the components in the trace elements formulation. The numbers in brackets are concentrations (in mg/L) in the complete media.

Trace elements					
$ZnSO_4 \cdot 7H_2O$	(10)	$Cr_2(SO_4)_3 \cdot 15H_2O$	(0.5)	$NiSO_4 \cdot 6H_2O$	(1.0)
$CuSO_4 \cdot 5H_2O$	(1.0)	H_3BO_3	(0.6)	$Na_2SeO_4 \cdot 10H_2O$	(1.0)
$MnSO_4 \cdot 4H_2O$	(1.0)	$Na_2MoO_4 \cdot 2H_2O$	(0.5)	$Na_2WO_4 \cdot 2H_2O$	(0.1)
$CoSO_4 \cdot 7H_2O$	(1.9)	$NaVO_3$	(0.1)		

2.2.1.1 Liquid media

2.2.1.1.1 Liquid media for aerobic acidophiles

Liquid media used in this study are listed in Table 2.5. Solutions containing all components, except ferrous sulfate and potassium tetrathionate, were adjusted to the desired pH using sulfuric acid, and heat-sterilized (120°C for 20 minutes). Separately filter-sterilized (through 0.2 µm nitrocellulose membranes; Whatman, U.K.) 1 M ferrous sulfate (pH adjusted to 2.0 with

sulfuric acid) and 100 mM potassium tetrathionate stock solutions were prepared, and added to the cooled media as indicated in Table 2.5.

Table 2.5. List of liquid media used in routine experiments. HBS = "heterotrophic" basal salts, HBS* = "modified heterotrophic" basal salts; TSB = tryptone soya broth; TE = Trace elements.

Liquid medium	Contents	pH
"Iron/TSB"	HBS*, TSB (0.025%)**, $FeSO_4$ (25 mM).	2.5
"Iron-tetrathionate/TSB"	HBS*, TSB (0.025%)**, $FeSO_4$ (25 mM), $K_2S_4O_6$ (2.5 mM).	2.5
"Iron"	HBS, TE, $FeSO_4$ (10 mM).	2
"Yeast extract 3"	HBS, TE, yeast extract (0.02%)**, $FeSO_4$ (0.5 mM).	3
"Yeast extract 4"	HBS, TE, yeast extract (0.02%)**, $FeSO_4$ (0.5 mM).	4
Fructose	HBS, TE, fructose (5 mM), $FeSO_4$ (0.1 mM)	3

** Values in percentages are weight per volume.

2.2.1.1.2 Liquid medium for acidophilic/acid-tolerant sulfate reducing bacteria (aSRB)

A liquid medium containing heterotrophic basal salts, trace elements and 5 mM K_2SO_4, was prepared, adjusted to pH 4.0 using 1 M H_2SO_4, and deoxygenated by gassing with oxygen-free nitrogen (OFN) for 30 minutes. The medium (20 ml) was poured into 25 ml universal bottles and the bottles were stoppered with foam bungs. The medium was then heat-sterilized and, when cool, glycerol, $FeSO_4$ and $ZnSO_4$ were added from 1 M sterile stock solutions to final concentrations of 5, 0.1 and 5 mM, respectively. A vitamin mixture was also added at 1 ml/L. The mixture used was that described by Widdel & Pfenning (1981) and contained: 4-aminobenzoic acid (0.004% w/v); D(+)-biotin (0.001% w/v); nicotinic acid (0.01% w/v); Ca-D(+)-pantothenate (0.005% w/v); pyridoxine·HCl (0.015% w/v); and thiamine·HCl (0.01% w/v). The bottles were inoculated with single colonies of aSRB or liquid cultures and incubated anaerobically as described in Section 2.2.1.3.2.

2.2.1.2 Solid media

2.2.1.2.1 Non-overlay solid media

The compositions of the non-overlay solid media used in this study are listed in Table 2.6. For acidic solid media, solutions containing all components, except for agarose, ferrous sulfate and fructose, were adjusted to the target pH with sulfuric acid prior to heat sterilization (120°C for 20 minutes). Agarose (Sigma, Type I) was heat-sterilized separately as a 2% (w/v) suspension. The two solutions were cooled to around 50°C and combined (in the ratio 3 basal salts:1 agarose) prior to addition of filter-sterilized ferrous sulfate and heat-sterilized fructose stock solutions (each 1 M). The media were then dispensed into sterile plastic Petri dishes. For neutral pH R2A medium, all components, including agar, were heat-sterilized together.

Table 2.6. List of non-overlay solid-media. HBS = heterotrophic basal salts; TE = trace elements.

Solid medium	Contents	pH
"Yeast extract 3"	HBS, TE, yeast extract (0.02%)*, $FeSO_4$ (0.5 mM).	3
"Yeast extract 4"	HBS, TE, yeast extract (0.02%)*, $FeSO_4$ (0.5 mM).	4
"Fructose"	HBS, TE, fructose (5 mM), $FeSO_4$ (0.1 mM).	3
R2A	Yeast extract (0.5%)*, peptone (0.5%)*, casein hydrolysate (0.5%)*, glucose (0.5%)*, soluble starch (0.5%)*, sodium pyruvate (0.3%)*, K_2HPO_4 (0.3%)*, $MgSO_4 \cdot 7H_2O$ (0.05%)*.	7

*Values in percentages are weight per volume.

2.2.1.2.2 Overlay solid media

2.2.1.2.2.1 Overlay solid media for aerobic acidophiles

Five different overlay solid media were used for cultivating aerobic acidophiles as listed in Table 2.7. For acidic media, the acidified basal salts-containing solutions were heat-sterilized separately from the gelling (agarose) suspensions to avoid hydrolysis of the polysaccharide. Solutions of heat-labile components (ferrous sulfate and potassium tetrathionate) were all filter-sterilized. These solutions were mixed when still warm (*ca.* 50°C) and split into two lots. To one of these solutions, an active culture of the underlay heterotroph (*Acidiphilium* SJH or *Acidocella* WJB-3) was added (at 0.5%, v/v), the suspension mixed, and aliquots of about 20 ml dispensed into each

sterile Petri plate. When the underlay gel had set, it was covered with about 20 ml of sterile molten medium (the "overlayer"). *Acidiphilium* SJH was regularly subcultured in a liquid medium containing heterotrophic basal salts, 0.025% (w/v) tryptone soya broth, 10 mM galactose and 25 mM ferrous sulfate (to inoculate "iron overlay" plates), and the same medium containing 2.5 mM potassium tetrathionate (for "iron-tetrathionate" and "iron/thiosulfate" plates). *Acidocella* WJB-3 was regularly subcultured in fructose liquid medium (Table 2.5).

Table 2.7. List of aerobic overlay solid media. HBS = heterotrophic basal salts, HBS* = modified heterotrophic basal salts, TSB = tryptone soya broth; TE = Trace elements.

Overlay medium	Contents (final concentrations)	pH	Inoculum
"Iron"	HBS*, TSB (0.025%)**, $FeSO_4$ (25 mM).	2.5	*Acidiphilium* SJH
"Iron-tetrathionate"	HBS*, TSB (0.025%)**, $FeSO_4$ (25 mM), $K_2S_4O_6$ (2.5 mM).	2.5	*Acidiphilium* SJH
"Iron/thiosulfate"	HBS*, TSB (0.025%)**, $Na_2S_2O_3$ (10 mM), $FeSO_4$ (5 mM).	4	*Acidiphilium* SJH
"Yeast extract 3"	HBS, TE, yeast extract (0.02%)**, $FeSO_4$ (0.5 mM).	3	*Acidocella* WJB-3
"Yeast extract 4"	HBS, TE, yeast extract (0.02%)**, $FeSO_4$ (0.5 mM).	4	*Acidocella* WJB-3

** Values in percentages are weight per volume.

2.2.1.2.2.2 Solid media for aSRB

Diluted heterotrophic basal salts, trace elements, 5 mM K_2SO_4 and 0.02% (w/v) yeast extract were combined, the pH adjusted to 3.5 using H_2SO_4, and heat-sterilized. Filter-sterilized $FeSO_4$ or heat-sterilized $ZnSO_4$ was added from 1 M stock solutions to give final concentrations of 5 mM. When $ZnSO_4$ was used, $FeSO_4$ was added to the media (to 0.1 mM). A gelling solution containing 2% (w/v) agarose was autoclaved separately and combined (1:3, v/v) with the salts solution. The molten medium was divided into two parts; one was inoculated with actively-growing *Acidocella* WJB-3 or *Acidocella* PFBC and about 20 ml aliquots dispensed into sterile Petri plate. When the underlay gel had set, it was covered with about 20 ml of sterile molten medium. The solid medium was incubated at room temperature overnight to

allow the heterotrophs to remove potentially growth-inhibiting small molecular weight organic compounds. Finally, 0.2 ml of 1 M glycerol solution was spread on the surface of each plate and allowed to soak into the gel. This resulted in the solid medium containing *ca.* 5 mM glycerol.

2.2.1.3 Bioreactor medium

A 2L bench-scale bioreactor system, Electrolab P350 (Electrolab, U.K.), fitted with pH and temperature monitoring and control was used in this research project. The liquid medium used routinely in bioreactor experiments contained heterotrophic basal salts, trace elements and 5 mM K_2SO_4. The pH of the medium was adjusted to 4.0 using 1 M H_2SO_4 prior to heat sterilization at 120°C for 20 minutes. After the medium had cooled, heat-sterilized glycerol and $ZnSO_4$ solutions were added to give final concentrations of 5 mM of each. A vitamin mixture (Section 2.2.1.1.2) was added (at 1 ml/L), as was $FeSO_4$ (to a final concentration of 0.1 mM). The medium was deoxygenated with OFN for 30 minutes before inoculation with pure or mixed cultures of aSRB. The bioreactor culture was incubated at 30°C and stirred at 60 rpm with a continuous stream of OFN. The pH of the bioreactor culture was maintained between set values (generally ± 0.1 pH unit) by automatic addition of 0.1 M NaOH or 0.1 M H_2SO_4.

2.2.1.4 Culture incubation conditions

Cultures grown in liquid or on solid media were incubated under aerobic, micro-aerobic or anaerobic conditions.

2.2.1.4.1 Micro-aerobic atmospheres

Micro-aerobic atmospheres, in which oxygen concentrations were less than 5%, were generated by the Campy*Gen*™ CN25 system (Oxoid Ltd., U.K.) in 2.5 L anaerobic jars. Campy*Gen*™ Compact CN020C (Oxoid) sachets were used to generate micro-aerobic conditions in plastic pouches (which each held 2-4 Petri plates).

2.2.1.4.2 Anaerobic atmospheres

To isolate and maintain anaerobic microorganisms, anaerobic atmospheres were generated using the AnaeroGen™ AN25 system (Oxoid) in 2.5 L jars. In this system, no hydrogen is generated and oxygen is removed as CO_2. To generate anaerobic condition in a plastic pouches, AnaeroGen™ Compact sachets (Oxoid) were used. Anaerobic conditions were monitored using Anaerobic Indicators (Oxoid) throughout culture incubation.

A second anaerobic system used on occasions was the "Oxoid Anaerobic System". In this system, water is added to a sachet containing sodium borohydride, thereby releasing hydrogen. A palladium catalyst (activated by pre-heating to 160°C) held in the lid of the 3.5 L jar unit catalyzes the reduction (and removal) of oxygen in the jar as:

$2H_2 + O_2 \rightarrow 2H_2O$ (2.1).

2.3 Determination of microbial biomass

2.3.1 Total bacterial counts using the Thoma counting chamber

Microbial cells in liquid cultures were dispersed thoroughly and a small drop (about 20 µl) was placed on a Thoma bacteria counting chamber (Weber Scientific International, England). The culture was covered with a cover slip and the number of cells was counted under a phase-contrast microscope with ×40 objective lens. A minimum of about 50 cells was counted under a phase-contrast microscope as described in Section 2.4.2 and the number of cells in 1 ml culture was calculated on the basis that the volume of each squared compartment was 50,000 μm^3. The mean number of cells was calculated and the number was multiplied by 2×10^7 (1 ml (=10^{12} μm^3)/5×10^4 μm^3) to obtain the number of cells in 1 ml of test liquid.

2.3.2 DAPI (4', 6-diamidino-2-phenylindole) staining

DAPI binds to DNA molecules and enables visualization of microorganisms in samples under UV irradiation. As DAPI stains any cells with DNA, this technique does not discriminate between active and non-active cells.

2.3.2.1 Sample fixation

For DAPI staining, samples were centrifuged for 5 minutes at 16,100 *g* to collect cell pellets. Each pellet was suspended in 250 µl of filter-sterilized (<0.2 µm) 1X phosphate buffer saline (PBS, containing 130 mM NaCl, 10 mM $Na_2HPO_4 \cdot 12H_2O$ and 3 mM $NaH_2PO_4 \cdot H_2O$; pH adjusted to 7.2 with NaOH or HCl). Next, 750 µl of 4% (w/v) paraformaldehyde (PFA) in PBS was added to the sample to fix the cells. The sample was incubated at 4°C for 2 hours and then centrifuged at 16,100 *g* for 5 minutes. The cell pellet was suspended in 1 ml PBS and centrifuged before it was re-suspended in PBS and ethanol (1:1) mixture. The fixed samples were stored at -20°C until use.

The PFA solution used in this protocol was prepared as follows: 33 ml of filter-sterilized MilliQ-grade water was heated to 60°C and 2 g of PFA and one drop of 2 M NaOH were added. The solution was stirred in a fume cupboard until the PFA dissolved completely and then 16.5 ml filter-sterilized 3X PBS (three times the concentration of 1X PBS) was added. The mixture was cooled to room temperature and the pH adjusted to 7.2 by adding NaOH or HCl. The solution was dispensed (750 µl) into 1.5 ml Eppendorf tubes and stored at -20°C.

2.3.2.2 DAPI staining

To determine the total number of cells in a sample, the fixed sample was suspended in 10 ml of filtered MilliQ-grade water and adsorbed onto 25 mm black polycarbonate filters (0.2 µm pore-size; Millipore, U.S.A.) using a 1225 sampling manifold (Millipore). The fixed sample was washed twice by drawing filtered MilliQ-grade water through the membrane before staining with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) for 10 minutes. If the sample had a high iron content, the fixed sample was washed with filtered MilliQ-grade water that was acidified to pH 2.0 with H_2SO_4, and then washed with MilliQ-grade water prior to DAPI staining. The sample fixed on the membrane was washed twice with filtered MilliQ-grade water after DAPI staining and placed onto a drop of CITIFLUOR^{TM} AF87 (Citifluor Ltd., U.K.) on a slide glass. In order to prevent fading of the dye, mounting medium (90% glycerol in 50 mM Tris pH 9.5 containing 23.3 mg/ml 1,4-

diazabicyclo[2.2.2]octane (DABCO)) was applied directly onto the membrane. Enumeration of DAPI-stained cells was carried out under U.V. light using a fluorescence microscope as described in Section 2.4.3.

2.4 Microscopy

2.4.1 Stereo-scan microscopy

Observation and enumeration of bacterial colonies grown on solid media was carried out using a stereo-scan microscope (Leitz-Wild M32, Switzerland). The magnification used was between ×50 and ×400 and images of the colonies were captured using an Olympus OM-10 camera (Olympus, Japan) attached to the microscope.

2.4.2 Phase-contrast microscopy

For investigation of microbial cell morphology (including spore formation and motility) and for enumerating cells using the Thoma counting chamber, a phase-contrast microscope Leitz Labolux (Switzerland) was used. The microscope was fitted with a Zenike condenser and objective to achieve a magnification of ×400.

2.4.3 Fluorescence microscopy

Microbial cells stained with DNA- or rRNA-binding dyes were viewed with an ECLIPSE E600 fluorescence microscope (Nikon, Japan) using ×1000 magnification. The microscope was fitted with a super high-pressure mercury lamp power supply, HB-10104AF (Nikon, Japan), and a digital camera COOLPIX (Nikon, Japan) was used to obtain digital images of samples. In order to view a sample stained with different dyes, appropriate filters were used. The filters and corresponding dyes used are listed in Table 2.8.

Table 2.8. List of dyes and filters used in this study.

Dyes	Filters
Fluorescein derivative Alexa-fluor 488	HQ FITC-LP
Cy3	HQCy3
DAPI	UV-1A

2.4.4 Scanning electron microscopy (SEM)

In order to obtain visual images under high magnifications, scanning electron microscopy was used. Sample preparation and microscopy technique are described in this section.

2.4.4.1 Dehydration and fixation

Liquid samples (ca. 2 ml) were adsorbed onto polycarbonate membranes (0.2 µm pore size, 13 mm diameter; Whatman) and placed in Swinnex filter units. Samples were fixed by extruding 1 ml of 4% (v/v) glutaraldehyde through the filter unit. Some glutaraldehyde solution was retained in the syringe and the pressure was applied so that the samples remained in the fixation solution for 15 minutes. Then samples were dehydrated by flushing with 5 ml aliquots of ethanol solutions: 30%, 60%, 90% and absolute ethanol. Samples were left in each ethanol solution for 15 minutes. Next, 5 ml dehydrated acetone (dried over anhydrous $CuSO_4$) was pushed through the filter and samples were stored in acetone overnight ahead of drying by critical point drying.

The protocol was modified for acid streamer samples. These were fixed in 4% PFA overnight and stored in 1X PBS/ethanol mixture (1:1 (v:v)) at -20°C. The streamers were dehydrated using a series of ethanol solutions (as above) by placing them in 1.5 ml Eppendorf tubes and changing solutions at intervals. Acid streamer samples were lastly stored in 1.5 ml Eppendorf tubes containing 1.5 ml dehydrated acetone until dried by critical point drying.

2.4.4.2 Critical point drying

The critical point is an occurrence known as a continuity of state for which there is no apparent difference between the liquid and gas state of a medium, and the surface tension between the liquid and gas interface is reduced to zero. The critical point of liquid CO_2, which was used in this study, is 31°C and 1072 p.s.i.. Liquid CO_2 can be converted to a gas at this point without causing surface tension, which often causes cell damage. Use of CO_2 in critical point drying allows dehydration of a specimen without causing any damage to its structure.

Prior to the critical point drying step, the filter unit was disassembled and the filter was placed in a sample basket which was filled with dehydrated acetone. The sample basket was placed in a specimen holder which was then placed in a chamber. The chamber, which was cooled below room temperature prior to the mounting of the specimen holder, was filled with liquid CO_2 until the pressure reached 800 p.s.i.. The acetone and liquid CO_2 mixture was drained repeatedly until the acetone had been completely removed. The chamber was drained and filled with liquid CO_2 every 30 minutes for 3 hours. When the chamber was free of acetone, it was warmed to 36°C and pressure was increased to 1400 p.s.i.. In order to avoid re-condensation of the liquid, the temperature was raised 5°C higher than the critical point of CO_2. The chamber was opened after slowly releasing the pressure and the specimen holder was removed from the chamber. The dried specimen was quickly placed in a sealed jar containing a desiccating agent (self-indicating silica gel) and stored at room temperature until viewed.

2.4.4.3 Viewing samples with a SEM

The dehydrated sample was placed on a carbon tape on an aluminium stub and sputter coated with gold using Polaron E5000 for 5 minutes immediately before viewed with Hitachi S-520 scanning electron microscope (Hitachi, Japan).

2.5 Analytical techniques

2.5.1 Determination of pH

An Accumet® 50 pH meter coupled to a pHase combination glass electrode (Fisher Scientific U.S.A.), calibrated between pH 1.00 and 7.00, was used to measure pH.

2.5.2 Determination of ferrous and total iron concentrations

To determine the concentrations of ferrous and total iron, the Ferrozine assay was carried out, following the method described by Lovley and Phillips (1987). Ferrozine reagent was prepared by adjusting pH of 50 mM HEPES buffer to 7.0 with KOH and adding 1 g of ferrozine (3-(2-pyridyl)5,6-bis(4-

phenyl-sulfonic acid)-1,2,3-triazine). Ferrozine reagent (950 µl) was added to 50 µl sample (diluted with distilled water, adjusted to pH 2.0 with sulfuric acid when necessary) and the absorbance was recorded at 562 nm against a reagent blank that contained no ferrous iron, using a Cecil CE1011 spectrophotometer (Cecil Instruments Ltd., England). A range of standards (0.01-1 mM) was prepared using 1 M $FeSO_4$ stock. A typical standard curve is shown in Figure 2.1.

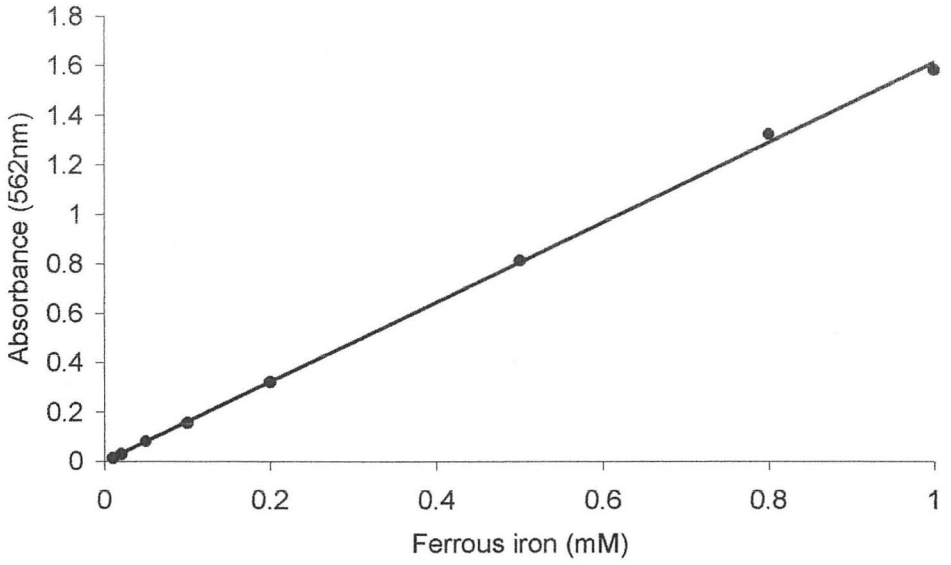


Figure 2.1. A typical standard curve for Ferrozine assay. The equation of the fitted line is y=1.61x+0.0004. R^2=1.

2.5.3 Determination of sulfate concentration using a turbidometric assay

Concentrations of sulfate in liquid samples were measured using a turbidometric assay (and also by ion chromatography, as described in section 2.5.6.1). With this, a sample (5 ml) was added to 400 µl conditioning reagent (consisting of 250 ml distilled water, 50 ml glycerol, 30 ml concentrated HCl, 100 ml 95% ethanol and 75 g NaCl) and mixed thoroughly. Fine-grained barium chloride (60 mg) was added, the solution was mixed using vortex for 30 seconds and absorbance was measured at 420 nm against a sulfate-free blank, using the Hydrocheck (WPA Ltd., U.K.) system.

When the sample volume was limited, a small-scale turbidometric assay was performed by following the method described by Kölmert et al. (2000). One

milliliter of sample solution and 1 ml conditioning reagent were mixed thoroughly and 60 mg fine grain barium chloride was added to the mixture. The absorbance was read at 420 nm against sulfate-free blank using a Cecil CE1011 spectrophotometer (Cecil instrument Ltd., England).

A range of standards (0.1-0.6 mM for a standard scale and 0.25-2.5 mM for a small scale) was prepared using a 1 M K_2SO_4 stock solution. A typical standard curve for a small-scale assay is shown in Figure 2.2.

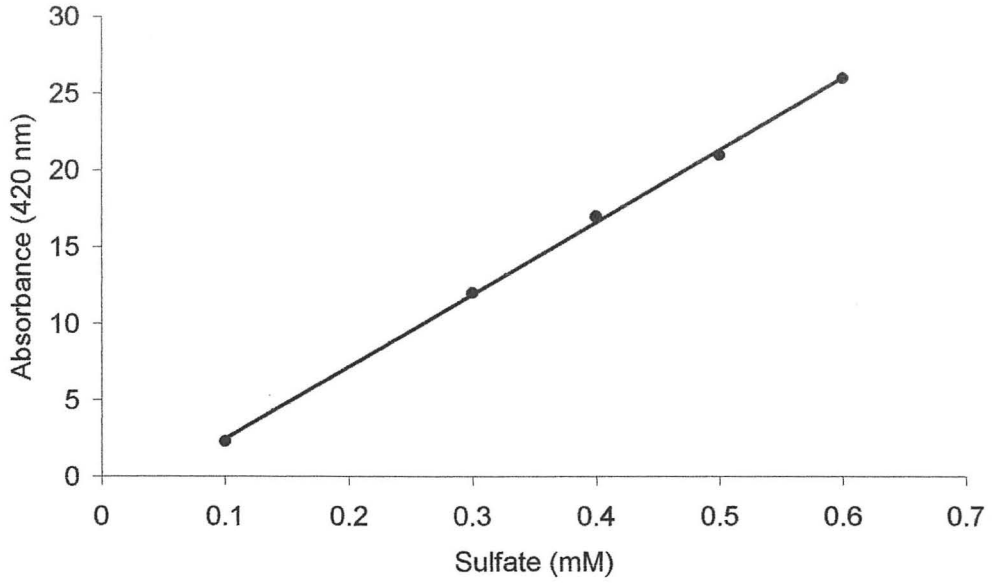


Figure 2.2. A typical standard curve for a small scale turbidometric sulfate assay. The equation of the fitted line is y=47.14x-2.25. R^2=1.

2.5.4 Atomic absorption spectrophotometry (AAS)

Concentrations of Al, As, Cu, Fe, Mn, and Zn in environmental samples were measured using a SpectrAA Duo atomic absorption spectrophotometer (Varian, U.K.). A drop (*ca.* 100 µl/20 ml) of concentrated nitric acid was added to the environmental samples immediately after collection and solutions were stored at –20°C. The sample solutions were combusted in N_2O/acetylene for Al and As measurements and air/acetylene for measurements of other elements. A range of standards was prepared for each element: 20-200 mg/L for Al, 10-100 mg/L for As, 1-10 mg/L for Cu, 25-250 mg/L for Fe, 1-10 mg/L for Mn and 0.2-2 mg/L for Zn.

Concentrations of Zn in samples from aSRB cultures were measured at 213.9 nm using a Pye Unicam SP2900 (Pye Unicam, England) double beam atomic absorption spectrophotometer fitted with a Pye Unicam SP9-10 gas controller unit using a lean air/acetylene flame. A range of standards was prepared each time samples were analyzed. A typical standard curve is shown in Figure 2.3.

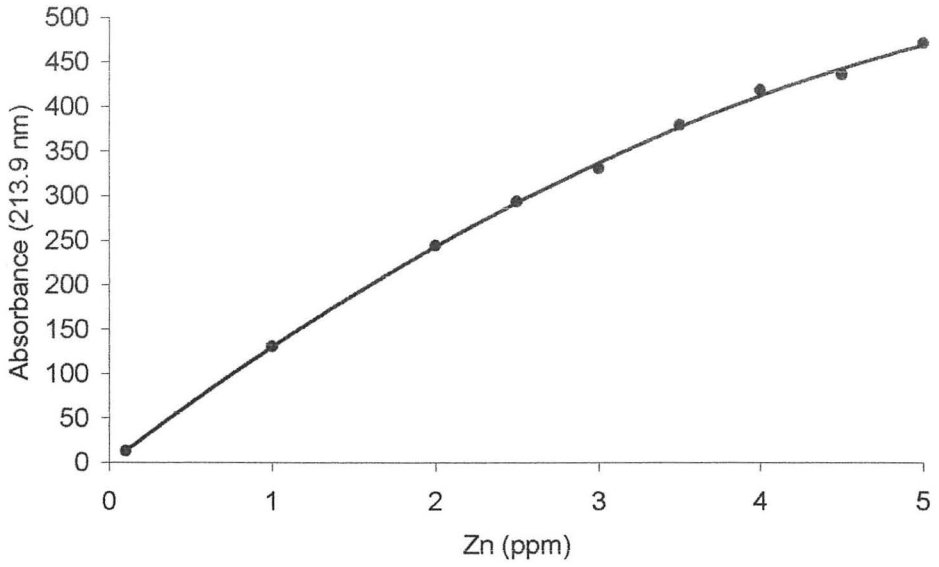


Figure 2.3. A typical standard curve for Zn analysis. The equation of the fitted line is $y=-9.34x^2+140.61x-0.65$. $R^2=1$.

2.5.5 Determination of dissolved organic carbon (DOC)

Liquid samples were filtered through 0.2 µm cellulose nitrate membranes and stored at -20°C. Concentrations of dissolved organic carbon were determined using a Protoc DOC analyzer (Pollution & Process Monitoring Ltd., U.K.). A standard curve (1-20 mg DOC/L) was prepared using KHP (anhydrous potassium hydrogen phthalate).

2.5.6 Ion chromatography

Concentrations of acetate, sulfate, glucose, glycerol and zinc in liquid samples were determined using a Dionex DX-320 ion chromatograph (Dionex, U.S.A.). The data obtained were analyzed using Chromeleon, version 6.40 SP2 Build 731 (Dionex, U.S.A.).

2.5.6.1 Anion analysis

Acetate and sulfate concentrations were determined by suppressed ion chromatography using an Ion Pac® AS11 column, an ASRS ULTRA 4 mm Suppressor and an IC 25 ion chromatograph fitted with a DS11 conductivity detector. A program was developed to analyze the two anions together, and the analytical conditions were set as follows. The pressure generated by a pump was maintained around 1,500 p.s.i. (lower limit was set to 200 p.s.i. and upper limit was set at 3,000 p.s.i.) and 250 µl sample was injected. The flow rate of the eluent and a sample was set at 1 ml/minute. The column temperature was maintained at 30°C and the suppressor current was set at 75 mA. The suppressor was used to minimize the background noise generated by temperature fluctuations in the detector. To maximize the peak separation, the eluent (KOH) was run as a gradient. The starting concentration of the eluent was set at 15 mM and it was set to increase incrementally for 5 minutes until it reached 45 mM. Then the concentration was set to decrease to 15 mM in 5 minutes. A range of standards (0.05-10 mM for acetate and 0.5-10 mM for sulfate) was prepared for each anion, and typical standard curves are shown in Figure 2.4 and Figure 2.5. To validate the accuracy of the technique, 1 mM standards of acetate and sulfate were run at the beginning and the end of each sequence of samples.

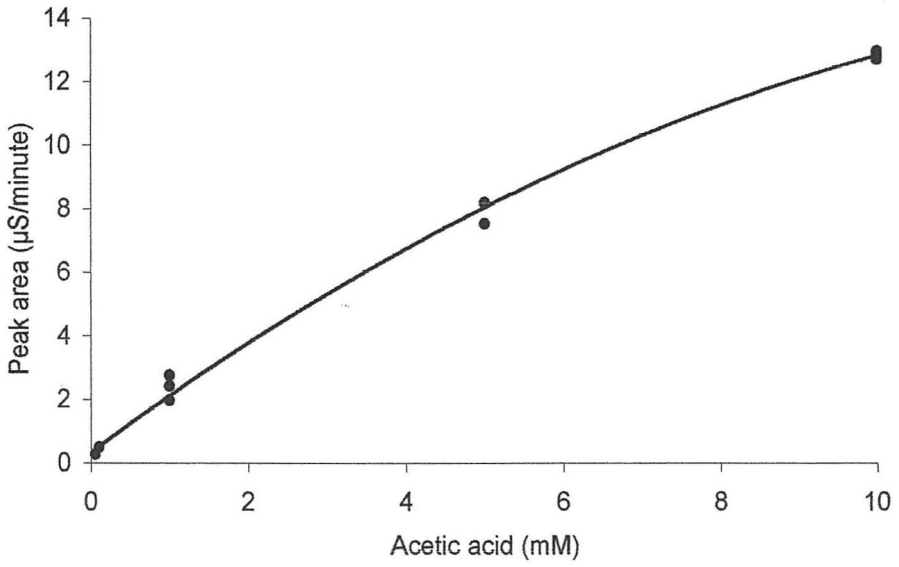


Figure 2.4. A typical standard curve for an acetate analysis. The equation of the fitted line is $y=-0.06x^2+1.83x+0.35$. $R^2=1$.

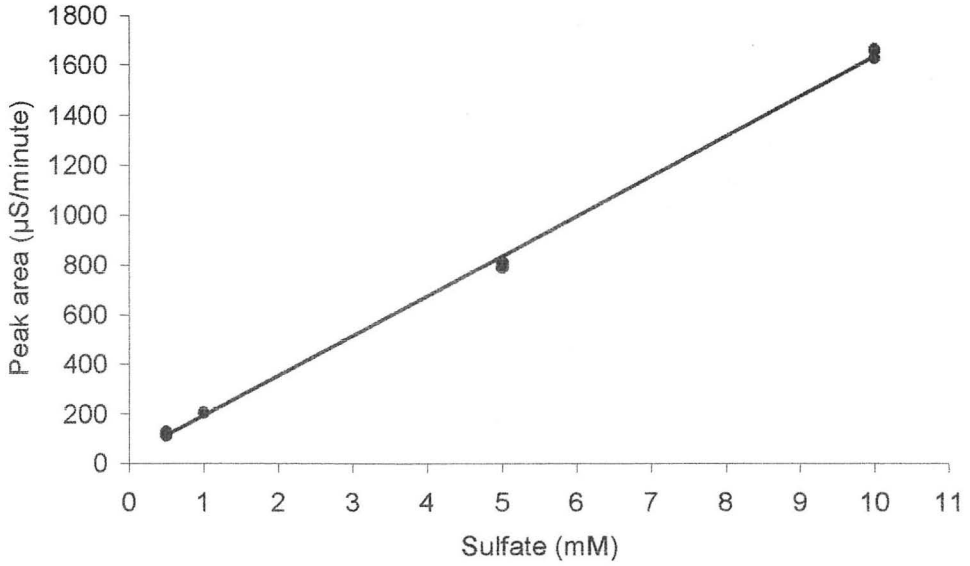


Figure 2.5. A typical standard curve for a sulfate analysis. The equation of the fitted line is y=160.37x+34.26. R^2=1.

In the latter stages of this study, increases in peak area over time became apparent, possibly due to the temperature increase within the detector compartment over time. To compensate for this drift, a new analytical method referred to as a "bracketed standard curve" was developed. In this method, a range of standards was analyzed before and after the sample sequence of 2-3 samples were run, and the standard curves were prepared specifically to samples in between, hence minimizing effects of peak area drift over time.

2.5.6.2 Organic compound analysis

Fructose, glucose and glycerol were analyzed using a Carbo Pac™ Pa10 column and an ED 40 Amperometric Detector. The analytical conditions, including column temperature, flow rate, and sample injection volume, were set as for anion analysis. The column pressure was maintained around 2,500 p.s.i. with a lower limit of 200 p.s.i. and an upper limit of 3,000 p.s.i.. The pH of the mixture of sample and eluent, which was measured in the detector compartment, was maintained between 10 and 13.

For glucose analysis, the concentration of the eluent (KOH) was maintained at 18 mM and the analysis was carried out over 15 minutes. A range of

standards was prepared (1-100 µM) each time a sample sequence was run, and a typical standard curve is shown in Figure 2.6.

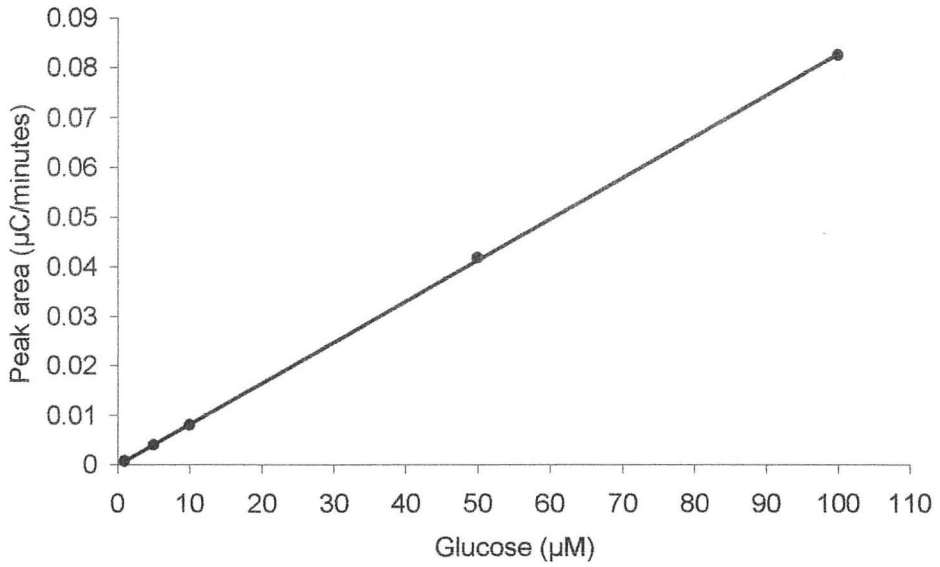


Figure 2.6. A typical standard curve for a glucose analysis. The equation of the fitted line is $y=8\cdot10^{-4}x-6\cdot10^{-5}$. $R^2=1$.

For glycerol analysis, the concentration of the eluent (KOH) was set at 30 mM and the sample analysis was carried out over 7 minutes. A range of standards was prepared (1-100 µM), and a typical standard curve is shown in Figure 2.7. A standard containing 1 mM glycerol was run every time a sample sequence was analyzed to validate the accuracy of the technique.

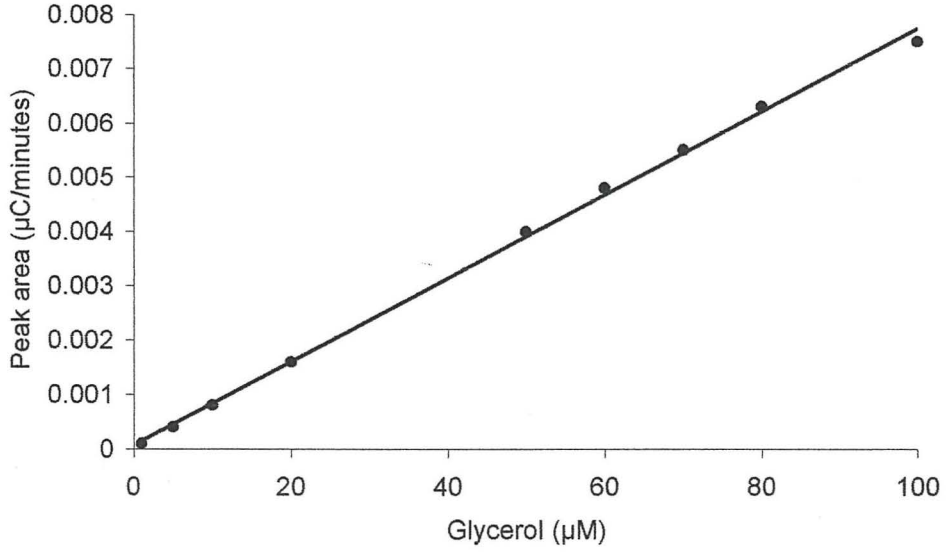


Figure 2.7. A typical standard curve for a glycerol analysis. The equation of the fitted line is $y=8\cdot10^{-5}x+7\cdot10^{-5}$. $R^2=1$.

A standard curve was not prepared for fructose as the "bracketed standard curve" method was used to analyze this sugar. The standards used were 0.1 and 1 mM fructose, and 5 samples were analyzed between the standards.

2.5.6.3 Zinc analysis by ion chromaography

Concentrations of zinc in aqueous solutions were also determined by ion chromatography using an Ion Pac® CS5A column, an AD25 absorbance detector and IP25 isocratic pump. The analytical conditions were set as follows: flow rate, 1.2 ml/minute; column pressure, ca. 1600 p.s.i., and sample injection volume, 250 µl. The eluent used for zinc analysis contained PDCA (pyridine-2,6-dicarboxylic acid; 1.4 mM), KOH (13.2 mM), K_2SO_4 (11.2 mM) and formic acid (14.8 mM). PDCA complexed the cationic metal (Zn^{2+}), forming an anionic compound that was retained for a specific duration within the column. Concentrations of zinc were determined by forming a derivative with PAR [4-(2-pyridylazo) resorcinol] and measuring changing in absorption at 520 nm. The PAR reagent was prepared by dissolving 0.06 g PAR in 500 ml solution containing 2-dimethylaminoethanol (1 M), ammonium hydroxide (0.5 M) and sodium bicarbonate (0.3 M). A bracketed standard curve (two points; 1.25 and 2.5 ppm) was used to determine zinc concentrations in the samples.

2.5.7 Energy dispersive analysis of X-ray (EDAX)

Precipitates formed within the bioreactor were analyzed using energy dispersive analysis of X-rays (EDAX). Samples were dehydrated by successive incubation for 3 minutes in 50, 80, and 95% (v/v) ethanol. Following air-drying, the samples were placed onto carbon tape on aluminium stubs and the elemental composition of the solid phase materials was determined using an Oxford Instruments Link Isis III EDAX System (Oxford Instrument, England) coupled to an Hitachi S-520 scanning electron microscope (Hitachi, Japan).

2.6 Biomolecular techniques

2.6.1 Preparation of template DNA

To obtain template DNA, cell lysates were prepared by centrifuging (16,100 *g* for 6 minutes) actively growing cultures and resuspending pellets in lysis solution (0.05 M NaOH and 0.25% x/v SDS). If the culture was an iron-oxidizer, the pellet was washed by resuspending it in 500 µl of 100 mM oxalic acid to remove any ferric iron precipitates. The solution was centrifuged and washed in 500 µl MilliQ-grade water before the pellet was suspended in lysis solution. The lysis solution was heated to 95°C for 10 minutes before180 µl of heat-sterilized MilliQ-grade water (pH adjusted to 7.5 by NaOH) was added. When lysates were made from cultures growing on solid media, a colony was transferred into the lysis solution and the lysate was prepared as described above. Lysates were stored at -20°C until required.

DNA from environmental samples was extracted using UltraClean™ Soil DNA Kits (MO BIO Laboratories, Inc. U.S.A.). The extracted DNA was stored at -20°C.

2.6.2 Polymerase chain reaction (PCR)

The 16S rRNA genes of eubacteria and archaea were amplified by touchdown polymerase chain reaction (Don et al. 1991) using oligonucleotide primers listed in Table 2.9. Each reaction used "PCR master mix" (24 µl, total volume) which was made up by combining 2.5 µl 10X reaction buffer, 2.5 µl 25 mM $MgCl_2$, 2.5 µl dNTPs (2 mM each dATP, dCTP, dTTP and dGTP), 0.5 µl of 10 pmol/µl forward primer (5 pmol/25 µl reaction), 0.5 µl of 10 pmol/µl reverse primer, 0.5 µl DMSO (to final concentration of 2% (v/v)), 0.05 µl *Taq* polymerase (0.05 unit/µl, Promega, U.S.A.) and 14.95 µl nuclease-free water. Alternatively, $MgCl_2$ (2.5 mM final concentration), 0.5 µl DMSO (2% v/v), 0.5 µl forward primer and 0.5 µl reverse primer were added to commercial PCR Master Mix (Promega, U.S.A.). Lysis solution (1.0 µl) was added to the PCR master mix. Touch down PCR was performed as follows: 95°C denaturation for 5 minutes ahead of 20 cycles at 95°C for 30 seconds, 57°C for 30 seconds (the temperature decreasing by 1°C every second cycle) and 72°C

for 90 seconds which was followed by 15 cycles of 95°C for 30 seconds, 47°C for 30 seconds and 72°C for 90 seconds.

Table 2.9. List of primers used in this study. r/R = reverse, f/F = forward. Primers listed without a reference were designed by other laboratory members.

Primer	Sequence (5'-3')	Target	Reference
27f	AGAGTTTGATC(A/C)TGGCTCAG	Eubacteria	Lane et al. 1991
27fG	GAGAGTTTGATC(A/C)TGGCTCAG	Eubacteria	
20f	TCCGGTTGATCC(T/C)GCC(A/G)G	Archaea	Orphan et al. 2000
530r	GTGCCAGC(AC)GCCGCGG	Eubacteria/ Archaea	Lane et al. 1991
1100r	GGGTTGCGCTCGTTG	Eubacteria/ Archaea	Lane et al. 1991
1392r	ACGGGCGGTGTGT(G/A)C	Eubacteria/ Archaea	Lane et al. 1991
1392Gr	GACGGGCGGTGTGT(G/A)C	Eubacteria/ Archaea	
1492r	TACGG(C/T)TACCTTGTTACGACTT	Eubacteria/ Archaea	Lane et al. 1991
1492rG	GTACGG(C/T)TACCTTGTTACGACTT	Eubacteria/ Archaea	
APS7-F	GGG(C/T)CT(G/T)TCCGC(C/T)ATCAA(C/T)AC	*aps* genes	Friedrich 2002
APS8-R	GCACATGTCGAGGAAGTCTTC	*aps* genes	Friedrich 2002
TRA3-20	TCCAGGTTATTCGCCTGA	clone TrefC7	This study
TF539	CAGACCTAACGTACCGCC	*At. ferrooxidans*	This study

2.6.3 Agarose gel analysis of DNA

PCR products were analyzed by running on a 0.7% agarose gel. The gel was prepared by melting electrophoresis-grade agarose (Sigma-Aldrich) in 0.5X TBE (5.4 g tris-hydroxymethyl-methylamine, 2.75 g boric acid and 2 ml of 0.5 M EDTA stock of which pH was adjusted to 8.0 with NaOH). TBE solution was made up to 1 L prior to heat sterilization using a microwave oven. The solution was cooled to around 50°C and poured into a mould to polymerize after 0.005% (v/v) ethidium bromide solution (10 mg/ml) was added to the gel solution. The PCR product was mixed with the 6X DNA loading buffer (0.25% (w/v) bromophenol blue in 30% glycerol) and loaded into a well in the

agarose gel alongside 1 Kb DNA ladder (100 µg/µl. Promega, U.S.A.). A variable electric current was applied to run the gel until the dye reached the desired position in the gel. The gel was viewed under UV light.

2.6.4 Restriction fragment length polymorphism (RFLP) analysis

PCR products were analyzed by RFLP to identify and screen 16S rRNA genes amplified by PCR. The RFLP reaction mix (supplied by Promega) contained 1 µl 10X reaction buffer, 0.1 µl bovine serum albumin acetylated (10 mg/ml) and 0.25 µl restriction enzyme. The enzymes used in this study are listed in Table 2.10 and were all supplied by Promega. The volume of PCR product added to the reaction mix was determined according to the amount of DNA in the PCR product. The reaction mix was made up to 10 µl by adding nuclease-free water and incubated at 37°C for 1-2 hours. The gene fragments were separated alongside 100 bp DNA ladder (500µg/µl. New England Biolab, U.S.A.) by electrophoresis using a 3% agarose gel, which was made by melting high-resolution electrophoresis-grade agarose in 1X TBE buffer as described in Section 2.6.3.

Table 2.10. List of restriction enzymes used, and their recognition sites.

Restriction Enzyme	Restriction sites
*Alu*I	5'AG▼CT3' 3'TC▲GA5'
*Cfo*I	5'GCG▼C3' 3'C▲GCG5'
*Hha*I	5'GCG▼C3' 3'C▲GCG5'
*Msp*I	5'C▼CGG3' 3'GGC▲C5'
*EcoR*I	5'G▼AATTC3' 3'CTTAA▲G5'

2.6.5 Terminal-restriction fragment length polymorphism (T-RFLP) analysis

Eubacterial or archaeal 16S rRNA genes were amplified by PCR, as described in Section 2.6.2, in three separate 25 µl PCR reactions using a fluorescent dye-labeled (with D4-phosphoramadite; ResGen, U.K.) 27f or 20f primer and non-labeled 1392r or 1492r primer. The three PCR products were

combined and run on an agarose gel to confirm successful PCR amplification (Section 2.6.3). The combined PCR product was purified using QIAquick® PCR Purification Kit (QIAGEN, Germany) and digested with a restriction enzyme (Section 2.6.4) - *Hha*I, *Cfo*I, *Msp*I or *Alu*I. The reaction was terminated after 2 hours by adding 5 µl "stop solution" (2 µl 5 M sodium acetate, 2 µl 100 mM EDTA and 1 µl 0.02% (w/v) glycogen) and 60 µl absolute ethanol, which had been stored at -20°C. The mixture was mixed briefly and centrifuged at 16,100 *g* for 20 minutes. The pellet was washed twice by adding 100 µl 70% (v/v) ethanol kept at 4°C and dried until the ethanol had completely evaporated. The dried pellet was resuspended in 40 µl molecular grade formamide (Beckman Coulter, U.S.A.) and 4 µl of this solution was added to a solution containing 36 µl formamide and 0.5 µl of a 600 bp size standard, labeled with fluorochrome D1 (RedGen, U.K.). The sample- and size standard-containing solution was analyzed by capillary electrophoresis using a CEQ8000 Genetic Analysis System (Beckman Coulter, U.S.A.). The fragment length of the sample was determined by referring to the size standard and the fragment was identified by referring to the fragment sizes of known microorganisms.

2.6.6 Cloning of 16S rRNA gene

2.6.6.1 Preparation of competent* Escherichia coli *DH5α

A frozen stock culture of *E. coli* DH5α was streaked onto a solid LB medium (1.5% (w/v) agar, 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl; pH adjusted to 7.0 with NaOH). One colony was used to inoculate 5 ml liquid LB medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl; pH adjusted to 7.0 with NaOH). The culture was grown overnight, shaken at 37°C, and 1 ml of the culture was used to inoculate 50 ml liquid LB medium (warmed to 37°C). The culture was shaken at 37°C for around 1 hour until the culture density reached an optical density of 0.5 at 550 nm. The culture was transferred onto ice and centrifuged at 4°C at 750 *g* for 5 minutes. The supernatant was removed and 15 ml filter-sterilized (0.2 µm nitrocellulose membranes) TFB1 was added to the pellet. TFB1 was prepared by mixing 1.473 g potassium acetate, 4.95 g $MnCl_2 \cdot H_2O$, 6.046 g RbCl, 0.74 g $CaCl_2 \cdot 2H_2O$, 75 ml glycerol and 400 ml MilliQ-grade water; the

pH was adjusted to 5.8 with diluted glacial acetic acid and the solution was made up to 500 ml with MilliQ-grade water. The culture was incubated on ice for 2 hours before being centrifuged at 4°C at 600 *g* for 5 minutes. The supernatant was removed and 2 ml filter-sterilized TFB2 was added. TFB2 was prepared by mixing 0.1075 g 3-[N-morpholino]propanesulfonic acid, 0.06 g RbCl, 0.55 g $CaCl_2 \cdot 2H_2O$, 7.5 ml glycerol and 40 ml MilliQ-grade water; the pH was adjusted to 7.0 with NaOH and the solution was made up to 50 ml with MilliQ-grade water. The culture was gently mixed by swirling and 30 µl DMSO was added. The culture was divided into 100 µl aliquotes, flash-frozen using liquid nitrogen, and stored at -70°C until required.

2.6.6.2 Ligation reaction

DNA extracted from environmental samples was amplified by three individual PCR reactions and the pooled products were purified as described in Section 2.6.5. The combined purified PCR product (3 µl) was added to a ligation mix containing 5 µl buffer, 1 µl T4 DNA ligase and1 µl pGEM®-T Easy Vector (Promega, U.S.A.) and incubated at 4°C overnight.

2.6.6.3 Transformation

To 100 µl of defrosted competent *E. coli* DH5α culture, 5 µl ligated vector containing solution was added and placed on ice for 20 minutes ahead of incubation at 42°C for 1 minute. The mixture was cooled on ice for 5 minutes and 900 µl of heat sterilized SOC medium was added before incubation at 37°C for 1 hour. SOC medium was prepared by mixing 20 g tryptone, 5 g yeast extract, 0.58 g NaCl, 0.19 g KCl, 2.03 g $MgCl_2 \cdot 6H_2O$, 2.47 g $MgSO_4 \cdot 7H_2O$ and 3.6 g glucose in 900 ml MilliQ-grade water; the pH was adjusted to 7.0 and the solution was made up to 1 L with MilliQ-grade water.

2.6.6.4 Screening of clone libraries and purification of plasmids

Aliquots of transformed *E. coli* cultures (50, 100 and 200 µl) were spread onto solid LB medium containing 50 mg/ml X-gal (Melford laboratories, U.K.) and incubated at 37°C overnight. White colonies, which had insert, were transferred into a liquid LB medium containing 100 µg/ml ampicillin and incubated overnight. The culture (10 µl) was centrifuged and a lysate was

prepared prior to amplification by PCR using SP6 (5'-ATTTAGGTGACACTATAGAA-3') and T7 (5'-TAATACGACTCACTATAGGG-3') primers. The PCR product was screened by RFLP analysis as described in Section 2.6.4 using *Hha*I, *Cfo*I, *Msp*I and *Alu*I. The restriction enzyme *EcoR*I was also added to the restriction enzyme digest reaction to remove plasmid sequences. Plasmids from selected cultures were purified using the Wizard® Plus SV Minipreps DNA Purification System (Promega).

2.6.7 Sequencing analysis

In order to identify isolates and cloned 16S rRNA genes obtained from environmental samples, purified PCR products and clone plasmids were sequenced using CEQ 2000 Dye Terminator Cycle Sequencing with a Quick Start Kit (Beckman Coulter, U.S.A.). The primers used for the sequencing analysis are listed in Table 2.9. The sequence data were analyzed using Chromas version 1.45 and compared with the gene sequences deposited in GenBank using BLAST (Altschul et al. 1997).

2.6.8 Fluorescent in situ *hybridization (FISH)*

To determine the proportion of a certain microorganism in a sample, fluorescent *in situ* hybridization (FISH) was carried out. In this technique, fluorescently-labeled (with either sulfoindocyanine, indocarbocyanine (Cy3), or the fluorescein derivative, Alexa-fluor 488) oligonucleotide probes targeting 16S rRNA were designed to target microorganisms of interest. Unlike DAPI staining, this technique only targets active cells, as the numbers of rRNA copies are usually too low in inactive cells to permit their detection. The cells stained with the Cy3-labeled probes that were specific to a group or groups of bacteria of interest were counted relative to cells stained with the fluorescein-labeled eubacteria-specific probe. To enumerate the relative number of total active bacteria in a sample, numbers of cells stained with eubacteria-specific probe were compared with those stained by DAPI. For the archaeal probe, cells were always counted relative to total (DAPI-stained) cells. All of the reagents used were filter-sterilized through 0.2 µm cellulose nitrate membrane before use.

2.6.8.1 Sample fixation

The samples for FISH analysis were fixed according to the method described in Section 2.3.2, and samples were stored at -20°C until use.

2.6.8.2 Slide preparation

Slides were cleaned by soaking overnight in ethanolic KOH (95 ml ethanol, 5 ml MilliQ-grade water and 10 g KOH). The slides were rinsed twice in MilliQ-grade water and dried. Next, the slides were immersed into a gelatin solution, made by dissolving 0.15 g gelatin and 0.02 g $CrK(SO_4)_2 \cdot 12H_2O$ in 200 ml MilliQ-grade water that had been heated to 70°C. The slides were dried and stored at 4°C. Samples (5-10 µl) were placed onto gelatin coated slides and dried prior to being immersed in series of 50, 80 and 95% ethanol solutions in sequence for 3 minutes each, to dehydrate the cells.

2.6.8.3 Hybridization

Hybridization buffers containing various concentrations of formamide were prepared as follows. The formamide concentration was varied to allow the hybridization reaction to be carried out at the uniform temperature of 46°C when using oligonucleotide probes that had different melting temperatures. The optimum concentration of formamide required in hybridization buffer for each probe was determined by testing over the concentration range of 0–60% (v/v). Hybridization buffer contained 40 µl 1M Tris-HCl (pH adjusted to 7.4 with HCl), 2 µl 10% sodium dodecyl sulfate (SDS), 360 µl 5 M NaCl and an appropriate volume of deionized formamide. The solution was made up to 2 ml by adding MilliQ-grade water. Next, a paper tissue was placed in a 50 ml conical-based plastic test tube and soaked with 1.8 ml hybridization buffer. The temperature of the tube was equilibrated in a hybridization oven (HIR4M; Grant Instruments Ltd. Cambridge) at 46°C for 30 minutes. Hybridization buffer (10 µl) containing 25 ng Cy3-labeled oligonucleotide probe was placed onto the sample smear on the prepared slide and a cover slip was placed on top. To determine a proportion of specific bacteria in a sample, 25 ng Cy3-labeled probe and 25 ng fluorescein-labeled eubacteria specific probe were added to the hybridization buffer. If the probe required helper oligonucleotides (Fuchs et al. 2000), which target rRNA immediately adjacent

to, and either side of, the probe target site, 25 ng of each helper oligonucleotide probe was added to the hybridization buffer. The probes used in the experiment are listed in Table 2.11. In order to protect the probes, which were light sensitive, the procedures involving them were carried out with minimum light exposure.

The glass slide was then placed into the equilibrated test tube and incubated at 46°C for 2 hours, after which it was removed and the cover slip was gently taken off. Then the slide was immersed in a 50 ml washing buffer solution, which had been equilibrated for 15 minutes at 48°C in a thermostatic bath (Y6-VFB; Grant Instruments Ltd., England). The washing buffer contained 50 µl 10% SDS, 1ml Tris-HCl (pH was adjusted to 7.4 with HCl), 0.5 ml 500 mM sodium ethylenediaminetetra-acetic acid (EDTA; pH adjusted to 8.0 using NaOH) and various concentrations of NaCl. The concentration of NaCl was determined by the concentration of formamide used in the hybridization buffer. The washing buffer solution was made up to 50 ml by adding MilliQ-grade water. The slide was removed from the washing buffer solution, washed twice in MilliQ-grade water, air-dried, and 10 µl of 1 µg/ml 4', 6-diamidino-2-phenylindole (DAPI) solution was placed onto the sample smear and covered with a cover slip. The slide was incubated in the dark for 10 minutes at room temperature and rinsed twice in MilliQ-grade water. After drying the slide, 10 µl of mounting medium (70% glycerol in 50 mM Tris pH 9.5 containing 23.3 mg/ml 1,4-diazabicyclo[2.2.2]octane (DABCO)) was placed onto the smear and again covered with a cover slip. The mounting medium was placed onto the sample to minimize the fading of dyes during observation under the microscope. Cell counts were carried out by taking mean cell numbers of 5-10 fields of views. The stained samples were viewed using a fluorescence microscope (Section 2.4.3).

Table 2.11. List of oligonucleotide probes used in FISH analysis. All probes except EUB338FI were labeled with the fluorochrome Cy3 at 5' end.

Probe Name	Target Organism	Sequence (5'-3')	Tm (°C)	Formamide (%)	Reference
EUB338Cy3	Eubacteria	GCTGCCTCCCGTAGGAGT	60.5	10-50	Amann et al. 1990
EUB338FI[a]	Eubacteria	GCTGCCTCCCGTAGGAGT	60.5	10-50	Amann et al. 1990
ARCH915	Archaea	GTGCTCCCCCGCCAATTCCT	63.5	40	Stahl & Amann 1991
ALF1B	α-*Proteobacteria* and some δ-*Proteobacteria*, most spirochetes	CGTTCG(CT)TCTGAGCCAG Competitor GCCTTCCCACTTCGTTT	56.4	20	Manz et al. 1992
BET42a[b]	23S rRNA of most β-*Proteobacteria*	GCCTTCCCACTTCGTTT Competitor CGTTCG(CT)TCTGAGCCAG	52.8	35	Manz et al. 1992
GAM41a[b]	23S rRNA of most γ-*Proteobacteria*	GCCTTCCCACATCGTTT	52.8	35	Manz et al. 1992
LGC0355	Low G+G gram-positive bacteria	GGAAGATTCCCTACTGCTG	56.7	20	Hallberg & Johnson unpubl.
FER656	*Ferroplasma*	CGTTTAACCTCACCCGATC	56.7	25	Edwards et al. 2000
LF581	*Leptospirillum* groups I and II[c]	CGGCCTTTCACCAAAGAC	56.0	15	Schrenk et al. 1998
LF1252	*Leptospirillum* group III[c]	TTACGGGCTCGCCTCCGT	60.5	35	Bond & Banfield 2001
LF655	*Leptospirillum* groups I, II and III[c]	CGCTTCCCTCTCCCAGCCT	63.0	35	Bond & Banfield 2001
ACM732	*Acidimicrobium* and relatives[d]	GTACCGGCCCAGATCGCTG	63.1	35	Bond & Banfield 2001
ACM995Cy3	*Acidimicrobium ferrooxidans*[d]	CTCTGCGGCTTTTCCCTCCATG	64.0	10	Norris unpubl.

Table 2.11 Continued

Probe Name	Target Organism	Sequence (5'-3')	Tm (°C)	Formamide (%)	Reference
SUL228	*Sulfobacillus* genus[e]	TAATGGGCCGCGAGCTCCC	63.1	30	Bond& Banfield 2001
GSMCy3	GSM[f]	GCTTTCCACTCCTTAGGTAG	57.3	40	Okibe et al. unpubl.
TF539	*At. ferrooxidans*	CAGACCTAACGTACCGCC	58	20	Schrenk et al. 1998
THC642	*At. caldus*	CATACTCCAGTCAGCCCGT	58.8	25	Edwards et al. 2000b
aSRBCY3*	acidophilic SRB M1[g]	CTGAAGAAACCGTTCTTCC Helper CTAAAAACAGTACTTTACAATCCG Helper GTCATGTCTCCTCCATATTC	54.5	30	Roberto et al. unpubl.
GALTS0084*	Cae Coch clone 4	CCACTAACCTGGGAGCAA Helper GCCCCCAGGCCCGTTCGA Helper GATATATTACTCACCCGTTCG	56.0	40	This study
BSC0459	Acid streamer clones MPKCSC9 and TrefC11	TCCAGGTTATTCGCCTGA	53.7	30	Kimura et al. unpubl.
TM1G0138	*Thiomonas* Group 1	GTAGTTATCCCCCATCACA	54.5	40	K.Coupland unpubl.
TM2G0138	*Thiomonas* Group 2	GCAGTTATCCCCCATCAAT	54.5	40	K.Coupland unpubl.
SPH120	*Sphingomonas*	GGGCAGATTCCCACGCGT	60.5	35	Eilers et al. 2000
Alk	*Acidiphilium*-WBW	CTTCAGACGCTCTCTCAAGC	59.4	40	K.Coupland unpubl.
FMR0732	*Ferrimicrobium*-KP1	GTGTCGGCCCAGATTGCTG	61.0	30	K.Coupland unpubl.
WJ20646*	γ-proteobacterium WJ2	TACCGTACTCCAGCAAGC Helper CAGTATCCACCGCCATT Helper GGGAATTCCACCTTTCTC	56.0	30	Hallberg unpubl.

Table 2.11 Continued

Probe Name	Target Organism	Sequence (5'-3')	Tm (°C)	Formamide (%)	Reference
S3BC3*	Cae Coch clone S3BC3	CGCCTTTCCTTCTTCACT Helper CATGCGAGCAAAGAGTCT Helper ATCAAGCGACGCCGTAG	53.7	30	This study
S3BC11*	Cae Coch clone S3BC11	CTTTCCTTCCTCTGCCAT Helper GCGGCAAAGGAAACCATT Helper TTGGACGGCGCCGTA	53.7	30	This study
AcaromCy3*	*"Acidocella aromatica"*	GTCGCTACGTATCAAAAG Helper AGTCCCCAGGTTGAGCCC Helper CAACCCTCTTCCTTACTCTA	54.5	20	Kimura et al. 2004
ABI1002*	*Acidobacterium*-like KP3	CTATTTCTAGGGGTGTCC Helper TGTACATTTCGAGCCCAG Helper ATACAGACCCATTGCTGG	53.7	10	K.Coupland unpubl.
ATT0223	*At. thiooxidans*	AGACGTAGGCTCCTCTTCC	58.8	40	Hallberg unpubl.

*Probe requires the use of helper oligonucleotide probes

a. This probe is labeled with the fluorescein derivative Alexa-fluor 488. b. As there is only one mismatch between the 2 probes, 10 times the amount of the unlabeled version of the other (called a competitor oligonucleotide) was added (Manz et al. 1992). c. The *Leptospirillum* grouping is based on phylogenetic analysis and group III is represented only by environmental clones from the Iron Mountain site. d. ACM732 targets *Am. ferrooxidans* as well as related clones from Iron Mountain and "*Ferrimicrobium acidiphilum*", but not Iron Mountain clones IMBA84 or TRA2-10 nor Wheal Jane isolate 25 ("WJ25"). ACM995 is specific to *Am. ferrooxidans*. e. This probe exactly matches the sequence from *Sb. thermosulfidooxidans* and has a 1 bp mismatch with the target in *Sb. acidophilus* but still hybridizes with the RNA from *Sb. acidophilus*. f. This probe targets GSM. g. This probe targets the acidophilic sulfate-reducing bacteria isolates P1, CC and M1. The target sequence is not present in any other *Desulfosporosinus* species, including the aSRB isolate PFB.

Chapter 3 A study of acid streamers found in a chalybeate spa, using a combined cultivation-based and cultivation-independent approach

3.1 Introduction

Acid streamers, which have been reported to be composed of microorganisms embedded in a polysaccharide matrix, have been found in a number of acid mine drainage-impacted sites (Johnson et al. 1979; Bond et al. 2000a; Bond et al. 2000b; López-Archilla et al. 2004). Previous studies have found that acid streamers contain a variety of organisms including ciliates, rotifers and fungi (Johnson et al. 1979), and also showed that prokaryotes dominate these communities. Prokaryotic populations were found to vary between streamers, depending on the physicochemical characteristics of the acid mine drainage water (Bond et al. 2000b). In this chapter, acid streamers found in a chalybeate (iron-rich) spa (the Trefriw Wells Spa) were studied using combination of culture-dependent and independent techniques.

3.2 Sampling location and sample collection

Streamer samples were collected at the Trefriw spa, which is located below the abandoned Cae Coch pyrite mine in the Conwy valley, North Wales (Grid reference SH 775 653). Most streamer samples were taken from within a small cave excavation at the site. Within the cave, acidic, iron-rich water percolating from rocks within the hillside above the Spa was fed into a small pool, about 1.5 m in diameter and 1 m deep. The overflow water from the pool was channeled through a shallow open drain in the cave and then into a second open drain outside the cave. Acid streamer growths were sampled at the end of the Feed pipe (Figure 3.1 A) that delivered water to the pool, from the main body (Figure 3.2 B) and from the bottom (Figure 3.2 C) of the pool ("Pool benthic" and "Pool planktonic" streamers), and from the drainage channels inside and outside the cave ("Drain 1" and "Drain 2", respectively).

Streamer samples were collected in sterile 20 ml sample tubes and brought back to the laboratory within two hours. The samples were stored at 4°C until

used. The water samples for chemical analyses were filtered through 0.2 µm nitrocellulose membrane at the site and stored at 4°C until used.

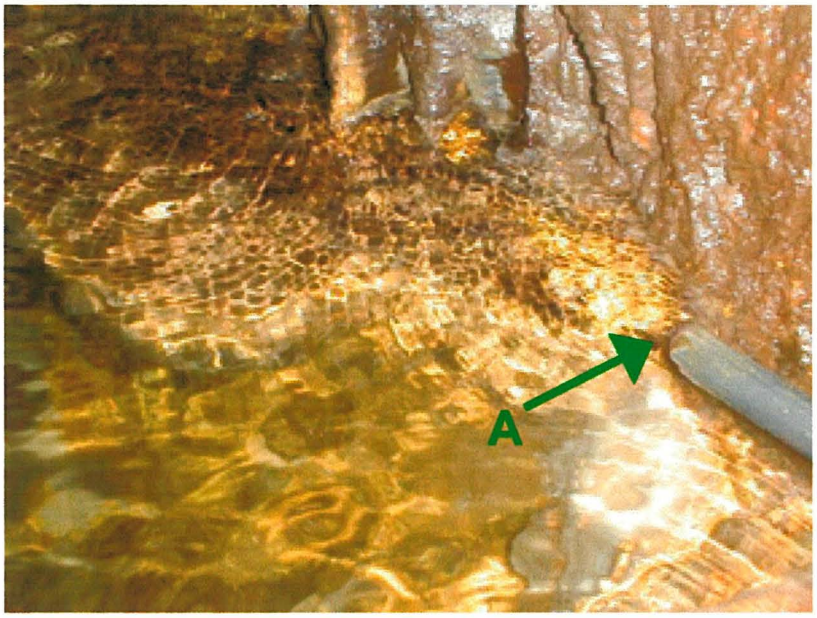


Figure 3.1. Trefriw spa sampling point A. Key: (A) Feed pipe.

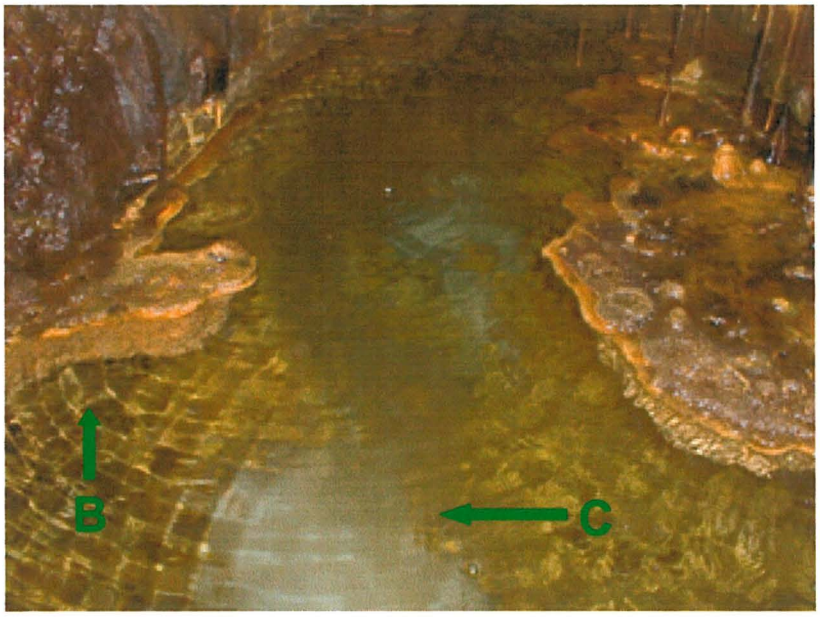


Figure 3.2. Trefriw spa sampling point B and C. Key: (B) Pool planktonic zone; (C) Pool benthic zone streamers.

3.3 Chemical analyses

3.3.1 Materials and Methods

On site analysis of pH, redox potential, temperature and conductivity was initially carried out using a Whatman Water Tester (Whatman, U.K.) and dissolved oxygen (DO) was measured using a D400 DO meter (Whatman, U.K.). Later, all of these parameters were measured using a YSI 556 MPS multimeter (YSI Environmental, U.S.A.).

Concentrations of dissolved metals in water samples were measured using atomic absorption spectrophotometry (Section 2.5.4) and ferrous, ferric and total iron was measured using the Ferrozine assay (Section 2.5.2). Concentrations of sulfate were measured using the turbidometric sulfate assay described in Section 2.5.3. Dissolved organic carbon was measured using PROTOC DOC analyzer (Pollution & Process Monitoring Ltd., U.K.; Section 2.5.5).

3.3.2 Results

The results of *in situ* analysis of waters, sampled in March 2002, are listed in Table 3.1 and the chemical characteristics of the water samples are listed in Table 3.2. Feed pipe and Pool waters had similar chemistries, though there was more ferric iron present in the pool than in the water that fed into it. The Pool planktonic zone water contained slightly more DO than the Feed pipe and Pool benthic water samples. Water in Drain 1 had the highest pH, redox, conductivity, DOC and temperature of all the water samples. In Drain 1 water, most of the dissolved iron was in the ferric form and the DO was higher than in the Feed pipe and Pool waters. In contrast, on this sampling occasion water in Drain 2 had the lowest pH, temperature and total iron concentration of all those analyzed, though it contained more DO and DOC.

Table 3.1.Results of *in situ* analysis of Trefriw spa water samples.

Sample	pH	DO (mg/L)	Temperature (°C)	Eh (mV)	Conductivity (ms/cm)
Feed pipe	2.7	0.6	10.1	+657	1.55
Pool planktonic zone	N.D.	0.9	N.D.	N.D.	N.D.
Pool benthic zone	2.6	0.5	9.8	+670	1.52
Drain 1	2.9	1.6	13.3	+813	1.64
Drain 2	2.5	3.9	9.5	+712	1.52

Table 3.2 Results of chemical analyses of Trefriw spa water samples. Values are in mM, except for DOC (mg/L).

Sample	Total Fe	Fe^{3+}	Fe^{2+}	Cu	Mn	SO_4^{2-}	Zn	DOC
Feed pipe	4.00	0.10	3.90	<0.03	0.02	7.3	0.007	4.5
Pool benthic zone	4.65	1.20	3.45	<0.03	0.02	10.3	0.003	4.3
Drain 1	1.80	1.76	0.04	N.D.	N.D.	11.4	N.D.	12.2
Drain 2	0.73	0.48	0.25	<0.03	0.03	7.4	0.004	8.0

3.4 Cultivation-based studies on acid streamer samples

3.4.1 Materials and Methods

For cultivation-based techniques, two streamer samples (taken from the Feed pipe and Drain 1) were selected. Wet streamer samples (500 mg) were added to 20 ml centrifuge bottles containing 10 ml heterotrophic basal salts solution, pH 3.5 (Section 2.2.1). The suspended samples were vigorously mixed to disperse the streamer materials, and then centrifuged briefly to remove fragments of undispersed materials. The supernatants were diluted (10^{-1}, 10^{-3} and 10^{-4}) in heterotrophic basal salts solution (pH 3.5) and 100 µl aliquots were used to inoculate solid media. A range of solid media was used to isolate indigenous microorganisms (Section 2.2.1.2): iron overlay, iron non-overlay, iron-tetrathionate overlay, yeast extract overlay (pH 3 and 4), yeast extract non-overlay (pH 3 and 4), iron thiosulfate overlay, R2A and aSRB (supplemented with 5 mM mannitol, 5 mM glycerol or 0.25 mM lactate). Gelled mine water (prepared by adding yeast extract (to 0.01%, w/v) to Pool water, filter-sterilizing and combining with an autoclaved agarose

solution) was also used. The inoculated solid media were incubated under aerobic, micro-aerobic or anaerobic conditions, at 20°C.

Following incubation, plates were examined and colonies were differentiated (on the basis of color and morphology) and counted. In cases where there were very large numbers of colonies on a plate, colonies in a known small area (0.785 cm^2; generally 10 of these were counted separately) were counted and the total numbers on the plate derived by extrapolation. Single representative colonies (i.e. showing distinct color and/or morphology) were inoculated into a liquid medium that contained the same components as the solid medium (apart from agarose) on which they grew originally. When grown, cells were harvested, lysates prepared (Section 2.6.1) and 16S rRNA genes were amplified by PCR (Section 2.6.2). Next, RFLP analysis was carried out on the PCR products to identify the isolates (Section 2.6.4). If the RFLP pattern was not identified from the existing laboratory database, the PCR product was purified using QIAquick® PCR purification kit (QIAGEN) and identified by 16S rRNA gene sequence analysis (Section 2.6.7).

3.4.2 Results

The results of colony counts of dispersed acid streamers sampled at the Feed pipe and Drain 1 are listed in Tables 3.3 and 3.6. Microaerobically-incubated yeast extract (pH 3 and 4) and yeast extract overlay (pH 3 and 4) solid media were infected with fungi and colonies on these solid media were not counted. No colonies grew on neutral pH R2A solid medium, or on iron thiosulfate solid medium (which was used to isolate any moderately acidophilic microorganisms present).

For the solid media inoculated with dispersed Feed pipe streamer, the largest number of colonies was recorded on microaerobically-incubated iron overlay solid medium. On these plates, ferric iron-encrusted colonies dominated. In contrast, no iron-encrusted colonies were observed on iron-tetrathionate overlay media incubated either aerobically or microaerobically. More colonies grew on yeast extract overlay media than on non-overlay media. About 10^3 times more colonies grew on yeast extract overlay (pH 3) medium than on non-

overlay medium. Colony counts on yeast extract overlay medium (pH 4) were similar to those on pH 3 overlay medium, but no colonies grew on non-overlay yeast extract (pH 4) medium. Some colonies grew on anaerobic solid medium containing mannitol, but these did not deposit black FeS, which is a characteristic of sulfate reducers.

In contrast to the dispersed Feed pipe streamer, the largest number of colonies with the dispersed Drain 1 streamer sample grew on aerobically-incubated yeast extract medium (pH 3), and again colony numbers were much greater on overlay than on non-overlay medium (by a factor of *ca.* 100). The second largest colony counts were obtained with iron-overlay medium incubated microaerobically though, interestingly, no colonies grew on iron-tetrathionate medium incubated either aerobically or microaerobically. As with the dispersed Feed pipe streamer, no colonies grew on either iron/thiosulfate or R2A plates.

Colonies displaying distinct morphologies were counted separately and representative colonies of each type were removed and put into liquid media. Unfortunately, not all of these grew successfully therefore it was not possible to identify the isolates (beyond them being iron-oxidizing or heterotrophic acidophiles) to the species level by analysis of their 16S rRNA genes. Only around 18% of isolates were successfully sub-cultured and those isolates from iron overlay and yeast extract overlay pH 4 plates proved particularly difficult to grow on, with only 8 and 9% of isolates, respectively, being successfully sub-cultured. Those which were identified to the genus/species levels are shown in Tables 3.5 and 3.8. It was confirmed that *Acidithiobacillus* sp. *Acidiphilium* sp and *Acidocella* sp. were present in acid streamers taken from both the Feed pipe and Drain 1

Isolate CCW27 (from the Feed pipe streamer), which formed a non-iron oxidizing colony on yeast extract pH 3 overlay solid medium, grew as long filaments on solid and in liquid media. The closest (93%) characterized relative of this isolate was "*Ferrimicrobium acidiphilum*" and was 98.5% identical to a clone sequence (TRA2-10) obtained from the Iron Mountain mine in California, U.S.A. (Edwards et al. 1998). Unfortunately, this isolate was lost

but other isolates (CS11 and TS1), which had 99% 16S RNA gene similarity to CCW27 and also to each other, were later isolated from iron non-overlay ferrous sulfate solid medium and on gelled mine water medium.

Table 3.3. Colony counts on various solid media inoculated with dispersed acid streamer from the Feed pipe at the Trefriw spa. Dilution factor 10^{-x} represents 10^{x}-fold dilution.

Solid medium	Dilution factor	Iron oxidizers	Non-iron oxidizers	Total
Aerobic incubation				
Iron overlay	10^{-1}	(5579)	(1610)	(7189)
	10^{-3}	58 *2**	24 *2**	82
Iron-tetrathionate overlay	10^{-1}	0	17 *1**	17
	10^{-3}	0	7 *1**	7
Yeast extract pH 3	10^{-1}	0	1 *1**	1
Yeast extract pH 4	10^{-1}	0	0	0
Yeast extract overlay pH 3	10^{-1}	0	(8545)	(8545)
	10^{-3}	0	17 *1**	17
Yeast extract overlay pH 4	10^{-1}	0	661 *2**	661
Iron thiosulfate overlay	10^{-1}	0	0	0
R2A	10^{-1}	0	0	0
Micro-aerobic incubation				
Iron overlay	10^{-1}	(7412)	(1397)	(8809)
	10^{-3}	588 *2**	20 *1**	608
Iron-tetrathionate overlay	10^{-1}	0	(1812)	(1812)
Anaerobic incubation				
None[#]	10^{-1}	0	0	0
Mannitol[#]	10^{-1}	0	20	20
Glycerol[#]	10^{-1}	0	0	0
Lactate[#]	10^{-1}	0	0	0

1. [#]aSRB solid media with or without additional organic substrates.

2. Numbers in parentheses are based on counts obtained by extrapolation (see materials and methods text).

3. Italicized numbers (followed by an *) refer to the numbers of distinct colony types on each plate.

Table 3.4. List of microorganisms isolated from the Feed pipe streamer sample.

Isolate Code	Isolation medium	Characteristics	Identification
CCO6	Feo/10^{-3} 57%	Large Fe^{3+}-encrusted colony Large rods/SP-/M-	-
CCO8	Feo/10^{-3} 43%	Small Fe^{3+}-encrusted colony Rods/SP-/M-	-
CCW10	Feo/10^{-3} 100%	White round colony Single rods and filaments/SP-/M-	+
CCW17	FeSo/10^{-3} 100%	White round colony Rods/SP-/M-	+
CCW30	YE3/10^{-3} 100%	White raised round colony Rods or chains/SP-/M-	+
CCP3	YEo3/10^{-1} 100%	Pink ellipsoid colony Rods/SP-/M-	+
CCW27	YEo3/10^{-3} 100%	White hard round colony Filaments/SP-/M-	+
CCW24	YEo4/10^{-3} 84%	Large white round colony Cocci/SP-/M-	-
CCW25	YEo4/10^{-3} 16%	Small white round colony Rods/SP-/M-	-
CS11	Fe	White round colony Filaments/SP-/M-	+
TS1	MW	Fe^{3+}-encrusted round colony Filaments/SP-/M-	+
CCO13	Feo/10^{-3} 24%	Large Fe^{3+}-encrusted round colony Rods/SP-/M+	-
CCO14	Feo/10^{-3} 76%	Small Fe^{3+}-encrusted round colony Rods/SP-/M-	+
CCW65	Feo/10^{-3} 100%	Large white round colony Rods/SP-/M-	-
CCW68	FeSo/10^{-3} 67%	White round colony Paired rods/SP-/M-	+
CCW69	FeSo/10^{-3} 33%	Greenish white colony Cocci/SP-/M-	-

1. Feo = iron overlay, Fe = iron sulfate non-overlay, YEo3 = yeast extract overlay pH 3, FeSo = iron-tetrathionate overlay, YE3 = yeast extract pH 3, MW = Mine water medium.

2. (+) identified; (-) lost during sub-culturing and not identified.

3. SP = spore formation, M = motility.

4. The"%" figure for each isolate refers to the relative abundance of the colony type (iron-encrusted or non iron-encrusted) on the plate on which it was originally isolated

Table 3.5. List of identified (from analysis of 16S rRNA genes) isolates from the Feed pipe streamer sample, and their nearest cultivated relatives and environmental clones.

Isolate	Length of the 16S rRNA gene sequenced (base pairs)	Closest relative	Identity* (%)
CCW10	684	*Acidithiobacillus* sp. NO-37	99.7
CCW17	720	*Acidithiobacillus* sp. NO-37	99.7
CCW30	664	*Acidocella* sp. NO-12	98.6
CCP3	594	*Acidiphilium* sp. NO-17	99.7
CCW27	361	Uncultured eubacterium TRA2-10	98.5
		"Ferrimicrobium acidiphilum"	93
CS11	1411	Uncultured eubacterium TRA2-10	99.1
		"Ferrimicrobium acidiphilum"	92.9
TS1	1395	Uncultured eubacterium TRA2-10	98.5
		"Ferrimicrobium acidiphilum"	91.5
CCO14	722	*Acidithiobacillus* sp. NO-37	99.6
CCW68	711	*Acidithiobacillus* sp. NO-37	99.3

* based on comparison of 16S rRNA gene sequences

Table 3.6. Colony counts on various solid media inoculated with dispersed acid streamer from the Drain 1 at Trefriw spa. Dilution factor 10^{-x} represents 10^{x}-fold dilution.

Solid media	Dilution factor	Iron oxidizers	Non-iron oxidisers	Total
Aerobic incubation				
Iron overlay	10^{-1}	(759) *2**	(719) *2**	(1478)
Iron-tetrathionate overlay	10^{-1}	0	354 *1**	354
Yeast extract pH 3	10^{-1}	0	27 *3**	27
Yeast extract pH 4	10^{-1}	0	375 *2**	375
Yeast extract overlay pH 3	10^{-1}	0	(2310) *3**	(2310)
Yeast extract overlay pH 4	10^{-1}	0	315 *2**	315
Iron thiosulfate overlay	10^{-1}	0	0	0
R2A	10^{-1}	0	0	0
Micro-aerobic incubation				
Iron overlay	10^{-1}	(1833) *2**	405 *1**	(2238)
Iron-tetrathionate overlay	10^{-1}	0	0	0
Anaerobic incubation				
Yeast extract overlay pH 3	10^{-1}	0	0	0
Yeast extract overlay pH 4	10^{-1}	0	0	0

1. Numbers in parentheses are based on counts obtained by extrapolation (see materials and methods text).
2. Italicized numbers (followed by an *) refer to the number of distinct colony types on each plate.

Table 3.7. List of microorganisms isolated from Drain 1 streamer sample.

Isolate	Solid medium	Characteristics	Identification
CCO3	Feo/10^{-3} 14%	Large Fe^{3+}-encrusted colony Cocci/SP-/M-	-
CCO5	Feo/10^{-3} 86%	Small Fe^{3+}-encrusted colony Rods/SP-/M-	-
CCT3	Feo/10^{-3} 83%	Small colony Rods/SP-/M-	-
CCW4	Feo/10^{-3} 17%	Medium pink colony Rods/SP-/M-	-
CCW19	FeSo/10^{-3} 100%	White round colony Rods/SP-/M-	+
CCP5	YE3/10^{-3} 7%	Pink ellipsoid colony Rods/SP-/M-	-
CCP6	YE3/10^{-3} 40%	Pink round colony Coccobacilli/SP-/M-	-
CCW32	YE3/10^{-3} 53%	White round colony Rods/SP-/M+	+
CCW34	YE4/10^{-3} 81%	White diffused colony Rods/SP-/M-	+
CCW35	YE4/10^{-3} 19%	Medium white colony Paired rods/SP-/M-	-
CCP4	YEo3/10^{-3} 5%	Pink round colony Rods/SP-/M-	+
CCW28	YEo3/10^{-3} 17%	White ellipsoid Rods/SP-/M-	-
CCW29	YE3o/10^{-3} 78%	White round colony Rods or chains/SP-/M-	+
CCW20	YEo4/10^{-3} 79%	Large white round colony Long chains/SP-/M-	-
CCW21	YEo4/10^{-3} 21%	White large diffused colony Rods/SP-/M+	-
CCO17	Feo/10^{-3} 11%	Large Fe^{3+}-encrusted colony Large rods/SP-/M-	-
CCO18	Feo/10^{-3} 89%	Small Fe^{3+}-encrusted colony Coccobacilli/SP-/M-	-
CCW67	Feo/10^{-3} 100%	Small white colony Rods/SP-/M-	-

1. Feo = iron overlay, Fe = iron non-overlay, YEo3 = yeast extract overlay pH 3, FeSo = iron-tetrathionate overlay, YE3 = yeast extract pH 3, MW = Mine water medium.
2. (+) identified; (-) lost during sub-culturing and not identified.
3. SP = spore formation, M = motility.
4. The"%" figure for each isolate refers to the relative abundance of the colony type (iron-encrusted or non iron-encrusted) on the plate on which it was originally isolated.

Table 3.8. List of identified (from analysis of 16S rRNA genes) isolates from the Drain 1 streamer sample, and their nearest cultivated relatives and environmental clones.

Isolate	Length of the 16S rRNA gene sequenced (base pairs)	Closest relative	Identity* (%)
CCP6	606	*Acidiphilium* sp. NO-17	99.7
CCP4	611	*Acidiphilium* sp. NO-17	99.8
CCW19	723	*Acidithiobacillus* sp. NO-37	99.7
CCW32	635	*Acidiphilium* sp. NO-17	99.7
CCW34	712	*Acidocella* sp. NO-12	98.7
CCW29	660	*Acidocella* sp. NO-12	98.9

* based on comparison of 16S rRNA gene sequences

Table 3.9. Plate counts of bacteria in dispersed Feed pipe and Drain 1 streamers. The numbers are expressed as colony forming units/ml of dispersed acid streamer suspensions.

	Feed pipe	Drain 1
Iron-oxidizing bacteria		
- extreme acidophiles	5.9×10^6	1.8×10^4
- moderate acidophiles	$<10^2$	$<10^2$
Heterotrophic acidophiles	2.4×10^5	2.3×10^4
Neutrophiles	$<10^2$	$<10^2$

3.5 Cultivation-independent analysis of acid streamer samples

3.5.1 DNA-based analyses

3.5.1.1 Terminal restriction fragment length polymorphism (T-RFLP) analysis

3.5.1.1.1 Materials and Methods

DNA was extracted from acid streamer samples as described in Section 2.6.1 and used for T-RFLP analysis (Section 2.6.6). T-RFLP analysis on DNA extracted from acid streamers taken from the Feed pipe, Pool (benthic and planktonic zones) Drain 1 and Drain 2, was carried out using two restriction enzymes *Hha*I and *Msp*I (Section 2.6.4).

3.5.1.1.2 Results

The results of T-RFLP analyses of DNA extracted from acid streamer samples are shown in Figures 3.3 and 3.4.

As the terminal restriction fragment (T-RF) acidophile database was, at the time, limited, most (80-100%) of the T-RFs could not be identified. Two separate digests (using *Hha*I and *Msp*I) were carried out, as it is possible that different species of acidophiles might produce the same T-RF when digested with only one restriction enzyme, but there is a far lesser probability that gene fragments of the same sizes will also be produced when a second enzyme, that targets a different site, is used. However, the only T-RFs that could be assigned to known microorganisms were those of *Thiomonas* spp. whose T-RF lengths were 566 and 489 nucleotides when treated with *Hha*I and *Msp*I, respectively.

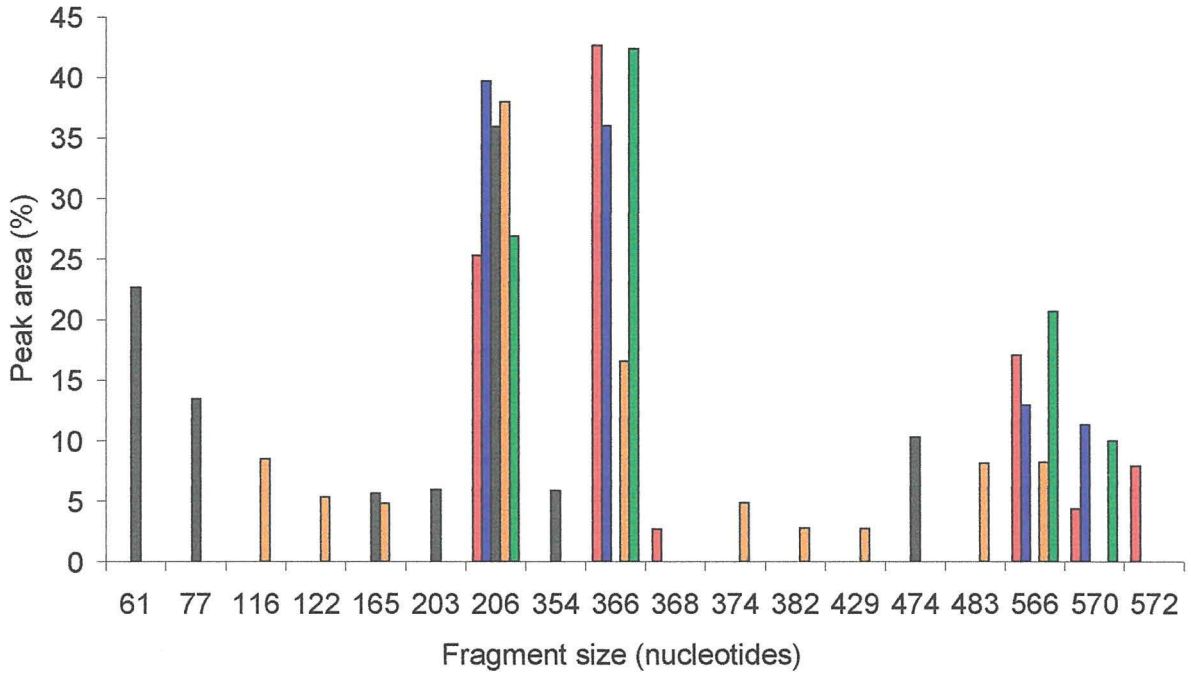


Figure 3.3. Results of T-RFLP analyses of Trefriw spa streamer samples using the restriction enzyme *Hha*I. Feed pipe=Blue, Pool benthic zone=Green, Pool planktonic zone=Orange, Drain 1=Red and Drain 2=Black.

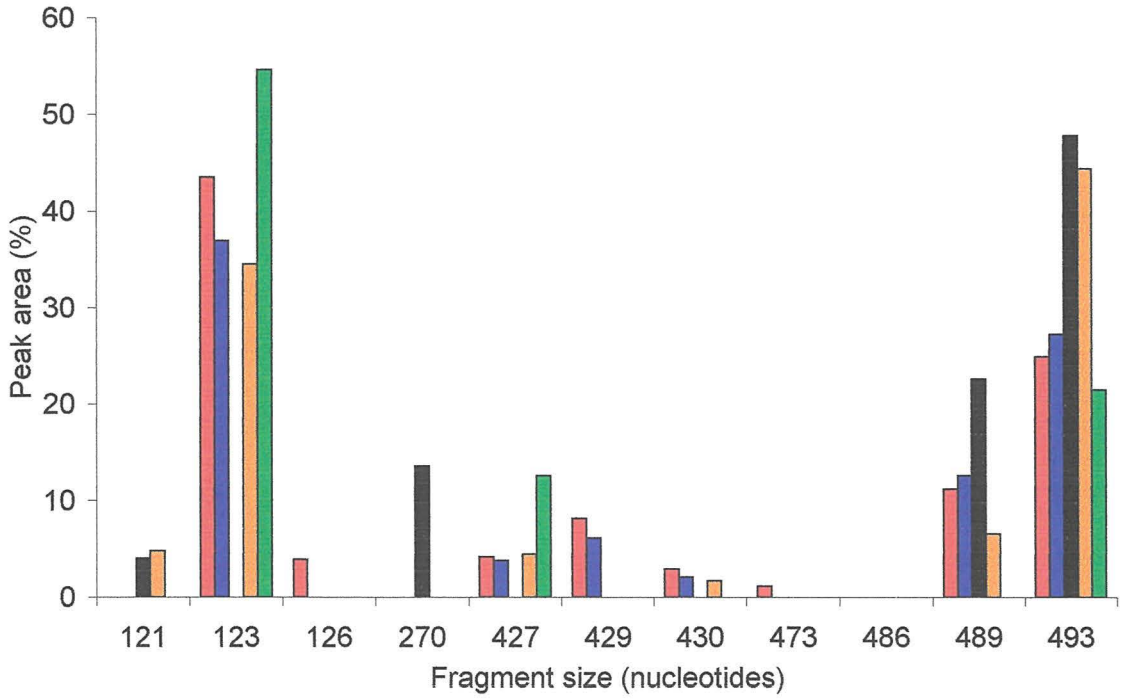


Figure 3.4. Results of T-RFLP analyses of Trefriw spa streamer samples using *Msp*I restriction enzyme. Feed pipe=Blue, Pool benthic zone=Green, Pool planktonic zone=Orange, Drain 1=Red and Drain 2=Black.

3.5.1.2 Construction of mini-clone libraries

3.5.1.2.1 Materials and Methods

A mini-clone library was constructed using DNA extracted from the Feed pipe streamer sample (Section 2.6.6). Ten clones were selected and sequences of eight clones were analyzed. Complete sequences of three clones were obtained using 27f, 1100r and 1492r primers and chimeric sequences were identified using the *Chimera Detection* program (Ribosomal Database Project, U.S.A.; http://rdp.cme.msu.edu/cgis/chimera). T-RFLP analyses on the clones were performed with separate digests using *Hha*I, *Msp*I and *Alu*I.

The second clone library was made with diluted (10-fold) DNA extracted from the Feed pipe streamer sample in order to obtain a non-chimeric sequence of the TrefC11 clone. A new clone (TrefC11) was chosen and its sequence was obtained using 27f, 1100r and 1492r primers.

3.5.1.2.2 Results

The results of the sequence analyses of eight clones are shown in Table 3.10 and a phylogenetic tree was constructed using the 16S rRNA gene sequences of these clones (Figure 3.5).

Table 3.10. Results of sequence analyses of clones obtained from the Feed pipe streamer mini-clone library. Numbers in brackets are NCBI accession numbers.

Clone	Length of the 16S rRNA gene sequenced (base pairs)	Closest relative	Identity* (%)
TrefC2	1435	Chimeric sequence	
TrefC3	727	GOUTB18(AY050601)	96.6
		Gallionella ferruginea (L07897)	95.9
TrefC4	1414	GOUTB18	97.0
(AY766001)		*Gallionella ferruginea*	95.4
TrefC5	1020	Chimeric sequence	
TrefC7	1428	Chimeric sequence	
TrefC8	1003	Chimeric sequence	
TrefC9	1016	Chimeric sequence	
TrefC10	1035	Chimeric sequence	

* based on comparison of 16S rRNA gene sequences.

From sequence analysis, there were two distinct clones present in the mini-clone library (TrefC4 group and TrefC2 group). The "TrefC4 group" (TrefC3 and TrefC4) was most identical to GOUTB18, which is an uncultured bacterium clone obtained from an *in situ* reactor system treating monochlorobenzene-contaminated groundwater (Alfreider et al. 2002). The closest characterized microorganism to this clone is *Gallionella ferruginea* to which the clone is 95.4% identical. The "TrefC2 group" sequences (TrefC2, TrefC5, TrefC7, TrefC8, TrefC9 and TrefC10) were all chimeric. Complete sequences of TrefC2 and TrefC7 were obtained and their sequences were found to be 99.4% identical to each other. Both sequences appeared to be composed of two segments: the first section (756 nucleotides) of TrefC2 and that (868 nucleotides) of TrefC7 were 98% and 99% (respectively) identical to the environmental clone TRA3-20 (NCBI accession no. AF047644). Clone TRA3-20 is a sequence of an uncultured eubacterium obtained from Iron Mountain (Edwards et al. 1998). The latter sections of TrefC2 (405 nucleotides) and TrefC7 (422 nucleotides) were both 99% identical to that of the TrefC4 sequence. An almost complete sequence (1465 nucleotides) of clone TrefC11 (NCBI accession number AY766003) obtained from construction of the second clone library was not chimeric and was 99.5% homologous to the environmental clone TRA3-20. Around the same time, a second highly related clone (MPKCSC9; 99% identity to TrefC11) was obtained from acid streamers at the former Mynydd Parys copper mine, north Wales (K. Coupland, unpublished data).

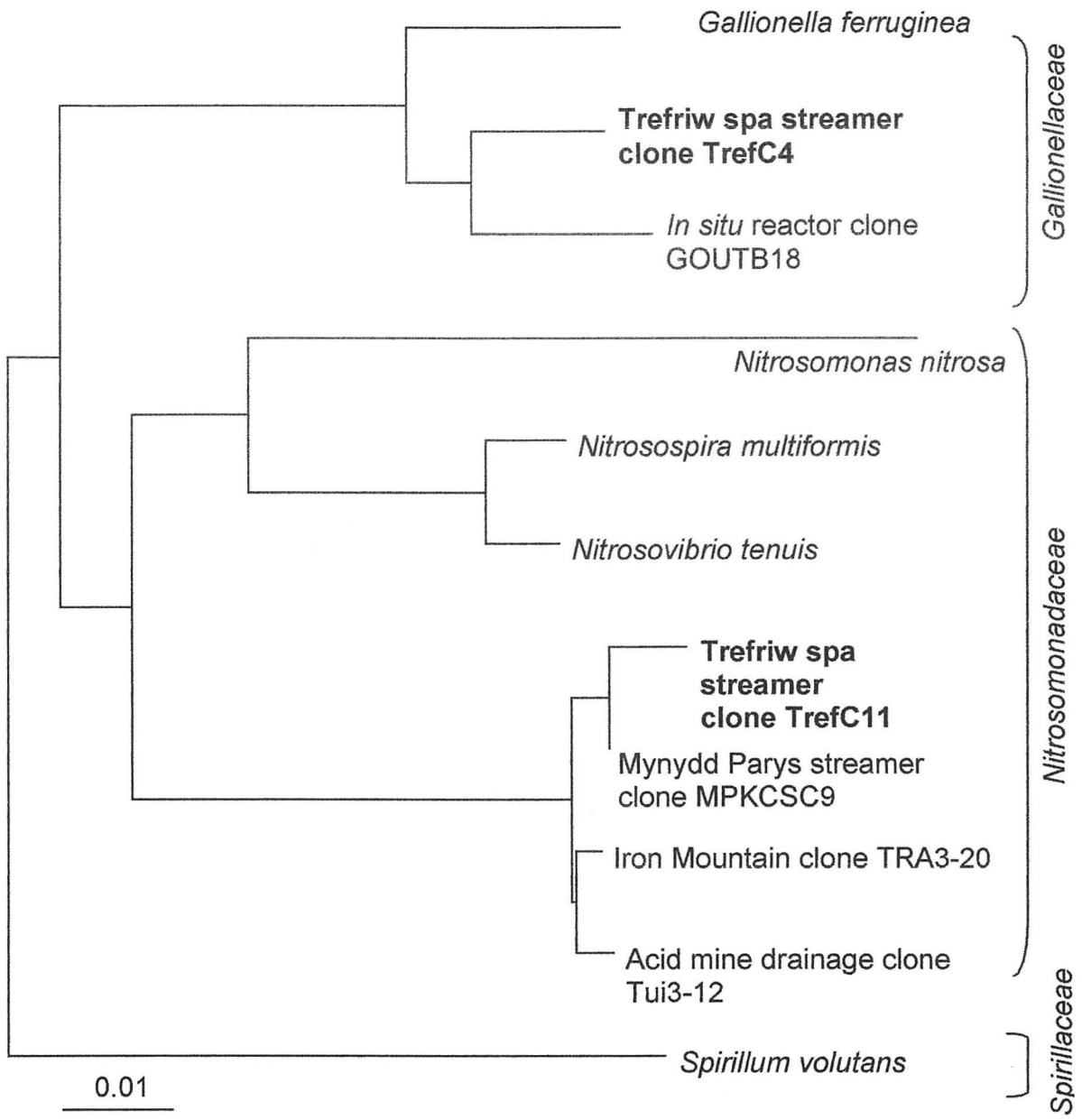


Figure 3.5. Phylogenetic relationships of Trefriw spa clones (TrefC4 and TrefC11) and Mynydd Parys clone (MPKCSC9), in bold, to the clones GOUTB18, Tui3-12 and TRA-3-20, and characterized members of the order *Nitrosomonadales* (the families *Gallionellaceae*, *Nitrosomonadaceae* and *Spirillaceae*). The bar represents 0.01 nucleotides substitution per 100 for the branch lengths. The tree was rooted with *Thiomonas cuprina* (not shown). T-RFLP was performed on clones TrefC4 and TrefC7 and their fragment sizes are shown in Table 3.11.

Table 3.11. Results of T-RFLP analyses on Clones TrefC4 and TrefC7.

Restriction enzyme	Clone TrefC4 length (nucleotides)	Clone TrefC7 length (nucleotides)
*Hha*I	366	206
*Msp*I	123	493
*Alu*I	233	170

Following T-RFLP analysis of these two clones, it was possible to identify the dominant fragments present in T-RF digests of the Trefriw spa streamer samples (Table 3.12 and Table 3.13). Most of the total T-RF peak area obtained with the Feed pipe streamer was accounted for by the two clones, TrefC4 and TrefC7 (76% of the *Hha*I digest). For the Pool benthic zone sample, TrefC4 T-RF accounted for a greater percentage of the total than TrefC7, whilst the reverse was the case with the Pool planktonic zone streamer. In these streamers, the combined peak areas of TrefC4 and TrefC7 accounted for up to 80% of the total. In Drain 1 streamer, both TrefC4 and TrefC7 T-RFs were again present and accounted for about 70% of the total peak area. However, no TrefC4 T-RF was detected in Drain 2 streamer sample digested with either *Hha*I or *Msp*I and the dominant T-RF (48% of the total) corresponded to that of TrefC7.

Table 3.12. List of peak areas of T-RFs assigned to TrefC4 in Trefriw spa acid streamers.

Restriction enzyme	Feed pipe (%)	Pool benthic (%)	Pool planktonic (%)	Drain 1 (%)	Drain 2 (%)
*Hha*I	36	42	17	43	0
*Msp*I	37	55	35	44	0

Table 3.13. List of peak areas of T-RFs assigned to TrefC7 in Trefriw spa acid streamers.

Restriction enzyme	Feed pipe (%)	Pool benthic (%)	Pool planktonic (%)	Drain 1 (%)	Drain 2 (%)
*Hha*I	40	27	38	25	36
*Msp*I	27	21	44	25	48

3.5.2 Microscopic analysis of acid streamer microorganisms

3.5.2.1 Analysis of acid streamers using scanning electron microscopy (SEM)

3.5.2.1.1 Material and Methods

About 0.2 g (wet weight) of streamer sample taken from the Pool (benthic zone) was fixed in 4% PFA in PBS for 48 hours at 4°C. The streamer sample was prepared for SEM analysis as described in Section 2.4.4 and viewed using a HITACHI S-520 scanning electron microscope.

3.5.2.1.2 Results

A scanning electron micrograph of the Pool benthic zone acid streamer is shown in Figure 3.6. In the streamer material, no eukaryotes were observed and the material appeared to be composed exclusively of bacteria. The length of the cells in the samples ranged from 1 to 2 µm and some were present as filaments of >5 µm length. The filaments seemed to be composed of chains of cells rather than tubular filaments. Although exopolymeric material was not preserved well as a result of the process used to prepare the sample, there appeared to be some exopolymers present as web-like materials attached to the cells.

3.5.2.2 Total microbial cell counts by DAPI staining

3.5.2.2.1 Materials and Methods

One milliliter aliquots of dispersed Feed pipe and Drain 1 streamer samples (Section 3.4.1) were fixed in 4% PFA in PBS and samples were used for total cell counts by DAPI staining (Section 2.3.2.2).

3.5.2.2.2 Results

The numbers of total microbial cells stained with DAPI in dispersed streamers were compared to the plate count data to calculate the efficiencies of the plating process. Total plate counts were obtained from adding the numbers of iron-oxidizing and non iron-oxidizing colonies (Table 3.3 and 3.6). The results obtained from the Feed pipe and Drain 1 streamers are shown in Table 3.14. In both cases, plating efficiency (7.18 and 0.07%) was very low.

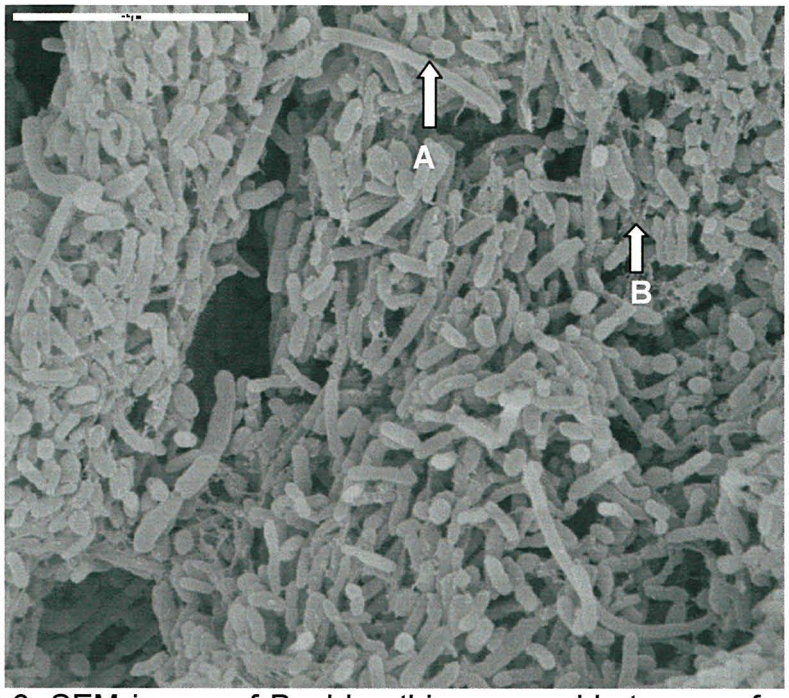


Figure 3.6. SEM image of Pool benthic zone acid streamer from the Trefriw spa. The bar represents 5 µm. Arrow A indicates a filament. Arrow B indicates web-like exopolymers.

Table 3.14. Comparison of DAPI counts and plate counts, and calculated plating efficiencies of dispersed Feed pipe and Drain 1 streamers.

Streamer sample	DAPI counts*	Plate counts*	Plating efficiency (%)
Feed pipe	8.5×10^{7}	6.1×10^{6}	7.18
Drain 1	5.8×10^{7}	4.1×10^{4}	0.07

* number of cells/ml dispersed streamer sample.

3.5.2.3 Fluorescent in situ *hybridization (FISH) analysis*

3.5.2.3.1 Materials and Methods

Fixed samples of Feed pipe and Drain 1 acid streamers (Section 3.5.2.1) were used for FISH analysis (Section 2.6.8).

3.5.2.3.2 Results

The results obtained from FISH analyses on Feed pipe and Drain 1 streamer samples are shown in Table 3.15. The proportions of EUB338Cy3- and ARCH915-stained cells were counted against cells stained with DAPI. The cells stained with other probes were enumerated against EUB338Cy3-stained cells.

Table 3.15. Results of FISH analyses of Feed pipe and Drain 1 acid streamers.

Probe	Feed pipe streamer sample (%)	Drain 1 streamer sample (%)
EUB338Cy3	88.9	89.2
ARCH915	0	0
ALF1B	0.10	0.13
BET42a	84.5	90.9
GAM41a	8.55	0.07
LGC0355	0	0
LF581	0	0
LF1252	0	0
LF655	1.80	*
ACM732	0	3.80
ACM995	0	0
TF539	2.30	0.07

* stained cells were detected but were not countable.

By comparing EUB338 counts against DAPI counts, it was found that about 90% of the total microorganisms present in the sample were active bacteria. The majority (91% in Feed pipe and 84.5% in Drain 1 streamer) of the cells in both samples was stained with the BET42a probe; few cells were stained with the ALF1B, GAM41a, LF655 (which target members of *Leptospirillum* groups I, II and III) and TF539 (which targets *Acidithiobacillus ferrooxidans*) probes. No archaea were detected by FISH analysis in either streamer sample. No cells were detected with the LF655 probe in a 20-fold diluted fixed sample, but a few cells in a 9-fold diluted Drain 1 streamer sample were stained by the probe. As these cells were present in a clump of streamer material that was not well dispersed, the relative number of cells stained with LF655 could not be accurately assessed. Positive staining with the ACM732 probe (which targets *Acidimicrobium ferrooxidans* and related actinobacteria) was recorded only for the acid streamer from Drain 1. Although most cells in both samples were *β-Proteobacteria,* they could not be identified any further with probes available at that time.

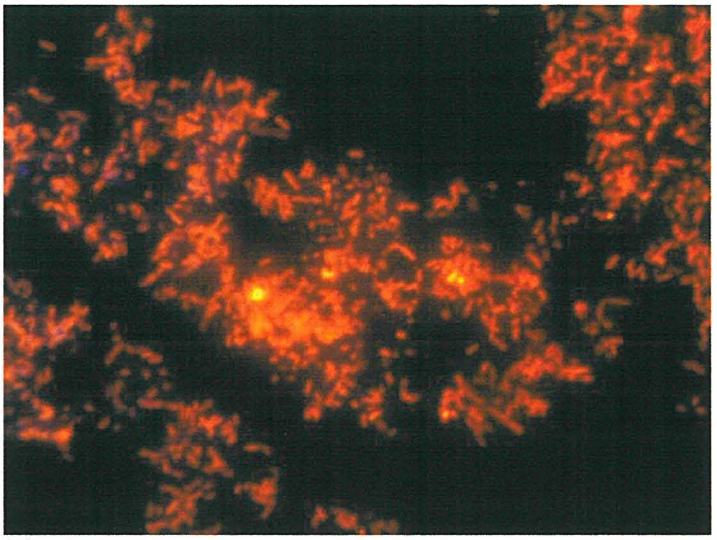
Based on the sequences of the clones obtained from the mini-clone library (Section 3.5.1.2), FISH probes that specifically targeted TrefC4 or TrefC2 groups were designed. The "GALTS" probe, which targets TrefC4, was made

by modifying the Gallprobe 1 (Hallbeck et al. 1993) that targets *Gallionella ferruginea* but not TrefC4. The sequence of the GALTS probe is 5' CCACTAACCTGGGGCAA 3' and the oligonucleotide database project (ODP) nomenclature (Alm et al. 1996) of the probe is S-S-TreC4-84-a-A-17. The probe was specific only to TrefC4. In order to enhance the probe signal, unlabeled helper oligonucleotide probes were designed to target sites prior (GALTS-1 5' GCCCCCAGGCCCGTTCGA 3') and subsequent (GALTS+1 5' GATATATTACTCACCCGTTCG 3') to the GALTS probe. TrefC2 was targeted by "BSC" probe, whose sequence is 5' TCCAGGTTATTCGCCTGA 3' and the ODP nomenclature is S-S-TreC7-459-a-A-18. The probe targeted sequences of TrefC2, the Mynydd Parys clone and the Iron Mountain clone TRA3-20. The results of FISH analyses using GALTS and BSC probes are shown in Table 3.16.

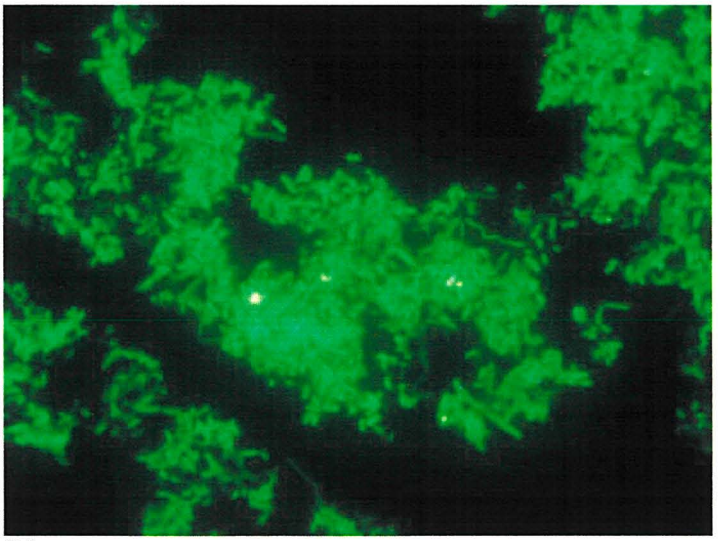
Table 3.16. Results of FISH analyses of Feed pipe and Drain 1 acid streamers using the GALTS and BSC probes.

Probe	Feed pipe streamer (%)	Drain 1 streamer (%)
GALTS	20.9	36.5
BSC	13.4	59.2

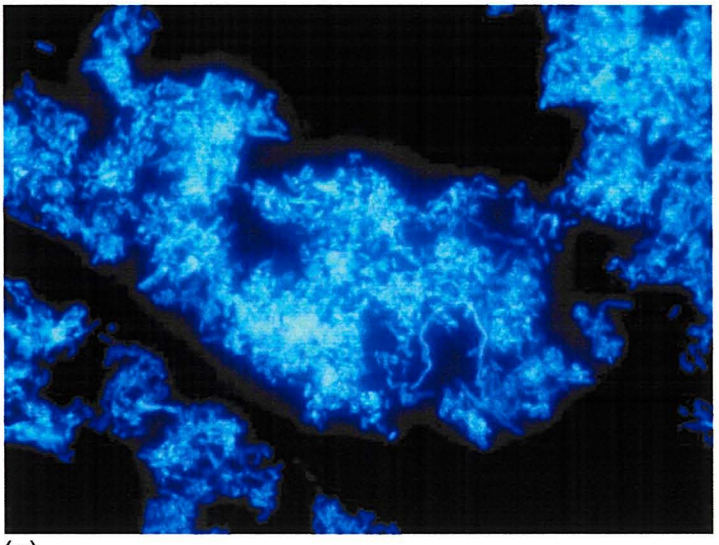
The results obtained from FISH analyses using the GALTS and BSC probes showed that both TrefC4 and TrefC7 were present in relatively large numbers in both Feed pipe and Drain 1 acid streamers. Images obtained from FISH analysis of Drain 1 streamer sample using the BSC probe are shown in Figures 3.7, while Figure 3.8 shows the cells stained with the GALTS probe in a Pool benthic zone streamer. In the Feed pipe streamer sample, TrefC4 comprised 20.9% and TrefC7 13.4% of the total active bacteria present. As both clones belong to the *β-Proteobacteria*, 41% of the cells stained with BET42a could be accounted for by TrefC4 and TrefC7. With the Drain 1 streamer sample, TrefC4 and TrefC7 comprised 36.5% and 59.2%, respectively, of the bacteria stained by the EUB338 probe and were therefore the dominant microorganisms in this particular acid streamer sample.



(a)

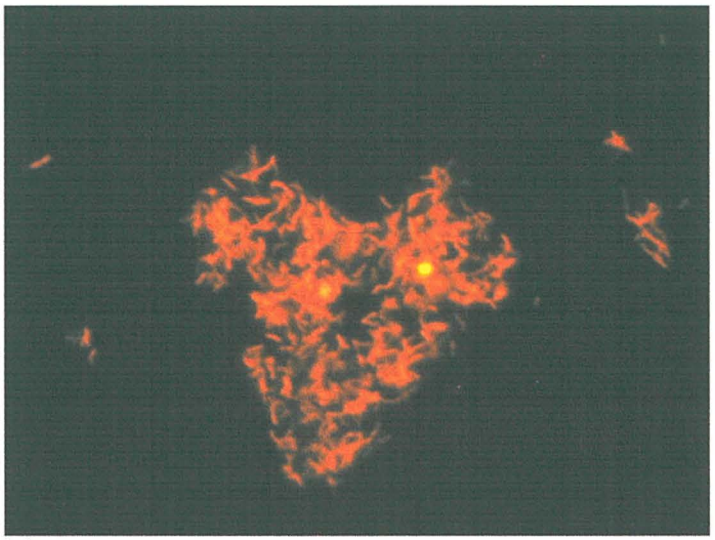


(b)

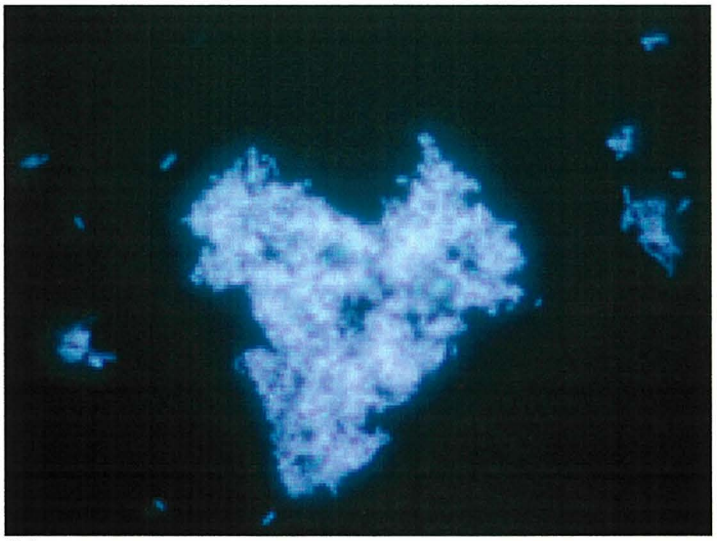


(c)

Figure 3.7. FISH analysis of Drain 1 acid streamer. (a) Cells stained with Cy3-labeled BSC probe; (b) Cells stained with the fluorescein-labeled eubacterial probe EUB338; (c) Cells stained with DAPI.



(a)



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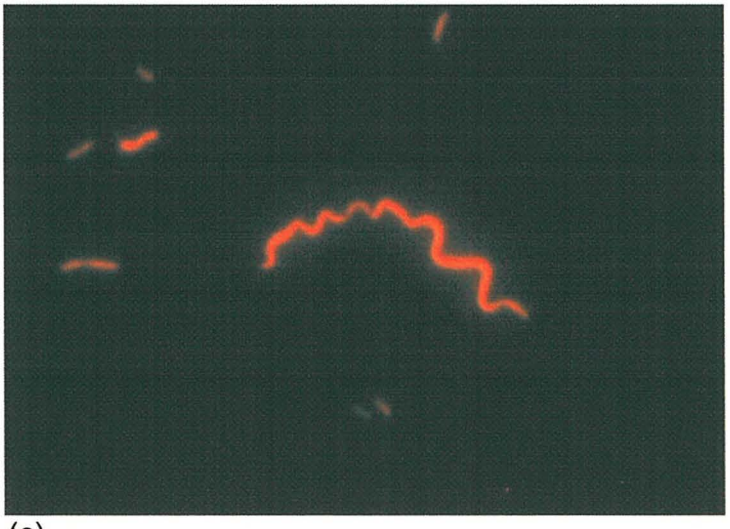


(c)

Figure 3.8. FISH analysis of Pool benthic zone acid streamer. (a) Cells stained with Cy3-labeled GALTS probe; (b) Cells stained with DAPI; (c) Cells stained with Cy3-labeled GALTS probe, showing a "corkscrew" morphology that is typical of the neutrophile *Gallionella ferruginea.*

3.6 Discussion

This study has highlighted the deficiencies in the use of cultivation-dependent approaches alone to study the microbial diversity of macroscopic acid streamer communities, and has demonstrated the potential and power of biomolecular techniques in microbial ecology.

No neutrophilic or moderately acidophilic microorganisms were isolated on the solid media used in this study. It was thought that pH might vary on the micro-scale level within acid streamers, which could allow neutrophiles and moderate acidophile to survive and grow, even though the pH of the water in which the streamers were bathing was very low (2.5-2.9). However, the absence of neutrophiles and moderate acidophiles amongst the isolates obtained would suggest either that they were not present in these acid streamers, or that they did not survive the dispersal/dilution phase (in pH 3.5 basal salts), or that they could not grow on the media used. It is interesting to note that a culture-independent study of acid streamers found in the Rio Tinto (López-Archilla et al. 2004) found that the dominant microorganisms were most closely related to neutrophilic bacteria (*Pseudomonas* and *Sphingomonas*). Neutrophilic bacteria were also isolated in earlier studies of acid streamers (Dugan et al. 1970; Johnson et al. 1979).

The water from which the Feed pipe streamer was sampled contained very little dissolved oxygen (0.6 mg/L), and it is interesting to note that greater counts of iron-oxidizing colony forming units (CFUs) were obtained on plates incubated under microaerobic than under aerobic conditions. There were fewer CFUs on iron-tetrathionate- than on iron-overlay plates, suggesting some of the microorganisms that grew on the latter were inhibited by tetrathionate. Most of colonies that grew on iron- and iron-tetrathionate-overlay media were identified as *At. ferrooxidans.* This acidophile has a versatile metabolic capability and can grow by oxidizing Fe^{2+}, S^{2-}, S^0, SO_3^{2-}, thiosulfate, tetrathionate, formate or H_2 (Friedrich 1998; Pronk et al. 1991; Drobner et al. 1990). It is also capable of anaerobic growth using elemental sulfur, tetrathionate, formate or H_2 as electron donors, coupled to ferric iron reduction. A study of an oxygen-depleted subterranean water body at

Mynydd Parys, Wales, showed that *At. ferrooxidans* was the dominant microorganism present (Coupland & Johnson 2004) indicating that *At. ferrooxidans* is a particularly significant bacterium in iron-rich, acidic environments that have low oxygen contents.

Although the DOC content of Feed pipe water was low (4.5mg/L), acidophilic heterotrophs, identified as *Acidocella* and *Acidiphilium* spp., were isolated from the dispersed streamer. These acidophiles were probably growing on organic compounds leaked from autotrophic primary producers (e.g *At. ferrooxidans*) within the streamer community, as has been shown elsewhere (Johnson et al. 1983; Johnson et al. 1992). The numbers of colonies on yeast extract-overlay media were much greater than on corresponding non-overlay plates. This further supports the hypothesis that heterotrophic, as well as autotrophic, acidophiles are strongly inhibited by small molecular weight organic compounds (such as pyruvic acid) that are present or are produced during incubation of agar-gelled media (Johnson & McGinness 1991; Johnson 1995).

Actinobacteria isolates (CCW27, CS11 and TS1) were isolated from various solid media and they oxidized iron on mine water solid medium supplemented with yeast extract. Isolate CCW27 was able to grow heterotrophically on yeast extract and (apparently) mixotrophically in ferrous sulfate/yeast extract liquid medium. The isolate was considered, on the basis of its morphology and physiology, to be identical to "CCH7", an isolate obtained from acid streamer growths in the Cae Coch mine, which is adjacent to the Trefriw spa, by Johnson et al. (1992). Isolate-CCH7 was shown to be a filamentous, obligately heterotrophic iron-oxidizer that, in contrast to autotrophic iron-oxidizers, grew better on non-overlay than on overlay iron/TSB solid medium. In liquid medium, CCH7 (and the Trefriw spa isolates) formed small acid streamer-like growths that became increasingly encrusted with ferric iron precipitates during incubation and then broke up. In view of its macroscopic filamentous growth, isolate CCH7 was considered by Johnson et al. (1992) to be an important member of acid streamer communities, though this was not supported by evidence from the present

study. However, CCH7 was not sequenced at the time and has since been lost. The microorganisms isolated from Trefriw spa streamer sample belong to the class *Actinobacteria* and the closest (93%) characterized microorganism is "*Ferrimicrobium acidiphilum*", which oxidizes iron in the presence of yeast extract, reduces ferric iron under anaerobic condition and cannot fix CO_2 (Johnson et al. 1995; Hallberg & Johnson 2001). The sequence of 16S rRNA gene of the Trefriw spa streamer isolate was most identical (99%) to that of an isolate from Mynydd Parys acid streamer (Coupland unpubl.) and that of the microorganism represented by a clone (TRA2-10) found in Iron Mountain mine. Together with these microorganisms found in acidic metal-rich environment, the Trefriw spa isolates are thought to form a unique genus in the class *Actinobacteria*.

In contrast to the Feed pipe streamer, more heterotrophs than iron-oxidizers were isolated on solid media inoculated with dispersed acid streamers from Drain 1. This might reflect the higher DOC content (*ca.* three times greater) of the water in Drain 1 and low iron (especially ferrous iron) content. The low iron content could have been due to iron oxidation by iron-oxidizing microorganisms at upstream of Drain 1. Since ferric iron precipitates out of solution at the pH values found in Trefriw spa, ferrous iron oxidation resulted in reduction in the amount of iron recorded in Drain 1 water sample. Although the DO content of Drain 1 water was relatively higher (1.6 mg/L) than that of Feed pipe water (0.6 mg/l), higher colony numbers were again recorded on microaerobically-incubated iron-overlay medium than on aerobically-incubated medium. This might suggest that, even though DO content was higher in Drain 1 water, oxygen availability within the acid streamer was still limited, hence many microorganisms (especially the iron-oxidizers) grew better under oxygen-limiting conditions. The higher colony numbers on the microaerobically incubated media could also be explained by the elevated concentration of CO_2 in the jar in which the microaerobic atmosphere was generated. In the CampyGen system, oxygen is partially removed by the formation of CO_2; this should benefit autotrophic acidophiles, which use CO_2 as their principal carbon source.

Many of the isolates obtained on solid media were lost during subsequent attempts to subculture them as pure cultures in liquid media. This could suggest that many of the streamer isolates require partner microorganisms to allow them to grow. Such relationships (mutualism, syntrophy etc.) may involve one organism providing essential growth factors for another, and/or (as in the overlay plates) removing substances that are toxic to others. Therefore, when partner microorganisms are eliminated during culture purification, the target microorganism is no longer able to grow. This phenomenon implies that there are likely to be complex relationships between members of the acid streamer microbial community.

Another problem encountered with the cultivation-based technique was the difficulty in dispersing the acid streamers. As the cells within them were tightly held together by exopolymeric substances, it was impossible to completely disperse the streamers without damaging the microorganisms. Therefore, acid streamers were only partially dispersed and some microorganisms would have remained in the undisrupted fragments. An unavoidable consequence of this is that plate counts and DAPI counts are shown as the number of cell/ml streamer cell suspension rather than /g streamer sample. Despite the fact that the cultivation approach used resulted in the successful isolation of microorganisms that have been found previously in acidic environments, comparison of DAPI counts showed that the plating efficiency was only 7.18% for the Feed pipe streamer and 0.07% for the Drain 1 streamer sample. This finding suggested that most microorganisms in the streamer sample could not be cultivated on the solid media used in this study.

As the majority of microorganisms present within the acid streamer communities at the Trefriw spa could not be cultivated with the available media, a culture-independent study was carried out to assess the biodiversity of these growths and to try, if possible, to identify the indigenous microflora. Initially, this involved PCR-based techniques. DNA was successfully extracted from the streamers and the 16S rRNA genes were amplified using bacterial primers. Amplification of 16S rRNA genes using the archaeal primer

was not successful, suggesting that the prokaryotes present were dominantly (or exclusively) bacteria, a fact later confirmed by FISH analysis.

The community structure of the acid streamers was analyzed using T-RFLP analysis of the amplified bacterial genes. This technique was selected in preference to alternatives (e.g. DGGE and TGGE) as it was thought that it might offer a greater degree of precision, as preliminary laboratory results had indicated that digested genes could be resolved to within ±one nucleotide using this approach (K. Hallberg, unpubl.). A drawback was that, so far as could be ascertained, T-RFLP had not been used previously in the study of extremely acidic environments, so that there was no available database of T-RFs corresponding to known acidophiles. The initial T-RFLP analyses performed on Trefriw spa streamers showed that there were two dominant T-RF peaks and numerous minor T-RF peaks present when digested with *HhaI* and *MspI*. Of these, only one of the minor peaks, which corresponded to a *Thiomonas* sp., could be identified.

During the first phase of FISH analysis of the acid streamers, it was found that the majority of cells (90.9% in Feed pipe and 84.5% in Drain 1 streamers) were stained with the probe targeting *β-Proteobacteria.* The only characterized acidophilic microorganism that belongs to the *β-Proteobacteria* class is *Thiomonas cuprina,* but no cells were stained with *Thiomonas*-specific probes (TMI and TMII). In the Drain 1 streamer sample, 3.8% of the cells were stained with ACM732 probe, which targets *Acidimicrobium ferrooxidans* and related actinobacteria, including *"Ferrimicrobium acidiphilum"* (Bond & Banfield 2001). However, no streamer cells were stained with the ACM995 probe, which only targets *Am. ferrooxidans*. The cells stained with the ACM732 probe were found to form long filaments, similar to the "CCH7"-like isolates CS11, TS1 and CCW27. Although no "CCH7"-like bacteria had been isolated from Drain 1 streamer, the cells stained by ACM732 in the dispersed sample from Drain 1 were considered to be actinobacteria, related to "*Ferrimicrobium*", and most probably identical to the earlier isolate, CCH7. With both the Feed pipe and Drain 1 streamers,

around 0.1% of the cells were stained with the probe specific to *α-Proteobacteria;* these were likely to include the *Acidiphilium* and *Acidocella* isolates that were the dominant culturable non iron-oxidizing heterotrophs in these streamers. With the Feed pipe streamer, 8.6% of cells were stained with a probe specific to *γ-Proteobacteria* and 2.3% of those stained with the *At. ferrooxidans*-specific probe. Few cells were detected with LF655, which targets *Leptospirillum* groups I, II and III. The number of LF655-stained cells in the Drain 1 streamer was particularly low and none was detected in highly diluted streamer suspensions. This precluded the accurate enumeration of *Leptospirillum*-like bacteria, as the cells were either only present in clusters, or there were too many unstrained cells present in the sample examined to give accurate background counts. In conclusion, the preliminary results obtained from FISH analyses concurred with the T-RFLP analyses that suggested the majority of microorganisms in the streamer samples were unknown.

In order to identify the unknown microorganism in the streamers, a mini-clone library was constructed. The library was composed of two types of clones (represented by TrefC4 and TrefC2) and the chimeric sequences (TrefC2 and others) comprised 75% of the mini-clone library. Recombination might have occurred at the early stage of PCR reaction leading to the amplification of the recombinant. The recombination could have been resulted from an excess amount of template that increased the chance of recombination. To obtain non-chimeric sequence of the microorganism represented by TrefC2 clone, a clone library was constructed later in the study using diluted template and a non-chimeric sequence was obtained as clone TrefC11. T-RFLP analyses of the two clone types confirmed that these were responsible for the two dominant T-RF peaks in the acid streamers. Although sequences of the TrefC2 group were chimeric, the first part (up to 493 nucleotides) of the sequence, which was critical for T-RFLP analysis, was not affected by the recombination. Therefore the T-RF length of the chimeric clone sequence was used to identify the T-RFs obtained from Feed pipe and Drain 1 streamer samples. Using the clone sequences, FISH probes specifically targeting the microorganisms represented by the two clone groups were

designed and the second phase of FISH analysis confirmed that the majority of the bacteria in the streamer communities was composed of these two microorganisms. In the Feed pipe streamer, around 34% of bacterial population was stained with either the GALTS (21%) or BSC (13%) probe. However, 50% of the cells that were stained with the *β-Proteobacteria*-specific probe could not be identified using the clone/species-specific probes. This could be due to enumeration error during FISH analysis since T-RFLP analysis of the streamer sample had indicated that about 70% of the total population was composed of TrefC4 and TrefC11. With the Drain 1 streamer sample, 37% of the cells were stained with the GALTS probe (which targets TrefC4) and 60% with the BSC probe (which targets TrefC11) suggesting the Drain 1 streamer was composed almost exclusively of the two microorganisms represented by these clones. Staining with FISH probes also enabled observation of cell morphologies. Cells stained with the BSC probe were short coccobacilli (Figure 3.7), while those stained with the GALTS probe were long, thin curved rods (Figure 3.8) and were often found in the inner parts of partially-dispersed streamers, suggesting that TrefC4 grows in more oxygen-limiting condition than TrefC11.

In an attempt to elucidate the growth characteristics of the two uncultured microorganisms (TrefC11 and TrefC4), T-RFLP analysis on acid streamers growing in different parts of the Trefriw spa cave, where physico-chemical conditions varied, was carried out. The T-RF peak representing TrefC4 was not present in Drain 2 streamer and the TrefC11 T-RF peak was not found in the Pool benthic zone streamer sample. The major differences between the water in these sites were the concentrations of DO, DOC and ferrous iron. Pool benthic zone water was lower in DO (0.5 mg/L) and DOC (4.3 mg/L) than Drain 2 (3.9 mg/L DO and 8.0 mg/L DOC). Pool benthic zone water also contained more ferrous iron (3.5 mM) than Drain 2 water (0.3 mM). These contrasting physico-chemical parameters suggested that the bacterium represented by clone TrefC4 thrives in low oxygen-containing waters containing little DOC and elevated concentrations of ferrous iron. This is in agreement with the description of *Gallionella ferruginea* (the closest characterized relative of TrefC4) which can grow autotrophically by ferrous

iron oxidation in low oxygen-containing waters, such as well waters (Hallbeck & Pedersen 1991). In contrast, the microorganism represented by clone TrefC11 appears to prefer more aerated waters containing higher concentrations of DOC, suggesting that it might be a heterotrophic aerobe. As these microorganisms can exist in the absence of each other, they do not appear to be obligately mutualistic, though they are likely to be interacting within the acid streamers. For example, in an environment where oxygen concentrations are too high for the TrefC4 microorganism, TrefC11 might grow as a biofilm around TrefC4, thereby limiting oxygen diffusion, while TrefC4 could, in return, providing organic substrates (in the form of cell exudates) to support the growth of TrefC11.

The closest characterized relative of TrefC4, *G. ferruginea*, is a neutrophilic bacterium that grows between pH 5.0-6.5. *G. ferruginea* can grow autotrophically via ferrous iron oxidation, but can also utilize glucose, fructose and sucrose (Hallbeck & Pedersen 1991, Hallbeck et al. 1993). It is also capable of using thiosulfate and sulfide as electron donors (Lutteres-Czekalla 1990). This bacterium is capable of growth under aerobic and micro-aerobic conditions but cannot grow under strictly anoxic conditions (Hallbeck & Pedersen 1990). Clone TrefC4 is, however, only distantly related to *G. ferruginea* (95% 16S rRNA gene sequence similarity) and certainly represents a novel species, if not genus. The fact that the bacterium represented by clone TrefC4 can grow in very acidic waters (as low as pH 2.6 in the present study) also indicates that it is different in at least one aspect of its physiology to the only currently classified *Gallionella* sp., *G. ferruginea*. However, it will be necessary to isolate the TrefC4 bacterium before it can be fully characterized and classified (possibly as *"Gallionella acidophilum"*).

The identity of the other major clone, TrefC11, is more elusive. Phylogenetically, it falls within the family *Nitrosomonadaceae* and members of this family are neutrophilic ammonium-oxidizing autotrophs. Attempts to enrich a streamer sample obtained from Mynydd Parys, which was composed almost exclusively of a microorganism identical (>99%) to

TrefC11, using ammonium sulfate-containing liquid medium, was unsuccessful (K. Coupland unpubl.). Furthermore, the amounts of ammonium present in acid mine waters are invariably low, so it is considered unlikely that the TrefC11 microorganism is an ammonium-oxidizer. Since ferrous iron is abundant in acidic mine waters, such as those in the Trefriw spa, it is more likely that the microorganism is obtaining energy from ferrous iron rather than ammonium oxidation. Circumstantial evidence for the TrefC11 bacterium being an iron-oxidizer also arises from the fact that concentrations of ferrous iron declined dramatically over a relatively short distance as the water flowed over the acid streamers, though other streamer bacteria, such as *At. ferrooxidans* and the putative *"Gallionella"* TrefC4 would also have contributed to the net iron oxidation. Little or no spontaneous oxidation of iron would have occurred at the low pH of the Trefriw spa water (Stumm & Morgan 1981). More direct evidence has arisen recently from sequence analysis of ferric iron-encrusted colonies obtained from AMD at a site (Bowden Close) in the north of England, which have been found to be closely related (*ca.* 98% 16S rRNA gene sequence identity) to clone TrefC11 (Johnson et al. unpubl.).

In conclusion, the acid streamers found in the Trefriw spa were composed of a variety of microorganisms. Two bacteria that have yet to be isolated and characterized, were dominant in these communities. Culturable streamer bacteria were all extreme acidophiles and included an iron-oxidizing autotroph (*At. ferrooxidans*), iron-oxidizing heterotrophs (the actinobacteria isolates CCW27, TS1 and CS11) and heterotrophs that do not oxidize iron (*Acidiphilium* and *Acidocella*).

The microbial composition of the acid streamers at the Trefriw spa were found to be very different from those that have been examined, using cultivation-independent techniques, at Iron Mountain, California (Bond et al. 2000a; Bond et al. 2000b), and in the Rio Tinto, Spain (López-Archilla et al. 2004).

Although cultivation techniques are important for characterizing microorganisms, only a minor fraction of the acid streamer communities were identified using the plating approach. Utilization of cultivation-independent techniques gave a more accurate picture of these acid streamer communities. T-RFLP analysis was a useful and relatively quick technique to examine the community structures of different acid streamers. The initial limitation of a poor database is now less significant as the technique has been used increasingly in the context of extremely acidic environments (e.g. Coupland & Johnson 2004) and the database has expanded greatly.

The construction of a mini-clone library facilitated the identification of the T-RFs of the dominant microorganisms in the streamers. However, as T-RFLP analysis is subject to distortion by differential DNA extraction and PCR bias (Head et al. 1998), accurate enumeration of microorganisms in the streamer communities could only be undertaken by FISH analysis. Given the availability of appropriate FISH probes, this technique is a very powerful tool to study microbial communities, since it allows the abundance, morphology and sometimes position of a particular microorganism in cell clusters to be studied. If, as in the present study, a probe targeting a specific microorganism is not available, it can be designed and synthesized from sequences obtained from clone libraries. Obstacles faced during FISH analyses were the difficulty in dispersing streamer samples and accessibility of probes to the target site. The GALTS probe, in particular, did not show a signal without the addition of "helper oligos", which made the target site accessible to the labeled probe. Though FISH probes are capable of fluorescing even in the presence of large numbers of non-target cells, accurate enumeration was sometimes not possible in such a situation. A solution to this problem, that could be examined in future work, is to disperse the streamers more thoroughly so that they could be analyzed in a flow cytometer.

Chapter 4 A study of the biodiversity of acid streamer communities within an abandoned pyrite mine

4.1 Introduction

Following the study of the microbial communities in acid streamers found in the Trefriw spa (Chapter 3), a related project was carried out in the abandoned Cae Coch pyrite mine (North Wales). This was a working mine during the 19th century but has been abandoned since 1918 (Johnson et al. 1979). Within Cae Coch, acid streamer growths are very extensive (estimated at >100 m^3), taking the forms of streamers, pipes (microbial stalactites) and slimes. Previous studies carried out on these acid streamers by Johnson (1979), Johnson et al. (1992) and McGinness & Johnson (1993) have used only cultivation techniques. In this study, a combined culture-dependent and culture-independent approach was used to elucidate streamer community structure and biodiversity.

4.2 Sampling location and sample collection

Streamer and water samples were collected at several sites within the Cae Coch mine on two separate occasions (June and October, 2003). On the first occasion, acid streamers and water samples were taken from seven locations (sites 1-7) within the mine sites and analyzed using T-RFLP. On the basis of these results, five of these sites (sites 1, 2, 3, 4 and 7) were selected for more detailed analysis, in which a combination of culture-dependent and culture-independent techniques (clone library and FISH analyses) was used, as described in Chapter 3. These five sampling sites are shown in Figures 4.1 - 4.5 and described in Table 4.1.

Figure 4.1. Sampling site 1 at the Cae Coch mine.

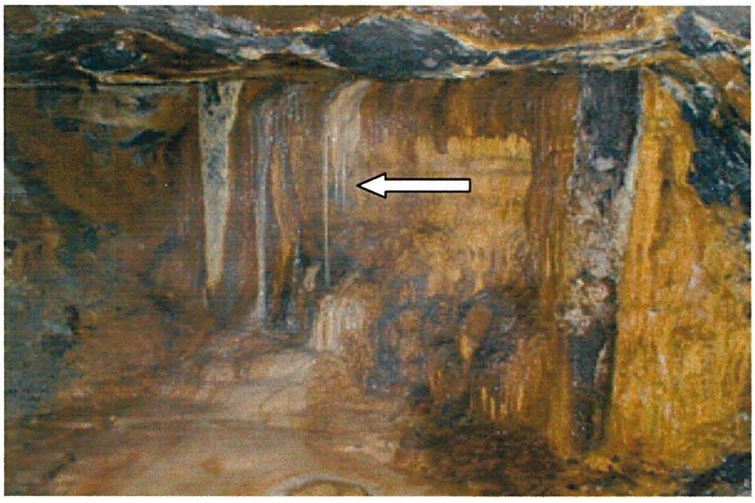


Figure 4.2. Sampling site 2 at the Cae Coch mine. The arrow indicates the microbial pipe.

Figure 4.3. Sampling site 3 at the Cae Coch mine.

Figure 4.4. Sampling site 4 at the Cae Coch mine.

Figure 4.5. Sampling site 7 at the Cae Coch mine.

Table 4.1. Description of sampling sites within the Cae Coch mine.

Sites	Sample code	Sample type	Sampling site description	Figure
Site 1	S1	Slime	Located mid-way between the mine entrance and Site 3.	4.1
Site 2	S2	Pipe	Stalactite hanging from a wooden retention structure.	4.2
Site 3	S3		Main drainage stream, located between sites 2 and 4.	4.3
	S3T	Streamer	Top layer (0-10 cm).	
	S3M	Streamer	Middle layer (30-40 cm)	
	S3B	Streamer	Bottom layer (50-60 cm)	
Site 4	S4		Isolated pool at the bottom of the mine	4.4
	S4T	Water	Surface water.	
	S4B	Water	Bottom water (30 cm deep)	
Site 7	S7	Streamer	Pool located between sites 2 and 3.	4.5

Streamer and water samples were collected in 20 ml sterile tubes and brought to the laboratory within five hours. Samples were stored at 4°C until required. The streamer samples for DAPI, FISH and SEM analyses were fixed immediately at the site in 12% PFA (w/v, 0.25 ml) and stored at -20°C until used. Free-swimming microorganisms were collected by filtering about 500 ml of mine water through 0.2 μm nitrocellulose membranes, and materials collected on the filters were used for DNA extraction. For DAPI counts and FISH analysis, water samples (2.5 ml) were put into 20 ml sample tubes containing 4% PFA (w/v, 7.5 ml) and the fixed water samples were stored at -20°C until required. Water samples used for chemical analyses were filtered into 10 ml plastic sample collection tubes containing one drop of 50% (volume/volume) nitric acid and stored at -20°C until used.

4.3 Chemical analysis of water samples

4.3.1 Materials and methods

In situ water analyses were carried out using YSI 556 multimeter (YSI Environmental, U.S.A.). Ferrous iron was determined using the Ferrozine assay (Section 2.5.2), sulfate by ion chromatography (Section 2.5.6.1), DOC using the Protoc DOC analyzer (Section 2.5.5) and concentrations of heavy metals, aluminium and arsenic by AAS (Section 2.5.4).

4.3.2 Results

The results of *in situ* analyses of water samples are summarized in Table 4.2 and laboratory analyses in Table 4.3.

Table 4.2. Results of *in situ* analyses of Cae Coch water samples.

Sample	pH	DO (mg/L)	Temperature (°C)	Eh (mV)	Conductivity (mS/cm)
S1	2.25	7.1	12.5	+655	3.9
S2	2.28	6.3	11.6	+671	3.5
S3T	2.26	3.4	9.5	+725	3.2
S3M	2.17	0.6	9.2	+679	4.3
S3B	2.11	0.3	9.1	+435	4.2
S4T	1.95	0.9	8.6	+654	9.7
S4B	1.84	0.2	9.4	+611	17.8
S7	1.90	2.9	9.5	+679	10.5

Table 4.3. Laboratory-based analyses of Cae Coch water samples.

Sample	Total Fe (mM)	Fe^{3+} (mM)	Fe^{2+} (mM)	Cu (μM)	Mn (μM)	Zn (μM)	Al (mM)	As (mM)	SO_4^{2-} (mM)	DOC (mg/L)
S1	22	18	4	2	40	30	1.3	<0.1	20	4.5
S2	18	5	13	6	60	20	1.6	<0.1	36	12.1
S3T	28	24	4	10	90	20	3.1	<0.1	43	5.7
S4T	199	129	70	18	190	70	9.8	<0.1	111	3.3
S4B	262	50	212	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
S7	78	48	30	9	90	30	3.8	<0.1	53	11.4

N.D. = not determined

All the water samples analyzed were found to be extremely acidic (pH 1.84-2.28) with those at sites 4 and 7 having particularly low pH values. The amount of oxygen in the water varied between the sites, with the deeper zones in site 3 (S3M and S3B) and site 4 (the isolated pool) being more oxygen-depleted than other sites. The water temperature at sites 1 and 2 were slightly higher than others due to their position (sites 1 and 2 are closer to the mine entrance). The conductivities measured at sites 4 and 7 (the two pools) were significantly higher than in other sites, indicating that concentrations of dissolved solutes were greater in standing than in flowing water within the mine. Interestingly, the two sampling zones (surface and bottom layers of one of the pools) sampled at site 4 differed in some of their physicochemical characteristics, implying that the pool was stratified. The dissolved iron in the water sampled in the surface layer (S4T) was predominantly ferric, whereas ferrous iron was the dominant form in the bottom water. The water sampled at site 4 contained higher concentrations of heavy metals, sulfate and aluminium than other sites, which was considered to be due to evaporation. There were differences in DOC concentrations between water samples, with those at sites 2 and 7 containing about twice those of other water samples.

4.4 DNA-based analysis of streamer and water samples

4.4.1 Extraction of DNA and amplification

4.4.1.1 Materials and methods

DNA was extracted from 0.5 g (wet weight) streamer samples (two DNA extractions were carried out with each streamer sample), following the

method described in Section 2.6.1. For water samples collected from sites S4T and S4B, DNA was extracted from microbial cells collected onto filters as described in Section 4.2. The extracted DNA was amplified by PCR using primers specifically targeting bacterial and archaeal 16S rRNA genes, and also APS genes (Section 2.6.2).

4.4.1.2 Results

The results of 16S rRNA gene and APS gene amplification by PCR using different primers are summarized in Table 4.4.

Table 4.4. Results of gene amplification of DNA extracted from acid streamers and mine waters samples from Cae Coch, using bacterial (16S rRNA gene)-, archaeal (16S rRNA gene)-, and APS gene-specific primers; (+) positive, (-) negative.

Streamer and water samples	Bacteria	Archaea	APS
S1	+	+	-
S2	+	+	-
S3T	+	+	-
S3M	+	+	-
S3B	+	-	-
S4T	+	+	-
S4B	+	+	-
S7	+	+	-

All of the streamer samples gave positive results for bacteria and also (except for those from site S3B) for archaea. In contrast, no APS genes were amplified from the extracted DNA, indicating that either that sulfate reducing prokaryotes possessing the APS gene were not present in streamer and water samples, or that the protocols and/or primers used could not detect them.

4.4.2 Terminal restriction fragment polymorphism (T-RFLP) analysis

4.4.2.1 Materials and methods

Amplified 16S rRNA genes obtained using the bacteria-specific primers (Section 4.4.1) were analyzed by T-RFLP using the restriction enzymes *Cfo*I, *Msp*I and *Alu*I. The codes used to denote the amplified 16S rRNA genes are

listed in Table 4.5. Three enzymes were used as some microorganisms could not be accurately differentiated by two enzymes alone. T-RFs were identified using the T-RFs database that included T-RFs of clones obtained by constructing clone libraries using streamer and water samples that was carried out later in the study (Section 4.4.3).

Table 4.5. List of codes used to denote amplified 16S rRNA genes obtained from DNA extracted from acid streamer and water samples collected at Cae Coch.

Sites	Sample code	Codes for amplified 16S rRNA genes
Site 1	S1	S1a/S1b
Site 2	S2	S2a/S2b
Site 3 (top layer)	S3T	S3Ta/S3Tb
Site 3 (middle layer)	S3M	S3Ma/S3Mb
Site 3 (bottom layer)	S3B	S3B
Site 4 (top layer)	S4T	S4T
Site 4 (bottom layer)	S4B	S4B
Site 7	S7	S7a/S7b

4.4.2.2 Results

The results obtained from T-RFLP analyses of DNA amplified with bacteria-specific primers are shown in Figures 4.6-4.29. The unlined (1) and lined (2) bars represent the replicate samples taken from each site (Table 4.4). T-RFs that could be identified are differentiated by colors; green = *At. ferrooxidans*, black = *Sphingomonas*-isolate S3MMN1, red = γ-proteobacterium clone S11C1, blue = β-proteobacterium clone S3B2C20, yellow = acidobacterium-clone S11C3, orange = bacillus-clone S3B2C3, pink = *L. ferrooxidans* clone S72C15, purple = *Ralstonia pickettii*-clone S4BC11 and gray = actinobacterium-clone S3B2C4.

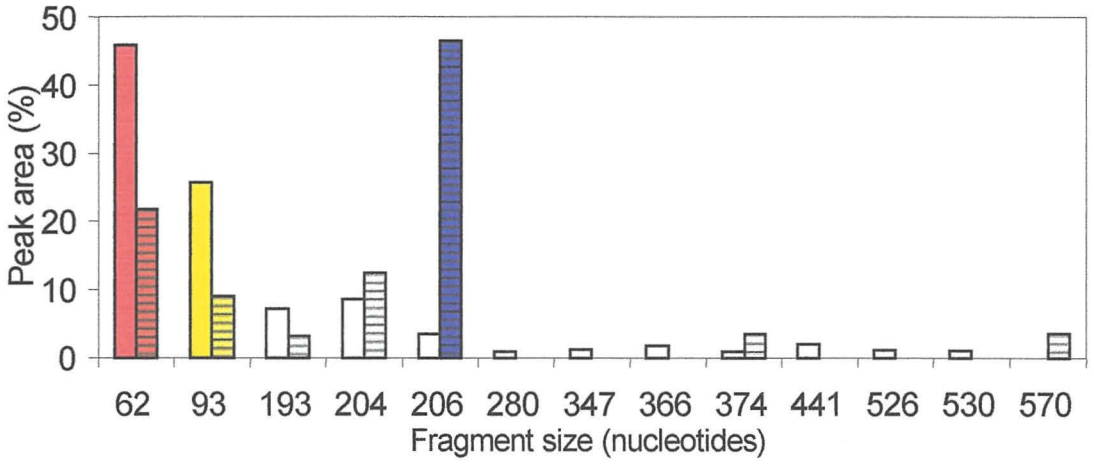


Figure 4.6. Results of T-RFLP analysis of sample S1 using the restriction enzyme *Cfo*I. The unlined and lined bars represent the replicate samples.

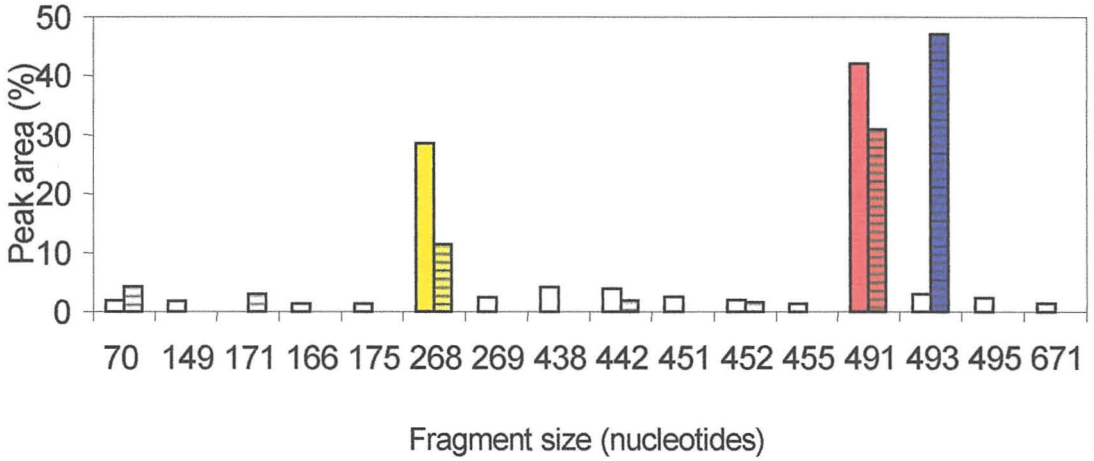


Figure 4.7. Results of T-RFLP analysis of sample S1 using the restriction enzyme *Msp*I. The unlined and lined bars represent the replicate samples.

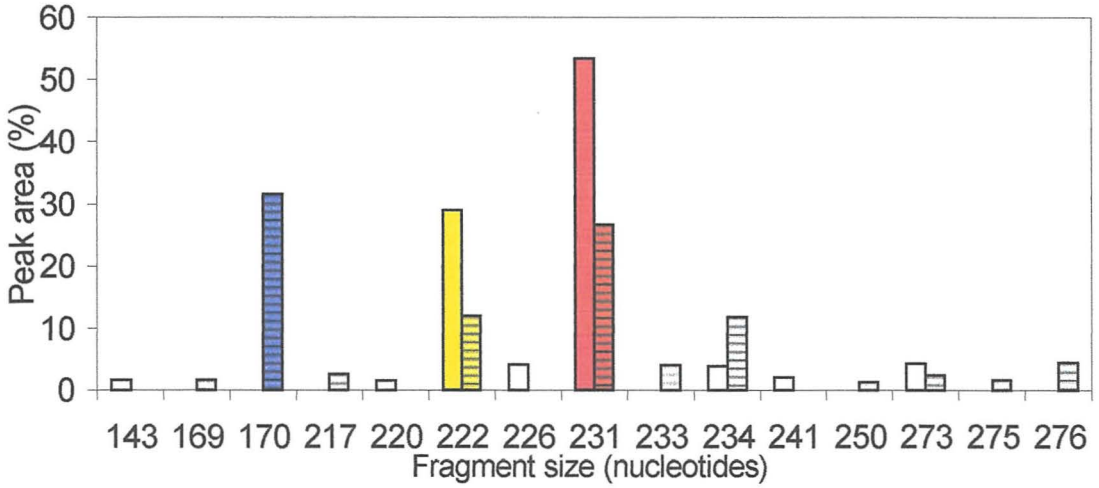


Figure 4.8. Results of T-RFLP analysis of sample S1 using the restriction enzyme *Alu*I. The unlined and lined bars represent the replicate samples.

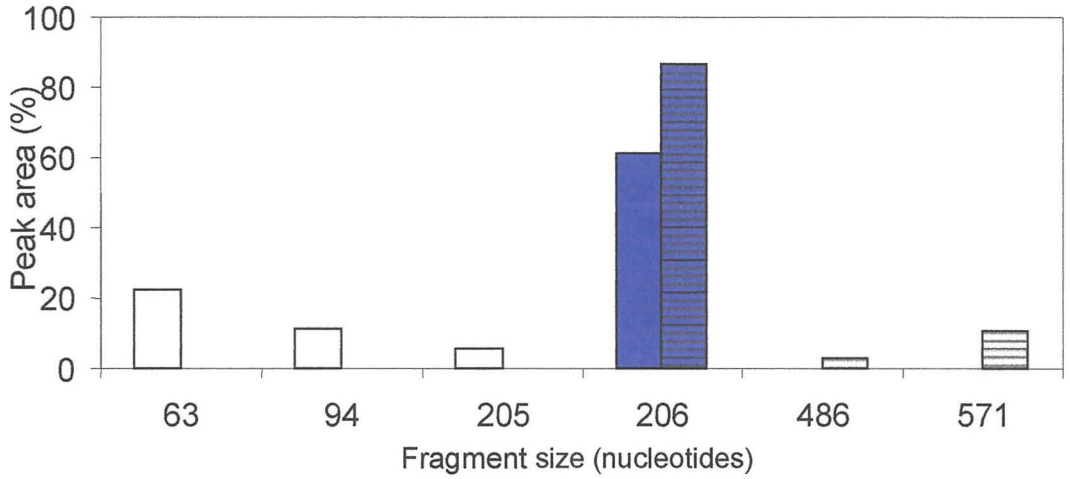


Figure 4.9. Results of T-RFLP analysis of sample S2 using the restriction enzyme *Cfo*I. The unlined and lined bars represent the replicate samples.

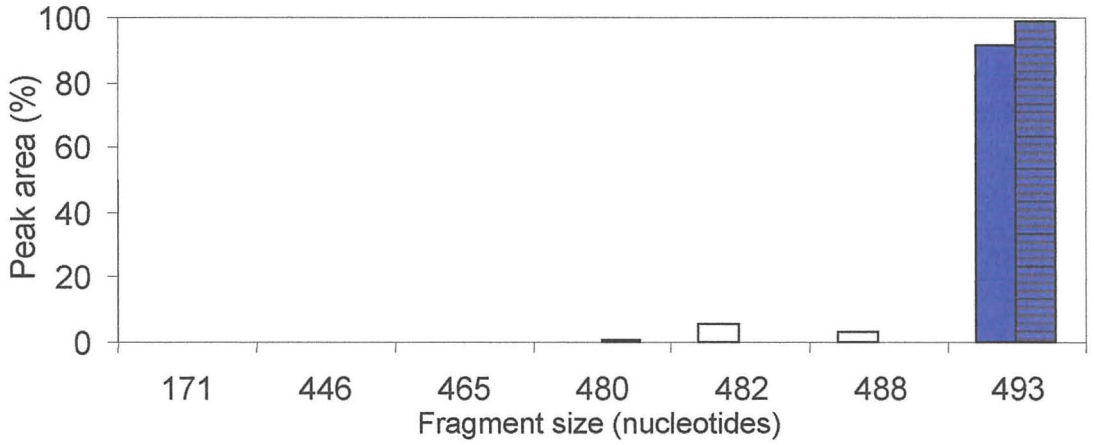


Figure 4.10. Results of T-RFLP analysis of sample S2 using the restriction enzyme *Msp*I. The unlined and lined bars represent the replicate samples.

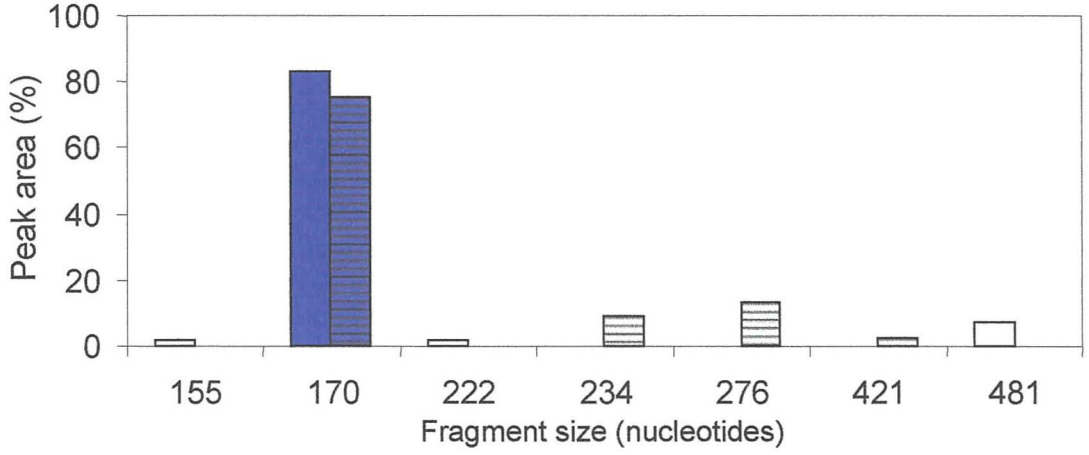


Figure 4.11. Results of T-RFLP analysis of sample S2 using the restriction enzyme *Alu*I. The unlined and lined bars represent the replicate samples.

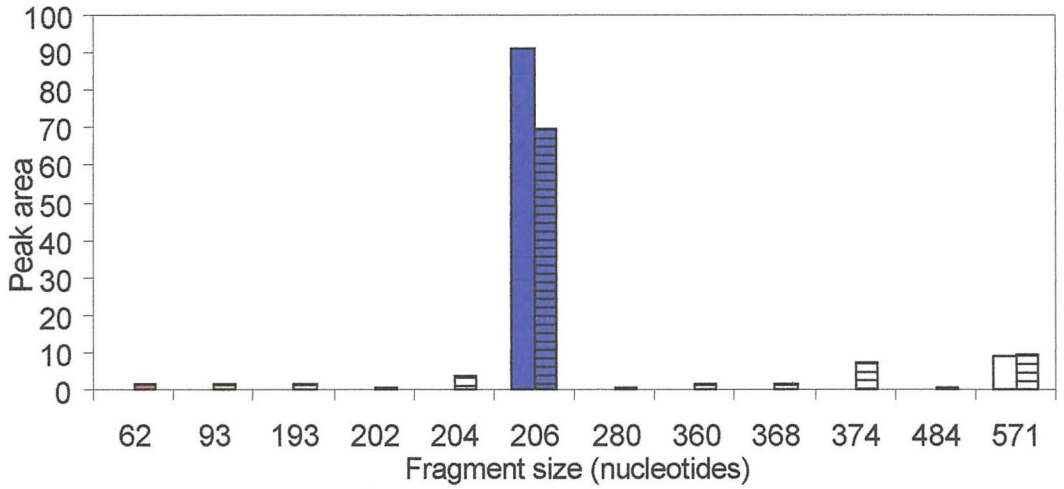


Figure 4.12. Results of T-RFLP analysis of sample S3T using the restriction enzyme *Cfo*I. The unlined and lined bars represent the replicate samples.

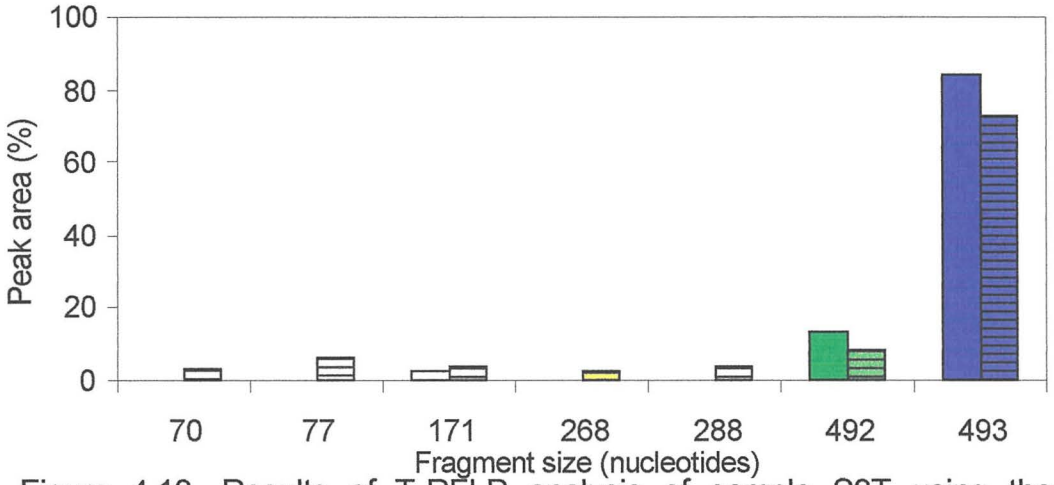


Figure 4.13. Results of T-RFLP analysis of sample S3T using the restriction enzyme *Msp*I. The unlined and lined bars represent the replicate samples.

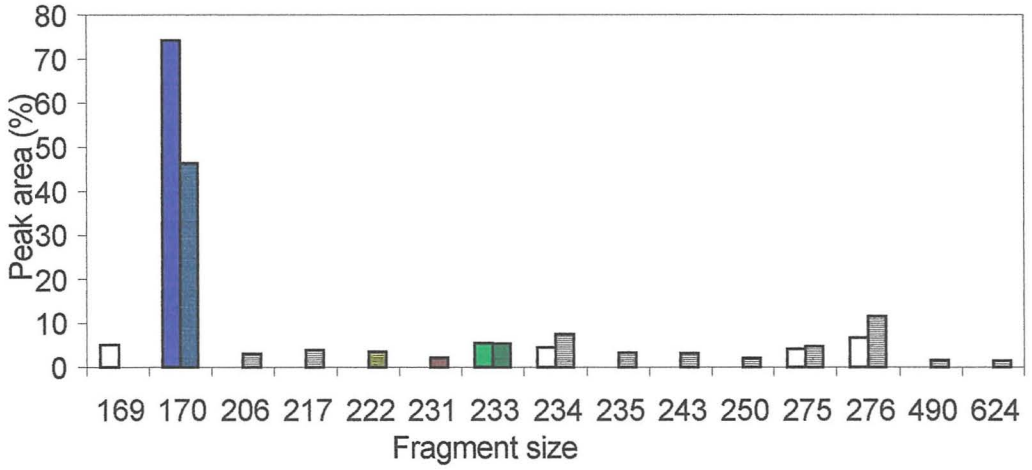


Figure 4.14. Results of T-RFLP analysis of sample S3T using the restriction enzyme *Alu*I. The unlined and lined bars represent the replicate samples.

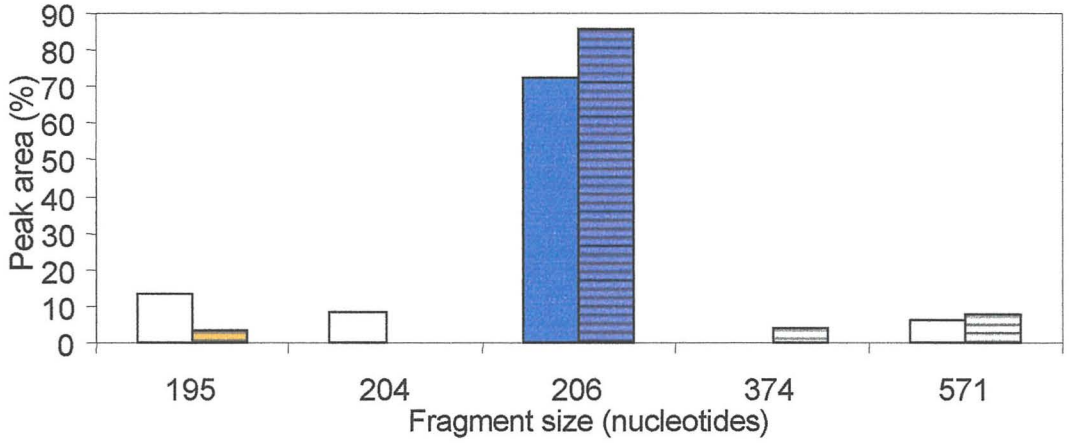


Figure 4.15. Results of T-RFLP analysis of sample S3M using the restriction enzyme *Cfo*I. The unlined and lined bars represent the replicate samples.

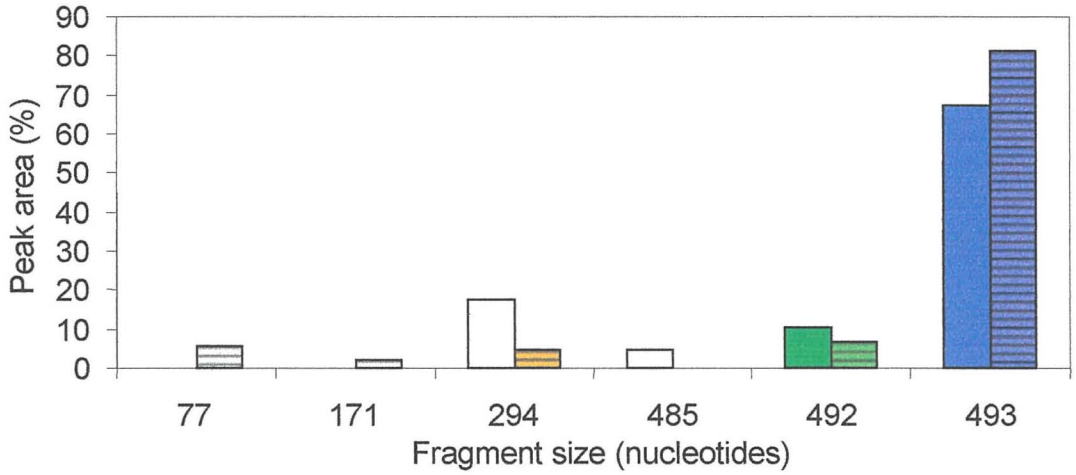


Figure 4.16. Results of T-RFLP analysis of sample S3M using the restriction enzyme *Msp*I. The unlined and lined bars represent the replicate samples.

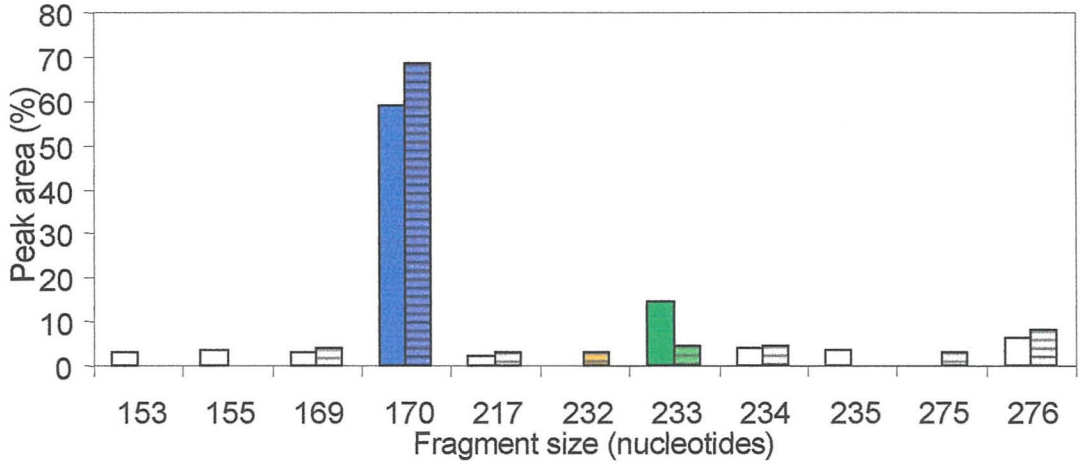


Figure 4.17. Results of T-RFLP analysis of sample S3M using the restriction enzyme *Alu*I. The unlined and lined bars represent the replicate samples.

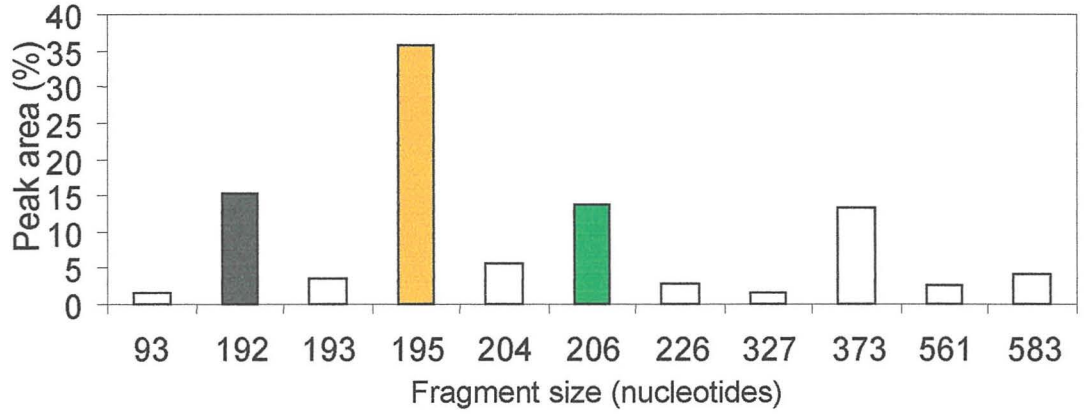


Figure 4.18. Results of T-RFLP analysis of sample S3B using the restriction enzyme *Cfo*I.

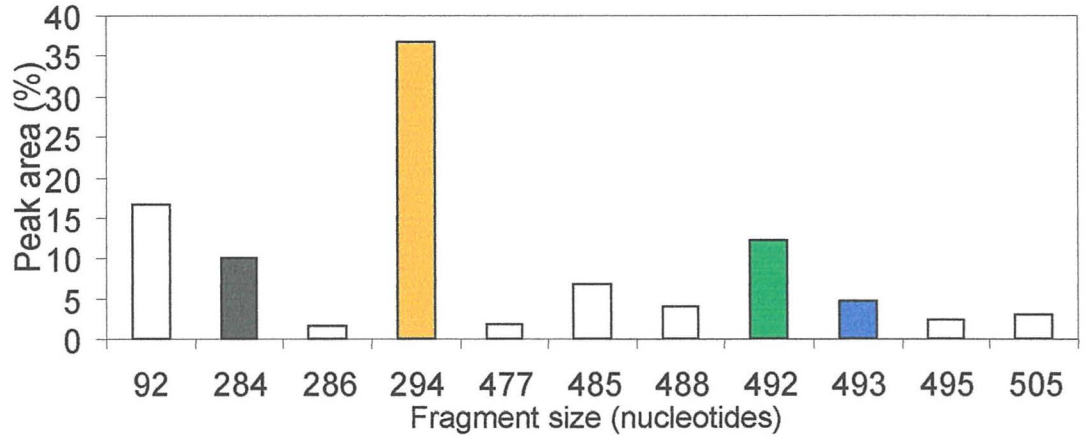


Figure 4.19. Results of T-RFLP analysis of sample S3B using the restriction enzyme *Msp*I.

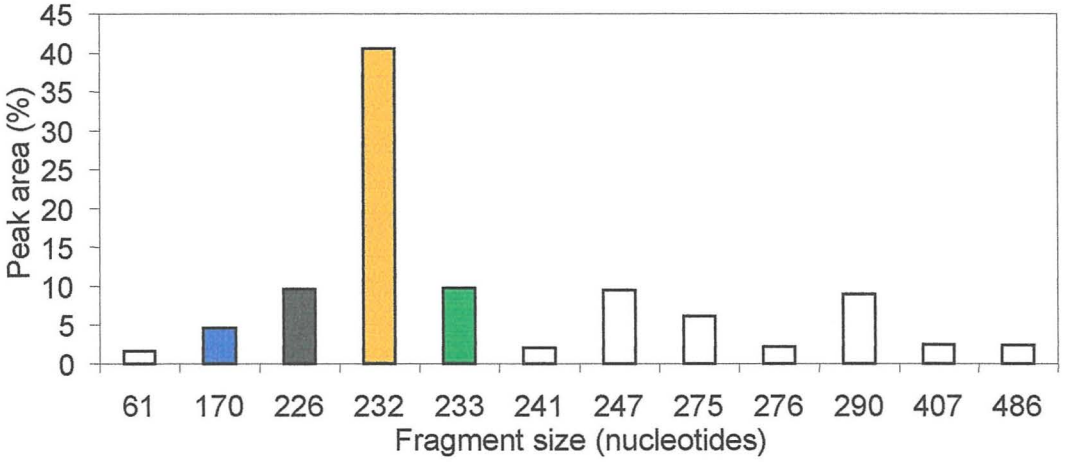


Figure 4.20. Results of T-RFLP analysis of sample S3B using the restriction enzyme *Alu*I.

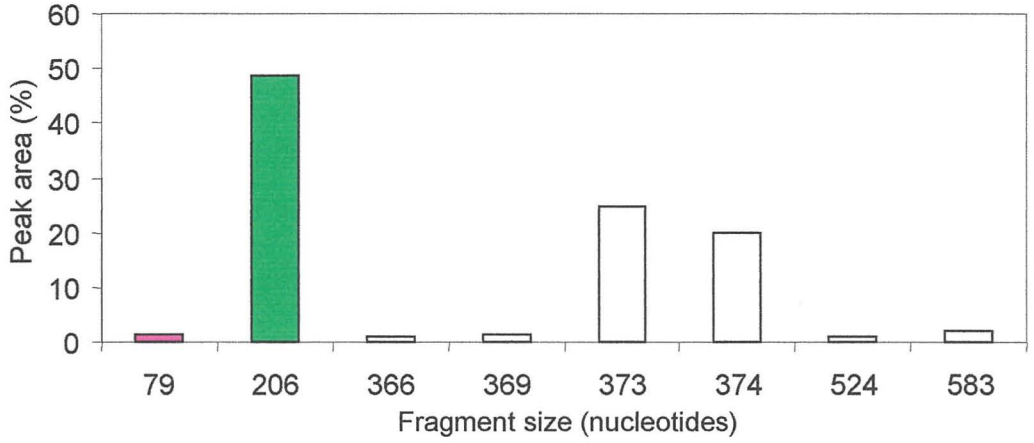


Figure 4.21. Results of T-RFLP analysis of sample S4T using the restriction enzyme *Cfo*I.

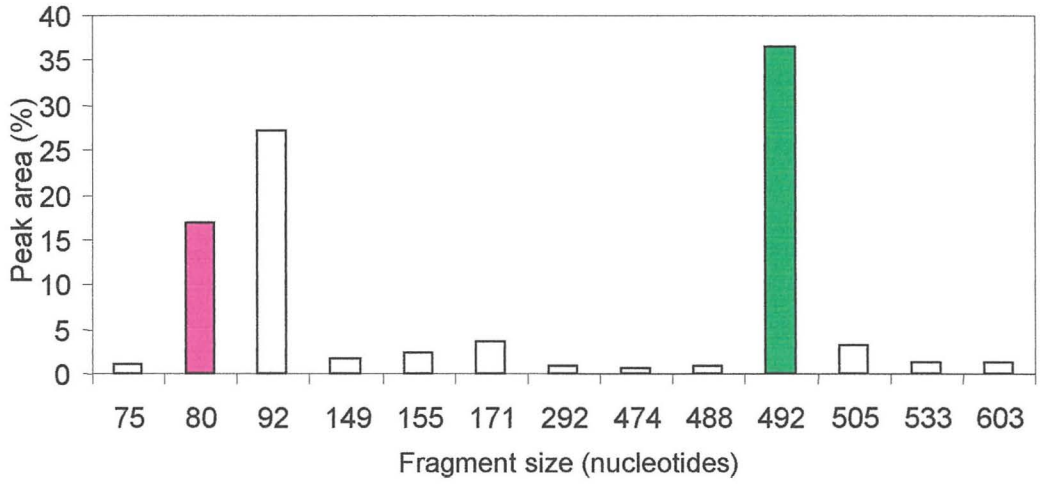


Figure 4.22. Results of T-RFLP analysis of sample S4T using the restriction enzyme *Msp*I.

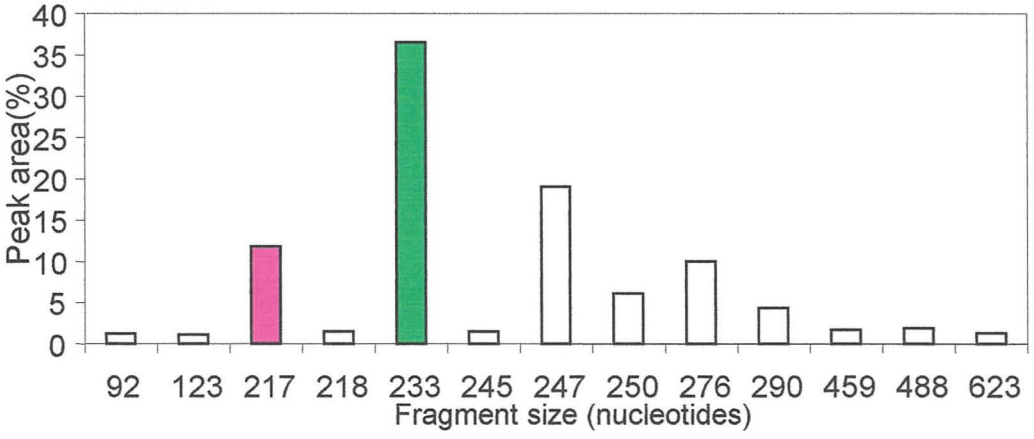


Figure 4.23. Results of T-RFLP analysis of sample S4T using the restriction enzyme *Alu*I .

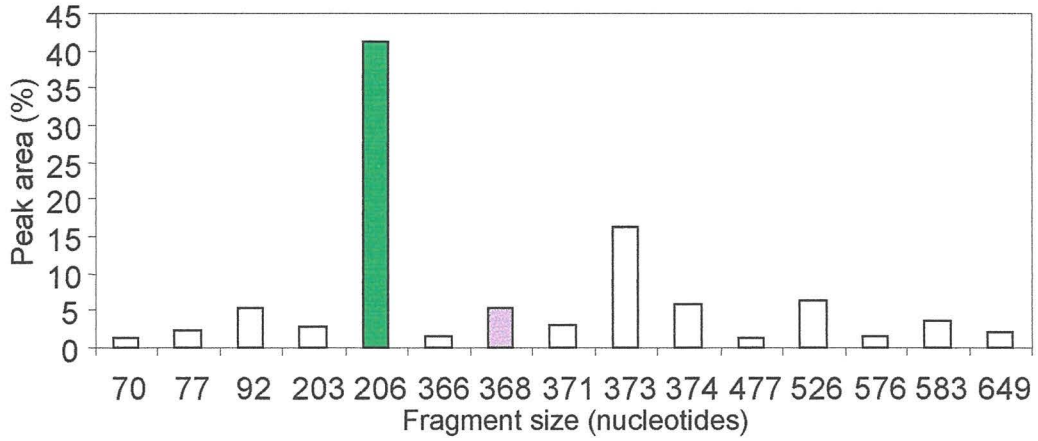


Figure 4.24. Results of T-RFLP analysis of sample S4B using the restriction enzyme *Cfo*I.

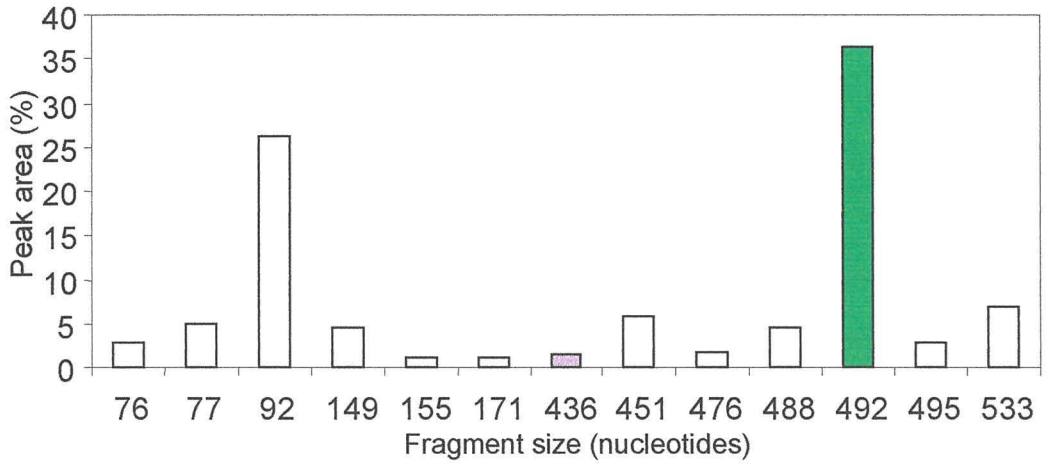


Figure 4.25. Results of T-RFLP analysis of sample S4B using the restriction enzyme *Msp*I.

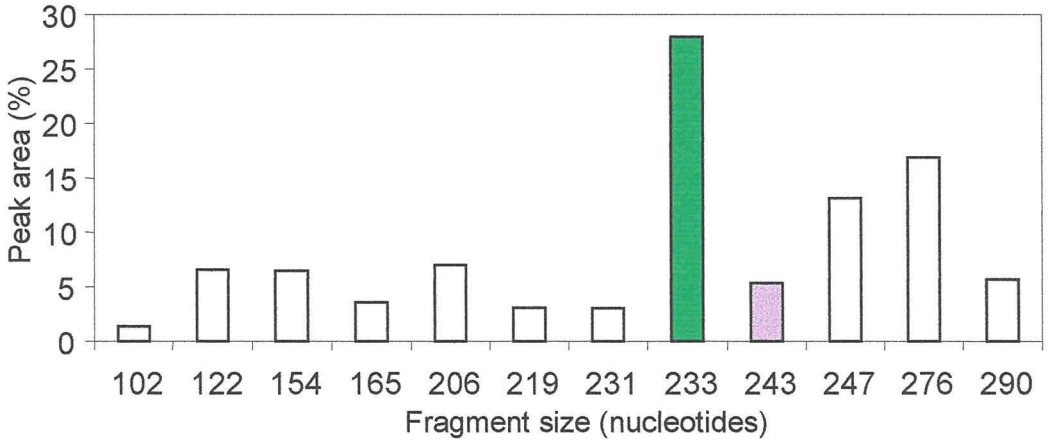


Figure 4.26. Results of T-RFLP analysis of sample S4B using the restriction enzyme *Alu*I.

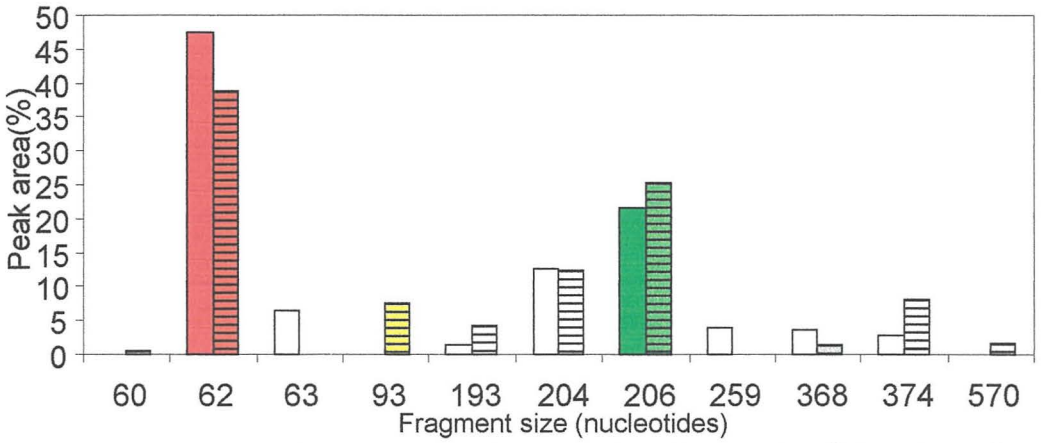


Figure 4.27. Results of T-RFLP analysis of sample S7 using the restriction enzyme *Cfo*I. The unlined and lined bars represent the replicate samples.

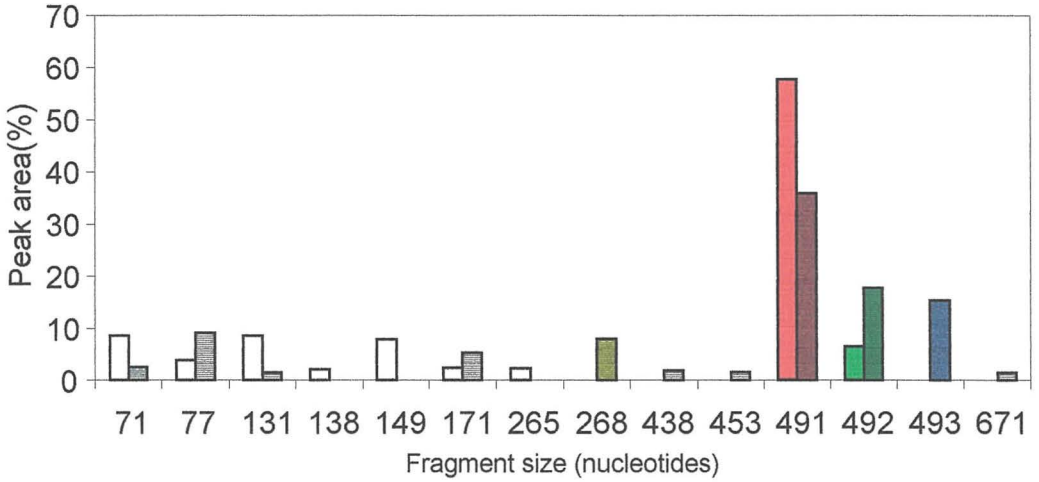


Figure 4.28. Results of T-RFLP analyses of sample S7 using the restriction enzyme *Msp*I. The unlined and lined bars represent the replicate samples.

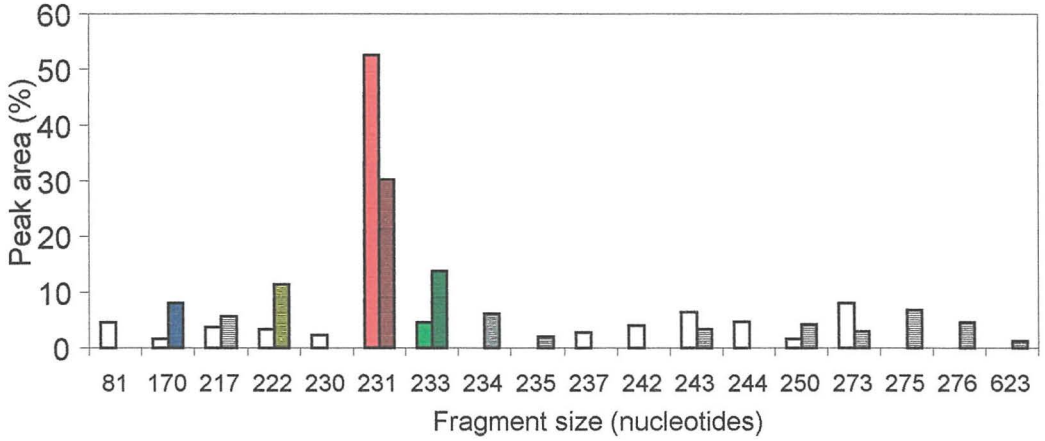


Figure 4.29. Results of T-RFLP analyses of sample S7 using the restriction enzyme *Alu*I. The unlined and lined bars represent the replicate samples.

The T-RFLP analyses of slime DNA from site 1 (Figures 4.6-4.8) showed that the replicate samples (S1a and S1b) taken from this site showed significant variation in their microbial compositions. There were two dominant T-RFs in S1a that were subsequently identified as clones S11C1 and S11C3. In contrast, whilst these two T-RFs were also present in S1b, the dominant T-RF was that of clone S3B2C20 which was not detected in S1a.

With the pipe (stalactite) sample from site 2, there was greater homology between the T-RFs of the two samples analyzed (S2a and S2b). Clone S3B2C20 was the dominant peak in both cases and there were several unidentified T-RFs that comprised 40% and 25% of the total peak area of S2a and S2b, respectively.

Acid streamers found at the surface of the main stream running through Cae Coch (site S3T) were dominated by clone S3B2C20 (Figures 4.12-4.14). The T-RF with the second largest peak area in both samples was identified as *At. ferrooxidans*. As the T-RF sizes of clone S3B2C20 and *At. ferrooxidans* were the same when digested with *Cfo*I, the 206 nt fragment in Figure 4.12 includes both bacteria. Although both streamer samples (S3Ta and S3Tb) were dominated by single bacterium, S3Ta showed greater biodiversity (i.e. more T-RFs) than S3Tb. Two of the minor T-RFs present in S3Tb were identified as clones S11C1 and S11C3, but other T-RFs were not identified. Acid streamers sampled at the same site as S3T, but *ca.* 40 cm deeper (site S3M), showed similar biodiversity (from T-RFLP analysis) as the surface streamers, with the same clone, S3B2C20, being the dominant peak (Figures 4.15-4.17). The minor T-RFs of the surface and mid-depth streamer sample were different, with peaks corresponding to clones S11C1 and S11C3 being present in S3T but not in S3M. There were several minor T-RFs in S3M streamer samples that could not be identified, except for the T-RF of clone S3B2C3 which was present only in one of the two samples analyzed. Streamers taken from the lowest depth (*ca.* 50 cm) in the main stream (S3B) were very different from those sampled in the surface and mid-depths (Figures 4.18-4.20). The dominant (around 40% of total peak area) T-RF peak was that of clone S3B2C3 and there were several minor T-RFs, three of

which were identified as *At. ferrooxidans*, isolate S3MMN1 and clone S3B2C20.

Analyses of surface water from pool S4 showed that there were four major T-RFs and several minor T-RFs in the sample (Figures 4.21-4.23). The largest T-RF peak area corresponded to *At. ferrooxidans* and one of the major T-RFs to clone S72C15. The other major T-RFs and all of the minor T-RFs were not identified. Water sampled from the bottom of this pool (S4B) showed broadly similar biodiversity, in that the dominant T-RF corresponded to *At. ferrooxidans* and two of the unidentified peaks found in S4T were also present in S4B (Figures 4.24-4.26). One difference was the presence of an additional, though minor, T-RF in S4B which was identified as corresponding to clone S4BC11.

The T-RFLP analyses on streamer samples (S7a and S7b) obtained from pool Site 7 are shown in Figures 4.27-4.29. As with the slime sample (site S1), the dominant T-RF corresponded to clone S11C1. T-RFs corresponding to *At. ferrooxidans* were also present in both samples analyzed. Other T-RFs identified were only present in S7b and represented clones S11C3, S3B2C20 and S3B2C4. None of the minor peaks in S7a was identified.

4.4.3 Construction of mini-clone libraries

4.4.3.1 Materials and methods

Mini-clone libraries were constructed using PCR products (samples S1a, S3Ba and S7b; Table 4.5), which were amplified with bacteria-specific primers. DNA extracted from water sampled at site S4B was amplified with archaeal-, as well as bacterial-, specific primers. However, the initial attempt to construct a clone library using amplified bacterial 16S rRNA genes from water sample S4B failed. To try to circumvent this problem, the PCR product was concentrated, as follows. To 60 µl PCR products, 6 µl of 5 M sodium acetate, 5 µl of 100 mM EDTA and 3 µl 0.02% (w/v) glycogen were added. Next, 180 µl absolute ethanol (stored at -20°C) was added to the solution after brief mixing using vortex. The solution was centrifuged at 16,100 *g* for 15 minutes before the pellet was washed twice with 100 µl of 70% (v/v)

ethanol. The washed pellet was then resuspended in 6 µl TE buffer, adjusted to pH 8.0 with 1 M HCl and 1 M KOH. The attempt to construct a clone library with amplified archaeal 16S rRNA genes from site S4B water was also unsuccessful. In this case, an alternative approach was used whereby 3 ml S4B water sample was centrifuged (16,100 *g* for 5 minutes), the cell pellet lysed (Section 2.6.1) and the lysate used as a template for PCR using the archaeal-specific primers. The PCR product was used to construct a clone library.

Clones (between 2 and 40) from each library were selected and inserts were amplified using SP6 and T7 primers (Section 2.6.6.4). The amplified products were analyzed by RFLP using *Msp*I (S3Ba and S7b), *Cfo*I (S4B bacteria), *Msp*I and *EcoR*I (S1a) or *Msp*I and *Alu*I (S4B archaea). Inserts in cloned plasmids possessing novel RFLP patterns were selected and plasmids were extracted (Section 2.6.6.4). The inserts were analyzed using the 27f bacterial primer or SP6 primer for archaeal 16S rRNA genes. If the sequence obtained was novel, primers (1100r and 1492r for bacterial, T7 and 530r for archaeal genes) were used to obtain a near complete gene sequence. Chimeric sequences were identified using The Chimera Detection program. T-RFLP analysis using *Cfo*I, *Msp*I and *Alu*I was carried out on any clones perceived to be novel.

4.4.3.2 Results

Tables 4.6, 4.8, 4.10 and 4.12 list the results from sequence analysis of clones acquired from clone libraries of samples S1a, S3Ba, S4B and S7b (bacteria) and of S4B (archaea). Tables 4.7, 4.9, 4.11 and 4.13 show results of T-RFLP analysis of the clones obtained from these libraries. These data were used to identify the T-RFs that were found in the T-RFLP analysis of the amplified 16S rRNA genes from different samples (Section 4.4.2). Phylogenetic trees of bacterial (Figure 4.30) and archaeal (Figure 4.31) clones were constructed from the 16S rRNA gene sequences of the clones.

Seventeen clones were selected from the S1a bacterial clone library; RFLP analysis showed that there were 6 different types present, represented by

clones S11C1, S11C2, S11C3, S11C12, S11C15 and S11C17. Sequence analysis (Table 4.6) showed that two of the ligated genes were chimeric (S11C2 and S11C15). The microorganism represented by clone S11C1 was most closely related (99.8%) to isolate WJ2 which belongs to the *γ-Proteobacteria*. Clones S11C3 and S11C17 were 92.7% identical to each other and 94.1% (S11C3) and 91.1% (S11C17) to *Acidobacterium capsulatum*. The closest match (98.5%) in the NCBI to clone S11C12 was an uncultured α-proteobacterium (MS5) found in acidic, geothermal springs of Montserrat (Burton & Norris 2000), and the closest (96.8%) characterized microorganism was the acidophilic heterotroph, *Acidisphaera rubrifaciens*.

Table 4.6. Sequence analysis of clones from the mini-clone library obtained from streamer sample S1a. Numbers in brackets are NCBI accession numbers.

Clone	Length of the 16S rRNA gene sequenced (base pairs)	Closest relative	Identity* (%)
S11C1	1466	γ-proteobacterium WJ2 (AY096032)	99.8
		Frateuria aurantia strain IFO13331 (AB091200)	92.0
S11C2	1423	Chimeric sequence	
S11C3	1415	Clone WD247 (AJ292581)	95.1
		Acidobacterium capsulatum (D26171)	94.1
S11C12	462	Uncultured bacterium MS5 (AF232921)	98.5
		Acidisphaera rubrifaciens (D86512)	96.8
S11C15	1396	Chimeric sequence	
S11C17	1403	*Acidobacterium capsulatum* (D26171)	91.1
		Clone FW45 (AF523984)	91.0

* based on comparison of 16S rRNA gene sequences.

Table 4.7. T-RFLP analysis on clones from the mini-clone library obtained from streamer sample S1a. Values shown are fragment lengths, in nucleotides.

Clone	Restriction enzyme		
	*Cfo*I	*Msp*I	*Alu*I
S11C3	93	268	222
S11C12	180	445	214
S11C17	93	268	222

Table 4.8 shows the sequence analysis results of the clone library constructed using streamer sample S3Bb. Twenty clones were selected and 12 of those had appropriate-sized inserts. The other eight clones had inserts

with smaller sizes than the 16S rRNA gene (*ca.* 1,500 bp), and these inserts were considered to be primer dimers. RFLP analysis showed there were 9 different clone types present, represented by clones S3B2C1, S3B2C2, S3B2C3, S3B2C4, S3B2C8, S3B2C9, S3B2C10, S3B2C11 and S3B2C20. Results of T-RFLP analysis of these clones are shown in Table 4.9. Sequence analysis of S3B2C2 and S3B2C10 failed, hence the clones were not identified, and sequences of S3B2C8, S3B2C9 and S3B2C11 were found to be chimeric. The sequence of the first 500 nucleotides of one of the chimeric insert of clone S3B2C11 was 90% identical to that of clone S3B2C3 but other chimeric sequences (S3B2C8 and S3B2C9) were not analyzed in detail. The 16S rRNA gene sequence of S3B2C1 was 99.8% identical to the γ-proteobacterium, WJ2, and 98.3% identical to clone S11C1. Clone S3B2C3 was most closely related to *Alicyclobacillus pomorum* and (92%) to the Gram-positive iron-oxidizing acidophile SLC66 (92% gene similarity, in both cases). S3B2C4 was most identical to clone RCP1-33, which was obtained from a forested wetland impacted by coal waste (Brofft et al. 2002), and the closest characterized microorganism was the actinobacterium, "*Ferrimicrobium acidiphilum*". The closest match (99% gene identity) to clone S3B2C20 was the microorganism represented by clone TrefC11, which was one of the major bacteria identified in acid streamers in the Trefriw spa (Chapter 3).

Table 4.8. Sequence analysis of clones from the mini-clone library obtained from streamer sample S3Bb. Numbers in brackets are NCBI accession numbers.

Clone	Length of the 16S rRNA gene sequenced (base pairs)	Closest relative	Identity* (%)
S3B2C1	813	γ-proteobacterium WJ2 (AY096032)	99.8
S3B2C3	1456	*Alicyclobacillus pomorum* (AB089840)	92.2
		Gram-positive iron-oxidizing acidophile SLC66 (AY040739)	92.1
S3B2C4	1422	Clone RCP1-33 (AF523914)	99.1
		"Ferrimicrobium acidiphilum" (AF251436)	92.6
S3B2C8	1465	Chimeric sequence	
S3B2C9	1422	Chimeric sequence	
S3B2C11	832	Chimeric sequence	
S3B2C20	813	TrefC11 (AY766003)	99.3

* based on comparison of 16S rRNA gene sequences.

Table 4.9. T-RFLP analysis on clones from the mini-clone library obtained from streamer sample S3Bb. Values shown are fragment lengths, in nucleotides.

Clone	Restriction enzyme		
	*Cfo*I	*Msp*I	*Alu*I
S3B2C3	195	294	232
S3B2C4	368	71	243

The results of sequencing analysis of clones obtained from S4B bacterial clone library are summarized in Table 4.10 and T-RFLP analysis results are listed in Table 4.11. Only two of the 12 clones had 16S rRNA gene inserts. Clone S4BC5 was found to be *At. ferrooxidans* as the product was obtained with PCR using *At. ferrooxidans*-specific primer (Section 2.6.2). S4BC11 was closest to *Ralstonia pickettii* (a β-proteobacterium).

Table 4.10. Sequence analysis of clones from the mini-clone library obtained from water sample S4B. Numbers in brackets are NCBI accession numbers.

Clone	Length of the 16S rRNA gene sequenced (base pairs)	Closest relative	Identity* (%)
S4BC11	1447	*Ralstonia pickettii* strain 2000032023 (AY268176)	99.7
S4BAC1	1374	Uncultured archaeon clone ASL1 (AF544224)	92.1
		Thermoplasma acidophilum (M38637)	88.9
		Thermoplasma volcanium (AF339746)	88.9

* based on comparison of 16S rRNA gene sequences.

Table 4.11. T-RFLP analysis on clones from the mini-clone library obtained from water sample S4B. Values shown are fragment lengths, in nucleotides.

Clone	Restriction enzyme		
	*Cfo*I	*Msp*I	*Alu*I
S4BC11	368	436	243
S4BAC1	320	505	408

An archaeal clone library was also constructed for sample S4B sample and 40 clones were analyzed. RFLP analysis using two different enzymes showed that all of these clones had identical RFLP patterns, and S4BAC1 was selected for gene sequencing (Table 4.9). The closest (92.1%) match was an uncultured archaeal clone ASL1, which was obtained from Iron Mountain (Baker & Banfield 2003). The closest characterized archaea were

Thermoplasma acidophilum and *Thermoplasma volcanium* (both *Euryarchaeota*), which were both 88.9% identical to S4BAC1.

From the S7b clone library, 20 clones were selected and 7 of those had 16S rRNA gene inserts. RFLP analysis showed that all of these clones were different and sequence analyses data are shown in Table 4.12. Although no product was obtained from PCR using *At. ferrooxidans*-specific primers, 16S rRNA gene sequences of clones S72C3 and S72C12 were found to be most similar to that of to *At. ferrooxidans* sp. NO-37. Clones S72C8 and S72C18 were most closely related to γ-proteobacterium WJ2. The 16S rRNA gene sequence of S72C10 and 14 was most identical to that of uncultivated eubacterium TRA2-10 obtained from Iron Mountain, and S72C15 had the highest similarity to group I *Leptospirillum ferrooxidans* strain CF12 which was isolated from Cobalt Mine, Cobalt, Idaho, U.S.A. (Blake & Johnson 2000).

Table 4.12. Sequence analysis of clones from the mini-clone library obtained from streamer sample S7b. Numbers in brackets are NCBI accession numbers.

Clone	Length of the 16S rRNA gene sequenced (base pairs)	Closest relative	Identity* (%)
S72C3	361	*Acidithiobacillus* sp. NO-37 (AF376020)	98.1
S72C8	648	γ-proteobacterium WJ2 (AY096032)	97.8
S72C10	1411	Uncultured eubacterium TRA2-10 (AF047642)	92.3
		"Ferrimicrobium acidiphilum" (AF251436)	86.8
S72C12	689	*Acidithiobacillus* sp. NO-37 (AF376020)	99.8
S72C14	762	S72C10	99.5
S72C15	736	*Leptospirillum ferrooxidans* group 1 strain CF12 (AF356834)	98.8
S72C18	738	γ-proteobacterium WJ2	98.8

* based on comparison of 16S rRNA gene sequences.

Table 4.13. T-RFLP analysis on clones from the mini-clone library obtained from streamer sample S7b. Values shown are fragment lengths, in nucleotides.

Clone	Restriction enzyme		
	*Cfo*I	*Msp*I	*Alu*I
S72C8	62	491	231
S72C10	206	465	233
S72C15	79	80	217

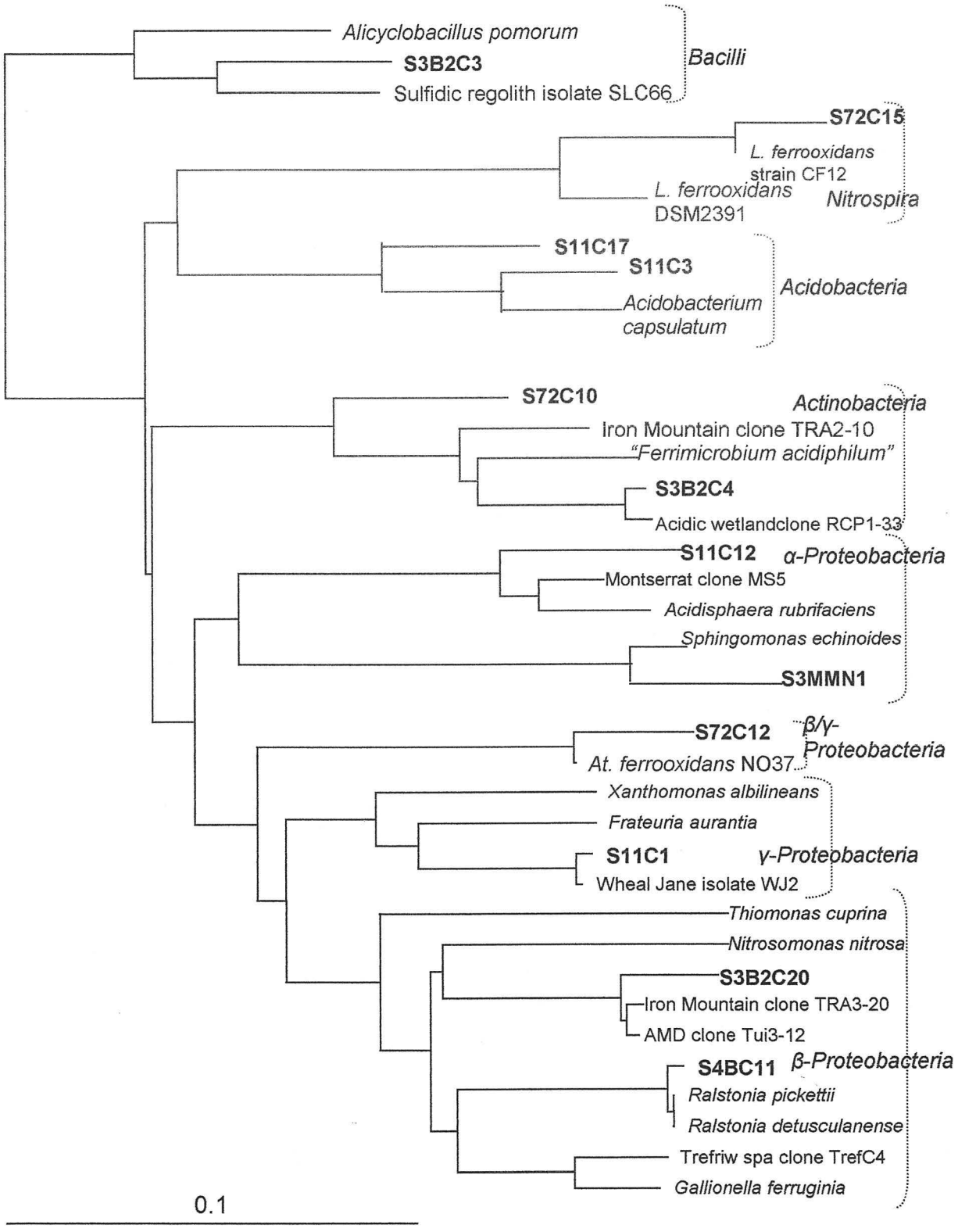


Figure 4.30. Phylogenetic relationships, based on 16S rRNA genes, of Cae Coch eubacterial clones (in bold) with clones and characterized bacteria in the classes *Acidobacteria, Actinobacteria, Bacilli, Nitrospira, α-Proteobacteria, β-Proteobacteria* and *γ-Proteobacteria*. The bar represents 0.1 nucleotide substitutions per 100 for horizontal branch lengths. The tree was rooted with Gram-positive *Desulfosporosinus orientis* (not shown).

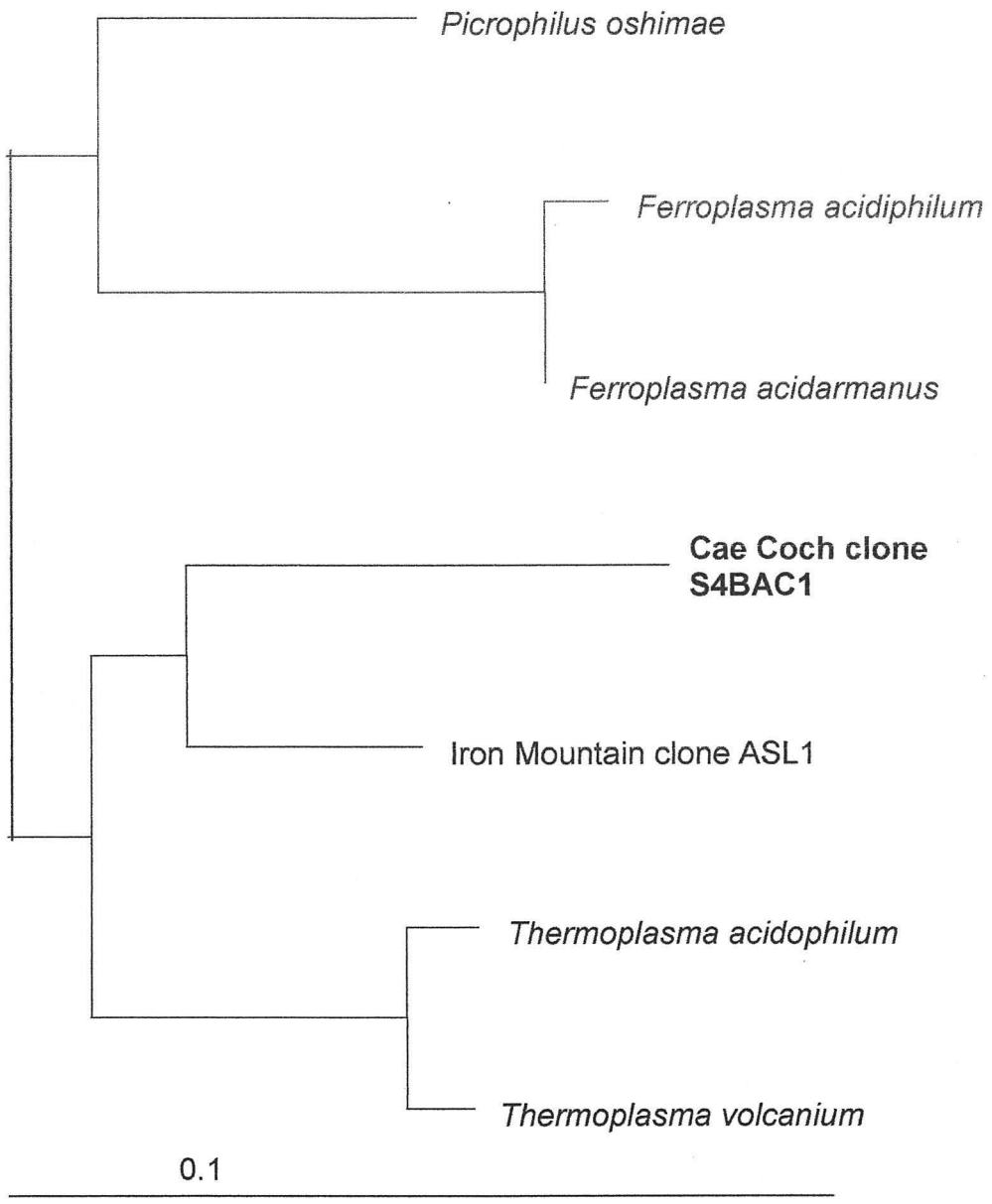


Figure 4.31. Phylogenetic relationships based on 16S rRNA genes of Cae Coch archaeal clone, S4BAC1 (bold), to clone ASL1 and characterized members of the order *Thermoplasmatales* (the families *Ferroplasmaceae, Picrophilaceae and Thermoplasmataceae*). The bar represents 0.1 nucleotide substitutions per 100 for horizontal branch lengths. The tree was rooted with *Acidianus infernus*.

4.5 Cultivation-based analysis of streamer samples

4.5.1 Materials and Methods

For cultivation-based techniques, one pipe (microbial stalactite, from site S2), four acid streamer samples (from sites S3T, S3M, S3B and S7) and two water samples (from sites S4T and S4B) were selected. The pipe and streamer samples were dispersed and diluted as described in Section 3.4. Water samples were diluted in heterotrophic basal salts solution (pH 3.5) and 100 µl aliquots were used to inoculate solid media (Section 2.2.1.2).

Colony forming units were counted prior to sub-culturing and purification of isolates. A single colony of each representative morphological type was used to inoculate liquid medium, and grown cultures were harvested and lysed (Section 2.6.1). The *At. ferrooxidans*-specific primer TF539 was used to identify ferric iron-encrusted colonies that were tentatively identified as *At. ferrooxidans* (Section 2.6.2). If no product was obtained using TF539r, the 16S rRNA gene was amplified using 27f and 1392r, followed by RFLP analysis (Section 2.6.4). If the RFLP pattern was not identified, the PCR product was purified and sequenced (Section 2.6.7).

4.5.2 Results

Colony count data are summarized in Tables 4.14 to 4.19. The microorganisms that grew on the solid media were categorized as extremely or moderately acidophilic iron-oxidizers, or heterotrophic acidophiles (Section 3.4). No colonies grew on yeast extract pH 4 or R2A media, incubated either aerobically or microaerobically, and no heterotrophic colonies grew on iron-thiosulfate plates.

Table 4.14 shows colony count data for a dispersed microbial pipe sample from site S2. CFUs of extremely acidophilic iron-oxidizers were greater than those of moderate acidophiles and of heterotrophic acidophiles. All colonies on iron-overlay media (incubated aerobically and microaerobically) and on iron-tetrathionate-overlay medium (incubated aerobically) were iron-encrusted. Three different colony morphologies of iron-oxidizers on micro

aerobically-incubated iron-tetrathionate-overlay were all identified as *At. ferrooxidans,* though a representative of the single colony type that grew on aerobically-incubated plates was not successfully subcultured. Similarly, colonies that grew on iron-overlay medium (microaerobic incubation) were found to be *At. ferrooxidans*. Only one type of colony morphology was found with heterotrophic isolates that grew on yeast extract pH 3 medium incubated either under aerobic or microaerobic conditions. The isolates were provisionally identified as *Acidocella* or *Acidiphilium* spp., on the basis of their cell and colony morphologies, but were not sequenced.

Table 4.14. Plate counts of dispersed S2 streamer. The numbers are expressed as CFUs/ml pipe (microbial stalactite) cell suspension.

	Aerobic incubation	Microaerobic incubation
Iron-oxidizing bacteria		
- extreme acidophiles	1×10^6	5×10^6
- moderate acidophiles	$<10^2$	4×10^4
Heterotrophic acidophiles	2×10^4	1×10^6
Neutrophiles	$<10^2$	$<10^2$

Colony counts of an acid streamer sample from the surface of the main stream in Cae Coch (S3T) are listed in Table 4.15. The isolates appeared to prefer microaerobic to aerobic conditions, though this might have been related to enhanced CO_2 levels in the former, and again the highest counts obtained were those of extremely acidophilic iron-oxidizers. All colonies that grew on iron-, iron-tetrathionate- and iron thiosulfate- overlay media were iron-encrusted. All of the iron-encrusted colonies, apart from one colony type (23% of total) on microaerobically-incubated iron-overlay medium and another colony type on microaerobically-incubated iron thiosulfate-overlay medium (2% of total), were identified as *At. ferrooxidans.* The isolate that grew as iron-encrusted colonies with a different morphology on microaerobically incubated iron-overlay medium was lost during purification. The iron-encrusted colony (isolate S3TT1M) found on microaerobically incubated iron-tetrathionate medium was almost identical (99%) to Clone S11C1 (isolate WJ2-like; Section 4.4.3). The isolates that grew on yeast extract pH 3 media were provisionally identified as *Acidocella* or *Acidiphilium*

spp., on the basis of their cell and colony morphologies, but were not sequenced.

Table 4.15. Plate counts of dispersed S3T streamer. The numbers are expressed as CFUs/ml streamer cell suspension.

	Aerobic incubation	Microaerobic incubation
Iron-oxidizing bacteria		
- extreme acidophiles	9×10^6	2×10^6
- moderate acidophiles	1×10^6	2×10^6
Heterotrophic acidophiles	1×10^6	6×10^6
Neutrophiles	$<10^2$	$<10^2$

Table 4.16 lists the colony counts of a streamer sample taken from 30-40 cm below the surface of the main stream in Cae Coch (S3M). In contrast to other streamer samples, CFUs of heterotrophic acidophiles exceeded those of iron-oxidizers. Those heterotrophic acidophiles were provisionally identified to be *Acidiphilium* based on colony and cell morphologies. All colonies that grew on iron-, iron-tetrathionate- and iron thiosulfate- overlay media, except for one colony type (14% of total) found on microaerobically incubated iron-overlay medium, were iron-encrusted. The dominant colony type (97% of total number of colonies found on aerobically incubated medium) on iron-tetrathionate media was lost during purification, but the less abundant colony type (3% of total) was identified as *At. ferrooxidans*. All the iron-encrusted colonies found on iron-overlay and iron-thiosulfate media were identified as *At. ferrooxidans* and the non iron-encrusted colony on iron-overlay medium was identified as a *Sphingomonas* sp..

Table 4.16. Plate counts of dispersed S3M streamer. The numbers are expressed as CFUs/ml streamer cell suspension.

	Aerobic incubation	Microaerobic incubation
Iron-oxidizing bacteria		
- extreme acidophiles	4×10^3	4×10^3
- moderate acidophiles	2×10^3	$<10^2$
Heterotrophic acidophiles	7×10^3	5×10^3
Neutrophiles	$<10^2$	$<10^2$

Table 4.17 lists the colony count results of the media inoculated with S3B streamer (50-60 cm below the surface of the main stream in Cae Coch) sample. Much greater numbers of extremely acidophilic iron-oxidizers were isolated than other types of microorganisms. All of the colonies on iron-, iron-tetrathionate- and iron thiosulfate-overlay media were iron-encrusted and most of these were identified as *At. ferrooxidans.* A different colony morphology (5% of total) was not successfully subcultured and was therefore not identified. The isolates found on microaerobically incubated yeast extract pH 3 media were identified as *Acidocella* (46% of total) or *Acidiphilium* (54% of total) spp., on the basis of their cell and colony morphologies, but were not sequenced.

Table 4.17. Plate counts of dispersed S3B streamer. The numbers are expressed as CFUs/ml streamer cell suspension.

	Aerobic incubation	Microaerobic incubation
Iron-oxidizing bacteria		
- extreme acidophiles	1×10^4	6×10^5
- moderate acidophiles	5×10^2	2×10^2
Heterotrophic acidophiles	$<10^2$	3×10^2
Neutrophiles	$<10^2$	$<10^2$

Colony count data for the surface and the bottom (*ca.* 30 cm deep) of the isolated pool (site S4) are shown in Table 4.18. No heterotrophic bacteria were isolated on media inoculated with surface water (S4T), and all of the colonies on iron-, iron-tetrathionate- and iron thiosulfate-overlay media were iron-encrusted. Amongst these, only the colonies that grew on iron-tetrathionate-overlay media were identified (as *At. ferrooxidans)* as others were lost during purification. With the water sample from the bottom of the pool, all colonies that grew on iron-, iron-tetrathionate- and iron thiosulfate-overlay media were again iron-encrusted, and CFUs of moderately acidophilic iron-oxidizers were greater in number than those of other groups. The dominant isolates on iron- and iron-tetrathionate media were again identified as *At. ferrooxidans.* All of the heterotrophic colonies that grew on yeast extract (pH 3) plates incubated under microaerobic conditions, was identified as an *Acidocella* spp..

Table 4.18. Plate counts (as CFUs/ml) of water samples collected at pool S4 (S4T and S4B).

	S4T	S4B	
	Aerobic incubation	Aerobic incubation	Microaerobic incubation
Iron-oxidizing bacteria			
extreme acidophiles	7×10^5	1×10^5	5×10^4
moderate acidophiles	2×10^5	8×10^5	3×10^2
Heterotrophic acidophiles	$<10^2$	$<10^2$	$3x10^4$
Neutrophiles	$<10^2$	$<10^2$	$<10^2$

The results obtained from colony counts of the media inoculated with dispersed acid streamer from site S7 are shown in Table 4.19. Again, all colonies that grew on iron-, iron-tetrathionate- and iron thiosulfate-overlay media were iron-encrusted, and the dominant isolate was *At. ferrooxidans.* Heterotrophic isolates with two distinct colony morphologies were found on microaerobically incubated yeast extract (pH 3 medium); one (83% of total) was a *Sphingomonas* sp., but the other was lost during purification and not identified. On aerobically-incubated yeast extract pH 3 medium, one *Acidiphilium*-like colony type was recorded.

Table 4.19. Plate counts of dispersed acid streamer from site S7. The numbers are expressed as CFUs/ml streamer cell suspension.

	Aerobic incubation	Microaerobic incubation
Iron-oxidizing bacteria		
- extreme acidophiles	7×10^5	1×10^6
- moderate acidophiles	9×10^5	6×10^4
Heterotrophic acidophiles	1×10^5	$6x10^4$
Neutrophiles	$<10^2$	$<10^2$

In an attempt to isolate and enumerate anaerobic microorganisms from streamer and water samples collected at S3T, S3M, S3B, S4B and S7, aSRB media (Section 2.2.1.2.2.2) were inoculated and incubated under anaerobic conditions. Microbial growth on these plates was diffuse, rather than as distinct colonies, and it was not possible to enumerate CFUs. Growth was obtained on all media variants, as areas of white- and orange-colored "colonies", which were successfully separated, purified and identified. All of the anaerobic isolates were found (from RFLP and gene sequence analysis)

to be *Sphingomonas,* identical to isolates S2F2M, S3MF1M and S731M that had been obtained on iron- and yeast extract-overlay plates, incubated microaerobically. The 16S rRNA gene sequence of one anaerobic isolate (S3BMN1) was sequenced (1357 bp) and found to be 99.4% identical to a *Sphingomonas*-like oral clone AV069 (NCBI accession number AF385529) and 99.2% identical to *Sphingomonas echinoides* (AJ012461). T-RFLP analyses of isolate S3MMN1 showed that the T-RF lengths were 192, 284 and 226 nucleotides when treated with *Cfo*I, *Msp*I and *Alu*I, respectively. Isolate S3MMN1 was unable to grow on R2A medium under aerobic, microaerobic or anaerobic conditions and also could not grow on aSRB medium supplemented with mannitol under aerobic conditions. The isolate grew as gelatinous orange-colored colonies on microaerobically- and anaerobically-incubated aSRB yeast extract medium supplemented with mannitol. The isolate also grew in mannitol/yeast extract liquid medium (adjusted to pH 4) under anaerobic conditions, producing copious amounts of exopolymeric slime.

4.6 Total microbial cell counts

4.6.1 Materials and Methods

The protocol followed for total microbial counts in dispersed acid streamers was as described in Section 3.4. For water samples, 2.5 ml aliquots were fixed in 7.5 ml 4% PFA. To remove iron precipitates from the fixed samples, oxalic acid (100 mM) was added until the sample solution became clear. The sample solution was centrifuged for 5 minutes at 16,100 *g* to collect pellet and the pellet was resuspended in 1 ml of 1X PBS. The sample solution was centrifuged again and the pellet was resuspended in a PBS/ethanol (1:1, v/v) mixture. The fixed sample was stored at -20°C and used for total cell count by DAPI staining (Section 2.3.2).

4.6.2 Results

The numbers of total microbial cells stained with DAPI were compared with the values obtained from the colony counts (Section 4.5.2), which were obtained by adding the numbers of extreme and moderate iron-oxidizers, and heterotrophic acidophiles, to calculate plating efficiencies (Table 4.20).

Table 4.20. DAPI- and colony counts, and plating efficiencies of acid streamer and water samples from the Cae Coch mine. DAPI- and colony counts are shown as numbers of cells/ml cell suspension of streamer or water samples.

Samples	DAPI counts	Plate counts	Plating efficiency (%)
S2	5×10^8	6×10^6	1.2
S3T	3×10^8	1×10^7	3
S3M	3×10^8	1×10^4	0.003
S3B	3×10^8	6×10^5	0.2
S4T	9×10^6	9×10^5	10
S4B	2×10^7	9×10^5	4.5
S7	8×10^8	2×10^6	0.25

As shown in Table 4.20, the plating efficiencies of the samples were variable, but were always low (<10%). The samples taken from S4T had relatively high plating efficiency, but >98% of the microorganism from sites S2, S3M, S3B and S7 were not cultivated using the protocols used in this study.

4.7 Fluorescent *in situ* hybridization analysis

4.7.1 Materials and Methods

Streamer and water samples fixed in 4% PFA (Section 4.2) were analyzed by FISH (Section 2.6.8). Streamer samples were treated with dilute alkali after fixation to improve dispersion as follows. A fixed streamer fragment was added to 1 ml NaOH (20 mM) and mixed using vortex for 15 minutes until the streamer was completely dispersed. The dispersed streamer material was centrifuged for 5 minutes at 16,100 *g* and the pellet was resuspended in 1 ml of 1X PBS. The sample was centrifuged and the pellet was resuspended in a PBS/ethanol (1:1, v/v) mixture. The fixed sample was stored at -20°C until required. As the cells targeted by the *Sphingomonas* specific-probe (SPH120) were only present in low numbers, fixed samples containing larger numbers of cells were used to detect these. However, this caused difficulties in enumerating targeted cells accurately.

Based on the sequences of the clones obtained from the S3Bb mini-library (Section 4.4.3), FISH probes specifically targeting clones S3B2C3 and S3B2C11 were designed. The sequence of the S3B2C3 probe was 5'

CGCCTTTCCTTCTTCACT 3' (ODP nomenclature S-S-S3B2C3-193-a-A-18; Alm et al. 1996). In order to enhance the probe signal, unlabeled helper oligonucleotide probes were designed to target sites prior (S3B2C3-1 5' CATGCGAGCAAAGAGTCT 3') and subsequent (S3B2C3+1 5' ATCAAGCGACGCCGTAG 3') to the labeled S3B2C3 probe. Although S3B2C11 was a chimeric sequence, the first *ca.* 500 nucleotides were 90% identical to that of clone S3B2C3. However, the S3B2C3-specific probe did not target clone S3B2C11, due to 4 base mismatches, and a new probe, specifically targeting clone S3B2C11, was designed. The sequence of this probe was 5' CTTTCCTTCCTCTGCCAT 3' (ODP nomenclature S-S-S3B2C1-190-a-A-18). Unlabeled helper oligonucleotide probes were also designed to target sites prior (S3B2C11-1 5' GCGGCAAAGGAAACCATT 3') and subsequent (S3B2C11+1 5' TTGGACGGCGCCGTA 3') to the S3B2C3 probe.

4.7.2 Results

The results obtained from FISH analysis of streamer and water samples from sites S2, S3T, S3M, S3B, S4T, S4B and S7, are shown in Table 4.21 and Figure 4.32. Cells targeted by ALF1B, SPH120, LF655 and LF581 were so low in number that enumeration was not possible in some samples. For samples S2, S3T, S3M, S3B, S4 and S7, no cells were stained by the archaea-specific (ARCH915) probe, but 70-88% of the DAPI-stained cells were stained by the bacteria-specific EUB338 probe. In contrast to other samples, only 24% of DAPI stained cells in pool water sample S4B was stained with EUB338, and 10% was stained by the ARCH915 probe.

In order to identify the cells stained with the archaea- and bacteria-specific probes, a variety of group- and species-specific probes were used. However, many probes failed to stain any cells in all of the samples examined. These were the low G+C Gram-positive (LGC0355), *Desulfosporosinus*-M1 (aSRB), *Sulfobacillus* genus (SUL228), *Acidiphilium*-WBW (Alk), *Thiomonas* group 1 and 2 (TM1G0138 & TM2G0138), *At. thiooxidans* (ATT0223), *Am. ferrooxidans* (ACM995), isolate KP3 (FMR0732), *Acidobacterium*-like KP1 (ABI1002) and *L. ferrooxidans* group III (LF1252) probes.

With the microbial pipe sample S2, 4% of EUB338-stained cells was stained with the *α-Proteobacteria* specific probe (ALF1B), which would have theoretically included those stained with the *Sphingomonas* probe (SPH120). However, cells stained with SPH120 could not be enumerated accurately as they were present in very low numbers in the sample. Most (94%) of cells in this sample were stained with the *β-Proteobacteria*-specific probe, BET42a. In theory, these should have included cells stained with the TrefC11 group-specific BSC probe (38%) and the TrefC4-specific GALTS (45%) probe. About 2% of cells was stained with the *γ-Proteobacteria*-specific probe, GAM42a, and these would have included cells stained with the *At. ferrooxidans*- and WJ2-specific probes. A small number of bacteria was also stained with the *Acidimicrobium/"Ferrimicrobium"*-specific probe (2%), and with the *Leptospirillum*-specific probe (1%).

About 1% of the cells in the S3T sample was stained with the ALF1B probe, while the BET42a probe stained 68% of active bacteria. Among the cells stained with the *γ-Proteobacteria*-specific probe (17% of total active bacteria), some would have been theoretically stained with the *At. ferrooxidans*- (15%) and WJ2- (7%) specific probes. The *Acidimicrobium/"Ferrimicrobium"*-specific probe (ACM732) stained 11% of cells, but few were stained by the *Leptospirillum*-specific probe LF655. A similar picture emerged from FISH analysis of a streamer sample from site S3M, though, a small number of cells were stained with the probes targeting the microorganism represented by clone S3B2C11 (Figure 4.34 A) and *Leptospirillum* spp.. *Sphingomonas* was also detected in FISH analysis of the S3B streamer sample (Figure 4.33) and about 5% of cell was stained with the S3B2C3 probe (Figure 4.34 B).

A very different picture emerged from the pool water collected at site S4. Again there were few *α-Proteobacteria* (1%), but in this case, no *β-Proteobacteria* were detected and, although *Leptospirillum* spp. were detected, they again accounted for only 1% of active bacteria. The majority of cells in water sample S4T was stained with either the *At. ferrooxidans*- or the *Acidimicrobium/"Ferrimicrobium"*-specific probes (77% and 49%,

respectively). With this sample, cells with very similar morphologies (rods in long chains) were stained with both TF539 and ACM732 probes and this phenomenon might have caused errors in enumeration. Archaea were detected by FISH analysis (10% of DAPI-stained cells) in the S4B sample, which made it unique among the Cae Coch samples tested. However, the identity of these cells could not be determined, except that they were not stained with the *Ferroplasma*-specific probe, FER656. In this sample, *At. ferrooxidans* (40%) and actinobacteria (24%) were the only bacteria detected (Figure 4.35).

With the acid streamer sample from site S7, no cells were detected by the ALF1B probe. The majority of active bacteria (63%) was stained with the BET42a probe, and 35% of those was theoretically targeted by the BSC probe. A significant proportion of cells was stained with the *At. ferrooxidans*-specific probe (29%) and the *Acidimicrobium*/"*Ferrimicrobium*"-specific probe (21%), and a relatively small number (3%) was stained with the WJ2-specific probe.

Table 4.21. Results of FISH analyses on Cae Coch streamer and water samples. The numbers are shown as percentage against EUB338 or DAPI (EUB338 and ARCH915) stained cells. (-) not determined, (*) detected but not enumerated.

	S2	S3T	S3M	S3B	S4T	S4B	S7
EUB338	80.9	86.6	86.2	85.2	68.5	23.8	88.3
ARCH915	0	0	0	0	0	9.9	0
FER656	-	-	-	-	-	0	-
LGC0355	0	0	0	0	0	0	0
aSRB	-	-	0	0	0	0	-
SUL228	0	0	0	0	0	0	0
S3B2C3	0	0	0	4.6	0	0	0
S3B2C11	0	0	*	0	0	0	0
ALF1B	3.4	0.9	*	0	1.1	0	0
Alk	0	0	0	-	0	-	-
SPH120	*	*	*	*	0	0	*
BET42a	93.5	68.3	69.5	69.8	0	0	63.4
BSC	37.8	77.2	78.6	33.8	0	0	34.8
GALTS	45.3	0	0	0	-	-	0
TM1G0138	0	0	0	0	0	0	0
TM2G0138	0	0	0	0	0	0	0
GAM41a	1.8	16.7	5.2	10.8	57.2	39.6	19.1
TF539	1.4	14.8	13.3	5.9	77.0	41.7	29.1
WJ20646	0.6	6.5	0	4.5	0	0	2.9
ATT0223	0	0	0	0	0	0	0
ACM732	1.6	10.5	6.5	15.1	49.1	23.8	21.8
ACM995	0	0	0	0	0	0	0
FMR0732	0	0	0	0	0	0	0
LF655	0.5	*	2.7	*	0.8	0	0
LF581	0	0	*	-	0	-	0
LF1252	0	0	0	0	0	0	0
ABI1002	0	0	0	0	0	0	0

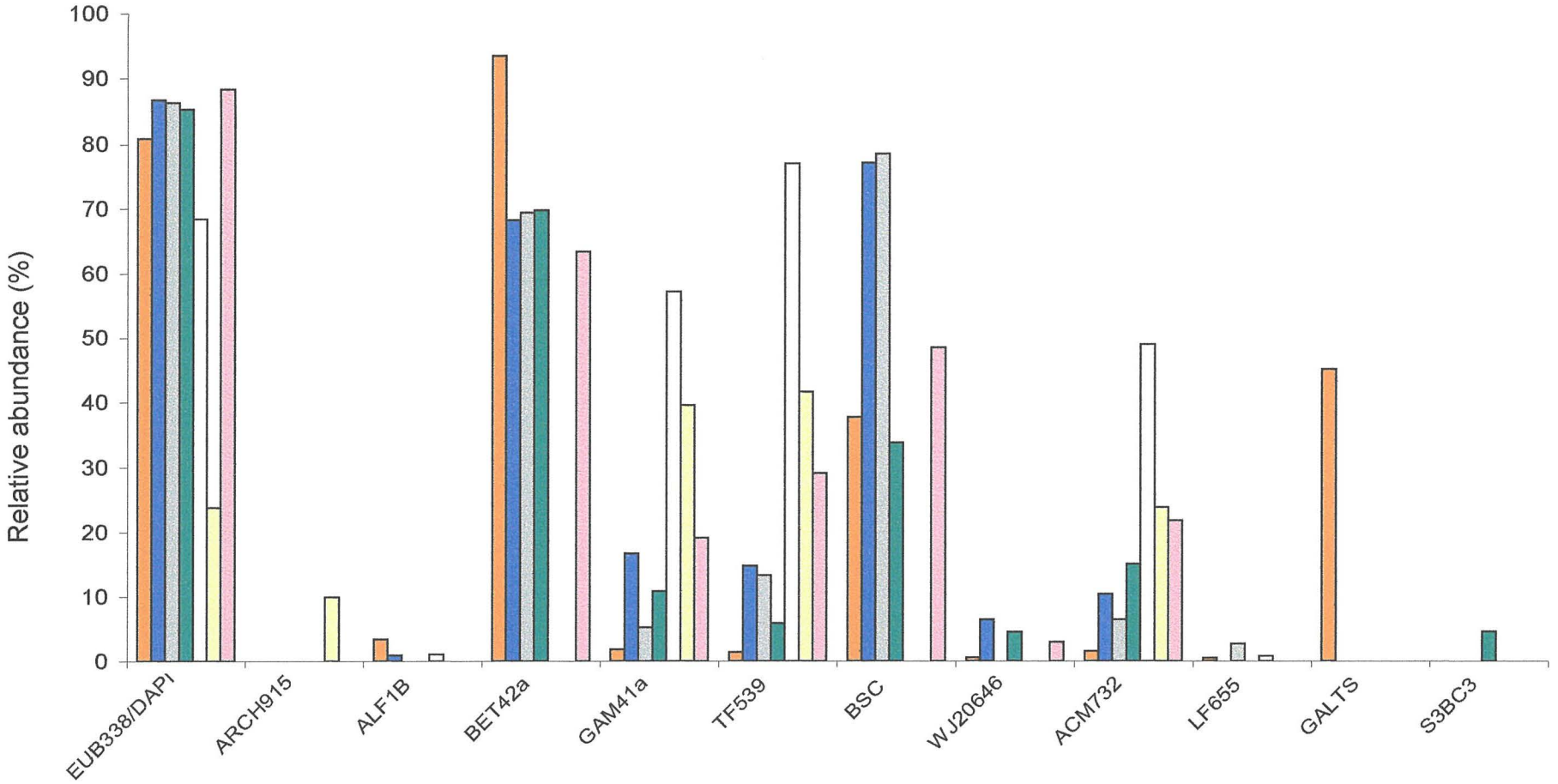
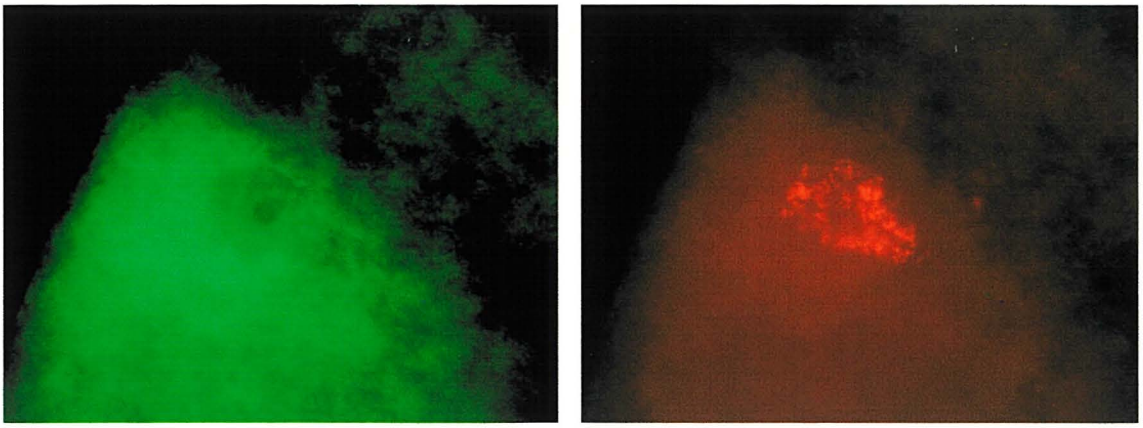
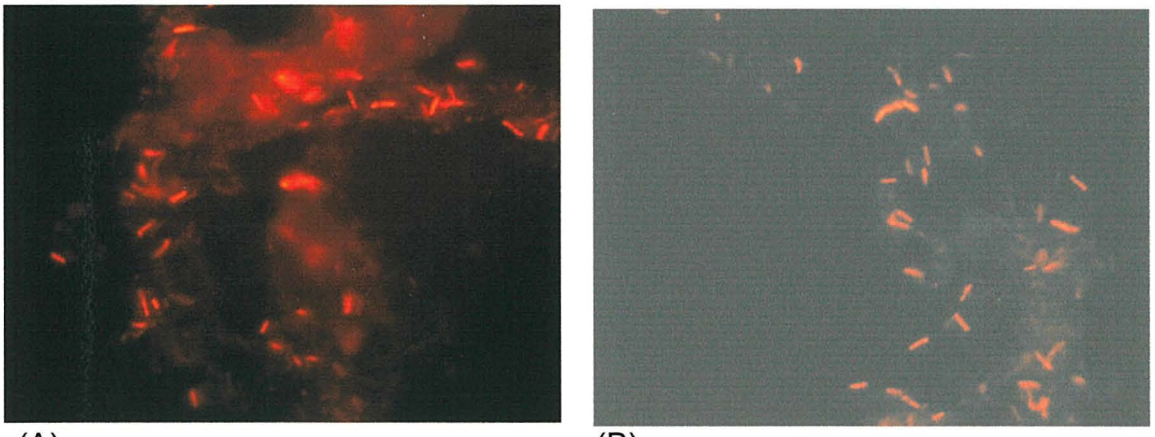


Figure 4.32. FISH analysis of Cae Coch acid streamer and water samples; S2 = red, S3T = blue, S3M = grey, S3B = green, S4T = white, S4B = yellow, S7 = pink. Probe targets; EUB338 = *Bacteria*, ARCH915 = *Archaea,* ALF1B= *α-Proteobacteria,* BET42a = *β-Proteobacteria*, GAM41a = *γ-Proteobacteria*, TF539 = *At. ferrooxidans,* BSC = clone TrefC11/S3B2C20, WJ20646 = γ-proteobacterium WJ2, ACM732 = *Acidimicrobium/"Ferrimicrobium",* LF655 = *Leptspirillum* spp., GALTS = clone TrefC4, S3B2C3 = clone S3B2C3.



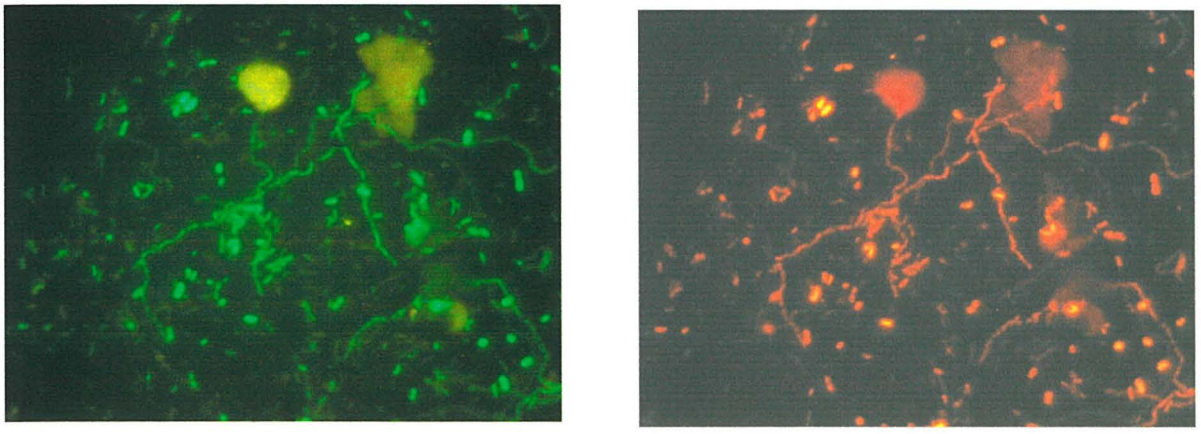
(A) (B)

Figure 4.33. FISH analysis of a cell aggregate from an acid streamer from site S3B; (A) stained with the fluorescein-labeled EUB338 probe, (B) stained with the Cy3-labeled SPH120 probe.



(A) (B)

Figure 4.34. Acid streamer sampled from 50-60 cm depth in the main stream (Site 3) within Cae Coch, stained with two probes targeting low G+C Gram-positive bacteria; represented by clones S3B2C3 (A), and S3B2C11 (B).



(A) (B)

Figure 4.35. Bacteria in pool water sample S4B, targeted by the fluorescein-labeled EUB338 probe (A), and the *Acidimicrobium*/"*Ferrimicrobium*"-specific probe ACM732 (B).

4.8 Analysis of a "pipe" (microbial stalactite) sample using scanning electron microscopy (SEM)

4.8.1 Material and Methods

About 0.2 g (wet weight) of a pipe streamer sample taken from S2 was fixed in 4% PFA in 1X PBS for 48 hours at 4°C. The sample was prepared for SEM analysis as described in Section 2.4.4 and viewed using a HITACHI S-520 SEM.

4.8.2 Results

Electron micrographs of S2 streamer sample are shown in Figures 4.36 and 4.37. The streamer-like material was composed of prokaryotic cells seemingly held together with exopolymeric substances, which appear as web-like dehydrated materials in Figure 4.37. The streamer-like material contained some inorganic material (small fragments of minerals that can be seen in Figure 4.36), but no eukaryotes, such as fungi, yeast or protozoa, were found.

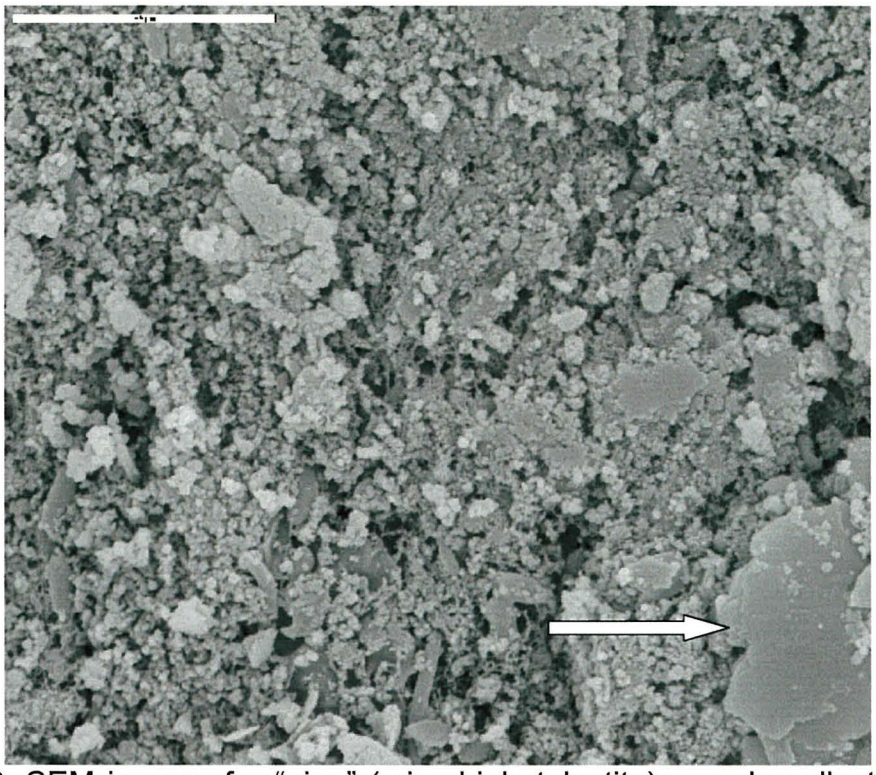


Figure 4.36. SEM image of a "pipe" (microbial stalactite) sample collected at Site 2 within Cae Coch. The streamer-like material was composed mainly of microbial cells, with some inorganic materials (arrowed). The bar represents 5 µm.

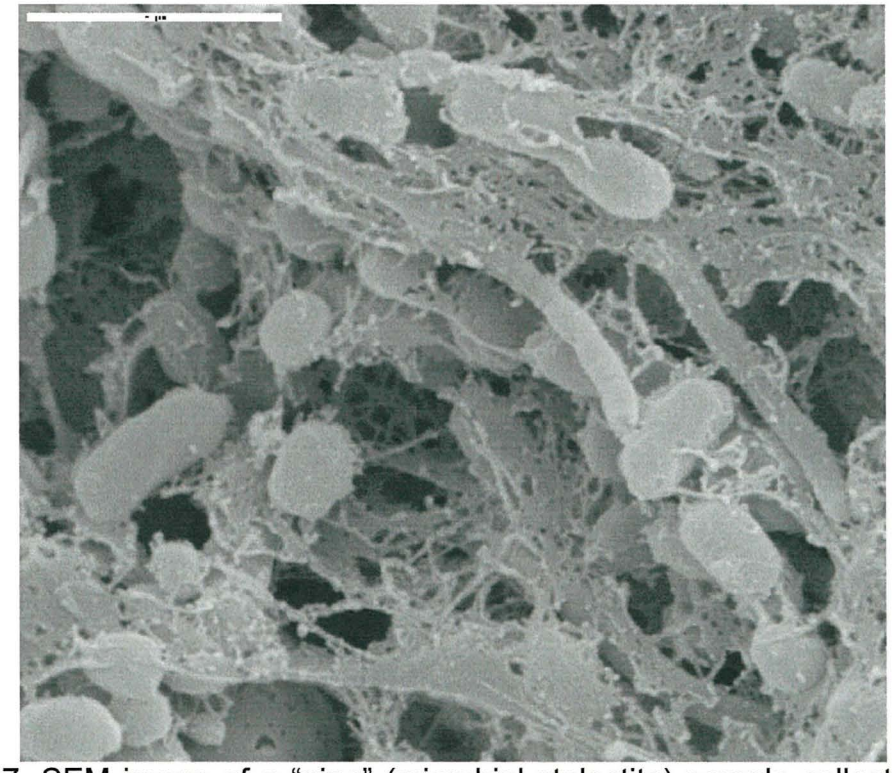


Figure 4.37. SEM image of a "pipe" (microbial stalactite) sample collected at Site 2 within Cae Coch. Microbial cells are seen, together with by web-like exopolymeric substances. The bar represents 2 µm.

4.9 Discussion

As with the study of acid streamers in the Trefriw Wells spa (Chapter 3), the combined use of culture-independent and cultivation-dependent techniques was successful in elucidating the biodiversity of streamer communities and water samples in the abandoned Cae Coch pyrite mine. Table 4.22 lists the microorganisms detected by these methods in samples collected from the sites within the Cae Coch mine and shows that the microbial communities varied greatly in composition between different sites within the mine.

Chemical analysis showed that all waters sampled in the mine were extremely acidic (pH range 1.84 to 2.28) and that water temperatures were typical of subterranean environments in the U.K. (8.6-12.5°C). There were, however, significant differences in redox potentials, conductivities, and concentrations of dissolved carbon, oxygen, metals and sulfate in waters sampled at different locations within the mine, and this would have been predicted to have an influence on the biodiversity of the indigenous microflora.

Microbial populations in Cae Coch streamers and water samples were found to be exclusively prokaryotic. No eukaryotes (fungi or protozoa) were found by DAPI staining and SEM examination, in contrast with an earlier report (Johnson et al. 1992) which found that protozoa and rotifers could be observed (*via* light microscopy) within acid streamers, actively consuming the bacteria.

The data from culture-dependent techniques suggested a somewhat limited biodiversity of the microbial communities within Cae Coch. The dominant isolates were the iron-oxidizers, *At. ferrooxidans* and *γ-proteobacterium* isolate S3TT1M (WJ2), and heterotrophic acidophiles (*Acidocella* and *Acidiphilium* spp.). The novel *Sphingomonas* isolate (S3MMN1) was also found to grow in acidic media. These results suggested that *At. ferrooxidans* is the dominant microorganism in Cae Coch streamers and water samples. However, comparison with total (DAPI) counts showed that the plating efficiencies of the media used in this study were very low (maximum 10%)

indicating that most microorganisms present were not isolated on the solid media used.

Table 4.22. Microorganisms detected by culture-dependent and culture-independent methods in acid streamer and water samples collected from the Cae Coch pyrite mine.

Microorganism detected	Sampling site						
	S2	S3T	S3M	S3B	S4T	S4B	S7
(a) Microorganisms identified by culture-dependent methods							
At. ferrooxidans	+	+	+	+	+	+	+
Acidocella-like	+	+	-	+	-	+	-
Acidiphilium-like	+	+	+	+	-	+	+
γ-proteobacterium WJ2-like	-	+	-	-	-	-	-
Sphingomonas	+	-	+	+	-	-	+
(b) Microorganisms identified by culture-independent methods							
Domains							
Archaea	+	+	+	-	+	+*	+
Bacteria	+	+	+	+	+	+	+
Classes							
α-Proteobacteria	+	+	+	+	+	-	-
β-Proteobacteria	+	+	+	+	+	+	+
γ-Proteobacteria	+	+	+	+	+	+	+
Bacilli	-	-	+	+	-	-	-
Actinobacteria	+	+	+	+	+	+	+
Acidobacteria	-	+	-	-	-	-	+
Nitrospira	+	+	+	+	+	-	+
Species							
Sphingomonas	+	+	+	+	-	-	+
Ralstonia	-	-	-	-	+	+	-
At. ferrooxidans	+	+	+	+	+	+	+
Acidimicrobium/ "Ferrimicrobium"	+	+	+	+	+	+	+
Leptospirillum	+	+	+	+	+	-	+
γ-proteobacterium WJ2-like	-	+	-	+	-	-	+
Clones							
β-proteobacterium clone S3B2C20	+	+	+	+	-	-	+
β-proteobacterium clone TrefC4	+	-	-	-	-	-	-
Bacillus clone S3B2C3	-	-	-	+	-	-	-
Bacillus clone S3B2C11	-	-	+	+	-	-	-

*Archaea in this sample were detected by FISH analysis and by 16S rRNA gene amplification using archaea-specific primers. In all other samples, archaea were only detected by 16S rRNA gene amplification using archaea-specific primers.

The inability to isolate *ca.*90% of microorganisms in the samples highlighted the importance of using culture-independent techniques to study these microbial communities.

Of the culture-independent techniques used, T-RFLP analysis proved to be the most straightforward method for obtaining information on community diversity. However, the lack of data in the T-RFs database limited the number of T-RFs in extracted DNA that could be identified. However, although this technique was useful for identifying only a small number of prokaryotes, it did provide an insight into the complexity of the microbial communities (i.e. the greater the number of T-RFs, the more complex the community). As PCR-based techniques, such as T-RFLP and clone library construction, are subject to bias (e.g. due to differential extraction of DNA and gene amplification; Head et al. 1998), the data obtained from these analyses gave information on which microorganisms were present in the samples but, at best, only semi-quantitative analysis of community structures. On the other hand, FISH enabled the relative proportion of a particular microorganism in a sample to be determined much more accurately, though a limitation of this technique was the size of the samples that were examined. FISH also facilitated the detection of microorganisms that were present in too low a number to be detected in mini-clone libraries or by T-RFLP. At the outset of this study, FISH was limited by the availability of appropriate probes and data obtained from clone libraries was essential in designing new probes for this aspect of the work. To summarize, the combined techniques used in this study were useful in obtaining an accurate picture of the different microbial communities within the Cae Coch mine.

In all the streamer, slime and water samples (except for sample S1), *At. ferrooxidans* and microorganisms targeted by the ACM732 FISH probe were detected. Since the ACM732 probe targets *Am. ferrooxidans* and related actinobacteria, including "*Ferrimicrobium*", the microorganisms found in the samples could not be identified at genus level. In addition, no bacteria in the samples were stained with either the *Am. ferrooxidans*-specific probe (ACM995) or the actinobacteria isolate KP3 probe (FMR0732). Since *Am.*

ferrooxidans is a moderate thermophile (Johnson & Roberto 1997), it is unlikely that it would be found in the cool waters within the Cae Coch mine. It is more likely that the bacteria that were detected by the ACM732 probe were the heterotrophic iron-oxidizing mesophile "*Ferrimicrobium acidophilum*", which has previously been isolated from this site (Johnson & Roberto 1997). Interestingly, both *At. ferrooxidans* and microorganisms detected by the ACM732 probe were found to be less abundant in streamer materials than in water samples.

Archaeal 16S rRNA genes were amplified from all samples except for S3B, but archaeal cells were only detected by FISH analysis in sample S4B, suggesting that they were present in small numbers in other samples. Although the materials examined were composed of a range of prokaryotes, the microorganism represented by clone S3B2C20 was the most abundant microorganism present in many of the streamer samples. The 16S rRNA gene sequence of clone S3B2C20 was almost identical to that of clones found in Trefriw spa (Chapter 3), Mynydd Parys, Iron Mountain (U.S.A.) and AMD in New Zealand, and it is likely that this uncultivated β-proteobacterium is a significant microorganism in many extremely acidic, metal-rich environments worldwide. As the microorganism represented by clone S3B2C20 was detected in streamers but not in water samples, this microorganism is probably less important as a planktonic acidophile and may have an obligate requirement for growing (possibly together with other acidophiles) in biofilms, slimes and streamers.

The *Sphingomonas* isolate (S3MMN1) was detected in all acid streamer samples, but only in low numbers. Visualization of probe-stained cells in partially-dispersed streamers using FISH showed that these bacteria occurred as clusters of cells, and never as single, discrete bacteria (Figure 4.33). The discovery of isolate S3MMN1 within acid streamers was somewhat surprising as most *Sphingomonas* spp. are aerobic, neutrophilic heterotrophs; many strains are able to degrade recalcitrant carbon sources (White et al. 1996). Attempts to grow isolate S3MMN1 on neutral pH R2A medium failed and this

Sphingomonas sp. was found to grow only in media of pH <4.0. The isolate grew better under microaerobic and anaerobic conditions than under aerobic conditions, which may reflect its occurrence within streamers where oxygen diffusion would be predicted to be restricted. Colonies of S3MMN1 were gelatinous, and liquid cultures were highly viscous, suggesting S3MMN1 produces exopolymeric materials, which is again a characteristic of other *Sphingomonas* spp. (White et al. 1996).

Although some specific prokaryotes were identified in all acid streamers examined, others were detected in only some of the samples. The T-RFLP data suggested that microorganisms represented by clones S11C1, S11C3 and S3B2C20 were dominant in the S1 slime sample. Microorganisms represented by S11C1 and S11C3 are both closely related to heterotrophic acidophiles. S11C1 is closely (99%) related to the γ-proteobacterium isolate WJ2, which was isolated from aerobic AMD treatment cells at the Wheal Jane mine, Cornwall, U.K. (Johnson & Hallberg, 2003), and was classified as a moderately acidophilic, iron-oxidizing eubacterium. S11C3 is most closely related (though only 94% gene similarity) to the moderately acidophilic obligate heterotroph *Acidobacterium capsulatum,* which does not oxidize either iron or sulfur (Kishimoto et al. 1991) but which can, apparently, grow anaerobically using ferric iron as electron acceptor (K, Coupland, University of Wales, Bangor, unpublished). Clone S11C12 was also detected in the S1 streamer sample, but no other streamers or water samples from Cae Coch; its 16S rRNA gene sequence is 97% identical to *Acidosphaera rubrifaciens,* which is another moderately acidophilic heterotroph (Hiraishi et al. 2000).

The S2 pipe/stalactite sample was found to be mainly composed of two microorganisms, represented by clones S3B2C20 and TrefC4. The latter is most closely related to the neutrophilic iron-oxidizer, *Gallionella ferruginea*, and was one of the most abundant bacteria found in streamer growths within the nearby Trefriw spa (Chapter 3). Interestingly, water collected from S2 microbial pipe was the least acidic of all those sampled within Cae Coch and also contained elevated concentrations of ferrous iron. On both counts, the water in which this microbial pipe was bathing was more similar to that in the

Trefriw spa than that at any other site sampled within Cae Coch, and this might have been reflected in a more similar microbial community structure.

The main stream that flows through the Cae Coch mine is, at some locations (e.g. sampling point 3), some 50-60 cm deep, and the whole water column is ramified with acid streamer growths. Three samples were taken from different depths at sampling point S3 to investigate the influence of oxygen concentrations and redox potentials (both of which declined with depth) on the streamer communities. It was found that a single bacterial species (represented by clone S3B2C20) made up a large proportion of the microbial biomass of streamer samples taken at the surface (S3T), and at 30-40 cm (S3M) and 50-60 cm (S3B) depth, though the relative abundance of this prokaryote decreased with sampling depth (77% of total bacteria in S3T, and 34% in S3B). However, about half of the bacteria in the S3B streamer sample that were targeted by the β-*Proteobacteria* probe were not detected by any genus-/species-specific probe available, implying that there were unknown β-*Proteobacteria* in this sample.

The iron/sulfur-oxidizing chemolithotroph, *At. ferrooxidans,* was detected in all three streamer samples from site 3, but the relative abundance decreased with depth. This trend would not be directly due to oxygen availability, as *At. ferrooxidans* is a facultative anaerobe (Pronk et al. 1992), but may have been due to lack of available electron donors (reduced sulfur, or hydrogen) that are used as electron donors coupled to iron reduction under anaerobic conditions (Pronk et al. 1992). Iron-oxidizing *Leptospirillum* spp. were also detected in all three streamer samples in site 3 and were most abundant in streamer sample S3M. The probe that targets *Leptospirillum* groups I and II detected cells only in streamer S3M, implying that *Leptospirillum* group III were present in the other streamers,.

Amongst the other clones identified (and corresponding bacteria detected by FISH) in site 3 streamers were S11C3 (94% similar to *Ab. capsulatum)* in surface streamers only, and a Gram-positive microorganism (detected only in streamer sample S3B and represented by clone S3B2C3) whose closest match (92% gene similarity) was the thermoacidophilic heterotroph,

Alicyclobacillus pomorum. A related Gram-positive bacterium (S3B2C11, which had 90% gene similarity to S3B2C3) was detected only in the S3M streamer sample. These findings suggest that both these Gram-positive bacteria favored oxygen-limited conditions. However, these microorganisms were not detected in the anaerobic water sample S4B (which is assumed to have a similar metal-content to S4T), though this might be due, at least in part, to the very elevated concentrations of dissolved metals and sulfate in that pool. This may imply that S3B2C3 and S3B2C11 either grow only in streamer communities in AMD, or else are more sensitive than some other indigenous bacteria to very high concentrations of metals and/or total dissolved solutes. The physiological characteristics of the bacteria represented by clones S3B2C3 and S3B2C11 await the isolation of these prokaryotes, though it seems unlikely that they would be thermophilic (like their closest known relatives, *Alicyclobacillus* spp.) as the temperature of the main stream in Cae Coch is constantly <10°C (McGinness & Johnson 1993).

The water in pool site S7 had physico-chemical characteristics similar to those of the main drainage stream (S3) though its pH was slightly less and DOC content slightly greater. The streamer community at site S7 was composed mainly of the bacterium represented by clone S3B2C20, *At. ferrooxidans* and actinobacteria targeted by probe ACM732; small numbers of bacterium S11C1 were also detected. As S7 water contained elevated concentrations of both ferrous and ferric iron, iron cycling (e.g. by *At. ferrooxidans* and "*Ferrimicrobium*") involving oxidation at the water surface and reduction at depth, might conceivably be operating within this pool. The relatively high concentration (11 mg/l) of DOC in site 7 would have been particularly favorable to the iron-oxidizing/reducing heterotroph, "*Ferrimicrobium*". Interestingly, the relative numbers of clone S3B2C20 were less, and those of *At. ferrooxidans* and actinobacteria greater, in the streamer sample from site 7 than that from site 3, suggesting that the uncultivated bacterium represented by clone S3B2C20 may not as tolerant to high concentrations of metals and low pH as *At. ferrooxidans* and the *Acidimicrobium*/"*Ferrimicrobium*"-like actinobacteria.

Site 4 was the only site where streamer-like growths were absent and the physico-chemistry of water in this pool was also markedly different from other sites. The S4 pool contained much greater concentrations of dissolved solutes, and this was thought due to the fact that there was no obvious flow into this small pool and that, over time, evaporation had reduced its volume. Both pH values and concentrations of DOC were the lowest recorded within Cae Coch. Unlike other samples, where most of DAPI-stained cells were active bacteria, only 70% of DAPI-stained cells in S4T and 24% in S4B sample were active bacteria. Even when numbers of cells that were stained with the archaea-specific probe were taken into account, more than 60% of DAPI stained cells in S4B were found to be inactive, suggesting that a significant proportion of the biomass was either moribund or dead, and that rates of decomposition in the bottom of the pool were slow. Both S4T and S4B microbial communities were mainly composed of *At. ferrooxidans* and actinobacteria, indicating that these bacteria are more tolerant of low pH, high metal and sulfate concentrations, than others found within Cae Coch. In particular, the more typical "acid streamer" bacteria (e.g. clone S3B2C20-type) were not detected by FISH analysis in the samples collected at Site 4. The surface and bottom waters in pool S4 differed in DO content, iron concentrations and conductivities, indicating that the pool was stratified. In S4T, which had a greater concentration of DO, but smaller concentrations of dissolved solutes and dissolved iron (predominantly ferric iron) than S4B, bacteria detected (other than *At. ferrooxidans* and *"Ferrimicrobium"*) included *α-Proteobacteria* and *Leptospirillum* spp., though these were minor components of the microbial community. In contrast, these were not detected in the lower pool water at S4, though a bacterium represented by clone S4BC11 (99.7% gene similarity to the neutrophilic heterotrophic β-proteobacterium, *Ralstonia pickettii)* was found. Although the DOC content of S4B water was relatively small, S4BC11 (a presumed heterotroph) might have been able to survive by scavenging dead cell material and lysates. The absence of *Leptospirillum* spp., which are aerobic iron oxidizers, in sample S4B can be explained by the low oxygen content of the water. Ten percent of cells in S4B was stained with the archaea-specific probe. An archaeal clone sequence obtained (S4BAC1) was related (89% gene similarity) to the

moderately thermophilic heterotrophic archaea, *Thermoplasma acidophilum* and *T. volcanium,* though there was a closer (92%) match to a clone that had been obtained at Iron Mountain. FISH analysis showed that archaea (assumed to be predominantly clone S4BAC1) were present only in significant numbers in sample S4B, suggesting that their preferred habitat is oxygen-limiting, metal- and sulfate-rich environment. This archaeon represented by clone S4BAC1 appears to be a new member of the order *Thermoplasmatales,* though isolation of the organism is necessary for full characterization to be carried out.

Comparison of Cae Coch streamer/slime microbial populations with those reported from Iron Mountain (U.S.A.) and Rio Tinto (Spain) showed that, although the microbial growths share similar gross morphologies, they differ greatly from each other in their microbial compositions. Iron Mountain is extremely acidic (pH 0.6-1.2) and temperatures within the mine are higher (up to 43°C) than within Cae Coch. Acid streamer and slime communities within Iron Mountain were found to be dominated by *Ferroplasma*, *Leptospirillum* and *Sulfobacillus*, and fungi were also detected (Bond et al. 2000b). As with the Cae Coch samples, streamers and slimes within Iron Mountain were heterogeneous, with those found in more extreme (lowest pH and highest conductivity) locations being dominated by *Archaea,* while *Leptospirillum* was the dominant prokaryote in less extreme sites. Although archaea were not detected in most materials sampled within Cae Coch, they were found in the lower waters of pool site S4, where physico-chemical conditions were the most extreme of those recorded within the mine.

In general terms, the Rio Tinto is more similar to stream and pool waters within Cae Coch than is the Iron Mountain site. The pH of the Rio Tinto is *ca.* 2.0 and the temperature shows seasonal variation. A study of long, filamentous streamer growths, based on construction and analysis of clone libraries, found no known acidophilic prokaryotes, such as *Acidithiobacillus, Acidiphilium, Leptospirillum* and *Ferroplasma* (López-Archilla et al. 2004). Instead, these researchers found that the microbial community was mainly composed of γ-*Proteobacteria* (*Pseudomonas*) and *α-Proteobacteria*

(*Sphingomonas*). Interestingly, "neutrophilic" bacterial genera were also detected in Cae Coch acid streamers, although these were not very abundant. These included the *Sphingomonas* isolate (S3MMN1) and the *Ralstonia* clone (S4BC11). Preliminary work with the *Sphingomonas* isolate confirmed that it is obligately acidophilic (growth occurred at <4.0 pH but not at pH 7.0) indicating that this is possibly a novel acidophilic species. The data from the Cae Coch streamers and slimes indicate that these microbial communities are more considerably more diverse and heterogeneous than has previously been thought.

In conclusion, the streamer and water samples obtained from Cae Coch were very different in their microbial composition to those reported at other locations. Some microorganisms were ubiquitous in the streamer materials examined, most notably the bacterium represented by clone S3B2C20. Conversely, some bacteria were found only in a limited number (or even one) of the streamer/slime communities examined. It was also found that streamer-like growths were absent in the most extreme environment found within Cae Coch (pool site 4), suggesting that "streamer" bacteria such as clone S3B2C20 type are unable to tolerate such extreme conditions (low pH and high concentrations of dissolved solutes) as found in that particular site. Even though only a small fraction of microbial population was cultivated, cultivation-independent techniques provided useful information about some of the "unculturable" microorganisms, which could be used in future work to devise new protocols to isolate these prokaryotes, and thereby to facilitate their study in the laboratory.

Chapter 5

A study of acid-tolerant sulfate reducing bacteria

5.1 Introduction

As elevated concentrations of soluble sulfate are commonly found in acidic environments where sulfidic minerals are actively oxidizing, dissimilatory sulfate reduction might be expected to be a common phenomenon in acidic anaerobic zones and there have been some reports of sulfidogenesis occurring in such environments (e.g. Tuttle et al. 1969; Gyure et al. 1990; Fortin et al. 2000). Although attempts have been made to isolate and cultivate acid-tolerant and acidophilic sulfate reducing bacteria (aSRB) from acidic environments, these have been mostly unsuccessful. Most mine water isolates to date are neutrophilic and are not active below pH 5.0 (Tuttle et al. 1969; Kusel et al. 2001). One of the reasons for the failure in isolating aSRB is the widespread use of inappropriate media. For example, some of the commonly used substrates utilized to isolate neutrophilic SRB, such as lactate (Postgate 1979) are toxic to microorganisms at low pH (Section 1.2.4). Even when substrates other than organic acids are used in enrichment cultures, problems may arise since some SRB are classed as "incomplete oxidizers" and generate and excrete acetate, which is again far more toxic in acidic than in neutral pH environments.

The use of non-acid substrates, such as alcohols, to isolate aSRB has had some success. Hard and Babel (1997) isolated an SRB from a Norwegian copper mine on solid media that contained methanol as carbon and energy source. The isolate had a pH range for growth of 4.0 – 9.0. The same authors also reported that a characterized SRB, *Desulfovibrio salexigens*, could also grow on methanol in media set at pH 4.5 and above.

To overcome the problem of organic acid toxicity at low pH, Sen and Johnson (1999) proposed a new solid medium formulation to isolate aSRB at low pH (3.5). An acidophilic heterotroph was used to remove small molecular weight organic compounds produced by acid hydrolysis of the gelling agent and those generated due to incomplete substrate oxidation by the aSRB, as

described in Section 1.6.1.2. Using this medium, aSRB were successfully isolated from sediment samples from Montserrat (West Indies) and Mynydd Parys (U.K.). Experiments designed to characterize one of the aSRB obtained in that study, isolate M1 (a Montserrat isolate), are described in this chapter.

5.2 Initial isolation, identification and cultivation of isolate M1

Of the four aSRB isolated by Sen (P1, CC1, M1 and PFB; Sen 2001), isolate M1 was selected for further research as this isolate displayed reproducible growth in liquid and on solid media containing glycerol, whereas consistent growth was not achieved with the other isolates.

Isolate M1 had originally been obtained from an enrichment culture inoculated with sediment taken from the White River, Montserrat. The pH of the enrichment was kept between pH 2.5 and 4.0, and glycerol (10 mM) was added as carbon/energy source. Liquid cultures were spread onto glycerol-containing aSRB solid medium and incubated anaerobically (Sen & Johnson 1999). Sen (2001) described isolate M1 as growing as straight to curved rods that displayed occasional motility, and which formed endospores. Sequence analysis of the 16S rRNA gene of this bacterium (partial product, 466 bp) showed that the most closely related characterized microorganism was a neutrophilic SRB, *Desulfosporosinus orientis* (94% sequence identity). The16S rRNA gene sequence of M1 was 99% identical to that of isolates P1 and CC1 (Sen 2001).

5.3 Modified growth media for aSRB and growth of isolate M1 under different incubation conditions

The liquid and solid media developed by Sen (2001) which contained heterotrophic basal salts, trace elements, 5 mM K_2SO_4, 5 mM $FeSO_4$ and 0.02% (w/v) yeast extract, were modified in this study. Yeast extract was replaced with vitamin mixture (Widdel & Pfenning 1981) in liquid medium but not in the solid medium. Superior growth (in terms of cell density) of M1 was observed in vitamin mixture-containing liquid medium compared to yeast extract-containing medium. No difference was seen with solid media,

however, and therefore yeast extract (rather than the vitamin mixture) was included in the general solid medium formulation.

It was noted by Sen (2001), and also during the early stages of the present study, that attempts to sub-culture aSRB in liquid medium, and occasionally on solid medium, were unsuccessful. There were two proposed reasons for this: (i) toxicity of sulfide, and (ii) increase in pH during sulfidogeneis (liquid cultures increased by >3 pH units during incubation). In an attempt to address these problems, zinc sulfate (at a final concentration of 5 mM) was added to liquid and solid media, in place of ferrous sulfate. Zinc forms a far less soluble sulfide phase than iron and consequently ZnS forms at lower pH than FeS. Formation of ZnS would remove toxic sulfide and also generate protons (thereby buffering culture media), as described in Section 5.9.

Following the isolation of the acidophilic heterotroph *Acidocella* PFBC (Chapter 6), it was decided to use this bacterium in the underlayer of aSRB solid medium, rather than either *Acidocella* WJB-3 or *Acidiphilium* SJH, which had been used previously. The modified aSRB media used in this study are described in detail in Sections 2.2.1.1.2 and 2.2.1.2.2.2.

To test the growth of isolate M1 under aerobic, micro-aerobic (oxygen concentration <5%: v/v) and anaerobic conditions, aSRB solid media described above were inoculated with M1. The inoculated plates were incubated under different conditions: (1) micro-aerobic (sealed plastic pouches containing Campy*Gen*™ Compact sachets (Section 2.2.1.4.1); (2) anaerobic (sealed plastic pouches containing Anaero*Gen*™ Compact sachets (Section 2.2.1.4.2); (3) aerobic (placed directly in an aerobic incubator). Plates were incubated at 30°C and were examined after 7 days. Colonies of M1 were observed only on plates incubated anaerobically.

5.4 Metal toxicity

5.4.1 Materials and methods

The tolerance of isolate M1 to two heavy metals, copper and iron, which are often present at elevated concentrations in AMD, was determined. As

another heavy metal of interest, zinc, was added routinely to liquid and solid media at 5 mM (which is well in excess of concentrations typically found in mine waters) with no apparent inhibitory effects to M1, no toxicity tests were carried out with this metal. The objective here was to assess whether heavy metal toxicity was likely to be problematic in the projected development of an on-line AMD treatment system in which AMD would be pumped directly into a sulfidogenic bioreactor containing isolate M1 (Johnson et al. 2004).

The concentrations of ferrous iron and copper used in this experiment were based on the typical concentrations found in AMD draining the Mynydd Parys mine (North Wales, U.K.). These were 12.2 mM Fe^{2+} and 0.6 mM Cu^{2+}. The AMD stream also contained 1.3 mM zinc, which was well below that used in synthetic media for cultivating M1. Tolerance of M1 to ferrous iron and copper was tested by adding various concentrations of $FeSO_4$ and $CuSO_4$ to aSRB liquid medium (20 ml in 20 ml universal bottle) containing 5 mM glycerol (Section 2.2.1.1.2). The metals were added to the final concentrations shown in Table 5.1 from sterile 1 M stock solutions, and media were inoculated with 1 ml of M1 culture grown in aSRB liquid medium (5 mM glycerol and 5 mM Zn^{2+}). The cultures were incubated for 42 days under anaerobic conditions in 2.5 L anaerobic jars using the Anaero*Gen*™ AN25 system and metal toxicity was determined by noting the presence or absence of growth (microscopic examination of cultures) on day 42.

5.4.2 Results

The results obtained from metal toxicity experiment are summarized in Table 5.1. No growth of M1 was recorded in the cultures containing 0.6 mM $CuSO_4$ while $FeSO_4$ (at 12.2 mM) did not inhibit the growth of isolate M1. In cultures containing ferrous iron and no copper, black precipitates (identified tentatively as FeS) were noted to form, presumably due to bacterial sulfidogenesis. However, no black precipitates were formed in cultures that contained both ferrous iron and copper, confirming the results from microscopic examination of these cultures. A subsequent experiment, in which lower concentrations (0.5, 0.4, 0.3 and 0.2 mM) of $CuSO_4$ (and copper-free controls) were added to M1 cultures, again resulted in no growth of isolate M1, except in the

controls. Therefore, it was concluded that the apparent minimum inhibitory concentration of copper to isolate M1 was <0.2 mM.

Table 5.1. The effect of ferrous iron and copper on the growth of isolate M1. Metal concentrations tested were those found typically in Mynydd Parys AMD. Key: (-) no growth; (+) growth.

Dissolved metal content		Growth
$FeSO_4$ (mM)	$CuSO_4$ (mM)	
12.2	0.6	-
0	0.6	-
12.2	0	+

5.5 Oxidation of glycerol

5.5.1 Materials and methods

In order to determine whether M1 oxidizes glycerol completely to CO_2 or incompletely to acetic acid, the bacterium was grown in aSRB medium (20 ml in a 20 ml universal bottles, pH 4.0) containing 5 mM glycerol and 5 mM Zn^{2+} (Section 2.2.1.1.2). The experiment was carried out in triplicate and cultures were incubated under anaerobic condition in 2.5 L anaerobic jars using the Anaero*Gen*™ AN25 system. Samples (3 ml) were removed from each culture immediately after inoculation and again 37 days later. Concentrations of sulfate, acetic acid and glycerol were measured as described in Sections 2.5.6.1 and 2.5.6.2. Concentrations of soluble zinc in cultures were determined using atomic absorption spectrometry (Section 2.5.4).

5.5.2 Results

The results obtained from this experiment are shown in Table 5.2. Oxidation of glycerol was coupled to the reduction of sulfate to sulfide, which reacted with soluble zinc to form ZnS. Increased concentrations of acetic acid paralleled the decrease in glycerol concentration. The stoichiometry of the reaction was 4.9 mmoles glycerol oxidized/L, 4.1 mmoles sulfate reduced/L, 4.3 mmoles soluble zinc precipitated/L and 4.8 mmoles acetic acid produced. These results indicated that the stoichiometry of glycerol oxidation to acetic acid production was 1:1, as in Equation 5.1:

$$4C_3H_8O_3 + 3SO_4^{2-} + 3Zn^{2+} \rightarrow 4C_2H_4O_2 + 3ZnS + 4CO_2 + 8H_2O \qquad (5.1)$$

Table 5.2. Changes in concentrations of glycerol, acetic acid, soluble zinc and sulfate during growth of isolate M1 in liquid medium containing 5 mM glycerol and 5 mM zinc. Data shown are mean values (n=3) and standard deviations at the start of the experiment and at day 37.

Time (days)	Glycerol (mM)	Soluble zinc (mM)	Acetic acid (mM)	Sulfate (mM)
0	5.4 ±0.1	5.3 ±0.9	0.03 ±0.02	15.4 ±0.7
37	0.4 ±0.6	1.0 ±0.8	4.88 ±0.80	11.3 ±1.7

5.6 Inhibition of sulfidogenesis by acetic acid

5.6.1 Materials and methods

In the above experiment, M1 was shown to oxidize glycerol incompletely to acetic acid. As acetic acid is known to be toxic to many microorganisms at low pH, the tolerance of M1 to different concentrations of acetic acid was tested. To aSRB medium (20 ml in 20 ml universal bottles, pH 4.0) containing 5 mM glycerol and 5 mM Zn^{2+}, various concentrations of potassium acetate were added from a heat-sterilized 0.5 M potassium acetate stock solution to give final concentrations of 2, 4, 5, 6 and 8 mM acetic acid. The medium was inoculated with 100 µl of M1 inoculum, prepared by dispersing colonies of the aSRB grown on solid medium supplemented with glycerol, to minimize the amount of acetic acid carried over from the inoculum. The cultures (one for each concentrations of acetic acid) were incubated for 37 days under anaerobic conditions in 2.5 L anaerobic jars using the Anaero*Gen*™ AN25 system. Bacterial growth and sulfidogenesis were monitored by microscopic examination of cultures and by monitoring the formation of zinc sulfide, respectively. For cultures containing 5 mM acetic acid, concentrations of acetic acid, glycerol, sulfate and soluble zinc were measured as described in Section 5.5.1.

5.6.2 Results

The results obtained from this experiment are shown in Table 5.3.

Table 5.3. The effect of different concentrations of acetic acid on sulfidogenesis by isolate M1, as evidenced by the formation of ZnS in zinc-containing media. Key: (+) ZnS present; (-) ZnS absent (after an incubation period of 37 days).

Initial concentration of acetic acid (mM)	ZnS formation
2	+
4	+
5	+
6	-
8	-

Formation of ZnS was not observed in M1 cultures to which acetic acid had been added at 6 and 8 mM, whereas both formation of ZnS and active M1 cells were observed in the cultures containing (initially) 2 and 4 mM acetic acid. Although ZnS formation was recorded in the culture containing (initially) 5 mM acetic acid, M1 cells were noticeably elongated, which was a general feature observed when cultures were stressed.

Changes in concentrations of glycerol, acetic acid, sulfate and soluble zinc in M1 cultures containing (initially) 5 mM acetic acid were measured, and results are summarized in Table 5.4.

Table 5.4. Changes in concentrations of acetic acid, glycerol, sulfate and soluble zinc in a culture of isolate M1 to which acetic acid had been added at 5 mM.

Time (days)	Glycerol (mM)	Soluble zinc (mM)	Acetic acid (mM)	Sulfate (mM)
0	5.8	6.4	4.9	15.9
37	0.3	1.7	7.6	11.7

The results showed that 5.5 mmoles glycerol/L had been oxidized by day 37, 4.2 mmoles sulfate/L reduced and 4.7 mmoles zinc/L had been precipitated, presumably as ZnS. In this case, although there was an increase in acetic acid concentrations, this was only 2.7 mmoles/L, i.e. significantly less than the amount predicted from Equation 5.1. It is possible that there had been some loss of volatile acetic acid from these cultures, though this was unlikely

as they had been incubated for the same length of time and at the same temperature as the earlier experiment where the glycerol/acetic acid stoichiometry was 1:1. The exact reason for this apparent discrepancy is unclear.

Changes in glycerol concentration were also measured in a culture containing (initially) 6 mM acetic acid. In this case, there was an apparent net increase of 0.4 mM glycerol over the time course of the experiment, confirming that addition of 6 mM acetic acid inhibited glycerol oxidation by isolate M1. The results of this experiment showed that acetic acid was toxic to isolate M1 when present at $\geq$6 mM.

5.7 Acetic acid utilization by M1

5.7.1 Materials and Methods

To test whether isolate M1 was able to metabolize acetic acid when provided as the sole electron donor, sub-toxic concentrations (2 and 4 mM acetic acid) were added to liquid aSRB medium containing 5 mM Zn^{2+} (in triplicate; 20 ml in 20 ml universal bottles). Sulfidogenesis was determined by monitoring the formation of ZnS in the cultures. A control culture, which was not inoculated with M1, was also incubated alongside M1 cultures to monitor any evaporation of acetic acid. Concentrations of acetic acid were measured using suppressed ion chromatography (Section 2.5.6.1).

5.7.2 Results

No decrease in acetic acid concentration (concentrations of acetic acid were noted to increase by about 0.3 mmoles/L in all cultures) and no ZnS formation were recorded in any M1 cultures containing 2 or 4 mM acetic acid after 62 days of incubation. The cells were only present in very low number ($<10^6$/ml) and most of the cells present were either elongated or sporulating. A slight increase (0.4 mmoles/L) in acetic acid concentration was also measured in the control culture.

5.8 Sulfidogenesis by isolate M1 using molecular hydrogen as electron donor and determination of the minimum pH for growth

5.8.1 Materials and methods

To test the ability of M1 to use hydrogen as an electron donor in media poised at different pH values, a liquid medium containing heterotrophic basal salts, trace elements and 5 mM K_2SO_4 was prepared. The pH of the medium was adjusted to 2.5, 3.0, 3.5 or 4.0 and 20 ml medium was poured into 20 ml universal bottles following deoxygenation (bubbling nitrogen gas through the medium for 20 minutes). The medium was heat-sterilized and $ZnSO_4$ (to 5 mM) and $FeSO_4$ (to 0.1 mM) were added from 1 M sterile stock solutions. A sterile mixed vitamin solution (Widdel & Pfenning 1981) was also added to the medium after heat sterilization.

An inoculum was prepared by growing M1 on a modified aSRB solid medium. This particular solid medium contained the defined vitamin mixture (added at 1 ml/L) in place of yeast extract, and inoculated plates were incubated in 3.5L anaerobic jars. Yeast extract was not added to the medium to exclude the possibility of M1 using this material as an electron donor for sulfidogenesis. Anaerobic atmospheres were generated using the Oxoid Anaerobic System (Section 2.2.1.4.2.). With this system, an excess of molecular hydrogen (greater than that required to reduce the oxygen in the jars) is generated, and this hydrogen can served as a potential electron donor. Colonies of M1 grew under these conditions, and around ten colonies were collected and suspended in liquid medium prepared as above. The suspension (100 µl) acted as the inoculum for the liquid cultures described above and these were again incubated in 3.5L jars containing the Oxoid Anaerobic System. Since ZnS was predicted not to precipitate out of culture solutions set at low pH (3.0 and 2.5), a 20 ml universal bottle containing 20 ml 0.3 M $CuSO_4$ was placed in the jar along with M1 cultures in an attempt to collect hydrogen sulfide gas produced during sulfidogenesis. The experiment was carried out in triplicate and changes in concentrations of sulfate and soluble zinc were monitored using ion chromatography (Section 2.5.6).

5.8.2 Results

The results from this experiment are shown in Figure 5.1. Microbial sulfidogenesis by pure cultures of M1 occurred in media initially poised at pH 4.0, 3.5 and 3.0, as evidenced by decreased concentrations of both sulfate and soluble zinc concentrations in pH 4.0 and 3.5 cultures, and decreased concentrations of sulfate in pH 3.0 cultures. When the AnaeroGen™ AN25 system, which does not produce hydrogen gas, was used in place of the Oxoid Anaerobic System, M1 was not capable of sulfidogenesis in vitamin-containing liquid aSRB medium that was not supplemented with organic substrates. Therefore, M1 was considered to have coupled molecular hydrogen oxidation to sulfate reduction in the hydrogen-containing anaerobic jars.

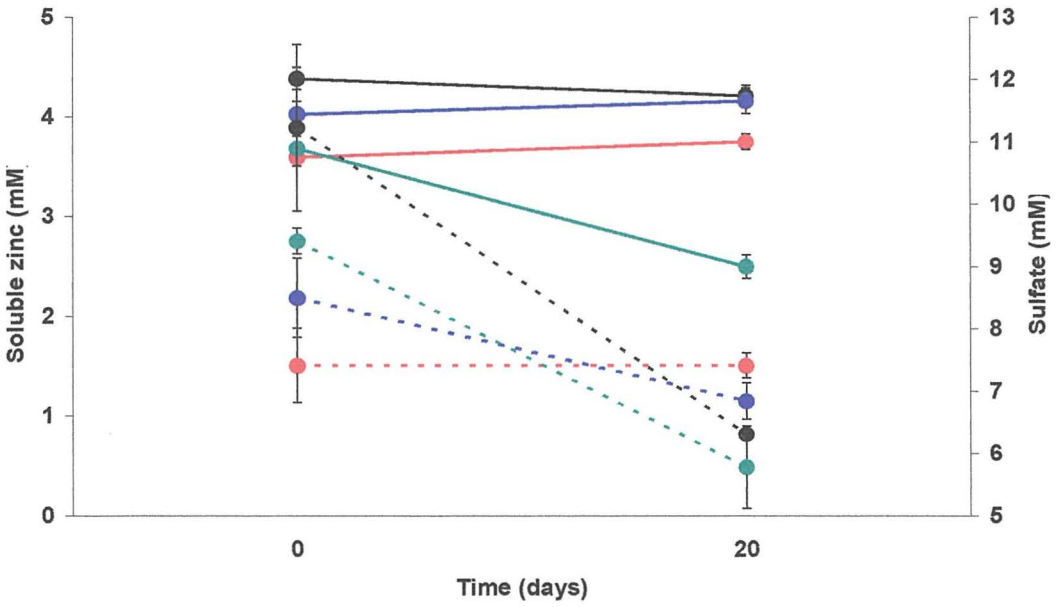


Figure 5.1. Changes in concentrations of soluble zinc and sulfate in cultures of isolate M1 poised at different initial pH values, and provided with hydrogen gas as electron donor. Key: (●) pH 2.5; (●) pH 3.0; (●) pH 3.5; (●) pH 4.0; (broken line) sulfate concentration; (solid line) soluble zinc concentration.

This experiment was performed in cultures set at various pH values (2.5, 3.0, 3.5 and 4.0) in order to determine the minimum pH for growth and sulfidogenesis by isolate M1. The results showed that sulfidogenesis occurred in cultures of M1 set at pH 3.0 and above, but not at pH 2.5. In the cultures set at pH 3.5 and 4.0, the concentrations of sulfate and soluble zinc

decreased, and pinkish-white ZnS precipitates were noted to accumulate during culture incubation. M1 cells had "normal" morphologies in pH 4.0 cultures, whereas some elongated cells, again characteristic of stress, were observed at pH 3.5. In addition, no soluble zinc removal was recorded in cultures set at pH 3.0 despite the evidence that sulfate reduction was occurring. The majority of M1 cells in pH 3.0 culture were elongated and therefore likely to have been stressed. By taking evaporation into account (by measuring changes in analyte concentrations in sterile control medium), small net increases (about 0.2 mM) in concentrations of both soluble zinc and sulfate were detected in M1 cultures set at pH 2.5.

5.9 Discussion

One of the restrictions in using sulfate reducing bacteria (SRB) for recovering dissolved metals from acidic mine waters and other industrial effluents is that SRB are, in general, highly sensitive to even moderate acidity. To get around this problem, neutrophilic SRB are usually grown in separate "off-line" bioreactors, where they are isolated from direct contact with acidic waste waters. A disadvantage of these off-line systems is that they require at least two separate reactors; one to produce sulfide and the other to precipitate dissolved metals, thereby adding to construction and operating costs. Biogenic sulfide produced must be stripped from the sulfidogenic reactor(s) by bubbling gas mixture (often H_2 and CO_2 mixture) and transferred to the metal precipitation reactor (Buisman et al. 1991). A full-scale sulfidogenic system utilizing neutrophilic SRB has been operating for a number of years at the Budelco zinc refinery in the Netherlands (Barnes et al. 1994) where zinc is being recovered from contaminated ground water, and the ZnS produced is smelted on site. Other bench- and pilot-scale systems have also been described (e.g. Tabak & Govind 2003; Tabak et al. 2003; Pott & Mattiasson 2004). In addition to off-line systems, laboratory-scale fluidized-bed reactors, in which the biomass is retained on an inert carrier material in the reactor, have been used to treat AMD in a bench-scale system (Kaksonen et al. 2003a). Unlike off-line bioreactors, acidic metal-containing water was pumped directly into the fluidized-bed reactors but the acidity was effectively

neutralized immediately within the reactor by the excess of alkalinity generated by the sulfidogenic bacteria (Kaksonen et al. 2003b).

The use of acid-tolerant or acidophilic sulfate reducing bacteria (aSRB), which could generate sulfide at low pH, would have several advantages in metal removal/recovery systems. By combining the sulfidogenic reactor and metal precipitating reactor in an "on-line" system, the costly sulfide transfer step would be eliminated. In addition, by poising on-line sulfidogenic bioreactors at different pH values, it would, in theory, be possible to recover heavy metals selectively, based on the different solubility products of their sulfides (Johnson et al. 2004).

Sen and Johnson (1999) successfully isolated aSRB from sediments collected from Montserrat (West Indies) and the Mynydd Parys copper mine (U.K.), using a specifically-designed solid medium. The nearest known relative of two closely-related aSRB (isolate P1 from Mynydd Parys and isolate M1 from Montserrat) was the Gram-positive neutrophile, *D. orientis,* with which the acidophilic isolates had 94% 16S rRNA gene sequence similarity. *D. orientis* is an "incomplete oxidizer" of organic substrates, such as lactate, and can grow autotrophically using hydrogen as electron donor (Lee et al. 1994).

The aSRB liquid medium developed by Sen (2001) was modified by replacing $FeSO_4$ with $ZnSO_4$ in this study. The rationale in adding zinc sulfate was that ZnS precipitates in moderately acidic liquors (*ca.* pH $\geq$3.5) whereas FeS precipitates at higher pH values (*ca.* pH >6). Liquid media used to cultivate neutrophilic SRB frequently contain ferrous sulfate and sulfide generated reacts with Fe^{2+} to form a black FeS precipitate. However, the solubility product of FeS ($4 \cdot 10^{-19}(mol/L)^{vA+vB}$; Vogel 1979) is significantly greater than that of ZnS ($1 \cdot 10^{-23}(mol/L)^{vA+vB}$; Vogel 1979) and consequently FeS forms at higher pH than ZnS. In the present study, and in agreement with data obtained by Sen (2001), FeS precipitates formed when the pH was *ca.* 6 and above. In contrast, ZnS precipitated in pH 4 liquors, which was the routine pH at which M1 was grown in liquid media.

This has two important consequences when cultivating SRB at low pH. Firstly, biogenic sulfide is removed, thereby eliminating its potential toxicity. Although SRB produce sulfide, they, as other microorganisms, are readily inhibited by small concentrations of H_2S (Oude Elferink et al. 1994). Secondly, ZnS formation helps maintaining the acidic pH of aSRB cultures. As shown in Equation 5.2, sulfidogenesis is an alkali-generating reaction and complete oxidation of glycerol coupled to sulfate reduction requires 3.5 H^+/glycerol oxidized,

$$4C_3H_8O_3 + 7SO_4^{2-} + 14H^+ \rightarrow 12CO_2 + 7H_2S + 16H_2O \quad (5.2)$$

whereas incomplete oxidation of glycerol to acetic acid consumes 1.5 protons/glycerol oxidized (Equation 5.3).

$$4C_3H_8O_3 + 3SO_4^{2-} + 6H^+ \rightarrow 4C_2H_4O_2 + 3H_2S + 4CO_2 + 8H_2O \quad (5.3)$$

Hydrogen sulfide is a weak acid, and has two dissociation constants:

$$H_2S \leftrightarrow HS^- \leftrightarrow S^{2-} \quad (pKa_1 = 6.9, pKa_2 = 14.2) \quad (5.4).$$

From Equation 5.4, it can be seen that, in acidic liquors, the dominant species would be undissociated hydrogen sulfide (H_2S). Reaction of soluble zinc with hydrogen sulfide releases two protons, as shown in Equation 5.5.

$$H_2S + Zn^{2+} \rightarrow ZnS + 2H^+ \quad (5.5)$$

Proton release due to zinc sulfide precipitation balances that consumed as a result of sulfidogenesis, independent of whether glycerol is completely (Equation 5.6) or incompletely (Equation 5.1) oxidized.

$$4C_3H_8O_3 + 3SO_4^{2-} + 3Zn^{2+} \rightarrow 4C_2H_4O_2 + 3ZnS + 4CO_2 + 8H_2O \quad (5.6)$$

The aim of establishing sulfidogenic bioreactor, which contains aSRB M1, is to carry out on-line treatment of acidic, metal-containing solutions (Johnson et al. 2004). However, unlike off-line systems, metal-laden solutions would be pumped into the sulfidogenic bioreactors, and therefore the aSRB would need to be able to generate sulfide in the presence of dissolved heavy metals. Some SRB are reported to be highly sensitive to some dissolved metals. For example, small concentrations (30 μM) of Ni^{2+} and Zn^{2+} were found to be toxic to *Desulfovibrio desulfuricans* (Poulson et al. 1997). In this study, tolerance of M1 to two heavy metals (Cu^{2+} and Fe^{2+}) that occur at

elevated concentrations in acidic mine water draining the abandoned Mynydd Parys copper mine was tested. This site has been targeted as the first pilot-scale demonstration of metal recovery using an acidophilic sulfidogenic system (Johnson et al. 2004). A third heavy metal, zinc, is also present in relatively high concentrations in Mynydd Parys AMD. However, the concentration of zinc (1.3 mM) is well below that routinely used in liquid media for cultivating isolate M1 (5 mM) and it was therefore assumed that zinc toxicity would not be an issue (for sulfidogenesis) at this mine site. Isolate M1 was not inhibited by ferrous iron at the concentration tested (12 mM) which is typical of what found in water draining Mynydd Parys. In contrast, copper (as low as 0.2 mM) was highly inhibitory to M1, suggesting that sensitivity to this metal might prove problematic. However, this should not be a significant problem in an active on-line sulfidogenic system. The solubility product of CuS is very small ($1 \cdot 10^{-44}(mol/L)^{vA+vB}$) and CuS precipitates in extremely acidic liquors (as low as pH 0). Hence if copper-containing AMD is pumped into a sulfidogenic bioreactor, CuS will precipitate instantly, providing that there is some soluble sulfide present in the reactor. Even though the mine water contains a mixture of heavy metals, the extremely small solubility product of CuS dictates that it will precipitate ahead of ZnS and FeS, even in circum-neutral pH liquors.

Utilization of glycerol by isolate M1 was assessed, specifically to determine whether this substrate was completely oxidized to CO_2 or incompletely oxidized to acetic acid. The results showed that M1 produced equimolar amounts of acetic acid from glycerol oxidation, suggesting that it is an incomplete oxidizer. However, some SRB that are "complete oxidizers" excrete acetic acid before subsequently oxidizing it to CO_2. This is due to the faster rate of acetyl-CoA formation than its terminal oxidation process. The excess acetyl-CoA is converted to acetate (Widdel & Hansen 1992) and excreted. In order to confirm that M1 is an incomplete-oxidizer (i.e. unable to oxidize acetic acid), its ability to utilize acetic acid was tested. Before such an experiment could be carried out, it was necessary to determine the lowest concentration at which acetic acid is toxic to isolate. M1 was found to be able to tolerate up to 5 mM acetic acid, while 6 mM completely inhibited

sulfidogenesis. However, cells that grew in cultures containing (initially) 5 mM acetic acid were noticeably stressed. As concentrations of <5 mM acetic acid were not toxic to M1, 2 and 4 mM acetic acid was included in liquid media (in place of glycerol) to examine whether this aSRB might oxidize this organic acid. The negative results obtained confirmed that M1 cannot use acetic acid. The inability of M1 to carry out complete oxidation of glycerol is problematic since acetic acid will accumulate in these cultures. This might explain why sub-culturing pure cultures of M1 was sometimes unsuccessful. As oxidation of 5 mmoles/L glycerol leads to production of 5 mmoles/L acetic acid, which caused notable stress to active M1 cultures, this might have caused a rapid mortality or resting of cultures.

The ability of M1 to use hydrogen as electron donor was tested as some SRB, including its closest characterized relative *D. orientis,* can grow on H_2 using CO_2 as a carbon source (Lee et al. 1994; Lee & Sublette 1994). Yeast extract was omitted from the medium to eliminate the possibility of M1 growing on yeast extract rather than molecular hydrogen. The results showed M1 is able to use molecular hydrogen as electron donor and to couple hydrogen oxidation to sulfate reduction (Equation 5.7).

$$4H_2 + SO_4^{2-} + 2H^+ \rightarrow 4H_2O + H_2S \qquad (5.7)$$

In these cultures, isolate M1 was seemingly growing chemolithotrophically. The only organic carbon present was that provided in the vitamin mixture, which was very small (0.45 mg C /L) and therefore unlikely to have been the carbon source used by isolate M1 in this case.

As M1 is capable of using hydrogen gas to fuel sulfidogenesis, it would be possible to use this acidophilic SRB in a reactor similar to the "second-generation" system that has been installed at the Budelco zinc refinery in the Netherlands. The first system at the site used ethanol as electron donor and carbon source. The "second-generation" system, however, uses a mixture of H_2/CO_2 derived from natural gas, to fuel sulfidogenesis by the neutrophilic SRB (Boonstra J. et al. 1999). The use of H_2/CO_2 mixturesfor sulfate reduction has been suggested to be more cost-effective than using organic electron donors (Tabak & Govind 2003).

The discovery that isolate M1 can use hydrogen as an electron donor allowed determination of the minimum pH for growth and sulfidogenesis by this aSRB. Glycerol was considered to be an inappropriate substrate with which to test low pH tolerance by isolate M1. As noted previously, M1 oxidizes glycerol to acetic acid, which accumulates in the culture medium. As pH declines, the undissociated (lipophilic) species becomes increasingly dominant, relative to the dissociated (acetate) species. As it is the former that is biotoxic, it would be anticipated that acetic acid toxicity becomes more acute as the pH falls. The results showed that the lowest pH, at which M1 could generate sulfide, using hydrogen as electron donor, was 3.0. However, although sulfidogenesis did occur in M1 cultures set at pH 3.0, the majority of cells were elongated (i.e. stressed); some cells also showed stress symptoms in pH 3.5 cultures. Sulfide toxicity may have contributed to the poorer growth of isolate M1 at very low pH, as there was no obvious formation of ZnS at pH 3.0, and the efficiency of Zn^{2+} removal at pH 3.5 was less than that recorded in pH 4.0 cultures. Isolate M1 was routinely grown on aSRB solid medium at pH 3.5, where hydrogen sulfide would be anticipated to freely diffuse away from growing bacterial colonies, and, in this situation, elongated cells were not observed. The presence of stressed cells in the pH 3.5 liquid cultures was therefore considered to be caused by accumulation of soluble hydrogen sulfide, rather than by low pH. When the samples were taken from pH 3.5 liquid cultures, a distinct smell of hydrogen sulfide was noticed. In theory, the sulfide toxicity problem at low pH could be avoided by incorporating a heavy metal that forms a more insoluble sulfide than ZnS. Copper is obviously not appropriate in view of its toxicity to M1, and other candidate metals, such as cadmium and silver, might also be toxic to the SRB, though this was not tested. It was encouraging that M1 could grow in media poised as low as pH 3.0. It is conceivable that regular subculturing in pH 3 medium could result in a better adaptation to extreme acidity, which could be reflected in faster growth rates etc..

In conclusion, aSRB M1 was shown to be an incomplete oxidizer of glycerol, producing equimolar amounts of acetic acid. Isolate M1 was able to tolerate acetic acid at up to 5 mM, but was not able to use this organic acid as a

substrate. M1 was also shown to be capable of growth using hydrogen as electron donor. The minimum pH at which M1 can carry out sulfidogenesis using hydrogen as electron donor is at 3.0.

Chapter 6 Sulfidogenesis at low pH by a defined bacterial consortium

6.1 Introduction

Hydrogen sulfide produced by SRB may be used to treat acidic waters containing heavy metals by precipitating metals as insoluble sulfides (Section 1.5.3.2). Since all SRB currently used in such systems are neutrophilic, sulfide generation and metal precipitation processes are usually carried out in separate tanks, as described previously (Poulson et al. 1997). Experiments carried out with pure cultures of the acidophilic *Desulfosporosinus*-like isolate M1 (described in Chapter 5) showed that, although this sulfidogen could use hydrogen as electron donor for sulfate reduction at low pH (down to pH 3.0), growth in glycerol-containing media was inconsistent. This was thought to be due to the accumulation of acetic acid, as M1 was shown to be an "incomplete oxidizer" of organic substrates. Previous to the current studies, sulfidogenesis and alkalinity production by an undefined mixed population of microorganisms had been demonstrated in a bioreactor maintained at pH 2.0 – 4.0 (Sen 2001). This culture was established by enriching a sediment sample obtained from Mynydd Parys, using glycerol as the carbon/energy source. In addition, sulfidogenesis could be readily demonstrated at low pH on overlay solid media, in which heterotrophic bacteria that are thought to remove inhibitory small molecular weight organic compounds are incorporated in the gel underlayer.

In this Chapter, the establishment and operation of a sulfidogenic bioreactor run at low pH, and a simple (two member) bacterial consortium used in the system, are described.

6.2 Composition of the acidophilic sulfidogenic consortium

Two bacteria, coded M1 and PFBC, were used in this study, as described below.

Isolate M1 is an acidophilic/acid-tolerant SRB (aSRB), most closely related to *Desulfosporosinus orientis,* and has been described in detail in Chapter 5. It can use glycerol or molecular hydrogen as electron donors. The lowest pH at

which sulfidogenesis can be carried out by M1 (using molecular hydrogen as electron donor) is 3.0. It oxidizes glycerol incompletely to acetic acid, and is incapable of using acetic acid as an electron donor.

Isolate PFBC is an acidophilic heterotrophic bacterium that had been isolated from a supposedly pure culture of *Desulfosporosinus*-isolate PFB. Colonies of PFBC were found to be growing adjacent to isolate PFB on anaerobically-incubated aSRB solid medium, supplemented with mannitol (Kimura & Johnson 2004). From its physiological characteristics (described below), PFBC was deduced to be identical to "*Acidocella aromatica*" isolate WJB3 (Hallberg et al. 1999). However, RFLP analysis (using the restriction enzyme *Msp*I) produced an RFLP pattern that was distinct from WJB3, though analysis of 16S rRNA gene sequences (partial products; 656 bp) showed that the two bacteria share 99% sequence similarity. "*Ac. aromatica*" has been described as an obligately aerobic acidophile that is able of catabolizing a variety of aromatic compounds, as well as small molecular weight organic acids, such as acetic acid (Gemmell & Knowles 2000; Hallberg et al. 1999). Like "*Ac. aromatica*", PFBC was found to grow aerobically on fructose, acetic acid and phenol, and was subsequently used as the under-layer acidophile in aSRB solid medium (Section 2.2.1.2.2.2).

6.3 Utilization of glycerol by a mixed culture of M1 and PFBC

6.3.1 Materials and methods

An aSRB liquid medium (20 ml in foam bung-stoppered universal bottles) containing 5 mM glycerol and 5 mM $ZnSO_4$ (Section 2.2.1.1.2) was inoculated with 1 ml of M1 culture (grown anaerobically in the same medium) and 1 ml of PFBC culture (grown aerobically in fructose liquid medium; Section 2.2.1.1.1). The experiment was carried out in triplicate and cultures were incubated under anaerobic conditions in a 2.5 L anaerobic jar using the Anaero*Gen*™ AN25 system. Concentrations of sulfate and acetic acid were measured at the start of the experiment and again 36 days later, using suppressed ion chromatography (Section 2.5.6.1) and glycerol was measured by ion chromatography using a Carbo Pac™ Pa10 column and an ED 40 amperometric detector (Section 2.5.6.2). The concentration of soluble

zinc in culture solutions was determined using atomic absorption spectrometry (Section 2.5.4).

6.3.2 Results

The concentrations of acetic acid, glycerol, sulfate and soluble zinc at the start and the end of the experiment are shown in Table 6.1.

Table 6.1. Changes in concentrations of glycerol, soluble zinc, sulfate and acetic acid during growth of the defined mixed culture of M1 and PFBC in a liquid medium containing 5 mM glycerol and 5 mM zinc. Data shown are mean values (n=3) and standard deviations at the start of the experiment and at day 36.

Time (days)	Glycerol (mM)	Soluble zinc (mM)	Sulfate (mM)	Acetic acid (mM)
0	4.4 ±1.0	6.7 ±1.2	14.3 ±2.2	0.5 ±0.1
36	0.02 ±0.02	0.05 ±0.03	9.8 ±0.6	0.6 ±0.2

The results show that oxidation of glycerol was coupled to sulfate reduction. The concentration of soluble zinc had decreased by day 36 as zinc reacted with hydrogen sulfide to form insoluble ZnS. In contrast to pure cultures of M1 grown on glycerol, where equimolar amount of acetic acid were produced (Section 5.5), there was virtually no accumulation of acetic acid (an 0.1 mmoles/L increase, corresponding to 4.4 mmoles/L glycerol oxidized) in the mixed culture. Overall, 4.4 mmoles/L glycerol was oxidized, 4.5 mmoles sulfate/L was reduced and 6.6 mmoles zinc/L was precipitated in these liquid cultures.

6.4 Bioreactor cultures of M1 and PFBC

6.4.1 Materials and methods

6.4.1.1 Culture set up

A 2L bioreactor medium (Figure 6.1), containing vitamin solution (1 ml/L), 0.1 mM $FeSO_4$, 5 mM K_2SO_4, 5 mM glycerol and 5 mM $ZnSO_4$ was prepared as described in Section 2.2.1.3, and inoculated with 60 ml of a mixed M1/PFBC culture grown in aSRB liquid medium containing 5 mM glycerol and 5 mM $ZnSO_4$. The pH of the bioreactor culture was maintained between 3.9 and 4.1

by automated addition of 0.1 M NaOH or 0.1 M H_2SO_4. One liter of culture was replaced with 1L of medium containing vitamin solution (2X), ferrous iron (0.2 mM), glycerol (10 mM) and $ZnSO_4$ (10 mM) each time a new batch experiment was set up. The culture liquor was sampled every day and 3 ml was filtered (through 0.2 µm nitrocellulose membranes) into two 1.5 ml tubes. The samples were filtered to remove small particulate matter, including cells and zinc sulfide, and were stored at -20°C until analyzed.

Figure 6.1. The mixed culture sulfidogenic bioreactor: (a) hydrogen sulfide trap (copper acetate); (b) bioreactor; (c) pH and temperature control system.

6.4.1.2 Total cell count by DAPI staining, and FISH analysis

For DAPI staining and FISH analysis, 2 ml culture aliquots were centrifuged and fixed in 4% PFA, as described in Section 2.3.2.1. Fixed samples were stored at -20°C until analyzed by DAPI staining (Section 2.3.2.2) and FISH analysis (Section 2.6.8). Three probes, fluorescein-labeled EUB338, AcaromCy3 and aSRBCy3, were used in FISH analyses, to target total active eubacteria, PFBC and M1, respectively. AcaromCy3 was designed to specifically target "*Ac. aromatica*" (including isolates WJB-3 and PFBC); its sequence is 5'GTCGCTACGTATCAAAAGC3', and its ODP nomenclature (Alm et al. 1996) is S-S-Acarm-0633-a-A-19. This probe required unlabeled helper oligonucleotides targeting either side of the target sequence. These

were AcaromCy3- (5'AGTCCCCAGGTTGAGCCC3') and AcaromCy3+ (5'CAACCCTCTTCCTTACTCTA3').

6.4.1.3 Chemical analyses

Concentrations of sulfate, acetic acid, glycerol and zinc were measured as described in Section 6.3.1.

6.4.1.4 Scanning electron microscope (SEM)

Cells in the bioreactor culture were filtered onto polycarbonate membranes (0.2 µm pore size) and fixed with 4% glutaraldehyde, as described in Section 2.4.4.1. The fixed sample was prepared for SEM analysis (Section 2.4.4.2) and viewed using a HITACHI S-520 scanning electron microscope (Section 2.4.4.3).

6.4.1.5 Energy dispersive analysis of X-rays (EDAX)

The precipitate formed inside the bioreactor was collected and prepared for EDAX analysis as described in Section 2.5.7. The sample was analyzed using an Oxford Instruments Link Isis III EDAX System (Oxford Instrument, England).

6.4.2 Results

Two batch experiments were carried out in the bioreactor at pH 4.0. Representative data from one of these runs (experiment 4.0a) are shown in Figure 6.2.

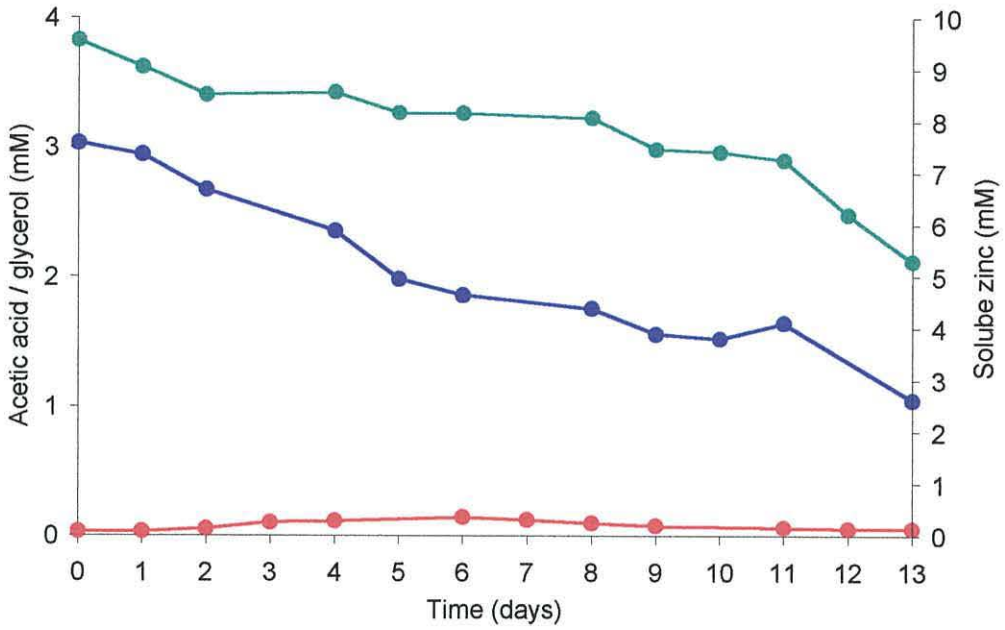


Figure 6.2. Relationship between glycerol utilization and precipitation of zinc in a mixed culture sulfidogenic bioreactor maintained at pH 4.0 (±0.1). Key: (●) acetic acid; (●) glycerol; (●) soluble zinc.

During the 14 day incubation period, 2.0 mmoles/L glycerol was oxidized and 4.2 mmoles/L soluble zinc was removed from solution. Changes in glycerol and soluble zinc concentrations were closely coupled. As shown in Figure 6.2, there were three distinct phases (days 0-6, 6-11 and 11-13) during incubation. During the first phase, the concentration of acetic acid increased while glycerol was being oxidized. This was followed by a period during which glycerol concentration remained almost unchanged while that of acetic acid declined. In the final phase of glycerol oxidation, no increase in acetic acid concentration was noted. Sulfate reduction was evaluated by measuring sulfate concentrations in the culture and correcting for the sulfuric acid that was added to the bioreactor to maintain pH control. However, the data were erratic, due to errors that were not identified, and are not shown in Figure 6.2.

SEM images from the bioreactor culture are shown in Figures 6.3 and 6.4. Figure 6.3 shows both M1 (relatively long rods) and PFBC (the smaller cells). The small (*ca.* 0.1 µm diameter) circular objects were assumed to be zinc sulfide precipitates. In Figure 6.4, cells are appeared to be held together by exopolymeric substances, which are shown as film-like materials in the

image, and inorganic materials, presumably ZnS, can be seen to precipitate on the bacterial surfaces. The mean sizes of M1 and PFBC were determined by measuring the lengths and widths of nine cells of M1 and nine of PFBC. From these measurements, mean volumes of M1 and PFBC cells were calculated ($r^2 \cdot \pi \cdot h$), and the results are listed in Table 6.2.

Table 6.2. The dimensions of isolates M1 and PFBC obtained from scanning electron micrographs.

Bacteria	Mean values	
M1	Length	3.25 μm
	Width	0.74 μm
	Volume	1.40 μm^3
PFBC	Length	0.98 μm
	Width	0.82 μm
	Volume	0.13 μm^3

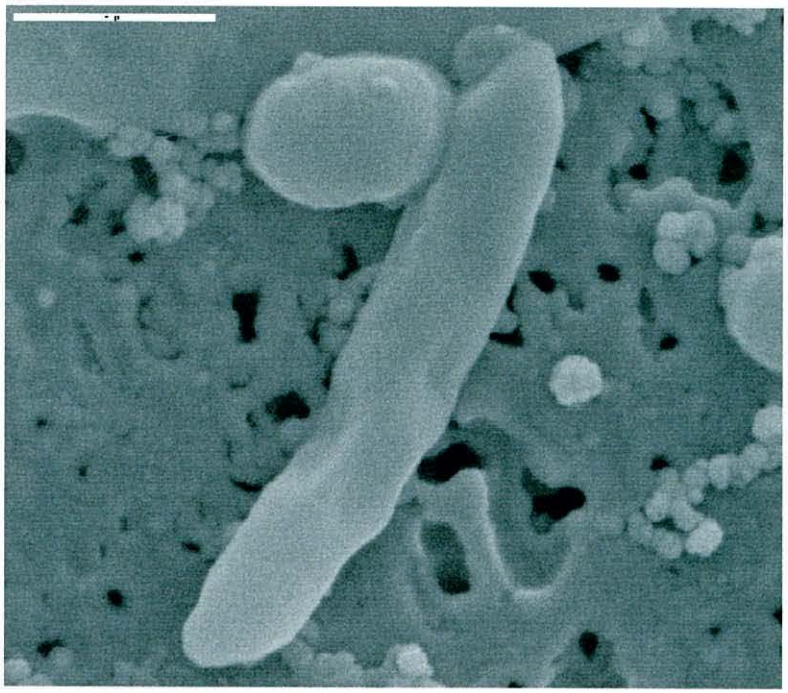


Figure 6.3. Scanning electron micrograph of bacteria in the bioreactor culture. The long rod is M1 and the smaller rodococcus is PFBC. The bar represents 1 µm.

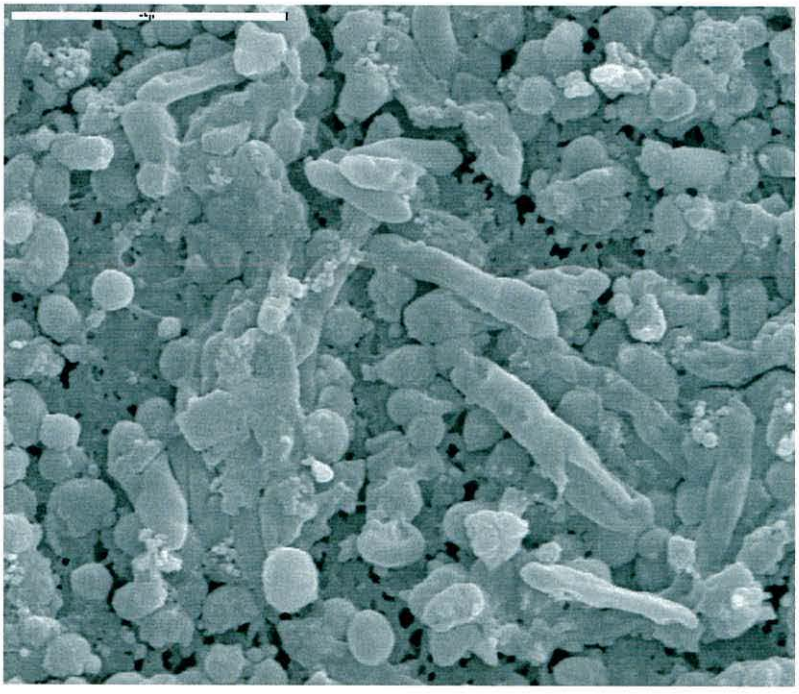


Figure 6.4. Scanning electron micrograph of bacteria in the bioreactor culture, showing microbial cells held together with exopolymeric substances. The bar represents 5 µm.

Data from FISH and DAPI analysis are shown in Figure 6.5. FISH data were obtained by counting the number of cells stained with probes specific to M1 or PFBC, relative to EUB338-stained cells. The results are shown as a bar chart in Figure 6.5. As the sizes of M1 and PFBC cells differ considerably, the relative biovolumes of M1 and PFBC in the culture were calculated. The mean volume of M1 was 10.8 times that of PFBC, hence the cell numbers of M1 was multiplied by 10.8 to obtain relative biovolume values. Throughout the experiment, more than 98% of cells stained by DAPI were also stained by EUB338, suggesting that almost all of the cells present were active. Figure 6.6 shows the cells stained with the AcaromCy3 probe, which targets PFBC, and Figure 6.7 shows the cells stained with the aSRBCy3 probe, which targets M1. These results showed that the relative biovolume of M1 increased gradually (to 70% of the total) during days 0-6 while glycerol was being oxidized, and that the largest increase in the total cell number occurred during this phase. After day 6, PFBC increased in relative abundance while the concentration of glycerol remained unchanged and the acetic acid concentration decreased. The total cell numbers decreased dramatically on day 8 and remained low until the end of incubation.

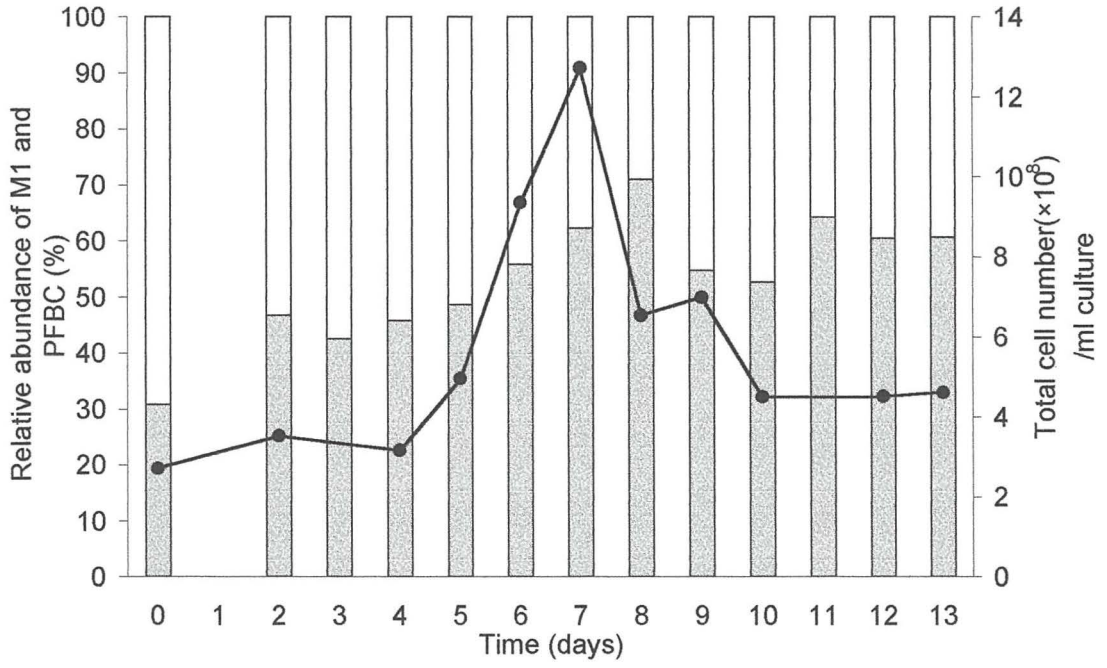


Figure 6.5. Microbial population dynamics in the pH 4.0a bioreactor culture. Relative abundance (from biovolumes) of PFBC (unshaded bars) and M1 (shaded bars) as determined by FISH analysis. The line graph indicates total bacterial numbers (the cell numbers are indicated as values×10^8/ml culture) determined by DAPI staining.

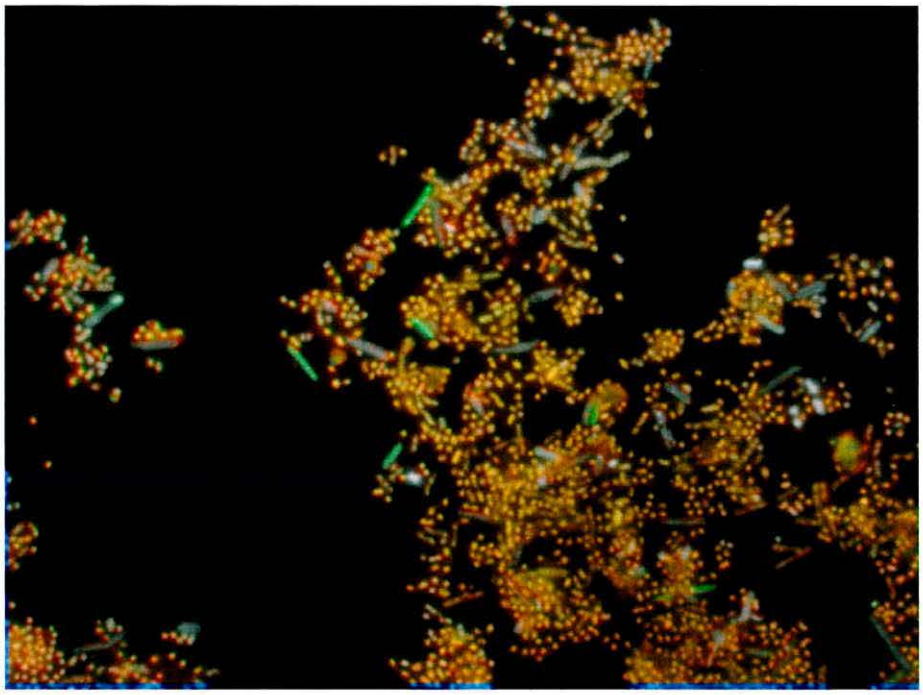


Figure 6.6. Image of bioreactor culture obtained from FISH analysis. The cells stained orange were targeted by the Cy3-labeled AcaromCy3 probe, and green cells were only stained with the fluorescein-labeled EUB338 probe. Gray cells were stained with DAPI only.

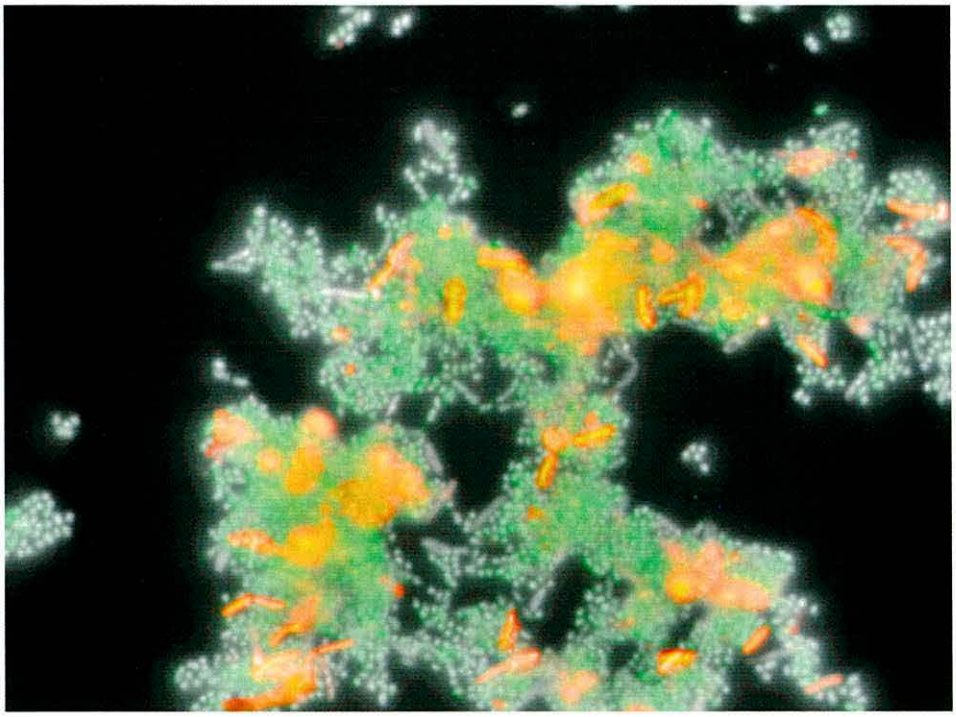


Figure 6.7. Image of bioreactor culture obtained from FISH analysis. The cells stained orange were targeted by the Cy3-labeled aSRB probe, and green cells were only stained with the fluorescein-labeled EUB338 probe. Gray cells were stained with DAPI only.

The pinkish-white precipitate that formed within the bioreactor culture was analyzed by EDAX and found to be mainly composed of ZnS, as the large peaks were identified to correspond to zinc (8.6 keV, 9.5 keV and 1.0 keV) and sulfur (2.4 keV). Although 0.1 mM iron was added to the bioreactor culture, there was no iron sulfide in the precipitate since no peaks corresponding to iron were observed (at 6.4 and 7.1 keV).

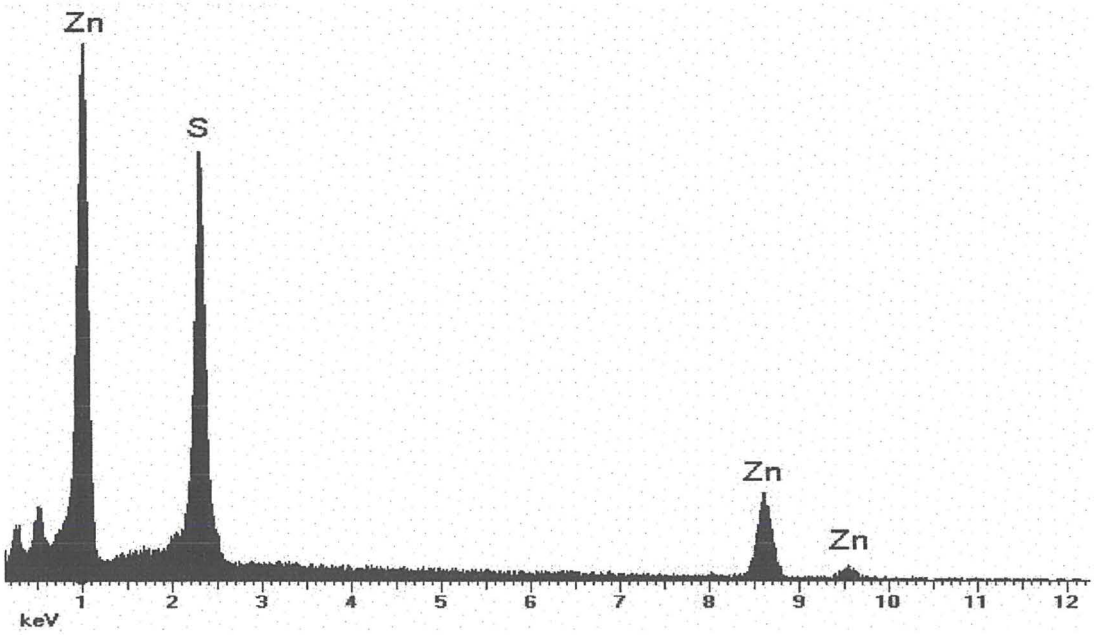


Figure 6.8. Composition of precipitates of the bioreactor culture determined by energy dispersive analysis of X-rays (EDAX). The Kα1 (8.6 keV), La1 (9.5 keV) and La2 (1.0 keV) peaks of zinc along with the Kα1 peak (2.4 keV) of sulfur have been labeled.

The pH 4.0 bioreactor experiment was repeated (experiment 4.0b), and results are shown in Figure 6.9. During the eight day incubation period, 3.7 mmoles/L, glycerol was oxidized, 5.0 mmoles/L sulfate reduced and 7.3 mmoles/L zinc was precipitated. As was found in the previous experiment (4.0a), changes in glycerol and soluble zinc concentrations were synchronized. Concentrations of soluble zinc and glycerol started to decrease on day 1 and continued until glycerol concentration reached 0.5 mM by day 5. The amount of sulfate reduced increased slowly from days 0-3, but then the rate of reduction increased dramatically before slowing down

from day 5. The acetic acid concentration remained very low throughout the incubation period; the maximum concentration (0.12 mM) occurred on day 4.

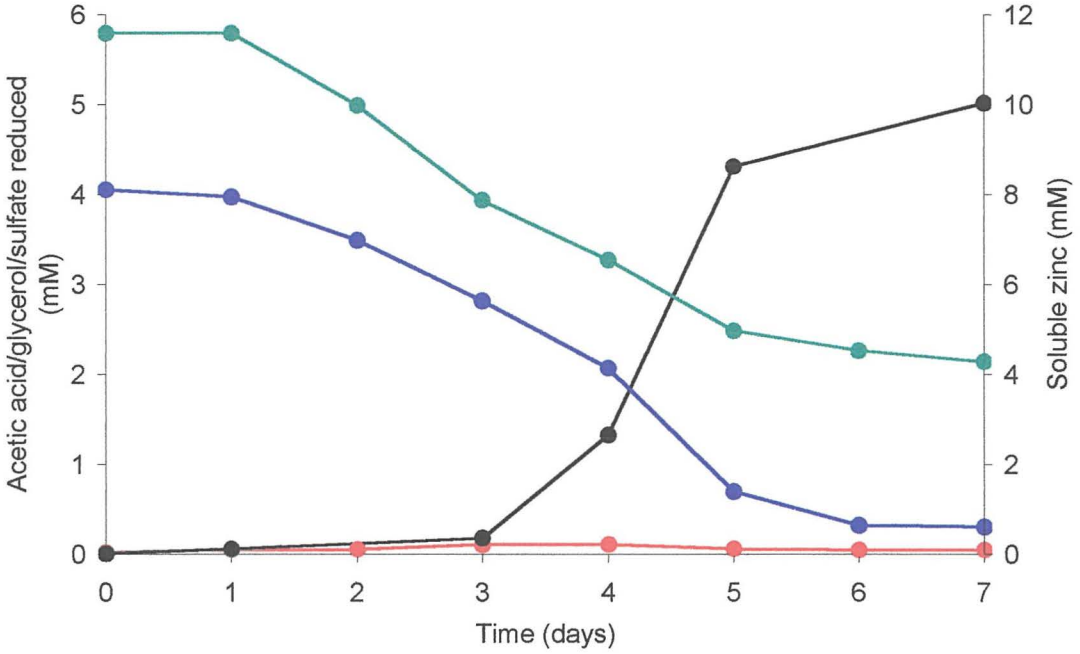


Figure 6.9. Relationships between glycerol utilization and precipitation of zinc in a mixed culture bioreactor (4.0b) maintained at pH 4.0 (±0.1). Key: (●) acetic acid; (●) glycerol; (●) sulfate reduced; (●) soluble zinc.

6.5 Effect of pH variation on the performance of the acidophilic sulfidogenic consortium

The acidophilic sulfidogenic culture was maintained at two other pH values to determine the effect this had on its overall performance.

6.5.1 Materials and methods

The bioreactor culture was set and maintained at either pH 3.8 or pH 4.2 by automated addition 0.1 M NaOH or 0.1 M H_2SO_4, using the same protocol as described in Section 6.3.1. Two batch culture experiments were carried out at pH 3.8 and three batch culture experiments were carried out at pH 4.2.

6.5.2 Results

The data obtained from the two batch experiments set at pH 3.8 showed very similar trends; Figure 6.10 shows the results obtained from one of these experiments. During the ten day incubation period, 1.9 mmoles/L glycerol

was oxidized, 1.9 mmoles/L sulfate was reduced and 3.7 mmoles/L zinc was precipitated. Again, whilst concentrations of glycerol changed throughout the incubation period, sulfate reduction progressed relatively slowly up to day 5. Concentrations of soluble zinc paralleled those of sulfate. The maximum acetic acid concentration measured (0.4 mM) was significantly greater than that found when the bioreactor was maintained at pH 4.0 (Section 6.4). As shown in Figure 6.10, acetic acid concentrations increased during days 0-3, but then stabilized before declining from day 6.

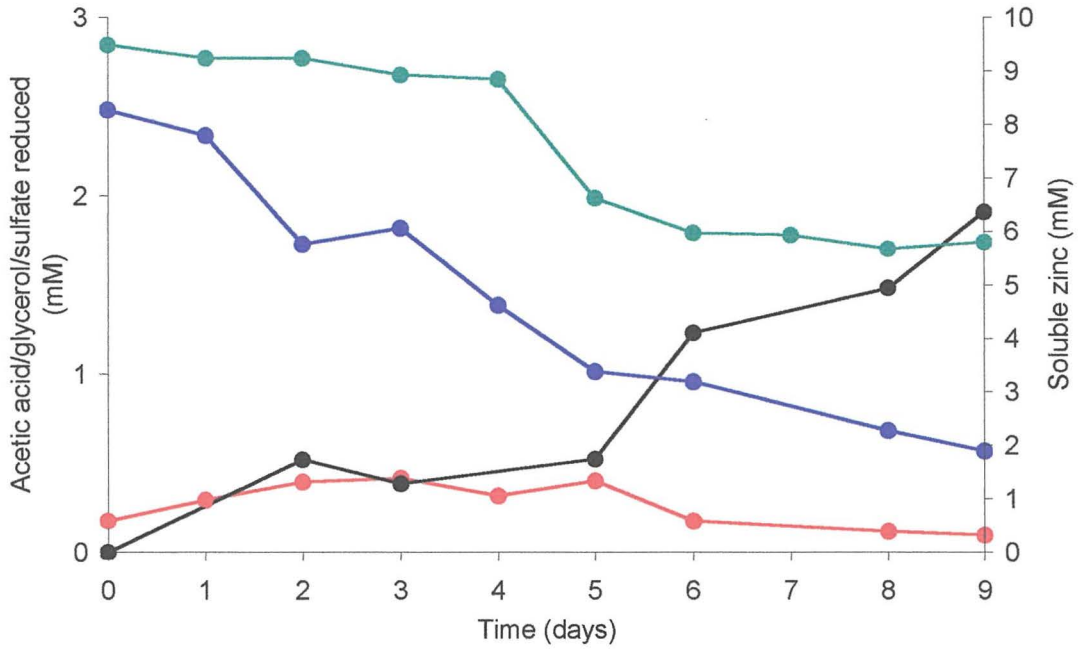


Figure 6.10. Relationships between glycerol utilization and precipitation of zinc in a mixed culture bioreactor maintained at pH 3.8 (±0.1). Key: (●) acetic acid; (●) glycerol; (●) sulfate reduced; (●) soluble zinc.

The bioreactor culture pH was then set and maintained at pH 4.2; results obtained are shown in Figure 6.11. In this experiment, the initial amounts of glycerol and soluble zinc were greater than in previous experiments, in order to examine what effect elevated concentrations may have on levels of acetic acid. During the ten day incubation period, 8.8 mmoles/L glycerol was oxidized, 6.1 mmoles/L sulfate was reduced and 9.6 mmoles/L soluble zinc was removed from the solution. Changes in glycerol and zinc concentrations were tightly coupled throughout incubation. The concentration of acetic acid increased gradually to day 7 (to a maximum of 1.4 mM) after which it

declined quite quickly. Although the amount of sulfate reduced was monitored, the results are not shown in Figure 6.11 as the data were erratic, due to unknown errors.

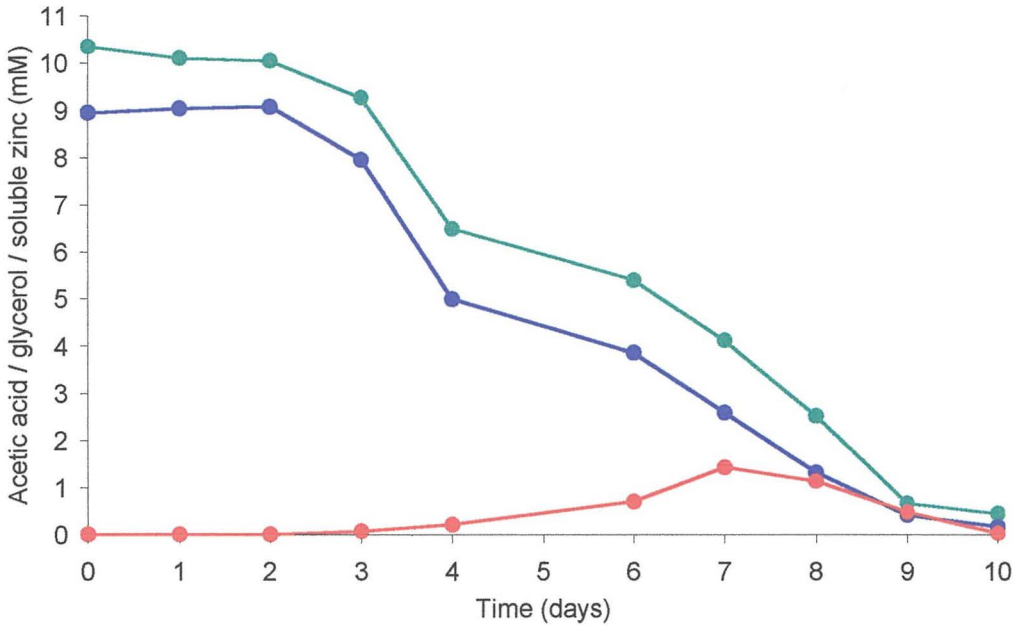


Figure 6.11. Relationships between glycerol utilization and precipitation of zinc in a mixed culture bioreactor maintained at pH 4.2 (±0.1). Key: (●) acetic acid; (●) glycerol; (●) soluble zinc.

6.6 Sulfidogenesis by the M1/PFBC consortium using acetic acid as electron donor: preliminary experiment

In contrast to data obtained from the pure cultures of the sulfate-reducer, M1 (Chapter 5), there was virtually no accumulation of acetic acid in the mixed cultures of M1 and PFBC when grown on glycerol. It was decided, therefore, to test the ability of the mixed culture to utilize acetic acid as sole carbon source to fuel sulfidogenesis.

6.6.1 Materials and methods

Aliquots of the M1/PFBC consortium (1 ml) were used to inoculate modified aSRB medium (20 ml in universal bottle) containing either 2 or 4 mM acetic acid and 10 mM $ZnSO_4$. The inocula were prepared by centrifuged and resuspending cells grown in glycerol medium in order to minimize carry over of any residual glycerol. The experiment was carried out in a triplicate and

cultures were incubated under anaerobic condition in a 2.5 L Anaerobic jar using the Anaero*Gen*™ AN25 system (Section 2.2.1.4.2). Cell-free control solutions were also incubated alongside inoculated cultures. Analysis of cultures was carried out as described previously (Section 6.4.1.3).

6.6.2 Results

The results obtained from the mixed culture containing either 2 or 4 mM acetic acid are shown in Table 6.3.

Table 6.3. Changes in concentrations of acetic acid, soluble zinc and sulfate during growth of the defined mixed culture of M1 and PFBC in a liquid medium containing 2 or 4 mM acetic acid and 10 mM zinc. Data shown are mean values (n=3) and standard deviations at the start of the experiment and at day 50.

Time (days)	Acetic acid (mM)	Soluble zinc (mM)	Sulfate (mM)
Culture containing 2 mM acetic acid			
0	2.1 ±0.2	7.5 ±1.2	14.4 ±0.6
50	1.5 ±0.7	5.7 ±1.4	13.3 ±0.7
Culture containing 4 mM acetic acid			
0	3.6 ±0.8	9.6 ±0.6	14.0 ±0.6
50	3.5 ±0.3	9.7 ±0.7	14.7 ±0.2

In the mixed culture containing 2 mM acetic acid, 0.6 mmoles acetic acid was oxidized/L, 1.1 mmoles sulfate/L reduced and 1.8 mmoles soluble zinc was removed/L. Zinc sulfide precipitates were observed in the culture containing, initially, 2 mM acetic acid. Although small decreases in the mean values of acetic acid, sulfate and soluble zinc concentrations were recorded, standard deviation values (shown in Table 6.3) of the mean values were relatively large. These data were therefore indicative that the mixed culture could utilize acetic acid for sulfate reduction, but were not conclusive. In contrast, no ZnS formation was observed in the culture containing 4 mM acetic acid and no decreases in the concentrations of acetic acid, sulfate or soluble zinc were detected.

6.7 Sulfidogenesis by the M1/PFBC consortium using acetic acid as electron donor: bioreactor culture

Since the preliminary results had indicated that the M1/PFBC consortium could utilize acetic acid to fuel sulfidogenesis, a bioreactor culture was established wherein acetic acid replaced glycerol as sole carbon source and electron donor.

6.7.1 Materials and methods

A 2L bioreactor culture was prepared as described in Section 2.2.1.3 and the pH was maintained at between 3.9 and 4.1. One liter of culture was replaced with 1L of medium containing vitamin solution (2X), ferrous iron (0.2 mM), acetic acid (4 mM) and $ZnSO_4$ (10 mM) every time a new batch experiment was set up. Analysis of the culture solution was as described in Section 6.4.1.3 and analysis of the microbial community was carried out as described in Section 6.4.1.2. There were two separate runs of this experiment.

6.7.2 Results

Changes in the concentrations of acetic acid, sulfate and soluble zinc in the bioreactor culture supplied with acetic acid (experiment AAa) are illustrated in Figure 6.12. Concentrations of acetic acid, sulfate and soluble zinc decreased throughout the incubation period. During this time, 1.3 mmoles/L acetic acid was oxidized, 1.2 mmoles/L sulfate were reduced and 0.8 mmoles/L soluble zinc were precipitated. The most rapid changes in the concentrations of acetic acid, soluble zinc and sulfate reduced were recorded between day 2 and 4. FISH and DAPI analyses were carried out on the fixed samples and results are shown in Figure 6.13. FISH analysis showed that the majority (88-99%) of cells was active throughout the incubation (i.e. cells were stained by the EUB338 probe). Although large changes in total cell numbers were not recorded, some changes in the relative abundance of M1 and PFBC were apparent. At the start of the experiment, 88% of cells (by number) was stained by the EUB338 probe, and PFBC was the dominant (>80% of cells by biovolume as calculated in Section 6.4.2) member of bioreactor culture. By day 1, the majority of cells were active (97% of cells by number) and the relative abundance of M1 increased to around 60% (cells by

biovolume). The relative abundance of PFBC population increased slightly between days 2 and 3 during which time concentrations of acetic acid declined relatively fast.

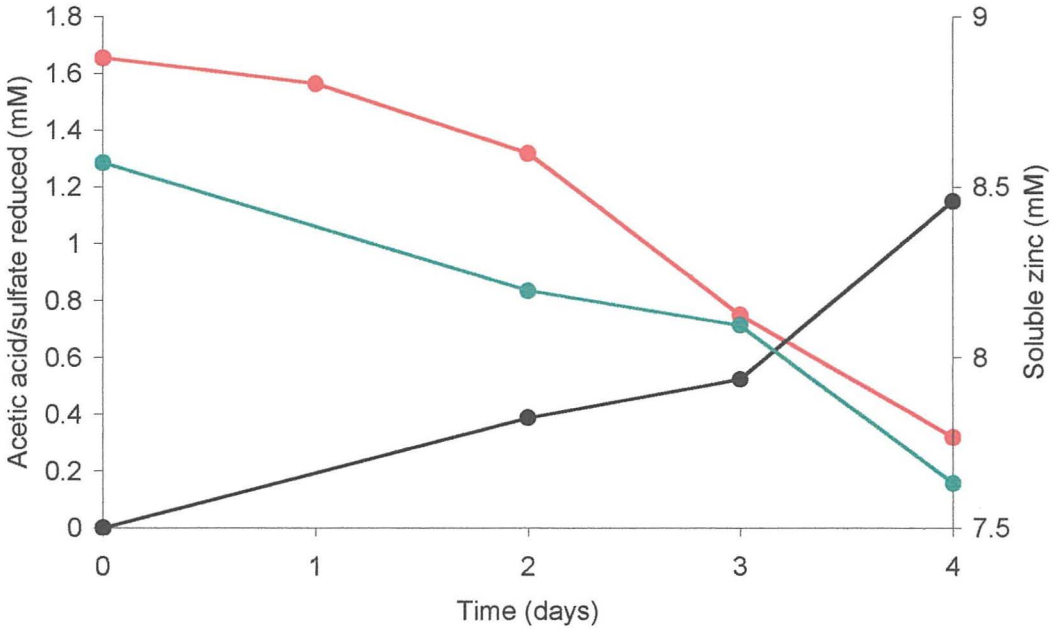


Figure 6.12. Relationships between acetic acid utilization, sulfate reduction and precipitation of zinc in a bioreactor culture of M1 and PFBC supplied with 2 mM acetic acid and maintained at pH 4.0 (±0.1; experiment AAa). Key: (●) acetic acid; (●) sulfate reduced; (●) soluble zinc.

The experiment was repeated (experiment AAb), and results are shown in Figure 6.14. During the five days of the experiment, 2.2 mmoles/L acetic acid was oxidized, 0.9 mmoles/L sulfate was reduced and 0.8 mmoles/L zinc was precipitated. Changes in the concentrations of acetic acid, soluble zinc and sulfate reduced followed very similar patterns. Interestingly, although acetic acid was depleted by day 4, there was some further reduction of sulfate and precipitation of zinc until day 5.

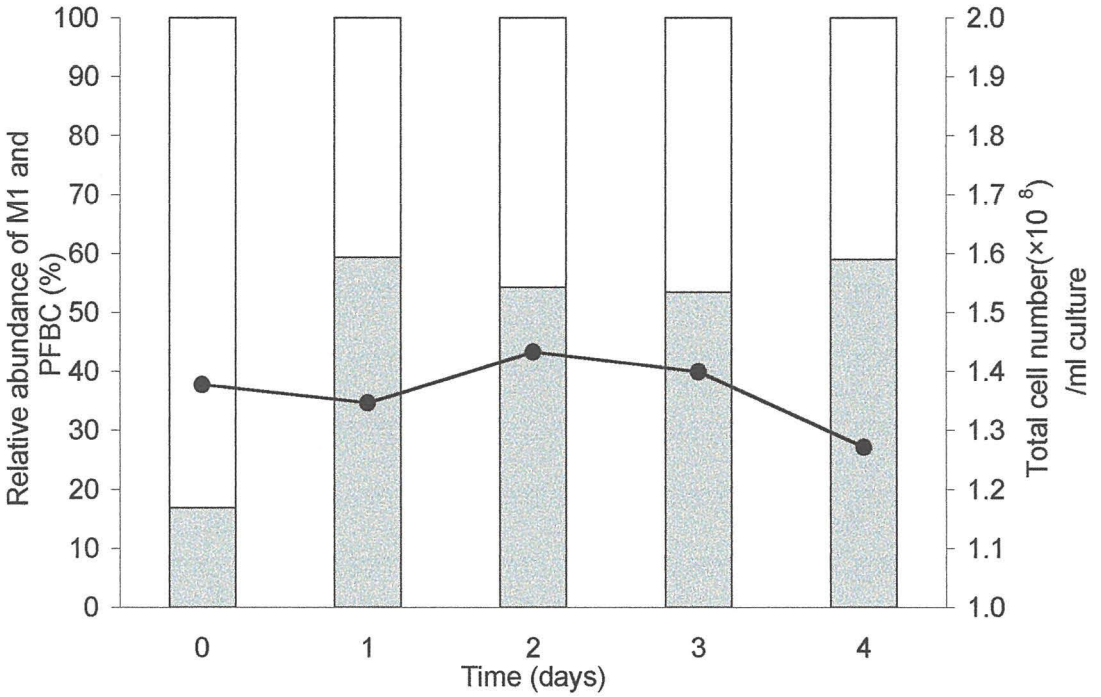


Figure 6.13. Microbial population dynamics in the bioreactor culture supplied with acetic acid maintained at pH 4.0 (±0.1; experiment AAa). Relative abundance of PFBC (unshaded bars) and M1 (shaded bars) as determined by FISH analysis; Line graph indicates total bacterial numbers (the cell numbers are indicated as values×10^8/ml culture) determined by DAPI staining.

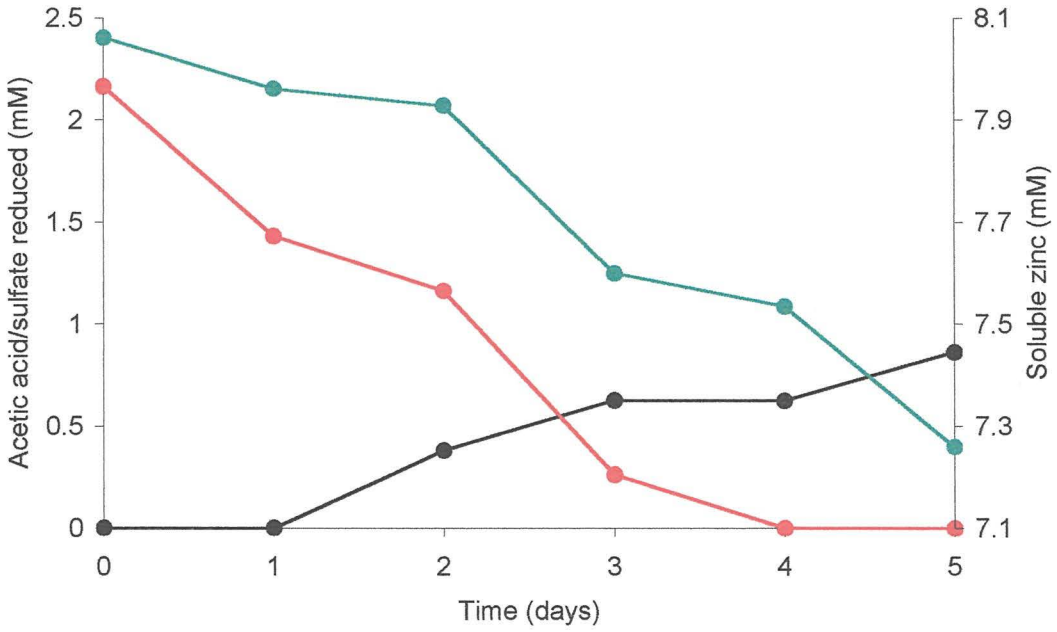


Figure 6.14. Relationships between acetic acid utilization and precipitation of zinc in a bioreactor culture supplied with acetic acid, maintained at pH 4.0 (±0.1; experiment AAb). Key; (●) acetic acid; (●) sulfate reduced; (●) soluble zinc.

6.8 Physiological studies of isolate PFBC

The *Acidocella*-isolate PFBC was obviously playing an important role in the sulfidogenic consortium. Previously, all *Acidocella* spp. have been classified as obligate aerobes, and to investigate the apparent anomaly that this acidophile was growing concurrently with the aSRB M1 in anaerobic cultures, and to characterize the bacterium in more detail, a series of experiments was carried out.

6.8.1 Isolation of Acidocella *PFBC*

PFBC was isolated from a supposedly pure culture of *Desulfosporosinus* isolate PFB, which was streaked onto mannitol-supplemented aSRB solid medium, and incubated anaerobically. Two colony forms were observed; larger colonies, typical of PFB (long, curved motile rods) and smaller colonies (composed of small, motile rods). Initial attempts to purify the latter (denoted PFBC) under anaerobic conditions failed. However, the "contaminant" was successfully purified by repeated single colony isolation using fructose-containing solid medium (Section 2.2.1.2.1) incubated aerobically.

6.8.2 Growth of Acidocella *PFBC under aerobic conditions*

6.8.2.1 Materials and methods

PFBC was grown routinely on fructose-containing liquid or solid media at pH 3.0 under aerobic conditions (Section 2.2.1.2.1) at 30°C. To assess its ability to grow aerobically on acetic acid and glycerol, an active culture of PFBC was inoculated in liquid medium containing 2, 3, 4 or 5 mM acetic acid, or 5 mM glycerol, and incubated at 30°C.

6.8.2.2 Results

PFBC was found to be able to grow aerobically on acetic acid, but not on glycerol. However, although some growth was observed in cultures initially containing 5 mM acetic acid, the majority of cells present were elongated, which is indicative of stress. From these results, the maximum concentration of acetic acid used in subsequent experiments was set at 4 mM.

Table 6.4. Results obtained from an experiment to test the ability of PFBC to grow on acetic acid (2, 3, 4 or 5 mM) or 5 mM glycerol, under aerobic conditions. Key: +, growth observed; -, no growth observed.

Substrate	Growth
Acetic acid (mM)	
2	+
3	+
4	+
5	+
Glycerol (mM)	
5	-

6.8.3 Growth of Acidocella *PFBC under anaerobic conditions*

6.8.3.1 Assessment of growth by fermentation

An experiment was carried out to test whether PFBC could grow on fructose, glycerol or acetic acid under anaerobic conditions in the absence of any extraneous electron acceptor (i.e. *via* fermentation). Although PFBC was found unable to grow on glycerol under aerobic conditions (Section 6.8.2), its ability to grow on glycerol under anaerobic conditions was tested as glycerol was the sole electron donor/carbon source added to the bioreactor mixed cultures described in Section 6.4.

6.8.3.1.1 Materials and methods

Inocula for the experiment carried out under anaerobic conditions were prepared by cultivating PFBC in corresponding liquid media, incubated under aerobic conditions. However, since PFBC did not grow on glycerol under aerobic conditions, fructose-grown cultures were used to inoculate glycerol-containing medium. The liquid medium was prepared by adding 2 mM fructose, 5 mM glycerol or 2 mM acetic acid to aSRB liquid medium (20 ml in 20 ml foam bung-stoppered universal bottles) and the media were inoculated with 1 ml aliquots of active PFBC cultures. These were incubated anaerobically at 30°C in 2.5 L anaerobic jars using the Anaero*Gen*™ AN25 system. An uninoculated control culture was also included. Fructose concentrations were measured on day 0 and 17 (Section 2.5.6.2) and concentrations of glycerol and acetic acid were measured on day 0 and 36 (Section 6.3.1). In addition, the cultures were streaked onto fructose solid

medium (incubated aerobically) to test whether viable bacteria were still present at the end of the incubation periods.

6.8.3.1.2 Results

No changes in concentrations of fructose (2.9 mM at days 0 and 17) or of glycerol (5.2 mM at days 0 and 36) were detected in anaerobically-incubated cultures. In addition, no viable bacteria were recovered from these cultures. Similarly, in the case of acetic acid, there was only a very slight decrease (0.1 mmoles/L) by the end of the incubation period, and again no viable bacteria were recovered by streaking cultures onto fructose solid media.

6.8.3.2 Assessment of growth by anaerobic respiration

Since it was apparent that *Acidocella* PFBC could not ferment fructose, glycerol or acetic acid, its ability to grow on fructose *via* anaerobic respiration was tested by including a variety of potential electron acceptors in the growth media.

6.8.3.2.1 Materials and method

A liquid medium containing heterotrophic basal salts, trace elements, 0.1 mM $FeSO_4$ and 2 mM fructose was prepared. The medium was adjusted to pH 3.0 and sterile solutions of the following potential electron acceptors were added: KNO_3 (to 2 mM), $Na_2S_2O_3$ (to 2 mM), $K_2S_4O_6$ (to 2 mM), $Fe_2(SO_4)_3$ (to 2 mM), 0.5 ml solution containing elemental sulfur (5 mg/ml) or 0.1 g schwertmannite (a poorly crystalline ferric iron mineral $[Fe_8O_8(OH)_6SO_4]$ which is common in AMD-impacted environments; added at an amount equivalent to *ca.* 2 mM Fe^{3+}). The medium was inoculated with 1 ml of PFBC culture (grown in fructose liquid medium; Section 2.2.1.1.1) in duplicate universal bottles. Cell-free control cultures were also prepared. The cultures were incubated anaerobically at 30°C in 2.5 L anaerobic jars using the AnaeroGen™ AN25 system. Concentrations of fructose were measured by ion chromatography (Section 2.5.6.2).

6.8.3.2.2 Results

The results obtained from the experiment to determine the ability of PFBC to grow anaerobically using a variety of potential electron acceptors are shown in Table 6.5. After 17 days of incubation, no significant decrease (maximum 0.1 mmoles fructose decrease/L) of fructose was measured in any of the inoculated cultures or cell-free controls.

Table 6.5. Change in concentration of fructose in PFBC cultures containing various potential electron acceptors, incubated under anaerobic conditions. The control cultures were not inoculated. The values shown are in mM unless stated otherwise, of duplicate cultures.

Time (days)	Cell-free controls	NO_3^-	$S_2O_3^{2-}$	$S_4O_6^{2-}$	$Fe_2(SO_4)_3$	Schwert -mannite	S^0
0	2.0/2.0	2.0/2.0	2.0/2.0	2.0/2.0	2.0/2.0	2.0/2.0	1.9/1.9
17	2.0/2.0	2.0/2.0	2.0/2.0	2.0/2.0	2.0/2.0	2.0/2.0	1.9/1.9

6.8.4 Anaerobic growth of PFBC on acetic acid in the presence of palladium

The experiments described above had shown that, although *Acidocella* PFBC could grow aerobically using either fructose or acetic acid as electron donor, it could not grow on these substrates in the absence of oxygen. However, the mixed culture (M1/PFBC) bioreactor experiments had shown that PFBC grew concurrently with the strict anaerobe, M1. In addition, the fact that acetic acid accumulated in pure cultures of M1 grown on glycerol, but not in mixed cultures, suggested that PFBC was responsible for metabolizing the acid. A theoretical mechanism for acetic acid metabolism, involving inter-species transfer of hydrogen, was proposed (detailed in Section 6.9). In this, the acetoclastic (i.e. acetate utilizing) reaction by which the acid is converted to hydrogen and carbon dioxide becomes thermodynamically viable (i.e. negative ΔG value) if the hydrogen is removed rapidly by the aSRB in the consortium. In order to test whether this could be mimicked in a pure culture of *Acidocella* PFBC, a simple experiment was set up whereby activated palladium (which adsorbs hydrogen gas) was added to liquid cultures containing acetic acid and incubated anaerobically.

6.8.4.1 Materials and methods

A liquid medium containing heterotrophic basal salts and trace elements (adjusted to pH 3.0 using 1 M H_2SO_4) was prepared, deoxygenated by bubbling with OFN, and 20 ml aliquots poured into universal bottles before heat-sterilization. Acetic acid and $FeSO_4$ were added (to 4 mM and 0.1 mM, respectively) from sterile stock solutions. Activated (by heating at 160°C overnight) granular palladium (0.1 g; Sigma-Aldrich) was added to the medium, which was then inoculated with an active culture of PFBC, grown aerobically on 4 mM acetic acid, in triplicate. The cultures, together with uninoculated controls were incubated anaerobically at 30°C for 15 days in 2.5 L anaerobic jars using the Anaero*Gen*™ AN25 system.

6.8.4.2 Results

The results obtained from this experiment are shown in Table 6.6. Although slight decreases (mean value 0.01 mmoles/L) in the amount of acetic acid were measured in cultures containing PFBC, similar changes was recorded in the control cultures, hence the decrease was likely to be caused by loss by volatility rather than by catabolism by PFBC. The number of PFBC cells in the culture decreased over 15 days of incubation suggesting PFBC was not capable of anaerobic growth on acetic acid in the presence of palladium.

Table 6.6. Changes in concentrations of acetic acid and cell numbers in cultures of *Acidocella* PFBC grown in liquid medium containing 4 mM acetic acid and activated palladium. Data shown are mean values (n=3) and standard deviations at the start of the experiment and at day 15.

Time (days)	Acetic acid (mM)		Cell numbers/ml
	PFBC	Uninoculated control	
0	3.3 ±0.1	3.4	9.7×10^6 ±1.8×10^6
15	3.3 ±0.1	3.3	1.3×10^6 ±3.2×10^5

6.9 Discussion

During the course of experimental work carried out with pure cultures of *Desulfosporosinus* M1 (Chapter 5), it was noted that sub-culturing aSRB through liquid media (containing glycerol as carbon and energy source) was often unsuccessful. As M1 was found to oxidize glycerol incompletely, the consequential accumulation of acetic acid in these cultures was thought to be the cause of this inconsistency. In contrast, sub-culturing of M1 on solid medium was far more reproducible. In the solid medium, the under-layer of the gel was inoculated with the heterotrophic acidophile, *Acidocella* PFBC, specifically to remove inhibitory small molecular weight organic compounds produced, for example, from on-going acid hydrolysis of agarose. The greater reproducibility of growth on solid media suggested that PFBC might also be facilitating growth of M1 in this situation, by removing acetic acid. However, there was an apparent anomaly that all *Acidocella* spp. have previously been shown to be obligate aerobes (Hallberg & Johnson 2001), which would imply that *Acidocella* PFBC should be metabolically inactive in the anaerobically-incubated plates.

When mixed cultures of M1 and PFBC were grown in liquid medium containing glycerol as sole electron donor, there was virtually no accumulation of acetic acid. The initial experiments were carried out in small-scale universal bottles, but the mixed culture behaved similarly when 2 L batch cultures were grown in pH- and temperature-controlled bioreactors. In these, glycerol oxidation was coupled to sulfidogenesis, which was indicated both by lowering of sulfate concentrations and by sulfide formation (precipitation of zinc as ZnS). Since isolate PFBC (as the proposed type strain of *"Ac. aromatica"*, WJB3) cannot use glycerol as an energy source (either under aerobic or anaerobic conditions), glycerol was necessarily being catabolized by the sulfate-reducer, M1. This being the case, acetic acid, as the product of incomplete substrate oxidation, should have accumulated in stoichiometric concentrations in these cultures. The fact that it did not suggested that acetic acid was being utilized by the other member of the consortium, *Acidocella* PFBC.

Analysis of the bioreactor mixed culture showed that PFBC was detectable, using specific probes, throughout the incubation period. FISH analysis can only detect actively growing cells, as the signals from non-active or slow growing cells are too low to be detected. In addition, numbers of PFBC increased during days 1-7 of the batch experiment, along with those of M1. It was conceivable that some slight ingress of air had allowed PFBC to catabolize the acetic acid produced by M1, coupled to oxygen as terminal electron acceptor. However, continuous monitoring of DO using an oxygen electrode confirmed that anaerobic conditions were maintained in the bioreactor. Since the tip of the electrode was located deep within the bioreactor vessel, it was possible that PFBC was scavenging oxygen at the surface of the culture. Opposing this was the observation by FISH analysis that revealed M1 and PFBC were physically closely associated with each other. Figures 6.6 and 6.7 show small flocs containing active cells of both M1 and PFBC. Since M1 cannot grow in the presence of oxygen (M1 failed to grow under microaerobic conditions: Section 5.3), the culture liquor in which these microbial aggregates were growing must have been anaerobic. However, the most convincing evidence that acetic acid catabolism was not coupled to oxygen reduction came from examination of the stoichiometry of sulfate reduction in the bioreactor experiments, as discussed below. The general conclusions from the bioreactor experiments was that glycerol oxidation coupled to sulfate reduction was carried out by the sulfidogen, M1, while acetic acid was being catabolized by *Acidocella* PFBC, by a mechanism that was not immediately apparent.

The large decrease in the number of total cell counts in the bioreactor culture at the mid-point of incubation (experiment 4.0a) was thought to be caused by a decline in numbers of planktonic phase bacteria. As illustrated by FISH analysis, M1 and PFBC formed flocs, and SEM analysis showed that ZnS precipitated on the bacteria within these flocs (Figure 6.4). The precipitation of ZnS on the microbial aggregates would have increased their densities, and might have caused them to sink in the bioreactor, which was stirred only gently. In addition, pinkish-white biofilms containing ZnS were seen to accumulate on the sides and the bottom of the bioreactor vessel, as shown in

Figure 6.1, and the majority of cells in the bioreactor culture was thought to be in these biofilms rather than in the culture liquor.

As no significant accumulation of acetic acid was recorded in experiment 4.0a, the close association of the two bacteria in flocs and biofilms was thought to have facilitated microbial interactions, resulting in a rapid removal of the acetic acid generated by M1. This was further supported by similar data for acetic acid recorded throughout the second bioreactor experiment (4.0b), which was set up a few days after experiment 4.0a had finished. In experiment 4.0b, there was a more steady decrease in soluble zinc and glycerol throughout the incubation period than in experiment 4.0a, probably because the bioreactor/biofilm community was more firmly established in the bioreactor by then. During the second experiment, far fewer planktonic phase cells were detected than in experiment 4.0a, and therefore no FISH analysis was carried out.

In order to analyze the precipitate formed inside the bioreactor, EDAX was carried out. The results showed that the precipitate was predominantly composed of Zn and S indicating the precipitate was mainly ZnS. There were some peaks (<1.0 KeV) that corresponded to carbon and oxygen, and these were assumed to be due to the presence of microbial biomass. EDAX analysis of the precipitate also confirmed that $Zn_3(PO_4)_2$ was not formed in the reactor culture, despite the fact that the solubility product of $Zn_3(PO_4)_2$ ($5 \cdot 10^{-36}(mol/L)^{vA+vB}$) is considerably smaller than that of ZnS ($1 \cdot 10^{-23}(mol/L)^{vA+vB}$). However, calculations based on these solubility products show, for any concentration of soluble zinc, the concentration of phosphate (as PO_4^{3-}) required to form $Zn_3(PO_4)_2$ is about six orders of magnitude greater than that of sulfide required to form ZnS. Furthermore, as the culture pH was maintained around 4.0, the dominant ionic species of phosphate would be $H_2PO_4^-$, rather than the PO_4^{3-} anion.

The findings from EDAX analysis illustrated the potential of an acidic sulfidogenic bioreactor to selectively precipitate zinc from liquors containing both soluble zinc and iron. EDAX analysis confirmed that the precipitate

formed did not contain FeS. As the solubility product of FeS is greater than ZnS (FeS = $4 \cdot 10^{-19}(mol/L)^{vA+vB}$, ZnS = $1 \cdot 10^{-23}(mol/L)^{vA+vB}$), a greater concentration of S^{2-} is required to precipitate FeS than ZnS. As noted previously (Section 5.9), for any concentration of total hydrogen sulfide plus free sulfide (i.e. $H_2S + HS^- + S^{2-}$), the concentration of S^{2-} is determined by solution pH. However, an important factor in these batch experiments was that the initial concentration of soluble zinc (*ca.* 5 mM) was considerably greater than that of ferrous iron (*ca.* 0.1 mM). In mine waters, such as that draining Mynydd Parys, the reverse situation is more usual, and this may have an impact on the purity of the ZnS precipitated from such waters.

In both bioreactor experiments (4.0a and 4.0b) and also in the experiment carried out in universal bottles, more sulfide was produced than would be predicted from the amount of glycerol oxidized, assuming partial oxidation of glycerol to acetic acid. As described in Equation 6.1, the oxidation of 1 mole of glycerol (to acetic acid) should result in the reduction of 0.75 mole of sulfate.

$$4C_3H_8O_3 + 3SO_4^{2-} + 6H^+ \rightarrow 4CH_3COOH + 4CO_2 + 3H_2S + 8H_2O \quad (6.1)$$

However, the amount of sulfate reduced (measured by both the amount of sulfate reduced and soluble zinc removed) by the mixed culture of M1 and PFBC always exceeded the amount of glycerol oxidized. For example, in bioreactor experiments 4.0a and 4.0b, between 1.4 and 2.1 mmoles/L sulfide were produced when 1 mmole glycerol was oxidized. The fact that more sulfide was generated than predicted by Equation 6.1, and the observation that PFBC was actively growing in the bioreactor culture, led to the development on an hypothesis to account for these observations.

In this model, PFBC is proposed to oxidize acetic acid to carbon dioxide and hydrogen under anaerobic conditions. If this reaction is carried out within the circum-neutral pH bacterial cytoplasm, it can be described by Equation 6.2.

$$4CH_3COO^- + 16H_2O \rightarrow 16H_2 + 8HCO_3^- + 4H^+ \quad (6.2)$$

The free energy (ΔG^0) of this reaction, calculated from the free energies of formation (ΔG_f) of the reactants and products (Dean 1973), is +418.24 kJ/mol

(or 104.5 kJ/mol of acetate). If, however, the reaction takes place within the periplasm where the pH (pH 4.0 in this case) is the same as the bathing liquor (i.e. analogous to ferrous iron oxidation by *At. ferrooxidans*), the reaction would be:

$$4CH_3COOH + 8H_2O \rightarrow 16H_2 + 8CO_2 \quad (6.3)$$

This is the case as pH 4.0 is lower than the *p*K*a* of both acetic acid (4.75) and the pKa_1 of carbonic acid (pH 6.4), so that acetic acid and carbon dioxide rather than acetate and bicarbonate are the dominant species involved. This has an effect on the net ΔG^0, which is now +328.4 kJ/mol (or +82 kJ/mol acetic acid).

In this model, protons are assumed to act as terminal electron acceptors, and are thereby reduced to hydrogen gas (Equation 6.4):

$$2e^- + 2H^+ \rightarrow H_2 \quad (6.4)$$

However, there are obvious theoretical problems with this hypothesis. Firstly, the redox potential (E_0') of the $2H^+/H_2$ couple (-410 mV; Madigan et al. 1997) is very low (similar to the value for ferrodoxin and lower than that of the NAD^+/H couple) so it is difficult to envisage how the electron transport chain would operate under such conditions. A similar scenario exists with sulfate as a terminal electron acceptor (the E_0' of the SO_4^{2-}/HSO_3^- couple is -520 mV) but SRB are known to circumvent this problem by "activating" sulfate (as APS; Section 1.4.2). It is important to note, however, that E_0' values are calculated on the basis of equimolar concentrations of the reduced and oxidized species. For example, the often-quoted value for the ferrous/ferric couple (+770 mV) applies at low pH (*ca.* 2) where both species are soluble. However, at higher pH, ferric iron is highly insoluble, and there is a marked shift in the redox potential towards the negative range (e.g. at pH 7, it is *ca.* +200 mV; Ehrenreich & Widdel 1994). Similarly, it could be argued that the redox potential of the $2H^+/H_2$ couple would be more positive in situations where proton concentrations are enhanced (i.e. acidic liquors) and where hydrogen partial pressures are very low. The second theoretical problem with the proposed model is that thermodynamic calculations show that the reactions described in Equations 6.2 and 6.3 are both endogonic, though

interestingly the reaction at pH 4.0 is somewhat less energetically unfavorable than that at pH 7.0. Indeed, the reverse reaction, which is energetically favorable, is carried out by acetogens, such as *Acetobacterium wodii,* to generate energy (Madigan et al.1997).

A key part of the hypothesis is that the hydrogen produced by PFBC could be utilized by M1 to fuel further sulfidogenesis (Equation 6.5):

$$16H_2 + 4SO_4^{2-} + 8H^+ \rightarrow 16H_2O + 4H_2S \quad (6.5)$$

This reaction is energetically favorable (ΔG^0 = -609.2 kJ/mol) and by coupling reactions 6.2 and 6.3 with reaction 6.5, the net reaction becomes exergonic (ΔG^0 = -190.96 kJ/mol at pH 7.0; -280.8 kJ/mol at pH 4.0). By combining Equations 6.1, 6.2/6.3 and 6.5, the complete oxidation of glycerol to carbon dioxide by the M1/PFBC consortium is accounted for (Equation 6.6):

$$4C_3H_8O_3 + 7SO_4^{2-} + 14H^+ \rightarrow 12CO_2 + 7H_2S + 16H_2O \quad (6.6)$$

The ΔG_f of the reaction shown in Equation 6.6 is –1238 kJ, or –309.5 kJ/mol glycerol. In this case, the stoichiometry between glycerol oxidized and sulfide produced is 1:1.75, which is similar to the values obtained in the mixed culture bioreactor (1:1.4-2.1 in bioreactor experiments 4.0a and 4.0b). The inclusion of PFBC in the sulfidogenic culture had therefore improved the efficiency of net sulfidogenesis, in that 2.3-times more sulfide was produced per glycerol oxidized than by pure cultures of M1, where acetic acid was produced. The model that was derived to account for the various observations noted in the M1/PFBC sulfidogenic consortium is summarized in Figure 6.15.

The hypothesis proposed involves a syntrophic relationship between M1 and PFBC. A syntrophic association occurs when the degradation of a substrate by one species is thermodynamically possible only through removal of the end product by another species (Schink 1997). Although rare in aerobic environments, syntrophy appears to be widespread in anaerobic environments, and usually involves interspecies transfer of hydrogen (Cord-Ruwisch et al. 1998).

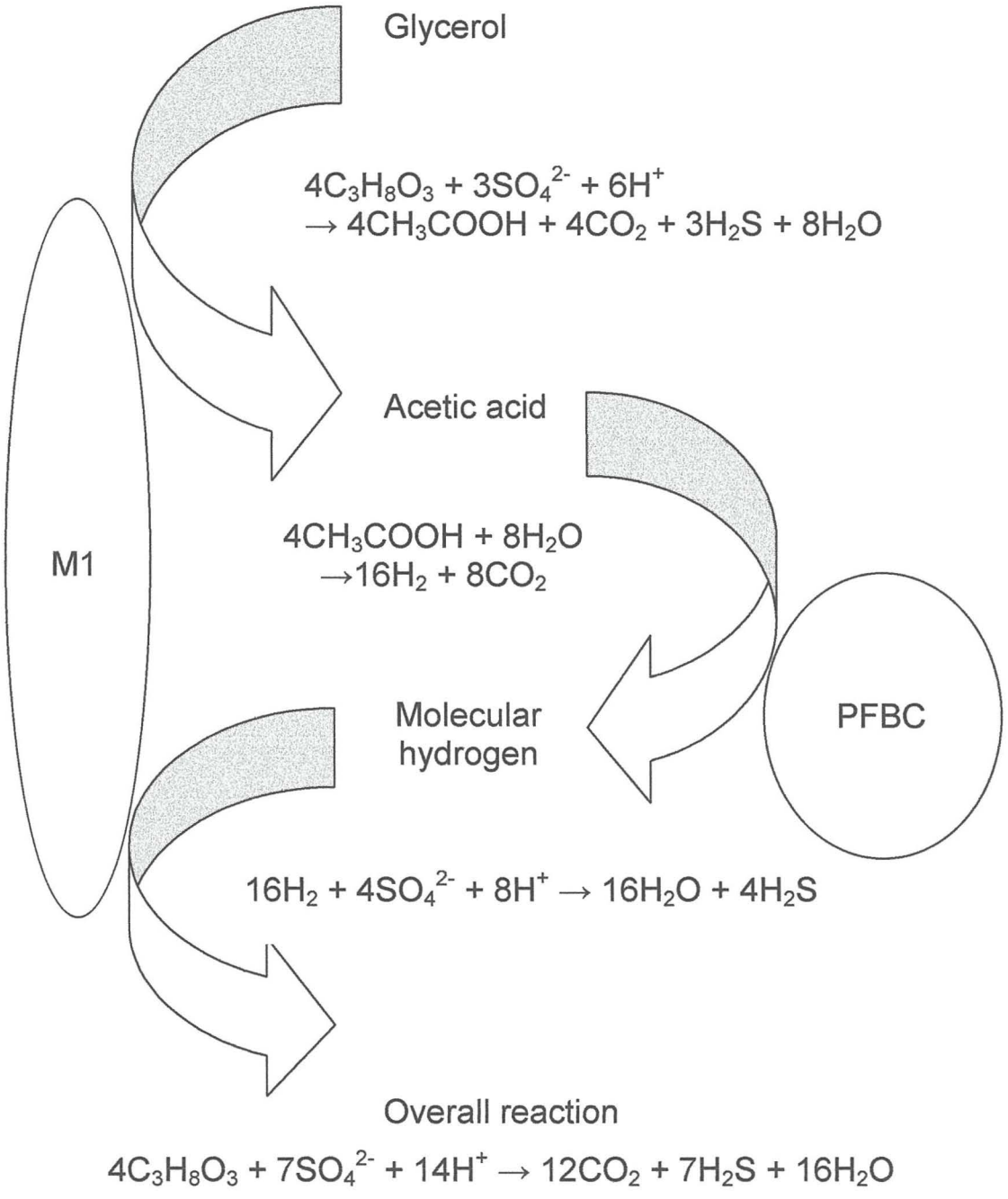


Figure 6.15. A schematic diagram showing proposed reactions carried out by the M1 and PFBC consortium in the sulfidogenic bioreactor.

This model predicts that the M1/PFBC consortium should be capable of using acetic acid as an electron donor for sulfate reduction, even though the aSRB are unable to use acetic acid directly (Section 5.7). This would be a two-stage reaction involving oxidation of acetic acid by PFBC, forming hydrogen which is used by the sulfate-reducer M1 as an electron donor for sulfate reduction. Although the formation of hydrogen from acetic acid is endogonic (Equation 6.2/6.3) the net reaction (Equation 6.7) is exergonic (ΔG^0 = –104.33 kJ/mol at pH 4.0):

$$CH_3COOH + SO_4^{2-} + 2H^+ \rightarrow 2CO_2 + H_2S + 2H_2O \quad (6.7)$$

In order to test the ability of the mixed culture of M1 and PFBC to carry out sulfidogenesis using acetic acid, as suggested by the hypothesis, acetic acid was supplied in place of glycerol. Evidence of sulfidogenesis was found in the small-scale (universal bottle) mixed culture experiment supplied with 2 mM acetic acid. Subsequently, experiments AAa and AAb were set up in a bioreactor to study acetic acid utilization by the consortium in more detail. The results obtained from these experiments showed that oxidation of acetic acid was coupled to sulfate reduction and caused zinc to precipitate (presumably as ZnS). Although 1 L of culture liquor was replaced with 1 L of fresh medium when a new bioreactor experiment was set up, most of the biofilm and precipitates remained in the bioreactor, and consequently the most bacteria in the vessel were sessile rather than planktonic. This caused difficulty in obtaining sufficient numbers of cells for DAPI staining and FISH analysis. Total cell counts (Figure 6.13) showed that there was no significant change in cell numbers compared to the results obtained from experiment 4.0a, which was carried out when the biofilm was not fully established. Although this could be due to low energy yield from acetic acid oxidation (ΔG^0 = –104.33 kJ/mol acetic acid) compared to glycerol oxidation ($\Delta G^{0'}$ = -309.5 kJ/mol glycerol), it is more likely due to the likelihood that most cells were attached to the surfaces of the reactor. Despite the difficulty in collecting large numbers of cells, FISH analysis showed both *Desulfosporosinus* M1 and *Acidocella* PFBC were active throughout incubation. On day 0 of experiment AAa, FISH analysis showed only 88% of

DAPI-stained cells were active (i.e. stained with the EUB338 probe); the inactive cells were identified (from their distinctive morphology) as *Desulfosporosinus* M1. However, on day one, most M1 cells were stained by both EUB338 and SRBCy3 probes (i.e. were metabolically active) and were found in relatively high numbers throughout incubation. According to the hypothesis, acetic acid oxidation by PFBC can only be carried out when H_2 is removed by M1, and it appeared that only a small amount of acetic acid was oxidized until *Desulfosporosinus* M1 became active. The results from experiments AAa and AAb showed that the amount of acetic acid that was apparently oxidized was slightly greater than the amount of sulfide produced (determined both from the amount of sulfate reduced and the amount of soluble zinc removed as ZnS). From Equations 6.2/6.3 and 6.5, equimolar amounts of sulfide should be produced from acetic acid oxidation. This discrepancy could have been due to loss of some acetic acid through volatilization; bubbling OFN through the cultures would probably have accentuated this loss.

Although syntrophic relationships between SRB and obligate and facultative anaerobes have been described (Cord-Ruwisch et al. 1998; Jackson et al. 1999; Boetius et al. 2000), there are currently no reports of syntrophic relationships between anaerobic and (supposedly) aerobic microorganism, as proposed in this study. *Acidocella* PFBC did not grow in pure culture under anaerobic conditions under any of the experimental conditions tested. Other work (K. Coupland, UWB, unpublished) has shown that *Acidocella* PFBC does cause the reductive dissolution of schwertmannite (presumably using ferric iron in the mineral as an electron sink) in cultures containing either fructose or phenol as carbon source incubated under microaerobic conditions. An analogous situation occurs with most *Acidiphilium* spp., which use ferric iron as a terminal electron acceptor under microaerobic but not under strictly anaerobic conditions (Hallberg & Johnson 2001). Current data suggest that *Acidocella* PFBC should, as other *Acidocella* spp., be classified as an obligate aerobe, under presently accepted guidelines.

Since *Acidocella* PFBC was shown to be unable to ferment acetic acid or to be incapable of anaerobic respiration, using the electron acceptors tested (NO_3^-, $S_2O_3^{2-}$, $S_4O_6^{2-}$, Fe^{3+} and elemental sulfur), it was considered to be growing by acetic acid oxidation as described previously (Equations 6.2, 6.3 and 6.4). The scheme shown in Figure 6.15 suggests that *Acidocella* PFBC should be able to grow on acetic acid under anaerobic conditions, provided that the partial pressure of hydrogen is maintained at a very low level (i.e. by oxidation by *Desulfosporosinus* M1 in the mixed culture). In order to test whether removal of hydrogen gas by an abiotic mechanism might also result in the anaerobic growth of *Acidocella* PFBC on acetic acid, activated palladium granules were added to liquid medium (Figure 6.16).

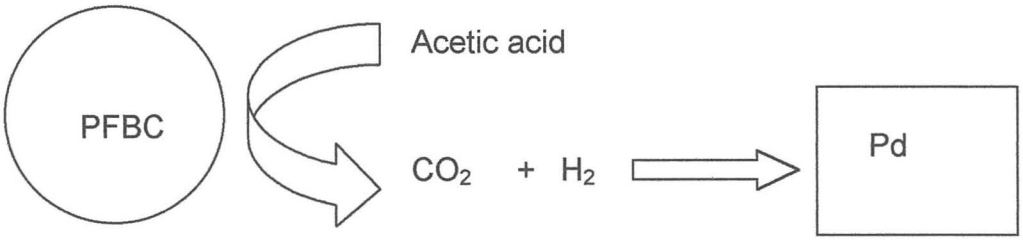


Figure 6.16. A hypothetical scheme showing H_2 removal by palladium in anaerobic cultures of *Acidocella* PFBC.

When heat activated, Pd is able to absorb molecular hydrogen up to 900 times its volume (at 80°C and under 1 atmosphere pressure; Hartley 1973). However, inclusion of palladium did not result in the growth of *Acidocella* PFBC. This may have been due to the design of the experiment, in that adding palladium granules to the liquid medium might well have removed their ability to adsorb hydrogen gas. Recent work at the University of Wales Bangor, in which palladium granules were suspended above acetic acid-containing cultures of *Acidocella* PFBC in anaerobic jars, has resulted in the growth of the bacteria (Owen Rowe, UWB, unpubl.). This observation supports the hypothesis that was developed to explain growth of the M1/PFBC consortium.

Since sulfidogenesis was successfully demonstrated using the mixed-culture of M1 and PFBC at pH 4.0, the effect of small variations in pH on the bioreactor mixed-culture was tested at pH 3.8 and 4.2 (±0.1 pH unit). This was designed to determine the effect of pH on members of the microbial consortium, and also to determine what effects small variation in pH had on precipitation of zinc. The results showed that sulfidogenesis occurred at both pH values and that there was again no significant accumulation of acetic acid in either case. Since the pH values in these experiments did fluctuate between the pre-set limits, these experiments showed that sulfidogenesis could be carried out by the defined mixed-culture at the pH ranges 3.7 to 4.3. Figure 6.17 illustrates that glycerol oxidation and soluble zinc removal occur at the pH range of 3.7-4.3 and the Table 6.7 shows the changes in glycerol and soluble zinc concentrations in the bioreactor cultures.

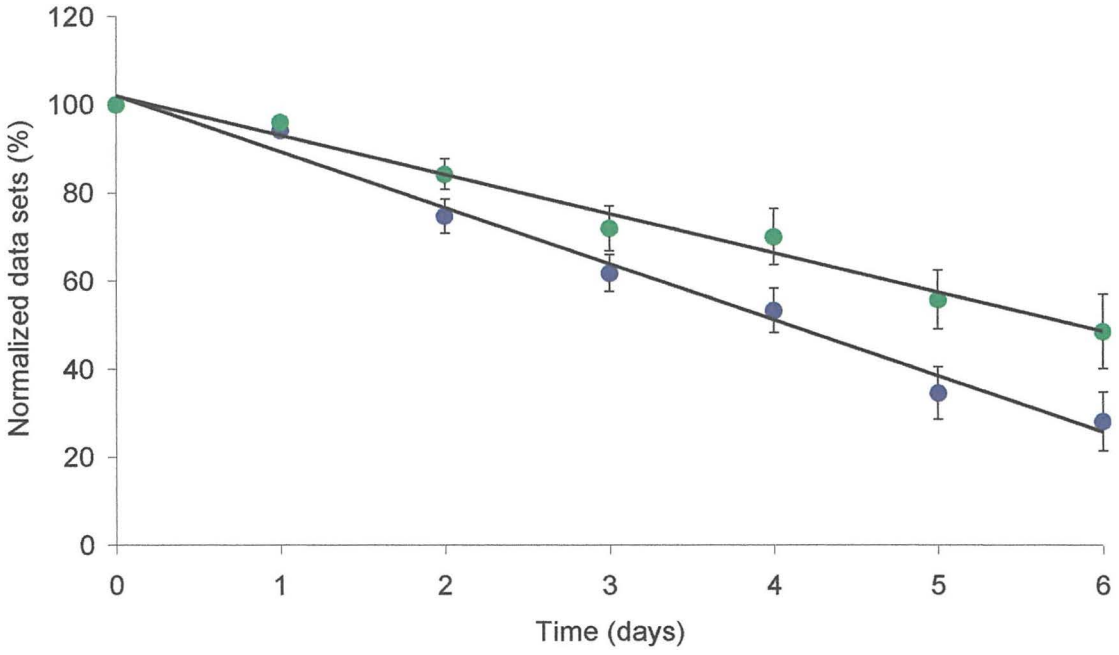


Figure 6.17. Relationships between glycerol utilization and precipitation of zinc in bioreactor cultures. The data were obtained by normalizing the amount of glycerol and soluble zinc in the bioreactor cultures (experiments carried out at pH 4.0, 4.2 and 3.8). The concentrations of glycerol and soluble zinc measured in each experiment are shown in Table 6.7. Key: (●) glycerol; (●) soluble zinc. The equation of the fitted line for soluble zinc is y=-8.90x+101.92 (R^2=0.98) and that for glycerol is y=-12.73x+102.01 (R^2=0.99).

Table 6.7. Changes in concentrations of glycerol and soluble zinc in mixed culture bioreactor experiments carried out at pH 4.0 (4.0a and 4.0b), 4.2 and 3.8. The values were obtained at the start of the experiment and on day 6 for experiments carried out at pH 4.0 and 3.8 and on days 2 and 8 for experiment carried out at pH 4.2.

Experiments	Glycerol (mM)		Soluble zinc (mM)	
	Day 0	Day 6	Day 0	Day 6
3.8	2.48	0.96	9.49	5.98
4.0a	3.04	1.86	9.57	8.17
4.0b	4.05	0.32	11.60	4.54
4.2	9.07	0.42	10.05	0.67

Zinc precipitation was not closely coupled to glycerol oxidation and sulfate reduction in the bioreactor culture set at pH 3.8, in contrast to cultures set at pH 4.0 and 4.2. Initially there was virtually no zinc removed from solution at pH 3.8, even though glycerol oxidation and sulfate reduction were detectable from the start of the experiment. The concentration of S^{2-}, which reacts with soluble zinc to form ZnS, depends on solution pH (the lower the pH value, the less sulfide exists as S^{2-}), and precipitation of insoluble compounds does not occur in a solution until concentrations of the component ions (in this case Zn^{2+} and S^{2-}) reach sufficient concentration to exceed the solubility product, *S*, of the solid phase product (ZnS). At pH 3.8, the concentration of S^{2-} corresponding to net concentration of sulfate reduced would be less than at pH 4.0 or 4.2, and was assumed to be insufficient to cause ZnS to precipitate in the early phase of the pH 3.8 experiment. However, as sulfate reduction progressed, the concentration of S^{2-} would also have increased, ultimately to the point at which ZnS was precipitated.

Although the pH of the bioreactor was maintained by adding acid or alkali, some fluctuations in culture pH were observed during incubation. Generally, the culture pH declined slightly during the early phase of each batch experiment (though this was generally less than the 0.1 set limit, so that no alkali was added) but increased in the later stages, and sulfuric acid was usually pumped in to maintain the pH within the pre-set boundaries. The reasons for these pH fluctuations are unclear, though net alkalinity could

have been generated by excretion of bicarbonate or HS^- by the bacteria into the acidic media (Equations 6.8 and 6.9):

$$HCO_3^- + H^+ \rightarrow CO_2 + H_2O \quad (6.8)$$

$$HS^- + H^+ \rightarrow H_2S \quad (6.9)$$

Mostly, the hydrogen sulfide excreted by M1 would have been used to precipitate ZnS. However, a slight excess in HS^- would have caused the culture pH to increase.

In conclusion, the mixed culture of *Desulfosporosinus* M1 and *Acidocella* PFBC was shown not to accumulate acetic acid, even though this was produced in equimolar amounts to glycerol oxidized in pure cultures of the aSRB. Furthermore, the mixed culture produced more sulfide than would be predicted by incomplete oxidation of glycerol by M1. Somewhat surprisingly, the supposedly obligate aerobe PFBC was found to be actively growing in the mixed culture alongside the obligate anaerobe M1. This suggests that *Acidocella* PFBC is capable of active growth under anaerobic condition in co-culture with *Desulfosporosinus* M1. These observations could be explained by the proposed syntrophic relationship between the two bacteria, involving interspecies transfer of hydrogen.

Although PFBC was not shown to grow by acetic acid oxidation in the presence of an abiotic H_2 sink (e.g. Pd) under anaerobic conditions in the present study, subsequent experimental work using a different experimental set-up, has shown that this can occur. The proposed hypothesis, outlined in Figure 6.15, was therefore supported by a number of independent experiments (hydrogen oxidation by *Desulfosporosinus* M1, acetic acid utilization coupled to sulfate reduction by the M1/PFBC consortium, and acetic acid oxidation by *Acidocella* in the presence of a hydrogen sink).

These findings have demonstrated that sulfidogenesis can be carried out at low pH using glycerol, and possibly other organic substrates, as electron donors, since virtually no acetic acid accumulates in mixed cultures containing the acidophilic acetotroph *Acidocella* PFBC. The lower pH limit for

the M1/PFBC mixed culture would be determined by the relative acid sensitivity of each partner bacterium. Pure cultures of *Desulfosporosinus* M1 grown on hydrogen grew at pH 3.0 and above (Chapter 5). The pH profile of *Acidocella* PFBC was not determined, though it is known that *Acidocella* spp. (including the closely related *"Ac. aromatica"*) have a lower pH limit of about 2.5 (Hallberg & Johnson 2001). Therefore, it is more likely that the pH limit of the consortium is likely to be determined by *Desulfosporosinus* M1, at about pH 3.0. At this pH, CuS will precipitate but ZnS will not (based on solubility calculation and experiments carried out in Chapter 5), and therefore a bioreactor culture maintained at *ca.* pH 3.0 could, in theory, be used to separate soluble copper and zinc in waste streams, such as the AMD at Mynydd Parys.

Chapter 7 A study of the growth of the thermo-acidophile *"Acidicaldus organovorus"* Y008 under oxygen-limiting conditions

7.1 Introduction

Acidophilic prokaryotes exhibit diverse physiological characteristics, as described in Section 1.2, and can be differentiated on the basis of various traits, such as temperature optima and means of carbon assimilation. A large number of characterized acidophilic prokaryotes are known to play major roles in iron- and sulfur-cycling by their abilities to oxidize and reduce iron and/or sulfur. For example, the most well-studied acidophile, *Acidithiobacillus ferrooxidans* can couple the oxidation of ferrous iron and various reduced inorganic sulfur compounds (and also hydrogen) to the reduction of oxygen (Friedrich 1998) and, in anoxic environments, the oxidation of elemental sulfur and reduced sulfur compounds (or hydrogen) to the reduction of ferric iron compounds (Pronk et al. 1991; Drobner et al. 1990). *Acidianus* spp. (thermo-acidophilic archaea) can reduce S^0 to sulfide using molecular hydrogen as an electron donor and can also oxidize S^0 under aerobic conditions (Segerer et al. 1986). Due in part to improvements in techniques for isolating and cultivating acidophiles, there has been a vast increase in characterized acidophilic prokaryotes in the past 20 years. However, relatively few moderately thermophilic acidophiles have been characterized. Many of these are mixotrophic iron- and/or sulfur-oxidizers and most are Gram-positive (Section 1.2.2). Currently, the only recognized obligately heterotrophic bacterial genus in this category is *Alicyclobacillus* (a Gram-positive, spore-forming rod-shaped bacterium), and many species of this genus are not extremely acidophilic (Norris & Johnson 1998).

Several moderately thermophilic acidophiles were isolated from geothermal sites (30-83°C; pH 2.7-3.7) in Yellowstone National Park, U.S.A. by Johnson et al. (2003). These were characterized and their 16S rRNA genes were sequenced for identification. The isolates included *Sulfobacillus*-like bacteria, an *Acidimicrobium* sp. and several Gram-negative isolates belonging to the class α-Proteobacteria (Johnson et al. 2001b). The latter grew heterotrophically but were also found to oxidize elemental sulfur. As

described below, a thermo-acidophile which was very closely related to these *α-Proteobacteria* was isolated from an anaerobic enrichment culture during the course of the present study. Experiments were carried out to test the ability of this apparently novel thermo-acidophile to grow under microaerobic and anaerobic conditions.

7.2 Isolation and identification of "*Acidicaldus*"-like bacteria

A water sample obtained from a geothermal site (80⁻C; pH 3.0) in Yellowstone National Park (Wyoming, U.S.A.) was inoculated into a medium containing 5 mM tetrathionate and 1 mM $FeSO_4$ (pH 2.3, adjusted with sulfuric acid). The inoculated medium was put into 25 ml sterile plastic syringes, the residual air expelled and the syringes sealed. The culture was incubated under these anaerobic conditions at 45°C for 2 years, with periodic replacement of half of the culture with fresh medium. The objective was to attempt to enrich for thermo-acidophiles that could grow by disproportionation of tetrathionate to sulfate and hydrogen sulfide (Section 1.4.2.2).

Plating of the enrichment culture onto iron-tetrathionate overlay plates (Section 2.2.1.2.2.1) over the two year period produced only one colony form (small, off-white colonies) and these grew best on plates that were incubated under aerobic conditions. After two years, biomass in the syringe liquor was harvested, cells lysed and the 16S rRNA gene(s) of the microorganism(s) present was amplified (Section 2.6.2). Gene sequencing showed that the single (or dominant) microorganism present was an α-proteobacterium which was closely related to the Gram-negative heterotrophic thermo-acidophiles described by Johnson et al. (2003; 99% gene similarity, of 540 bp analyzed, to isolate Y008). As with Y008 and similar isolates, the isolate from the tetrathionate enrichment was found not to be able to oxidize ferrous iron, and was found to be able to oxidize elemental sulfur only in media containing organic carbon. Since it was essentially identical to isolate Y008, the latter isolate (the proposed type species of a new genus) was used in further work.

7.3 Growth factor requirements of isolate Y008

Isolate Y008 and related bacteria were subcultured routinely in the laboratory in yeast extract-containing liquid or solid media. The requirement of the isolate Y008 for growth factors to support growth on glucose was tested.

7.3.1 Materials and Methods

A heterotrophic basal salts/trace elements-containing medium (Section 2.2.1) was adjusted to pH 2.2 with 1 M sulfuric acid, and heat-sterilized. To this medium was added: (i) glucose (10 mM, final concentration); (ii) 10 mM glucose and 0.01% (w/v) yeast extract; (iii) 10 mM glucose and a vitamin mixture (0.1% v/v; Section 2.2.1.1.2); or (iv) 10 mM glucose and 0.01% (w/v) casein hydrolysate. Triplicate shake flasks (150 ml containing 50 ml medium) were prepared and inoculated with 1 ml of Y008 culture grown in a medium containing 10 mM glucose and 0.01% (w/v) yeast extract. The cultures were incubated at 45°C and optical densities were measured at 600 nm over 9 days of incubation.

7.3.2 Results

Results obtained from this experiment are shown in Figure 7.1. Although small increases in cell numbers were recorded in cultures containing only glucose, addition of yeast extract, vitamins or casein hydrolysate all greatly enhanced bacterial growth.

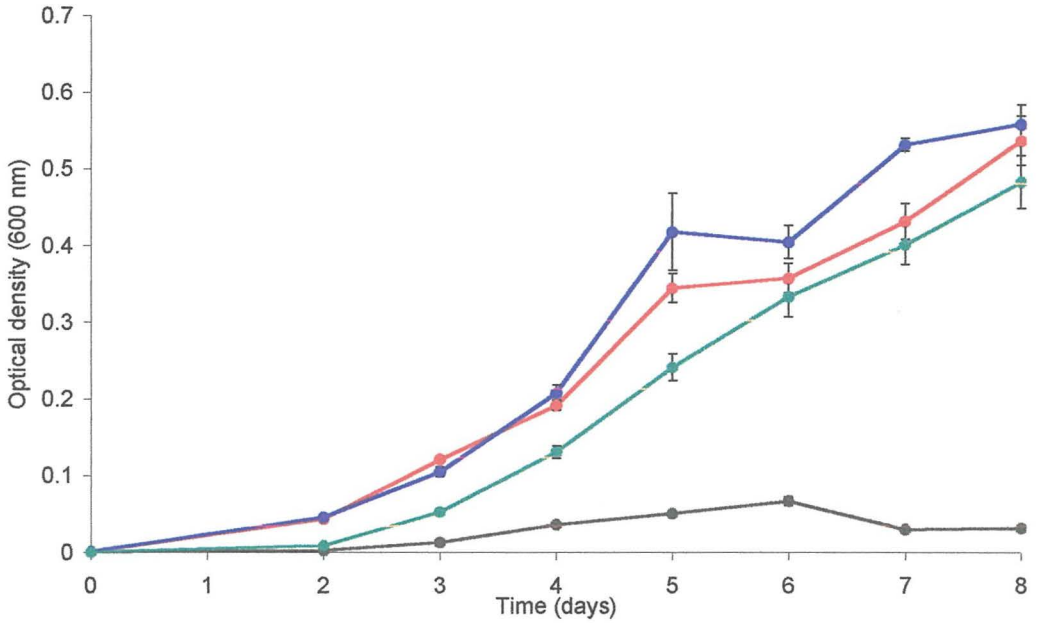


Figure 7.1. Effect of yeast extract, casein hydrolysate and vitamins on the growth of isolate Y008. Key: (●) yeast extract-supplemented cultures; (●) casein-supplemented cultures; (●) vitamin-supplemented cultures; (●) glucose only cultures.

7.4 Growth of isolate Y008 under microaerobic conditions

The ability of isolate Y008 to grow in a medium containing glucose and ferric iron under microaerobic conditions was studied in this experiment.

7.4.1 Materials and methods

A liquid medium, containing heterotrophic basal salts, trace elements and 0.01% (w/v) yeast extract, was adjusted to pH 2.2 with 1 M sulfuric acid. The medium was deoxygenated by gassing with OFN for 20 minutes, and $Fe_2(SO_4)_3$ and glucose were added to the medium from 1 M stock solutions to give final concentrations of 20 and 5 mM, respectively. The pH of the medium was readjusted to 2.2 prior to filter sterilization and 50 ml aliquots were dispensed into sterile 100 ml serum bottles. Duplicate bottles were inoculated with 1 ml of Y008 culture, grown aerobically on glucose, and were placed in a 2.5 L anaerobic jar (Section 2.2.1.4.1). A cell-free control culture (50 ml in 50 ml bottle), and a culture to which 1 mM ferrous iron was added instead of ferric iron, were also placed in the jar. A microaerobic atmosphere (<5% oxygen) was generated in the jar *via* a Campy*Gen*™ CN25 sachet

(Section 2.2.1.4.1) and the cultures were incubated at 45°C. Concentrations of glucose were measured by ion chromatography (Section 2.5.6.2), and ferrous iron using the Ferrozine assay (Section 2.5.2). Cells were enumerated using a Thoma bacteria counting chamber (Section 2.3.1).

7.4.2 Results

Results from this experiment are shown in Figure 7.2. There were parallel increases in cell numbers and ferrous iron during incubation, indicating that the isolate was both growing and reducing ferric iron under oxygen-limiting (microaerobic) conditions. Rather surprisingly, although isolate Y008 oxidizes glucose aerobically, no oxidation of glucose was recorded in microaerobically incubated cultures. No significant abiotic ferric iron reduction was observed in uninoculated control culture. In addition, there was a slight increase of *ca.* 1 mM glucose in the cell-free culture and in the culture without ferric iron. It was also found that bacterial numbers did not increase in media that did not contain ferric iron.

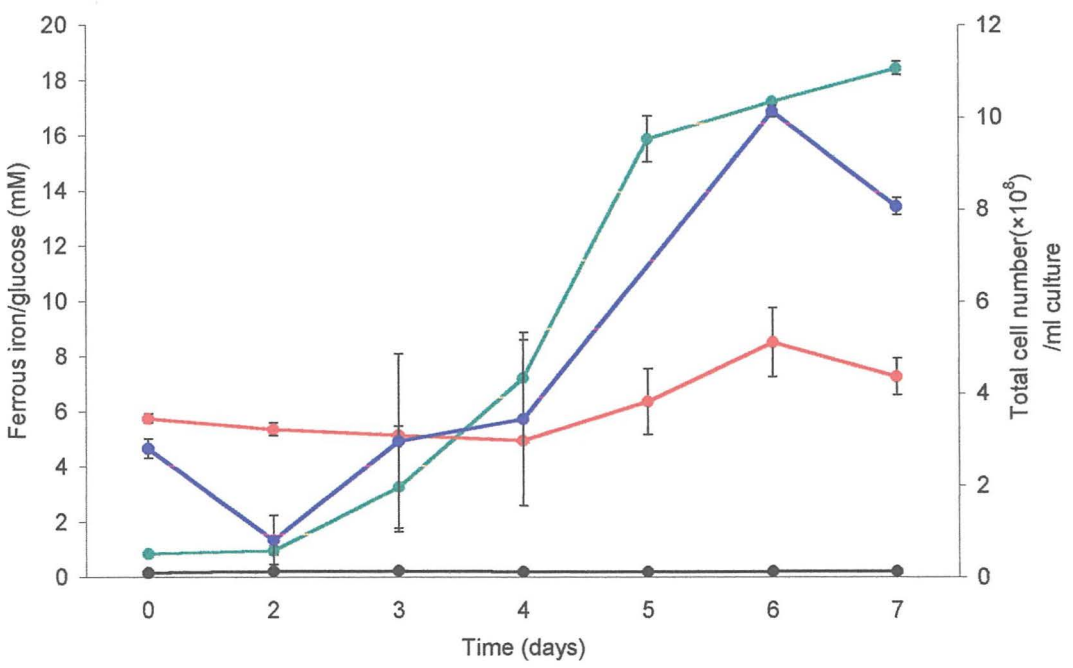


Figure 7.2. Growth of Y008 under microaerobic conditions. Key: (●) glucose; (●) ferrous iron (inoculated cultures); (●) ferrous iron (control culture); (●) bacterial numbers.

7.5 Growth of isolate Y008 under anaerobic conditions

7.5.1 Anaerobic respiration using ferric iron as electron acceptor

7.5.1.1 Materials and methods

A liquid medium was prepared as described in Section 7.4.1. Triplicate serum bottles were inoculated with 1 ml of Y008 culture grown aerobically on glucose and were incubated under anaerobic atmospheres generated using Anaero*Gen*™ AN25 sachets (Section 2.2.1.4.2) in an anaerobic jar. Control cultures (one non-inoculated, and the other inoculated but containing 1 mM ferrous iron in place of ferric iron) were also included. Changes in cell numbers and concentrations of glucose and ferrous iron were monitored as described in Section 7.4.1.

7.5.1.2 Results

Results from this experiment are shown in Figure 7.3. As with microaerobically incubated Y008 cultures, ferric iron reduction (measured as increase in ferrous iron concentration) correlated with cell number increase. In anaerobically-incubated cultures, lag phases were greater than those observed under microaerobic conditions. Again, no glucose utilization was measured in the cultures and amount of ferric iron reduced in the cell-free control culture was insignificant. In the culture with no added ferric iron, a slight increase in glucose concentration was recorded (*ca.* 0.5 mM) and no growth (increase in numbers) of isolate Y008 was observed.

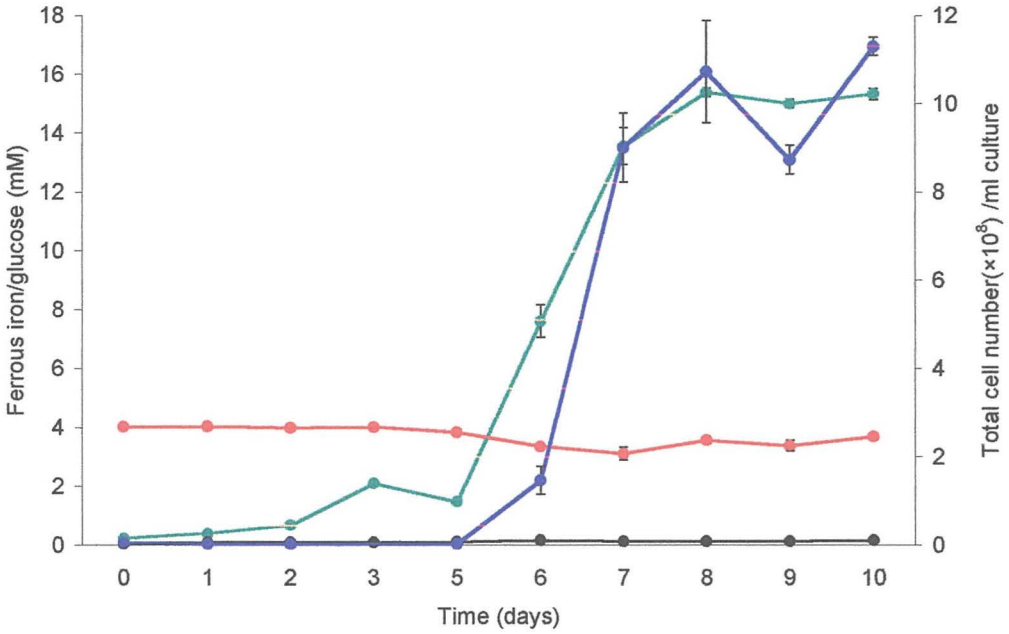


Figure 7.3. Growth of Y008 under anaerobic conditions. Key: (●) glucose; (●) ferrous iron (inoculated cultures); (●) ferrous iron (control culture); (●) bacterial numbers.

7.5.2 Growth via disproportionation of tetrathionate

Since an isolate very closely related to Y008 survived for two years in seemingly anaerobic cultures containing only tetrathionate as a potential electron donor, the ability of Y008 to grow under strictly anoxic conditions by disproportionating tetrathionate was examined.

7.5.2.1 Materials and methods

A liquid medium containing heterotrophic basal salts, trace elements, 5 mM tetrathionate and 1 mM $FeSO_4$ (adjusted to pH 2.3 by adding sulfuric acid) was prepared, deoxygenated (with OFN) and 20 ml aliquots dispensed into 20 ml universal bottles in triplicate. A second medium containing 5 mM tetrathionate and 5 mM ferric iron was also prepared. Both media were inoculated with 1 ml of Y008 culture and incubated anaerobically as described above. Cultures were observed at regular intervals, over 3 months, using a phase contrast microscope, but accurate counts were not carried out.

7.5.2.2 Results

No changes in cell numbers were observed in cultures containing only tetrathionate, though numbers in tetrathionate/ferric iron medium appeared to show a slight increase during the protracted incubation period. It was concluded, therefore, that Y008-like thermo-acidophiles cannot grow by disproportionation of tetrathionate.

7.6 Discussion

Isolate Y008 is the first thermo-acidophilic, obligate heterotroph within the class *Proteobacteria* to be characterized. The closest characterized relative of Y008 is the mesophilic moderate acidophile *Acidisphaera rubrifaciens,* which was first isolated from acidic hot springs in Japan (Hiraishi et al. 2000). *As. rubrifaciens* is an obligate aerobic heterotroph which synthesizes photosynthetic pigments, and has a pH range for growth of 3.5-6.0 (optimum 4.5 to 5.0) and temperature range of 20-40°C (optimum 30-35°C). In contrast, isolate Y008 had been shown to be thermophilic (growth temperature range 40-65°C; optimum temperature 53-55°C) and extremely acidophilic (growth pH range 1.75-3.0; optimum pH 2.5-3.0). Isolate Y008 and *Acidisphaera rubrifaciens* share only 93% 16S rRNA gene similarity, indicating that they are species of different genera.

An experiment to determine the growth factor requirement of Y008 showed that cell numbers were significantly greater when either yeast extract, casein hydrolysate or a vitamin mixture was added to the glucose/basal salts medium. The three different supplements were added to determine what particular growth factor was stimulating growth of the thermo-acidophile: an amino acid(s) (casein hydrolysate), vitamin(s) or both (yeast extract). In the event, all three additives enhanced growth of Y008 to a similar extent, so it was not possible to identify any particular growth stimulant. The experiment confirmed that Y008 grows relatively poorly in glucose/basal salts medium.

Y008 was found to grow under both microaerobic and anaerobic conditions in media containing ferric iron, though it failed to grow in both situations in the absence of ferric iron. Under both conditions, ferric iron was reduced to

ferrous. In the anaerobic cultures, growth was considered to occur *via* anaerobic respiration, in which ferric iron was used as terminal electron acceptor. It is somewhat curious that ferric iron reduction also occurred under microaerobic conditions where oxygen, although present in smaller concentrations than in aerobic cultures, might be expected to be the sole or major electron sink. Interestingly, growth of isolate Y008 was observed only in cultures containing ferric iron that were incubated under microaerobic conditions. Ferric iron is soluble (and therefore readily available) at low pH and the ferric/ferrous iron couple has a relatively high Eh (+770mV), which is close to oxygen/water couple (+820 mV), so that ferric iron is an attractive alternative electron acceptor to oxygen in extremely acidic environments and appears to be reduced concurrently with oxygen in micro-aerobic situations.

Although glucose was present in the micro-aerobic and anaerobic cultures of isolate Y008, glucose oxidation did not appear to be coupled to ferric iron reduction. It was concluded that yeast extract acted as both energy and carbon sources in these cultures, but the reason for the non-utilization of glucose was not resolved. It is conceivable that there was an error in glucose analysis in this case.

A bacterium, which was essentially identical to isolate Y008, survived in "anaerobic" syringe cultures containing tetrathionate and ferrous iron and maintained at 45⁻C for 2 years. As there was no apparent electron donor other than tetrathionate and no electron acceptor other than sulfate (and there was no evidence of sulfidogenesis in the syringe cultures), the possibility that the bacteria were growing *via* disproportionation of tetrathionate, which does not require an extraneous electron acceptor, was tested. However, subsequent experiments under more tightly regulated anaerobic conditions failed to find evidence of such growth with isolate Y008 itself. The 2-year enrichment experiment was also carried out using medium to which no organic carbon had been added, suggesting the possibility that the Y008-like isolate was fixing CO_2. However, other experiments have

shown clearly that Y008 is incapable of autotrophic growth (Johnson et al. unpubl.).

It is now thought that the Y008-like isolate was able to survive for long periods in the syringe cultures probably because of some ingress of oxygen, so that conditions were not strictly anoxic. In addition, although organic carbon was not added to the liquid medium, sufficient trace quantities of organic materials may have been present in the "inorganic" liquid medium for the Y008-like bacterium to survive. There is also the possibility that the acidic liquor might have caused some release of organic matter from the plastic syringes. Whilst any such materials would be expected to be recalcitrant, other research has shown that Y008 is able to degrade a wide range of organic materials, including aromatic compounds such as phenol (D.B. Johnson unpubl.).

In conclusion, isolate Y008 is a novel thermo-acidophile, and the first α-proteobacterium of this type to be described. It appears to be auxotrophic, though the required growth factor(s) was not identified. The bacterium is a facultative anaerobe, using either oxygen or ferric iron as terminal electron acceptor. Sulfate is not reduced to sulfide, and reduced inorganic sulfur compounds are not disproportionated, as was originally supposed. Isolate Y008 is the proposed type strain of "*Acidicaldus organovorus*", gen. nov., sp. nov., the description (Johnson et al. unpubl.) of which follows:

Description of *Acidicaldus* gen. nov.

Acidicaldus (A.ci.di.cal.dus. N. L. n. *acidum* an acid; N. L.. adj. *caldus*, warm; a (moderately) thermophilic acid-requiring microorganism).

Cells are motile rods, Gram-negative, and do not form spores. Obligately heterotrophic. Acidophilic (grows between pH 1.75 and pH 3.0) and thermophilic (40-65^{-}C). Facultative anaerobes, growing by ferric iron respiration in the absence of oxygen. Bacteriochlorophyll and carotenoids are absent. Grow on simple organic compounds such as sugars and small

molecular weight alcohols. Aliphatic acids inhibit growth at mM concentrations. Capable of dissimilatory oxidation of elemental sulfur, though not of autotrophic growth on sulfur in organic-free media. A growth factor (or factors), in the form of yeast extract, vitamins or casein hydrolysate, is (are) required for growth. Sequence analysis of the 16S rRNA gene places the genus within the α-subclass of the *Proteobacteria,* and the G+C content of the genomic DNA is *ca.* 72 mol%. The type species is *Acidicaldus organivorus*.

Description of *Acidicaldus organivorus* gen. nov. sp. nov.

Acidicaldus organivorus (or.gan.i.vo.rus; N. L.. n. *organum*, organic compound; L. v. *voro*, to consume; N. L. adj. *organivorus*, microorganisms that use organic substrates).

Cells are rod-shaped, 1.2-1.5 μm in length and 0.5 μm diameter, motile and do not form endospores. Gram-negative. Growth occurs between 40 and 65ºC (optimum 50-55ºC and pH 1.75 to >3.0 (optimum 2.5-3.0). Colonies on acidic (pH 2-3) yeast extract-containing media solidified with agarose are small, off-white/cream in colour. Cells grow aerobically with oxygen as terminal electron acceptor and anaerobically *via* ferric iron respiration. Fermentative growth on glucose does not occur. Elemental sulfur is oxidised to sulfate. Growth is enhanced by sulfur oxidation, but autotrophic growth on sulfur does not occur. Glucose, galactose, fructose, xylose, galacturonic acid, mannitol, ethanol, glycerol and glutamic acid all serve as good carbon and energy sources for aerobic growth. More limited growth occurs with mannose, ribose, arabinose, rhamnose, methanol and glycine. Small molecular weight aliphatic acids (acetic, pyruvic, lactic, succinic and citric) are highly inhibitory to growth, at low concentrations. Phenol also serves as carbon and energy source, with optimum concentrations being <5 mM. An unknown growth factor(s) supplied by yeast extract, vitamins or casein hydrolysate is (are) required for growth. The G+C content of the chromosomal DNA is 71.8 ∓ 0.9 mol%. Habitats include geothermal springs and soils. The type strain is strain Y008^T and has been deposited within the

Deutsche Sammlung von Mikroorganismen und Zellkulturen and the American Type Culture Collection (DSM 16953 /ATCC BAA-1105). The 16S rRNA gene sequence (1340 bp) has been deposited in GenBank (accession number AY140238).

8.1 General discussion and conclusions

Metal-rich acidic environments are widely distributed throughout the world and are created by both natural and anthropogenic processes. In such environments, a wide variety of prokaryotic life may exist, and these microorganisms may form complex inter-relationships. The study of microbial communities is important in understanding the ecology of metal-rich acidic environments, and in turn microbial community dynamics are important for optimizing the unique abilities of microorganisms in biotechnological processes, such as mineral leaching and water treatment technologies. In the current project, the microorganisms found in macroscopic streamer growths, which are frequently encountered in extremely acidic sites, were studied using culture-dependent and culture–independent methods. In addition, the phenomenon of microbially-catalyzed sulfidogenesis at low pH was investigated, and a novel acidophilic consortium capable of efficient reduction of sulfate to sulfide was discovered.

Microbial populations found in "acid-streamers" in extremely acidic sites at Trefriw spa and the abandoned Cae Coch pyrite mine were studied by plating onto selective solid media and also by using a variety of cultivation-independent techniques, some of which (clone library construction and T-RFLP analysis) are based on PCR (with its well-documented inherent problems of bias) whilst another (FISH analysis) was independent of PCR and, as such, was the most reliable quantitative technique for analyzing microbial populations used in the present study. The techniques showed that acid streamers at both sites were composed of prokaryotic cells embedded in expolymeric substances. By comparing the two methods, >90% of microorganisms in the samples was found to be unculturable using the solid media that have been developed to promote the growth of known acidophiles, such as *At. ferrooxidans. Leptospirillum* spp. and *Acidiphilium* spp.. In the event, many of the acidophiles that were cultivated in the present study were identified as previously-characterized bacteria, though others (e.g. the *Sphingomonas*-like isolate S3BMN1 and the actinobacterium isolate CCW27) appear to represent novel species or even novel genera.

From the culture-independent study of streamer samples in the Trefriw spa, the microbial populations were found to be dominated by up to two distinct bacteria belonging to class *β-Proteobacteria*. These were identified as members of the families *Nitrosomonadaceae* and *Gallionellaceae*, and one or both microorganisms were found in all samples. Minor members (in terms of relative abundance) of the streamer populations consisted of members of the *α-Proteobacteria, γ-Proteobacteria*, *Leptspirillum* spp. and actinobacteria. Although the dominant microorganisms could not be cultivated for characterization purposes with the cultivation techniques used in this study, some aspects of their physiology could be deduced from the physico-chemistry of the waters in which the streamers were found. One of the dominant microorganisms (the *Nitrosomonadaceae*) was found in greater relative abundance in the more oxygen-enriched drainage waters in the spa. In addition, ferrous iron concentrations in the water were found to decrease as the water flowed over the acid streamers. Therefore, this unknown microorganism was deduced to be an aerobic, iron-oxidizing acidophile. The other dominant microorganism (the *Gallionellaceae*) was found in streamers growing in waters containing relatively low concentrations of both DO and DOC. Hence the microorganism was deduced to be similar in aspects of its physiology to its closest characterized microorganism, *Gallionella ferruginea* which is known to oxidize iron in oxygen-limiting, neutral pH environments.

Acid streamer and water samples collected from sites with more varying physico-chemical characteristics (in terms of pH, DO, DOC and metal contents) in the abandoned Cae Coch pyrite mine showed a greater degree of microbial heterogeneity. As with Trefriw spa streamers, a non-cultivated *β-Proteobacterium* (*Nitrosomonadaceae*) was found to dominate most of the streamer samples in this mine, though in one case (site 7) *Acidithiobacillus ferrooxidans* and actinobacteria comprised about half of the bacteria in the streamer community. This was unusual, as these latter two acidophiles tended to be more numerically dominant in mine water samples than in the gelatinous streamer growths. The heterogeneity of the streamer community was illustrated by the discovery of other bacteria (*α-Proteobacteria, γ-*

Proteobacteria, *Actinobacteria, Nitrospira*, *Acidobacteria* and *Bacilli*) in these macroscopic growths within Cae Coch.

Microbial populations in water samples collected at Cae Coch were found to be considerably different from that of streamer samples. Mine waters in two pools sampled within Cae Coch contained much greater concentrations of dissolved metals, especially Al and Fe, and lower DOC contents, than the flowing waters in which most of the acid streamers were found. The microbial population in the most extreme (in terms of pH, solute concentration etc.) of these pools was dominated by *At. ferrooxidans* and actinobacteria, and no β-proteobacteria were detected. In addition, archaea were detected in this pool by FISH analysis. By comparing the streamer and water samples collected in Cae Coch, microbial diversity was found to decrease as the site became more extreme (low pH, high metal content and low DOC) and *At. ferrooxidans* and actinobacteria became increasingly dominant (and β-proteobacteria less abundant) in these conditions.

The results from the study of streamer and water samples collected at the Trefriw spa and the Cae Coch mine showed the microbial communities were very different to those found both in Iron Mountain (Bond et al. 2000b) and River Tinto (López-Archilla et al. 2004). Conditions within Iron Mountain, though variable, were in general more acidic and temperatures were higher (by up to *ca*. 30⁻C) than in the two sites in north Wales that were studied in the current project, and extremely acidophilic and thermotolerant iron-oxidizing prokaryotes (e.g. *Ferroplasma* and *Leptospirillum* spp.) were often found to dominate streamers and slimes in that abandoned metal mine. In contrast, the Rio Tinto was more similar to AMD in Trefriw spa and Cae Coch, though no acidophiles were detected in streamers by López-Archilla et al. 2004, though *At. ferrooxidans, Acidiphilium* and other acidophilic bacteria have been found within the river water itself (Gonzalez-Toril et al. 2003)..

Also in the current project, sulfidogenesis at low pH by a pure culture of aSRB and by a defined mixed-culture of aSRB and another acidophilic heterotroph was investigated. The aSRB studied in the current project was found to oxidize glycerol incompletely, producing acetic acid as a waste metabolite, which had negative feedback on its growth (as acetic acid was toxic to isolate M1 at >5 mM). However, isolate M1 could also grow on hydrogen, and in this case acetic acid was not produced and the aSRB was able to carry out sulfidogenesis at pH as low as 3.0. An attempt to grow isolate M1 in pure culture in a bioreactor in which glycerol was supplied as the carbon/energy source was unsuccessful, possibly due to the toxicity of acetic acid. The inclusion of *Acidocella* PFBC to cultures of the aSRB M1 resulted in little, if any, accumulation of acetic acid, and subsequent use of the defined mixed culture of M1 and PFBC resulted in successful demonstration of sulfidogenesis in a bench-scale (2L) bioreactor between pH 3.7 and 4.3. The mixed culture was monitored closely and PFBC, which is supposedly an obligate aerobe, was found, rather unexpectedly, to be active in the culture, growing concurrently with the obligate anaerobe, M1. The bioreactor culture was found to be producing more sulfide than would be anticipated if glycerol was only partially oxidized to acetic acid. These findings lead to the development of a hypothesis, in which a syntrophic relationship between M1 and PFBC, involving interspecies transfer of hydrogen, results in the complete oxidation of glycerol to carbon dioxide, and an increased efficiency of sulfate reduction compared to pure cultures of M1, in agreement with observations from experiments.

There are a number of examples of syntrophic microbial relationships in the literature. For example, Cord-Ruwisch et al. (1998) showed that *Geobacter sulfurreducens* grew syntrophically with either *Wolinella succinogenes* or *Desulfovibrio desulfuricans*, using acetate as sole carbon source and nitrate as an electron acceptor. In pure culture, *G. sulfurreducens* can oxidize acetic acid using fumarate, ferric iron and elemental sulfur (but not nitrate) as an electron acceptor (Coccavo et al. 1994), and hydrogen (but not acetate) is used as an electron donor by both *W. succinogenes* and *D. desulfuricans*, using nitrate as an electron acceptor (and also sulfate, in the case of *D.*

desulfuricans; Krekeler & Cypionka 1995). In mixed cultures (*G. sulfurreducens* plus *W. succinogenes* or *D. desulfuricans*) containing acetate and nitrate, acetate was oxidized and nitrate was reduced. Cord-Ruwisch et al. (1998) proposed that *G. sulfurreducens* oxidized acetate to bicarbonate and hydrogen (as in Equation 6.2) and then the partner bacteria coupled the oxidation of hydrogen to the reduction of nitrate. Although acetic acid fermentation is endergonic, the combination of acetic acid fermentation and nitrate reduction (Equation 8.1) is exergonic ($\Delta G^{0'}$ = -599.6 kJ/mol).

$$CH_3COO^- + NO_3^- + 3H^+ \rightarrow 2CO_2 + H_2O + NH_4^+ \quad (8.1)$$

Cord-Ruwisch et al. (1998) also tested sulfate reduction by the co-culture of *G. sulfurreducens* and *D. desulfuricans* by replacing nitrate with sulfate, but no evidence of growth by sulfate reduction was recorded. However, an acetate-decomposing sulfidogenic syntrophic association has been described comprising the SRB *Desulfotomicrobium apsheronum* and an unidentified partner bacterium (Rozanova et al. 1990).

There have been other reports of syntrophic associations involving SRB and non-SRB, in which SRB enabled energetically-unfavorable substrate utilization by non-SRB to proceed by removing H_2. Jackson et al. (1999) described syntrophy involving *Syntrophus aciditrophicus* and *Desulfovibrio* strain G11. The oxidation of benzoate by *Syntrophus aciditrophicus* was only possible when hydrogen was removed by hydrogen-utilizing SRB, as the benzoate degradation reaction (Equation 8.2) is energetically unfavorable ($\Delta G^{0'}$ = +71 kJ/mol benzoate).

$$C_7H_5O_2^- + 7H_2O \rightarrow 3CH_3COO^- + HCO_3^- + 3H^+ + 3H_2 \quad (8.2)$$

However, in the presence of H_2-utilizing SRB, the net reaction (Equation 8.3) is energetically favorable ($\Delta G^{0'}$ = -43 kJ/mol benzoate):

$$4C_7H_5O_2^- + 3SO_4^{2-} + 16H_2O \rightarrow 12CH_3COO^- + 4HCO_3^- + 3HS^- + 9H^+ \quad (8.3)$$

A syntrophic association between SRB and archaea has also been proposed to exist in microbial aggregates found in the crest of the southern Hydrate Ridge (located off-shore of Oregon, U.S.A.). FISH analysis of these aggregates revealed methanogenic archaea surrounded by SRB, related to *Desulfosarcina variabilis* (Boetius et al. 2000). In this consortium, the

microbial population was assumed to be sustained by anaerobic oxidation of methane and sulfidogenensis (Equation 8.4).

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O \quad (8.4)$$

Boetius et al. (2000) proposed that reverse methanogenesis by methanogenic archaea was supported by an effective removal of the end products, such as H_2 and acetate, by SRB.

The findings from the current project illustrate that microbial interactions in acidic environments are complex and that microbial communities in these situations are best studied by using a variety of techniques. This was the first instance where a syntrophic association involving acidophiles has been described, and the data help explain previous anomalies concerning sulfate reduction in low pH environments (i.e. that it occurs in the environment, but had not been previously demonstrated in the laboratory). The results should help underpin future developments in the expansion of sulfidogenic biotechnologies at low pH.

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In preparation

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